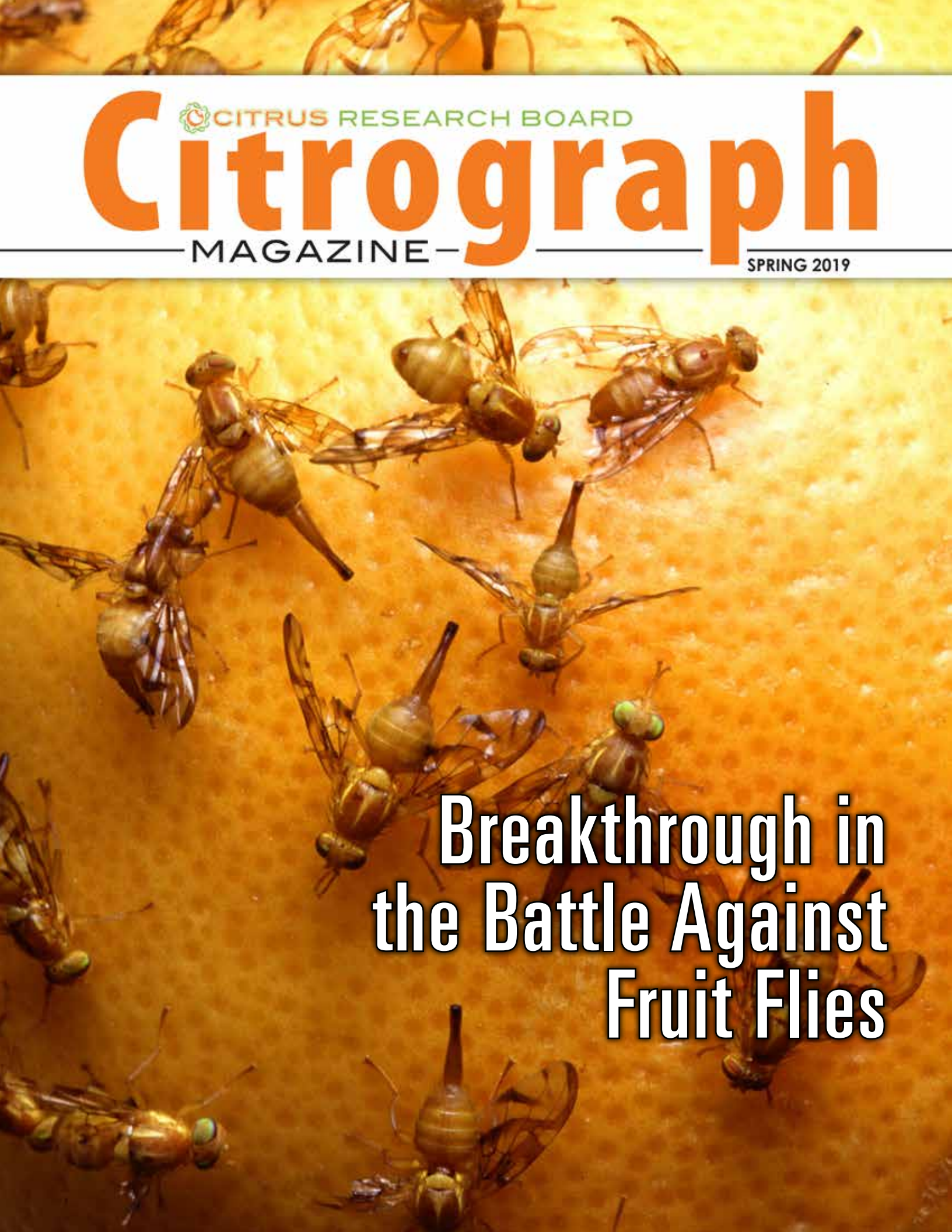
CITRUS RESEARCH BOARD
Citrograph
MAGAZINE
SPRING 2019



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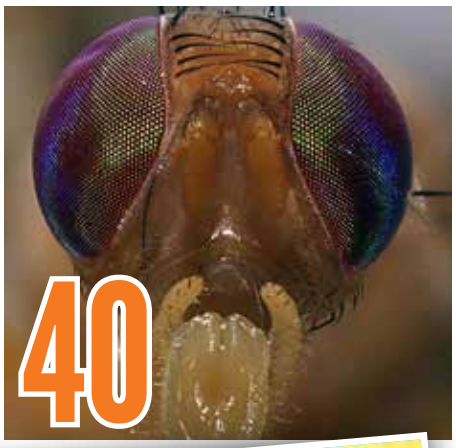

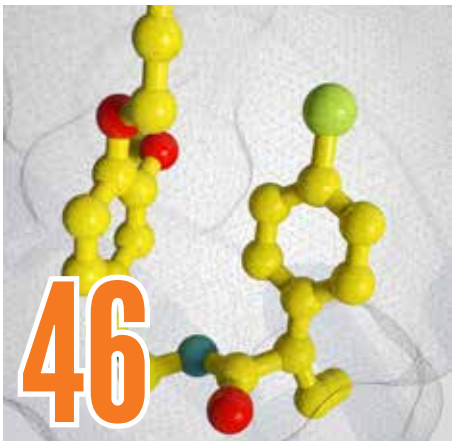

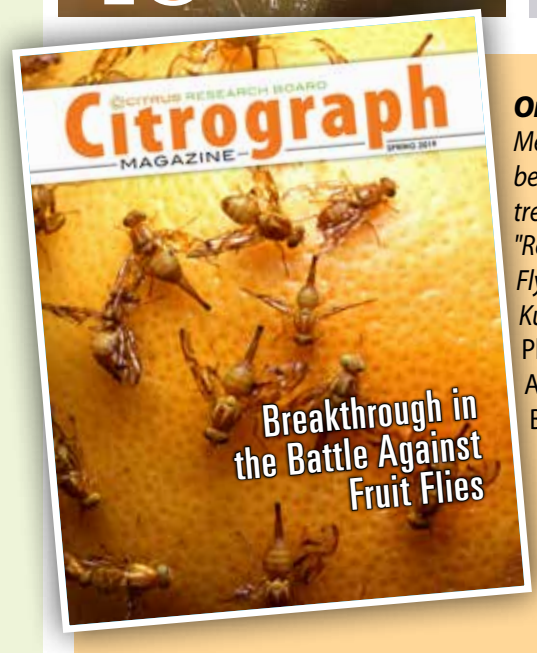

On the Cover:

Mexican fruit flies lay eggs in grapefruit before a test of the reduced-oxygen treatment. For more information, see "Recent Advances Toward a Mexican Fruit Fly Lure" by Spencer Walse, Ph.D., and Dan Kuzmich, Ph.D., on page 38.

Photo Credit: Jack Dykinga, USDA Agricultural Research Service, Bugwood.org

In This Issue

Spring 2019 | Volume 10 • Number 2 The Official Publication of The Citrus Research Board

Citrograph's mission is to inform citrus producers and other industry members of research progress and results that will help ensure the sustainability of California citrus.

CITRUS RESEARCH BOARD

The Mission of the Citrus Research Board:

Ensure a sustainable California citrus industry for the benefit of growers by prioritizing, investing in and promoting sound science.

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By District 2018-2019

(Terms Expire September 30)

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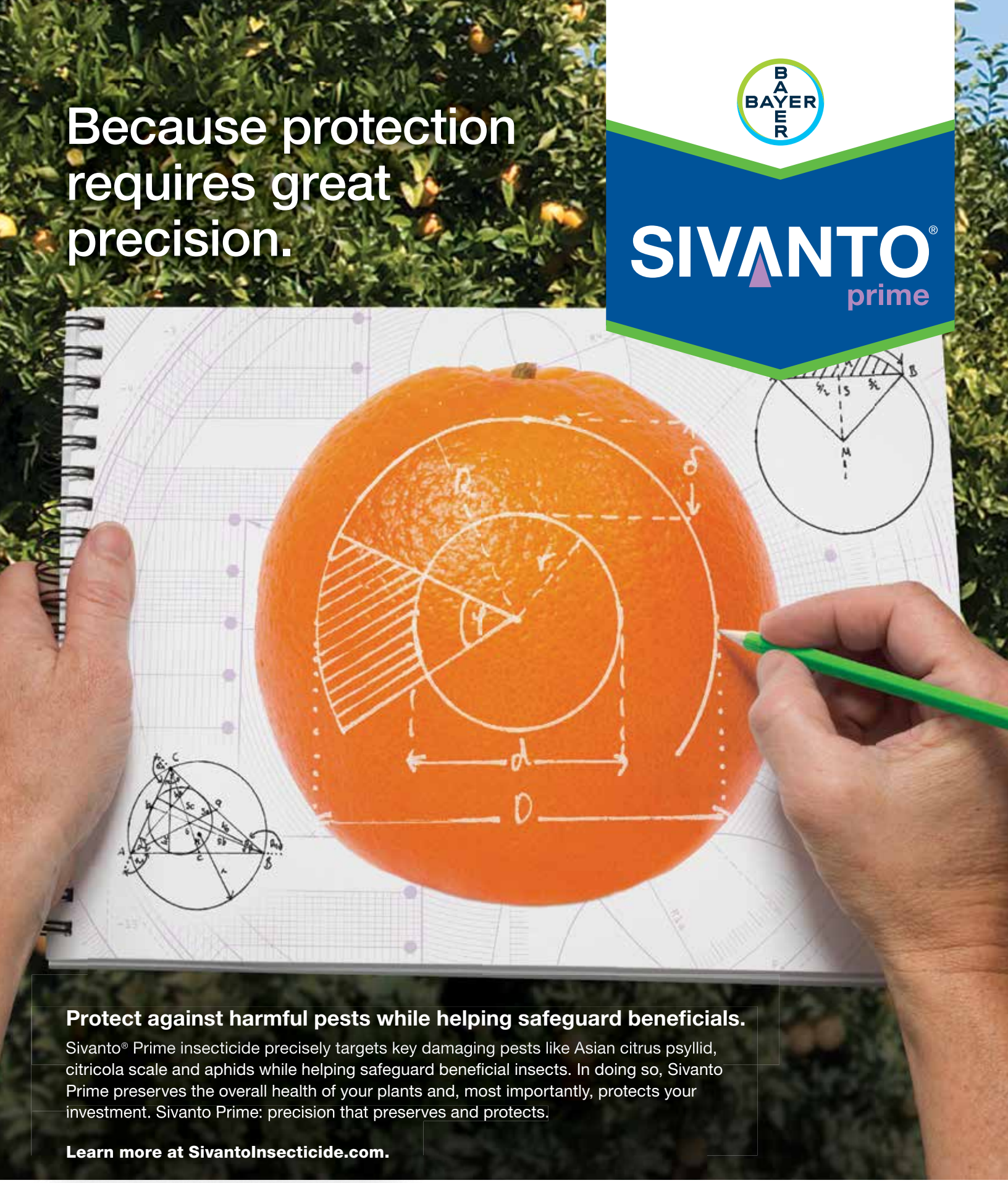

CALENDAR OF EVENTS 2019

March 7

California Citrus Mutual (CCM) Citrus Showcase
Visalia Convention Center, Visalia, California.
For more information, contact (559) 592-3790 or visit *www.cacitrusmutual.com*

March 10-15

Joint Conference of the International Organization of Citrus Virologists (IOCV XXI) and 6th International Research Conference on Huanglongbing (IRCHLB VI)
Riverside Convention Center, Riverside, California.
For more information, visit *http://irchlb.com*

March 12

Citrus Pest and Disease Prevention Committee (CPDPC) Meeting
Riverside, California. For more information, visit *www.cdfa.ca.gov/citruscommittee*

March 28

California Citrus Quality Council (CCQC) Board Meeting
Doubletree Hotel, Bakersfield, California. For more information, visit *http://ccqc.org*

April 24-25

40th Annual CRB Citrus Post-harvest Pest Control Conference and Citrus Food Safety Forum
Hyatt Centric Santa Barbara, Santa Barbara, California. For more information, contact (559) 738-0246, or visit *www.citrusresearch.org*

May 7

Citrus Research Board (CRB) Meeting
Ventura, California. For more information, contact (559) 738-0246, or visit *www.citrusresearch.org*

May 8

Citrus Pest and Disease Prevention Committee (CPDPC) Meeting
Ventura, California. For more information, visit *www.cdfa.ca.gov/citruscommittee*

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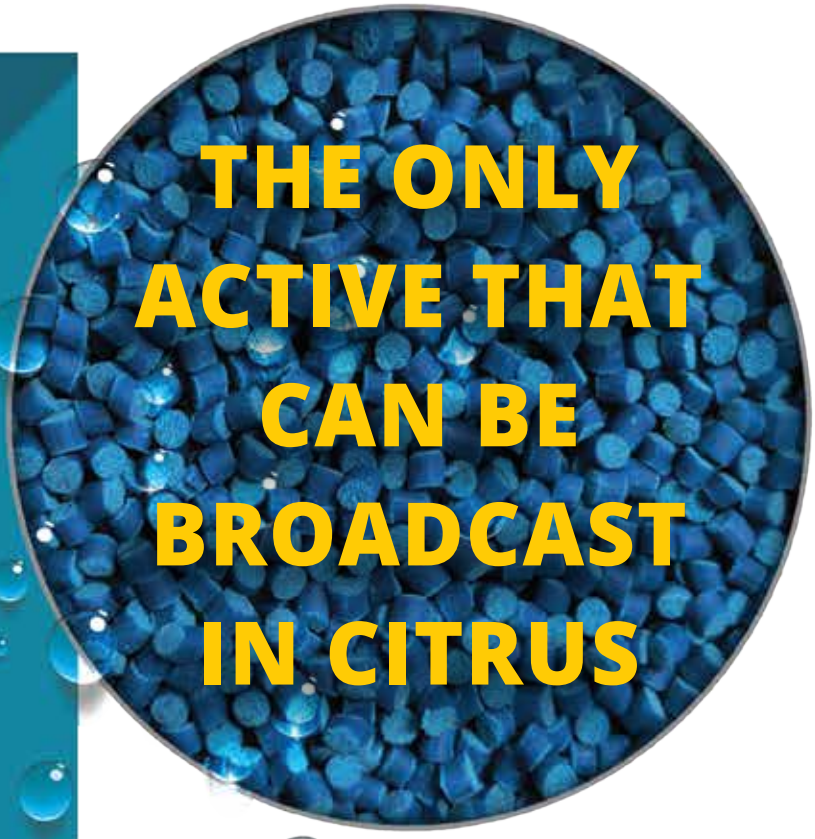

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2019 Citrus Showcase

March 7th

At the
Visalia Convention Center

Schedule

7:30 AM Doors open. Coffee and Pastries Sponsored by *Valley Packline Solutions*

8:00 - 9:00 AM Workshop A: "Strategies for Surviving SGMA"

9:00 - 10:00 AM Tradeshow Open

10:00 - 11:00 AM Workshop B: How Citrus Research Informs the Regulatory Process and How the Current Regulations Affect the Citrus Industry. *Sponsored by the Citrus Research Board*

11:00 AM - 12:00 PM Tradeshow Open

12:00 PM Luncheon Program Sponsored by: *Gless Ranch Nursery*
Lunch Tickets can be purchased for $40 by calling California Citrus Mutual at 559.592.3790

1:30 - 2:30 PM Tradeshow Open

2:30 - 3:30 PM Workshop C: "*Selling Citrus* - It's Not 2018 Anymore"

$0
Admission

Sponsored By: Accu-Label International | BASF | Bayer | Corteva | Farm Credit | Fruit Growers Supply | Giffin-Parker Insurance Brokers | Pace International | Packline Technologies | Sinclair Systems International | Suterra | Syngenta | Valent USA |

California Citrus Mutual

40th ANNUAL CITRUS POST-HARVEST PEST CONTROL CONFERENCE

A technical two-day citrus conference for packinghouse and industry personnel, service company representatives and researchers to provide updates on recent developments in post-harvest disease control, export requirements and food safety.

Conference Highlights

- Fruit quality and decay management
- Auditing for food safety compliance following GWP and GAP guidelines
- International MRL compliance
- Update on CCQC activities
- Update on research and control of ACP/HLB

Registration Fee: $500

Registration fee includes two continental breakfasts, four refreshment breaks, two lunches, evening reception and conference materials.
(Does NOT include hotel room.)
Deadline to Register: April 10, 2019

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April 25th | 1:00-4:00 PM
Citrus Food Safety Forum
Facilitator: Mr. Ted Batkin

For more information visit: www.citrusresearch.org

Sponsorship opportunities available: Contact Carolina Evangelo at carolina@citrusresearch.org or (559) 738-0246

CITRUS RESEARCH BOARD

University of California
Agriculture and Natural Resources

Summit on Interstate Movement of Citrus Material

Report from Denver

Melinda Klein

There's a lot to do in Denver, Colorado, in late October – enjoy beautiful fall color, visit a Denver Broncos or Denver Nuggets game, early season skiing – but for 36 state and federal regulators, researchers, nursery representatives and citrus research funding agency representatives from across the U.S., their trip to Denver this past October 25-26 was planned to discuss the finer regulatory points of citrus budwood, pollen and seed movement across state lines.

The "Summit on Interstate Movement of Citrus Material" was held to address some of the federal and state regulatory barriers identified at a previous Denver meeting in February 2018. The earlier meeting reviewed the current research process for developing new and/or improved citrus germplasm and varieties, including huanglongbing (HLB)-resistant materials; identified current barriers at the research, regulatory, intellectual property and technology transfer, and funder-related levels; and then identified potential solutions to achieve more effective coordination and collaboration to speed the process.

The issues highlighted within the regulatory group at the February meeting included:

- delays experienced by researchers in moving HLB-tolerant or -resistant research and advanced materials between states,
- the different state regulatory requirements for movement, which can be contradictory and confusing,
- lack of information on the risks associated with moving citrus propagative materials for research purposes and
- a lack of understanding among scientists regarding the regulatory requirements for genetically-engineered citrus propagative materials.

One of the suggested solutions to address these concerns was better communication – to convene a conference specifically to review the process for interstate movement of citrus breeding materials for research purposes. During the next eight months, a working group that included the U.S. Department of Agriculture (USDA)-Agricultural Research Service, USDA-Animal and Plant Health Inspection Service (APHIS), University of California Riverside, Citrus Research Board, Citrus Research and Development Foundation and nursery representatives met via bi-weekly conference calls to design and prepare a meeting to tackle these issues head on. With the approval of funds from the HLB-Multi-Agency Coordination (HLB-MAC) Group for this summit, the meeting was a go. To prepare

attendees for the meeting, a questionnaire was sent out prior to the meeting to review some of the potential scenarios involving interstate movement of citrus material as a way to consider some of the larger regulatory issues at play.

With the stage set, the October meeting brought together attendees from across the U.S. – including California, Florida, Texas, Arizona, Louisiana and Washington D.C. As outlined, the goal of the summit was to create protocols that would account for the phytosanitary safety and containment concerns of HLB while simultaneously allowing for the timely interstate transfer of citrus material to allow research to proceed more rapidly.

On the first day of the summit, attendees were split into small groups of participants with a mix of expertise and home states to address one of three levels of risk questions previously given to participants for consideration:

Question #1 (Low Risk):
What are the minimum containment conditions in the recipient state under which you would accept propagative material from a pathogen-free plant program in another state?

Question #2 (Medium Risk):
What are the minimum containment conditions under which you would accept material maintained in an ACP-exclusion greenhouse or lab located in another state?

Question #3 (High Risk):
What are the minimum containment conditions under which you would accept plant material collected from field grown trees in another state?

After an initial break-out session for discussion within the smaller groups, presentations were made to the larger group with specific attention paid to comments and suggestions from the state regulators. The three groups then broke out again, developed their plans further based on the feedback received and provided a final protocol to the larger group.

The second day of the summit began with a discussion by the entire group with state regulators to understand existing state processes and listen to their suggestions on how to quickly move forward through the current regulatory system. Utilizing the permit processes, especially in California where the distribution of HLB is limited, was emphasized. Keeping in mind the needs of other states when developing new guidelines (especially those that accept budwood from other states' clean budwood programs) also was highlighted in the discussion. A final series of action items was developed that will be available for review when the final report is released (it was in the final stages of being written when this article was submitted for publication).

The priority action items that came out of this meeting included a review of risk associated with moving different sources of plant material for breeding purposes from different research facilities (including materials that originate from lab, greenhouse or field). A work group was formed to streamline the permit process to help researchers move materials from state to state in coordination with other research groups. Ongoing discussions between summit participants will be facilitated by APHIS to update the group on progress with the action items as the risk assessments move forward.

HLB is a serious citrus disease, and the process involved in developing new citrus varieties is not a fast one. There is a real and significant need to speed the variety development process and provide long-term disease-resistance options for all U.S. citrus. This summit provided a chance for state and federal regulators, researchers, funding agency representatives and citrus grower and nursery representatives to meet and address these long-standing issues regarding interstate citrus material movement in order to identify a way forward. Special thanks to all summit participants, the USDA HLB-MAC Group for financial support, meeting facilitator Jim Kastama and to the working group for their assistance in planning this summit.

***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.**

Using Social Media to Connect with Californians

California Pest and Disease Prevention Outreach Committee

As the threat huanglongbing (HLB) poses to California citrus continues to rise, the Citrus Pest and Disease Prevention Program (CPDPP) works continuously to find new ways – and innovate tried-and-true tactics – to reach residents. While social media sites like Facebook, Twitter and YouTube are not "new" to the average person, they are consistently evolving and allow the CPDPP to connect with communities, individuals and organizations in new ways.

Californians are engaging with social media sites like never before. These sites offer easily accessible settings for discussion and information sources. According to the Pew Research Center, 73 percent of U.S. adults watch videos on YouTube, two-thirds use Facebook and 68 percent claim they get some of their news from social media.

These on-line platforms are essential tools the CPDPP uses to reach a variety of audiences with information related to the Asian citrus psyllid (ACP) and HLB. While the potential for reaching audiences on social platforms is rich, it is not a stand-alone communications channel – making it just one of the many outreach methods used to encourage California residents to take action and protect the state's citrus trees.

A Look at Platforms and Uses

The CPDPP has official organization pages on Facebook, Twitter and YouTube – all of which are used for different purposes.

Facebook allows the CPDPP to maintain a presence on one of the world's largest social media networks and engage with its audience through multimedia posts and comments. The CPDPP's YouTube page is used to house videos – like public service announcements, best practices for farm labor contractors and other informational content – that are easy for others to view and share.

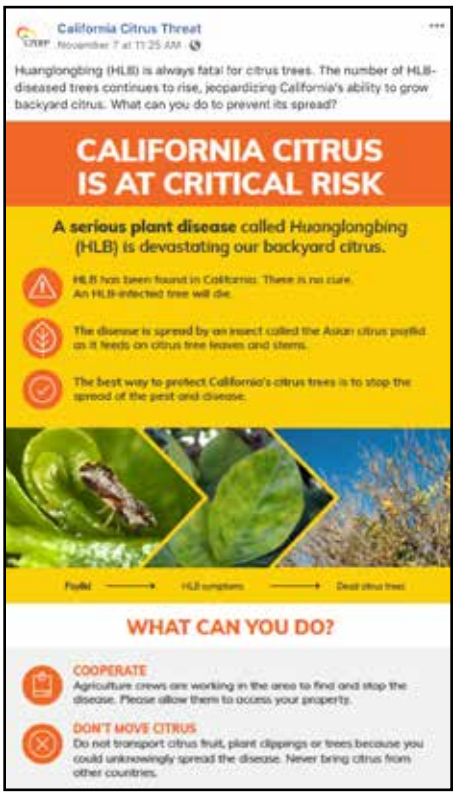
The program's Twitter presence has evolved as an effective one-on-one tool to engage with influential reporters, partners and other like-minded organizations. Additionally, Twitter allows the outreach team to monitor keywords and

issues in real time, helping manage and anticipate potential issues.

The types of information shared on the CPDPP's Facebook, Twitter and YouTube accounts include:

- timely and accurate articles about ACP and HLB detections,
- tips for proper care of backyard citrus trees,
- videos and infographics that communicate the urgency of the issue and best practices to prevent the spread of HLB,
- links to helpful resources like the University of California Integrated Pest Management website and quarantine maps and
- posts that recognize program partners – like master gardeners and city government employees – who share information on the issue with their networks.

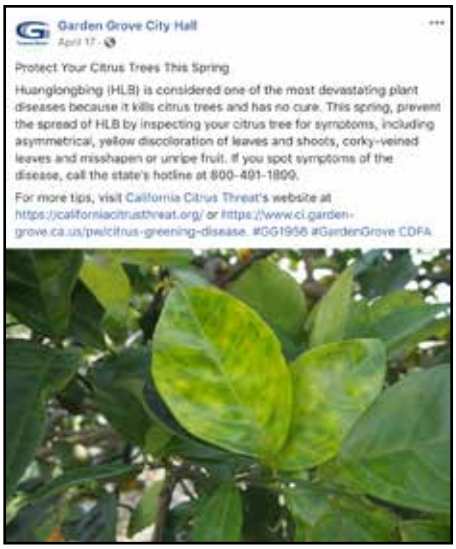
Over the last year, these social media pages, which regularly share content about ACP and HLB, have garnered more than one million impressions.



One example of an infographic that the CPDPP outreach team shares on social media.

Engaging with Influential Partners on Social Media

The CPDPP outreach team has been exploring new ways to connect and motivate California residents to protect the state's citrus trees, including partnering with elected officials and local government employees. By providing them with ready-to-post content about ACP and HLB, they easily can share information on their own social media profiles and with their constituents. More than 15 city governments, all of which are in or near the HLB quarantine zone, have shared the information provided by the outreach team with their constituents on social media.



The City of Garden Grove shared important information with their residents as the number of HLB detections in the city increased.

The program also has started identifying individuals such as master gardeners, University of California and other researchers and farm advisers, citrus growers and packers, trade organizations and others who are passionate about growing healthy citrus trees, gardening and saving California's citrus. The outreach team works with these individuals to obtain their photos and quotes about why citrus is important to them. These photos and quotes are shared across CPDPP's social media channels – introducing social media users to the people who are or would be impacted by HLB.

While the outreach team continues to explore new ways to expand its social media presence, the citrus industry and its supporters can play a role. Visit the program's social media pages, follow them and share their posts with your own social media networks. Additionally, if you would like to be featured on the program's Facebook and Twitter pages, please email *info@citrusinsider.org* and the CPDPP will work with you to gather the information needed.

There is no one better to advocate about the danger of ACP and HLB than you. Your voice can be powerful and motivating to your friends, families, colleagues and California residents. The CPDPP is diligently working to keep the disease at bay, but by working together to share information about HLB, we can help protect our state's citrus for years to come.

CONNECT WITH US

Facebook.com/CaliforniaCitrusThreat

Twitter.com/CitrusThreat

Search "Citrus Pest & Disease Prevention Program"

***For more information about the program's social media outreach, contact* info@citrusinsider.org.**

California Marketing Programs

How these public/private partnerships work for you

Kacie Fritz

The Citrus Research Board (CRB) is a marketing program overseen by the California Department of Food and Agriculture. Many of you may be wondering what this means exactly. Here is a brief history of how these programs came into existence, what oversight exists and why this benefits the citrus industry.

The California Department of Food and Agriculture

The California Department of Food and Agriculture (CDFA) traces its roots back to 1880 when the Legislature appointed a seven-member State Board of Viticulture to protect grapevines from phylloxera-caused decline and death. Social and technological changes around the turn of the last century created the need for additional agricultural protection and consumer assurance. Work was done to keep the Mexican fruit fly out of the United States, and border inspections began in response to the introduction of the Mediterranean fruit fly and alfalfa weevil. By 1919, the California Legislature created a single department responsible for protecting and promoting agriculture. The Legislature believed the prosperity of agriculture was essential to the general health and well-being of all Californians. For 100 years, the CDFA has fulfilled its mission in a manner that encourages farming, ranching and agribusiness, while protecting consumers and natural resources.

Marketing Programs

What is a marketing program?

California marketing programs are instruments of the State of California that:

- aid agricultural producers and handlers in preventing economic waste in marketing their commodities;
- develop more efficient and equitable production and marketing methods;
- aid producers in maintaining their purchasing power at a more adequate, equitable and reasonable level and
- function as "self-help" programs by which producers or handlers within the state can work together to solve marketing problems they cannot solve individually.

Examples of Benefits Generated by Marketing Programs

Benefits to the public		Benefits to agriculture generated by marketing programs
Generated by agriculture	**Generated by marketing programs**	
• Production of abundant supply of agricultural products • Sales of ag-related products and services • Exports that help U.S. balance of trade • Jobs • Tax revenue	• Encouragement of agriculture's responsible stewardship of land, water and marine resources • Health and nutrition research • Consumer and general public education • Establishment of quality standards to ensure wholesome and safe food supply • Implementation of State policy in support of industry-funded self-help programs • Enhanced contribution of agriculture industry to state economy • Research: improved productive farming practices • Research: new food products and/or new farm product uses	• Representation of the State's continuing commitment to the agricultural industry • Economic viability of agriculture through coordination of collective action by farmers • Enhanced image of agricultural products grown in California • Expanded domestic and international markets for California farm products • Research to improve farming and handling practices • Market and economic research • Management of issues that affect the ability of California agriculture to compete nationally and internationally

Marketing programs were first made available under California law in 1933 and equally apply to all producers of an agricultural commodity. Programs authorized by legislation were based on the previous experience of growers and handlers who had come together to improve marketing conditions. Voluntary programs were developed by industry groups as early as 1900. However, these programs had no effective means of requiring compliance, which meant that they broke down as a result of noncompliance by an ever-increasing number of individuals. The greater initial success that a program had, the greater the incentive was for individuals to market their products without complying with the terms of the program (free-riders).

The Agricultural Prorate Act, the California Agricultural Adjustment Act and federal legislation providing for uniform application of a program all were enacted in 1933. An industry now could develop a program with the assurance that all persons affected would be treated equally.

The marketing program concept as it exists today originated with the California Marketing Act of 1937, which authorized the Department to provide administrative direction and oversight for marketing programs' governance and operations. The Marketing Act does not impose regulations over the marketing of a commodity, rather it provides the authority under which an industry can develop programs to address its own needs and marketing issues.

Marketing programs serve the industry and the public by helping producers and handlers manage issues that affect them. The government's reasons to authorize marketing programs are to stimulate the agricultural economy and to ensure a continuous and abundant supply of food and agricultural products. To the extent that marketing programs fulfill these purposes, the well-being of California agriculture is inseparable from the well-being of consumers. The table above summarizes the benefits that marketing programs generate for the public and the agricultural commodities. Because all affected parties stand to gain from marketing program activities, every represented agricultural producer and handler must abide by relevant statutory provisions and share the cost of implementing them.

What is the main benefit of marketing programs?

The Marketing Act provides for state enforcement, which is the main benefit over voluntary programs. However, this benefit means it is necessary that all actions under a marketing order are in accord with the general administrative public policy followed by state agencies.

While the constitutionality of the Marketing Act's basic concepts has been upheld by many court decisions, the manner in which programs are administered is subject to court action at any time. Members of an industry must bear in mind that every action under a marketing order must be consistent with the terms and provisions of the act and the marketing order.

This is where the CDFA comes in…

A marketing program's daily business can be run by its board and staff. However, the board must submit recommendations regarding program plans and expenditures to the Secretary of Food and Agriculture for review and approval. Any action the board takes that is not consistent with its marketing order or is not in the public interest, can place the marketing order and other commodity marketing orders in jeopardy if challenged in court. On behalf of the Secretary, the CDFA's Marketing Branch oversees and liaises with individual marketing programs. The Marketing Branch staff provides administrative direction and oversight through their participation in program activities, decision-making and meetings. To promote compliance, the CDFA also notifies programs of changes to applicable laws, regulations and policies.

This is why we call marketing programs like the Citrus Research Board "quasi-governmental" organizations. The staffers at these programs are considered public employees, but not civil servants. They have some autonomy, but must speak with "one voice" with the CDFA.

Citrus Research Board

Where does the CRB fit in to all of this? The Citrus Research Program is a marketing program under the State's Marketing Act. The original "California Citrus Improvement Program" became effective on October 24, 1968. Like most commodity programs, it came into being as the result of an industry petition and vote. The original advisory board had a total of 11 members, alternate members from three districts and three at-large positions. It had the authority to carry out a quality assurance program on agricultural chemical residues, a variety improvement research program and general production research. In 2008, the industry conducted a referendum to change the marketing order to add pest detection and disease control authority after the first detections of huanglongbing in North America.

Because the CRB is a marketing program, the citrus industry is authorized to collect an assessment from all citrus growers. Through this assessment, the Board has:

1. created the Citrus Clonal Protection Program,
2. established the California Citrus Quality Council,
3. opened the Jerry Dimitman Diagnostic Laboratory and
4. conducted a significant amount of beneficial research.

The CRB also is able to partner with other state and federal entities to receive additional funding from outside sources to address the many challenges the industry faces – whether that is huanglongbing and the Asian citrus psyllid, tristeza, fruit flies, production practices, diseases or other threats.

The citrus industry, through the CRB, is a prime example of how an industry can come together effectively through a state marketing program to collectively solve problems that threaten the entire commodity.

***Kacie Fritz is an associate agricultural economist in the marketing branch of the California Department of Food and Agriculture in Sacramento, California, where she oversees Citrus Research Board activities. For additional information, contact* Kacie.Fritz@cdfa.gov.**

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Fiugre 1. A citrus grove in California's central valley.

DATOC: Data Analysis and Tactical Operations Center

Holly Deniston-Sheets, Sara García Figuera and Neil McRoberts

Project Summary

The mission of the Data Analysis and Tactical Operations Center (DATOC) is to provide analyses and science-based guidance in the fight against huanglongbing (HLB). A diverse team of experts fulfills the need for rapid, flexible and responsive analyses and interprets the complex issues surrounding Asian citrus psyllid (ACP) and HLB control.

Introduction

There can be no doubt that the presence of HLB in California is a significant threat to the long-term sustainability of the state's citrus industry. Whether new plantings of citrus, like those pictured in **Figure 1**, will last their projected lifespan without becoming infected by the HLB-associated bacterium '*Candidatus* Liberibacter asiaticus' (CLas) is uncertain. This should be a major cause for concern for everyone, from those involved in commercial citrus production to those who own just a few trees for personal use, or even those who simply love to enjoy the diverse variety of California citrus. One thing is certain, inaction by the citrus industry will not be to blame if HLB escapes the urban centers where it is currently detected and spreads to commercial citrus. The response to HLB in California, coordinated by the Citrus Pest and Disease Prevention Committee (CPDPC), is one of the most concerted and sustained efforts in plant disease suppression ever attempted in the U.S. – but it didn't start out that way.

Jack Williams, who provided the inspiration for what DATOC was to become, explained, "When I first joined the Citrus Research Board in 2013, California's HLB strategy was evolving. It was being developed and implemented by a loose confederation of universities, state, federal and local governments and industry organizations. Each entity had its own priorities and disciplinary/operational focus and was separated geographically from the others by hundreds, even thousands of miles. While ostensibly this was a collective effort, it was not an ideal architecture, especially when we soon would be faced with very complex problems at the tactical/field level. I thought what California really needed was as a kind of 'war room' similar to what is shown in movies: generals supported by a room full of military and intelligence experts and administrative staff, all standing around a tabletop depiction of the battlefield."

With this idea in mind, a collaboration between the Citrus Research Board (CRB) and University of California (UC) scientists began, and DATOC was formed in 2016.

The diverse team that makes up DATOC aims to interpret the best science-based evidence available to help guide operational decisions for combatting HLB and ACP, as well as to provide comprehensive situational awareness updates to CPDPC, the California Department of Food and Agriculture (CDFA) and other stakeholders in the citrus industry.

"To me, DATOC is a kind of field operations center where you have a multidisciplinary, multi-sector group of scientists, economists, regulators and growers using common sources of the most recent and best research, field data and analysis. The goal is to provide key decision-makers with timely, well-informed, alternative solutions tempered by economic and political realities." -Jack Williams

Typically, the analyses provided by DATOC are prompted by requests from the CPDPC or its Operations or Science subcommittees. However, topics for analysis also can be raised internally by experts on the DATOC panel or come from third-party groups, such as grower liaisons, citrus pest control districts and task forces, individual growers, companies or federal government agencies.

Results to Date

Several DATOC briefing papers have been submitted to the CPDPC, and suggestions put forward by DATOC for operational guidelines have been adopted. One key briefing paper, "Recent changes in the ACP/HLB invasion in California and implication for regional quarantines," produced in November 2017, served as the basis for the CPDPC to enact quarantine zones and truck tarping, rules that likely have significantly limited ACP movement around the state, thereby protecting citrus production in areas that are not yet heavily infested.

More recently, in October 2018, DATOC presented two recommendations to the CPDPC Operations committee, one of which advised against allowing hand-removal of leaves as a mitigation measure before bins of harvested fruit can be moved. The second summarized available evidence to help guide treatment activities following ACP detection.

DATOC also launched a new website (*www.DATOC.us*) for desktop and mobile applications (**Figure 2**), where all past policy briefings (such as the two listed above) are available, as well as current activity updates.

Current Projects

HLB Exposure

There are a few aspects of HLB that make control particularly difficult, including a long latency period and unequal distribution of the pathogen within a tree. The long latency period means that the disease can exist within the tree long before visual symptoms appear; and because of unequal distribution, leaves collected from an infected tree to check for HLB may test negative for CLas. An infected tree, therefore, can remain in the ground for some time acting as a source of CLas for the ACP to spread. This causes significant logistical problems for HLB management, including rising laboratory and survey costs, as the number of undetected, but infected, trees increases (**Figure 3**).

Figure 3. Neil McRoberts, Ph.D., (right) presents DATOC's preliminary findings on exposure to HLB in southern California as CRB Board member Jim Gorden listens.

An alternative way of approaching management could emphasize whether a tree had been exposed to CLas as the first step in decision-making, rather than confirmatory testing for the bacterium. This is the focus of a DATOC project to be

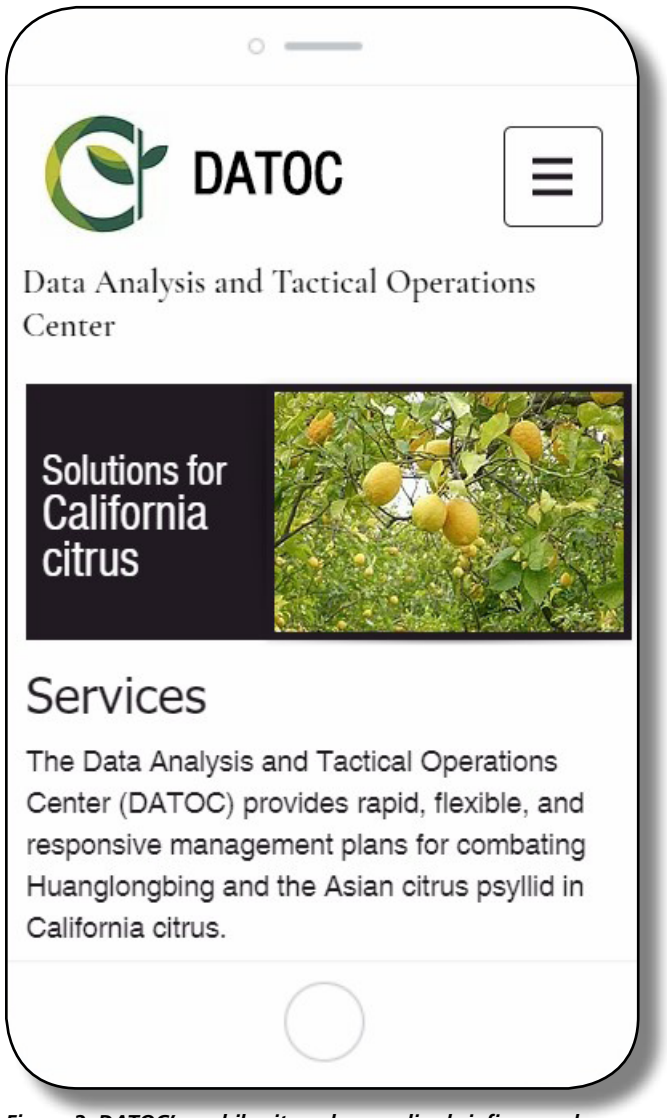

Figure 2. DATOC's mobile site, where policy briefings and activity updates are available.

Figure 4. Current quarantine zones in place for movement of bulk citrus. Modified from CDFA 2018 ACP Bulk Citrus Movement – Overview Map.

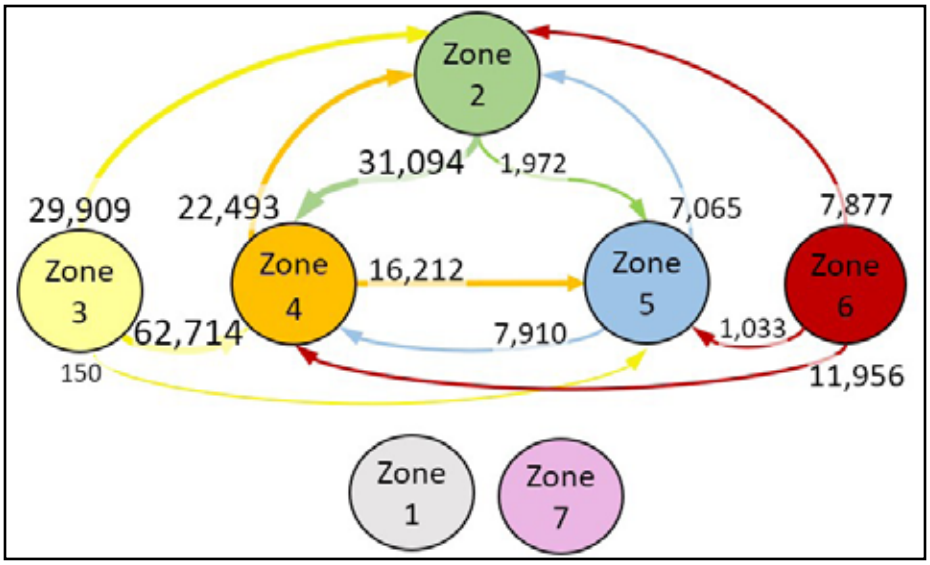

Figure 5. The volume of citrus fruit (as 900 lb. bins) moved between ACP quarantine zones during the 2017-18 growing season. Data provided by the CDFA.

completed shortly, which is evaluating the parameters under which a citrus tree becomes exposed to CLas.

Transportation Risk

Limiting dispersal of ACP is a crucial component of protecting California citrus from HLB. To this end, DATOC is collaborating with a CPDPC regulatory task force to evaluate the risk of inadvertently moving ACP and CLas out of a quarantine zone in bulk fruit shipped for processing and packing. **Figure 4** shows the quarantine zones currently in place, and **Figure 5** shows the known volumes of bulk fruit transported between zones during the 2017-18 season (as 900 lb. bins). This information is being used in conjunction with other known factors to calculate a qualitative assessment of risk. Results of this project will inform CPDPC decisions regarding transport regulations.

Cost-benefit analysis

A second model also is under development to provide a formal method for the CPDPC to evaluate activities within a cost-benefit framework. The project was started following the strategic review of the Citrus Pest and Disease Prevention Program in 2017-18 and is intended to help the CPDPC make strategic decisions about allocation of resources among the various program activities, such as risk-based surveying, geographic extent of infected areas and removal of infected trees. This will help guide HLB management policy by comparing the costs and probabilities of different courses of action, allowing the CPDPC to choose the most cost-effective, lowest-risk methods of managing HLB.

Analysis of Disease Incidence Expansion

An advisory panel has recommended to the CPDPC that disease prevalence be considered in operations planning for HLB management. The recommendation is that the CPDPC alter their management tactics if disease incidence crosses a threshold beyond which the current management strategy is no longer feasible or practical. DATOC is currently analyzing the increase of CLas-positive trees (**Figure 6**) to calculate the growth rate on a regional basis.

State of the State

The "State of the State" is a standing item on DATOC's agenda. This paper is a comprehensive summary of the ongoing statewide development of the HLB epidemic and the expansion of ACP over time using several different indicators of disease intensity and spread. Work currently is underway to produce a second edition of the paper, which then will be updated every six months. The latest version of the document (and all of the other briefings DATOC produces) will be available for viewing and downloading from the DATOC website.

CRB Research Project #5300-182

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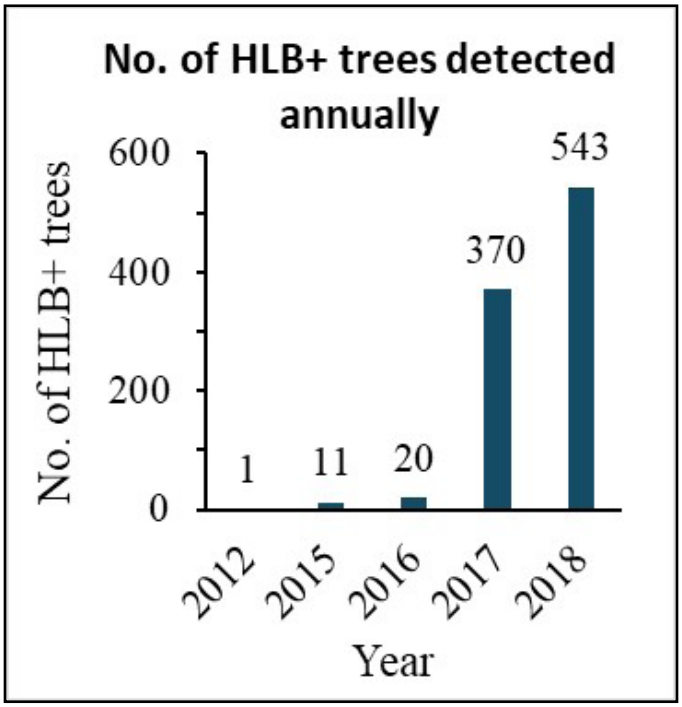

Figure 6. The number of HLB-positive trees detected annually in California from 2012-2018.

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The Heck lab is involved in host-vector-pathogen interaction research and vector biology methods development to address issues impacting agriculture and biological research.

The Hunt for Early HLB Biomarkers

Michelle Heck

Herbert Wetzel was a Ph.D. student at Cornell in 1904; and by 1907, he became the first chair of Cornell's new Plant Pathology Department in the New York State College of Agriculture. He was known for his precise expression of terms. Wetzel suggested that control measures for plant pathogens can be grouped into four categories: exclusion, eradication, protection and immunization – concepts now accepted and widely used by plant pathologists.

Huanglongbing (HLB) is a citrus disease involving citrus host trees, the Asian citrus psyllid (ACP) and a bacterium known as *'Candidatus* Liberibacter asiaticus' (CLas). HLB is a plant pathologist's worst nightmare and is considered the most devastating of all citrus diseases. No resistance has been found in any commercial citrus variety to date, nor is there an adequate protection strategy. HLB exclusion and eradication have eluded growers in Florida for more than ten years, and the disease has significantly affected that state's commercial citrus production. In California, the citrus industry is working to maintain exclusion through extensive monitoring and by eradication when CLas-positive trees (thus far in residential areas) have turned up. Based on the successful management of plant pests and diseases in the U.S., such as plum pox virus and the golden or pale cyst nematode, maintaining exclusion and eradication in California will be the best way to manage the disease.

Methods to pinpoint possible CLas-infected trees before they become CLas-positive by gene-detection techniques may be the most effective way for California to remain in these modes. Such early detection methods may streamline and hasten decisions on tree removal, sampling and quarantine areas. Strategies to achieve pre-symptomatic detection focus on sampling the plant or ACP. Early detection tests can be

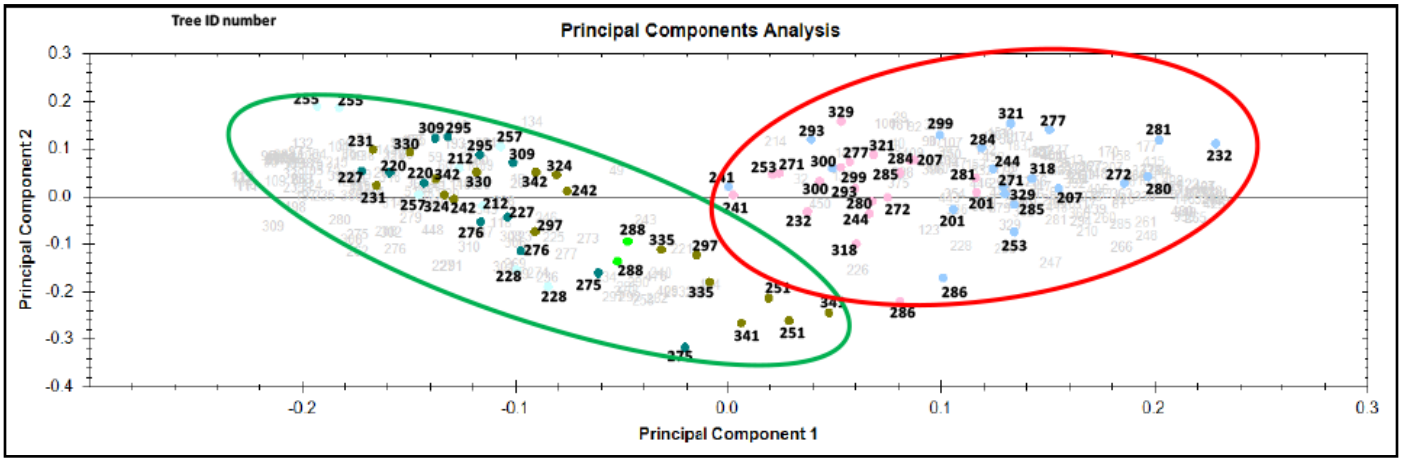

Figure 1. Principal Components Analysis (PCA) of citrus protein data from field-collected, healthy and '*Candidatus *Liberibacter asiaticus' (CLas)-positive citrus. The PCA plot shows leaf sample numbers and protein numbers. The major source of variation in the experiment can be attributed to the tree's HLB status, either healthy (encircled in green) or infected (encircled in red) and visualized along the first principal component (PCA 1), in this case, the horizontal axis. The protein ID numbers at either end of the PCA plot are most informative for distinguishing CLas positive and negative samples from this experiment. These protein biomarkers must be validated in other groves. Among the CLas-infected samples encircled in red, the blue samples were symptomatic and the pink samples were asymptomatic. Samples were provided to us for analysis by Carolyn Slupsky, Ph.D., at the University of California, Davis. Future work will focus on developing these protein biomarkers into antibody-based tests that growers can use in the field for indirect detection of CLas in their citrus trees.

direct, which measures specific CLas genes, or indirect, which relies on measuring the immune response from the tree or insect infected with the HLB pathogen. Our goal is to ensure each type of test has an appropriate place in the eradication of HLB from California.

Our grant has produced notable achievements to advance early detection research. We performed a longitudinal study to identify HLB biomarkers in different citrus varieties in collaboration with Carolyn Slupsky, Ph.D., Kris Godfrey, Ph.D., and the staff at the University of California, Davis Contained Research Facility. Publications on that research are forthcoming. The results from these studies show that the effects of CLas infection in different citrus varieties target several core metabolic pathways of the tree and that the timing of these effects is citrus species-specific. Our team also showed that a method to measure specific, citrus leaf proteins led to the ability to distinguish between healthy and CLas-infected samples from the field. A principal component analysis, which is a method to measure sources of variation in an experiment, shows that CLas infection status is the major source of variation in the type and amounts of proteins from these field-collected leaf samples. These proteins are sufficient to delineate healthy samples from CLas-positive tree samples (**Figure 1**). As a part of this research, we deposited improved genomic resources for citrus on *http://www.citrusgreening.org/*.

In future research, these systemic protein variants can form the basis of a protein-based indirect detection method for the early indication of CLas-infected trees. My team of scientists is partnering with a commercial entity to develop antibody-based tools for indirect detection of HLB and will test their usefulness in other groves and citrus varieties. As part of a comprehensive strategy for HLB eradication, I hypothesize that protein-based methods could be used alongside other detection methods to determine the infection status of trees.

From the ACP side, new research from our program and others supports the idea that psyllid nymphs remain the sentinels for HLB (Lee et al. 2015; Ammar et al. 2016; Ramsey et al. 2017; Mann et al. 2018), and future research should focus on ways to accurately sample ACP nymphs for CLas. CLas has very different effects on the nymphs as compared to the adults, and more research is needed to understand the interaction between CLas and ACP nymphs (Ramsey et al. 2017; Mann et al. 2018). The proteomic work done during our project suggests that the nymphs have an attenuated immune response to CLas even though CLas titers can be measured and quantified in nymphs at the 4th and 5th instar stage. ACP nymphs do not have wings and cannot fly. Thus, if CLas-positive nymphs are found on a tree, the bacteria must have been acquired from that specific tree. Screening for nymphs may catch a new CLas infection before the pathogen moves systemically throughout the tree. A strategy focused on nymph screening is of pressing importance for California, considering new research that shows ACP can acquire and transmit CLas directly from the flush within a single generation, prompting rapid spread of CLas during the asymptomatic stage (Lee et al. 2015). Ongoing proteomics research in the Heck lab on the molecular interactions

between CLas and ACP nymphs may lead to protocols that are faster and cheaper than those currently being used for HLB detection, as well as novel CLas transmission control strategies.

CRB Research Project #5300-155

References

Ammar, E.-D.; Ramos, J.E.; Hall, D.G.; Dawson, W.O.; Shatters, Jr., R.G. 2016. Acquisition, Replication and Inoculation of *'Candidatus* Liberibacter asiaticus' following various acquisition periods on huanglongbing-infected citrus by nymphs and adults of the Asian citrus psyllid. *PLoS One* 11(7): e0159594.

Lee, J.A.; Halbert, S.E.; Dawson, W.O.; Robertson, C.J.; Keesling; J.E.; Singer, B.H. 2015. Asymptomatic spread of huanglongbing and implications for disease control. *Proceedings of the National Academy of Sciences U S A* 112(24): 7605-7610.

Mann, M.; Fattah-Hosseini, S.; Ammar, E.D.; Stange, R.; Warrick, E.; Sturgeon, K.; Shatters, R.; Heck, M. 2018. *Diaphorina citri* nymphs are resistant to morphological changes induced by *'Candidatus* Liberibacter asiaticus' in midgut epithelial cells." *Infection and Immunity* 86(4):e00889-17

Ramsey, J.S.; Chavez, J.D.; Johnson, R.; Hosseinzadeh, S.; Mahoney, J.E.; Mohr, J.P.; Robison, F.; Zhong, X.; Hall, D.G.; MacCoss, M.; Bruce, J.; Cilia, M. 2017. Protein interaction networks at the host-microbe interface in *Diaphorina citri*, the insect vector of the citrus greening pathogen. *Royal Society Open Science* 4(2): 160545.

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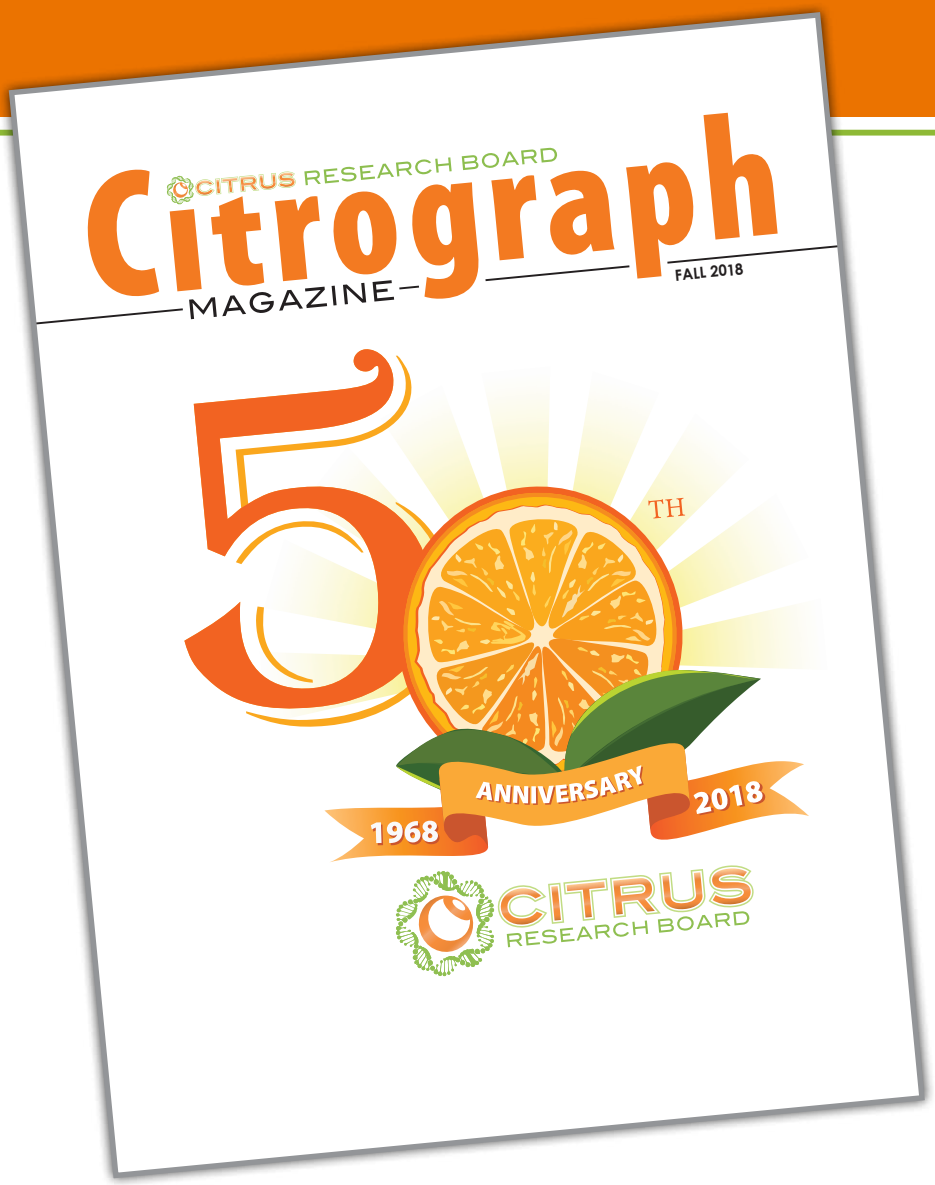

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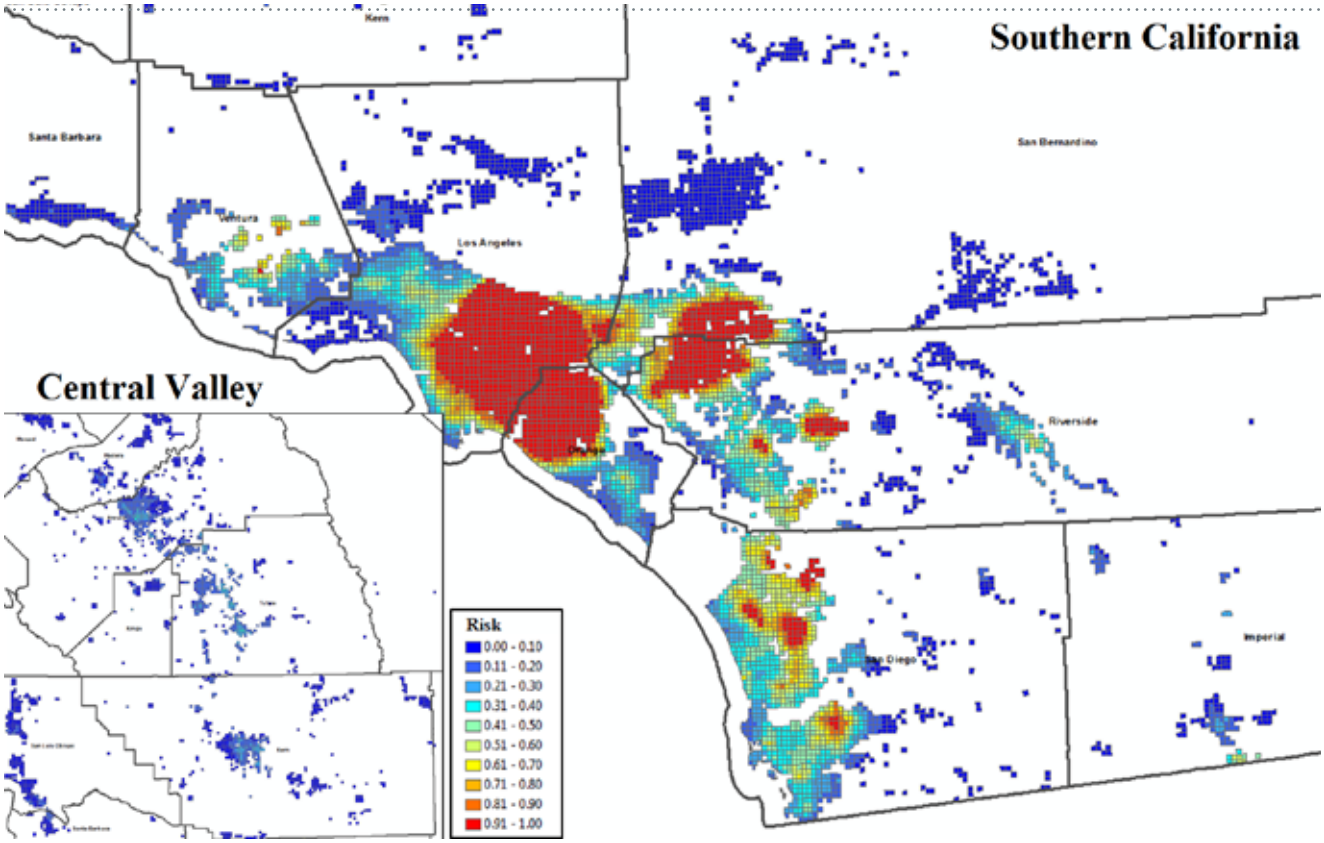

Figure 1. 2018 Risk-based residential survey map for southern and central California. Risk is calculated on a 0 to 1.0 scale for each one-square-mile area as denoted by the imbedded risk key. Risk has changed dynamically over time with ACP spread and HLB-positive tree finds.

Risk-based HLB Surveys for California

Optimizing delimiting survey distance

Tim Gottwald, Weiqi Luo and Neil McRoberts

Project Summary

Evidence from citrus-producing areas worldwide clearly demonstrates that huanglongbing (HLB) is not a disease that can be managed effectively by individual growers. Rather, it is a disease that must be managed simultaneously by regulatory agencies and the whole production industry. The importance of an effective early HLB detection system for California is increased by the existence of the large and diverse commercial citrus industry, whose landscape is intermixed with an extensive residential citrus population. Since 2013, we have developed and continuously refined a risk-based survey that addresses the urgent need to respond to the HLB threat. Rather than waiting for the disease to appear locally, the survey anticipates pathogen introduction and spread statewide. The risk-based survey captures the current HLB situation, identifies high-risk areas, predicts where the disease will occur and directs surveyors to these areas to more efficiently search for the disease. Such early searches aid disease mitigation and management when the disease is at low incidence, thereby suppressing a severe impending epidemic.

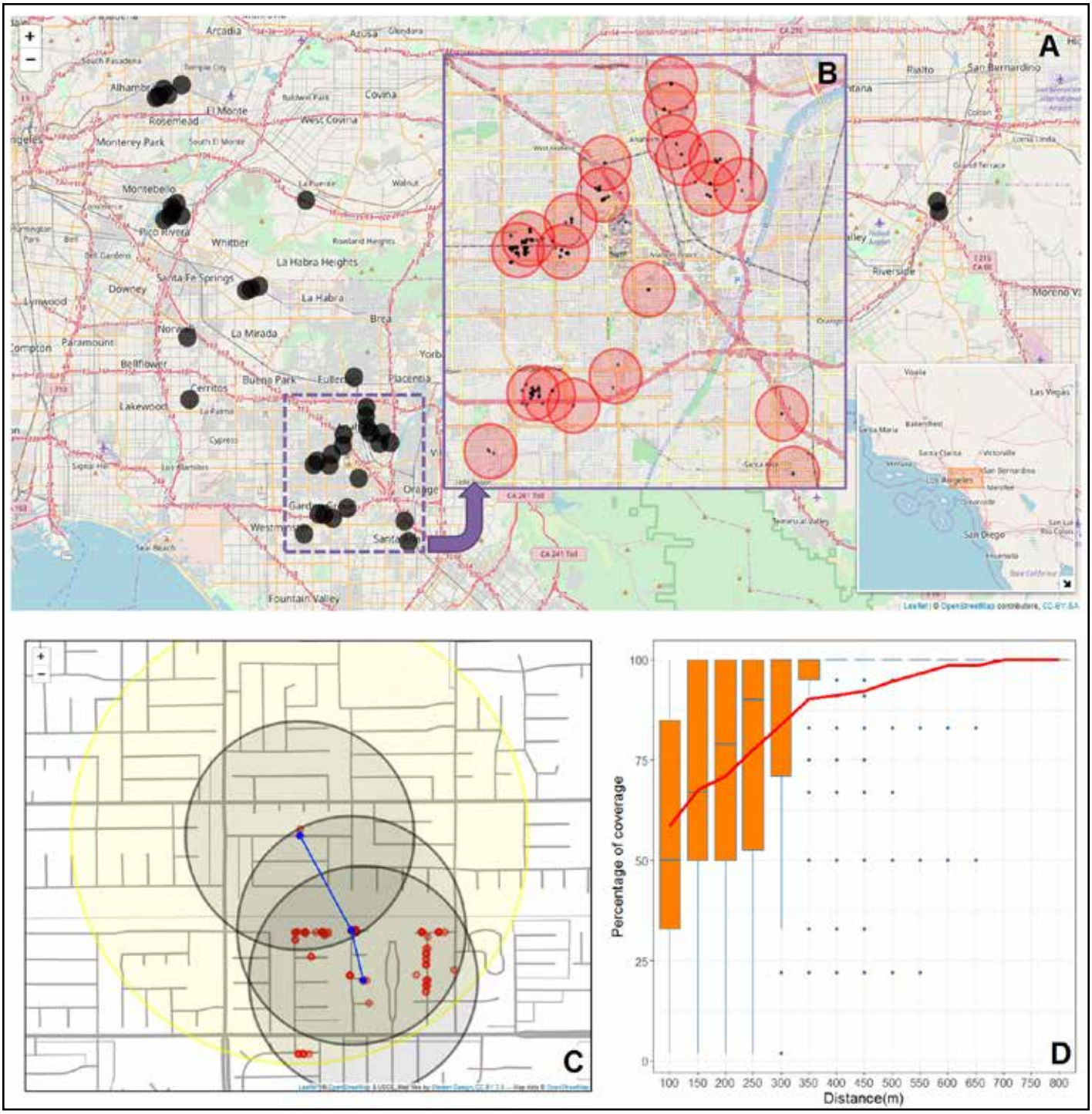

Figure 2. (A) Space-time cluster analysis identified 41 huanglongbing (HLB)-infected clusters in residential southern California when using an 800-meter delimitation distance. (B) High resolution inset image of HLB clusters in Orange County. (C) Reconstructed timeline of CLas-positive tree detections in one infection cluster in Anaheim, California. The yellow circle identifies the original 800-meter delimitation survey; the black circles demonstrate a 400-meter delimitation survey, starting with the initial find and subsequent resurvey finds, capturing all confirmed CLas-positive trees. (D) Summary results of simulation experiments showing the cumulative percentage of HLB detections by delimitation surveys of different radii utilizing all known HLB detection data from California from 2012 through December 2017.

Risk-based Survey Map 2018 Updates

As of November 2018, the risk-based survey implemented by the California Department of Food and Agriculture (CDFA) played an important role in the discovery of nearly 1,000 HLB-affected trees from residential areas of the Los Angeles basin in southern California. In 2017-18, the disease occurred in urban neighborhoods and spread into Orange and Riverside counties with 10-30 new weekly confirmations. Concurrently, the number of Asian citrus psyllids (ACPs) found to be positive for *'Candidatus* Liberibacter asiaticus' (CLas) increased substantially with a strong spatial correlation

with CLas-positive tree finds, indicating the potential identification of new HLB clusters. We continuously refine the survey model of HLB/ACP for urban and commercial citrus. Also, we have extended the model to integrate the potential use of emerging early detection technologies (EDTs). This will allow us to predict (and regulatory agencies to combat) HLB outbreaks and detect the subclinical infections most likely to lead to new HLB outbreaks, allowing the industry to optimize resources.

The updated 2018 final risk map (**Figure 1**) and current survey protocols were delivered to CDFA in March 2018. We continuously refine the survey model of HLB/ACP for urban and commercial citrus to optimize risk predictions of disease spread. Numerous factors change over time as the epidemic evolves and are revisited frequently to ensure an up-to-date representation of the California HLB/ACP situation. In collaboration with the CDFA, we have explored different survey strategies to keep an appropriate balance between the CDFA survey, total lab capacity for CLas diagnostic testing and increasing emphasis on proximity to commercial citrus.

Optimizing Delimiting Survey Distance

Following CLas-positive tree discoveries, it is necessary to design a high intensity, targeted survey, usually referred to as a delimitation survey, to maximize detection of additional CLas-positive trees in new outbreaks to prevent further spread. In association with the risk map, we have investigated more cost-effective approaches for these high-intensity survey efforts for improved resource allocation and early detection optimization through two simultaneous approaches.

First, we performed "space-time cluster" analyses of HLB outbreaks to optimize the delimiting survey distance (**Figure 2**). Initial analyses indicated that the 800-meter survey distance can be significantly reduced to 400 meters while still capturing more than 90 percent of new infections emanating from centers of existing inoculum sources. Such a reduction would free significant resources to be used elsewhere in the program. Additionally, the risk-based model assigns higher risk to new outbreaks in surrounding areas. Thus, any infections beyond the lesser delimiting distance, i.e., 400 meters, still should be identified by the intensified risk-based survey in the area, further compensating for the reduced delimiting survey.

Second, we integrated an epidemiological model using data from prior survey results (i.e. CLas-positive confirmations, inconclusive and negative results) to anticipate potential subsequent CLas spread. This approach will further assist in prioritizing survey deployment and sampling resources for delimiting surveys and provide another risk layer for the risk-based survey design (i.e., future risk outlook for allocating resources).

Subsequently, the CDFA and California Pest and Disease Prevention Committee reviewed our analyses and adopted the reduced HLB delimiting survey distance and quarantine treatment area to 400 meters from a CLas source tree.

CRB Research Project # 5300-154

Acknowledgements

This research is funded by grants from the Citrus Research Board. We also thank the diligent efforts of CDFA inspectors, administrators and laboratory personnel for data sharing on the risk-based and targeted surveys, plus USDA and APHIS colleagues for their continuing support of this project and efforts.

References

Gottwald, T.R.; Luo, W.; McRoberts, N. (2017) Risk-based HLB surveys: for Southern California, statewide and targeted. *Citrograph*, 9 (2):38-43.

Gottwald, T.R.; Luo, W.; McRoberts, N. (2014) Risk-Based Residential HLB/ACP Survey for California, Texas and Arizona. *Citrograph*, 5 (2): 52-59.

Gottwald, T.R.; Luo, W.; McRoberts, N. (2013) Risk-Based Residential HLB/ACP Survey for California, Texas and Arizona. Plant Management Network (Webcast). http://*www.plantmanagementnetwork.org/edcenter/seminars/Outreach/Citrus/HLB/*

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UNTIL THEY FIND A CURE... WE CAN PROVIDE A CONTROL

BEFORE – OCT 2006

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While we completely support the future possibilities of research, ***UNTIL THEY FIND A CURE... WE CAN PROVIDE A CONTROL.***

To find out more about K-PHITE 7LP and K-PHITE 7LP Programs, contact your California Plant Food Systems Representative directly: Mark Brady – Western Marketing Manager 559 731-1267.

Developing Improved Gene-editing Technologies for Citrus

Yannick Jacob, Vivian Irish, Fei Zhang, Chantal LeBlanc, Yewei Wang, Alan May and Josefina Mendez

Project Summary

CRISPR[1]/Cas9[2] editing is a powerful and versatile approach that is revolutionizing the agricultural industry due to its ability to precisely and rapidly create new genetic variants. We have adapted this technology for use in citrus, by optimizing the vectors and transformation protocols required for Cas9 and guide RNA (gRNA) expression in citrus, as well as improving the frequency of CRISPR/Cas9-induced mutant generation by subjecting plants to a series of heat stresses. We have shown that our optimized CRISPR/Cas9 system is highly efficient in generating targeted mutations in the LCY1 *and* LCY2 *genes of Carrizo Citrange and Cara Cara orange, which results in increased levels of lycopene production in plants. In addition, we started exploring ways to use CRISPR/Cas9 to create edited, but non-GMO citrus plants. These achievements provide the citrus industry with a key molecular tool for rapid precision breeding, which can be used to combat diseases like huanglongbing (HLB) and also to improve fruit quality.*

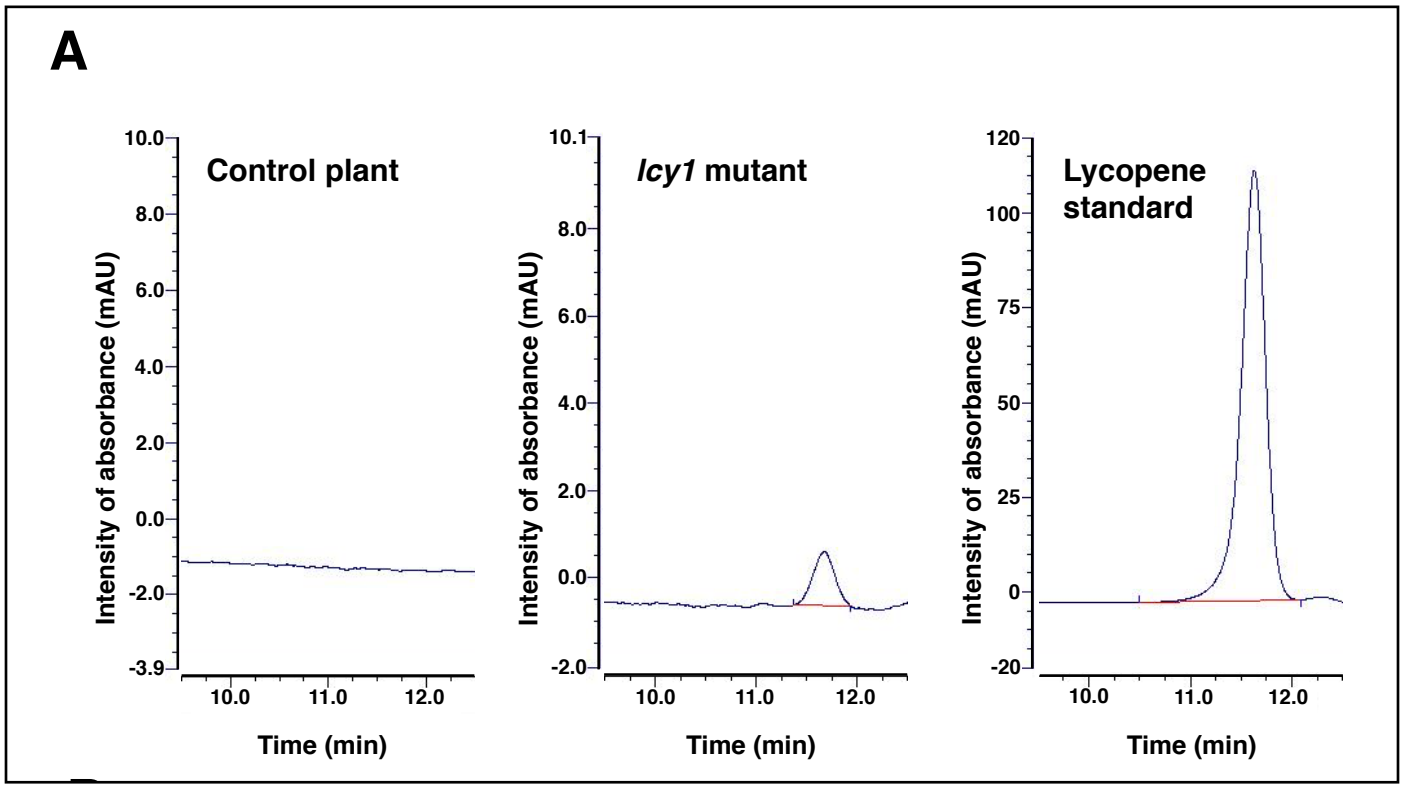
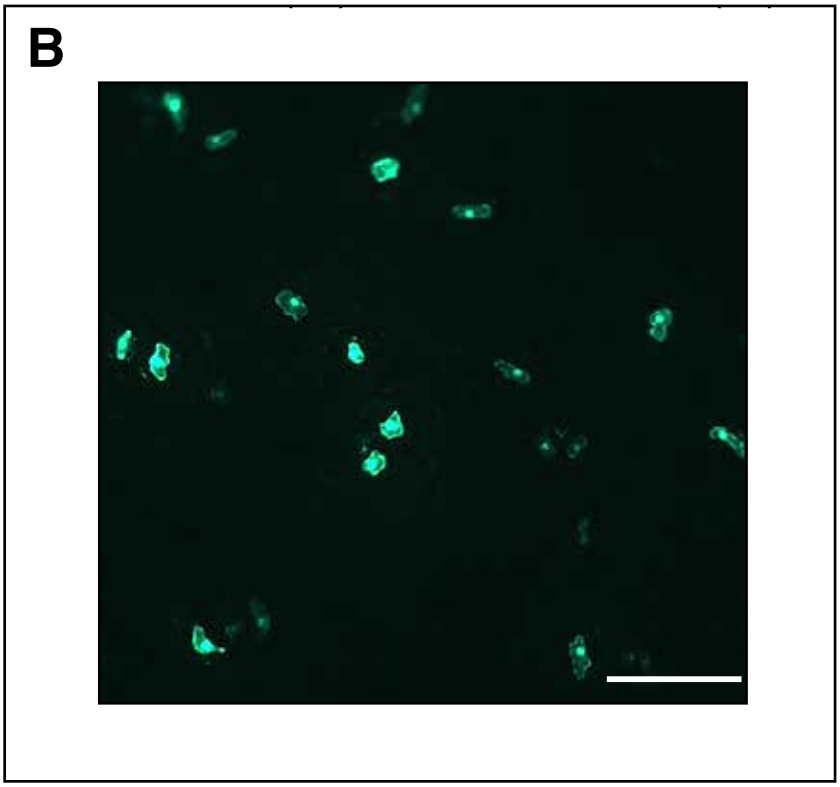


Figure 1. (A) Lycopene levels in citrus extracts from a control plant, a lcy1 *mutant and a lycopene standard determined by high-performance liquid chromatography. The* lcy1 *mutant shows an increased level of lycopene compared to that in the control plant. (B) Fluorescence microscopy of a leaf from 'Pineapple' sweet orange after biolistic particle bombardment using metal beads coated with a DNA plasmid coding for a green fluorescent protein. Bar = 50µm (approximately 0.002 inches).*



CRISPR/Cas9 Technology Development for Citrus Gene Editing

The CRISPR/Cas9 gene-editing technology allows researchers to make precise and specific modifications in the genomes of plant and animal cells (Jinek et al. 2012). CRISPR/Cas9 acts like molecular scissors that can cut DNA at a defined location in the genome, which usually results in mutations at that site. This powerful gene-editing system has two components: gRNA and the Cas9 enzyme responsible for cutting DNA. Cas9 and the gRNA interact in cells to form a molecular complex that recognizes and cleaves a target DNA sequence that is complementary to the gRNA sequence. Because the gRNA sequence can be precisely designed by researchers to be complementary to most sites in the genome, Cas9 can be targeted to any gene of interest to initiate a molecular cut that will lead to a mutation in that gene.

In general, CRISPR/Cas9 is a fairly efficient, versatile and precise gene-editing system, which accounts for its rising popularity as a molecular tool for basic research in plants and animals. However, CRISPR/Cas9 gene editing is not equally effective in all organisms, and initial results from other researchers showed that this molecular tool was not very efficient for editing genes in citrus (Jia and Wang 2014). Therefore, our research group at Yale University, with support from the Citrus Research Board (CRB), initiated work on adapting and optimizing this new gene-editing system for use in citrus. This was an important goal to achieve, as an effective CRISPR/Cas9 system in citrus would allow researchers to easily create new cultivars with value-added traits of interest to citrus growers. We initially optimized binary vectors[3] and transformation protocols for inducing high levels of expression of Cas9 and gRNAs in citrus (Zhang et al. 2017). In addition, we discovered that exposing citrus plants containing Cas9 and gRNAs to heat stress was extremely effective in increasing the recovery of mutations at targeted genes (LeBlanc et al. 2018). The optimized CRISPR/Cas9 system and protocols that we designed are making it possible to rapidly generate citrus plants that are completely edited at targeted genes.

Application of CRISPR/Cas9 to Create New Cultivars with Higher Lycopene Levels

The next phase of the research project focused on demonstrating the utility of the optimized CRISPR/Cas9 system to edit citrus genes affecting a trait of interest to growers. The genes *LCY1* and *LCY2* encode lycopene β-cyclase enzymes, which regulate levels of the antioxidant lycopene responsible for the red color of many fruits and vegetables. In citrus, the LCY1 is active in most tissues, while LCY2 is specifically produced in fruit. Naturally occurring mutations in the *LCY2* gene are associated with loss of enzymatic activity and an increase in lycopene content in 'Star Ruby' red grapefruit (Alquézar et al. 2009). We used our optimized CRISPR/Cas9 system to edit *LCY1* and *LCY2* in Carrizo citrange and Cara Cara orange, and recovered gene-edited citrus plants lacking functional lycopene β-cyclase enzymes. High-performance liquid chromatography[4] (HPLC) was used to measure the levels of lycopene in citrus plants edited at the *LCY1* gene, and we observed increased lycopene levels in the CRISPR/Cas9-edited *lcy1* mutant plants compared to wild-type plants (**Figure 1A**). We currently are growing plants with the gene-edited forms of the *lcy2* gene; when these plants flower, we will test fruit-specific levels of lycopene in them. Our results with *LCY1* demonstrate that CRISPR/Cas9 can be used effectively in citrus to rapidly create new genetic variants with value-added traits.

Optimization of the Technology to Produce Non-GMO Gene-edited Citrus Cultivars

An additional goal of this project was to start exploring how CRISPR/Cas9 can be used in citrus without relying on genomic integration of the Cas9 and gRNA genes. Non-integrative and transient means of introducing Cas9 and gRNAs in citrus would allow for the production of non-GMO gene-edited citrus plants, as defined by the U.S. Department of Agriculture. We initiated this work by testing the efficacy of biolistic particle bombardment[5] (BPB) in delivering Cas9 and gRNA into citrus cells. Our preliminary results show that DNA can be transiently introduced in citrus leaf cells using BPB (**Figure 1B**). However, it remains to be determined if CRISPR/Cas9 edits can be recovered when DNA coding for Cas9 and a gRNA are introduced in citrus cells with BPB. In addition, we also have used BPB to introduce the CRISPR/Cas9 machinery into callus cells. Callus cells may be better suited for these experiments as callus cells are highly competent for regeneration, an important step in developing gene-edited cultivars using this approach (Vardi et al. 1982). To date, our results indicate a possible path for achieving the goal of using non-integrative CRISPR/Cas9 in citrus to produce edited plants that are not considered GMOs.

Future Directions

More work will be needed to establish a transient CRISPR/Cas9 system in citrus to create non-GMO edited plants, but our results over the last year support the feasibility of this goal. To achieve this, it will become important for citrus researchers to understand the genetic and epigenetic[7] mechanisms regulating citrus regeneration from different commercial cultivars. An additional future direction resulting from this work is to combine strategies to accelerate flowering in citrus with gene-editing approaches, so that fruit-specific enhancements using CRISPR/Cas9 can be studied more rapidly.

CRB Research Project #5200-151

References

Alquézar, B.; Zacarias, L.; Rodrigo, M.J. 2009. Molecular and functional characterization of a novel chromoplast-specific lycopene beta-cyclase from *Citrus* and its relation to lycopene accumulation. *Journal of Experimental Botany* 60(6):1783-1797.

Jia, H. and Wang, N. 2014. Targeted genome editing of sweet orange using Cas9/sgRNA. *PLoS One* 9(4):e93806.

Jinek, M., et al. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337(6096):816-821.

LeBlanc, C., et al. 2018. Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants using heat stress. *The Plant Journal* 93(2):377-386.

Vardi, A.; Spiegel-Roy, P.; Galun, E. 1982. Plant regeneration from Citrus protoplasts: Variability in methodological requirements among cultivars and species. *Theoretical and Applied Genetics* 62(2):171-176.

Zhang, F.; LeBlanc, C.; Irish, V.F.; Jacob, Y. 2017. Rapid and efficient CRISPR/Cas9 gene editing in *Citrus* using the YAO promoter. *Plant Cell Reports* 36(12):1883-1887.

Glossary

[1]CRISPR: An acronym for Clustered Regularly Interspaced Short Palindromic Repeats, referring to a series of short repeated DNA sequences, interspersed with short target DNA sequences, that together are transcribed into a guide RNA (gRNA). It is part of a molecular complex, with Cas9 (below), used for precise gene editing.

[2]Cas9: An enzyme found in many bacteria that specifically cleaves DNA complementary to the guide RNA.

[3]Binary vector: A two-component DNA vector system used to transform plant cells. The first vector has the genes of interest and the second is a helper plasmid to improve the insertion of genes into the plant genome.

[4]High-performance liquid chromatography (HPLC): Analytical technique in which components of a liquid mixture are separated by passage through a column filled with an adsorbant. Each component passes through at a different rate, allowing for the amounts of a substance in a complex mixture to be identified.

[5]Biolistic particle bombardment: Technique used to deliver DNA, RNA and/or proteins to different types of cells, including plant cells. A particle of a heavy metal coated with a biological molecule is projected under pressure directly into cells, where the biological molecule is ultimately released.

[6]Callus cells: Unorganized cells that have the ability to rapidly grow and potentially regenerate a new plant.

[7]Epigenetic: Heritable changes in gene expression that do not result from changes to the DNA.

Yannick Jacob, Ph.D., and Vivian Irish, Ph.D., are professors, Fei Zhang, Ph.D., is a post-doctoral associate, Chantal LeBlanc and Yewei Wang are research associates and Alan May and Josefina Mendez are undergraduate students, all in the Department of Molecular, Cellular and Developmental Biology at Yale University. For additional information please contact yannick.jacob@yale.edu or vivian.irish@yale.edu.

In grapefruit, as well as many other fruits, one female Mexican fruit fly can deposit large numbers of eggs – up to 40 eggs at a time, 100 or more a day and about 2,000 throughout her lifespan. **Photo Credit: Jack Dykinga, USDA Agricultural Research Service, Bugwood.org**

Recent Advances Toward a Mexican Fruit Fly Lure

Spencer Walse and Dan Kuzmich

Project Summary

The male-produced sex and aggregation pheromones of Mexican, Caribbean and South American fruit flies have been known for nearly 40 years. Recent Citrus Research Board-funded research has led to a major breakthrough – an efficient synthetic process to yield the quantities required for commercial-scale testing and ultimately for area-wide trapping systems. Field studies currently are underway across the Americas, from remote jungles in Surinam to commercial orchards in Guatemala, Florida, Texas and California. Key local, state, federal and international regulatory agencies expect to boost quarantine programs historically void of effective traps and feasible post-harvest treatments for these species.

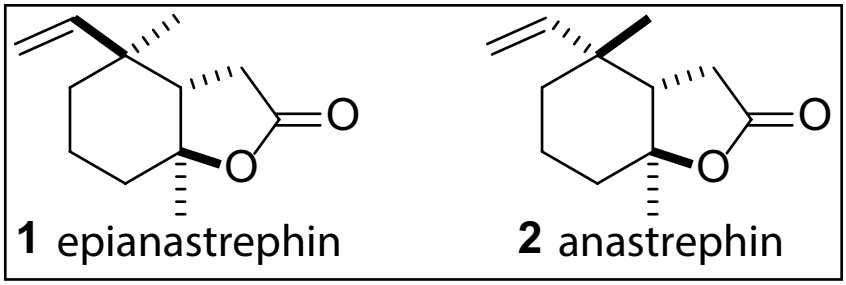


Figure 1. Pheromones epianastrephin (1) and anastrephin (2) are naturally emitted by Mexican fruit fly males to attract females. A new synthetic route affords the opportunity to start exploring the potential for control in commercial orchards, as well as in remote areas where populations persist.

In most agriculturally important regions of the United States, outbreaks of Mexican (*Anastrepha ludens*) and Caribbean (*Anastrepha suspensa*) fruit flies are regulated with quarantines to minimize potential damage of commercially valuable host fruit – none more important than citrus. Most in the California and Florida citrus industries are familiar with the extensive (and expensive) Sterile Insect Technique (SIT)[1] that the U.S. Department of Agriculture-Animal Plant and Health Inspection Service (USDA-APHIS) employs to suppress populations of Mexican fruit flies. In Mexico and countries to its south, the South American fruit fly (*Anastrepha fraterculus*) is an issue, hopefully one that the California citrus industry never will have to combat.

Improved field-level control of these *Anastrepha* pests has been studied for a very long time, and an increasing number of scientists have worked on this for many years. Currently, trapping and monitoring devices rely on food-based lures, which are non-selective and not nearly effective enough. Moreover, post-harvest treatments – including methyl bromide fumigation – are not particularly effective at controlling the internally feeding larvae of these flies, at least under conditions where the fruit remains marketable.

For 40 years, chemists have been attempting to develop a lure based on the male-produced sex and aggregation pheromones (**Figure 1**) (Lima et al. 2001; Nation 1972 and 1975), with the potency of trimedlure and methyl eugenol for the Mediterranean fruit fly and Oriental fruit fly, respectively (Tan et al. 2014). No less than a dozen synthetic processes to produce epianastrephin and anastrephin have been published, but not a single one satisfied the mass production requirements for formulation studies, field trials and commercialization (Teal and Walse 2012).

Dan Kuzmich, who has a Ph.D. in organic chemistry from Ohio State University, joined the Walse group at the U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS), San Joaquin Valley Agricultural Sciences Center in 2013 with extensive experience in small molecule synthesis, and virtually no idea what a fruit fly was – or where citrus was grown. In five years – the limitation for temporary positions at a USDA-ARS facility - Kuzmich devised a four-step process to yield the pheromones at a kilogram scale, blowing open the doors of opportunity. With ingenuity to overcome some tricky structural conformations, the dedication and genuine interest displayed by Kuzmich was truly commendable, a highly focused effort in problem solving for the citrus industry.

These pheromones are now being produced commercially and deployed in trapping studies supervised by USDA-ARS-APHIS, as well as the Food and Agricultural Organization of the United Nations. The pheromones may work best as lures in attract-and-kill traps, or potentially mating disruption programs, or even as hormone supplements to increase the mating viability of the irradiated SIT flies. We will find out in the coming seasons, hopefully adding to the chest with tools to eliminate these pests from citrus production areas.

CRB Research Project #5500-209

References

Lima, I.S.; House, P. E.; do Nascimento, R. R. 2001. Volatile substances from male *Anastrepha fraterculus* Wied. (Diptera: Tephritidae): Identification and behavioural activity. *Journal of the Brazilian Chemical Society.* 12(2):196-201.

Nation, J.L. 1972. Courtship behavior and evidence for a sex attractant in the male Caribbean fruit fly, *Anastrepha suspensa*. *Annals of the Entomological Society of America*. 65(6):1364-1367.

The male Mexican fruit fly produces large quantities of pheromones in the clear abdominal glands, using his wings to both fan the scent and call females.

Mexican Fruit Fly. **Photo Credit: Susan Ellis, USDA APHIS PPQ, Bugwood.org**

Nation, J.L. 1975. The sex pheromone blend of Caribbean fruit fly males: isolation biological activity, and partial chemical characterization. Environmental Entomology. 4(1):27-30.

Tan, K. H.; Nishida, R.; Jang, E. B.; Shelly, T. E. 2014. Pheromones, male lures, and trapping of tephritid fruit flies. p. 15-74. In: Shelly, T. E.; Epsky, N. D.; Jang, E. B.; Reyes-Flores, J.; Vargas, R. I. (Eds.), *Trapping and the detection, control, and regulation of tephritid fruit flies.* Springer, Dordrecht, Netherlands.

Teal, P. E. A. and Walse, S. S. 2012. *U.S. Patent No. 8,128,948*. Washington, DC: U.S. Patent and Trademark Office.

Glossary

[1]Sterile Insect Technique: An approach to suppress insect pest populations where male insects are irradiated at levels that cause sterility, then released into the field to limit successful reproduction of female insects present in the environment.

Spencer Walse, Ph.D., is a research chemist at the United States Department of Agriculture-Agricultural Research Service, San Joaquin Valley Agricultural Sciences Center, as well as an adjunct professor in the Environmental Toxicology Department at the University of California, Davis. Dan Kuzmich, Ph.D., is a synthetic organic chemist, who recently started a consulting company in the Fresno area. For additional information, please contact **spencer.walse@ars.usda.gov.**

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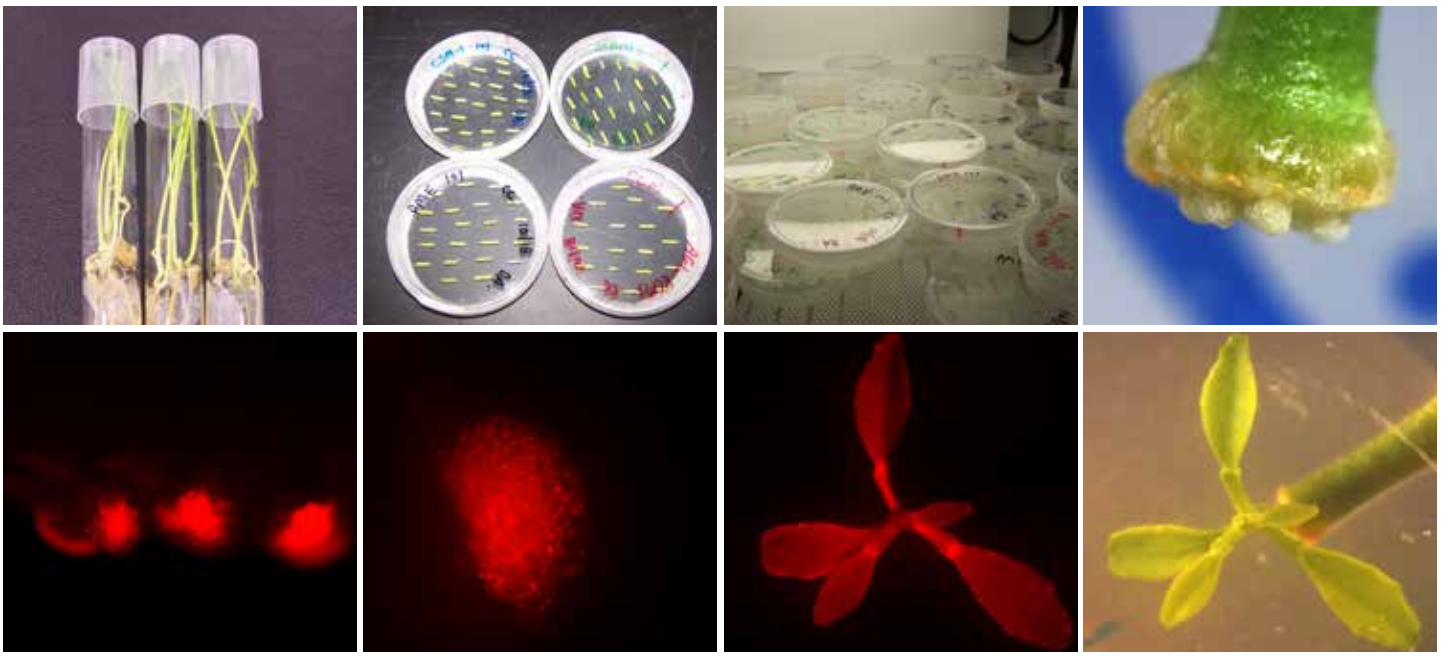

Figure 1. Demonstrates the transformation process where disease resistance genes are added to the citrus (Carrizo) genome.

Development of Mature Citrus Tissue Transformation Technology

Min Shao, Diaa Alabed and James G. Thomson

Plant tissue transformation is a process that allows propagation and modification of plant DNA to expand material or produce something unique. This can be relatively simple if clonal (identical to mother plant) seeds are available. However, this is not always the case, nor do many desirable elite cultivars even produce seeds. This project seeks ways to enhance stem (budwood) tissue transformation to provide options for modifying cultivars with desirable traits such as pest and disease resistance. Huanglongbing (HLB), which has severely impacted citrus production around the world, including Florida, is the first disease that comes to mind these days, and it is a major concern of the California citrus industry. Because of this, we are developing methods to improve citrus transformation and plan to add known disease-resistance genes to citrus genomes for potential resistance.

These potential resistance genes were previously discovered by other scientists (Belknap et al. 2015; Crouzet et al. 2006; Dong et al. 1999; Dutt et al. 2015; Hao et al. 2016; Wang et al. 2013; Zhang et al. 2014) and shown to be useful against specific diseases – but not necessarily HLB (**Table 1**). Since there is no known resistance to HLB in commercial citrus varieties, we decided to make and evaluate these unique genes in citrus for imparting resistance to HLB. This has been done by adding one, two and even three different resistance genes together in hopes of finding additive or possibly a synergistic combination that will

Table 1. Disease-resistance genes introduced into Carrizo and Mexican Lime.

Gene name	Gene Function	Plant Source	HLB tolerance demonstrated?	Reference
CaPME-pectin methylesterase	Cell wall stiffening	Pepper	Not determined	Wang et al. 2013
AtNPR1-Arabidopsis Nonexpressor of PR Genes 1	Regulator of (SA)-acquired resistance (SAR)	*Arabidopsis*	Yes	Dutt et al. 2015
CsCSM-1-calcineurin B-like protein	Novel gene. Calcium response to disease	Citrus	Yes	Unpublished
CsSCAMPPS-Small Cyclic AMPhipathic PeptideS	Novel gene. Ionophore function	Citrus	Not determined	Belknap et al. 2015
AtPDR1.2-pleiotropic drug resistance (PDR)	Plant defensin	*Arabidopsis*	Not determined	Crouzet et al. 2006
Harpin	Elicitor of hyper sensitive response	Rice	Not determined	Dong et al. 1999
mThionin	Antimicrobial proteins plant defensin	Tobacco	Yes	Hao et al. 2016
Sn1	Novel gene. Antimicrobial proteins plant defensin	Potato	Not determined	Unpublished
Sn2	Novel gene. Antimicrobial proteins plant defensin	Potato	Not determined	Unpublished
CapA1	Novel gene. Antimicrobial proteins plant defensin	Pepper	Not determined	Unpublished
CapA2	Novel gene. Antimicrobial proteins plant defensin	Pepper	Not determined	Unpublished
CapG1	Novel gene. Antimicrobial proteins plant defensin	Pepper	Not determined	Unpublished
CitGrp1	Novel gene. Antimicrobial proteins plant defensin	Citrus	Not determined	Unpublished
CitGrp2	Novel gene. Antimicrobial proteins plant defensin	Citrus	Not determined	Unpublished

prove helpful against HLB. More than 100 unique Carrizo and Mexican lime citrus lines have been produced from 30 different disease-resistance gene combinations. Plants produced using these disease-resistance genes have been shipped to the University of California, Davis (UC Davis) Contained Research Facility for Asian citrus psyllid (ACP) feeding and *'Candidatus* Liberibacter asiaticus' transmission studies (**Figure 1**).

Progress toward citrus transformation has been made as new artificial growth media have been developed for *in vitro* shoot regeneration from stem tissue. The number and type of hormones that stimulate tissue growth have been examined, and four different mixtures have been found that give enhanced rates of growth. Growth medium 'DS3' appears to give the best results to date. Out of the 31 lines tested, 84 percent responded with increased tissue in culture to the hormone treatment, and 58 percent grew best on the DS3 growth media compared to the other tested media.

Other progress has been made in the design of a genetic system that provides extra genes to help boost rates of transformation. Some genes help reduce cell death under tissue culture conditions, resulting in longer life for plant tissue under artificial conditions. Other genes help the tissue regenerate and produce new shoots. Our current effort is to find the correct genetic switch to turn on these regeneration genes just long enough to stimulate growth without overgrowth (**Figure 2**). Transformation efficiency for these lines is being assessed using the regeneration of healthy plants expressing the *DsRed* gene as a measurement of efficiency. (**Figures 1** and **2**). The *DsRed* gene encodes a nontoxic protein that glows red under certain wavelengths of light. This allows quick identification of plants that actually are transgenic vs. those that have "escaped" the selection process and are not transformed.

A final portion of this project has been the design of a "FasTraK" citrus rootstock. This novel rootstock is designed to induce early flowering of grafted scions, whether from juvenile or mature

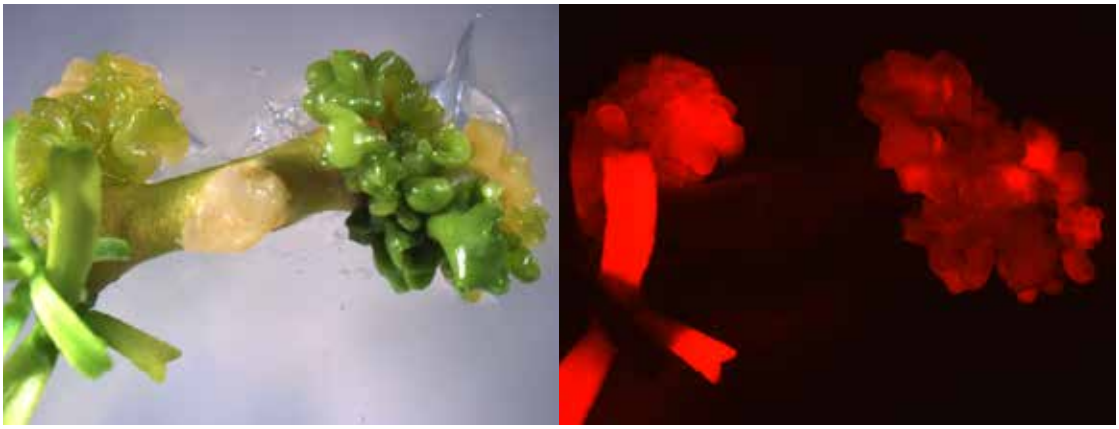

Figure 2. Demonstration of enhancement genes producing an abundance of regenerated tissue.

starting material. The original idea was to help scientists to speed their research for any study that requires juvenility to break. However, the idea also has become popular with nursery operators who see the potential in developing early flowering varieties for commercial sale. The ideal rootstock would provide all the benefits normally seen in a rootstock, plus the added early flowering trait. Our lab has chosen to use US-942 from Kim Bowman's U.S. Department of Agriculture lab in Florida. US-942 appears to have a broad range of soil tolerance, pH and innate disease resistance to *Citrus tristeza virus* (CTV) and *Phytophthora-Diaprepes* weevil complex (PDC), as well as increased fruit yield. As a further benefit to scientists, this cultivar is very easy to transform and root, thus making its genetic manipulation much easier than other existing cultivars (unpublished results). To date, our lab has tried five different combinations of genes for early flowering. We still have another three, which have been suggested by our collaborators in Florida. Currently, we are waiting for the transformed US-942 to get large enough for flowering gene testing.

Conclusion

Methods to improve citrus transformation are being investigated for use on a broad array of citrus cultivars by using stem tissue and an enhanced transformation system. To date, a number of improvements have been made to tissue culture growth media (for overall tissue culture health) and the use of specific genes to stimulate plant growth. During the development of this technology, more than 100 citrus plants containing potential HLB-resistance genes were produced and currently are being tested in the Contained Research Facility at the University of California, Davis. Finally, a number of early flowering rootstock lines have been produced to potentially bypass the long juvenile stage of citrus for both research and commercial production. Results from this study will facilitate future opportunities for citrus biotechnology research and provide molecular tools leading to improved citrus cultivars. The materials generated from this research will provide valuable resources for the citrus community and are available upon request.

CRB Research Project #5200-165

References

Belknap, W.R.; McCue, K.F.; Harden, L.A.; Vensel, W.H.; Bausher, M.G.; Stover, E. 2015. A family of small cyclic amphipathic peptides (SCAmpPs) genes in citrus. *BMC Genomics*. 16(1):303.

Crouzet, J.; Trombik, T.; Fraysse, Å.S.; Boutry, M. 2006. Organization and function of the plant pleiotropic drug resistance ABC transporter family. *FEBS Letters.* 580(4):1123-1130.

Dong, H.; Delaney, T.P.; Bauer, D.W.; Beer, S.V. 1999. Harpin induces disease resistance in Arabidopsis through the systemic acquired resistance pathway mediated by salicylic acid and the NIM1 gene. *The Plant Journal*. 20(2):207-215.

Dutt, M.; Barthe, G.; Irey, M.; Grosser, J. 2015. Transgenic citrus expressing an *Arabidopsis* NPR1 gene exhibit enhanced resistance against Huanglongbing (HLB; Citrus Greening). *PLoS One*. 10(9): p.e0137134.

Hao, G.; Stover, E; Gupta, G. 2016. Overexpression of a modified plant thionin enhances disease resistance to citrus canker and huanglongbing (HLB). *Frontiers in Plant Science*. 7:1078.

Wang, M.; Yuan, D.; Gao, W.; Li, Y.; Tan, J.; Zhang, X. 2013. A comparative genome analysis of PME and PMEI families reveals the evolution of pectin metabolism in plant cell walls. *PLoS One*. 8(8): p.e72082.

Zhang, L.; Du, L.; Poovaiah, B.W. 2014. Calcium signaling and biotic defense responses in plants. *Plant Signaling & Behavior*. 9(11): p.e973818.

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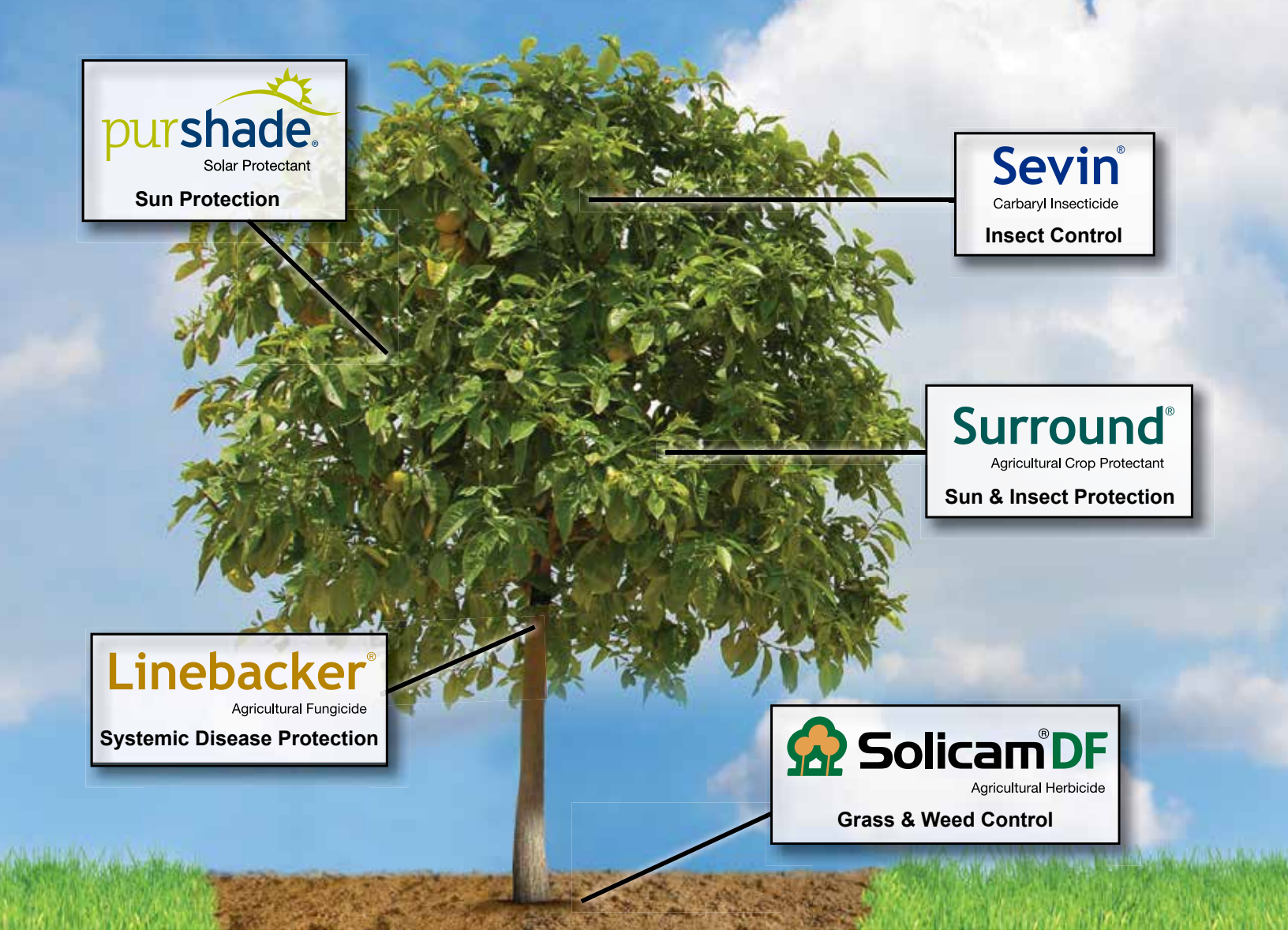

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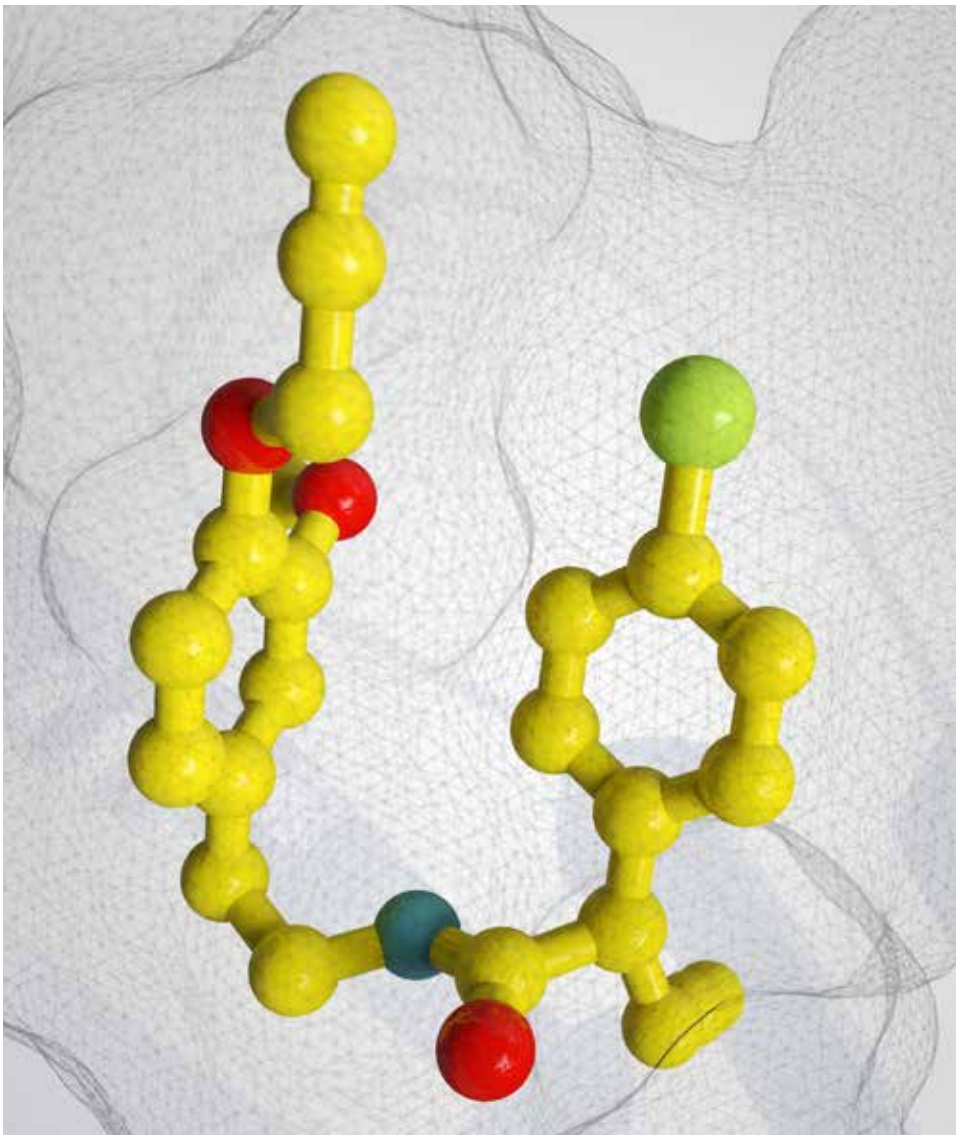

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A Sci-fi Future for Citrus

Flowering at the flick of a switch

Sean Cutler, Mikeal Roose and Sang-Youl Park

Project Summary

Citrus geneticists have brought many exciting new varieties to market over the years, but the biology of citrus limits how quickly geneticists can respond to market needs. Citrus plants grown from seed possess a juvenile period of five or more years before they reach sexual maturity – this adds many years onto each breeding cycle as opposed to an annual crop like maize, which reaches sexual maturity within months and can be improved much more quickly. In this project, we are using state-of-the-art tools and concepts from the growing field of synthetic biology[1] to accelerate citrus breeding. We are engineering "inducible flowering" rootstocks that can be used to trigger flowering in juvenile scions and, ultimately, shave years off of each breeding cycle.

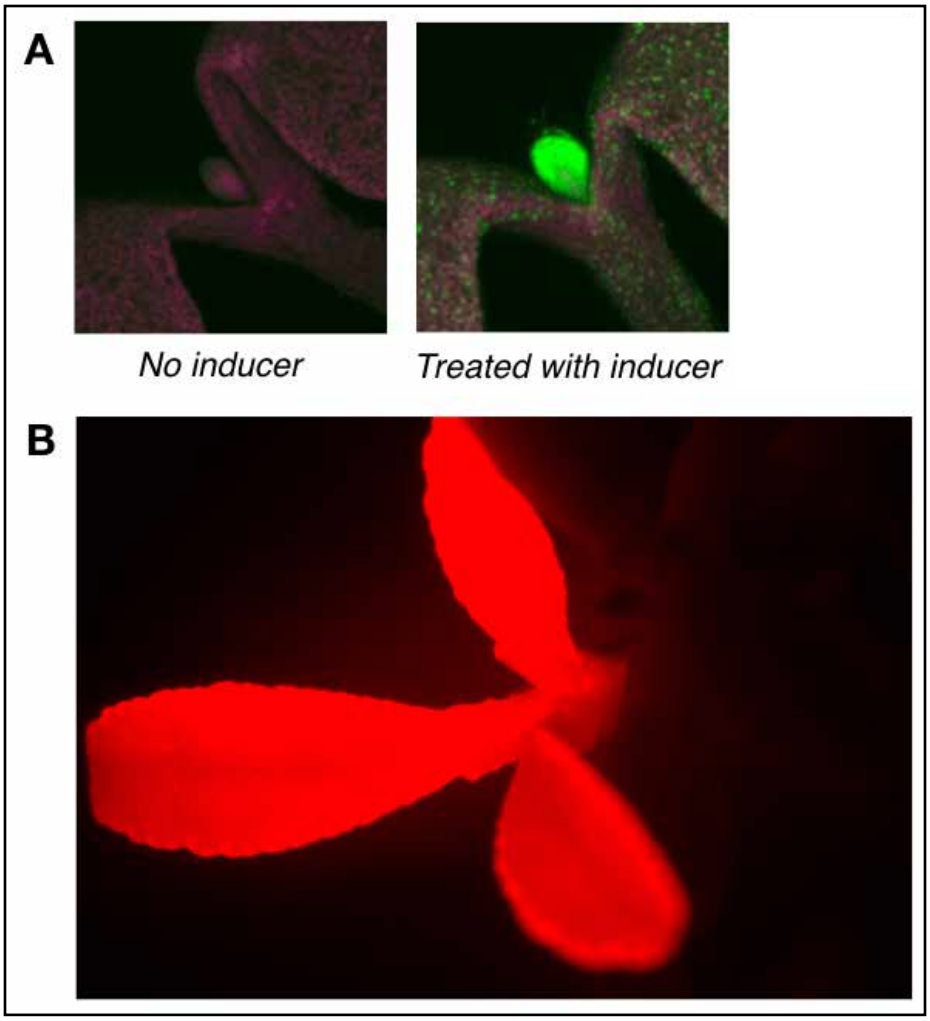

Figure 1. (A) Example of inducible gene expression following agrochemical application. Both panels show transgenic Arabidopsis plants harboring a flowering gene fused to green fluorescent protein (GFP). Gene expression remains limited (left panel) until plants are treated with the inducer Revus™, upon which cells then produce GFP and appear green under fluorescent light (right panel). (B) Transgenic citrus plants harboring an inducible gene construct. The inducible* Flowering Locus T (FT[3]) *construct designed for our experiments has been successfully transformed into Carrizo, as indicated by the expression of a red fluorescent protein (a transformation marker). Quantification of gene expression in this and other transgenic citrus materials are underway.

Our inducible strains are being built using a new genetic "switch" that is controlled by an inexpensive agrochemical called Revus™. This system is designed to allow genes of interest to be turned on at will in a grower's field safely and economically. Our project will yield immediate benefits to citrus geneticists and breeders by shortening the time to flower. On the long-term horizon, we envision using our new genetic switch to design cultivars with traits that can be activated by growers as needed.

Imagine this – it's an usually cold winter in the Central Valley. There are predictions of a damaging freeze. A large section of your acreage has young trees; you're pretty sure they won't survive a frost, and you're rightfully worried. What if you could protect your trees by flicking on a genetic switch borrowed from cold-hardy crops? Or imagine another scenario. Market trends have shifted recently, and you anticipate financial benefit if you can get your crop to market a month later than usual. No problem – you flick a different genetic switch this time and delay flowering by a month. Both of these scenarios currently live in the realm of farmland science-fiction, but concepts and tools from the new field of synthetic biology could help bring this bold future to California's citrus growers, thanks in part to Citrus Research Board (CRB)-funded research.

The immediate goal of our project is to accelerate citrus breeding by developing strains whereby flowering can be induced whenever we desire. Citrus plants possess a long juvenile period before they reach sexual maturity – this adds many years to each breeding cycle relative to annual crops. We are developing a system that will allow us to activate flowering in juvenile plants and thus accelerate breeding cycles. To reach this goal, though, we will first need to be able to selectively time when genes are turned on in citrus crops to avoid flowering when young plants are still developing leaves and roots in tissue culture or as seedlings. In the language of synthetic biology – we first need to develop a system for inducible gene expression[2] that works in citrus.

Inducible gene expression systems are used extensively in microbial biotechnology. For example, the insulin used by people with diabetes is mass-produced in bacterial strains that synthesize insulin by inducible expression. Inducible systems are essential for insulin production because if the bacteria produced insulin all of the time, rather than inducibly, they would grow slowly, since producing insulin is energetically costly leaving engineers struggling to produce sufficient yields (Farmer and Liao, 2000). Inducible systems allow organisms to live two lives – one life as healthy and fast-growing bacteria that can be grown to high densities, and another life as production factories that are activated once those high-growth densities have been reached.

Although microbial engineers have used inducible systems for many years, there are few options for regulating gene expression in plants and none with the right combination of properties that would allow a grower to control gene expression in a field. For example, the systems for gene induction that have been developed in plants are designed

for laboratory research labs and are toxic and expensive, prohibiting their use in agriculture (Ordiz et al. 2012). One frequently used inducer in plants is the steroid dexamethasone, which would not be suitable for use in "real-world" scenarios due to its effects on humans. To address this limitation, we have developed a new gene induction system that is controlled by an inexpensive agrochemical called mandipropamid (sold commercially as Revus and used for controlling oomycete pathogens). Revus already has undergone the extensive safety testing needed for government registration, and its registration for use on citrus in California is pending – it can, therefore, be used in real-world production environments. To date, we have tested our new induction system in the model plant *Arabidopsis thaliana* (a relative of canola and other mustard plants) and demonstrated its effectiveness (**Figure 1**). Our CRB project, now starting its second year, is currently working to validate, optimize and deploy the system for use in citrus.

Assuming that our system works as expected, how will we use it to induce flowering? Fortunately, a great deal is known about the control of flowering from years of research by plant geneticists. It is now well-established that a gene called *Flowering Locus T (FT)* is a key regulator of flowering in essentially all flowering plants (Turck et al. 2008). Prior CRB-funded research from other research groups demonstrated that *FT* over-expression in transgenic citrus plants caused early flowering, so much so that the transgenic plants flowered while still in tissue culture and then died. This illustrates why inducibility is such a critical feature – to be useful, plants are needed that flower at specific times as opposed to all of the time.

FT encodes a protein that moves long distances within a plant to signal flowering. It is synthesized in leaves at high levels immediately before flowering and then moves throughout the plant body in the phloem, which is the specialized plant tissue that transports sugars from leaves to shoot apices and fruits. FT is carried in the phloem to cells in the shoot apical meristem[4] where it instructs the meristem to make flowers. FT is unique because it acts far away from its site of synthesis. In this way, FT is similar to a hormonal signal, a property which makes FT very powerful for manipulating flowering. Many investigators have shown that FT can move across graft junctions (Turck et al. 2008), which means that an FT-producing rootstock can, in principle, be used to induce flowering in a non-transgenic scion grafted onto the rootstock (we are using Carrizo). Thus, we can use engineered transgenic Carrizo to induce high levels of FT, which will act across a graft junction and induce flowering in a non-transgenic scion. If successful, this approach could speed up any existing citrus breeding program with access to the inducible flowering lines. We eventually will test this directly, using juvenile hybrids from the Roose laboratory's citrus program, with a focus on developing new varieties with improved HLB-resistance or tolerance.

Synthetic biology is rapidly changing how biological engineers approach many problems. To be useful to the citrus industry, we will need a whole suite of tools, including those for controlling gene expression. We are developing a new technological platform for citrus synthetic biology and will use it, in the short-term, to engineer inducible flowering lines and accelerate breeding. Over the long term, we are optimistic that new traits such as inducible frost tolerance or disease resistance will move from fantasy to reality.

CRB Research Project #5200-156

References

Farmer, W. R. and Liao, J. C. 2000. Improving lycopene production in *Escherichia coli* by engineering metabolic control. *Nature Biotechnology* 18(5): 533–537.

Ordiz, M. I.; Yang, J.; Beachy, R. N. 2012. Trait Control (Gene Switch Technology). p. 67–84. In: M. J. Oliver and Y. Li (eds.), *Plant Gene Containment*. John Wiley & Sons, Chichester, UK.

Turck, F.; Fornara, F.; Coupland, G. 2008. Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. *Annual Review of Plant Biology* 59: 573–594.

Glossary

[1]Synthetic biology: A field of study that merges principles from biology and engineering to design organisms with specific traits.

[2]Inducible gene expression: Rapid and specific expression of genes in response to an external stimulus.

***[3]FT*:** *Flowering Locus T* is a gene encoding a small protein that moves throughout the plant to induce flowering.

[4]Apical meristem: Small clusters of cells at the growing tips of organs, including shoots, that continually produce structures such as leaves and/or flowers as the shoots grow.

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Figure 1. Symptoms of Colletotrichum Dieback on clementine mandarins: (A) shoot dieback symptoms; (B) gumming symptoms on an infected shoot; (C) branch dieback symptoms; and (D) wood discoloration and canker.

Colletotrichum Dieback of Mandarins and Navel Oranges in California

A new twig and shoot disease in the Central Valley

Akif Eskalen, Greg W. Douhan, Craig Kallsen and Joey S. Mayorquin

Project Summary

Recently, a twig and shoot dieback disease of clementine mandarins and navel oranges has occurred in the main citrus growing regions of California's Central Valley. The disease was first noticed in 2012 by several growers and nurserymen in various Central Valley orchards; however, the source of this dieback disease remained unknown. The purpose of the study was to determine the cause(s) of the twig and shoot dieback in Central Valley mandarins and navel oranges. Field surveys determined that Colletotrichum gloeosporioides *and* C. karstii *were among several fungi recovered from symptomatic tissues. Tests confirmed* C. karstii *as a pathogen of citrus, making this the first report of this citrus pathogen in California. Studies also determined that spores of* Colletotrichum *spp. generally are most abundant during rainy months. Although no management strategies exist for this new citrus disease, preliminary field trials determined that products containing pyraclostrobin were capable of reducing disease prevalence of Colletotrichum Dieback (CD).*

CD symptoms include leaf chlorosis, gumming on twigs and shoot dieback (**Figures1A** and **1B**) and, in severe cases, branch dieback of trees (**Figures 1C** and **1D**). The most characteristic symptoms of this disease are the gum pockets, which appear on young shoots either alone or in clusters, and the dieback of twigs and shoots (**Figures 1B** and **1C**). These symptoms primarily were reported by Farm Advisors and Pest Control Advisors from clementine, mandarin and navel oranges, but also have been seen on additional citrus varieties (data not shown). At this time, it is unclear which, if any, variety or cultivars are more susceptible or more resistant to this disease. Field observations indicate that symptoms initially appear during the early summer months and continue to be seen until the early fall. Trees showing dieback and gumming symptoms characteristic of this disease usually are spread out sporadically within an orchard, and generally only a few twigs or shoots are affected within a tree.

More than 100 species of *Colletotrichum* have been described, of which there are three well-known "species" of *Colletotrichum* based on traditional morphology – *C. gloeosporioides, C. acutatum and C. boninense*. With respect to citrus, two species of *Colletotrichum, C. gloeosporioides* and *C. acutatum*, have been associated with anthracnose diseases of citrus. These diseases, which include post-harvest anthracnose, post-bloom fruit drop (PFD) and key lime anthracnose (KLA), are of great economic importance (Timmer et al. 2000). However, recent evidence suggests that additional, previously unknown species of *Colletotrichum* are causing citrus diseases globally, particularly from the *C. boninense* species complex.

Colletotrichum karstii increasingly has been associated with anthracnose symptoms of citrus worldwide (Aiello et al. 2014; Huang et al. 2013; Ramos et al. 2016) and often occurs in association with other *Colletotrichum* spp., particularly *C. gloeosporioides*, which generally predominates within citrus hosts. *C. karstii* increasingly has been reported from anthracnose diseases of other crops like avocado (Silva-Rojas and Vila-Quezada 2011) and is considered the most common and widely distributed species of the *C. boninense* species complex (Damm et al. 2010). Although *C. karstii* has been reported from citrus in China, Italy and Portugal, it has not been reported from citrus species in the U.S.

To date, *C. gloeosporioides* has been the only species associated with anthracnose diseases of California citrus. Therefore, the objectives of this study were to:

1. identify *Colletotrichum* species associated with twig and shoot dieback, as well as branch canker of *Citrus* spp. in the Central Valley of California;
2. assess the pathogenicity of *Colletotrichum* spp. in twigs of *Citrus* spp.; and
3. determine when and under what environmental conditions spores of *Colletotrichum* spp. are dispersed within Central Valley citrus orchards based on spore trapping.

How the study was conducted

Field surveys were conducted in ten commercial citrus orchards throughout Madera County (one orchard), Tulare County (four orchards) and Kern County (five orchards) beginning in the spring of 2014 to fall 2015. Citrus orchards were sampled once during the spring and re-sampled during the fall of that same year. In spring 2017, two Tulare County and two Kern County orchards that had been previously surveyed during 2014-2015 were resurveyed. The citrus varieties surveyed in citrus orchards from Kern and Tulare counties were clementine (cv. Clemenules) and navels (cv. Fukumoto and Washington). The citrus varieties surveyed in citrus orchards from Madera County were Valencia oranges and navels (cv. Fisher). The average age of all surveyed orchards was 11 years. Approximately 16 trees were sampled from each orchard during each sampling period, with twigs and shoots collected from trees showing signs of blighted twigs and shoots and cankered shoots.

Field Survey and Fungal Identification

By studying the physical characteristics of the fungi, as well as sequencing a specific genomic region, two distinct species of *Colletotrichum* (*Colletotrichum karstii* and *C. gloeosporioides*) were associated with twig and shoot dieback in the orchards. Interestingly, these *Colletotrichum* species also were isolated from cankers in larger branches. Other fungi isolated from symptomatic tissues were identified as *Alternaria* spp., *Penicillium* spp., *Fusarium* spp., *Quambalaria* spp., *Botryosphaeria* spp. and Diatrypaceae species. *Botryosphaeria* spp. were recovered from 27 of the 274 samples collected, and Diatrypaceae species were recovered from eight of the

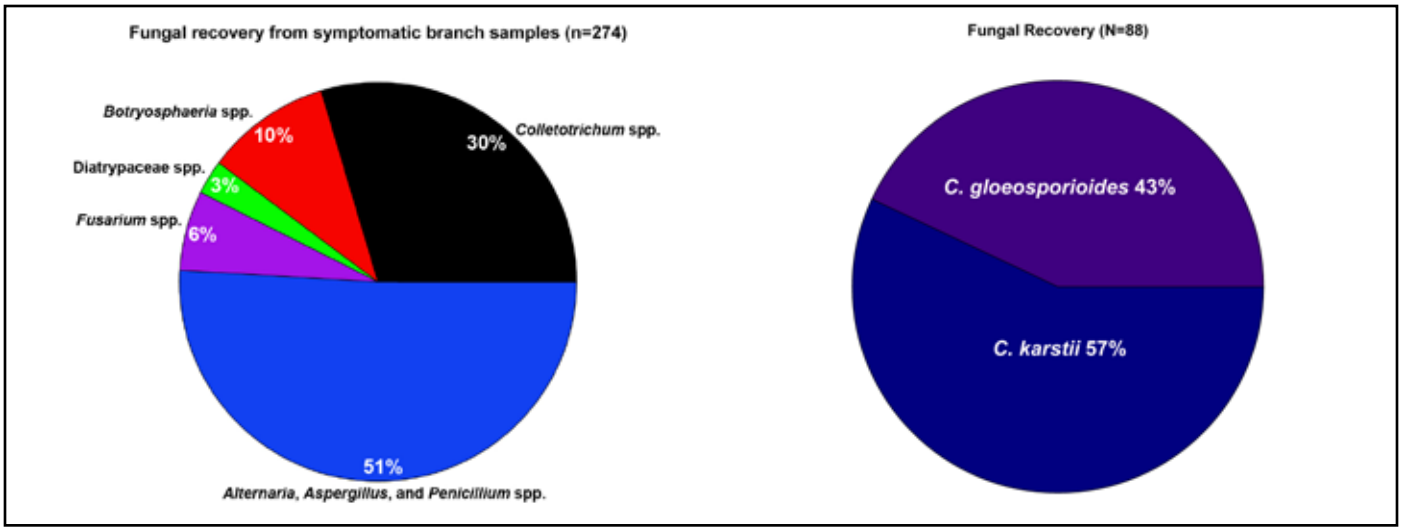

Figure 2. Fungal species recovered from twig and shoot samples.

274 samples. Both *Botryosphaeria* and Diatrypaceae species, known canker pathogens associated with citrus, were never co-isolated with species of *Colletotrichum* (**Figure 2**).

Pathogenicity and Fruit Decay Studies

Pathogenicity tests using clementine (cv. 4B) indicated that both *C. karstii* and *C. gloeosporioides* can produce lesions following inoculation of stems. *C. karstii* was the most aggressive fungal species producing the longest lesions after 15 months (**Figure 3**). Although *C. gloeosporioides* is known to cause anthracnose on citrus (a post-harvest disease causing fruit decay), it has not been reported to cause shoot dieback of citrus (Benyahia et al. 2003). *C. karstii*, however, has not been reported previously from citrus in California, and our laboratory confirmed the pathogenicity of this species in citrus. These findings confirm *C. karstii* as a new pathogen of California citrus.

It is known that *C. gloeosporioides* causes a post-harvest disease in citrus; however, it was unclear if *C. karstii* isolated from this study also could cause a post-harvest decay. Fruit decay studies using navel (cv. Washington), mandarin (cv. Murcott) and lemon (cv. Lisbon) revealed that when unwounded, neither *C. gloeosporioides* nor *C. karstii* caused any decay. When fruit were wounded, both *Colletotrichum* spp. caused decay, however a higher incidence and severity of decay was observed in fruit inoculated with *C. gloeosporioides*, particularly lemon and mandarin.

Spore Trapping

Relative humidity and precipitation in California citrus orchards play an important role in the epidemiology of *Colletotrichum* infection, whereby fungal spores dispersed by rain and humidity are conducive to pathogen spread. Based on spore trapping results, *Colletotrichum* species occurred most frequently during the months with the highest precipitation (January through May) (**Figure 4**); however, *Colletotrichum* spp. were not always correlated with rainfall. Wounding is also known to predispose plants to infection by

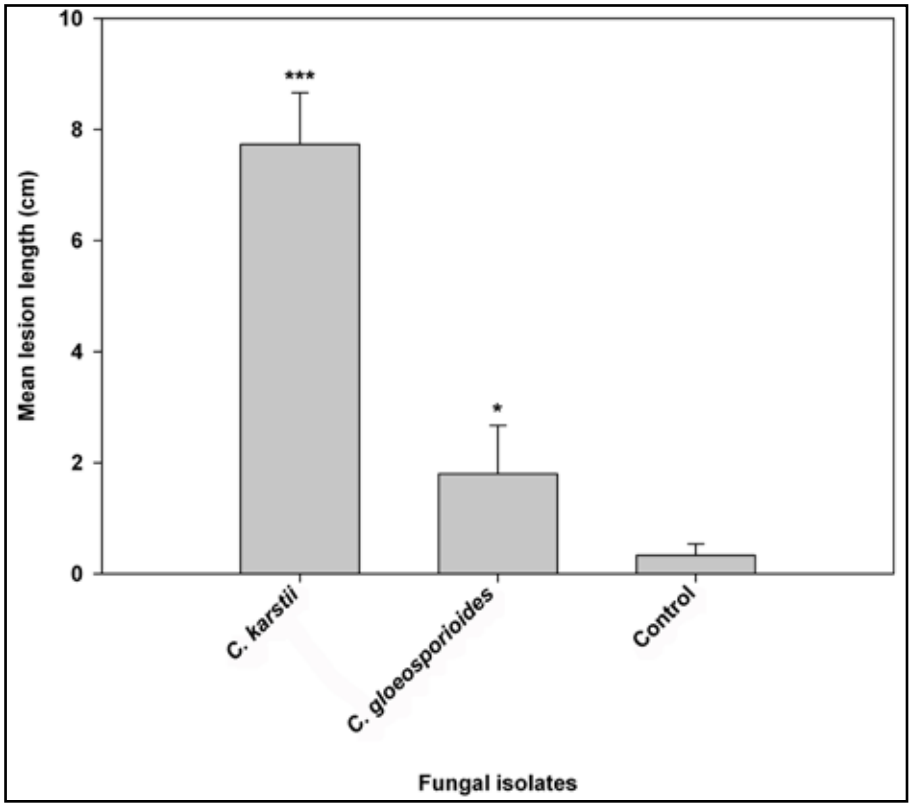

Figure 3. Pathogenicity of* Colletotrichum *spp. on clementine mandarin (cv. 4B) after 15 months. Vertical lines represent standard error of the mean. Asterisks represent significance as follows: '*' P < 0.05 and '' P < 0.001.***

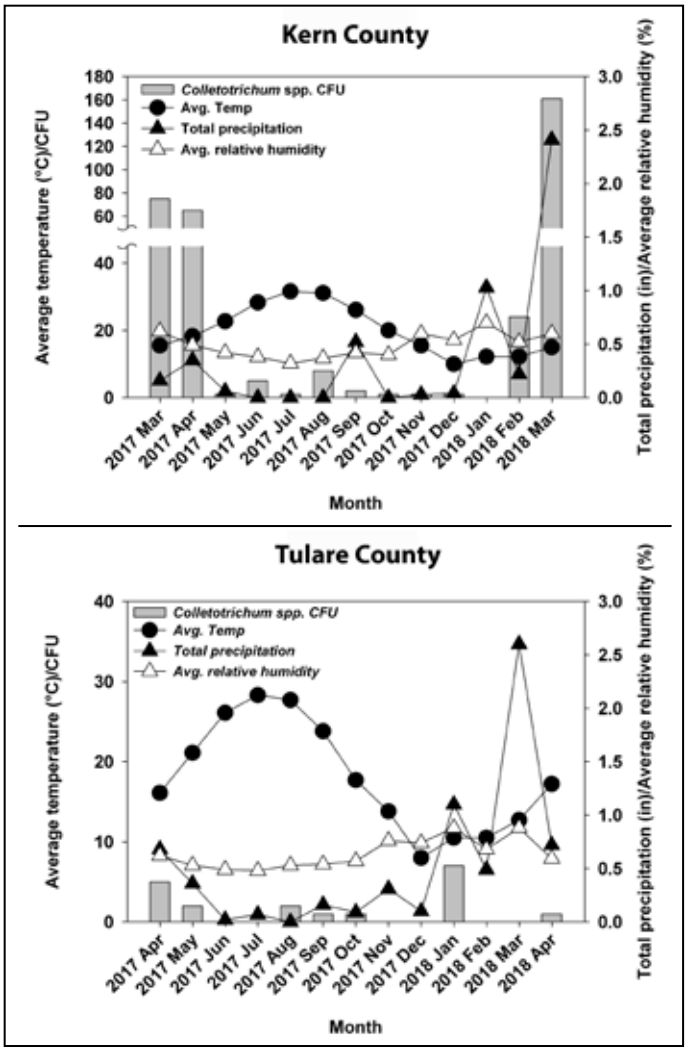

Figure 4. Relationships of monthly spore trap counts to temperature (°C), precipitation (mm), and relative humidity (%) for (a) Kern and (b) Tulare counties. Vertical bars represent total colony forming units (CFU) counted from each citrus orchard by month. Lines represent average monthly temperature (°C), relative humidity (%) and total monthly precipitation (mm).

Colletotrichum spp., and typical agricultural practices and the environment in California citrus groves (pruning, shearing, wind/sand damage) give *C. gloeosporiodes* and *C. karstii* the opportunity to colonize citrus trees. During this study, symptoms were observed during the late spring and summer months, with no new symptoms being observed in fall, winter or early spring. This suggests that young, tender tissues developing in the late spring likely are necessary for initial pathogen colonization.

Fungicide Sensitivities and Field Trial

Germination of *Colletotrichum* spp. spores was inhibited *in vitro* at concentrations below labeled rates for fungicides tested: azoxystrobin, pyraclostrobin, trifloxystrobin (all strobilurins) and fenbuconazole (a triazole) (**Table 1**). The half-maximal effective concentration (EC_{50}), as used in this

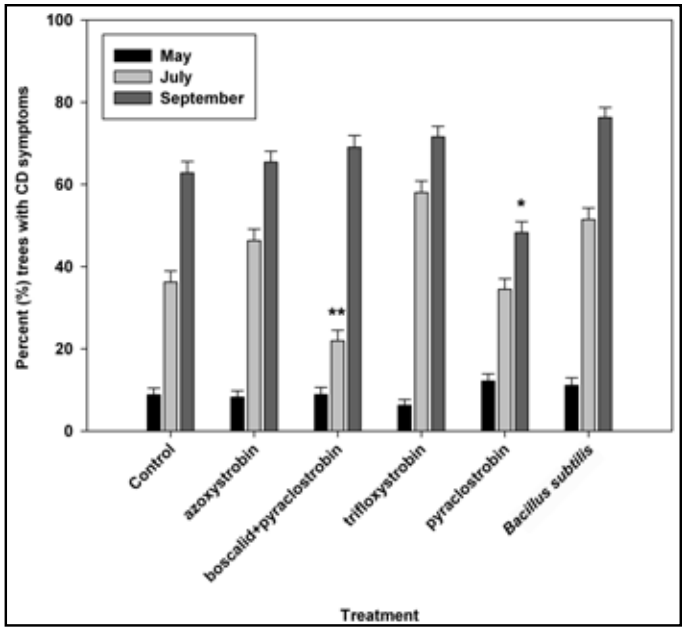

Figure 5. Prevalence of Colletotrichum Dieback (CD) symptoms in citrus trees by month after fungicide application in June. Vertical bars represent the mean percent of trees displaying symptoms of CD. Asterisks denote the following significance: '*' P < 0.05 and '**' P < 0.01.

report, refers to the dose of a fungicide that causes 50 percent inhibition of spore germination. Strobilurins had EC_{50} values (between 10 to 50 parts per billion) approximately 100-fold lower than EC_{50} values (between 500 to 600 parts per billion) of fenbuconazole. At the request of citrus growers, copper also was studied to determine its inhibitory effects on spore germination of *Colletotrichum* spp. *In vitro* experiments determined that copper concentrations of 150 parts per million or greater were completely inhibitory to spore germination of all isolates tested.

During a one-season field trial, single applications of commercial fungicides containing pyraclostrobin reduced CD symptoms for at least one month when tested in the field (**Figure 5**). Treatment with a premix of boscalid and pyraclostrobin showed a significant reduction ($P < 0.01$) in CD symptoms during the month of July, while treatment with pyraclostrobin alone showed a significant reduction ($P < 0.05$) in CD symptoms for September when compared to untreated control trees. All other treatments showed higher disease prevalence when compared to untreated controls for the months of July and September. It is important to note that the results of this field trial are preliminary and further field trials will be conducted to confirm the efficacy of these fungicides in managing CD.

Conclusions

Adherence to cultural practices recommended for the management of canker and dieback pathogens should be followed. These practices include maintaining trees in good condition through appropriate irrigation regimens and

Table 1. EC_{50} values of several fungicides tested for spore germination inhibition of *Colletotrichum karstii* and *C. gloeosporioides in vitro*.

Fungicide A.I.	Concentration applied (µg/ml)	EC_{50} values (µg/ml)			
		C. karstii		*C. gloeosporioides*	
		Mean±SD	Range	Mean±SD	Range
Fenbuconazole	500	0.518±0.201 a	0.238-1.056	0.622±0.252 a	0.302-1.190
Azoxystrobin	10	0.039±0.019 b	0.020-0.099	0.055±0.021 b	0.020-0.111
Trifloxystrobin	10	0.008±0.004 c	0.004-0.017	0.008±0.004 c	0.004-0.018
Pyraclostrobin	10	0.002±0.001 d	0.001-0.004	0.002±0.001 d	0.001-0.005

Values were determined from four isolates per fungal species. No inhibition is indicated by NI. Levels connected by the same letter are not significantly different using Tukey's honest significant difference (HSD) at $\alpha = 0.05$.

proper fertilization, removal of infested branches and pruning debris during dry periods followed by immediate disposal of infested material and sanitizing pruning equipment. Chemical management using fungicides is being investigated, and these methods may become part of an integrated pest management strategy for controlling this disease. Additional work also is being conducted to investigate the diversity of both *C. gloeosporiodes* and *C. karstii* to better understand the epidemiology of these pathogens and thus gain further insights into controlling these pathogens.

CRB Research Project #5400-152

Acknowledgements

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References

Aiello, D.; Carrieri, R.; Guarnaccia, V.; Vitale, A.; Lahoz, E.; Polizzi, G. 2015. Characterization and Pathogenicity of *Colletotrichum gloeosporioides* and *C. karstii* Causing Preharvest Disease on Citrus sinensis in Italy. *Journal of Phytopathology*. 163(3):168–177.

Benyahia, H.; Ifi Jr., A.; Smaili, C.; Afellah, M.; Lamsetef, Y.; Timmer, L.W. 2003. First report of *Colletotrichum gloeosporioides* causing withertip on twigs and tear stain on fruit of citrus in Morocco. *Plant Pathology*. 52(6):798.

Damm, U.; Cannon, P.; Woudenberg, J.; Johnston, P.; Weir, B.; Tan, Y.; Shivas, R.; P. Crous. 2012. The *Colletotrichum boninense* species complex. *Studies in Mycology*. 73:1-36.

Huang, F.; Chen, G.Q.; Hou, X.; Fu, Y.S.; Cai, L.; Hyde, K.D.; Li, H.Y.; 2013. *Colletotrichum* species associated with cultivated citrus in China. *Fungal Diversity*. 61(1):61–74.

Ramos, A.P.; Talhinhas, P.; Sreenivasaprasad, S.; Oliveira, H. 2016. Characterization of *Colletotrichum gloeosporioides*, as the main causal agent of citrus anthracnose, and *C. karstii* as species preferentially associated with lemon twig dieback in Portugal. *Phytoparasitica*. 44(4):549-561.

Silva-Rojas, H.V. and Vila-Quezada, G.D. 2011. Phylogenetic and morphological identification of *Colletotrichum boninense*: a novel causal agent of anthracnose in avocado. *Plant Pathology*. 60(5):899-908.

Timmer, L.W.; Garnsey, S.M.; J. H. Graham. eds. 2000. Compendium of Citrus Diseases, Second Edition. American Phytopathological Society Press. Saint Paul, Minnesota.

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Greenhouse citrus used for immune response assays.

Developing Immune Response Assays for Rapid Citrus Evaluation

Jessica Franco, Chandrika Ramadugu and Gitta Coaker

Project Summary

Major bottlenecks in developing huanglongbing (HLB)-resistant citrus by breeding include long generation times and the lack of assays to rapidly evaluate germplasm. We sought to develop a plate assay that could test the ability of diverse citrus varieties to actively perceive the HLB-associated bacterium and Asian citrus psyllid (ACP). We screened a panel of susceptible citrus varieties and resistant/tolerant wild relatives and hybrids. Our results illustrate a potential marker that breeders may use to accelerate breeding efforts by rapidly screening citrus material for response to pathogen perception.

Introduction

HLB is a devastating disease plaguing the citrus industry worldwide and is associated with the bacterium *'Candidatus* Liberibacter asiaticus' (CLas), which is spread by the ACP in California. Currently, there is no cure, so removal of infected trees and pesticide applications to decrease ACP populations are used to mitigate disease spread in California. To prevent further losses due to HLB, the development of resistant citrus germplasm/rootstocks is essential. All commercial citrus varieties are susceptible to HLB, but previous work has shown that Australian wild citrus relatives, which are sexually compatible with cultivated citrus, are more HLB-tolerant (Ramadugu et al. 2016).

Disease tolerance refers to the ability to limit infection or delay disease onset, while resistance is the inability of the pathogen to establish successfully. In perennial crops, related

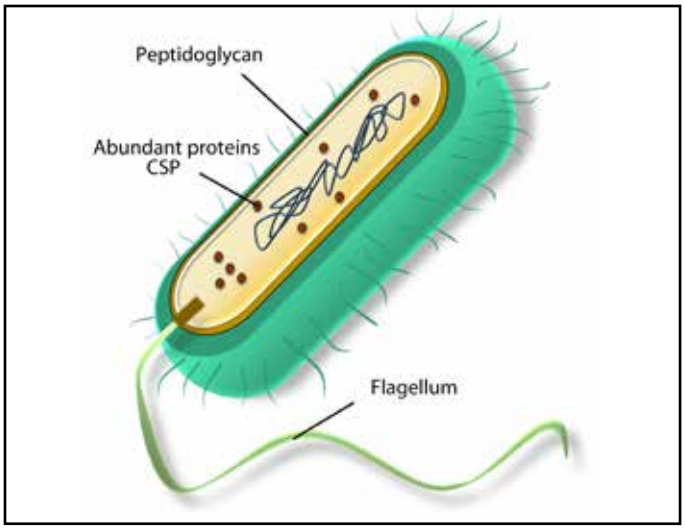

Figure 1: Pathogen-associated molecular patterns present in 'Candidatus Liberibacter asiaticus' that are perceived in plants. **Plants recognize bacteria by components not present in plant cells, such as flagella and bacterial proteins (e.g. cold shock protein, CSP) and peptidoglycan (a bacterial cell wall component).**

wild species have provided breeders the genetic diversity for incorporation into crop plants to overcome diseases. However, slow breeding cycles, lack of knowledge about the basis of resistance, and limited tools to screen germplasm all provide barriers in generating resistant citrus varieties. A heightened understanding of how resistance is conferred in citrus will lead to development of enhanced screening tools to speed up the process for identifying resistant germplasm.

Plant recognition of a pathogen as an invading organism can activate defense responses, which can lead to disease resistance. Plants perceive pathogens through the use of surface-localized receptors known as pattern recognition receptors[1] (PRRs). PRRs recognize unique, non-host plant molecules, termed pathogen-associated molecular patterns (PAMPs)[2] on the surfaces and cell membranes of bacteria. PAMPs are essential for pathogen growth and survival (Zipfel 2014) **(Figure 1**). The PRR repertoire varies across different plant species, and this diversity has been used to select resistant species to introduce into breeding populations. Plant host defense activation includes production of reactive oxygen species (ROS)[3] (Boutrot and Zipfel 2017) within 30-60 minutes. ROS are highly reactive molecules containing oxygen and include superoxide (O_2) and hydrogen peroxide (H_2O_2). ROS production is required for cell wall reinforcement, resistance to bacterial pathogens, cell-to-cell signaling and systemic resistance (Boutrot and Zipfel 2017).

Not all ROS signals exhibit the same pattern in different species, and the range of ROS responses has not yet been explored in citrus. For example, the timing and amount of ROS produced can vary. Our goal was to develop ROS-based assays to analyze the range of PAMP perception in citrus. These assays can be used to compare the PAMP responsiveness of citrus varieties or citrus relatives with differential HLB susceptibility to identify correlations between PAMP perception and tolerance/resistance. We hypothesize that the identification of PAMPs correlating with HLB tolerance/resistance will provide markers to enable breeders to rapidly identify promising material, as well as attain a greater understanding of the basis of resistance.

Results

To test the perception of previously characterized PAMPs in citrus, a light-based assay was used to quantify ROS production in diverse citrus species (Zipfel 2014). The advantage of this assay is that it can be performed on leaf tissue from mature or young seedlings. One individual can test 24 citrus varieties within two days as long as they have access to a luminometer[4] (Boutrot and Zipfel 2017). It has been demonstrated that a small 22-amino acid fragment from flagellin, cold shock protein and chitin are sufficient to trigger defenses in *Arabidopsis* and tomato (Felix et al. 1999; Wang et al. 2016). We chemically synthesized small fragments of flagellin from CLas ($flg22_{CLas}$) and *Pseudomonas syringae* (flg22) (Zhang and Zhou 2010), cold shock protein (csp22) and chitin to screen susceptible citrus and resistant relatives (**Figure 1**). To conduct these assays, we optimized the protocols for leaf age and PAMP concentrations using leaf discs from different citrus varieties and wild citrus relatives in 96-well microplates (**Figure 2**). Leaf disks were treated with a solution containing PAMPs, luminol and horseradish peroxidase that would produce light if ROS were produced in the leaf disk (following exposure to PAMPs). Light production was measured over time as a surrogate for ROS production.

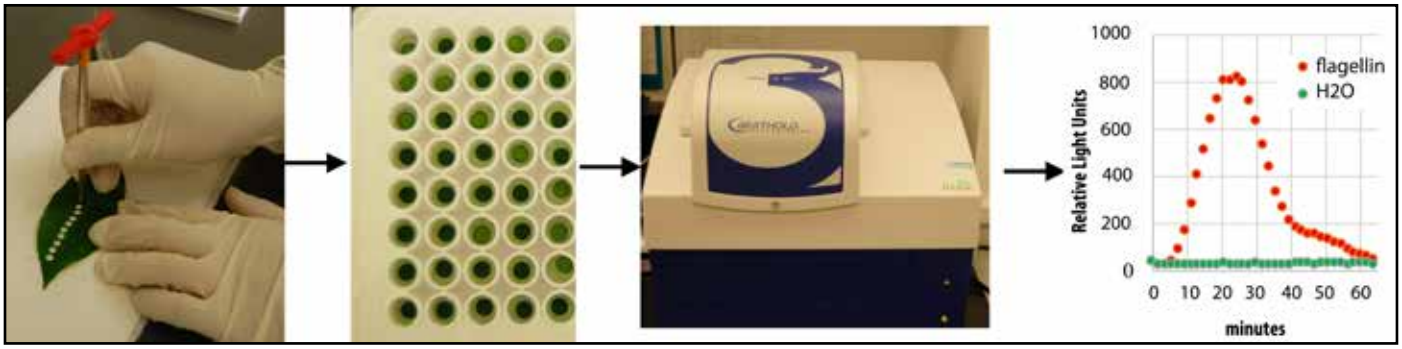

Figure 2: Measuring pathogen-associated molecular pattern-induced reactive oxygen species (ROS) burst in citrus. A microplate assay is used to test the production of ROS in citrus in response to a small fragment (flg22) of flagellin from **Pseudomonas syringae.** ***(A) Leaf disks are taken surrounding the midvein using a corkborer. (B) Leaf disks are placed in a 96-well plate and floated on water overnight to remove the wound signal. (C) To measure the ROS burst, the water solution is removed and replaced with compounds that produce light if ROS were produced in the leaf disk and a PAMP to test for ROS production. A luminometer is used to measure ROS production over 60 minutes. (D) The trace of relative light units is graphically depicted. To determine significant differences between genotypes, the sum of luminescence under the curve is quantified and graphed.***

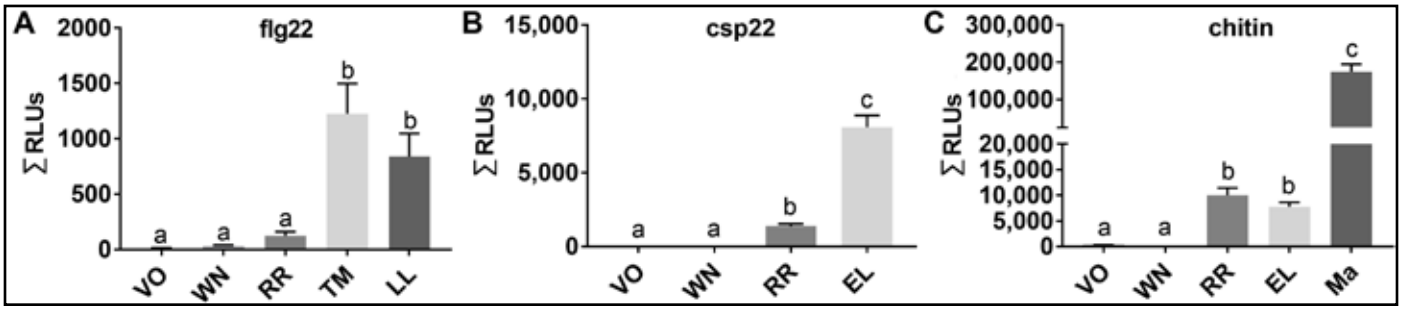

Figure 3: Differential pathogen-associated molecular pattern recognition. Graphs represents the reactive oxygen species (ROS) burst response to (A) 100 nM flg22, (B) 200 nM csp22 and (C) and 10 uM acetylated chitin. ROS is quantified as the sum of relative light units (Σ RLUs) over 30-90 minutes. VO=Valencia orange, WN=Washington navel, TM=Tango mandarin, RR=Rio Red grapefruit, LL=Lisbon lemon, EL=Eremolemon, Ma=*Microcitrus australasica*. Different letters indicate significant differences after ANOVA and Tukey's test.

Our results indicate that citrus varieties and citrus relatives show different responses to the tested PAMPs. Washington navels and Valencia oranges were the least responsive following treatment with each of the three PAMPs tested. Other varieties such as Lisbon lemons and Tango mandarins have a more robust response when treated with flg22 (**Figure 3A**). The HLB-resistant Australian citrus relative, *Microcitrus australasica*, had the most robust response when treated with chitin compared to commercial citrus varieties tested (**Figure 3C**). *Microcitrus australasica* also exhibits lower adult ACP feeding preference compared to current citrus cultivars, which correlates with its enhanced response to chitin treatment (Westbrook et al. 2011). The HLB-resistant variety Eremolemon, a hybrid of *Eremocitrus glauca* (an HLB-resistant Australian wild citrus relative) and Meyer lemon (an HLB-susceptible commercial variety), also responded strongly to recognition of csp22 and chitin (**Figure 3B-C**). Although we tested $flg22_{CLas}$, the citrus varieties that we tested did not show a response based on the ROS assay. There may be another region of the CLas flagellin protein that is more widely recognized in wild citrus. Our results are consistent with previous work illustrating that sweet oranges succumb to HLB more quickly than grapefruit and mandarins (Ramadugu et al. 2016; Shi et al. 2015). This study demonstrated a promising PAMP that may correlate with HLB tolerance. In the long term, screening more PAMPs in diverse citrus species will help identify additional markers for resistance.

CRB Research Project #5200-157

References

Boutrot, F.; Zipfel, C. 2017. Function, discovery and exploitation of plant pattern recognition receptors for broad-spectrum disease resistance. *Annual Review of Phytopathology*. 55:257-286.

Felix, G.; Duran, J. D.; Volko, S.; Boller, T. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *The Plant Journal*. 18(3):265-276

Ramadugu, C.; Keremane, M.L.; Halbert, S.E.; Duan, Y.P.; Roose, M.L.; Stover, E.; Lee, R.F. 2016. Long-term field evaluation reveals Huanglongbing resistance in *Citrus* relatives. *Plant Disease*. 100 (9):1858-1869.

Shi, Q.; Febres, V.J.; Jones, J.B.; Moore, G.A. 2015. Responsiveness of different citrus genotypes to the *Xanthomonas citri* ssp. citri-derived pathogen-associated molecular pattern (PAMP) flg22 correlates with resistance to citrus canker. *Molecular Plant Pathology*. 16(5):507-520.

Wang, L.; Albert, M.; Einig, E.; Fürst, U.; Krust, D.; Felix, G. 2016. The pattern-recognition receptor CORE of Solanaceae detects bacterial cold-shock protein. *Nature Plants*. 2(12):16185.

Westbrook, C. J.; Hall, D. G.; Stover, E.; Duan, Y. P.; Lee, R. F. 2011. Colonization of *Citrus* and *Citrus*-related germplasm by *Diaphorina citri* (Hemiptera: Psyllidae). *HortScience* 46(7):997-1005.

Zhang, J.; Zhou, J. M. 2010. Plant immunity triggered by microbial molecular signatures. *Molecular Plant*. 3(5):783-793.

Zipfel, C. 2014. Plant pattern-recognition receptors. *Trends in Immunology*. 35(7):345-351.

Glossary

[1]Pattern Recognition Receptor (PRR): A receptor capable of recognizing pathogen components and triggering defense.

[2]Pathogen Associated Molecular Patterns (PAMPs): Unique, non-host plant molecules that are essential for pathogen growth and survival.

[3]Reactive Oxygen Species (ROS): Chemically reactive molecules containing oxygen that have important roles in defense response and signaling.

[4]Luminometer: A sensitive photometer used for measuring very low light levels. Plant production of reactive oxygen species can be measured using a luminometer.

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Figure 1. Conducting wide crosses. Unopened citrus buds are chosen (A), emasculated (B, C), and pollen from the selected '*Candidatus *Liberibacter asiaticus'-resistant citrus relative is brushed on the stigma of the emasculated citrus flower using a paint brush (D). Fruit that develop from this flower are carefully collected, seeds germinated and confirmed to be hybrids. **Photo credit: Dan Willey.**

Novel Citrus Hybrids with HLB Resistance

Chandrika Ramadugu, Manjunath L. Keremane, Richard F. Lee, David G. Hall, Thomas G. McCollum and Mikeal L. Roose

Project Summary

Huanglongbing (HLB)-resistant citrus cultivars are desirable for long-term, sustainable citrus cultivation. Toward this goal, we generated hundreds of novel citrus hybrids by crossing mandarin cultivars with HLB-resistant Australian citrus relatives. The resulting hybrids were evaluated rigorously for HLB disease response, and many have been found to be disease-free two years after exposure to the pathogen. Using wild relatives to develop HLB resistance is necessary since resistance to the disease is not found in cultivated citrus species.

Introduction

HLB is associated with the bacterium, '*Candidatus* Liberibacter asiaticus' (CLas) and is spread by the Asian citrus psyllid (ACP). In Florida, where HLB has negatively impacted citrus production, application of antimicrobial substances, thermal therapy of infected trees and nutritional treatments were reported to alleviate the symptoms and extend the productive life of infected trees (Ramirez et al. 2016; Yang et al. 2016). In California, where the disease has been spreading, HLB-positive trees are removed, and disease management includes vector suppression. Aggressive psyllid management could be effective in reducing the spread of CLas, but can be prohibitive due to the costs and effort involved. In addition to reduction of the inoculum and vector control, development of commercially acceptable HLB-tolerant and HLB-resistant citrus cultivars will be needed for a sustainable citrus industry.

Citrus has been cultivated for many centuries, and new cultivars are generated primarily to improve horticultural characteristics. Vegetative propagation[1] of the cultivars limits the introduction of new genetic material and has resulted in a narrow genetic base for commonly cultivated varieties. This can be detrimental when the plants are challenged with new diseases, since they may not have any resistance to the new pathogens. Often in such cases, resistance is incorporated into cultivated varieties from wild crop relatives through breeding. The first step of "pre-breeding"[2] introduces desirable traits from the relative species and reduces genetic uniformity. Identification of disease-resistant plants from the pre-breeding step will facilitate further breeding aimed at generating useful commercial cultivars (Sleper and Poehlman, 2006).

In a previous Citrus Research Board-funded study, we identified HLB-resistant citrus relatives in a six-year Florida field trial (Ramadugu et al. 2016a). In this study, 90 accessions[3] belonging to the sub-family Aurantioideae were included. We observed immunity to CLas infection in only two accessions (in immune plants, the pathogen is never detected), and resistance was reported in six accessions (temporary replication of the pathogen usually is detected immediately following a CLas challenge, but the pathogen does not become successfully established). Various levels of tolerance were observed in 14 accessions. The tolerant plants generally have lower pathogen levels than susceptible plants and exhibit reduced symptoms; some plants have a high pathogen titer initially, but the titer reduces later, while certain types of tolerant plants have delayed symptom expression. Unlike resistant plants, tolerant plants are likely to carry a low pathogen load that may contribute to disease spread in the surrounding citrus. Susceptible plants exhibit symptoms to varying degrees, including death within a couple of years in the most vulnerable plants. Most citrus was classified as susceptible in our previous study, which included representatives of 21 related genera (Ramadugu et al. 2016a). Certain CLas-tolerant accessions with citron parentage have healthy canopies and showed good growth despite having substantial pathogen titers (Miles et al. 2017). Since most citrus accessions do not have appreciable resistance to HLB, we chose to breed resistance into citrus using sexually compatible, HLB-resistant citrus relative accessions.

Our main long-term goal is to generate HLB-resistant citrus hybrids. Several types of scion and rootstock hybrids will be needed for different citrus growing regions with a wide range of agronomical and ecological situations. Incorporation of resistance traits into citrus is first attained through pre-breeding. We have achieved germplasm[4] enhancement through wide crosses. The HLB-resistant intermediate hybrids generated in this project will be essential for future cultivar development through further crosses.

Results

We have identified four Australian limes as resistant to or tolerant of CLas infection and/or HLB disease development and suitable as parents for breeding since they also are sexually compatible with many citrus types. All of the Australian limes that we used have mono-embryonic[5] seeds, and the seedlings that are true-to-type are resistant to CLas. The following citrus relative genotypes were used:

1. *Eremocitrus glauca* (synonym *Citrus glauca*) or the 'Australian Desert lime,' is a small desert tree native to Australia. The tree has grey-green leathery foliage and bears yellow fruit that is about one-two centimeters (cm) in diameter with an exceptional lime-like flavor. This accession was identified as one of the most HLB-resistant Australian limes (Ramadugu et al. 2016b).
2. *Microcitrus australasica* (synonym *Citrus australasica*) or 'Australian Finger lime,' has finger-like seven-cm long fruit with a variety of flesh colors and acidic flavor. Pure finger limes are highly resistant to HLB.
3. *Microcitrus inodora* (synonym *Citrus inodora*), commonly known as 'Large leaf Australian wild lime' or 'Russell River Lime,' has oblong to elliptical, yellowish-green fruit with pulp vesicles similar to those of citrus. The fruit is five cm long, three cm in diameter and very seedy. In its native Australia, *M. inodora* is a wild plant with no known commercial use. True-to-type seedlings are highly resistant to HLB.
4. *Microcitrus australis* (synonym *Citrus australis*) or 'Australian Round lime,' is HLB-tolerant with spherical green fruit, two-five cms in diameter. The pulp vesicles often contain acrid oil droplets.

Many of the Australian limes are precocious and can yield fruit in three or four years under favorable conditions, a desirable trait for conventional breeding. We have crossed several mandarins and trifoliates (usually as seed parents) with HLB-resistant Australian limes (**Figure 1**; **Table 1**). The breeding population was evaluated either in Florida or in the Contained Research Facility at the University of California, Davis (required due to quarantine regulations).

Table 1. Representative crosses conducted with HLB-resistant citrus relative genera are shown.

No.	Seed parent	X	Pollen parent
1	Encore mandarin	X	*Eremocitrus glauca*
2	Encore mandarin	X	*Microcitrus australasica*
3	Encore mandarin	X	*Microcitrus inodora*
4	Encore mandarin	X	Sydney hybrid (*Microcitrus australis* X *M. australasica*)
5	Encore mandarin	X	Eremolemon (*Eremocitrus* X Meyer lemon)
6	Fortune mandarin	X	*Microcitrus australasica*
7	Fortune mandarin	X	*Microcitrus australis*
8	Fortune mandarin	X	*Microcitrus inodora*
9	Fortune mandarin	X	Sydney hybrid (*M. australis* X *M. australasica*)
10	Wilking mandarin	X	*Eremocitrus glauca*
11	Wilking mandarin	X	*Microcitrus australasica*
12	Wilking mandarin	X	*Microcitrus inodora*
13	Wilking mandarin	X	*Microcitrus australis*
14	Wilking mandarin	X	Sydney hybrid (*M. australis* X *M. australasica*)
15	Wilking mandarin	X	Eremolemon (*Eremocitrus* X Meyer lemon)
16	Fallglo mandarin	X	*Eremocitrus glauca*
17	Fallglo mandarin	X	*Microcitrus australasica*
18	Fallglo mandarin	X	*Microcitrus inodora*
19	Fallglo mandarin	X	C146 trifoliate hybrid
20	Fallglo mandarin	X	Eremolemon (*Eremocitrus* X Meyer lemon)
21	Temple tangor	X	*Eremocitrus glauca*
22	Flying Dragon trifoliate	X	*Microcitrus australasica*
23	Pomeroy trifoliate	X	*Microcitrus inodora*
24	*Microcitrus australasica*	X	Rich 16-6 Trifoliate

The hybrid seedlings in Florida were evaluated by exposing them to CLas-positive psyllids for four weeks in a small greenhouse with free flying psyllids (**Figure 2**). In parallel experiments, propagations of the hybrid plants grafted on standard rootstocks were enclosed in plastic containers and exposed to 20 CLas-positive psyllids in a no-choice situation to ensure that each hybrid plant is exposed to the HLB pathogen (**Figure 3**). After the CLas challenge, the plants were treated with pesticide to destroy psyllid adults, nymphs and eggs, raised in greenhouses and evaluated for about two years. Both hybrid seedlings on their own roots and clones propagated by grafting on rootstock were analyzed. In California, the hybrids were challenged with grafts of CLas-positive tissue from lemon plants infected with the Hacienda Heights isolate of CLas (**Figure 4**).

No CLas was detected by qPCR or digital droplet PCR in several of the challenged hybrid plants, and no HLB symptoms developed within two years under greenhouse conditions. To ensure that the graft-inoculated plants in which CLas was not detected by PCR were not escapes, two or three repeat graft challenges were made at six-month intervals. Resistance can be validated when the promising hybrids are exposed to

Figure 2. Hybrid seedlings under observation after exposure to free flying, 'Candidatus Liberibacter asiaticus' -positive psyllids for four weeks in a small greenhouse. Psyllids colonized the plants, presumably transmitting the pathogen.

Figure 3. No-choice inoculations of hybrid plants was conducted in Fort Pierce, Florida (Hall Lab) by releasing 20 Liberibacter-positive psyllids inside the plastic enclosure covering the hybrid seedlings for two weeks. The psyllids and progeny were eliminated, and then plants were incubated in a greenhouse for evaluations.

Figure 4. HLB-resistant (top panel, A and B) and susceptible (bottom panel, C and D) hybrids generated from a cross of Wilking mandarin and* Microcitrus inodora*. Plants were inoculated with '*Candidatus *Liberibacter asiaticus' using bud grafts from CLas-positive lemon trees maintained at the UC Davis Contained Research Facility.

different isolates of the pathogen in various situations. Field evaluations are essential since the psyllid is continuously feeding on the plant in the field, and constant, low-level re-inoculation will be needed to accurately determine the host response to HLB in the field. We currently have many novel hybrids being evaluated in experimental Florida groves. However, each of the hybrids generated in this project are now under evaluation either in Florida or in California, but not in both regions. At present, we do not have a rapid mechanism for interstate movement of budwood. Efforts now are being made to make this possible from a regulatory standpoint so that thorough disease challenges can be conducted for each hybrid in multiple locations to confirm HLB resistance.

Some of the hybrids we generated in this project yield lime-like fruit. These fruit from the novel hybrids are acidic (pH 3.0), juicy and have a hint of *Microcitrus* flavor. The fruit from mandarin X *Microcitrus inodora* crosses may be acceptable as substitutes for limes after one more generation. According to past research, the volatile oils of Mexican lime were described as very similar to Australian finger lime (Scora and Kumamoto 1983). A disease-resistant citrus hybrid with lime-like fruits may be useful in regions where the lime industry has been devastated by HLB.

Breeding for specific types of citrus hybrids can be challenging. Most cultivated citrus types are a result

of repeated hybridization events, resulting in complex genomes with genes from different parents (Ramadugu et al. 2017). Generating an HLB-resistant hybrid similar to a sweet orange following traditional breeding methods will be time consuming and complicated. Methods to speed up this process will involve identification of HLB-resistance-associated traits by studying currently available populations and introducing these traits into elite cultivars by biotechnological methods. Development of genomic-based information to identify and select resistant progeny will accelerate conventional breeding for generating HLB resistant cultivars.

Conclusions

During the course of this project, we conducted about 4,000 wide crosses using the four HLB-resistant/tolerant Australian citrus. About 800 hybrid seedlings were generated, and around 600 were evaluated in greenhouses. Several hybrids were found to be promising, since we could not detect HLB pathogen two years after exposure to CLas under greenhouse conditions. Extended evaluations under field conditions and challenging the hybrids with multiple pathogen strains will be necessary to confirm resistance. In the near future, some of the hybrids generated in our breeding program may be useful as acceptable lime substitutes. Further breeding to generate disease resistant plants with edible fruit quality is in progress.

CRB Research Project #5200-147

References

Miles, G.P.; Stover, E.; Ramadugu, C.; et al. 2017. Apparent tolerance to huanglongbing in *Citrus* and *Citrus*-related germplasm. *HortScience*. 52:31-39.

Ramadugu, C.; Keremane, M.L.; Halbert, S.E. et al. 2016a. Long-term field evaluation reveals huanglongbing resistance in *Citrus* relatives. *Plant Disease*. 100:1858-1869.

Ramadugu, C.; Keremane, M.L.; McCollum, T.G.; et al. 2016b. Developing Resistance to HLB. *Citrograph* 7(2):46-51.

Ramadugu, C.; Razi, M.F.; Keremane, M.L.; et al. 2017. Systematic Classification, Distribution and Botany. p:12-36. In: Khan, M.M., Al-Yahyai, R. and Al-Said, F. (eds), *The Lime. Botany, Production and Uses*. CABI, Oxfordshire, UK.

Ramirez, D.; Chaparro, J.; Wright, A.; et al. 2016. Mitigation of huanglongbing effects on grapefruit trees using enhanced nutritional programs. *Proceedings of the Florida State Horticultural Society* 129: 51-55.

Scora, R.W.; Kumamoto, J. 1983. Chemotaxonomy of the genus *Citrus*. p:343-351. In: Walterman, P.G. and Grundon, M.F. (eds), *Chemistry and Chemical Taxonomy of the Rutales*. Academic Press, London, UK.

Sleper, D.A.; Poehlman, J.M. 2006. *Breeding Field Crops*. 5th edition. Blackwell Publishing, US. pp.424.

Yang, C.; Powell, C.A.; Duan, Y.P.; et. al. 2016. Mitigating citrus huanglongbing via effective application of antimicrobial compounds and thermotherapy. *Crop Protection* 84:150-158.

Glossary

[1]Vegetative propagation: Asexual methods of propagation. Citrus is cultivated as a grafted plant leading to genetic uniformity.

[2]Pre-breeding: Generating an intermediate set of plants with desirable characteristics (resistance to HLB in our project) for later use in further rounds of breeding required for cultivar development.

[3]Accession: A plant material from a single species collected at one time from a specific location. Accession numbers are unique identifiers.

[4]Germplasm: Living plants of a taxonomic group that represent the natural genetic resources. Genetically diverse germplasm is considered valuable for development of new cultivars.

[5]Mono-embryonic: Seeds have a single embryo that develops into one seedling. Typically such seedlings are not genetically identical to the mother tree.

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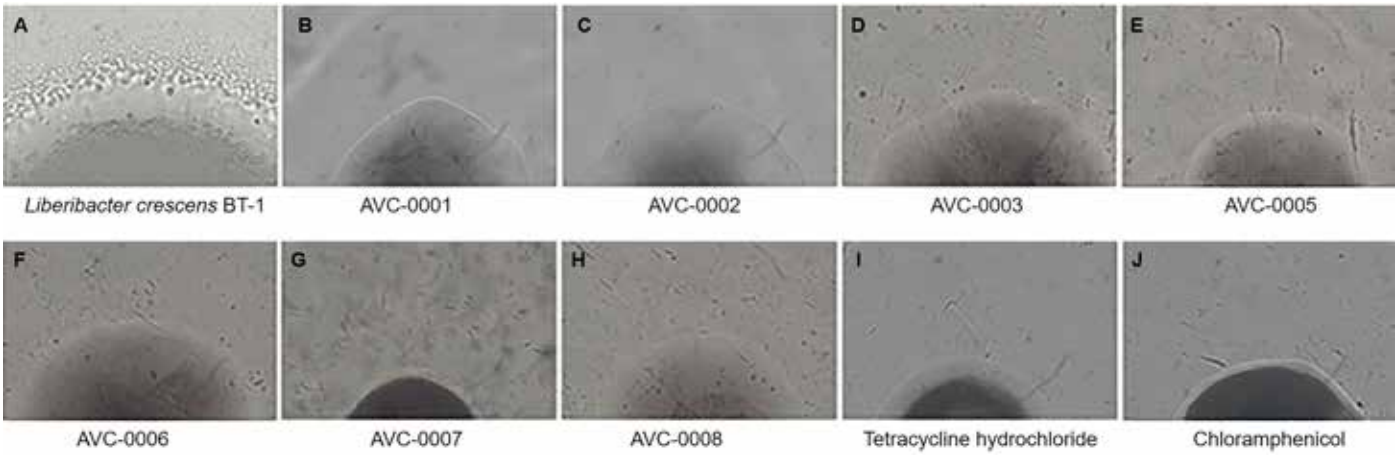

Figure 1. The evaluation of inhibitive effectiveness of the anti-virulence compounds, 50 µM (micromolar) on the colony peripheral fringes of CLas-complemented* L. crescens *(Lcs). Lcs cells were spotted on the surface of BM7 agar plates and grown for 10 days at 28°C. (A) Lcs colonies with typical peripheral fringes around the margins. (B-H) Treatment with anti-virulence compounds (AVCs) inhibits the peripheral fringes of colonies. (I-J) Lcs colonies treated with the antibiotics tetracycline hydrochloride and chloramphenicol with no peripheral fringes.

Molecular Therapy Targets for Huanglongbing

Hong Lin, Yongping Duan and Xianyang Shi

Project Summary

*The goal of this research was to develop a short-term practical approach to mitigating huanglongbing (HLB). We proposed to identify and functionally characterize key virulence genes/factors of the bacterium, '*Candidatus *Liberibacter asiaticus' (CLas) associated with HLB. We developed a molecular-based strategy to deliver anti-virulence molecules into host plants that block the activity of virulence traits and reduce the virulence of CLas. During this project, we screened a chemical library and identified potential anti-virulence compounds through* in vitro *bioassays. In addition, we validated the efficacy of selected anti-virulence molecules through greenhouse experiments in which HLB-affected citrus and CLas-infected periwinkle plants were foliar-sprayed with various concentrations of the selected anti-virulence compounds. Plants treated with anti-virulence compounds showed alleviated symptoms and new growth. CLas cell titers also were reduced compared to untreated plants as measured by quantitative polymerase chain reaction (qPCR). Based on these results, an anti-CLas virulence therapeutic approach could suppress and mitigate HLB.*

Background and Research Strategy

HLB is one of the most devastating diseases of citrus worldwide. In the U.S., it is associated with a phloem-limited bacterium CLas transmitted by the Asian citrus psyllid (ACP). HLB is now threatening the entire U.S. citrus industry (Gottwald 2010). Although various control strategies have been implemented to curtail the spread of CLas, the disease already has spread to most citrus growing counties in Florida and is a looming threat to California and Texas.

Currently, no effective measures are available to effectively control the disease. Affected trees irreversibly decline because all commercial citrus varieties are susceptible to HLB (Quarles 2013). So far, no naturally immune or resistant cultivars have been found in *Citrus* species. Even if resistant germplasm is identified, it will take a considerable amount of time to incorporate resistance genes into most current citrus cultivars through conventional breeding methods to cope with the current HLB issue. Thus, a new short-term strategy to slow or mitigate disease development is urgently needed. Antibiotics or bactericides have been widely used for controlling bacterial plant diseases. While these compounds usually are effective, repeated use of antimicrobial drugs could result in the development of drug resistant strains. Additionally, these approaches may have negative effects on the ecological community of microorganisms and are frequently associated with phytotoxicity[1] and food safety.

A novel target-based therapeutic strategy to interfere with diseases recently has been developed (Allen et al. 2014, Rasko and Sperandio 2010). This approach is based on the hypothesis that disarming key virulence[2] gene functions will knock out the pathogen's ability to cause disease and, therefore, eliminate development of the disease.

Bacteria possess an array of virulence genes/factors required for disease development in their hosts. These include adhesins, which bind to host cells for colonization, and toxins, which alter and interfere with a host's metabolic pathways or directly kill host cells. In addition, pathogenic bacteria use specialized secretion systems to deliver effectors (protein molecules expressed by pathogens to facilitate infection of specific plant species). Since the production of these virulence gene products are metabolically expensive, the expression of these virulence traits is intricately regulated and only occurs when they invade into the hosts.

After pathogenic bacteria enter their host, they sense the host environment and trigger a series of processes to activate virulence traits. These traits are regulated by bacterial virulence regulators. Thus, by disrupting or blocking the functional domains of virulence genes or factors with specific anti-virulence compounds, we will disarm bacterial virulence traits and make bacteria less capable of colonization or lose mobility in order to control the disease (Heaslip et al. 2010, Mahmood et al. 2016). This strategy, in contrast to antibiotic treatment, aims to disrupt bacterial pathogenicity rather than directly kill bacteria and is unlikely to lead to the development of resistant strains as there is no or minimal selection pressure imposed on bacterial survivors. As such, the proposed methodology could provide sustainable effectiveness for HLB management.

Additionally, anti-virulence compounds specifically target virulence genes and thus are less likely to impact other beneficial microbial organisms and beneficial insects. The anti-virulence compounds function as molecular ligands[3] and are less likely to be toxic or to have environmental consequence. We, therefore, propose this novel strategy to specifically block or disrupt the virulence traits of CLas required for disease development in citrus trees and thus stop the development of HLB.

We conducted in *silico*[4] analysis and identified several key virulence genes/factors in the CLas genome, using an orthologous gene[5] replacement technique to characterize the utility of virulence targets. These virulence genes or factors were selected because of their involvement in cell motility, cell-cell aggregation and biofilm formation[6], which are essential virulence traits for plant pathogenic bacteria. With the functional confirmation of target CLas genes, we identified small molecules that potentially target functional domains of virulence genes or factors. Using *in vitro* screening methods, we identified several compounds that showed potential candidates for anti-virulence. These putative anti-virulence compounds were further subjected to *in planta* evaluation whereby HLB-affected trees were foliar-sprayed with various concentrations of compounds to evaluate their effect on disrupting the activity of virulence traits of CLas and on mitigating HLB development.

Research Progress

Using an orthologous gene replacement technique, we characterized several virulence genes in the CLas genome, including genes regulating twitching motility[7], biofilm formation and cell-cell aggregation. A new gene evaluation system using a culturable bacterium *Liberibacter crescens* (Lcs) was recently developed. Since Lcs and CLas belong to the same genus, *Liberibacter*, both genomes possess high degrees of similarity in gene contents. While Lcs is not a pathogenic bacterium to citrus, it serves well for molecular characterization of gene functions (e.g., orthologous gene replacement).

Those key virulence genes/factors of CLas that were selected for potential control targets were subjected to functional validation tests. A custom-made anti-virulence compound library has been constructed from a commercial core library stock based on the types of chemical structures that consist of various ligand molecules potentially targeting the functional domains of key CLas virulence genes or factors. A few anti-virulence chemical compounds that have a strong effect on the suppression of bacterial virulence

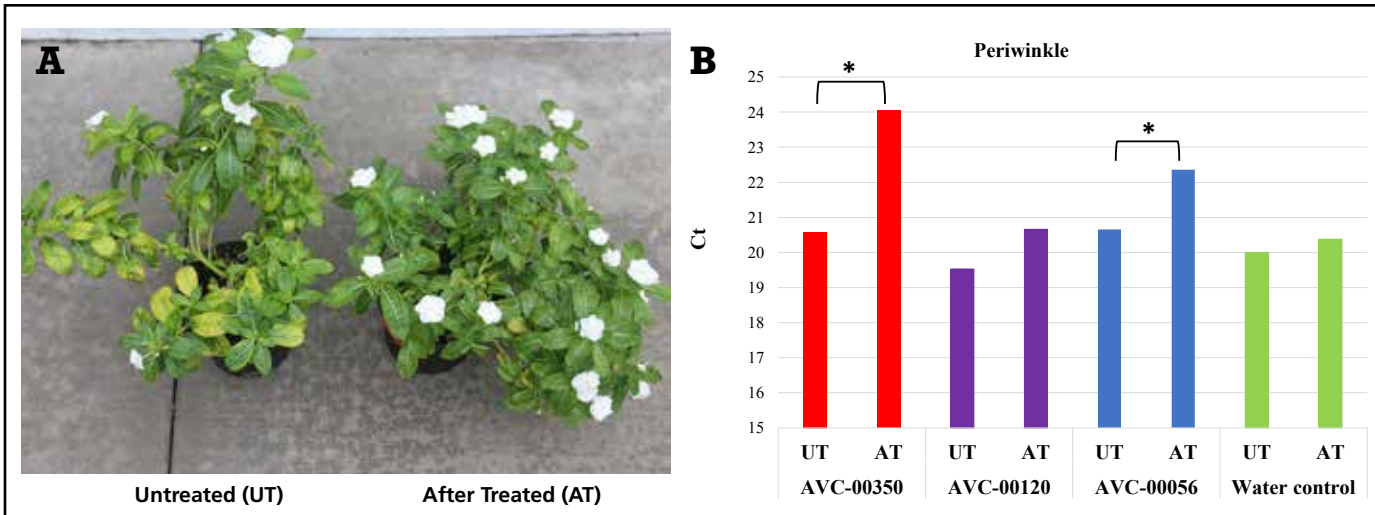

Figure 2. (A) CLas-infected periwinkle plants were foliar-sprayed with 50 µM (micromolar) anti-virulence compounds (AVC) once a week for six weeks. Control plants were sprayed with water. Plants treated with AVC showed reduced leaf yellowing compared to untreated plants. (B) Comparison of untreated (UT) and AVC-treated (AT) leaves of periwinkle plants, CLas-infected periwinkle plants treated with anti-virulence compounds AVC-00350 and AVC-00056 had significantly lower (denotes ΔCt >2) bacterial titers than those of the untreated plants.*

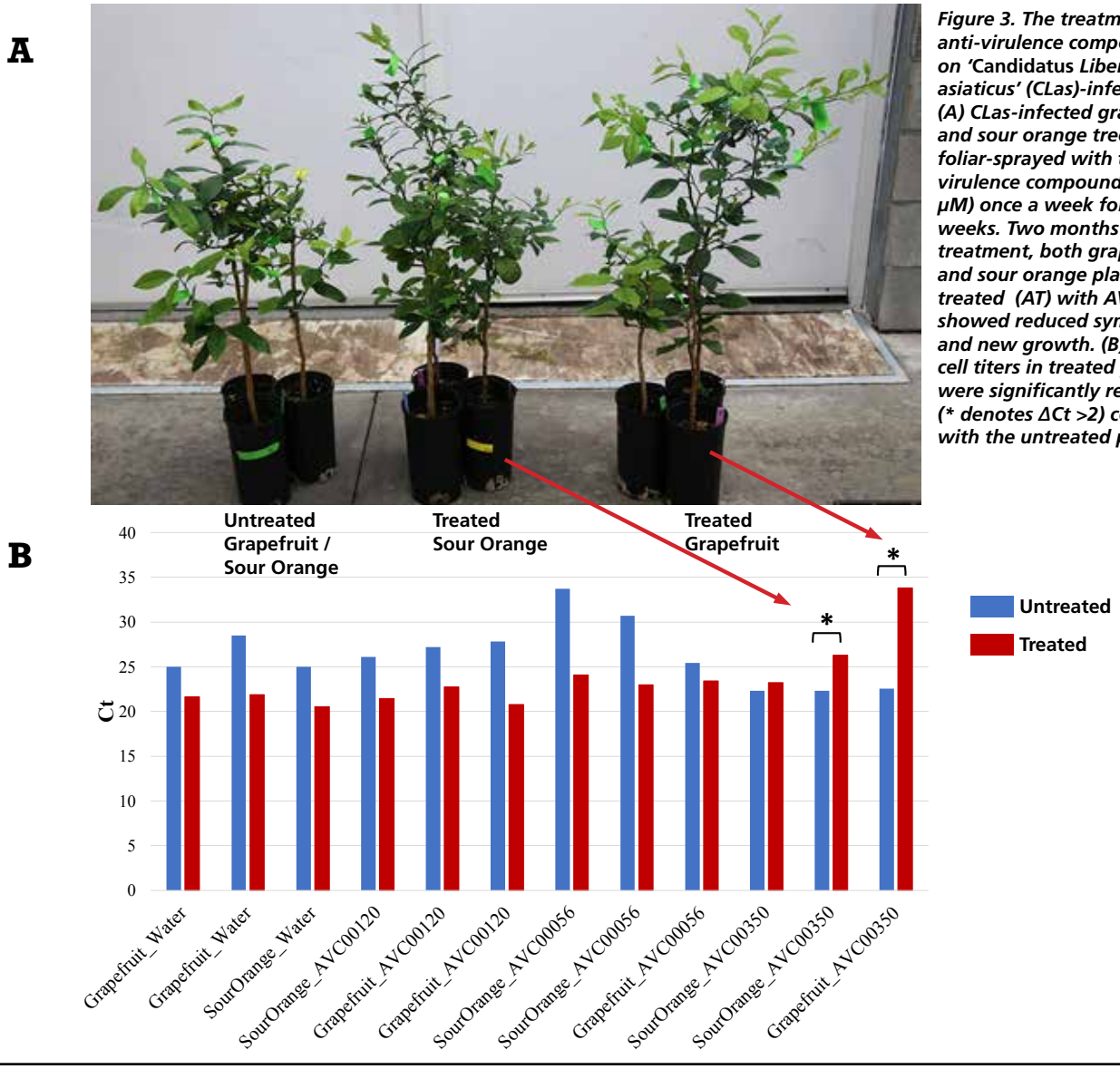

Figure 3. The treatments of anti-virulence compounds on 'Candidatus Liberibacter asiaticus' (CLas)-infected citrus. (A) CLas-infected grapefruit and sour orange trees were foliar-sprayed with the anti-virulence compounds (100 µM) once a week for eight weeks. Two months after treatment, both grapefruit and sour orange plants as treated (AT) with AVC 00350 showed reduced symptoms and new growth. (B) CLas cell titers in treated plants were significantly reduced (denotes ΔCt >2) compared with the untreated plants.*

traits, such as cell-cell aggregation, twitching motility and biofilm formation, have been identified by library screening using CLas gene-complemented *Xylella fastidiosa (Xf)* cells. For example, peripheral fringes[8] are structural features of colonies of bacteria with a twitching phenotype. Colonies lacking a peripheral fringe are designated as having a twitching defect. Several compounds, including DL-3-aminobutyric acid, showed strong effectiveness in suppressing the twitching motility of CLas *pilG*, a gene that regulates cell-twitching motility. **Figure 1** shows that the treatment of bacteria with anti-virulence molecules causes inhibition of peripheral fringe formation. Our results showed that the effective concentrations of anti-virulence compounds that suppressed the virulence traits such as biofilm formation, cell-cell aggregation and virulence gene expression were equivalent or even lower than that of antibiotics such as tetracycline and chloramphenicol. Based on the *in vitro* screening assays, anti-virulence compounds may interact with CLas virulence genes/factors resulting in a reduction or loss of pathogenicity.

Eight anti-virulence compounds that showed notable effectiveness were further evaluated on HLB-affected plants in the U.S. Horticultural Research Laboratory greenhouse in Florida. Periwinkle plants also were included in this study, as they respond well to CLas infection and chemical treatments. CLas-infected periwinkles grown in the greenhouse were foliar-sprayed with selected compounds once a week for two months. Yellowing symptoms were observed on untreated periwinkle, but treated plants showed new growth and reduced leaf-yellowing symptoms. (**Figures 2A**). Similarly, CLas-infected citrus (grapefruit and sour orange) (**Figure 3A**) showed reduced severity of yellowing symptoms after weekly treatments for two months with AVC-00350 (**Figure 3B**). Reduced symptoms also were seen on the young leaves that developed from new growth during this period. In both cases, qPCR assays showed CLas titers were reduced in treated CLas-infected periwinkle, grapefruit and sour orange plants compared with untreated plants (**Figures 2B** and **3B**). Greenhouse experimental data has suggested that several anti-virulence compounds, including AVC-00350, have considerable inhibitory effects on CLas-infected periwinkle and citrus plants and may mitigate HLB.

With the results of the effect of anti-virulence compounds on suppressing HLB in *in planta* experiments, we expect this strategy can lead to the development of a practical HLB management strategy. Our next goals are to validate this strategy on HLB-affected citrus under field conditions and to develop a practical delivery system to effectively introduce the compounds into plants to suppress and or mitigate HLB.

Conclusion

HLB management based on the use of anti-CLas virulence compounds is a potential new short-term, practical

approach to reduce symptom severity in HLB-diseased citrus. Use of anti-CLas virulence compounds could enhance the effectiveness of existing ACP treatment or other HLB management strategies. The long-term management effectiveness of this anti-virulence strategy[9] to suppress and mitigate the development of HLB largely will depend on the optimization of application procedures and the efficacy of the delivery of anti-virulence compounds into citrus trees under field conditions.

Considering the current situation of HLB in the U.S., a pest and disease management system balanced with short-term and long-term goals is needed. We envision this strategy as a novel short-term approach to supplement current HLB management programs.

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References

Allen, R.C.; Popat, R.; Diggle, S.P.; Brown, S.P. 2014. Targeting virulence: can we make evolution-proof drugs? *Nature Reviews Microbiology*. 12(4):300-308.

Gottwald, T.R. 2010. Current epidemiological understanding of citrus huanglongbing. *Annual Review of Phytopathology* 48:119–139.

Heaslip, A.T.; Leung, J.M.; Carey, K.L.; Catti, F.; Warshaw, D.M.; et al. 2010. A small-molecule inhibitor of *T. gondii* motility induces the posttranslational modification of myosin light chain-1 and inhibits myosin motor activity. *PLoS Pathogens* 6(1):e1000720.

Mahmood, H.Y.; Jamshidi, S.; Sutton, J.M.; Rahman, K.M. 2016. Current advances in developing inhibitors of bacterial multidrug efflux pumps. *Current Medicinal Chemistry* 23(10):1062-1081.

Quarles, W. 2013. IPM for Asian citrus psyllid and huanglongbing disease. *IPM Practitioner* 34:1–7.

Rasko, D.A. and Sperandio, V. 2010. Anti-virulence strategies to combat bacteria-mediated disease. *Nature Reviews Drug Discovery* 9:117-128.

Glossary

[1]Phytotoxicity: An adverse effect on plant function by a compound.

[2]Virulence: The degree of the ability of a pathogen to cause disease.

[3]Molecular ligands: A small molecule that binds or interacts with a larger molecule or protein, typically leading to functional changes of the larger molecule.

[4]*In silico*: Biological experiments conducted or produced through computer simulations or models.

[5]Orthologous gene: Genes derived from a common ancestor gene that retain similar sequences and functions across different organisms.

[6]Biofilm formation: The process by which microorganisms attach and grow on a surface, forming a three-dimensional matrix with altered growth rates and gene transcription.

[7]Twitching motility: A form of bacterial movement over surfaces similar to "crawling" in which hair-like filaments extend from the cell's exterior, attach and retract.

[8] Peripheral fringes: A physical feature of a bacteria colony edge associated with a "twitching" behavior.

[9]Anti-virulence strategy: A strategy to interfere with pathogen virulence to reduce disease development.

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