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Summer 2022 | Volume 13 • Number 3  The Official Publication of The Citrus Research Board

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### CITRUS RESEARCH BOARD MEMBER LIST

By District 2021-2022  (Terms Expire September 30)

#### DISTRICT 1 – NORTHERN CALIFORNIA

<table>
<thead>
<tr>
<th>MEMBER</th>
<th>EXPIRES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andrew Brown</td>
<td>2022</td>
</tr>
<tr>
<td>Justin Golding</td>
<td>2022</td>
</tr>
<tr>
<td>Zac Green</td>
<td>2022</td>
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<tr>
<td>Jason Reynolds</td>
<td>2022</td>
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<tr>
<td>Henk Griffin</td>
<td>2023</td>
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<tr>
<td>Megan Morreale</td>
<td>2023</td>
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<tr>
<td>Jason Orlopp</td>
<td>2023</td>
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<tr>
<td>Joe Stewart</td>
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<tr>
<td>Ram Uckoo</td>
<td>2023</td>
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<tr>
<td>Justin Brown, Chairman</td>
<td>2024</td>
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<td>Scott Carlisle</td>
<td>2024</td>
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<td>Greg Galloway</td>
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<td>Justin Huffman</td>
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<tr>
<td>John Konda, Vice Chairman</td>
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<tr>
<td>Etienne Rabe</td>
<td>2024</td>
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#### DISTRICT 2 – SOUTHERN CALIFORNIA – COASTAL

<table>
<thead>
<tr>
<th>MEMBER</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Chris Boisseranc</td>
<td>2023</td>
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<tr>
<td>John Gless III</td>
<td>2023</td>
</tr>
<tr>
<td>Gabriel Olmos</td>
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#### DISTRICT 3 – CALIFORNIA DESERT

<table>
<thead>
<tr>
<th>MEMBER</th>
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</tr>
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<tbody>
<tr>
<td>Mark McBroom, Secretary/Treasurer</td>
<td>2022</td>
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<td>Craig Armstrong</td>
<td>2023</td>
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#### PUBLIC MEMBER

<table>
<thead>
<tr>
<th>MEMBER</th>
<th>EXPIRES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melissa Cregan</td>
<td>2022</td>
</tr>
</tbody>
</table>

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Citrus Research Board  | 217 N. Encina St., Visalia, CA 93291
PO Box 230, Visalia, CA 93279
(559) 738-0246  | FAX (559) 738-0607
Info@citrusresearch.org  | www.citrusresearch.org

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.
NEW GENERATION K-PHITE 7LP
BRINGS SUPERIOR EFFICACIES TO
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- *Alternaria alternata*
- *Anthracnose*
- *Botryosphaeria dothidea*
- *Fusarium*
- *Hyphoderma sambuci*
- *Phytophthora (soil borne and aerial phases including brown rot)*
- *Pseudomonas syringae*
- *Pythium*
- *Rhizoctonia*
- *Xanthomonas ssp. (including citri)*

K-PHITE 7LP is a clear, pH neutral, linear polymer potassium phosphite exhibiting molecular stability and pathogenic activities that common materials do not display.

K-PHITE 7LP contains no sodium or chlorides for safe and compatible applications without rind stain. RE-NEW can be tank mixed with most pesticides, including fungicidal cop-per (maintain pH >6.2).

For more information including research results and scientific publications, contact;
Mark Brady, Western Marketing Manager, Plant Food Systems, Inc. (559) 731-1267
CALENDAR OF EVENTS

JUNE
7
2022 Citrus Research Board Webinar Series
For more information and additional Webinar Series dates, see page 22.

JULY
13
Citrus Pest and Disease Prevention Committee (CPDPC) meeting
For more information, visit www.cdfa.ca.gov/citruscommittee.

AUGUST
9
Citrus Research Board (CRB) meeting
For more information, contact the CRB at (559) 738-0246 or visit www.citrusresearch.org

SEPTEMBER
20
Citrus Research Board (CRB) Annual Meeting
For more information, contact the CRB at (559) 738-0246 or visit www.citrusresearch.org

SEPTEMBER
21
Citrus Pest and Disease Prevention Committee (CPDPC) meeting
For more information, visit www.cdfa.ca.gov/citruscommittee

NOVEMBER
9
Citrus Pest and Disease Prevention Committee (CPDPC) meeting
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As we look back on the past year, the Citrus Research Board (CRB) remains steadfast in adapting to the ever-changing industry and providing value to California’s citrus industry. By evaluating our operational structure and our research priority-making decision process, we continue to push ourselves to ensure the sustainable and long-term viability of the citrus industry. However, the answers we seek through research are not always found through simple solutions.

Researchers have been seeking solutions for decades in areas that we’re all familiar with. Including identifying emerging technologies that will shape our future growing operations, how new varieties will play a role in our industry, and finding solutions to the devastation wielded by vectored diseases, to name a few. We hope that through the research we fund, we’ll be able to create pathways of knowledge to generate California-centric solutions that will benefit future generations of industry members.

Just as the industry must remain nimble, so must the CRB. Our committee structure and collaboration with growers and other industry members allow us to address the industry’s challenges and seek solutions to the problems you’re seeing in your operations head-on while keeping emerging trends top of mind. In February 2022, the CRB’s Board reconvened in a workshop to revisit the CRB’s strategic plan developed in 2020 with a fresh perspective. The Board reviewed the challenges, threats, and opportunities identified during the 2020 strategic planning meeting to reevaluate how things may have changed throughout the last two years. Our ability to envision the best possible future for citrus growers requires awareness of the key driving factors. Ensuring the organization is aligned with research that considers both applied and basic research needs and the current economy while retaining an advocacy component for our growers.

As a grower-funded organization, we understand that the CRB must demonstrate its commitment to being good stewards of the dollars entrusted to us. In this issue of Citrograph, I invite you to review CRB’s annual financial report on page 12, including a comprehensive overview of the previous season’s yearly budget and a summary of all funded projects. I am proud to share that we’ve maintained a reduced assessment rate for the second year, and we hope to continue this upward trend of allocating and adjusting our resources in plans for the future.

Marcy L. Martin serves as the president of the Citrus Research Board, based in Visalia, California. She also is the executive editor of Citrograph. For more information, please contact marcy@citrusresearch.org
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A Sumitomo Chemical Company
These data represent the financial statement of the Citrus Research Board for the years ended September 30, 2019, 2020 and 2021. You are welcome to visit us at any time to discuss any elements of the program and explore our portfolio of work. This program is paid through your grower assessment dollars, and the board welcomes your feedback.

-Marcy L. Martin, CRB President
These data represent the financial statement of the Citrus Research Board for the years ended September 30, 2019, 2020 and 2021. You are welcome to visit us at any time to discuss any elements of the program and explore our portfolio of work. This program is paid through your grower assessment dollars, and the board welcomes your feedback.

-Marcy L. Martin, CRB President
On September 21, 2021, the Citrus Research Board (CRB) approved the funding of all new and continuing research projects for the 2021-22 fiscal year at the CRB annual meeting. This year, seven new projects, 13 continuing projects, four core programs (including a new core program) and one United States Department of Agriculture (USDA) sub-award were approved for funding by the Board for a total of $4,396,646 (Tables 1 and 2).

Huanglongbing (HLB)-related projects are one focus of the CRB research portfolio, which also includes projects to create and evaluate improved citrus varieties and projects that further potential disease control efforts. Work exploring and refining new tools for effective bacterial and vector management of both the...
Table 1: Listing of CRB-funded research projects for the 2021-22 fiscal year by category. See table 2 for a breakdown by project, including those funded through grants from CDFA California Pest & Disease Protection Program (CPDPP) and USDA Technical Assistance for Specialty Crops (TASC).

CRB-funded research projects are overseen by one of four CRB Research Committees – New Varieties, Vectored Diseases, Production and Post-harvest Technology, and Pest Management. The Citrus Clonal Protection Program (CCPP), a core program, is overseen by its own CRB committee.

New Varieties Research (5200) Committee

Research projects within this committee focus on improving the development and evaluation steps needed for the creation of new varieties, as well as the production of new citrus varieties to meet the market demands of the California citrus industry. Six continuing research projects, including the core Integrated Citrus Breeding and Evaluation Program,
Table 2: Listing of all CRB-funded research projects for the 2021-22 fiscal year.

<table>
<thead>
<tr>
<th>Project Number</th>
<th>Project Title</th>
<th>Principal Investigator</th>
<th>Affiliation</th>
<th>Approved Budget</th>
</tr>
</thead>
<tbody>
<tr>
<td>5200 - New Varieties</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5200-168</td>
<td>Refinement and application of greenhouse methods to evaluate scion and rootstock tolerance to CLas</td>
<td>Kim Bowman</td>
<td>USDA-ARS</td>
<td>$83,538</td>
</tr>
<tr>
<td>5200-169</td>
<td>Field testing to identify elite rootstocks that can mitigate or prevent HLB in scions commercially</td>
<td>Jude Grosser</td>
<td>Univ. of Florida</td>
<td>$28,000</td>
</tr>
<tr>
<td>5200-170</td>
<td>Development of mature citrus tissue transformation technology</td>
<td>James Thomson</td>
<td>USDA-ARS</td>
<td>$135,462</td>
</tr>
<tr>
<td>5200-173</td>
<td>Breeding for generating HLB-resistant citrus, and field evaluation of selected HLB-tolerant hybrids</td>
<td>Chandrika Ramadugu</td>
<td>UC Riverside</td>
<td>$140,000</td>
</tr>
<tr>
<td>5200-175</td>
<td>Engineering citrus for HLB resistance using gene-editing technologies</td>
<td>Vivian Irish</td>
<td>Yale University</td>
<td>$160,703</td>
</tr>
<tr>
<td>5200-201</td>
<td>CORE: Integrated citrus breeding and evaluation for California</td>
<td>Tracy Kahn &amp; Mikeal Roose</td>
<td>UC Riverside</td>
<td>$901,404</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>5300 - Vectored Diseases</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>5300-182ª</td>
<td>DATOC: Data Analysis and Tactical Operations Center (CPDPP)</td>
<td>Neil McRoberts</td>
<td>UC Davis</td>
<td>$180,408</td>
</tr>
<tr>
<td>5300-199ª</td>
<td>Risk-based survey for decision making in the management of Huanglongbing: Phase II (CPDPP)</td>
<td>Weiqi Luo</td>
<td>North Carolina State Univ.</td>
<td>$242,283</td>
</tr>
<tr>
<td>5300-205</td>
<td>Phase 2 of high-throughput sequencing as a CCPP routine diagnostic tool for variety introduction</td>
<td>Georgios Vidalakis</td>
<td>UC Riverside</td>
<td>$73,057</td>
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<tr>
<td>5300-211</td>
<td>Phage hydrolases for Liberibacter biofilm control</td>
<td>Jennie Fagen</td>
<td>North Carolina State Univ.</td>
<td>$33,967</td>
</tr>
<tr>
<td>5300-212ª</td>
<td>Predicting the likelihood of ACP/HLB dispersal into California commercial citrus groves via scenario (CPDPP)</td>
<td>Drew Posny</td>
<td>North Carolina State Univ.</td>
<td>$100,000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>New Projects</th>
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</thead>
<tbody>
<tr>
<td>5300-214</td>
<td>Field evaluation of a novel virus-like RNA as an expression vector for HLB and Tristeza management</td>
<td>Kiran Gadhave</td>
<td>UC Riverside</td>
<td>$83,337</td>
</tr>
</tbody>
</table>

ªFunding provided by the CDFA California Pest and Disease Protection Program (CPDPP)
ªFunding provided by the USDA Technical Assistance for Specialty Crops (TASC)
Table 2: Listing of all CRB-funded research projects for the 2021-22 fiscal year.

<table>
<thead>
<tr>
<th>Project Number</th>
<th>Project Title</th>
<th>Principal Investigator</th>
<th>Affiliation</th>
<th>Approved Budget</th>
</tr>
</thead>
<tbody>
<tr>
<td>5100-154</td>
<td>Citrus dwarfing of commercial varieties using TsnRNAs</td>
<td>Georgios Vidalakis</td>
<td>UC Riverside</td>
<td>$75,000</td>
</tr>
<tr>
<td>5400-165</td>
<td>Disease development, epidemiology and management of Colletotrichum dieback and Alternaria rot</td>
<td>Themis Michailides</td>
<td>UC Davis</td>
<td>$56,948</td>
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<tr>
<td>5400-166</td>
<td>A survey of wood rots in California desert citrus</td>
<td>Glenn Wright</td>
<td>Univ. of Arizona</td>
<td>$49,241</td>
</tr>
<tr>
<td>5400-167</td>
<td>Developing molecular markers to identify citrus rootstocks in the field</td>
<td>Greg Douhan</td>
<td>UC Cooperative Extension</td>
<td>$26,000</td>
</tr>
<tr>
<td>5400-168</td>
<td>Identification of a surrogate for use in citrus specific validation studies</td>
<td>Amanda Lathrop</td>
<td>California Polytechnic State University SLO</td>
<td>$90,472</td>
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<tr>
<td>5400-401</td>
<td>CORE: Pre- and post-harvest citrus disease management</td>
<td>James Adaskaveg</td>
<td>UC Riverside</td>
<td>$289,490</td>
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<td>5500-217</td>
<td>Transgenesis tools for the control of the Asian citrus psyllid</td>
<td>Omar Akbari</td>
<td>UC San Diego</td>
<td>$100,000</td>
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<tr>
<td>5500-220</td>
<td>Characterizing earwig damage to citrus fruits, and damage prevention using trunk barrier treatments</td>
<td>Jay Rosenheim</td>
<td>UC Davis</td>
<td>$64,346</td>
</tr>
<tr>
<td>5500-501</td>
<td>CORE: Citrus IPM program</td>
<td>Sandipa Gautam</td>
<td>UC Agriculture and Natural Resources</td>
<td>$200,598</td>
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<tr>
<td>5500-225</td>
<td>Developing and testing an IPM approach for managing roof rats in citrus</td>
<td>Roger Baldwin</td>
<td>UC Davis</td>
<td>$14,726</td>
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<tr>
<td>5500-226</td>
<td>California adapted Tamarixia radiata to support ACP biological control</td>
<td>Raju Pandey</td>
<td>CRB</td>
<td>$129,100</td>
</tr>
<tr>
<td>5050-010b</td>
<td>Breaking critical pest-related trade barriers for California citrus exports (TASC)</td>
<td>Spencer Walse</td>
<td>USDA-ARS</td>
<td>$444,979</td>
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<tr>
<td>6100</td>
<td>Citrus Clonal Protection Program (CCPP)</td>
<td>Georgios Vidalakis</td>
<td>UC Riverside</td>
<td>$693,587</td>
</tr>
<tr>
<td></td>
<td><strong>Total:</strong></td>
<td></td>
<td></td>
<td><strong>$4,396,646</strong></td>
</tr>
</tbody>
</table>

*Funding provided by the CDFA California Pest & Disease Protection Program (CPDPP)

bFunding provided by the USDA Technical Assistance for Specialty Crops (TASC)
received support this year with a budget of $1,449,107 (Tables 1 and 2). Continuing projects are focusing on standardizing greenhouse methods for scion and rootstock variety testing, field evaluations of rootstocks for HLB mitigation, developing protocols for enhanced genetic transformation of mature citrus tissues, development and evaluation of HLB-tolerant and -resistant citrus hybrids by conventional methods and the creation of new citrus varieties via genetic engineering for early flowering and HLB resistance or tolerance. This research committee also oversees the Integrated Citrus Breeding and Evaluation core program, led by Tracy Kahn, Ph.D., and Mikeal Roose, Ph.D., at the University of California, Riverside (UCR), which develops and evaluates new scion and rootstock cultivars for the California citrus industry.

**Vectored Diseases Research (5300) Committee**

This committee’s research projects focus on detection, eradication, control and management strategies and tools for insect-vectored diseases to minimize crop damage and economic losses. One new project and five continuing projects are receiving support this year in an amount of $713,052 (Tables 1 and 2). The new project is evaluating the mobility of a newly discovered plant-associated RNA virus for potential use as a vector for ACP/HLB control in several commercial citrus varieties. Work in this project also is testing the potential movement of this RNA to other citrus plants via pollen and aphid movement. The first continuing project is collecting information to seek regulatory approval and implement high-throughput sequencing protocols to speed up and reduce the costs of citrus pathogen detection. A second continuing project is evaluating enzymes derived from prophages present in California strains of CLas that may disrupt biofilm formation.

With the support of the Citrus Pest and Disease Prevention Program (CPDPP), the CRB continues to oversee three additional continuing projects. One is a risk-based model used to identify likely areas of HLB infection across the state. Another CPDPP-supported project will create predictive models for ACP and HLB movement between residential and commercial areas. Finally, in coordination with the CPDPP, the Data Analysis and Tactical Operations Center (DATOC) project continues to address operations-based questions for the industry in a data-driven manner.

**Production and Post-harvest Technology Research (5400) Committee**

These research projects focus on horticultural factors and production methods (both in the grove and in the packinghouse) that impact fruit quality for the California citrus industry. Maximizing food safety and minimizing trade barriers to maintain foreign and domestic market accessibility are committee priorities, as well.

Five new projects and one continuing project are receiving support this year for a total of $587,151 (Tables 1 and 2). The continuing project focuses on field evaluation of a citrus dwarfing viroid in common California citrus varieties to address citrus production needs (e.g., labor costs, water shortages, land reduction). New projects approved under this committee include a project assessing and mitigating the impact of anthracnose and Alternaria Rot on production, surveying southern California desert citrus to determine the prevalence and cause of various wood rots, a project developing molecular markers to help growers identify citrus rootstocks in the field and research efforts to identify non-pathogenic bacteria as a surrogate species for food safety studies in packinghouse research.

This year, a new core program, the Pre- and Post-harvest Citrus Disease Management core program, led by James (Jim) Adaskaveg, Ph.D., at UCR was endorsed by the committee. This core program will focus on determining economic thresholds, forecasting infection periods for diseases and timing of fungicide applications, developing sampling methods, registering new fungicides, applying fungicides in an appropriate manner and developing alternatives to fungicide treatments such as biopesticides and biological controls for pre- and post-harvest diseases of citrus caused by fungi, fungal-like organisms and non-fastidious bacteria. Research efforts this year are focusing on anthracnose, Septoria spot, Phytophthora brown rot and root rot, Sweet Orange Scab, green and blue molds, whisker mold and sour rot. New and emerging diseases will be incorporated into the program as needed in subsequent years.

**Pest Management Research (5500) Committee**

Pest Management research projects focus on eradication, control or management strategies and tools against insect pests to minimize crop damage and to maintain foreign and domestic market accessibility. Two continuing projects, two new projects and the Integrated Pest Management core program were funded for the 2021-22 fiscal year at $508,770 (Tables 1 and 2). ACP-related projects include infrastructure support for ACP colonies at the Mount Rubidoux Production Facility for research projects and biocontrol programs (with CPDPP assistance) and developing methods for genetic engineering of ACP. Non-ACP projects include a continuing project looking to characterize earwig damage and host preference and to develop management practices for earwigs and a new project looking to develop a cost-effective IPM program for roof rat control in citrus orchards.
The core Citrus IPM Program has undergone a change in research leadership with the recent departure of Monique Rivera, Ph.D., from UCR, and is currently led by Cooperative Extension Area Citrus IPM Advisor Sandipa Gautam, Ph.D., at the Lindcove Research and Extension Center. This core program conducts long-term research applying IPM strategies to manage major citrus pests in California, including ACP.

The CRB also continues to oversee a cost-sharing grant with the USDA Technical Assistance for Specialty Crops (TASC) Program providing additional research funds to researchers to develop systems-based control measures for pests of export concern (i.e., bean thrips, Fuller rose beetle, California red scale, ACP, flat mites, *Phytophthora*) in Korea, China, Australia and New Zealand.

**Citrus Clonal Protection Program (CCPP)**

The CCPP, led by Georgios Vidalakis, Ph.D., at UCR continues work to provide a safe mechanism for the introduction and distribution of clean citrus varieties to the California citrus industry and residents. The 2021-22 budget disbursement to CCPP is $693,587 (*Tables 1* and *2*).

**Summary**

The CRB remains committed to prioritizing, investing in and promoting research that improves the sustainability and profitability of the California citrus industry. At this time, HLB is a significant area of focus with 14 of these new and continuing CRB research projects actively engaged in HLB and ACP or related research projects. Other CRB-funded projects support the ongoing core programs and address current export and production needs of the California industry. The CRB continues to also identify, develop and join collaborations with other funding agencies to maximize California citrus grower investments in research. The projects underway support a sustainable California citrus industry by taking a proactive stance on identifying and implementing short-, medium- and long-term solutions to the threats and concerns of the California citrus environments and markets.

*Joey S. Mayorquin, Ph.D., is a research associate with the Citrus Research Board in Visalia, California, and serves as associate science editor of Citrograph. Melinda Klein, Ph.D., is Chief Research Scientist at the Citrus Research Board in Visalia, California, where she also serves as scientific editor of Citrograph. For additional information, contact melinda@citrusresearch.org*
Californian citrus producers in Districts 1 (Northern California) and 3 (California Desert) should make plans to attend the appropriate Citrus Research Board (CRB) nomination meetings. Four positions in District 1 and one position in District 3 expire on September 30, 2022. The public nomination meetings will be conducted by officials of the California Department of Food and Agriculture (CDFA) and the CRB.

In addition to the elected positions mentioned above, the Board will have the opportunity to consider the extended appointment of the public member at its annual meeting on September 20, 2022.

The detailed list of seats expiring this September may be found on page 6, where the current Board member roster appears by name, district and year of term expiration. Member terms are for three years.

Board Member Responsibilities

The bulk of the Board’s time is spent considering a broad portfolio of citrus research proposals and projects totaling approximately $10 million. Members are involved in:

- developing research priorities and requests for proposals,
- prioritizing responses, awarding funds,
- devising successful implementation strategies,
- assessing progress and
- providing critiques of project results.

The 21-member CRB is served by 13 staff with headquarters in Visalia and two laboratories in Riverside. There is a relatively high time commitment compared to many other volunteer commodity boards, but those involved with the CRB are integral in directing the response to critical citrus research needs in California. Members are expected to attend Board meetings and to serve on research and/or administrative committees. A typical fiscal year has five Board meetings in various geographic locations, occasional committee meetings and the opportunity to attend a number of citrus-related conferences and events.

Voter Qualifications

(as provided by the CDFA Marketing Branch)

- Any owner, officer or employee of an entity in California in the business of producing, or causing to be produced for market, 750 or more standard field boxes (or the equivalent) of any variety of citrus is qualified to participate in the nomination proceedings.
- If you wish to nominate a person at a nomination meeting to serve on the Board, you should determine the candidate’s eligibility and willingness to serve prior to the nomination meeting.
- An individual person is entitled to represent only one legal entity at a nomination meeting.
- In the case of a partnership, only one of the partners may vote.
- In the case of a corporation, a person affiliated with the corporation, preferably an officer, may represent the corporation.
- A married couple operating a production entity is entitled to just one vote, unless each spouse owns and operates separate and distinct entities.
- To participate in a district’s nomination meeting, a business entity must have citrus production within that district. Any entity with production in more than one district must choose a single district in which to participate to vote. If a separate production entity can be proven as the operating entity in another district, the person qualified to act as the representative of that entity may vote in that district, even if he/she has voted as a representative of another entity in another district. Essentially, each separate citrus-producing business entity is entitled to one vote in the district in which it operates.
- Voting by proxy is not permitted.

For more information, the California Citrus Research Program Marketing Order may be viewed on-line at: http://www.cdfa.ca.gov/mkt/mkt/pdf/Laws/CitrusResearchMarketingOrder.pdf Questions may be directed to CDFA Marketing Branch Senior Agricultural Economist Mirek Wilczek at (916) 900-5018 or CRB President Marcy L. Martin at (559) 738-0246. ☯

Caitlin Stanton is the communications coordinator with the Citrus Research Board and also serves as the editorial assistant on Citrograph. For more information, please contact caitlin@citrusresearch.org
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CITRUS RESEARCH BOARD WEBINAR SERIES STREAMING THIS MONTH

Caitlin Stanton

The Citrus Research Board (CRB) is looking forward to hosting the 2022 Citrus Growers Educational Webinar Series this month. The four one-hour webinars are scheduled for June 7, June 14, June 21 and June 28. Each session will highlight a specific CRB-funded research area and provide technical insight for growers. Previous series were successfully held in October 2020 and June 2021 and featured an impressive line-up of extension and industry professionals. Past topics included an update on pesticide laws and regulations, insight into citrus IPM, a report on California’s water situation and a preview of new cultivar’s being developed through the CRB’s Core Breeding Program.

Continuing education units will be available through the California Department of Pesticide Regulation and Certified Crop Advisers, pending approval.

For up-to-date information—including speakers, topics and CEU information—please visit www.citrusresearch.org

Caitlin Stanton is the communications coordinator with the Citrus Research Board and also serves as the editorial assistant on Citrograph. For more information, please contact caitlin@citrusresearch.org
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As I sit here in mid-March, we are left wondering what is next? We are exiting the pandemic with caution as to whether we will truly ever get back to normal. We had to modify our Citrus Showcase to be able to put on the show regardless of the everchanging state and local health requirements, only to see the mandates fade just before the show. It was great to see the industry together two years after our in-person Showcase.

California Citrus Mutual (CCM) is a voluntary non-profit trade association whose mission is “to protect and enhance the viability of California’s citrus growers.”

Lieutenant Governor of California Eleni Kounalakis visited with exhibitors to learn more about each facet of the citrus industry.
We leave one crisis, and another one appears. The war is undoubtedly on people’s minds as the impacts extend far beyond the borders of Ukraine. The supply chain is disrupted yet again with significant uncertainty related to fuel prices and fertilizer availability. All of this is compounding the inflationary pressures that continue to impact the costs to grow, pack and transport citrus.

Lemon pricing has been declining with higher volumes this year in California and escalating imports. Navel and mandarin prices are high, but will growers see a justifiable return with lower volumes and significantly higher costs? The U.S. Department of Agriculture (USDA) ceased enforcement of the grapefruit marketing order standards that reside in Florida, causing a significant increase in Mexican grapefruit imports – all without any notice to the affected industry. The relationship between China and the United States is volatile and has the potential to further disrupt important citrus exports.

Another year of drought is upon us despite December’s record precipitation. Groundwater Sustainability Agencies (GSAs) are beginning to curtail pumping to be compliant with the state’s Sustainable Groundwater Management Act (SGMA). All the while, the biological opinions in the delta are being rewritten in court to further enhance species protection and to the detriment of communities and agriculture that rely on that supply.

An agricultural coalition that included CCM was able to convince the Governor to veto damaging labor legislation more commonly known as “Card Check.” Will it be back again? Of course, and we must be ready for it. Attorneys are continuing to go after employers under the misguided and abused labor law known as the Private Attorney General Act (PAGA). This allows attorneys to hold employers hostage and extort large settlements. A ballot measure supported by CCM is gathering signatures to reform the law and diminish the ability of private attorneys to extort businesses.

The federal government is banning chlorpyrifos, putting the rest of the country on par with California’s bad policy. The state is moving forward with pilot pesticide notification programs and has released a second draft regulation for neonicotinoids. A report from the Sustainable Pest Management Working Group, scheduled to be completed in the summer, could set the path toward additional pesticide regulation and/or additional support for agricultural programs that have been continuously cut from the state budget.
So, to honestly answer the question of what is next – the truth is no one really knows. What we do know is what is in front of us now, and there is no shortage of issues that threaten your ability to grow citrus in California. We also know that we must be focused on citrus-specific issues and work effectively with others on the more general agricultural problems. This allows us to address citrus growers’ challenges more effectively according to the strategic direction developed by the Board with input from staff.

Will it be successful? The answer to that depends on your definition of success. We can set our ambitions too high and guarantee failure, or too low and guarantee success. A wise CCM board member properly identified our scope as the “hard, but doable.” This requires a true understanding of the positive or negative trajectory of any given issue and putting the right amount of attention to bend that trajectory in the desired direction. This kind of “success” always brings criticism that more could have been accomplished.

We welcome that criticism, as our true goal is to always do more and better for all citrus growers as success or failure for a collective of growers is not a finite issue, but an infinite one. Wins or losses are for finite games that can be measured, like at the end of nine innings or four quarters. Infinite games are ones that cannot be won or lost but are defined by being able to stay in the game. They require persistence and an attitude of never giving up. That is what is embodied in this industry and will continue to define our future success. So, it is not “what’s next,” but “when!” And when it does, you will have advocates at CCM to protect and enhance the viability of California’s citrus growers.

Casey D. Creamer is the president and chief executive officer of California Citrus Mutual in Exeter, California. For additional information, contact Casey@cacitrusmutual.com
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As the new outreach subcommittee chairman for the Citrus Pest & Disease Prevention Committee, it is my hope to follow the path and the efforts laid in place by my predecessor, Mark McBroom, who is now serving as the Committee’s newest chairman. As we work in tandem to guide the outreach efforts of the Citrus Pest & Disease Prevention Program (CPDPP), we will support citrus industry members through ongoing outreach to residents, industry members and elected officials focused on the importance of working together to prevent the spread of huanglongbing (HLB) in California.

Responding to Growers’ Concerns

At the end of 2021 and earlier this year, we heard directly from citrus growers along the Central Coast region about their concerns regarding the sharp increase of Asian citrus psyllid (ACP) populations in residential areas near commercial operations. Kicking our outreach efforts into high gear, the CPDPP’s outreach contractor, Nuffer, Smith, Tucker, worked with local industry members, media outlets and journalists in these key areas to build residents’ awareness of the devastating impacts ACP and HLB could have on their backyard citrus trees. To best connect with homeowners on the importance of this issue, the CPDPP’s outreach team worked with the following goals in mind:
quickly notify individuals of the dangerous pest,
• highlight the consequences should HLB take hold of local citrus trees,
• encourage cooperation with agricultural officials as they inspect citrus trees and
• ask residents to inspect their own trees for signs of the pest and disease and report potential sightings to the California Department of Food and Agriculture (CDFA) pest hotline.

Through an opportunity developed with a broadcast group in the Santa Barbara area, our outreach team coordinated multiple interviews with a variety of stakeholders to highlight the severity of what increasing populations of the dangerous pest could mean for the Central Coast. Featuring interviews with local area grower Zach Rissel and Victoria Hornbaker, director of the Citrus Pest & Disease Prevention Division (CPDPD), the broadcast segment discussed the implications of ACP populations in the area, what it would mean for homeowners and growers alike should HLB make its way to the Central Coast and what residents can do to help. The pre-recorded interview aired several times across three different news stations throughout Santa Barbara County and garnered more than 25,000 impressions.

Building on Outreach Success via Multiple Methods

To capitalize on the success of local media outreach, the CPDPD outreach team deployed a range of additional tactics to ensure the program’s key messages remained top of mind for residents. CPDPD agricultural crews often serve as our “boots on the ground” eyes and ears in residential areas for the CPDPD as they travel through neighborhoods surveying and inspecting citrus trees. Because of their work, the CPDPD team is able to connect with hyperlocal communities with higher refusal rates or homeowners who do not allow CPDPD staff on their property to inspect their citrus trees. The team can arrange to utilize additional outreach tools, including customizable door hangers and targeted social media ads with messaging focused on the importance of cooperating with agricultural officials and why they’re there to help. Thanks to the efforts of CPDPD staff and industry members alike, our outreach team is able to respond to issues that are occurring on a more localized level.
As we look toward the months ahead, the CPDPP outreach program will continue to work together with the industry to identify fresh narratives and strategies to quickly share important and relevant news about HLB and ACP throughout the state and the growing presence of ACP along the Central Coast. California’s citrus is part of our livelihood, and the widescale effort to preserve backyard and commercial citrus in California has reached a critical point – it hinges on public education, outreach and awareness about the disease, and preventive measures about ACP and the spread of HLB. That said, as detections of the disease creep closer to major commercial citrus areas, we must recognize that we all need to work together toward preventing the spread of the pest and disease. If you’re interested in learning more or would like to collaborate on future outreach opportunities in your area, please feel free to connect with me or with the CPDPP via CitrusInsider.org.

Kevin Ball is the outreach subcommittee chair for the Citrus Pest & Disease Prevention Committee. For additional information, contact Kevin at kevin.ball@aglandca.com.
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This issue of Citrograph is focused on innovations in new varieties and the growers who continue to move the industry forward. We recently asked several citrus industry members for their insights on new varieties and current industry issues in California.

JOHN S. GLESS
Gless Ranch
Riverside, Tulare and Kern Counties

What is your main concern with growing citrus today in California?

This can be answered by one word: water. Without water, anything else that we’re doing doesn’t matter. There is no issue that even comes close to water.

What innovations have you seen or heard about that you think will provide a significant benefit to the citrus industry in the near future?

As far as new innovations in the field, I don’t see many that are largely affecting me. Equipment we were using 20 years ago is the same or better than what we have now. Most of the new innovations are focused on environmental compliance. As a grower, the new equipment is much more difficult to maintain and repair. In the packinghouses, there
are incredible innovations, including automation and sorting techniques that maximize utilization and quality to get us better returns as growers. There are also new pruning techniques and innovative ways of “training” the trees, which are helping with yield and quality.

**Where would you like to see research efforts focused in the upcoming years and why?**

The number one area I would like to see efforts focused is water storage. Without water, there is no future. Disease-resistant or -tolerant rootstocks against HLB are extremely important, but without water, it doesn’t matter. We also need new chemistries to fight red scale, thrips and ACP. They keep taking materials away from us, and we are running out of tools in our tool chest as we are seeing resistance to the chemistries that we have.

**KENNY WILEMAN**  
Porterville Citrus  
Tulare County

**What is your main concern with growing citrus today in California?**

I am concerned by the rising costs across the board. Water issues are also concerning, but they are encompassed by these rising costs.

**What innovations have you seen or heard about that you think will provide a significant benefit to the citrus industry in the near future?**

The citrus industry is lacking a lot of technology, which may be due to a lack of belief in technological innovations on the growers’ side. One area where there has been more innovation is in rootstocks. Recently, I have been hearing about more tolerant rootstocks that require less water and are HLB/ACP resistant.

**Where would you like to see research efforts focused in the upcoming years and why?**

I would like to see more money go toward HLB/ACP resistance; and, as we try to use less water, the possibility of a drought-tolerant rootstock should be explored.

**Caitlin Stanton is the communications coordinator with the Citrus Research Board and also serves as the editorial assistant on Citrograph. For more information, please contact caitlin@citrusresearch.org**
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Project Summary

The introduction of navel oranges to California in the late 1800s is among the most important plant introductions to the country and is recognized as initiating the citrus industry in Riverside, California. Production of navel oranges spread to neighboring communities and eventually throughout the state.

Today, navel oranges are still the most widely grown variety of citrus in California. Seedlessness is one of the many desirable characteristics of navel oranges, but it requires navel varieties to be maintained by clonal propagation. As a result, all navels are nearly genetically identical and can trace their ancestry back to two trees planted in Riverside in 1873. One of those two historic trees, known as the ‘Parent Washington’ navel, is still alive today. While clonal propagation of navels ensures that important quality characteristics are preserved for generations, it also increases their susceptibility to changing climate conditions, including increasing temperatures, extended periods of drought and the introduction of exotic pests and pathogens. To ensure navel oranges are protected from these challenges in the future, we developed a set of resources, including a high-quality genome sequence of the ‘Parent Washington’ navel. This genome sequence will enable investigation into the genetic basis of traits that distinguish navel varieties, including flesh color and maturity, and serve to facilitate their genetic improvement into the future.
Introduction

Navel oranges are the most widely grown type of citrus in California. Recent estimates indicate that navel oranges are cultivated across 117,000 acres, which amounts to more than 40 percent of citrus-bearing acreage in the state (CDFA 2020). The precise origin of navel oranges is uncertain, but ‘Washington’ navel oranges are believed to derive from a bud or limb sport identified on a cultivar of sweet orange in Bahia, Brazil (Roistacher 2013). Historical records indicate that the first attempt to share propagated material with the U.S. Department of Agriculture in Washington, DC failed as the budwood was inviable upon arrival (Roistacher 2013). Eventually, grafted navel orange trees were successfully imported in the early 1870s and later made their way by train from Washington, DC to Riverside, California (Roistacher 2013). Eliza and Luther Tibbets planted two or three of these trees on their property in Riverside (Roistacher 2013). Fruit from the ‘Washington’ navel was first showcased at a local citrus fair in 1879.

Navel oranges are valued for having large, seedless, deep-colored orange fruit with rich flavor (Reuther et al. 1967). The superiority of navels was quickly recognized, and demand for ‘Washington’ navel budwood ballooned, kickstarting the citrus industry in the state.

The importance of the introduction of the ‘Washington’ navel to California was recognized early on. In 1902, one of the original trees was transplanted from the Tibbets land to a small park located on the corner of Magnolia Avenue and Arlington Avenue in the city of Riverside. This marked the beginning of significant efforts to preserve the historic tree, and these efforts have continued for the past century. One plaque located in the park identifies the tree as “one of two original trees from which all navel oranges in California have descended” and another commends Eliza Tibbets for “her good work in planting in Riverside in 1873 the first ‘Washington’ navel orange trees.” The ‘Parent Washington’ navel, as it is commonly called, is alive and well and at least 149 years old as of this writing.

Many navel varieties with unique characteristics have been identified through the years. The University of California, Riverside Givaudan Citrus Variety Collection maintains a collection of more than 70 navel varieties. Previous work...
has reported that at least 20 varieties have been grown commercially (Kahn et al. 1995). Navel orange varieties are presumed to trace their ancestry to the ‘Parent Washington’ navel and have been identified as bud or limb sports with distinct characteristics. Because navel oranges do not produce seeds, the identification of sport varieties is the primary mechanism for genetic improvement. In 2020, nearly a quarter of navel acreage in California was planted with varieties derived previously from sports including Cara Cara, a variety with deep pink flesh, and late maturing varieties (e.g., Lane Late or Powell) (CDFA 2020). The ‘Parent Washington’ navel established the California citrus industry; and it continues, together with associated sport varieties, to be the leading citrus type grown in the state.

Research Overview and Future Directions

Although navel fruit are valued for a combination of characteristics, the trees themselves have been noted to have only moderate vigor and a range restricted by sensitivity to heat during critical phases, including flowering (Reuther et al. 1967). Clonally propagated varieties, like the navel, are especially sensitive to changing environmental conditions, including the introduction of new pests and pathogens, and can only be improved through the identification of naturally occurring limb sports as discussed above or by induced mutation², including gene editing. We set out to develop the resources needed to support navel improvement and its continued success in our warmer, drier state.

An essential resource for timely genetic improvement in any crop species is a genome³ sequence. A genome includes a near complete reconstruction of the DNA sequence of an individual, including the location and sequence of genes. These sequences can expedite directed improvement of cultivars like the ‘Washington’ navel and provide insight into the nature of spontaneous mutations that give rise to limb sports. We produced a high-quality genome for the ‘Parent Washington’ navel using state-of-the-art long-read sequencing technologies (347X coverage). The assembly is 335 million base pairs (Mb) in length with the nine longest sequences representing the nine chromosomes typical in citrus species. These nine sequences contain a large portion of the total assembly sequence (80 percent), indicating that the reconstruction of the genome is highly contiguous. Identification of the location and sequence of genes is also complete. Approximately 33,000 genes were detected by integrating in silico gene identification methods with measurements of gene expression levels. Two additional navel varieties, Cara Cara and Powell, also were sequenced at lower coverage to compare to the ‘Parent Washington’ navel. With this genome sequence, we can explore what distinguishes a navel orange from other sweet oranges and also begin to dissect the genetic control of characteristics of navel orange varieties derived from the parent tree. For example, is there a mutation responsible for seedlessness or that gives rise to the characteristic navel structure at the apex of the fruit?

It also has been previously noted that the ‘Parent Washington’ navel has given rise to a surprising number of limb sports (Reuther et al. 1967). We have initiated efforts to characterize the nature of spontaneous mutations, both in the parent tree itself and in commercially important navel varieties including Cara Cara and Powell, a late-season cultivar. By characterizing the number and types of mutations in these cultivars, we can begin to understand the mechanism responsible
for naturally occurring mutations and potentially leverage this to develop improved navel varieties in the future.

Finally, the genome sequence will support targeted improvement of navel oranges using gene editing, including CRISPR/Cas9 (Wada et al. 2020). CRISPR/Cas9 is a technology that enables precise manipulation of the DNA sequence. Knowledge of the location and sequences of genes in navels will expedite the identification of sequence targets for editing and support cultivar improvement using these methods. The genome of the historic ‘Parent Washington’ navel is the foundation for future improvement of navel oranges and can support their continued success in California.

GLOSSARY

1Bud or limb sport: A part of a plant with a stable morphological difference. The underlying cause of this difference is typically attributed to a spontaneous genetic mutation.

2Mutation: A change in DNA sequence.

3Genome: An organism’s complete set of genetic instructions encoded by DNA.

REFERENCES


Danelle Seymour, Ph.D., is an assistant professor of genetics, Emmanuel Avila de Dios is a post-doctoral researcher, and Taylor Beaulieu is a doctoral candidate in plant biology, all at the University of California, Riverside. For more information, contact danelle.seymour@ucr.edu
Project Summary

Citrus hobbyists in California would like to grow varieties for which budwood currently is unavailable from the Citrus Clonal Protection Program (CCPP). Propagation and movement of citrus trees that originate from budwood that has not completed CCPP’s therapy and variety indexing¹ (VI) procedures threatens citrus in California due to the potential introduction and spread of multiple pathogens, including ‘Candidatus Liberibacter asiaticus’ (CLas). This project identified 20 such citrus varieties and initiated their introduction into the CCPP for the production of therapied, pathogen-free VI budwood sources.

Introduction

Through the hobbyist audience of my website fruitmentor™ and YouTube channel (Willey 2016), I learned there was a demand for budwood of citrus varieties that were unavailable from the CCPP and that trees of unavailable varieties already were being propagated and moved into different parts of California. Beginning in 2015, I visited a number of small nurseries in various California locations including San Jose, Orange County, Riverside and San Diego. I discovered trees of exotic Vietnamese pummelo varieties that were unavailable from the CCPP being sold in Orange County (Figure 1). The trees had been grafted using budwood from a Riverside backyard. I learned of a number of citrus varieties that were for sale including the following pummelo varieties: Buoi Oi (Figure 2), Bien Hoa, Thanh Tra and Shatian. I found this especially disturbing because the first documented case of huanglongbing (HLB) in California was in 2012, and it was believed that the likely source was a
I discussed my findings with CCPP Director Georgios Vidalakis, Ph.D., and volunteered to send some of these varieties to the CCPP. Vidalakis provided the steps needed to submit budwood to the CCPP for therapy and VI testing. Buoi Oi budwood was collected from a backyard tree in San Jose and sent to the CCPP for cleanup. The CCPP citrus variety introduction process uses an in vitro tissue culture procedure (Navarro et al. 1975) involving shoot-tip grafting to remove any pathogens such as bacteria, viruses and viroids that the original tree and budwood may contain. Because shoot-tip grafting produces citrus plantlets using microscopic pieces of budwood tissue, the survival rate of the produced plants can be low, and none of the shoot-tip grafted plants from my first three Buoi Oi budwood shipment attempts survived. After discussing my failed Buoi Oi introduction with Vidalakis and other citrus experts, this project was proposed to the Citrus Research Board (CRB) in 2019.

### Identifying and Introducing Citrus Varieties

The first task was to identify which varieties should be introduced in addition to those that already had been identified. California citrus hobbyists were surveyed to get input on varieties via my email list, articles on my website and my YouTube channels. The surveys took place first through email and later via more sophisticated survey software. English, Spanish, Chinese and Vietnamese speakers were surveyed. They were asked several questions, including what varieties they would like to grow that currently are unavailable from the CCPP. Hundreds of email messages and online survey responses were received. Zoom meetings with California fruit hobbyist groups provided further insight into desired varieties.

Surveys revealed a range of unavailable varieties of interest including those currently being grown by hobbyists. This was especially helpful in the case of the Vietnamese pummelo varieties Bien Hoa and Thanh Tra because no other source was known. A hobbyist who is growing these pummelo varieties introduced budwood to the CCPP for
therapy and VI testing. To improve the chances of successful introduction, in addition to the in vitro tissue culture of the original budwood, the CCPP grafted back-up plants of Bien Hoa, Thanh Tra and Buoi Oi in its quarantine greenhouse using a traditional grafting technique. The back-up plants then could be used for additional shoot-tip grafts if the plantlets produced from the original budwood failed. In determining the varieties to be introduced, four criteria were considered (Table 1):

1. Have trees of the variety ever been sold in California?
2. Is the variety available in other HLB-infested states (i.e., could diseased budwood or trees be easily smuggled into California)?
3. Is the variety currently being grown by hobbyists in California (i.e., could California hobbyists trade diseased budwood)?
4. Was the variety requested by more than one person?

A large number of varieties was suggested, and using these criteria helped to limit introduced varieties to those whose introduction would most reduce the spread of disease. Any variety that did not meet at least one of the four criteria was eliminated from the candidate list for CCPP introduction.

In researching the varieties identified in the surveys, a California website was discovered that was offering for sale budwood of 49 different citrus varieties, seven of which were not available from the CCPP. This was reported to the California Department of Food and Agriculture (CDFA), and it was observed a few weeks later that these 49 varieties were no longer available for online ordering. The seven varieties from this website that were not available through CCPP were included in the set of varieties introduced in this project.

Altogether, the project has initiated the introduction of the following 20 unavailable varieties into the CCPP so that California citrus hobbyists will be able to grow them without the risk of spreading disease:

1. **Buoi Oi** is a low-acid Vietnamese pummelo with dry flesh and has been sold in many small nurseries all over California.

2. **Bien Hoa** is a Vietnamese pummelo that has been propagated in Riverside and has been sold in Orange County and San Jose.

3. **Thanh Tra** is another Vietnamese pummelo that has been propagated in Riverside and has been sold in Orange County and San Jose.

4. **Sa Teen Yau Pummelo** or 沙田柚 (Shatian) is a low-acid pummelo whose albedo and flavedo are used in Cantonese cuisine. There have been many requests for this variety. Trees said to be of this variety were sold in Hacienda Heights before HLB was discovered in the area.

5. **Poorman Orange** (also known as New Zealand Grapefruit, Golden Grapefruit and other names) is a Pummelo hybrid popular among hobbyists in northern California because it does well in the cool climate there. Hobbyists want to propagate it, but seeds produced are hybrids rather than clones of the mother tree.

6. **Hyuganatsu** is a Japanese pummelo-like fruit that may be a sweet orange hybrid. It was requested by hobbyists.

7. **Kinkoji** (also known as Bloomsweet Grapefruit) is a hybrid of pummelo and mandarin that came from Japan and is being grown by hobbyists in northern California and Texas. The fruit has a pleasant flavor, and the trees are cold-hardy.

8. **Ichang Lemon** (or Hsiang yuan) is believed to be a hybrid of papaya and pummelo. It has high-quality juice, like lemon juice, and a delightfully fragrant rind. It is grown by hobbyists in California. Budwood and trees have been sold in California despite the lack of a CCPP VI budwood source.

9. **Shinn Satsuma** is a variety of Satsuma mandarin imported from Japan in the late 19th century and sold in Fremont, possibly through the 1960s. It is believed to be different from Owari.

10. **Shiikawaasa** or Citrus Depressa, requested by many people, is a small-fruited sour mandarin that is very popular in Japan.

11. **Tankan** is a tangor originally from China that spread to Taiwan and Japan. It came into the U.S. from Taiwan. Hobbyists in California have attempted to grow it from seed, and trees are available for purchase in Florida.

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1 Variety information was compiled from the University of California, Riverside Givaudan Citrus Variety Collection, “Hardy Citrus for the Southeast” Southeastern Palm Society and from information collected through this project.
12. **Ten Degree Tangerine** may be a cross of Yuzu and a mandarin and came into California from Texas. It is valued by hobbyists for its cold-hardiness. Budwood has been sold in California despite the lack of a CCPP VI budwood source.

13. **Allspice Tangelo** (Figure 3) is a cross between 'Imperial' grapefruit and 'Willowleaf' mandarin. The fruit has tender and juicy flesh and a rich, tart and spicy flavor and aroma. The fruit is grown by hobbyists in northern California and other hobbyists are attempting to grow it from seed.

14. **Sunquat** is said to be a hybrid between lemon and kumquat, but it may be a mandarinquat. It is being grown by hobbyists in California, Texas and other states and is sold by nurseries outside California.

15. **Morton Citrange** is a hybrid of sweet orange and *Poncirus*. It is valued by hobbyists for its cold-hardiness. The tree is said to have ornamental value and the fruit is said to have grapefruit-like flavor. Budwood and trees have been sold in California despite the lack of a CCPP VI budwood source.

16. **Orton Englehart Hybrid** appears to be a hybrid of orange and grapefruit. The juice is delicious. The variety was bred and sold in the Glendora area by Orton Englehart, a citrus farmer. This variety is also traded by hobbyists.

17. **Feminello Sfusato** (Figure 4) is a major lemon variety in Italy. Its juice is used in Italian cuisine and its skin is used to make the liqueur limoncello. Feminello Sfusato was a very common survey response and was one of the most requested varieties. Some people requested it as ‘Sfusato Amalfitano’ or ‘Sorrento lemon.’ People on internet forums suggested that they would be willing to smuggle or engage in other reckless behavior to acquire it.

18. **Ichang Papeda** is said to be the most cold-resistant of all the evergreen species in the orange sub-family, making it of interest to hobbyists. Budwood has been sold in California despite the lack of a CCPP VI budwood source.

19. **Ujukitsu** (Figure 5) may be a sweet orange hybrid and was requested by many people. Ujukitsu trees are readily available in the U.S. The fruit has a nice lemony flavor, but it is not sour.

20. **Sanbokan** is a Japanese citrus fruit of unknown origin. It has a sweet lemony flavor and was requested by hobbyists. It has been grown in California and also is available in other states with HLB.

Another objective of the project was to make the public aware of introduced varieties when they become available. To do this, fruit of some of the varieties were picked at the University of California, Riverside Givaudan Citrus Variety Collection. Photos and videos of these varieties were taken and will be published when the varieties are available.
When the varieties introduced via this project are made available, hobbyists will be able to grow desired varieties without resorting to smuggling and/or propagations thereby reducing the risk of further spread of HLB and other diseases in California.

**CRB Research Project #5200-172**

**Glossary**

¹Variety Index (VI): A comprehensive set of diagnostic tests, including bio-indexing and laboratory tests, to detect all known graft transmissible pathogens of citrus that are required before release from quarantine can be requested.

**References**


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I thank all of the individuals from the CCPP and the UC Riverside Givaudan Citrus Variety Collection for their support of this project.

*Dan Willey is the founder of fruitmentor™, a company that is using Silicon Valley technology to reduce the spread of citrus diseases. For additional information, contact dan@fruitmentor.com*
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Project Summary

Increasingly sophisticated genetic technologies allow scientists to precisely insert beneficial genes into plant genomes and accurately predict the activity of the new genes in the host plant. However, public acceptance of genetically modified plants has not kept up with these technological advancements. Research at the U.S. Department of Agriculture (USDA) Western Regional Research Center, in conjunction with the Citrus Research Board (CRB) through project #5200-141, has developed Lilac Limes using advanced genetic technology. A series of control elements (promoters) were discovered to investigate the possibility of imparting characteristics specifically in the fruit. These elements were used to produce the anthocyanin giving a purple coloration (Lilac) to limes as a proof of concept using citrus-derived DNA. Based on the analyses of the fruit, there were no differences in pH, Brix or flavor. This striking purple fruit could help lead to dialogue with consumers and to encourage greater acceptance of the use of genetic technology in food plants. The Lilac Limes may further be used to learn from federal regulators and develop a plan specific for citrus deregulation.

Introducing Lilac Limes

The deep purple and red colors of some citrus varieties, such as blood oranges, are due to natural pigments deposited in the fruit tissue as they develop. These citrus varieties are popular with consumers and provide health benefits through the antioxidant properties of the pigments (Proteggente et al. 2009). Once the federal deregulation process is complete, consumers can look forward to a new variety of colored citrus – the Lilac Lime – which, as its name suggests, is an attractive purple fruit encased in a green peel. In addition to their aesthetic qualities, Lilac Limes also offer additional health benefits over their green counterparts through the addition of anthocyanin (Proteggente et al. 2009).
However, Lilac Limes are more than just an interesting new variety of citrus. They represent the culmination of years of genetic modification technology research, and their success offers promising new avenues to genetically modify crops to benefit consumers and farmers. The Lilac Lime was produced by transferring the pigment genes from the blood orange Moro and promoter control element from the sweet orange Valencia into Mexican Lime plants. Thus, the pigment produced in Lilac Limes originates from other edible citrus varieties and is specifically expressed in the fruit. Limiting the color to the fruit provides a crisp burst of eye-pleasing color. The USDA team hopes these qualities will aid in the deregulation process and encourage consumer confidence in the limes. The Lilac Lime lines moving forward in the testing process contain a citrus promoter and gene essentially taken from one cultivar put back into another, which is possible through breeding. We think this process – called intragenics – may ease the deregulation process since native citrus DNA is being manipulated and put back into citrus. In addition to developing and refining the technology to make Lilac Limes possible, the team also aims to navigate the complex regulatory process of bringing the fruit to consumers.

Genetic Technology and the Public

Modern genetic modification – in which beneficial genes are transferred from one organism to another – has been used to improve crop plants for many years (Turnbull et al. 2021). Previously, new plant lines were created using modern genetic modification technology predominantly for the benefit of farmers (i.e., Roundup Ready® crops).

While these genetically modified (or ‘transgenic’) plant lines are vitally important for farmers to keep up with the food requirements of a growing global population, a key area that has received less attention is increasing public trust in transgenic foods. Lilac Limes provide an innovative approach to introducing consumers to a transgenic food variety. Having a visible characteristic may help to increase the transparency of genetic technology and improve public perception of transgenic food varieties. With agricultural pests and diseases increasing in range and abundance, gaining consumer confidence in transgenic foods may become increasingly important.

However, taking transgenic foods to the market is a difficult process due to the strict regulations surrounding these foods. Deregulation of transgenic food varieties – or removing government restrictions that prevent transgenic foods from reaching the market – is notoriously complex. Therefore, a significant portion of the Lilac Lime project is devoted to navigating the deregulation process to take Lilac Limes to field trials and then finally to farmers and consumers. Both the USDA Animal and Plant Health Inspection Service-Biotechnology Regulatory Services (APHIS-BRS) and the Food and Drug Administration (FDA) have been contacted. Discussions with regulators from these agencies have led to a framework of objectives that need to be completed prior to writing the deregulation request. These include, but are not limited to:

- isolation of a single cultivar for studies to revolve around, based on field data;
- determination of the genomic placement of the inserted T-DNA, its copy number, its final configuration and if any native genes were disrupted during the integration process;
- analysis of the T-DNA’s final configuration and whether any known toxins and/or allergens were accidentally produced as the fruit needs to have compositional analysis to determine if the native chemical make-up has been disturbed in a negative fashion and
- the effect on the American diet through the addition of anthocyanin to lime, such as how much will be added, ingested and absorbed by the average American.

Determining the exact procedure for getting approval is part of the learning process for improving future transgenic citrus projects, so there may be multiple attempts before all questions from APHIS-BRS and FDA have been answered. Developing a clear procedure could vastly improve the deregulation process for future transgenic citrus varieties and possibly facilitate deregulation of other transgenic crop varieties.

Production and Evaluation of Transgenic Citrus

The gene that produces the rich purple color in Lilac Limes was obtained from the blood orange and inserted into Mexican Lime plants (Figure 1). The scientific team also identified control elements (promoters) that act to keep the pigment production confined to the fruit tissues. Lilac Limes still produce green peels and green leaves and reach...
The use of tissue-specific promoters produce fewer fruit, thus excluding pigments throughout their tissues. Transgenic lines that produce these wasting their finite energy resources on Confining pigment production to the anthocyanin to fruit.

Based on analyses, there good litmus scale to determine overall and sour of flavor (Figure 3). As seen in both Figure 1C and Figure 3, there can be a large gradation in the color observed.

The successful Lilac Lime lineages produced are an improvement, even in comparison to other purple citrus varieties already grown commercially. These include the blood orange varieties such as Moro, Tarocco and Sanguinello. In traditional citrus varieties, pigment production requires hot days followed by cold nights during fruit development for the color to develop fully. Thus, farmers are at the mercy of weather conditions during each season. With climate change altering average temperatures and weather conditions in many citrus-growing regions, the availability of these darker citrus varieties may decline in the future without the introduction of transgenic citrus lineages that produce pigmented fruit that don’t require cycling hot/cold periods. Following the success of Lilac Limes, this team has the opportunity and means to use this genetic modification technology to create other reliably pigmented transgenic citrus varieties.

**Figure 2.** BRIX value (also known as total soluble solids) provides the total sugar content of the fruit from individual Lilac Lime lines. * - average value for two season analyses.

their fruiting stage similar to traditional lime varieties. The fruit has been investigated exhaustively for quality traits important to citrus. At the top of the list are BRIX and pH, or the sweet and sour of flavor (Figure 2). While these two measurements indicate much more than just flavor, they do act as a good litmus scale to determine overall fruit quality. Based on analyses, there were little to no changes in the BRIX and pH values due to the addition of anthocyanin to fruit.

Confining pigment production to the inner fruit prevents the plants from wasting their finite energy resources on producing pigment in all their tissues. Transgenic lines that produce these pigments throughout their tissues would typically grow smaller and produce fewer fruit, thus excluding them from commercial viability. The use of tissue-specific promoters produces plants that are not “overtaxed” by producing purple pigmentation throughout the plant without benefit, and possibly detriment, to growth. The purple coloration is produced by a metabolite called anthocyanin and is considered a healthy antioxidant compound found in many fruits and vegetables. Five novel promoters (control elements) originating from citrus and one from plum were investigated for the ability to produce anthocyanin in citrus fruit (Dasgupta et al. 2020). Two of the five promoters (both from citrus) have been shown to be functional for citrus fruit expression. The remaining three promoters had no discernible function with regards to anthocyanin production. It was necessary to measure the level of anthocyanin produced from each transgenic Lilac Lime line for federal deregulation purposes and to provide the FDA a quantifiable amount for commercialization (Figure 3).

**Genetic Technology Behind Lilac Limes**

Lilac Limes also were developed using a new genetic modification approach, which removes some of the element of chance prevalent with other genetic modification technologies. Using a series of enzymes that work in harmony with each other, the insertion of the beneficial genes into the host plant’s DNA can be rigorously controlled and offers a method to insert multiple beneficial genes at once, called ‘gene stacking’. This lab has produced hundreds of ‘Founder Lines’ – plant lines with the required DNA sequence that the enzymes recognize – predominantly in citrus. These lines provide the basis for the Lilac Lime varieties, but also are a basis for future transgenic varieties, such as citrus that is resistant to new or emerging diseases, as well as abiotic stresses. While Lilac Limes have a single visible characteristic aimed
**Anthocyanin Absorbance @520nm**

Figure 3. Colorimetric assay to determine anthocyanin content of the fruit from individual Lilac Lime lines. * - are the average value for two season analyses.

at adding value for consumers, they also are Founders lines. Therefore, every independent Lilac Lime line is an independent Founder line. This means that as new resistance genes are discovered for citrus pests, diseases and abiotic stresses, they could be incorporated into the Lilac Lime. This technology provides an unprecedented ability to adapt to new threats and challenges that arise, providing that the beneficial genes are available.

**Conclusion**

Currently, research is poised to take Lilac Limes through federal deregulation, and we are scaling up efforts to produce more fruit for analysis by cloning preferred lines and through field trials to determine which are the most robust and dependable. With this novel purple fruit, the scientific team hopes to attract the attention of consumers and thus open a dialogue with the public about genetic technology in citrus and in food plants in general. Currently, we have determined three potential Lilac lime lines that have a single transgene insertion, provide strong purple color and have robust growth and fruit production in the greenhouse. These lines are being field evaluated for performance and to gather fruit for chemical compositional analysis. We are further sequencing the genome to determine placement and final transgene configuration. All data generated will be compiled for federal regulators to examine and to determine if this was the correct course needed for deregulation or if more information is required. Once a final action plan is agreed upon, this process will be written in full and provided to the CRB for future scientific endeavors. If deregulation is successful, this could pave the way to acceptance of other transgenic citrus lineages and transgenic foods in general. Consumers may have to wait a little longer before Lilac Limes reach grocery stores, but they soon could be looking forward to other varieties of transgenic citrus that benefit everyone from farmers to consumers.

**CRB Research Project #5200-155**

**References**


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Project Summary

The introduction of foreign genes in citrus has been accomplished for about three decades; however, when a gene of horticultural interest is introduced in a plant, a few other non-related genes also must be introduced in the plant. In this project, a gene to confer antibiotic resistance and a gene to give a visual cue was used to select for transgenic plants. Furthermore, to be able to remove all non-needed genes and leave the transgenic plant with only the gene of horticultural importance, we had to optimize a technology called Recombinase Mediated Cassette Exchange (RMCE), which requires several additional genes to be inserted into the plant. Most of the molecular markers and genes for visual selection, as well as genes needed for RMCE, are non-plant genes, sometimes with sequences sourced from viruses and bacteria, and are not well received by consumers. In this project, we were able to produce transgenic “Carrizo” citrange that initially had all genes introduced in it, but we were able to remove all non-needed genes and ultimately ended up producing 28 transgenic “Carrizo” citrange containing only the citrus gene CSM-1, which triggers a series of genes for broad disease resistance. The optimization of this technology for citrus may assist efforts to improve other commercial citrus varieties that lack a specific trait.
Introduction

The introduction of the Asian citrus psyllid in California and the arrival of the bacterial disease huanglongbing (HLB) pose a serious threat to the California citrus industry. For any disease, resistant/tolerant varieties are one option to an economical and sustainable agriculture.

Breeding citrus is not an easy task. When a cross is made, the new hybrid can take eight to 15 years to mature and flower for the first time. Also, the trees are large and require many acres for trials and additional years for evaluation. The advances in genetic manipulation and the ease of introducing new genes into crops without disturbing their general traits allows for an existing, desirable variety that lacks an important trait to be improved. One drawback of introducing a gene of horticultural interest into plants is that additional, non-related genes also are introduced. These genes serve as tools for scientists to identify and regenerate a plant with a modified genome. Some of these genes are molecular markers that give the plant resistance to antibiotics (nptII gene) or herbicides or make the plants emit light when seen under a microscope (mCherry gene). In addition to molecular markers, other genes like isopentenyl transferase (ipt gene), are introduced to improve the regeneration of plants. These genes are not well accepted by regulators or consumers because they are non-plant genes, many times from bacteria and viruses.

For a gene to work, a DNA element called a promoter is needed. Promoters tell genes where, when and how much protein to be produced. Many of the promoters used in genetic transformation are not derived from plants. To avoid regulatory and consumer concerns, it is best that all components of the gene of interest being introduced in a plant are derived from plants and that any unnecessary genes be removed.

In a previous project, we developed a technique for citrus transformation called Recombinase Mediated Cassette Exchange (RMCE) (Righetti et al. 2014). This technique eliminates unnecessary genes but maintains the gene of interest in the transformed plant material. However, that method was not efficient, since there were no genes that allowed us to visually identify the transgenic plants or a gene to improve the regeneration of plants. Furthermore, we were using non-plant DNA to drive the genes.

In this project, our aim was to improve the method of eliminating unnecessary genes from transgenic citrus plants, leaving only a gene with the potential for broad spectrum disease resistance. We also planned to test plant promoters so that the final transgenic citrus would have a plant gene with a plant promoter for improved regulatory and consumer acceptance. We previously reported (Louzada and Thomson 2019) the testing of seven promoters, and here we expanded with an additional six plant promoters.

RMCE-mediated excision of genes from plant DNA is a process that can be activated in a transgenic plant. We first introduced all genes into a “Carrizo” citrange plant including the disease resistance gene, the marker genes and the gene to improve regeneration of plants. Afterward, we removed the unnecessary genes to leave behind only the disease resistance gene. The method also requires a gene called recombinase (CinH) that directs the removal of DNA, including itself, from the plant genome. To do this, the CinH is activated by an inducer chemical when excision is desired. The chemicals dexamethasone (DEX) and estradiol (ETD) were chosen; and multiple publications have demonstrated that they work effectively in plants (Caddell et al. 2015; García-Almodóvar et al. 2012; Timerbaev et al. 2019).

Accomplishments

1. Optimization of the RMCE method for gene removal

The introduction of a foreign gene in a plant is not a new thing; in addition, a bacterium called Agrobacterium tumefaciens does this in nature. Agrobacterium has in its cell a DNA transfer system (termed tDNA), that contains important genes for their long-term survival that are inserted into the host plant. Successful insertion leads to the plant forming a gall in which the Agrobacterium can live. Scientists can remove the gall-forming genes and introduce any other gene of interest into plants.

We reported previously (Louzada and Thomson 2019) on experiments performed to verify the efficiency of RMCE and to test seven plant promoters. We verified that the RMCE method needed additional improvement, including reconstruction of plasmids to replace all non-plant promoters with plant promoters and to test additional plant promoters.

We tested 13 plant promoters from citrus and other plant species (seven previously tested, plus six new promoters) and compared them with the Cauliflower Mosaic Virus (CaMV) promoter (a strong plant virus promoter). The most important criterion for the selection of the promoters was that they would induce expression in the whole plant. The plasmids were designed to be expressed in citrus protoplasts and to contain only the mCherry gene driven by each of the promoters to be tested and without the need of an inducer. The brighter the red the protoplast was, the stronger the promoter was. The plasmid DNA was introduced into the protoplasts by electroporation and evaluated after three days according to its brightness on a scale of one to five, with five as the brightest and one as the least bright. Protoplasts in each scale category were recorded. Six million protoplasts were electroporated for each plasmid, but this number was drastically reduced after the electroporation. Each experiment was replicated three times. Although some promoters (CsEF1a #2) yielded high numbers of protoplasts expressing MCherry, very few were rated in high bright intensity (4 and 5) scales. Promoters Cit354, AtUBI10 and StUBI7m were the best and even were superior to the strong CaMV promoter. With this information, new plasmids including the disease resistance gene, the marker genes and the gene to improve regeneration of plants. Afterward, we removed the unnecessary genes to leave behind only the disease resistance gene. The method also requires a gene called recombinase (CinH) that directs the removal of DNA, including itself, from the plant genome. To do this, the CinH is activated by an inducer chemical when excision is desired. The chemicals dexamethasone (DEX) and estradiol (ETD) were chosen; and multiple publications have demonstrated that they work effectively in plants (Caddell et al. 2015; García-Almodóvar et al. 2012; Timerbaev et al. 2019).
were constructed into a new *Agrobacterium tumefaciens* system for a more precise insertion of multiple genes into “Carrizo” plants and to better excise non-wanted genes. Furthermore, the isopentenyl transferase gene (*ipt*) was introduced in the plasmid to increase plant regeneration.

2. Molecular Characterization

Using the new plasmid, several “Carrizo” citrange plants were produced by genetic transformation. Two kinds of plantlets were produced, some not expressing the bright red light from the gene *mCherry*, but that had originated from embryos expressing *mCherry* (Figure 2) and plantlets that expressed *mCherry* the same way as the embryos. To verify that a transgenic plant contained a specific gene, we used polymerase chain reaction (PCR) to detect the presence of specific DNA. We generated primers⁴ for the molecular characterization of the individual molecular marker genes and for the gene of interest. We selected 67 transgenic plants that were a mix of shoots that emitted red light, indicating the presence of the marker gene *mCherry*, and those that did not and assayed them by PCR. The molecular characterization indicated that 28 plants (Figure 1) out of the 67 were free from any molecular marker or unwanted gene and contained the gene for

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Figure 1: Embryos expressing the *mCherry* gene protein (bright red color) and shoots not expressing *mCherry* (white arrows), demonstrating that shoots lost markers (*mCherry* absent).

Figure 2. Marker-free transgenic “Carrizo” citrange plants growing in the greenhouse.
disease resistance. This is a substantial improvement in the methodology to eliminate unwanted genes when compared to the results obtained in the previous project. Furthermore, the improved methodology offers a more precise means to improve other citrus varieties.

Benefit to the California Citrus Growers

The development of new varieties is the heart of any fruit industry. Citrus has been challenged with many pests and diseases that cause losses to growers and increases inputs to make this industry profitable. Conventional breeding may not deliver all that is needed for the development of new varieties, so the addition of the improved RMCE technology described here may help in accelerating the development of new improved varieties. We produced 28 new marker-free transgenic “Carrizo” citrange (Figure 2); and when they reach suitable size, they will be challenged with citrus pathogens, including the HLB pathogen. Transgenic grapefruit plants previously transformed with the CSM-1 gene were shown to be resistant to Phytophthora nicotianae (root rot) and Xanthomonas citri subsp. citri (citrus canker - unpublished data). If the “Carrizo” citrange developed under this project shows any tolerance/resistance to HLB or other diseases, it would be a great benefit to the industry. These plants may be sent to California for tests.

CRB Research Project #5200-153

Glossary

²Genome: The whole DNA of an organism.
²Protoplast: Citrus cells without a cell wall.
²Electroporation: Method of introducing DNA into protoplasts using electric pulses.
²Primer: Small pieces of DNA sequence used in PCR to amplify specific genomic DNA sequences.

References


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Project Summary

Huanglongbing (HLB) is an imminent threat to the California citrus industry. HLB first was reported from a backyard tree in southern California in 2012; to date, more than 3,000 HLB-positive trees have been identified and removed. Incorporating genetic resistance through breeding can be a promising approach to providing long-term HLB solutions. We are developing novel HLB-resistant¹/tolerant² varieties via conventional breeding by crossing citrus accessions with disease-resistant Australian limes. We have evaluated 400 novel hybrids of the first generation in field trials in Florida and identified about 60 HLB-resistant hybrids. Development of novel cultivars with resistance to HLB and a predominantly citrus fruit flavor is in progress. Our previous research identified several citrus relative genera with resistance/tolerance to HLB. If disease-resistant plants grafted (i.e., interstocks and top grafting) to susceptible scions can impart tolerance traits to commercial citrus, they can provide interim HLB management strategies. We also have generated inarched³ citrus trees and identified ‘Orange jasmine’ as potentially valuable for imparting HLB tolerance to existing trees.
Introduction

HLB occurs in many citrus-growing regions globally, and effective disease control has been difficult (NAS 2018). Control of the psyllid populations currently is the most effective disease management strategy available (Grafton-Cardwell et al. 2013). Availability of disease-resistant/tolerant citrus cultivars will provide sustainable solutions to the industry (NAS 2018). This project focused on breeding scion and rootstock varieties for HLB resistance and imparting disease tolerance through inarching with known resistant plants. When exotic diseases threaten cultivated plants, wild relatives often are utilized to incorporate resistance or tolerance traits into crop cultivars (Mammadov et al. 2018). Narrow genetic diversity in cultivated citrus and lack of resistance to the HLB-associated pathogen ‘Candidatus Liberibacter asiaticus’ (CLas) prompted us to utilize wild relatives to develop HLB resistance. In field trials conducted in Florida, we have identified HLB-resistant plants that are closely related to cultivated citrus (Ramadugu et al. 2016). Australian limes, specifically ‘Australian Desert lime,’ ‘Australian Finger lime’ and other related species were selected as sources for developing disease resistance. Pre-breeding of various citrus cultivars with Australian limes produced first generation (F1) hybrids with disease resistance (Ramadugu et al. 2019).

Inarching with selected rootstocks can improve tree vigor and yield (Lu et al. 2019). In Brazil, citrus sudden death disease killed millions of citrus trees grafted on ‘Rangpur’ lime rootstock, ideal for the extreme drought prevalent in certain regions. Trees inarched with disease tolerant rootstocks like ‘Cleopatra’ mandarin exhibited tolerance to sudden death (Futch et al. 2020). Since we already have identified HLB-resistant accessions (Ramadugu et al. 2016), we utilized four non-citrus taxa for inarching and evaluated the inarched plants for HLB tolerance.

Results

Breeding for HLB resistance involved conducting crosses using citrus varieties and Australian limes as parents. The progeny of these wide crosses (F1 generation) was evaluated for HLB disease resistance in a greenhouse by grafting with CLas-positive bark patches or exposing to Asian citrus psyllids carrying CLas (Figure 1). In the greenhouse, test plants were challenged either through grafts or psyllids. Hybrid plants exposed to the pathogen were observed for up to two years for disease symptoms, cycle threshold (Ct) values from quantitative polymerase chain reaction (qPCR) tests were conducted to detect CLas, and the plants were scored as resistant, tolerant or susceptible. Symptoms were scored independently by two researchers. Disease status was determined based on Ct value for CLas and symptom expression. Test plants were assigned as susceptible, tolerant or resistant. The exact symptoms vary between different genotypes. The surviving hybrids were further evaluated in the field.

Figure 1. Novel hybrids evaluated for huanglongbing (HLB)-resistance response in Fort Pierce, Florida. The top panel shows HLB susceptible hybrids, and the bottom panel shows resistant hybrids.
Field evaluation of promising hybrids is essential for identifying stable HLB resistance. We selected plants that survived the inoculation challenges in greenhouse tests and planted 400 trees (previously inoculated in the greenhouse) in the experimental fields in Fort Pierce, Florida. These field plants previously were inoculated in the greenhouse test and were exposed to CLas-positive psyllids in the field where HLB is endemic. The field plants were evaluated at least once a year to monitor disease symptoms and to determine the CLas titer by qPCR. After three years of field exposure, we determined, based on Ct values and symptomatology, that 60 hybrids were resistant (CLas Ct 38-40), 120 were deemed tolerant (Ct 33-38) and the rest susceptible to HLB (Ct 18-32).

Generating breeding progeny from representative citrus types ensures the availability of hybrids that approximate commercially relevant cultivars. In this project, we have generated both scion and rootstock hybrids. Hybrids with adequate resistance/tolerance and good fruit flavors (lack of off-flavors) are chosen as parents in advanced breeding. Field trials provide information on other horticultural traits (overall growth/vigor, number of fruit, etc.), identify potential problems associated with specific hybrids and assist pre-selection (choosing hybrids that appear suitable as parents for cultivar development). Figures 2, 3 and 4 show representative fruit types obtained from F1 hybrids in California. If a sufficient number of advanced hybrids are evaluated for fruit quality, it may be possible to obtain edible, citrus-like fruits from the novel hybrids after two or three generations. Selected hybrids can be used as parents in advanced breeding designed to produce resistant hybrids with fruit traits similar to the citrus parent and minimize off-flavors originating from the disease-resistant parent. Rigorous testing of advanced hybrids for resistance, assessment of organoleptic properties and field trials to confirm stable resistance will be needed before the hybrids are considered useful. The goal of our breeding program is to incorporate disease resistance traits into commercial citrus cultivars.

Inarching resistant plants to susceptible citrus scions was useful for mitigation of citrus sudden death disease prevalent in Brazil (Futch et al. 2020). If a similar approach can diminish HLB symptoms, it may provide a short-term disease management strategy for infected trees. With help from Brite Leaf citrus nursery in Florida, we generated a set of grafted plants with ‘Kuharske’ rootstock and ‘Valencia’ scion. These plants were then inarched above the bud union with small, rooted seedlings of four different non-citrus taxa previously determined to be resistant/tolerant to HLB. We monitored CLas titer and symptom development in the scion for disease scoring (Ramadugu et al., 2016). The four plants

Figure 2. Three representative examples of F1 hybrids with Microcitrus parentage and lemon-like flavor. 1A and 1B: Hybrid fruit and cross section of the fruit; 2A and 2B: Hybrid fruit and cross section of the fruit; 3A and 3B: Hybrid fruit and cross section of the fruit. Evaluation for huanglongbing resistance is in progress. Fruit size varies from 4.5-8 cm length, 3.5-7 cm width; BRIX 8-16; Juice 13-46 percent; some without juice. Seeds 0-10. Most have lemon-like flavor; many have off-flavors.
used for inarching were: ‘Eremolemon,’ ‘Australian finger lime,’ ‘Large leaf Australian wild lime’ and ‘Orange jasmine.’ ‘Eremolemon,’ a naturally occurring hybrid of ‘Australian desert lime’ and ‘Meyer lemon,’ is resistant to HLB; on a disease scale of 1-8, with 1 being immune and 8 being most susceptible (scoring is based on Ct values and symptom expression), ‘Eremolemon’ scored a 2, a resistant response. Since ‘Eremolemon’ produces nucellar seedlings that are identical to the mother plant, the progeny has only HLB-resistant individuals. ‘Australian Finger lime’ and ‘Large leaf Australian wild lime’ were considered HLB-tolerant with a score of 3. Seedlings from open-pollinated Microcitrus species growing in the Givaudan Citrus Variety Collection (where citrus and Microcitrus are growing in close proximity), are expected to have variable HLB disease response. ‘Orange jasmine’ is resistant to CLas and has a disease score of 2; all the seedlings in the progeny show HLB resistance.

The experiment consisted of 108 inarched plants evaluated for up to one year in three locations – a research field and a greenhouse in Fort Pierce, Florida, and an organic grove in Clermont, Florida. The inarched plants were challenged with bark patches from a CLas-positive plant in the greenhouse. In the field, exposure to CLas was via psyllid feeding. Composite grafted plants inarched with ‘Eremolemon,’ ‘Australian Finger lime’ and ‘Large leaf Australian wild lime’ developed HLB symptoms indicating that these HLB-resistant or tolerant accessions did not impart this trait to the grafted plant. However, ‘Orange jasmine’ used as an inarch did offer protection against CLas if the inarched stem was securely attached to the grafted plant. Out of 22 plants inarched with ‘Orange jasmine,’ 20 showed low titers of CLas (Ct 35-38) and did not develop symptoms when the inarch was intact. However, due to the difference in the growth rates of citrus and ‘Orange jasmine,’ the inarch was dislodged after a few months. We have modified the technique and are continuing experiments to confirm the ability of ‘Orange jasmine’ to impart disease tolerance.

**Conclusions and Future Directions**

Wide crosses between citrus and Australian limes resulted in some resistant hybrids in the F1 progeny. We continue breeding to generate advanced hybrids with disease resistance and acceptable fruit quality comparable to commercial citrus. A total of two to three generations may be sufficient to select varieties suitable for cultivar development if sufficient progeny is screened for resistance and evaluated for fruit traits. Novel disease-resistant citrus types can be
made available to citrus growers in the future. Inarching disease-resistant plants to reduce HLB disease response in the scion variety will be valuable to manage HLB in the short term. Preliminary results indicate that HLB-resistant ‘Orange jasmine’ can impart tolerance to scions grafted on ‘Kuhaske’ rootstock.

CRB Research Project #5200-154

Glossary

1Resistance: The pathogen can multiply transiently in resistant plants but does not become established.

2Tolerance: The pathogen establishes in the host but does not reach high titers as in susceptible plants; tolerant plants have reduced symptoms and typically have lower yields compared to healthy plants.

3Inarching: A second rootstock grafted onto the citrus scion generating a composite plant with two root systems.

4Pre-breeding: Generating an intermediate set of plants with desirable characteristics (example, HLB resistance) to be used later in cultivar development.

5Organoleptic: Pertaining to the sensory properties of the fruit, e.g. the taste, color, odor and feel.

References


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ENGINEERING CITRUS USING GENE EDITING TECHNOLOGY

Vivian Irish, Fei Zhang, Yewei Wang, Archana Khadgi, Corina Vernon and Yannick Jacob
Project Summary

CRISPR¹/Cas9² is a versatile gene editing system that is revolutionizing the agricultural industry by allowing researchers to modify, delete or correct specific regions of DNA in the genome. Our goal is to develop a suite of strategies for effective citrus gene editing in order to engineer disease resistance in already well-established, horticulturally important varieties. In this project, we focused on using CRISPR/Cas9 to generate alterations that we expect will cause early flowering in citrus, and also to develop approaches for multiplex gene editing. These approaches either can be used alone or in conjunction with each other, depending on the desired outcomes and can pave the way for moving basic research results directly to field studies and commercial applications. Achieving these goals will support the citrus industry by providing a key tool for “molecular breeding,” not just for disease resistance, but for a wide variety of traits.

The CRISPR/Cas9 Gene Editing System

CRISPR/Cas9 is a gene-editing tool that has revolutionized biomedical and agricultural research. The system consists of the CRISPR guide RNA (gRNA) and the Cas9 enzyme, which act together as “molecular scissors” inside a cell, with the enzyme performing the cutting of DNA and the gRNA directing where the cuts should be made. By engineering specific gRNAs, Cas9 can be targeted to the corresponding specific genes and will cut the host target DNA sequence at that site (Figure 1). The natural DNA-repair mechanisms present in cells frequently fail to repair these cleavage sites appropriately, resulting in DNA mutations at that site. In general, this system is extremely efficient and easy to manipulate, allowing researchers to generate mutations at target sequences at a high frequency with high fidelity. However, CRISPR/Cas9 gene editing can be less effective in some species, including many plant species, presumably due to differences in genome structure or in other biological parameters. Our overall goal is to improve CRISPR/Cas9 to make it an effective tool for gene editing in citrus species. In our previously funded work from the Citrus Research Board, we developed a highly efficient CRISPR/Cas9 system for citrus that relies on:

1. increasing the expression of Cas9 to improve the frequency of DNA cleavage (Zhang et al. 2017),
2. developing a bifunctional selectable marker containing both an antibiotic resistance gene and a visually detectable GFP marker to identify transgenic citrus plants with high expression of Cas9 (Zhang et al. 2017) and
3. employing heat stress to increase the efficiency of the Cas9 enzyme to cleave DNA (LeBlanc et al. 2018).

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Figure 1. The CRISPR/Cas9 system. The Cas9 enzyme (blue) is positioned along the target DNA by the guide RNA (orange), which is specific for a particular sequence of DNA. The Cas9 enzyme then cuts the DNA (scissors), which is then repaired by the normal cellular machinery. However, that repair mechanism is prone to error and can result in mutations at the site of the cut. Top, conventional CRISPR/Cas9; bottom, multiplex CRISPR/Cas9, in which multiple sequences can be targeted using multiple different guide RNAs simultaneously.
Together, these approaches have resulted in up to 75 percent of transgenic Carrizo citrange citrus plants having mutations at the target gene of interest (Zhang et al. 2017; LeBlanc et al. 2018).

Using CRISPR/Cas9 to Promote Early Flowering

While a number of researchers have developed approaches to induce early flowering using transgenes that overexpress or induce high levels of expression of the flowering regulator gene FT, we sought to develop a complementary strategy. Using our efficient citrus CRISPR/Cas9 genome editing system, we decided to eliminate the function of candidate genes, whose loss of function by mutation have been shown in other species to accelerate flowering. By mutating these genes in citrus, we should be able to create citrus lines that flower precociously. One of the big advantages of using CRISPR/Cas9 to generate mutations is that even if we introduce the CRISPR/Cas9 machinery via a transgene¹, we can obtain transgene-free mutations via simple segregation through breeding and still maintain the attributes of a particular variety. Thus, this approach is fast and efficient, can be applied in principle to any variety of interest and can eliminate concerns associated with producing a transgenic commodity.

The genes TFL1 and CEN have been shown in other species to maintain indeterminate and continued growth (Bradley et al. 1997). Early flowering ensues when either TFL1 or CEN
function is lost via mutation. Based on what we know in other species, the loss of TFL1 or CEN function should result in early, but not excessively precocious flowering. The goal is to identify a mechanism by which early flowering is achieved in about two years, so that plants are robust and can provide seed, but not so accelerated that plant growth and fruit development are compromised. Indeed, naturally occurring mutations of TFL1- or CEN-like genes in crop plants such as tomato, soybean and cotton have been selected for during domestication because such mutations promote maturation and early flowering (Eshed and Lippman 2019).

We generated CRISPR/Cas9 constructs to mutate either the TFL1 or CEN genes, which should result in early flowering. We introduced these constructs into Carrizo citrange, Pineapple sweet orange and Jackson grapefruit using Agrobacterium-mediated transformation of hypocotyl tissue from seedlings (Figure 2). We screened the resulting lines for mutations in the TFL1 and CEN genes by sequencing each line. Through this approach, we obtained a number of lines that contained mutations for the TFL1 or CEN genes. We also used multiplex CRISPR/Cas9 gene editing to concurrently eliminate both TFL1 and CEN function. We have six TFL1 Carrizo citrange mutant lines, 17 CEN Carrizo citrange mutant lines, 11 Carrizo citrange lines in which TFL1 and CEN are both mutated, one such line in Pineapple sweet orange and two such lines in Jackson grapefruit.

These lines are now growing, and our oldest lines are about 20 months old. They appear healthy, with the TFL1 mutant lines looking completely normal, while the CEN mutant lines show an increase in the numbers of thorns produced (Zhang et al. 2021). We eagerly await these plants to grow further and hope that when they are two to three years old, they will flower. If so, this CRISPR/Cas9 approach would provide a new strategy for robust early flowering and could be an effective means of rapidly developing new breeding stock.

Developing a Multiplex CRISPR/Cas9 Approach

By incorporating multiple gRNA into a CRISPR/Cas9 construct, it is possible to carry out “multiplex” gene editing to mutate several genes at once (Figure 1). Multiplex gene editing would allow researchers to stack desirable mutations together simultaneously, potentially saving years of time in breeding programs.

To carry out multiplex gene editing, we first designed a new vector into which we could insert multiple gRNA sequences. Using this vector, we generated a construct containing multiple gRNAs to simultaneously target two genes for mutation, TFL1 and CEN. We also used multiplex gene editing to mutate three PP2 genes at once.

We chose three PP2 genes as targets for multiplex gene editing as they are prime candidates linked to the development of HLB symptoms. PP2 genes encode phloem proteins, and higher levels of PP2 genes have been measured in infected, symptomatic sweet orange plants (Kim et al. 2009). Thus, by mutating multiple PP2 genes and eliminating their function, we potentially could generate citrus plants that show alleviation of HLB symptoms. Using this multiplex approach, we have 30 confirmed lines in which the PP2-B10, PP2-B14 and PP2-B15 genes are mutated alone or in combination. Thus, we now have multiple plant lines that can be used to further investigate whether elimination of PP2 gene activity is sufficient to confer tolerance to HLB. We are currently growing these plants to a stage at which we can collect budwood to be shipped to California and tested for HLB tolerance.

Future Directions

We now have an arsenal of molecular genetic tools with which to engineer targeted mutations in citrus genomes that potentially can alleviate a variety of biotic and abiotic stresses. Using CRISPR/Cas9 as a method to alter citrus traits is faster than conventional breeding. Furthermore, this approach does not affect the valuable horticultural characteristics of commercial varieties. The CRISPR/Cas9 machinery can be removed by breeding, leaving just the mutation or mutations of interest in the plant genome (similar to what a conventional breeding program accomplishes). Accordingly, this approach also may alleviate consumer and regulatory concerns associated with genetically-modified organisms. This has brought us to the stage where we can use these tools to explore introducing one or more mutations into a specific variety and then assess the effects of such mutations on horticultural traits. We are optimistic that the CRISPR/Cas9 approaches used in this research to eliminate PP2 gene activity will lead to alleviation of HLB-related symptoms and provide an additional tool to manage or mitigate HLB.

CRB Research Project #5200-166
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).

2. **Cas9**: An enzyme, found originally in the bacterium *Streptococcus pyogenes*, that specifically cleaves DNA complementary to the guide RNA.

3. **Transgene**: A gene transferred from one organism to another.

4. **Stack, stacking**: Sometimes referred to as gene stacking or pyramiding, this refers to the process of combining two or more genes, mutations or traits of interest into a single plant.

References


Vivian Irish, Ph.D, is a professor, Fei Zhang, Ph.D., is a research associate, Yewei Wang is a research assistant, Archana Khadgi, Ph.D., is a post-doctoral associate, Corina Vernon is a research assistant and Yannick Jacob, Ph.D., is a professor, all in the Department of Molecular, Cellular and Developmental Biology at Yale University. For additional information, contact vivian.irish@yale.edu
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