Development of HLB-Tolerant Citrus Varieties and Rootstocks

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SWFREC- Immokalee
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- Identification of Natural Variation for HLB Sensitivity
- Breeding and Potential Mutant Selection
- Characterization of Tolerance Mechanisms by Genetic and Anatomical Analyses
- Potential Applications of CRISPR Technologies and Associated Challenges

Topics for Today

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Hope or hopeless?

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- Utilization of currently available tolerant germplasm

- Breeding of new scion and rootstock cultivars for tolerance or resistance

- Traditional genetic engineering, to overcome the bacteria and vector

- New modification technologies applied to specifically identified genetic targets

- Other therapeutic approaches based on host or pathogen genetic and genomic information

Genetic Options

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http://www.freshfromflorida.com/pi/chrp/greening/cgphotos.html

There is no typical resistance or simple cure for HLB!

Sugar Belle® near Vero Beach, HLB+ >8 years !

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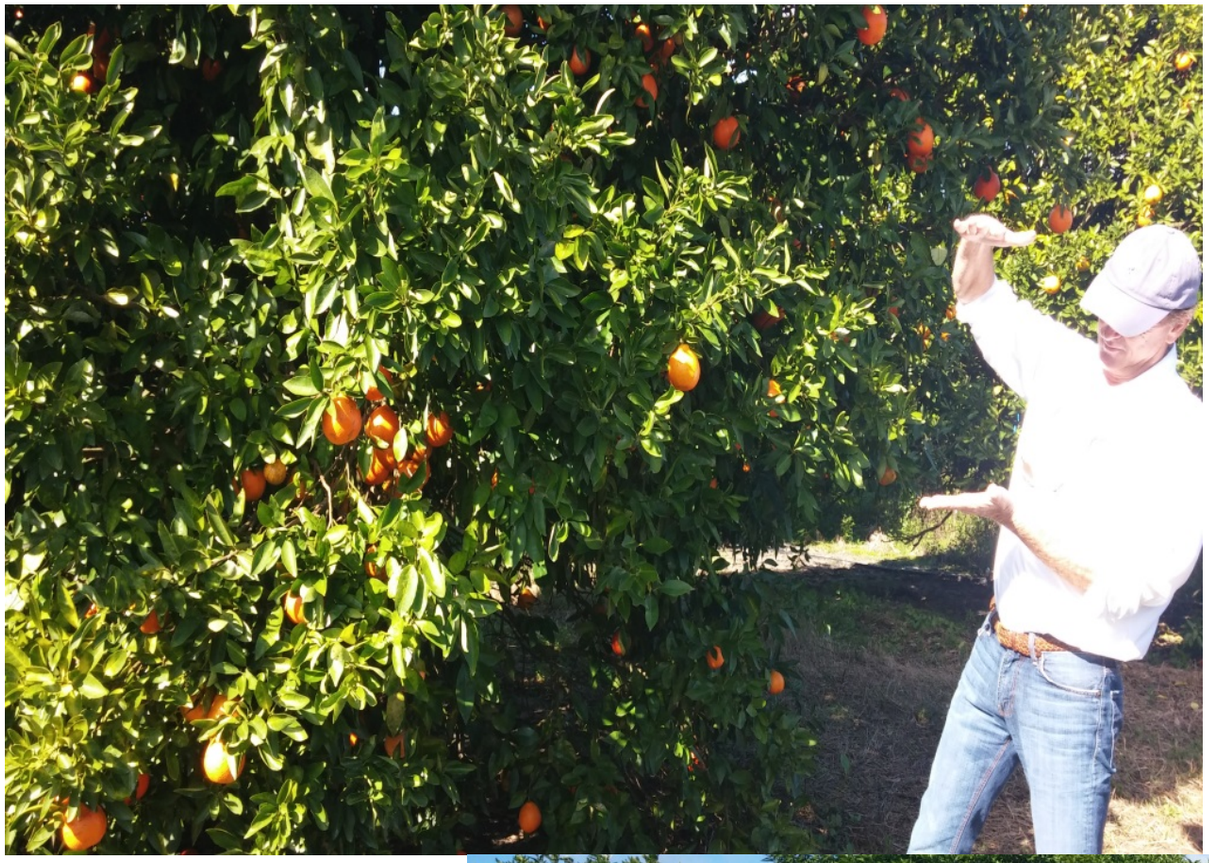
The HLB Tolerance of LB8-9; Sugar Belle®



The HLB Tolerance of LB8-9; Sugar Belle®

Pummelo and Seedling Variation

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A Grand Experiment in Natural Selection

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- **Most sensitive**:
  - Sweet orange
  - Some mandarin hybrids (Murcott tangor, W. Murcott, etc.)
  - Grapefruit
  - Some pummelo cultivars
- **Less sensitive**:
  - Other mandarin hybrids (Nova tangelo, Shatangju, etc.)
- **Some tolerance**:
  - Lemon; Persian lime
  - *Citrus latipes*
  - Other pummelo varieties
  - Mandarins hybrids such as LB8-9 (Sugar Belle)
- **Tolerant or resistant**:
  - *Poncirus trifoliata* and some hybrids with *Citrus*

The Range of Sensitivity to HLB

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The War Mentality

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Survivor Trees

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- Several dozen trees reported
- Scion propagations made, tested, infected
- Rootstocks fingerprinted, rare zygotics
- Rootstocks propagated, tested, infected
- Until now, no resistant trees found

Survivor Trees

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Irradiated Valencia Trees

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Valencia Mutant

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SERENDIPITY

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LB8-9 (Sugar Belle®)

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LB8-9 (Sugar Belle®) transmits tolerance to its offspring

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Sweet orange-like hybrids

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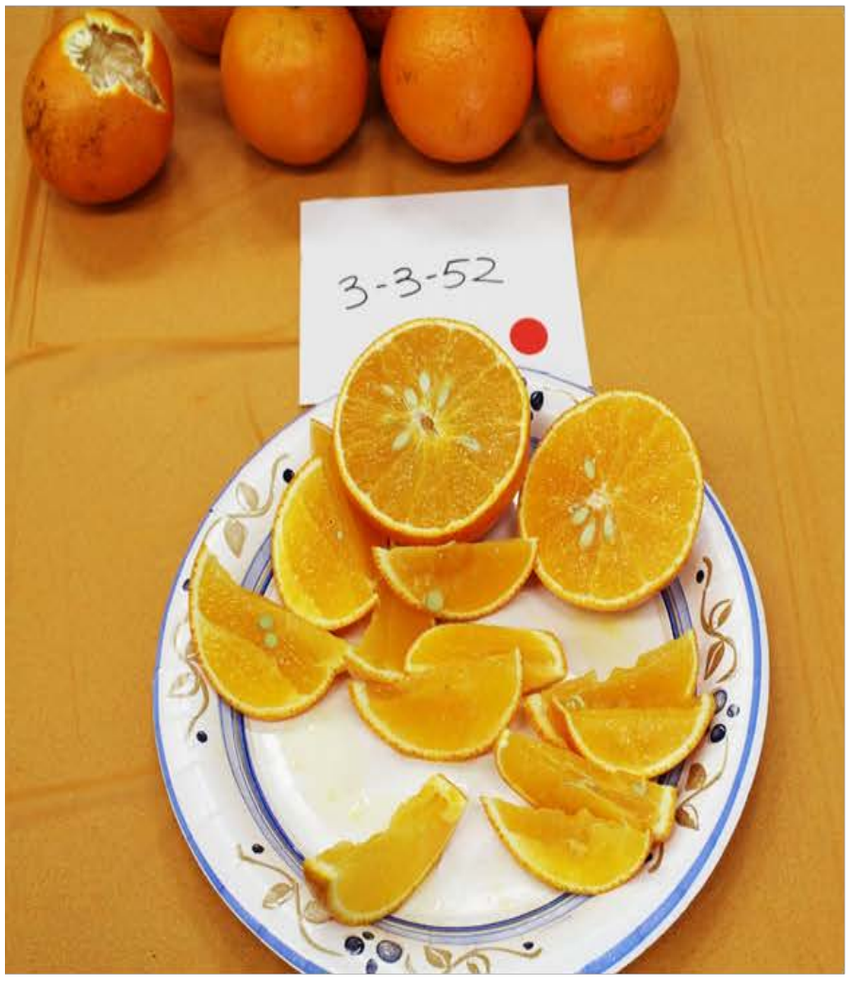
An orange-like hybrid **3-3-52** appears tolerant and was ranked highest in flavor at the CREC Fruit Display Day in February 2016 and 2017



Nursery “dirty house” summary

Scion	No HLB Found	Total	% of No HLB
18A-2-43	86	98	87.76
3-3-52	26	26	100.00
6-2-55	55	63	87.30
7-9-31	26	28	92.86
C4-14-51	64	115	55.65
C4-14-53	58	92	63.04
C7-12-19	78	81	96.30
18A-2-31	52	90	57.78
LB9-4	56	75	74.67
OLL-DCS-3-36	45	45	100.00
OLL-DCS-3-40	99	101	98.02
RBA-21-36	44	47	93.62
RBA-22-29	68	68	100.00
Total	**757**	**929**	

- **Several hundred hybrid families produced over the past 30 years**
- **Great genetic diversity**
- **Nearly 9000 trees have been assessed**
- **~4.3% superior in response to *C*Las**
- **Next steps: genotyping and association**

Toward Genomic Selection

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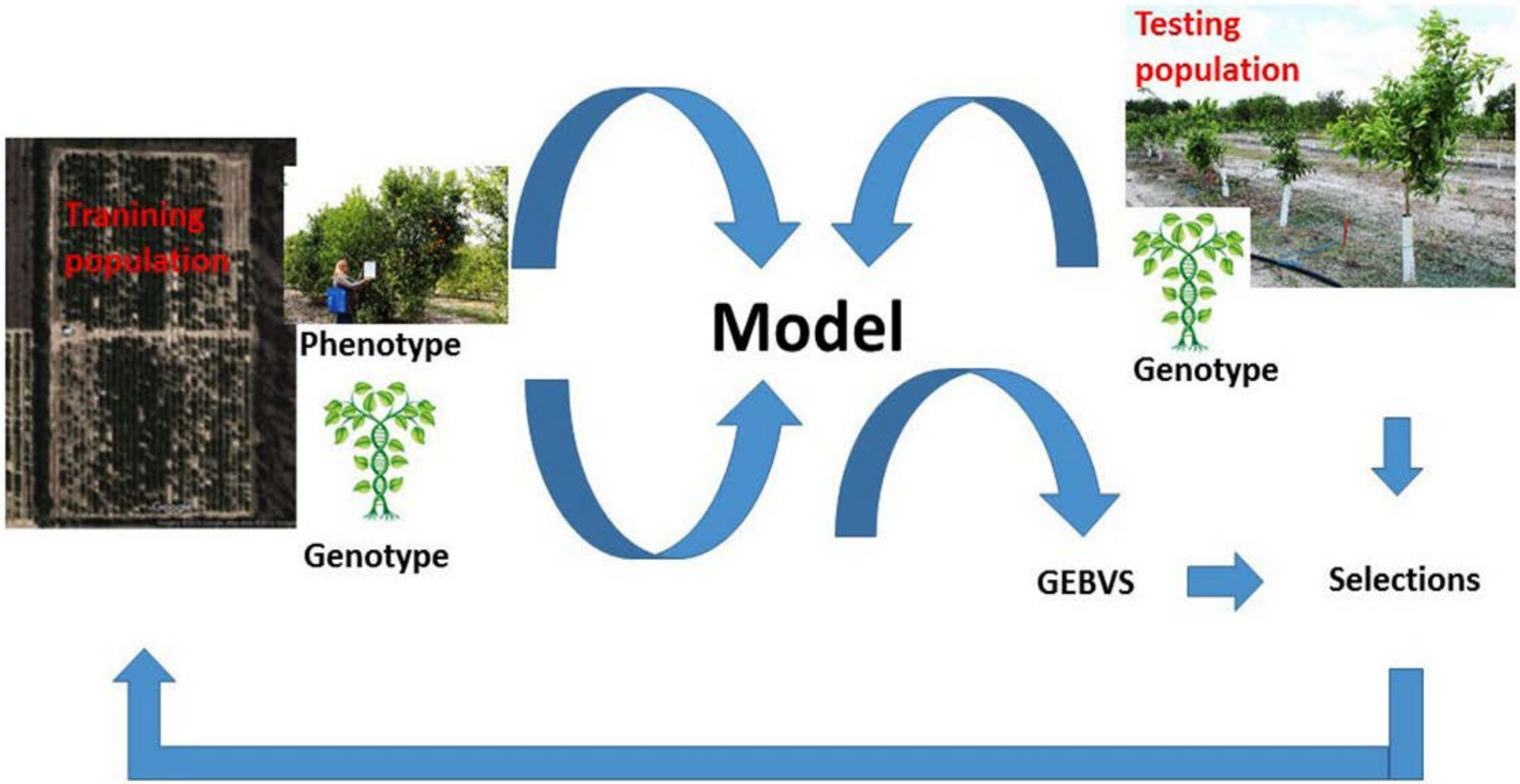
Genomics assisted-breeding



192 progenies were selected based on the speed of HLB infection in a mandarin breeding population

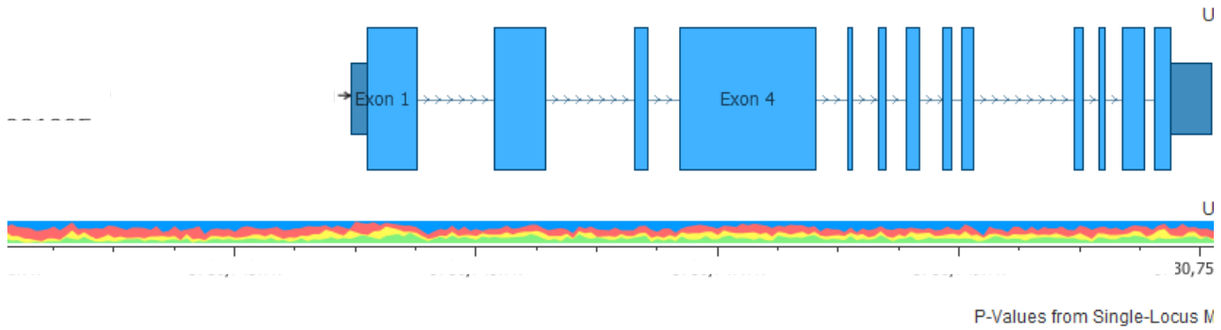
Pummelo X (Nova+Succari) planted in 2001

Temple X Valencia (4X) planted in 1996

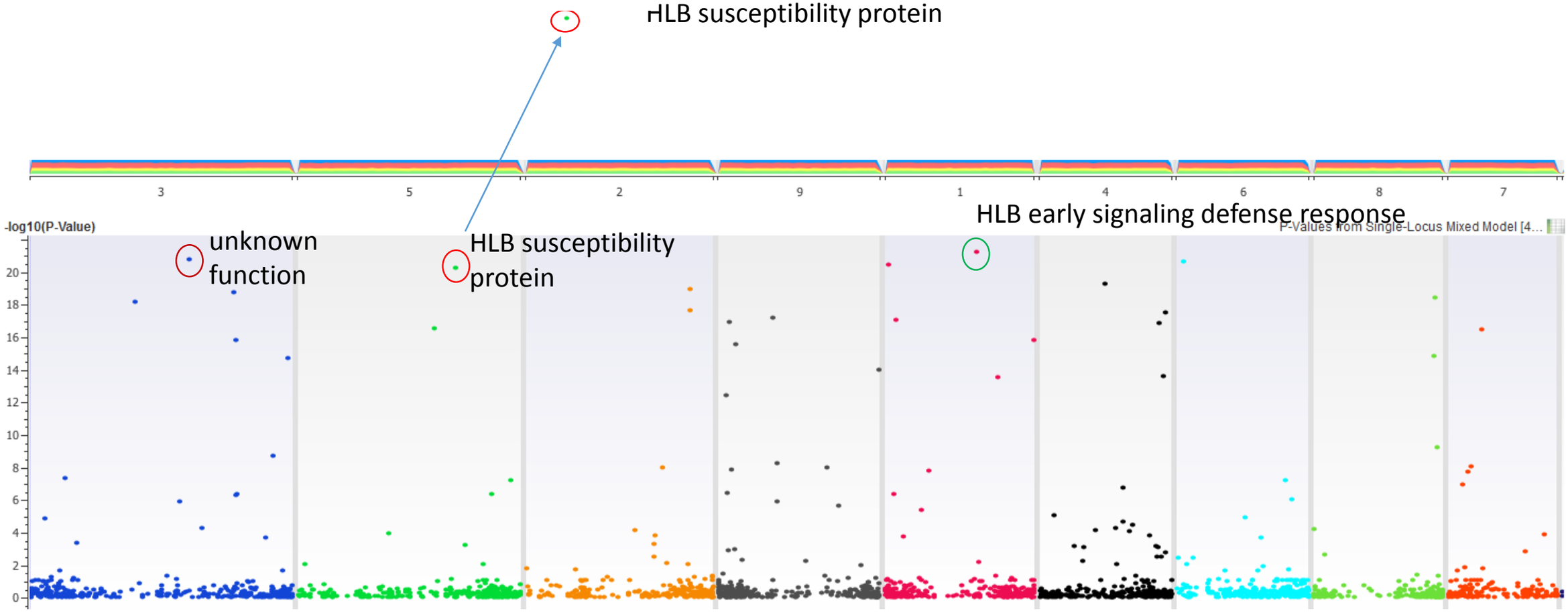
Temple X (Nova+Succari) planted in 2002

Temple X Valencia (4X) planted in 1996

“Manhattan Plot” showing SNPs responsible for HLB on citrus nine chromosomes



HLB susceptibility protein



WHAT ARE THE GENETIC MECHANISMS THAT UNDERLIE APPARENTLY TOLERANT PHENOTYPES ?

WHAT GENES CAN BE TARGETED TO INCREASE TOLERANCE OR RESISTANCE IN COMMERCIAL CITRUS CULTIVARS?

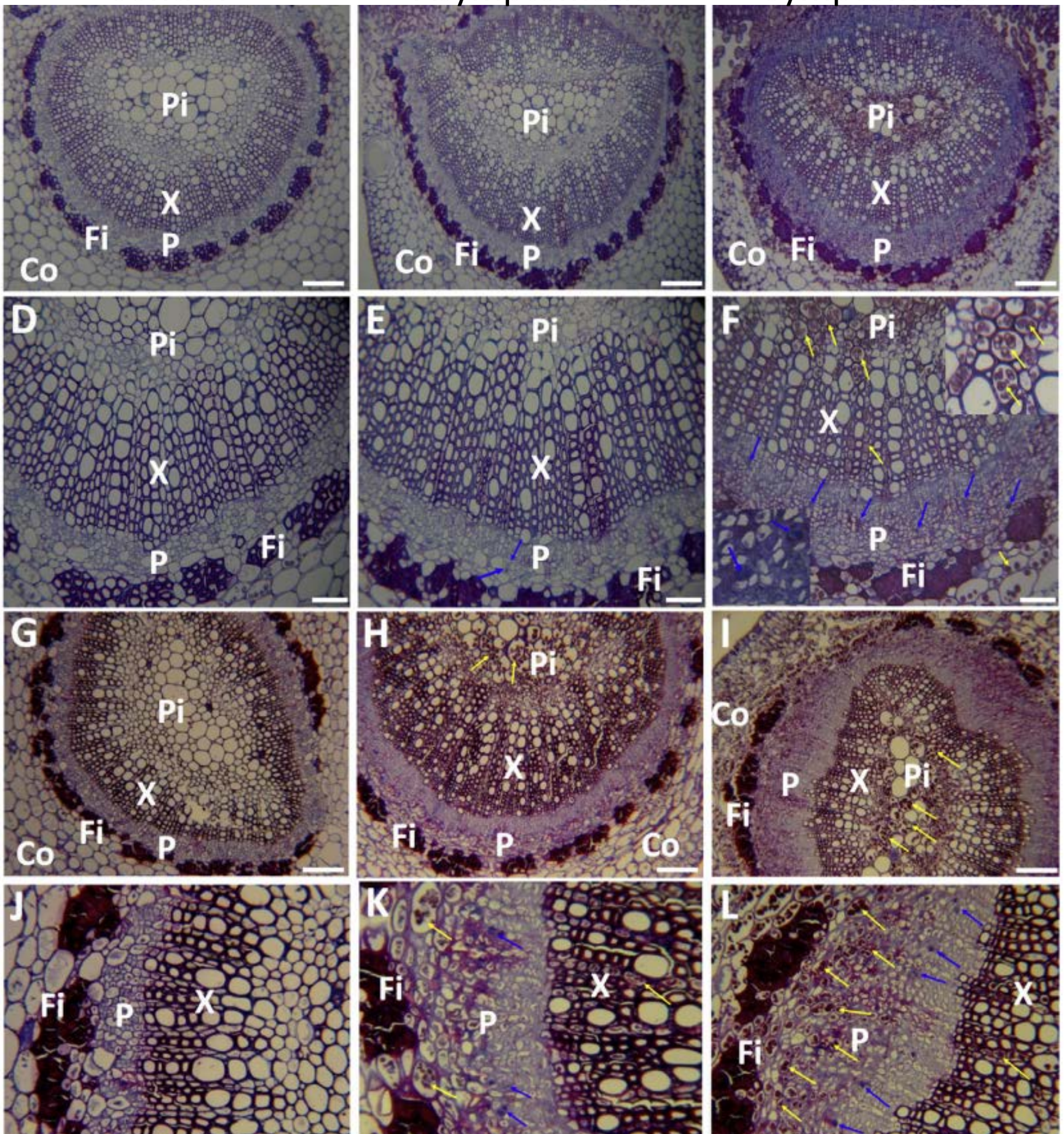
Control

Asymptomatic

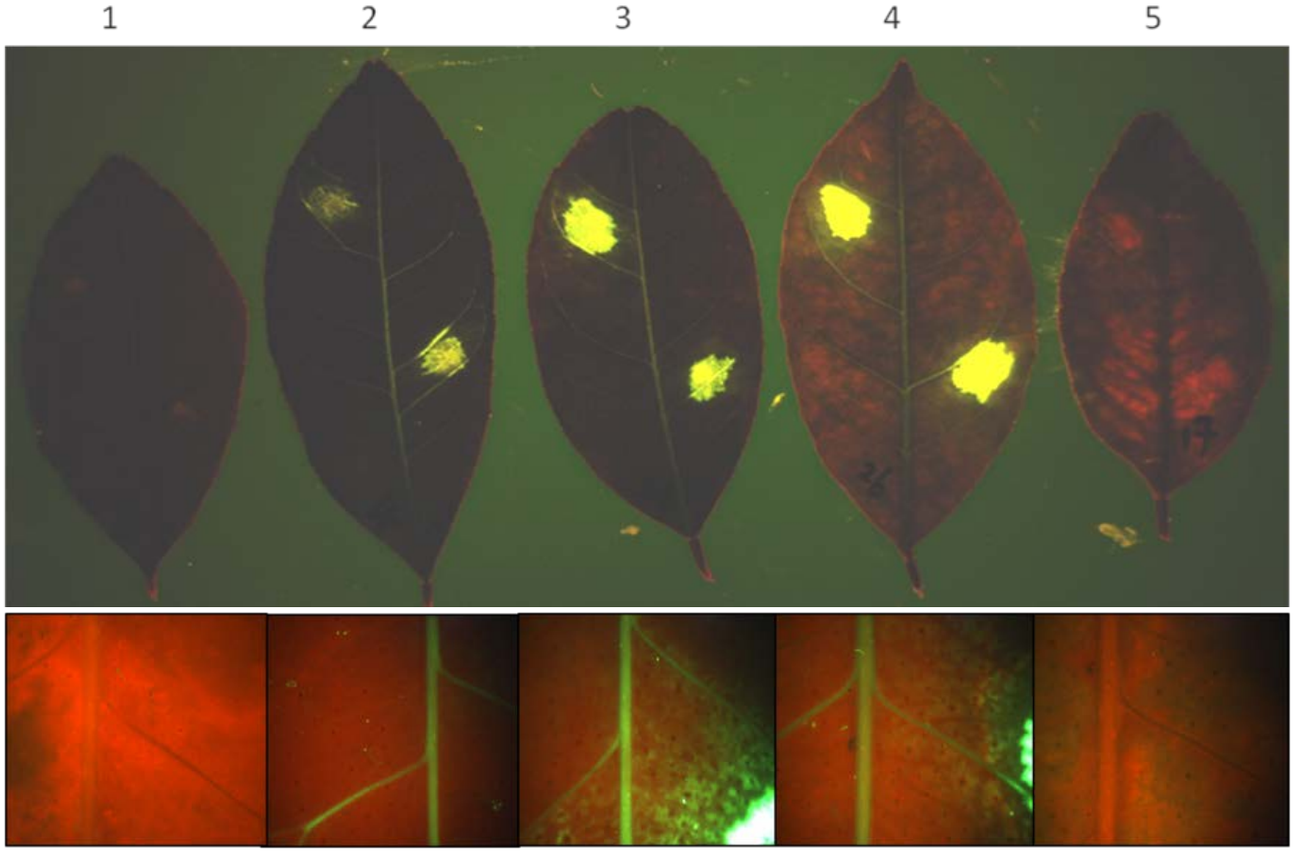
Symptomatic

Rough Lemon

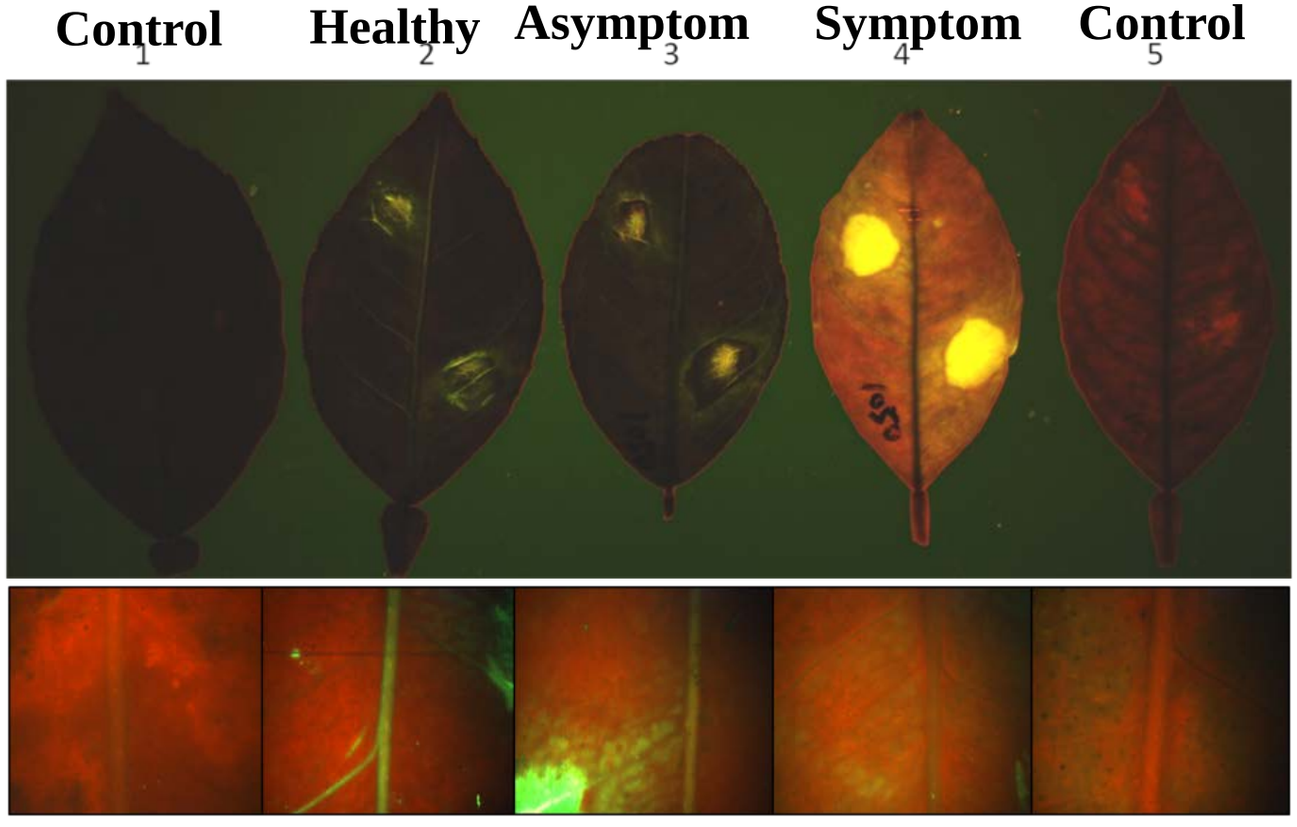
Sweet Orange



Rough lemon



Sweet orange



- Continued to explore anatomical differences between sensitive and tolerant types, including Sugar Belle and true lemon

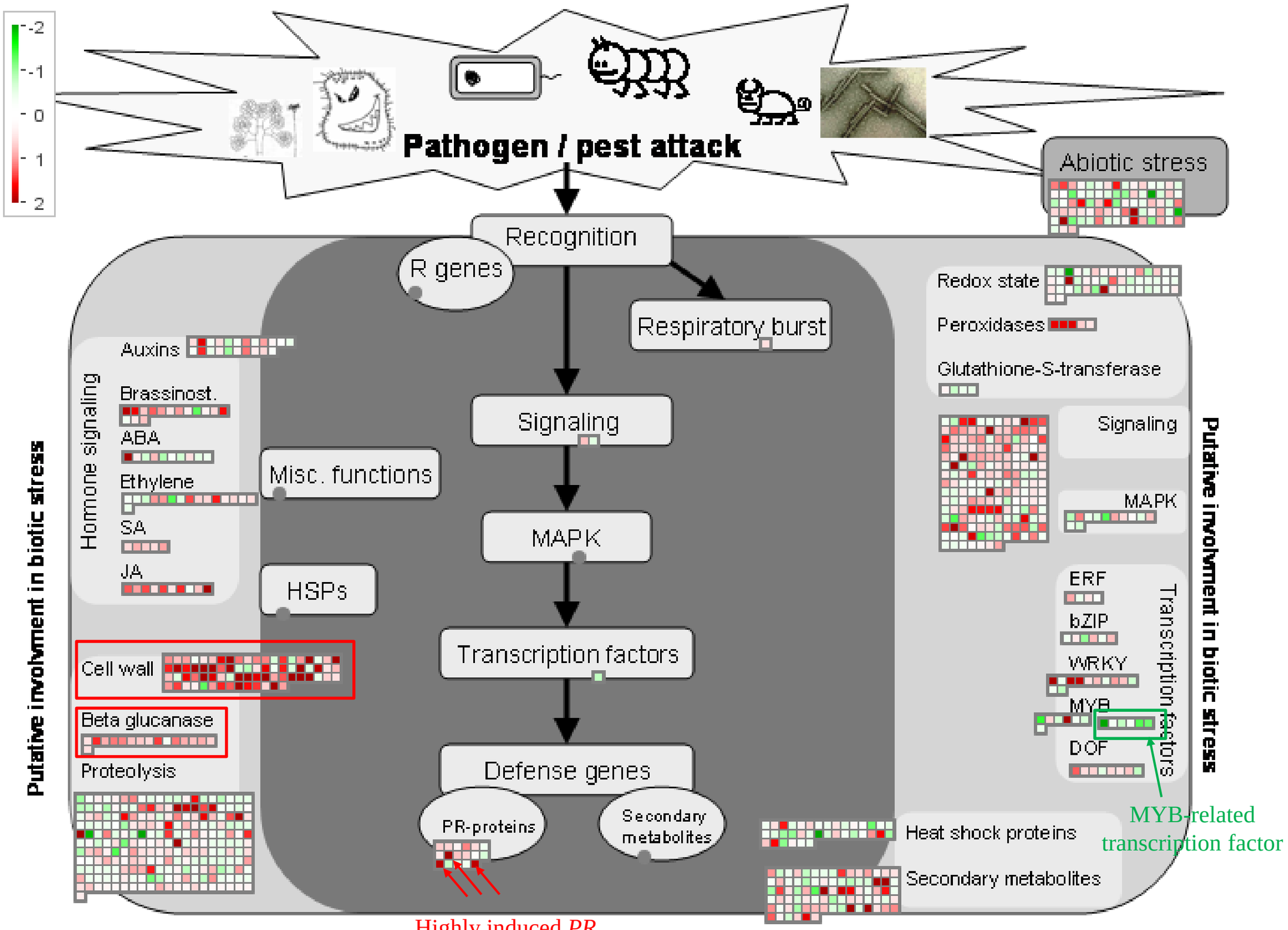
- Phloem destruction and disorganization is more pronounced in sensitive types

- Cambial zones of tolerant types become active and produce replacement phloem

- Cambium degenerates and produces mainly collapsing or necrotic replacement phloem

- In other words, tolerance is associatedwith new plumbing

Implications of Anatomical Studies



Rough lemon: Affymetrix Array Study

- **Focus on identifying gene targets for modification**
  - **Gene co-expression networks (Du et al., 2015)**
  - **Meta-analysis, co-expression, miRNA nested network analysis (Rawat et al. 2015)**
  - **Validation and characterization**
  - **Association studies across germplasm**
  - **More genome sequencing**

- **Development and utilization of genome editing technologies**

Seeking Silver Bullets

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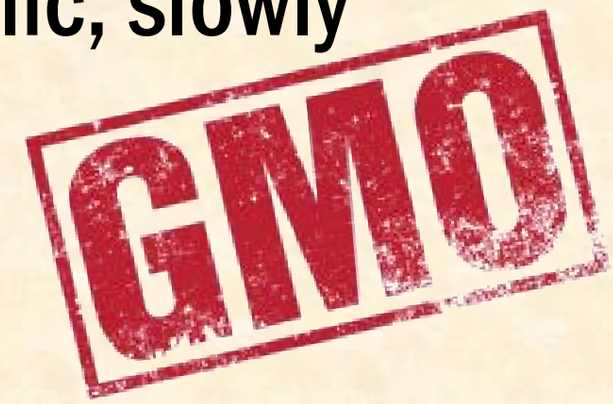
About GMO

- Genetically modified organism (GMO) contains foreign genes
- Discovery of DNA double-helix in 1950s & recombinant DNA technology in 1970s
- GMO bacteria, plants, and animals in research and industry
- GMO food is being accepted by the public, slowly



Overview of GMO crops

- First GMO crop (tomato) for sale in the U.S. in 1994
- GMO of staple crops such as corn, cotton, soybean, canola etc.
- Release and planting of GMO crops increased rapidly




GMO vs traditional breeding

Methods of Plant Breeding

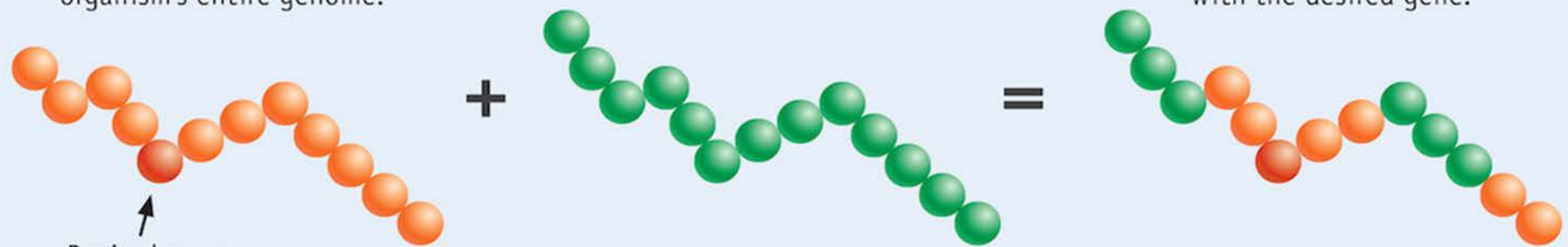
Traditional

The traditional plant breeding process introduces a number of genes into the plant. These genes may include the gene responsible for the desired characteristic, as well as genes responsible for unwanted characteristics.

Donor Variety DNA Strand
DNA strands contain a portion of an organism's entire genome.

Recipient Variety DNA Strand

New Variety DNA Strand
Many genes are transferred with the desired gene.



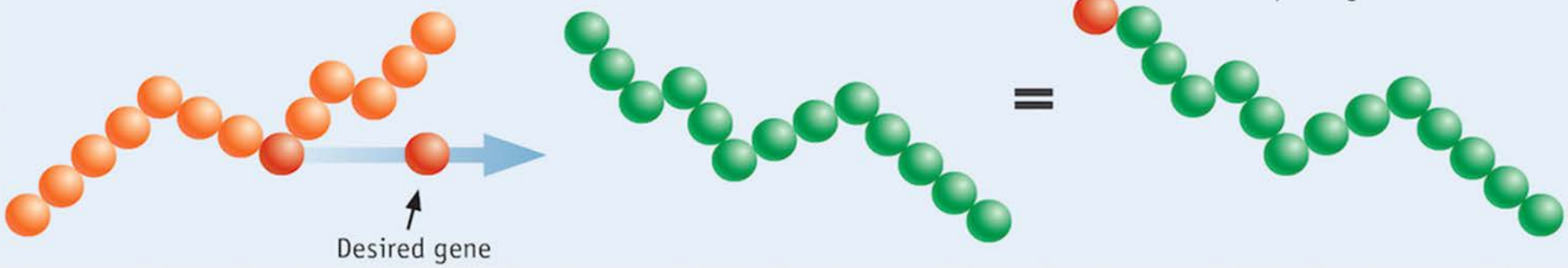
Genetic Engineering

Genetic engineering enables the introduction into the plant of the specific gene or genes responsible for the characteristic(s) of interest. By narrowing the introduction to one or a few identified genes, scientists can introduce the desired characteristic without also introducing genes responsible for unwanted characteristics.

Donor Organism DNA Strand
The desired gene is copied from the donor organism's genome.

Recipient Variety DNA Strand

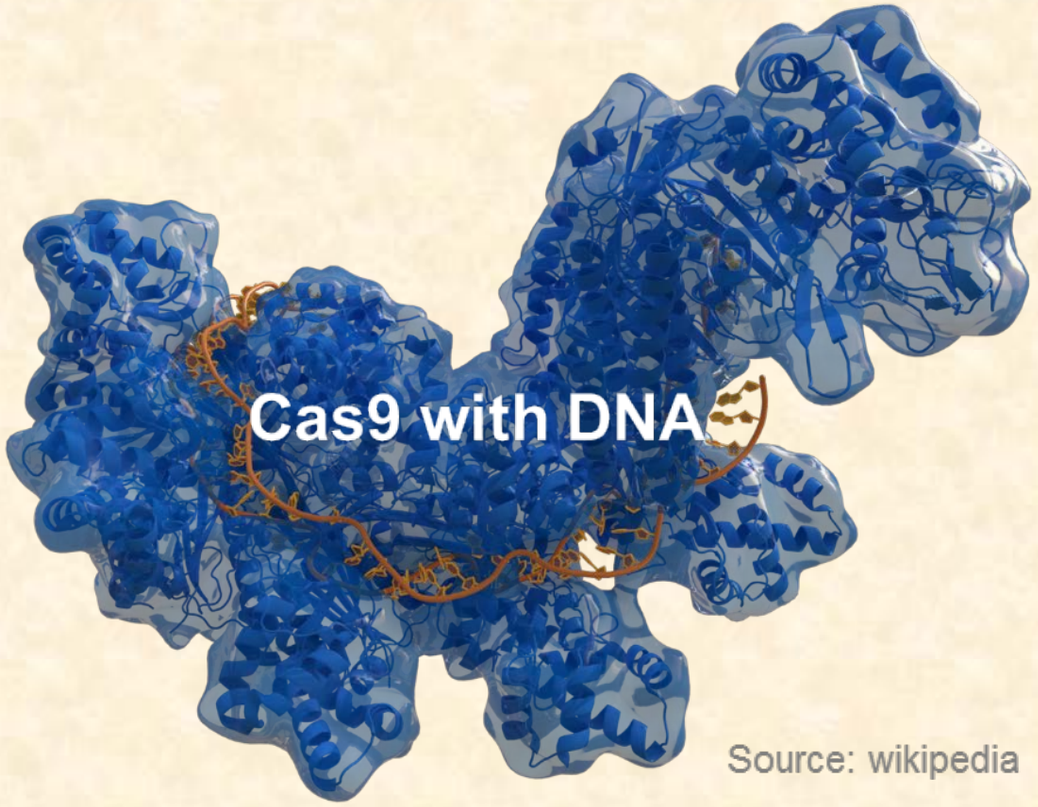
New Variety DNA Strand
Only the desired gene is transferred to a location in the recipient genome.



Source: FDA

CRISPR-Cas9

The new opportunity and challenge



Source: wikipedia

Huge advantages of the CRISPR system

Easy design to change almost any single genes

Or change multiple redundant genes simultaneously

Precise gene editing by homologous recombination

High-throughput functional genomics applications

Option to leave no fingerprint after making changes

Not subject to regulation if only small changes are made

No introduction of foreign/bacterial DNA like in GMO crops

BIOTECHNOLOGY

Gene-edited CRISPR mushroom escapes US regulation

A fungus engineered using CRISPR–Cas9 can be cultivated and sold without oversight.

BY EMILY WALTZ

The US Department of Agriculture (USDA) will not regulate a mushroom that has been genetically modified with the gene-editing tool CRISPR–Cas9, the agency has confirmed. The long-awaited decision means that the mushroom can be cultivated and sold without passing through the agency's regulatory process — making it the first CRISPR-edited organism to receive a green light from the US government.

"The research community will be very happy with the news," says Caixia Gao, a plant biologist at the Chinese Academy of Sciences Institute of Genetics and Developmental Biology in Beijing, who was not involved in developing the mushroom. "I am confident we'll see more gene-edited crops falling outside of regulatory authority."

Yinong Yang, a plant pathologist at Pennsylvania State University (Penn State) in University Park, engineered the fungus — the common white button mushroom (*Agaricus bisporus*) — to resist browning. The effect is achieved by targeting the family of genes that encodes polyphenol oxidase (PPO), an enzyme that causes browning. By deleting just a hand-

STUART MCCALL/GETTY

The common white button mushroom (*Agaricus bisporus*) has been modified to resist browning.

Source: Nature magazine

CRISPR-edited crops free to enter market, skip regulation

The first CRISPR-edited crops presented to the US regulatory system can be cultivated and sold without oversight by the US Department of Agriculture (USDA), the agency said in a pair of letters posted in April. The decisions could reduce by millions the cost of development of the crops: an anti-browning mushroom and a waxy corn genetically modified with the gene editing tool CRISPR-Cas9. Some scientists hailed the decision as a step in the right direction, although media outlets and other interested parties said it illustrates the murky state of US biotech regulations.

© Dinodia Photos / Alamy Stock Photo

DuPont Pioneer's high amylopectin corn is the first CRISPR-edited plant likely to bypass USDA oversight.

Johnston, Iowa–based DuPont Pioneer engineered the waxy corn to contain starch composed exclusively of the branched polysaccharide amylopectin—a commodity in processed foods, adhesives and high-gloss paper. Company researchers achieved the effect by shutting down production of cornstarch's other long-chain polysaccharide, amylose. Using the gene-editing tool CRISPR-Cas9, the team knocked out the endogenous waxy gene *Wx1*, which encodes the endosperm's granule-bound starch synthase responsible for making amylose.

DuPont Pioneer, currently undergoing a merger with The Dow Chemical Company, says it expects the CRISPR-edited variety to have higher yields than conventional waxy corn. The company plans to commercialize the plant within five years and follow it with many more CRISPR-edited crops. "This is just the beginning," said Neal Gutterson, vice president of R&D, in a statement released to coincide with the USDA's response.

necessary tool in biotech. Plant pests have served as the trigger for USDA oversight since the 1980s, when the US government wrote the regulatory framework for biotech products.

Newer genetic engineering (GE) techniques that don't involve plant pests are quickly supplanting the old ones, and the USDA appears to be saying it does not have the authority to regulate the products of these techniques. The letters to DuPont and Yang were the agency's first decisions on CRISPR-edited crops. The agency ruled similarly on plants transformed with other gene-editing techniques, such as zinc-finger nuclease and transcription activator-like effector nuclease systems.

Such letters from USDA have become "essential" to small companies attempting to bring to market GE plants, says

Source: Nature Biotechnology

CRISPR proven to work in citrus

OPEN ACCESS Freely available online

PLOS | ONE

Targeted Genome Editing of Sweet Orange Using Cas9/sgRNA

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Abstract

Genetic modification, including plant breeding, has been widely used to improve crop yield and quality, as well as to increase disease resistance. Targeted genome engineering is expected to contribute significantly to future varietal improvement, and genome editing technologies using zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9/single guide RNA (sgRNA) have already been successfully used to genetically modify plants. However, to date, there has been no reported use of any of the current genome editing approaches in sweet orange, an important fruit crop. In this study, we first developed a novel tool, Xcc-facilitated agroinfiltration, for enhancing transient protein expression in sweet orange leaves. We then successfully employed Xcc-facilitated agroinfiltration to deliver Cas9, along with a synthetic sgRNA targeting the *CsPDS* gene, into sweet orange. DNA sequencing confirmed that the *CsPDS* gene was mutated at the target site in treated sweet orange leaves. The mutation rate using the Cas9/sgRNA system was approximately 3.2 to 3.9%. Off-target mutagenesis was not detected for *CsPDS*-related DNA sequences in our study. This is the first report of targeted genome modification in citrus using the Cas9/sgRNA system—a system that holds significant promise for the study of citrus gene function and for targeted genetic modification.

Citation: Jia H, Wang N (2014) Targeted Genome Editing of Sweet Orange Using Cas9/sgRNA. PLoS ONE 9(4): e93806. doi:10.1371/journal.pone.0093806

Editor: Manoj Prasad, National Institute of Plant Genome Research, India

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Challenges and limitations

Transformation efficiency lower than non-CRISPR plasmids

Plant genomes can be more complex (polypoid)

Plant cell walls make it harder to reach inside cells

Optimize Cas9 codon for plants

Minimize off-target effects of Cas9 cleavage

<u>Identification of relevant targets for HLB resistance</u>

However, this tool can enable very precise, potentially unregulated, changes to the citrus genome, allowing trait-targeted modifications

Fruit quality, resistance to other diseases, etc.

- **8 to 9 members in sweet orange, Clementine, & *Poncirus* genomes**
- **2 members expressed in *Poncirus* leaves, increased expression after *C*Las inoculation**
- **Similar genes conferred resistance to bacteria in Arabidopsis & rice**
- **Secreted to intercellular spaces in those plants**
- **Suspected to produce a mobile signal**
- **Induced local & systemic defense responses**
- **Being cloned for transformation**

1st Group of Candidate Genes

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THANK YOU!

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