Updates on improving citrus resistance against HLB via CRISPR genome editing

-four citrus plants ready for Florida citrus industry to test against HLB

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08/20/2025

Take home message

- ✓We have found the genetic evidence that HLB is pathogen triggered immune disease.
- ✓The identified genes can be used for genome editing of citrus to improve disease resistance against HLB.
- ✓Non-transgenic *Eds1*-edited and *Dmr6*-edited Hamlin and Valencia were generated and have received regulatory approvals by USDA APHIS and EPA.

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What to consider for generating HLB-resistant citrus varieties using the CRISPR technology

- ✓<u>Suitable target genes</u>
- ✓Disease resistance
- ✓No significant negative side effects
- ✓Highly efficient biallelic or homozygous mutations in the T0 generation
- ✓No off-target
- ✓<u>Non-transgenic, or Non-GMO</u>
- ✓Bypassing juvenility if possible

The regulatory status of gene-edited organisms under proposed US legislation

Are genome-edited organisms a regulated article?			
	USDA-APHIS	EPA	FDA
YES	If the edit introduces additional nucleic acids	If the edit results in the introduction of a plant-incorporated protectant (PIP)	If it's an animal[1]
NO	1. If the edit is a deletion of any size 2. If the edit is a single base-pair substitution 3. If the edit introduces naturally occurring nucleic acid sequences	If the edit does not constitute a PIP	No, if it's a crop. But a voluntary consultation process

[1]Full risk assessment and market approval as an animal drug.

New regulation
EPA Exempt
published on
May 25 2023

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Overall progress on CRISPR genome editing for resistance/tolerance against HLB

> 200 lines were generated for 40 target genes.

- **Suppressing HLB symptoms by targeting HLB susceptibility gene**
- **Killing CLas by upregulating plant defense**

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What are the suitable target genes for HLB resistance/tolerance via CRISPR genome editing?

Citrus Huanglongbing is a pathogen-triggered immune disease

CLas does not contain typical pathogenicity factors that are directly responsible for disease damages.

CLas triggers systemic and chronic immune responses, including reactive oxygen species (ROS) production and callose deposition, leading to phloem cell death and HLB symptoms.

The genetic evidence for this disease model is unavailable.

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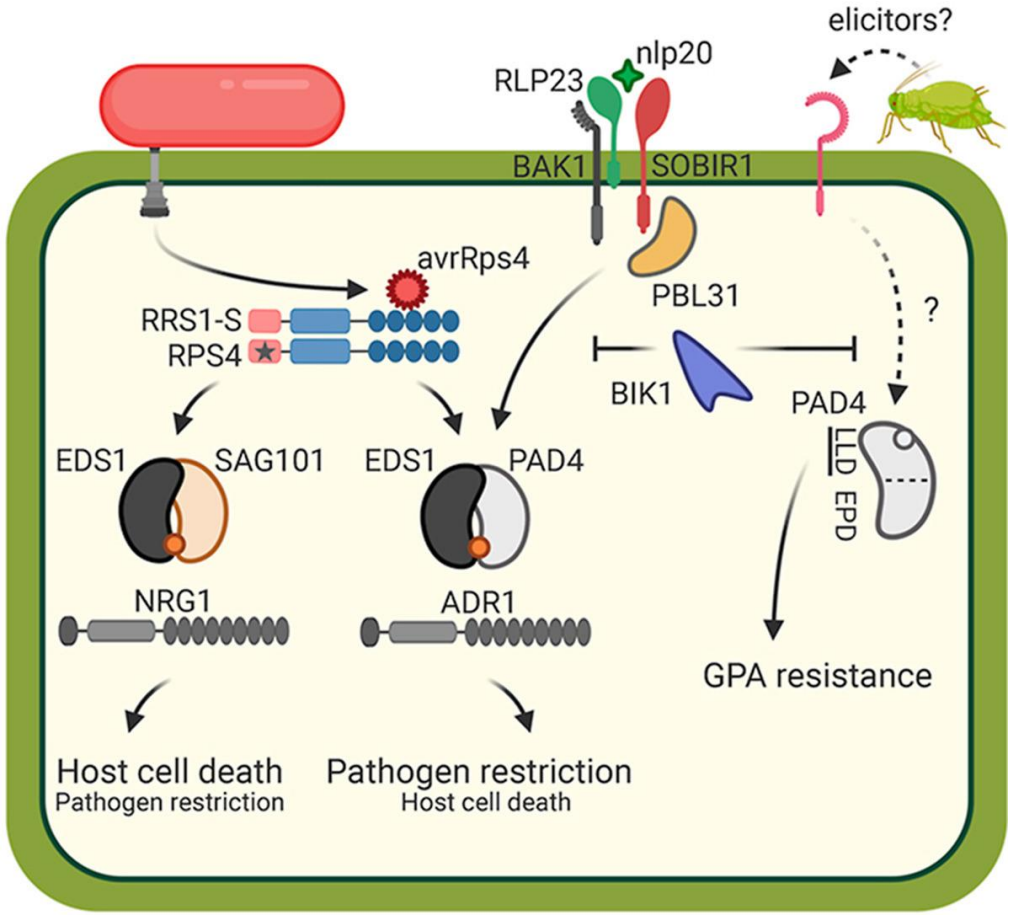
What are the immune signaling genes that drive *Ca.* Liberibacter triggered chronic immune disease?



PTI: BIK1/SOBIR1

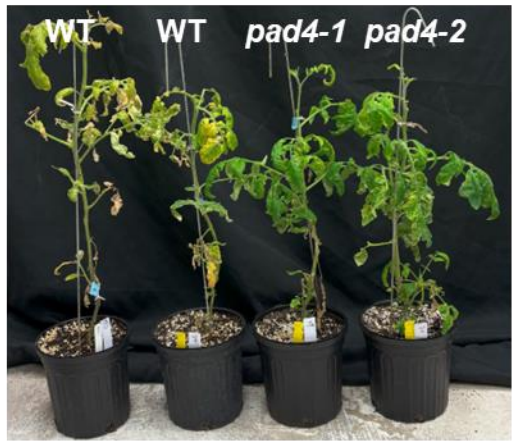
ETI branch 1: EDS1/PAD4/ADR1

ETI branch 2: EDS1/SAG101/NRG1

Dongus and Parker 2021

What are the immune signaling genes that drive *Ca*. Liberibacter triggered chronic immune disease?

Ct 24.1 | Ct 25.6 | Ct 26.5 | Ct 23.5 | Ct 25.9



Ct 19.8 | Ct 29.1 | Ct 21.2 | Ct 19.5

- ***Ca*. L. psyllaurous (Lpsy, synonym *Ca*. L. solanacearum) causes increased leaf yellowing, ROS production, cell death, phloem callose deposition and starch accumulation in tomato, similar to that seen in HLB.**

- **Knockout of tomato *Eds1* and *Pad4* but not *RbohB*, *Bik1* and *Sobir1* reduces disease symptoms, ROS production, callose deposition, and phloem cell death caused by *Ca.* Liberibacter**

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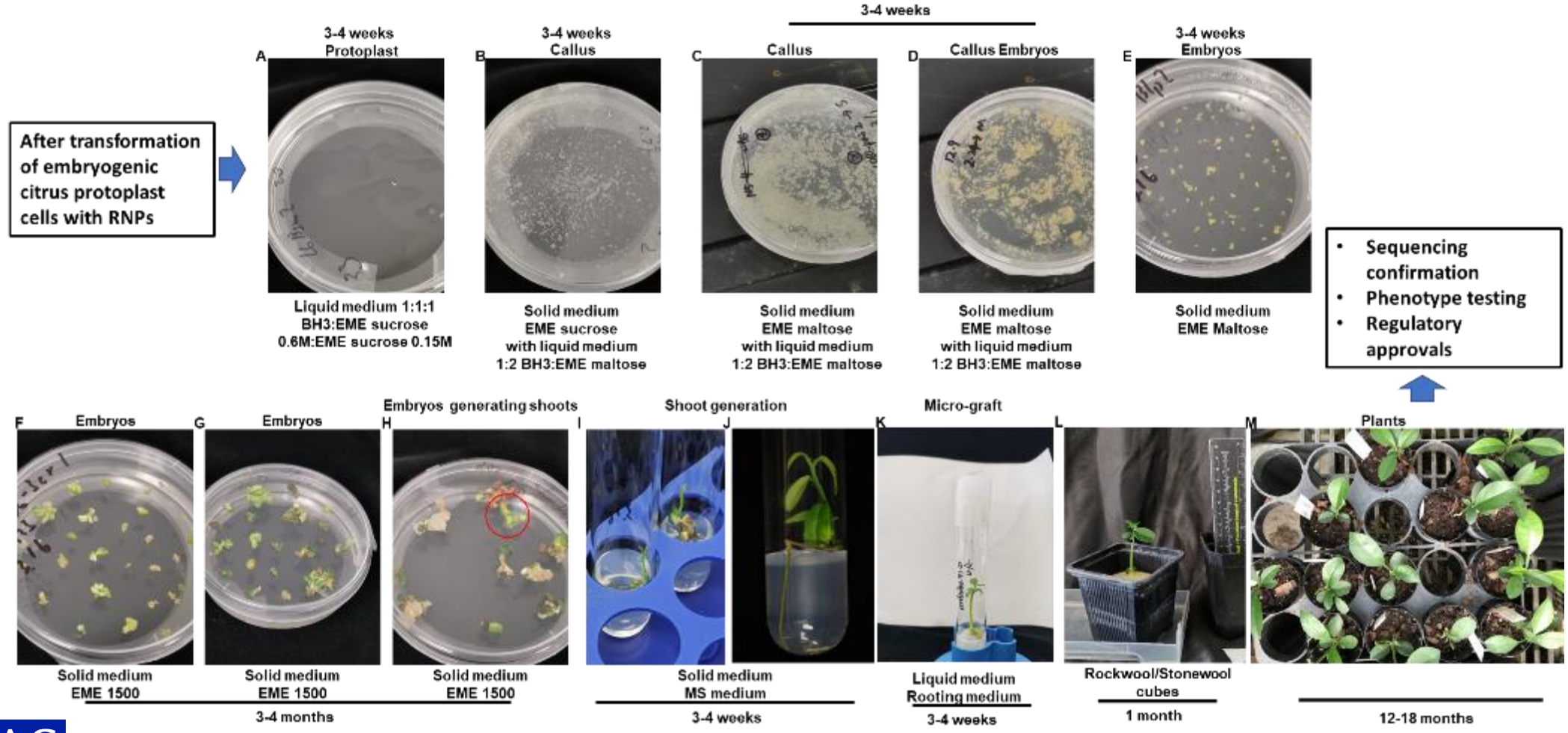
unpublished

How do we conduct non-transgenic CRISPR genome editing for citrus?

Non-transgenic genome editing of citrus using CCas12a/crRNA ribonucleoprotein transformation of embryogenic protoplasts



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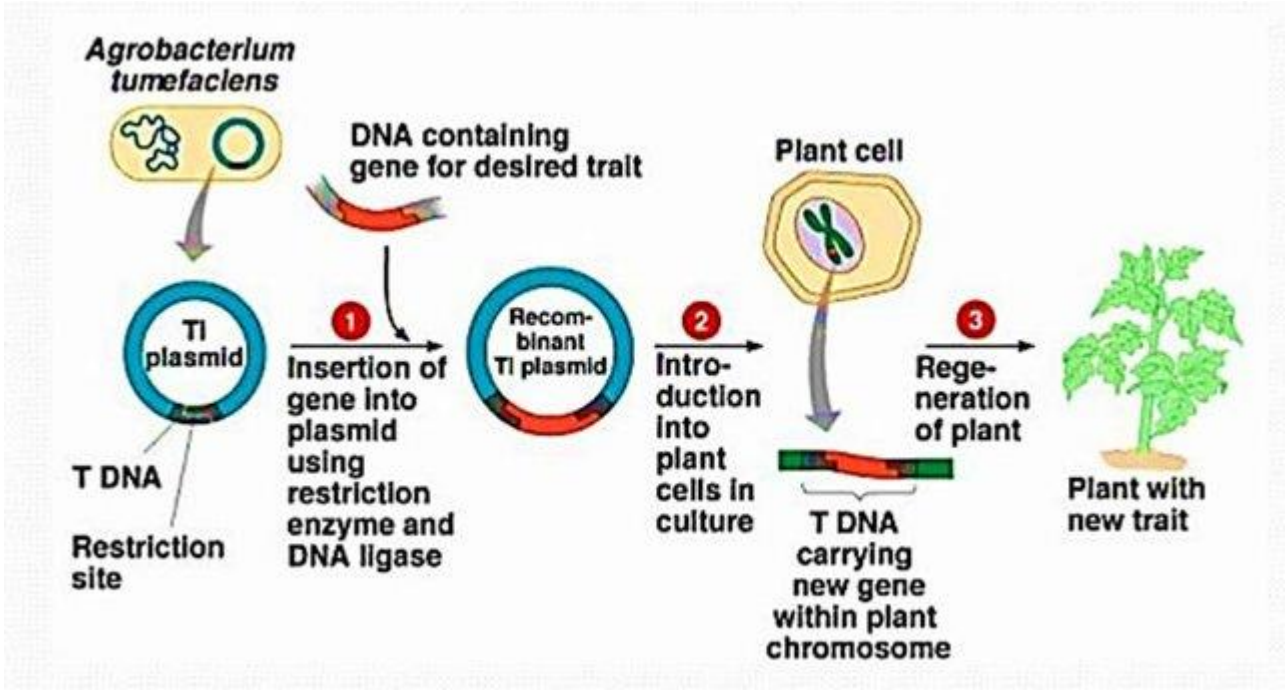
Transgene-free genome editing in plants in the T0 generation based on *Agrobacterium-mediated* co-editing strategy



nature plants

Brief Communication

https://doi.org/10.1038/s41477-023-01520-y

Transgene-free genome editing of vegetatively propagated and perennial plant species in the T0 generation via a co-editing strategy

Received: 4 March 2023

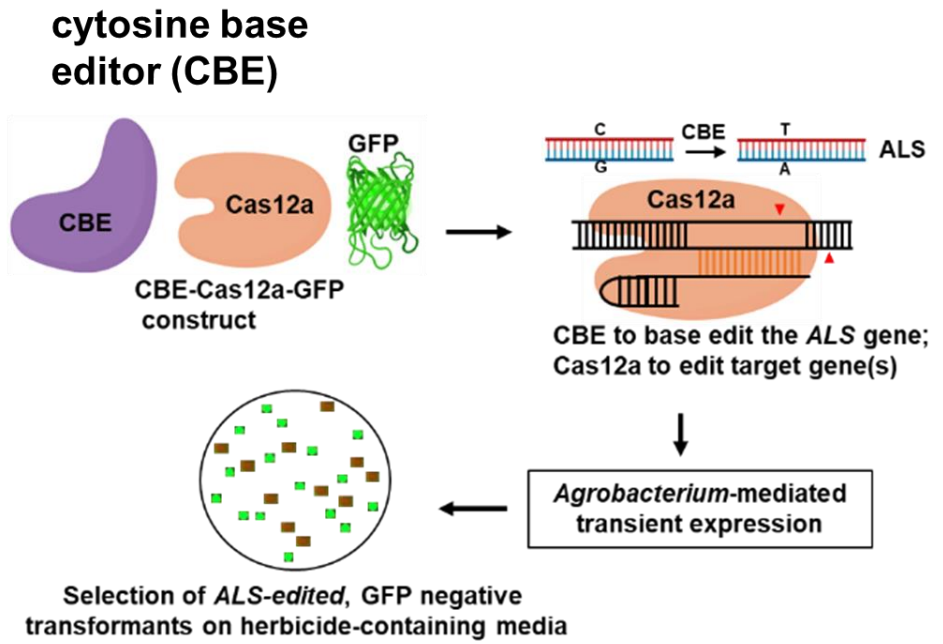
Accepted: 22 August 2023

Published online: 18 September 2023

Check for updates

Xiaoen Huang[1,3], Hongge Jia[1,3], Jin Xu[1], Yuanchun Wang[1], Jiawen Wen[2] & Nian Wang[1]

Transgene-free plant genome editing in the T0 generation is highly desirable but challenging[1,2]. Here we achieved such a goal using a co-editing strategy via *Agrobacterium*-mediated transient expression of cytosine base editor to edit *ALS* encoding acetolactate synthase to confer herbicide chlorsulfuron resistance as a selection marker, Cas12a/CRISPR RNA for editing gene(s) of interest, and green fluorescent protein for selecting transgene-free transformants. The biallelic/homozygous transgene-free mutation rates for target genes among herbicide-resistant transformants ranged from 1.9% to 42.1% in tomato, tobacco, potato and citrus. This co-editing strategy is particularly useful for transgene-free genome editing of vegetatively propagated and perennial plant species in the T0 generation.



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Target 1: Our first batch of non-transgenic genome edited (*Eds1*) Valencia and Hamlin sweet orange have received regulatory approvals by APHIS and EPA

- **APHIS regulatory filing: first file on 4/24/25, Not a regulated article (Non-GMO/non-transgenic) under 7 CFR part 340: May 1, 2025**
- **EPA approval was submitted on April 24, 2025, approved on 6/4/25**
- **FDACS/DPI New Budwood program: plants submitted on July 15, 2025 to permit moving plants to nurseries for propagation through fast-track.**

***Eds1*-edited**

Hamlin **Valencia**

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Why are the non-transgenic *Eds1-edited* sweet orange potentially a good choice against HLB?

- Editing of *Eds1* abolishes CLas induction of ROS production, phloem callose deposition and phloem cell death, thus the plants are likely HLB tolerant.
- Have received both APHIS (non-transgenic) and EPA approvals.
- No effects on growth and fruit quality!
- The plants are ready for testing by growers (need propagation by nurseries).

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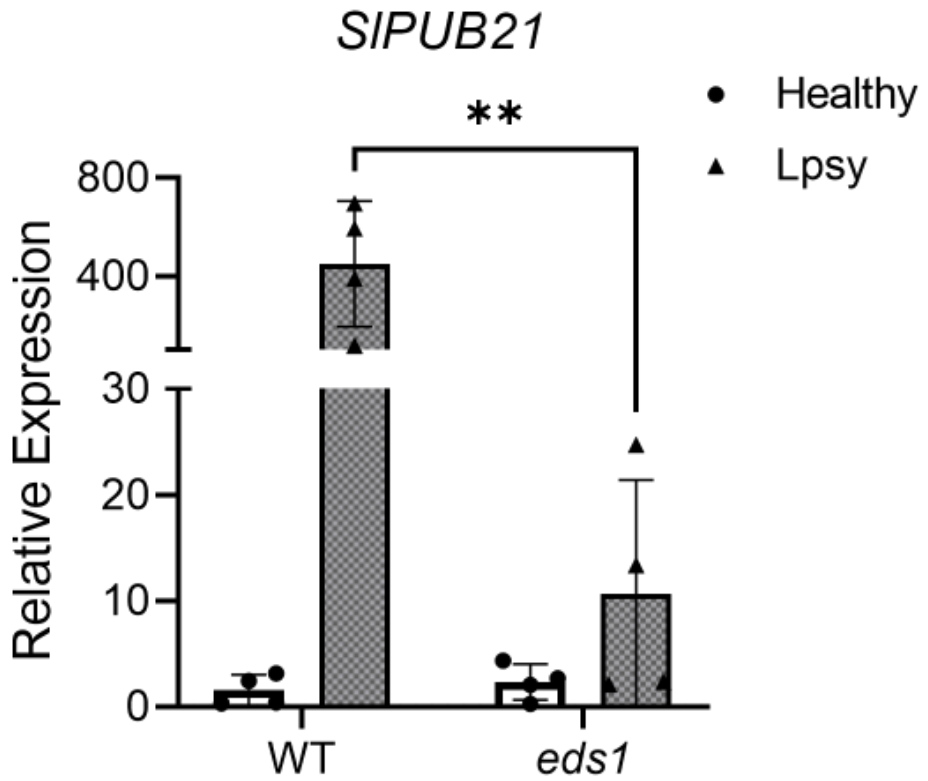
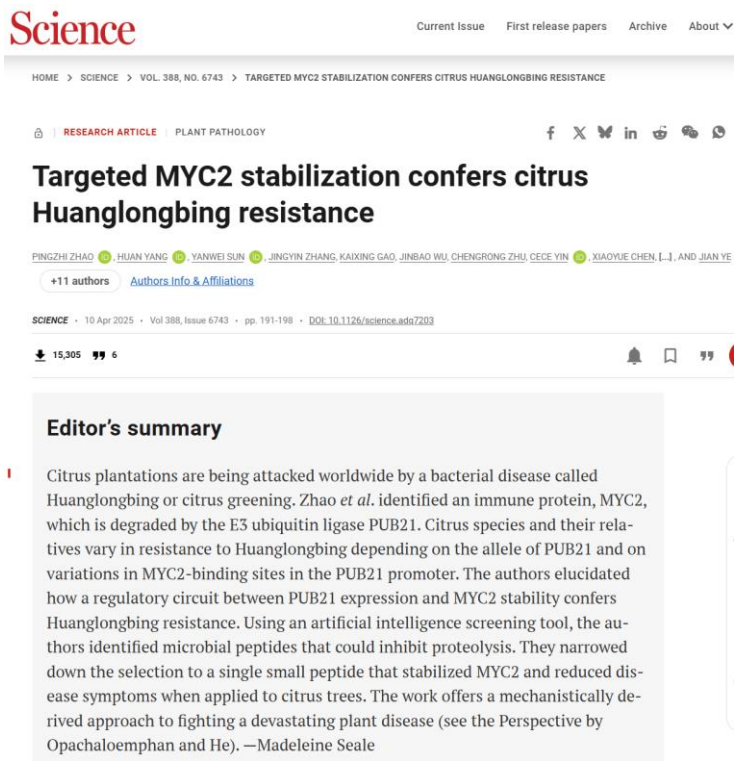
Eds1 positively regulates the recently identified HLB susceptibility gene PUB21

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RESEARCH ARTICLE | PLANT PATHOLOGY

Targeted MYC2 stabilization confers citrus Huanglongbing resistance

PINGZHI ZHAO, HUAN YANG, YANWEI SUN, JINGYIN ZHANG, KAIXING GAO, JINBAO WU, CHENGRONG ZHU, CECE YIN, XIAOYUE CHEN, [...], AND JIAN YE

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15,305 6

Editor's summary

Citrus plantations are being attacked worldwide by a bacterial disease called Huanglongbing or citrus greening. Zhao *et al.* identified an immune protein, MYC2, which is degraded by the E3 ubiquitin ligase PUB21. Citrus species and their relatives vary in resistance to Huanglongbing depending on the allele of PUB21 and on variations in MYC2-binding sites in the PUB21 promoter. The authors elucidated how a regulatory circuit between PUB21 expression and MYC2 stability confers Huanglongbing resistance. Using an artificial intelligence screening tool, the authors identified microbial peptides that could inhibit proteolysis. They narrowed down the selection to a single small peptide that stabilized MYC2 and reduced disease symptoms when applied to citrus trees. The work offers a mechanistically derived approach to fighting a devastating plant disease (see the Perspective by Opachaloemphan and He). —Madeleine Seale

***PUB21* is an *S* gene for citrus HLB**

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Cautions in adopting the non-transgenic *Eds1-edited* sweet orange against HLB and how to address them

- Mutation of *Eds1* likely reduces accumulation of salicylic acid, thus reducing disease resistance against several biotrophic pathogens such as citrus canker pathogen but increase resistance against *Phytophthora*.

- How to address this issue:
- This compromise is partially alleviated by rootstocks, and most commercial citrus rootstocks are resistant or tolerant against most biotrophic pathogens.

- The benefit of improving disease tolerance against HLB using this approach likely outweighs the cost because most scion diseases except HLB have effective control approaches.

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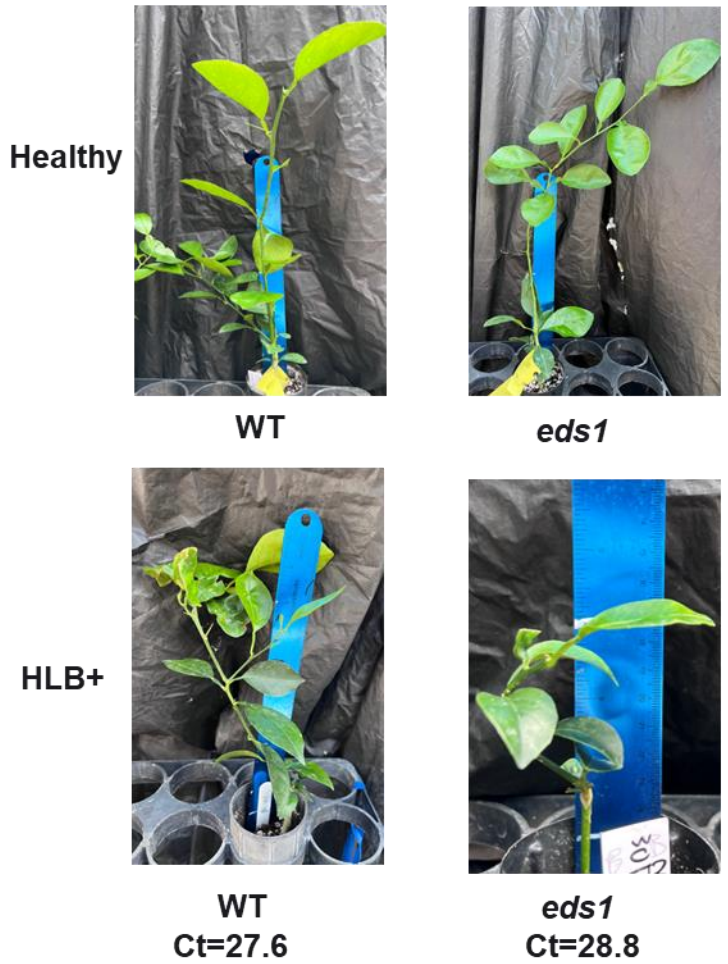
Next step for non-transgenic *Eds1* edited Valencia and Hamlin

- CLas inoculation test ongoing.
- Field trial needs to be done!
- Nursery propagation (8/26).
- To overcome the juvenility.



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Because CLas is a biotrophic pathogen, promoting immune response can increase citrus resistance against HLB by reducing CLas titers.

Target 2: Our 2nd batch of non-transgenic genome edited Valencia and Hamlin plants (*Dmr6, a broad range disease S gene*) have received regulatory approvals by APHIS and EPA

Hamlin

Dmr6-edited | *Dmr6*-edited | Wild type

Valencia

Dmr6-edited

The *Dmr6*-edited plants grow slightly slower than the wild type initially, but there are no major growth defects so far.

APHIS regulatory filing: filed on 6/02/25, “approved” on 6/24/25.

EPA: filed on 06/09/25, approved on 7/2/25.

FDACS/DPI New Budwood program: plants submitted on July 15, 2025 to permit moving plants to nurseries for propagation and testing through fast-track.

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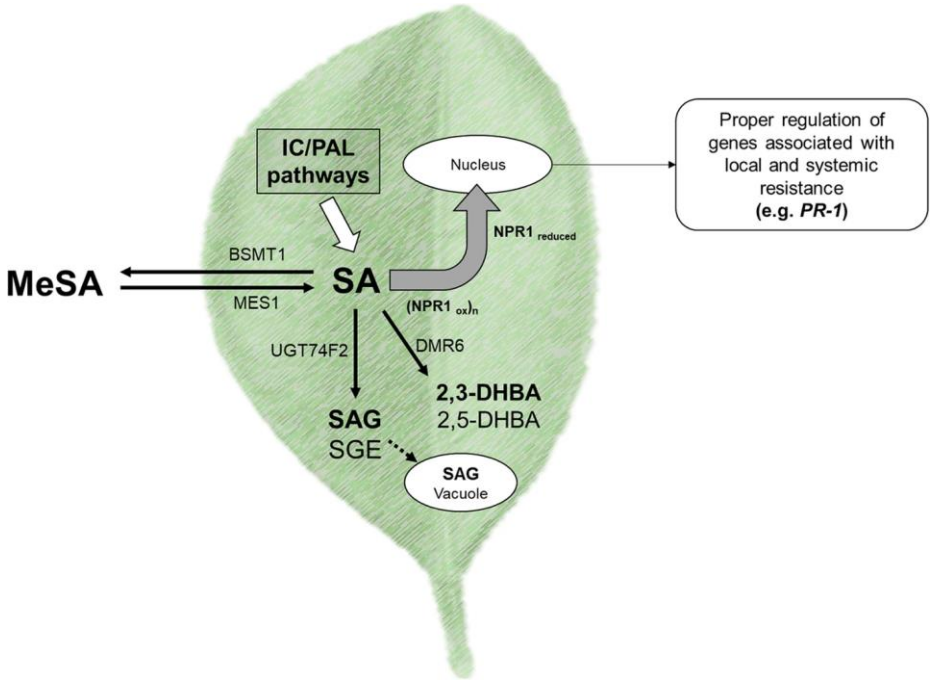
Why are the non-transgenic *Dmr6*-edited sweet orange potentially a good choice for citrus growers against HLB?



Ibanez et al. 2019

- ***DMR6*** **encodes SA-5 hydroxylase that degrades SA.**
- **Inactivation of *DMR6* results in increased SA levels and confers resistance to different classes of pathogens, including bacteria and oomycetes.**
- **It is expected genome editing of citrus DMR6 will enhance the killing effect of plant defense against CLas.**

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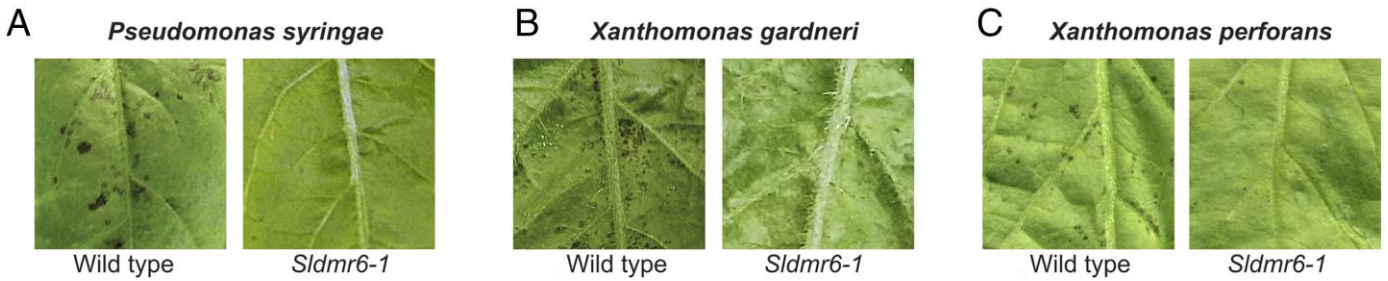
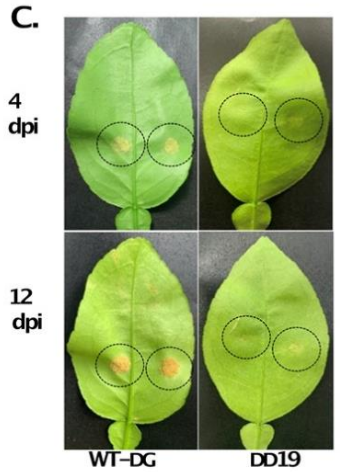
Inactivation of *DMR6* confers resistance to multiple bacterial pathogens



. 2021

Dmr6 **Wild type**

Banana *Xanthomonas campestris* pv. musacearum



grapefruit *Xanthomonas citri subsp. citri*

Parajuli et al. 2022

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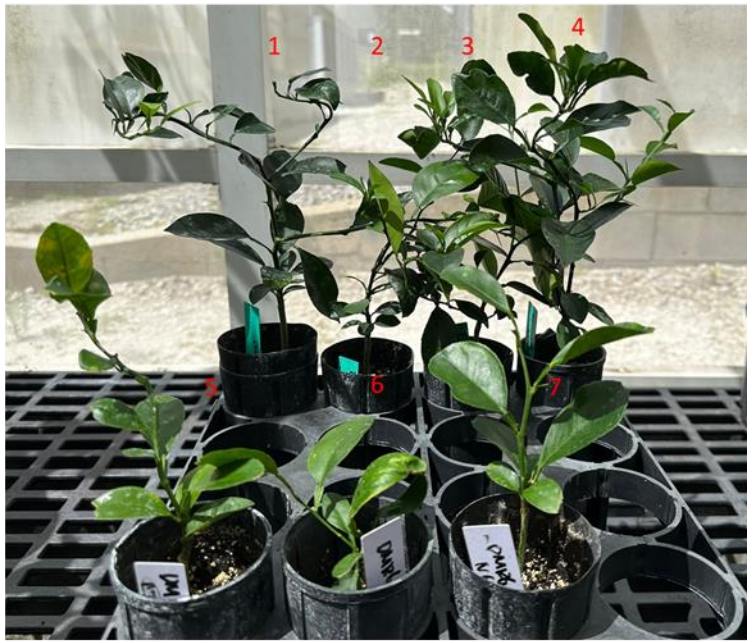
Next step for non-transgenic *Dmr6* edited Valencia and Hamlin

- CLas inoculation test ongoing.
- Field trials need to be done!
- Nursery propagation (8/26).
- To overcome the juvenility.



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Timeline for the non-transgenic *Eds1* and *Dmr6* edited citrus plants to be available to Florida citrus growers for testing

	2025	2026	2027	2028	2029
APHIS	Done				
EPA	Done				
HLB testing (greenhouse and field trial)	Ongoing/to be done				
DPI New Budwood Program	July 15, 2025				
Nursery propagation	to be done	v	v	v	v
Trees available for growers testing*		small scale	small scale	large scale	large scale

It is recommended that small scale field trials must be done by growers and the scientific community before large scale planting!

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Conclusion

- Citrus HLB is a pathogen-triggered chronic immune disease via Eds1 and Pad4 mediated chloroplastic ROS production and phloem callose deposition, leading to phloem cell death and subsequent disease development.

- We have generated non-transgenic citrus varieties by genome editing the putative HLB susceptibility gene *Eds1* and *Dmr6* that have received regulatory approvals by APHIS and EPA and are ready for testing by citrus growers.

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