Case studies for disease resistant/tolerant citrus varieties generated via non-transgenic CRISPR genome editing

Nian Wang
Citrus Research and Education Center
Department of Microbiology and Cell Science
Institute of Food and Agricultural Sciences
University of Florida
03/31/2026

Outline

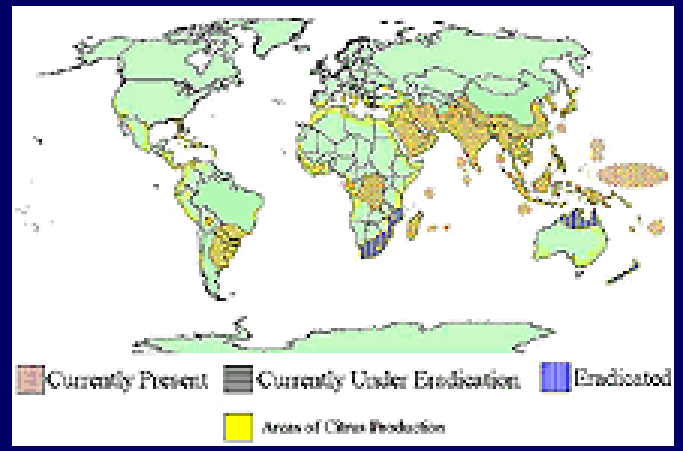
Regulatory approvals/exemptions by APHIS and EPA for non-transgenic genome edited citrus generated by either transformation of embryogenic protoplasts with ribonucleoprotein (RNP) or *Agrobacterium*-mediated co-editing method.

- ✓ **Case study 1. Citrus canker**
- ✓ **Case study 2. Citrus HLB**

Engineering canker resistance of citrus cultivars through transgene-free genome editing

Gram negative,
xanthomonadin, xanthan gum

fruit drop, defoliation, yield losses 5-30%, fruit marketability



Gottwald 2002

Present in Florida, Louisiana, Texas, Alabama, Georgia

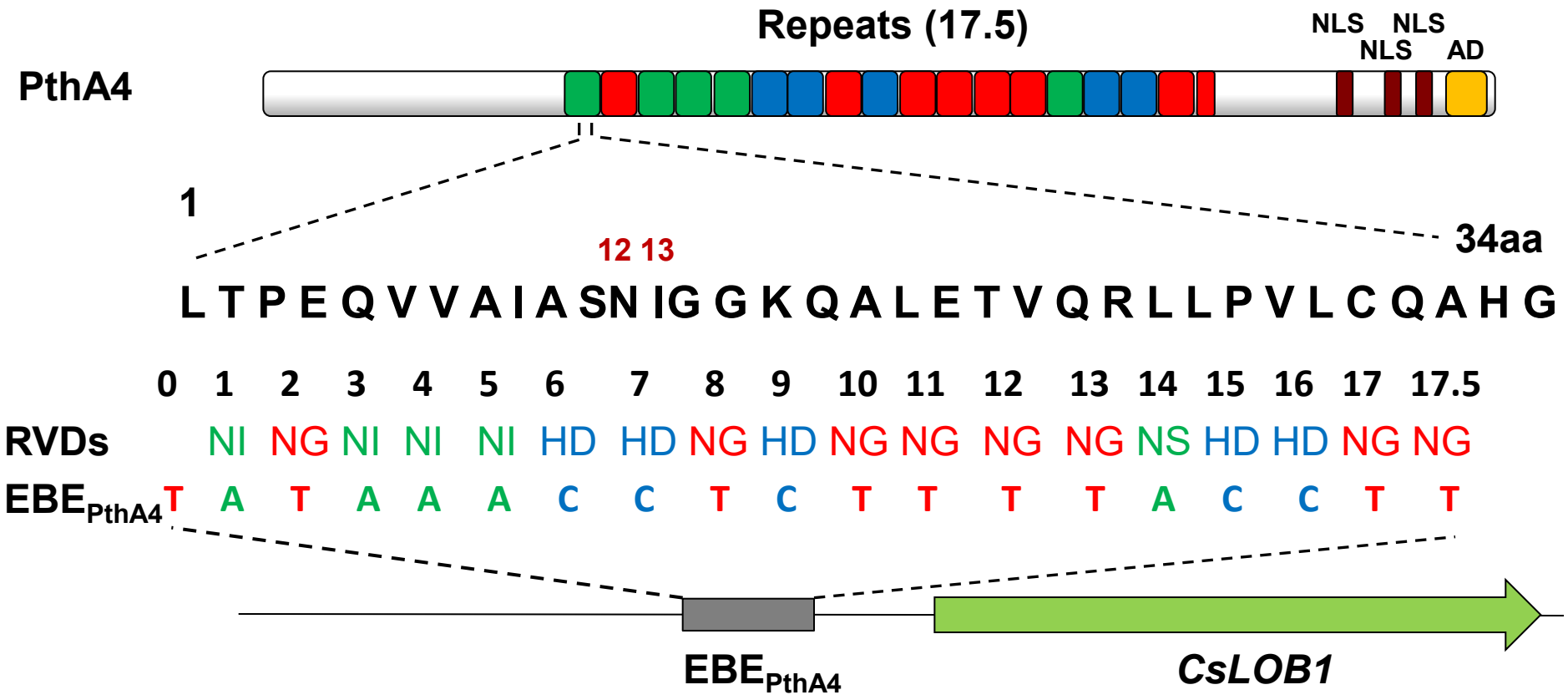
PthA is a transcription activator-like (TAL) effector that triggers canker symptoms by inducing expression of canker susceptibility gene *LOB1*


Yang Hu


Hongge Jia


Jeff Jones


Frank White



Hu et al. 2014 PNAS

TALEN (TAL Effector Nucleases (Fok1)) in genome editing in 2011, CRISPR genome editing 2013

Developing canker resistant citrus varieties via genome editing the EBE_{PthA4} and coding region of *CsLOB1*


Hongge Jia


Jeff Jones


Frank White

- *CsLOB1* is a disease susceptibility gene for citrus canker disease.

Genome editing of the EBE of *CsLOB1* promoter region or the coding region via transgenic approach has been successfully used to generate canker resistant citrus plants

Mutation of the coding region or the promoter region of *CsLOB1* enables citrus resistance against canker

grapefruit

Plant Biotechnology Journal

Plant Biotechnology Journal (2017) **15**, pp. 817–823 doi: 10.1111/pbi.12677

Genome editing of the disease susceptibility gene *CsLOB1* in citrus confers resistance to citrus canker

Hongge Jia[1], Yunzeng Zhang[1], Vladimir Orbović[2], Jin Xu[1], Frank F. White[3], Jeffrey B. Jones[3] and Nian Wang[1,*]

Pummelo

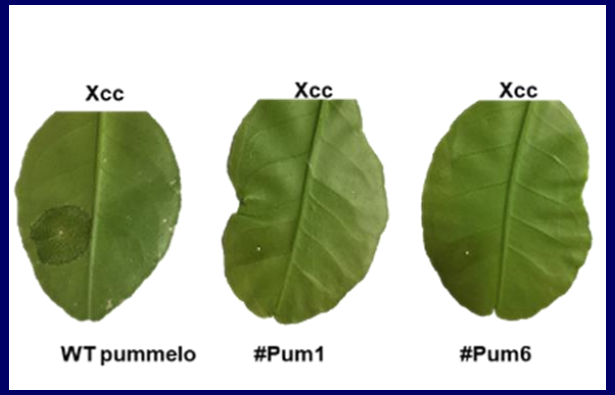
Plant Biotechnology Journal

Plant Biotechnology Journal (2020) **18**, pp. 1990–1992 doi: 10.1111/pbi.13375

Brief Communication

Generation of homozygous canker-resistant citrus in the T0 generation using CRISPR-SpCas9p

Hongge Jia and Nian Wang



frontiers in Plant Science

ORIGINAL RESEARCH published: 11 January 2022 doi: 10.3389/fpls.2021.769907

Highly Efficient Generation of Canker-Resistant Sweet Orange Enabled by an Improved CRISPR/Cas9 System

Xiaoen Huang†, Yuanchun Wang and Nian Wang†*

Sweet orange

Phytopathology® · 2022 · 112:308-314 · https://doi.org/10.1094/PHYTO-04-21-0144-R

Genetics and Genomics of Resistance

e-Xtra*

Biallelic Editing of the *LOB1* Promoter via CRISPR/Cas9 Creates Canker-Resistant 'Duncan' Grapefruit

Hongge Jia,[1] Ahmad A. Omar,[2,3] Vladimir Orbović,[2] and Nian Wang[1,†]

grapefruit

Genome editing of the EBE region of *LOB1* of *C. sinensis* cv. Hamlin has no observable negative effect on plants (so far)

Lob1 **edited *C. sinensis* cv. Hamlin 2 years old**

Wild type *C. sinensis* cv. Hamlin 2.5 years old

The edited lines are transgenic. Due to regulatory issues, it has not been commercialized.

We have generated non-transgenic canker resistant sweet orange cv. Hamlin by genome editing the codin region of *CsLOB1*

nature communications

Article

https://doi.org/10.1038/s41467-023-39714-9

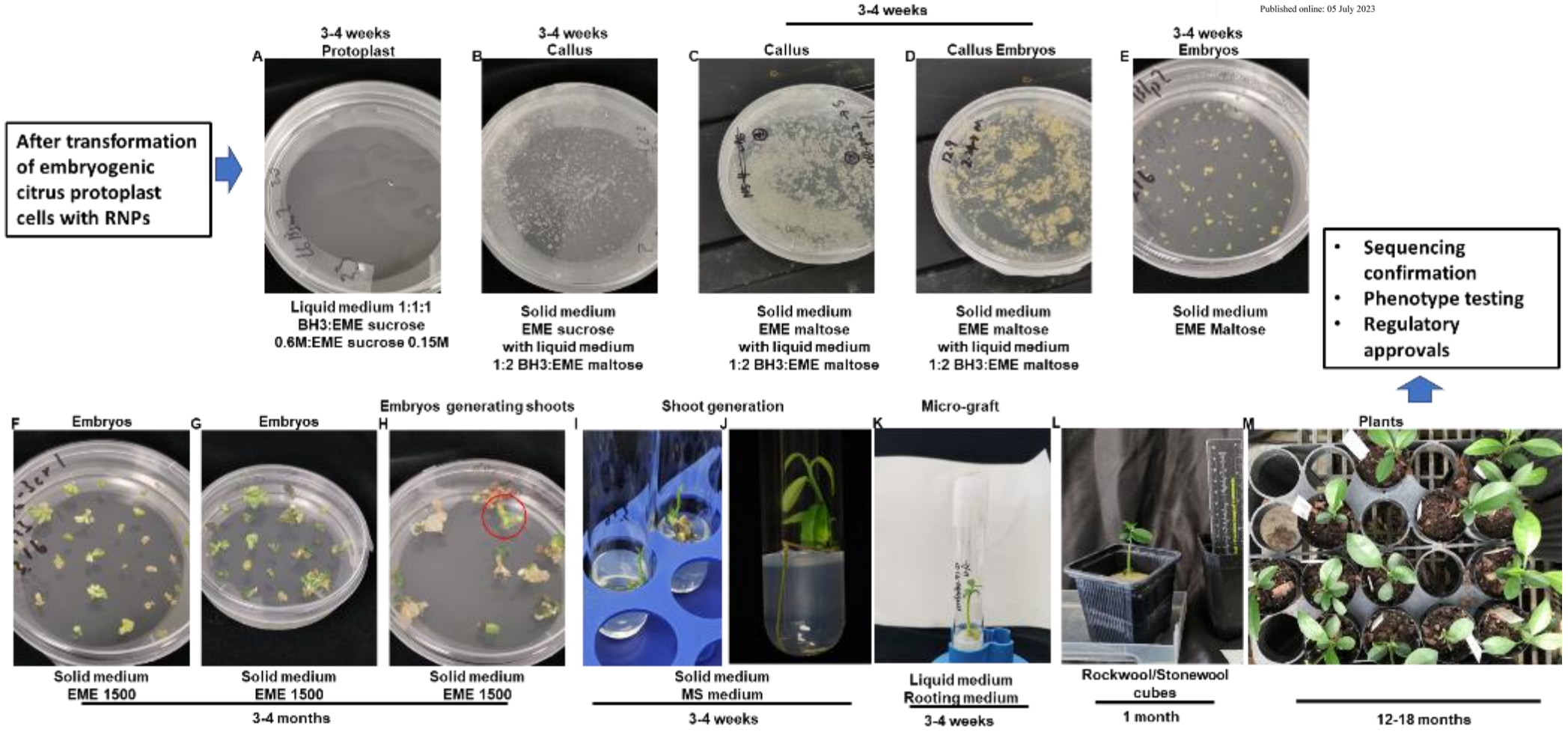
Generation of the transgene-free canker-resistant *Citrus sinensis* using Cas12a/crRNA ribonucleoprotein in the T0 generation

Received: 13 March 2023

Accepted: 26 June 2023

Published online: 05 July 2023

Hang Su[1], Yuanchun Wang[1], Jin Xu[1], Ahmad A. Omar[2,3], Jude W. Grosser[2], Milica Calovic[1], Liyang Zhang[4], Yu Feng[1], Christopher A. Vakulskas[4] & Nian Wang[1]



Non-transgenic canker-resistant *Citrus sinensis* cv. Hamlin

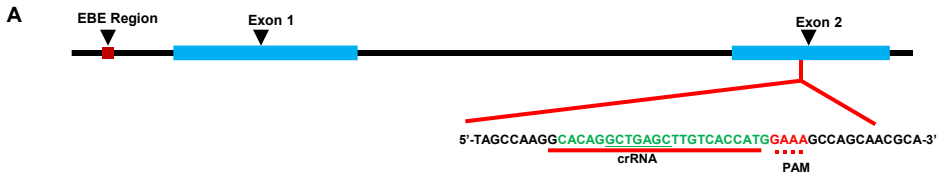
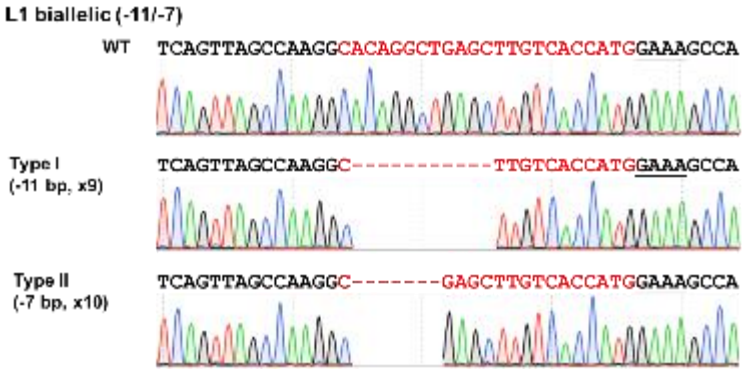
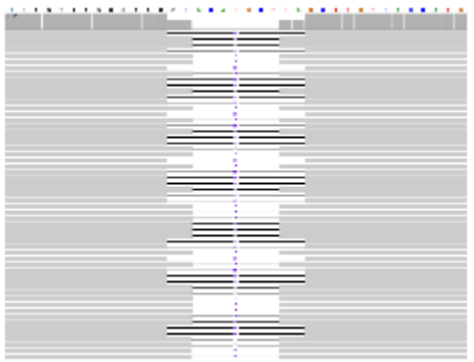


Table 1. Summary of transgene-free *CsLOB1*-edited *C. sinensis* cv. Hamlin lines generated by LbCas12aU/crRNA RNP transformation of embryogenic citrus protoplasts.

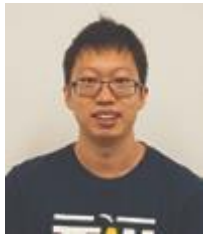
Types of regenerated lines	Mutation types	Mutations of two alleles	Number of regenerated lines
L1	Biallelic	-11/-7	4
L2	Biallelic	-7/-2	4
L3	Biallelic	-14/-7	10
L4	Biallelic	-19/-7	1
L5	Homozygous	-7/-7	8
L6	Biallelic	-7/-4	4
L7	Biallelic	-7/-7 (different)	1
L8	Biallelic	-8/-4	1
L9	Biallelic	-7/-6	1
L10	Biallelic	-4/-3	2
L11	Biallelic	-11/-9	1
L12	Biallelic	-9/-6+348	1
L13	Wild type		1





PCR and sequencing
Whole genome sequencing using Illumina
No off-target mutations

Non-transgenic canker-resistant *Citrus sinensis* cv. Hamlin



Hang Su

Yuanchun Wang

Ahmad Omar

Jude Grosser

"Am I Regulated" by APHIS for the non-transgenic *LOB1*-edited canker-resistant *C. sinensis* cv. Hamlin

- ✓ **APHIS: regulatory approval (1st submission, 11/4/2022, resubmission 12/6/2022, approval 12/19/2022)**

11/04/2022
Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98
Riverdale, MD 20737

Re: Confirmation that transgene-free, CRISPR-edited *Citrus sinensis* cv. Hamlin is not a regulated article

Relevant technical information:
Transformation method: protoplast transformation
Construct: ribonucleoprotein and gRNA
Recipient organism: *Citrus sinensis*
Donor organisms: No DNA was used in this procedure and no foreign DNA was integrated in the edited Hamlin lines.
Description of both design and evaluation methods used to ensure target specificity of the gRNA in the citrus genome: gRNA, off target analysis based on deep sequencing and whole genome sequencing.

“Am I Regulated” by APHIS for the non-transgenic *LOB1*-edited canker-resistant *C. sinensis* cv. Hamlin

Regulatory exemption requested:
Because the multiple CRISPR-edited Hamlin lines contain no foreign DNA sequences, and the single genetic modification of *LOB1* gene is a change resulting from cellular repair of a targeted DNA break in the absence of an externally provided repair template, the exemption that the plants we developed qualifies for § 340.1(b)(1).

Date: 12/19/2022

Professor Nian Wang
University of Florida
Institute of Food and Agricultural Sciences
Citrus Research and Education Center
700 Experiment Station Road
Lake Alfred, FL 33850

Dear Professor Wang:

Thank you for your letter received on December 6, 2022 (22-311-01cr) requesting confirmation that your plant is exempt from regulation pursuant to 7 CFR § 340.1(b)(1). Your letter describes multiple sweet orange (*Citrus sinensis*) lines modified for providing resistance against citrus canker disease caused by *Xanthomonas citri* subsp. citri.

The Plant Protection Act of 2000 (PPA) provides USDA with broad authority to protect U.S. agriculture, the environment, and the economy by, among other things, regulating the movement of plants and articles to prevent the introduction or dissemination of a plant pest within the United States. As such, USDA, through the Animal and Plant Health Inspection Service, regulates the "Movement of Organisms Modified or Produced through Genetic Engineering," as described in 7 CFR part 340. These regulations do not apply to plants that contain a modification of a type listed in § 340.1(b) or § 340.1(c).

In your letter you state that the single modification in each of your sweet orange lines is a deletion in the citrus canker susceptibility gene *LATERAL ORGAN BOUNDARIES 1* (*LOB1*) resulting from cellular repair of a targeted DNA break to one pair of homologous chromosomes in the absence of an externally provided repair template. Your letter describes transient delivery methods used to generate the genetic modification without introducing any foreign DNA sequences into the citrus genome. You also describe methods used to verify that you made the intended modification with no unintended modifications.

Based on our review of the representations in your letter, USDA confirms the sweet orange lines modified for resistance against citrus bacterial canker disease meet the exemption described in § 340.1(b)(1) and are exempt from regulation under 7 CFR part 340. Plants with modifications that are exempt pursuant to § 340.1(b)(1) are achievable by conventional breeding and unlikely to pose an increased plant pest risk relative to their conventionally bred counterparts.

Although your modified sweet orange is not regulated under 7 CFR part 340, it may be subject to other USDA regulations or other regulatory authorities. For example, importation of your plant, may be subject to Plant Protection and Quarantine (PPQ) permit and/or quarantine requirements. For further information, you may contact the PPQ general number for such inquiries at (877) 770-5990. To inquire about the regulatory status of your plant with the Environmental Protection Agency, please contact Alan

Exemption from EPA for the non-transgenic *LOB1*-edited canker-resistant *C. sinensis* cv. Hamlin

- ✓ **EPA: Loss-of-function (LoF) Plant-Incorporated Protectants (PIPs) self-determination exemption (submission 08/21/2023, “exemption” 04/9/2024).**

- ✓ **Subject: Letter of self-determination per 40 CFR 174.90(a)(2)**

GEPER Package

Package ID: GEPER188

Package Information

Package Name	GEPER-188-Mon Aug 21 17:46:56 UTC 2023
Description	
Email	nianwang@ufl.edu
Fee Waiver Requested	No
Payment Tracking Number	N/A
Payment Amount	N/A
Company Name	University of Florida
Company Number	103160

Documents for the Package

Document Type	Contains CBI?	Document Name
Submission Cover Letter	No	EPA letter of self determination Wang UF 2023.pdf
Document Type	**Contains CBI?**	**Document Name**
Supplemental Study Data	No	transgene-free genome editing RNP canker resistance NC Wang 2023.pdf

Application List

Application: PreApp-GEPER-000001

PIP Name	Transgene-free canker resistant sweet orange (Citrus sinensis) cv. Hamlin lines	**Contains CBI?** No
Genus and Species of Recipient Plant	Citrus x sinensis (Orange)	**Contains CBI?** No
Entrez Gene ID of the Unmodified Gene	102622391	**Contains CBI?** No
PIP Exemption Type	174.27 - Loss-of-function PIP	
Request Type	SelfDetermination	

Documents for the Application

Document Type	Contains CBI?	Document Name
Supplemental Study Data	No	transgene-free genome editing RNP canker resistance NC Wang 2023.pdf

CBI Substantiation for the Application

Submitter Information

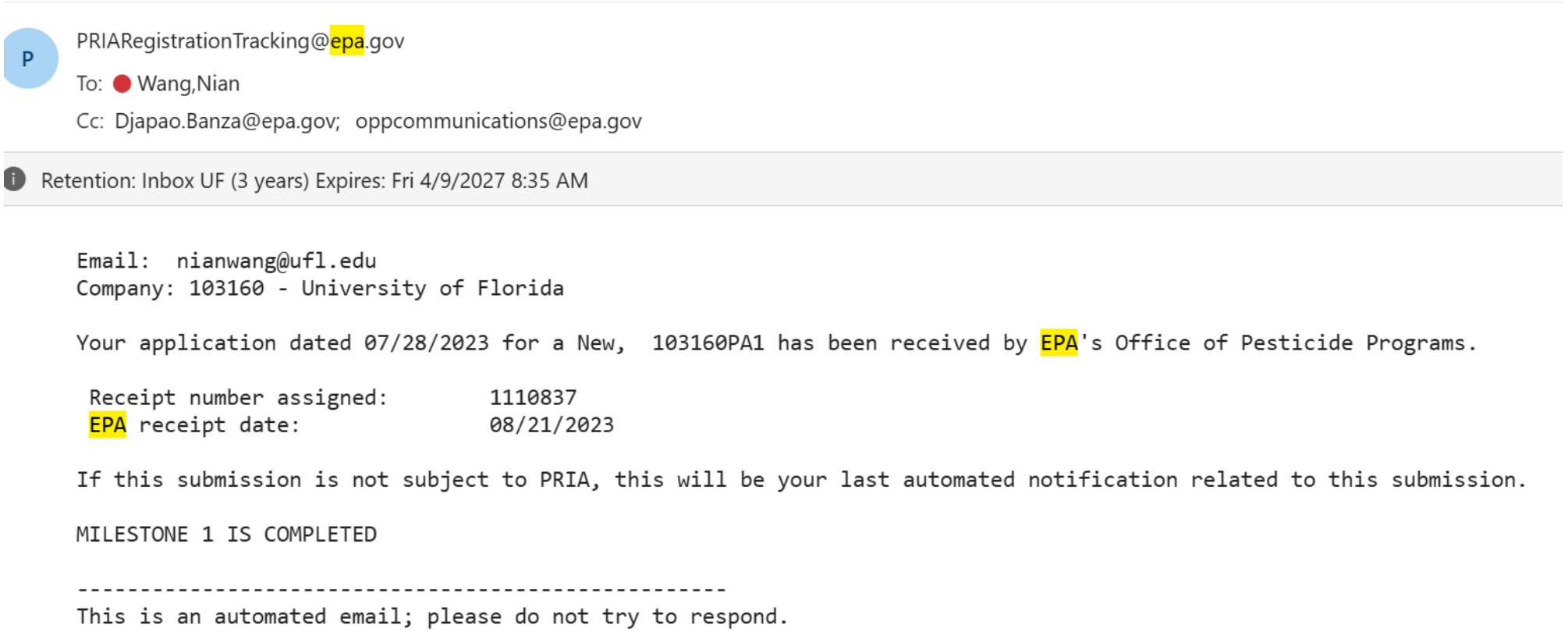
Submitter	Nian Wang
Submitted Date	2023-08-21T17:46:56.692+00:00

☑ I, **Nian Wang**, as the duly authorized agent of **University of Florida**, am submitting this Plant-Incorporated Protectant Exemption Eligibility Determination consistent with the provisions of 40 CFR part 174. I hereby certify that the plant-incorporated protectant(s) known as is/are eligible under 40 CFR § 174.21 to be exempt from the requirements of FIFRA, except as provided under 40 CFR §§ 174.71 and 174.73.

I understand that it is a violation of 18 U.S.C. § 1001 to willfully make any false statement to the EPA. I further understand that if this self-determination is not consistent with the provisions of 40 CFR part 174, this plant-incorporated protectant product may not be exempt from the requirements of FIFRA, and I and/or **University of Florida** may be subject to enforcement and penalties under FIFRA sections 12, 13, and 14, 7 U.S.C. §§ 136j, 136k, and 136l. Moreover, I also understand that if this determination is not consistent with 40 CFR part 174, the residues of this plant-incorporated protectant may not be exempt from the requirement of a tolerance under the FFDCA, and that I and/or **University of Florida**, as well as foods containing such residues, may be subject to enforcement and penalties under Chapter III of the FFDCA, 21 U.S.C. 331 et seq.

Moreover, I also understand that any information not claimed as Confidential Business Information pursuant to 40 CFR 174.9 constitutes a waiver of confidentiality for the information submitted, and the information may be made available to the public, subject to section 10(g) of FIFRA, with no further notice to the submitter.

EPA response for the self-determination application of the non-transgenic *LOB1*-edited canker-resistant *C. sinensis* cv. Hamlin



P

PRIARegistrationTracking@epa.gov

To: Wang,Nian

Cc: Djapao.Banza@epa.gov; oppcommunications@epa.gov

Retention: Inbox UF (3 years) Expires: Fri 4/9/2027 8:35 AM

Email: nianwang@ufl.edu
Company: 103160 - University of Florida

Your application dated 07/28/2023 for a New, 103160PA1 has been received by EPA's Office of Pesticide Programs.

Receipt number assigned: 1110837
EPA receipt date: 08/21/2023

If this submission is not subject to PRIA, this will be your last automated notification related to this submission.

MILESTONE 1 IS COMPLETED

This is an automated email; please do not try to respond.

For EPA self determination process, the receipt on 4/9/2024 serves as the proof of exemption.

Where are we with the non-transgenic *LOB1*-edited canker-resistant *C. sinensis* cv. Hamlin?

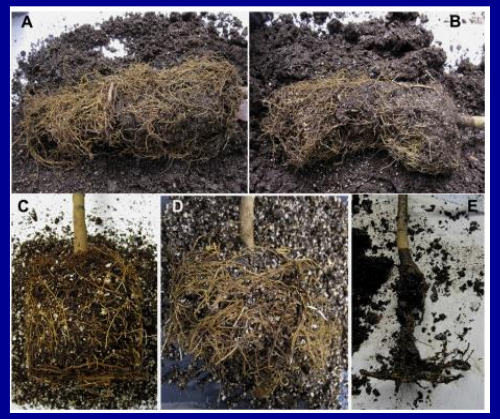
- ✓ **APHIS: exemption 12/19/2022**
- ✓ **EPA: “approval” 04/9/2024**
- ✓ **Florida DPI Citrus Budwood Registration program (2 years are needed, 4 lines were sent to the budwood program on 7/20/23)**
- ✓ **FDA: initiated some discussion regarding how to prepare for FDA application when fruit are ready.**
- ✓ **Being propagated by two nurseries for field trials and commercialization.**

Outline

- Case study 1. Citrus canker
- **Case study 2. Citrus HLB**

Citrus HLB is the most devastating citrus disease

Candidatus **Liberibacter asiaticus (CLas), not cultured on artificial media, can't make mutants.**



Improving citrus resistance/tolerance against HLB via non-transgenic CRISPR genome editing

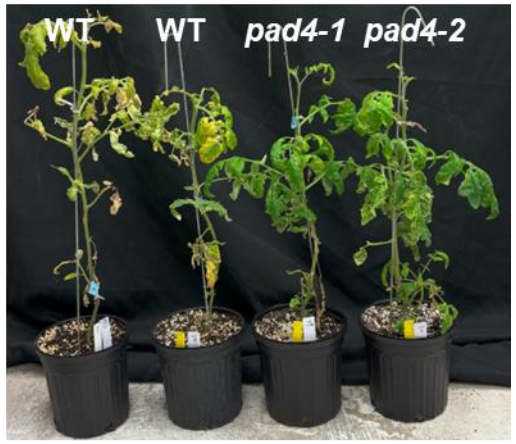
- Suppressing HLB symptoms
- Killing CLas

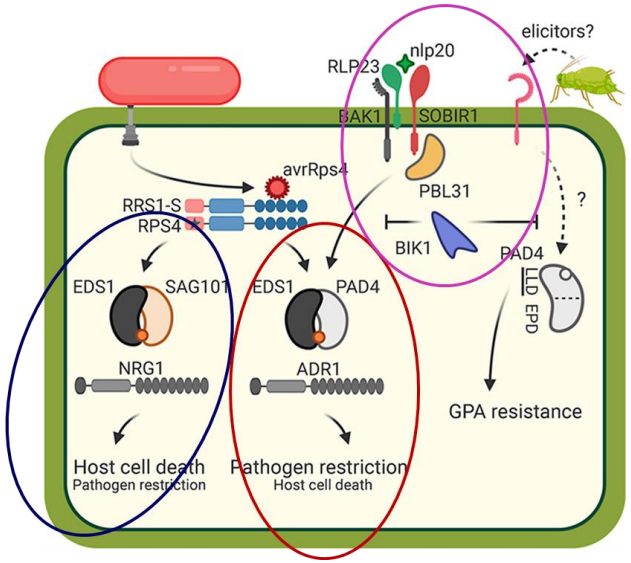
Citrus Huanglongbing is a pathogen-triggered immune disease

CLas is a biotrophic pathogen and causes disease by triggering phloem cell death.

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

EDS1 drives *Ca*. Liberibacter triggered chronic immune disease





- *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

- Non-transgenic genome edited (*EDS1*) Valencia and Hamlin sweet orange were generated in 2024 by transformation of embryogenic protoplasts with Cas12a ribonucleoprotein complex

unpublished

UF IFAS UNIVERSITY of FLORIDA

“Am I regulated” by APHIS for the non-transgenic genome edited (*EDS1*) Valencia and Hamlin sweet orange

- **APHIS regulatory filing “Am I regulated”: first filed on 4/24/25.**
- **Response and correction 4/30/25: “Please remove the paragraph under (Regulatory exemption requested:) . Due to the December 2, 2024 vacatur of the 7 CFR part 340 regulations, we are now under the 2019 7 CFR part 340 regulations and no longer have the exemptions and confirmation process that was previously at § 340.1.”**
- **Not a regulated article (Non-GMO/non-transgenic) under 7 CFR part 340: May 1, 2025**

***EDS1*-edited**

Hamlin **Valencia**

Exemption by EPA for the non-transgenic genome edited (*EDS1*) Valencia and Hamlin sweet orange

- ✓ **EPA: Loss-of-function (LoF) Plant-Incorporated Protectants (PIPs) self-determination exemption**
- ✓ **Subject: Letter of self-determination per 40 CFR 174.90(a)(2)**
- ✓ **April 24, 2025, first response back for revision: 5/01/25, and revision submitted on 5/21/25, "exemption/approved based on self determination" on 6/4/25**

OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION
WASHINGTON, D.C. 20460

July 2, 2025

Nian Wang
Citrus Research and Education Center
Department of Microbiology and Cell Science
University of Florida
700 Experiment Station Rd.
Lake Alfred, FL 33850

SUBJECT: Receipt of self-determination letters for exemption of plant-incorporated protectants

Dear Dr. Wang:

EPA understands that you have submitted multiple self-determination letters for different loss-of-function plant-incorporated protectants (PIPs) in citrus. EPA also understands that for these loss-of-function PIPs in citrus, you have certified that your PIPs meet the exemption criteria.

The provision at 40 CFR 174.91 explains that a developer must submit the letter of self-determination prior to engaging in activities that would be subject to FIFRA for the proposed PIP (*e.g.,* distribution and sale of the PIP at issue). As specified in 40 CFR 174.90(b), self-determination letters must be submitted electronically. Self-determination letters will not be submitted under FIFRA section 33 and will not be subject to application fees under the Pesticide Registration Improvement Extension Act of 2022 (PRIA 5). The exemption does not apply until EPA confirms receipt of the self-determination, but since the submission of the self-determination letter is made electronically, the receipt confirmation by the Agency is supposed to occur automatically upon submission and is considered equivalent to written confirmation of receipt.

As we are currently having difficulties with our electronic system, this letter serves as confirmation of receipt for submissions:
CDX_GEPER_2025_GEP-0769
CDX_GEPER_2025_GEP-0758
CDX_GEPER_2025_GEP-0747
CDX_GEPER_2025_GEP-0716

We note that this citrus may also be subject to other regulatory authorities such as the United States Department of Agriculture (USDA) or the Food and Drug Administration (FDA).

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

- ***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.**

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 generated in 2024 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, exemption based on self-determination on 7/2/25.

UF IFAS UNIVERSITY of FLORIDA

Where are we with the non-transgenic *EDS1*-edited and *DMR6-edited Citrus sinensis* cvs. Hamlin and Valencia

- ✓ **Florida DPI Citrus Budwood Registration Program: submitted non-transgenic *EDS1*-edited and *DMR6-edited Citrus sinensis* cvs. Hamlin and Valencia on July 15, 2025.**

- ✓ **Being propagated by the Brite Leaf Citrus Nursery (9/2/25) for field trials and potential commercialization.**

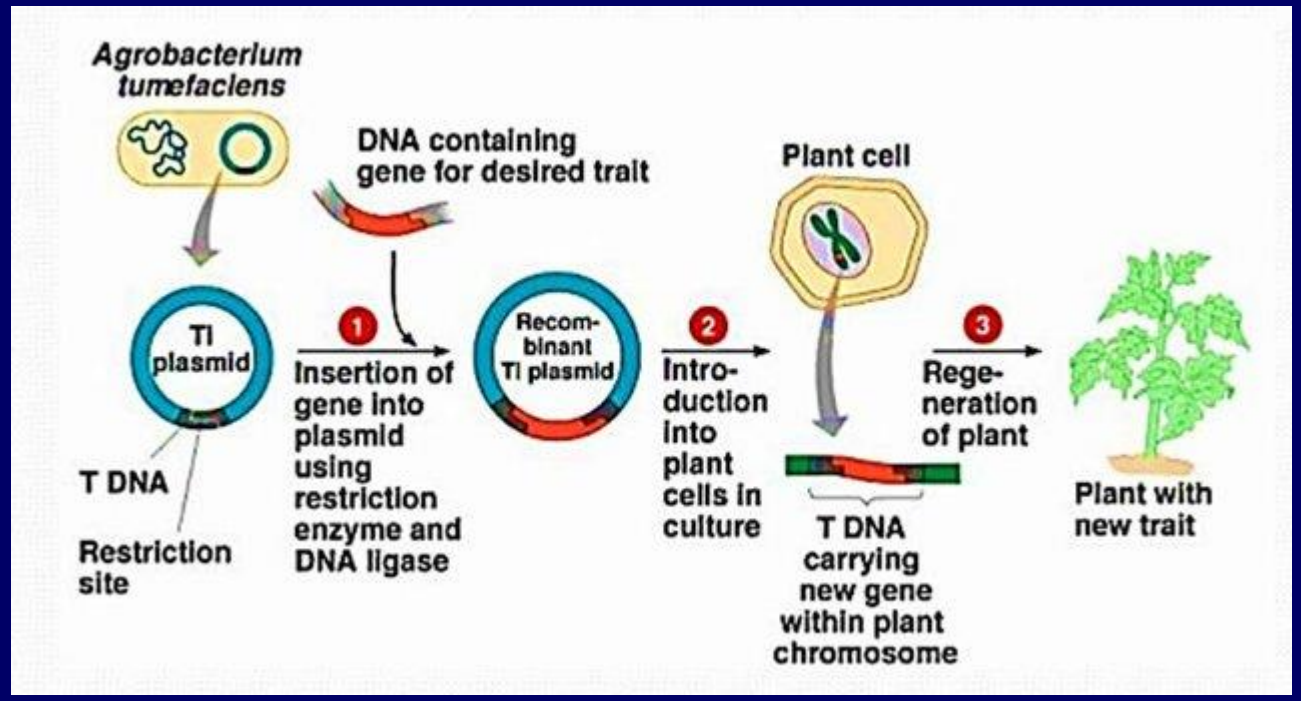
Updates on regulatory approvals for other non-transgenic genome-edited *Citrus sinensis* cvs. Hamlin and Valencia generated via the RNP method

- ✓ **On Jan 29, 2026, we have field self-determination exemption per 40 CFR 174.90(a)(2) to EPA for non-transgenic genome-edited *Citrus sinensis* generated via the RNP method for 21 target genes and received exemption response on 2/5/26.**

- ✓ **On Jan 30, 2026, we have filed "Am I regulated" to APHIS for non-transgenic genome-edited *Citrus sinensis* generated via the RNP method for 21 target genes, received approval on 3/5/2026.**

Regulatory approvals for non-transgenic genome edited citrus generated via *Agrobacterium-mediated* co-editing strategy

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



Sabu et al. 2020

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.

This approach is commonly used for transgenic!

Approaches to remove selection marker or CRISPR sequence

Annual crops

Perennials plants

Genetic segregation via backcrossing or selfing

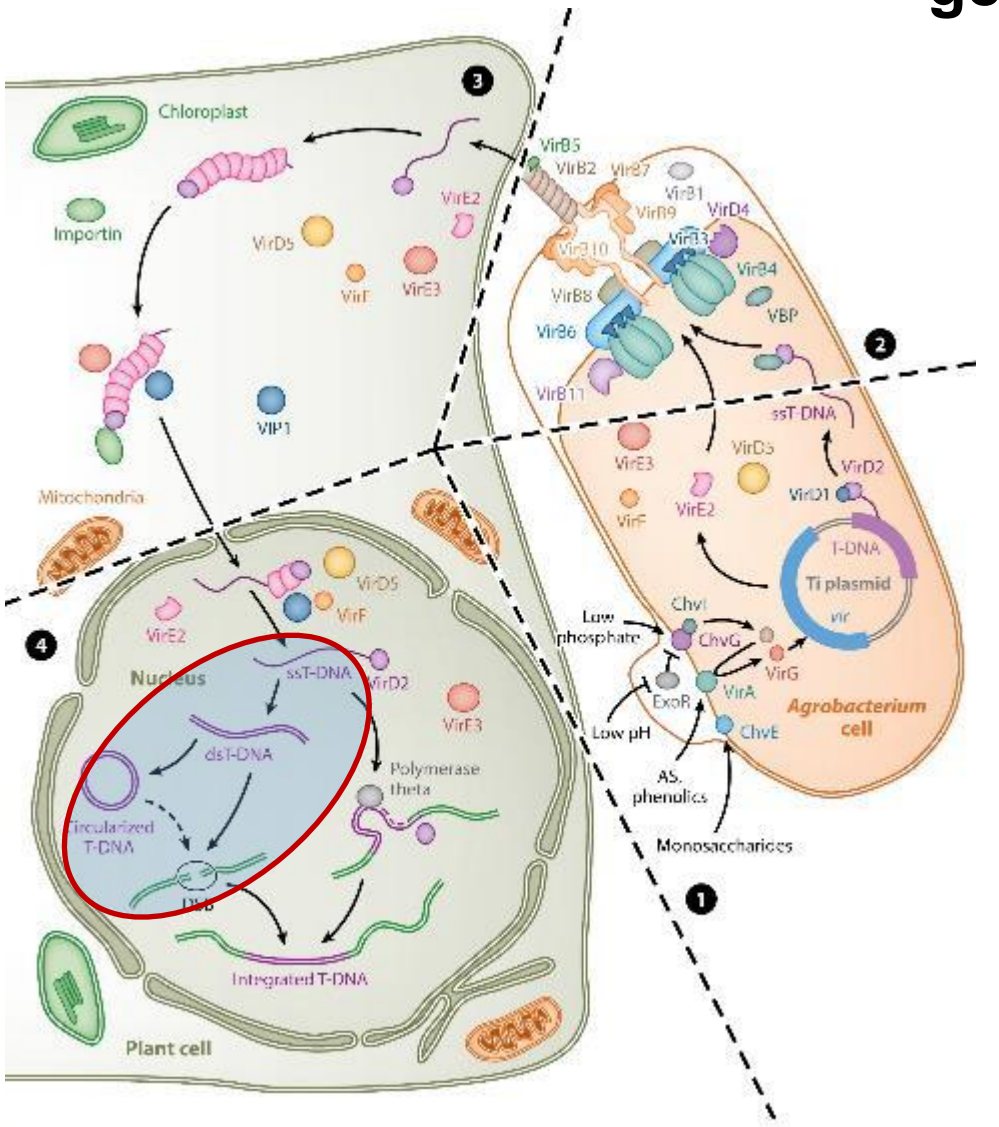
✓

laborious and time-consuming

High heterozygosity, polyembryony, long juvenility

✗

Transient expression via *Agrobacterium*-mediated transformation in genome editing



Lacroix B, Citovsky V. 2019.
Annu. Rev. Phytopathol. 57:231–51

Most T-DNAs are not integrated into the host chromosomes

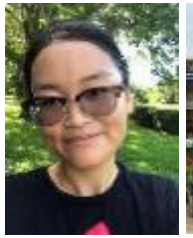
***Agrobacterium*-mediated transient expression was used for transgene-free genome editing: tomato, tobacco, and potato**

Without selection pressure, making it difficult, laborious and time-consuming to differentiate

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

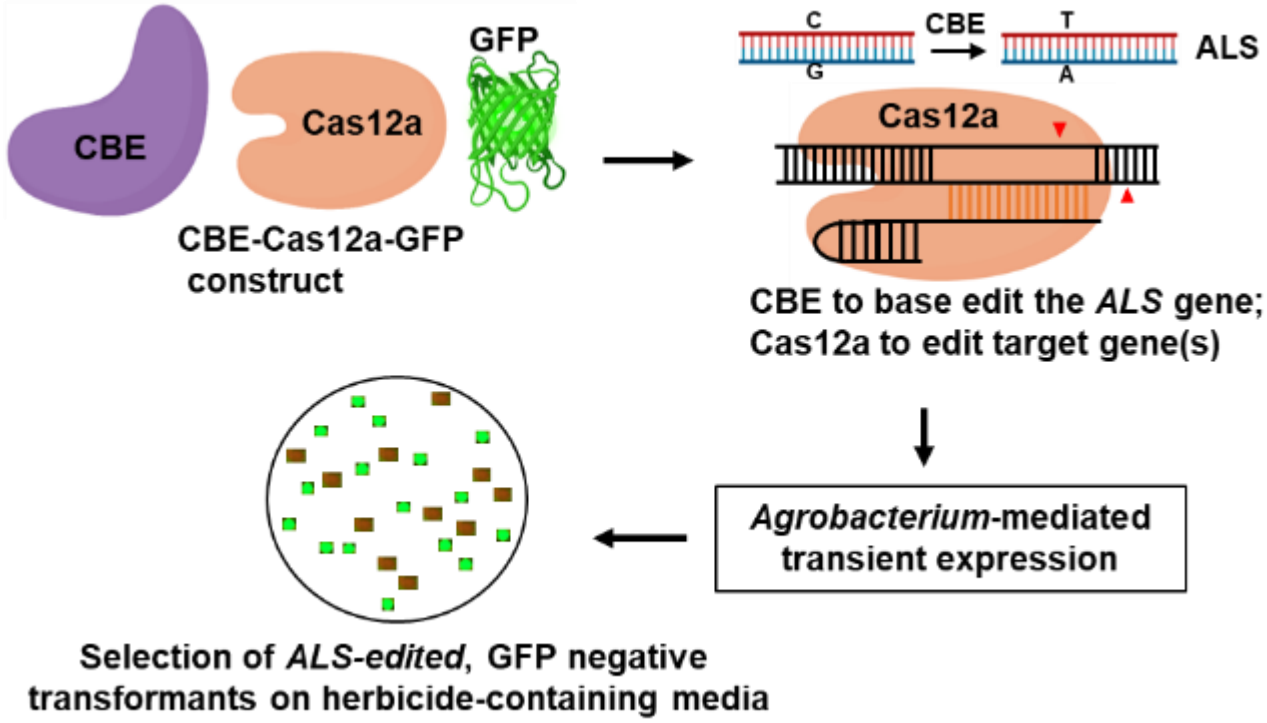

Xiaoen Huang

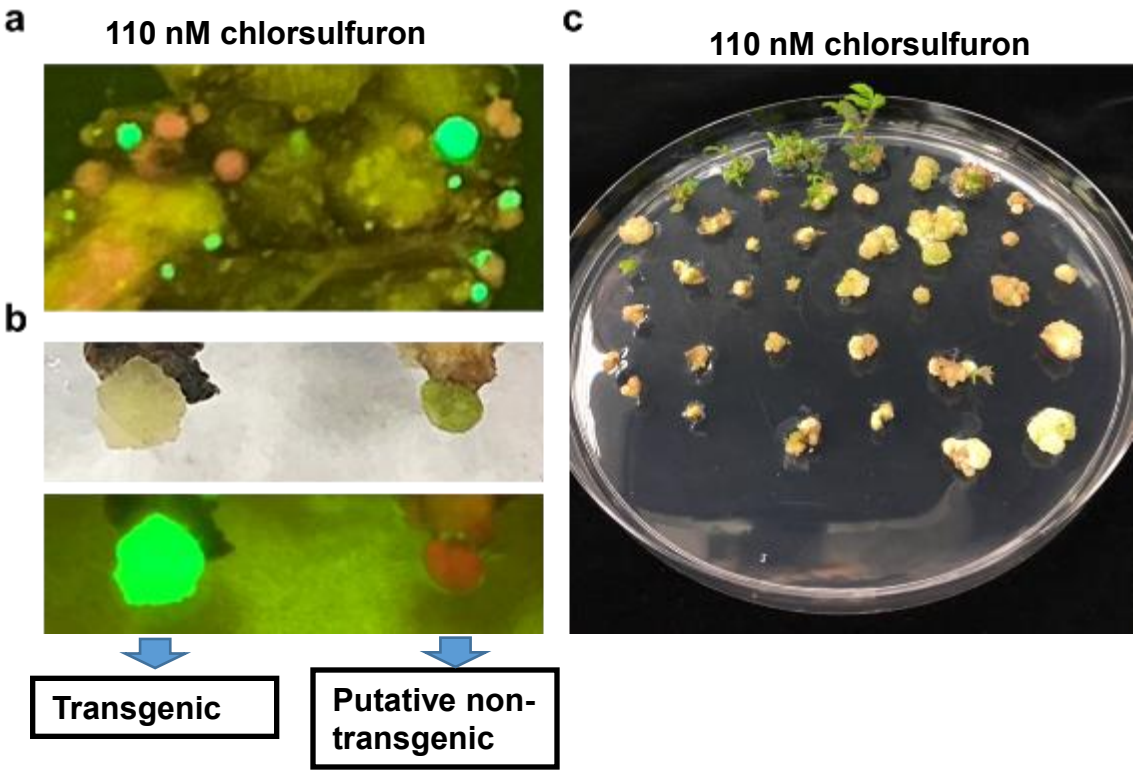

Hongge Jia


Yuanchun Wang


Jin Xu

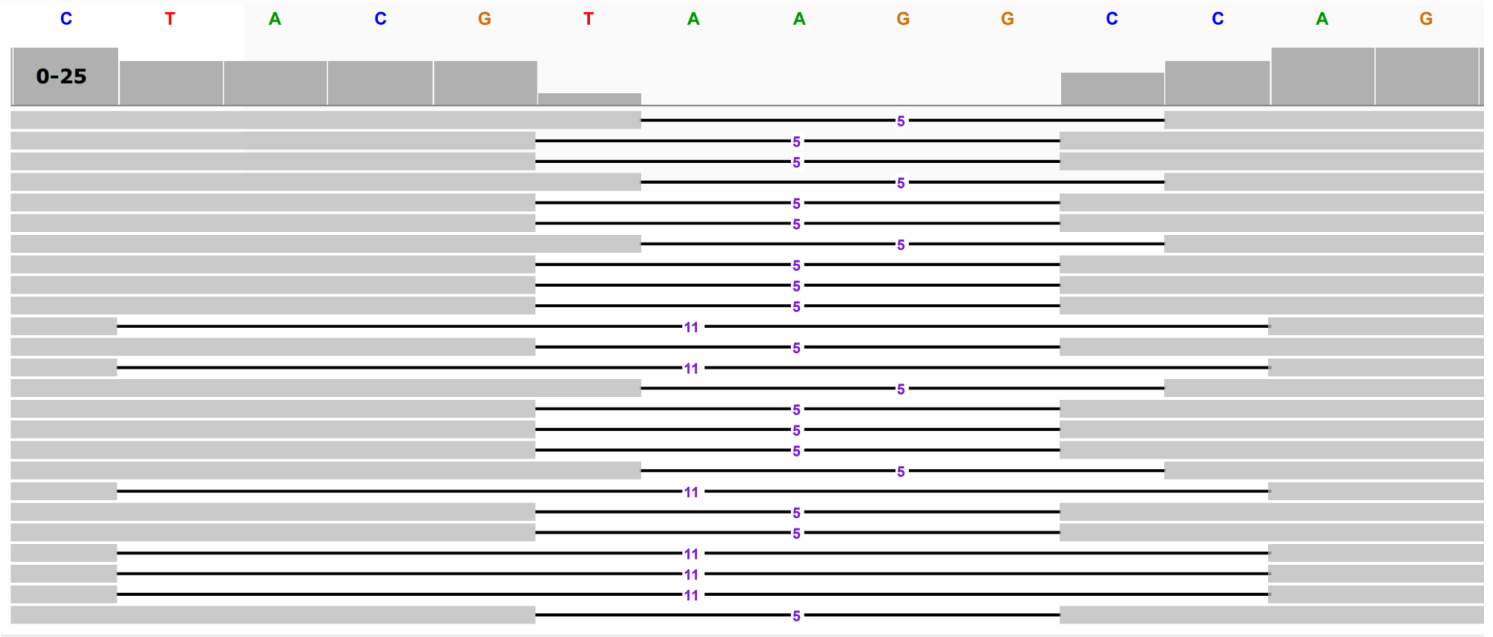
cytosine base editor (CBE)





Final confirmation of non-transgenic genome editing: whole genome sequencing

Confirmation of non-transgenic genome editing of *DMR6* via whole genome sequencing



Sample	Total raw data (Gb)	Total high quality data (Gb)	Sequencing coverage	Construct sequence
DMR6-3	13.31	13.05	39.54	No

Updates on regulatory approvals for the non-transgenic genome-edited citrus generated via *Agrobacterium-mediated* co-editing strategy

- ✓ **On Feb 12, 2026, we have filed "Am I regulated" to APHIS for non-transgenic genome-edited citrus generated via the co-editing method for 2 target genes and received approval on 3/5/2026.**

- ✓ **On Feb 12, 2026, we have field self-determination exemption per 40 CFR 174.90(a)(2) to EPA for non-transgenic genome-edited citrus generated via the co-editing method for 2 target genes and received exemption response on 2/12/2026.**

Summary

We have generated non-transgenic *lob1* edited sweet orange via the RNP method, which is canker resistant and has received regulatory "approval/exemption" by both APHIS and EPA.

We have generated non-transgenic genome edited sweet orange for 23 target genes including *eds1 or dmr6* via the RNP method, which are putatively resistant/tolerant against HLB and have received regulatory "approval/exemption" by both APHIS and EPA.

We have generated non-transgenic genome edited sweet orange for two target genes including *DMR6* via *Agrobacterium*-mediated co-editing method, received regulatory "approval/exemption" by both APHIS and EPA.

Acknowledgements

Special thanks to APHIS, EPA, and FDA for their outstanding job!

J. R. (Rip) Graves Endowment
USDA NIFA Center of Excellence

PBI program (USDA-NSF)
SCRI-CDRE Program