CITRUS ADVANCED TECHNOLOGY PROGRAM

QUARTERLY & FINAL PROGRESS REPORT FORM: Control of Citrus Greening, Canker & Emerging Diseases of Citrus

▶**PROJECT QUARTER END** November 2024 ◉ *Quarterly Report* ○ *Final*

Proposal Title

Generation of non-transgenic HLB-resistant sweet orange varieties using CRISPR-Cas technology

Today's Date: 11/05/2024

Sponsoring Organization (drop-down): Citrus Research and Development Foundation

Category (drop down): Plant Improvement

ABSTRACT (Executive Summary-public report-do not disclose proprietary information or intellectual property)

1. Please state project objectives and what work was done this quarter to address them:

The purpose of this project is to generate non-transgenic HLB resistant Valencia and Hamlin sweet orange plants using CRISPR-Cas technology.

Objective 1. Generate non-transgenic HLB resistant/tolerant Valencia and Hamlin sweet orange plants by mutation of HLB susceptibility genes. In total, six putative S genes were proposed to be edited and we have lately added more targets. Constructs needed for CRISPR genome editing are being made. Multiple edited lines were generated for ACD2 gene. However, further confirmation demonstrated none of them was biallelic/homozygous mutant. We are genenerating more lines for ACD2 and other target genes.

To speed up the process, we have further optimized the transgene-free CRISPR genome editing using Cas12a/crRNA ribonucleoprotein. We have developed an efficient co-editing strategy for generating transgene-free, gene-edited plants via *Agrobacterium*-mediated transient expression of cytosine base editor (CBE)/gRNA-Cas12a/crRNA-GFP *in planta*. Specifically, CBE/gRNA was used to base edit the *ALS* gene to confer resistance to herbicide chlorsulfuron as a selection marker, which has no negative effects on plant phenotypes; Cas12a/crRNA was used for editing genes(s) of interest; GFP was used for selecting transgene-free transformants. Using this approach, transgene-free genome-edited plants can be relatively easily generated for citrus in the T0 generation. Whole genome sequencing further confirmed transgene-free and absence of off-target mutations in the edited plants. We are also using this strategy for genome editing of our target genes. This has been published by Nat. Plants (9, 1591–1597, 2023. https://doi.org/10.1038/s41477-023-01520-y).

Among the 8 target genes, we have done transformation again on 5/18/23, 5/25/23, 6/25/23, 7/27/23, 8/17/23, 11/16/23, 12/07/23, 1/18/24, 2/15/2024, 2/22/2024, 3/7/2024, 4/11/2024, 4/25, 5/9, 5/23, 6/6, 6/20, 7/5, 7/19, 8/2, 8/16, 8/30, 9/13, 9/27, 10/18. The transformants done before August 2023 have successufully generated shoots for target genes with high efficacy. We are conducting more sequencing confirmation. Deep sequencing of the edited protoplasts showed a mutation rate of 16-99.8%. We have successfully generated non-transgenic lines for four of the S genes.

In addition, we have generated Ray Ruby embryogenic protoplasts which are being tested for efficacy of transformation, editing, and regeneration.

Objective 2. Generate cisgenic genome modified Valencia and Hamlin sweet orange plants by knock-in the gene encoding MaSAMP from Microcitrus.

We have demonstrated the knock-in works using GFP as an insert. We have conducted knock-in for MaSAMP on 5/18/23, 8/17/23, 11/16/23, 12/07/23, 1/18/24, 2/15/2024, 2/22/2024, 3/7/2024, 4/11/2024, 5/23, 6/20, 7/19 which are under

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Organization: CREC, IFAS/UF

Sponsor Project Number: 21-028

Project Duration (years): 3 *Year of Project* 3

% Completion of Objectives (FDACS requirement): 94.00%

CITRUS RESEARCH AND DEVELOPMENT FOUNDATION, INC.