Citrus INDUSTRY Est. 1920

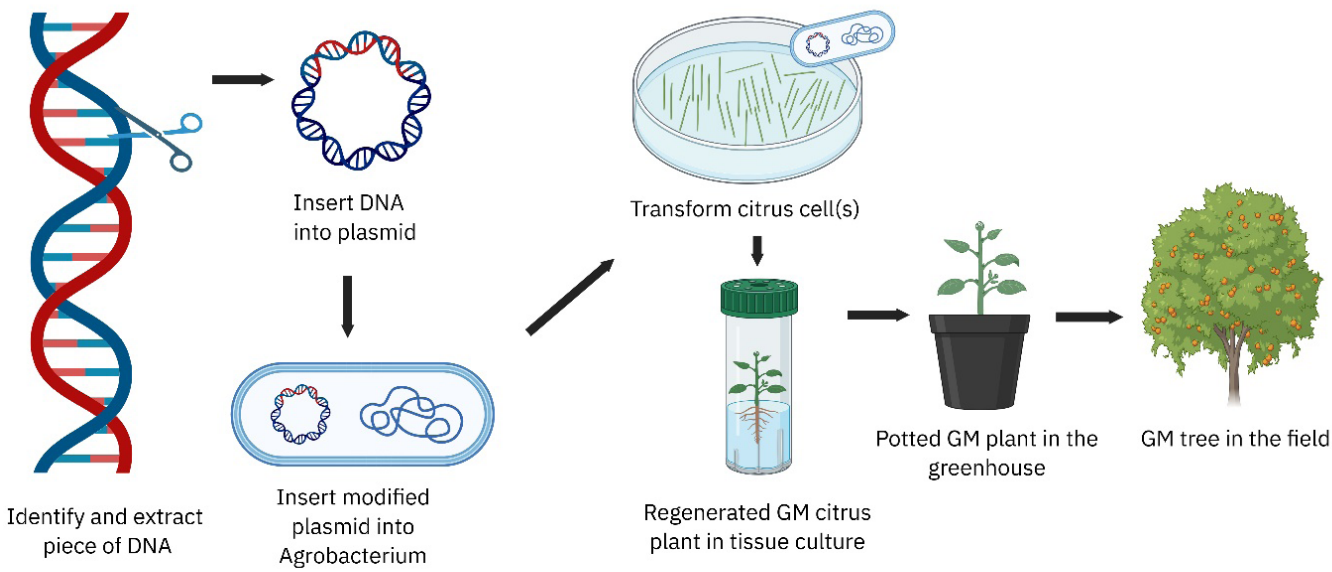

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Figure 1. The process of developing GM citrus trees

Improving citrus using genetic tools

By Manjul Dutt

Editor's Note: This is the first in a series of two articles on plant improvement. Next month will feature the timeline for CRISPR-developed trees.

Traditional methods for improving citrus, such as crossbreeding different varieties, can be time-consuming due to the creation of a random mix of genes from both parents through a process called genetic introgression, leading to offspring with unpredictable characteristics. This process may require extensive screening and considerable time to identify a suitable offspring.

Newer plant improvement tools, such as genetic transformation and genome editing using CRISPR, enable scientists to alter a plant's genetic makeup. Understanding the complexities of genetic transformation and CRISPR is essential for developing innovative solutions in biotechnology, such as creating genetically modified crops and implementing strategies against huanglongbing (HLB). This article discusses the processes of genetic transformation and genome editing using CRISPR and the timeframe to create genetically modified plants.

GMO vs. CRISPR

Genetic transformation results in the creation of genetically modified organisms (GM/GMOs), while CRISPR is a gene-editing technique used to alter the DNA of living organisms. Although both methods aim to modify genetic material, they differ in their approaches and outcomes.

GM plants are typically created by inserting genes from different species into an organism. For instance, a

Genetic transformation enables scientists to add a specific trait to trees without altering the rest of their characteristics.

disease-resistance gene from tobacco can be introduced into citrus plants to make them tolerant to HLB. This process is less precise and may lead to random DNA insertions within the plant's genome.

In contrast, CRISPR is a process that allows scientists to directly edit specific genes without necessarily adding foreign DNA. It functions like molecular scissors, precisely cutting DNA at a specific location to delete, repair or replace a gene. Although both techniques seek to improve traits such as disease resistance or crop yield, CRISPR is generally more accurate, as it can create changes that could also occur naturally.

Genetic transformation is the process of altering a plant's DNA by introducing a new gene to enhance beneficial traits, such as disease resistance, drought tolerance or improved fruit quality. Typically, this process does not change the existing genetic characteristics of the plant variety being transformed. Genetic transformation enables scientists to add a specific trait to trees without altering the rest of their characteristics, making the process faster and more precise compared to traditional breeding, which mixes traits from two different trees. Elite citrus cultivars that undergo genetic transformation can acquire new traits while maintaining the other qualities that make them valuable.

CREATING A GMO PLANT

There are two main methods that scientists use to change the genes in citrus trees:

1. Using Bacteria (*Agrobacterium* Method)

This method involves using a natural soil bacterium called *Agrobacterium tumefaciens*, which can insert its DNA

into plants. Scientists take advantage of this ability by removing the harmful components of the bacterium's DNA and replacing them with beneficial genes.

To begin, scientists use either young citrus tissue (such as small, etiolated stems) or pieces of mature citrus stems. They then mix this tissue with the engineered bacteria, which can subsequently insert the new genes into the citrus plant's DNA.

Once the DNA insertion is complete, scientists can grow the cells containing the modified DNA into new plants in a laboratory setting. These new plants are maintained in a greenhouse and tested to ensure that they possess the desired DNA modifications. The evaluation process may involve testing the modified plants in either a greenhouse or a field setting to assess tolerance to HLB.

2. Using Protoplasts (Direct DNA Insertion Method)

A protoplast is a plant cell that has had its outer cell wall removed, leaving only the inner part of the cell, which is surrounded by a thin membrane. When scientists want to introduce new DNA into a plant, using protoplasts simplifies the process because there is no tough cell wall to contend with.

To create protoplasts, scientists take plant cells and use special enzymes to dissolve the cell walls. Then, they mix the DNA containing the desired trait (such as a gene that may confer disease resistance) with the protoplasts and a chemical called polyethylene glycol. This chemical makes the cell membrane more flexible, allowing the DNA to enter the cell more easily. Once the DNA has successfully entered the protoplast, the cell is grown in a special nutrient-rich medium that enables it to rebuild its cell wall, divide and eventually develop into a new plant that incorporates the new DNA.

GMO DEVELOPMENT TIMELINE

1. Gene Identification, Gene Insertion and Lab Development (1–2 Years)

Scientists identify a trait of interest, such as tolerance to HLB. They identify and isolate a potential gene that could make the plant tolerant to HLB. This gene is inserted into citrus plants using *Agrobacterium*-mediated transformation or protoplast techniques. The modified plants, referred to as transformants, are then generated and screened. Laboratory testing is conducted to ensure that the gene is expressed correctly and is stably inherited in the citrus plants.

2. Greenhouse and Field Trials (3–7 years)

The best genetically modified lines of citrus trees are clonally propagated and screened for HLB tolerance in the greenhouse or tested in the field under endemic HLB conditions. Scientists also collect necessary data for regulatory submissions.

Citrus trees developed from juvenile tissues typically take five to seven years to flower and bear fruit, requiring field observations during this period. In contrast, trees created through the mature transformation process can flower and produce fruit within two years of transformation.

3. Regulatory Approval and Certification (2–3 years)

Once a specific plant line has been identified, the developer submits budwood to the Florida Department of Agriculture and Consumer Services Citrus Budwood Program to certify that the budwood is free from viruses and graft-transmissible diseases. The developer submits a comprehensive dossier containing scientific data required for evaluation by the U.S. Department of Agriculture, Environmental Protection Agency and Food and Drug Administration. This process typically includes public consultations and requests for additional data.

4. Commercialization (2–3 years)

Once approved, the GM line is officially released and clonally propagated for large-scale planting. Marketing and outreach to growers, which began earlier, is intensified, and consumers are informed about the new trees.

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Genetics Offers Solutions

By Rick Dantzler, CRDF chief operating officer

This is the third column in a four-part series on germplasm which might represent salvation for the Florida citrus industry. The first three focused on the existential threat of huanglongbing (HLB). The fourth, which will run in November, will be on advancements in dealing with the scourge of diaprepes.

The first in the series was on non-genetically modified, HLB resistant/tolerant citrus trees that Nian Wang's team at the University of Florida Institute of Food and Agricultural Sciences (UF/IFAS) are working on. They have successfully knocked out two genes — EDS1 and DMR6 — from Hamlin and Valencia.

EDS1 compromises phloem function, so by knocking it out, callose deposition, sieve element plugging and reactive oxygen species are all reduced. This is believed to bestow HLB resistance or at least a high level of tolerance.

DMR6 reduces or eliminates SA-5 hydrolase, an enzyme which degrades salicylic acid. By reducing or eliminating SA-5 hydrolase, the level of salicylic acid is increased so much that resistance to several pathogens, including *C*Las, is conferred by enhancing the plant's natural defenses.

The second column explained the work of Zhonglin Mou, Manjul Dutt, Jude Grosser and their colleagues at UF/IFAS who have overexpressed the gene NPR1 in citrus plants by adding the NPR1 gene from the plant Arabidopsis. This makes the genetically modified tree particularly potent at fighting off *C*Las. Trees which have been in the ground since 2019 are showing no ill effects and are loaded with fruit this year.

This column focuses on a tree developed by the company Soilcea, which is receiving a lot of industry interest because of how good it looks in comparison to control trees. Soilcea claims a 17,000-fold reduction in *C*Las titer. This reduction is achieved by negatively impacting the interactions of effector proteins, which are secreted by *C*Las, that interact with specific parts of the citrus genome to turn off the tree's natural defense pathways to create a more favorable environment for infection. Wang and his colleagues discovered the specific target proteins that *C*Las effector proteins could potentially interact with to promote susceptibility.

Mirroring the evolutionary process of gradual adaptation and natural selection, Soilcea is using CRISPR to restore the natural ability of citrus trees to defend against HLB with a new rootstock — CarriCea T1 — which is precisely edited for HLB resistance. It resists *C*Las by targeting two negative regulator proteins, preventing the suppression of the defense response and restoring proper cell death signaling. Additionally, interrupting another effector-interacting protein prevents the suppression of paralogous gene expression and activity in the plant, enhancing resistance and mitigating disease. These combined changes enable the citrus tree to defend itself and reduce HLB infection. While the rootstock of the tree is genetically modified (GM), non-GM scions are grafted onto the rootstock, so the tree is not considered GM.

Soilcea acquired the licensure rights to underlying target proteins and the CRISPR process in citrus, representing a good example of how public sector research works. The public sector creates the knowledge, and the private sector develops the trees and commercializes it for the benefit of growers.

CRDF Grower Dollars at work for you

Column sponsored by the Citrus Research and Development Foundation