Update on CRISPR research for citrus improvement

By Nian Wang, Fred Gmitter and Manjul Dutt

Nearly all commercially important citrus types — oranges, grapefruits etc. — have originated by mutations that have accumulated over several hundreds to thousands of years, from what once was an original, individual sweet orange tree and a single grapefruit tree. The many different cultivars that we know today are descended from those original trees, differing only by minor mutations to change color, season of maturity, etc. But the genetic package (the "genome sequence") that the originals possessed is nearly 100 percent conserved. Whatever good or bad traits they possessed are likewise still found in the modern varieties.

Plant breeders generally make crosses to bring in genes for other traits or to remove deleterious genes from the next generation. This is not an obvious option, however, if we want to keep nearly everything the same in our orange or grapefruit. This is further reinforced by regulations developed to manage marketing and commerce by strictly limiting the definitions of these important citrus types.

Genetically modified citrus plants were developed to introduce genes from other organisms to solve monumental challenges like citrus canker and huanglongbing (HLB). Some strategies have seen reasonable success in achieving these goals. However, the global marketplace remains mostly reluctant to accept such citrus products.

In recent years, genome editing, using a system called CRISPR (clustered regularly interspaced short palindromic repeats) has raised incredible new possibilities for citrus improvement. CRISPR is being used to make small changes in the DNA sequence of citrus trees, resulting in specifically targeted mutations. Major advances in the ability to decipher the genetic blueprint of any living organism, including citrus, have been made possible by the development of new genome-sequencing technologies and powerful computers. Many commercially important citrus types have been or are in the process of being sequenced.

Scientists can now identify the specific DNA sequences in regions of their genomes responsible for some important traits, good or bad. The ability to "edit" the DNA using CRISPR

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technology, essentially changing just one or a few of the letters of the genetic code in specific genes, gives the potential to "repair" some of the problems. These changes are no different than mutations that might occur naturally and spontaneously, given enough time for evolution. Current research is leading to new editing methods through which nothing foreign is introduced into the citrus genome; it is pure citrus!

CUTTING OUT PROBLEMS

CRISPR technology is commonly referred to as molecular scissors. It is necessary to have different scissors to do different jobs. Currently, three different CRISPR scissors (SpCas9, SaCas9 and Cas12a) have been successfully developed to cut the citrus genome by scientists at the University of Florida Institute of Food and Agricultural Sciences (UF/IFAS) Citrus Research and Education Center (CREC). These different scissors allow precise cutting of selected trouble-maker genes or specific sites of those genes. The scissors now can cut both short and long fragments from the citrus genome.

How sharp are the scissors? At the early stage of the CRISPR technology development for citrus, the mutation rate generated using the Cas9/sgRNA system was lower than 10 percent, comparable to rusty scissors. With the development of different tool kits and optimization of their application, scientists have achieved an efficacy of more than 40 percent, indicating that the CRISPR-mediated citrus genome editing technology is mature and could be implemented in citrus genetic improvement as a viable tool in practice.

THE ROAD TO DISEASE RESISTANCE

One of the urgent needs for the citrus industry is to develop HLB-resistant citrus varieties. This task remains to be accomplished. Currently, the CRISPR tool has been successfully used to generate canker-resistant citrus varieties, which might offer some relief and hope to solve the HLB problem. Canker resistance was achieved by deactivating the canker susceptibility gene (a gene that makes citrus vulnerable to the canker pathogen, Figure 1, page 24).

At least two grapefruit lines with high mutation rates and two pure mutation lines of pummelo have been generated that are resistant to canker as evidenced in greenhouse assays. It is important to note that the aforementioned genome-modified citrus plants contain foreign DNA sequences, thus considered to be genetically modified organisms.

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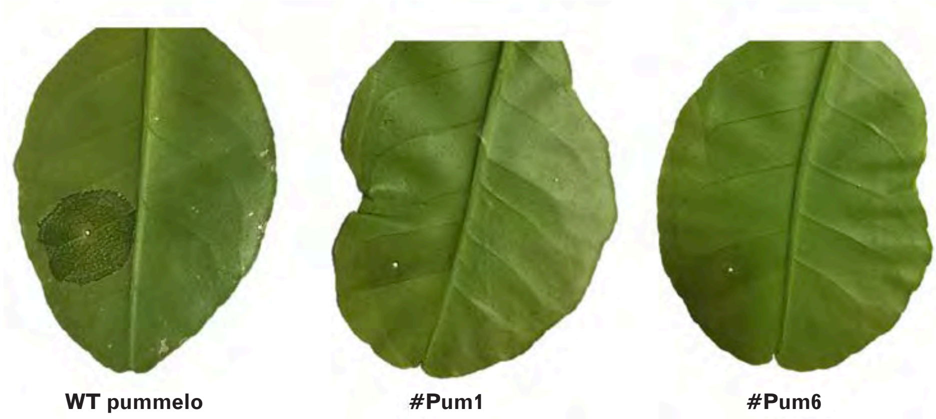

Figure 1. Five days after *Xanthomonas citri* inoculation, citrus canker symptoms were observed on wild type (WT) pummelo. However, citrus canker symptoms were not observed on genome-modified pummelo #Pum1 and #Pum6.

National Institute of Food and Agriculture, the Citrus Research and Development Foundation and state funds, non-transgenic canker-resistant grapefruit and sweet orange varieties are being developed. For development of HLB-resistant citrus varieties, the challenge now is not the actual CRISPR technology, but identifying the right genes to cut.

This explains why the federal and state agencies are funding and need to continue to fund some fundamental research (such as identification of HLB susceptibility genes), which does not provide the direct solution itself, but is critical to the eventual solution of HLB. The inability to cultivate the bacteria that causes HLB in pure culture has made it very difficult to pinpoint the genes that make citrus vulnerable to HLB. Despite those challenges, some putative HLB susceptibility genes have been identified and are being targeted.

To remove or deactivate a susceptibility gene is probably one of the easiest approaches to get regulatory approval for the genome-edited citrus varieties. On the other hand, the CRISPR technology has the potential to insert a piece of DNA sequence into the citrus genome to make it immune to the pathogens. Ongoing research aims to identify genes responsible for resistance against HLB or Asian citrus psyllids (ACP).

In addition to developing HLB- and canker-resistant citrus trees, CRISPR technology may be utilized to make citrus resistant to other diseases, such as citrus tristeza virus, alternaria, citrus black spot and citrus scab.

ADDITIONAL APPLICATIONS

The CRISPR technology is a powerful tool that can be used to modify the citrus genome to help solve a myriad of other issues affecting citrus. For example, the long juvenile phase is a major limiting factor that hampers the breeding process and significantly delays the development of new improved cultivars. Genome editing of a negative regulator, TFL1, that plays a role in juvenility, could potentially result in a shorter juvenile phase.

Tree architecture can also be modified to produce compact trees by editing the tiller angle control 1 (TAC1) gene. This gene functions by promoting the horizontal growth of branches. TAC1-edited citrus trees are more compact in stature when compared to unedited trees. Such compact trees could result in high-density plantings in advanced citrus production systems.

Additionally, editing genes such as CBF1 (a gene that can control expression of several other genes that respond to low temperature) or GI (a gene that negatively affects salt-stress regulation) may improve cold and salt tolerance in citrus, respectively.

Maintaining the flavor in fresh and juice citrus, especially during the HLB era, is important. It is well known that phytochemicals like naringin result in the bitter taste of citrus products. Likewise, limonoids make the citrus peels bitter, which is not desirable in OJ processing. Eliminating or significantly reducing these phytochemicals could help to improve the taste of citrus, benefiting consumers and thus driving demand for citrus products.

Decreasing or eliminating phytochemicals such as furanocoumarin levels in grapefruit would decrease the potential for negative effects associated with certain medications. Thus, grapefruit juice consumption could increase in the absence of those negative side effects.

In summary, the use of CRISPR genome-editing technology not only improves citrus tree health and reduces disease susceptibility, it provides many benefits to both producers and consumers of citrus.

OBSTACLES TO ACCEPTANCE

How and when will CRISPR-edited citrus trees find their way into Florida's citrus groves? When will the benefits become reality? These are not simple questions. As with any approach to genetic improvement, there is the inescapable need to test and validate performance in the field broadly. A greenhouse experiment or two and a handful of trees in the field are not enough to support the mass adoption of the new varieties.

Furthermore, there remain questions regarding the regulation of CRISPR-edited plants. Important steps have been taken in the United States and some other countries to greatly ease the regulatory burdens. However, current European Union regulations prohibit the use of this powerful technology in agriculture. Though genome editing is far less innocuous than transgenics, there are obstacles to acceptance that remain. But research to exploit genome editing in citrus will proceed, while the societal arguments over the technology conclude.

Nian Wang (nianwang@ufl.edu) and Fred Gmitter (fgmitter@ufl.edu) are professors and Manjul Dutt (manjul@ufl.edu) is a research assistant scientist, all at the UF/IFAS CREC in Lake Alfred.