What is CRISPR and what does it mean for citrus?

By Fred Gmitter, Yi Zhang and Jude Grosser

It is very likely that you have heard about the use of CRISPR technology and its great potential for addressing human health issues, as well as the promise it holds for providing solutions for major agricultural challenges, particularly for huanglongbing (HLB) in citrus. Granting agencies are supporting many citrus research projects aimed at developing resistance to HLB, through genome editing using CRISPR technology.

Although there is public awareness of the technology, and CRISPR has become a part of the citrus industry's vocabulary, there are quite a few questions about this subject that we hope to answer for you in a simple and understandable way in this article. What is CRISPR and how does it work? What kind of objectives can be addressed, what is necessary for successful application of the technology and what are the limitations? How is it different from GMO (genetically modified organism) technology? Finally, what are the prospects for global commercialization of citrus products developed using genome editing technology?

CRISPR EXPLAINED

The most widely used genome editing system is CRISPR-Cas9, which stands for clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9. This is a short piece of bacterial DNA that is part of the defense mechanism used to fight invading viruses, by recognizing them and "cutting" their DNA or RNA.

By clever molecular biology techniques, this bacterial defensive machinery has been used now to make small changes in the DNA sequences of genomes of many different organisms, including fungi, plants, animals and even humans (Figure 1). The concept has been evolving since the late 1980s

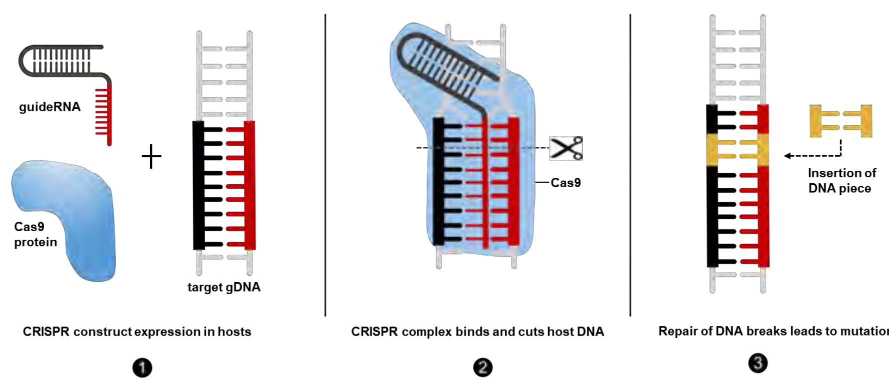

Figure 1. This simplified diagram shows how CRISP/Cas9 works to edit the genome. The guide RNA is based on the DNA sequence of the target gene. This guide is complexed with the Cas9 protein, which cuts the host DNA. Then, changes are made to the gene either by deleting or adding a few nucleotides to the gene sequence. This changes the "spelling" of the gene, thereby also altering its function.

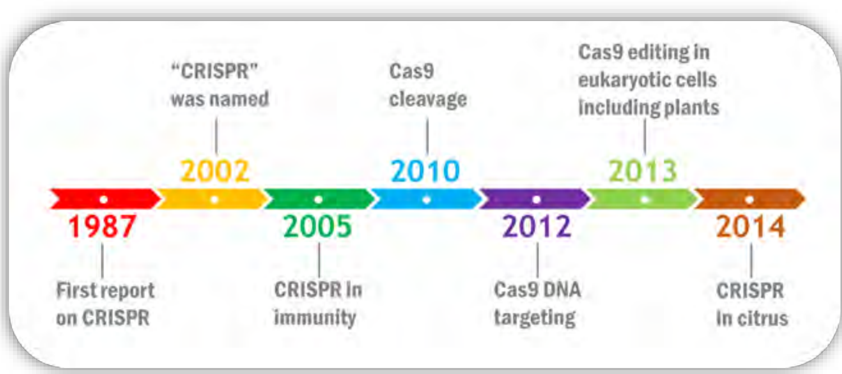

Figure 2. CRISPR technology has been under development since the late 1980s. It was first demonstrated to be effective in citrus in 2014. Since then, many research projects have been initiated to use CRISPR in hopes of making HLB-resistant plants.

when such bacterial sequences were first identified. But it wasn't until just five years ago, in 2013, when the first use of CRISPR for editing eukaryotic cells, including those from plants, was reported. In 2014, Nian Wang's lab at the University of Florida Institute of Food and Agricultural Sciences Citrus Research and Education Center first published on successfully editing the citrus genome using Cas9 (Figure 2).

To target any specific gene in the citrus genome that we wish to affect, the exact DNA sequence of that gene must be known. Until now, most citrus researchers relied on the publicly available reference genomes, such as the Clementine mandarin that was developed by the International Citrus Genome Consortium, to find the full sequences of their targeted gene.

An RNA "guide" molecule is then produced based on the specific gene sequence and complexed with the Cas9 protein, to find the gene's exact location in the genome. Once the complex is aligned with the gene, Cas9 does its job by cutting both strands of the DNA molecule near that location. These cuts then can be repaired, and the strands rejoined, but with either some missing or some additional DNA sequence to change the "spelling" of the gene. By doing so, a given gene can be made to be non-functional or to have its function and/or expression changed in some way different from the natural condition. The simple idea for making a susceptible plant resistant to a pathogen is to target the host gene that responds to something from the pathogen, some receptor for example, that leads then to the development of disease in the plant. Knock out the receptor and you prevent the disease.

CHALLENGES AND LIMITATIONS

Sounds simple, but it can be far more difficult in practice, particularly with a complicated disease such as HLB or other aspects of the plant that are not under the control of a single gene, but rather are influenced by many different genes. The greatest challenge to solving HLB through genome editing is the identification of the most likely specific target.

Much research has taken place to understand the mechanism of the

Committed to Clientele-Focused Leadership

By John Arthington

Hello. I want to take this opportunity to introduce myself and my new role at the Citrus Research and Development Foundation (CRDF). As many of you are aware, Harold Browning retired as chief operations officer (COO) to pursue other opportunities in private industry. At that time, Larry Black (CRDF president) and Jack Payne [University of Florida Institute of Food and Agricultural Sciences (UF/IFAS) senior vice president] reached out to me and asked that I serve an interim role during the search to hire a new permanent COO.

The timing was appropriate. I'm currently in the middle of a 1-year special administrative assignment with Payne. The major emphasis of that appointment is aimed at fostering improved relationships between UF/IFAS and our production-agriculture stakeholders. This is a focal area that has always been central to my professional life. I joined UF/IFAS more than 20 years ago as a faculty member at the Range Cattle Research and Education Center in Ona where today I serve as the director. The center's faculty programs seek science-based solutions to help solve problems impacting cattle and forage production in Florida. Although I've been in this role for over a decade, I'm only the fourth center director in 75 years. This clientele-focused history emphasizes the commitment to long-held relationships between the center director and the agricultural clientele they serve. I'm proud of that legacy.

The mission of CRDF is to "Advance disease and production research and product development activities to insure the survival and competitiveness of Florida's citrus growers through innovation." In many ways, I believe the key to successfully achieving this mission will be based on a commitment to clientele-focused leadership. As a steward of grower funds and state and federal support, CRDF must focus on aligning industry needs with the creative skill and talent of the scientific community. In this relationship, our clientele are defined as both citrus industry and scientific community professionals. In obvious ways, these two groups differ significantly, which further emphasizes the importance of clientele-focused leadership at CRDF.

Citrus remains Florida's flagship agricultural industry. Although we are living in a trying and uncertain time, our future continues to be promising. Pioneering agricultural families coupled with 100+ years of research partnerships have made our citrus industry a Florida icon. The strength of this partnership has never been more critical. Today, CRDF is engaged in a number of important processes that will help shape future directions for the funding of research. To date, more than 400 projects have been supported. These investments help to improve our understanding of HLB and methods directed at improving the ability of citrus to tolerate HLB infection.

A new investment in research funding began this year. In late April, CRDF began to solicit new research proposals. Funding priorities will be based on a collection of inputs derived from stakeholder listening sessions, researcher inputs and a National Academy of Sciences review. Additionally, improved methods for communicating these research efforts and associated outcomes are being sought.

I encourage you to learn more about CRDF and our efforts to advance citrus science. Visit the CRDF website at https://citrusrdf.org/ for more information and recent updates.

John Arthington is interim chief operations officer of CRDF.

CRDF Grower Dollars at work for you

Column sponsored by the Citrus Research and Development Foundation disease in citrus plants, by studying gene expression in tolerant and more HLB-sensitive citrus varieties in leaves, stems, fruit and roots. Researchers also have used microscopy to visualize what is happening within the phloem tissues. They have measured enzyme activity in various important metabolic pathways in the plants. They are comparing apparent tolerant mutants with their normal types, looking for clues. But because HLB is associated with many changes in infected citrus trees, it has been very difficult to identify the most likely genes to target.

A further complication to solving the HLB problem by genome editing is that although the Clementine genome sequence is of very high quality, and there are several others also publicly available, none of these genomes are complete and perfect. So, when researchers look for the full and complete sequence of their potential gene target, they may not find all that is needed to design an effective RNA guide molecule.

These sequences were produced using what was the state of the art several years ago, but now with new sequencing and genome assembly tools, nearly perfect and complete genome sequences are closer to reality. It will be of great value to the effort to have these new genome technologies applied to our most common citrus varieties as well, so we can target precisely what we need to edit within their genomes. The sequence of the target gene may be different in oranges than it is in Clementine, grapefruit, etc.

There are other challenges and limitations to the technology, in addition to the central problem of identifying the relevant targets and having their full and correct sequences. CRISPR does rely on genetic transformation to get the complex into the plant and to have it perform its functions. This can be more difficult to do than standard genetic transformations to inset foreign DNA into the plants. In so doing, a consequence with some constructs used for CRISPR is that they still carry some of the telltale signatures of standard GMOs, such as antibiotic-resistant genes or the green fluorescent protein used for selection of modified plants.

So, researchers are working in citrus and with many other plant systems to develop better constructs and new approaches to improve the efficiency of transformation, and to remove all of the extra baggage of the construct.

Genome editing can be accomplished in such a way that there is absolutely nothing foreign that remains in the plant.

The end goal is that there is nothing else at all in the plant genome except the plant's own original DNA and the changed "spelling" that codes for the gene of interest.

And because many genes are members of gene families with very similar sequences through much of their structure, it is possible that the guide can be less than specific and target other areas of the genome that are not intended. However, compared with traditional gene knock-out methods, CRISPR is superior in that it can aim at multiple gene targets in one go so it may greatly shorten the time needed to modify multiple genes to achieve desirable results — for instance, the resistance to HLB. Because there is a great deal of research into tool development outside of the citrus research community, the CRISPR system is constantly being upgraded and improved, and many of these technical limitations are being resolved.

GENOME EDITING VS. GMOS

Genome editing differs from standard GMO plants in a very fundamental way. GMOs involve the incorporation of foreign DNA from other organisms such as bacteria, viruses, other plants, even insects and animals. By contrast, genome editing can be accomplished in such a way that there is absolutely nothing foreign that remains in the plant, only its own natural DNA sequences with a small change in the "spelling."

Such changes in the DNA code are very common and happen all the time, naturally. If you were to have your own genome sequenced multiple times, and you used different cells from your bones, skin, muscles, etc. each time, you would find that there are literally hundreds to thousands of single letter changes to your own genome. This could be tremendously significant as the regulatory agencies consider how to deal with edited plants and as they have their day in the court of public opinion.

Already, CRISPR-edited, non-browning mushrooms have been developed. The U.S. Department of Agriculture made the decision that these mushrooms are *not* subject to regulation, as would be a GMO mushroom with the same trait. It will be important, not only for the citrus industry and its consumers, but for all of agriculture and its ability to feed the ever-expanding human population on our planet, that a rational approach to this technology evolves.

As citrus scientists come to understand better the genetic control, not only of disease resistance or susceptibility, but of the many other important traits and characteristics (such as citrus fruit and juice color, flavor, quality, abiotic stress tolerance and seediness), genome editing holds great promise for targeted improvements of citrus. That is not to say that it can or will replace traditional breeding approaches entirely, because as stated above, many important characteristics of citrus trees are under the control of a large number of genes and their interactions. Traditional plant breeding techniques have a long and proven record of success for such goals and objectives. However, genome editing by CRISPR and other emerging techniques provide powerful new tools in the plant breeders' tool box and might speed up the breeding process.

Fred Gmitter and Jude Grosser are professors, and Yi Zhang is a post-doctoral research associate — all at the University of Florida Institute of Food and Agricultural Sciences Citrus Research and Education Center in Lake Alfred.

The conference included two full days of seminars.

International Citrus Business Conference tackles industry issues

By Tacy Callies

The HLB infection rate in commercial citrus groves is 100 percent in Florida and approximately 17 percent in Brazil.

China is the largest citrus-producing nation, growing 26 percent of the world's citrus.

Florida orange production could be as little as 43 million boxes in 10 years.

Those were just a few of the facts and figures presented at the International Citrus Business Conference held in Daytona Beach on March 27–28.

More than 300 members of the global citrus industry attended the event presented by Morris Agribusiness Services and AgNet Media. Nearly all segments of the citrus industry were represented, including growers, processors, brokers, byproducts and flavor companies and research institutions.

MARKETING MATTERS

In the opening presentation, Will Polese of Markets and Markets discussed the strong growth in the citrus-flavored functional beverages segment being driven by North American consumers who want healthy drinks with low sugar, low calories and great taste. He said the biggest demand for nutritional beverages comes from the very young and the elderly.

Larissa Popp of Citrus BR spoke about the success of Brazil's juice marketing program in Europe. The "Fruit Juice Matters" science-based initiative began two years ago to address declining OJ consumption. Conducted in partnership with the European fruit juice associations, bottlers and packagers, the program is targeting the scientific community, health care professionals and the media to build allies and dispel myths about orange juice. Early results of the campaign have already resulted in a significant decline in negative media coverage of juice.

Popp

Allen Morris of Morris Agribusiness Services, along with former executive director of the Florida Department of Citrus, Dan Santangelo, discussed restoring growth in the U.S. orange juice market. "We've lost 50 percent of the market since 2002, and we continue to lose that market at the rate of about 7.5 percent a year," reported Morris. He said 60 percent of the sales decline is due to price while "40 percent is due to irrational fear of natural sugar content of juice, along with consumers not

Morris