PLANTS IN THE CRISPR

Sarah Webb explores how researchers are using CRISPR/Cas9 to solve agricultural problems.

From gene-edited human embryos to disease-free pigs for donor organs, applications of CRISPR/Cas9 technology are filling the headlines. But the impact of this gene-editing technique isn't limited to biomedical research: Plant biologists are also using CRISPR to study molecular mechanisms underlying plant function, fight disease, and enhance plant productivity.

"The CRISPR craze has pretty much swept through plant biology," says Dan Voytas of the University of Minnesota. "I would say most groups doing plant gene editing are using CRISPR or similar reagents." As a result, CRISPR/Cas9 could prove pivotal in addressing the challenge of feeding the world's growing population, which is expected to approach 10 billion by 2050.

New plant breeding

Transgenic plants (also known as genetically modified organisms or GMOs) have been around for decades. But the insertion of foreign genes and DNA to produce desirable traits has prompted controversy as well as rejection of these plants by some consumers. In recent years, biologists have been developing more tailored methods for altering genomes that complement traditional plant breeding strategies and dovetail with new genetic tools. Until the advent of CRISPR within the past 5 years, one of the more promising gene-editing technologies was TAL effector nucleases (TALENs), which were developed from building blocks that occur naturally in plants.

However, CRISPR/Cas9 has largely overtaken other gene-editing techniques. Researchers tell similar stories: A few years ago, they started working on projects using both TALENs and CRISPR/Cas9 side-by-side, but quickly settled on CRISPR. While both techniques offer precise editing, TALENs are

large, complex proteins that must be newly synthesized for each mutation, says Becky Bart of the Donald Danforth Plant Science Center in St. Louis. But using CRISPR/Cas9, a researcher needs only to develop new guide RNAs, she says, and "very quickly you can test a bunch of constructs right in the lab." As a result, CRISPR is both cheaper and faster, says Bing Yang of Iowa State University. And combining CRISPR with a traditional plant breeding program offers the most potential for making precise changes quickly.

That doesn't mean TALENs and other methods are completely out of the picture though. With the continuing uncertainty surrounding the patents and licensing of CRISPR technology, many companies are still centering their work around technologies such as TALENs and meganucleases, where the intellectual property rights are clear, says Voytas, who was one of the early developers of TALENs and is the Chief Science Officer of Calyxt—a Minnesota-based plant gene-editing company focusing on that technology (see "Legal and regulatory hurdles" sidebar).

Mining mutations

Scientists have long mined natural plant mutants that show up in fields or used random mutagenesis as a tool for understanding gene function in crops. "Hopefully, you hit a gene; hopefully, you get a change in the phenotype of interest, the trait of interest, and then you try to pin down which gene is broken," says Zach Lippman of Cold Spring Harbor Laboratory in New York. His laboratory focuses on understanding the flowering process, particularly in tomatoes and the related Solanaceae (nightshade) family, so that they can ultimately manipulate the process to improve agriculture.

An example of tomato mildew, one of the bacterial diseases that researchers are looking to eliminate in plants through the use of CRISPR/Cas9 technology. Credit: S. Kamoun.

CRISPR has enhanced Lippman's work. The power of the technology in plants, he says, is the ability to create guided chromosomal breaks in genes. By disrupting the coding sequences of genes and producing non-functional proteins, functional studies to look at the effects of specific genes on the flowering process are possible (1). "We can now use CRISPR to mutate those genes directly and in a very fast and efficient way, which was never before possible," he says.

Blake Meyers of the Danforth Center has been using CRISPR to introduce single-nucleotide changes in plant microRNAs in *Arabidopsis*. "It gives us a very powerful tool to make very small changes, kind of what we might think of as subtle changes that can have dramatic effects on processing," he says. He's also been collaborating on a project with Yang where they've been looking at the impact of cutting out 70-kilobase chunks of the maize genome, which they can do with single-nucleotide precision. "With CRISPR-generated mutants, we can get anywhere from a single base, which can cause a frame-shift mutation, to multiples of three that give us in-frame deletions, to much larger deletions, all from the same original construct, and so it's given us a lot of allelic diversity," he says.

But gene knockouts aren't the only way CRISPR can be applied. Mammalian researchers have developed screening techniques using CRISPR with an inactivated Cas9 protein that can't cleave DNA. Here, binding of the mutant Cas9 either activates or represses gene expression, serving as a type of dimmer switch,

rather than simply turning expression on and off. This strategy could also be useful in plants. Many traits Lippman studies in tomatoes are quantitative reproductive traits, and a targeted technique that modulates gene expression could help tease out more details of the flowering and fruiting processes.

CRISPR technology is also allowing researchers to explore new ways to domesticate plants that have agricultural potential. "There are some species of plants in the Solanaceae family that make wonderful, edible fruits that have never been domesticated," Lippman notes. People eat the fruits collected in the wild, but they're not suitable for farms or gardens because the plants might be large, but the fruits they produce are too small, or they might take too long to flower. However, with CRISPR, researchers can think about modifying genes in these wild species that are homologs or orthologs to tomatoes, or use a "dimming strategy" to change levels of gene expression.

"I think that's very exciting because now you're talking about creating new crops," Lippman says. But as some start thinking about cultivating new fruits, others are thinking about how to protect existing domesticated plants.

Stoking disease resistance

Plant pathogens, which deliver disease-causing molecules known as effectors to their hosts, can devastate a farmer's crop, often causing financial ruin or food insecurity within a region. While the plant's immune system works to clear these effector molecules (TAL effectors are one example of these plant pathogen effectors), conserved sequences within specific plant genes can prove to be weak points, and the pathogen's effectors can exploit them to cause disease. Once established within the plant's genome, such sequences are known as susceptibility genes.

"If you remove the targets of effectors, then the pathogen would struggle in causing disease and modifying the plant to make it susceptible," explains Sophien Kamoun, who studies plant-pathogen interactions at the Sainsbury Laboratory in Norwich, United Kingdom. CRISPR offers a convenient tool for both identifying such genes and producing plants resistant to the disease. Kamoun and his colleagues recently removed a portion of a susceptibility factor in a tomato plant using CRISPR. The resulting non-transgenic plants, which were fully resistant to the fungal disease powdery mildew, were developed quickly, within 10 months (2).

Yang, who has been a key figure in the development of CRISPR technology in plants, is focused on bacterial blight in rice. This severe disease in South Asia and Africa takes advantage of binding to the promoter of sucrose transporter genes, SWEET genes, to induce susceptibility. Using CRISPR, Yang is able to make multiple changes to these promoters to produce the equivalent of a plant vaccine. Nian Wang and his colleagues at the University of Florida Citrus Research and Education Center have successfully modified yet another known susceptibility gene, for a bacterial disease citrus canker, in a species of grapefruit (3). They are currently looking for susceptibility genes in another destructive citrus disease, citrus greening, also known as Huanglongbing.

Bart, in collaboration with Voytas, is doing related research with cassava, a hearty tuberous root vegetable that serves as a food security crop in sub-Saharan Africa, South American, and Asia. Funded by the Bill and Melinda Gates Foundation, the two researchers and their team are looking at various mutations that would protect these plants from a bacterial disease and two viral diseases. To date, they've successfully screened for mutations that abolish susceptibility genes for two of the diseases, and they've regenerated plants with mutations that they'll soon be testing for disease tolerance.

As with diseases in other organisms, pathogens are constantly adapting and changing. A disease that rapidly devastates a critical crop in one part of the world could lead to widespread famine, such as the Irish potato famine of the 1840s. CRISPR could provide a way to outpace those mutations or to generate plants with broad-spectrum resistance, according to Lippman. "Previously, if you had a disease that popped up and started to really knock out a crop or really hurt crop yields, you'd have to look for natural resistance that would exist in a wild species, or you'd have to use old-fashioned genetic engineering techniques, which may or may not work."

Overcoming technical challenges

Some technical challenges remain in applying CRISPR in plants. Removing DNA snippets is relatively easy, but while changing sequences or introducing genes at specific locations are also possible, according to Kamoun, "It's just not always easy." One challenge in plant biology, he says, will be to make other types of edits as routine as simple deletions.

Still other technical challenges come from the plant itself. The plant cell wall can be a formidable barrier to cross: In some plants it's difficult or even impossible to get the gene-editing machinery into a plant cell. In cassava, there is a robust set of strategies for transforming the cells, Bart says. But she also works on cotton, a plant with no gene-editing options because transformation is so difficult.

In some cases, researchers might use *Agrobacterium*, viral delivery, or plasmid bombardment to deliver the gene-editing components. But recent innovations are overcoming some of these obstacles. A new technology from DuPont Pioneer uses a gene gun to blast ribonucleoprotein particles into plant cells. As a result, they can transform almost any variety of corn, Lippman says. The company can also deliver factors along with the Cas9 machinery that prompt edited somatic cells to directly form an embryo-like structure that can germinate into a tiny plant (4).

Skipping DNA entirely avoids another problem: The removal of the Cas9 enzyme after editing, which is a priority as such plants move toward the mainstream. With some crops, researchers can use conventional breeding strategies to segregate transgenes that include Cas9 for removal. Kamoun and his colleagues used this strategy in their tomato study. However, some plants, including cassava, don't form seeds and are difficult to cross, which makes removing a transgene that encodes Cas9 more difficult.

Legal and regulatory hurdles

Even with its potential, legal and regulatory hurdles remain before CRISPR-altered plants make it to farmer's fields. Ongoing battles between the University of California at Berkeley and the Broad Institute of MIT and Harvard over the patents associated with CRISPR/Cas9 have led to muddiness surrounding intellectual property, says Voytas. His company, Calyxt, is focused on TALENs, while other companies have focused on meganucleases, where the commercial path forward is more certain.

In addition, agencies around the globe haven't worked out how plants edited with these technologies will be regulated. Regulatory costs can crush small biotechnology companies

trying to bring a product to market, says Blake Meyers, so they'd like to employ strategies that minimize or avoid those hurdles. A central issue may be whether regulatory agencies focus on the end product or on how it was made, says Sophien Kamoun. European regulatory agencies tend to focus on process, while U.S. regulators tend to focus on the end product.

Unlike transgenic crops, which introduce whole genes that remain within the organism, plant biologists can use breeding, segregation, and other strategies to eliminate the gene-editing machinery from plants. If researchers avoid transgenes, CRISPR-edited plants are often indistinguishable from plants that ac-

quired genetic mutations naturally. The traceability question may prove important for regulation over the long term, Kamoun says. "Unlike transgenics you really don't have a way to demonstrate that it went through the particular process of genomic editing."

Regulatory guidance is evolving. The USDA recently held a comment period on new proposed guidelines, but so far the USDA has allowed more than 2 dozen transgene-free plant products with knockout mutations to move forward without regulatory oversight (5). "They've been very consistent in allowing people to go out into the field with those products," Voytas says. -- S.W.

Blake Meyers from the Danforth Center has been using CRISPR/Cas9 to introduce small changes in plant microRNAs. Credit: Donald Danforth Plant Science Center.

Limitless possibilities

Despite the technical hurdles, CRISPR/Cas9 is changing plant biology as fast as it is revolutionizing other fields. Just a few years ago, a research article might have highlighted the ability to mutate plant genes using CRISPR, but now the title touts a better understanding of plant architecture, with CRISPR embedded in the Materials and Methods section. “I’m not saying that we don’t have a lot of work yet to do on technology development,” Voytas says, but he adds that it’s satisfying to see this shift. “It’s become the tool and not the story.”

With the investments made by researchers and industry, Kamoun sees CRISPR-based gene-editing technology as maturing relatively rapidly in plants. “I think the challenge now becomes about finding the traits,” he says. For a long time, plant biologists didn’t have the tools to apply the knowledge that they’d gained about interesting plants genes and then deliver those results to farmers. But now they have the technology, he says. “We need more research to actually understand what all of the genes are doing in plants and how we can tweak them and improve them.”

After years of mostly reading genomes, researchers are editing and moving toward rewriting those genomes in increasingly sophisticated ways, Voytas says. Synthetic biology, though rudimentary right now, could help modify plant genes to produce rare metabolites or even pharmaceuticals of interest. Such technologies could allow researchers to modify nutrient content to lower gluten levels in bread or optimize the fatty acid content in cooking oil. “The possibilities are limitless, but the editing allows us to start to harness and control those metabolic pathways,” he concludes.

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