CRISPR Technology and HLB-resistance

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Citrus Research & Education Center
Outline

Review of GMO crops

Introduction to CRISPR

CRISPR applications in plants

Current work of CRISPR on citrus and perspectives on HLB
• Genetically modified organism (GMO) contains foreign genes

• Discovery of DNA double-helix in 1950s & recombinant DNA technology in 1970s

• GMO bacteria, plants, and animals in research and industry

• GMO food is being accepted by the public, slowly
A gene is a piece of DNA encoding a biological function. DNA sequence of a gene determines its specific function. In plant genetic engineering, the DNA sequence is modified to achieve desirable changes in traits, usually by insertion of foreign DNA.
Overview of GMO crops

- First GMO crop (tomato) for sale in the U.S. in 1994
- GMO of staple crops such as corn, cotton, soybean, canola etc.
- Release and planting of GMO crops increased rapidly
Global GMO crops production by 2013

GE crops on world’s 12% cropland
40% of global GE crops in the U.S.
Annual GMOs releases by USDA

Number of releases of genetically engineered (GE) organisms varieties approved by APHIS, 1985-2013* (Includes permits and notifications)

*As of September 24, 2013.
Authorizations for field releases of GE organisms (mostly plant varieties) are issued by USDA’s Animal and Plant Health Inspection Service (APHIS) to allow technology providers to pursue field testing.
Traits of released GMOs

- Bacterial resistance, 224
- Fungal resistance, 1,191
- Virus resistance, 1,425
- Marker gene, 1,892
- Other, 1,986
- Agronomic properties, 5,190
- Product quality, 4,896
- Insect resistance, 4,809
- Herbicide tolerance, 6,772
- Nematode resistance (NR), 149

*As of September 24, 2013.*
**GMO vs traditional breeding**

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**Methods of Plant Breeding**

**Traditional**

The traditional plant breeding process introduces a number of genes into the plant. These genes may include the gene responsible for the desired characteristic, as well as genes responsible for unwanted characteristics.

- **Donor Variety DNA Strand**
  - DNA strands contain a portion of an organism’s entire genome.
  - Desired gene

- **Recipient Variety DNA Strand**

- **New Variety DNA Strand**
  - Many genes are transferred with the desired gene.

**Genetic Engineering**

Genetic engineering enables the introduction into the plant of the specific gene or genes responsible for the characteristic(s) of interest. By narrowing the introduction to one or a few identified genes, scientists can introduce the desired characteristic without also introducing genes responsible for unwanted characteristics.

- **Donor Organism DNA Strand**
  - The desired gene is copied from the donor organism’s genome.
  - Desired gene

- **Recipient Variety DNA Strand**

- **New Variety DNA Strand**
  - Only the desired gene is transferred to a location in the recipient genome.

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Source: FDA
GMO to contain citrus Huanglongbing

Traditional breeding to make HLB-resistant citrus

GMO method to introduce HLB resistance/tolerance

• Proven to effectively curb disease for staple crops
• Safe for growers, consumers, and the environment
• Introducing new traits from sources other than citrus
• New technology for more effective and faster delivery

CRISPR is the latest method for creating desirable plants
CRISPR-Cas9

The new opportunity and challenge

Source: wikipedia
First, how to make GMO plants:

1. Make bullets of foreign DNAs (including CRISPR)
2. These usually contain selectable markers
3. Prepare host plants so they can take foreign DNAs
4. Agrobacterium-mediated plant transformation, protoplasts, or biolistics

What is CRISPR? (Clustered Regularly Interspaced Short Palindromic Repeats)

- Piece of bacterial DNA containing short repetitive sequences
- Bacteria use it to chop up the invading viruses
- Could work in plants to make small changes at most places in the genome
CRISPR research timeline

1987
First report on CRISPR

2002
“CRISPR” was named

2005
CRISPR in immunity

2010
Cas9 cleavage

2012
Cas9 DNA targeting

2013
Cas9 editing in eukaryotic cells including plants

2014
CRISPR in citrus
How CRISPR-Cas9 works to modify DNA

gRNA (guide RNA, 20-nt), based on the TARGET GENE, directs where Cas9 goes and cuts the DNA at the specific location.

Cas9 (CRISPR associated protein 9) binds and cuts DNA.

The two DNA ends produced by Cas9 are then repaired and reconnected.

In the repairing process, addition or deletion of DNA fragment could occur.

“Spelling” of the gene is altered and usually becomes non-functional.
Huge advantages of the CRISPR system

Easy design to change almost any single gene

Or change multiple redundant genes simultaneously

Precise gene editing by homologous recombination

High-throughput functional genomics applications

Option to leave no fingerprint after making changes

Not subject to regulation if only small changes are made

No introduction of foreign/bacterial DNA like in GMO crops
Gene-edited CRISPR mushroom escapes US regulation

A fungus engineered using CRISPR–Cas9 can be cultivated and sold without oversight.

BY EMILY WALTZ

The US Department of Agriculture (USDA) will not regulate a mushroom that has been genetically modified with the gene-editing tool CRISPR–Cas9, the agency has confirmed. The long-awaited decision means that the mushroom can be cultivated and sold without passing through the agency’s regulatory process — making it the first CRISPR-edited organism to receive a green light from the US government.

“The research community will be very happy with the news,” says Caixia Gao, a plant biologist at the Chinese Academy of Sciences Institute of Genetics and Developmental Biology in Beijing, who was not involved in developing the mushroom. “I am confident we’ll see more gene-edited crops falling outside of regulatory authority.”

Yinong Yang, a plant pathologist at Pennsylvania State University (Penn State) in University Park, engineered the fungus — the common white button mushroom (Agaricus bisporus) — to resist browning. The effect is achieved by targeting the family of genes that encodes polyphenol oxidase (PPO), an enzyme that causes browning. By deleting just a hand-
CRISPR-edited crops free to enter market, skip regulation

The first CRISPR-edited crops presented to the US regulatory system can be cultivated and sold without oversight by the US Department of Agriculture (USDA), the agency said in a pair of letters posted in April. The decisions could reduce by millions the cost of development of the crops: an anti-browning mushroom and a waxy corn genetically modified with the gene editing tool CRISPR-Cas9. Some scientists hailed the decision as a step in the right direction, although media outlets and other interested parties said it illustrates the murky state of US biotech regulations.

Johnston, Iowa–based DuPont Pioneer engineered the waxy corn to contain starch composed exclusively of the branched polysaccharide amylpectin—a commodity in processed foods, adhesives and high-gloss paper. Company researchers achieved the effect by shutting down production of cornstarch’s other long-chain polysaccharide, amylose. Using the gene-editing tool CRISPR-Cas9, the team knocked out the endogenous waxy gene Wx1, which encodes the endosperm’s granule-bound starch synthase responsible for making amylose.

DuPont Pioneer, currently undergoing a merger with The Dow Chemical Company, says it expects the CRISPR-edited variety to have higher yields than conventional waxy corn. The company plans to commercialize the plant within five years and follow it with many more CRISPR-edited crops. “This is just the beginning,” said Neal Guttenson, vice president of R&D, in a statement released to coincide with the USDA’s response.

necessary tool in biotech. Plant pests have served as the trigger for USDA oversight since the 1980s, when the US government wrote the regulatory framework for biotech products.

Newer genetic engineering (GE) techniques that don’t involve plant pests are quickly supplanting the old ones, and the USDA appears to be saying it does not have the authority to regulate the products of these techniques. The letters to DuPont and Yang were the agency’s first decisions on CRISPR-edited crops. The agency ruled similarly on plants transformed with other gene-editing techniques, such as zinc-finger nuclease and transcription activator-like effector nuclease systems.

Such letters from USDA have become “essential” to small companies attempting to bring to market GE plants, says...
<table>
<thead>
<tr>
<th>Plant</th>
<th>Target genes</th>
<th>Traits</th>
<th>Delivery methods</th>
<th>Reference</th>
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<tbody>
<tr>
<td>西红柿</td>
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<td>Solanum tuberosum</td>
<td>sp5G, sp, SIIIA9, SIAGL6, PSY1, Mlo, GABA-TP1, GABA-TP2, GABA-TP3, CAT9, and SSADH</td>
<td>Plant development, Parthenocarpic, Fruit color, Powdery mildew, g-GABA synthesis</td>
<td>Agrobacterium, Agrobacterium, Agrobacterium, Agrobacterium</td>
<td>Soyk et al., 2017, Ueta et al., 2017, Klap et al., 2017, Li et al., 2017</td>
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<td>Solanum pimpinellifolium</td>
<td>SP, SP5G, SICLV3, SIWUS, SIGGP1</td>
<td>fruit development</td>
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<td>Li et al., 2018</td>
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<td>Lactuca sativa 萝卜</td>
<td>BIN2</td>
<td>Plant development</td>
<td>PEG protoplasts</td>
<td>Woo et al., 2015</td>
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<td>Cucumis sativus 黄瓜</td>
<td>eIF4E</td>
<td>Virus resistance</td>
<td>Agrobacterium</td>
<td>Chandrasekaran et al., 2016</td>
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<td>Grape</td>
<td>VvPDS, VvWRKY52</td>
<td>Carotenoid biosynth</td>
<td>Agrobacterium</td>
<td>Nakajima et al., 2017</td>
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<td>Citrus</td>
<td>CsPDS, CsLOB1</td>
<td>Botrytis inerea resis, canker resistance, Carotenoid biosyn</td>
<td>Agrobacterium, Agrobacterium, Agrobacterium</td>
<td>Wang et al., 2017, Peng et al., 2017; Jia et al., 2017, Jia and Wang, 2014</td>
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<td>Chrysanthemum</td>
<td>CpYGFP</td>
<td>Fluorescence</td>
<td>Agrobacterium</td>
<td>Kishi-Kaboshi et al., 2017</td>
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<tr>
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<td>Carotenoid biosynth</td>
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<td>Salvia miltiorrhiza 丹参</td>
<td>SmCPS1</td>
<td>Tanshinone biosyn</td>
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<td>Li et al., 2017</td>
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<td>Fragaria vesca</td>
<td>TAA1, ARF8</td>
<td>Plant development</td>
<td>Agrobacterium</td>
<td>Zhou et al., 2018</td>
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Prerequisite: No foreign DNA sequences such as Cas9 gene present in edited plants. CRISPR relies on genetic transformation, and initially carries the same “baggage”.

USDA Will Not Regulate CRISPR-Edited Crops

Restrictions will remain on transgenic plants, which contain artificially inserted genes from other species.
A widely used method to produce non-transgenic CRISPR-mediated mutant plants

Stable expression of CRISPR genes to create mutants

Self-pollination to
Segregate CRISPR genes from the mutations

T1 non-transgenic mutant plants  Transgenic plants
Dear Editor,

CRISPR gene-editing technology has successfully generated targeted mutations in rice and many other plant species (Ma et al., 2015). Assessment of heritability and phenotypic stability of CRISPR-edited plants requires the elimination of the CRISPR construct. The presence of the CRISPR construct makes it difficult to distinguish the mutations transmitted from the previous generation from newly generated mutations by the CRISPR construct at the current generation. The existence of the CRISPR generated, making it very laborious and time-consuming to identify edited plants. Here, we report the development of a technology that can actively and automatically eliminate any plants containing the CRISPR/Cas9 construct but still allows enough time for the CRISPR/Cas9 construct to perform targeted gene modification before its removal. We employ a pair of suicide transgenes that effectively kills all of the CRISPR/Cas9-containing pollen and embryos produced by T0 plants. Our strategy effectively eliminates the CRISPR/Cas9 transgenes in all of the T1 plants, greatly reducing the labor and time needed to identify
However, strategies for annual crops do not work well for woody or perennial crops that have a long juvenile phase and heterozygous
**Approach 1:** Delivering Cas9/sgRNA complex into protoplasts and then regenerate whole plants

However, regenerating plants from protoplasts is difficult or not possible for many crop plants at this time
Approach 2: Deliver Cas9/sgRNA genes using a gene gun method and screen for non-transgenic plants (August 2016)

Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA

Yi Zhang¹,²,*, Zhen Liang¹,²,*, Yuan Zong¹,²,*, Yanpeng Wang¹,², Jinxing Liu¹, Kunling Chen¹, Jin-Long Qiu³ & Caixia Gao¹

Editing plant genomes is technically challenging in hard-to-transform plants and usually involves transgenic intermediates, which causes regulatory concerns. Here we report two simple and efficient genome-editing methods in which plants are regenerated from callus cells transiently expressing CRISPR/Cas9 introduced as DNA or RNA. This transient expression-based genome-editing system is highly efficient and specific for producing transgene-free and homozygous wheat mutants in the T0 generation. We demonstrate our protocol to edit genes in hexaploid bread wheat and tetraploid durum wheat, and show that we are able to generate mutants with no detectable transgenes. Our methods may be applicable to other plant species, thus offering the potential to accelerate basic and applied plant genome-engineering research.
Approach 3:

Agrobacterium-mediated transient expression of Cas9 and sgRNA to produce transgene free mutant plants
T-DNA from *Agrobacterium* can be transiently expressed.
Agrobacterium-mediated Transient Gene Expression
(expression with no stable transgene integration)

**A: Transient + Stable**

2d | 3d | 4d | 5d | 6d

**B: Stable**

2+5d | 3+5d | 4+5d | 5+5d | 6+5d

**Transient:** T-DNA genes are not inserted into the plant genome

**Stable:** T-DNA genes are inserted into the plant genome

**Transient gene expression activity:** Difference between A and B
Agrobacterium mediates transient expression of T-DNA genes in citrus (Valencia sweet orange)

The GUS activities in the left panel are largely due to transient expression of GUS gene in T-DNA.
How to identify mutants if no selection for transformants?
We developed a two-step method to identify mutants

1. Agrobacterium infection and shoot regeneration with no selection pressure
2. Deep DNA sequencing analysis
3. High resolution melt analysis

- 1,000 or more shoots
  - 42 shoots per group
  - 7 shoots per group
  - 1 shoot + 1 WT shoot
A method for the production and expedient screening of CRISPR/Cas9-mediated non-transgenic mutant plants

Longzheng Chen¹,², Wei Li¹, Lorenzo Katin-Grazzini¹, Jing Ding³, Xianbin Gu¹, Yanjun Li¹, Tingting Gu³, Ren Wang¹, Xinchun Lin¹,⁴, Ziniu Deng⁵, Richard J. McAvoy¹, Frederick G. GmitterJr.⁶, Zhanao Deng⁷, Yunde Zhao⁸ and Yi Li¹,³

Abstract
Developing CRISPR/Cas9-mediated non-transgenic mutants in asexually propagated perennial crop plants is challenging but highly desirable. Here, we report a highly useful method using an Agrobacterium-mediated transient CRISPR/Cas9 gene expression system to create non-transgenic mutant plants without the need for sexual segregation. We have also developed a rapid, cost-effective, and high-throughput mutant screening protocol based on Illumina sequencing followed by high-resolution melting (HRM) analysis. Using tetraploid tobacco as a model species and the phytoene desaturase (PDS) gene as a target, we successfully created and expediently identified mutant plants, which were verified as tetra-allelic mutants. We produced PDS mutant shoots at a rate of 47.5% from tobacco leaf explants, without the use of antibiotic selection. Among these PDS plants, 17.2% were confirmed to be non-transgenic, for an overall non-transgenic mutation rate of 8.2%. Our method is reliable and effective in creating non-transgenic mutant plants without the need to segregate out transgenes through sexual reproduction. This method should be applicable to many economically important, heterozygous, perennial crop species that are more difficult to regenerate.
Targeted Genome Editing of Sweet Orange Using Cas9/sgRNA

Hongge Jia, Nian Wang*

Citrus Research and Education Center, Department of Microbiology and Cell Science, University of Florida, Lake Alfred, Florida, United States of America

Abstract

Genetic modification, including plant breeding, has been widely used to improve crop yield and quality, as well as to increase disease resistance. Targeted genome engineering is expected to contribute significantly to future varietal improvement, and genome editing technologies using zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9/single guide RNA (sgRNA) have already been successfully used to genetically modify plants. However, to date, there has been no reported use of any of the current genome editing approaches in sweet orange, an important fruit crop. In this study, we first developed a novel tool, Xcc-facilitated agroinfiltration, for enhancing transient protein expression in sweet orange leaves. We then successfully employed Xcc-facilitated agroinfiltration to deliver Cas9, along with a synthetic sgRNA targeting the CsPDS gene, into sweet orange. DNA sequencing confirmed that the CsPDS gene was mutated at the target site in treated sweet orange leaves. The mutation rate using the Cas9/sgRNA system was approximately 3.2 to 3.9%. Off-target mutagenesis was not detected for CsPDS-related DNA sequences in our study. This is the first report of targeted genome modification in citrus using the Cas9/sgRNA system—a system that holds significant promise for the study of citrus gene function and for targeted genetic modification.


Editor: Manoj Prasad, National Institute of Plant Genome Research, India

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Competing Interests: The authors have declared that no competing interests exist.

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Potential gene targets being investigated

<table>
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<tr>
<th>Gene Group</th>
<th>Target Gene</th>
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<tbody>
<tr>
<td>Plant genes for detecting pathogens</td>
<td>FLS2 (Flagellin sensitive 2)</td>
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<td>Chintin receptors</td>
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<td>Plant defense genes</td>
<td>NPR1 (Nonexressser of PR genes 1)</td>
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<td>Thionin (anti-bacterial protein)</td>
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<td></td>
<td>DMR6 (Downy mildew resistant 6)</td>
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<td>CDR (Constitutive disease resistant)</td>
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<tr>
<td>Clas virulence genes</td>
<td>LAS 5315 (effector protein)</td>
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<td></td>
<td>SDE (Sec-delivered effector)</td>
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<td>And many more ...</td>
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Challenges and limitations

Transformation efficiency lower than non-CRISPR plasmids

Plant genomes can be more complex (polyploid)

Plant cell walls make it harder to reach inside cells

Optimize Cas9 codon for plants

Minimize off-target effects of Cas9 cleavage

**Identification of relevant targets for HLB resistance**

However, this tool can enable very precise, potentially unregulated, changes to the citrus genome, allowing trait-targeted modifications

  Fruit quality, resistance to other diseases, etc.
Many challenges for using genome editing technologies to improve perennial vegetatively propagated plants

-- How to produce non-transgenic mutants without sexual reproduction?

-- How to more efficiently identify mutants if no selection transgenic cells/plants?

-- How to avoid chimera if no transgenic selection?

-- How to edit plants that are difficult to regenerate?

-- How to alter expression patterns of target genes?

-- Consumer/commercial acceptance: COMPLEXITY!
Sugar Belle® near Vero Beach, HLB+ >8 years!
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Funding
Questions

CRISPR/Cas9

DNA

gRNA

CRISPR/Cas9 GMO Questions

gRNA

DNA

CRISPR/Cas9

gRNA

DNA

CRISPR/Cas9

gRNA