CRISPR Technology and HLB-resistance

Fred Gmitter
University of Florida
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ICBC International Citrus & Beverage Conference

Outline

Review of GMO crops

Introduction to CRISPR

CRISPR applications in plants

Current work of CRISPR on citrus and perspectives on HLB

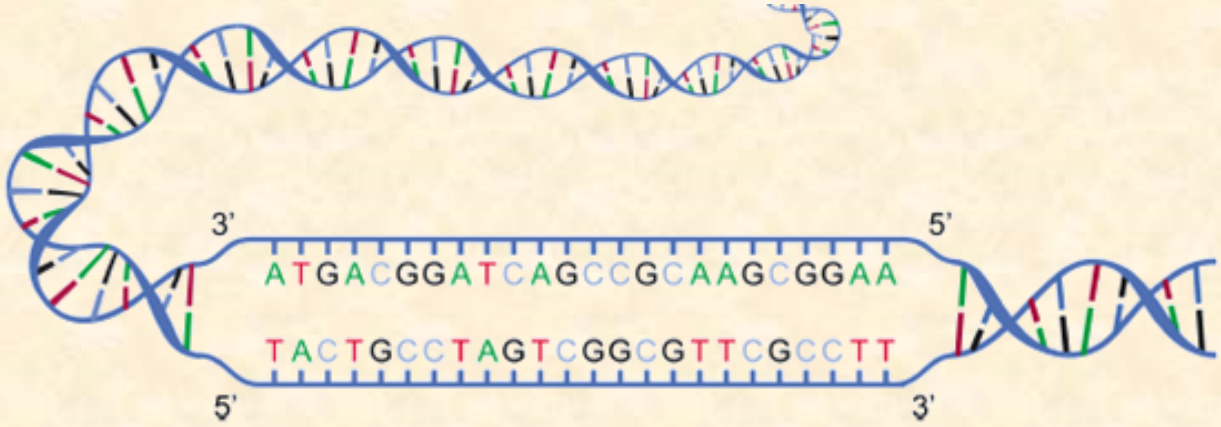
About GMO

- 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

Behind GMO: Genetic Engineering

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.**

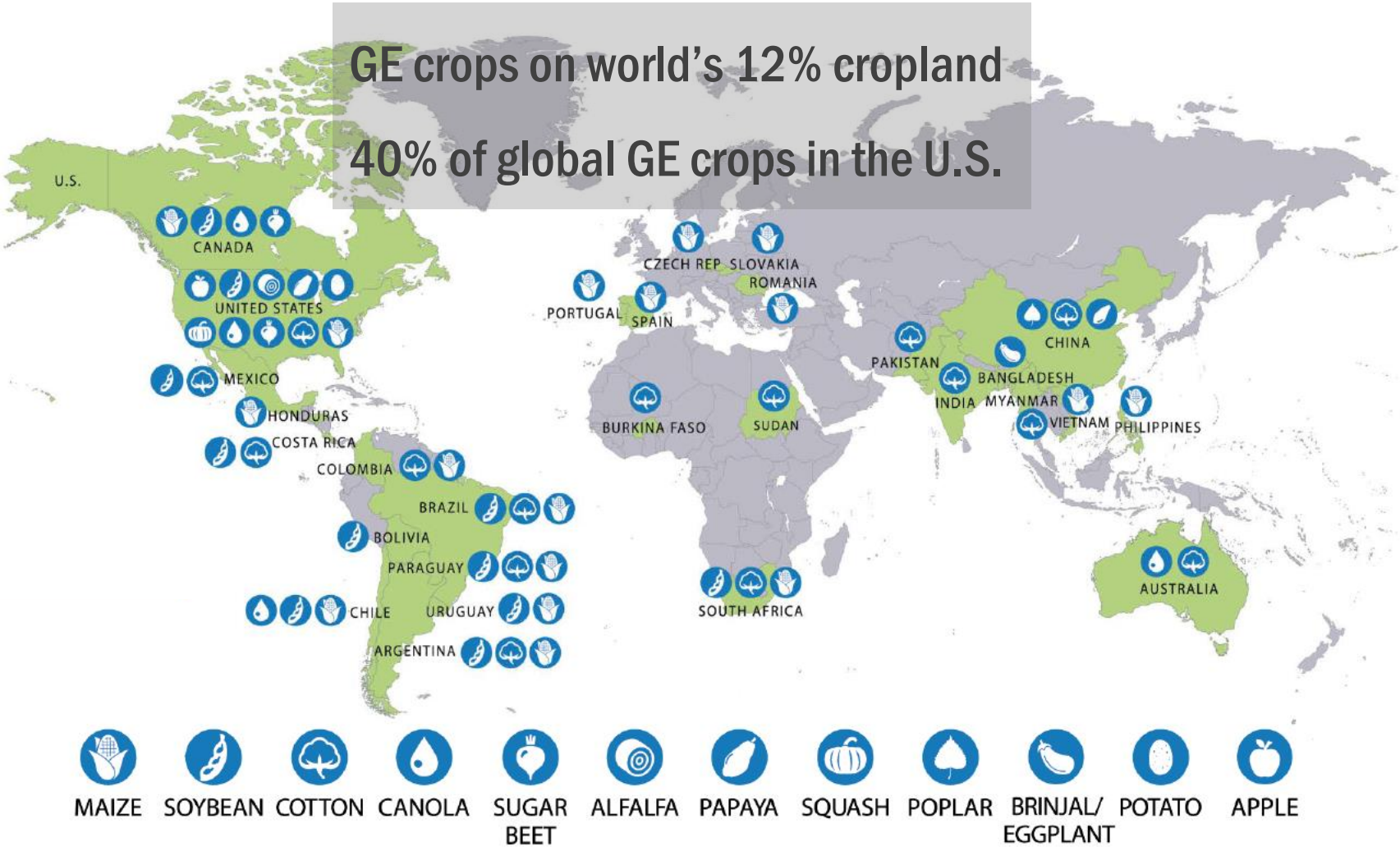


Overview of GMO crops

- 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



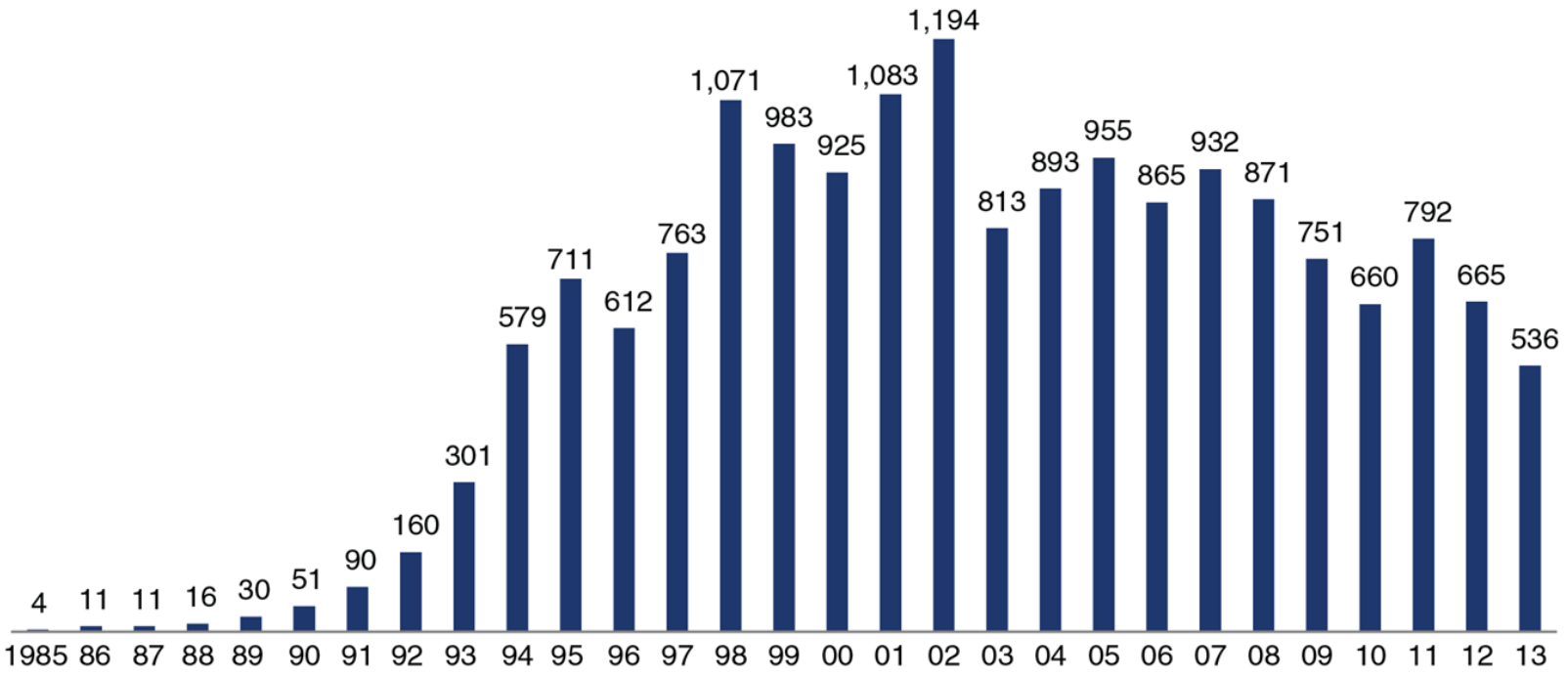

Global GMO crops production by 2013



James, Clive. 2014. Global Status of Commercialized Biotech/GM Crops: 2014

Annual GMOs releases by USDA

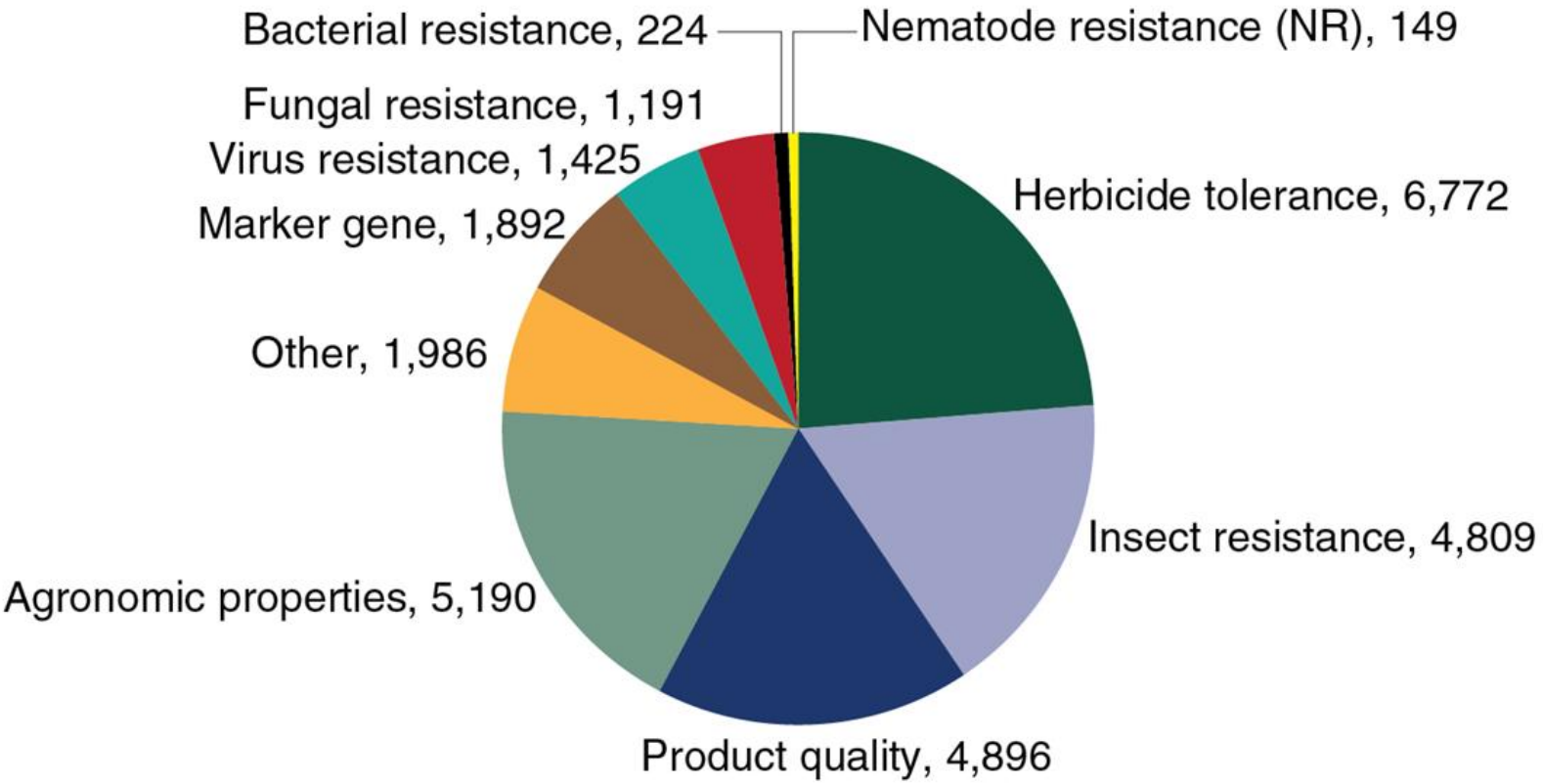
Number of releases of genetically engineered (GE) organisms varieties approved by APHIS, 1985-2013* (Includes permits and notifications)



*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.

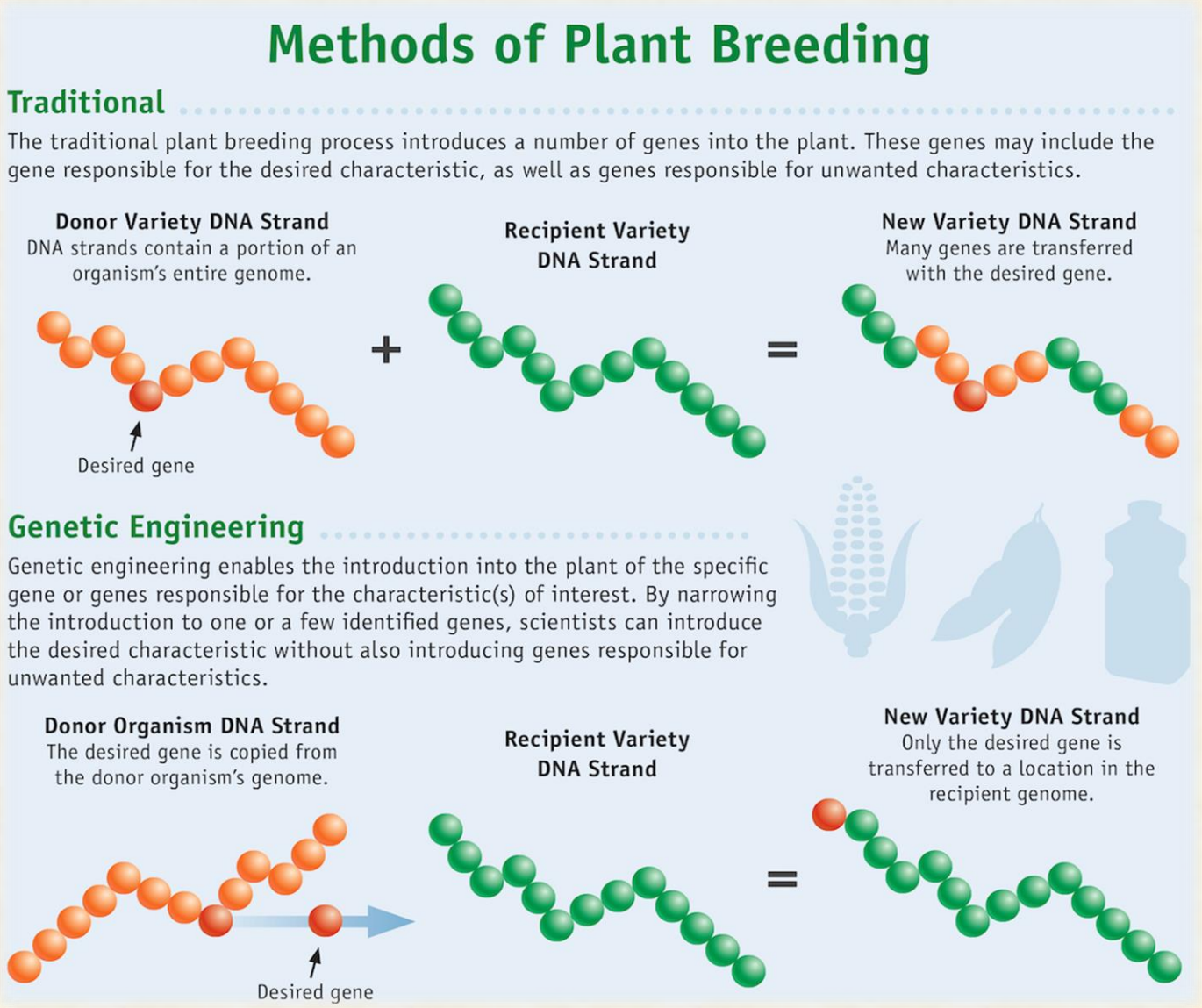
Source: Information Systems for Biotechnology (ISB, 2013).

Traits of released GMOs



*As of September 24, 2013.

GMO vs traditional breeding



Source: FDA

GMO to contain citrus Huanglongbing

Traditional breeding to make HLB-resistant citrus

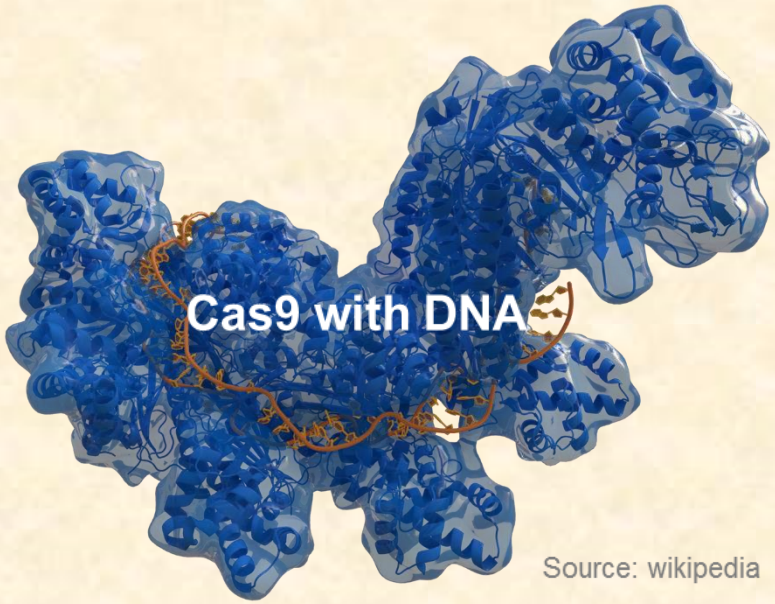
GMO method to introduce HLB resistance/tolerance

- 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

<u>CRISPR is the latest method for creating desirable plants</u>

CRISPR-Cas9

The new opportunity and challenge



Source: wikipedia

CRISPR overview

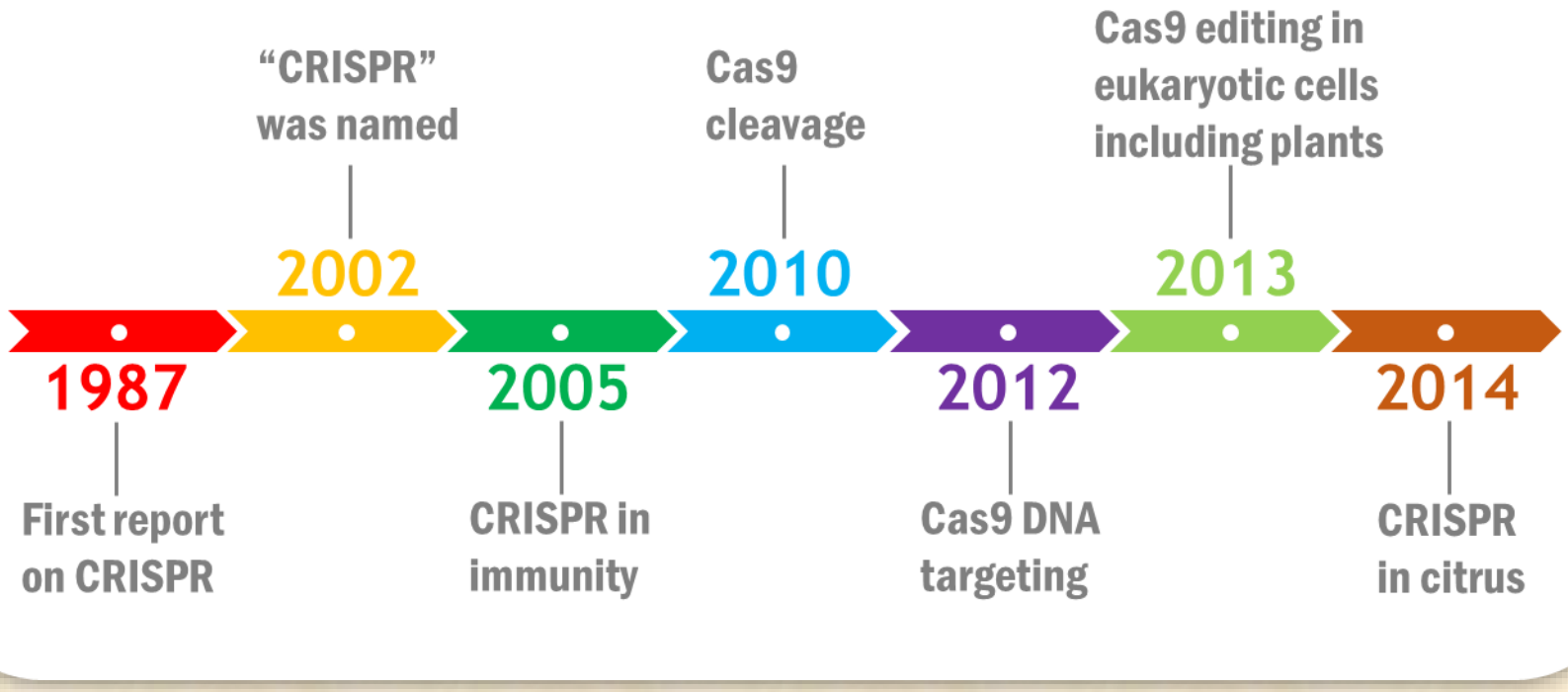
First, how to make GMO plants:

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 biolisitics

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



How CRISPR-Cas9 works to modify DNA

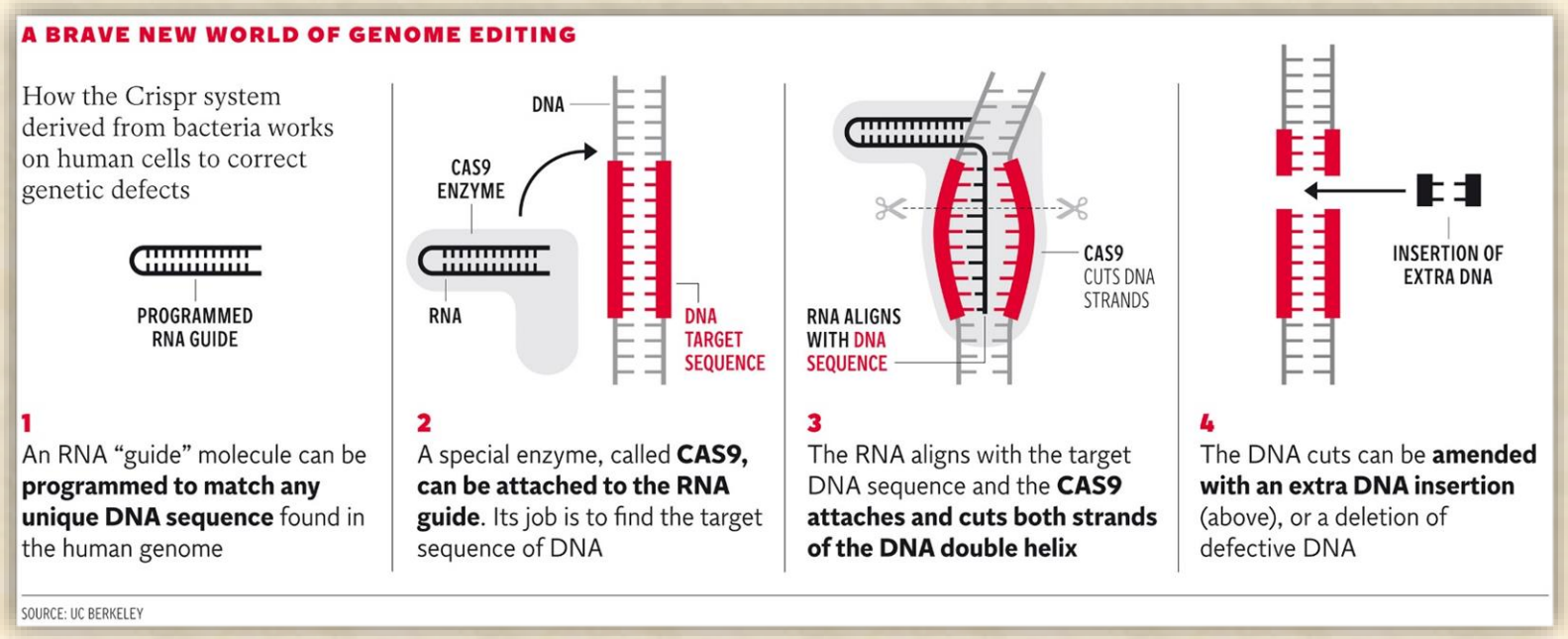
gRNA (guide RNA, 20-nt), based on the TARGET GENE, directs where Cas9 goes and cuts the DNA at the specific location

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

BIOTECHNOLOGY

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-

STUART MCCALL/GETTY

The common white button mushroom (*Agaricus bisporus*) has been modified to resist browning.

Source: Nature magazine

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.

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DuPont Pioneer's high amylopectin corn is the first CRISPR-edited plant likely to bypass USDA oversight.

Johnston, Iowa–based DuPont Pioneer engineered the waxy corn to contain starch composed exclusively of the branched polysaccharide amylopectin—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 Gutterson, 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

Source: Nature Biotechnology

Summary of Genome Edited Horticultural Plants （a partial list）

Plant	Target genes	Traits	Delivery methods	Reference
Solanum lycopersicum	*SlAGO7*	Leaf development	Agrobacterium	Brooks et al., 2014
西红柿	*SHR*	Root development	Agrobacterium	Ron et al., 2014
	RIN	Fruit ripening	Agrobacterium	Ali et al., 2015
	ANT1	Anthocyanin biosynth	Agrobacterium	Cermak et al., 2015
	SlPDS, SlPIF4	Carotenoid biosynthesis	Agrobacterium	Pan et al., 2016
	SlBOP	Inflorescence	Agrobacterium	Xu et al., 2016
	sp5G, sp	Plant development	Agrobacterium	Soyk et al., 2017
	SlIAA9	Parthenocarpy	Agrobacterium	Ueta et al., 2017
	SlAGL6	Parthenocarpy	Agrobacterium	Klap et al., 2017
	PSY1	Fruit color	Agrobacterium	Hayut et al., 2017
	Mlo	Powdery mildew	Agrobacterium	Nekrasov et al., 2017
	GABA-TP1, GABA-TP2, GABA-TP3, CAT9, and SSADH	g-GABA synthesis	Agrobacterium	Li et al., 2017
Solanum pimpinellifolium	*SP, SP5G, SlCLV3, SlWUS, SlGGP1*	fruit development	Agrobacterium	Li et al., 2018
Solanum tuberosum	*StMYB44*	Phosphate transport	Agrobacterium	Zhou et al., 2017
	StALS1	Herbicide resistance	Agrobacterium	Butler et al., 2016
	GBSS	Starch quality	Agrobacterium	Andersson et al., 2017
	VInv	Reduction of sugar	Agrobacterium	Clasen et al., 2016
	StALS, StGBSS	Plant development	PEG-protoplast	Zong et al., 2018
Brassica oleracea	*BolC.GA4.a*	fruit dehiscence	Agrobacterium	Lawrenson et al., 2015
Lactuca sativa 莴苣	*BIN2*	Plant development	PEG protoplasts	Woo et al., 2015
Cucumis sativus 黄瓜	*eIF4E*	Virus resistance	Agrobacterium	Chandrasekaran et al., 2016
Grape	*VvPDS*	Carotenoid biosynth	Agrobacterium	Nakajima et al., 2017
	VvWRKY52	Botrytis inerea resis	Agrobacterium–	Wang et al., 2017
Citrus	*CsPDS*	Carotenoid biosyn	Agrobacterium	Jia and Wang, 2014
	CsLOB1	canker resistance	Agrobacterium	Peng et al., 2017; Jia et al., 2017
Chrysanthemum	*CpYGFP*	Fluorescence	Agrobacterium	Kishi-Kaboshi et al., 2017
Watermelon	*ClPDS*	Carotenoid biosynth	Agrobacterium	Tian et al., 2017
Salvia miltiorrhiza 丹参	*SmCPS1*	Tanshinone biosyn	Agrobacterium	Li et al., 2017
Fragaria vesca	*TAA1, ARF8*	Plant development	Agrobacterium	Zhou et al., 2018

TheScientist
EXPLORING LIFE, INSPIRING INNOVATION

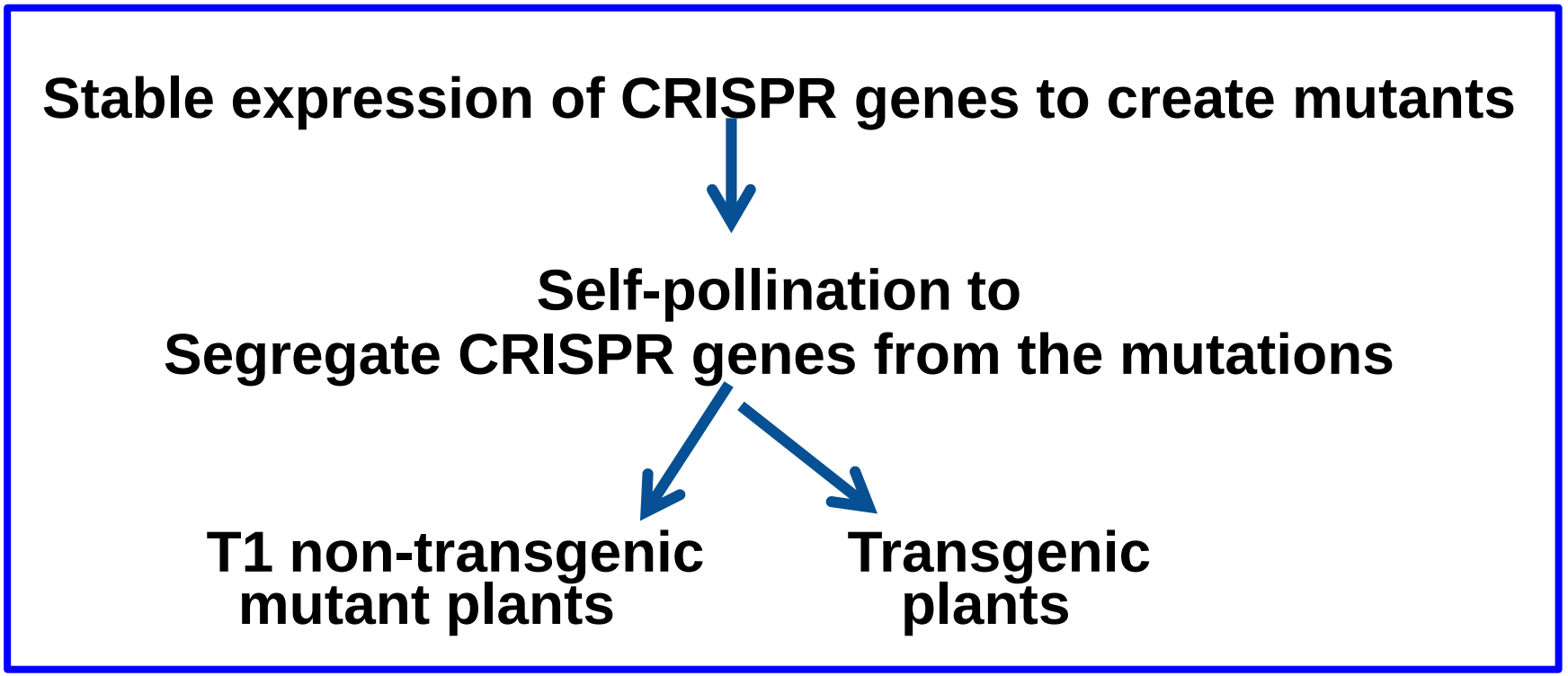
USDA Will Not Regulate CRISPR-Edited Crops

Restrictions will remain on transgenic plants, which contain artificially inserted genes from other species.

Apr 2, 2018
DIANA KWON

Prerequisite: No foreign DNA sequences such as Cas9 gene present in edited plants.
CRISPR relies on genetic transformation, and initially carries the same "baggage".

A widely used method to produce non-transgenic CRISPR-mediated mutant plants



Dr. Yunde Zhao's Lab: Self-driven elimination of transgenes for sexually propagated plants

CellPress
PARTNER JOURNAL

Molecular Plant
Letter to the Editor

Programmed Self-Elimination of the *CRISPR/Cas9* Construct Greatly Accelerates the Isolation of Edited and Transgene-Free Rice 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

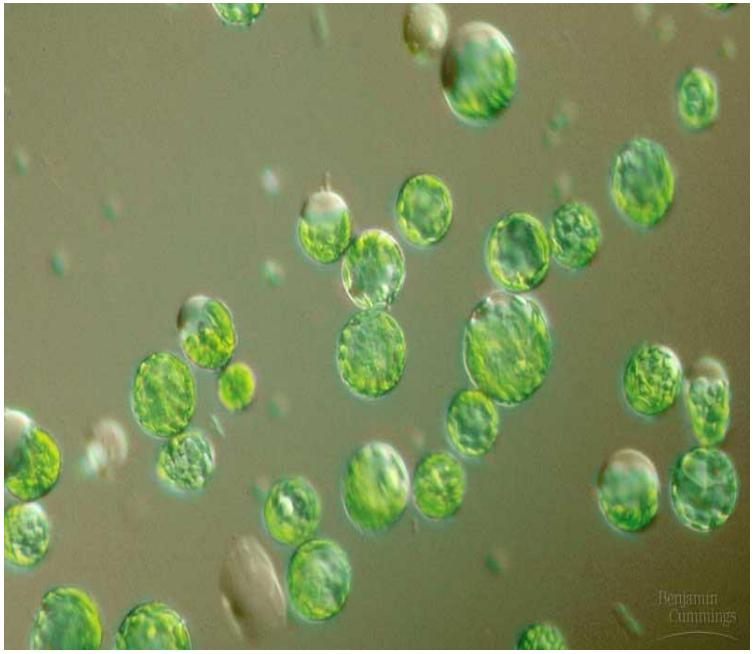
Received 19 May; accepted 23 September; published online 19 October 2015;

nature biotechnology

DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins

Je Wook Woo[1,7], Jungeun Kim[2,3,7], Soon Il Kwon[1], Claudia Corvalán[4], Seung Woo Cho[3,6], Hyeran Kim[2], Sang-Gyu Kim[2], Sang-Tae Kim[2], Sunghwa Choe[1,4,5] & Jin-Soo Kim[2,3]

Editing plant genomes without introducing foreign DNA into cells may alleviate regulatory concerns related to genetically modified plants. We transfected preassembled complexes of purified Cas9 protein and guide RNA into plant protoplasts of *Arabidopsis thaliana*, tobacco, lettuce and rice and achieved targeted mutagenesis in regenerated plants at frequencies of up to 46%. The targeted sites contained germline-transmissible small insertions or deletions that are indistinguishable from naturally occurring genetic variation.



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)

nature COMMUNICATIONS

Received 29 Apr 2016 | Accepted 18 Jul 2016 | Published 25 Aug 2016

DOI: 10.1038/ncomms12617 OPEN

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

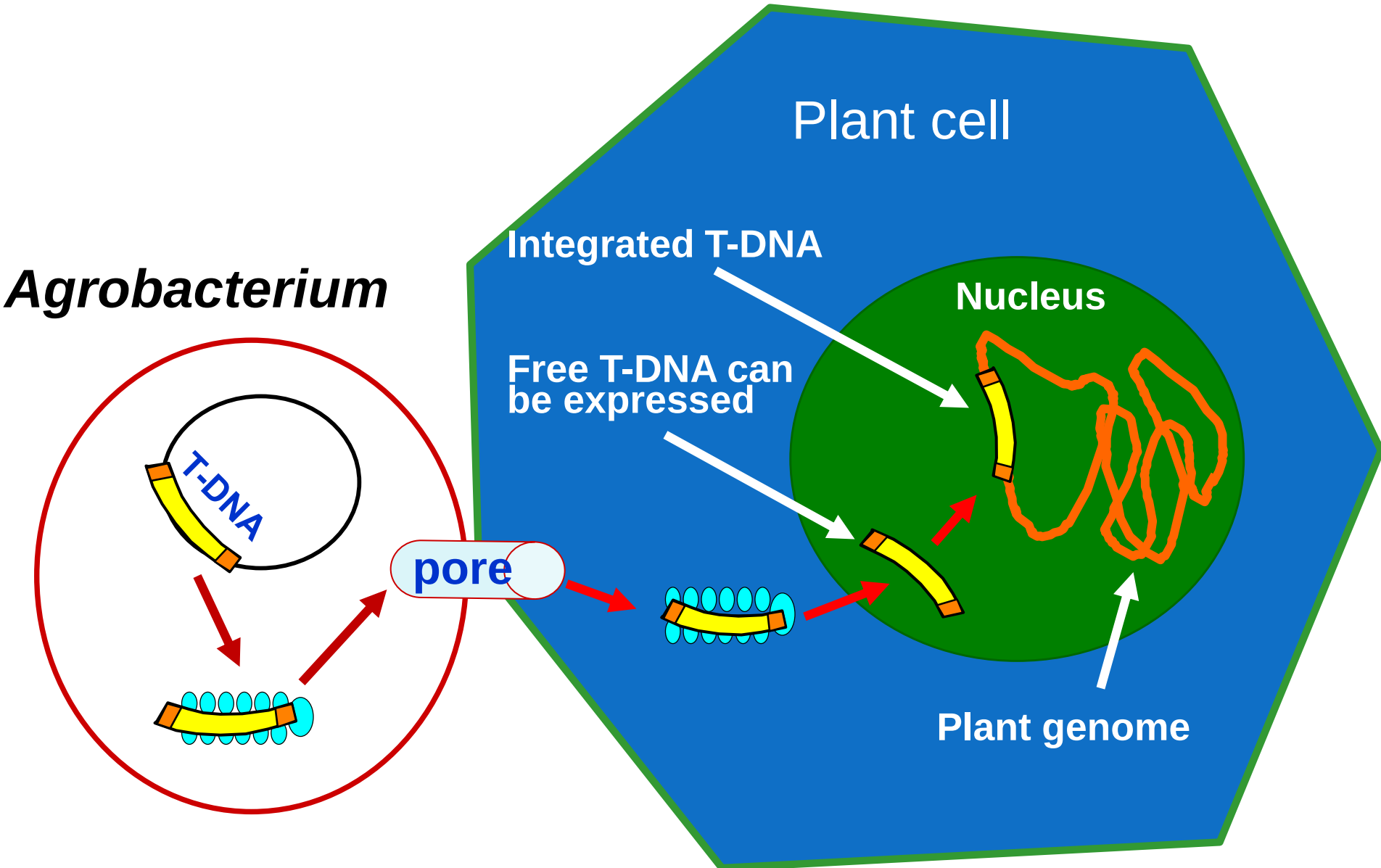
Yi Zhang[1,2,*], Zhen Liang[1,2,*], Yuan Zong[1,2,*], Yanpeng Wang[1,2], Jinxing Liu[1], Kunling Chen[1], Jin-Long Qiu[3] & Caixia Gao[1]

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

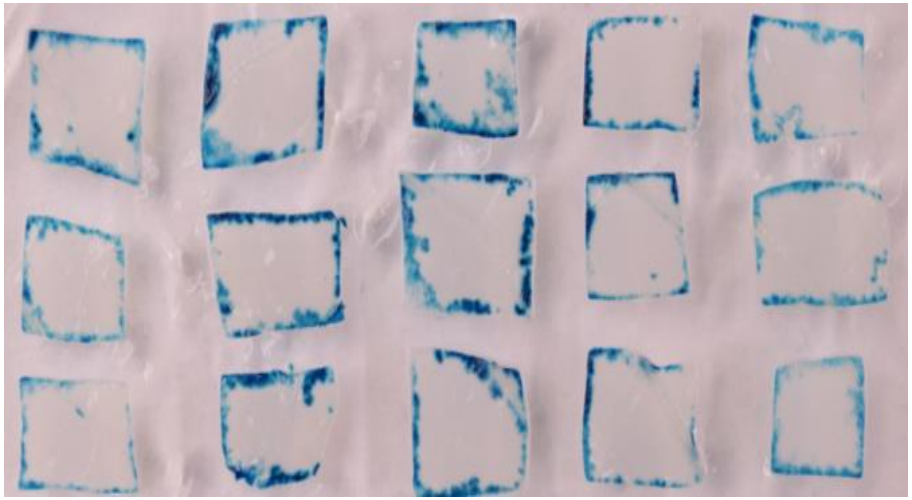
T-DNA from *Agrobacterium can be transiently expressed*



Agrobacterium-mediated Transient Gene Expression (expression with no stable transgene integration)

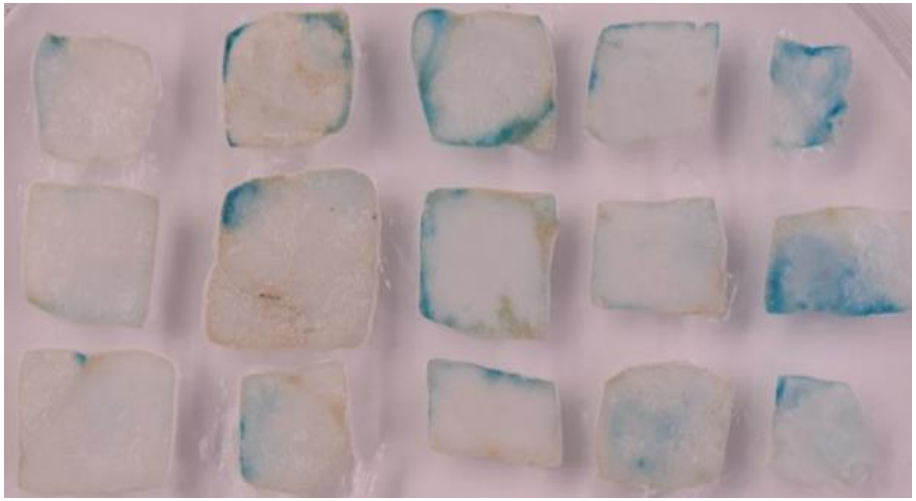
A: Transient +Stable

2d 3d 4d 5d 6d



B: Stable

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



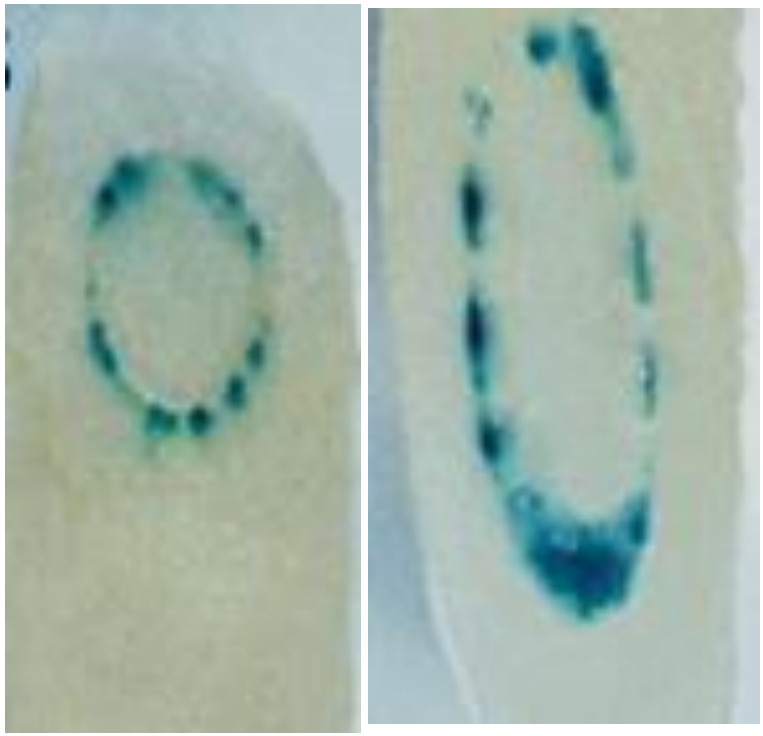
Transient: T-DNA genes are not inserted into the plant genome

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

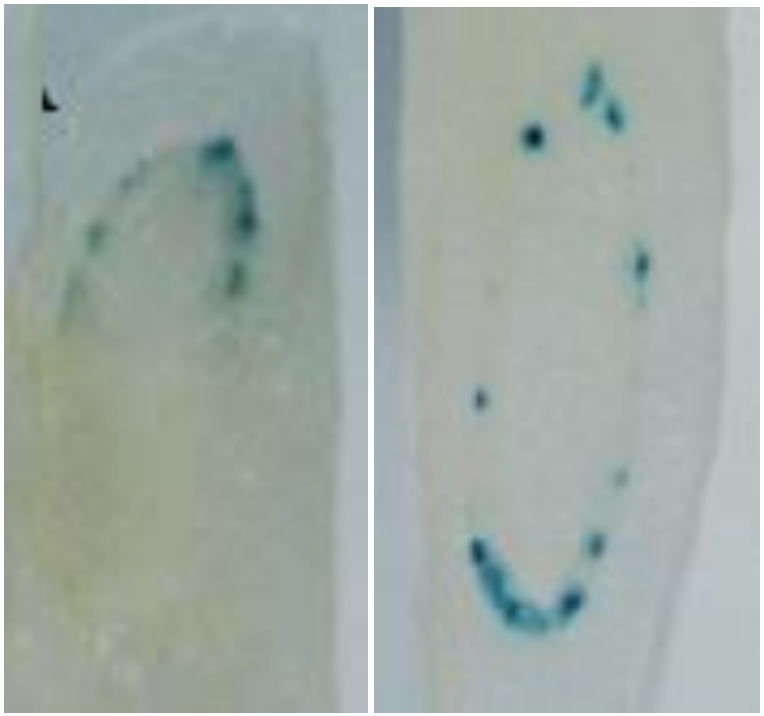
Transient gene expression activity: Difference between A and B

Agrobacterium mediates transient expression of T-DNA genes in citrus (Valencia sweet orange)

Transient & Stable

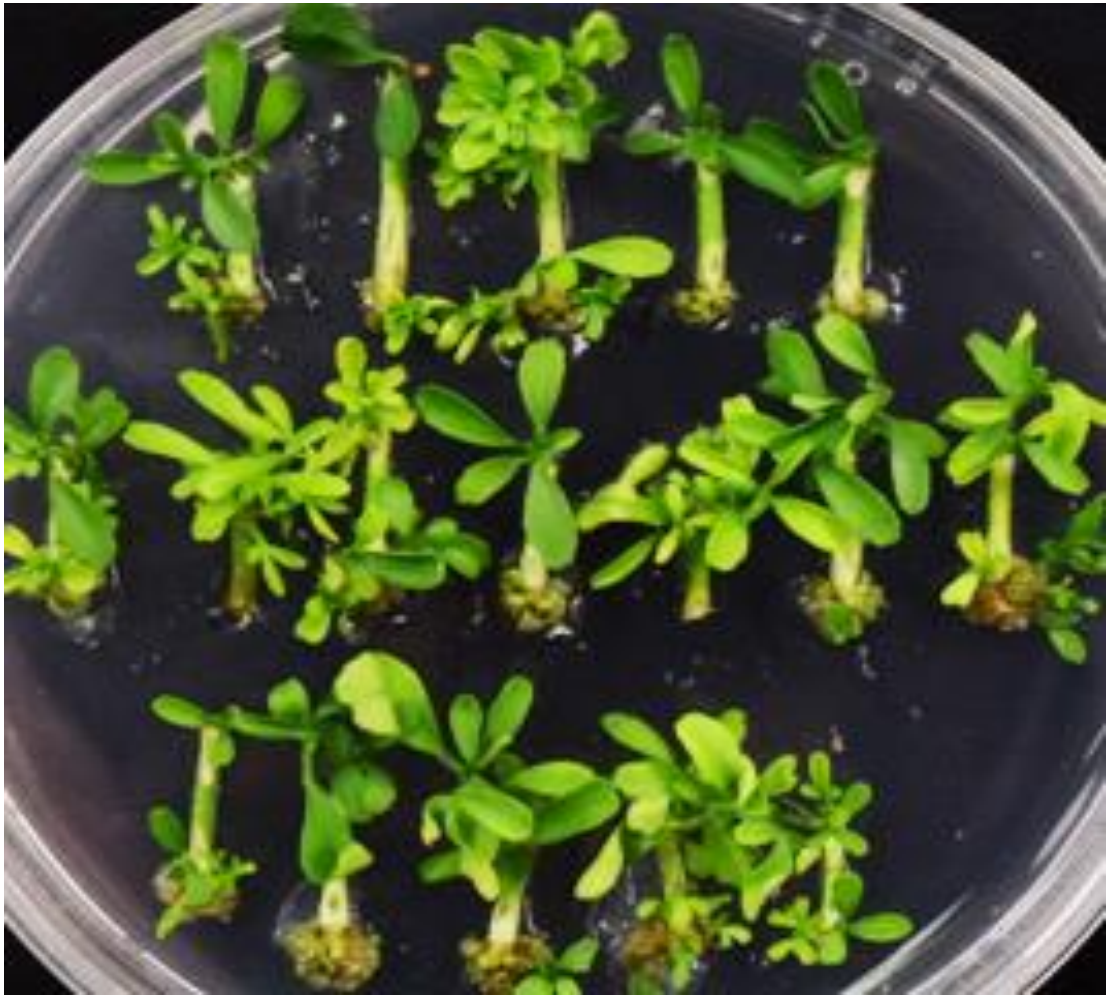


Stable

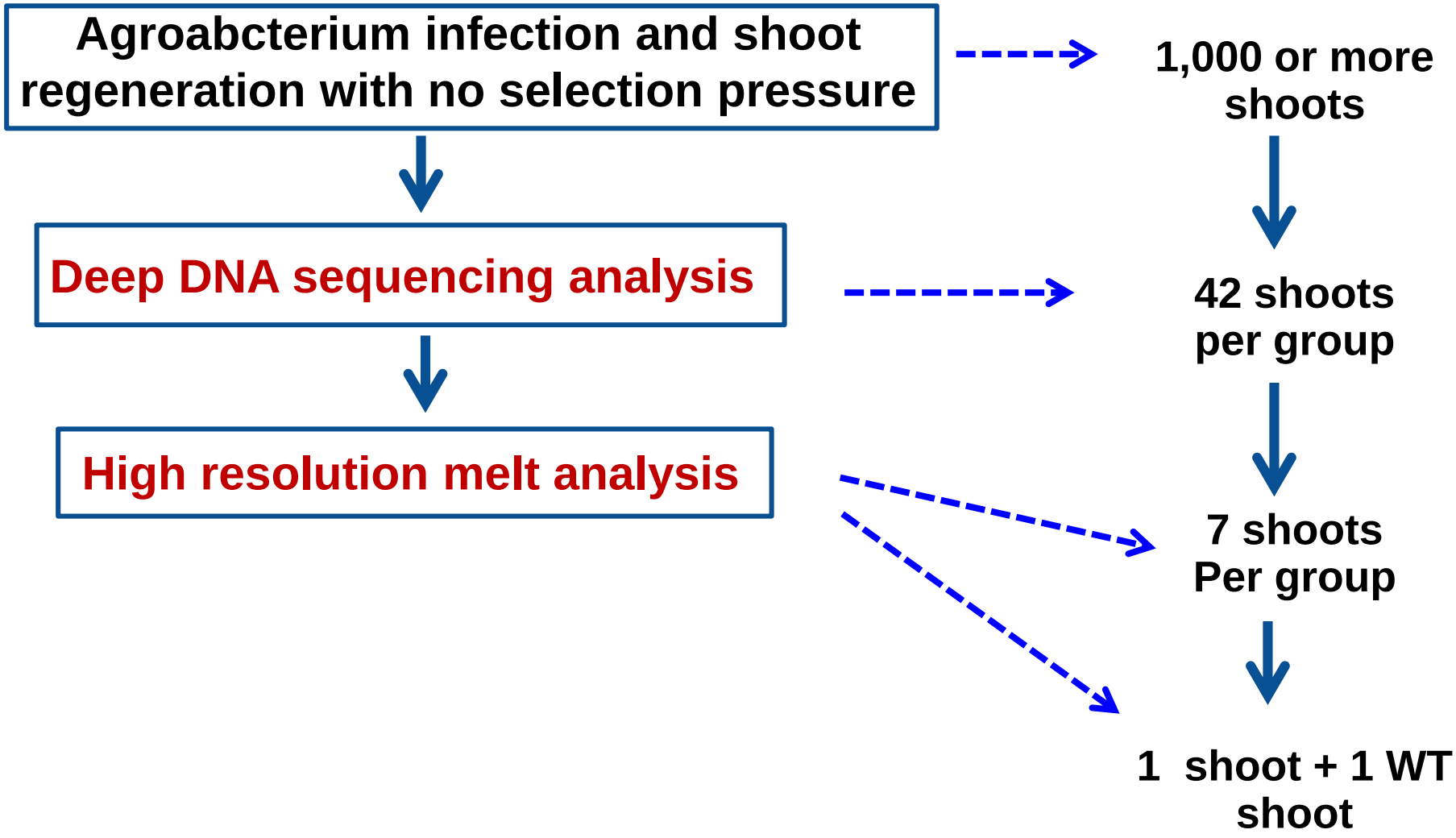


The GUS activities in the left panel are largely due to transient expression of GUS gene in T-DNA.

How to identify mutants if no selection for transformants?



We developed a two-step method to Identify mutants



Horticulture Research

ARTICLE Open Access

A method for the production and expedient screening of CRISPR/Cas9-mediated non-transgenic mutant plants

Longzheng Chen[1,2], Wei Li[1], Lorenzo Katin-Grazzini[1], Jing Ding[3], Xianbin Gu[1], Yanjun Li[1], Tingting Gu[3], Ren Wang[1], Xinchun Lin[1,4], Ziniu Deng[5], Richard J. McAvoy[1], Frederick G. GmitterJr.[6], Zhanao Deng[7], Yunde Zhao[8] and Yi Li[1,3]

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.

CRISPR proven to work in citrus

OPEN ACCESS Freely available online

PLOS ONE

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.

Citation: Jia H, Wang N (2014) Targeted Genome Editing of Sweet Orange Using Cas9/sgRNA. PLoS ONE 9(4): e93806. doi:10.1371/journal.pone.0093806

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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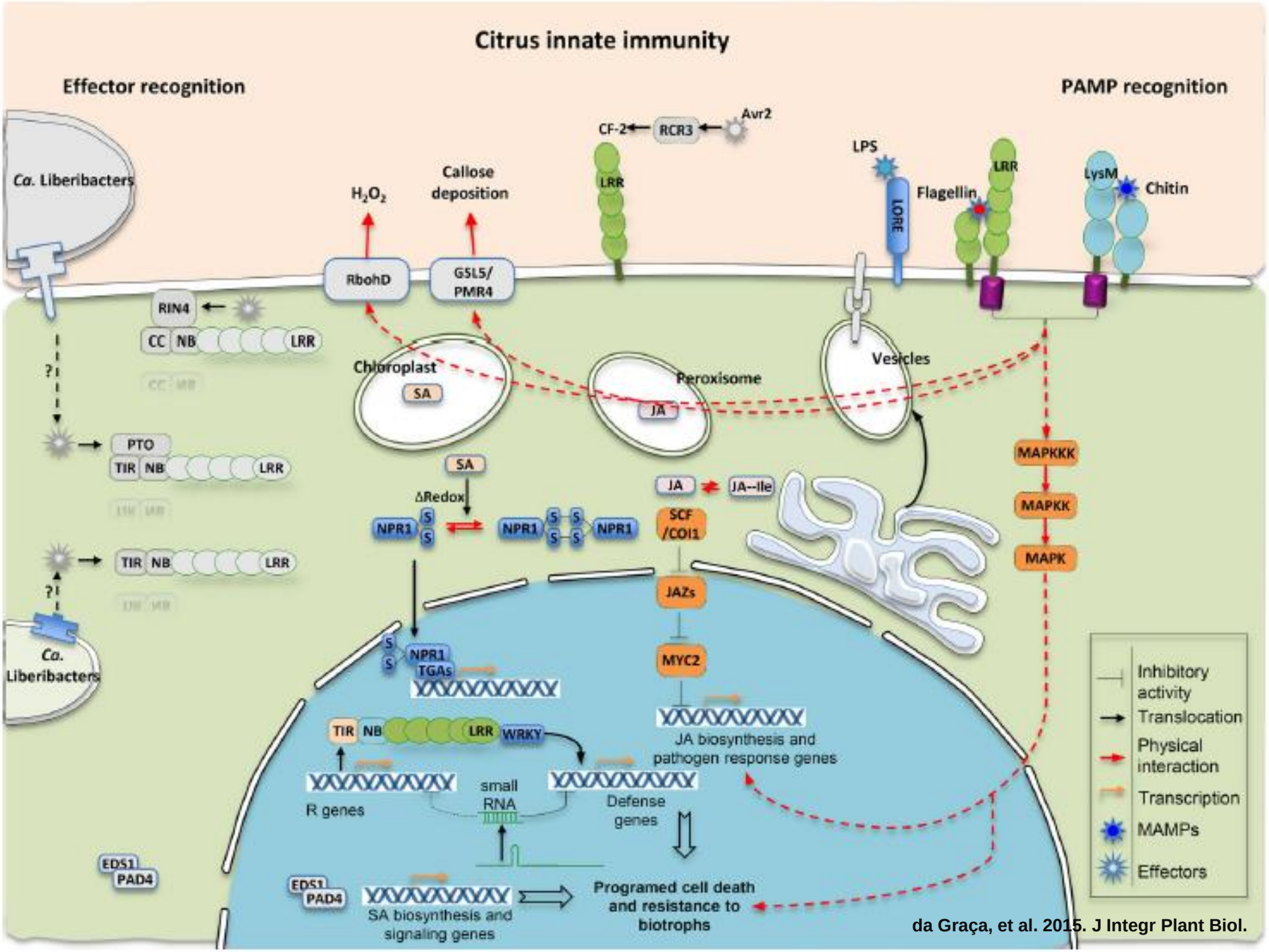

da Graça, et al. 2015. J Integr Plant Biol.

Potential gene targets being investigated

Gene Group	Target Gene
Plant genes for detecting pathogens	FLS2 (Flagellin sensitive 2) Chintin receptors
Plant defense genes	NPR1 (Nonexpresser of PR genes 1) Thionin (anti-bacterial protein) DMR6 (Downy mildew resistant 6) CDR (Constitutive disease resistant)
Clas virulence genes	LAS 5315 (effector protein) SDE (Sec-delivered effector)

And many more …

Challenges and limitations

Transformation efficiency lower than non-CRISPR plasmids

Plant genomes can be more complex (polypoid)

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 !

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

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Univ. of Florida: Fred Gmitter & Zhanao Deng

Univ. of California-San Diego: Yunde Zhao

Funding

NIFA

CRDF

BARENBRUG GreenEarth GRASS SEEDS

Questions?
gene
CRISPR/Cas9
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citrus
sequence
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crops
GMO
vector
immune
human
PAM
protein
spacer
nucleus
cells
repeats
defense
off-target
regulation