RNAi and genome editing for modifying reproductive traits in forest trees

Beijing, China

Steve Strauss

Oregon State University / USA

Why reproductive modification?

- **Goals are diverse**
  - Containment: Regulatory and social acceptance
  - Nuisance tissue reduction: Ornamentals, allergens
  - Improved wood production
- **Why containment?** Regulatory, market, and public acceptance with genetically engineered and exotic trees are difficult in many parts of the world – even for research
  - Long distance gene flow, incomplete domestication, wild or feral relatives
  - Public: Perception of forests as wild

Diverse approaches under study

- **Advantage of RNAi:** No toxic genes like barnase used (which can be unstable and harm vegetative development), degree of suppression can be varied, and can be highly stable
- **Advantage of repressor overexpression**: No flowering at all, trees remain juvenile, most rapid vegetative growth?
- **Advantage of gene editing:** Expected to give strongest loss of function, and be most efficient, predictable, and stable

Specific approaches

- Bisexual sterility: Target is intensely managed, vegetatively propagated elite forest tree varieties (clones), thus targeting master regulators of sexual development
  - No further breeding, or create asexual restorer systems
- Suppress or mutate: Floral organ identity gene *AGAMOUS* and floral meristem identity gene *LEAFY*
- Repressor overexpression: Use of natural floral suppressor or dominant negative form of natural activator

Agenda

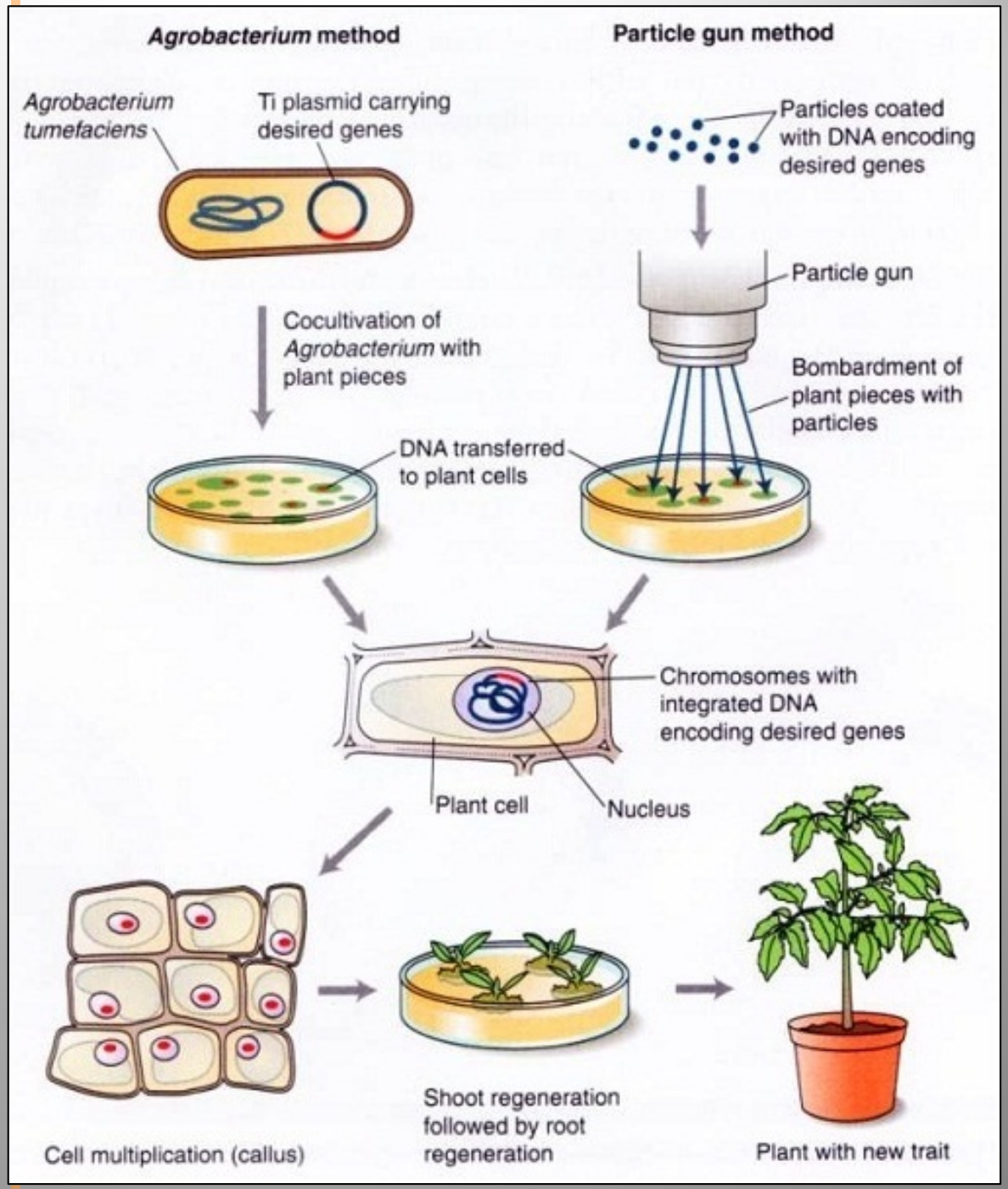
1. Genetic engineering defined (brief)
2. Genome editing explained
3. Gene editing examples in agriculture
4. Our work
   - RNAi
   - Repressors
   - CRISPR-Cas
5. Society, regulation, markets

What is genetic engineering (GE)

- Direct modification of DNA
  - Vs. indirect modification in breeding and genomic selection
- Asexually modified in somatic cells
  - Then regenerated into whole organisms, usually starting in Petri dishes

Steps to create a GE plant

- ***Agrobacterium*-mediated transformation**
- **Biolistics or gene gun**



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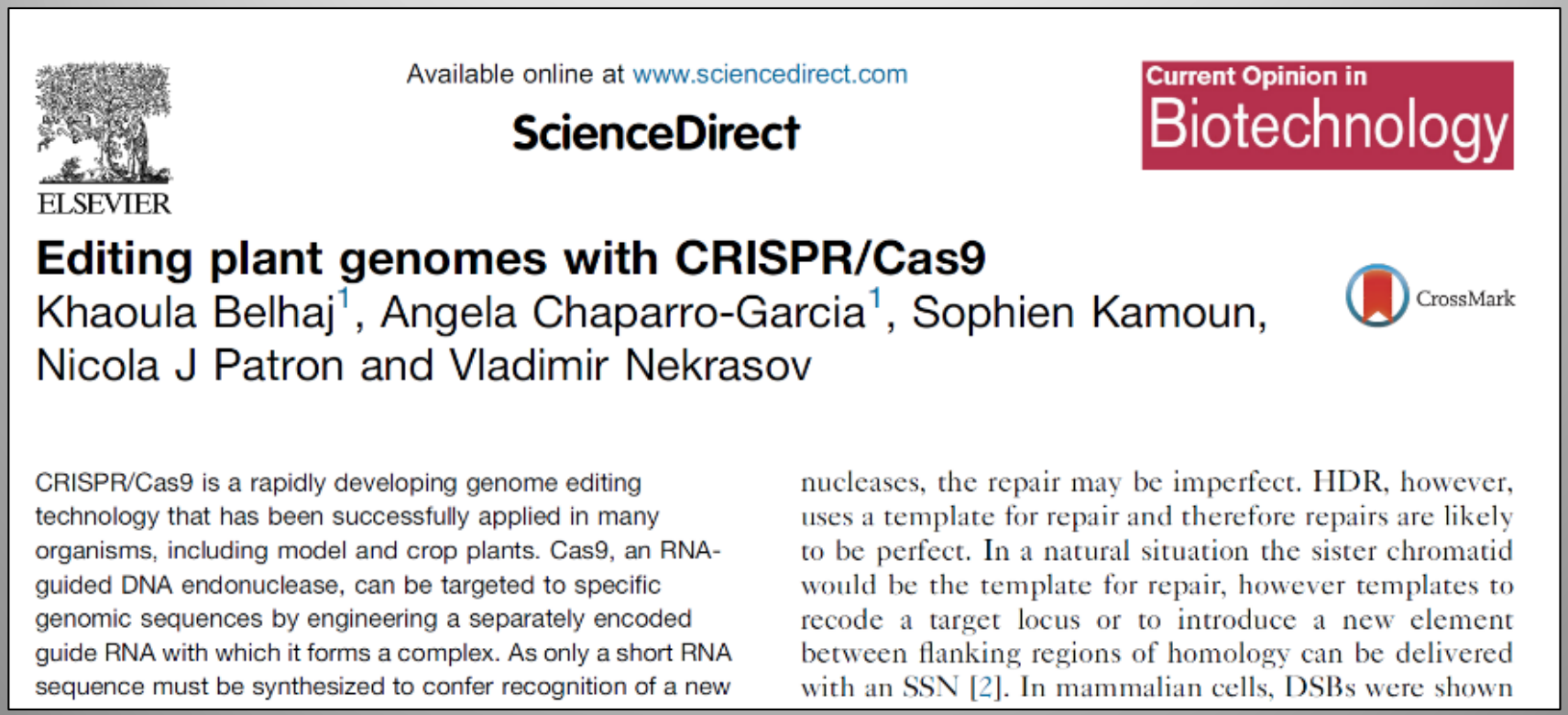
A big deal?

- Ability to modify native genes efficiently
- The theoretical becomes practical



Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

ELSEVIER

Editing plant genomes with CRISPR/Cas9

Khaoula Belhaj[1], Angela Chaparro-Garcia[1], Sophien Kamoun, Nicola J Patron and Vladimir Nekrasov

CrossMark

CRISPR/Cas9 is a rapidly developing genome editing technology that has been successfully applied in many organisms, including model and crop plants. Cas9, an RNA-guided DNA endonuclease, can be targeted to specific genomic sequences by engineering a separately encoded guide RNA with which it forms a complex. As only a short RNA sequence must be synthesized to confer recognition of a new

nucleases, the repair may be imperfect. HDR, however, uses a template for repair and therefore repairs are likely to be perfect. In a natural situation the sister chromatid would be the template for repair, however templates to recode a target locus or to introduce a new element between flanking regions of homology can be delivered with an SSN [2]. In mammalian cells, DSBs were shown

"CRISPR/Cas9 is a game-changing technology that is poised to revolutionize basic research and plant breeding."


Breakthrough of the Year, 2015
Science
AAAS


TIME
The Gene Machine
What the CRISPR experiments mean for humanity By Alice Park

Gene editing described

- Technique that allows specific changes to the genome
- Employs methods of genetic engineering but generally does not leave the editing agent in the genome
  - Editing agent enters cell but does not become part of genome
  - Editing agent sexually segregated away (progeny chosen with the edit, but not the editing agent)
  - Or agent somatically excised after editing

CRISPR gene editing system can be used for multiple purposes

- Mutations to destroy gene function
- Directed changes to sequence to change function
  - Proteins, RNAs, regulatory regions
- Gene or chromosome scale rearrangements (inversions, translocations)
- Ability to readily multiplex and mutate numerous genes at once
- Gene insertions directed at specific places
- Very low off-target rate in plants
- Conversion of alleles in successive generations (gene drive) – a useful means for control of serious diseases, pests, invasive exotic species?

Multiplex CRISPR:62 genes targeted

Science AAAS

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REPORT

Genome-wide inactivation of porcine endogenous retroviruses (PERVs)

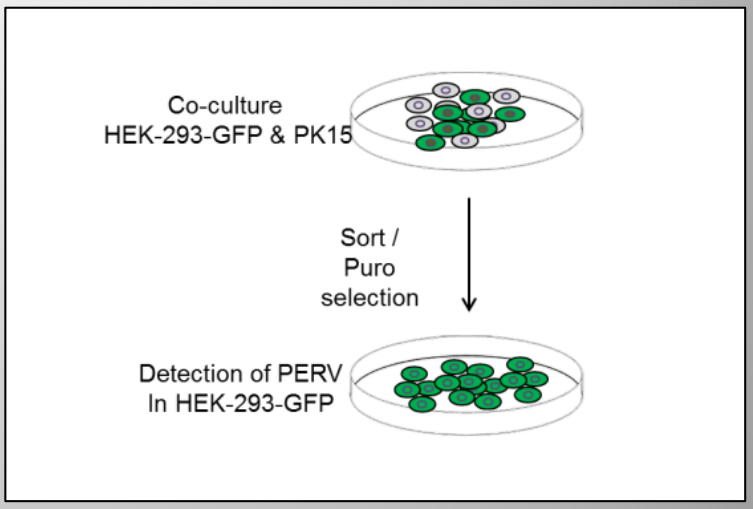
Luhan Yang[1,2,3,*,†], Marc Güell[1,2,3,†], Dong Niu[1,4,†], Haydy George[1,†], Emal Lesha[1], Dennis Grishin[1], John Aach[1], Ellen Shrock[1], Weihong Xu[6], Jürgen Poci[1], Rebeca Cortazio[1], Robert A. Wilkinson[5], Jay A. Fishman[5], George Church[1,2,3,*]

+ Author Affiliations

*Corresponding author. E-mail: gchurch@genetics.med.harvard.edu (G.C.); luhan.yang@egenesisbio.com (L.Y.)

† These authors contributed equally to this work.

Science 27 Nov 2015:
Vol. 350, Issue 6264, pp. 1101-1104
DOI: 10.1126/science.aad1191



Virally cleansing the pig genome

Transplants from pigs could be a solution to a shortage of human organs for transplantation. Unfortunately, porcine endogenous retroviruses (PERVs) are rife in pigs and can be transmitted to humans, risking disease. L. Yang *et al.* integrated CRISPR-Cas into the pig cell genome, where continuous induction of the Cas9 editing enzyme resulted in the mutation of every single PERV reverse transcriptase gene. This prevented replication of all copies of PERV, viral infection, and transmission to human cells.

Science, this issue p. 1101

Polyploid gene editing is effective

Plant Cell Reports
January 2017, Volume 36, Issue 1, pp 117–128

Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts

Authors | Authors and affiliations

Mariette Andersson, Helle Turesson, Alessandro Nicolia, Ann-Sofie Fält, Mathias Samuelsson, Per Hofvander

Open Access | Original Article

First Online: 03 October 2016

DOI: 10.1007/s00299-016-2062-3

Cite this article as:
Andersson, M., Turesson, H., Nicolia, A. et al.
Plant Cell Rep (2017) 36: 117.
doi:10.1007/s00299-016-2062-3

Abstract

Key message

Altered starch quality with full knockout of *GBSS* gene function in potato was achieved using CRISPR-Cas9 technology, through transient transfection and regeneration from isolated protoplasts.

CRISPR/Cas systems are the dominant form of gene editing

- CRISPR stands for Clustered, Regularly Interspaced, Short Palindromic Repeats
- The CRISPR/Cas system is an adaptive defense system in prokaryotes to fight against alien nucleic acids

Defense system in nature

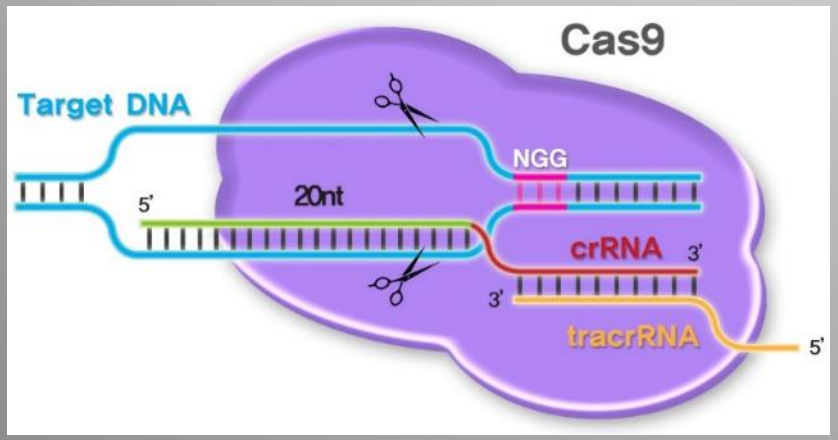
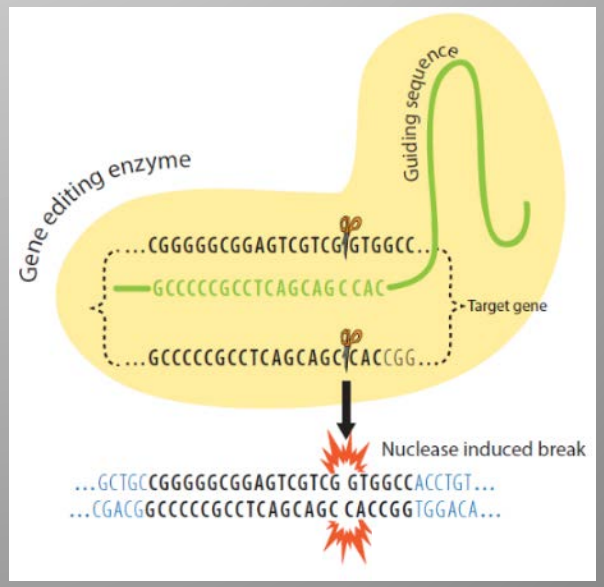


Image credit: http://pnabio.com/products/RGEN.htm

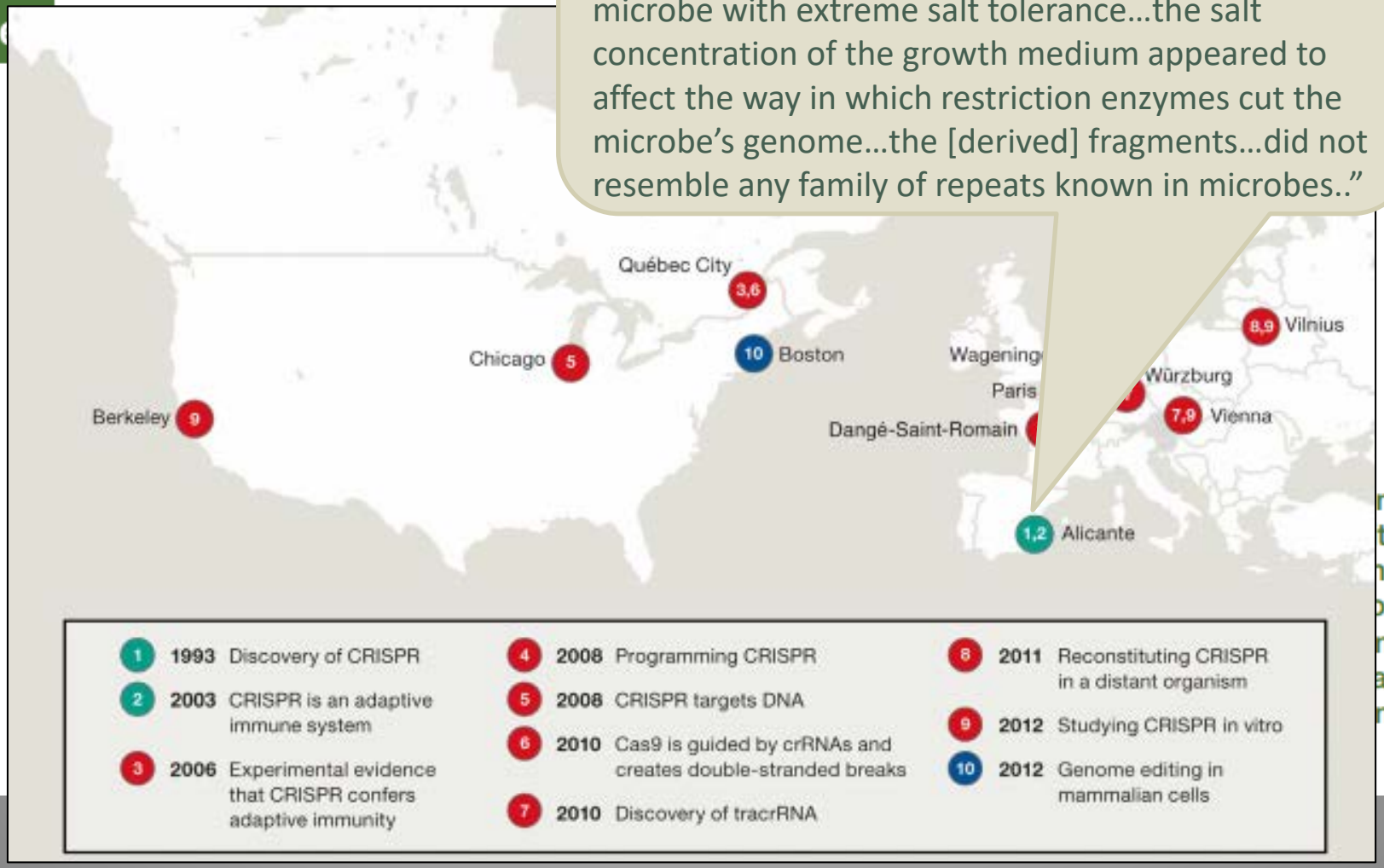
Synthetic nuclease system



A global scientific achievement

20 years of CRISPR

"The story starts in the Mediterranean port of Santa Pola on Spain's Costa Blanca, …in a laboratory working on *Haloferax mediterranei*, an archaeal microbe with extreme salt tolerance…the salt concentration of the growth medium appeared to affect the way in which restriction enzymes cut the microbe's genome…the [derived] fragments…did not resemble any family of repeats known in microbes.."



http://www.cell.com/cell/pdf/S0092-8674(15)01705-5.pdf

Lessons from CRISPR discovery

(Lander 2006, Cell)

- Breakthroughs often emerge from unpredictable origins
  - Motivations included curiosity, and military and industrial applications
- Growing “hypothesis-free” omic discovery
- Best science work early in career – often pre-30
- Seminal work not from eminent research centers
- Leading journals rejected all the early papers
- Science is a slow, global enterprise, with multiple authors and institutions contributing

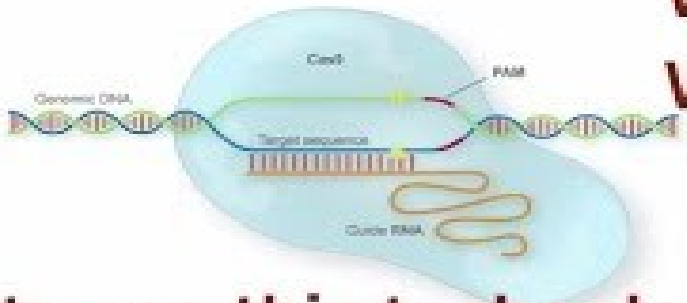
Science journalist Carl Zimmer explains CRISPR DNA editing in 90 seconds

A video with a more technical look at CRISPR

CRISPR | Cas9

What is CRISPR?

What is Cas9?



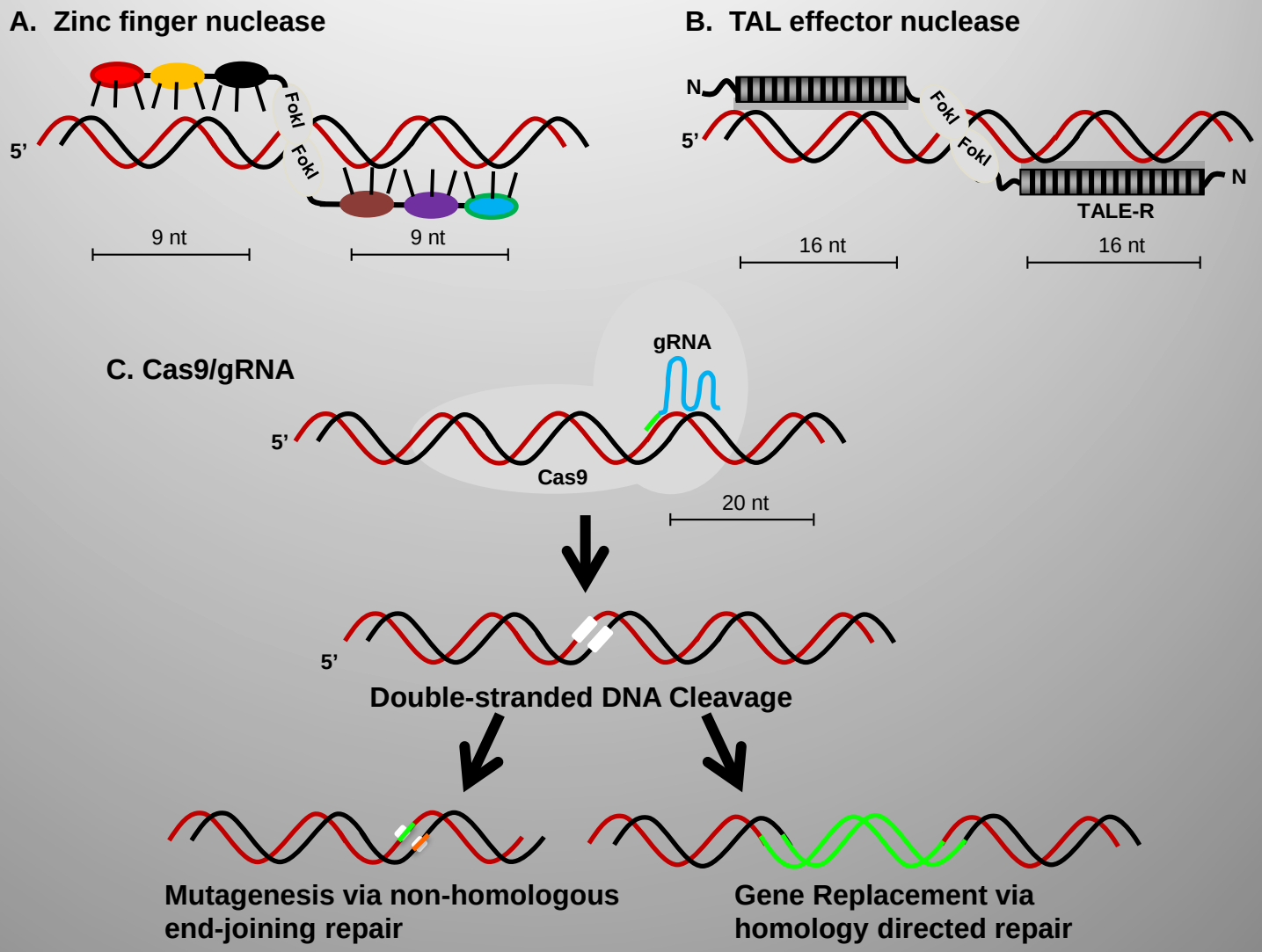
How to use this technology?

Learn from this 4-min Video

ORIGENE
Your Gene Company

Sandman CRISPR !

Genome editing is based on targeted DSBs



Slides courtesy of Bing Wang, Iowa State University

Delivery systems for genome editing

DNA constructs:

Ubi:Cas9:T U6:gRNA:T

Homo. L Homo. R

Template DNA

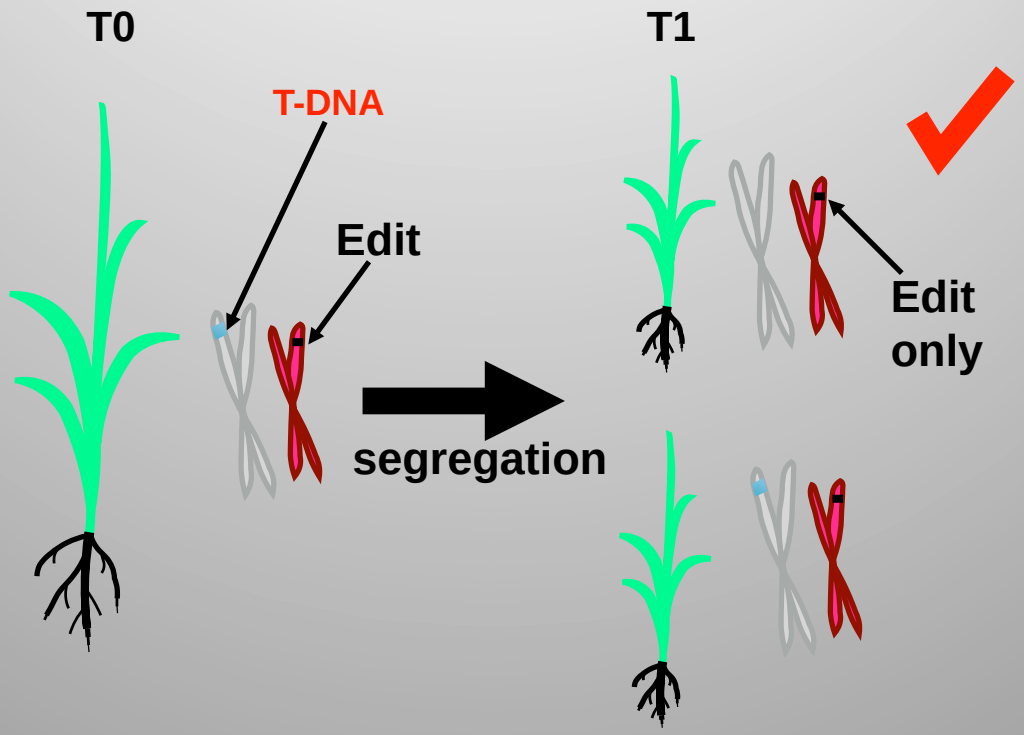
mRNA molecules:

Cas9 mRNA sgRNAs

Ribonucleoproteins (RNP):

Cas9/gRNA (preassembled mixture)

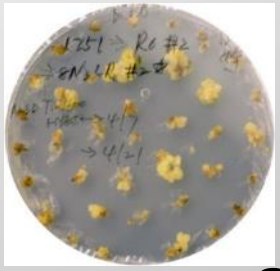
Transgene-free, gene-edited plants can be obtained from a segregating population, if sexual reproduction useful



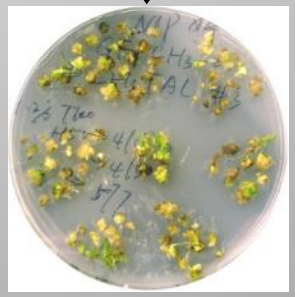
Plant genome editing requires transgenics

Callus initiation

Nuclease
gene transfer



Selection

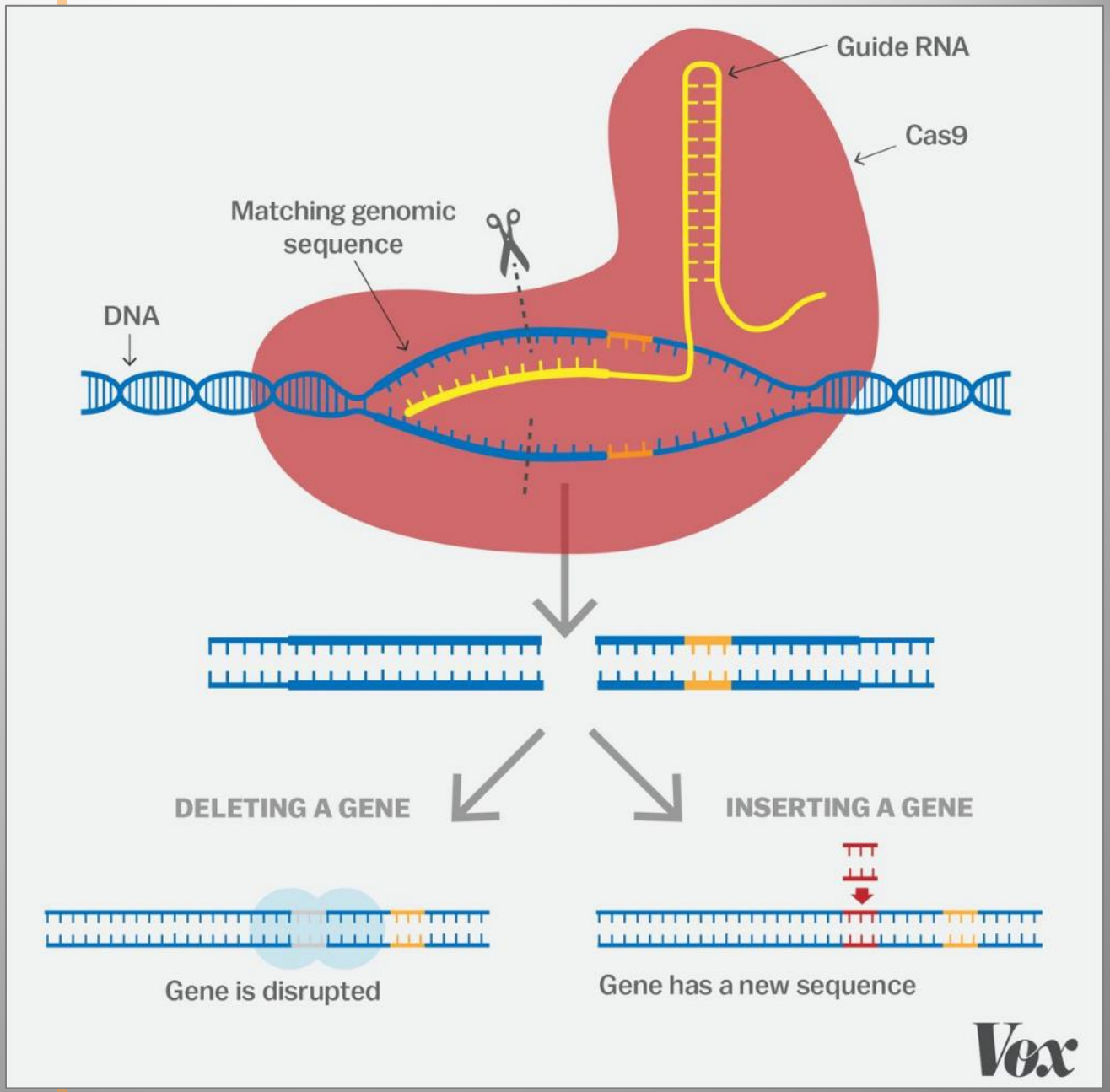


Regeneration

Planting

Summary of CRISPR Cas-mechanism

Two major types of edits



Many new constructs, approaches under development

Plant Cell Advance Publication. Published on May 18, 2017, doi:10.1105/tpc.16.00922

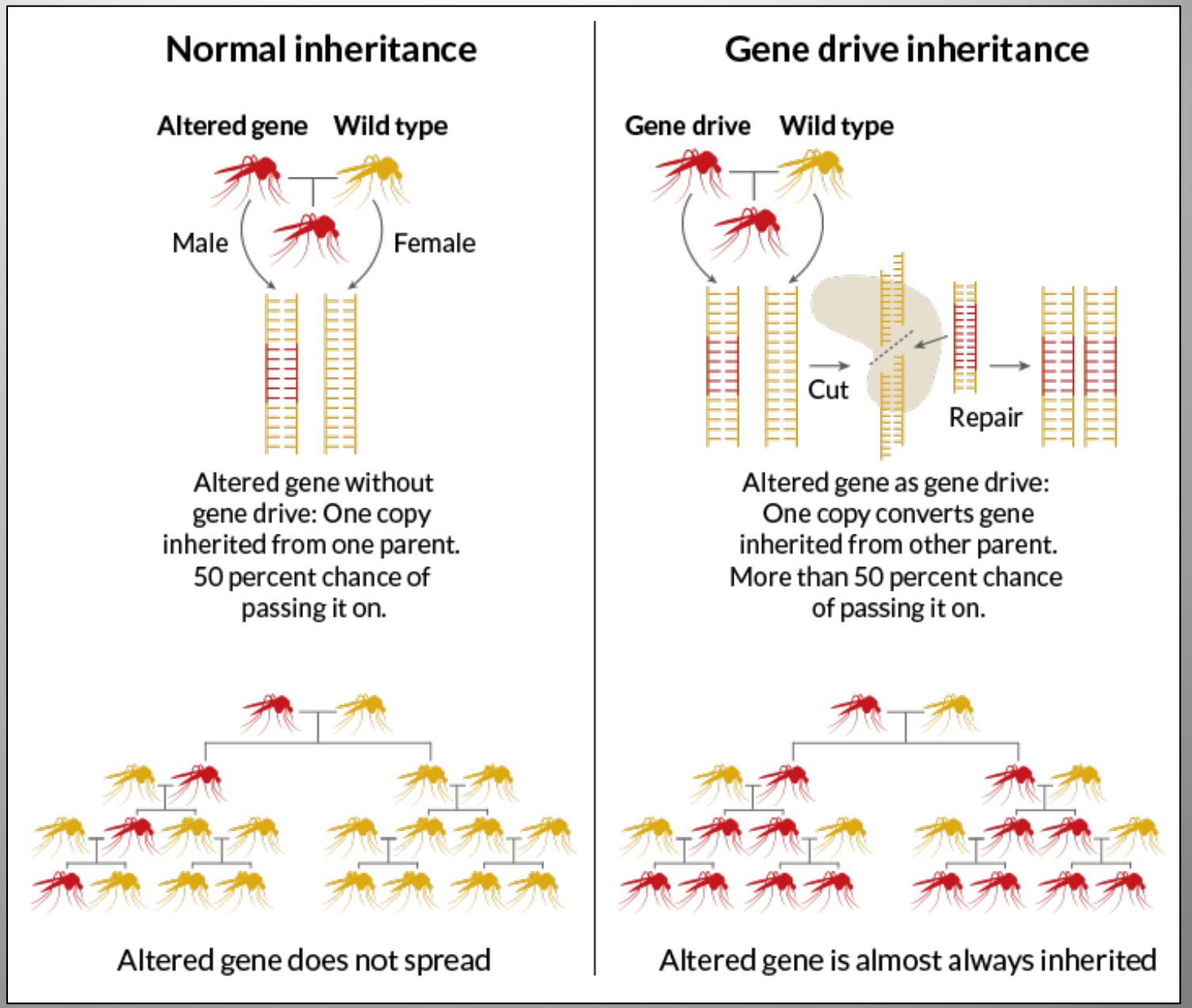
LARGE-SCALE BIOLOGY ARTICLE

A Multi-purpose Toolkit to Enable Advanced Genome Engineering in Plants

Tomáš Čermák[1], Shaun J. Curtin[2,3,4], Javier Gil-Humanes[1,5], Radim Čegan[6], Thomas J. Y. Kono[3], Eva Konečná[1], Joseph J. Belanto[1], Colby G. Starker[1], Jade W. Mathre[1], Rebecca L. Greenstein[1], Daniel F. Voytas[1]

[1]Department of Genetics, Cell Biology & Development and Center for Genome Engineering, University of Minnesota, Minneapolis, MN 55455

Gene drives for suppression of crop pests?



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Major plant species targeted for gene editing with engineered nucleases

Plant	Mega-nuclease	ZFNs	TALENs	Cas9/gRNA
Arabidopsis	√	√	√	√
Canola		√		
Cotton	√			
Potato			√	√
Soy bean		√	√	√
Tobacco		√	√	√
Tomato			√	√
Barley			√	√
Maize	√	√	√	√
Rice			√	√
Sorghum				√
Wheat			√	√

CRISPR non-browning mushroom with mutated polyphenol oxidase (PPO) gene

nature International weekly journal of science

NATURE | NEWS

Gene-edited CRISPR mushroom escapes US regulation

A fungus engineered with the CRISPR–Cas9 technique can be cultivated and sold without further oversight.

Emily Waltz

14 April 2016

Waltz, E. (2016). Nature News *532*.

Non-bruising PPO- and invertase-mutant potatoes produced by gene editing

Crop Biotech Update

A weekly summary of world developments in agri-biotech for developing countries, produced by the Global Knowledge Center on Crop Biotechnology, International Service for the Acquisition of Agri-biotechApplications SEAsiaCenter (ISAAA).

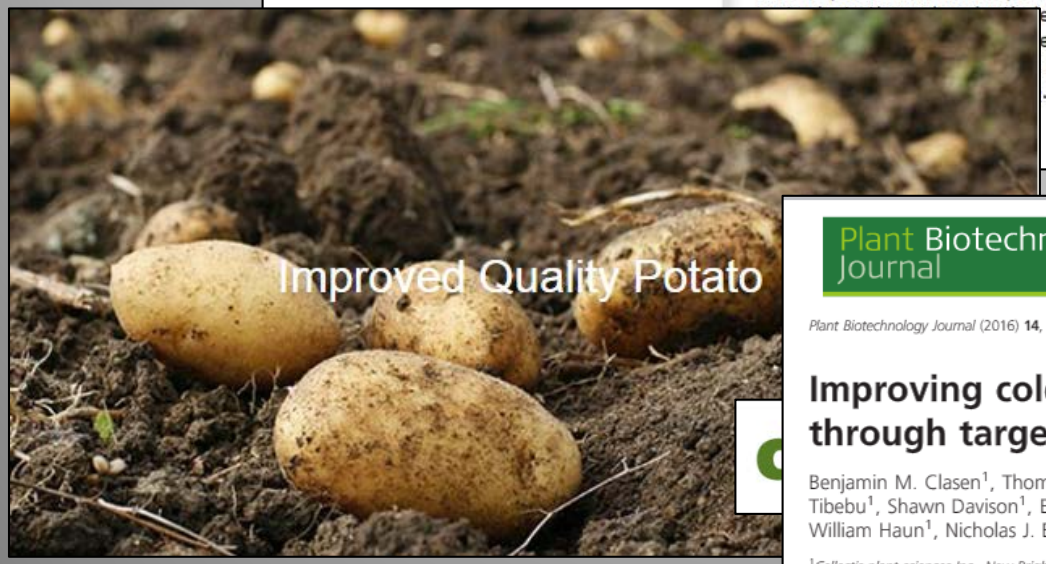
www.isaaa.org/kc/cropbiotechupdate/ www.isaaa.org

Articles in the November 3, 2016 Issue of Crop Biotech Update

NON-BRUISING GE POTATO CLEARED FOR SALE BY USDA

The GE potato that withstands bruising and browning has been cleared for sale by the U.S. Department of Agriculture. According to USDA, the GE potato is not considered as "regulated article" under federal law because it does not contain genes from plant pests.

The GE potato was developed by Calyxt, Inc. by introducing a TALEN reagent into potato ene glycol mediated transformation followed by temporary ent to achieve PPO gene knockout and regeneration of protoplast plants. Thus, there is no foreign genetic material inserted into .



Plant Biotechnology Journal

Plant Biotechnology Journal (2016) **14**, pp. 169–176 doi: 10.1111/pbi.12370

Improving cold storage and processing traits in potato through targeted gene knockout

Benjamin M. Clasen[1], Thomas J. Stoddard[1], Song Luo[1], Zachary L. Demorest[1], Jin Li[1], Frederic Cedrone[2], Redeat Tibebu[1], Shawn Davison[1], Erin E. Ray[1], Aurelie Daulhac[1], Andrew Coffman[1], Ann Yabandith[1], Adam Retterath[1], William Haun[1], Nicholas J. Baltes[1], Luc Mathis[1], Daniel F. Voytas[1] and Feng Zhang[1,*]

[1]*Cellectis plant sciences Inc., New Brighton, MN, USA*
[2]*Cellectis SA, Paris, France*

Pioneer's CRISPR-edited *waxy* corn of high commercial value, unregulated

© Dinodia Photos / Alamy Stock Photo

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

Waltz, E. (2016). Nat Biotech *34*.

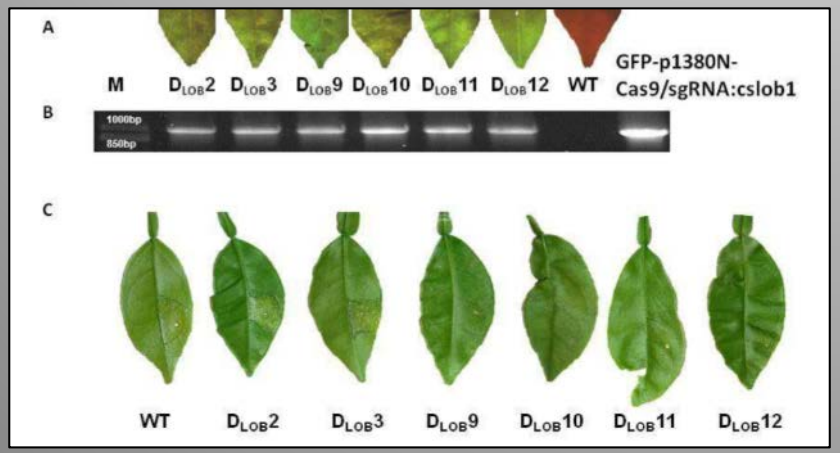
CRISPR- modified grapefruit resistant to citrus canker

Plant Biotechnology Journal

Plant Biotechnology Journal (2016), pp. 1–7 doi: 10.1111/pbi.12677

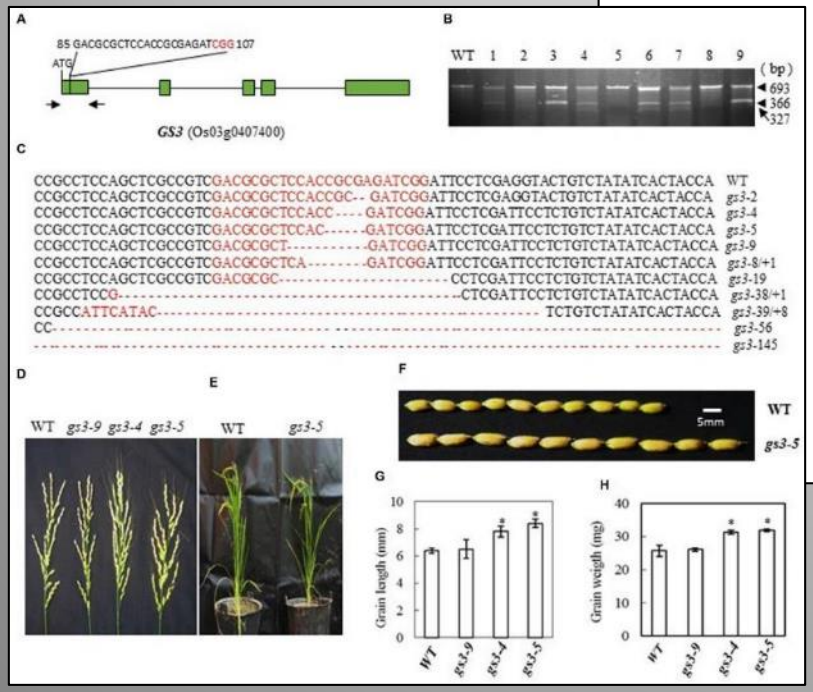
Genome editing of the disease susceptibility gene *CsLOB1* in citrus confers resistance to citrus canker

Hongge Jia[1], Yunzeng Zhang[1], Vladimir Orbović[2], Jin Xu[1], Frank F. White[3], Jeffrey B. Jones[3] and Nian Wang[1,*]



- Other examples:
  - Blight resistant rice (Zhou et al., 2014, Nucl. Acids Res.)
  - Fungus-resistant wheat (Wang et al., 2014, Nat Biotech)
  - Virus-resistant cucumber (Chandrasekaran et al., 2016, Molecular Plant Pathology)

Mutated yield-related genes in wheat leads to larger and more numerous grains



frontiers in Plant Science

ORIGINAL RESEARCH
published: 30 March 2016
doi: 10.3389/fpls.2016.00377

CrossMark

Reassessment of the Four Yield-related Genes *Gn1a*, *DEP1*, *GS3*, and *IPA1* in Rice Using a CRISPR/Cas9 System

Meiru Li[1,2†], Xiaoxia Li[3†], Zejiao Zhou[3], Pingzhi Wu[1,2], Maichun Fang[1,2], Xiaoping Pan[1,2], Qiupeng Lin[3], Wanbin Luo[3], Guojiang Wu[1,2] and Hongqing Li[3*]*

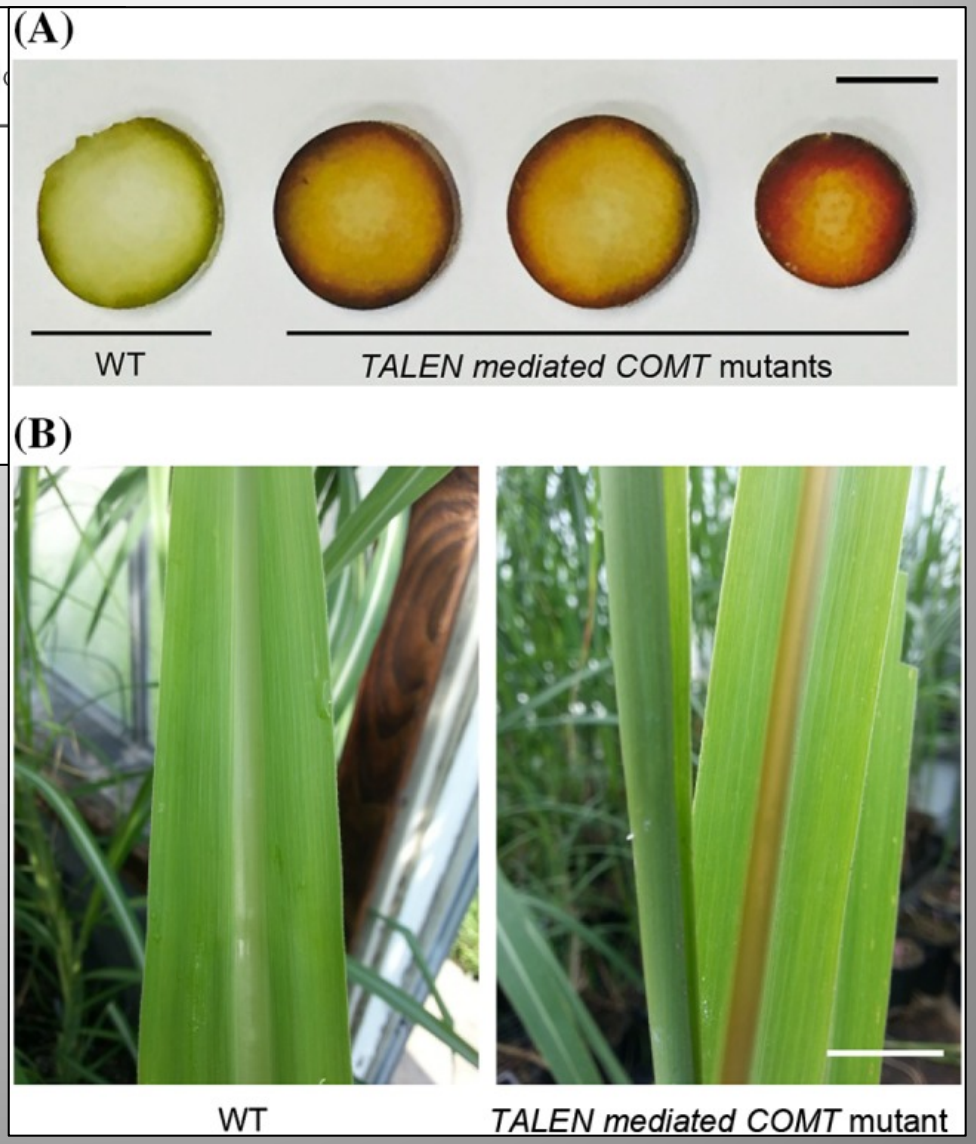
[1] Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, China, [2] Guangdong Provincial Key Laboratory of Applied Botany, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, China, [3] Guangdong Provincial Key Lab of Biotechnology for Plant Development, South China Normal University, Guangzhou, China

Sugarcane, a polyploid, with lower lignin for use as biofuel

Plant Mol Biol (2016) 92:131–142
DOI 10.1007/s11103-016-0499-y

TALEN mediated targeted mutagenesis of the *caffeic acid O-methyltransferase* in highly polyploid sugarcane improves cell wall composition for production of bioethanol

Je Hyeong Jung[1,4] · Fredy Altpeter[1,2,3]



- Brown coloration seen in plants with high lignin reduction (22 to 32% reduction)
- Altpeter, 2016, Plant Molecular Biology

Many other applications…..

- Soybean seeds with improved fatty acid content/profile (Haun et al. 2014, Plant Biotechnol Journal)
- Tobacco with improved glycosylation profiles for safer/faster production of pharmaceutical proteins (Li et al., 2016, Plant Biotechnol Journal 14)

Livestock too…..Recombinetics creates hornless cattle

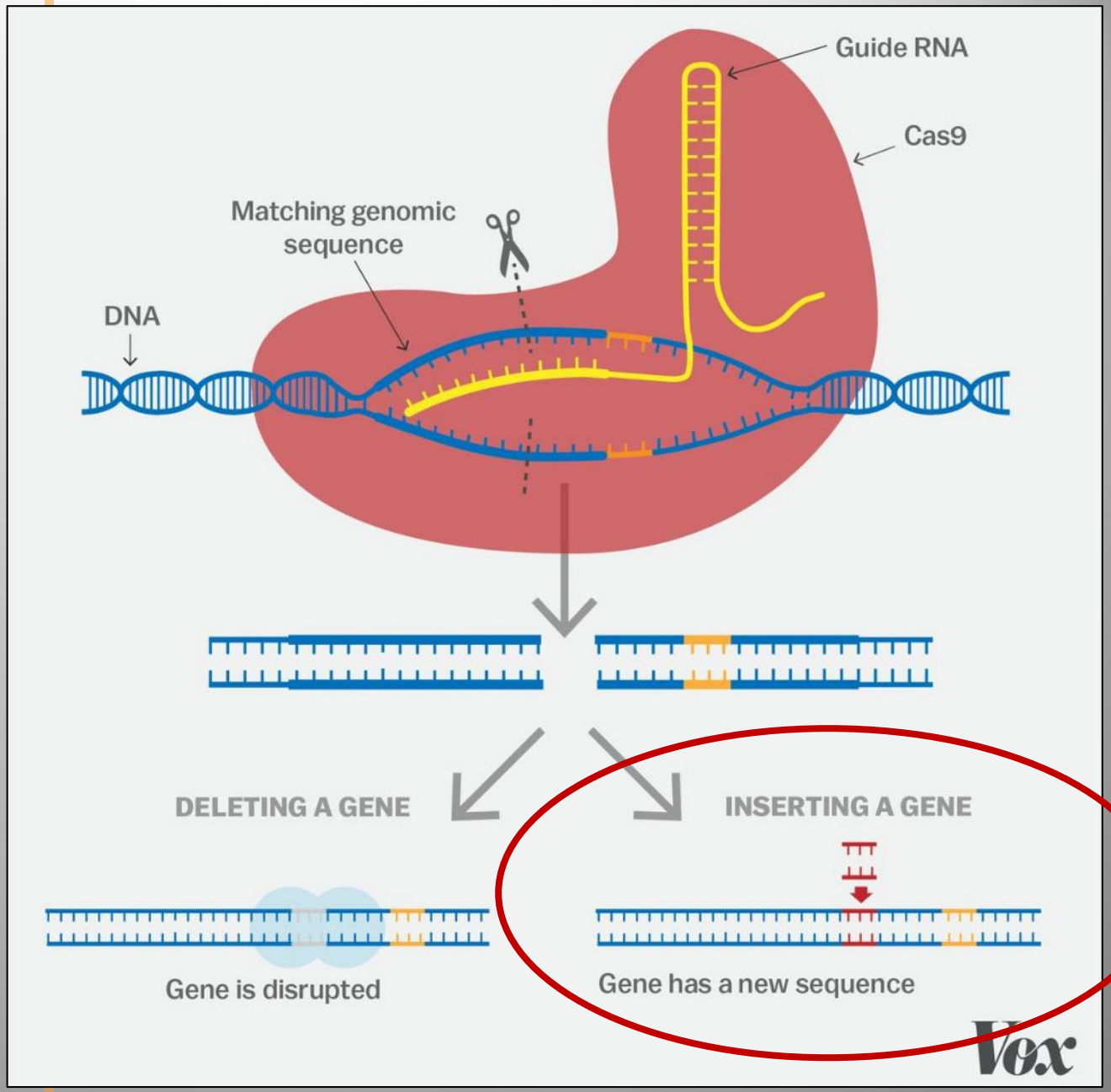
Open Season Is Seen in Gene Editing of Animals

By AMY HARMON NOV. 26, 2015

A calf, left, approximately the same age as the first two genetically modified calves to have their DNA edited so that they do not grow horns, right. Jenn Ackerman for The Nev

The New York Times

Directed gene modification



Homology-directed repair leads to herbicide-resistant crops

- Chlorsulfuron-resistant maize (Svitashev et al., 2015, Plant Physiolgy)
- Chlorsulfuron-resistant potato (Butler et al., 2016, Front. Plant Science)
- Chlorsulfuron-resistant soybean (Li et al., 2015, Plant Physiology)
- Chlorsulfuron-resistant rice (Sun et al., 2016, Molecular Plant)
- Glyphosate-resistant rice (Li et al., 2016, Nature Plants)

Promoter replacement using CRISPR increased grain yield in maize

Plant Biotechnology Journal Open Access aab SEB Society for Experimental Biology

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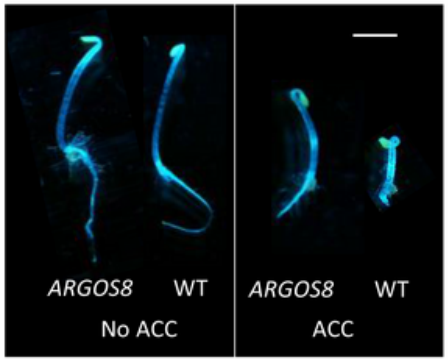
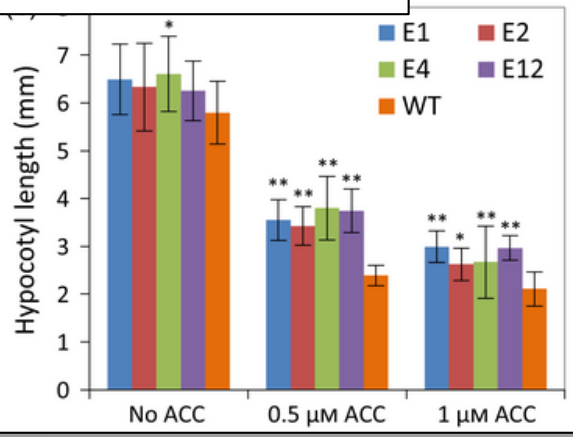
Research Article

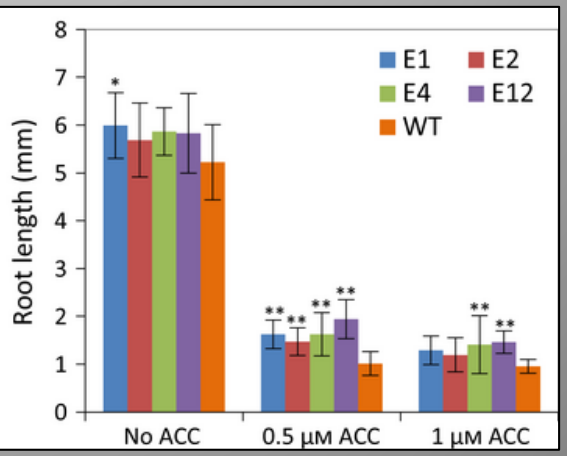
ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions

Jinrui Shi ✉, Huirong Gao, Hongyu Wang, H. Renee Lafitte, Rayeann L. Archibald, Meizhu Yang, Salim M. Hakimi, Hua Mo, Jeffrey E. Habben

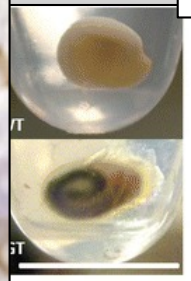
Promoter replacement of the endogenous *ARGOS8* gene leads to increased grain yield during flowering stress and no yield loss during well-watered condition







A strong promoter, modified by gene editing, increased anthocyanin biosynthesis in tomato



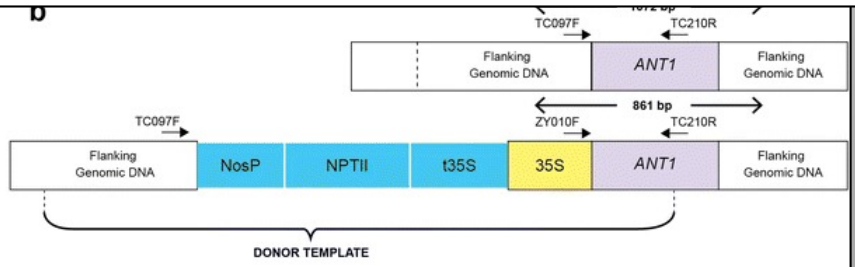
Čermák et al. Genome Biology
DOI 10.1186/s13059-015-0796-9

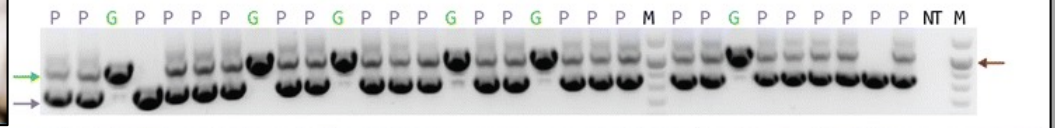
Genome Biology

RESEARCH Open Access

High-frequency, precise modification of the tomato genome

Tomáš Čermák[1], Nicholas J. Baltes[1], Radim Čegan[2], Yong Zhang[3] and Daniel F. Voytas[1*]





Čermák, T. et al., 2015, Genome Biol *16*.

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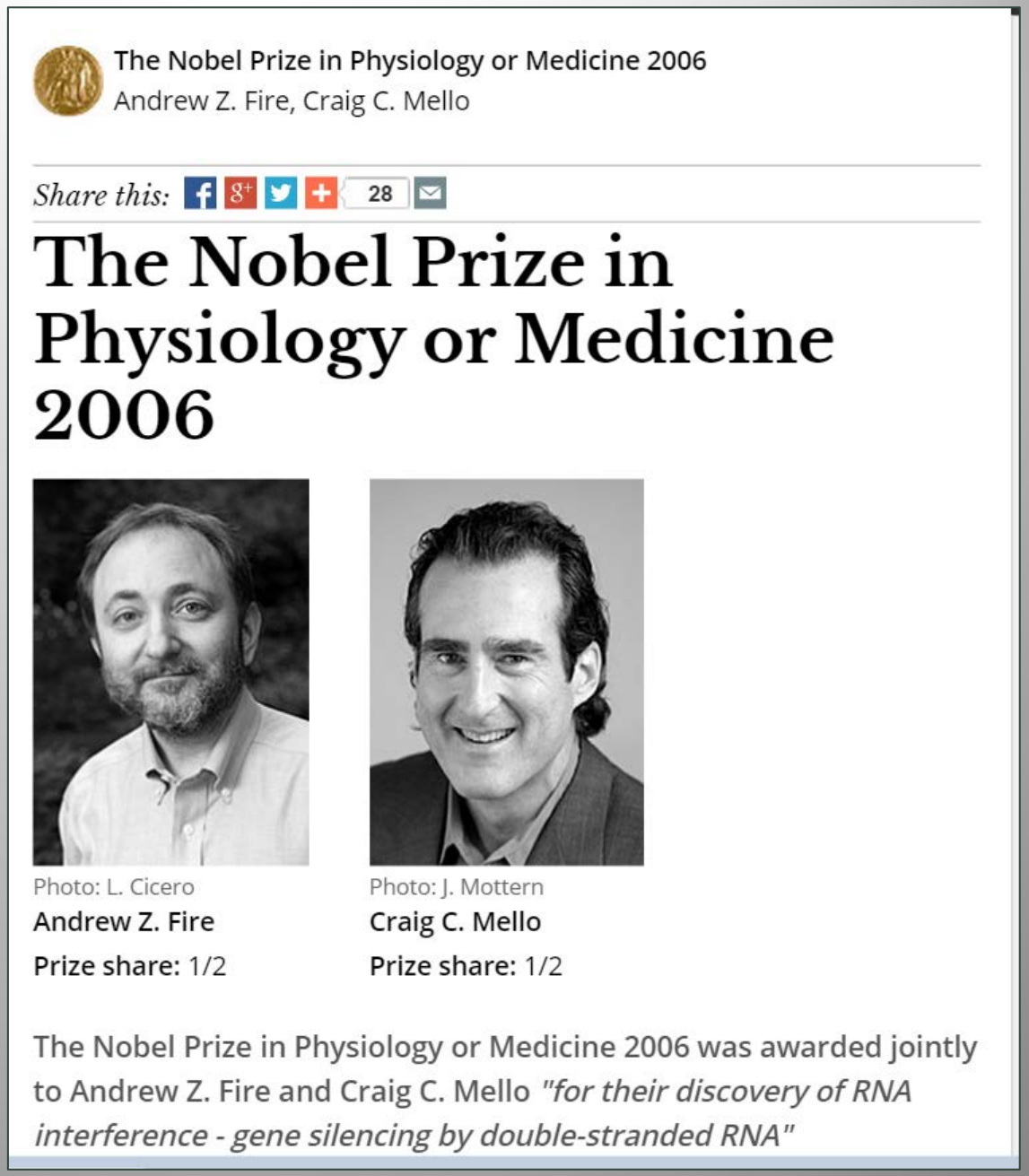
RNA interference (RNAi)

A natural mechanism of gene suppression.

`

Many products on the market. Gene editing products do similar things without the transgene present



The Nobel Prize in Physiology or Medicine 2006
Andrew Z. Fire, Craig C. Mello

Share this: 28

The Nobel Prize in Physiology or Medicine 2006

Photo: L. Cicero
Andrew Z. Fire
Prize share: 1/2

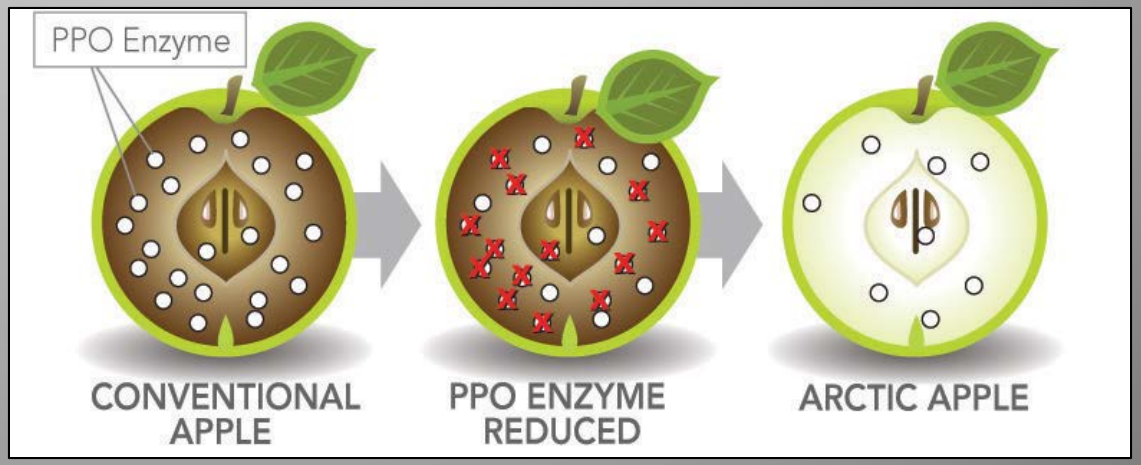
Photo: J. Mottern
Craig C. Mello
Prize share: 1/2

The Nobel Prize in Physiology or Medicine 2006 was awarded jointly to Andrew Z. Fire and Craig C. Mello *"for their discovery of RNA interference - gene silencing by double-stranded RNA"*

Non-browning “Arctic Apple”

RNAi suppression of native polyphenol oxidase gene expression

Courtesy of Jennifer Armen, Okanagan Specialty Fruits, Canada



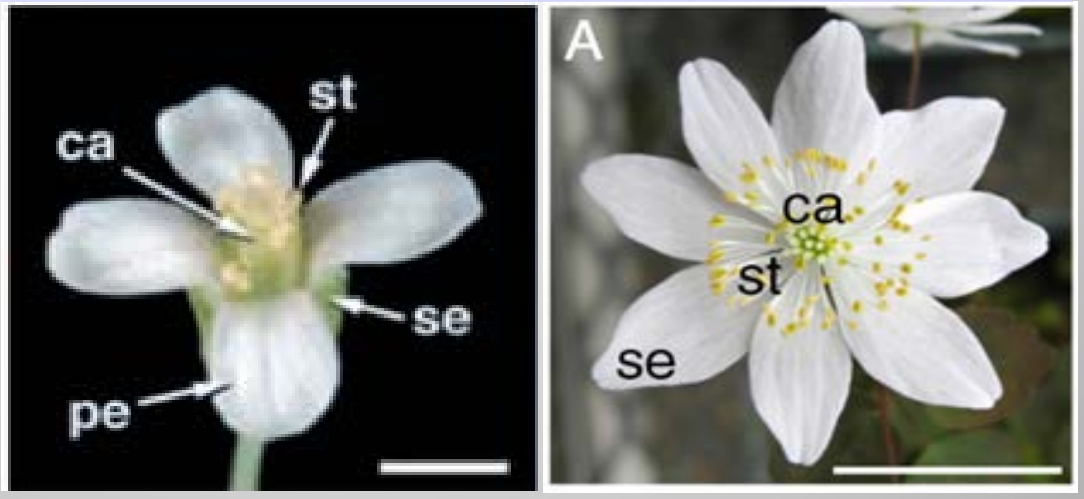
Target genes for bisexual sterility

- *LEAFY* – floral meristem prior to organ differentiation
- *AGAMOUS* – Male and female organ development and floral determinacy

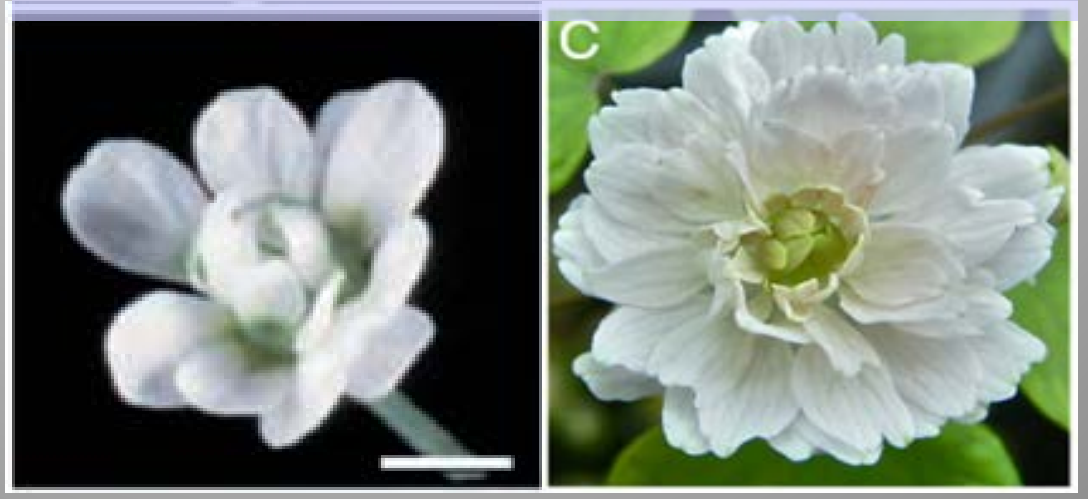
Flowers in strong *ag* mutants lack both stamens and carpels, and are indeter-minate

Arabidopsis Ranunculid

Wild type



ag mutants

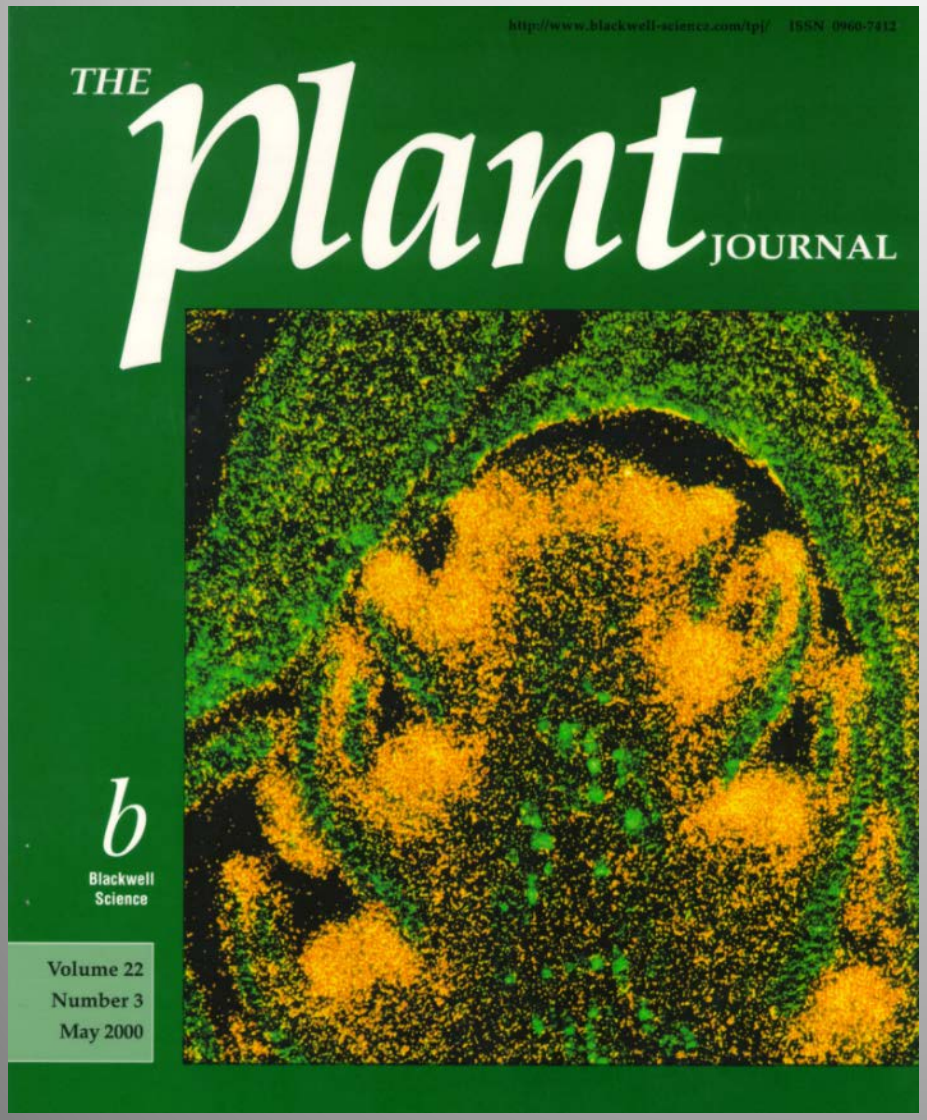


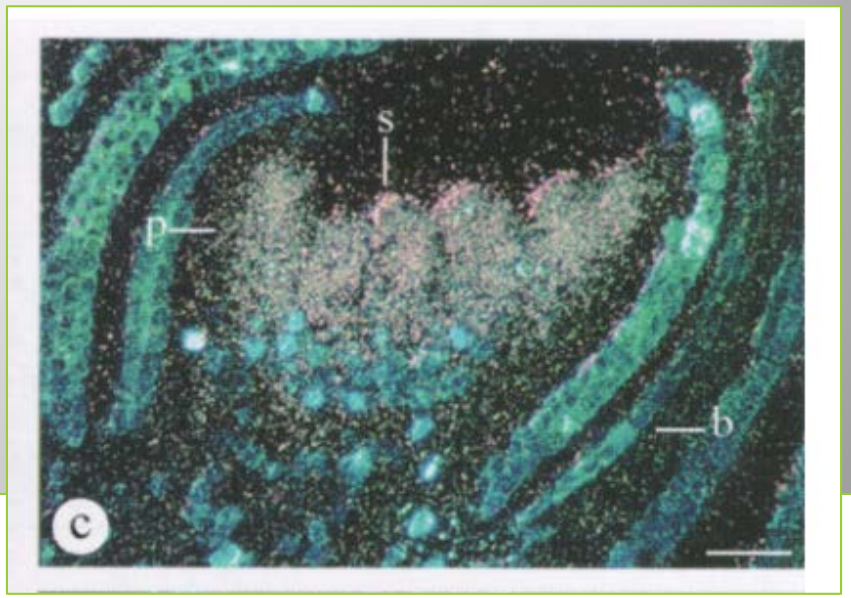
Parcy et al. 2002; Galimba et al. 2012

Strong *lfy* mutants appear to have no flowers

Parcy et al. 2002; Moyroud et al. 2010

LEAFY and *AGAMOUS* homologs in poplar studied in prior work





Plant Molecular Biology 44: 619-634, 2000. © 2000 Kluwer Academic Publishers. Printed in the Netherlands.

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Structure and expression of duplicate AGAMOUS orthologues in poplar

Amy M. Brunner, William H. Rottmann[1], Lorraine A. Sheppard[2], Konstantin Krutovskii, Stephen P. DiFazio, Stefano Leonardi[3] and Steven H. Strauss*

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Field trials of RNAi-poplars

Experimental overview

1

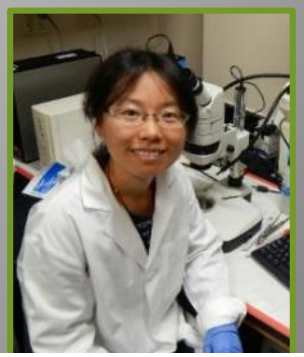
Create RNAi constructs based on the reference sequence from *Populus trichocarpa*

2

Produce transgenic poplars (*P. alba* genotype 6K10, Marizio Sabbati, Univ. Viterbo, Italy)

3

Evaluate phenotypic changes in field (*FT* accelerated flowering impeded RNAi effects)



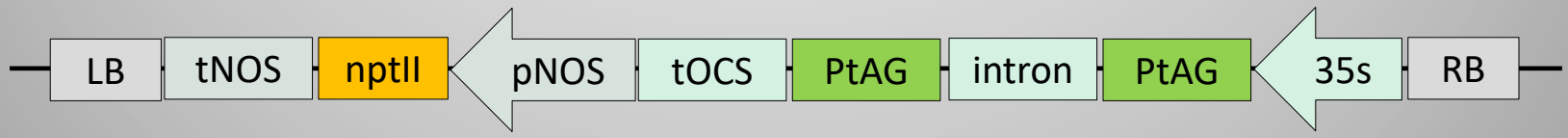
Haiwei Lu, PhD student, OSU

4

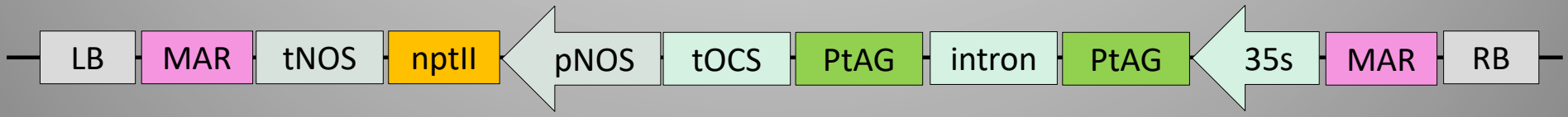
Evaluate gene expression

Two *PtAG*-RNAi constructs, with and without matrix attachment regions (MARs)

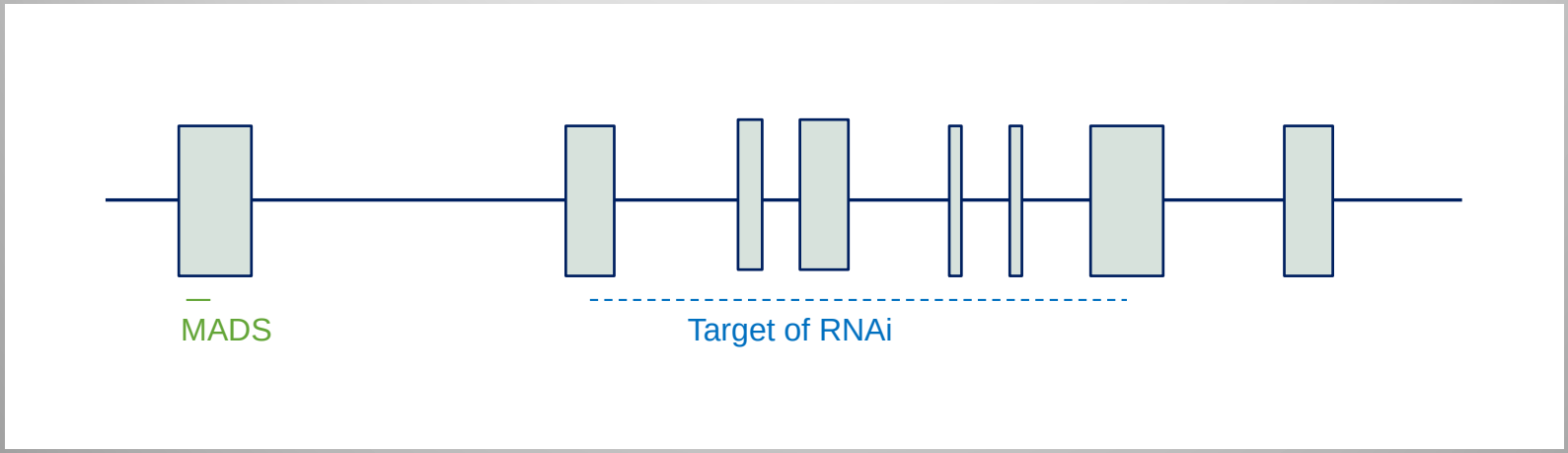
- PTG



- MPG



RNAi constructs contained an inverted repeat that targeted 393 bp of the non-MADS region



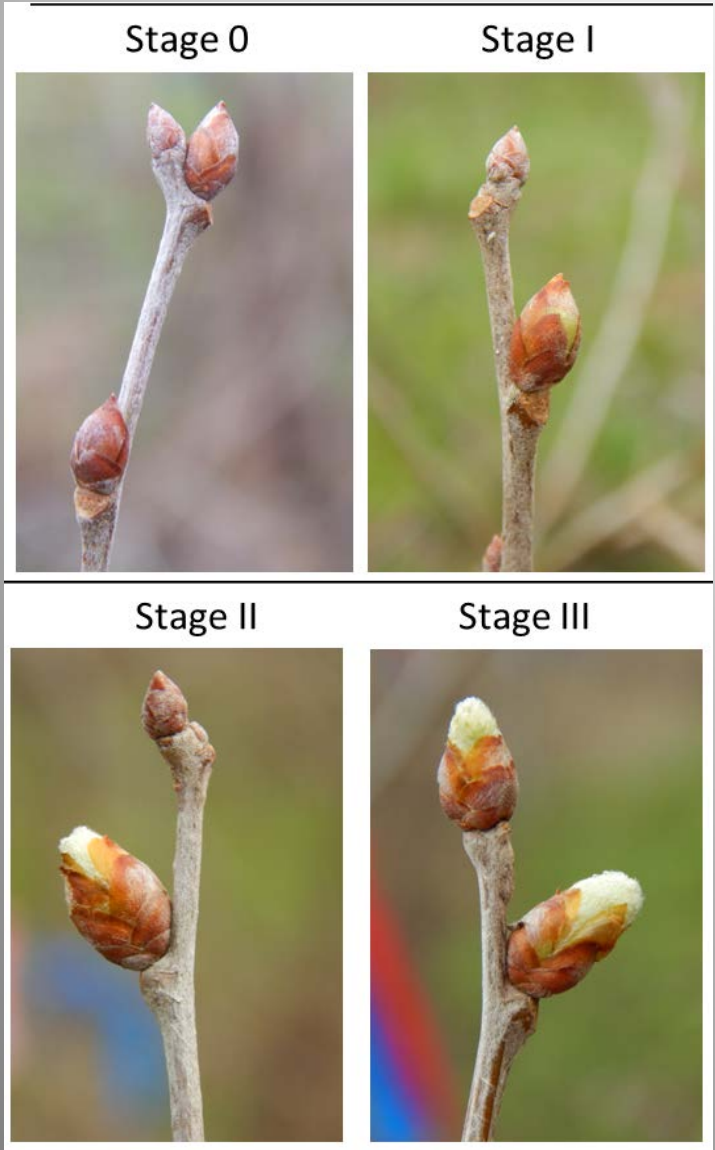
Targeting two paralogous (duplicated) highly similar *PtAG* genes in poplar

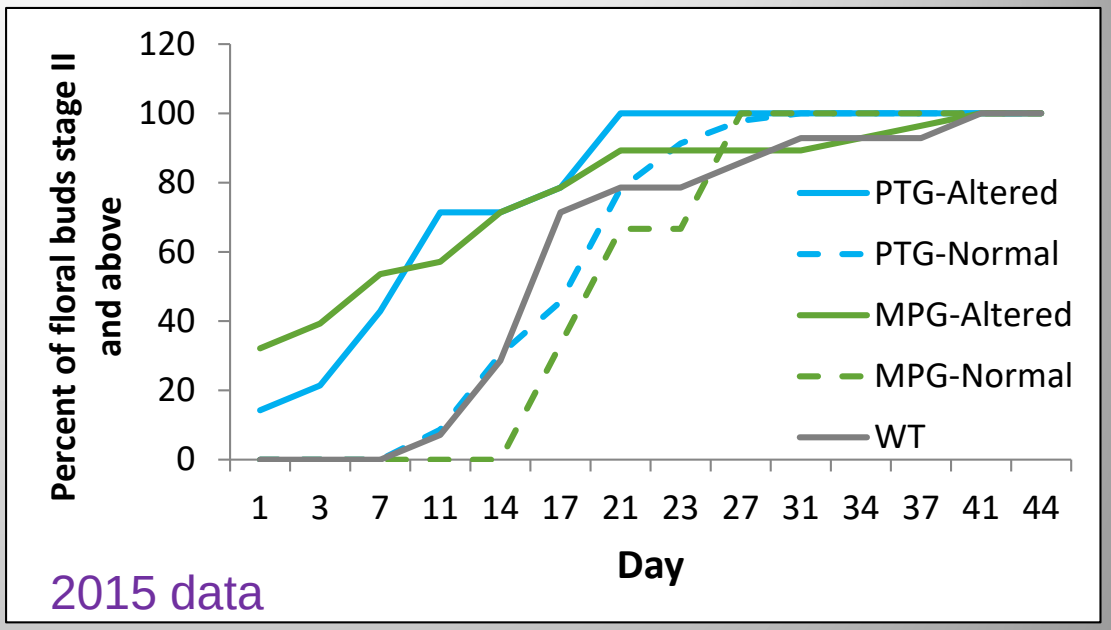
Summary of floral modifications

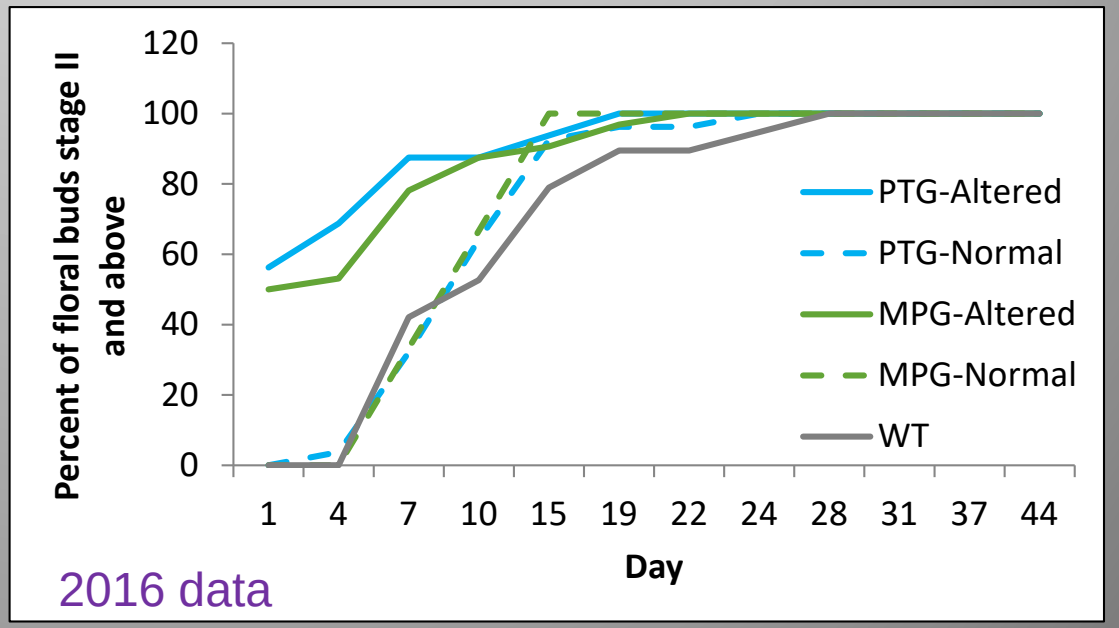
Construct ID	No. of insertion events	No. of events that flowered by 2017	No. of events with altered floral morphology
PTG	22	22	6 (27%)
MPG	13	12	11 (92%)
WT-CTR	24	19	0 (0%)

The MAR elements more than tripled RNAi suppression frequency

Floral buds on altered events flushed early - unexpected





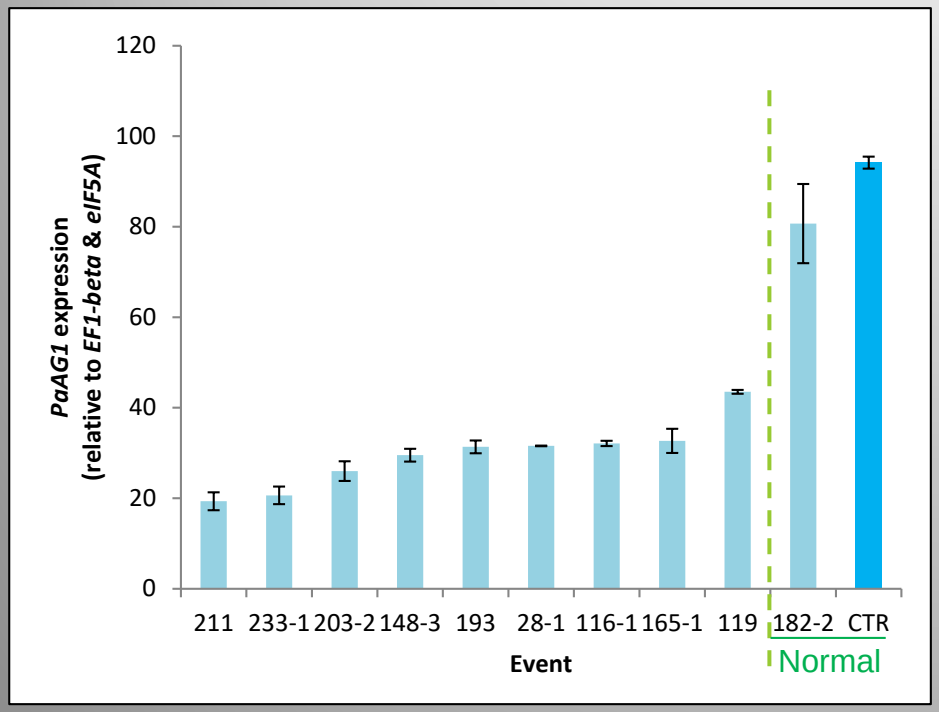


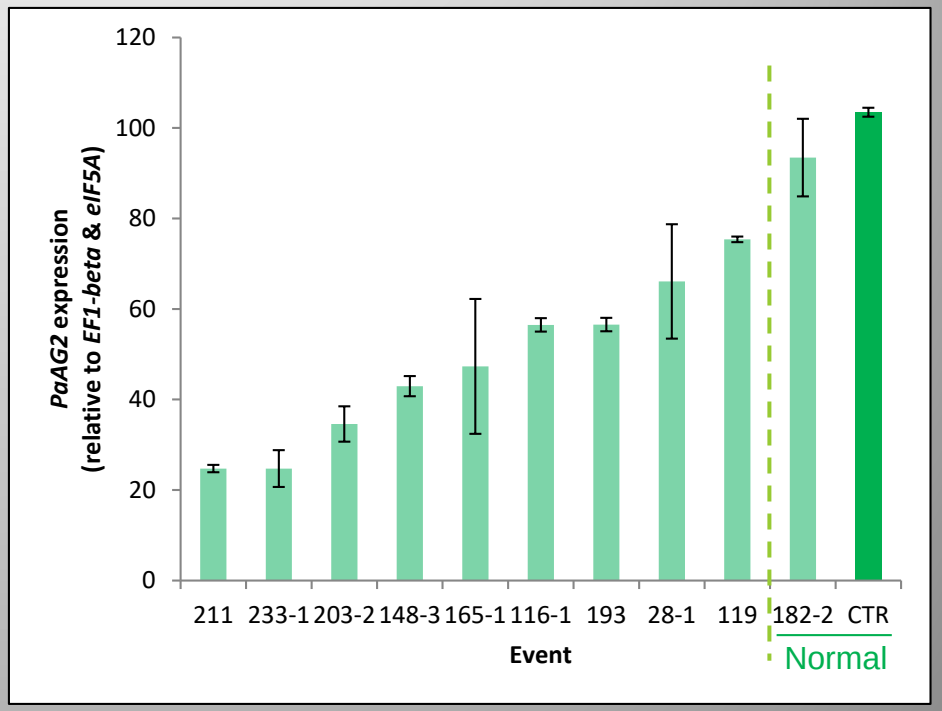
Altered events had highly modified, sterile flowers

Strongly altered events were stable within and among trees over 4 years

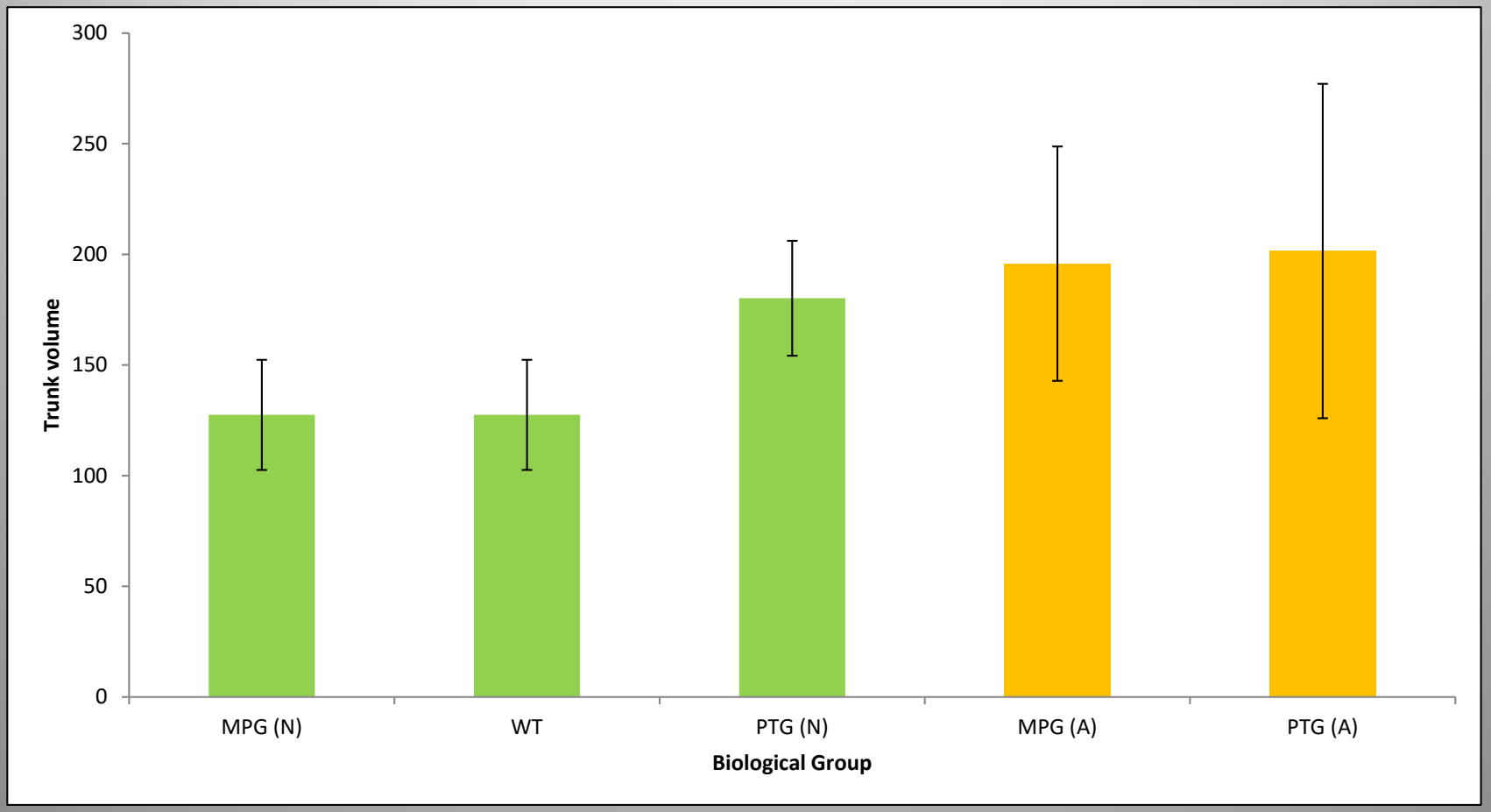
12 fully sterile events (2/3), 50 trees examined

Mild, correlated suppression of the two *PaAG* paralogs were associated with floral modification





Strong *AG*-RNAi trees showed normal vegetative growth as well as sterility



- A= Altered, N=Normal, Bars = SE of the mean

Sterility, normal growth of *LEAFY*-RNAi poplars over four growing seasons

limited, in large part owing to concerns over transgene flow into wild or feral tree populations[1–4]. Unlike other crops, trees are long-lived, weakly domesticated and their propagules can spread over several kilometers[5]. Although male sterility has been engineered in pine, poplar, and eucalyptus trees grown under field conditions by expression of the barnase RNase gene in anther tapetal cells[6,7], barnase can reduce rates of genetic transformation and vegetative growth[6]. Furthermore, barnase expression may not be fully stable[8]. Bisexual sterility would allay concerns over seed dispersal, could be used to control invasive exotic trees, and might increase wood production[9]. We

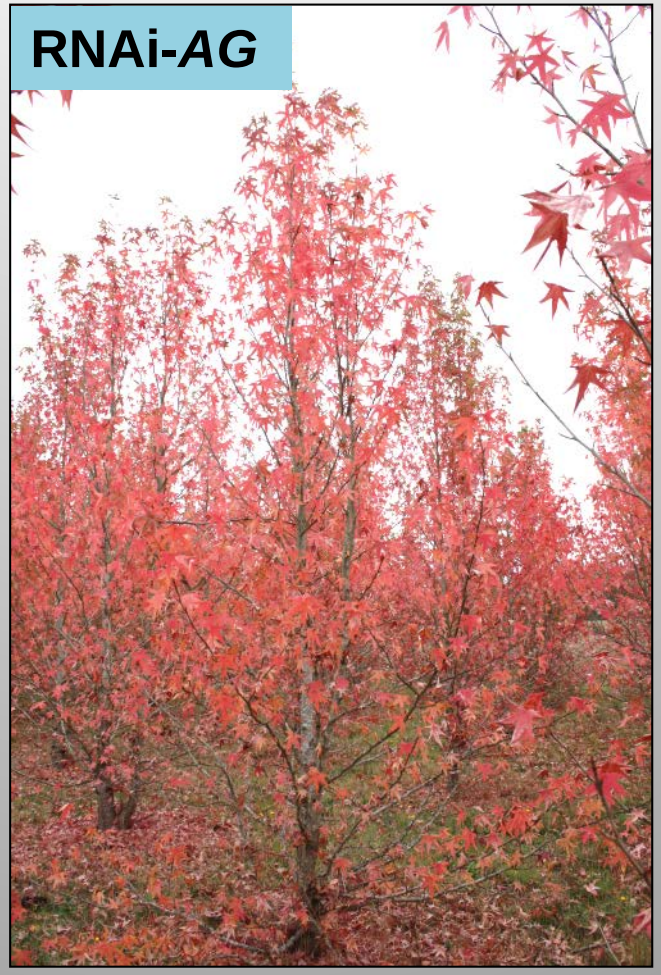
poplar.

RNAi has been used to reduce gene expression in many plant species[10,11], and the reduction in gene expression that RNAi confers is highly stable in trees under field conditions[12]. *LFY* is required for the early stages of male and female floral organ formation in plants, and encodes a transcription factor that promotes floral meristem identity[13,14]. In *Arabidopsis thaliana*, loss of *LFY* function results in the formation of vegetative structures instead of floral meristems, whereas reduction of *LFY* expression decreases floral abundance and results in partial conversion of floral organs to leaf-like structures[13,14]. We selected *LFY*

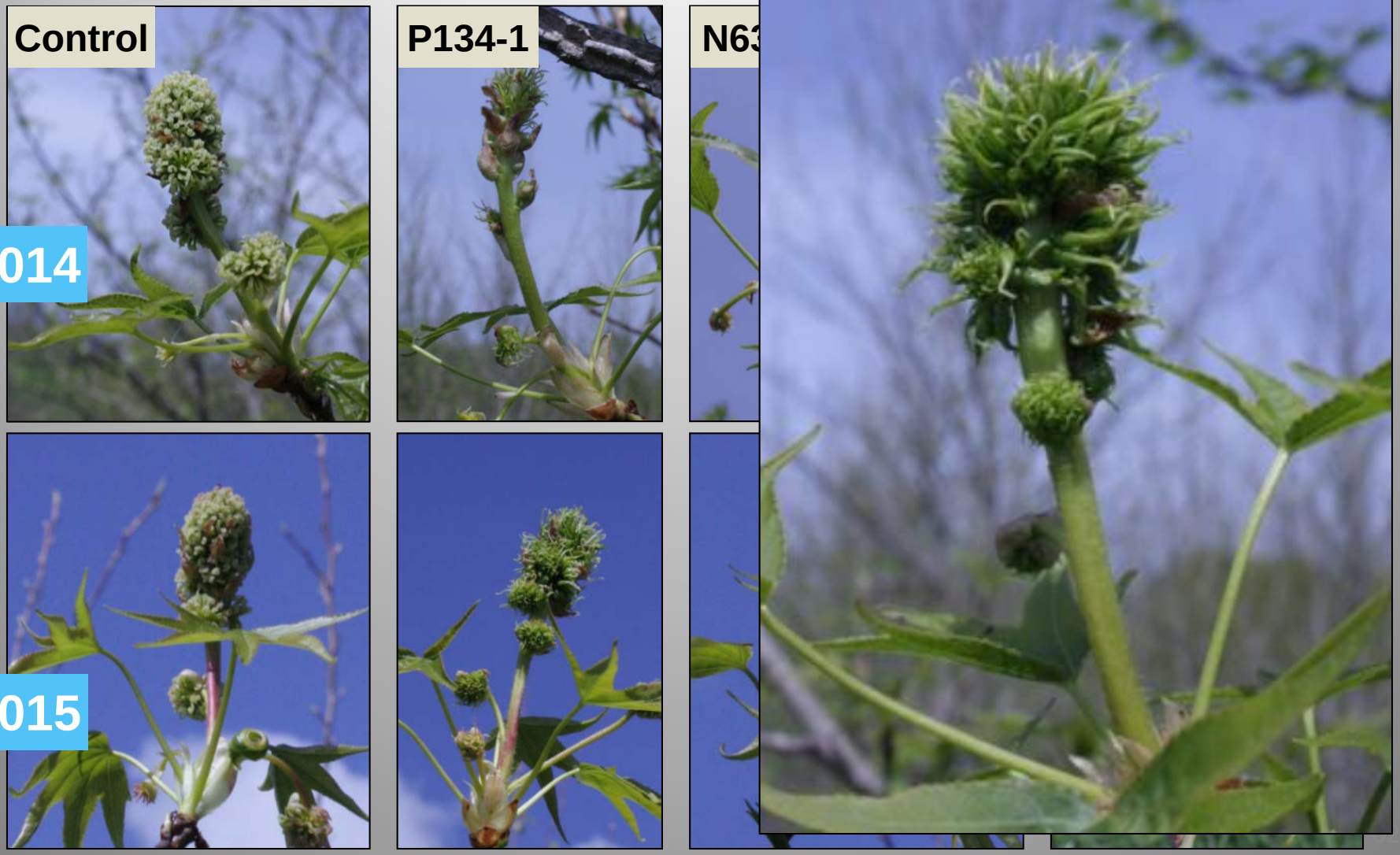
Klocko et al. 2016, *Nature Biotechnology*

Sweetgum RNAi-*AGAMOUS* plantation (Sept 2016)

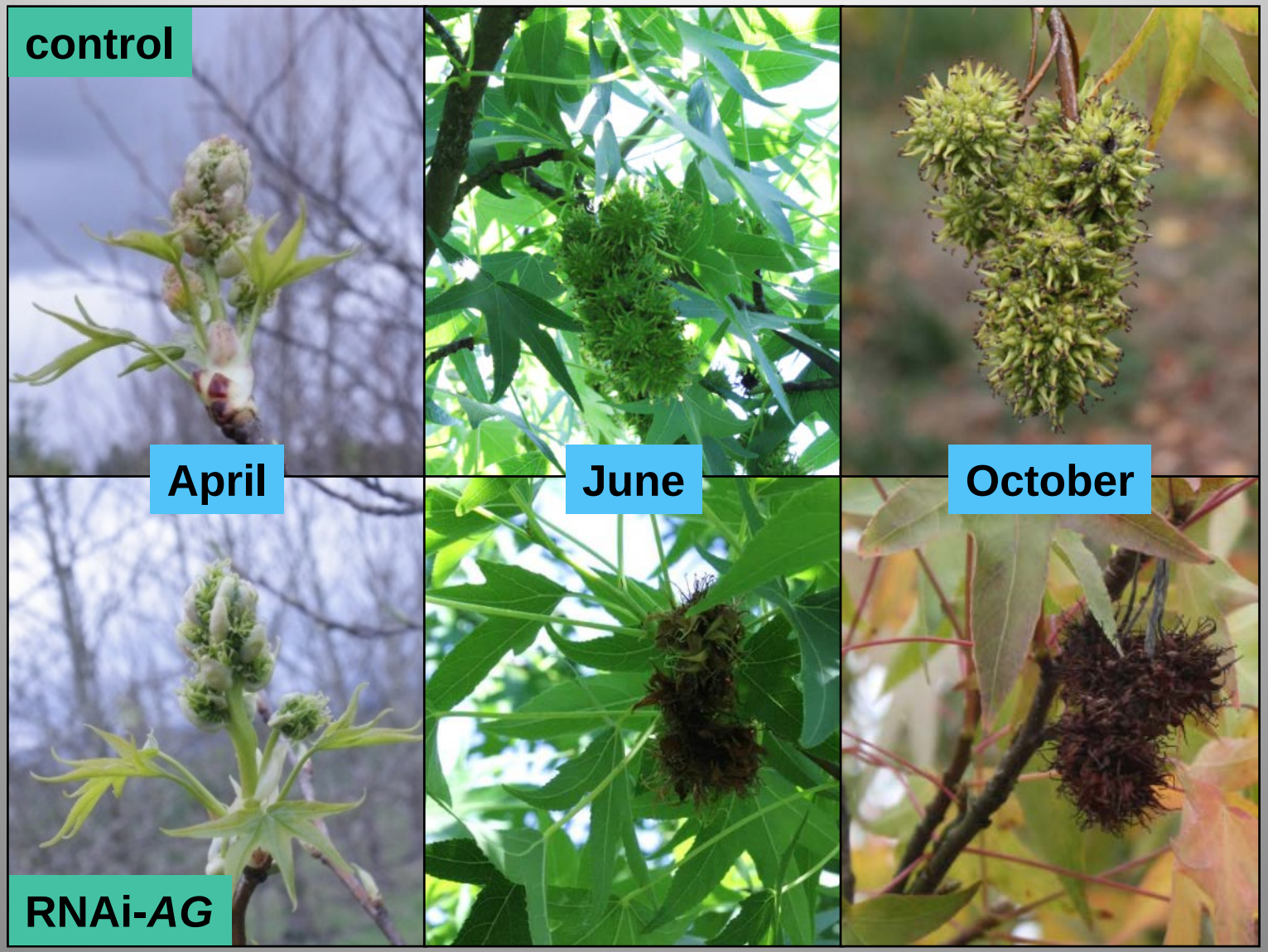
RNAi-AG trees had leaves and bright fall foliage like those of wild type ~8 years



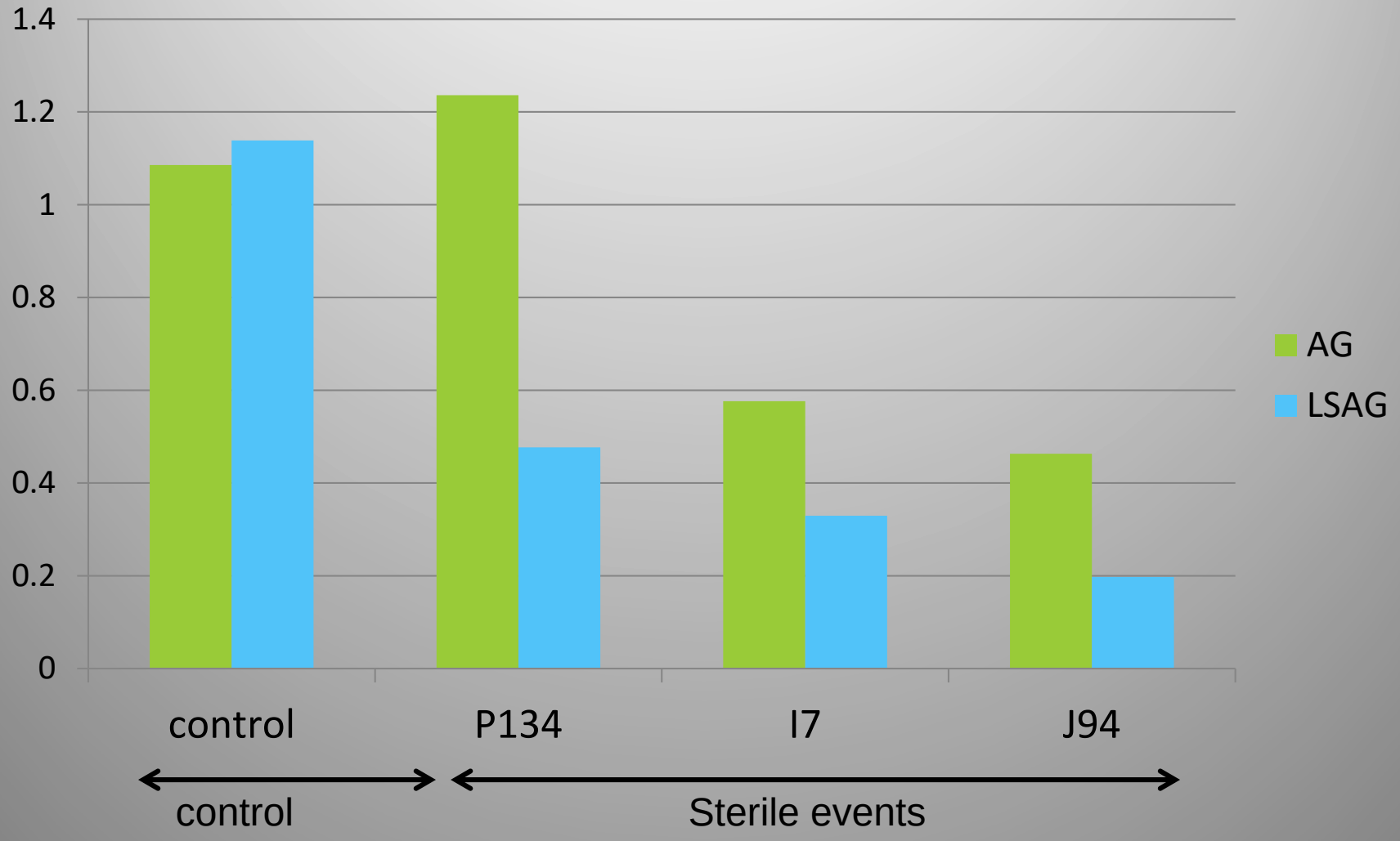
Altered phenotypes of RNAi-AG sweetgum were stable over 3 years



RNAi-*AG* flowers matured into sterile, brown papery fruits



qPCR shows sterile events have strong suppression of one or both AG-like genes



Relative expression in floral buds, 2 biological and 3 technical replicates

PLOS ONE

RESEARCH ARTICLE

Transgenic Suppression of *AGAMOUS* Genes in Apple Reduces Fertility and Increases Floral Attractiveness

Amy L. Klocko[1], Ewa Borejsza-Wysocka[2], Amy M. Brunner[3], Olga Shevchenko[1], Herb Aldwinckle[2], Steven H. Strauss[1]*

1 Department of Forest Ecosystems and Society, Oregon State University, Corvallis, Oregon, United States of America, **2** Section of Plant Pathology and Plant-Microbe Biology, School of Integrative Plant Science, Cornell University, Geneva, New York, United States of America, **3** Department of Forest Resources and Environmental Conservation, Virginia Tech, Blacksburg, Virginia, United States of America

* steven.strauss@oregonstate.edu

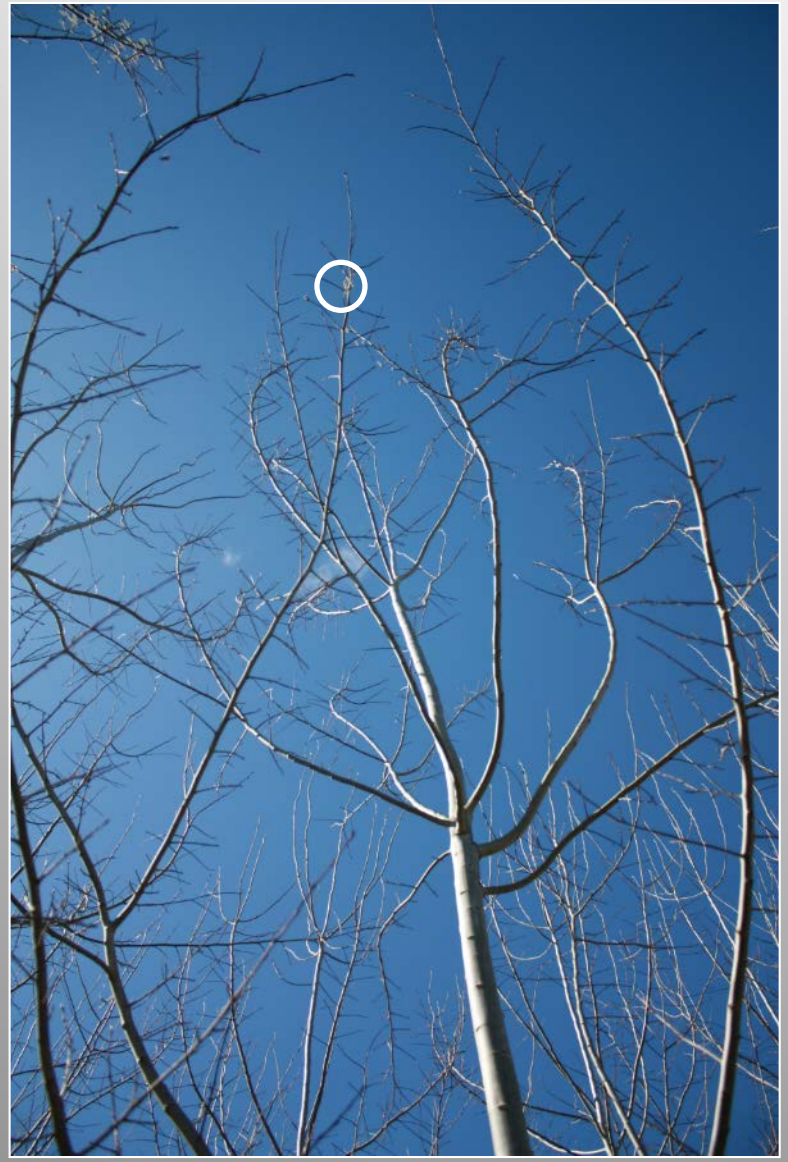
Abstract

We investigated the ability of RNA interference (RNAi) directed against two co-orthologs of *AGAMOUS* (*AG*) from *Malus domestica* (domestic apple, *MdAG*) to reduce the risks of

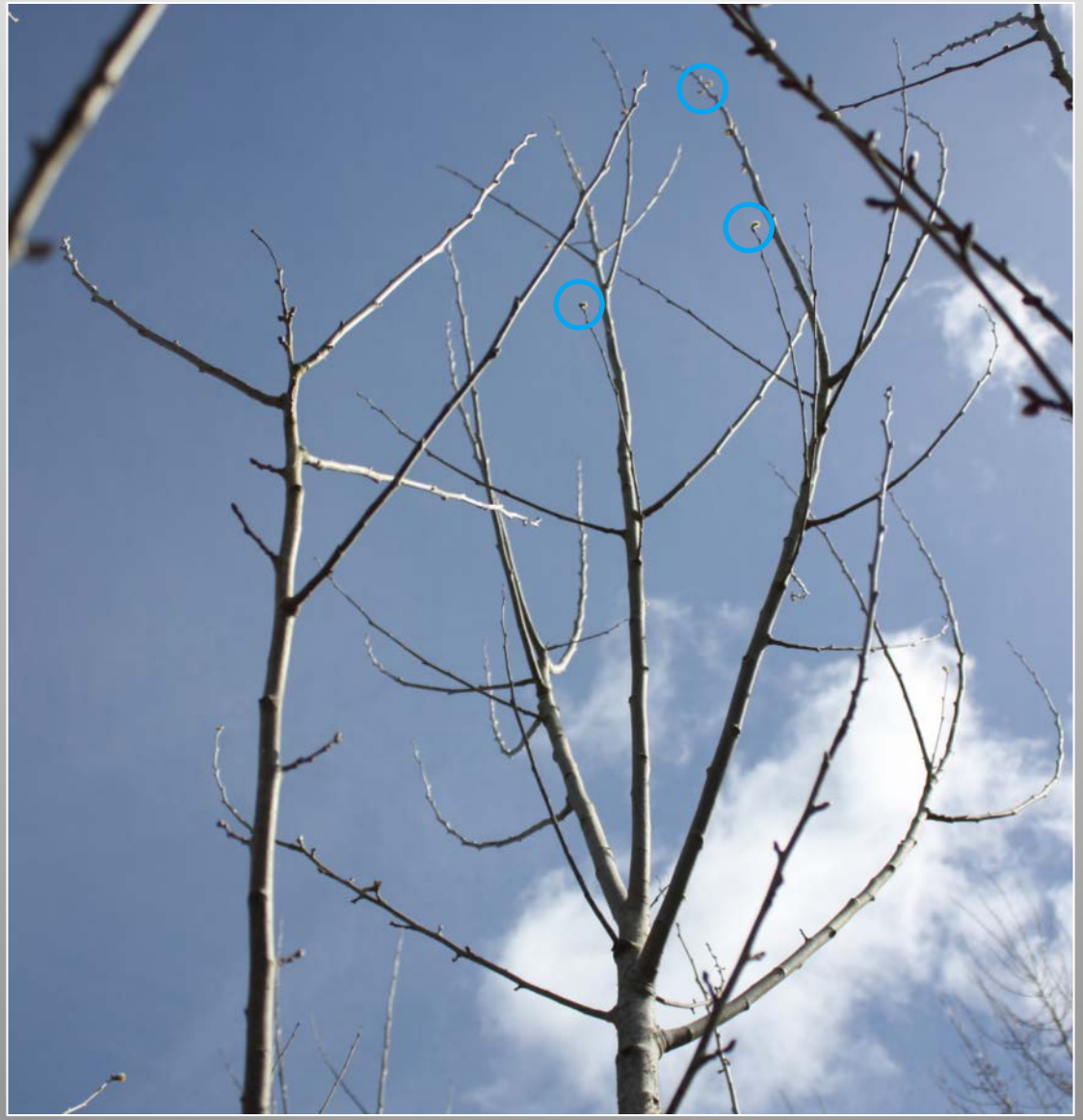
Floral suppressors: Scored extent of flowering in all trees

Score of 0

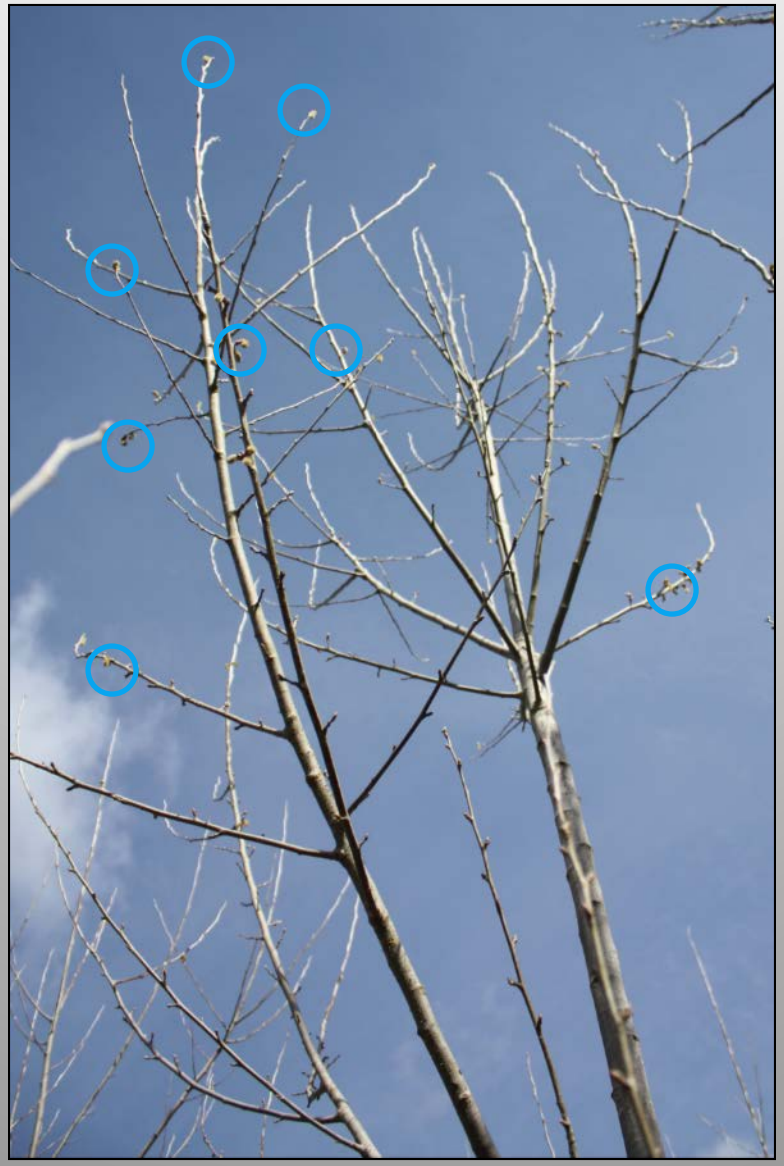
Score of 1



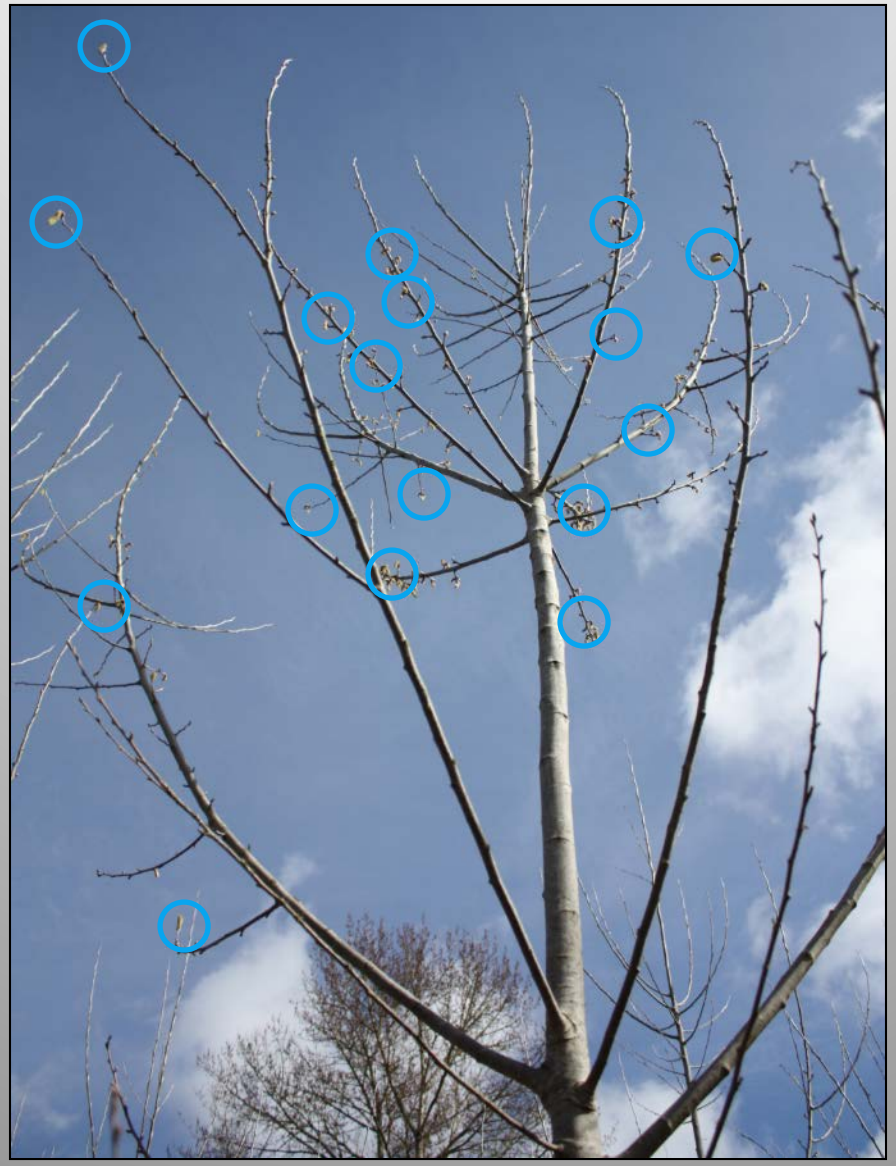
Score of 2



Score of 3

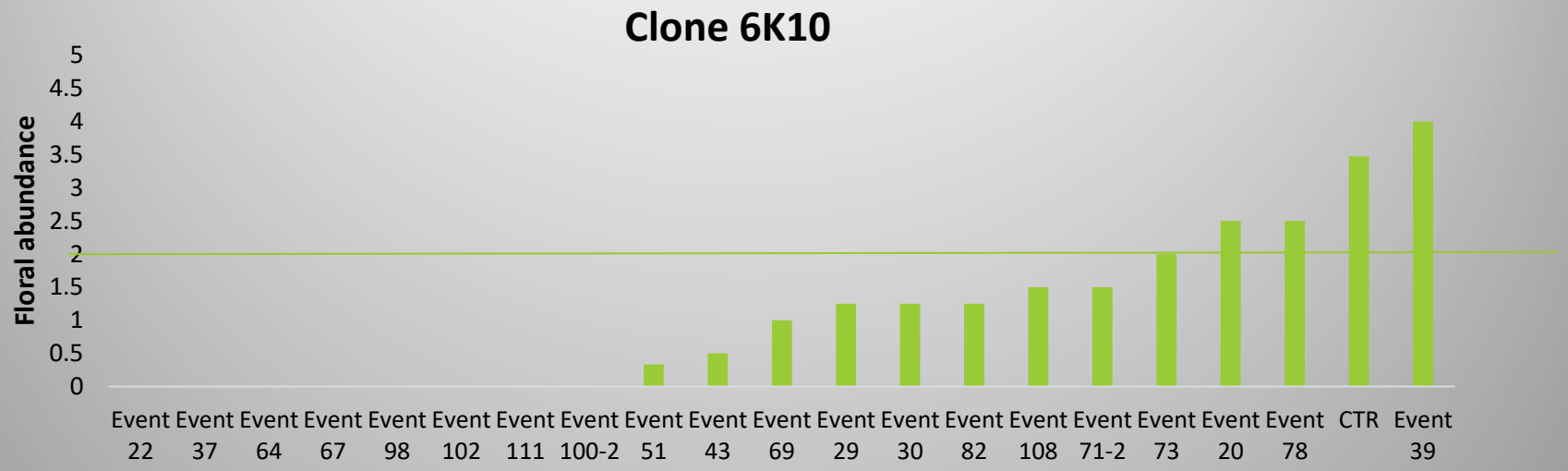


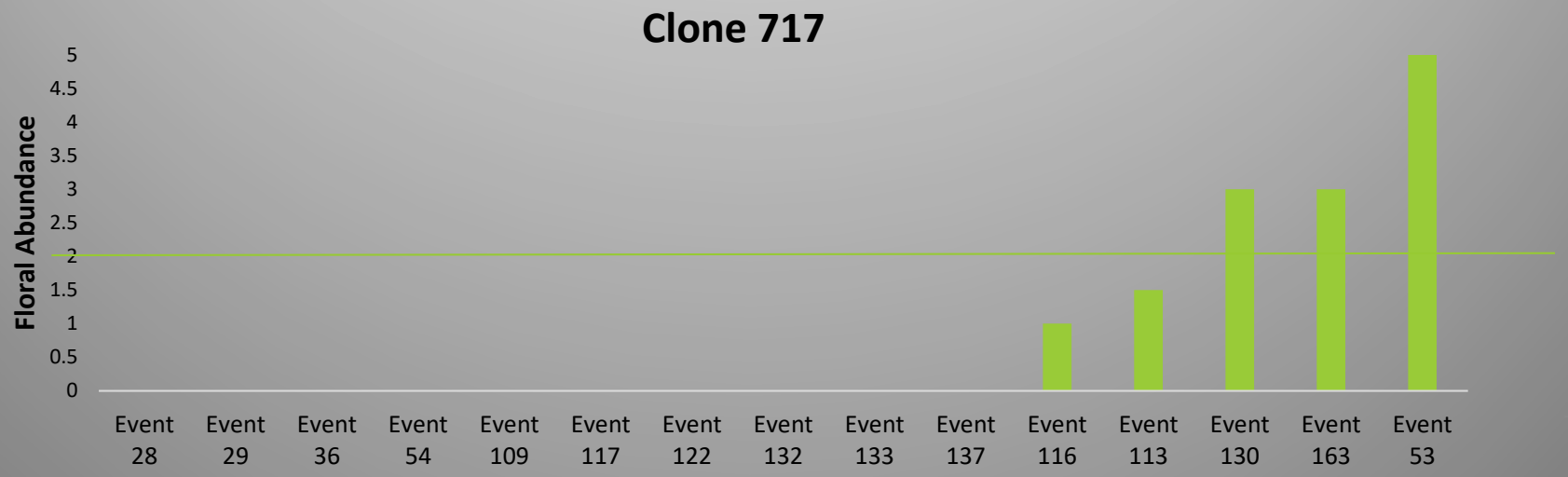
Score of 4



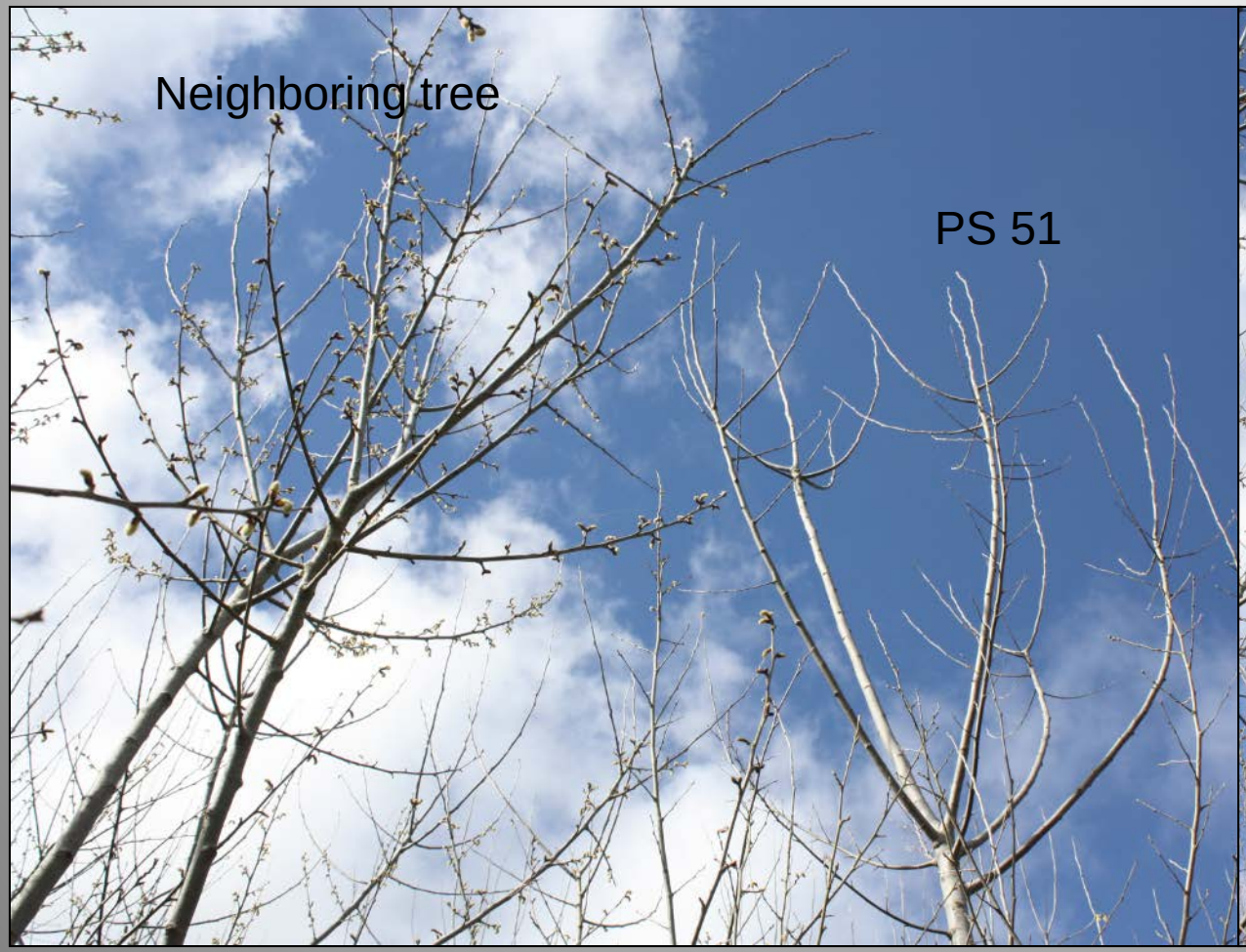
Score of 5

80% of all SVP-OE events showed floral abundance scores of less than 2

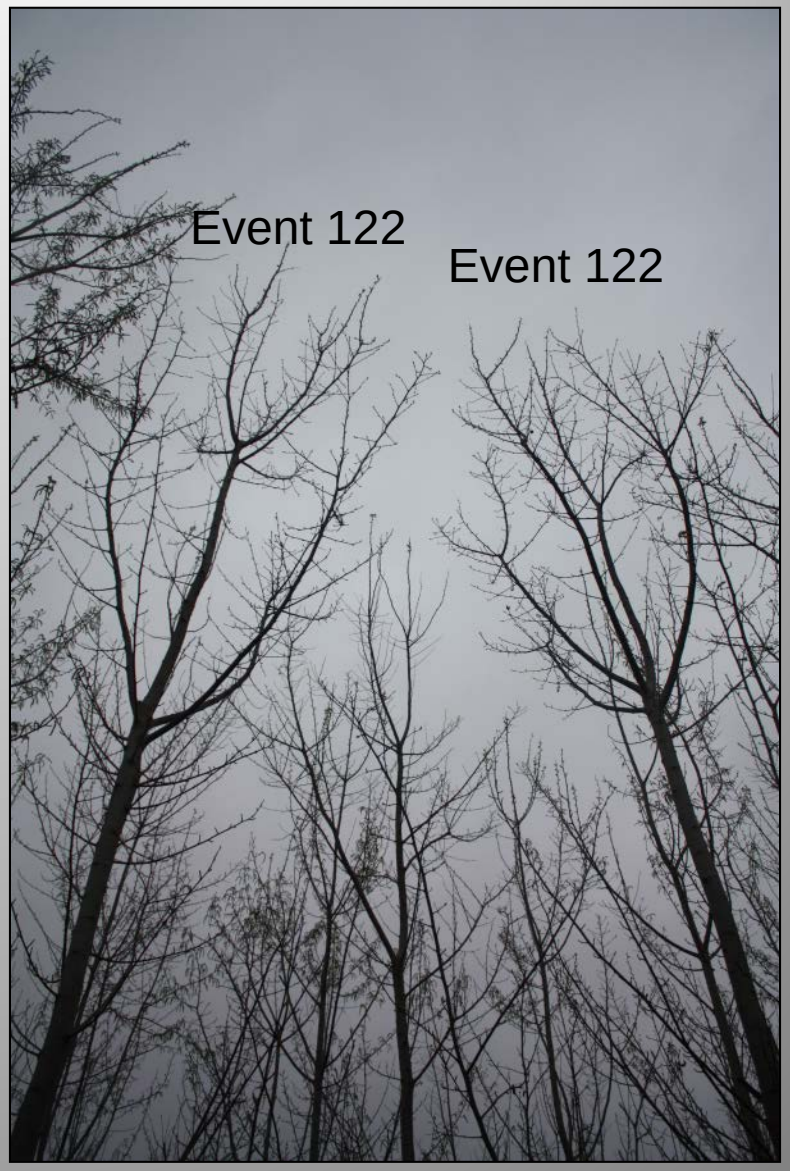




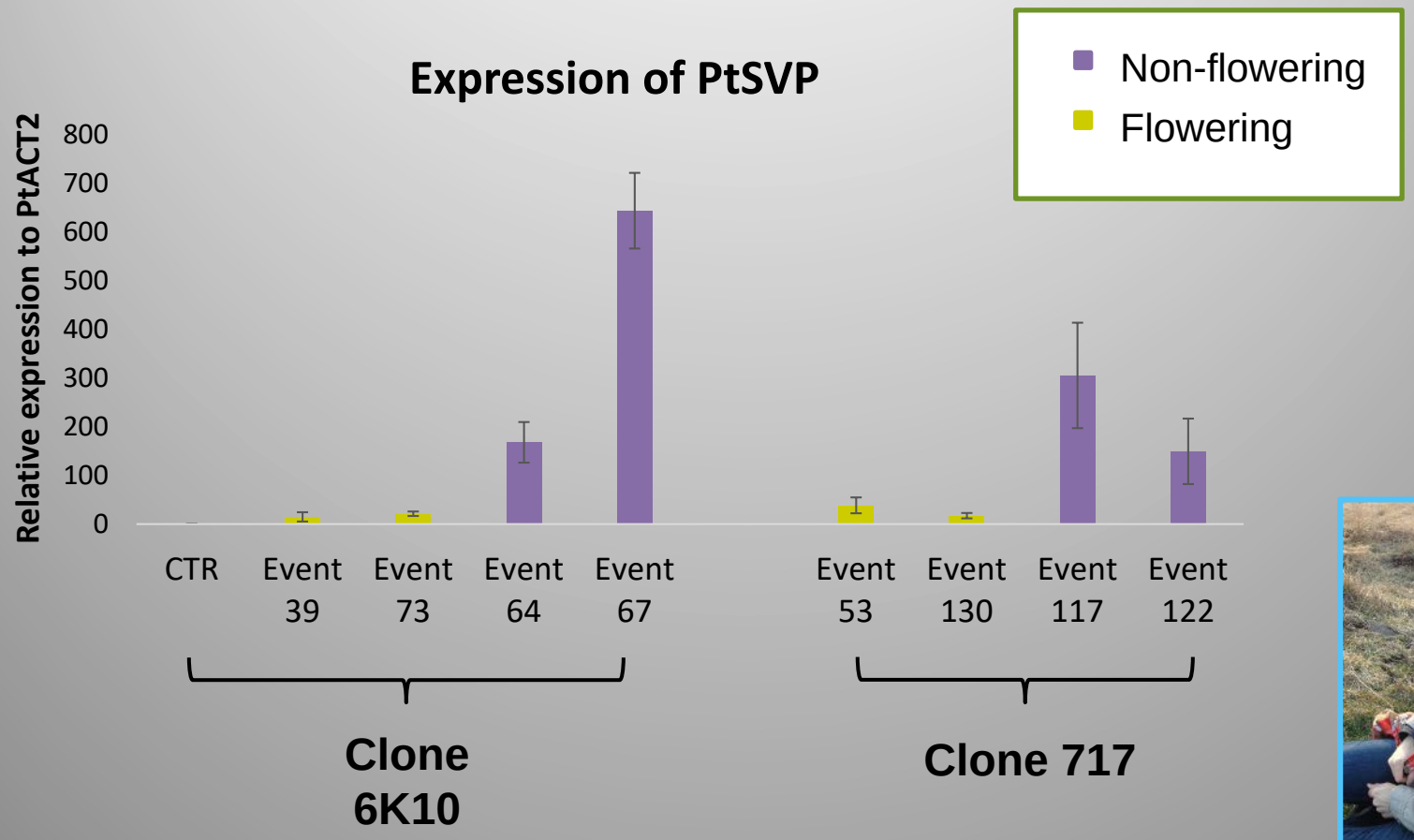
Striking differences among flowering vs. non-flowering adjacent events



717 SVP event 122 no flowers



Non-flowering events had high expression of *PtSVP* in leaves

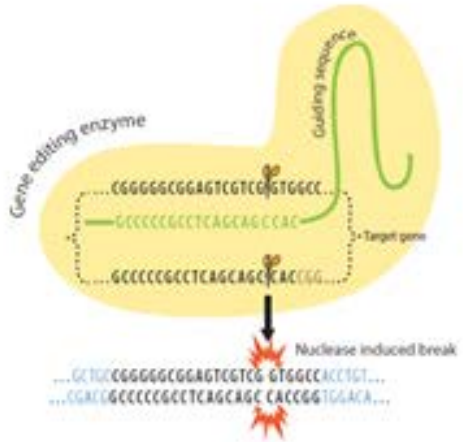


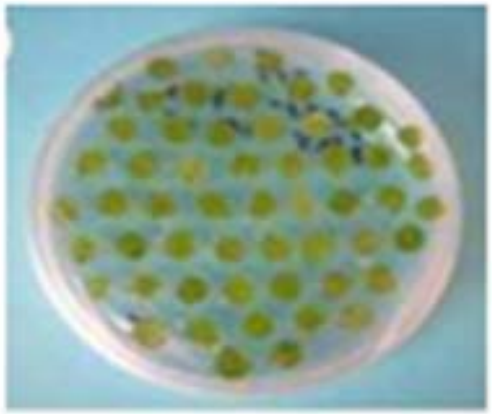
Emily Helliwell, Former postdoc

Future of floral suppressor studies

- Study of two additional successful suppressors based on mutated *AP1* gene
- Studies of growth effects underway – some appear likely
- Superior method likely to be CRISPR promoter engineering vs. simple 35S overexpression

Overview of CRISPR methods in poplar



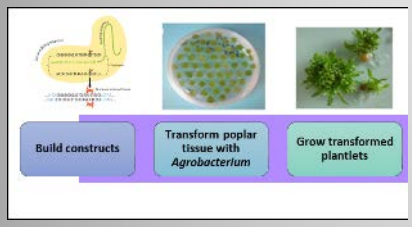


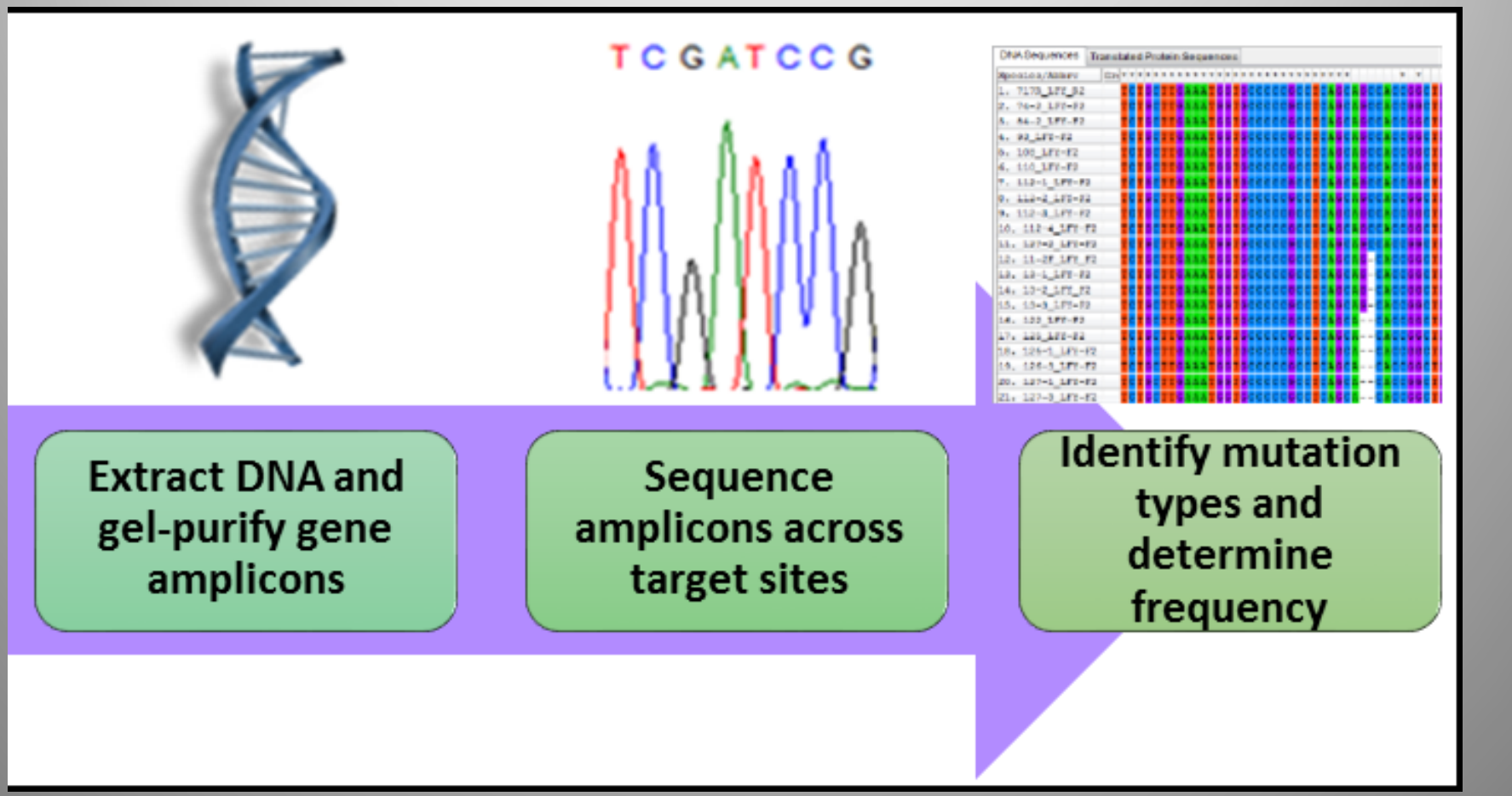
Build constructs

Transform poplar tissue with *Agrobacterium*

Grow transformed plantlets

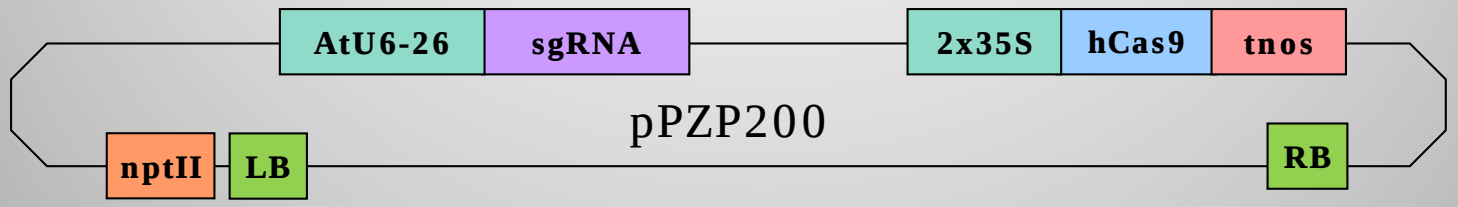
Overview of CRISPR methods in poplar

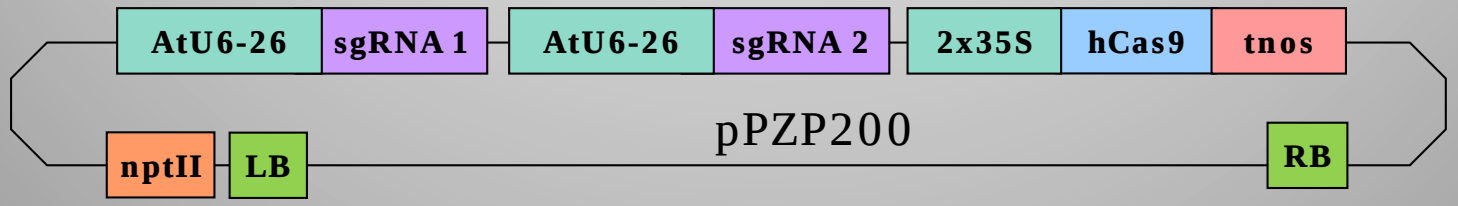




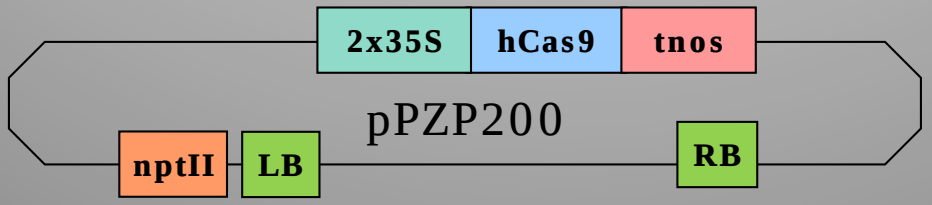
Experimental constructs – single and double targets per gene

Nuclease constructs





Control construct



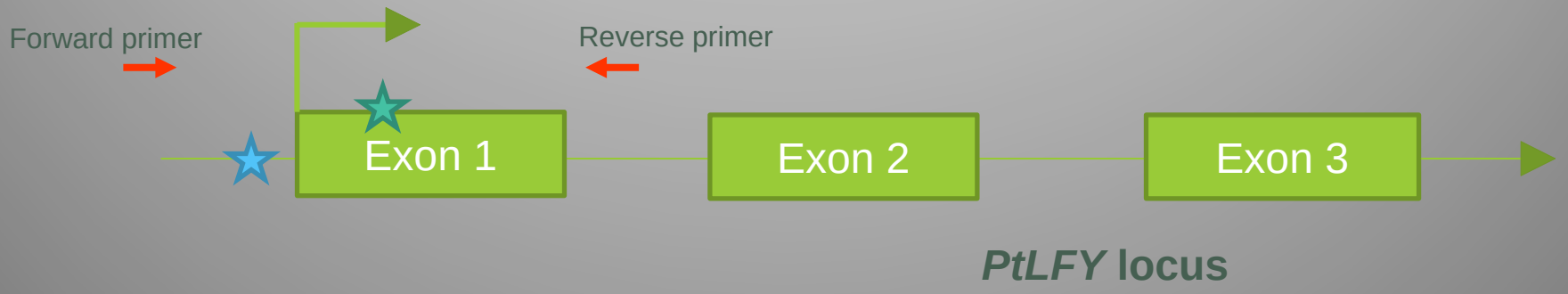
Targeting two sites in the single-copy LFY gene in poplar

Target site for LFYsg2

>PtLFY

…CATGCACCAGTGAAA**GATCACAGAGAGAGAGACAA**GGGGGCAGATAGAT**ATG**GATCCGGAGGCTTT
CACGGCGAGTTTGTTCAAATGGGACACGAGAGCAATGGTGCCACATCCTAACCGTCTGCTTGAAATGGT
GCCCCCGCCTCAGCAGCCACCGGCTGCGGCGTTTGCTGTAAGGCCAAGGGAGCTATGTGGGCTAGAGG
AGTTGTTTCAAGCTTATGGTATTAGGTACTACACGGCAGCGAAAATAGCTGAACTCGGGTTCACAGTGA…

Target site for LFYsg1 (in exon 1)



Targeting two identical sites in the two paralogous AG genes in poplar

>PtAG1

…GGATCAGCTAGCTAGACTGCAGCT**ATG**GAATATCAAAATGAATCCCTTGAGAGCTCCCCCCTGAGGAAGC

TAGGAA**GGGGAAAGGTGGAGATCAAG**CGGATCGAGAACACCACCAATC**GCCAAGTCACTTTCTGCAAA**

AGGCGCAGTGGTTTGCTCAAGAAAGCCTACGAATTATCTGTTCTTTGCGATGCTGAGGTTGCACTCATCG…

Target site for AGsg2

Target site for AGsg1

>PtAG2

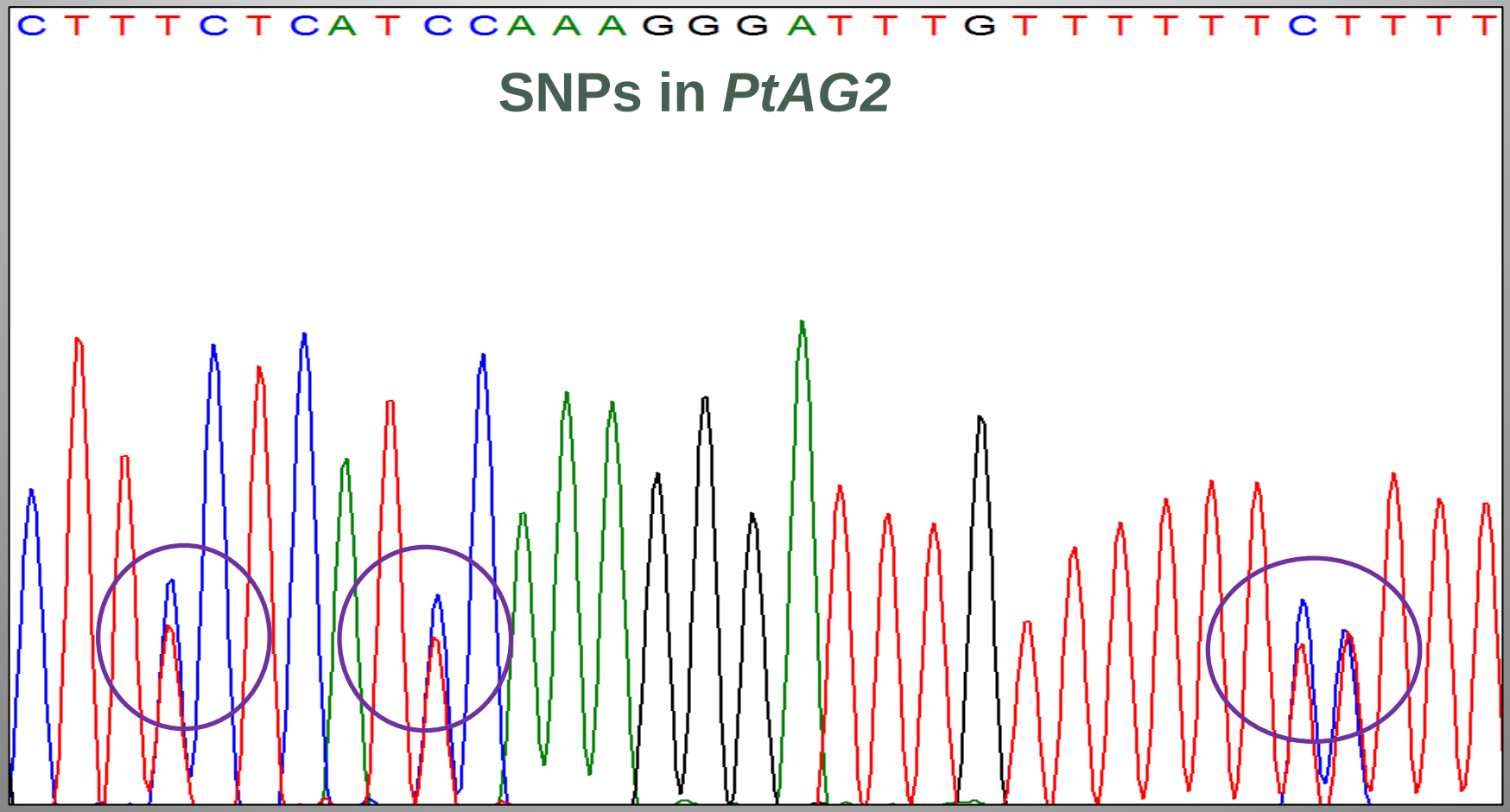
…GATCAGCTAGCTAGGCAGCAGCT**ATG**GCATACCAAAATGAATCCCAAGAGAGCTCCCCCCTGAGGAAGC

TGGGRA**GGGGAAAGGTGGAGATCAAG**CGGATCGAGAACACCACAAATC**GYCAAGTCACTTTCTGCAAA**

AGGCGGAATGGTTTGCTCAAGAAAGCCTATGAATTATCTGTTCTTTGCGATGCTGAGGTTGCACTCATCG…

***PtAG1* and Pt*AG2* loci**

Natural SNPs facilitate direct PCR, allele-specific PCR, or cloned amplicons for identifying knock-outs



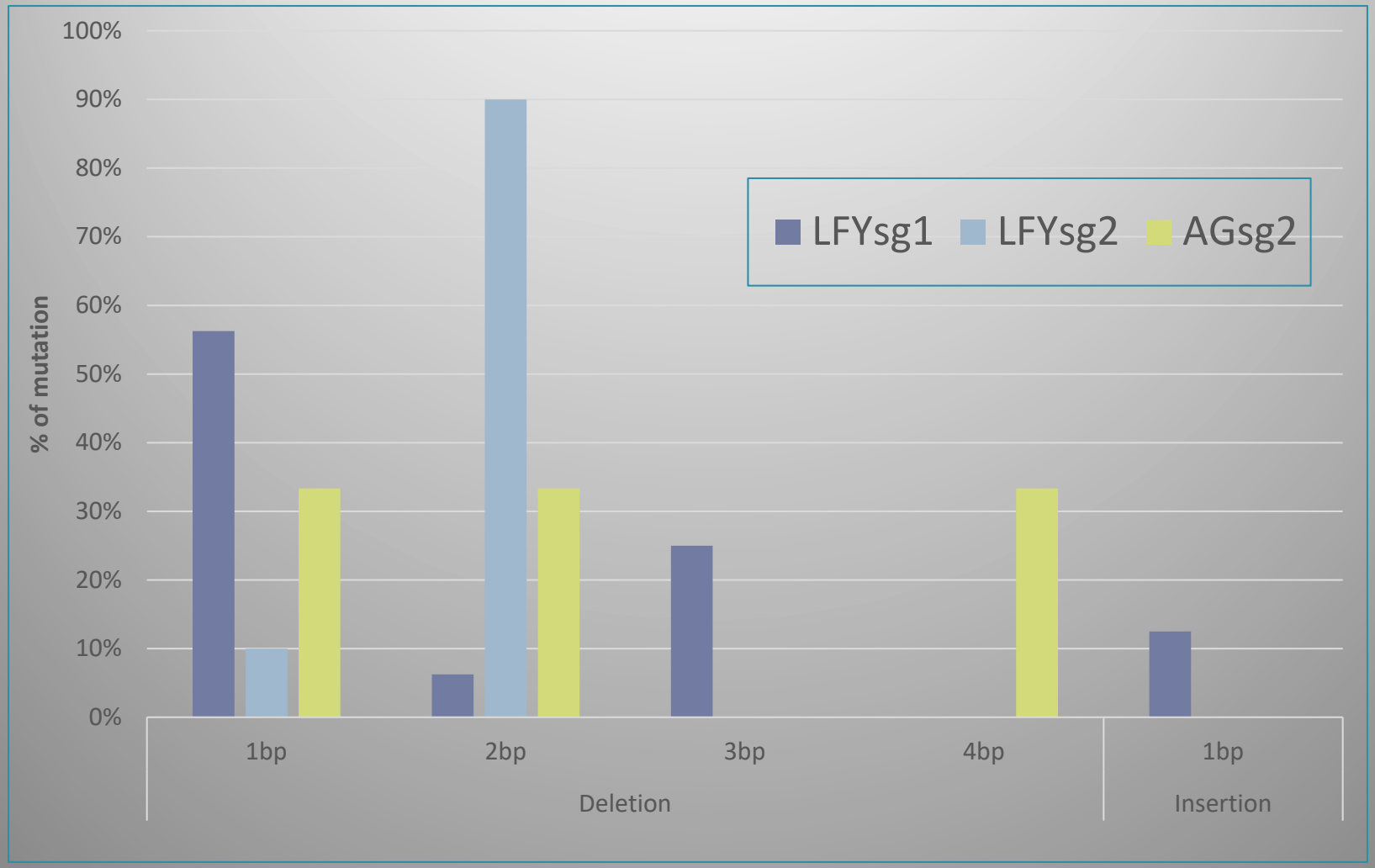
CRISPR causes high knock-out frequency in poplar

Population	Total events	Mutation	# events	Frequency
LFY-CRISPR 717	256	Biallelic KO	168	65%
		WT	88	35%
LFY-CRISPR 353	38	Biallelic KO	27	71%
		WT	11	29%
AG-CRISPR 717	159	Biallelic KO	133	84%
		WT	26	16%
AG-CRISPR 353	35	Biallelic KO	29	83%
		WT	6	17%
Cas9 control 717	33	Biallelic KO	0	0%
		WT	33	100%
Cas9 control 353	17	Biallelic KO	0	0%
		WT	17	100%
All poplar	**488**	**Biallelic KO**	**357**	**73%**
		WT	**131**	**27%**

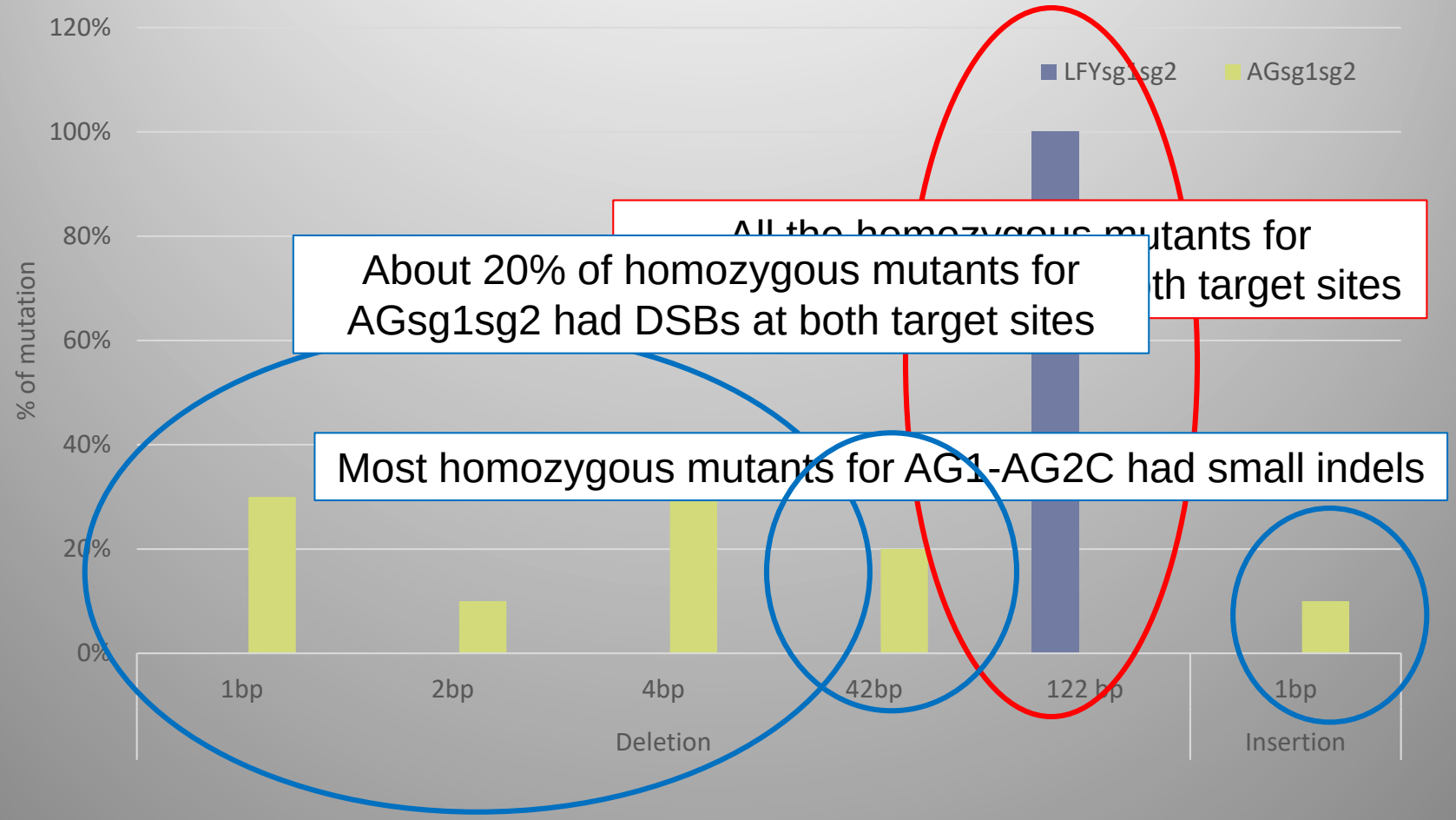
CRISPR causes high knock-out frequency in eucalypts

Population	Total events	Mutation	# events	Frequency
FT LFY-CRISPR	60	Biallelic KO	58	97%
		WT	2	3%
FT Cas9 control	10	Biallelic KO	0	0%
		WT	10	100%
SP7 LFY-CRISPR	10	Biallelic KO	10	100%
		WT	0	0%
SP7 Cas9 control	2	Biallelic KO	0	0%
		WT	2	100%
All eucalypt	**70**	**Biallelic KO**	**68**	**97%**
		WT	**2**	**3%**

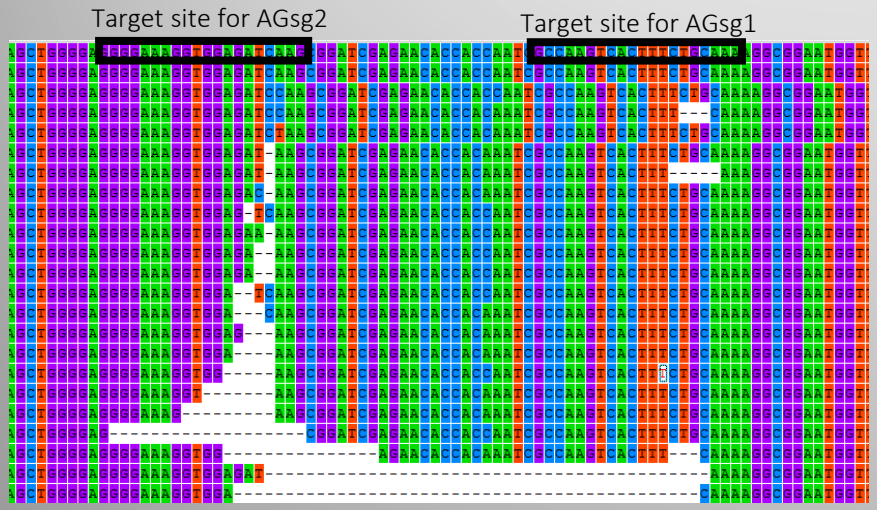
Small indels were frequent for single target nucleases

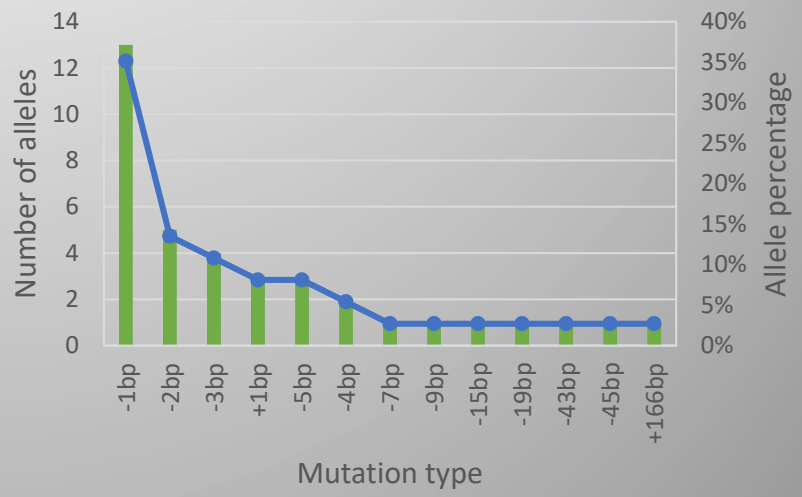


Large mutations were common among active double nucleases

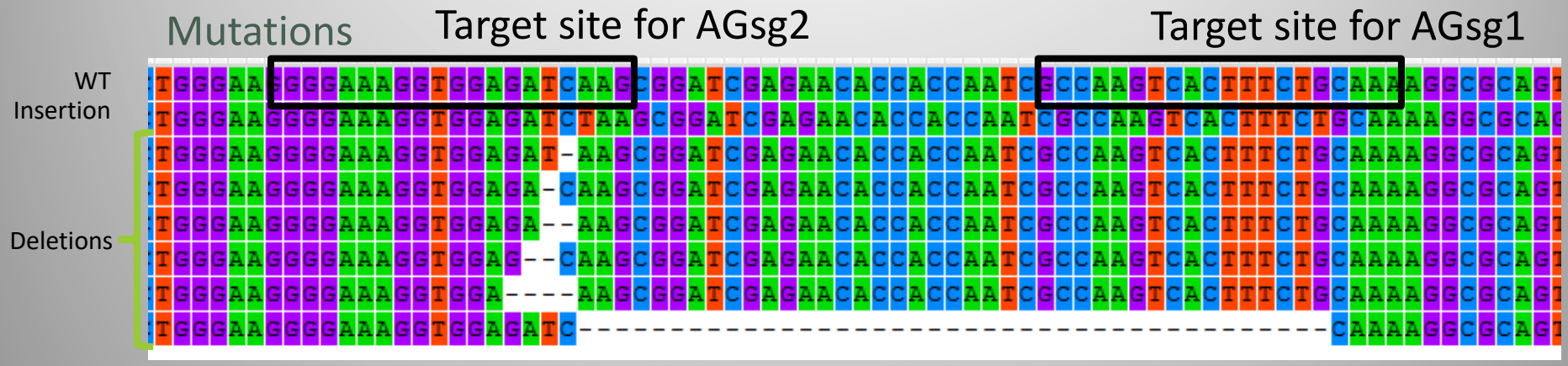


Diversity in mutation type and number from allele-specific PCR in hybrid male aspen 353

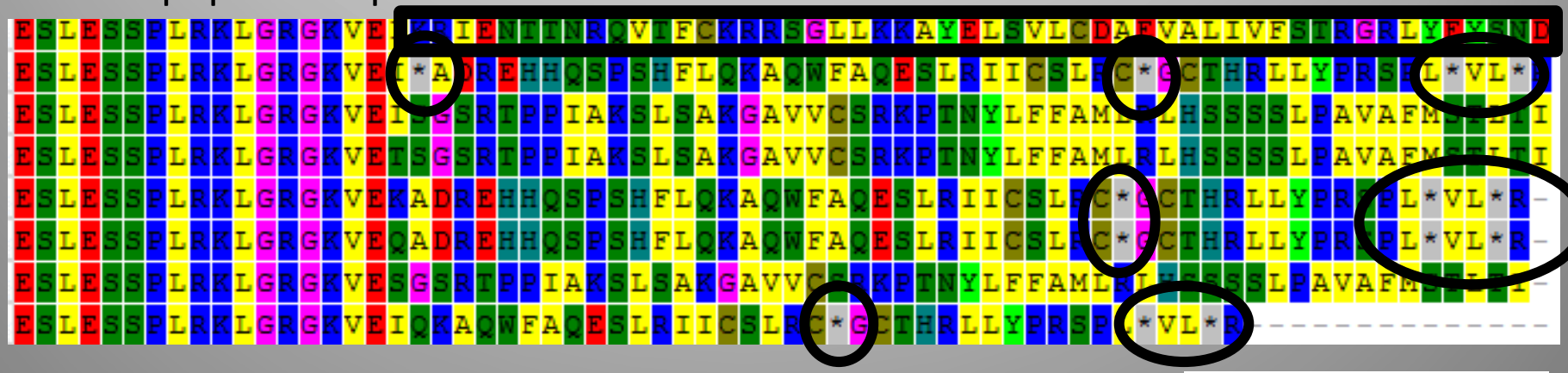




Knock-out events expected to have non-functioning *PtAG1*



Partial peptide sequence



Early stop codons

Summary: High CRISPR mutation rates observed in poplar and eucalypts

- Cas9-only control events
  - No mutations (62 events, poplar and eucalypts)
- CRISPR-Cas events
  - Poplar: 73% of events were knock-outs (488 events tested, *AG* and *LFY*)
  - Eucalypts: 97% knock-outs (70 events, *LFY*)
- Off-target studies underway

Estefania Elorriaga, PhD student

LFY knock-out in rapid flo background

Agenda

1. Genetic engineering defined (brief)
2. Genome editing explained
3. Examples and applications in crops
4. Our work
   - RNAi
   - Repressors
   - CRISPR-Cas
5. Society, regulation, markets

Social dimensions

- Largest barriers to use are social, not technical
- Ethics: Is the method OK to use at all?
  - Impossible to trace with certainty
  - Do we need to add tracer DNA?
- Should it be regulated and labeled like GMOs?
- Should all mutagenesis uses of gene editing be excused from regulation?
- What about market forces and self-labeling?

Global regulatory quandry

PUBLISHED: 8 JANUARY 2015 | ARTICLE NUMBER: 14011 | DOI: 10.1038/NPLANTS.2014.11

comment

Regulatory uncertainty over genome editing

Huw D. Jones

Genome editing opens up opportunities for the precise and rapid alteration of crops to boost yields, protect against pests and diseases and enhance nutrient content. The extent to which applied plant research and crop breeding benefit will depend on how the EU decides to regulate this fledgling technology.

We are at the dawn of a new paradigm in plant breeding. Classical approaches to crop improvement based on hybridization and selection can now be complemented by targeted genome editing that exploits knowledge of specific gene sequences in a systematic way. Unlike conventional genetic modification that results from the insertion of large pieces of exogenous DNA, or maize renders the plants highly resistant to lepidopteron pests; these lepidopteron-resistant crops are grown around the world. However, this technique cannot be used to make small edits to existing genes, and can lead to the random disruption of native genes because the destination of the inserted DNA cannot be dictated.

In contrast to traditional genetic modification, genome editing makes use of one or a few bases at the cut site, resulting in a mutation. Mutations generated in this way are indistinguishable from those that occur naturally and drive evolution, as well as from those induced through the application of chemical mutagens or radiation, as employed in mutation breeding programmes since the 1940s.

Here, I focus on the potential applications and regulation of this simple 'cut and repair'

USA GMO labeling law in place

Home › News Center › New US Labeling Law for Bioengineered Foods

NEW US LABELING LAW FOR BIOENGINEERED FOODS

September 28, 2016

Building on the work of individual states in preparing and implementing laws to regulate the labeling of bioengineered foods, the USA has enacted a federal law providing countrywide protection and consistency for consumers.

Jim Cook, SGS Food Scientific and Regulatory Affairs Manager explains in more detail.

On July 1, 2016, the USA's first labeling law, the Vermont Genetically Engineered (GE) food labeling law Act 120 became effective but as of July 29 when President Obama signed the National Bioengineering Food Disclosure Law [1] it

New USDA proposal on Jan 19, 2017

DEPARTMENT OF AGRICULTURE

Animal and Plant Health Inspection Service

7 CFR Part 340

[Docket No. APHIS–2015–0057]

RIN 0579–AE15

Importation, Interstate Movement, and Environmental Release of Certain Genetically Engineered Organisms

AGENCY: Animal and Plant Health Inspection Service, USDA.

ACTION: Proposed rule.

SUMMARY: APHIS is proposing to revise its regulations regarding the importation, interstate movement, and environmental release of certain genetically engineered organisms in order to update the regulations in response to advances in genetic engineering and understanding of the plant pest and noxious weed risk posed by genetically engineered (GE) organisms, thereby reducing burden for regulated entities whose organisms pose no plant pest or noxious weed risks. This would be the first comprehensive revision of the regulations since they were established in 1987.

DATES: We will consider all comments that we receive on or before May 19, 2017.

SUPPLEMENTARY INFORMATION:

Background

Overview of the Current Regulations

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) administers regulations in 7 CFR part 340, ''Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which are Plant Pests or Which There is Reason to Believe are Plant Pests'' (referred to below as the regulations). The current regulations govern the introduction (importation, interstate movement, or release into the environment) of certain genetically engineered (GE) organisms that are considered ''regulated articles.''

Under the current regulations, a GE organism is considered to be a regulated article if the donor organism, recipient organism, vector, or vector agent [1] is a plant pest or if the Administrator has reason to believe the GE organism is a plant pest. A *plant pest* is defined in § 340.1 as ''Any living stage (including active and dormant forms) of insects, mites, nematodes, slugs, snails, protozoa, or other invertebrate animals, bacteria, fungi, other parasitic plants or reproductive parts thereof; viruses; or any organisms similar to or allied with any of the foregoing; or any infectious agents or substances, which can directly or indirectly injure or cause disease or

article pursuant to 7 CFR part 340.

Agency Actions Following Promulgation of the Current Regulations

APHIS first issued these regulations in 1987 under the authority of the Federal Plant Pest Act of 1957 (FPPA) and the Plant Quarantine Act of 1912 (PQA), two acts that were subsumed into the Plant Protection Act (PPA, 7 U.S.C. 7701 *et seq.*) in 2000, along with other provisions. Since 1987, APHIS has amended the regulations six times, in 1988, 1990, 1993, 1994, 1997, and 2005, to institute exemptions from permitting for certain microorganisms and *Arabidopsis,* to institute the notification, petition, and extension procedures referenced above, and to exclude plants engineered to produce industrial compounds from the notification process.

Although, as discussed above, the current regulations have various functions, their primary function to date has been as a means for APHIS to authorize the importation, interstate movement, and introduction of certain GE organisms via the permit and notification procedures referred to above. Permits and notifications are collectively known as ''authorizations.'' To date, APHIS has issued more than 18,000 authorizations for the environmental release of GE organisms in multiple sites, primarily for research and development of improved crop varieties for agriculture. Additionally, APHIS has issued more than 12,000

Gene editing is GE…

- “For the purposes of this rule, APHIS is proposing to consider genome editing to be within the definition of genetic engineering. “
- Does that mean every editing line must come before USDA?
- How much data and confinement is required during research and breeding?
- Is it essentially a GMO?

But…..the definition of GE is further restricted…

- … an organism will not be considered a genetically engineered organism if:
- (1) The genetic modification to the organism is solely a deletion of any size or a single base pair substitution which could otherwise be obtained through the use of chemical- or radiation-based mutagenesis; or

But…..the definition of GE is further restricted…

- … an organism will not be considered a genetically engineered organism if:
- (2) The genetic modification to the organism is solely introducing only naturally occurring nucleic acid sequences from a sexually compatible relative that could otherwise cross with the recipient organism and produce viable progeny through traditional breeding…or

But…..the definition of GE is complex

- … an organism will not be considered a genetically engineered organism if:
- (3) The organism is a ‘‘null segregant,’’ that is, the progeny of a GE organism where the only genetic modification was the insertion of donor nucleic acid into the recipient’s genome, but the donor nucleic acid is not passed to the recipient organism’s progeny

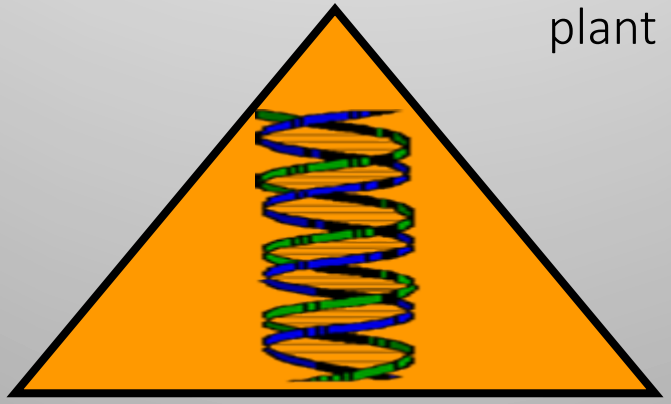
What does this mean?

- “For the purposes of this rule, APHIS is proposing to consider genome editing to be within the definition of genetic engineering. “
- Does that mean every editing line must come before USDA?
- How much data and confinement is required during research and breeding?
- Is it essentially a GMO up until the time of final approval, which should be expedited?

Regulation of gene editing depends on decisions of three agencies – coordinated but distinct

USDA – US Department of Agriculture:

Agricultural products of plant or animal origin



EPA - Environmental Protection Agency:

Biopesticides

FDA - Food & Drug Administration:

Food consumed by humans or animals

FDA is proposing to regulate gene edited animals as animal "drugs" – a very costly and difficult regulatory hurdle to clear

#187

Guidance for Industry

Regulation of Intentionally Altered Genomic DNA in Animals

Draft Guidance

(This guidance is a revision of Guidance #187, "Regulation of Genetically Engineered Animals," which has been revised to update information concerning the products of different technologies used to produce such animals, and to provide new weblinks.)

Submit comments on this draft guidance by the date provided in the *Federal Register* notice announcing the availability of the draft guidance. Submit electronic comments to http://www.regulations.gov. Submit written comments to the Division of Dockets Management (HFA-305), Food and Drug Administration, 5630 Fishers Lane, Room 1061, Rockville, MD 20852. All comments should be identified with the Docket No. FDA-2008-D-0394.

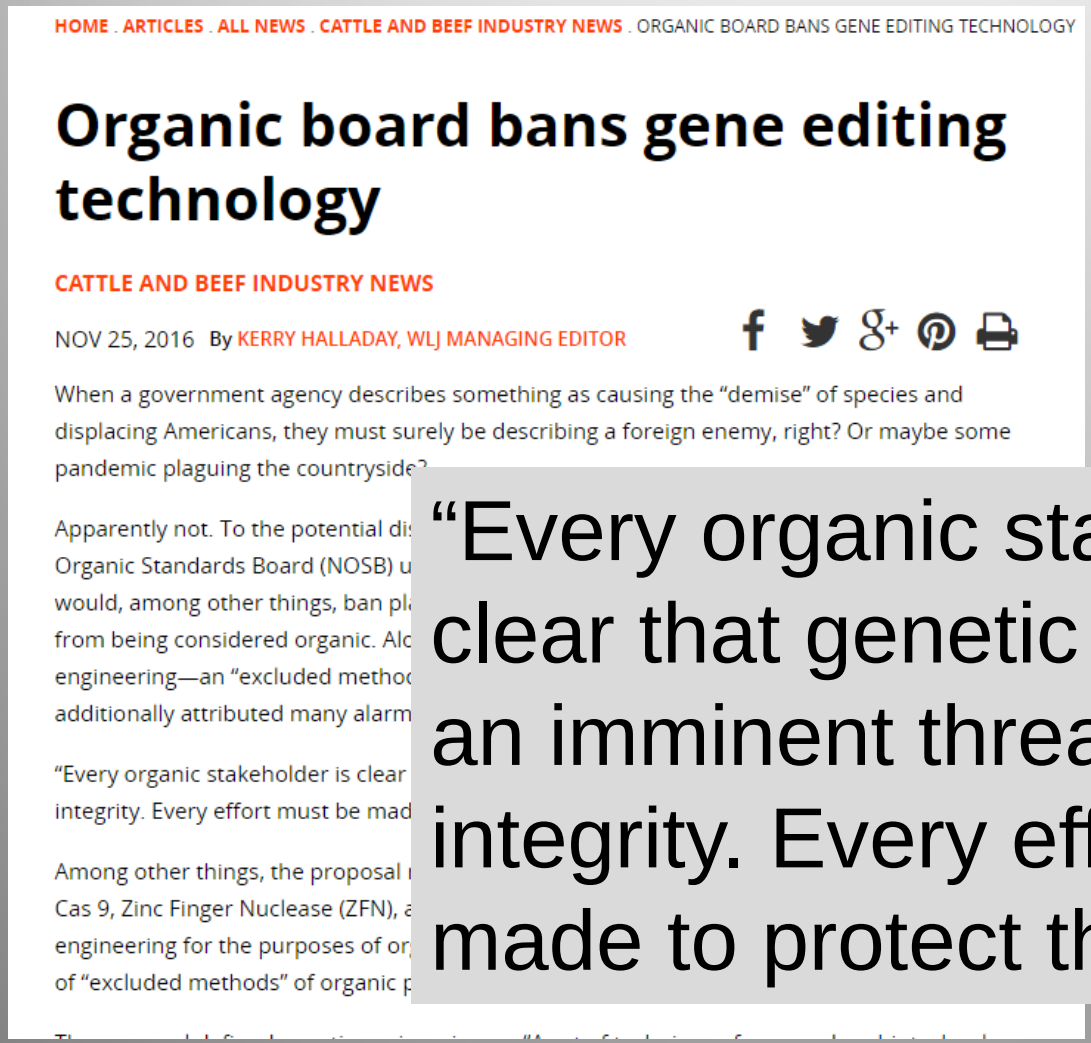
For further information regarding this document, contact Laura R. Epstein, Center for Veterinary Medicine (HFV-1), Food and Drug Administration, 7519 Standish Place, Rockville, MD 20855, 301-796-8558, email: Laura.Epstein@fda.hhs.gov.

Additional copies of this guidance document may be requested from the Policy and Regulations Staff (HFV-6), Center for Veterinary Medicine, Food and Drug Administration, 7519 Standish Place, Rockville, MD 20855, and may be viewed on the Internet at either http://www.fda.gov/AnimalVeterinary/default.htm or http://www.regulations.gov.

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Veterinary Medicine
January 2017

Markets are another thing….

The National Organic Standard Boards has **banned** gene editing technologies



HOME . ARTICLES . ALL NEWS . CATTLE AND BEEF INDUSTRY NEWS . ORGANIC BOARD BANS GENE EDITING TECHNOLOGY

Organic board bans gene editing technology

CATTLE AND BEEF INDUSTRY NEWS

NOV 25, 2016 By KERRY HALLADAY, WLJ MANAGING EDITOR

When a government agency describes something as causing the "demise" of species and displacing Americans, they must surely be describing a foreign enemy, right? Or maybe some pandemic plaguing the countryside?

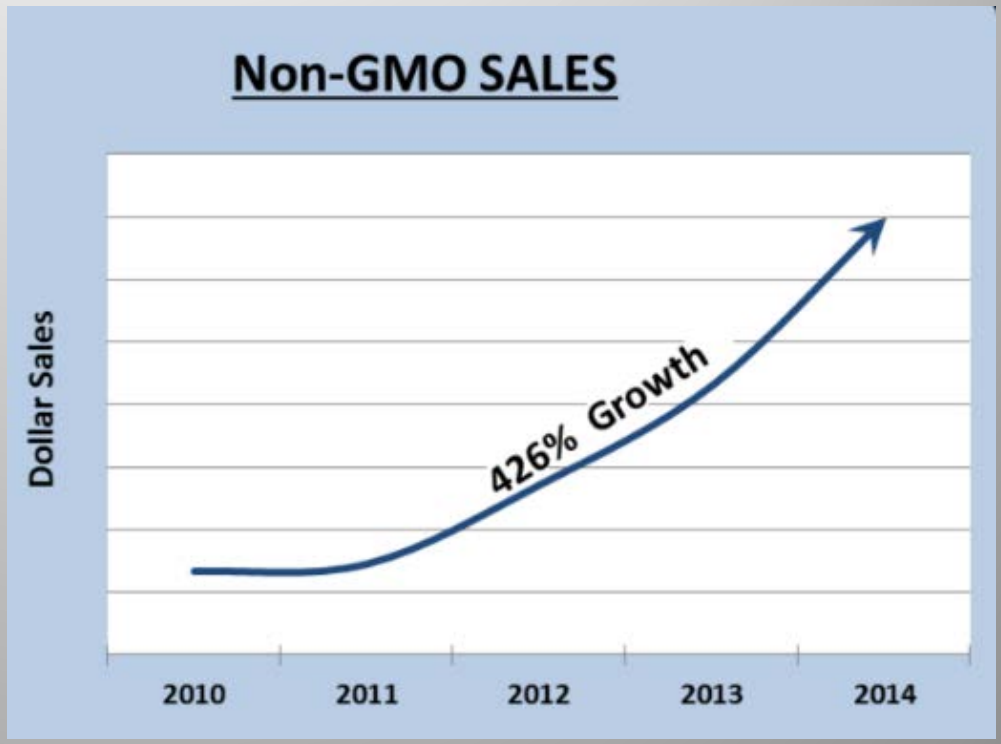
Apparently not. To the potential di
Organic Standards Board (NOSB) u
would, among other things, ban pl
from being considered organic. Alc
engineering—an "excluded metho
additionally attributed many alarm

"Every organic stakeholder is clear
integrity. Every effort must be mad

Among other things, the proposal
Cas 9, Zinc Finger Nuclease (ZFN), a
engineering for the purposes of or
of "excluded methods" of organic p

"Every organic stakeholder is clear that genetic engineering is an imminent threat to organic integrity. Every effort must be made to protect that integrity,"

Proliferation of self-defined no-GMO labels likely to exclude gene editing?



https://www.aphis.usda.gov/stakeholders/downloads/2015/coexistence/Errol-Schweizer.pdf

Summary

- Gene editing works well everywhere its been tried
  - This one is not hype!
- Still depends on capability for GE
  - Difficult in many species and genotypes
  - Methods to avoid insertion complex (protoplasts, RNA-proteins)
- Significant social issues and uncertainties
  - Ethics, regulation, market exclusions, labeling, and patents
  - Key determinants of whether this technology will matter a lot or not so much
- Highly effective in poplar and eucalypts, could be used for many traits beyond sterility
  - Key constraints are science knowledge, genetic engineering capability, and regulation/markets

Who did the work? Flowering research team 2016-17

Cathleen Ma, Transformation & Greenhouse Experiments

Anna Magnuson, Program & Field Manager

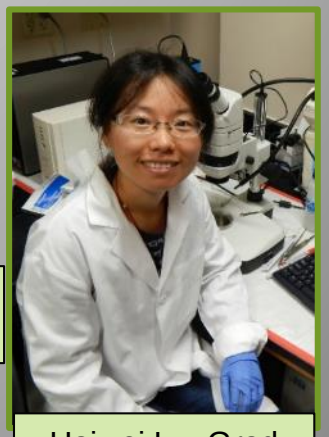
Ximin An, Visiting Scienist, Beijing Forestry University

Emily Helliwell, Post-Doc, Genomics and Bioinformatics

Amy Klocko, Postdoc, Flowering

Estefania Elorriaga, Grad Student, CRISPRs

Michael Nagle, Grad Student, CRISPRs



Haiwei Lu, Grad Student, RNAi

Sarah Higgins, Technician, Floral Analysis

Jeremy Jacobson, Undergraduate Research

Sources of grant support

Futuragene, SAPPI, SweTree, U. Pretoria, Arborgen