Enabling GMOs in forestry
CRISPRs as tools to promote coexistence

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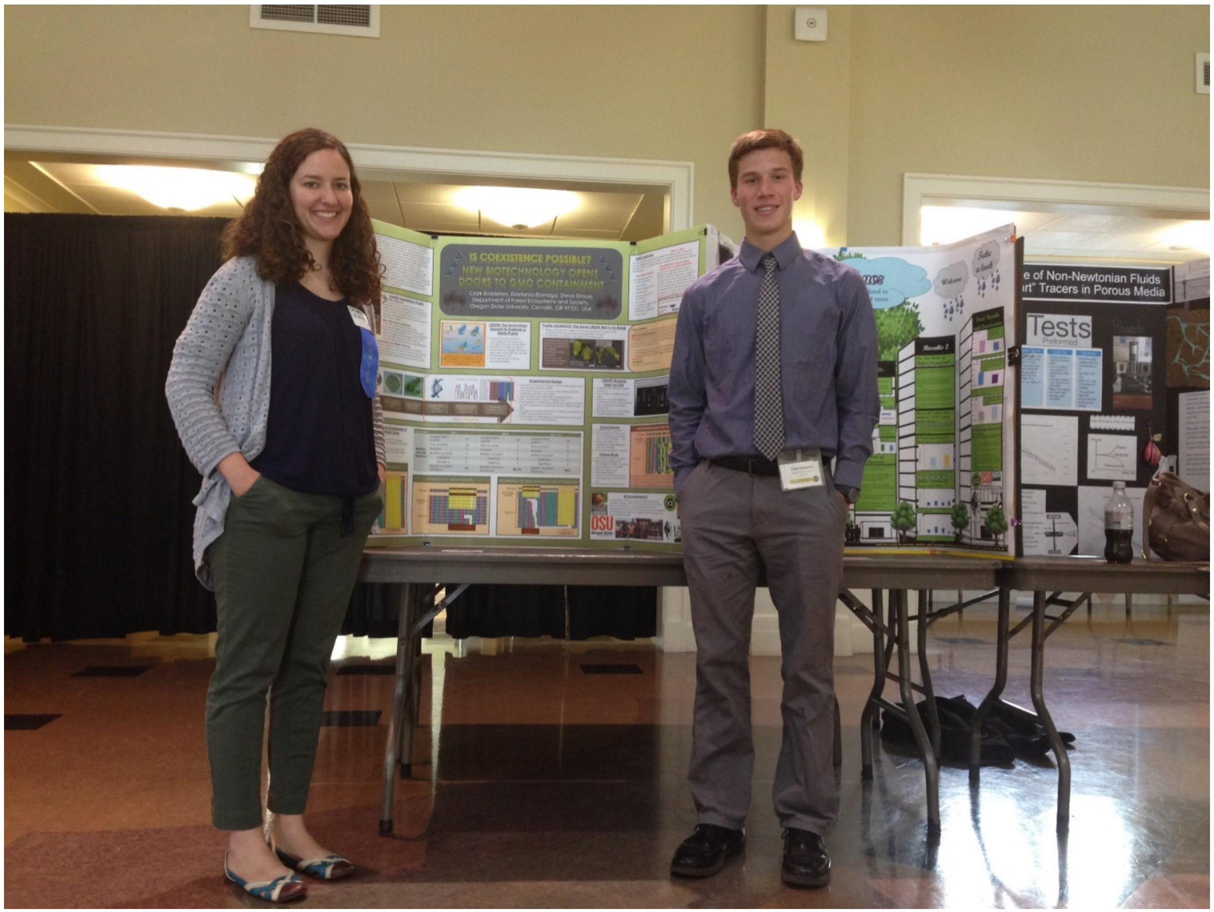

USDA

United States
Department of
Agriculture

National Institute
of Food and
Agriculture

TBGRC

Sappi, Arborgen, Futuragene
Swetree, U. Pretoria

NSF

The National
Science Foundation

Advanced Hardwood Biofuels Northwest

Roadmap

- Why gene flow is an immense problem
- Evolving technology options - CRISPR/Cas9 to the rescue?
- Progress in making it work for poplar trees

Gene flow is ubiquitous in agriculture and forestry – with or without GMOs – pollen, seed, and vegetative

Slides courtesy of Wayne Parrott, Univ. Georgia

Gene flow tends to be greater for forest trees vs. ag crops

Molecular Ecology (2009) **18**, 357–373

doi: 10.1111/j.1365-294X.2008.04016.x

Extensive pollen flow in two ecologically contrasting populations of *Populus trichocarpa*

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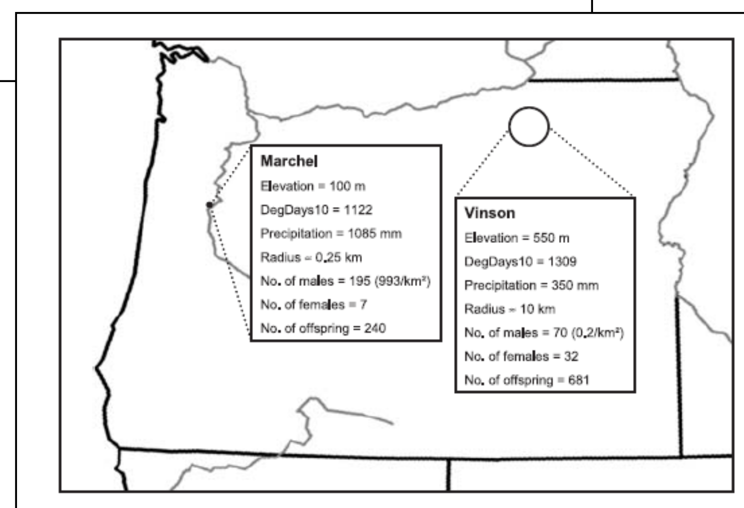


In poplar, paternity analsysis showed that ~50% of pollen comes from >1 km to >10 km

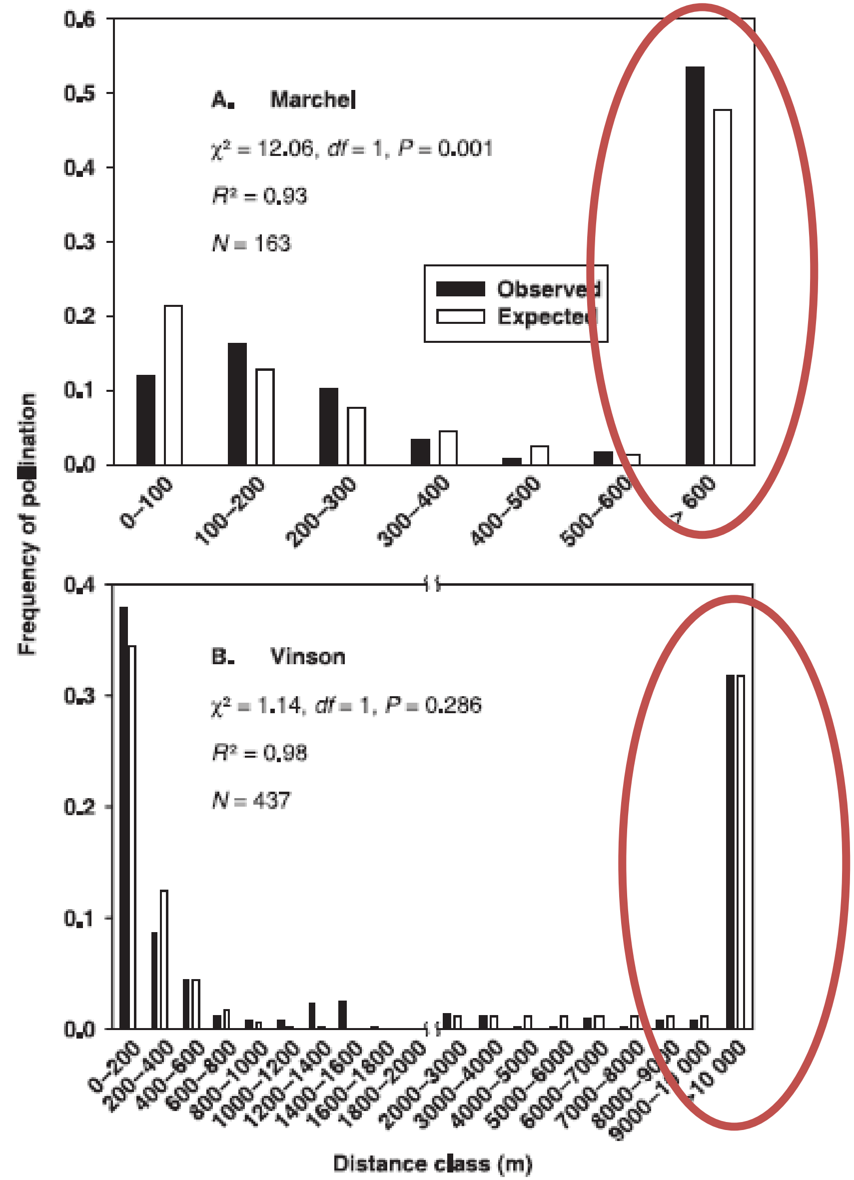


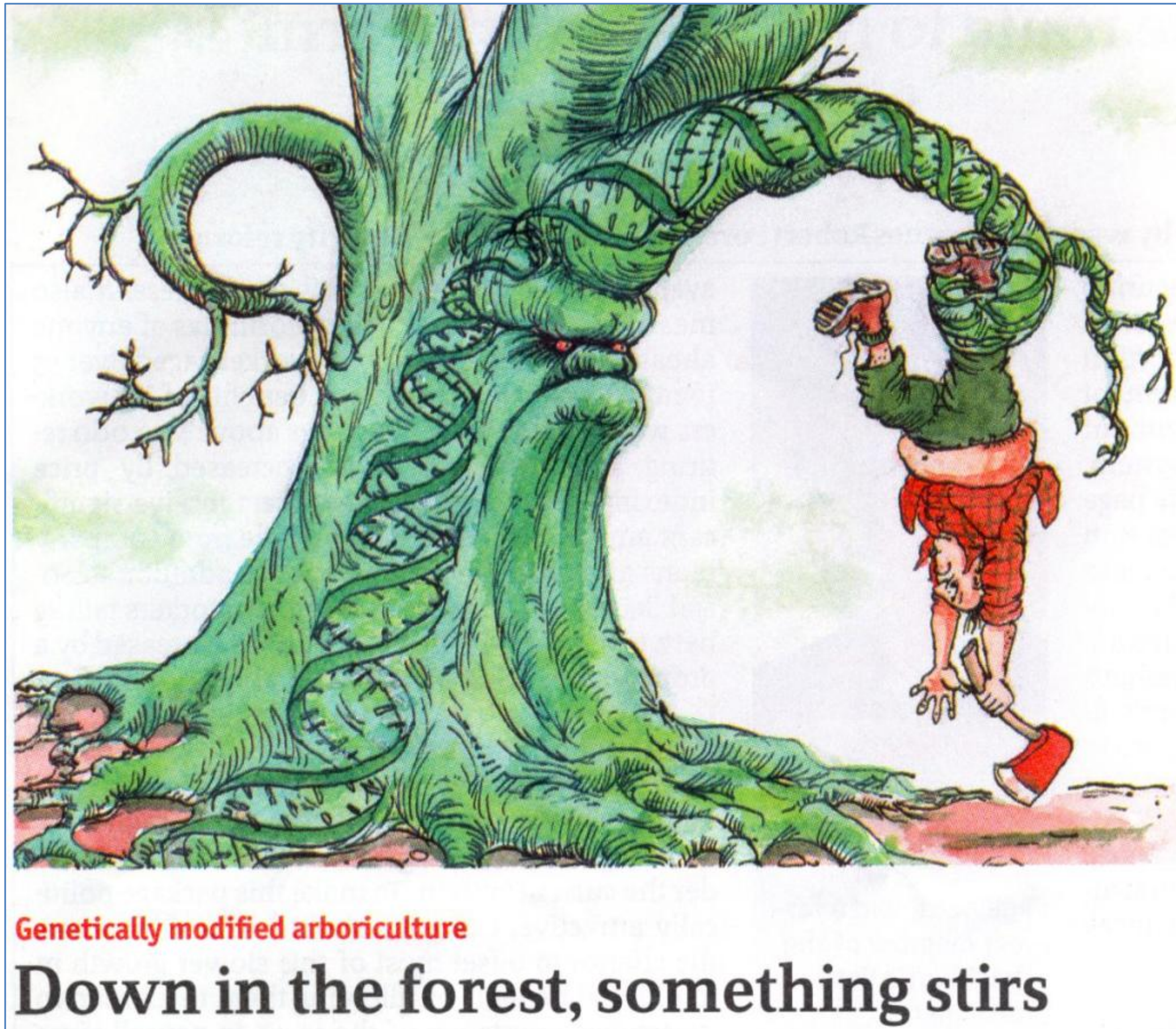
Fig. 5 Observed vs. expected pollination frequencies based on predictions from a mixed probability density function, whose parameters were estimated based on paternity analysis data.

Seeds can fly, float, and be carried far too

Tree gene flow extensive

- Long distances
  - Wind, insect, and animal pollinated
  - Wind and animal seed dispersal
- Less domesticated than many crops – establishment and persistence in wild expected
- Ecological impacts may be large
  - Often keystone species – ecologically dominant so with potential effects on many other organisms
- Regulatory and social approval challenging
  - Difficult to estimate effects, fitness during contained field studies
  - Ethical discomfort at ~irreversiby modifying wild organisms

Forest trees with significant anti-GMO activism



The Economist, 2005

Market barriers large

"Green" certification of forests create severe barriers to field research, markets

Plantation Certification & Genetic Engineering

FSC's Ban on Research Is Counterproductive

Steven H. Strauss, Malcolm M. Campbell, Simon N. Pryor, Peter Coventry, and Jeff Burley

ABSTRACT

Genetic engineering, also called genetic modification (GM), is the isolation, recombinant modification, and asexual transfer of genes. It has been banned in forest plantations certified by the Forest Stewardship Council (FSC) regardless of the source of genes, traits imparted, or whether for research or commercial use. We review the methods and goals of tree genetic engineering research and argue that FSC's ban on research is counterproductive because it makes it difficult for certified companies to participate in the field research needed to assess the value and biosafety of GM trees. Genetic modification could be important for translating new discoveries about tree genomes into improved growth, quality, sustainability, and pest resistance.

Keywords: biotechnology; entomology and pathology; ethics; genetics; silviculture

Genetic engineering, commonly called genetic modification (GM) in much of the world, is the use of recombinant DNA and asexual gene transfer methods to breed more productive or pest-resistant crops. It has been the subject of considerable controversy, with concerns raised from biological, socioeconomic, political, and ethical perspectives. Some of the issues are similar to those raised by the use of molecular biology and genetic engineering in medicine, which we see in the news headlines daily. However, genetic modification in agriculture and forestry raises environmental issues as well.

GM crops, mainly herbicide- and pest-resistant varieties of soybeans, maize, or cotton, have been vigorously adopted by farmers in North America because they are easy to manage and they improve yields, reduce costs, or reduce pesticide ecotoxicity (Carpenter and Gianessi 2001). However, the controversy, primarily embodied in regulatory barriers to trade of GM crops with Europe and Japan, has slowed their adoption considerably in recent years.

If GM trees are used in forestry in the near future, they are likely to occur primarily in intensively managed environments, such as urban forests or plantations. In urban forestry, genetic modification is expected to help trees adapt to the stresses and special demands of human-dominated systems. Examples would be trees that are more tolerant of heavy metals or other pollutants, resist urban pests or diseases, grow slower, or do not produce fruits when these create hazards in street environments (Brunner et al. 1998).

Plantations, although very different from natural forests in structure and function, are considered part of the spectrum of methods in sustainable forest management (Romm 1994). Plantations can relieve pressure on natural forests for exploitation and can be of great social value by supplying community and industrial wood needs and fueling economic development. The environmental role of plantations is recognized by the Forest Stewardship Council (FSC), an international body for certification of sustainably managed forests. FSC Principle 10 states that plantations should "complement the management of, reduce pressures on, and promote the restoration and conservation of natural forests" (FSC 2001).

FSC has certified some of the most intensively managed plantations in the world, including poplar plantations and the intensive pine and eucalypt plantations of the Southern Hemisphere. Although many environmental mitigations are built into these certified plantation systems, within the areas dedicated to wood production they function as tree farms. Such intensive plantation systems often use highly bred genotypes, possibly including exotic species, hybrids, and clones, as well as many other forms of intensive silvicultural management. It is in the context of these biointensive systems that the additional expense of GM trees is likely to be worthwhile.

However, FSC currently prohibits all uses of GM trees, and is the only certification system to have done so

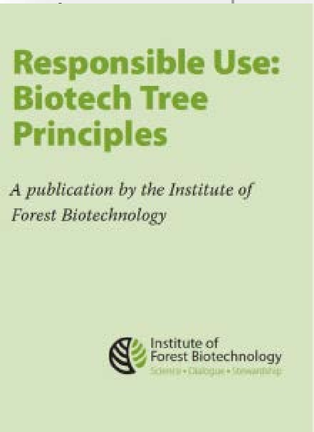
FSC

Forest Stewardship Council

"…genetically modified trees are prohibited…"

Forest certification systems universally ban all GM trees – no exemptions

System	Region	GM Tree Approach / Reason
PEFC : Programme for Endorsement of Forest Certification	International	**Banned** / Precautionary approach based on lack of data
FSC : Forest Stewardship Council	International	**Banned** / Precautionary approach based on lack of data
CerFlor : Certificação Florestal	Brazil	**Banned** via PEFC registration / No additional rationale
CertFor : Certficación Forestal	Chile	**Banned** via PEFC registration / No additional rationale
SFI : Sustainable Forestry Initiative	North America	**Banned** via PEFC registration / Awaiting risk-benefit data
ATFS : American Tree Farm System	USA	**Banned** via PEFC registration / No additional rati
CSA : Canadian Standards Association	Canada	**Banned** via PEFC regi Allows public to determi
CFCC : China Forest Certification Council	China	**Banned** via PEFC regi No additional rati



Adam Costanza, Institute for Forest Biotechnology

International treaties used to push for stringent regulations

Strangled at birth? Forest biotech and the Convention on Biological Diversity

Steven H Strauss, Huimin Tan, Wout Boerjan & Roger Sedjo

Against the Cartagena Protocol and widespread scientific support for a case-by-case approach to regulation, the Convention on Biological Diversity has become a platform for imposing broad restrictions on research and development of all types of transgenic trees.

The Convention on Biological Diversity (CBD) has become a major focus of activist groups that wish to ban field research and commercial development of all types of genetically modified (GM) trees. Recent efforts to influence CBD recommendations by such groups has led to the adoption of recommendations for increased regulatory stringency that are inconsistent with the views of most scientists and most of the major environmental organizations. We suggest that the increasingly stringent recommendations adopted by the CBD in recent years are impeding, and in many places may foreclose, much of the field research needed to develop useful and safe applications of

A convention co-opted

Negotiated under the United Nations (UN) Environment Program, CBD was adopted in June 1992 and subsequently entered into force in December 1993. The CBD has been signed by 191 of the 192 members of the UN, making it one of the largest international treaties. The aim of the CBD is to promote the conservation and sustainable use of biodiversity, and the fair and equitable sharing of benefits from the use of genetic resources. Because transgenic organisms have the potential to affect biodiversity, special provisions of the CBD cover the use and trade in living modified organisms (LMOs, also known as genetically modified organisms; GMOs).

In 2000, the Cartagena Protocol on Biosafety … mandate in the CBD

NATURE BIOTECHNOLOGY VOLUME 27 NUMBER 6 JUNE 2009

Need both technical and policy solutions (August 2015, Science)

Traces of the emerald ash borer on the trunk of a dead ash tree in Michigan, USA. This non-native invasive insect from Asia threatens to kill most North American ash trees.

BIOTECHNOLOGY

Genetically engineered trees: Paralysis from good intentions

Forest crises demand regulation and certification reform

By **Steven H. Strauss[1*], Adam Costanza[2], Armand Séguin[3]**

Intensive genetic modification is a long-standing practice in agriculture, and, for some species, in woody plant horticulture and forestry (*1*). Current regulatory systems for genetically engineered

recently initiated an update of the Coordinated Framework for the Regulation of Biotechnology (*2*), now is an opportune time to consider foundational changes.

Difficulties of conventional tree breeding make genetic engineering (GE) methods relatively more advantageous for forest trees than for annual crops (*3*). Obstacles

Although only a few forest tree species might be subject to GE in the foreseeable future, regulatory and market obstacles prevent most of these from even being subjects of translational laboratory research. There is also little commercial activity: Only two types of pest-resistant poplars are authorized for commercial use in small areas in China and two types of eucalypts, one approved in Brazil and another under lengthy review in the USA (*5*).

METHOD-FOCUSED AND MISGUIDED. Many high-level science reports state that the GE method is no more risky than conventional breeding, but regulations around the world essentially presume that GE is hazardous and requires strict containment

Forthcoming related essay in Forestry Source in November

Roadmap

- Why gene flow is an immense problem
- **Evolving technology options - CRISPR/Cas9 to the rescue?**
- Progress in making it work for poplar trees

Many options for containment technologies – V-GURTs

Plant Biotechnology Journal

Plant Biotechnology Journal (2014), pp. 1–11 doi: 10.1111/pbi.12242

Review article

Genetic use restriction technologies: a review

Luca Lombardo*

Department of Crop Systems, Forestry and Environmental Sciences, University of Basilicata, Potenza, Italy

Received 9 March 2014;
revised 16 July 2014;
accepted 17 July 2014.
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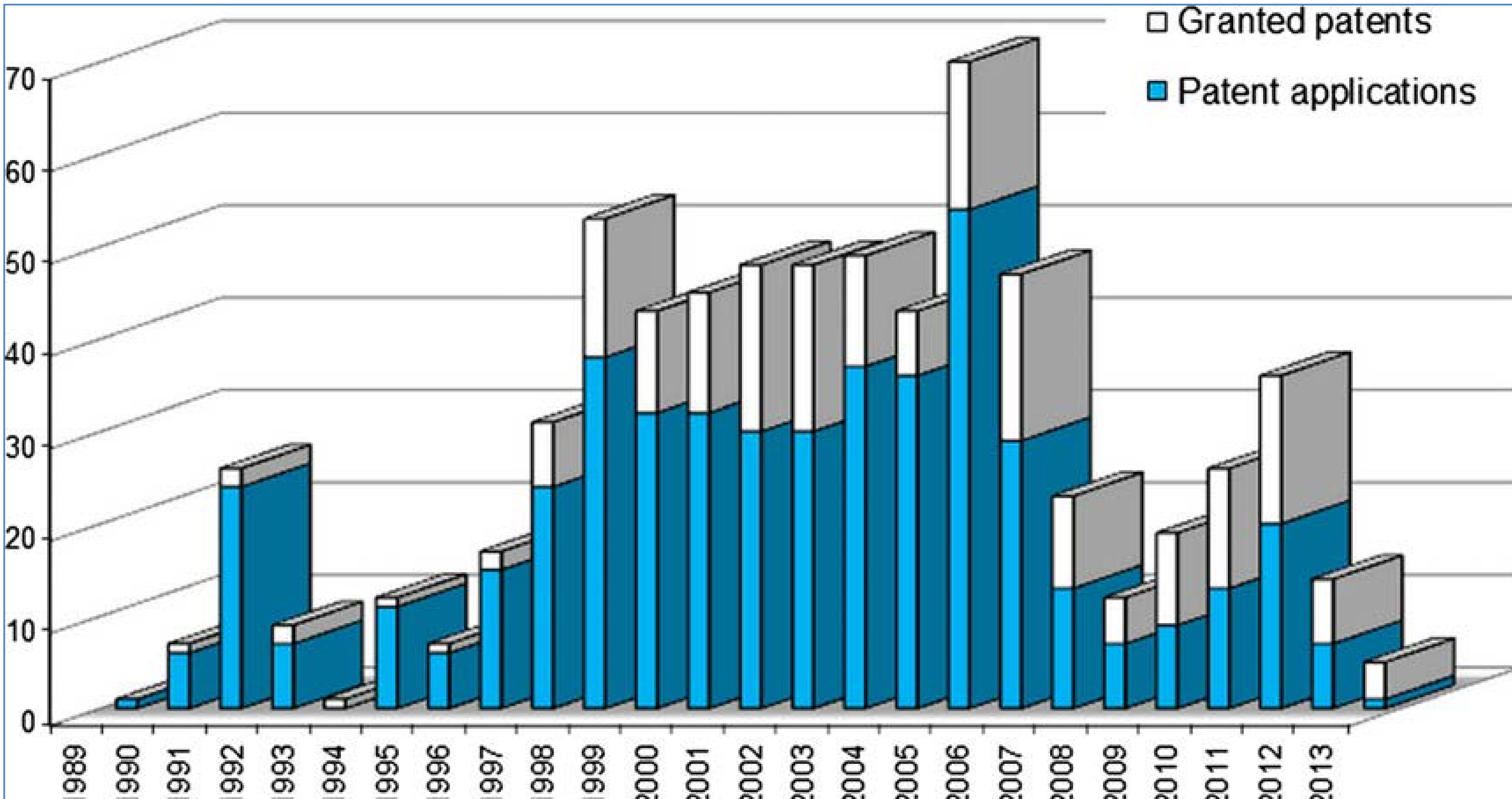
Keywords: V-GURTs, T-GURTs, intellectual property, seed saving.

Summary

Genetic use restriction technologies (GURTs), developed to secure return on investments through protection of plant varieties, are among the most controversial and opposed genetic engineering biotechnologies as they are perceived as a tool to force farmers to depend on multinational corporations' seed monopolies. In this work, the currently proposed strategies are described and compared with some of the principal techniques implemented for preventing transgene flow and/or seed saving, with a simultaneous analysis of the future perspectives of GURTs taking into account potential benefits, possible impacts on farmers and local plant genetic resources (PGR), hypothetical negative environmental issues and ethical concerns related to intellectual property that have led to the ban of this technology.

Investment in GURTs have rapidly declined, little field research, no commercial use to date



Lombardo 2014 / Plant Biotechnology Journal

Unpopularity of gene flow restriction technologies

"The Destruction of Our Food - GMO and Terminator Seeds….

"Ever since I found out about terminator seeds, I have understood how famine could take over the planet as predicted in the Bible."

'TERMINATOR'

Focus on genetic containment via complete bisexual sterility – vegetative propagation, vegetative harvest – poplar, eucalypts, pine

Options for genetic containment via complete, constitutive, bisexual sterility

- Controlled cell/tissue *ablation*
  - Floral developmental promoter driving cell toxin
- Floral gene malfunction
  - RNA suppression (RNAi)
  - Protein disruption (dominant negative)
  - Directed gene mutation (ZFN, TALEN, CRISPR)

Site directed mutagenesis might be an ideal method for containment

- Reported highly efficient – biallelic mutations achievable?
  - Complete loss of gene function without inbreeding
- Physical damage to floral gene/s should be far more reliable than modified/suppressed gene expression or protein function
- More predictable from new regenerant to flowering tree to speed breeding, avoid regulatory problems
- Inducible recombinases enable asexual removal if needed?

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Current Opinion in Biotechnology

CrossMark

Editing plant genomes with CRISPR/Cas9

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

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

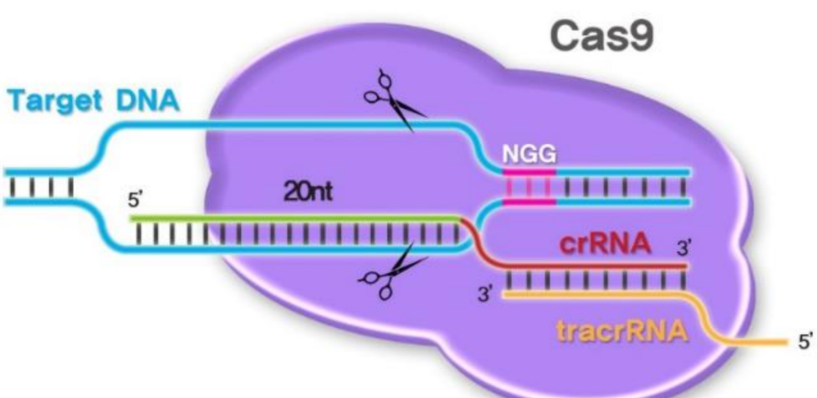
Current Opinion in Biotechnology 2015, 32:76–84

“CRISPR/Cas9 is a game-changing technology that is poised to revolutionise basic research and plant breeding.”

What are CRISPR-Cas systems?

- 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



Synthetic nuclease system

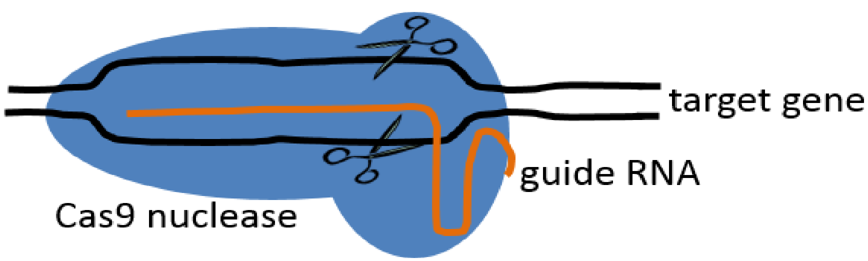


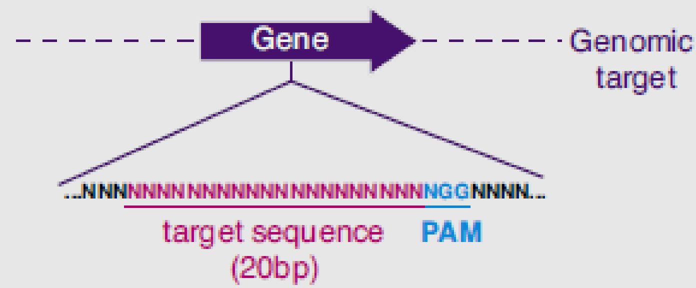
Image credit: http://pnabio.com/products/RGEN.htm

Overview: CRISPR/Cas9 construct creation

1. Select genomic target

a. 20 bp sequence followed by the PAM (NGG)

b. Use online tools to minimize off-targeting

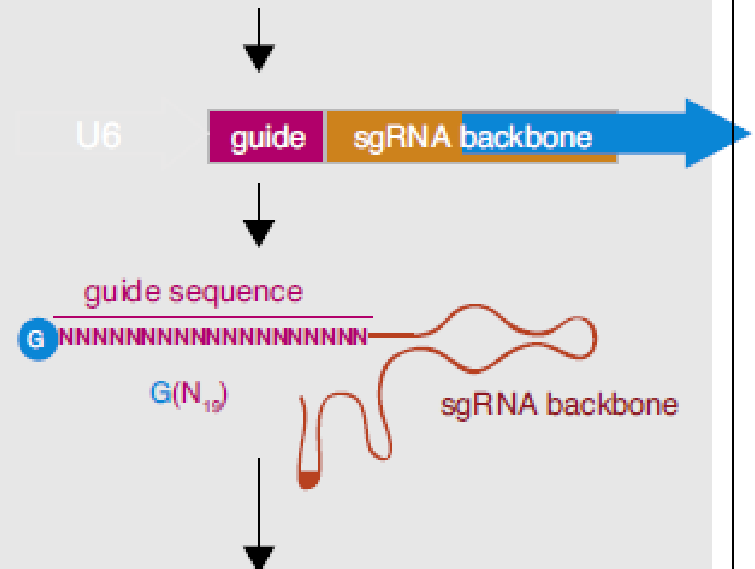


2. Design sgRNA

a. sgRNA is expressed using a small RNA promotor, such as U6p or U3p

b. First nucleotide in the guide sequence is a “G”, if U6p is used, or an “A”, if U3p is used

c. Guide sequence should match the target, except for the first nucleotide (5’ G or A) that does not have to match



3. Assemble Cas9 / sgRNA construct

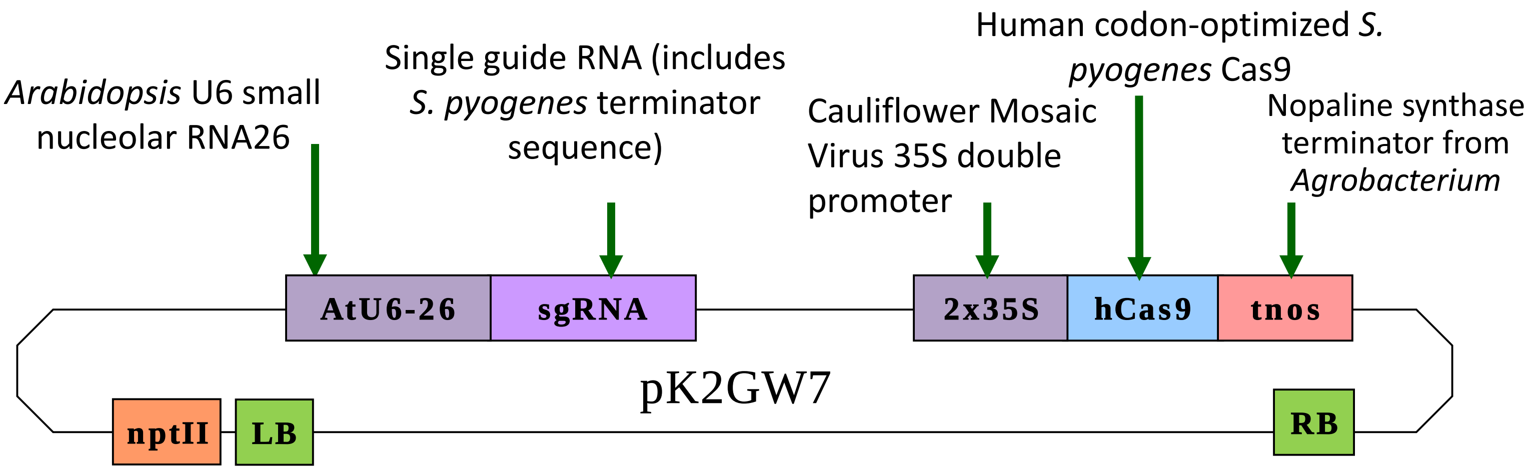
Belhaj et al, Current Opinion in Biotechnology 2015, 32:76–84

Roadmap

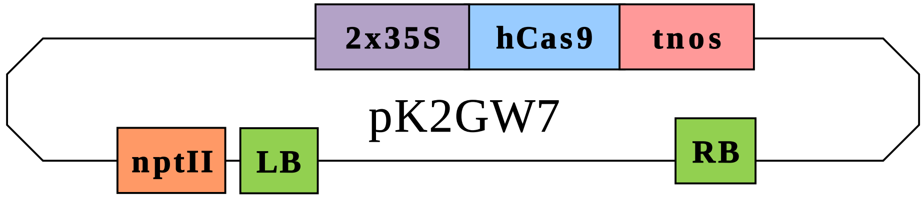
- Why gene flow is an immense problem
- Evolving technology options - CRISPR/Cas9 to the rescue?
- **Progress in making it work for poplar trees**

CRISPR-Cas construct maps

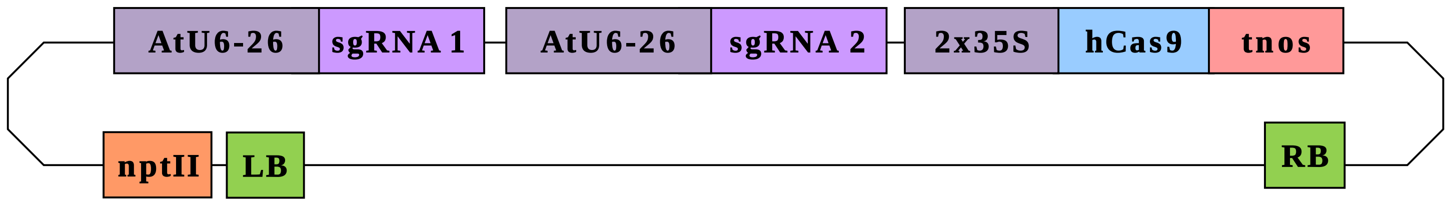
- Nuclease constructs



- Control construct

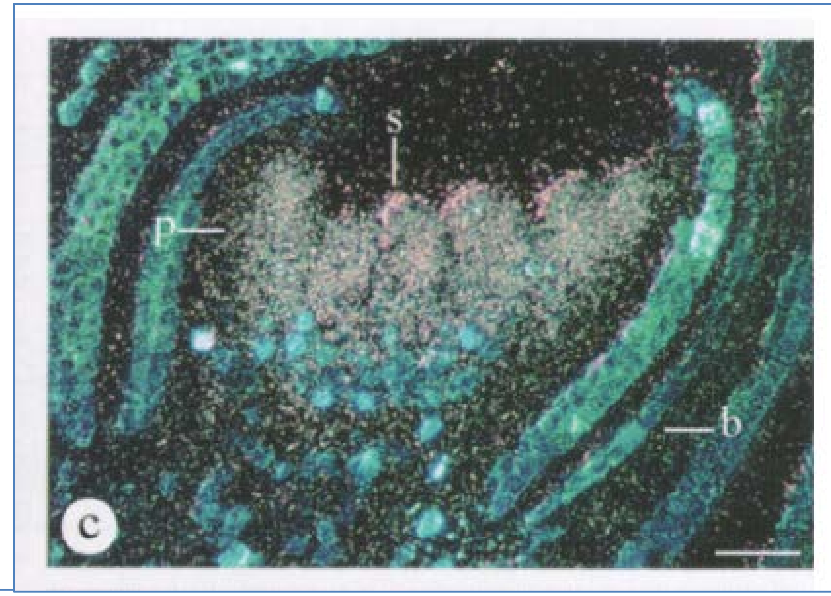


Double gRNA CRISPR/Cas construct for generating deletions



Gene targets *LEAFY* and *AGAMOUS*

Structure & expression in poplar studied previously



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

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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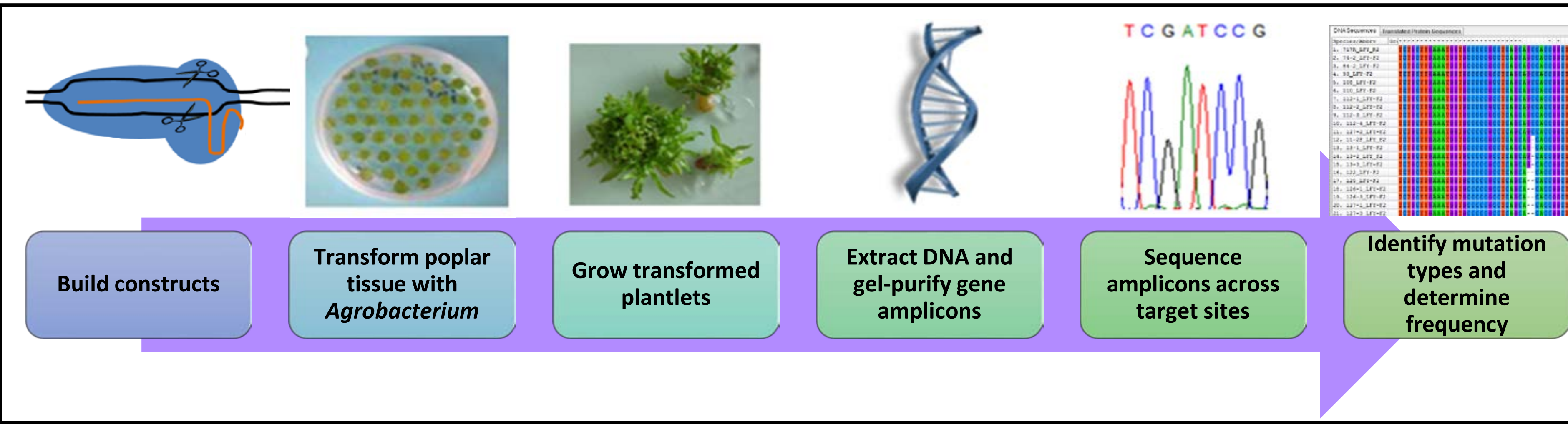
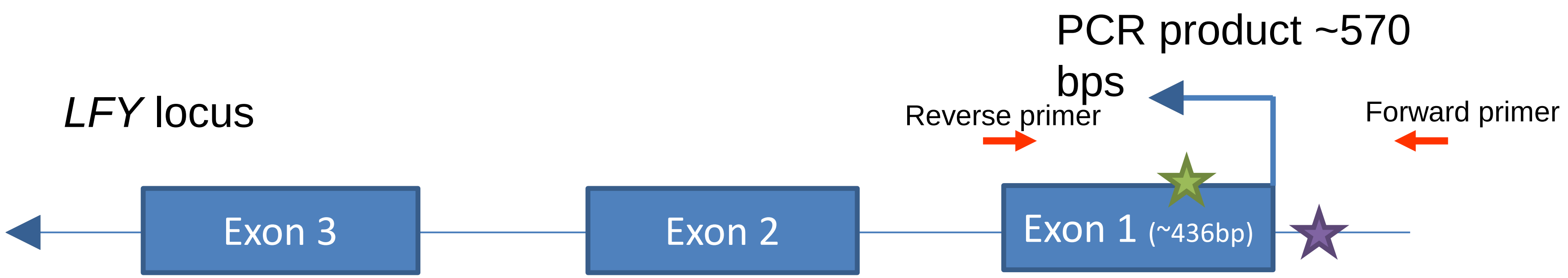
Work flow



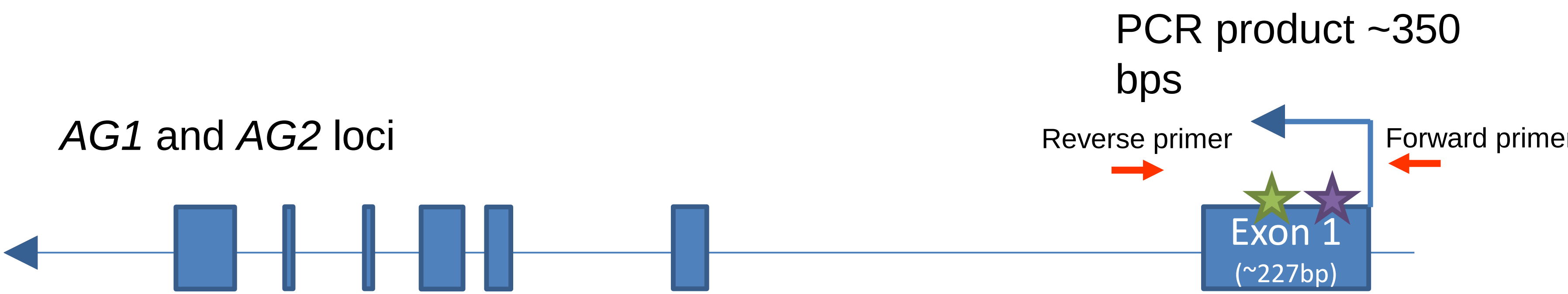
T C G A T C C G
Build constructs
Transform poplar tissue with *Agrobacterium*
Grow transformed plantlets
Extract DNA and gel-purify gene amplicons
Sequence amplicons across target sites
Identify mutation types and determine frequency

PCR amplification for mutation detection: *Pt-LFY*



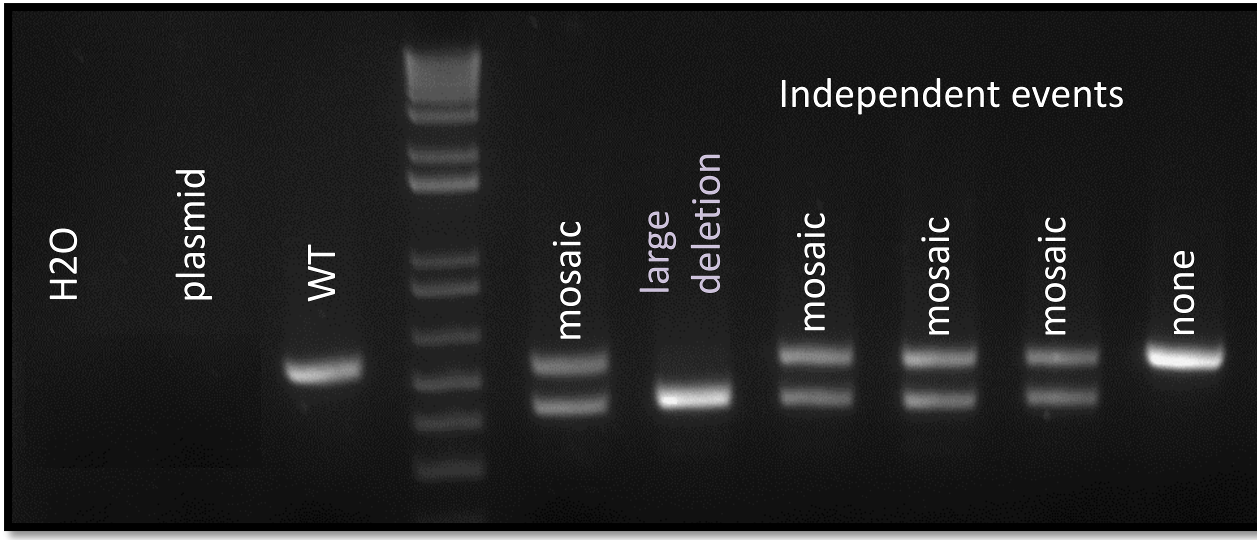
- Distance between forward primer and first target ( ): 70 bps
- Distance between first ( ) and second ( ) target: 120 bps
- Distance between second target ( ) and reverse primer: 313 bps

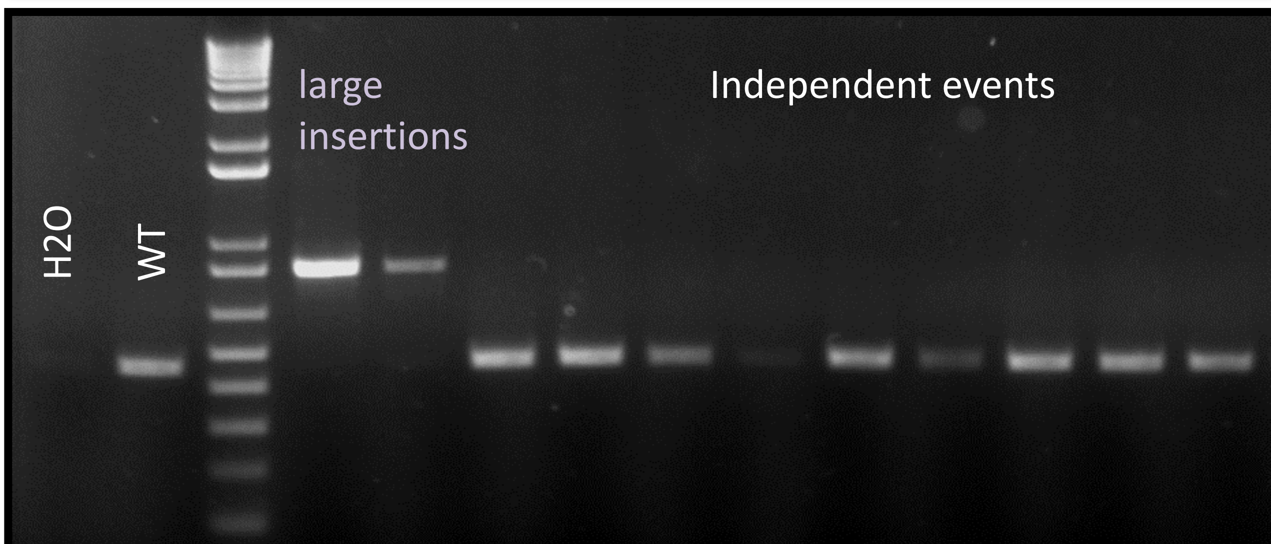
PCR amplification for mutation detection: *Pt-AG*



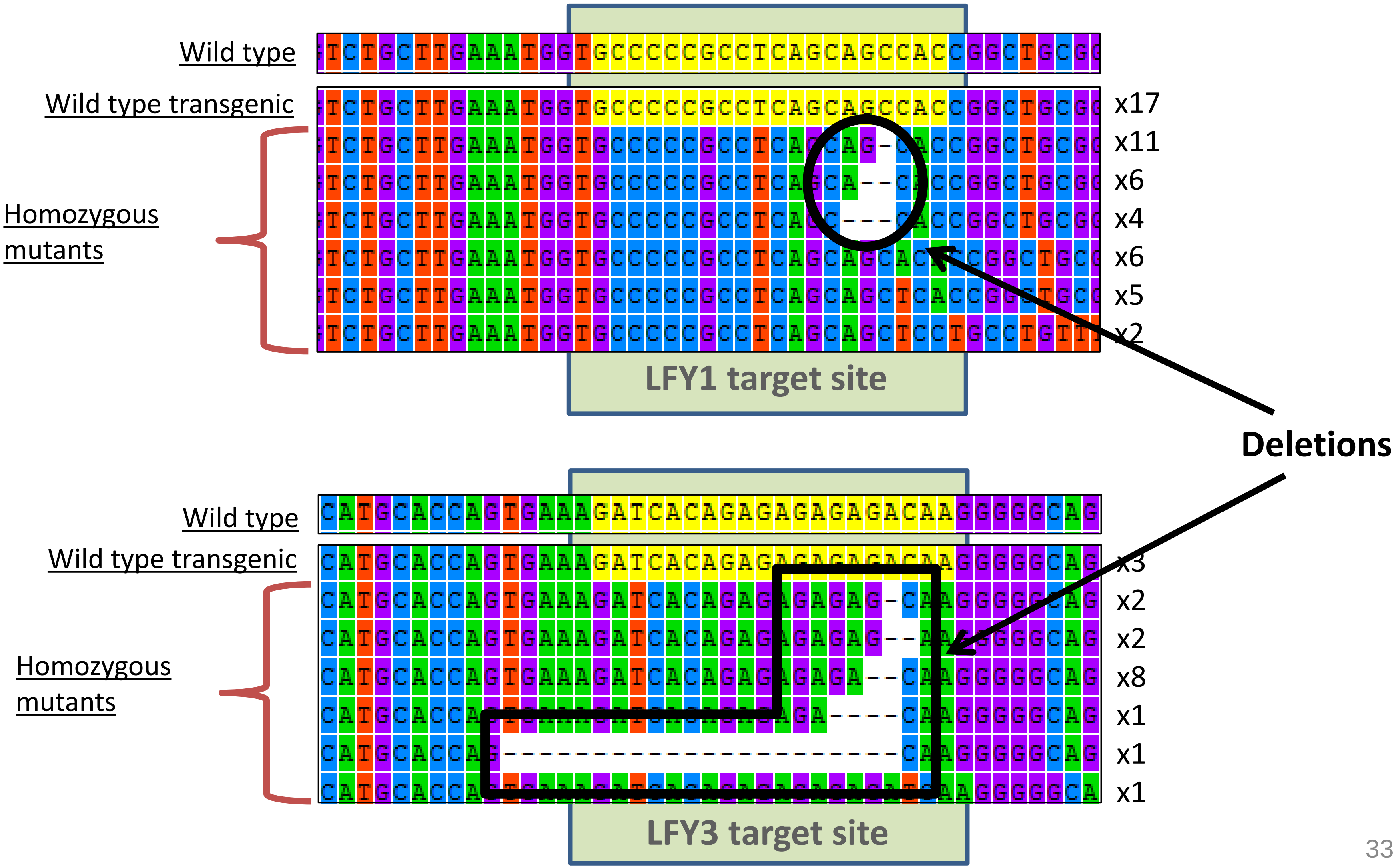
- Distance between forward primer and first target (★): 70 bps
- Distance between first (★) and second (★) target: 42 bps
- Distance between second target (★) and reverse primer: 174 bps

Gel analysis: Large mutations easy to spot

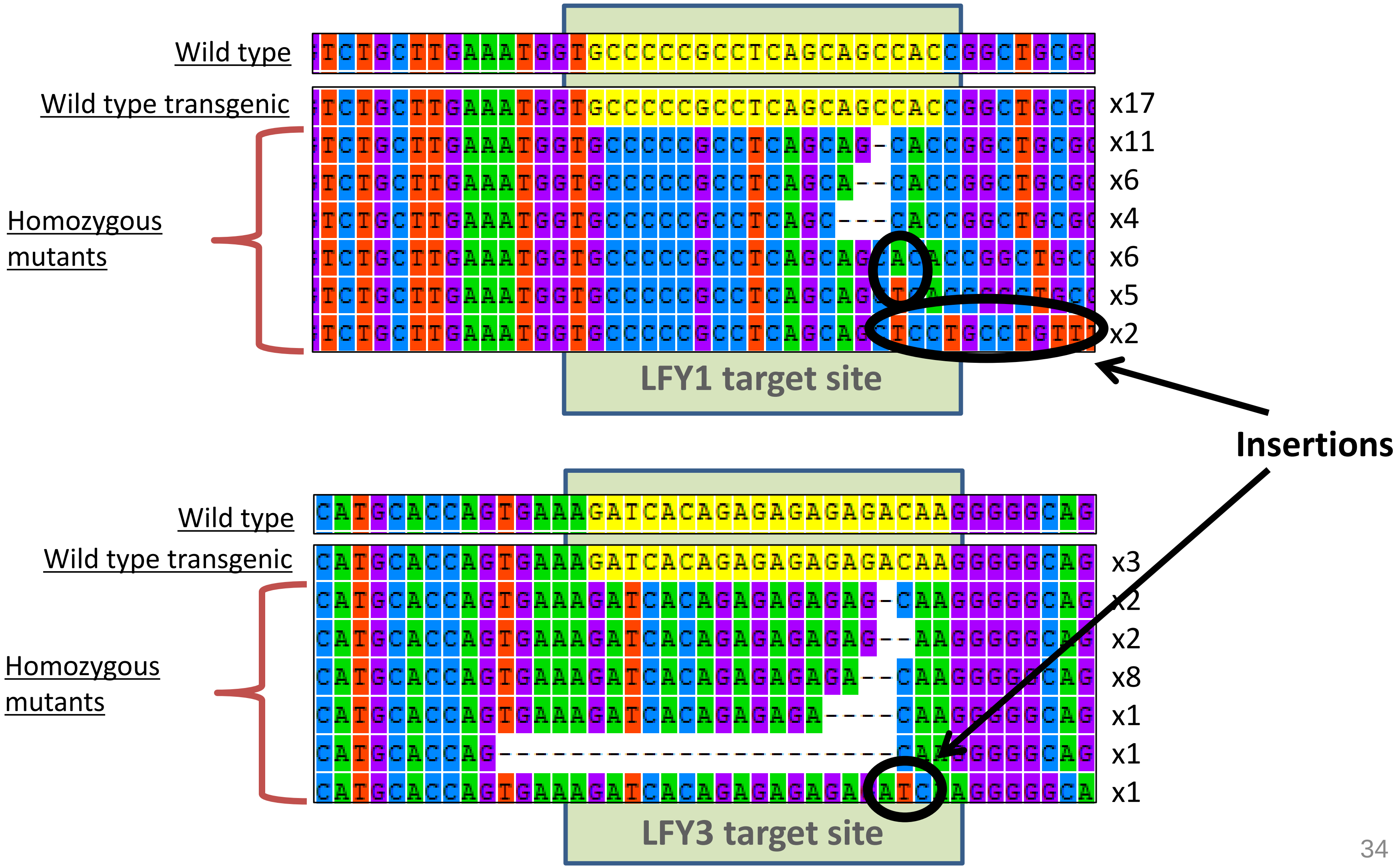




Many deletions

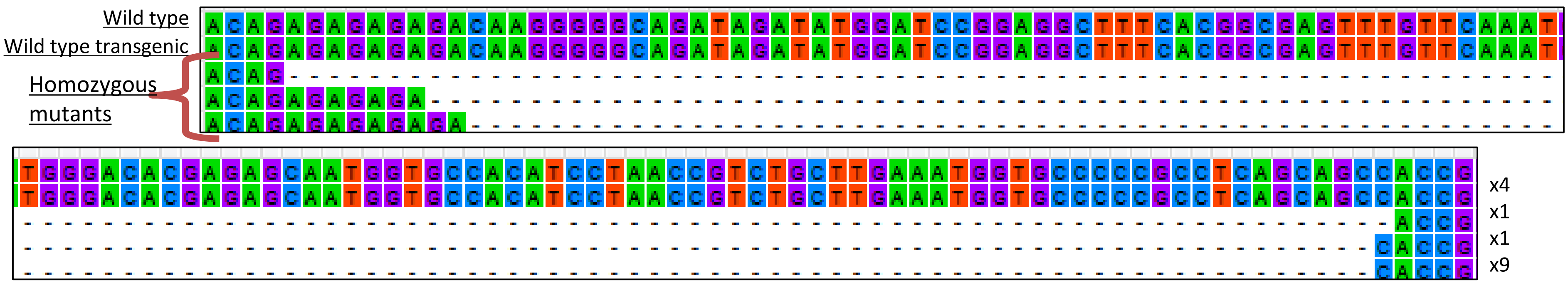


Many insertions

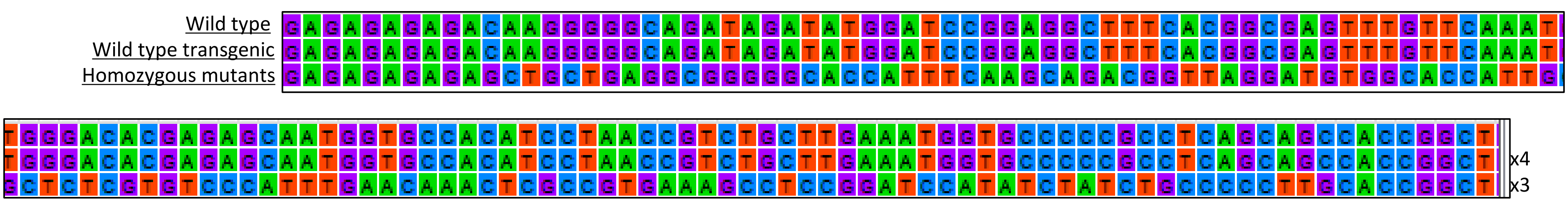


Double *LFY* CRISPR leads to large deletions and also inversions

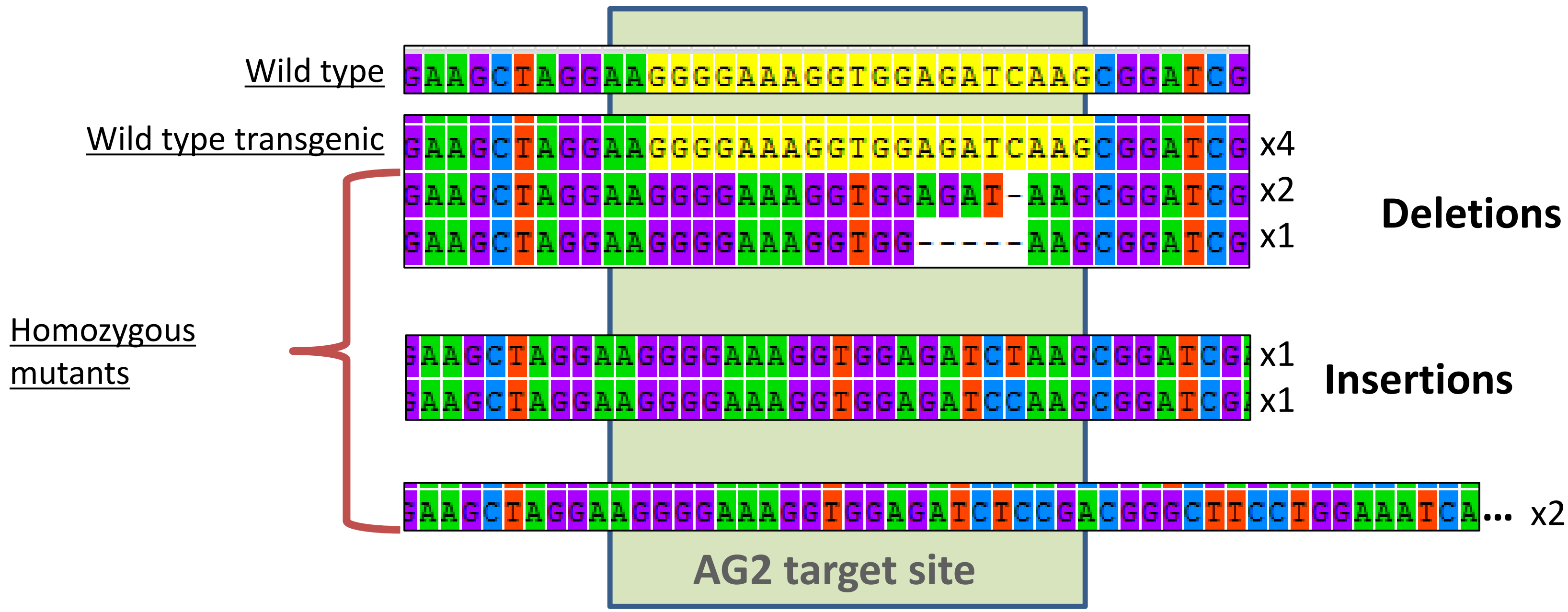
Large deletions



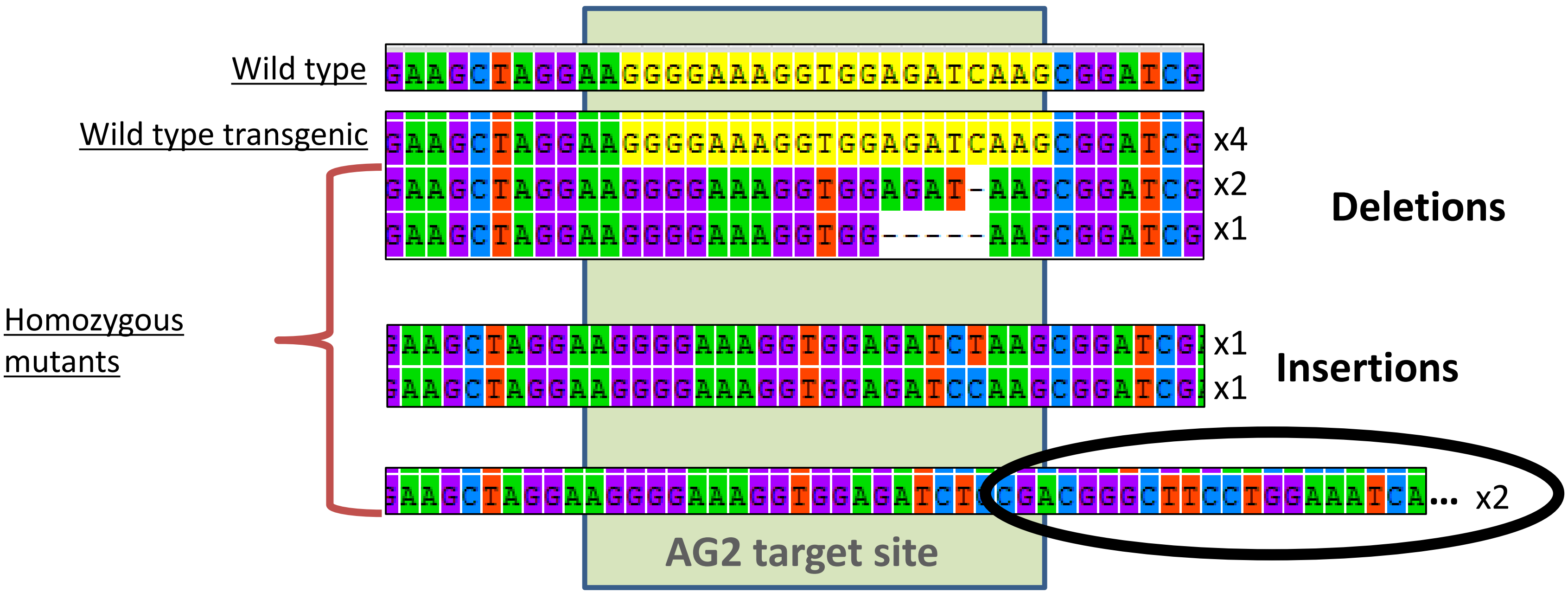
Inversion



Many mutants seen at one *AGAMOUS* target site

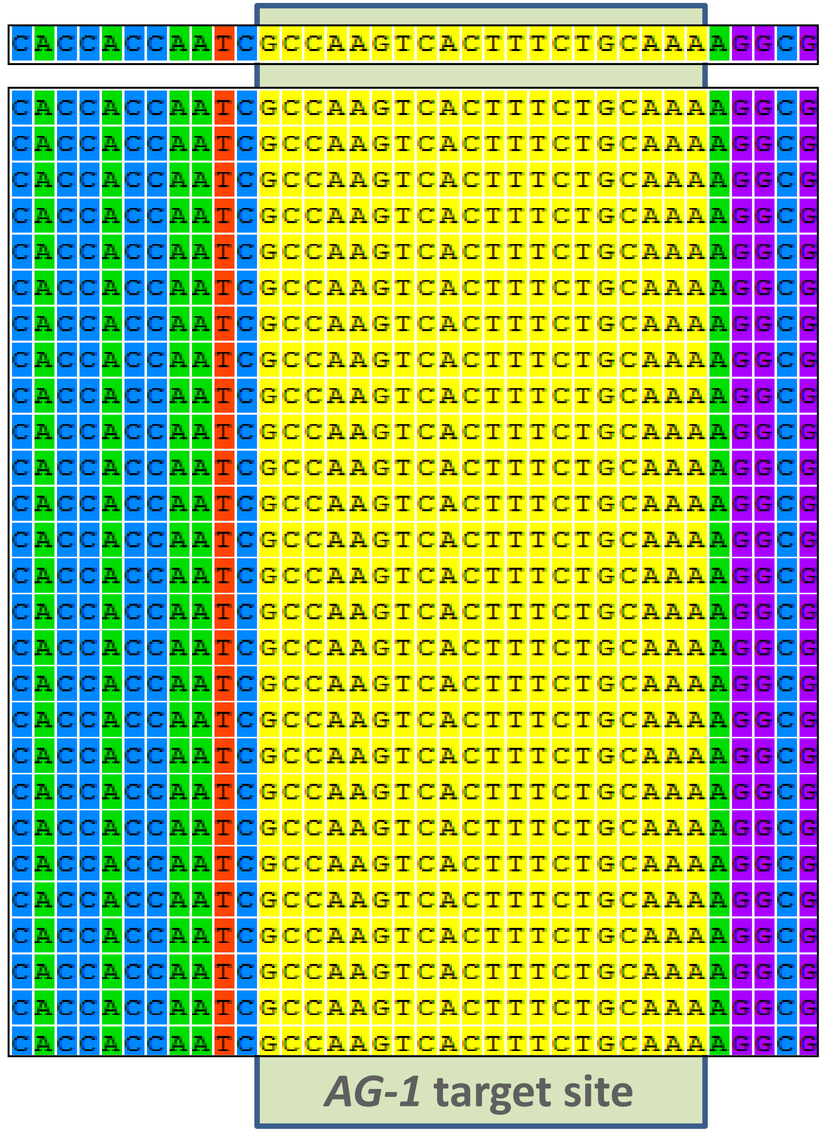


Two >400bp insertions seen to date



Very low mutation rate at other AG target site

Wild type

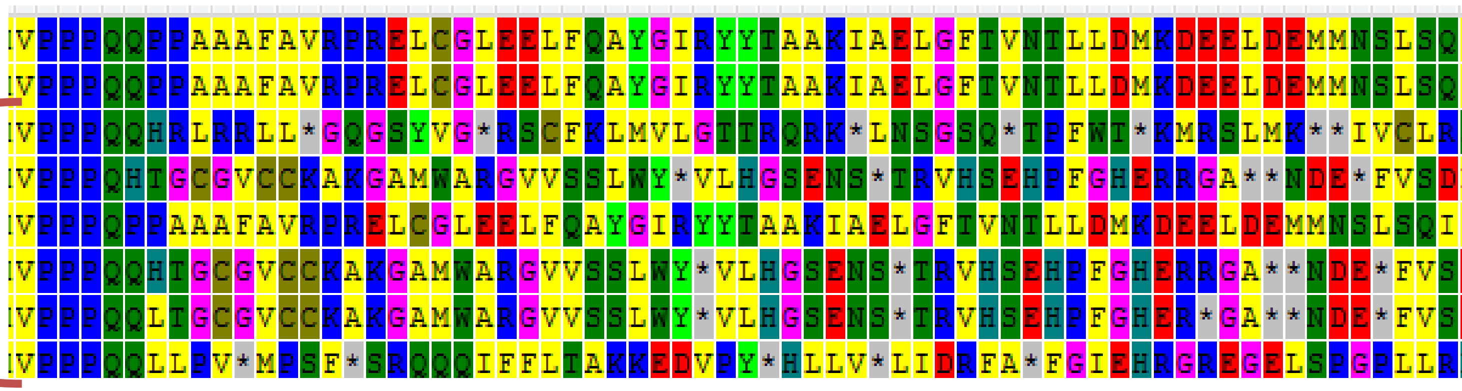


Most *LFY* mutations have completely disturbed the final protein

Partial LEAFY peptide sequence

Wild type
Wild type transgenic

Homozygous mutants



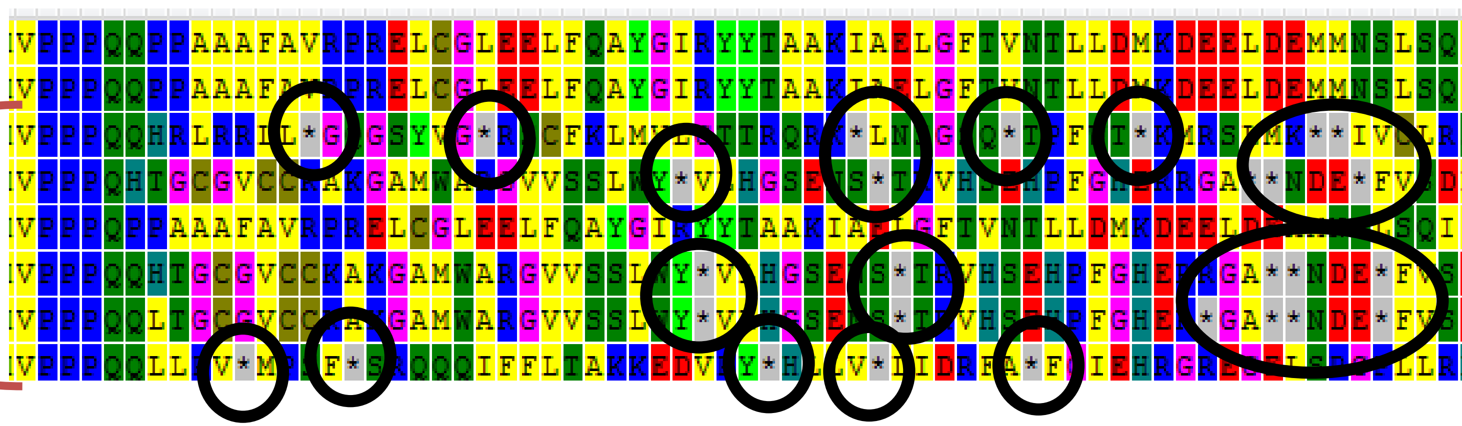
All the other mutants will have very short *LFY* proteins

Partial LEAFY peptide sequence

Wild type

Wild type transgenic

Homozygous mutants



SO MANY early stop codons

Summary: ¼ homozygous mutants, ½ mosaic mutants, no control mutants

Construct	GE events sequenced	Type of mutation	# of events (%)
Single LFY1C	102	Homozygous	34 (33%)
		Mosaic	51 (50%)
		None	17 (17%)
Single LFY3C	46	Homozygous	15 (32%)
		Mosaic	28(61%)
		None	3 (7%)
Double LFY1C-LFY3C	59	Homozygous	11 (19%)
		Mosaic	44 (74%)
		None	4 (7%)
Single AG1C	33	Homozygous	0 (0%)
		Mosaic	7 (21%)
		None	26 (79%)
Single AG2C	12	Homozygous	7 (58%)
		Mosaic	1 (8%)
		None	4 (34%)
Double AG1C-AG2C	80	Homozygous	19 (24%)
		Mosaic	45 (56%)
		None	16 (20%)
Cas (empty vector)	14	None	14 (100%)
Total (w/out control)	**332**	**Homozygous**	**86 (26%)**
		Mosaic	**176 (53%)**
		None	**70 (21%)**

What will phenotypes be?

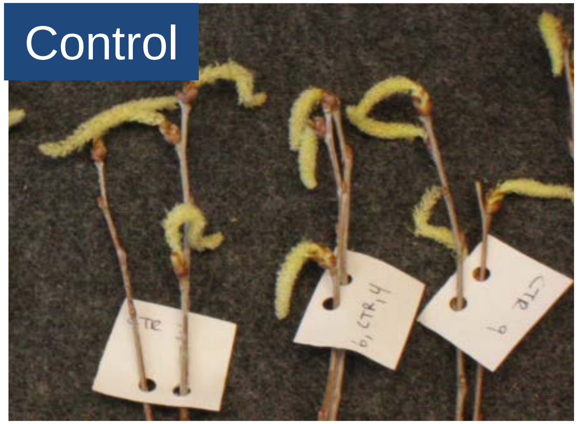
RNAi field studies give a good indication

RNAi field trial of poplar in Oregon (photo from 2013)

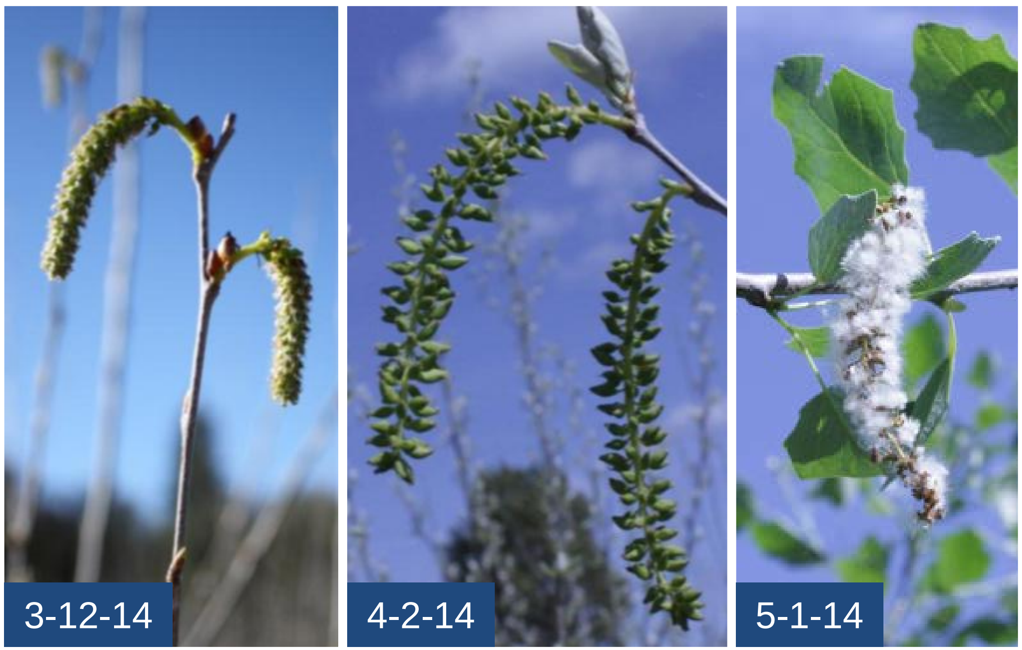
25 constructs, 3 genotypes, 4,000 trees, 9 acres

Trees are getting big of late

Flushing of dormant buds in lab uncovered modified catkin morphology



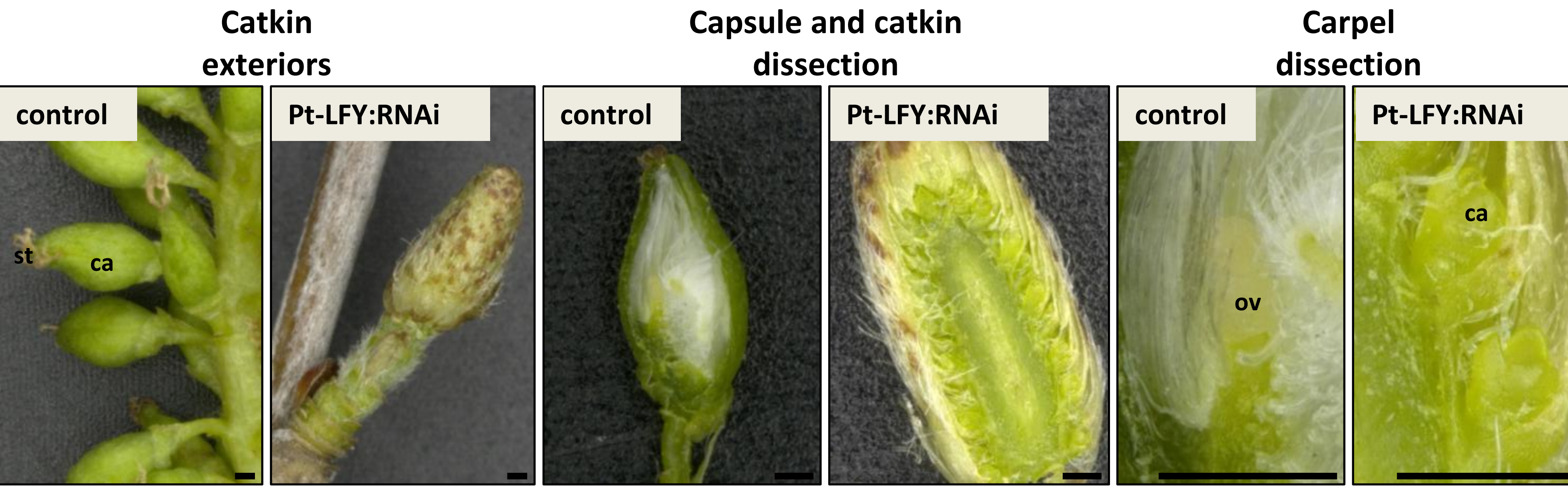
After field maturation, RNAi:*LFY* catkins remained tiny and did not produce seeds or cotton during two years of study



Control

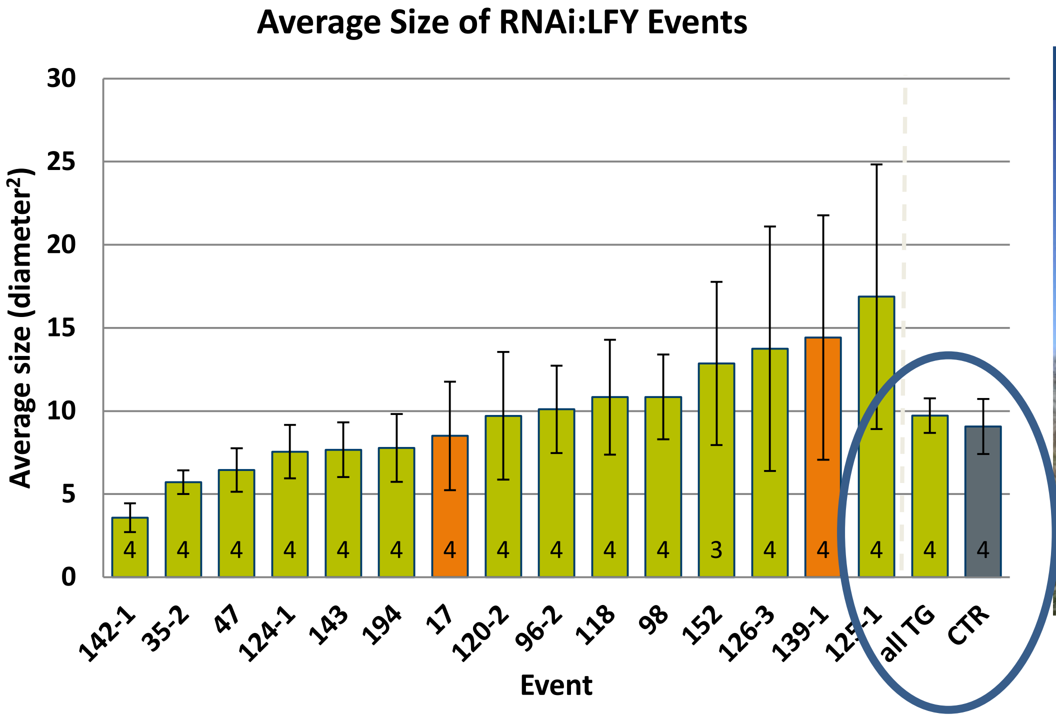
RNAi-LFY

Tiny RNAi:*LFY* catkins lacked stigmas, ovules, and cotton



An absence of pleiotropy?

RNAi:*LFY* trees had normal vegetative growth



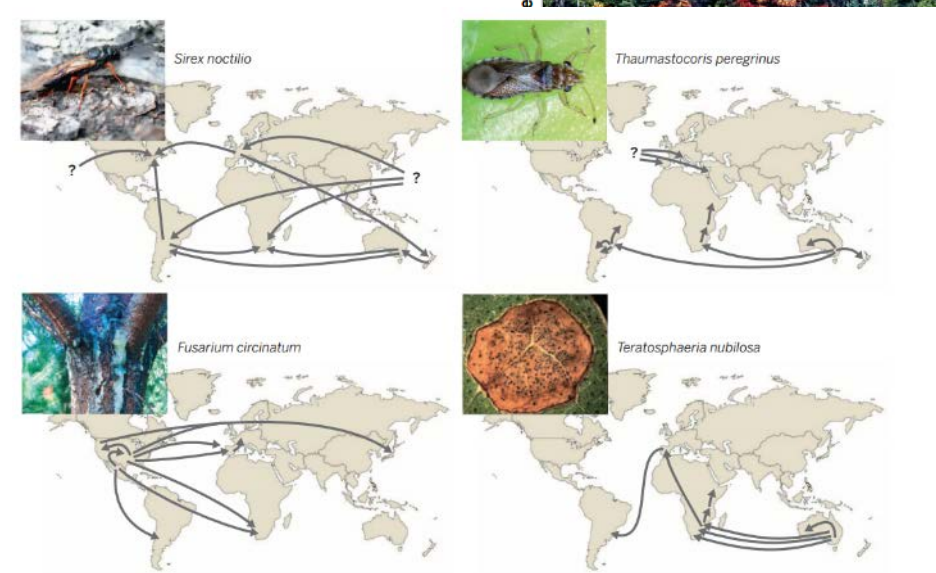
Work ahead on CRISPR mutants

- Flowering and vegetative phenotypes
  - FT retransformation to accelerate flowering
  - Transformation of early flowering genotype for field trials
- Study of off-target mutagenesis
- Cumulative mutagenesis/reversions with active CRISPR gene present?
- CRISPR removal/deactivation system for biological or social reasons?
- Understand effects on biodiversity from flower/seed removal
- Public engagement to promote a non-GMO designation for CRISPR mutants, or reduced regulatory stringency?

Summary

- Gene flow extensive in trees, a major GMO issue for society
- For clonally propagated trees, complete and reliable sexual sterility may be a solution
- CRISPR/Cas9 works incredibly well in poplar (and many other organisms)
- Numerous knock-out homozygous mutants (indels, large deletions)
- Healthy, non-flowering phenotypes seem feasible based on field RNAi knock-downs of the poplar *LFY* gene

Threats to forest health and productivity are massive, global, and growing



REVIEW

Planted forest health: The need for a global strategy

M. J. Wingfield,[1*] E. G. Brockerhoff,[2] B. D. Wingfield,[1] B. Slippers[1]

tree genera are used in planted forests worldwide, and these represent valuable
rces. Planted forests are increasingly threatened by insects and microbial
which are introduced accidentally and/or have adapted to new host trees.
n has hastened tree pest emergence, despite a growing awareness of the
proved understanding of the costs, and an increased focus on the importance of
To protect the value and potential of planted forests, innovative solutions and a
dinated global approach are needed. Mitigation strategies that are effective only in
ntries fail to contain invasions elsewhere in the world, ultimately leading to global
lutions to forest pest problems in the future should mainly focus on integrating
nt approaches globally, rather than single-country strategies. A global strategy to
t issues is vitally important and urgently needed.

er 8, 2015

Fig. 2. Examples of invasion routes of pests of planted forests that illustrate an apparently common pattern of complex pathways of spread to new environments, including repeated introductions and with either native or invasive populations serving as source populations (*18*). Invasion routes of the pine pitch canker pathogen *Fusarium circinatum* (origin in Central America) (*39*), eucalypt leaf pathogen *Teratosphaeria nubilosa* (origin in southeast Australia) (*40*), the pine woodwasp *Sirex noctilio* (origin in Eurasia) (*23*), and the eucalypt bug *Thaumastocoris peregrinus* (origin in southeast Australia) (*41*) were determined through historical and genetic data. [Photo credits: (top left) Brett Hurley; (top right) Samantha Bush; (bottom left) Jolanda Roux; (bottom right) Guillermo Perez]

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In the face of these enormous threats, why keep tools as powerful as GMOs on the shelf?

Traces of the emerald ash borer on the trunk of a dead ash tree in Michigan, USA. This non-native invasive insect from Asia threatens to kill most North American ash trees.

BIOTECHNOLOGY

Genetically engineered trees: Paralysis from good intentions

Forest crises demand regulation and certification reform

By **Steven H. Strauss[1], Adam Costanza[2], Armand Séguin[3]**

Intensive genetic modification is a long-standing practice in agriculture, and, for some species, in woody plant horticulture and forestry (*1*). Current regulatory systems for genetically engineered

recently initiated an update of the Coordinated Framework for the Regulation of Biotechnology (*2*), now is an opportune time to consider foundational changes.

Difficulties of conventional tree breeding make genetic engineering (GE) methods relatively more advantageous for forest trees than for annual crops (*3*). Obstacles

Although only a few forest tree species might be subject to GE in the foreseeable future, regulatory and market obstacles prevent most of these from even being subjects of translational laboratory research. There is also little commercial activity. Only two types of pest-resistant poplars are authorized for commercial use in small areas in China and two types of eucalypts, one approved in Brazil and another under lengthy review in the USA (*5*).

METHOD-FOCUSED AND MISGUIDED. Many high-level science reports state that the GE method is no more risky than conventional breeding, but regulations around the world essentially presume that GE is hazardous and requires strict containment