Genome editing for biological containment in engineered tree crops:
The miracle we have been waiting for?

Steve Strauss / Oregon State University / USA

IGI Berkeley – April 2019

General messages

- Social and technical innovations needed
  - Including BE and GE – don’t throw BE under the bus
- OCD on gene flow a great obstacle to research and trade
- Technical innovations needed
  - Trait-gene linkages and modulation system science
  - Transformation-editing systems for diverse and recalcitrant species
- People are fearful -- will be hard, conflict ridden work -- success by no means assured

Agenda

- The social context for gene editing in trees and forestry
  - Breeding/management
  - Public/ethics
    - Gene editing
  - Market access
  - Regulation
- Gene editing for mitigation of gene flow in eucalypts
  - Target genes
  - Research system
  - Some results

GE trees: Diverse applications and biology

- Orchards/horticulture – clones, high value products
- Short rotation forestry plantations (agricultural, short harvest cycles, clones)
  - Domesticate materials, intensify outputs, economize, reduced environmental impact
- Mixed stands, long rotations (use for special problems such as new diseases, rarely cloned)
  - Douglas-fir in Oregon, Loblolly pine in the southeastern USA
- Preserves/wild stands
  - For very special problems, conservation and restoration, such as Chestnut blight (if sustainable, acceptable, fundable)

Poplar in Oregon an example of ag-like forestry

Eucalypts in Brazil another example of ag-like forestry

Forests and sustainable intensification

Plantation forests occupy 5% of all forests and deliver 35% of industrial roundwood, usually with diversity preserves

More yield = less potential impact on wild/conservation forests

American chestnut was an iconic, keystone forest tree in the USA

It was extirpated as a forest tree by Chestnut Blight

Also subject of GE -- for restoration

Genetic diversity in wild trees large, but often hard to see – extensive genetic variation

Wild aspen stand – genetics obscure

Clonal rows of cottonwood – genetics striking

Sometimes nature reveals its secrets – wild aspen clones

Conventional breeding has powerful effects for quantitative (polygenic) improvement, now intensified by genomic selection

One generation of breeding Monterey pine in New Zealand made striking changes in growth & form

Tree breeding works most of the time,
but is slow, polygenic, gene-anonymous
–
Can bioengineering (BE)
or gene editing (GE) help?

Looking back a bit to breakthroughs in tree bioengineering methods

- Due to long generation times, intolerance of inbreeding, and lack of genic science – Mendelian breeding was non-existent for forest trees
- Transformation capacity
  - Leaf disc general plant transformation – 1984-85
  - Poplar transformation and regeneration - 1987
- Antisense and RNAi
  - Expression of single genes can be specifically modified for the first time – 1990s
- Gene editing revolution
  - Beyond ZFNs and TALENs – The CRISPR-Cas miracle age of today

A Simple and General Method for Transferring Genes into Plants

Abstract. *Transformed petunia, tobacco, and tomato plants have been produced by means of a novel leaf disk transformation-regeneration method. Surface-sterilized leaf disks were inoculated with an* Agrobacterium tumefaciens *strain containing a modified tumor-inducing plasmid (in which the phytohormone biosynthetic genes from transferred DNA had been deleted and replaced with a chimeric gene for kanamycin resistance) and cultured for 2 days. The leaf disks were then transferred to selective medium containing kanamycin. Shoot regeneration occurred within 2 to 4 weeks, and transformants were confirmed by their ability to form roots in medium containing kanamycin. This method for producing transformed plants combines gene transfer, plant regeneration, and effective selection for transformants into a single process and should be applicable to plant species that can be infected by* Agrobacterium *and regenerated from leaf explants.*

Efficient methods for introducing cloned genes into plants are important for understanding and controlling plant gene expression. The ability to manipulate genes could lead to rational, deliberate alterations of the genome of crop plants for improvement of their agronomic performance. Production of morphologically normal plants that contain and express foreign genes has been made possible by use of the natural gene-transfer capacity of *Agrobacterium tumefaciens*, a soil bacterium that causes crown gall disease in plants (*1*). Modified *A. tumefaciens* strains were used in which the tumor-inducing (Ti) genes had been deleted from the transferred DNA (T-DNA) and replaced with chimeric genes for bacterial antibiotic resistance that had been engineered to express in plant cells (*2*).

In previous studies the transformed plants were regenerated from calli derived from protoplasts (single cells without a cell wall) transformed by cocultivation with *A. tumefaciens* cells (*1*). However, the protoplast culture method has certain limitations: not all species of plants can be readily regenerated from protoplasts; the entire process can take up to 6 months from protoplast to plant; and plants derived from protoplasts can be subject to mutations or chromosomal abnormalities (*3*). Protoplast culture technology can also be difficult to reproduce in a new laboratory or to control from one experiment to the next. Transformation of stem or root explants in vitro is a simple substitute for cocultivation (*4*) but is laborious for large scale experiments and not easy to use with modified Ti plasmids that lack the tumor-inducing genes.

To overcome these limitations, we have developed an approach to transfor- of an *A. tumefaciens* strain (GV3Ti11SE) containing a modified octopine Ti plasmid (pTiB6S3SE) in which all phytohormone biosynthetic genes and the T_L-DNA right border sequence have been deleted has been described (*2*). Formation of a cointegrate between pTiB6S3SE and the intermediate vectors pMON120 or pMON200 results in a functional, avirulent T-DNA (*2, 7*). Plasmid pMON200 is a derivative of pMON120, which contains a translationally-improved chimeric NOS/NPTII/NOS gene for kanamycin resistance and confers a high degree of resistance to aminoglycoside antibiotics on transformed plant cells (*8*). The vectors also contain the nopaline synthase gene, which provides a second marker in the transformed plant cells (*1*).

Disks were punched from surface-sterilized leaves with a paper punch (6 mm in diameter) and submerged in a culture of *A. tumefaciens* grown overnight in luria broth at 28°C. After gentle shaking to ensure that all edges were infected, the disks were blotted dry and incubated upside-down on nurse culture plates prepared as described (*7*) containing medium that induces regeneration of shoots of the species being transformed. The age and titer of the bacterial inoculum had little influence on the effectiveness of the transformation; however, it was important to avoid excessive soaking of the internal tissues of the leaf disk by the bacterial culture. After 2 to 3 days, the disks were transferred to petri plates containing the same medium but without feeder cells or filter papers and containing carbenicillin (500 μg/ml) and kanamycin (300 μg/ml).

After 2 to 4 weeks, shoots that developed were excised from calli and transplanted to appropriate root-inducing medium containing carbenicillin (500 μg/ml) and kanamycin (100 μg/ml). Rooted plantlets were transplanted to soil as soon as possible after roots appeared. *Nicotiana tabacum* varieties Samson and Havana 425 (*9*) and a first-generation cross-fertilized (F_1) hybrid of *Petunia hybrida* (*10*) were easily transformed by this system. L2 tomato plants (*11*) responded better when the feeder plate medium was modified by reducing the amount of inorganic salts to one-tenth the usual concentration.

Uninoculated petunia leaf disks and those inoculated with *A. tumefaciens* strains containing pTiB6S3SE::pMON-120 (which lacks the chimeric gene for kanamycin resistance) did not produce calli or shoots on medium containing 300 μg of kanamycin per milliliter (Fig. 1). In contrast, leaf disks inoculated with *A.*

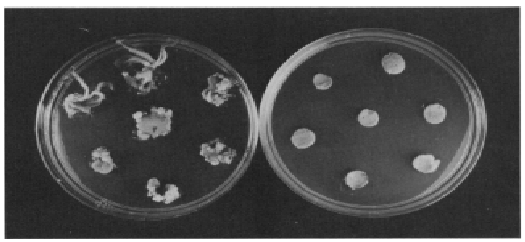

Fig. 1. Leaf disk transformation and selection of antibiotic-resistant cells. Leaf disks were punched from a surface-sterilized leaf of *Petunia hybrida* (Mitchell), inoculated with *Agrobac-*

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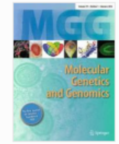


Molecular and General Genetics MGG
February 1987, Volume 206, Issue 2, pp 192–199 | Cite as

Agrobacterium mediated transformation and regeneration of *Populus*

Authors | Authors and affiliations

JoAnne J Fillatti, James Sellmer, Brent McCown, Bruce Haissig, Luca Comai

The Nobel Prize in Physiology or Medicine 2006

"for their discovery of RNA interference - gene silencing by double-stranded RNA"

Photo: L. Cicero/Stanford

Andrew Z. Fire

Photo: R. Carlin/UMMAS

Craig C. Mello

History of RNA Interference

the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

Free Access

Populus CEN/TFL1 regulates first onset of flowering, axillary meristem identity and dormancy release in *Populus*

Rozi Mohamed, Chieh-Ting Wang, Cathleen Ma, Olga Shevchenko, Sarah J. Dye, Joshua R. Puzey, Elizabeth Etherington, Xiaoyan Sheng, Richard Meilan, Steven H. Strauss, Amy M. Brunner

First published: 11 May 2010 | https://doi.org/10.1111/j.1365-313X.2010.04185.x | Cited by: 86

Science great, but unclear potential for applications of BE/GE for forest trees

The biotech world has shown much promise

- Herbicide tolerance
- Resistance against pests
- Flowering acceleration
- Resistance to abiotic stresses
- Fine tuned wood quality

In Vitro Cellular & Developmental Biology - Plant (2018) 54:341–376
https://doi.org/10.1007/s11627-018-9914-1

INVITED REVIEW

CrossMark

Genetic engineering of trees: progress and new horizons

Shujun Chang[1] · Elizabeth L. Mahon[2] · Heather A. MacKay[2] · William H. Rottmann[3] · Steven H. Strauss[4] · Paula M. Pijut[5] · William A. Powell[6] · Vernon Coffey[6] · Haiwei Lu[4] · Shawn D. Mansfield[2] · Todd J. Jones[1]

Received: 5 February 2018 / Accepted: 20 June 2018 / Editor: Marie-Anne Lelu-Walter / Published online: 5 July 2018

Abstract

Genetic engineering of trees to improve productivity, wood quality, and resistance to biotic and abiotic stresses has been the primary goal of the forest biotechnology community for decades. We review the extensive progress in these areas and their current status with respect to commercial applications. Examples include novel methods for lignin modification, solutions for long-standing problems related to pathogen resistance, modifications to flowering onset and fertility, and drought and freeze tolerance. There have been numerous successful greenhouse and field demonstrations of genetically engineered trees, but

Science great, but unclear potential for applications of BE/GE for forest trees

The biotech world has shown much promise

- Herbicide tolerance
- Resistance against pests
- Flowering acceleration
- Resistance to abiotic stresses
- Fine tuned wood quality

The real world has big obstacles

- Knowledge
- Transformation
- Product/economics

- Ethics
- Market access
- Regulation

Ethics / public acceptance

One rude message for this naïve scientist….

Conference at Oxford in 1999 / Vandalism against lignin modified trees to “welcome” conferees, Euro-press attacks

FRANKENSTEIN’S FOREST

The tree-top protesters, who confounded the Government’s road-building programme by camping in the path of bulldozers, are now poised to target the very trees they might once have called home.

Whilst public attention has been focused on the threat of ‘Frankenstein Fo
same corporations who are forcing us
genetically modified (GM) meals ha
quietly perpetrating yet anothe
against the environment.

The biotech industry has been unc
ably tight-lipped about its latest pha
genetic revolution. But it is currentl
ing to take over the world’s forests –
left of them – and grow regimentec
genetically engineered (modified) tre

Big deals are currently being st
tween forest and biotech corporation
Monsanto. By replacing plantations
over with GM trees, they are plannin
in on faster growth rates and higher
of saleable wood pulp.

But what’s good for Monsanto and their cronics is not necessarily good for the environment. Campaigners fear that GM trees will sap up water, nutrients and light, leaving indigenous trees to die out along with the host of insects, plants and fungi which rely upon them. In turn, birds and animals would lose many of their natural prey. Those surviving

Conc
threat l
Year’s E

1997. The trees, engineered by the University of Derby, to be disease- and insect-resistant were destroyed by removing the bark. A growing spate of raids on food crops caused AstraZeneca to make a statement to the press before a GenetiX Snowball action earlier this

vention, which governs global emissions of greenhouse gases, came into force after the 1997 Kyoto conference, industrialised countries have been forced to clean up. However, the corporations argue that by planting more trees, they should be awarded ‘carbon credits’,
s absorb carbon dioxide.
naturally rich native forests have
chainsaw, only to be replaced by
eign plantation species such as
To the undiscerning eye, one forest
uishable from another, allowing
s to boast about how well they are
heir operations. Look behind the
and companies such as Shell are
trees with new traits.
cular biologist Viola Sampson of

Whilst public attention has been focused on the threat of ‘Frankenstein Foods’, the same corporations who are forcing us to ingest genetically modified (GM) meals have been quietly perpetrating yet another crime against the environment.

“Eco” vandalism close to home

Pacific Northwest (2001)

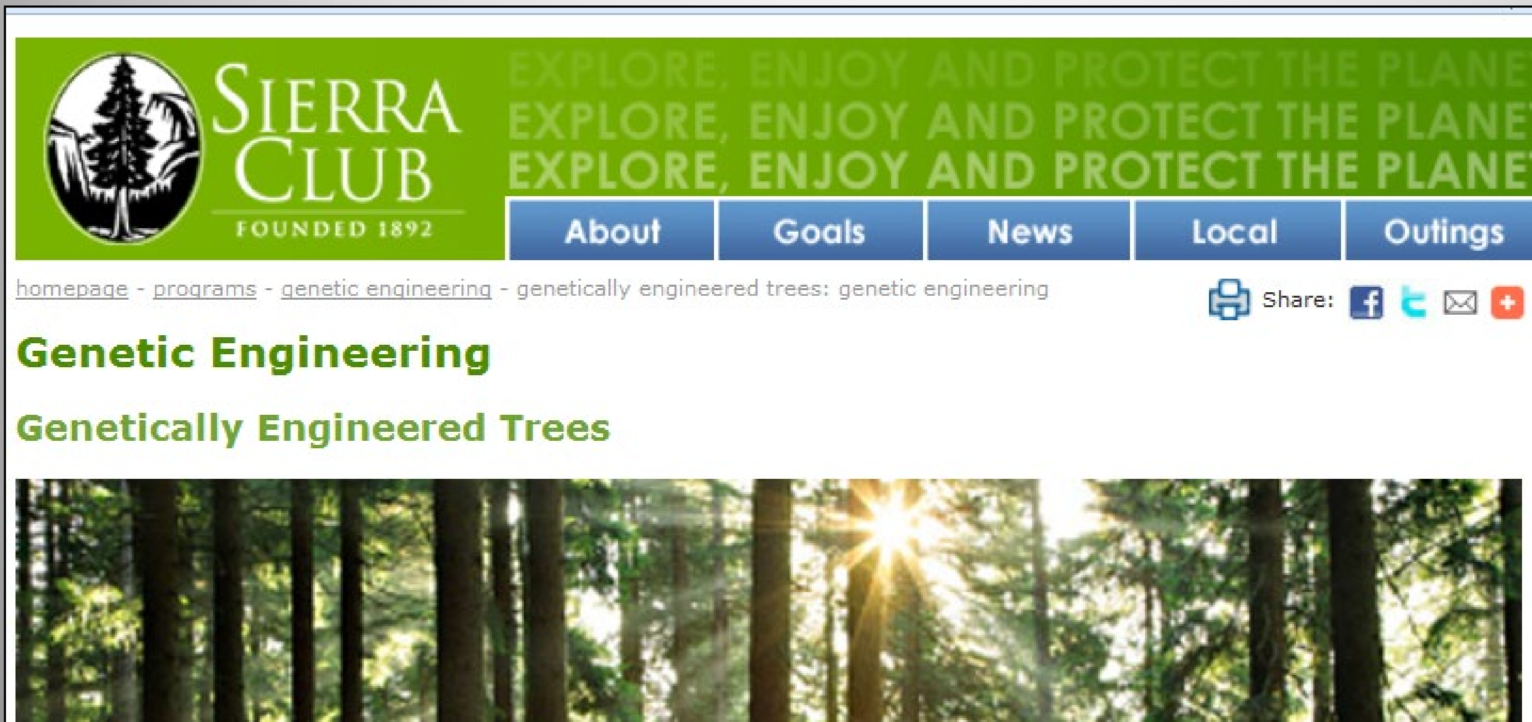
U Wash

Oregon State

2015 vandalism against collaborating company in Brazil

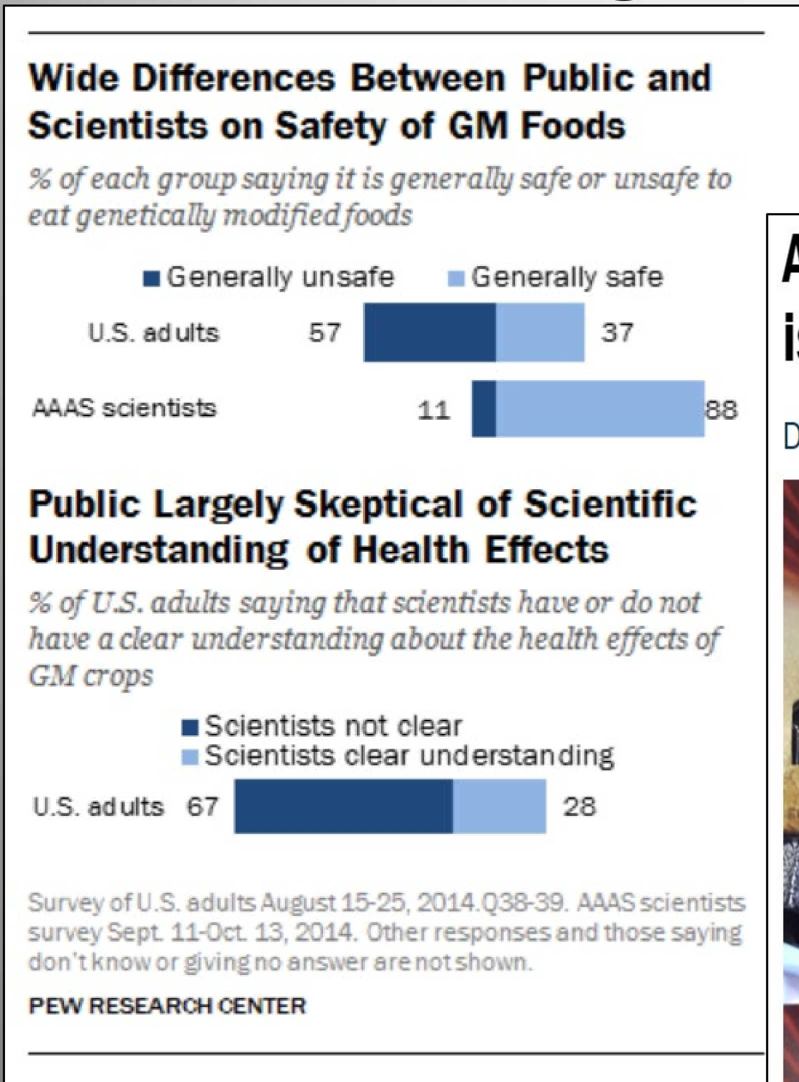
March 5, 2015: 1,000 women of the Brazil Landless Workers' Movement (MST) vandalized Suzano/FuturaGene's GE eucalypts greenhouse at Itapetininga, in São Paulo

Political forces hostile -- Major environmental groups promoting wild forests dislike or ignore GE trees



"The possibility that the new genes spliced into GE trees will interfere with natural forests isn't a hypothetical risk but a certainty. …genetic engineering may do as much damage to forests and wildlife habitat as chain saws and sprawl." (11/10/13)

People, their fears and their understanding scary for science



Americans don't trust scientists' take on food issues

Dan Charles · NPR · Dec 2, 2016 Health

Social reception for genome editing scary

CAST® Issue Paper

Number 60
July 2018

Genome Editing in Agriculture: Methods, Applications, and Governance

A paper in the series on
The Need for Agricultural Innovation to Sustainably Feed the World by 2050

The power of genome editing suggests that, if conducive social and regulatory conditions are in place, it can substantially increase the positive impacts of plant and animal breeding on human welfare and sustainability. (Shutterstock photos from Yaroslava [corn], vchal [gene manipulation], and Shyamalamuralinath [calf].)

Abstract

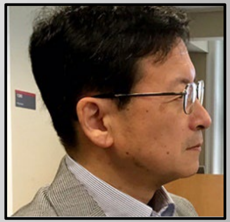
Genome editing is the process of making precise, targeted sequence

limitations of the approach. The paper also presents an overview of the current landscape of governance of genome editing, including existing regulations, inter-

decrease socioeconomic disparities, mitigate barriers to trade, and moderate political and market dependencies), the paper aims to provide a conceptual and

Public sentiment? Survey results about gene editing in Japan are sobering

(Prof. Masashi Tachikawa, Nagoya University, Japan)



		no need to regulate
Conventional Breeding	researchers (n=197)	56.3%
	consumers (n=3000)	46.5%
Genome Editing	researchers (n=197)	7.6%
	consumers (n=3000)	9.4%
GM	researchers (n=197)	3.0%
	consumers (n=3000)	8.1%

General conclusions from focus groups in Japan (Tachikawa)

- Too much unnatural food around us
- Long term impact should be counted
- Not Natural as far as genetic manipulation involved
- Scientists may provide biased information
- Protect ourselves through choice
- Labeling is key

"Green" markets not expected to love gene editing?

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?

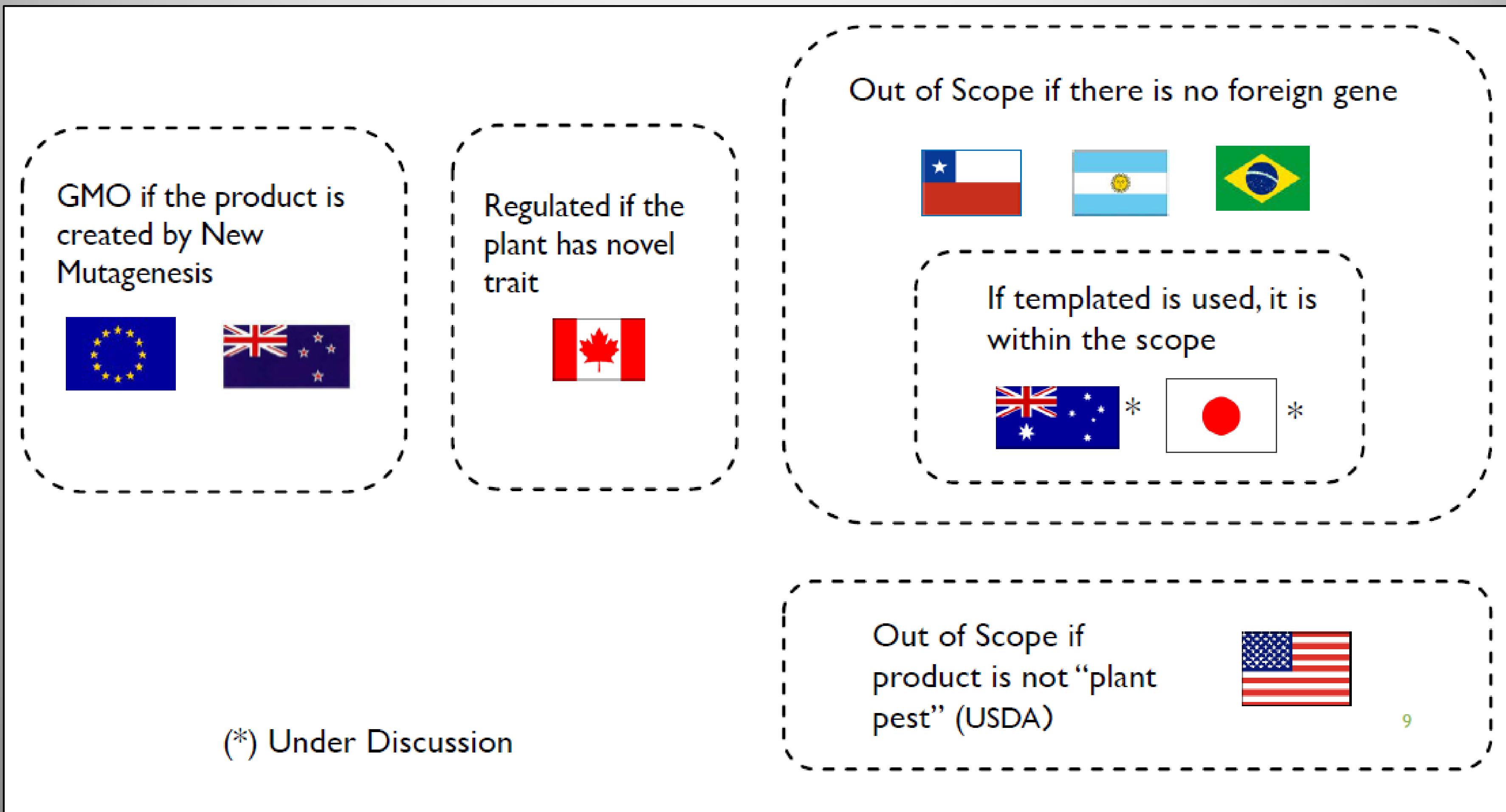
Apparently not. To the potential disappointment of the scientific community, the National Organic Standards Board (NOSB) unanimously voted to approve a proposal on Nov. 18 that would, among other things, ban plants and animals produced using gene editing technology from being considered organic. Along with defining new genetic technologies as genetic engineering—an "excluded method" for
additionally attributed many alarming a

"Every organic stakeholder is clear that
integrity. Every effort must be made to

Among other things, the proposal rules
Cas 9, Zinc Finger Nuclease (ZFN), and v
engineering for the purposes of organic
of "excluded methods" of organic produ

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

Messy and diverse global regulatory landscape for genome editing (Tachikawa, Japan)



Potentially large problems for trade, tracking, adventitious presence?

Market acceptance

Strict market barriers to BE trees in much of the world – like organic certification

Includes research applications

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 health a major and growing concern

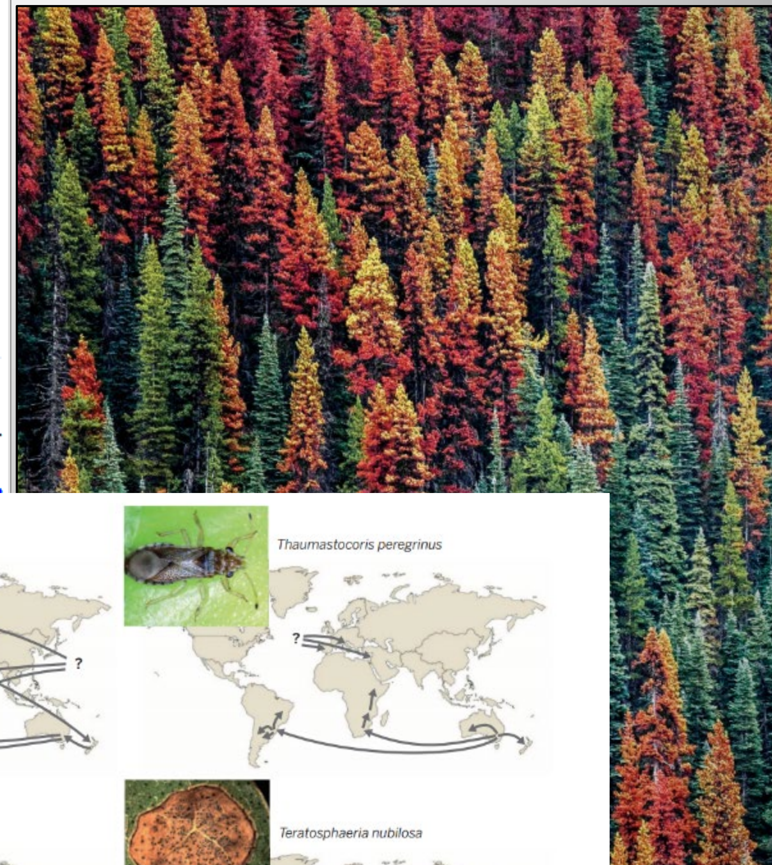
REVIEW

Planted forest health: The need for a global strategy

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

Several key tree genera are used in planted forests worldwide, and these represent valuable global resources. Planted forests are increasingly threatened by insects and microbial pathogens, which are introduced accidentally and/or have adapted to new host trees. Globalization has hastened tree pest emergence, despite a growing awareness of the problem, improved understanding of the costs, and an increased focus on the importance of … orests, innovative solutions and a … strategies that are effective only in … e world, ultimately leading to global … hould mainly focus on integrating … try strategies. A global strategy to … ed.

… separated from their natural enemies. … when plantation trees are reunited with … olved pests, which may be introduced … lly, or when they encounter novel pests … they have no resistance, substantial … r loss can ensue (7). The longer these



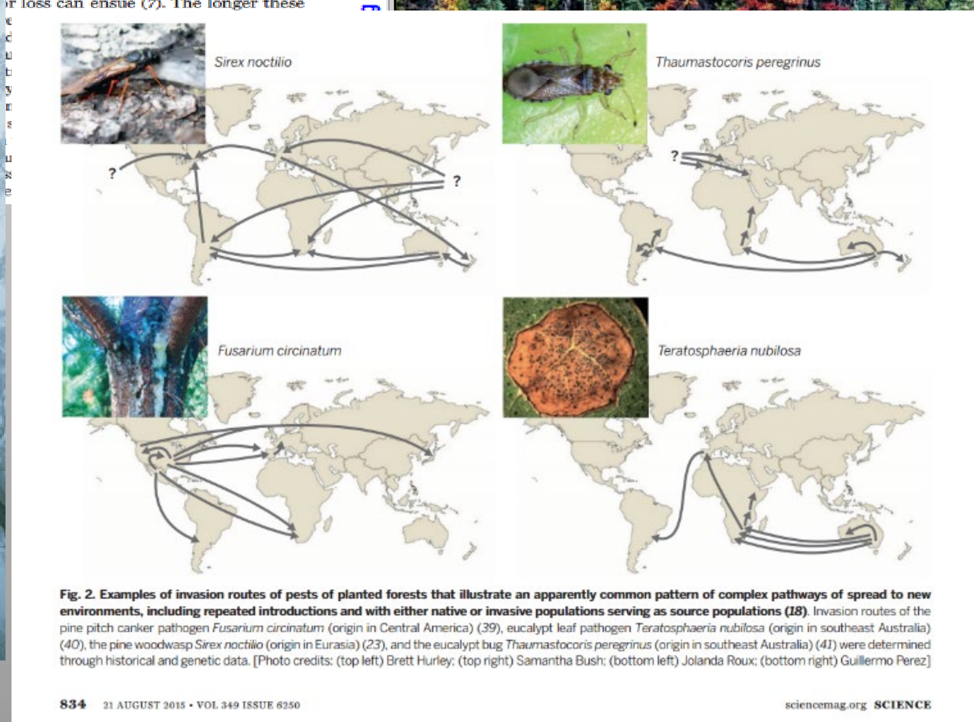


Fig. 2. Examples of invasion routes of pests of planted forests that illustrate an apparently common pattern of complex pathways of spread to new environments, including repeated introductions and with either native or invasive populations serving as source populations (18). Invasion routes of the pine 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]

Use of GE trees for forest health: Big constraints

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

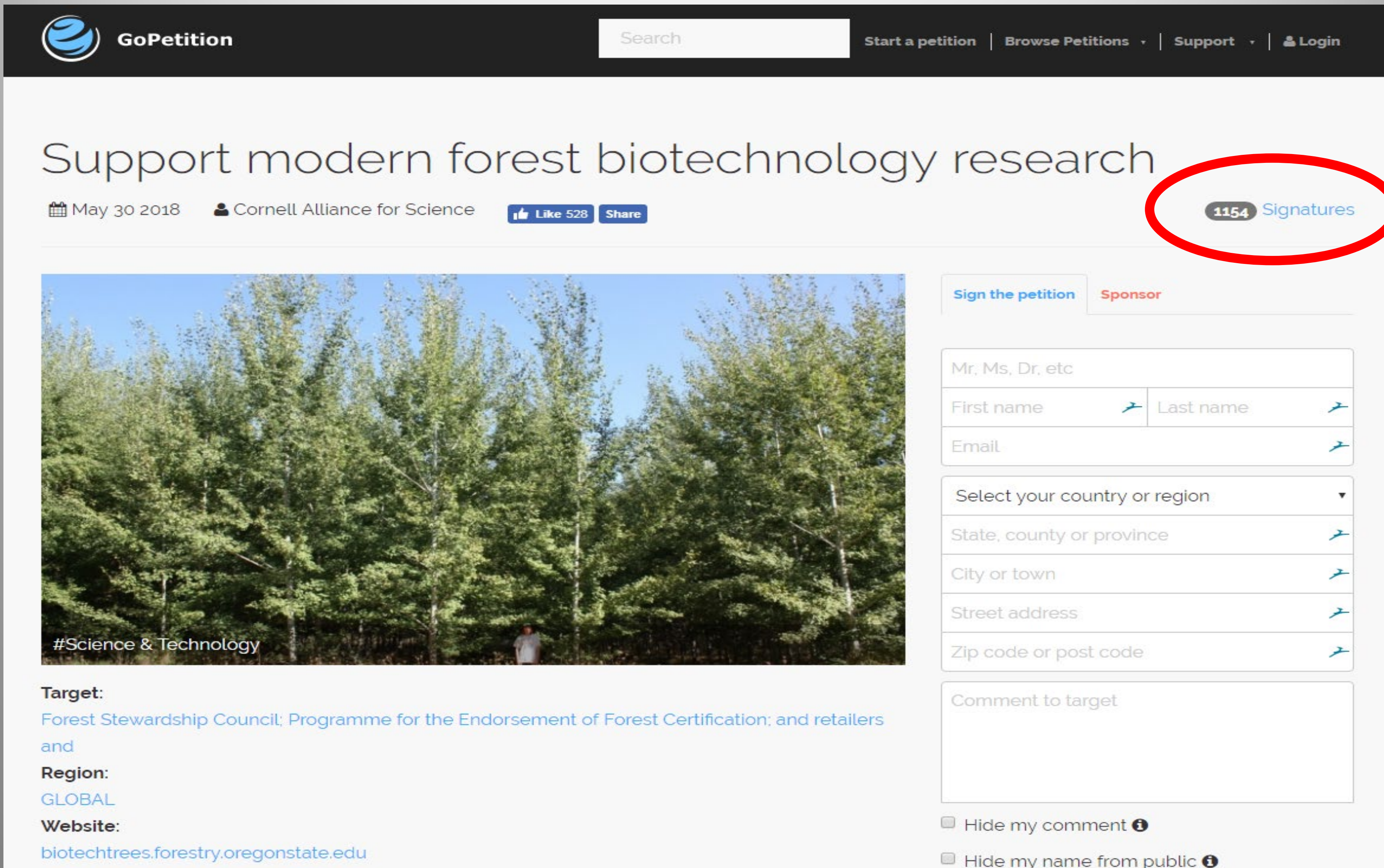
recently initiated an update of the Coordinated Framework for the Regulation of Biotechnology (2), now is an opportune time to consider foundational changes.

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

Petition to encourage research exemption by certifiers



Workability of regulations: Gene flow and gene editing

Regulations that presume the method is a hazard until proven innocent makes field and adaptive research very difficult

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.

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Articles

Far-reaching Deleterious Impacts of Regulations on Research and Environmental Studies of Recombinant DNA-modified Perennial Biofuel Crops in the United States

STEVEN H. STRAUSS, DREW L. KERSHEN, JOE H. BOUTON, THOMAS P. REDICK, HUIMIN TAN, AND ROGER A. SEDJO

October 2010 / Vol. 60 No. 9 • BioScience 729

Gene flow - Regulation and ethics a major obstacle, especially for trees

- Wild/feral populations
- Record of invasiveness of many exotic trees/shrubs
- Keystone species / Large role in providing ecosystem services
- Long distance pollen and/or seed movement
- Limited domestication
- Scientific uncertainty - Introgression experiments costly or impossible to do, models speculative
- Public view of forests as natural or wild: “contamination, impurity”

Poplar pollen and seed dispersal

The gene flow problem

Juvenile trees workable, but when research moves beyond juvenile, "boutique" research phase – very hard to completely isolate GE trees from wild or feral populations

Can society get beyond this and allow BE or GE adaptive research?

Example of RNAi-lignin-modified trees valued for ethanolic biofuels (Boerjan et al, Belgium)

Needs extensive adaptive, field research to get right

PNAS PNAS PNAS PNAS

Improved saccharification and ethanol yield from field-grown transgenic poplar deficient in cinnamoyl-CoA reductase

Rebecca Van Acker[a,b], Jean-Charles Leplé[c], Dirk Aerts[d], Véronique Storme[a,b], Geert Goeminne[a,b], Bart Ivens[a,b], Frédéric Légée[e], Catherine Lapierre[e], Kathleen Piens[f], Marc C. E. Van Montagu[a,b,1], Nicholas Santoro[g], Clifton E. Foster[g], John Ralph[h], Wim Soetaert[d], Gilles Pilate[c], and Wout Boerjan[a,b,1]

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Orléans, France; [d]Centre
Mixte de Recherche 1318
Ghent University, 9000 G
and [h]Departments of Bi
Research Center, Univers

Contributed by Marc C.

Lignin is one of the
enzymatic processing
tremula x *Populus alb*
tase (CCR), the enzym
specific branch of the
field trials in Belgium
ture. Wood samples
of the red xylem col
regulation. Saccharifi
conditions (none, tw
simultaneous sacchar
that wood from the
161% increased etha
rial from the compl
trees, including bark
yielded ~20% more
down-regulation of C
that *CCR* down-regul
improve biomass pro
and the yield penalty

bioethanol | GM | secon

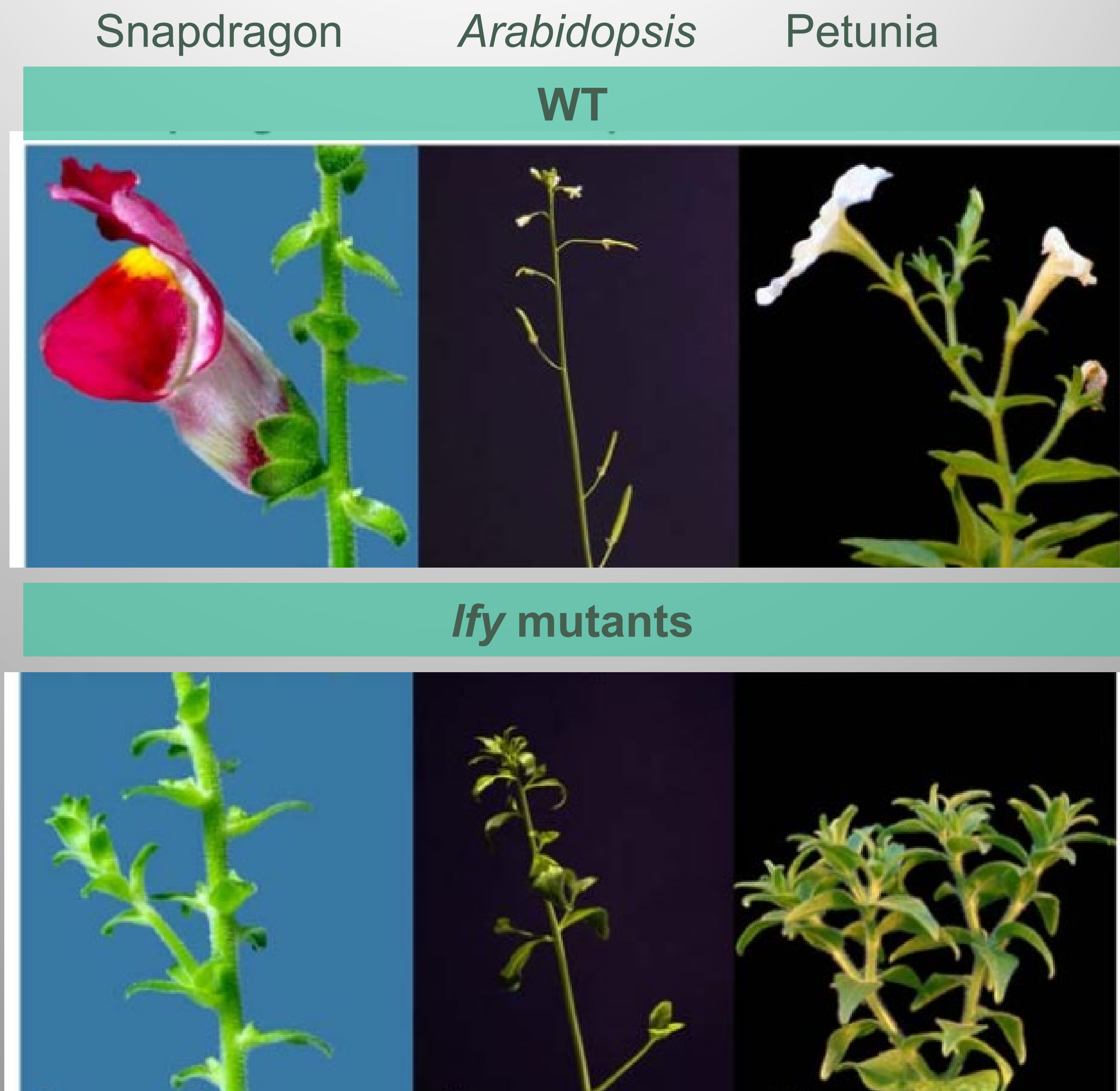
Global warming
major impetus
ergy sources. Liquid biofuels, bioethanol in particular, are cur-

In the transition from a fossil-based to a bio-based economy, bioethanol will be generated from the lignocellulosic biomass

Gene editing for genetic containment

- Socially mandated?
- Important for novel, risky applications
  - Advanced gene editing and synthetic biology
  - High value exotic species
- Many sterility options
  - Ploidy modification to BE to GE
  - Most are leaky, unreliable
- Focus on <u>bisexual</u> and <u>permanent</u> sterility for vegetatively propagated species
  - Take a great clone/variety, tweak with BE and GE, contain with GE
- Focus on floral developmental genes: *LEAFY* and *AGAMOUS*

Strong *lfy* mutants appear to have no flowers



Parcy et al. 2002; Moyroud et al. 2010

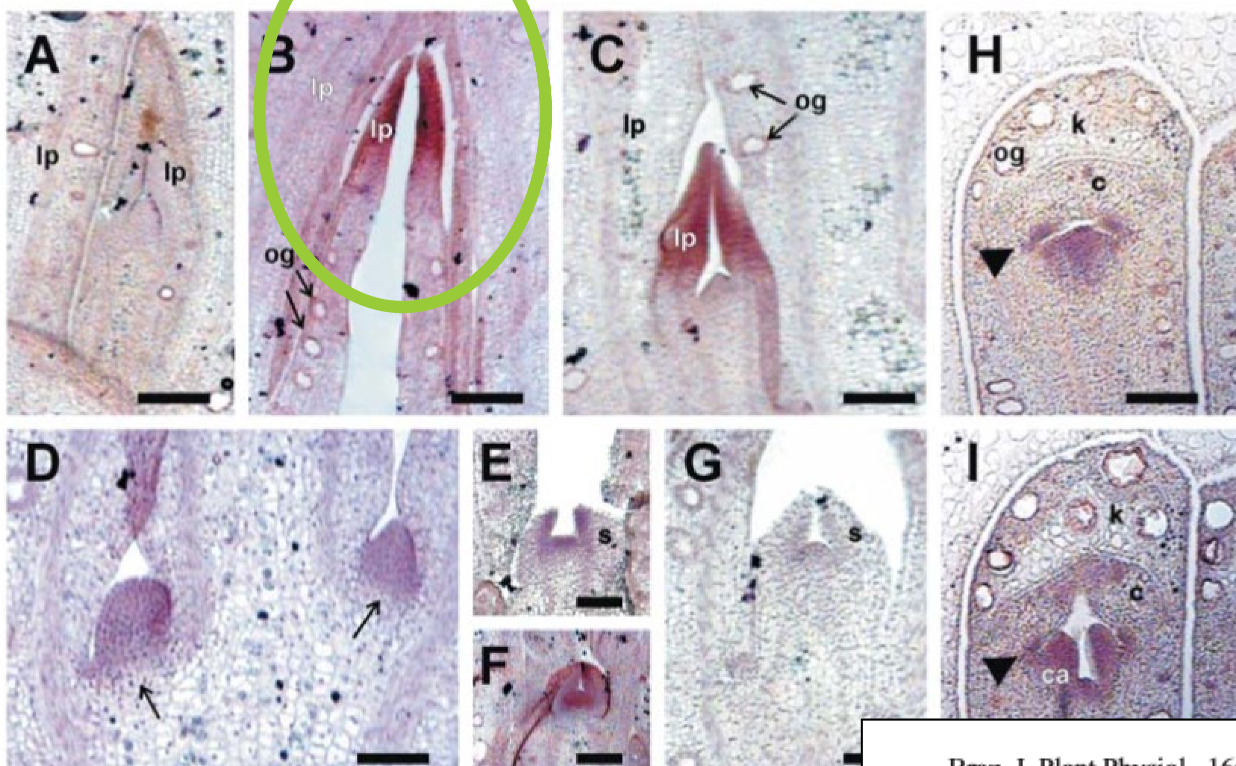
Though well studied for floral biology, the full biological role of *LFY* unknown

- Discovery studies did not have significant analysis of vegetative/productivity effects
  - An absence of studies of gene mutation/knock-out in the field
- No studies in the very divergent floral types of important forest tree taxa
  - Often parts of gene families
- Found to have vegetative as well as floral expression
  - Meristematic vegetative cell expression

Eucalyptus *LFY* vegetative expression

***EgLFY*, the *Eucalyptus grandis* homolog of the *Arabidopsis* gene *LEAFY* is expressed in reproductive and vegetative tissues**

Marcelo Carnier Dornelas[1*], Weber A. Neves do Amaral[2] and Adriana Pinheiro Martinelli Rodriguez[1]

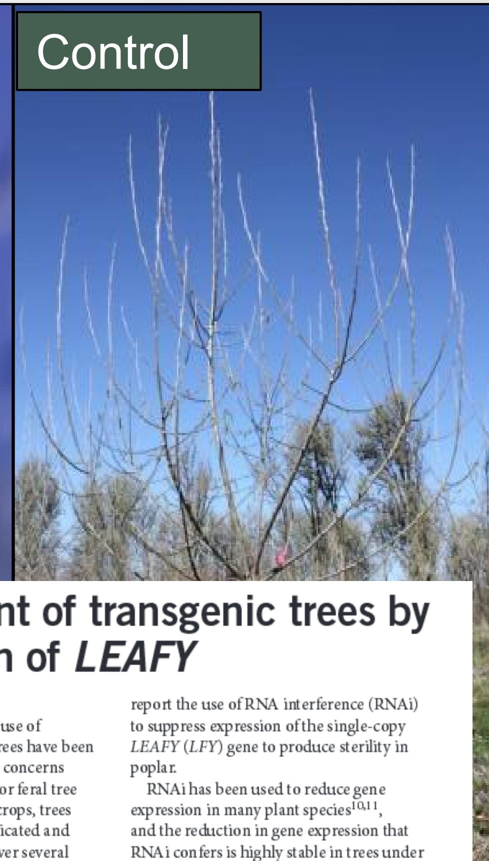


Braz. J. Plant Physiol., 16(2):105-114, 2004

4 ha field trial of RNAi approaches in Oregon – *LFY, AG* and other genes

Summer 2016

Sterility, normal growth of *LEAFY*-RNAi poplars



Containment of transgenic trees by suppression of *LEAFY*

To the Editor:

Field studies and commercial use of genetically engineered (GE) trees have been 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 report the use of RNA interference (RNAi) to suppress expression of the single-copy *LEAFY* (*LFY*) gene to produce sterility in 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*

Two other LFY-RNAi poplar clones tested: **NADA**

Clone 717 female

Clone 353 male

Clone 6K10 female

June 2017

Eucalypt RNAi-LFY also tested in a field trial: **NADA**

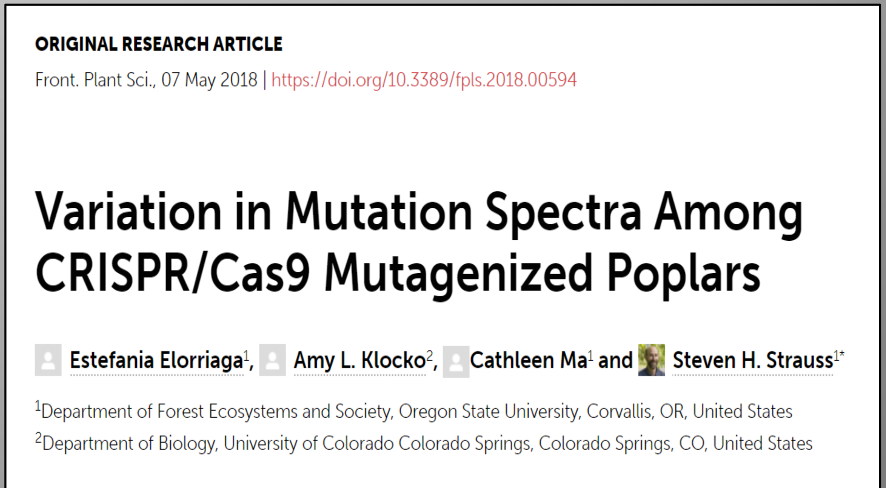
Eucalypt *LFY* CRISPR knock-outs

- Gene mutation/deletion the strongest and most stable form of genetic containment
  - Regulator and public confidence in containment?

The miracle we have been waiting for?

Eucalypt *LFY* CRISPR methods

- Created single- and two-sgRNA constructs
- Transformed into wild type and also into early flowering *E. urophylla x grandis* hybrid
- Conducted allele-specific target PCR followed by gel isolation and sequencing
  - High knock out and deletion rate: **97% of transgenic events** (indels and also larger deletions)
- Examined in greenhouse for growth rate and flowering/sterility


ORIGINAL RESEARCH ARTICLE

Front. Plant Sci., 07 May 2018 | https://doi.org/10.3389/fpls.2018.00594

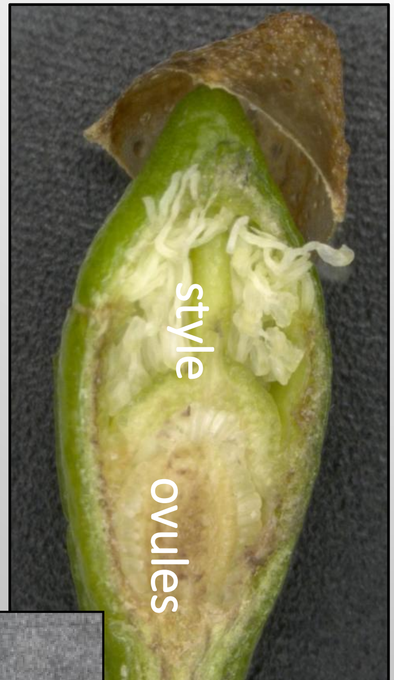
Variation in Mutation Spectra Among CRISPR/Cas9 Mutagenized Poplars

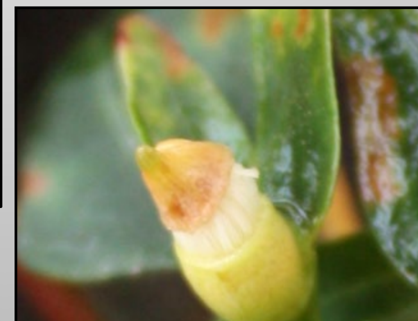
Estefania Elorriaga[1], Amy L. Klocko[2], Cathleen Ma[1] and Steven H. Strauss[1*]

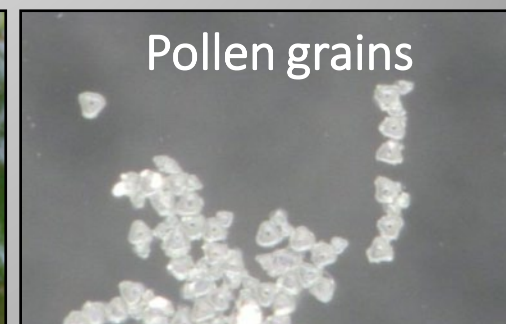
[1]Department of Forest Ecosystems and Society, Oregon State University, Corvallis, OR, United States

[2]Department of Biology, University of Colorado Colorado Springs, Colorado Springs, CO, United States

Early flowering in eucalypts to speed phenotyping







Plant Biotechnology Journal

Plant Biotechnology Journal (2016) **14**, pp. 808–819 doi: 10.1111/pbi.12431

FT overexpression induces precocious flowering and normal reproductive development in *Eucalyptus*

Amy L. Klocko[1], Cathleen Ma[1], Sarah Robertson[1], Elahe Esfandiari[1], Ove Nilsson[2] and Steven H. Strauss[1,*]

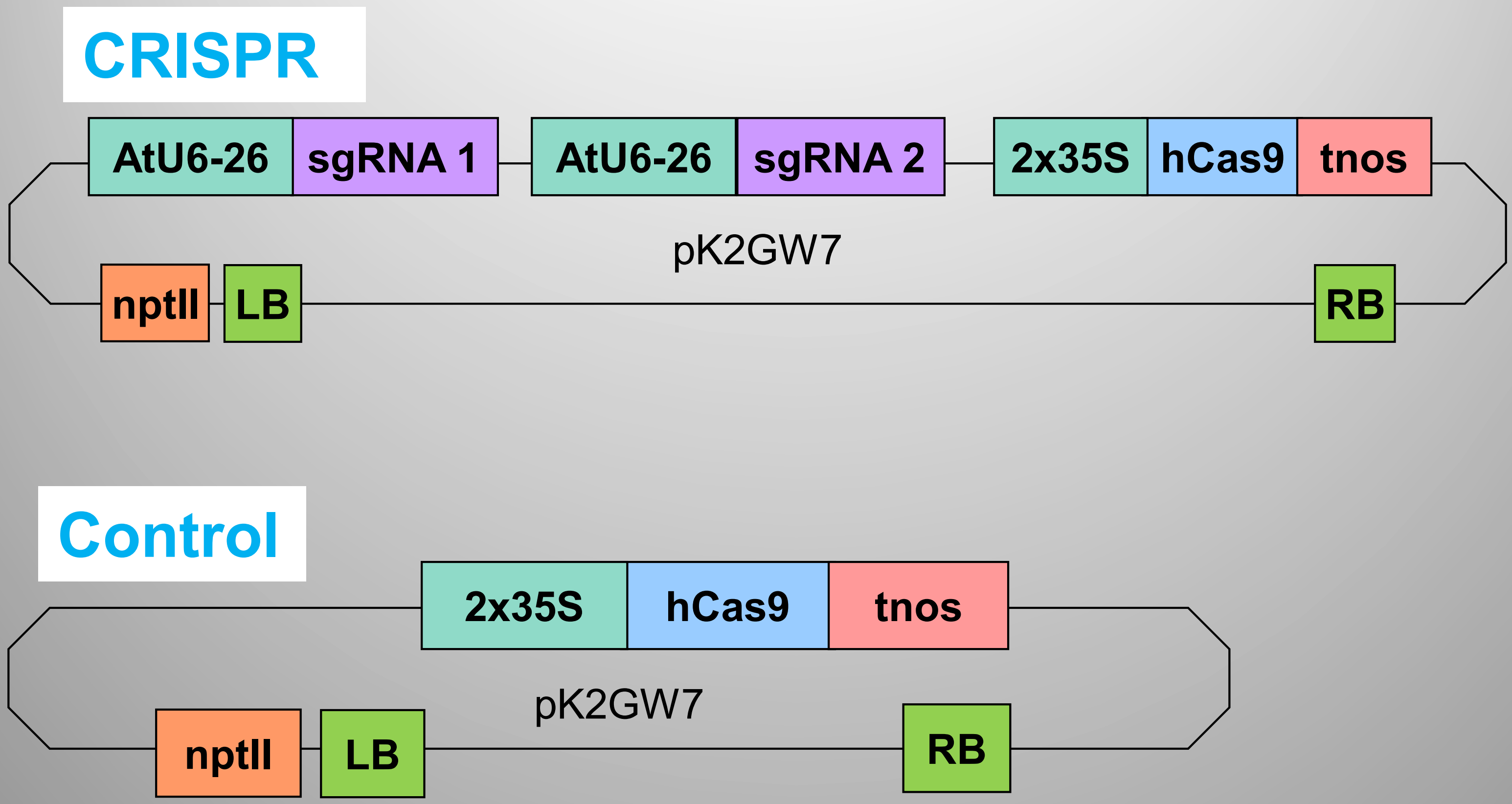
[1]Department Forest Ecosystems & Society, Oregon State University, Corvallis, OR, USA
[2]Department of Forest Genetics and Plant Physiology, Umeå Plant Science Centre, Swedish University of Agricultural Sciences, Umeå, Sweden

Received 8 April 2015;
revised 29 May 2015;
accepted 10 June 2015.

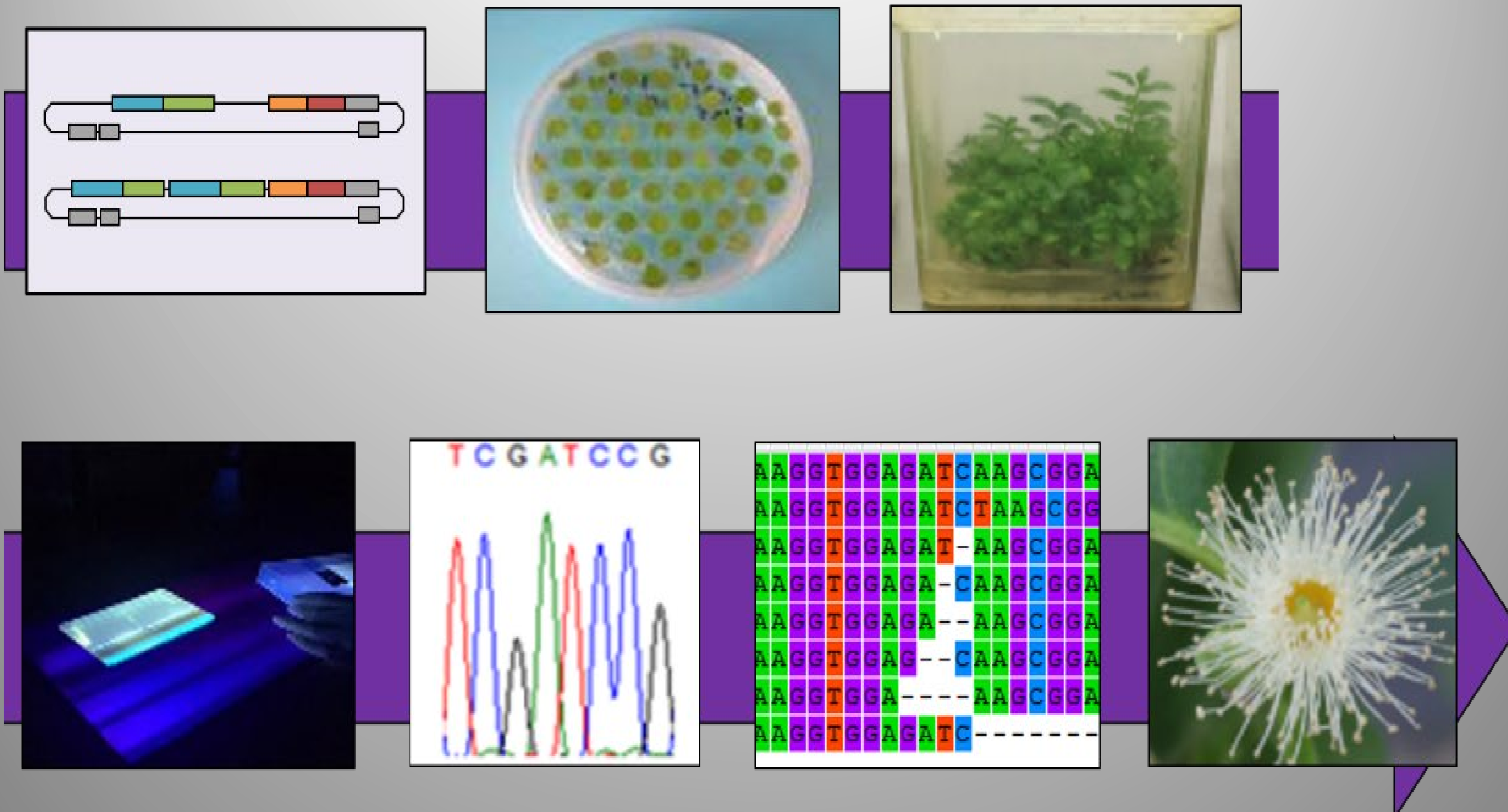
Summary

Eucalyptus trees are among the most important species for industrial forestry worldwide. However, as with most forest trees, flowering does not begin for one to several years after

Constructs employed

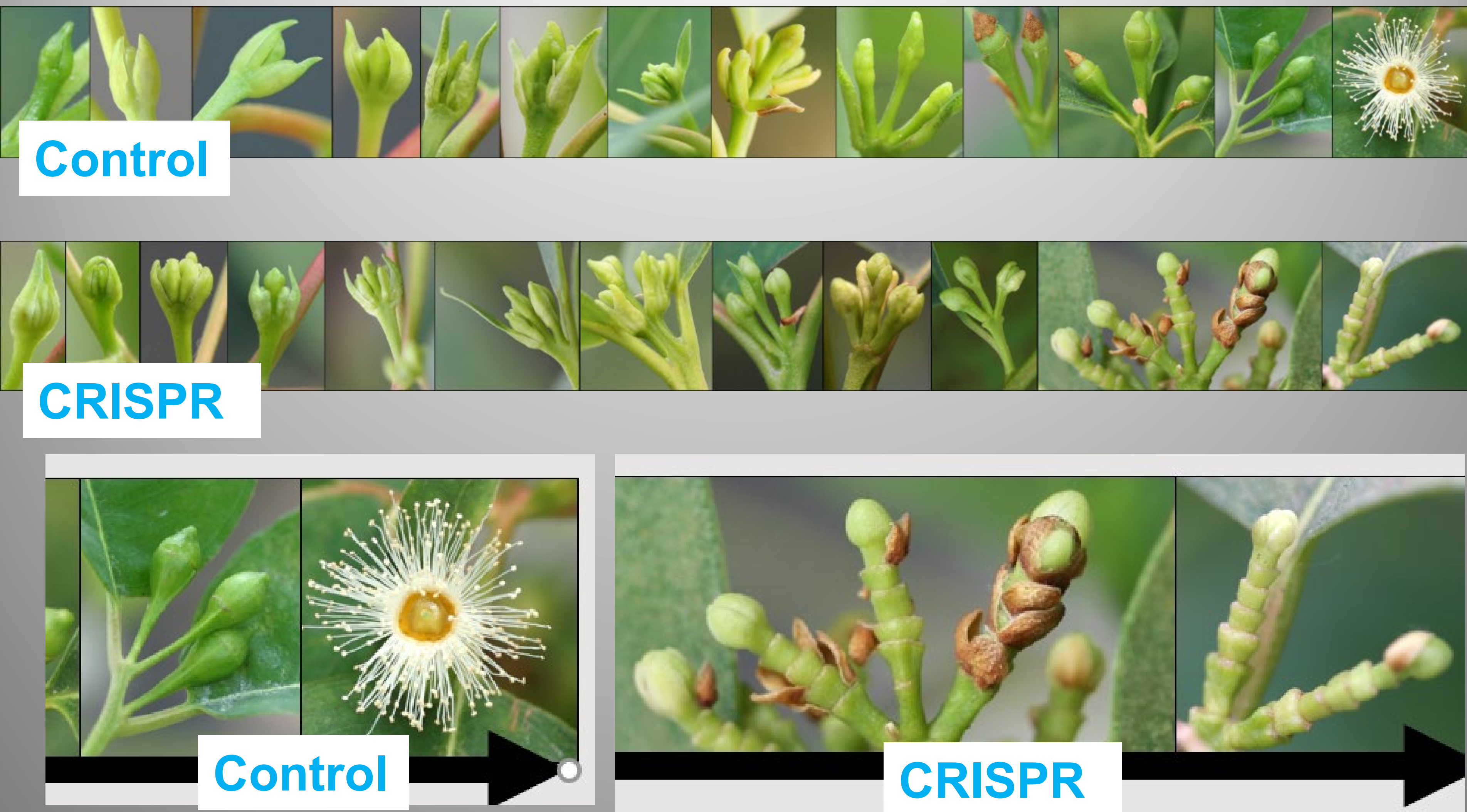


CRISPR pipeline

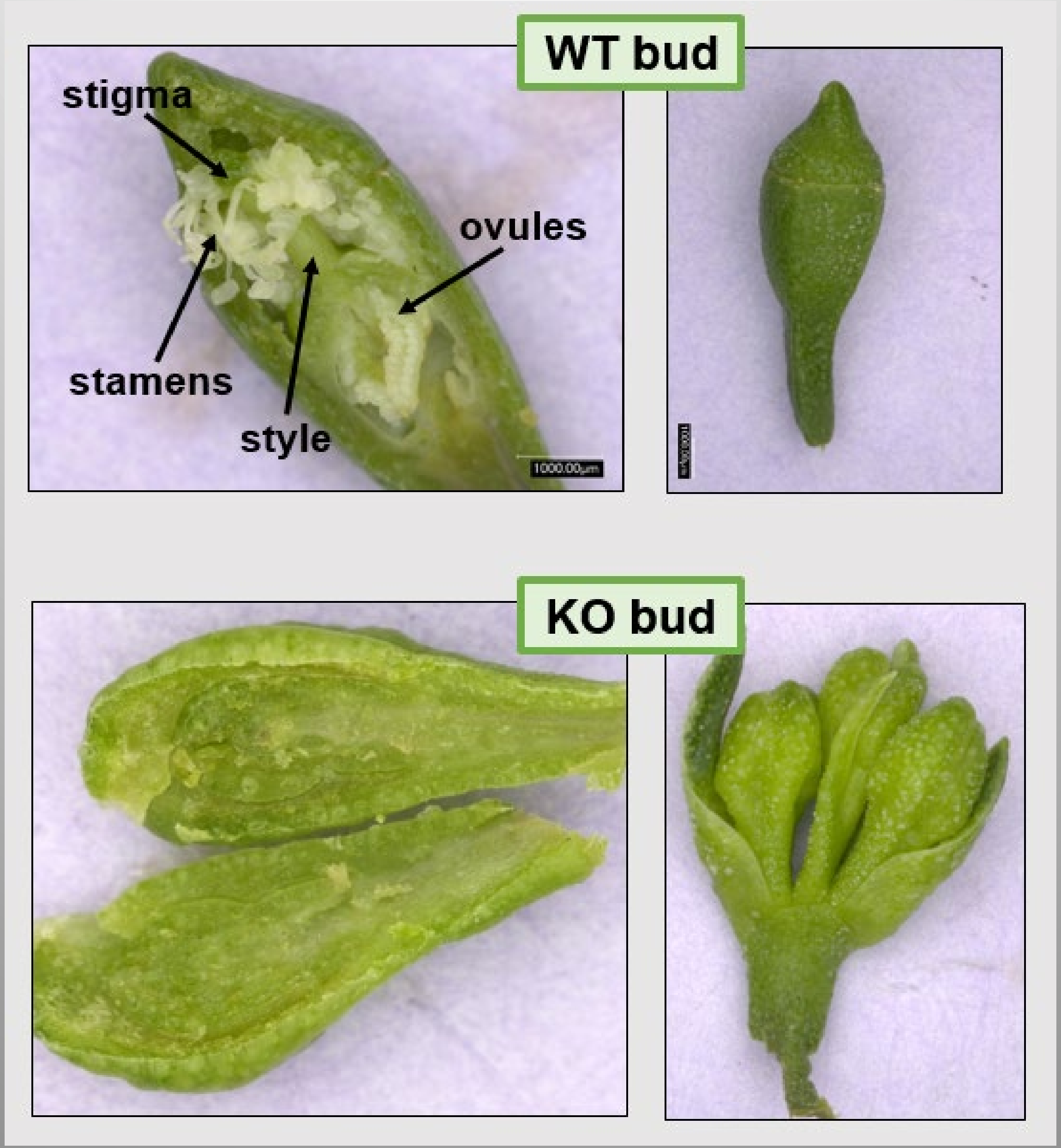


No detectable effects of *LFY* knockout on non-FT vegetative growth in greenhouse

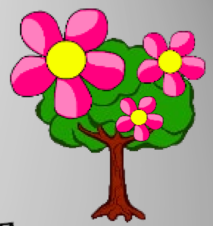
Knockouts in early flowering genotypes had no stamens or carpels, shoots partially indeterminate

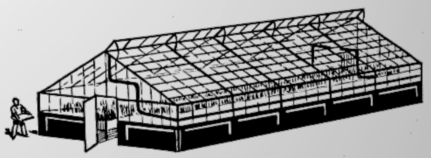


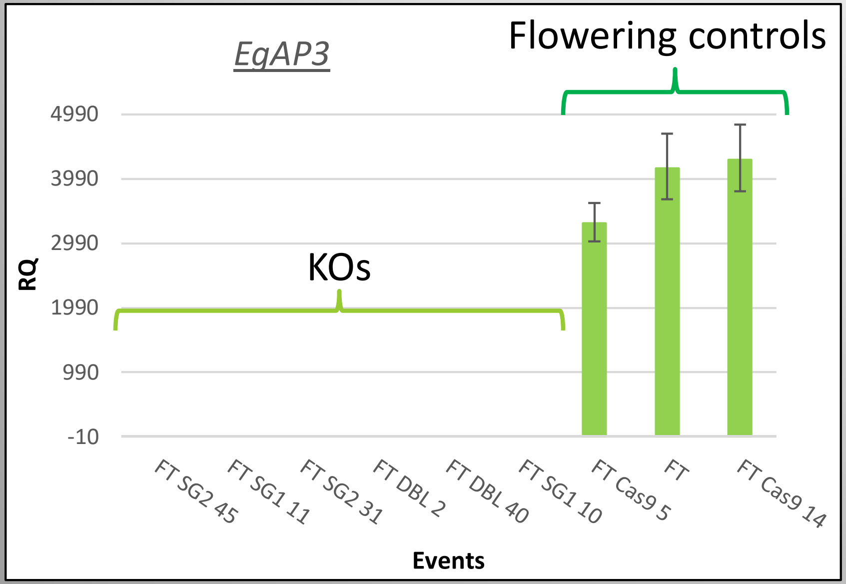
Knockout buds devoid of floral organs

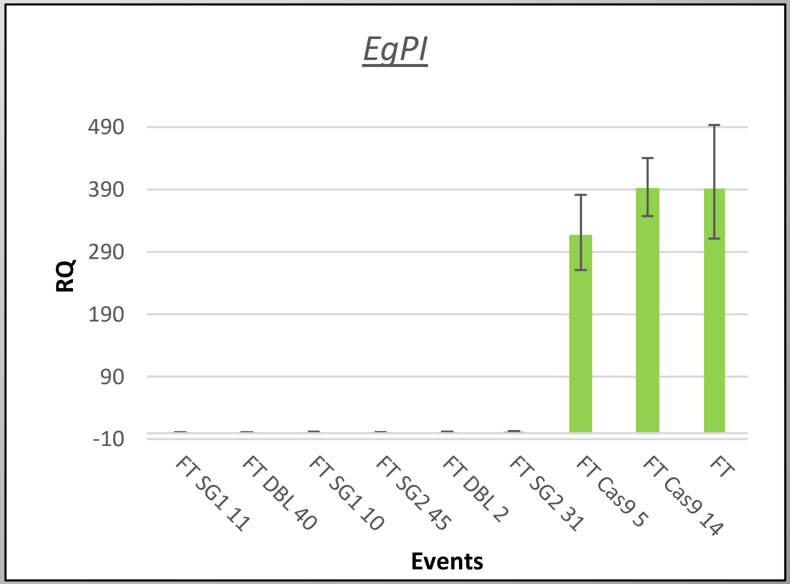


Knockouts have near absence of expression of floral organ identity gene homologs

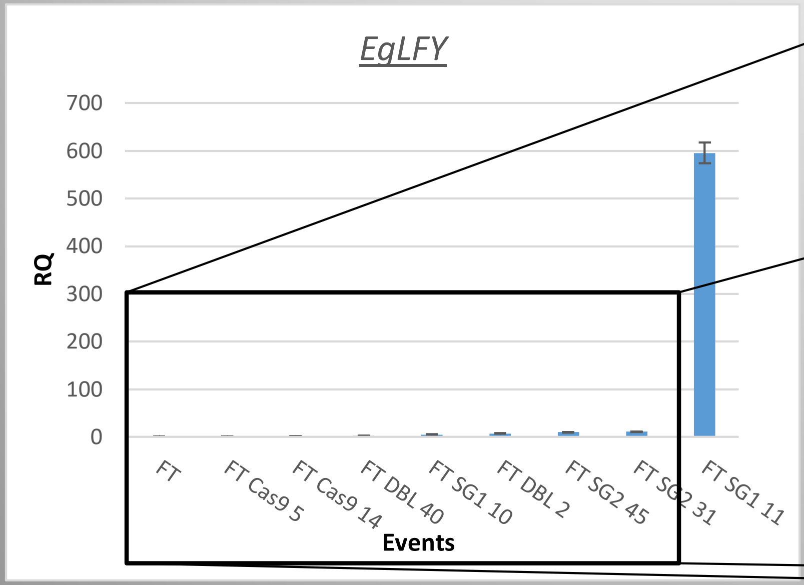


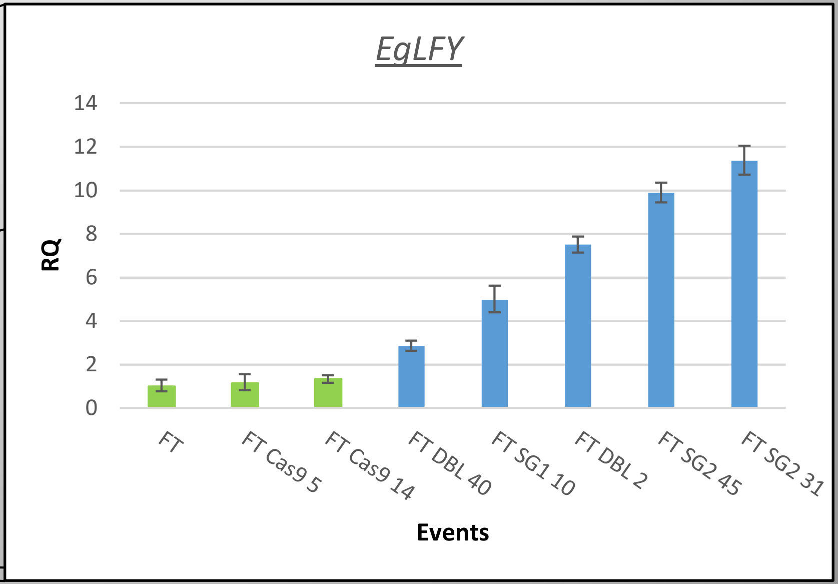






All KOs had very high *LFY* RNA expression





Key results – *LFY* CRISPR in Eucalyptus

- Nearly 100% knockout rate
- Flower buds devoid of reproductive structures
- Partially indeterminate inflorescences
- No detectable vegetative effects
- Work underway
  - Test efficiency of transient expression methods for “clean” knock-outs in eucalypts
  - To study genome scale off-target rate under continuous Cas9-sgRNA expression
  - To create system for developmentally triggered CRISPR excision

Sterility genes are tools – to be used with discretion and management and careful communication, or not at all

2017 Tansley Review

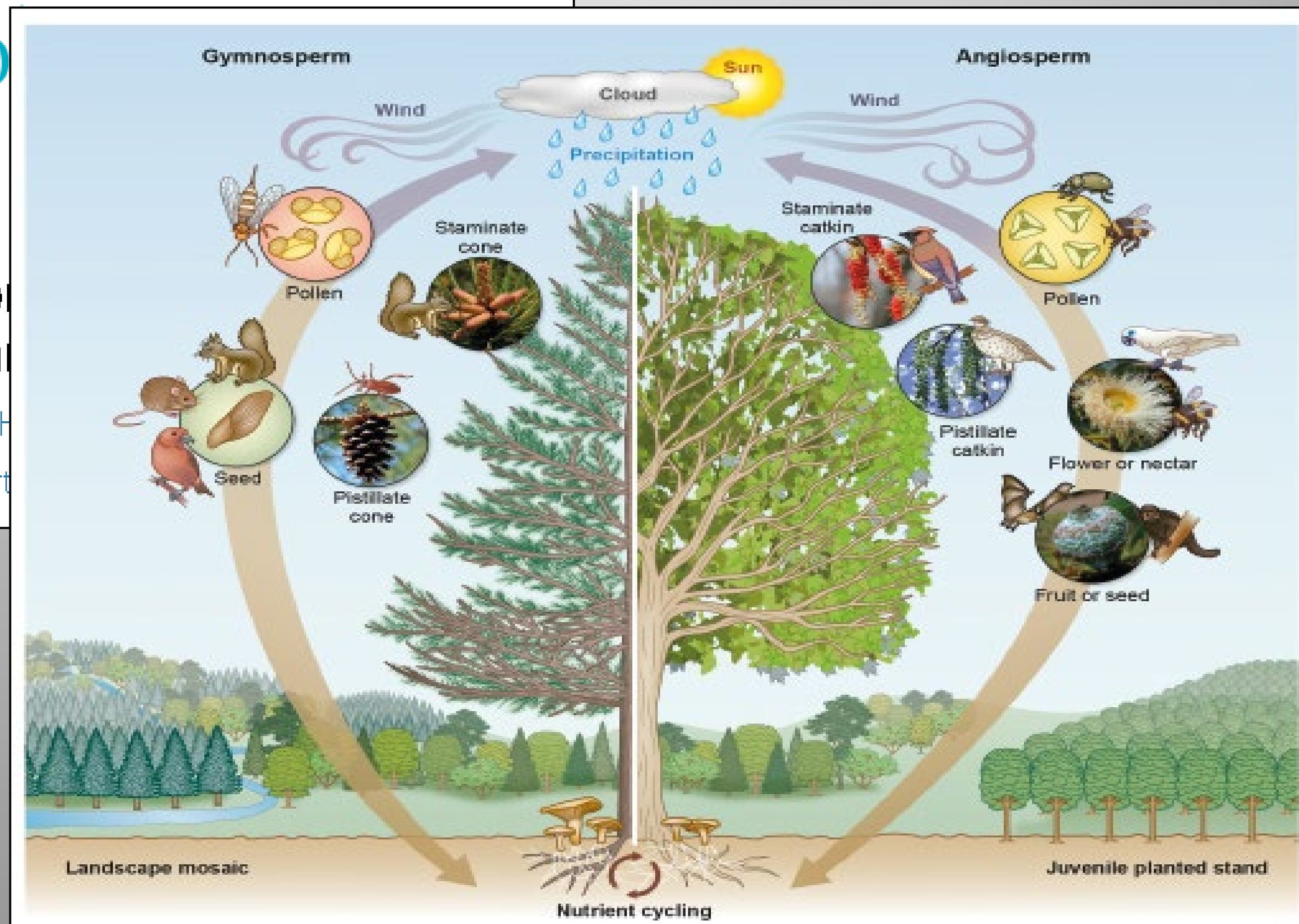
New Phyto

Tansley review

Reproductive modificatio impacts on biodiversity a

Steven H. Strauss, Kristin N. Jones, H

Matthew G. Betts, Berry J. Brosi, Robert



General messages

- Social and technical innovations needed
  - Including BE and GE – don't throw BE under the bus
- OCD on gene flow a great obstacle to research and trade
- Technical innovations needed
  - Trait-gene linkages and modulation system science
  - Transformation-editing systems for diverse and recalcitrant species
- People are fearful -- will be hard, conflict ridden work -- success by no means assured

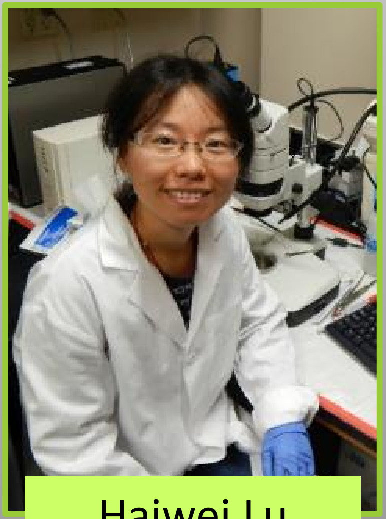
Thanks to these key people and many more over the years

Amy Klocko

Amy Brunner

Cathleen Ma

Estefania Elorriaga



Haiwei Lu

Anna Magnuson

Thanks for financial support

TBGRC

NSF
The National
Science Foundation

USDA

Futuragene, SAPPI, SweTree,
U. Pretoria, Arborgen