CRISPR, Gene Drive, and Regulatory Perspectives

Webinar Presentation to National Research Council Committee on Regulation of Gene Drives

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Agenda

- My background in transgenic field trials
- CRISPR work and status
- Regulatory concepts for gene drives

Will assume perspective of how to rationalize regulations to enable applied and field research

Goal is to avoid high costs to society of generically stringent forms seen in GMO world

Conducted dozens of regulated field trials in USA – mostly *Populus* and flowering modification

Trees must get large to see effects on wood, productivity, adaptation, and flowering behavior — such tests are long term and costly

Current *Liquidambar* (sweetgum) field trial – 8 years old and has only begun to flower in last two years

Test of different constructs for the genetic containment of an exotic, potentially invasive, and messy hardwood street tree

Field trials of Bt and herbicide tolerant trees in collaboration with forest and biotech industries in Oregon (2001)

NRC Research Press

ARTICLE

Bt-Cry3Aa transgene expression reduces insect damage and improves growth in field-grown hybrid poplar

Amy L. Klocko, Richard Meilan, Rosalind R. James, Venkatesh Viswanath, Cathleen Ma, Peggy Payne, Lawrence Miller, Jeffrey S. Skinner, Brenda Oppert, Guy A. Cardineau, and Steven H. Strauss

Abstract: The stability and value of transgenic pest resistance for promoting tree growth are poorly understood. These data are essential for determining if such trees could be beneficial to commercial growers in the face of substantial regulatory and marketing costs. We investigated growth and insect resistance in hybrid poplar expressing the *cry3Aa* transgene in two field trials. An initial screening of 502 trees comprising 51 transgenic gene insertion events in four clonal backgrounds (*Populus trichocarpa* × *Populus deltoides*, clones 24-305, 50-197, and 198-434; and *P. deltoides* × *Populus nigra*, clone OP-367) resulted in transgenic trees with greatly reduced insect damage. A large-scale study of 402 trees from nine insertion events in clone OP-367, conducted over two growing seasons, demonstrated reduced tree damage and significantly increased volume growth (mean 14%). Quantification of Cry3Aa protein indicated high levels of expression, which continued after 14 years of annual or biannual coppice in a clone bank. With integrated management, the *cry3Aa* gene appears to be a highly effective tool for protecting against leaf beetle damage and improving yields from poplar plantations.

Résumé : La stabilité et la valeur de la
bien connues. Ces données sont essen
être profitables pour des producteurs
marché de tels arbres. Les auteurs o

Can. J. For. Res. 44: 28–35 (2014) dx.doi.org/10.1139/cjfr-2013-0270

Published at www.nrcresearchpress.com/cjfr on 28 October 2013.

Field trials are essential for scientific progress as traits become more complex

Wide variance between greenhouse/field results

Tree Genetics & Genomes (2015) 11:127
DOI 10.1007/s11295-015-0952-0

CrossMark

ORIGINAL ARTICLE

Recombinant DNA modification of gibberellin metabolism alters growth rate and biomass allocation in *Populus*

Haiwei Lu[1] · Venkatesh Viswanath[1,4] · Cathleen Ma[1] · Elizabeth Etherington[1,5] · Palitha Dharmawardhana[1,6] · Olga Shevchenko[1,7] · Steven H. Strauss[1] · David W. Pearce[2] · Stewart B. Rood[2] · Victor Busov[3]

Received: 4 September 2015 / Revised: 26 October 2015 / Accepted: 28 October 2015

Abstract Overexpression of genes that modify gibberellin (GA) metabolism and signaling have been previously shown to produce trees with improved biomass production but highly disturbed development. To examine if more subtle types of genetic modification of GA could improve growth rate and modify tree architecture, we transformed a model poplar ge- transgenes (from sexually incompatible species), and studied their effects under greenhouse and field conditions. In the greenhouse, four out of the eight tested genes produced a significant and often striking improvement of stem volume, and two constructs significantly modified the proportion of root or shoot biomass. Characterization of GA concentrations

Agenda

- Background in transgenic field trials
- CRISPR work and status
- Considerations of containment / release of transgenic plants and trees

ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

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

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.”

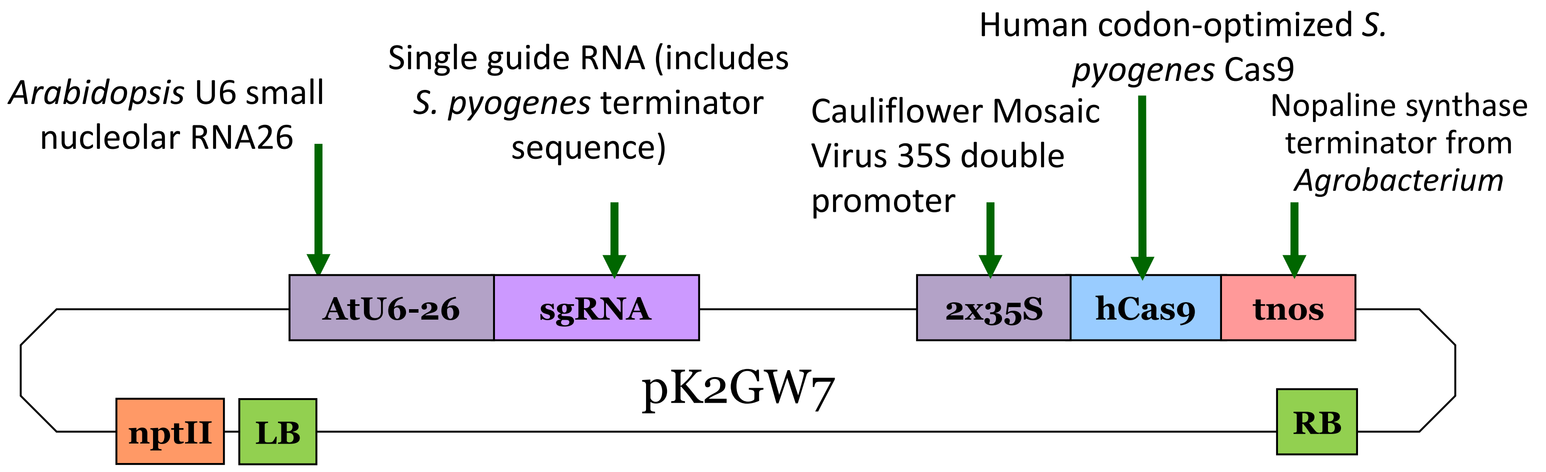
To promote coexistence and compliance, a primary focus in my lab is on genetic containment via complete bisexual sterility – vegetative propagation, vegetative harvest – poplar, eucalypts, pine

Site directed mutagenesis ideal as a method for containment ?

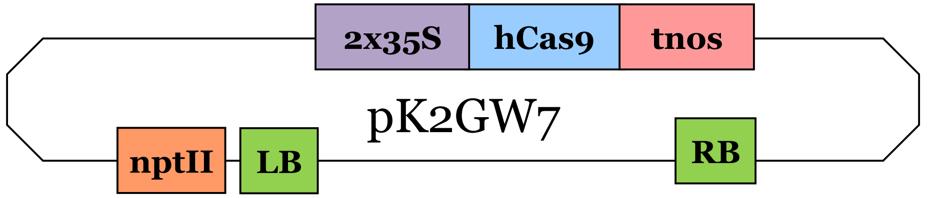
- Avoid cytotoxins like barnase / pleiotropy
- Physical damage to floral gene/s should be far more reliable than modified/suppressed gene expression or protein function
- Reported highly efficient – biallelic mutations
  - Complete loss of gene function without inbreeding
- Highly predictable from new regenerant to flowering tree to speed breeding, avoid regulatory problems
- Strong transient expression, or inducible recombinases, should avoid CRISPR presence (if needed)

Single and double CRISPR-Cas constructs studied to date

- Nuclease constructs

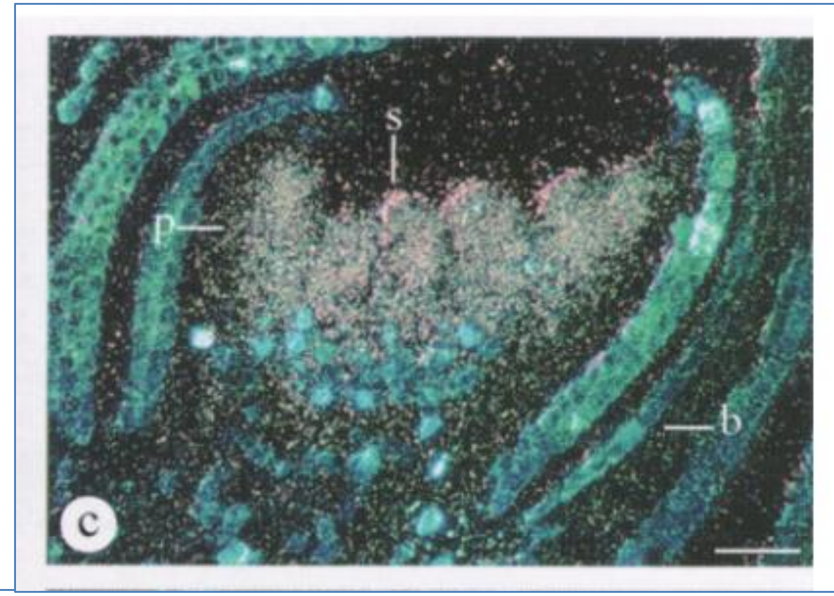


- Control construct



Gene targets *LEAFY* and *AGAMOUS*

Structure & expression in poplar studied previously



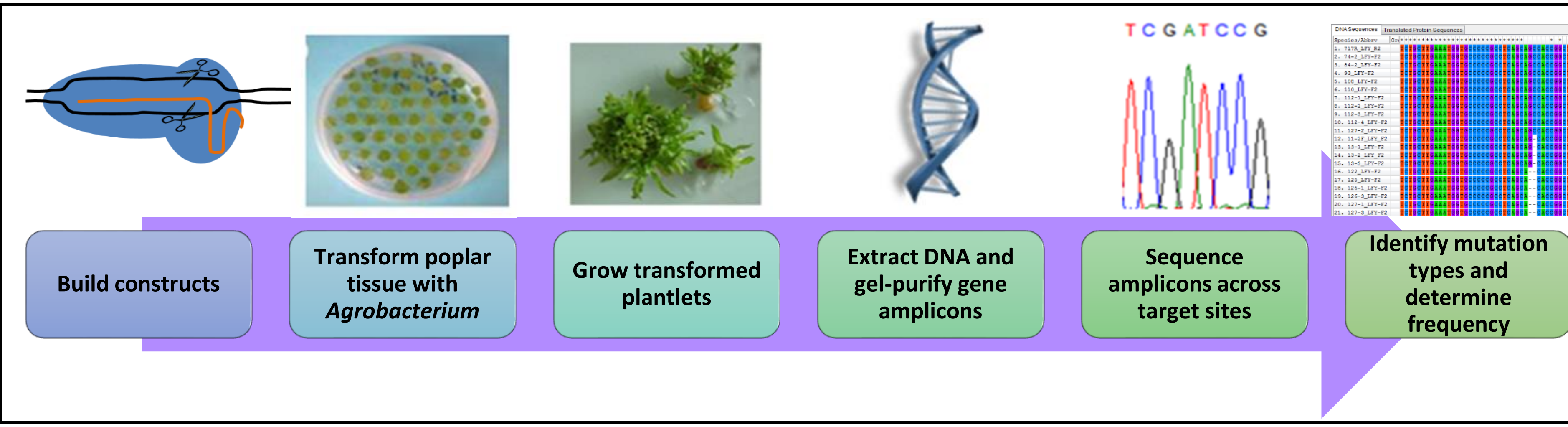
Plant Molecular Biology 44: 619-634, 2000. © 2000 Kluwer Academic Publishers. Printed in the Netherlands.

Structure and expression of duplicate AGAMOUS orthologues in poplar

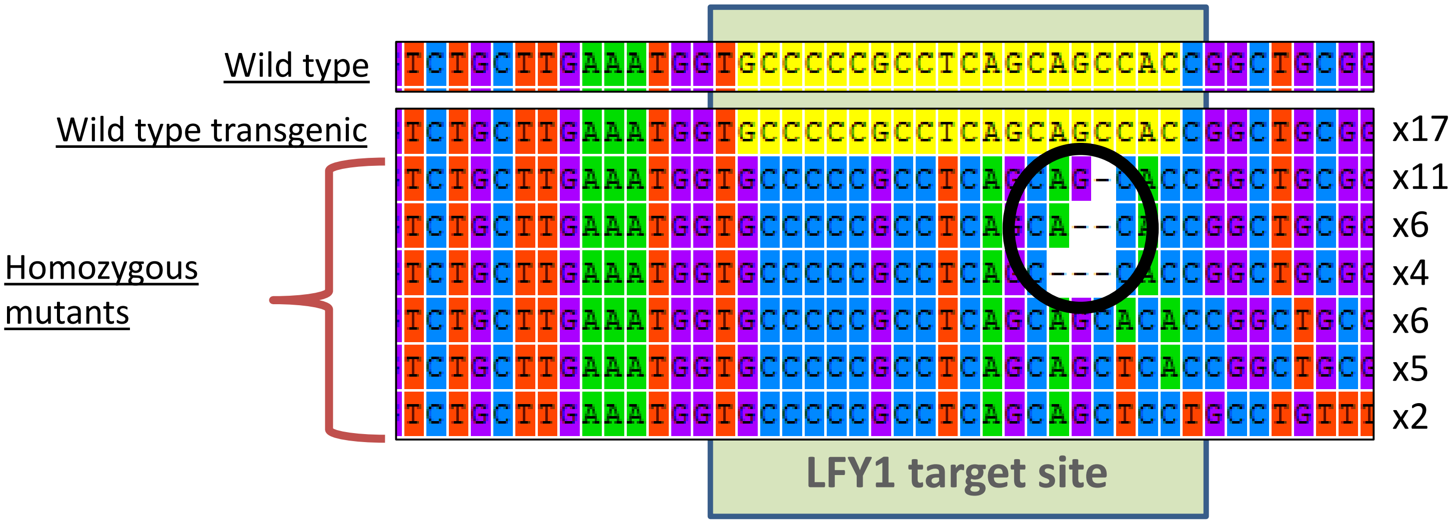
Amy M. Brunner, William H. Rottmann[1], Lorraine A. Sheppard[2], Konstantin Krutovskii, Stephen P. DiFazio, Stefano Leonardi[3] and Steven H. Strauss*

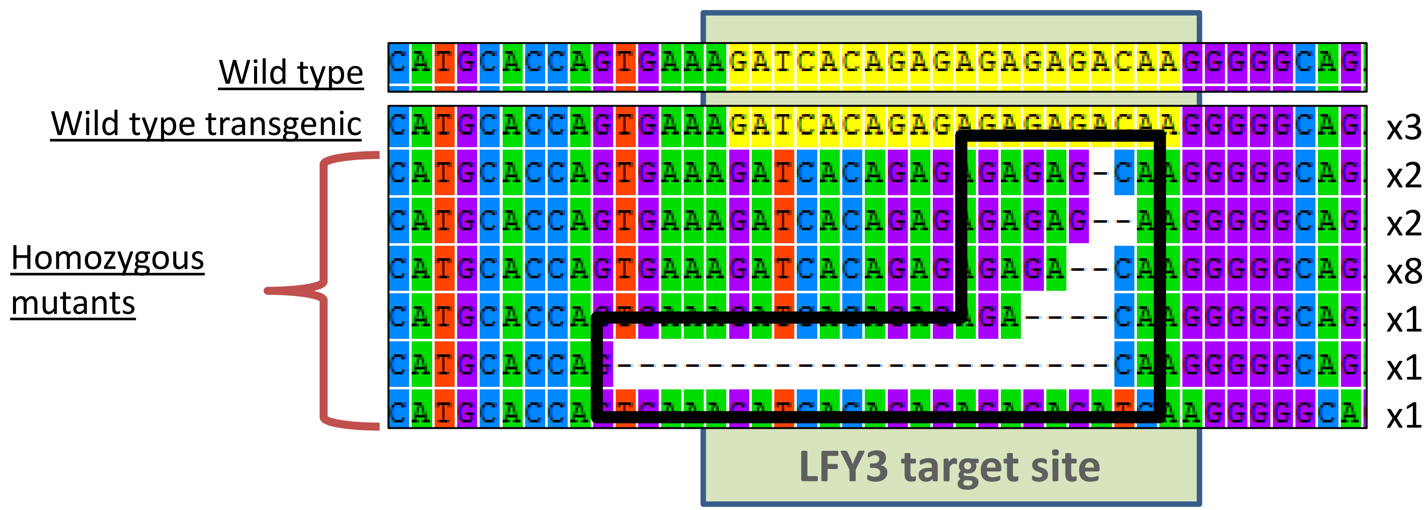
*Department of Forest Science, Oregon State University, Corvallis, OR 97331, USA (*author for correspondence; e-mail: strauss@fsl.orst.edu); present addresses: 1 Westvaco Forest Science and Technology, PO. Box 1950, Summerville, SC 29484, USA; 2Institute of Forest Genetics, USDA Forest Service c/o Department of Environmental Horticulture, One Shields Ave., University of California, Davis, CA, 95616, USA; 3Department of Environmental Science, University of Parma, Parco Area delle Scienze 33a, 43100 Parma, Italy*

Work flow


TCGATCCG
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

Frequent insertions and deletions– as well as longer mutations





Data from
Estefania Elorriaga,
PhD student

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%)**

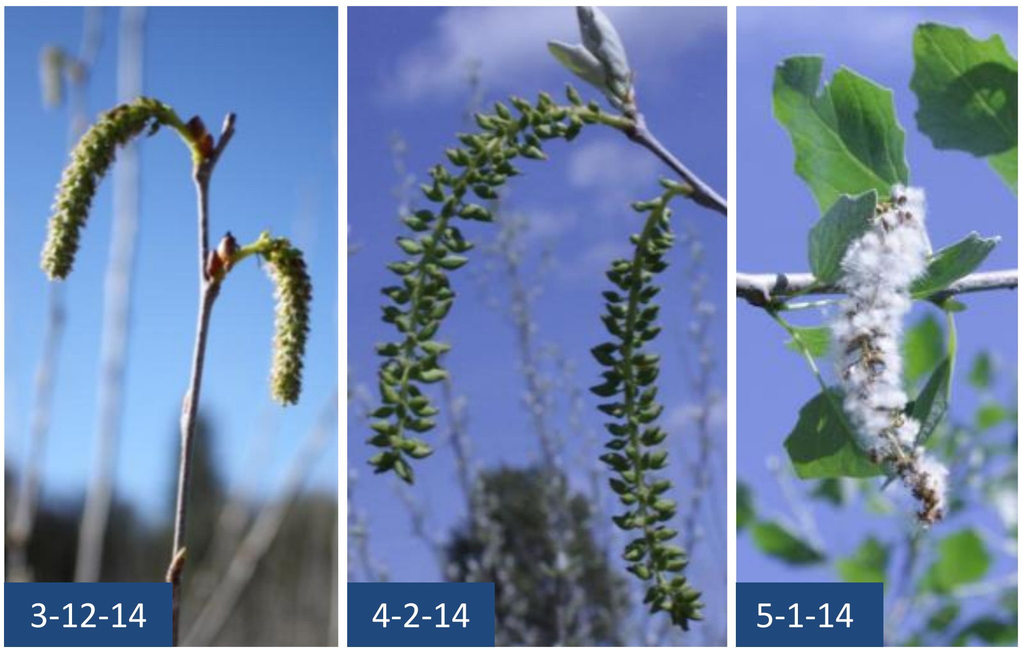
What will phenotypes be?

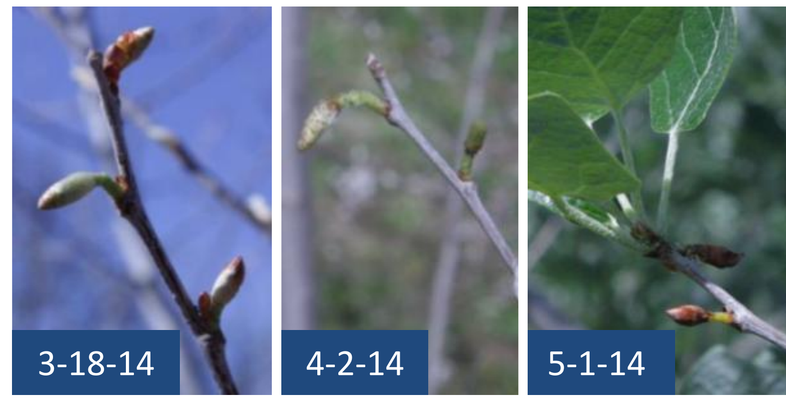
RNAi field studies give a good indication

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

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

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



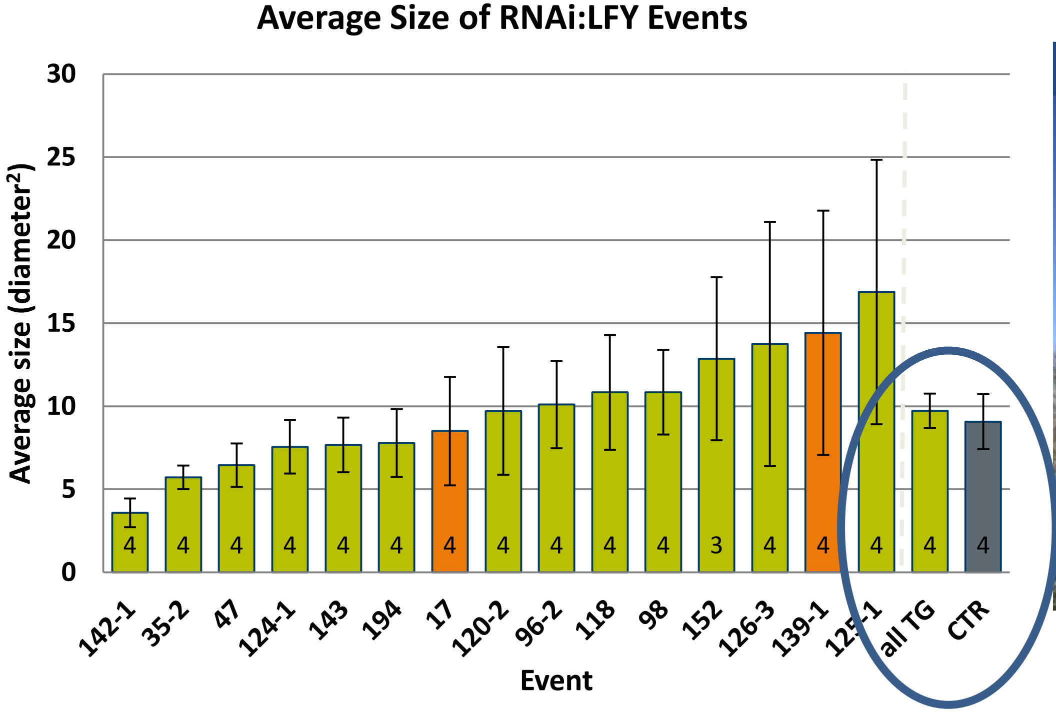


RNAi-LFY

Control

An absence of pleiotropy?

RNAi:*LFY* trees had normal vegetative growth



If approved, first crop of CRISPR mutants should be ready for field planting in 2016

Agenda

- Background in transgenic field trials
- CRISPR work and status
- Regulatory adequacy / concepts

How should CRISPRs be regulated?

- Market / adventitious presence criteria
- I will focus on biological / innovation efficiency criteria
- Will follow National Research Council and others: “Product not process”
- Avoid stringent, simple trigger to regulation if possible (i.e., based on CRISPR presence and drive possibility alone)


OREGON'S ACCIDENTAL CROP

The discovery of GE wheat highlights regulatory failures

BRENT WOJAHN/THE OREGONIAN

Wheat grows near Condon in north-central Oregon. Rogue genetically modified wheat found in an eastern Oregon field has prompted debate over how to contain, and how the U.S. regulates, genetically engineered crops.

By KRISTINA HUBBARD and NEVA HASSANEIN

The recent news that genetically engineered wheat never approved for sale was growing in an eastern

trials, ensure that performance standards are met and trace back the source of contamination that might occur as a result of GE experiments. This lack of basic information not only

I have shared similar thoughts in detail elsewhere

POLICY FORUM

GENETIC TECHNOLOGIES

Genomics, Genetic Engineering, and Domestication of Crops

Steven H. Strauss

Genomic sequencing projects are rapidly revealing the content and organization of crop genomes (*1*). By isolating a gene from its background and deliberately modifying its expression, genetic engineering allows the impacts of all genes on their biochemical networks and organismal phenotypes to be discerned, regardless of their level of natural polymorphism. This greatly increases the ability to determine gene function and, thus, to identify new options for crop domestication (*2*). The organismal functions of the large majority of genes in genomic databases are unknown.

portant to agricultural goals, but poorly represented in breeding populations because they are rare or deleterious to wild progeni-

huge numerical obstacle that is normally provided by extant wild and domesticated gene pools. Despite the great diversity of genes that can comprise GGTs, many of the modified traits are familiar, having a long history of domestication and consequent reduced fitness through artificial selection. Male sterility, seedless fruits, delayed spoilage, and dwarf stature are familiar examples.

GGTs that improve abiotic stress tolerance

Confinement level	Type 1 field trials (exploratory)	Type 2 field trials (precommercial)	Examples
High	Biological and physical confinement—detailed data		Highly toxic or allergenic pharmaceuticals and proteins
Medium	FSC, basic data	FSC, detailed data	Novel pest-management genes, low toxicity pharmaceuticals and proteins
Low: Stress tolerance	FSC, basic data	FSC, detailed data	Genomics-guided transgenes
Low: Domesticating	Petition for exemption?	FSC, basic data	

Categories of confinement and monitoring for small- and large-scale transgenic field trials. Biological confinement includes genetic mechanisms to preclude spread and/or reproduction. Physical confinement requires use of geographical isolation or physical barriers. FSC, farm-scale confinement; use of spatial isolation within and between farms and border crops, combined with ...oring. Detailed data include surveys of gene flow away from the site. Basic data ...hment of confinement mechanisms.

And recently updated…

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

Why getting regulation right matters…..

The strange case of the upright summer catkin

Summer “flowering” of ~200 semi-dwarf transgenic poplar trees in a field trial

The upright summer "catkins"

This was not the intended trait for this regulated trial - What to do?

- Being a good soldier, I faithfully and immediately reported this “unexpected occurrence”
- Then discussed what to do about it with APHIS regulatory science contacts for several days
- We wanted to leave it be for study of the novel and partial flowers
- Risk seemed to be zero and it would be difficult to remove all of them

I argued my case….

- I pointed out the layers of safety from the genes (dwarfism, fitness reduced) and biology (lack of pollen or receptive females in summer, unsuitable habitat, no seed dormancy)
- The APHIS scientists agreed, but they felt, legally, they need to report it to the **compliance branch** as a technical violation of our permit conditions…


802
© AP Photo/Rogelio V. Solis

A strange tip saved the day….

- Thankfully I was alerted that the report to Compliance had occurred prior to "a friendly visit"
- So rather than risk arrest, fines, and who knows what else by federal agents….
- Including what would be sure to be highly publicized as major disregard for the rules and the environment by our anti-GMO friends, and thus a call for even stricter and more costly regulations…
- The same day, all students in our lab were dispatched to manually remove every "catkin"..
- And the same in spring and beyond…

Students removing catkins from transgenic trees in spring

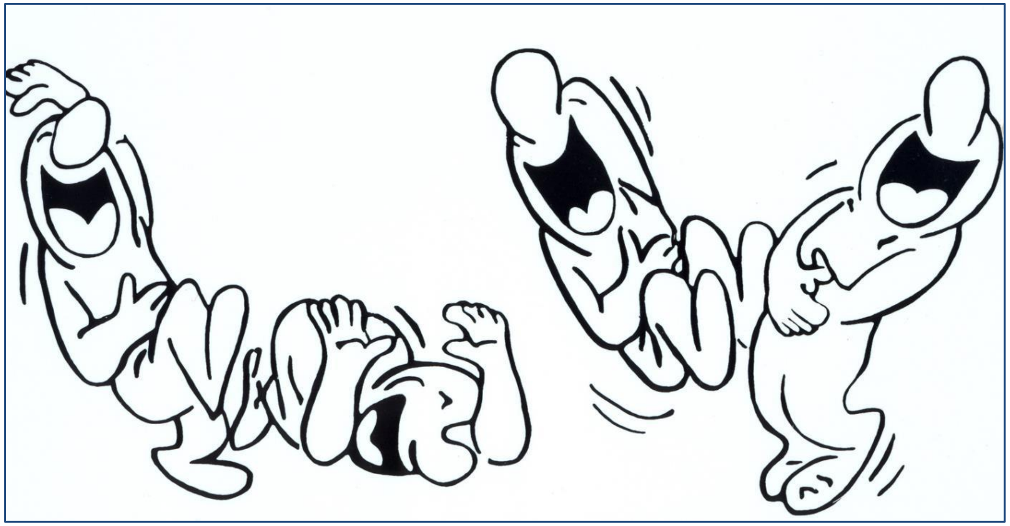
We documented for APHIS that “All removed flowers were collected and brought back to the lab, then autoclaved”

A lesson about science vs. law…

- Thank goodness, the federal agents never came to fine me or arrest me over this grave “violation”
- **A powerful lesson about the letter of the law, and the reality that GE methods are considered evil and dangerous until proven otherwise, period**
- Biology, safety, and intended benefit are irrelevant as written today
- Had the rules been based on product rather than process, these trees would probably not have been regulated at all
  - Safety of trait
  - Ecological and economic value of trait for coexistence
  - Exceedingly small risk from a field trial release

Wait, that’s not all…..

- One answer could be to deregulate the research trial for science
  - So we can study without risk from unexpected types of flowering
  - Science: Several constructs, dozens of insertion events
- So I visited APHIS and suggested this given the safety and benefits of the trait and associated knowledge



It just don't work that way kid…

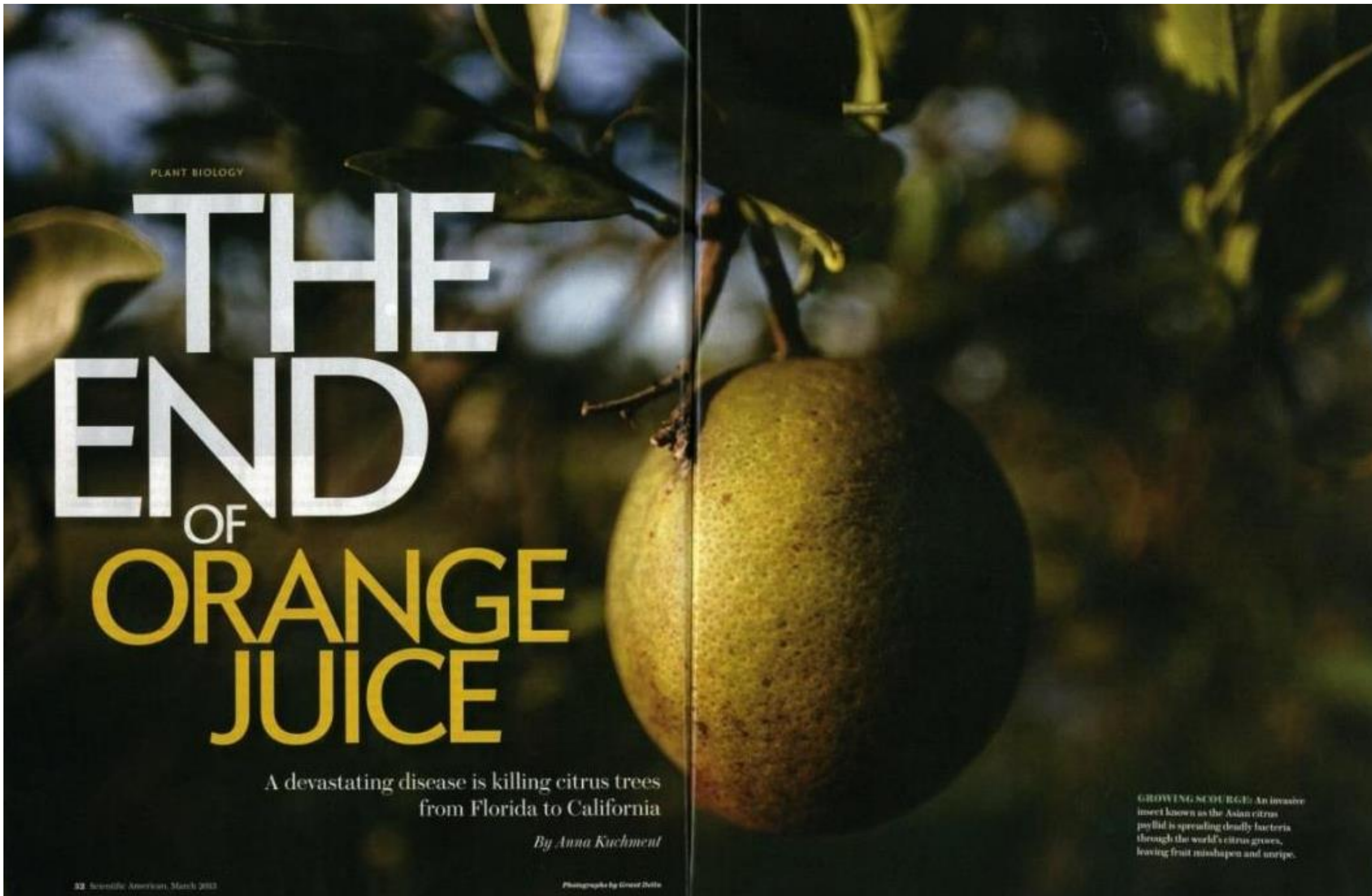
- They discussed how **each event** needs a pile of data, and now certainly an environmental impact statement (EIS), to withstand lawsuits
- And getting this data requires the years of research (that is what we are trying to find a way to do!)
- Do we want the same or more for all CRISPR and gene drive applications?
- Bottom line:
  Critical to make regulations risk/benefit and not method based — thus workable for public sector or small company involvement — and for projects other than those with billion dollar company profits

How should regulations for gene drives intended to solve plant problems be structured under a product not process system?

What are the targets we might expect can be done with a high level of safety during field research and application?

- Herbicide tolerant plants whose drive is focused on the new or now prevalent resistance alleles
- Damaging invasive plants without compatible wild relatives
- Damaging, exotic insect pests and pathogens such as of wild and cultivated trees
- Is strict regulation of field trials needed for any of these cases?
  - Exemptions? Registration but not full containment?

Solution to pests like devastating ‘citrus greening’ ?



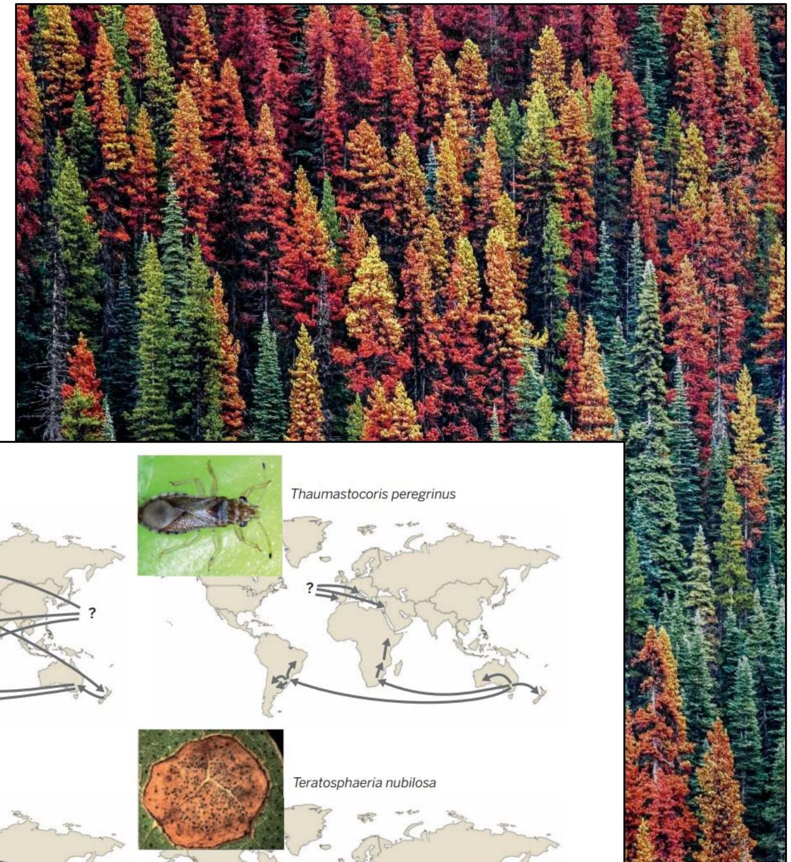
Help with forest health - A major and growing concern with climate change


REVIEW

Planted forest health: The need for a global strategy

gfield,[1] B. Slippers[1]

worldwide, and these represent valuable
reatened by insects and microbial
r have adapted to new host trees.
spite a growing awareness of the
an increased focus on the importance of
nted forests, innovative solutions and a
igation strategies that are effective only in
e in the world, ultimately leading to global
ture should mainly focus on integrating
e-country strategies. A global strategy to



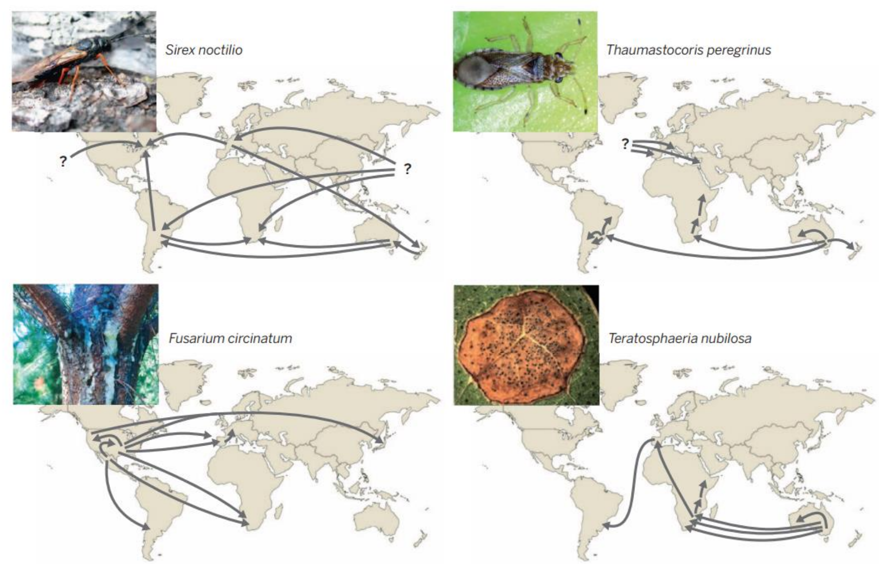


Fig. 2. Examples of invasion routes of pests of planted forests that illustrate an apparently common pattern of complex pathways of spread to new environments, including repeated introductions and with either native or invasive populations serving as source populations (18). Invasion routes of the pine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

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

It was extirpated as a forest tree by Chestnut Blight

Hemlock in US under siege today

Corrected 2 September 2015; see full text. *FOREST HEALTH* **SPECIAL SECTION**

BATTLING A GIANT KILLER

The iconic eastern hemlock is under siege from a tiny invasive insect

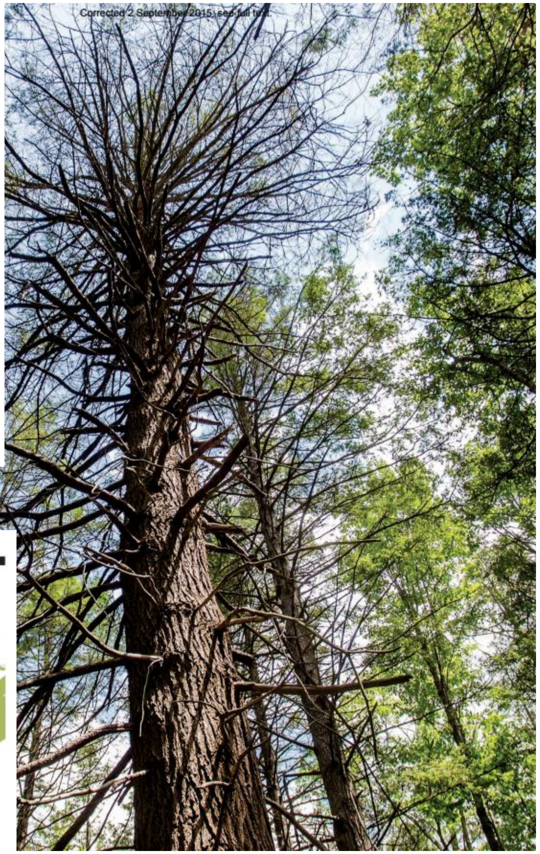
By **Gabriel Popkin** *in Highlands, North Carolina; photography by* **Katherine Taylor**

On a frigid morning this past March, arborist Will Blozan snuck behind a small church here and headed down into a gorge thick with rhododendron. He crashed through the shrubs until he spotted the gorge's treasure: the world's largest

park, "are in intensive care." Like the family of a gravely ill patient, ecologists are also preparing for the possibility that these efforts will fail, and the eastern forest will lose one of its defining species.

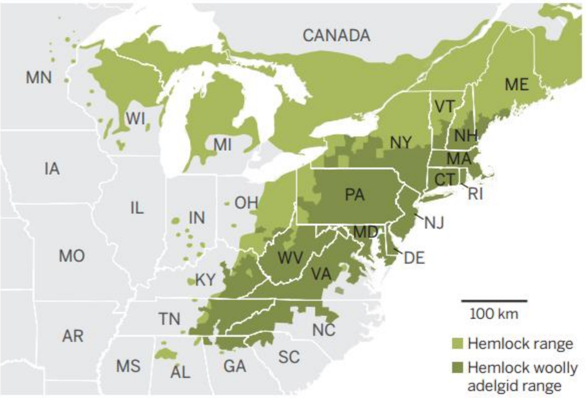
TSUGA CANADENSIS is one of eastern

branches, creating a thick canopy that blocks up to 99% of sunlight. Few plants grow in the gloom, but a hemlock seedling can bide its time for decades or more, waiting for a sunlit opening. Hundreds of species of insects, mites, and spiders appear to live primarily or exclusively in hemlock forests, and some



A creeping conflict

The hemlock woolly adelgid now infests about half of the eastern hemlock's range, and has been spreading by about 15 kilometers per year.



Emerald Ash Borer killing ~all ashes in USA – costing billions

Thriving Ash Trees in 2006 | **Emerald ash borer larva (26–32 mm long)** | **Dead Ash Trees in 2009**

The emerald ash borer was first detected in North America in 2002. Native to Asia, the beetle has proven to be highly destructive in its new range. Since its arrival, it has killed tens of millions of ash trees and continues to spread into new areas.

Photo credits - Trees: Daniel A. Herms, The Ohio State University - Borer larva: Dr. Robert Lavallée, Natural Resources Canada

What are mitigating factors that suggest a high level of safety during field research and application?

- Small releases such as from typical field trials
  - Impacts in the near term usually will take massive and repeated releases
- Gene drives in organisms where control methods also available should something bad happen later
  - Deactivation systems
- Gene drives that powerfully suppress fitness
  - Reproductive sterility an example

What are the targets that require more scrutiny during field research and application?

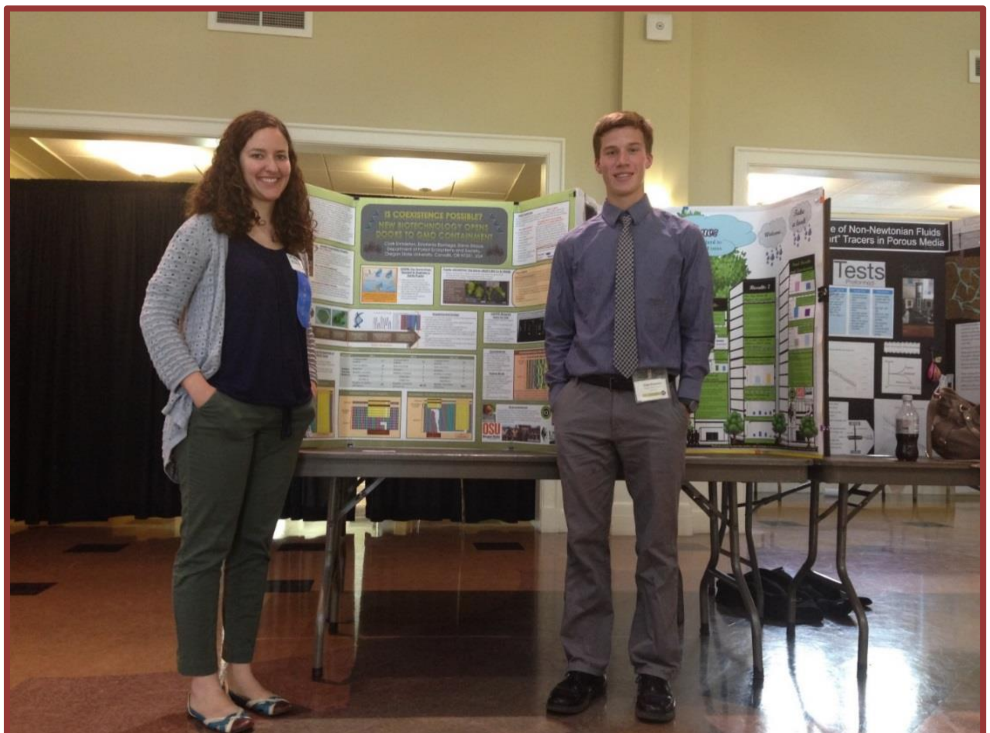
- Herbicide tolerant plants whose drive is focused on essential genes and have common, ecologically important, and sexually compatible wild relatives
- Damaging invasive plants whose drive is focused on essential genes and also have broadly compatible and ecologically important wild relatives
- Damaging, exotic insect pests and pathogens that have ecologically important wild relatives

Summary messages

- Burden of GE regulations can be large, costly to society
  - Many have argued that current regulations greatly retard public sector and small company use of all forms of GE biotech – slowing innovation and reducing public benefit
  - We need a smarter way
- "Product not process" concept to inform regulatory tiers
  - In both research and application
  - Also a part of regulatory triggers to avoid unnecessary GMO-esque baggage
- Such tiers seem feasible with gene drives

THANKS

Estefania Elorriaga, PhD student
Clark Embleton, ASE high school student
Cathleen Ma, Transformation
Amy Klocko, Postdoc, Floral molecular biology
Kori Ault, Field trial management



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