Enabling GMOs in forestry
CRISPRs as tools to promote coexistence

Steve Strauss, Professor
College of Forestry, Oregon State University
Steve.Strauss@OregonState.Edu
Estefania Elorriaga, PhD student
Clark Embleton, ASE high school student
Cathleen Ma, Transformation
Amy Klocko, Postdoc, Floral molecular biology
Kori Ault, Field trial management
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
In poplar, paternity analysis showed that ~50% of pollen comes from >1 km to >10 km
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 ~irreversibly modifying wild organisms
Forest trees with significant anti-GMO activism

Genetically modified arboriculture
Down in the forest, something stirs

The Economist, 2005
Market barriers large
“Green” certification of forests create severe barriers to field research, markets

Forest Stewardship Council

“...genetically modified trees are prohibited…”

Plants can relieve pressure on natural forests for exploitation and can be of great social value by supplying community and industrial wood needs and adding 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 natural forests and promote the restoration and conservation of natural forests (FSC 2000).

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

However, FSC currently prohibits all novel GM trees, and is the only certification system to have done so.
Forest certification systems universally ban all GM trees – no exemptions

<table>
<thead>
<tr>
<th>System</th>
<th>Region</th>
<th>GM Tree Approach / Reason</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEFC: Programme for Endorsement of Forest Certification</td>
<td>International</td>
<td>Banned / Precautionary approach based on lack of data</td>
</tr>
<tr>
<td>FSC: Forest Stewardship Council</td>
<td>International</td>
<td>Banned / Precautionary approach based on lack of data</td>
</tr>
<tr>
<td>CerFlor: Certificação Florestal</td>
<td>Brazil</td>
<td>Banned via PEFC registration / No additional rationale</td>
</tr>
<tr>
<td>CertFor: Certificación Forestal</td>
<td>Chile</td>
<td>Banned via PEFC registration / No additional rationale</td>
</tr>
<tr>
<td>SFI: Sustainable Forestry Initiative</td>
<td>North America</td>
<td>Banned via PEFC registration / Awaiting risk-benefit data</td>
</tr>
<tr>
<td>ATFS: American Tree Farm System</td>
<td>USA</td>
<td>Banned via PEFC registration / No additional rationale</td>
</tr>
<tr>
<td>CSA: Canadian Standards Association</td>
<td>Canada</td>
<td>Banned via PEFC registration</td>
</tr>
<tr>
<td>CFCC: China Forest Certification Council</td>
<td>China</td>
<td>Banned via PEFC registration / No additional rationale</td>
</tr>
</tbody>
</table>

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 transgenic organisms.

An 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 was adopted to ensure that parties are provided with an additional mandate in the CBD
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. Strauss1, Adam Costanza2, Armand Séguin2

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 (4).

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

Review article

Genetic use restriction technologies: a review

Luca Lombardo*

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

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.

Keywords: V-GURTs, T-GURTs, intellectual property, seed saving.
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.”
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?
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
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 promoter, 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
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

- Arabidopsis U6 small nucleolar RNA
- Single guide RNA (includes S. pyogenes terminator sequence)
- Human codon-optimized S. pyogenes Cas9
- Cauliflower Mosaic Virus 35S double promoter
- Nopaline synthase terminator from Agrobacterium

• Control construct
Double gRNA CRISPR/Cas construct for generating deletions
Gene targets **LEAFY** and **AGAMOUS**

Structure & expression in poplar studied previously
Work flow

- 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

**LFY locus**

- Exon 1 (~436bp)
- Exon 2
- Exon 3

PCR product ~570 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

Wild type

Wild type transgenic

Homozygous mutants

LFY1 target site

Deletions

Homozygous mutants

LFY3 target site

Wild type transgenic
Many insertions

Wild type transgenic

Homozygous mutants

Wild type

Wild type transgenic

Homozygous mutants

LFY1 target site

LFY3 target site

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

**Large deletions**

- **Wild type**
- **Wild type transgenic**
- **Homozygous mutants**

**Inversion**

- **Wild type**
- **Wild type transgenic**
- **Homozygous mutants**
Many mutants seen at one AGAMOUS target site

Wild type

Wild type transgenic

Homozygous mutants

Deletions

Insertions

AG2 target site
Two >400bp insertions seen to date

- Wild type
- Wild type transgenic
- Homozygous mutants

AG2 target site

Deletions
- x4
- x2
- x1

Insertions
- x1
- x1
- x2
Very low mutation rate at other AG target site

Wild type

AG-1 target site
Most LFY mutations have completely disturbed the final protein.
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

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

Control

Most events were normal

Unexpanded

AG/LFY and LFY

Replicated

AG
After field maturation, RNAi:LFY catkins remained tiny and did not produce seeds or cotton during two years of study.
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.
In the face of these enormous threats, why keep tools as powerful as GMOs on the shelf?