Somatic Transgene Excision Strategies for Gene Editing in Clonally Propagated Plants

Inducible Excision Performance in Transgenic Poplar

Greg S. Goralogia and Steven H. Strauss
Department of Forest Ecosystems and Society
Oregon State University

Thanks to postdoc Greg Goralogia who is the force behind this work

Agenda

1. Gene editing complications in trees and clonal crops
2. Design of a developmental excision system
3. Proof of concept semi-dwarf/sterile trees
4. New solutions for excision of gene editing machinery

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1. **Gene editing complications in trees and clonal crops**
2. Design of a developmental excision system
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4. New solutions for excision of gene editing machinery

CRISPR transgenic machinery is simple to remove in most annual crops

Agrobacterium-mediated transformation

Identify transgenic plants

Genotype for desired edits

Self-fertilize

Segregate away CRISPR/Cas9 transgene

Edited, transgene-free plants

Researchers Use CRISPR to Create Compact Tomato Plants

Dec 26, 2019 by News Staff / Source « Previous | Nex

Published in
Genetics

Tagged as
CRISPR-Cas9
DNA
Gene
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Solanaceae
Tomato

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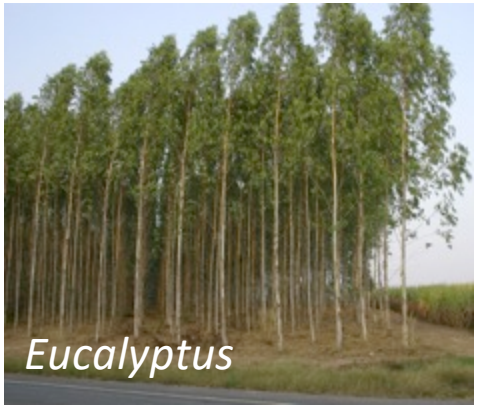
Scientists Discover Gene that Controls Flowering in Cacao

Researchers

An international team of scientists has used the CRISPR-Cas9 genome-editing technology to restructure vine-like tomato plants into extremely compact, early yielding plants suitable for urban agriculture and even spac missions.

But eliminating gene editing machinery is a big problem in asexually propagated plants



- **High heterozygosity**
- **Wide interspecific crosses**
- **Intolerant to inbreeding**
- **Many years to maturity**
- **Induced or natural sterility**

Sterility traits for containment in short rotation forestry emphasize need for somatic CRISPR/Cas9 removal

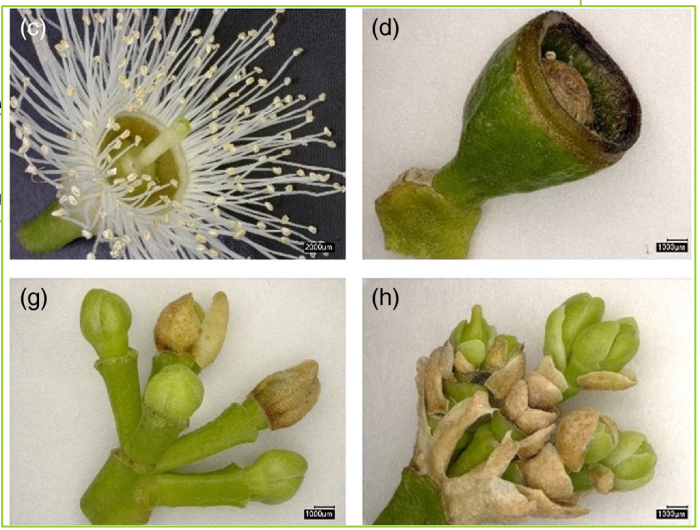
Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2021), pp. 1–13 doi: 10.1111/pbi.13588

Genetic containment in vegetatively propagated forest trees: CRISPR disruption of *LEAFY* function in *Eucalyptus* gives sterile indeterminate inflorescences and normal juvenile development

Estefania Elorriaga[1,a], Amy L. Klocko[2], Cathle
Steven H. Strauss[1,*]

[1]Department of Forest Ecosystems and Society, Oregon State Un



Ongoing field trial of *leafy* and *agamous* mutant hybrid poplar near Corvallis, Oregon

Transient editing methods can avoid the need for segregation or other means of removal – but tough in clonally propagated plants

- In press review: Of 87 studies on gene editing in trees and clonal crops, the large majority used transgene integration

Gene editing in tree and clonal crops: Progress and challenges

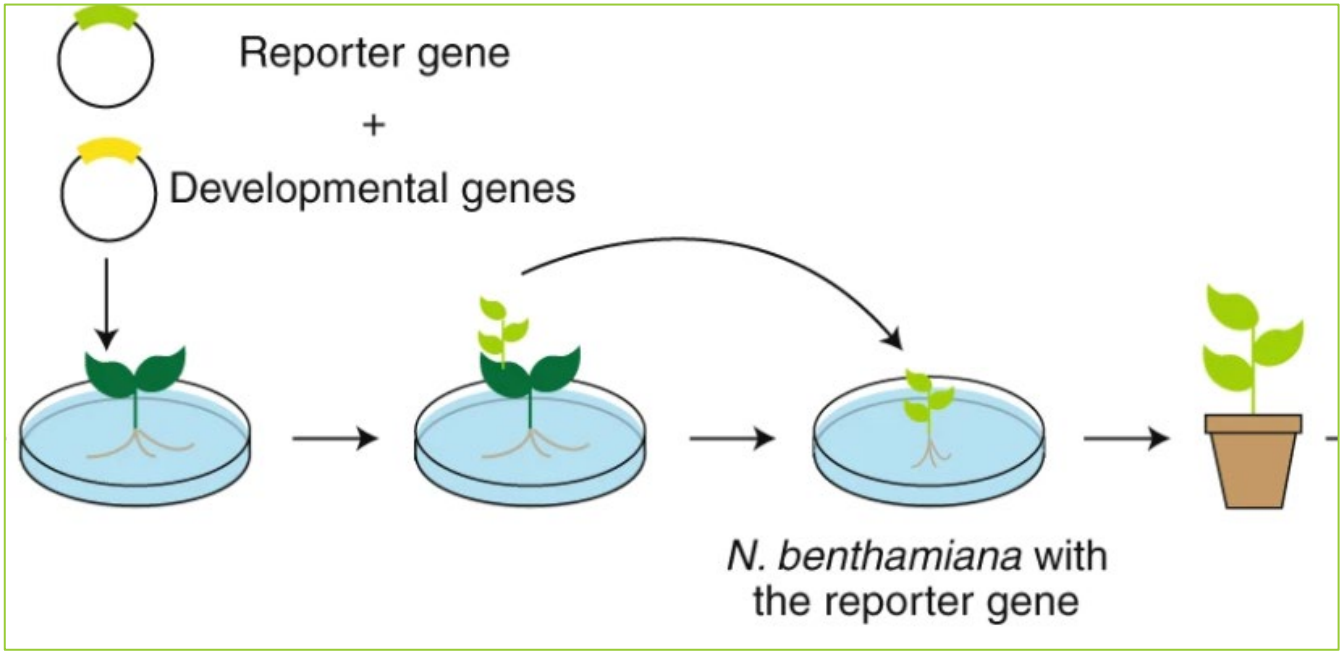
Greg S. Goralogia[1], Thomas P. Redick [2], and *Steven H. Strauss*[1]

1. Department of Forestry Ecosystems and Society, Oregon State University, Corvallis, OR 97331, USA
2. Global Environmental Ethics Counsel (GEEC), LLC, 17170 Laura St. Spring Lake MI 49456

Corresponding author: SH Strauss, Steve.Strauss@OregonState.Edu

In press: *in vitro* Cell and Developmental Biology - Plant

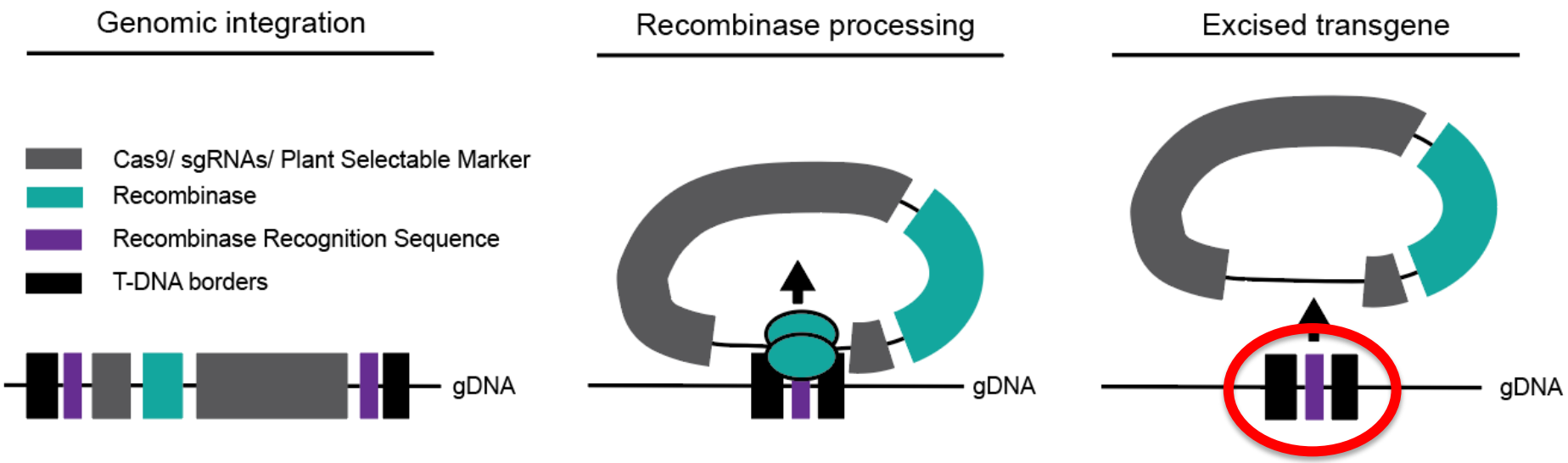
Many clonal crops difficult to transform: Morphogenic regulator ("developmental") genes could help but most will also need to be removed



Morphogenic regulator genes include ipt, WUS, BBM, LEC1, GRF4-GIF, and others

Dong and Ronald, 2020 PMID:31844295

Site-specific recombinases are long-known means for transgene removal - but not a general and reliable tool, and its excision footprint prevents “clean” edits



- Cre, FLP, R, others have been used for transgene excision in plants
- Efficient at excision over long distances
- High fidelity for recognition site

Under USDA SECURE regulations, classes of modified crops, not just insertion events, can be “exempted” – suggesting that footprints could be considered “inerts”

Implementing the SECURE Rule

Last Modified: Jun 2, 2020

Print

The SECURE rule is final on the day it is published in the Federal Register. The new rule’s provisions become effective on key dates over the next 18 months. The biotechnology community will have to learn some new processes and meet new requirements in accordance with the implementation schedule. We are available to support you through this process. It is our goal to minimize regulatory burden and help you comply with our regulations.

Heat shock and chemical induction systems are most common – but often with poor induction, high chimerism, and not widely tested

The Plant Journal (2000) **24**(2), 265–273

TECHNICAL ADVANCE

An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants

Jianru Zuo, Qi-Wen Niu and Nam-Hai Chua
Laboratory of Plant Molecular Biology, The Rockefeller University, 1230 York Avenue, N

Received 2 May 2000; revised 1 August 2000; accepted 1 August 2000.
*For correspondence (fax +1 212 327 8327; e-mail chua@rockvax.rockefeller.edu).

Vitis **49** (4), 201–208 (2010)

Comparing 17-β-estradiol supply strategies for applying the XVE-*Cre/loxP* system in grape gene transfer (*Vitis vinifera* L.)

L. Dalla Costa, M. Mandolini, V. Poletti and L. Martinelli

Research and Innovation Centre, Fondazione Edmund Mach-IASMA, San Michele all'Adige, Italy

Plant Cell Tiss Organ Cult (2016) 124:471–481
DOI 10.1007/s11240-015-0907-z

ORIGINAL ARTICLE

Efficient heat-shock removal of the selectable marker gene in genetically modified grapevine

Lorenza Dalla Costa[1] · Stefano Piazza[1] · Manuela Campa[1,2] · Henryk Flachowsky[3] · Magda-Viola Hanke[3] · Mickael Malnoy[1]

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2. **Design of a developmental transgene excision system designed for gene editing**
3. Proof of concept testing to produce semi-dwarf/ sterile trees of horticultural interest
4. Future directions for transgene excision strategies

Developmental excision methods an inspiration for our plans of to combine with CRISPR editing

Plant Cell Rep (2009) 28:1509–1520
DOI 10.1007/s00299-009-0750-y

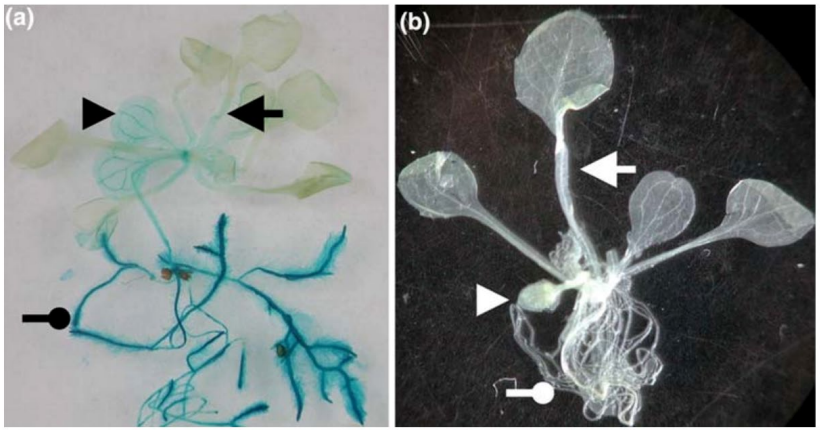
ORIGINAL PAPER

Evaluation of seven promoters to achieve germline directed Cre-*lox* recombination in *Arabidopsis thaliana*

Frédéric Van Ex · Dimitri Verweire · Martine Claeys · Ann Depicker · Geert Angenon

LoxP-35S-GUS-LoxP T_0

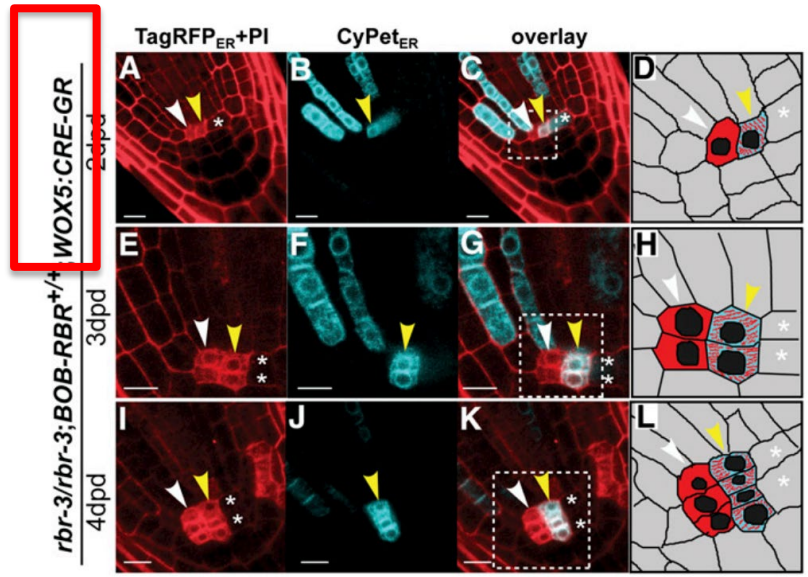
LoxP-35S-GUS-LoxP T_1 X *pCLV3*:Cre



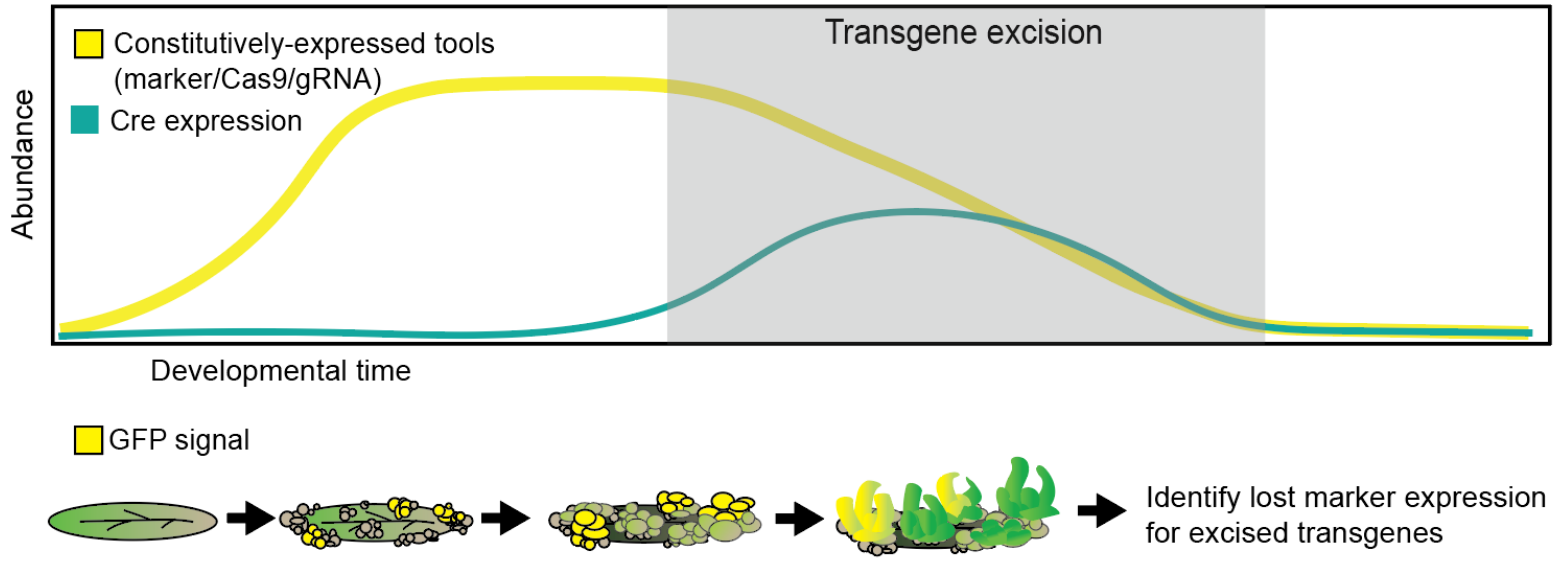
The Plant Cell, Vol. 23: 2581–2591, July 2011, www.plantcell.org © 2011 American Society of Plant Biologists.

Distinct Cell-Autonomous Functions of *RETINOBLASTOMA-RELATED* in *Arabidopsis* Stem Cells Revealed by the Brother of Brainbow Clonal Analysis System

Guy Wachsman, Renze Heidstra, and Ben Scheres[1]
Department of Biology, Utrecht University, 3584 CH Utrecht, The Netherlands



Concept of coupling recombinase activity with development during *in vitro* transformation and indirect regeneration?



Shoot meristem-expressed genes would seem ideal for driving recombinase expression

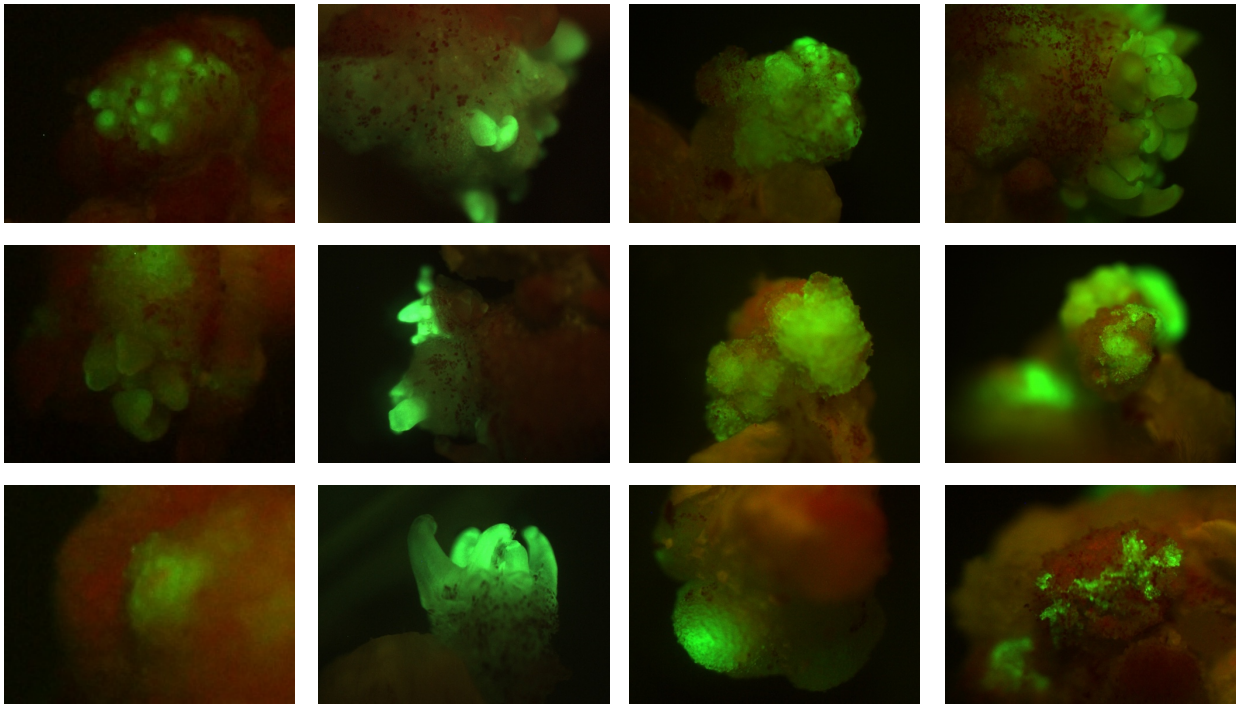
11 potential shoot meristem promoters to drive Cre were analyzed for tissue-specific expression

Promoter	Length (from TSS)
AtWUS	2.2kb
PtWUS2	2.5kb
AtSTM	3.6kb
PtSTM	2.5kb/1.3kb
AtCSP3	1.3kb
AtER	1.3kb
AtYAO	1.4kb
AtESR1/DRN	1.6kb
GmHSP17.5	450bp
AtUBQ10	1.3kb
PtUBQ10	1.5kb

- Selected known promoters
- Cloned minimal Arabidopsis and poplar homologs for most
- Promoter:GFP constructs
- Highly transformable 717-1B4 *P. tremula x alba*
- Most with disappointing and highly variable expression patterns when inserted transgenically
- Focused on *pWUS:GFP*, *pSTM:GFP*, and ***pCSP3:GFP*** for characterization

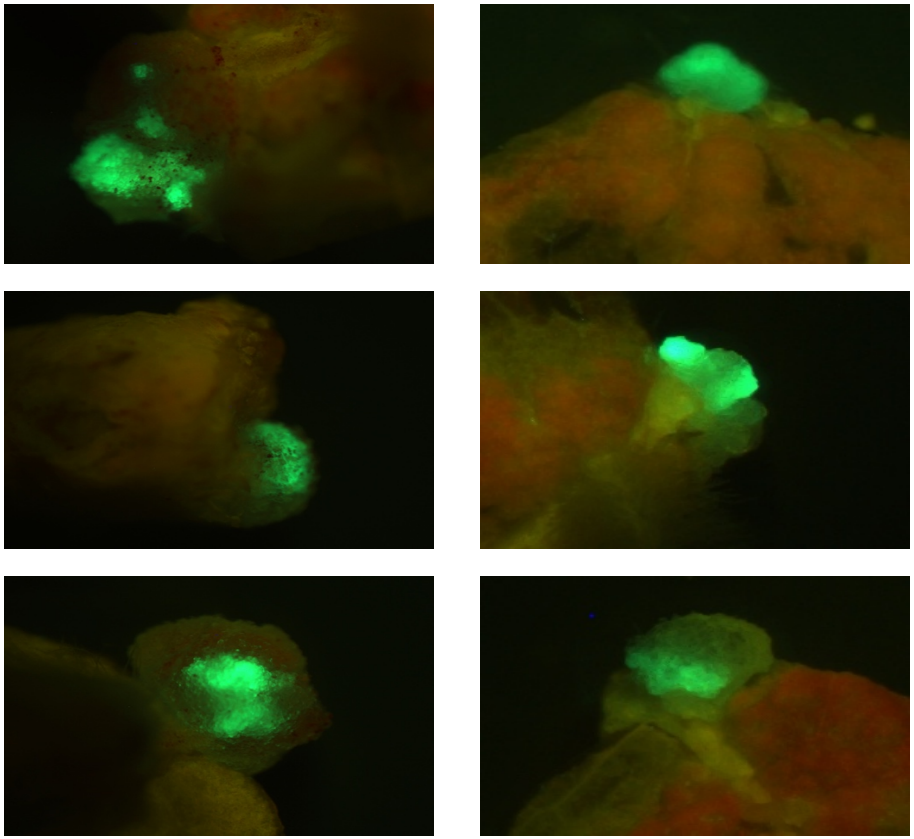
Arabidopsis *COLD SHOCK PROTEIN 3 (CSP3)* promoter showed strong and broad expression in shoot primordia and organized meristems of poplar

Shoot primorida emerging in shoot induction medium

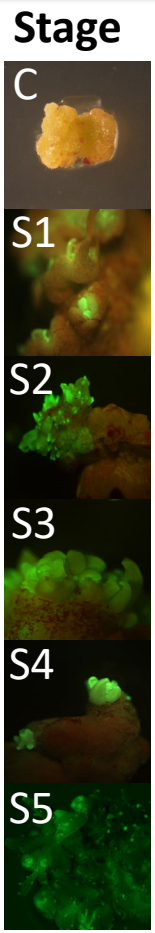


- 1.3kb fragment upstream of the *AtCSP3* TSS
- Note variation in expression among insertion events (columns)

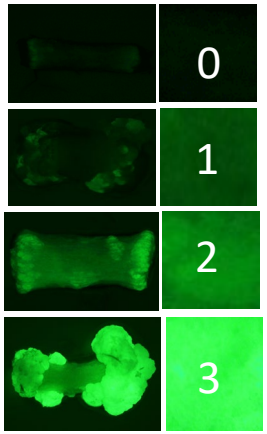
But it also showed strong callus expression – a critical problem for indirect regeneration/transformation systems

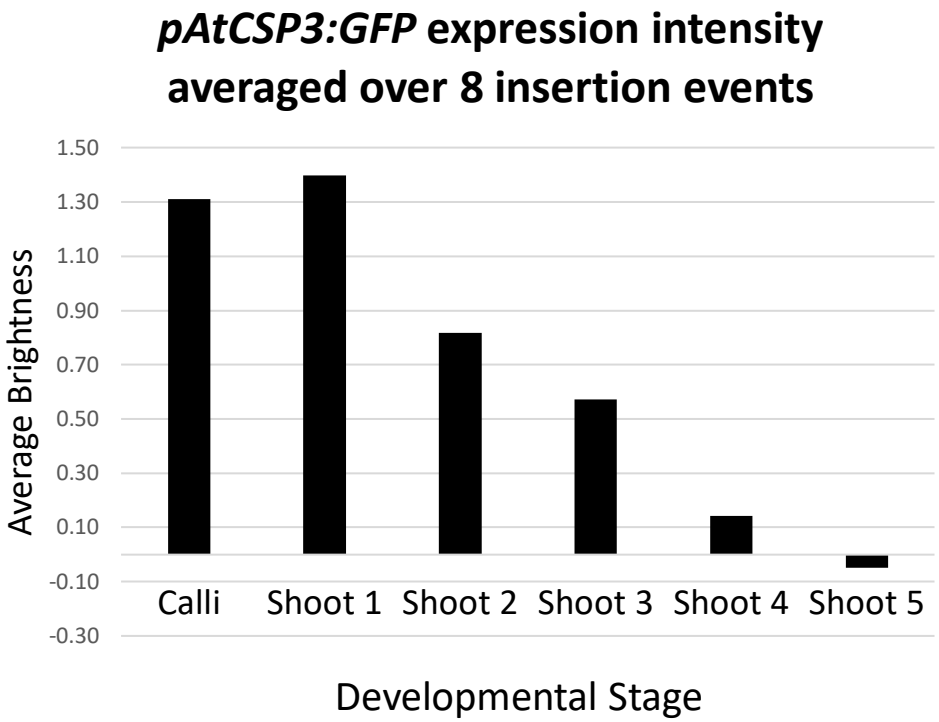


pCSP3:GFP stable transgenics have consistent and strong expression in both callus and shoot primordia



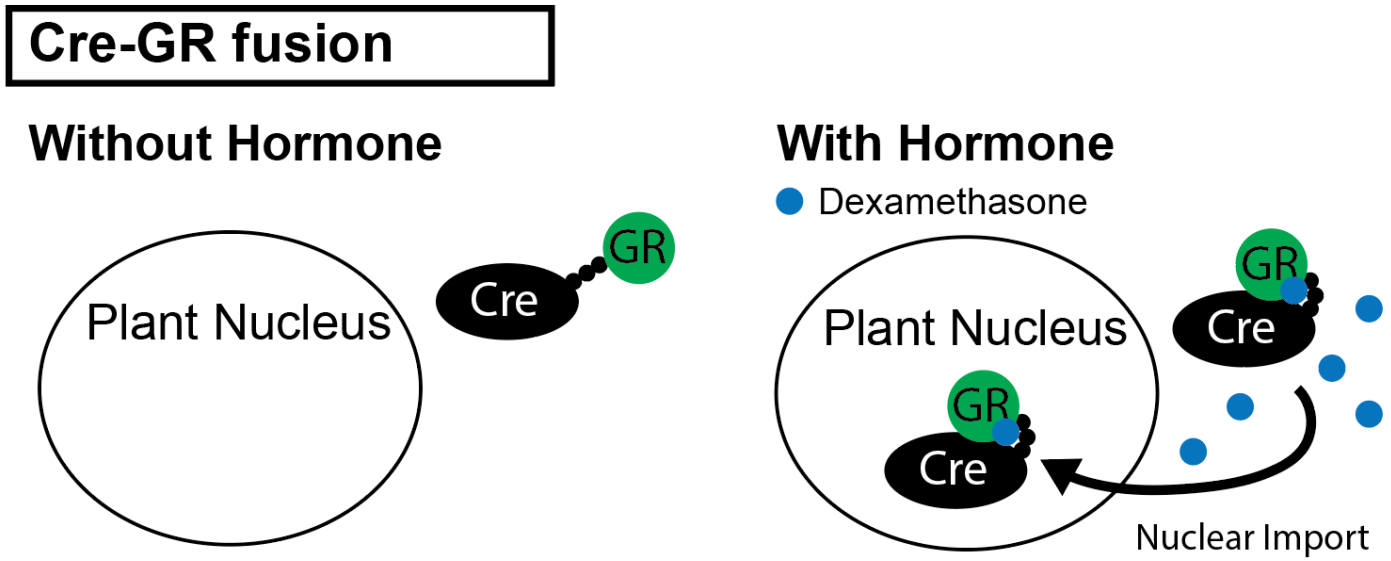
GFP intensity scoring system





Anna Brousseau

Glucocorticoid receptor (GR)-based chemical control system added to compensate for callus expression



- With this 2-stage control system, explants could be treated with DEX upon transfer to shoot induction media, and prevent premature excision in callus

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3. **Proof of concept testing to produce semi-dwarf/ sterile trees of horticultural interest**
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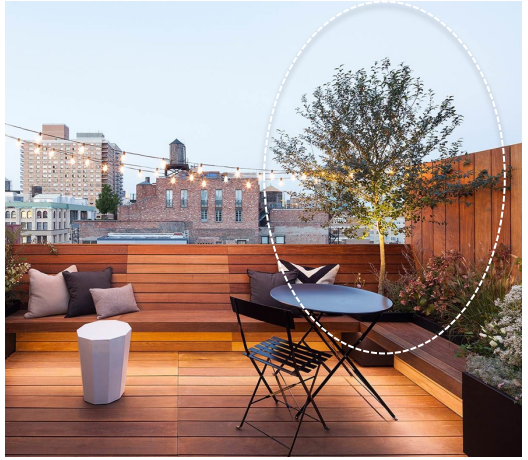
Proof-of-concept demonstration to generate semi-dwarf and sterile trees of horticultural interest – and engage regulatory agency

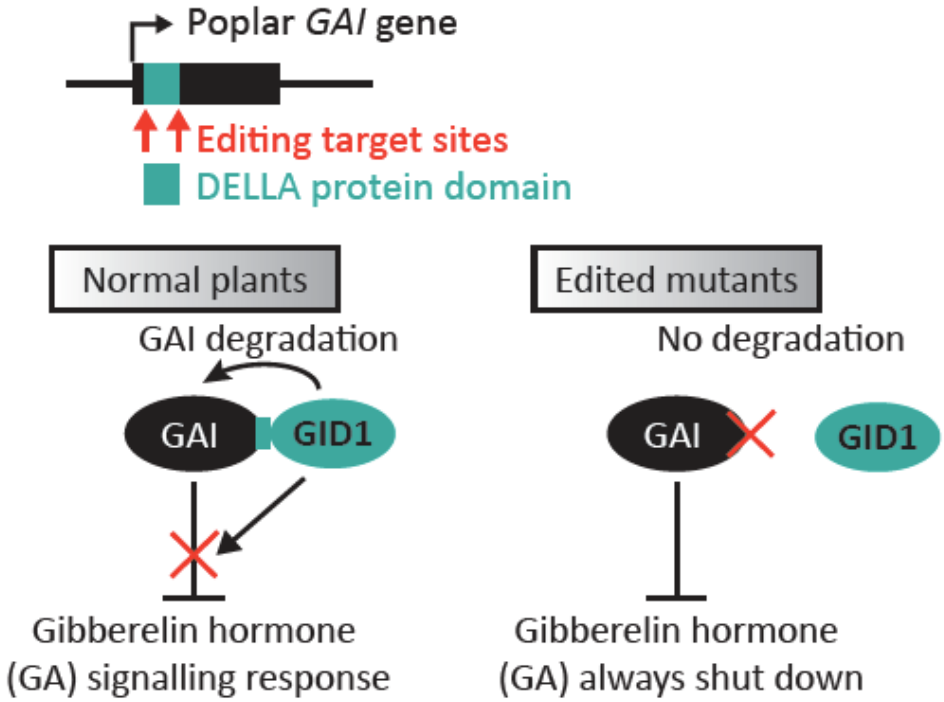


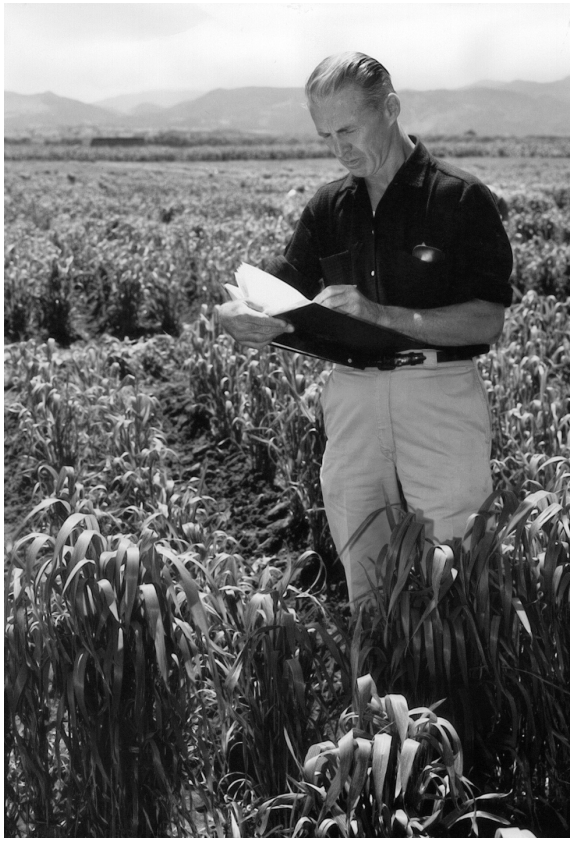



- Urban horticulture applications – semi-dwarfism
- Elimination of pollen and seed dispersal to reduce allergy and debris, enhance regulatory and public acceptance
- Test efficiency, legacy, and broad legal acceptance of recombinase excision system

USDA

Our dominant mutant approach mimics famous Green Revolution semi-dwarfism genes

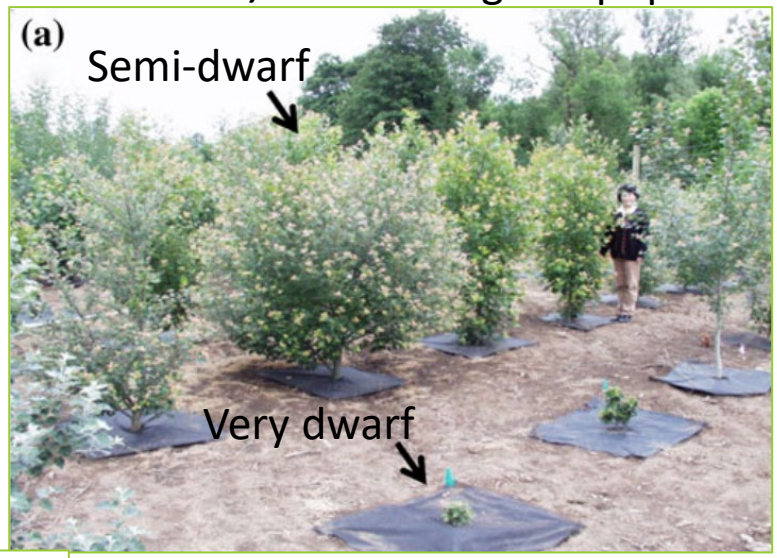




Mutant DELLA domain *GIBERELLIC ACID INSENSITIVE (GAI/RGA)* homologues previously shown to be effective for stature reduction in poplar field trials

Dwarfism Genes for Modifying the Stature of Woody Plants: A Case Study in Poplar

35S:ΔGAI/RGL1 transgenic poplars

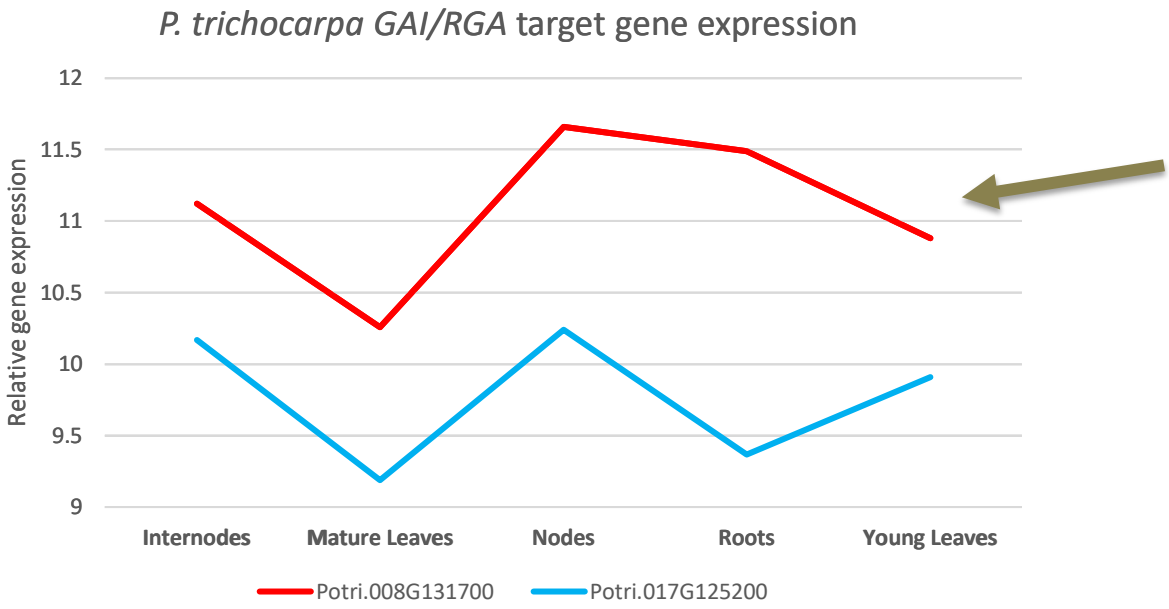


Green Revolution Trees: Semidwarfism Transgenes Modify Gibberellins, Promote Root Growth, Enhance Morphological Diversity, and Reduce Competitiveness in Hybrid Poplar[1[C][W][OA]]

Plant Physiology®, October 2012, Vol. 160, pp. 1130–1144,

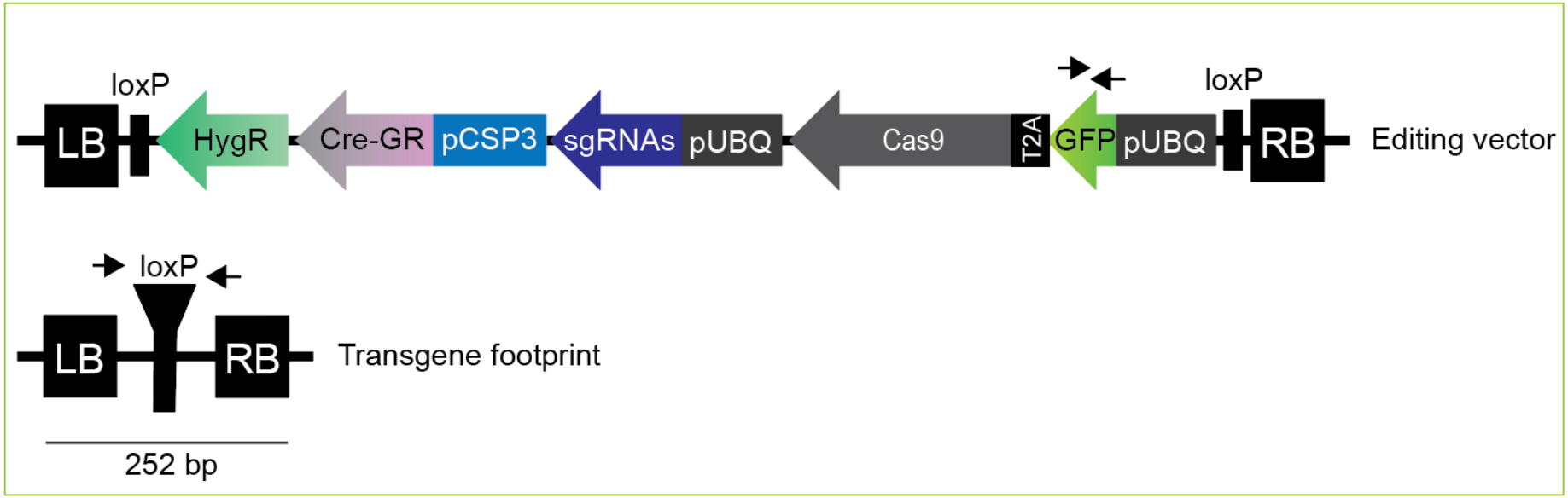
Ani A. Elias[2], Victor B. Busov, Kevin R. Kosola, Cathleen Ma, Elizabeth Etherington, Olga Shevchenko, Harish Gandhi, David W. Pearce, Stewart B. Rood, and Steven H. Strauss*

Two *RGA1*/DELLA homologs with different native expression levels chosen for study



- First focused on stronger *GAI* gene on chromosome 8
- Constructs targeting Chromosome 17 gene currently being analyzed (weakest DELLA-containing homolog)

Binary vector and expected excision footprint



Overall transformation workflow

Agrobacterium-mediated transformation

3 weeks callus induction with antibiotic selection

3 weeks shoot induction with antibiotic selection

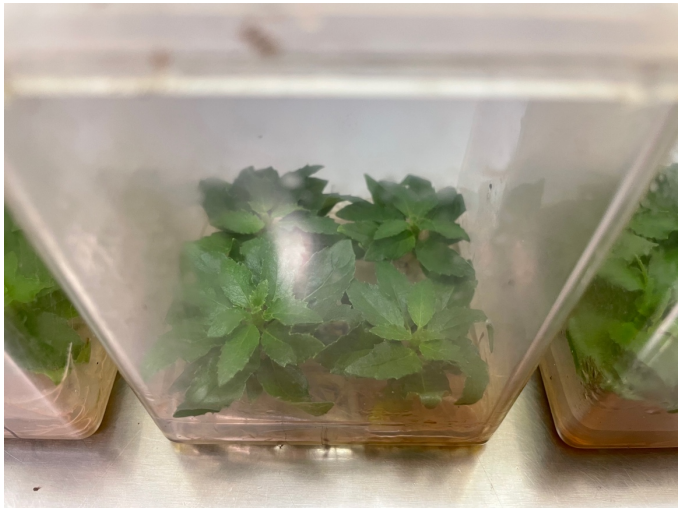
1 month shoot induction with DEX (20uM), no AB

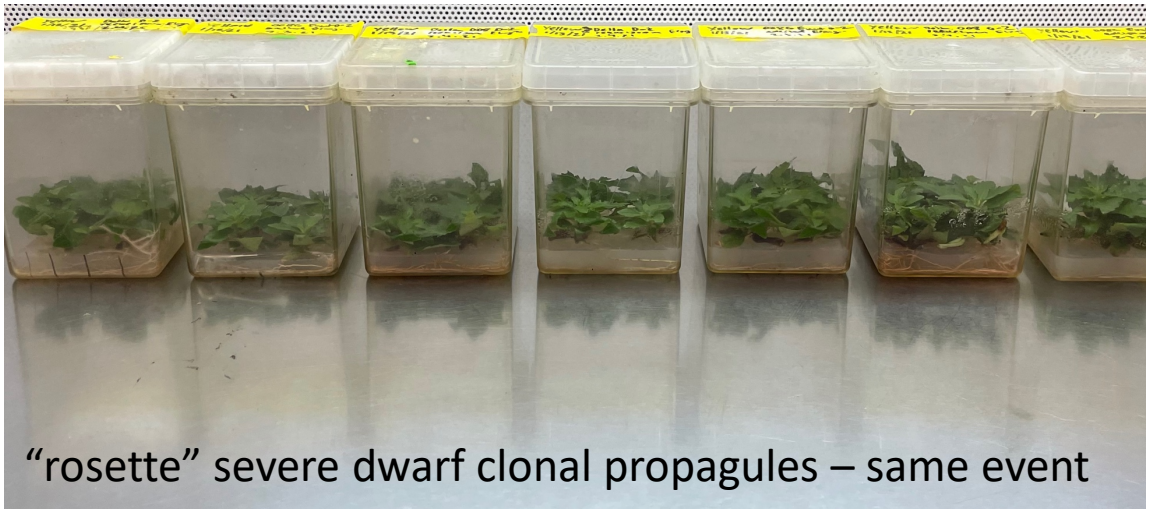
Elongation and propagation (GFP+ and GFP- retained)

Analyze transformants

High rates of transgene escapes but desirable edited events were obtained

- 87 total shoots recovered
- 13 transgenic events found (15%)
- 5 events had desired *gai* deletion fragment verified by PCR
- 3 events had severe dwarf phenotype



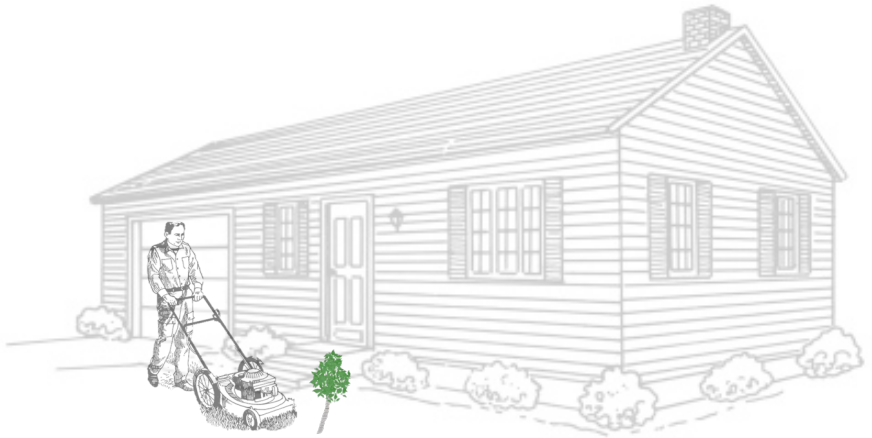


"rosette" severe dwarf clonal propagules – same event

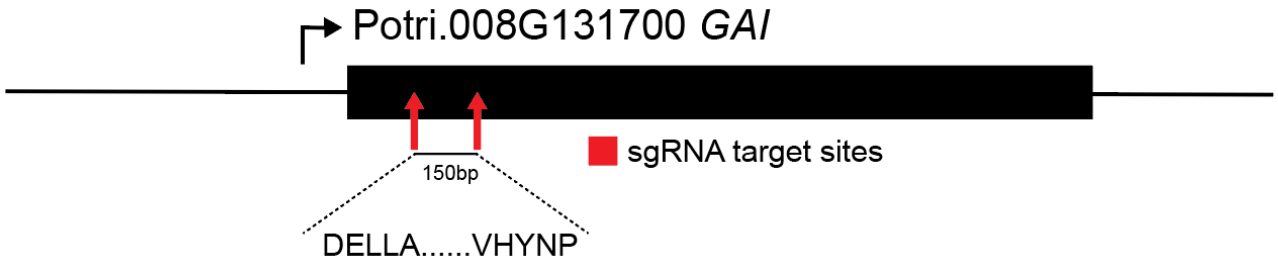
Future adjustment required for ideal semi-dwarf phenotype – hopefully our lower expressed *GAI* gene or heterozygote will do it

Desired phenotype

Our CRISPR/Cas9 poplars



Desired editing outcomes at GAI and LEAFY were achieved in target events



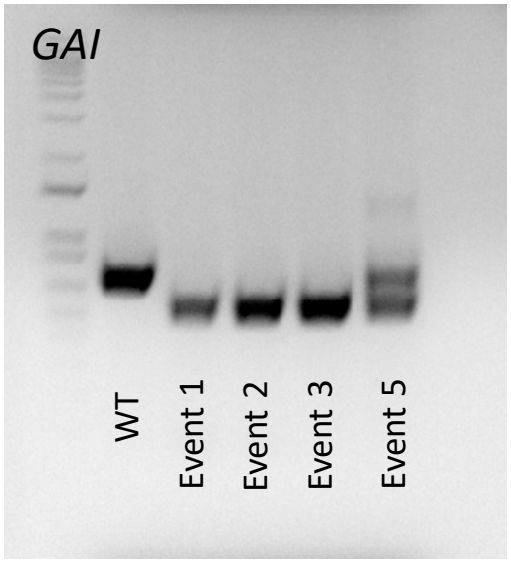
Event 1: -165/-165 (Δ55AA in-frame)
Event 2: -165/-165 (Δ55AA in-frame)
Event 3: -165/-165 (Δ55AA in-frame)
Event 4: -161/-161 (frameshift KO)
Event 5: -1 net/ -164 (frameshift KO)

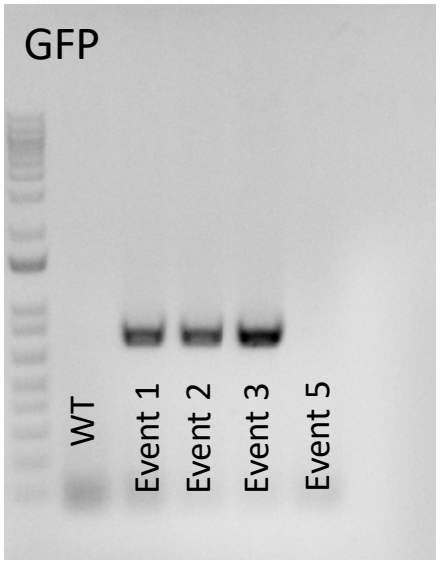
Desired editing outcomes

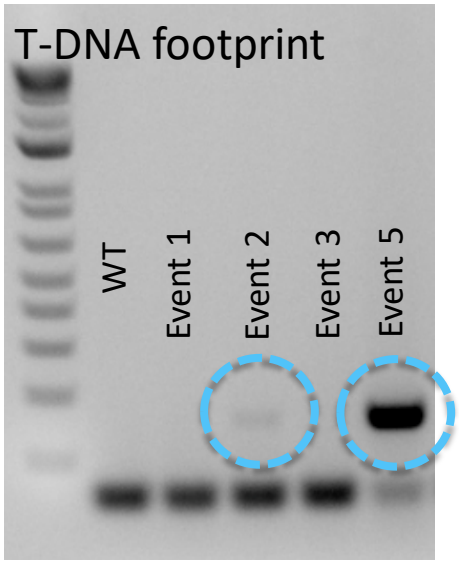
Event 1: +1/+1 (frameshift KO)
Event 2: +1/+1 (frameshift KO)
Event 3: -2060/-2060 (KO)
Event 4: +1/+1 (frameshift KO)
Event 5: +1/unknown

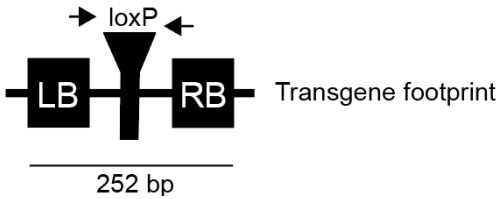
Desired editing outcomes

20% (1/5) deletion events fully excised, remainder were unexcised or chimeric



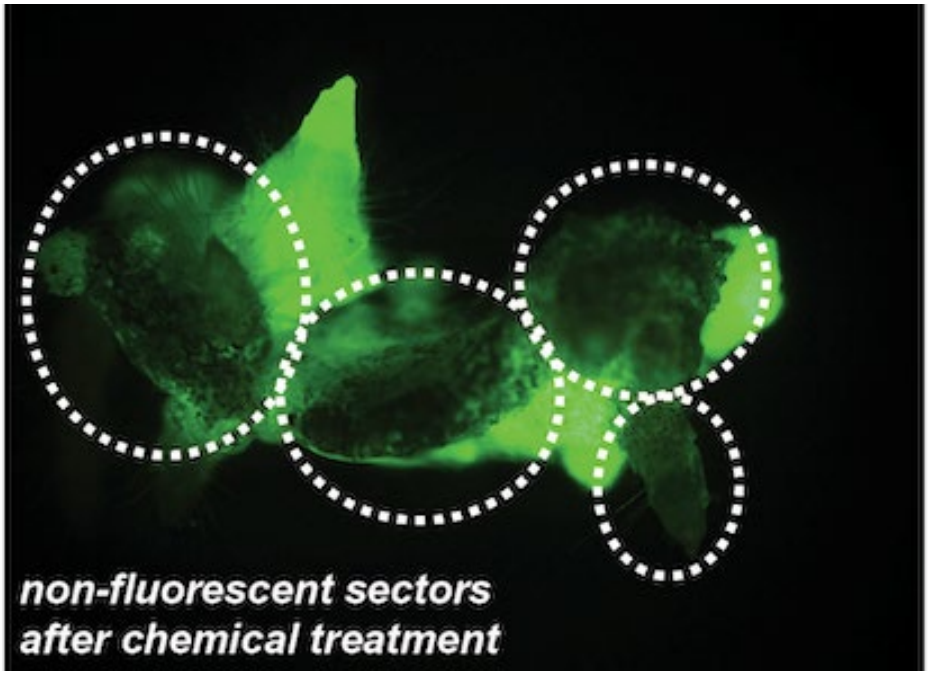




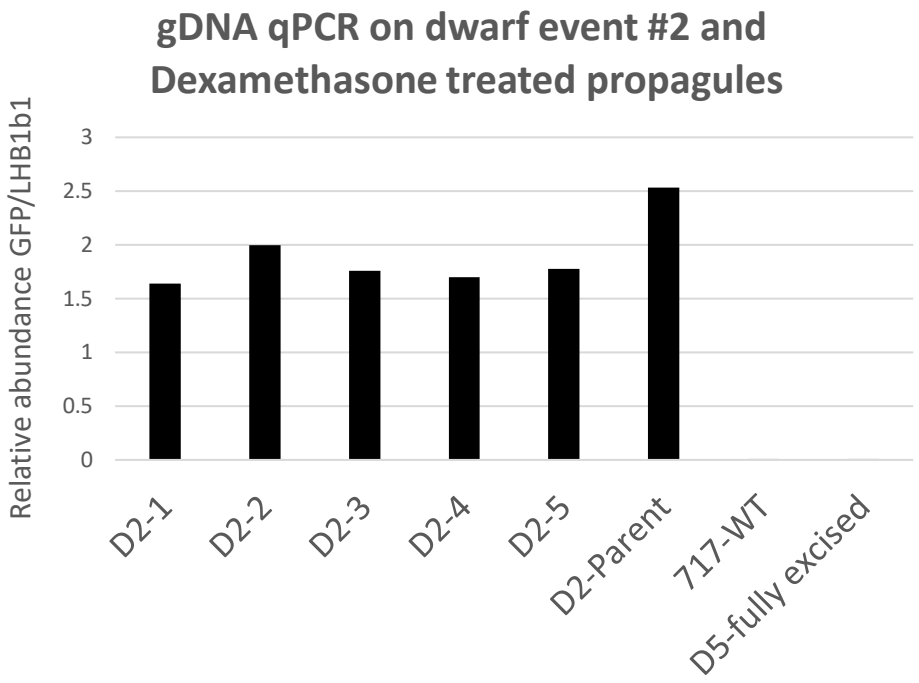


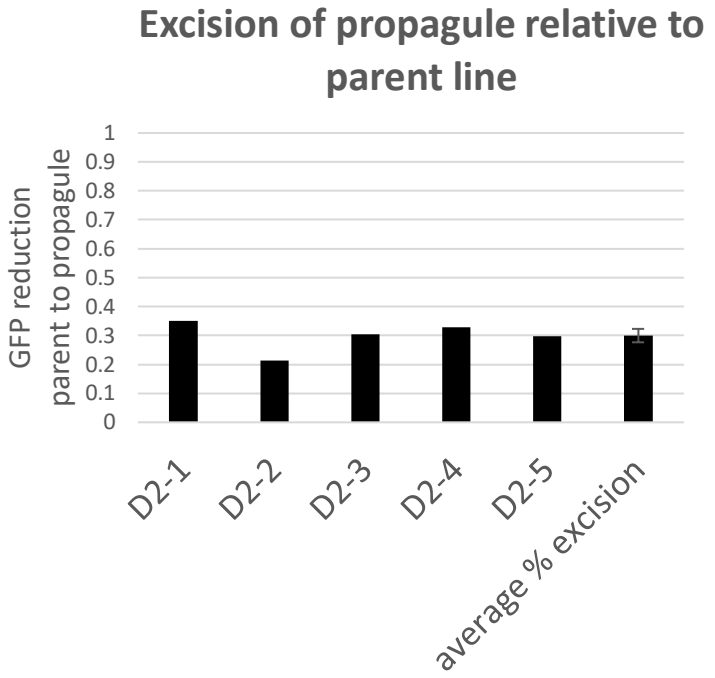
To try to obtain fully excised events, we re-regenerated explants on dexamethasone containing media

- 2-stage excision not ideal for rapid recovery of fully excised desired events
- 100uM Dex treatment – high level of reported induction spectrum
- Loss of fluorescent signal visible during re-regeneration
- Populations of 30 propagules isolated for excision analysis



Re-regeneration + Dex gave a quantitative reduction in transgene abundance, but not complete excision





30% reduction of transgene signal seen in Dex treated propagules

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Several alternatives under study

- Dual (positive/negative) fluorescent reporters to better understand spatial extent of excision and silencing
- Further chemical induction treatments – Dex and estradiol
- Heat induction – leaky but extensively used in our lab, and found to be most reliable in fruit crops (Dalla Costa et al.)
- Demethylation treatments – azacytidine


Plant Cell Tiss Organ Cult (2016) 124:471–481
DOI 10.1007/s11240-015-0907-z

ORIGINAL ARTICLE

Efficient heat-shock removal of the selectable marker gene in genetically modified grapevine

Lorenza Dalla Costa[1] · Stefano Piazza[1] · Manuela Campa[1,2] · Henryk Flachowsky[3] · Magda-Viola Hanke[3] · Mickael Malnoy[1]


New Phytologist

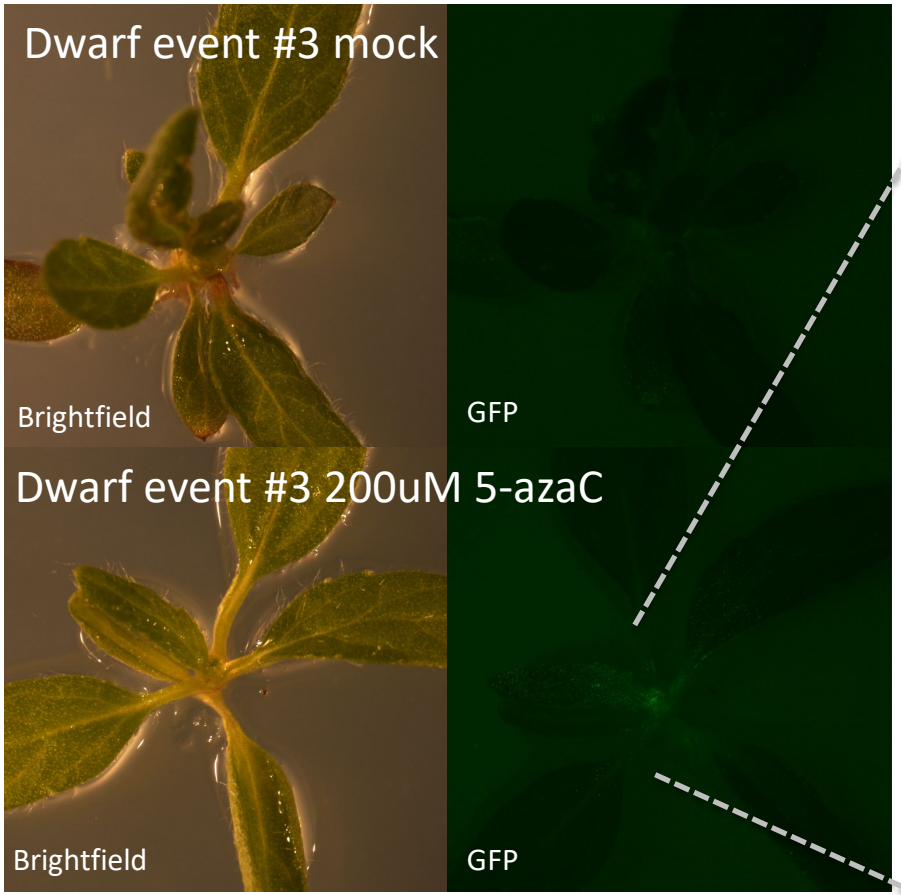
Full Paper | Full Access |

DNA methylation occurring in Cre-expressing cells inhibits loxP recombination and silences loxP-sandwiched genes

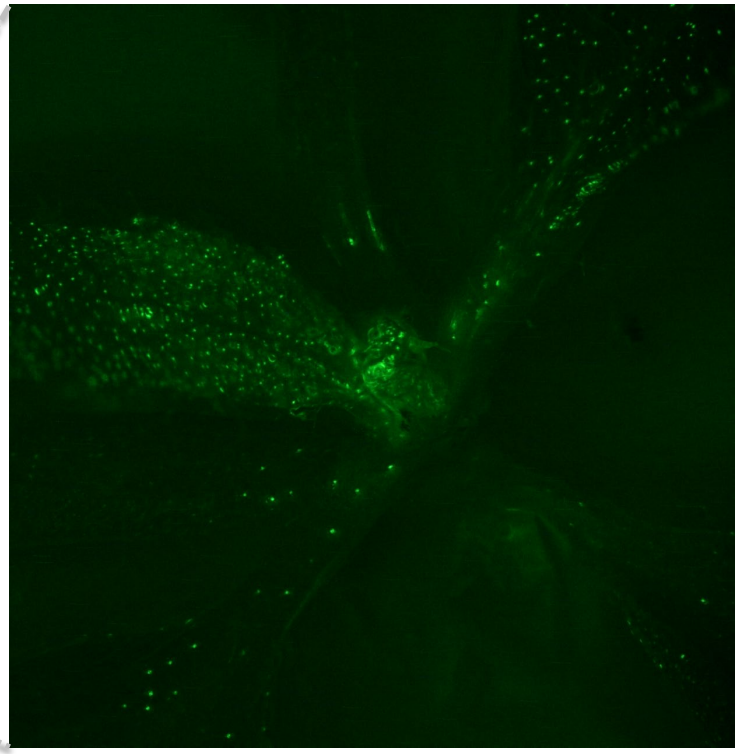
Ruochen Liu, Qin Long, Xiuping Zou, You Wang, Yan Pei

First published: 20 March 2021 | https://doi.org/10.1111/nph.17353

Preliminary experiments suggest DNA methylation-dependent silencing may be a major cause poor excision



- 5-azaC prevents methylation when incorporated after cell division
- 1 week treatment (Mock/ 200uM) on propagated nodes – 100% had GFP signal recovery n=8 shoots



Summary

- High callus expression during indirect regeneration impedes developmental Cre induction system
- Glucocorticoid enhancement of this system only partially effective
- Prototype semi-dwarf, sterile, excised tree in development, but with very low efficiency and extreme dwarfism – new GAI target under study
- Demethylation shows promise for relief from silencing and improving Cre induction with Dex – further studies underway
- Heat shock, CRISPR, and other methods for improving excision rates under study
- **Gene excision a long-standing method in plant biology, but needs much developmental research to become a general, reliable, and efficient tool for gene editing and genetic engineering in trees and clonally propagated plants**

Thank you

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Daniel Casey-Hain
Cathleen Ma

Estefania Elorriaga
Michael Nagle
Michael Gordon
Cathleen Ma
Bahiya Zahl
Ekaterina Premyslova

Corteva Agriscience
Bill Gordon-Kamm
Todd Jones

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Pr. No: 2017-03820 and 2020-33522-32316

NSF Plant Genome Research
No: 1546900

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GREAT TREES industrial cooperative at OSU