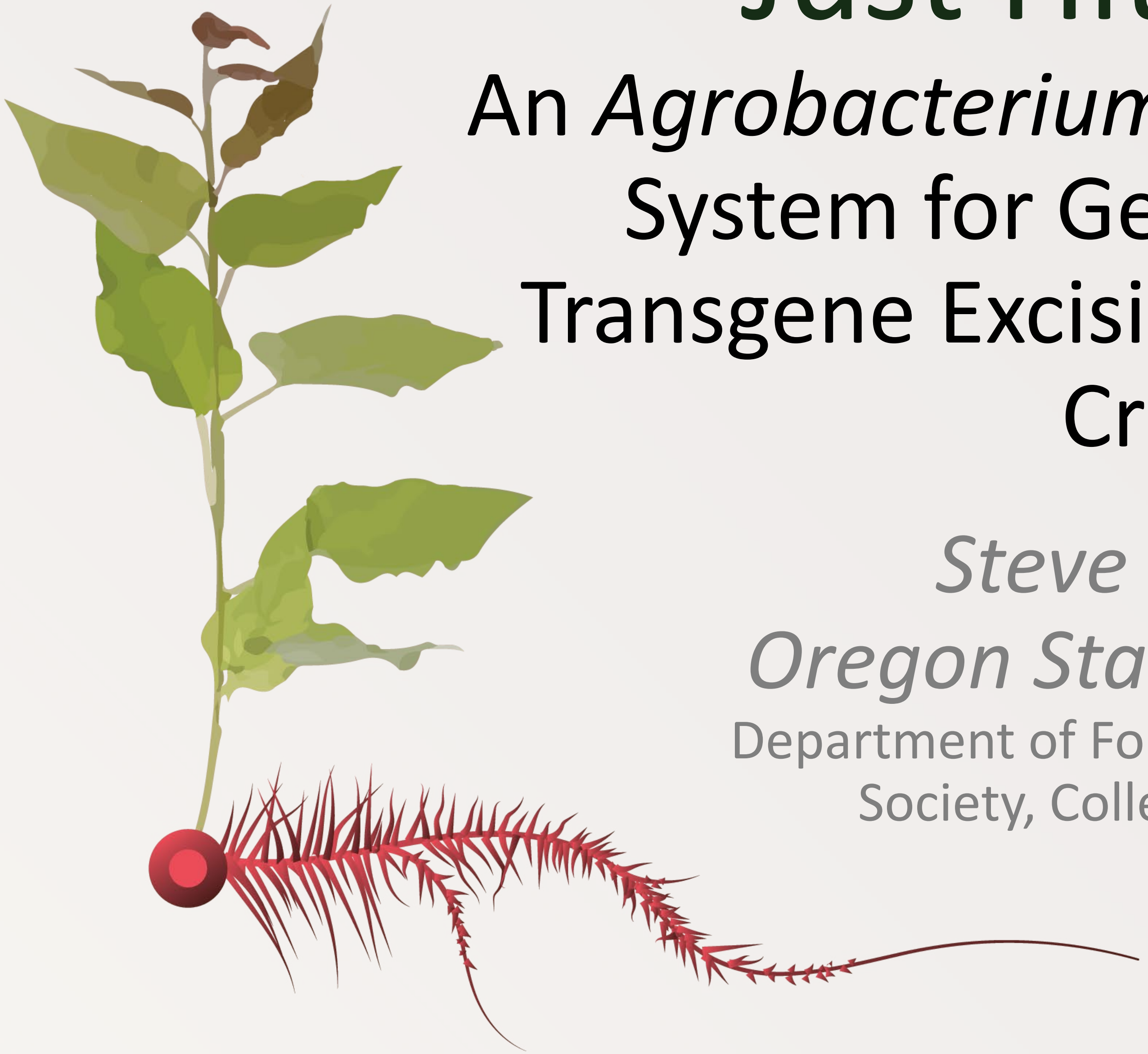
Just Hit RESET!

An *Agrobacterium rhizogenes*-based System for Gene Editing and Transgene Excision in Recalcitrant Crops



Steve Strauss

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Greg Goralogia, postdoc

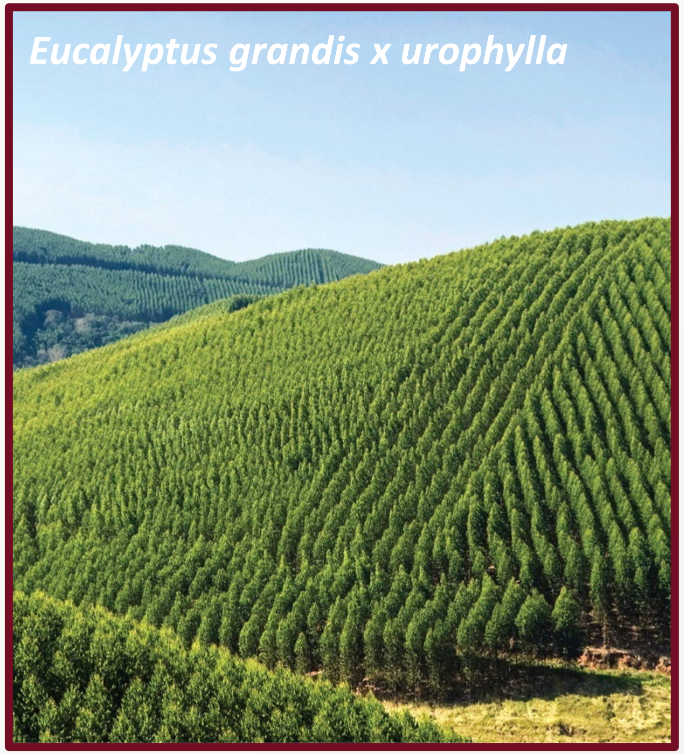
Agenda

- Background
- The basic RESET system
- Activities to improve it

Agenda

- **Background**
- The basic RESET system
- Activities to improve it

Focus on translational research in Populus and Eucalyptus

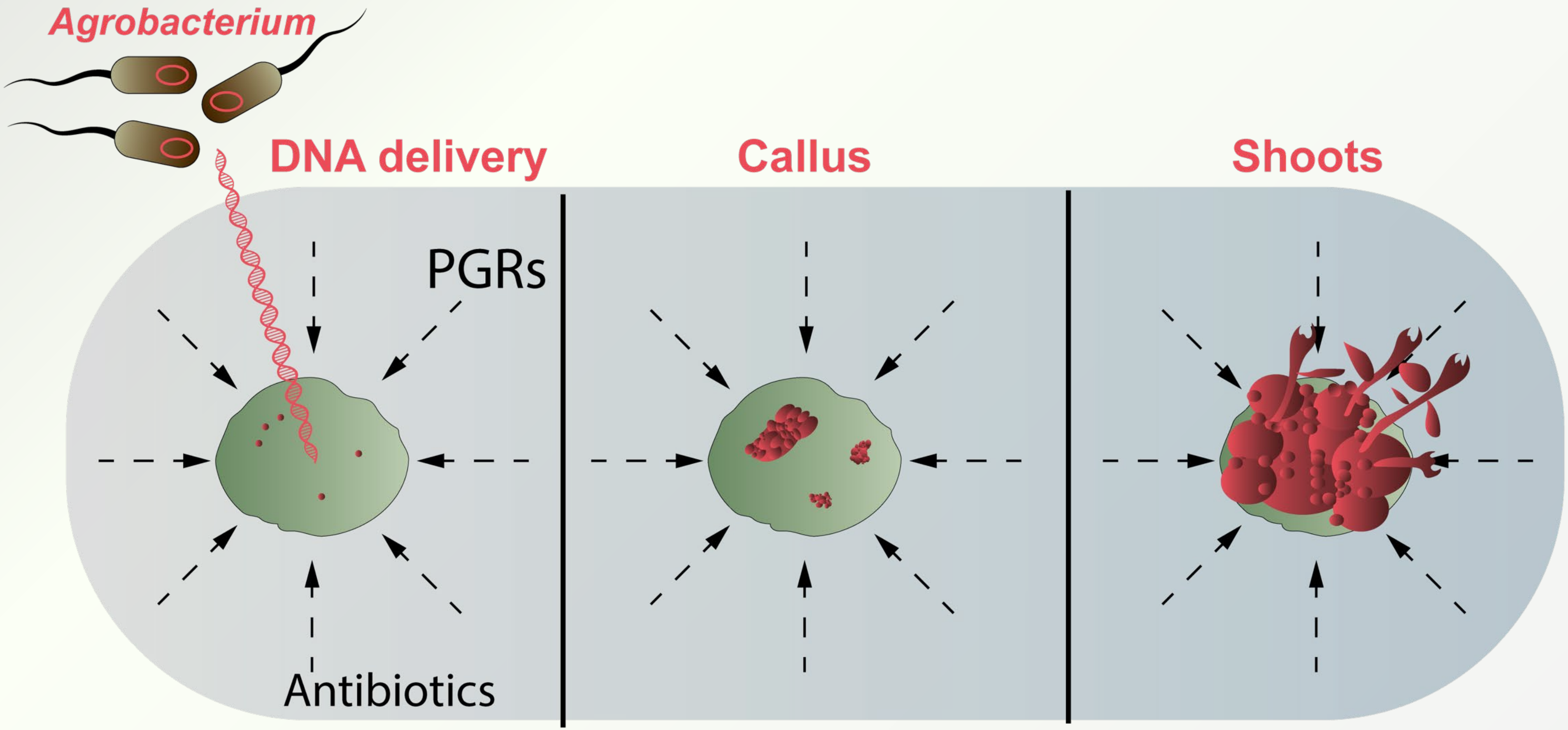


Most tree species are recalcitrant to transformation

- Often working with elite clones, not seed-derived
- High heterozygosity: each genotype a new adventure *in vitro*
- High physiological/epigenetic diversity
- Strong defense response to Agrobacterium

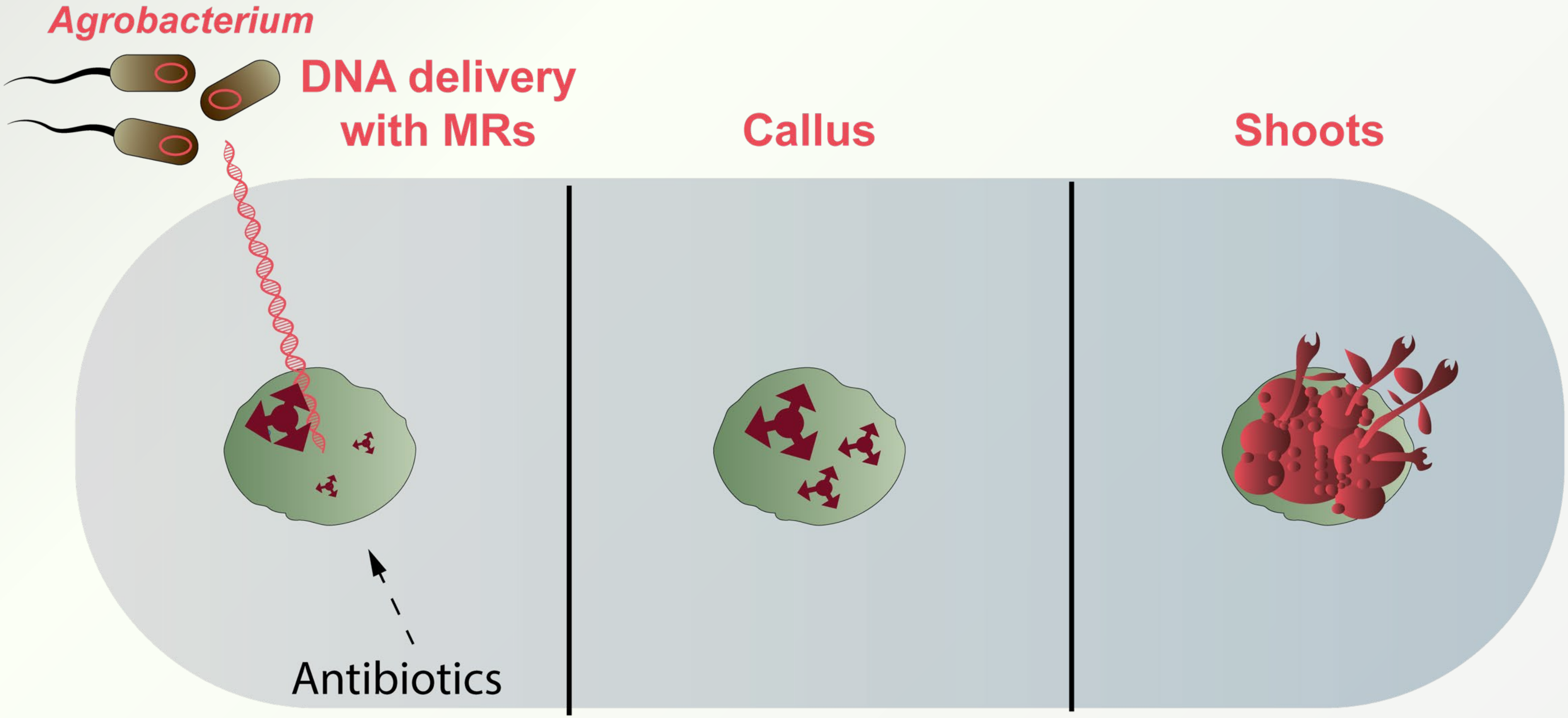
Eucalyptus grandis x urophylla

Conventional transformation methods rely on exogenous phytohormones supplied in the culture medium



PGRs = Plant growth regulators, hormones

Morphogenic regulators (MRs) spur developmental reprogramming via delivered DNA – sometimes in the absence of exogenous PGRs



Types of MR genes we have studied in poplars or eucalypts – many both *in vitro* and *in planta*

- LEC 1, 2 – LEAFY COTYLEDON
- EBB1 - EARLY BUD BREAK 1 (ESR family)
- BBM – BABY BOOM
- WOX 5, 11 -- WUSCHEL RELATED HOMEOBOX
- WUS – WUSCHEL
- IPT – ISOPENTYL TRANSFERASE
- GRF-GIF – GROWTH REGULATOR FACTOR 4 and GRF INTERACTING FACTOR 1

Most have failed with simple overexpression, or given highly genotype-specific enhancement or inhibition

Instead, built upon considerable prior work with Agrobacterium MR genes

- LEC 1, 2 – LEAFY COTYLEDON
- EBB1 - EARLY BUD BREAK 1 (ESR family)
- BBM – BABY BOOM
- WOX 5, 11 -- WUSCHEL RELATED HOMEOBOX
- WUS – WUSCHEL
- IPT – ISOPENTYL TRANSFERASE
- GRF-GIF – GROWTH REGULATOR FACTOR 4
- ***Agrobacterium* growth promoting genes**
  - *Shoot-inducing*
  - *Hairy root-inducing*

Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2025) **23**, pp. 3841–3850

doi: 10.1111/pbi.70159

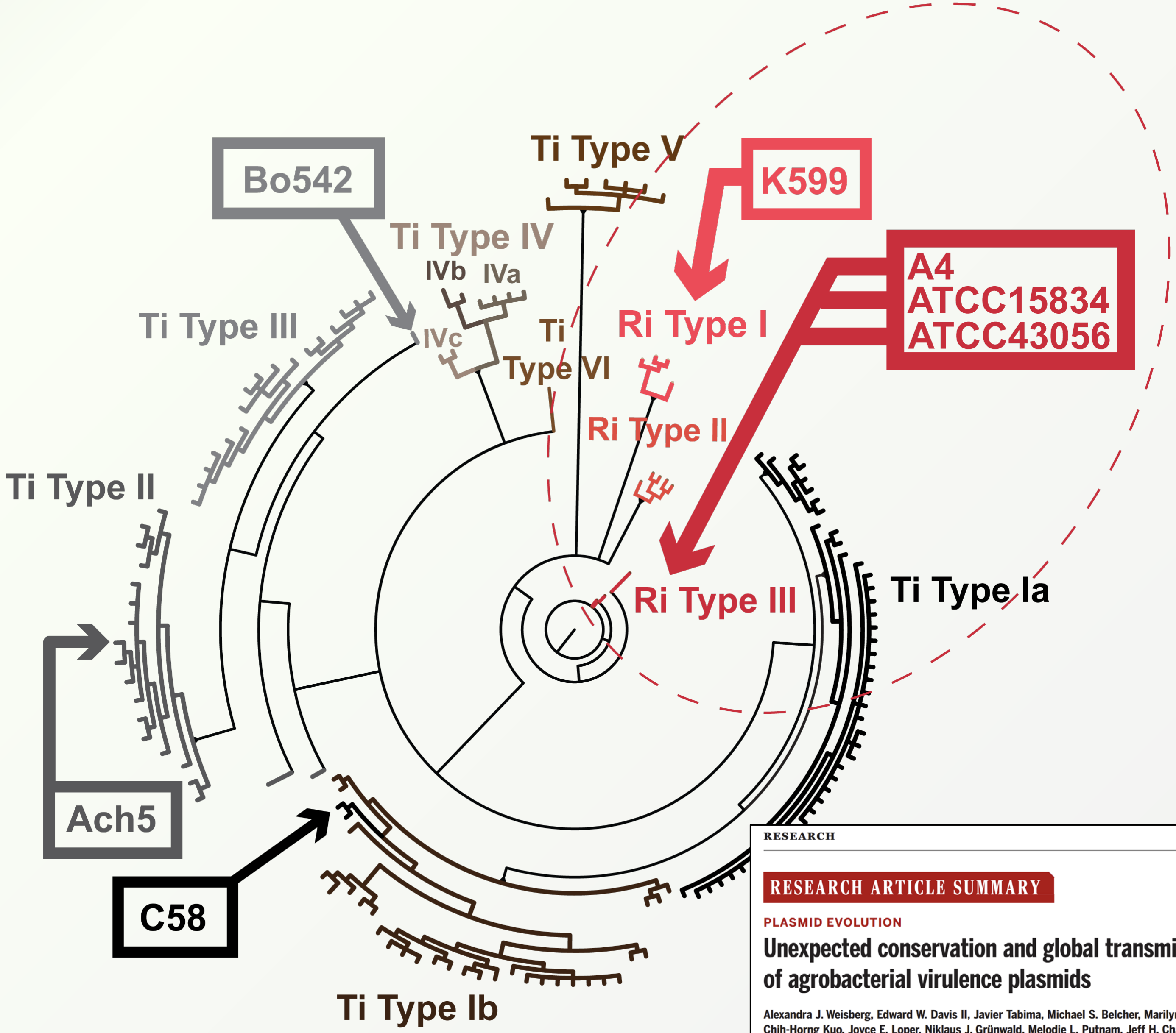
Co-transformation using T-DNA genes from *Agrobacterium* strain 82.139 enhances regeneration of transgenic shoots in *Populus*

Greg S. Goralogia, Cathleen Ma, David S. Taylor, Abigail Lawrence, Victoria Conrad, Ekaterina Peremyslova and Steven H. Strauss*

Department of Forest Ecosystems and Society, Oregon State University, Corvallis, Oregon, USA

Hairy root disease is caused by unique T-DNA genes contained in Ri plasmids

Hairy roots in hydroponically grown tomato



RESEARCH

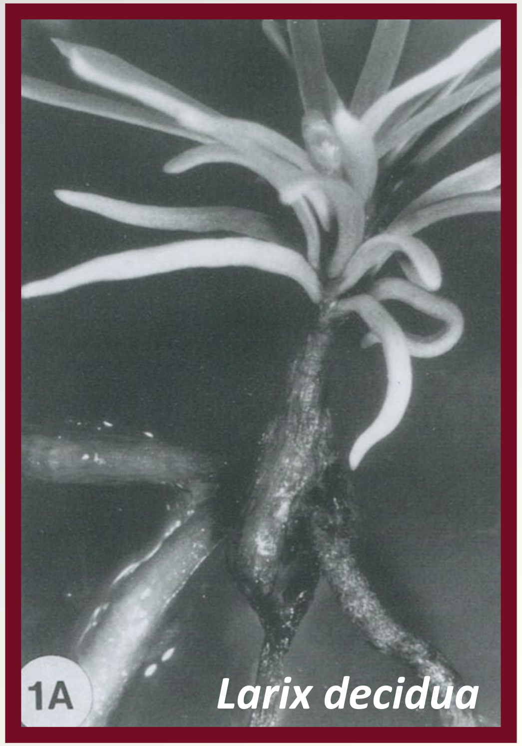
RESEARCH ARTICLE SUMMARY

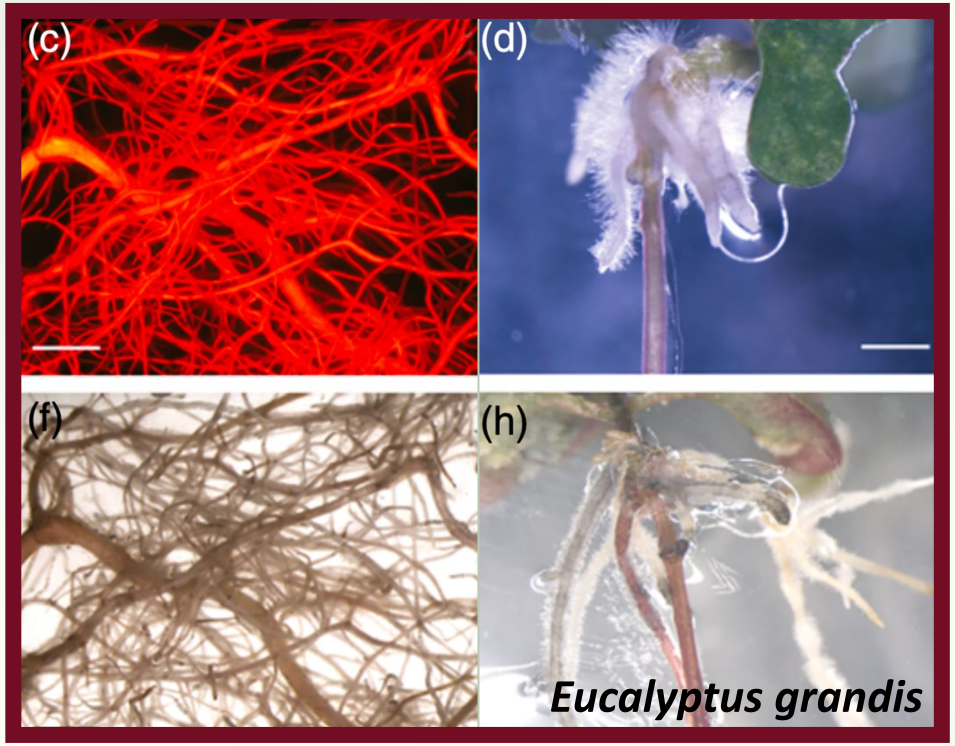
PLASMID EVOLUTION

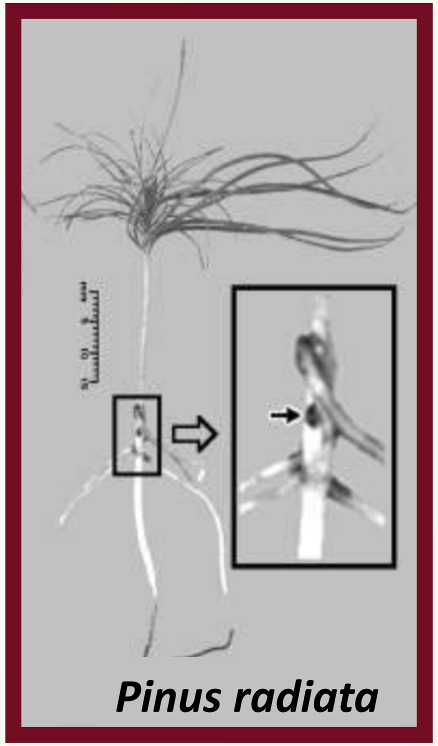
Unexpected conservation and global transmission of agrobacterial virulence plasmids

Alexandra J. Weisberg, Edward W. Davis II, Javier Tabima, Michael S. Belcher, Marilyn Miller, Chih-Horng Kuo, Joyce E. Loper, Niklaus J. Grünwald, Melodie L. Putnam, Jeff H. Chang*

Hairy root *rol* genes are an effective way to generate transgenic tissues across diverse tree species

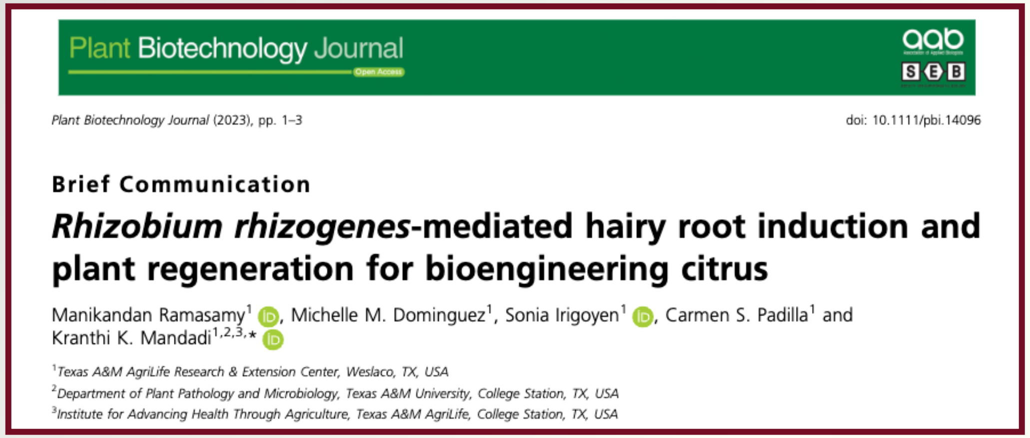






Huang et al. 1991 *IVCDB-Plant*, Placencia et al. 2016. *Plant Biotech J.*, Li et al. 2003 *EJ Biotech,* Gomes et al. 2019 *FIPS,* Du et al. 2025 *PNAS*.

Hairy root transformation is becoming popular for genotype-independent generation of "mostly" normal looking transgenic plants



Plant Biotechnology Journal

Open Access

Plant Biotechnology Journal (2023), pp. 1–3

doi: 10.1111/pbi.14096

Brief Communication

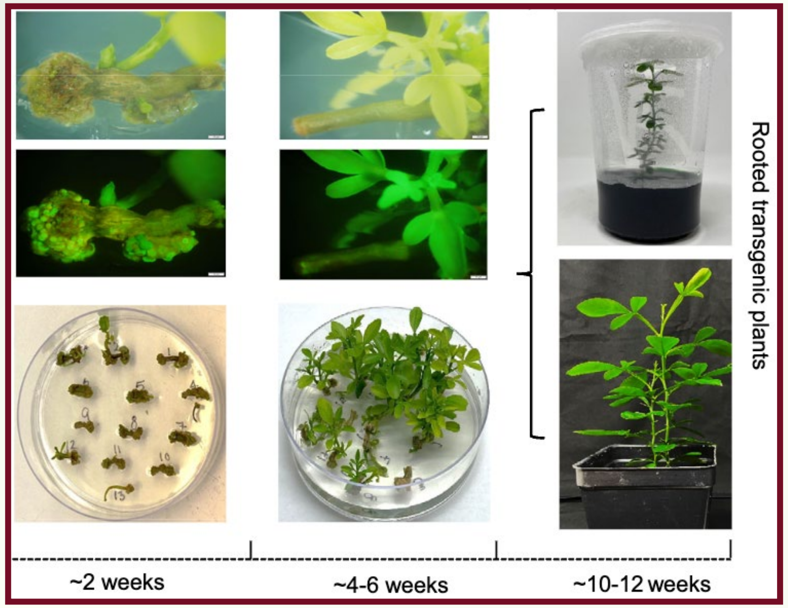
***Rhizobium rhizogenes*-mediated hairy root induction and plant regeneration for bioengineering citrus**

Manikandan Ramasamy[1], Michelle M. Dominguez[1], Sonia Irigoyen[1], Carmen S. Padilla[1] and Kranthi K. Mandadi[1,2,3,*]

[1]Texas A&M AgriLife Research & Extension Center, Weslaco, TX, USA

[2]Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX, USA

[3]Institute for Advancing Health Through Agriculture, Texas A&M AgriLife, College Station, TX, USA

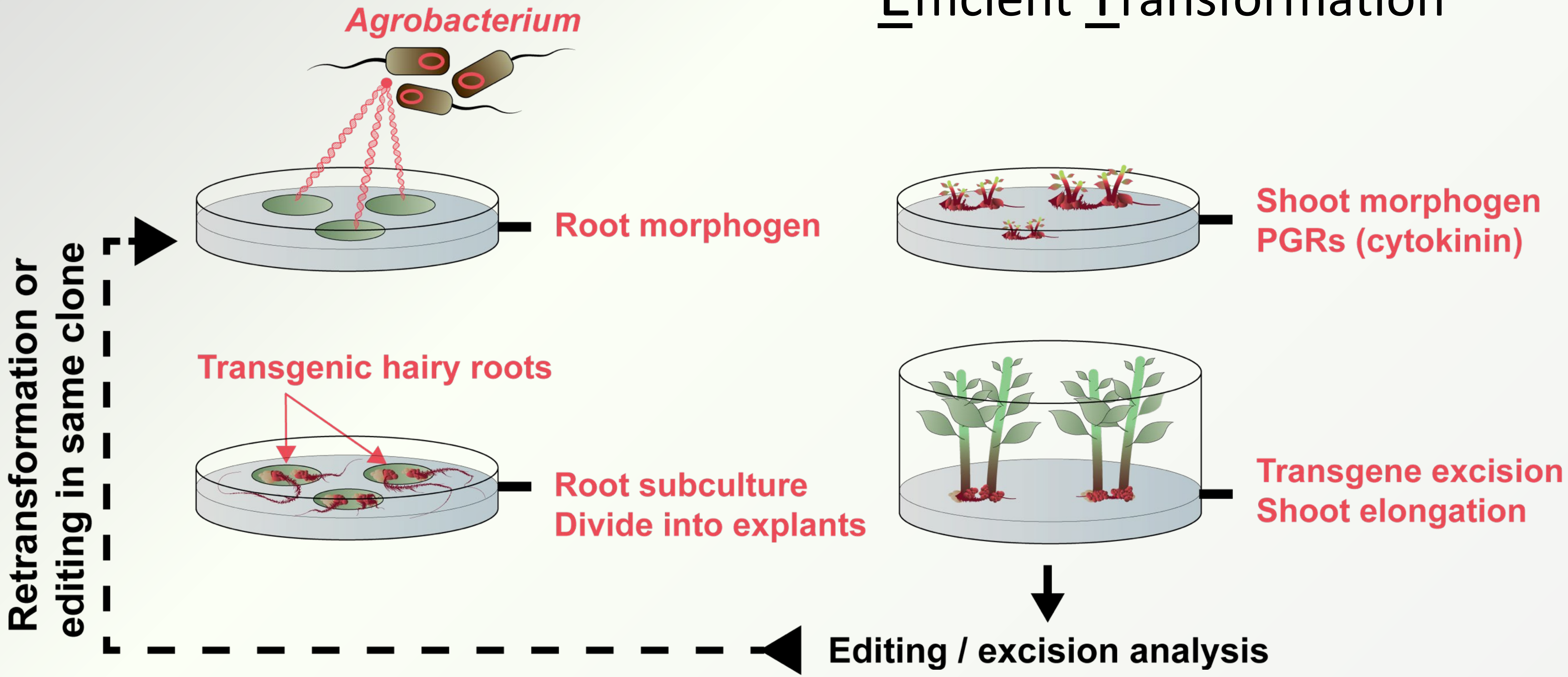


Osteospermum fruticosum (cape daisy)

rol transgenics often show dwarfism and changed floral timing and architecture

Desmet et al. 2021 *Plant Cell Rep.*

A concept for Root Excision System for Efficient Transformation



Root to shoot regeneration, followed by excision, is an elaboration of older ideas

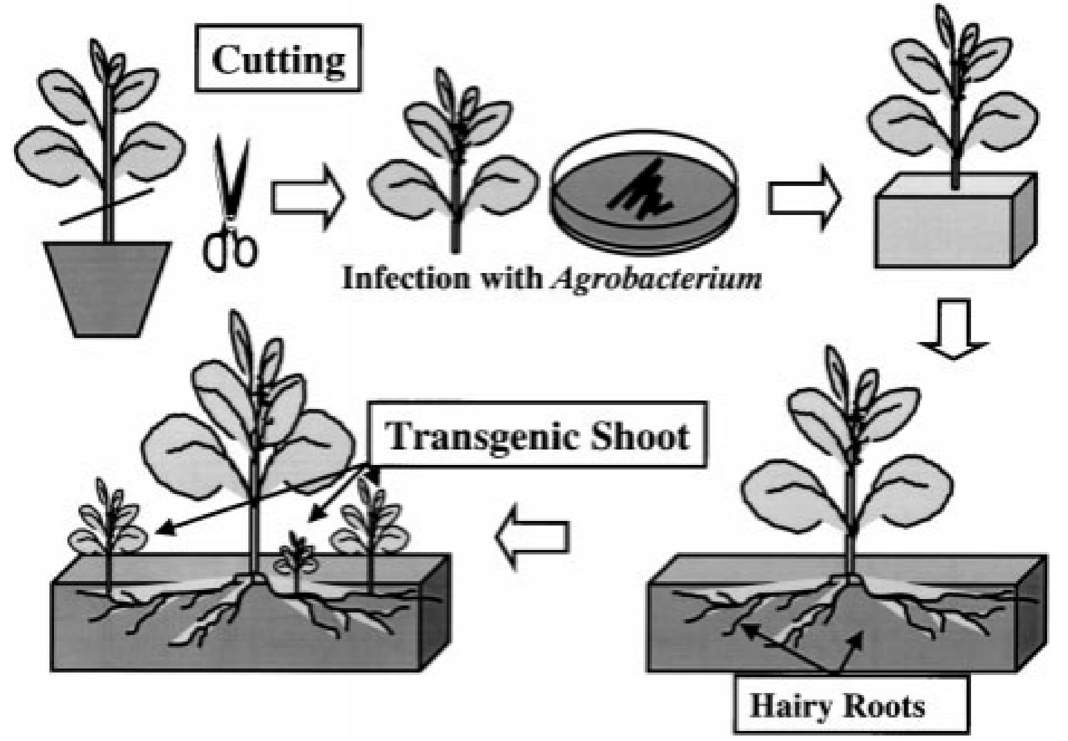
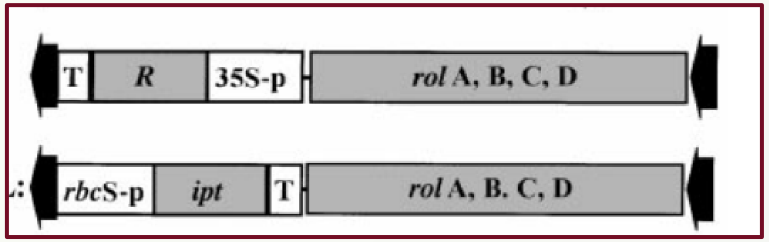


FIG. 8. Outline of *in vivo* transformation using cuttings. Cuttings are infected with *Agrobacterium* containing the *rol*-type MAT vector. After their rooting, marker-free transgenic shoots are induced from hairy roots by the light.



from Ebinuma and Komamine,
2001 In vitro cell and developmental biology -Plant

Hiroyasu Ebinuma
(Shinsu U. em,
Nippon Paper Co.)

GAANTRY system enabled an ambitious T-DNA design

(Gene Assembly in Agrobacterium by Nucleic acid Transfer using Recombinase technology)

the plant journal SEB Society for Experimental Biology

The Plant Journal (2018) **95**, 573–583 doi: 10.1111/tpj.13992

TECHNICAL ADVANCE

A versatile and robust Agrobacterium-based gene stacking system generates high-quality transgenic Arabidopsis plants

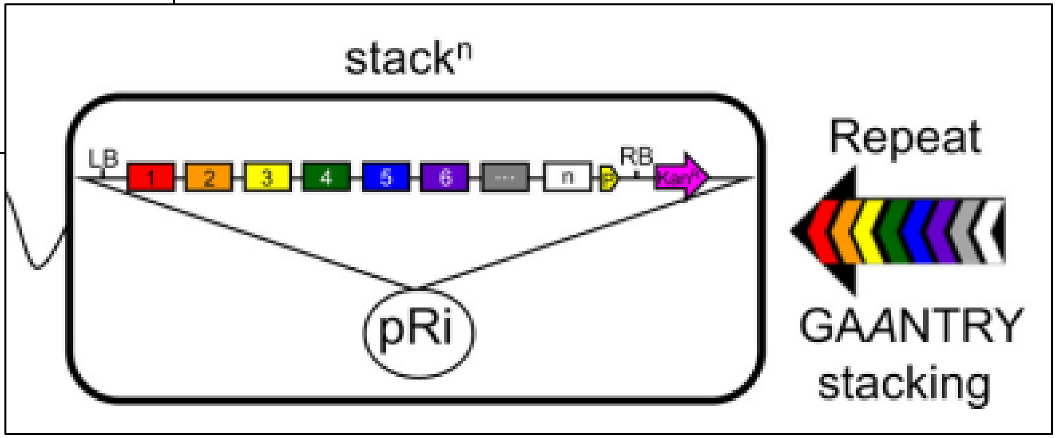
Ray Collier[†], **James G. Thomson*** **and Roger Thilmony***

United States Department of Agriculture-Agriculture Research Service, Western Regional Research Center, Crop Improvement and Genetics Research Unit, Albany, CA 94710, USA

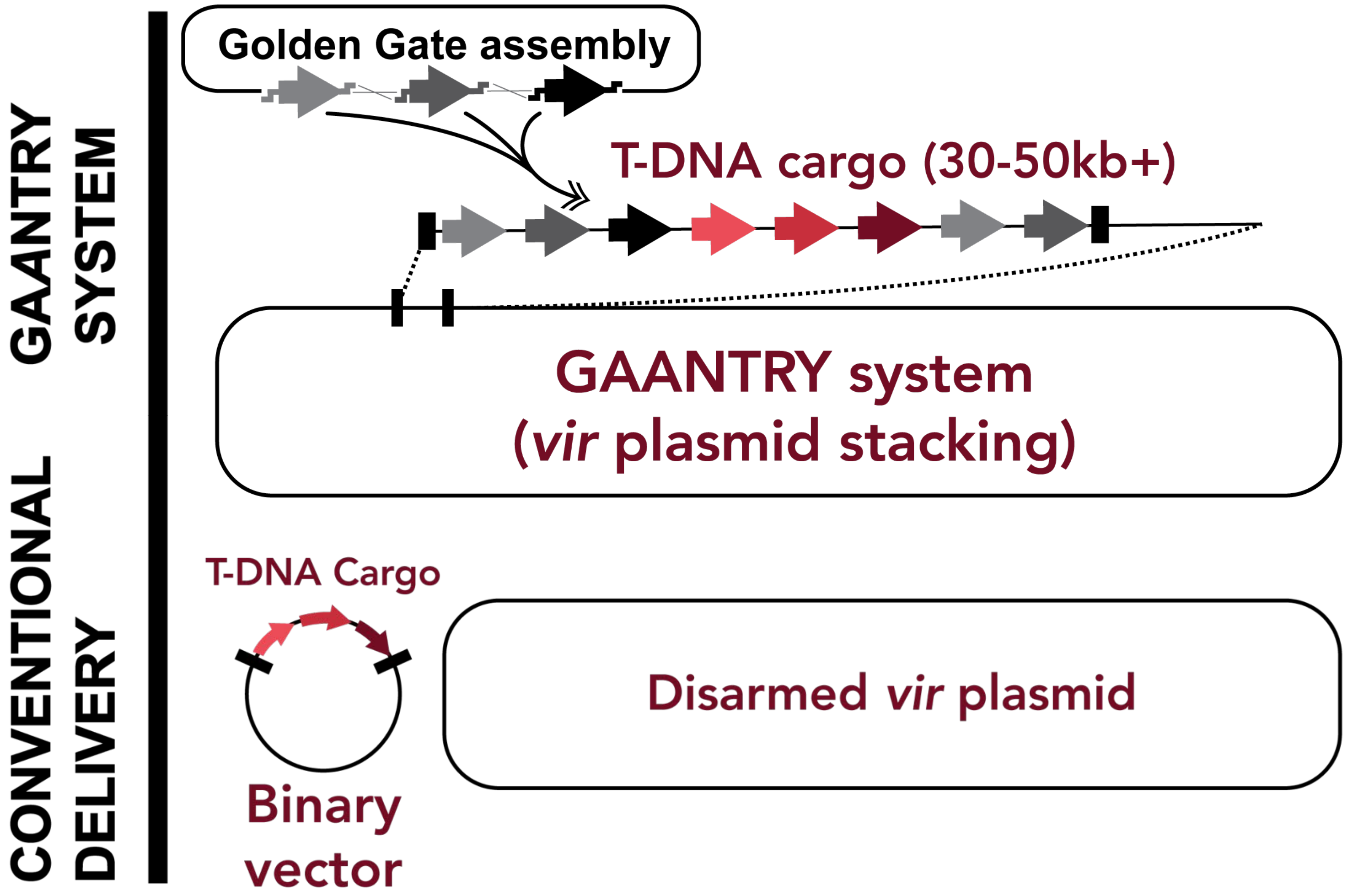
Received 9 April 2018; revised 15 May 2018; accepted 18 May 2018; published online 14 June 2018.

*For correspondence (e-mails Roger.Thilmony@ars.usda.gov or James.Thomson@ars.usda.gov).

[†]Present address: Wisconsin Crop Innovation Center, University of Wisconsin-Madison, 8520 University Green, Middleton, WI 53562, USA.



Assembly of our constructs would be next to impossible without GAANTRY

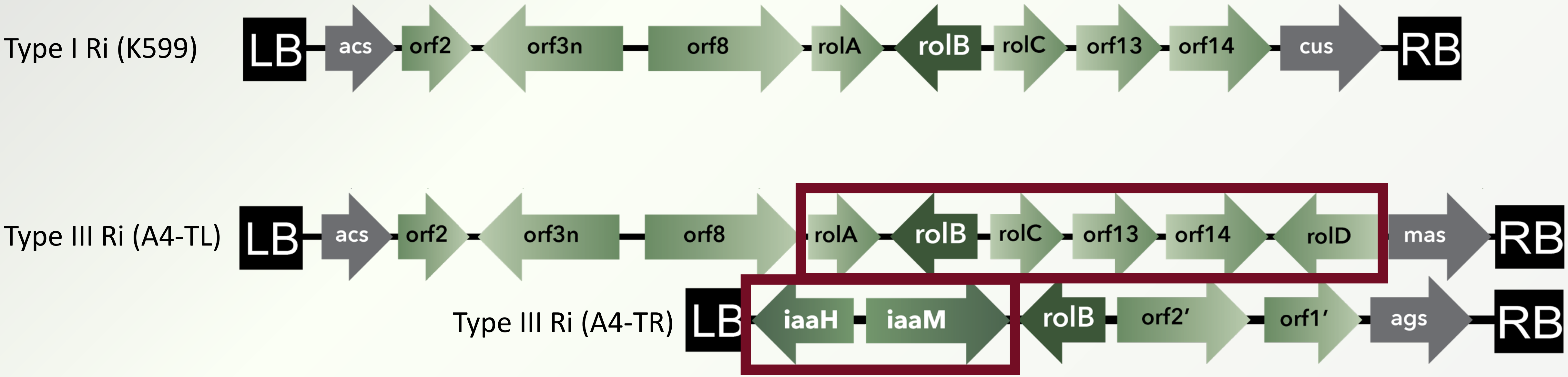


Agenda

- Background
- **The basic RESET system**
- Activities to improve it

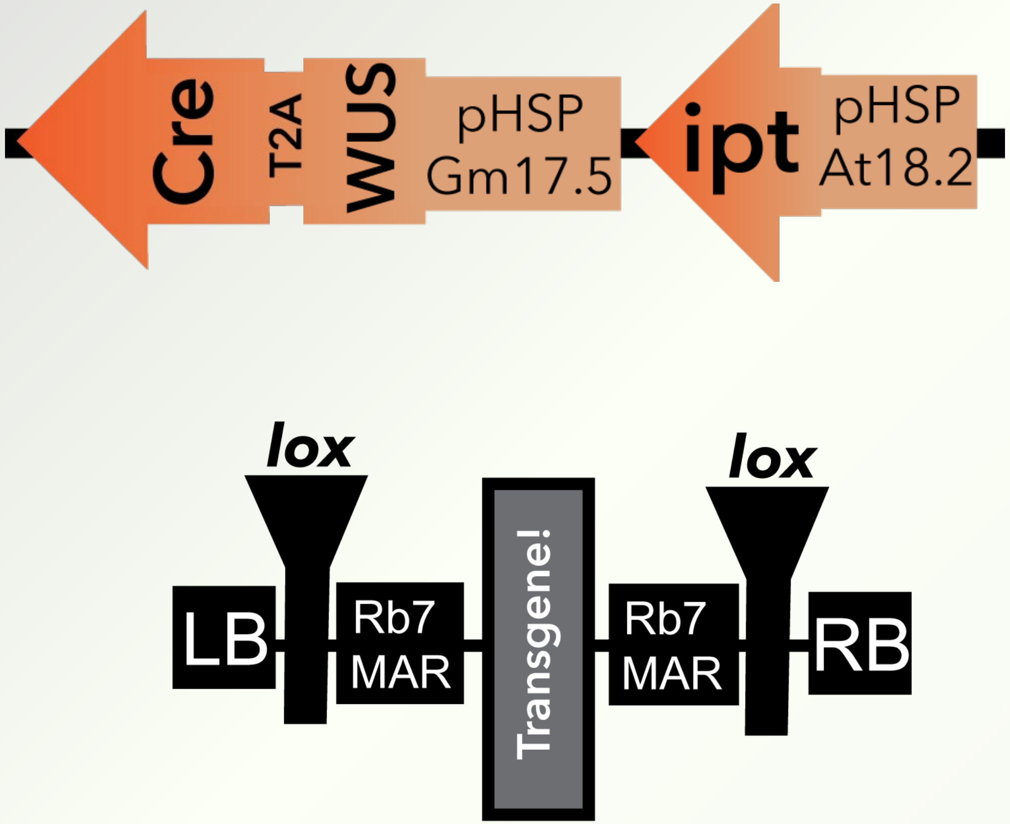
Let's go shopping for parts!

We selected a set of six *rol* genes from strain A4,
a Type III Ri plasmid which is known to work in tree species



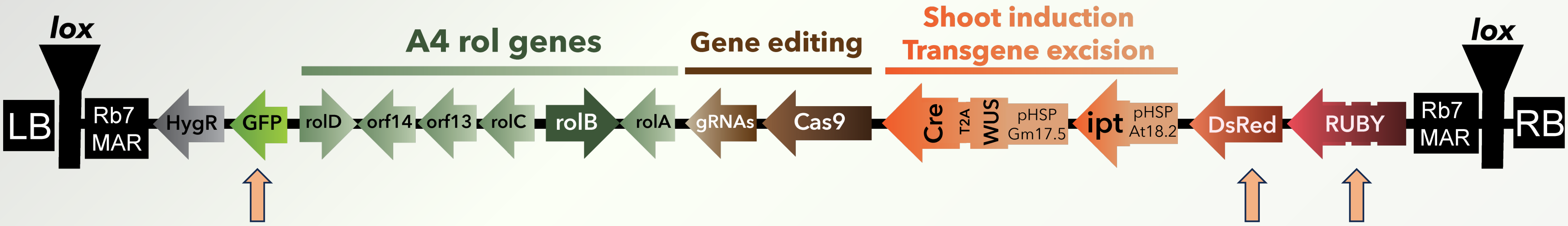
- *rolB* is required for hairy root formation
- Others including *rolA,B,C,D, orf13* and *orf14* quantitatively increase hairy root formation in many species

We selected ***WUSCHEL*** and ***ipt*** as shoot morphogens, **Cre-lox** for excision and chose heat shock as our induction system



- *WUS* shown to induce shoot trans-differentiation from roots
- *ipt*, an *Agrobacterium* T-DNA gene which produces cytokinins and works well in our experimental system
- Heat shock induction is leaky, but effective in poplar
- Cre recombinase used to induce transgene excision, construct flanked by lox sites
- Rb7 MAR elements known to reduce DNA – methylation dependent transgene silencing

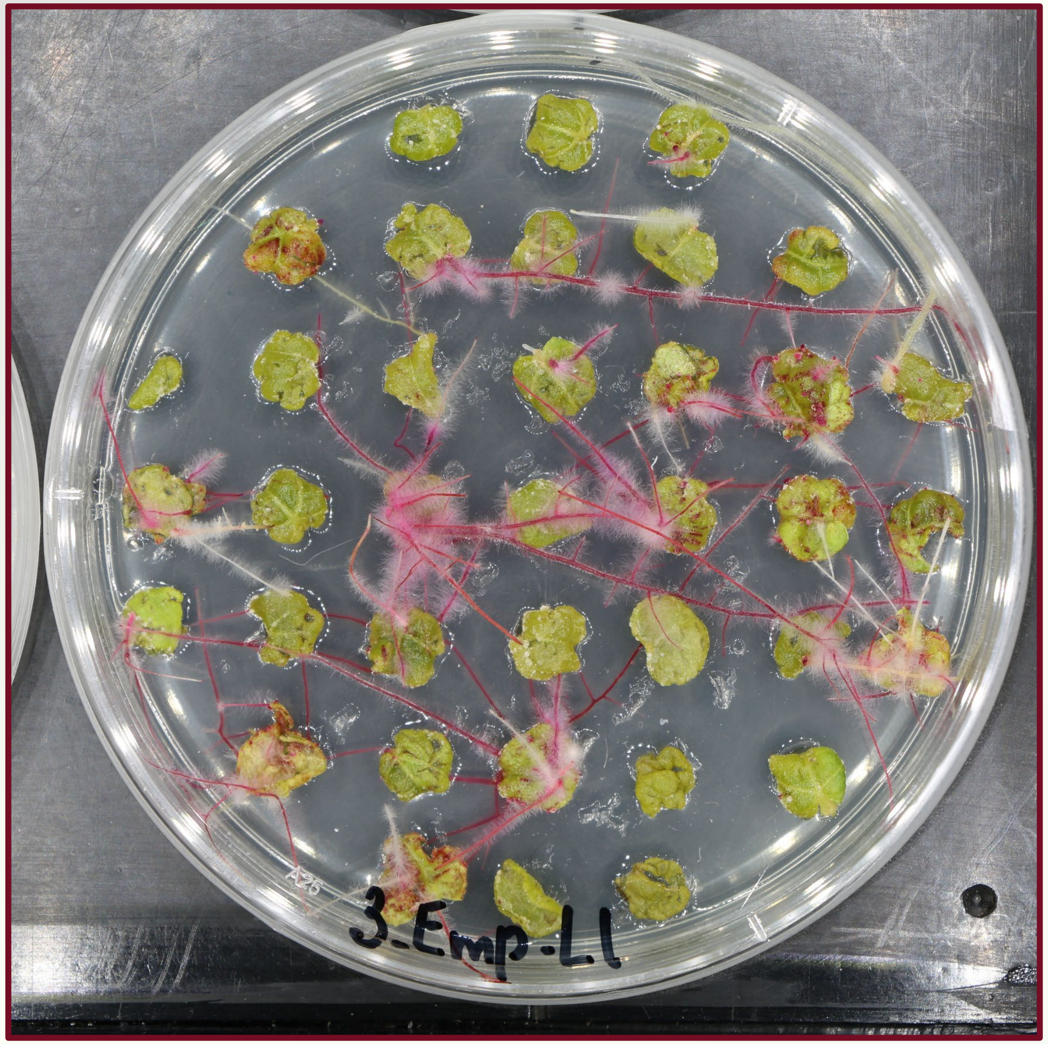
The "kitchen sink" RESET construct

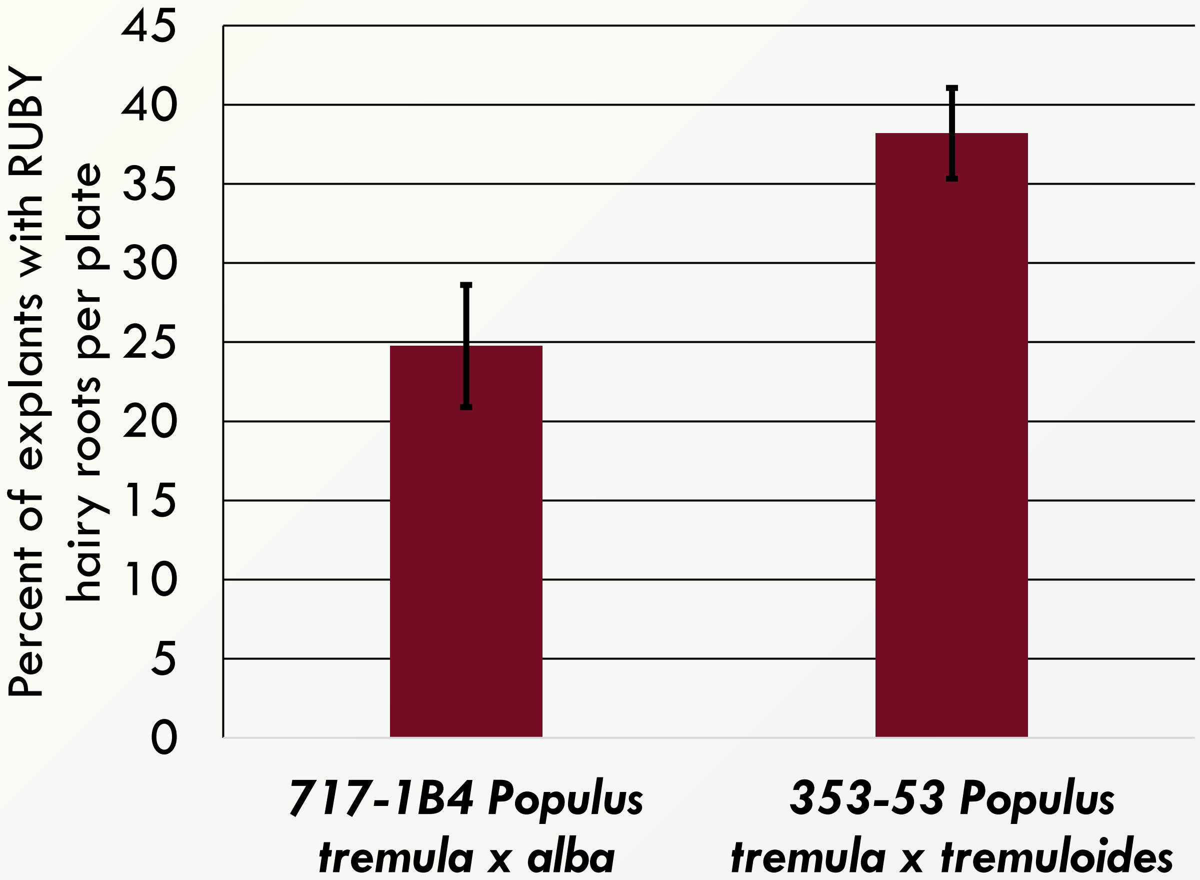


- 39kbp in size in this configuration
- 14 transcriptional units
- 16 independent peptides

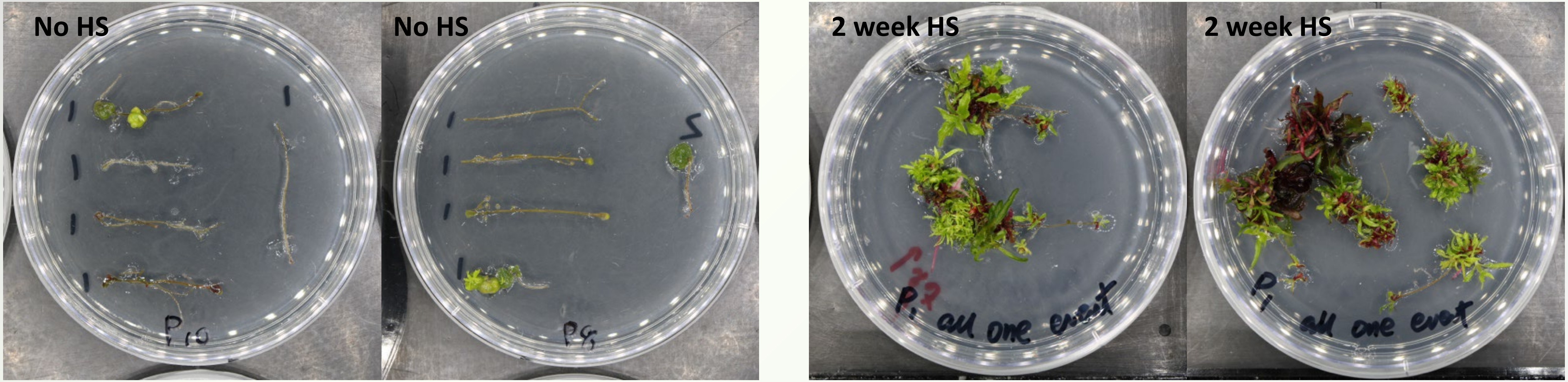
- Included 3 marker genes to identify transgene insertion (GFP, DsRed, and RUBY), excision, and truncations
- Includes gene editing through CRISPR-Cas9
    - tRNA-arrays targeting *RGA1* gene for semi-dwarfism
- Hygromycin selection gene present but no selection was used in these experiments

We efficiently generated transgenic hairy roots in two poplar genotypes for regeneration analysis



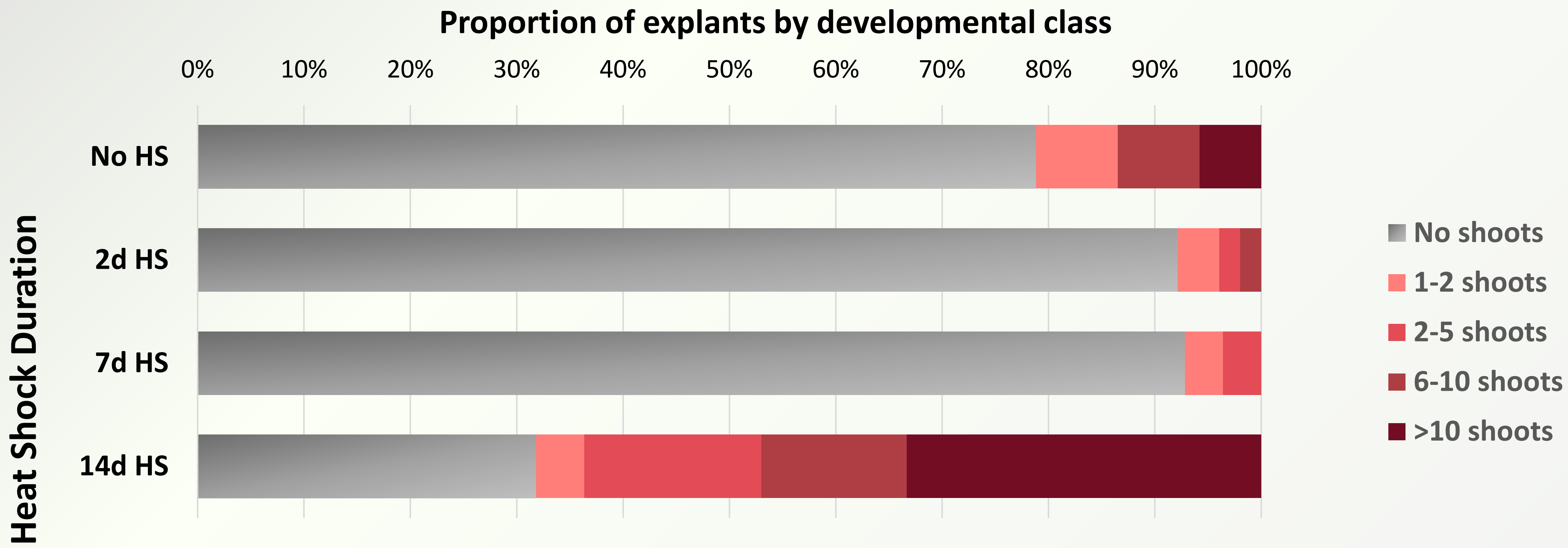


Two weeks of pulsed heat shock resulted in efficient shoot regeneration and excision from hairy roots

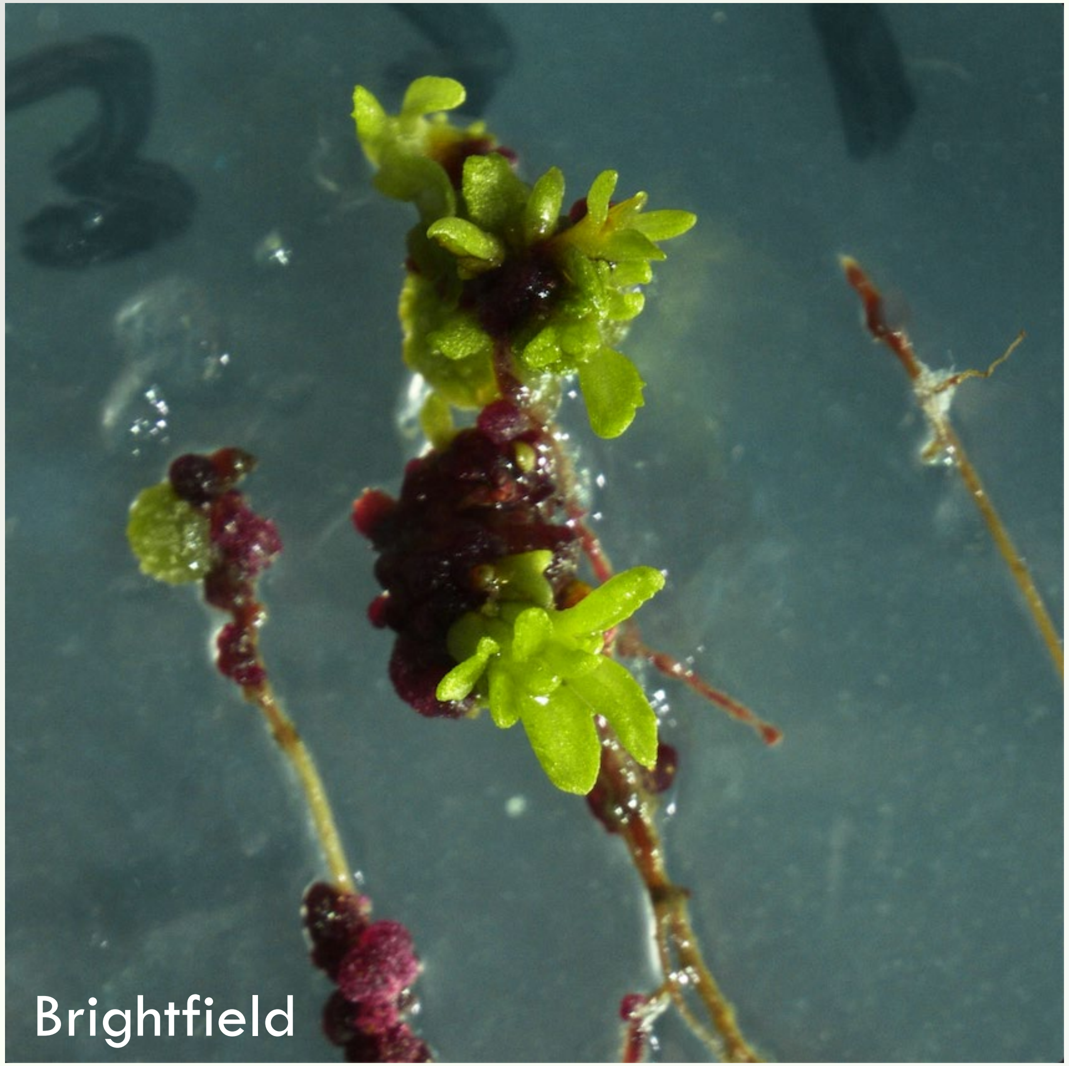


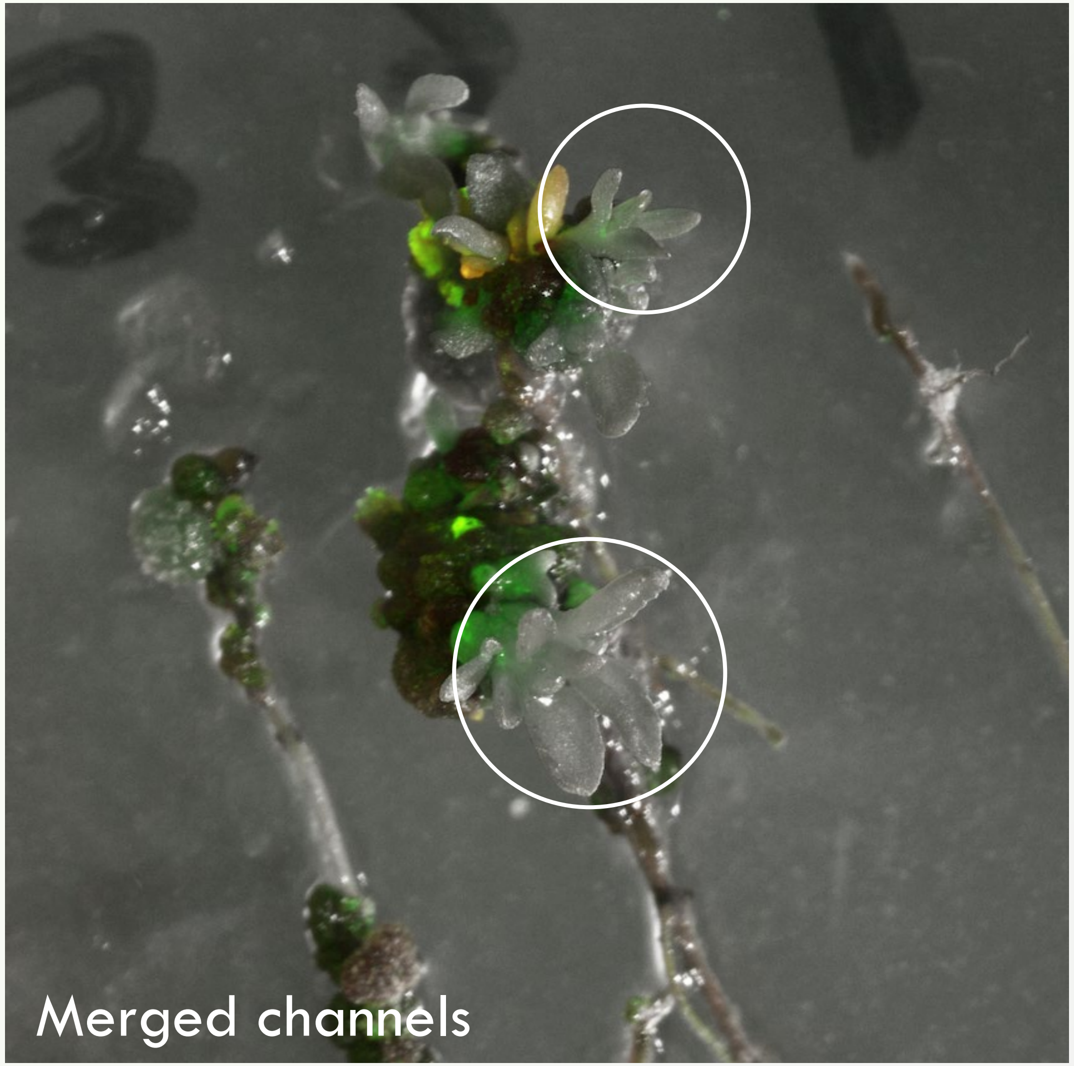
- Four hours heat shock at 39 degrees Celsius
- RUBY vs. non-ruby shoots can be easily identified for propagation, then other reporters examined by fluorescent microscopy after isolation

Longer heat-shock (HS) duration improved shoot regeneration rates per explant (segments of hairy roots)

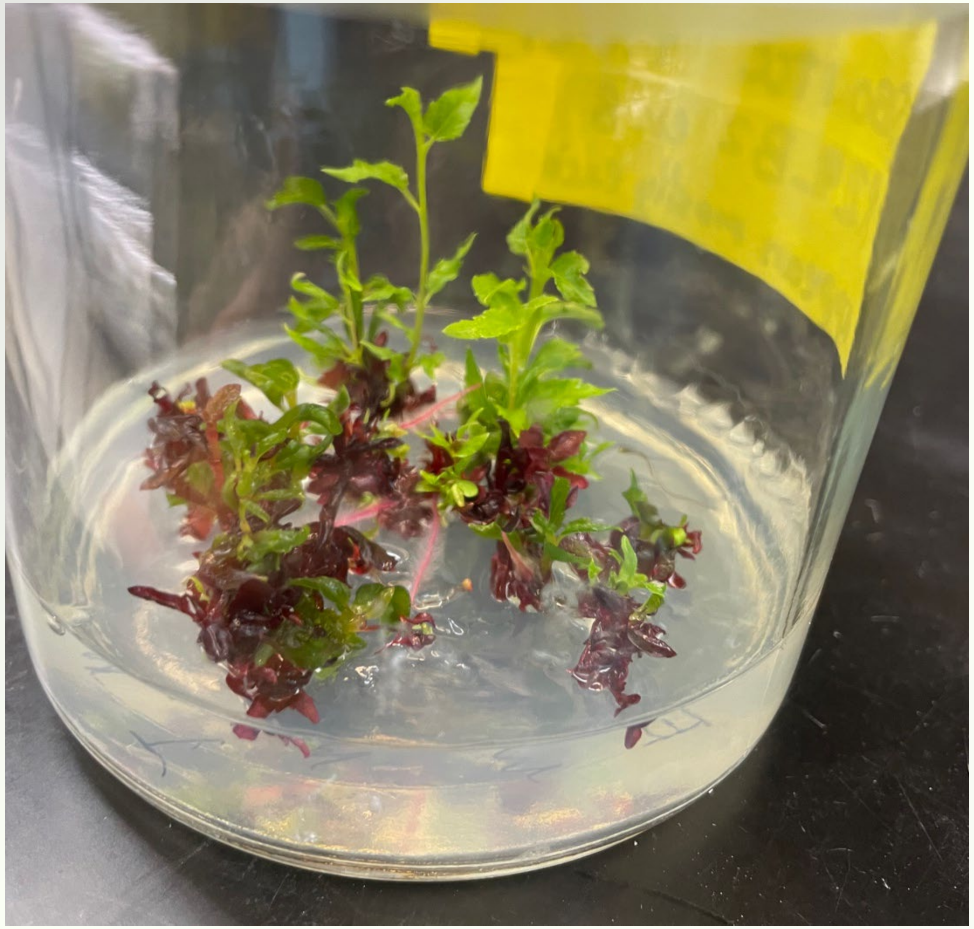


Using multiple reporters (GFP, DsRed and RUBY), we can find shoots with excised transgenes





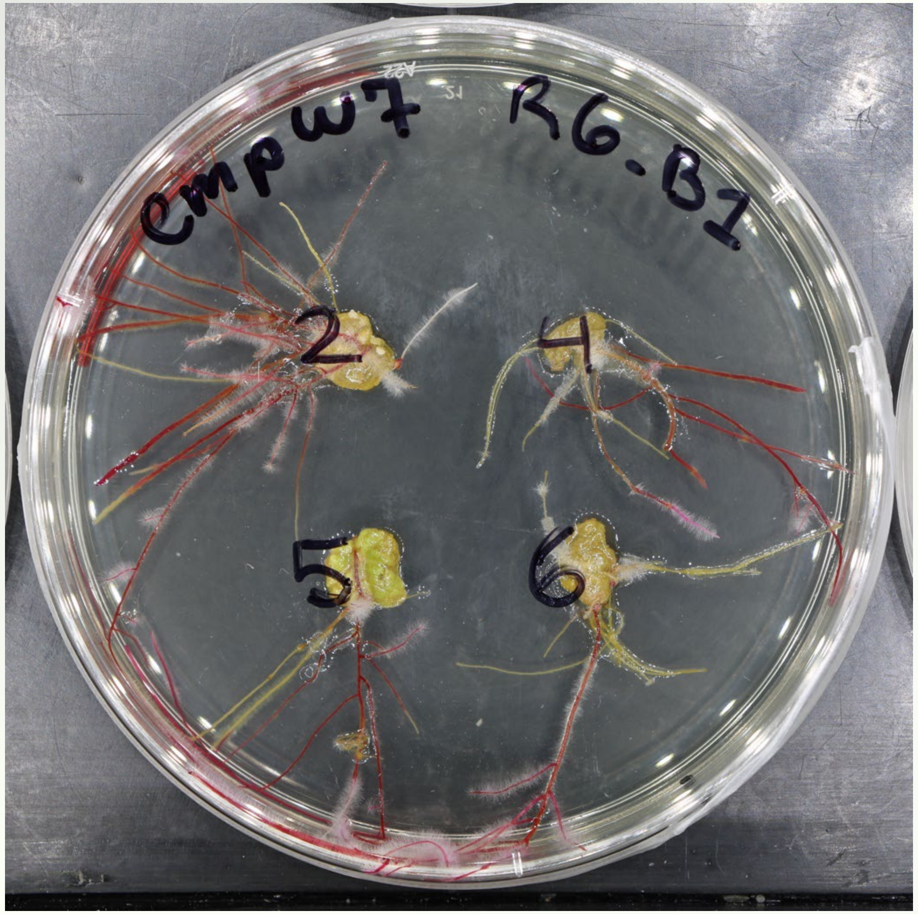
After transfer of regenerating root explants, elongated shoots without marker genes can be identified



Mixtures of RUBY and green RESET shoots in late-stage propagation

Populations of putative excised RESET shoots for further molecular analysis

We performed new transformations with the goal of estimating frequency and integrity of excision more reliably



Tracked individual hairy root insertion events through the transformation and excision process

Cre excision methods have been a constant frustration in this and other related projects

Full Paper

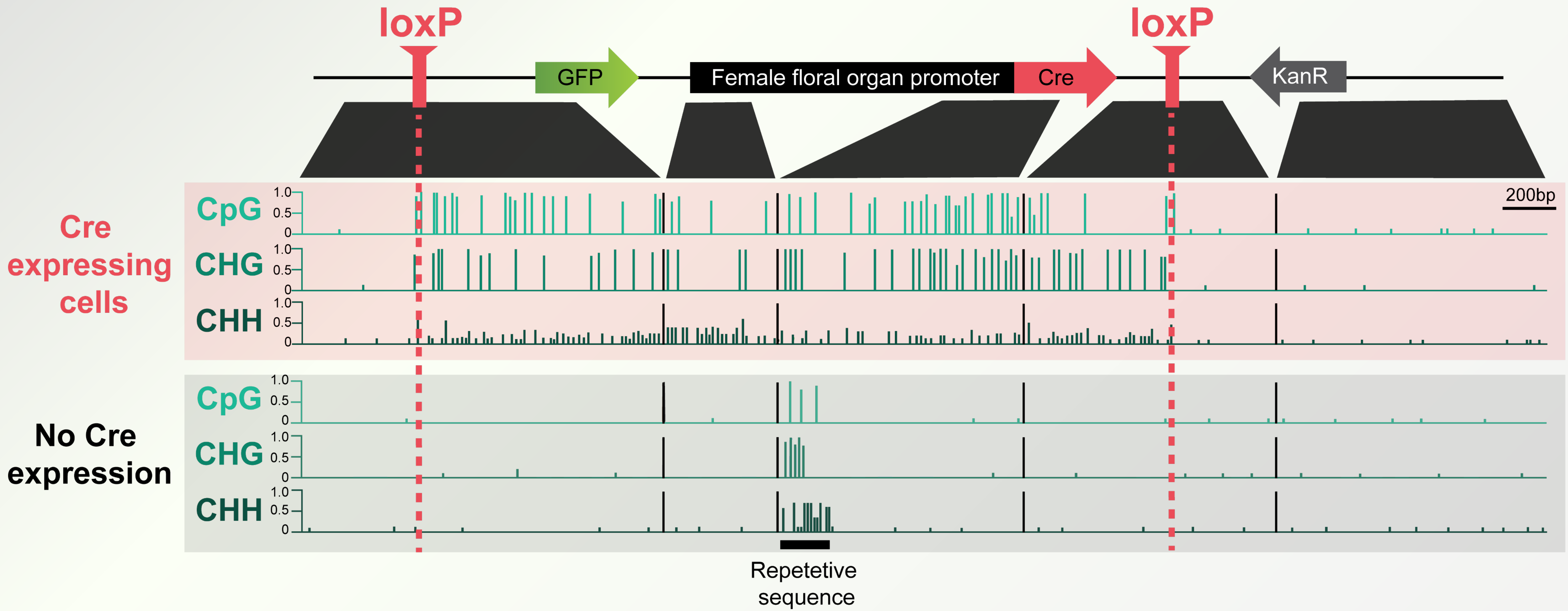
New Phytologist

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

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

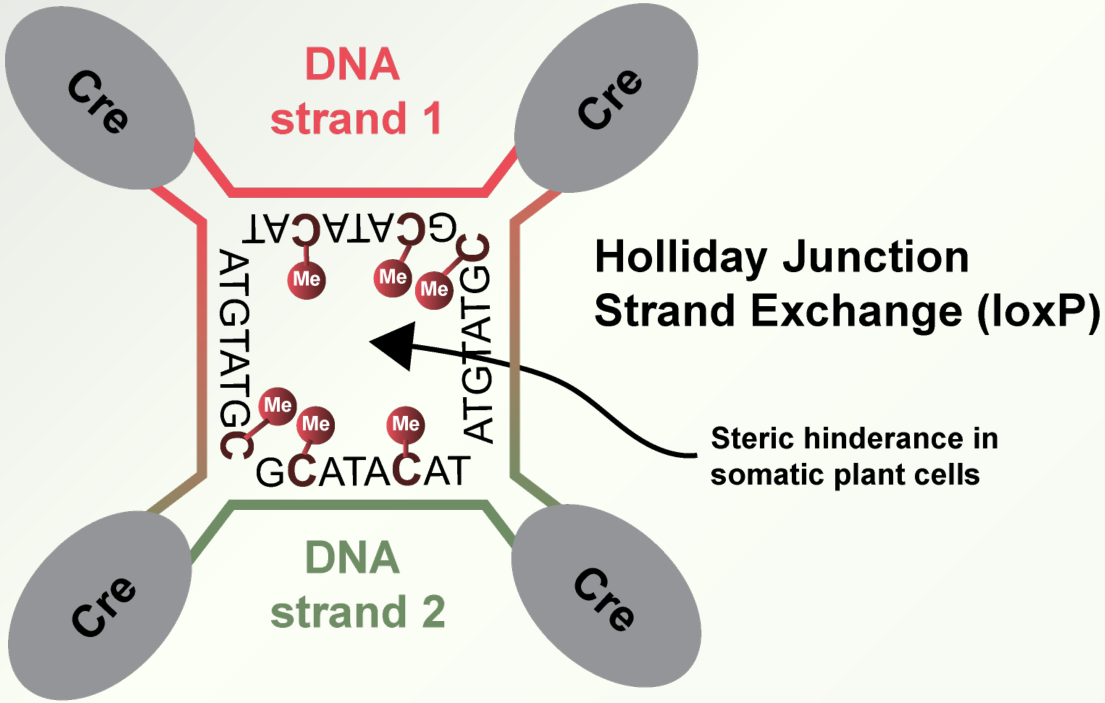
Chongqing Key Laboratory of Application and Safety Control of Genetically Modified Crops; Biotechnology Research Center, Southwest University, No. 2 Tiansheng Road Beibei, Chongqing 400715, China

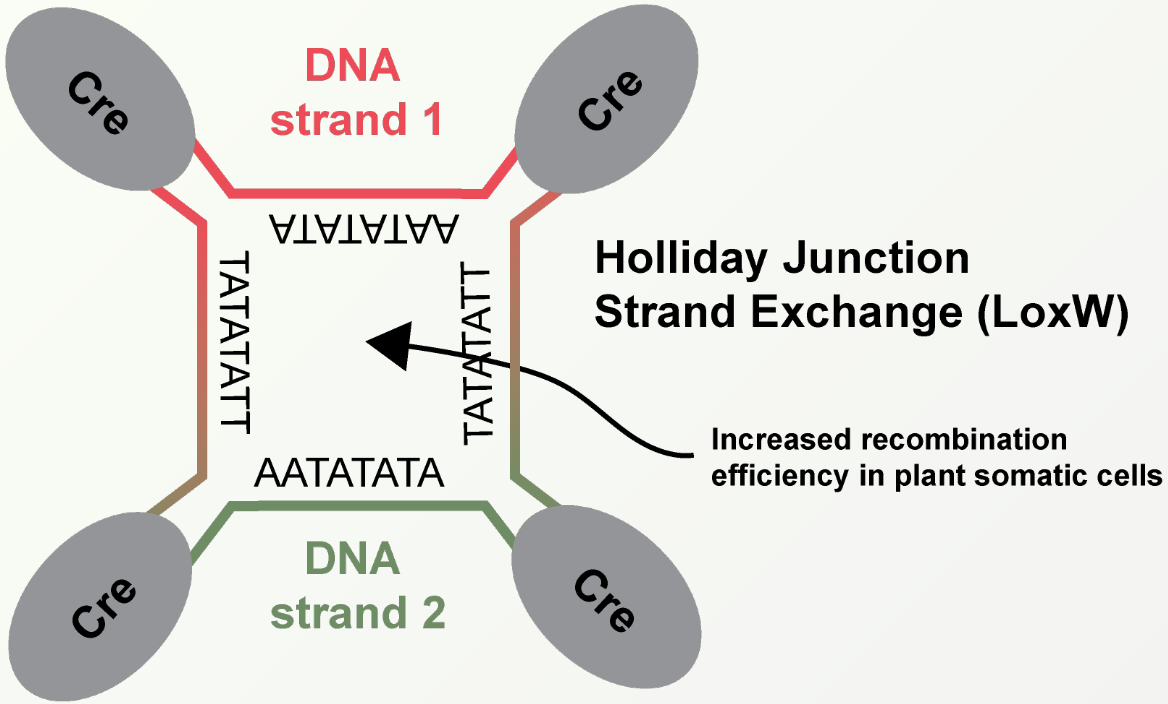
Cre can methylate transgene regions flanked by its recognition site loxP



Modified from Liu et al. 2021 *New Phytologist*

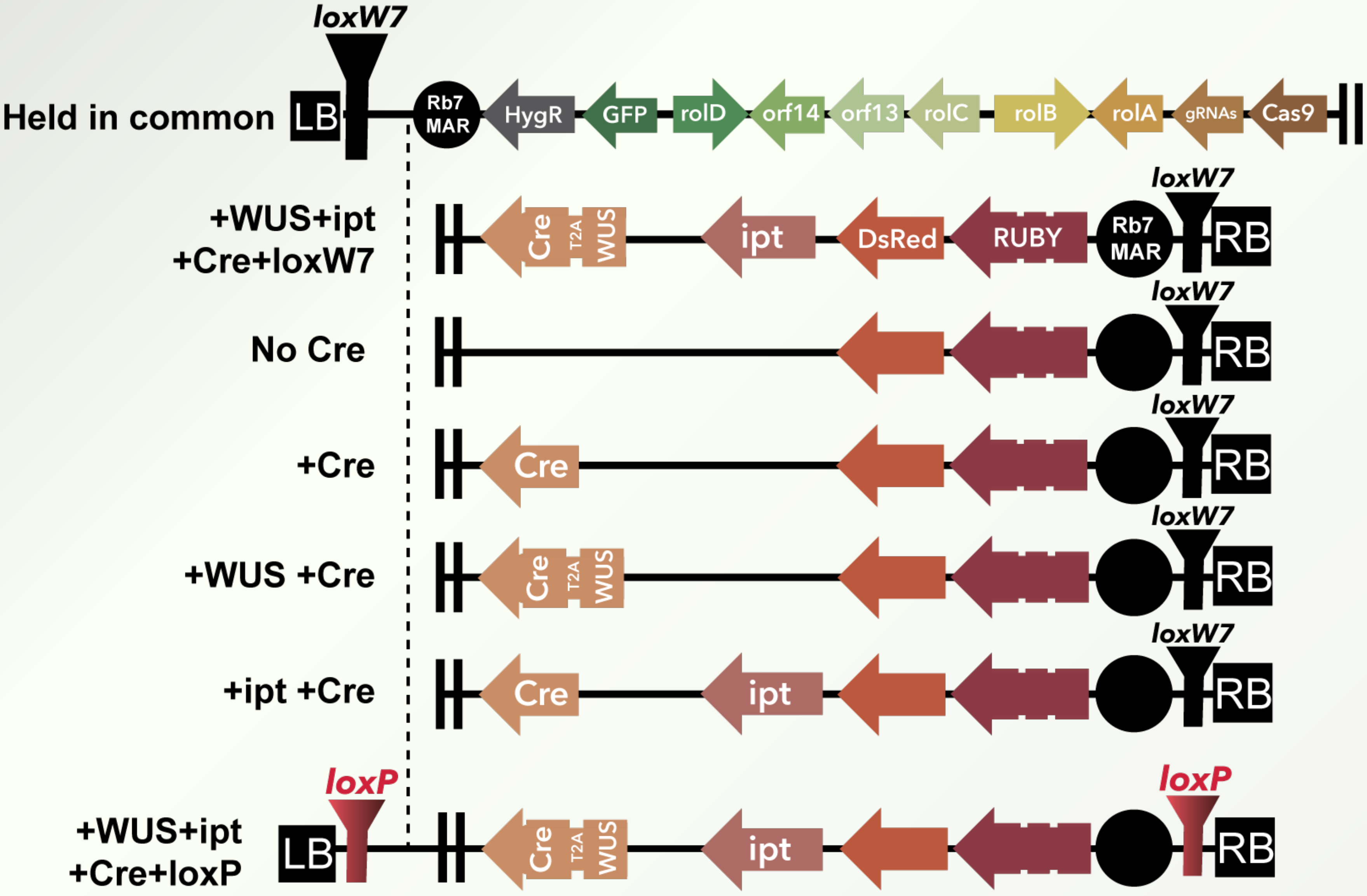
We made several variations of RESET constructs, including changes to flanking lox sites designed to be resistant to DNA methylation



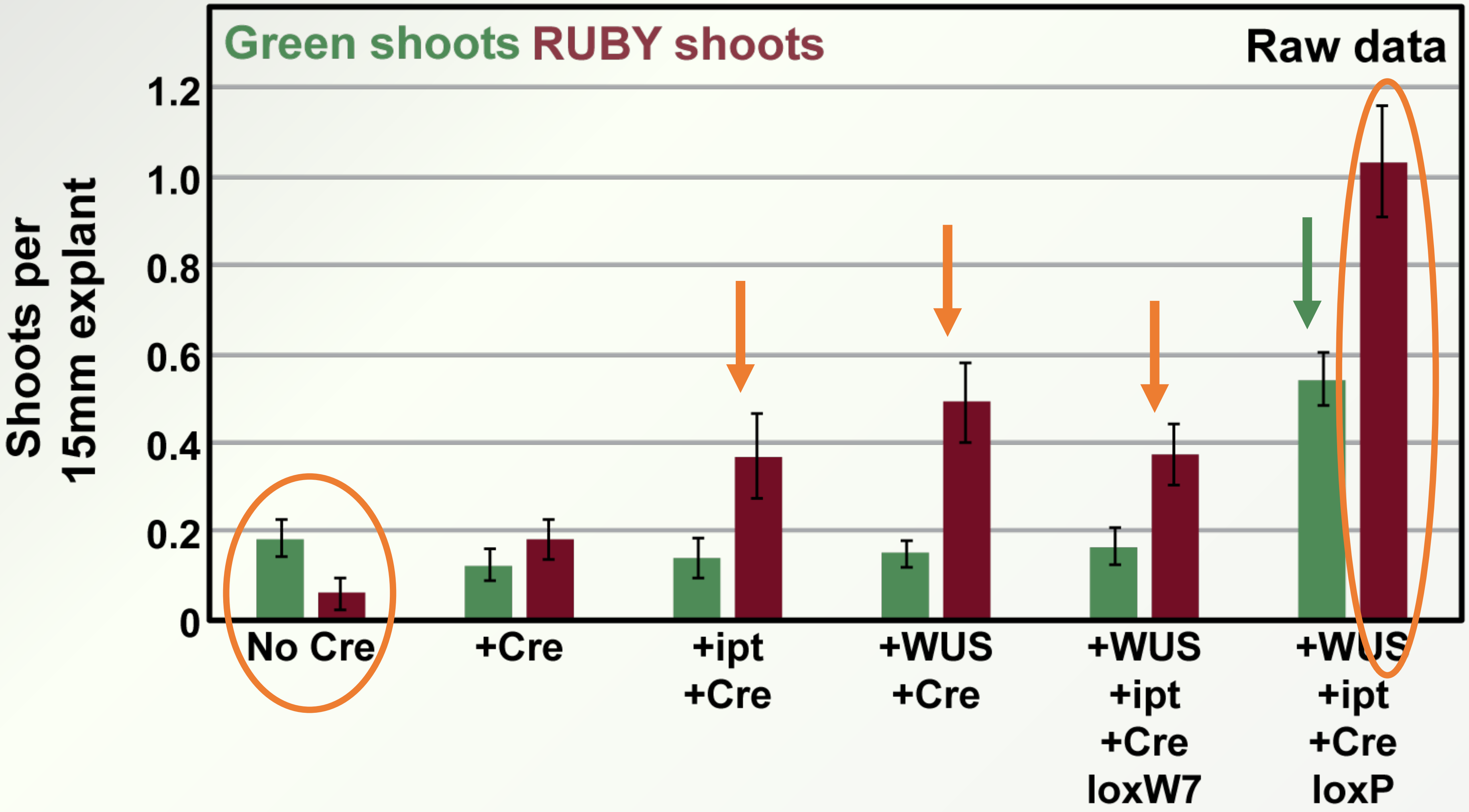


(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)
(19) World Intellectual Property Organization
International Bureau
(43) International Publication Date
07 November 2024 (07.11.2024)
WIPO | PCT
(10) International Publication Number
WO 2024/229458 A2

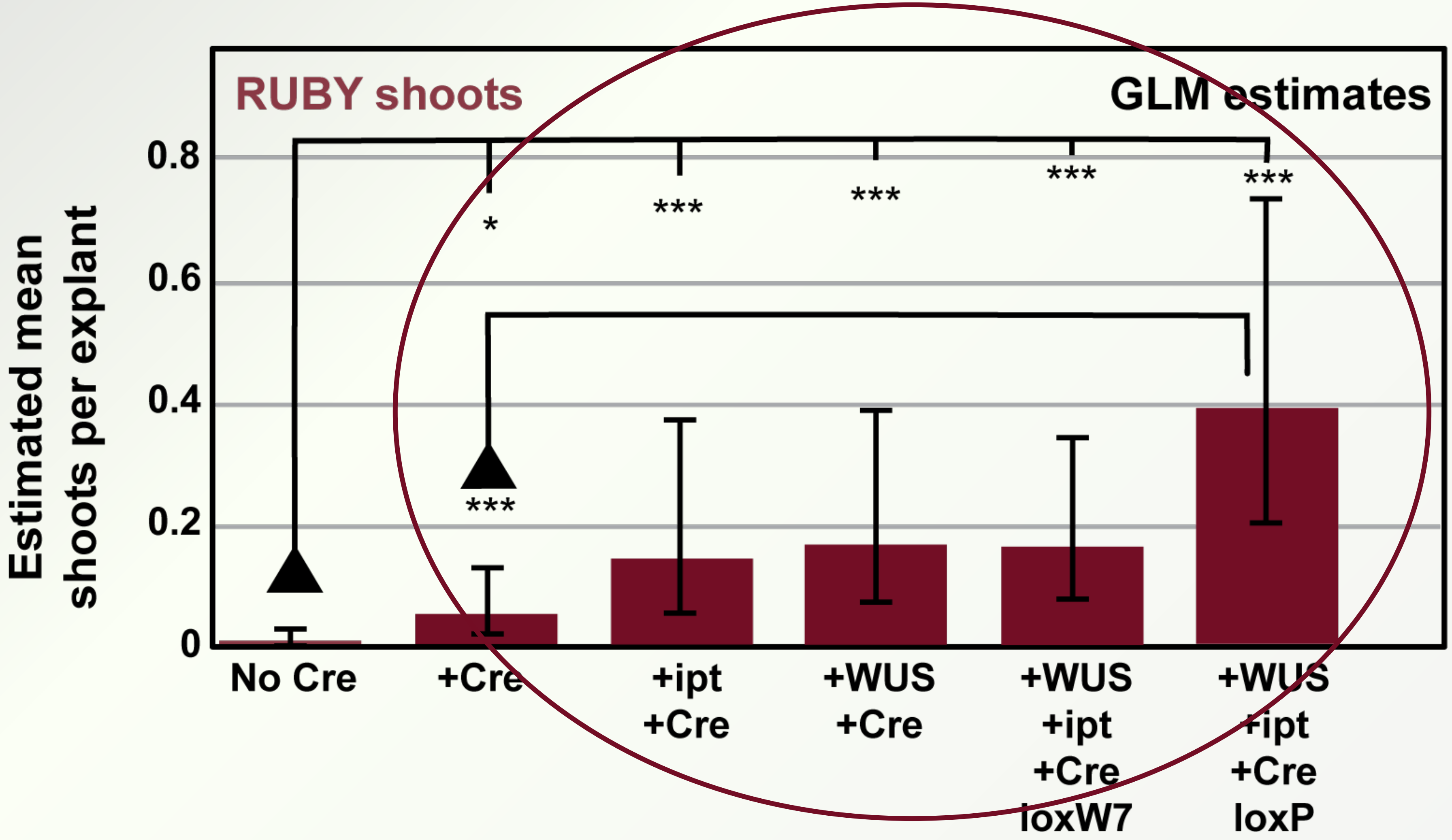
We also compared the presence of *WUS* and *ipt* morphogens



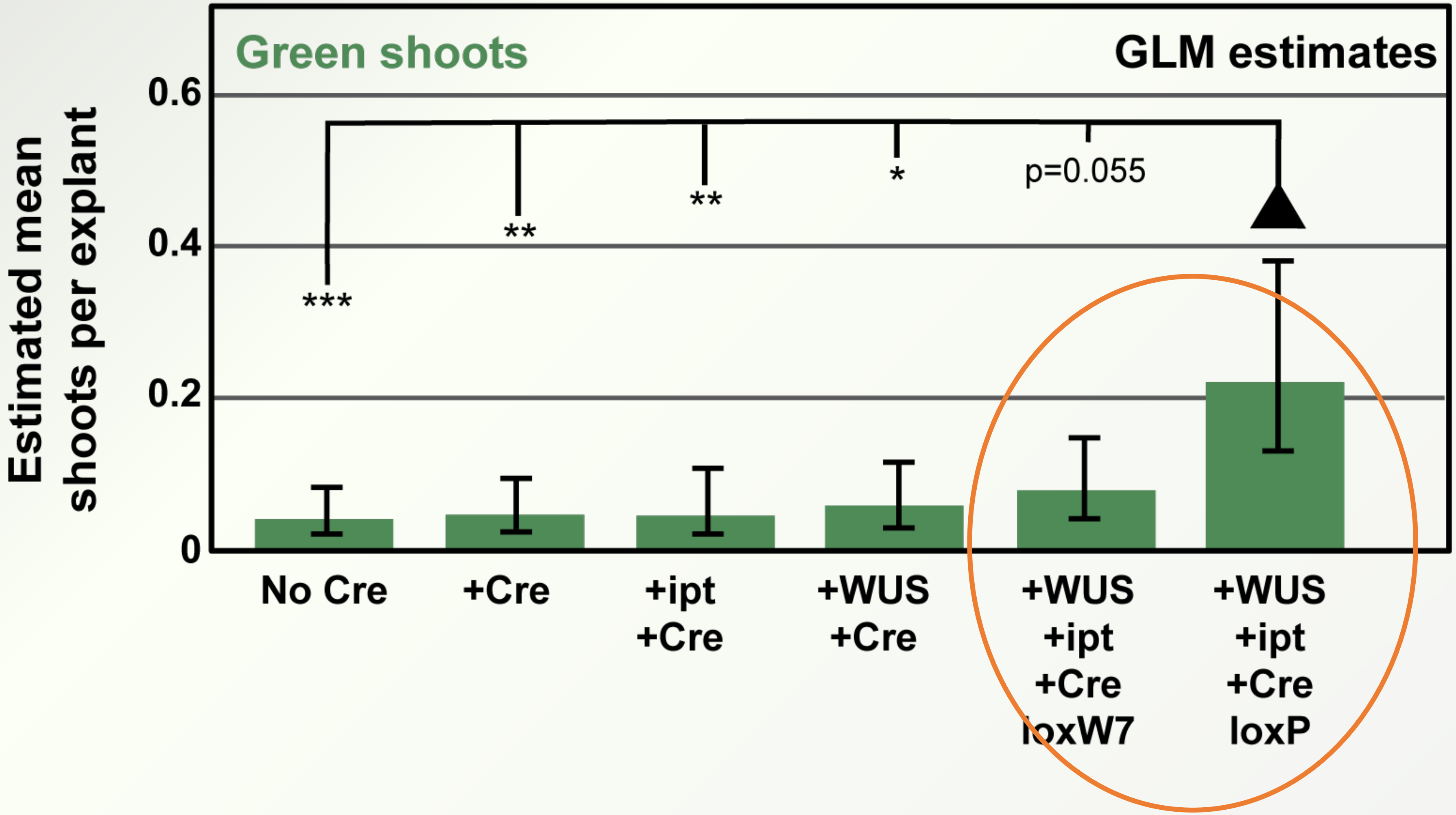
loxP-flanked RESET constructs gave the highest shoot regeneration after heat shock induction of transgenic roots



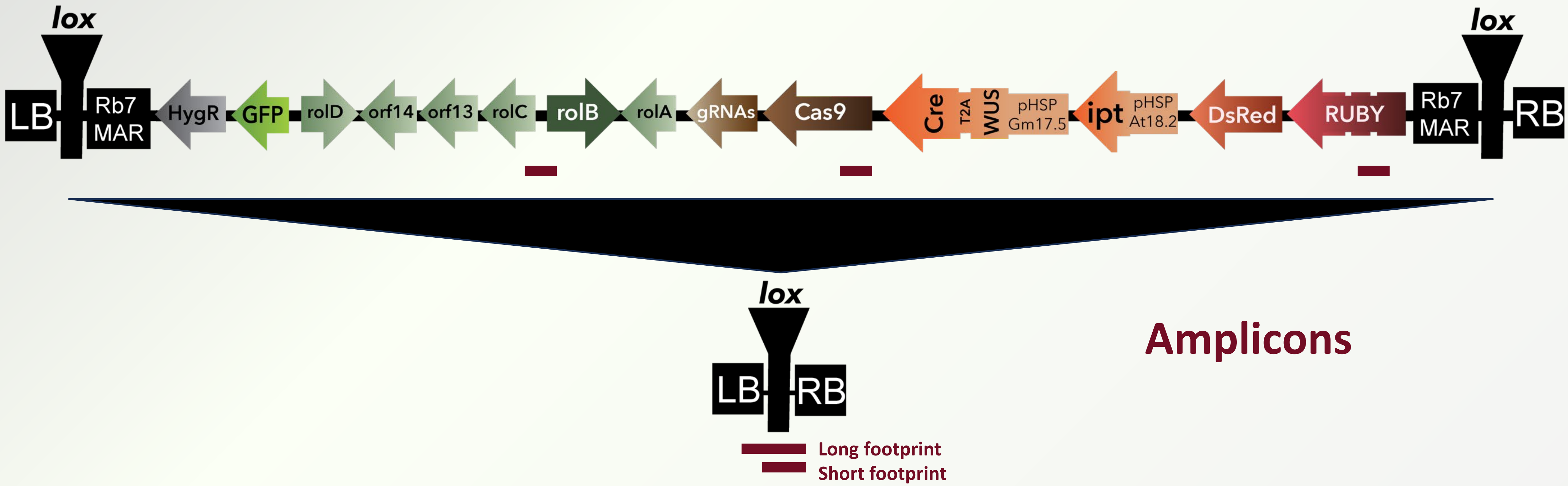
Cre expressing RESET constructs had a significant benefit for transgenic shoot regeneration



loxP-flanked RESET constructs had superior numbers of excised shoots versus loxW7 variants

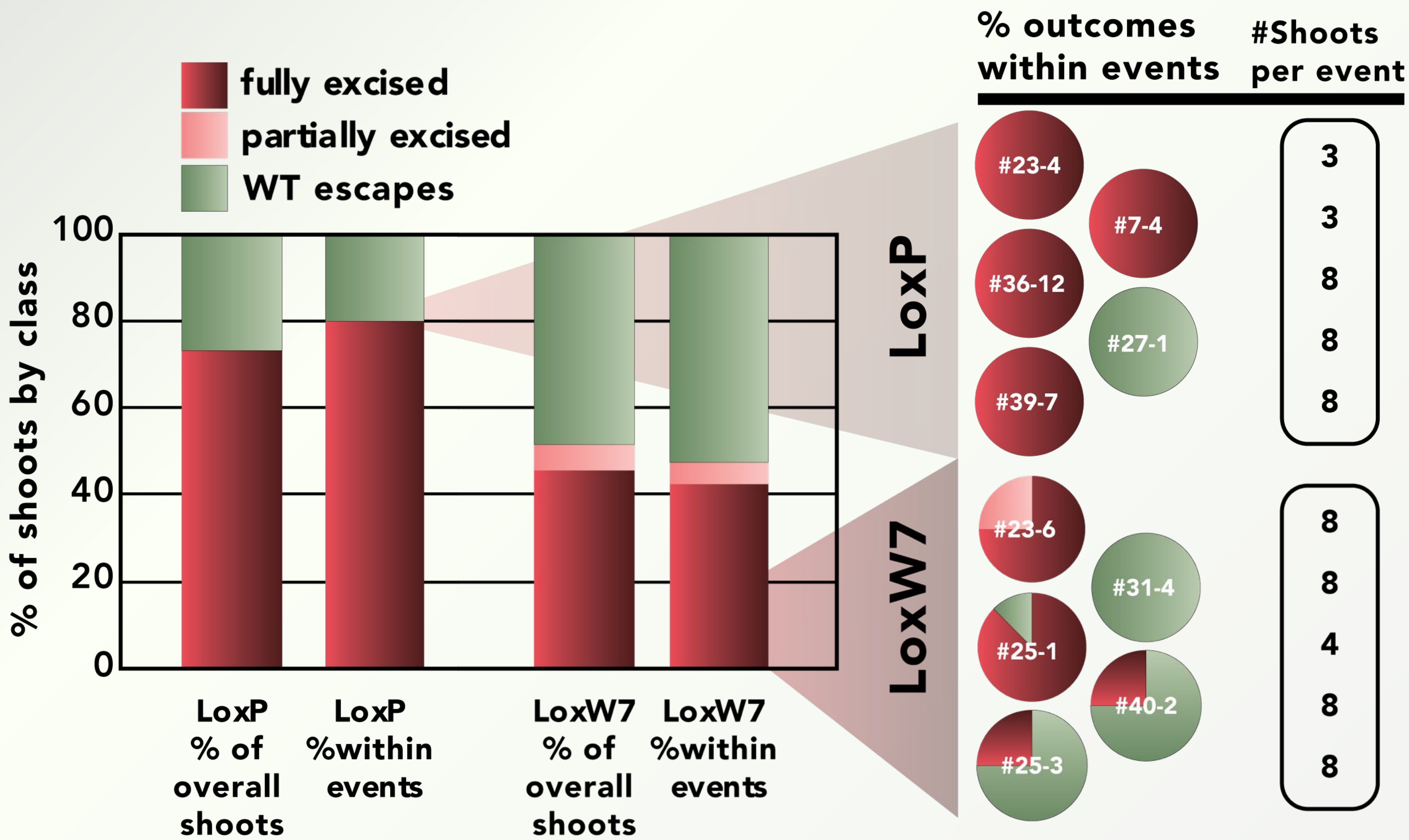


To figure out if the shoots without RUBY, GFP, and DsRed were completely excised, we used a panel of PCR amplicons

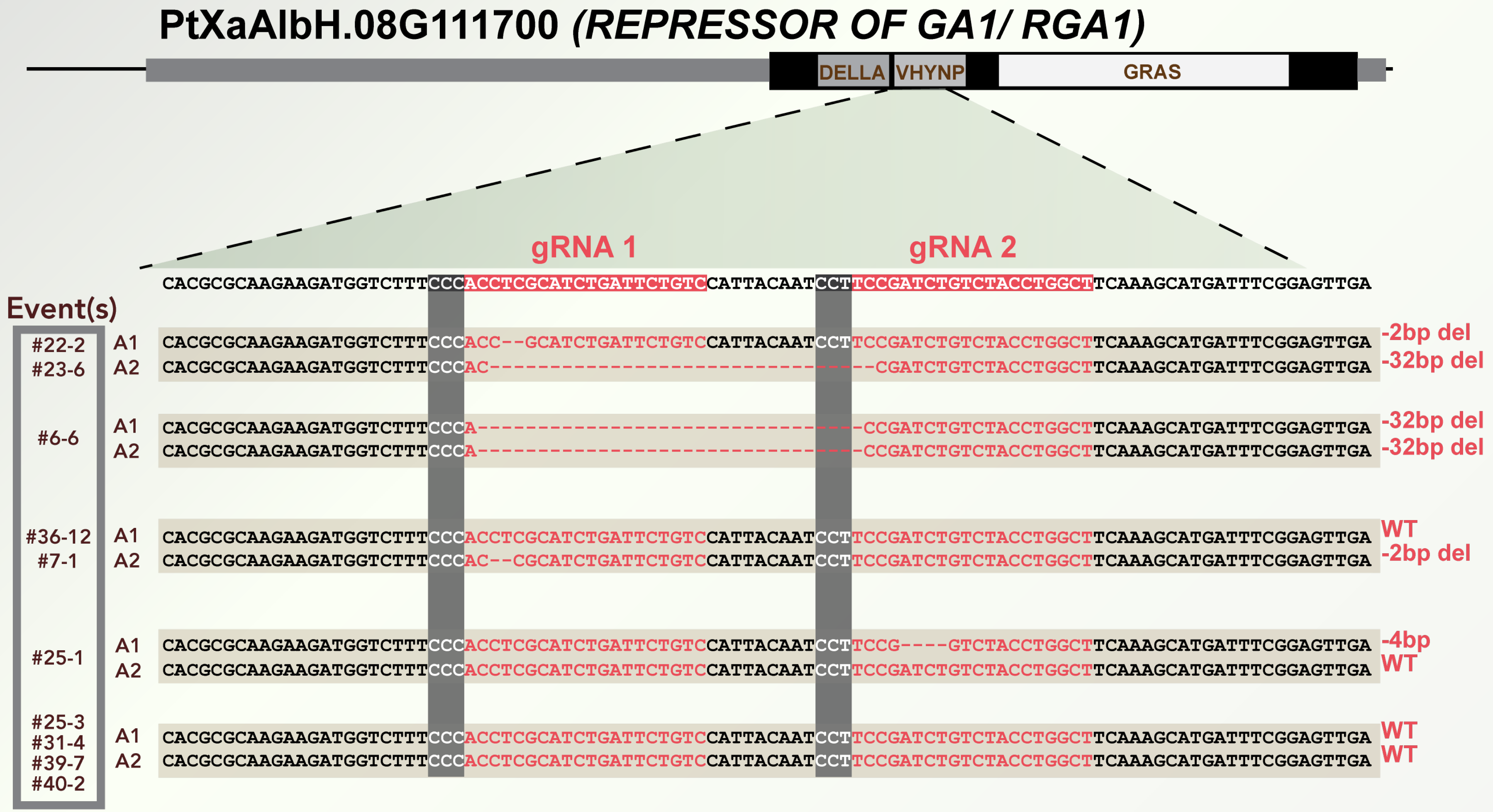


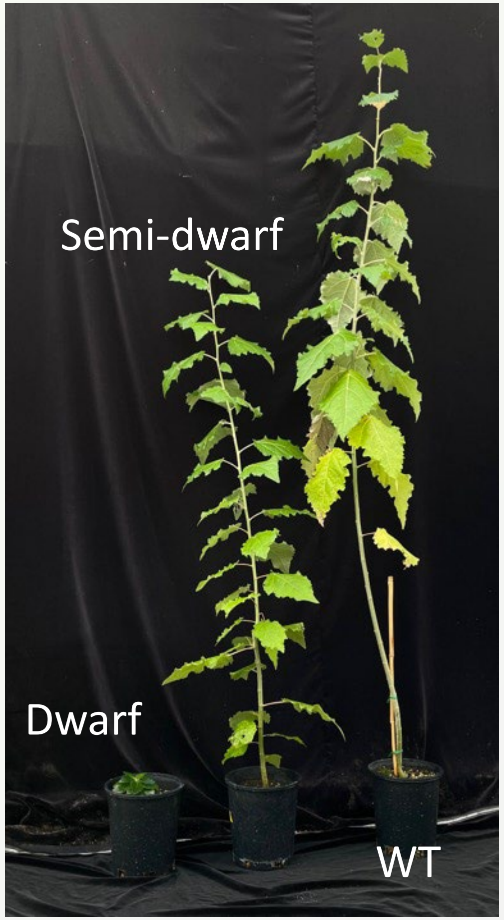
- The presence of any transgene band eliminated a shoot as being categorized fully excised (partial chimera)
- Two footprint amplicons were included in case of truncation of the T-DNA left border

While many escape shoots were found, high rates of complete excision were found in product shoots

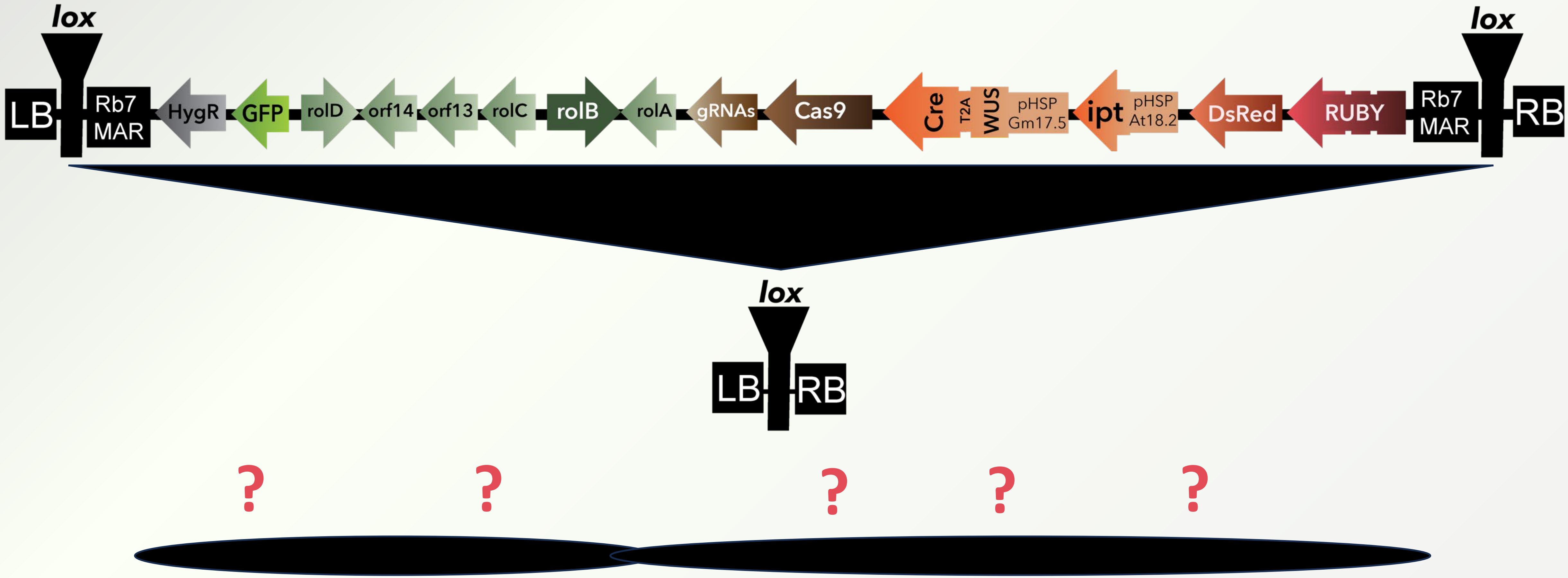


60% of excised events showed editing in at least one allele of the target locus, which is typical for poplar

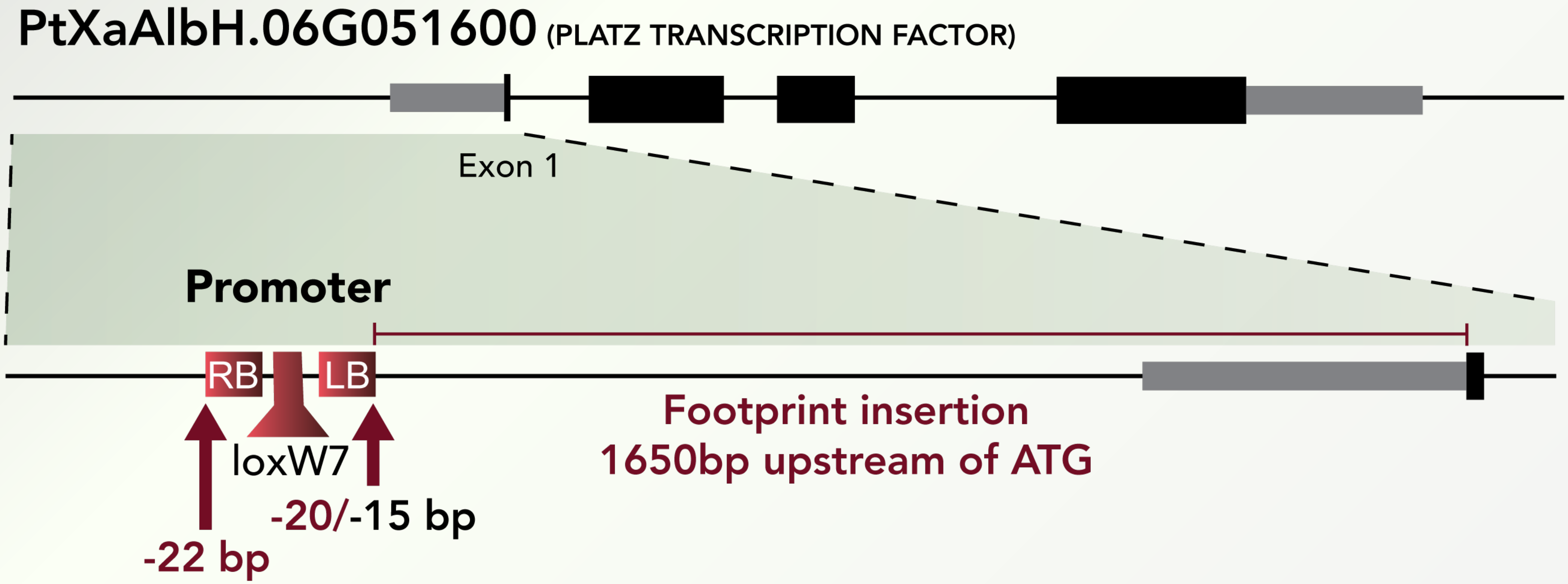




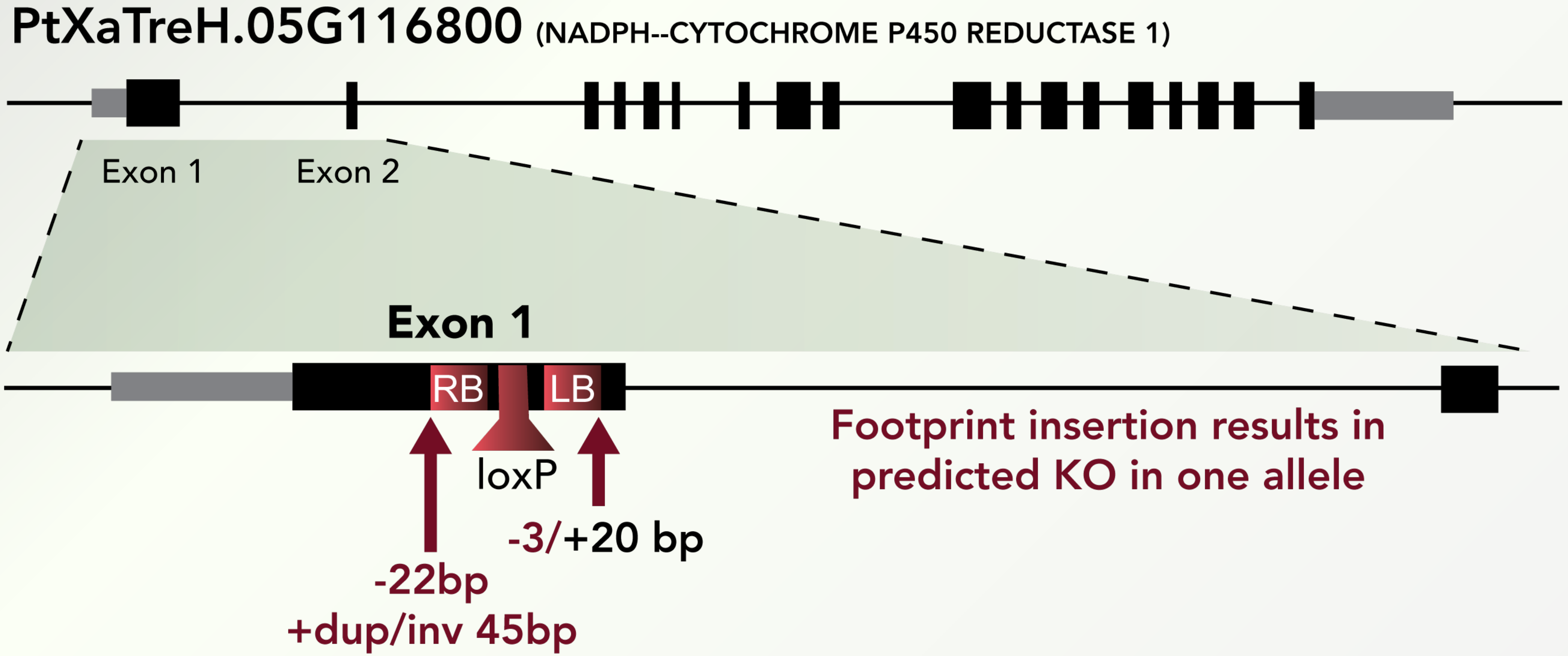
Where did our transgenes insert? How did the excision process resolve the footprints into final configurations?



We used TAIL-PCR and WGS to find where transgenes landed and resolved into smaller footprints



We did find some events where the footprint inserted into an exon of one allele



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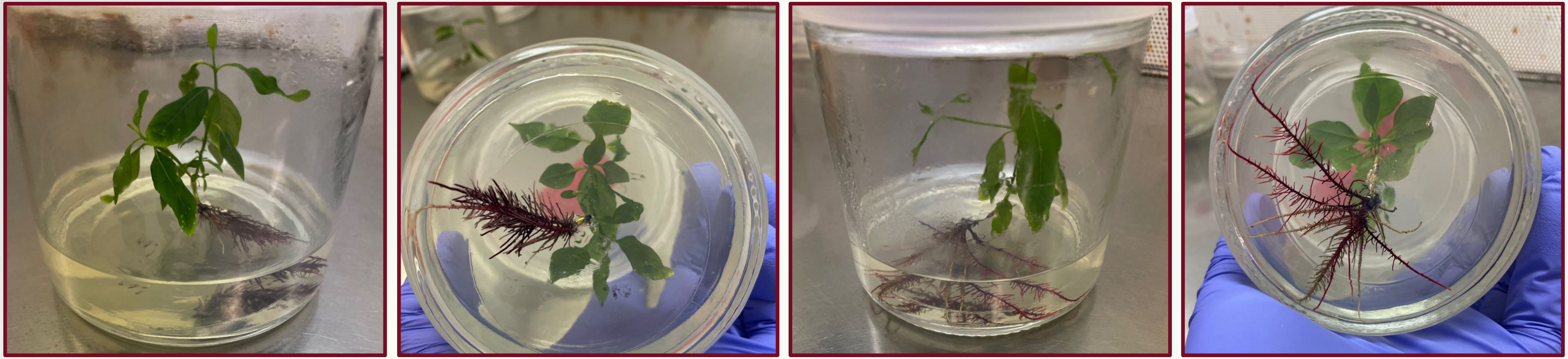
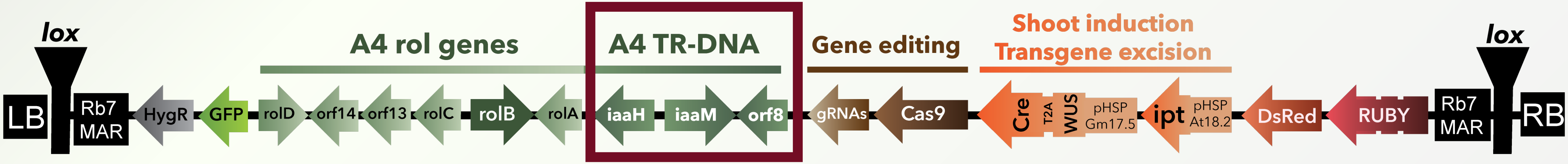
- **Activities to improve it**

Does this work in other, more recalcitrant species?

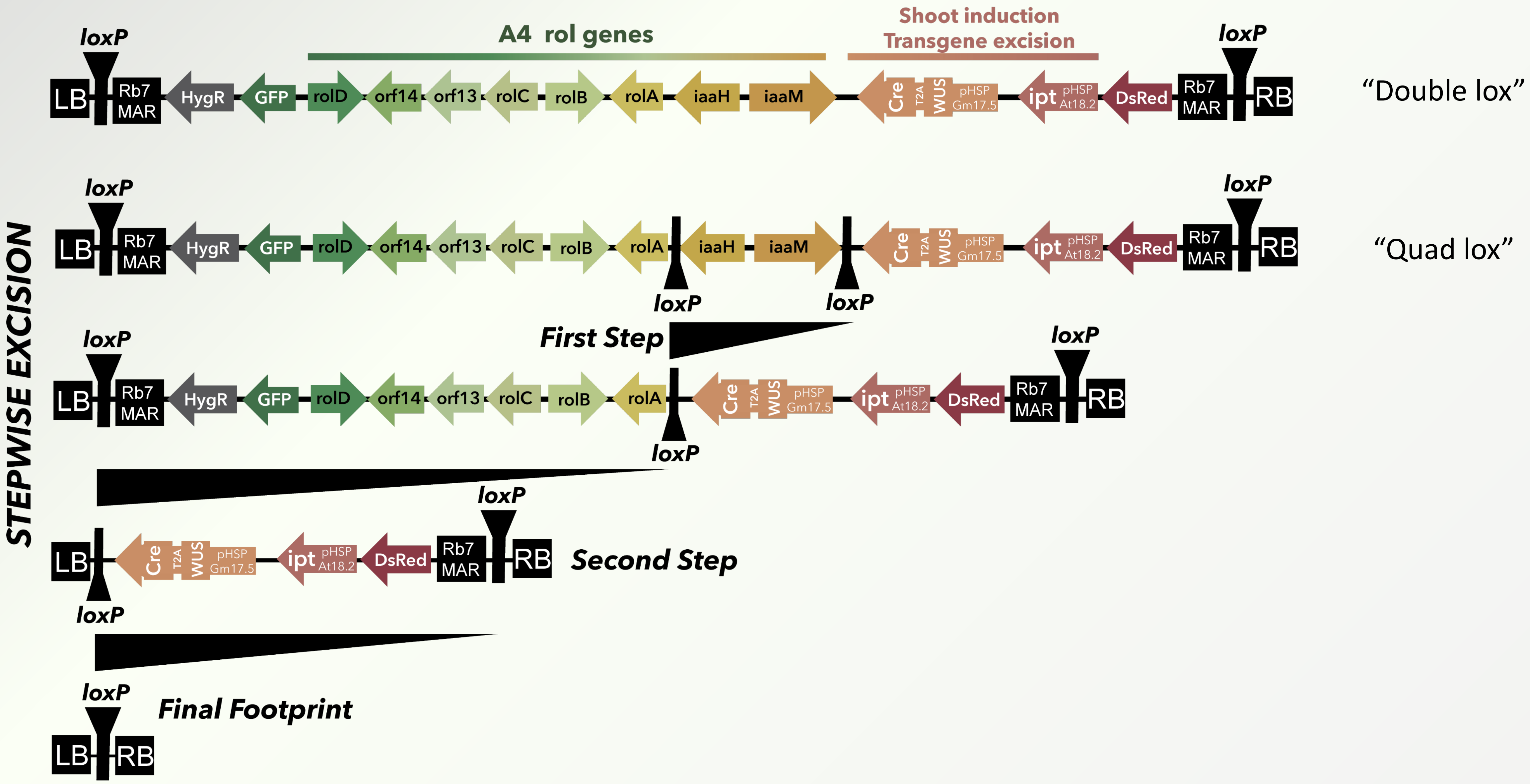


Eucalyptus grandis x urophylla RESET composite plants



So far, adding the iaaH/M genes is essential for hairy-roots, but also seems to interfere with shoot development

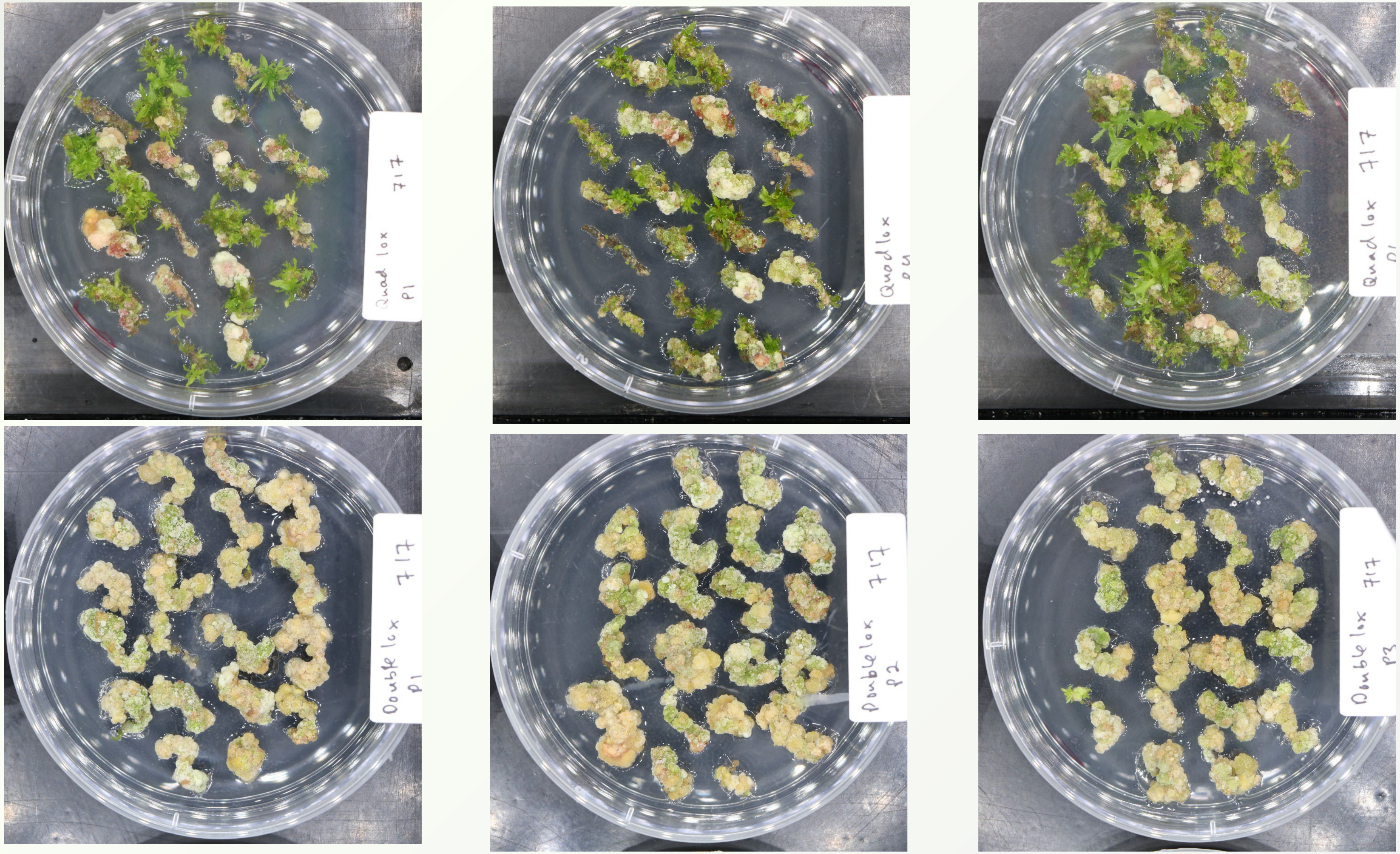
We are focusing on excision improvement in new variants



QuadLox showed improve shoot regeneration over double lox configurations

“QuadLox”

“Double lox”

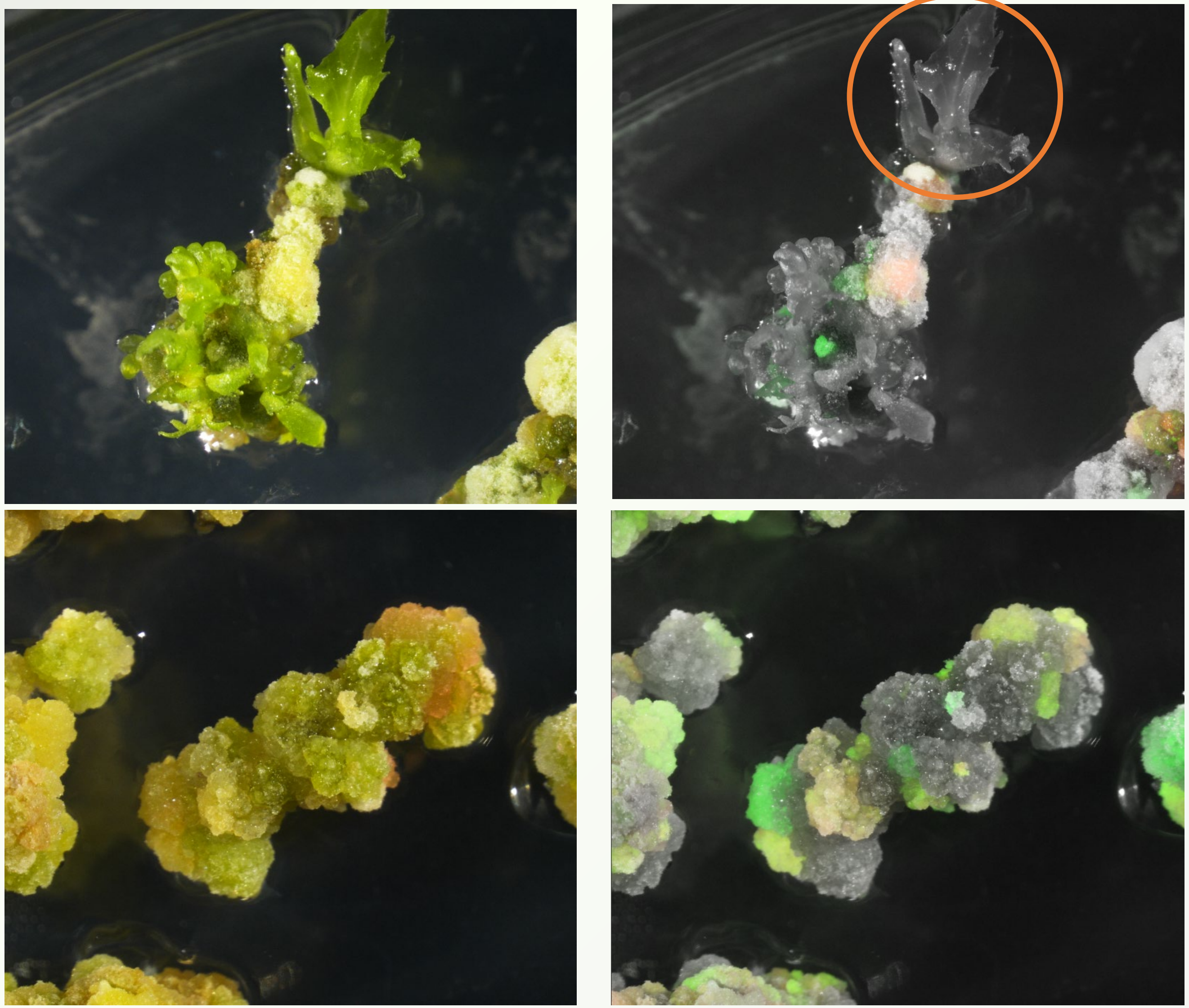


QuadLox construct showed impressive excision rates

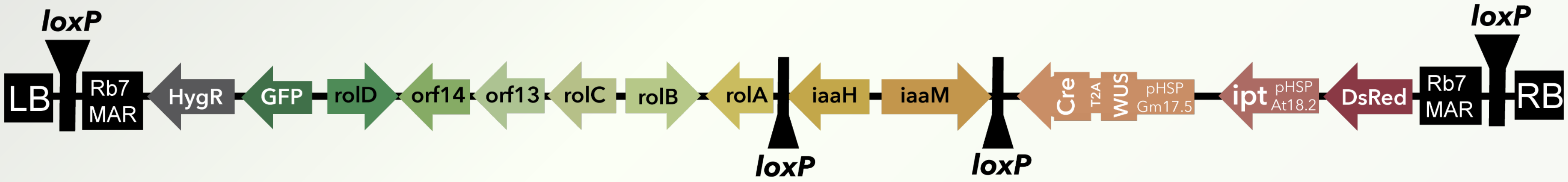
Brightfield | GFP and DsRed

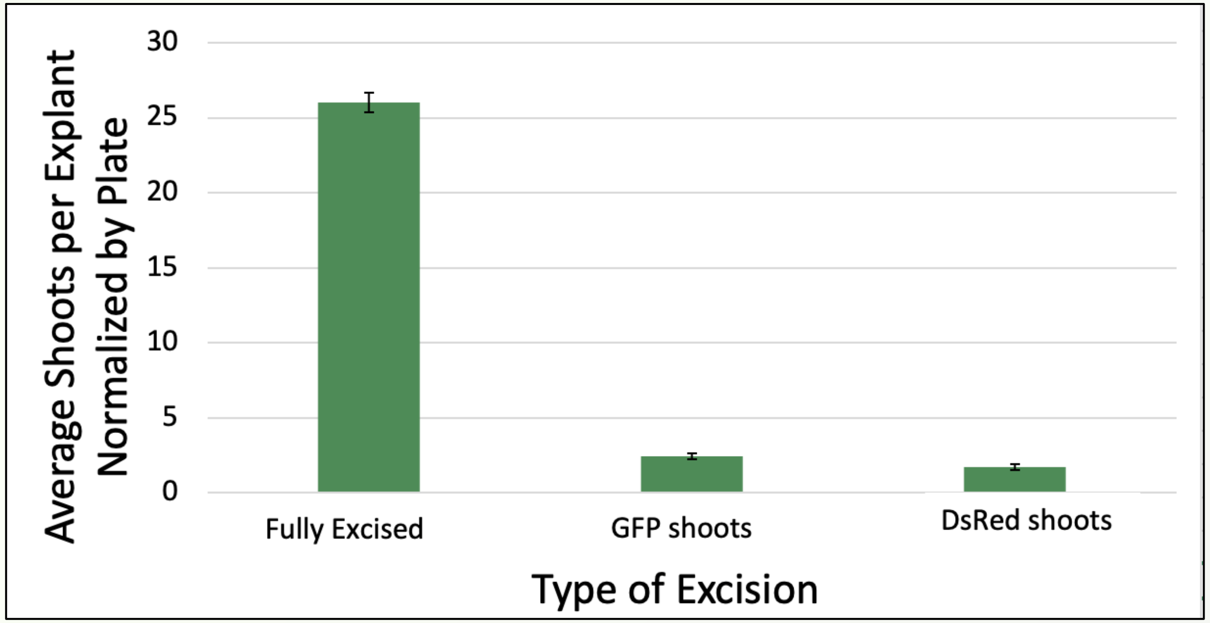
“QuadLox”

“Double lox”

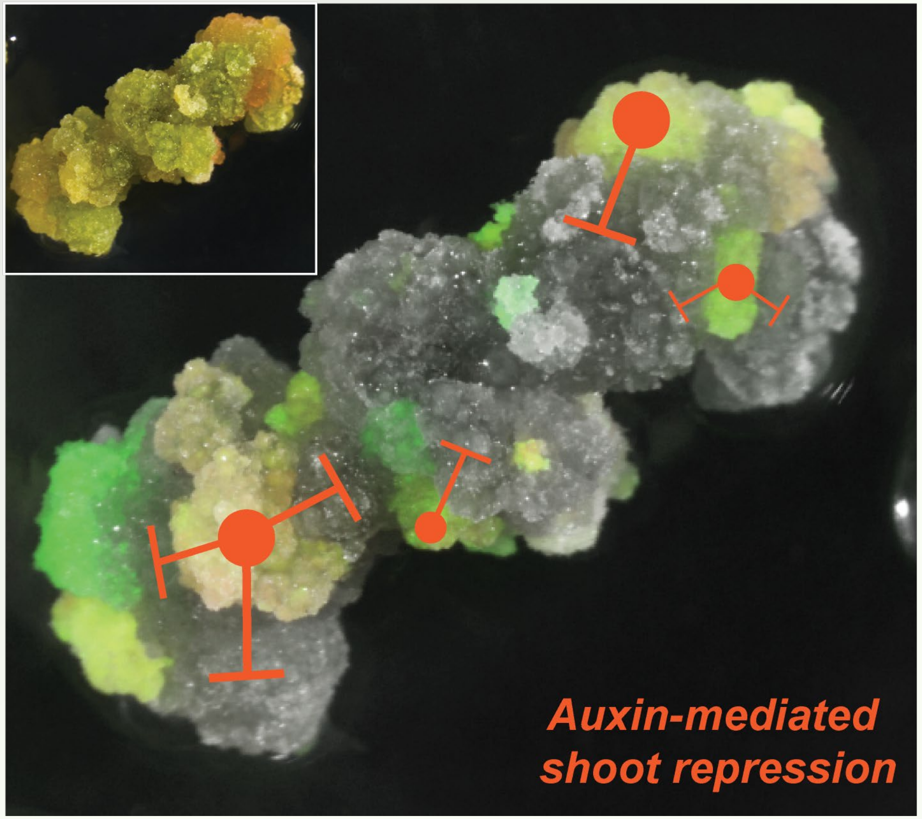


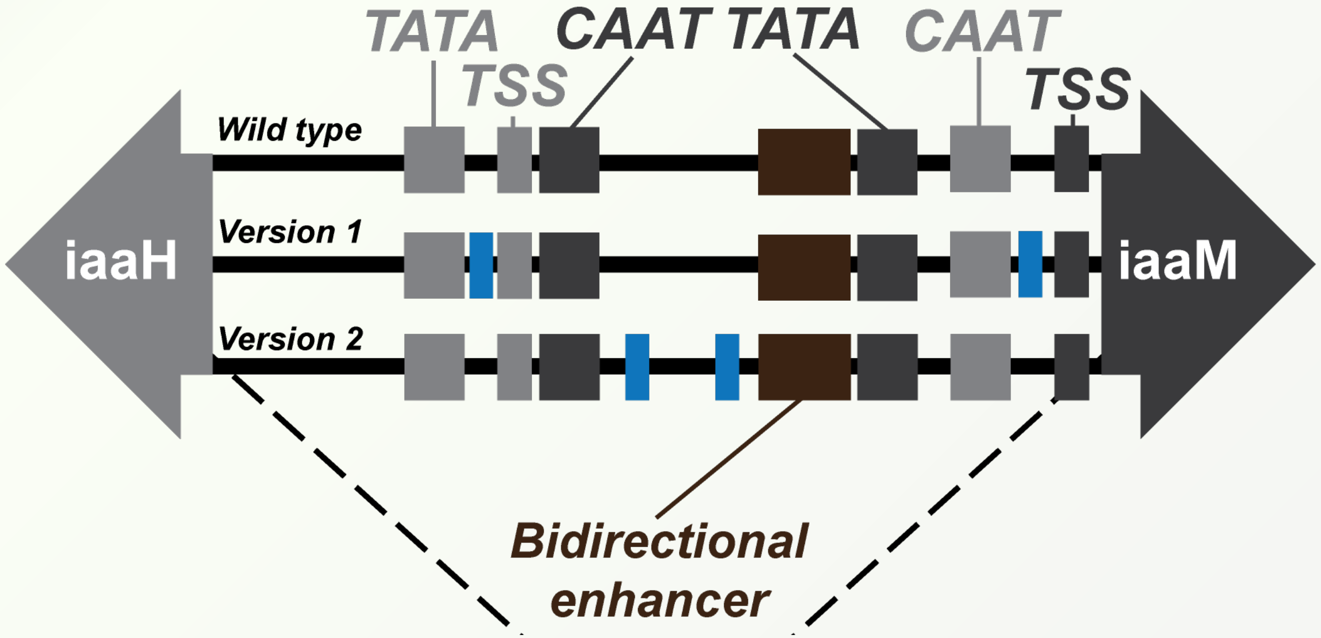
Final excision has the potential to remove Cre too early
- but found low rate of partially excised tissues with that outcome





We are working on means to turn off iaaH/M activity after hairy root culture, to aid shoot regeneration and reduce callus size





Using bacterial transcription factors and incorporated cis elements for light-inducible repression of bidirectional iaaH/M promoter


PLANT SCIENCE

Synthetic genetic circuits as a means of reprogramming plant roots

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We are also working on alternative morphogens such as gene 6b isolated from a "shooty" strain of Agrobacterium

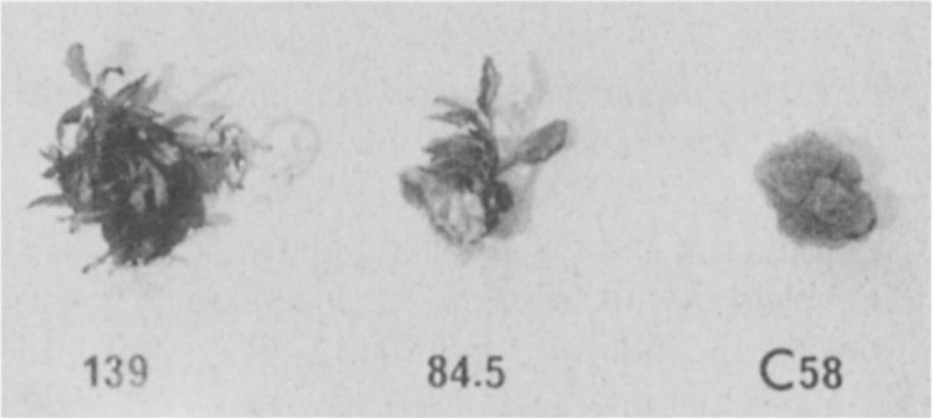

Fig. 1. Tumors and shoot differentiation from poplar tumors induced by *A. tumefaciens* strains 82.139, 84.5 and C58 and cultivated on MS medium, 6 weeks after inoculation.

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Co-transformation using T-DNA genes from *Agrobacterium* strain 82.139 enhances regeneration of transgenic shoots in *Populus*

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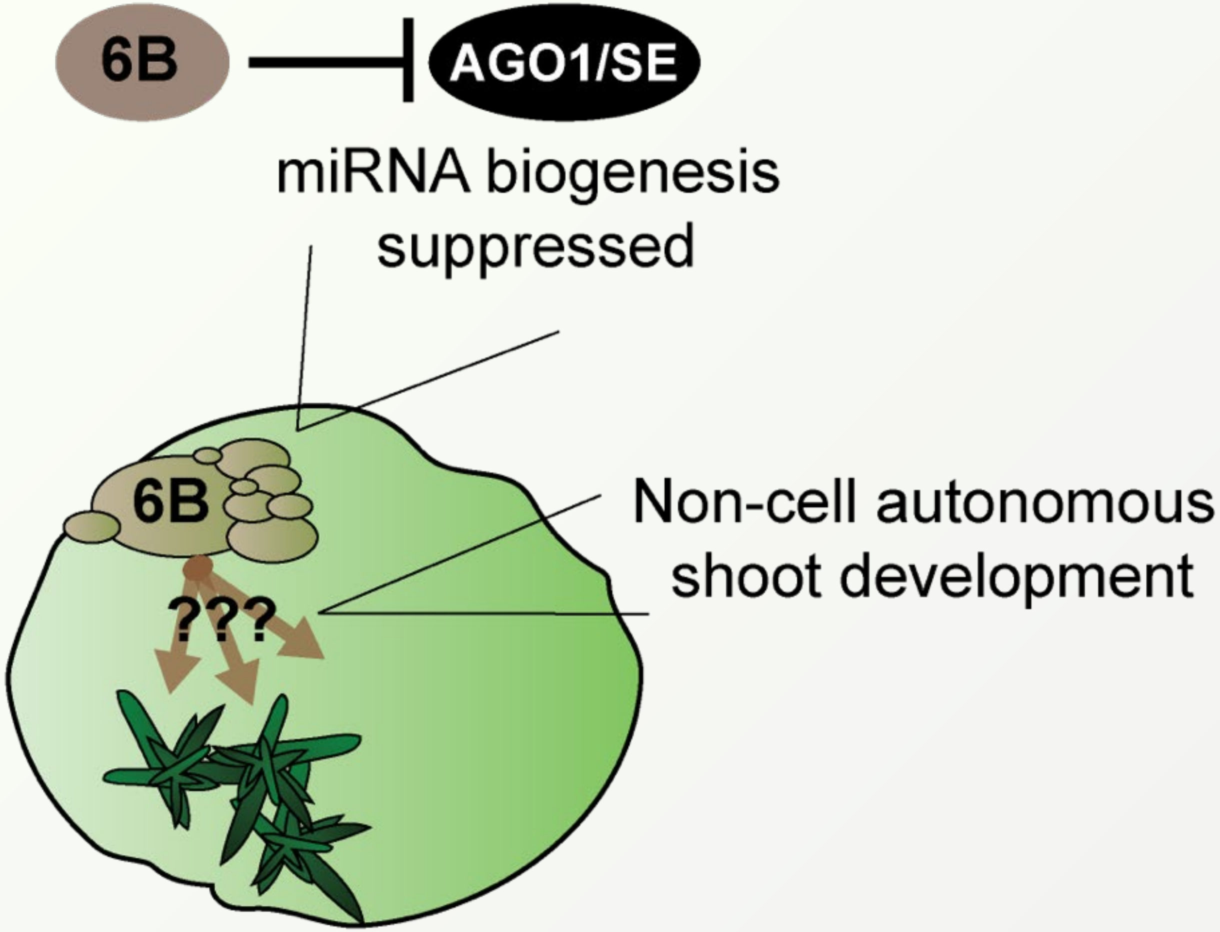
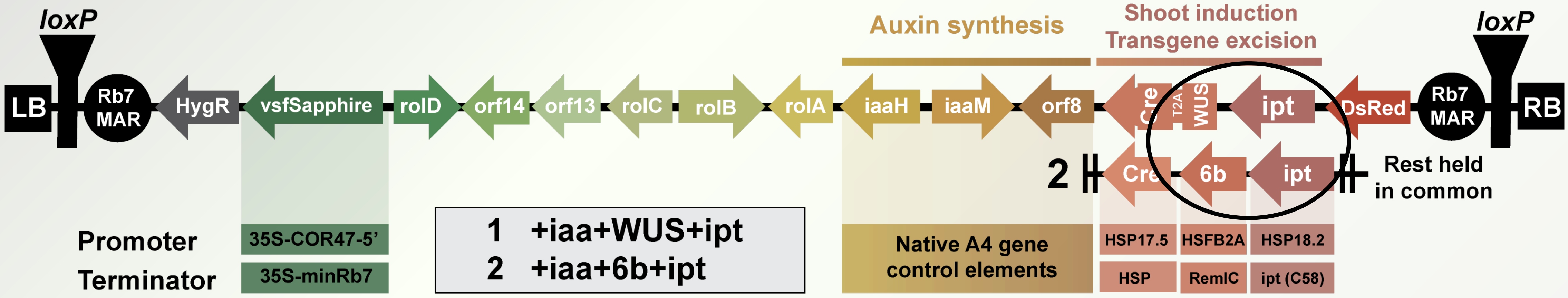
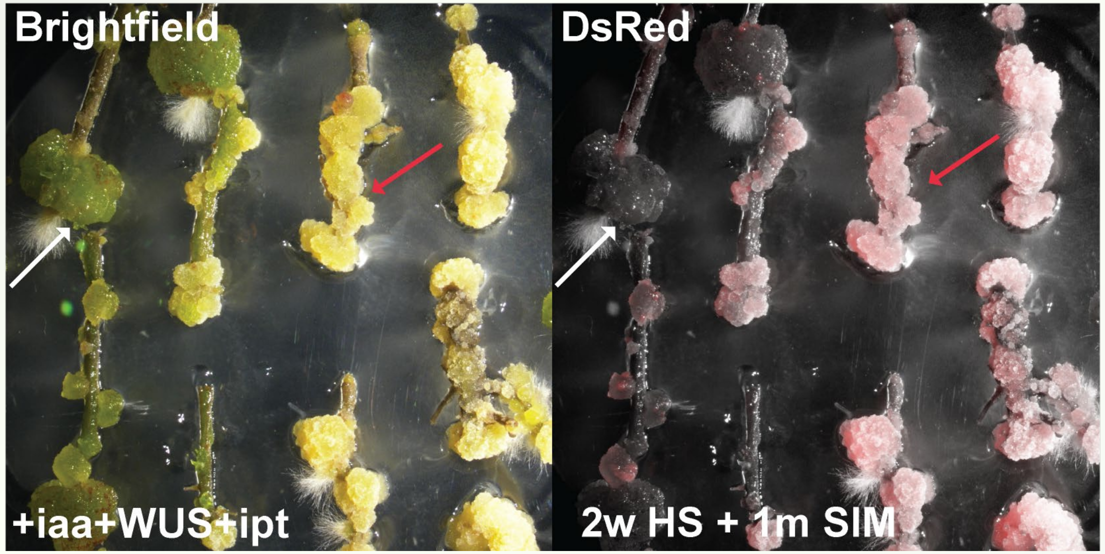
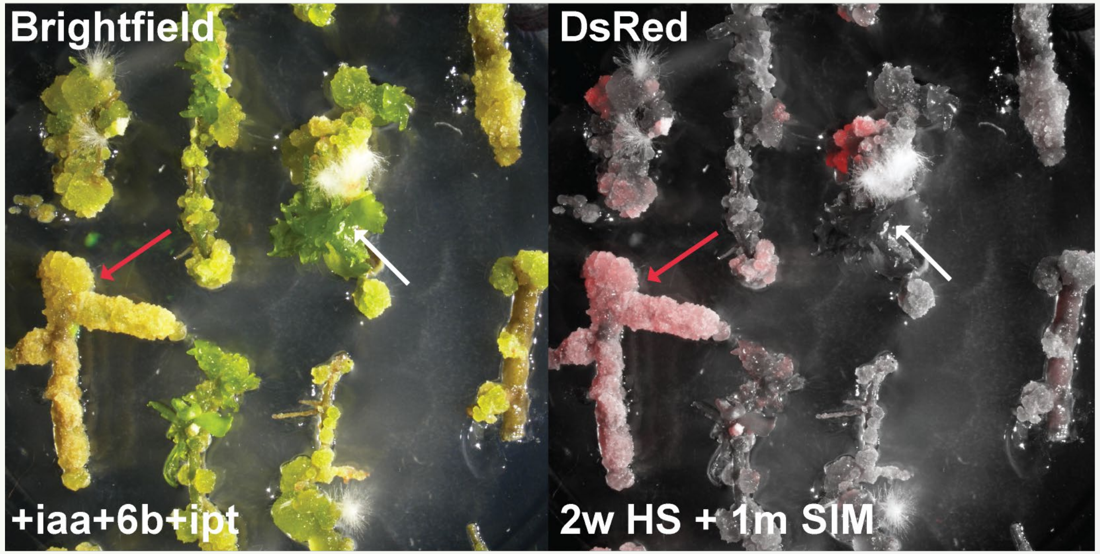


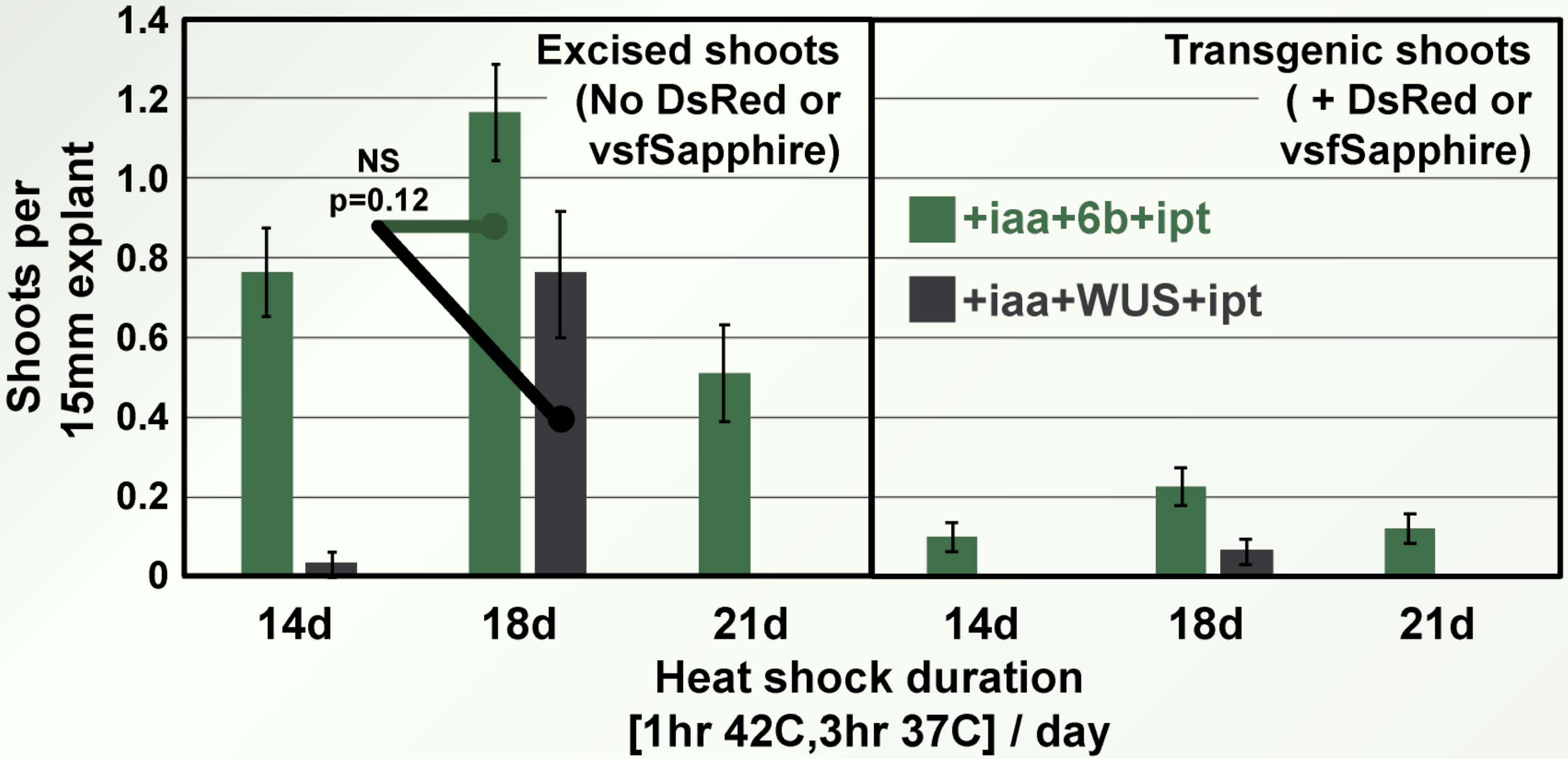
Gene 6b showed improved excision and shoot regeneration under some heat shock conditions vs. WUS







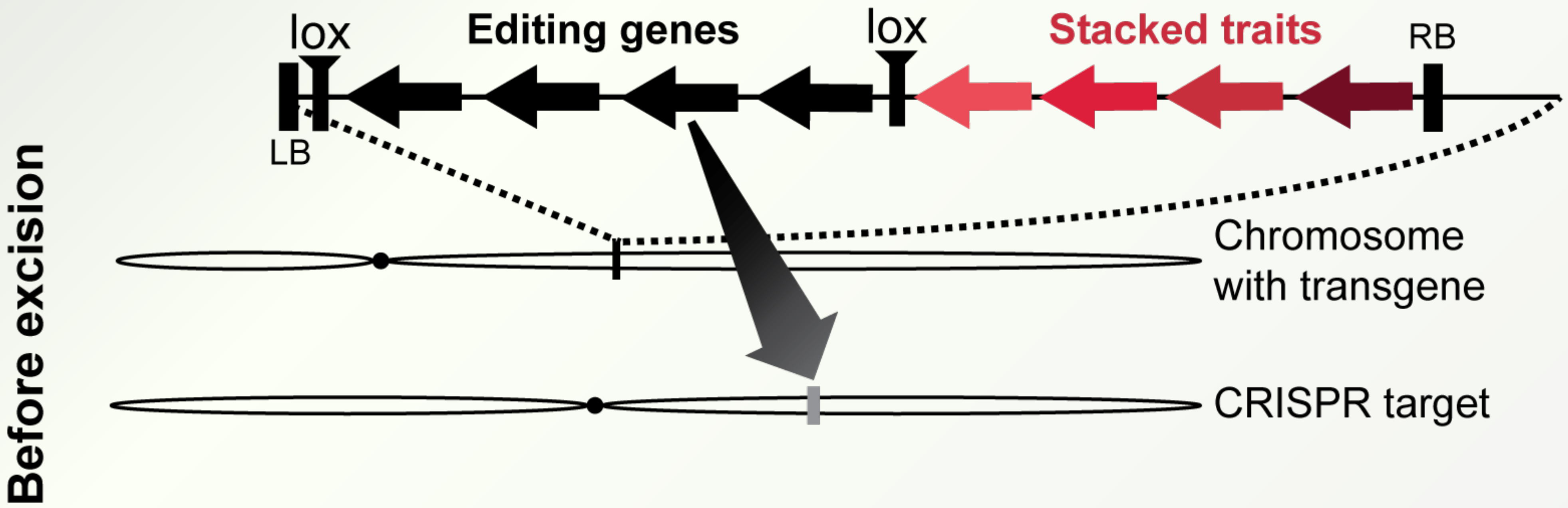
Gene 6b showed improved excision and shoot regeneration under most heat shock conditions vs. WUS



In summary: Hairy root-to-shoot methods are functional for editing and transformation in clonally propagated plants

- RESET system gave high rates of hairy root transformation
- High rates of editing seen in excised shoots
- Significant numbers of escape shoots, but easily eliminated through selection during hairy root subculture?
- We hope this system will function broadly in clonal woody plant species – testing ongoing

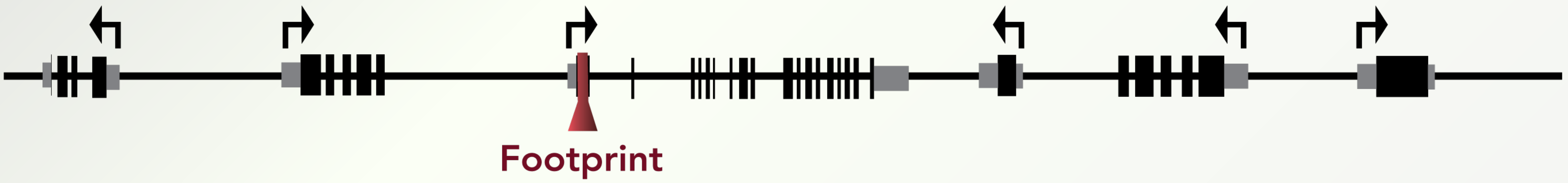
Enables multiple rounds of simultaneous editing and transgene insertion to a locus?



Questions remaining…

- How big can these T-DNAs be? Have used 60 kb to date…
- How will these large constructs affect transformation rate and integrity for recalcitrant genotypes?
- Can alternative *rol* genes, and related hormone genes, improve the rate of hairy root production?
- What morphogenic regulator genes, combinations, and expression control are best?
- Can we attenuate auxin synthesis and stimulate higher rates of shoot induction from hairy roots?
- What are the prospects for excised plants to be considered “clean” from a regulatory viewpoint? What does that mean?

Could these one day be considered clean edits for regulatory purposes? Are surrounding genes impacted?



- <u>Expression and epigenetics</u>: Analysis of surrounding genes
- <u>Chimerism</u>: ddPCR to detect residual transgenic cells

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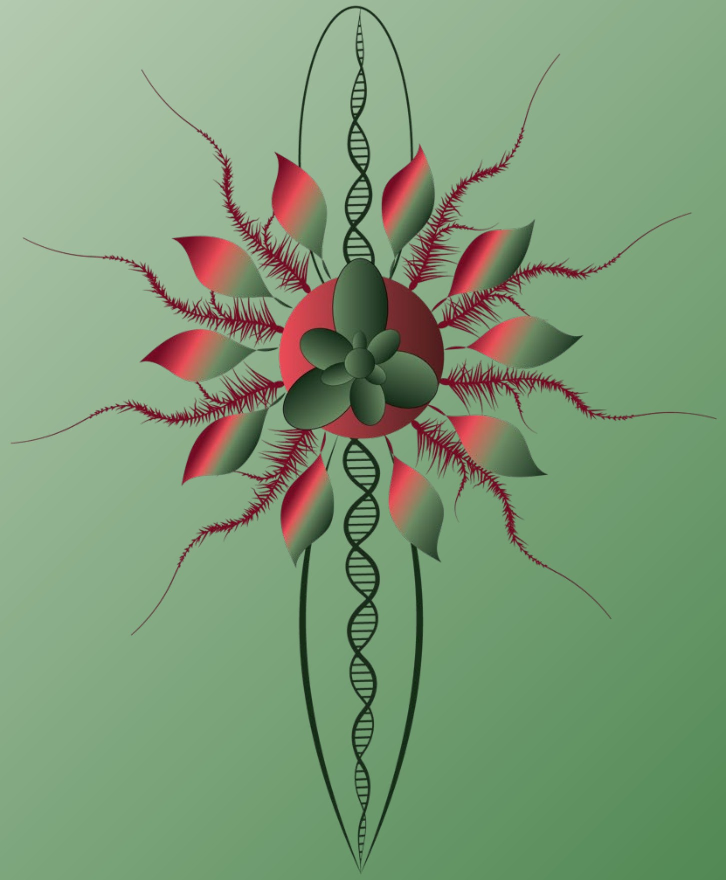

Thank you