

# RESET:

A root-to-shoot genetic circuit for transformation and editing in clonally propagated crops

Greg Goralogia

Laboratory of Steve Strauss

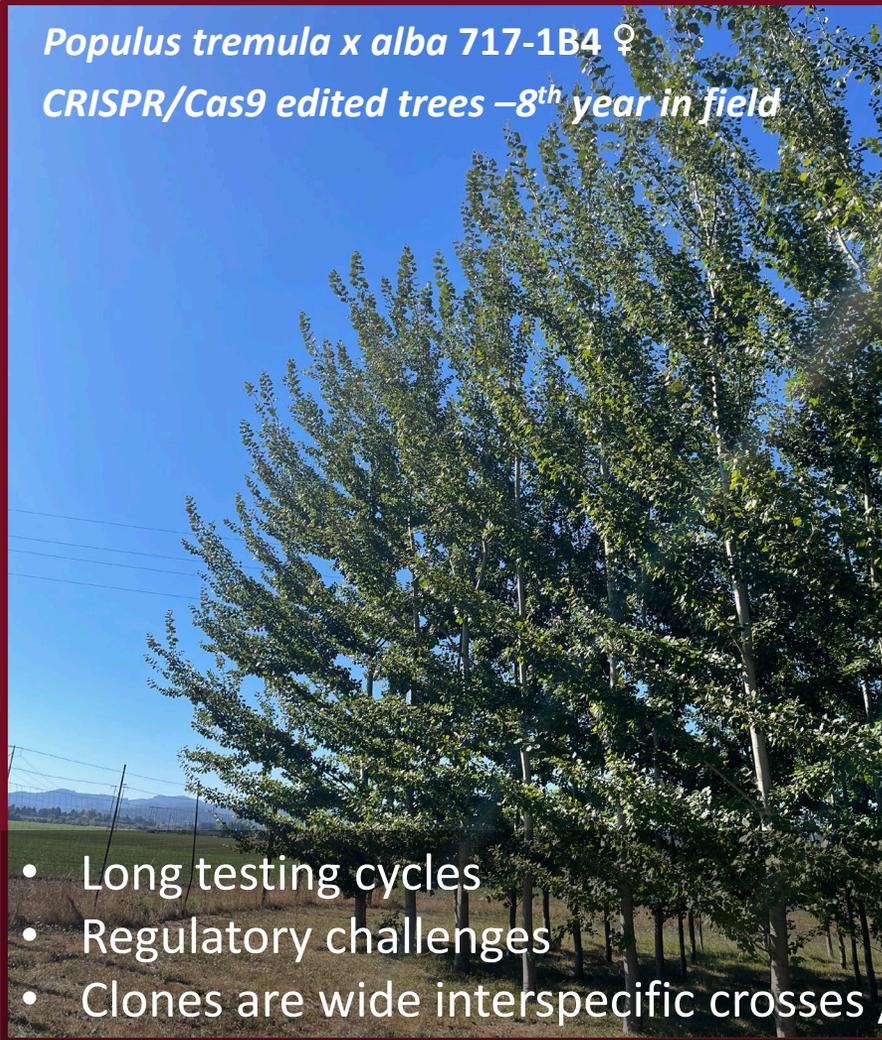
Oregon State University

College of Forestry

Forest Ecosystems and Society

# We work in tree species..... Many unique challenges for biotechnology!

*Populus tremula x alba* 717-1B4 ♀  
CRISPR/Cas9 edited trees –8<sup>th</sup> year in field

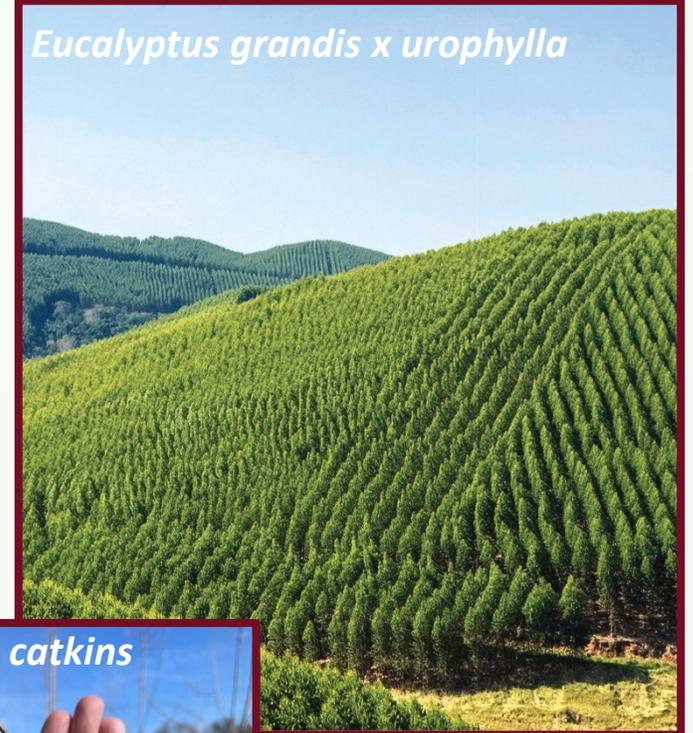


- Long testing cycles
- Regulatory challenges
- Clones are wide interspecific crosses / long breeding programs

*Populus tremula x tremuloides* ♂  
353-53 Feb. 2025



*Eucalyptus grandis x urophylla*

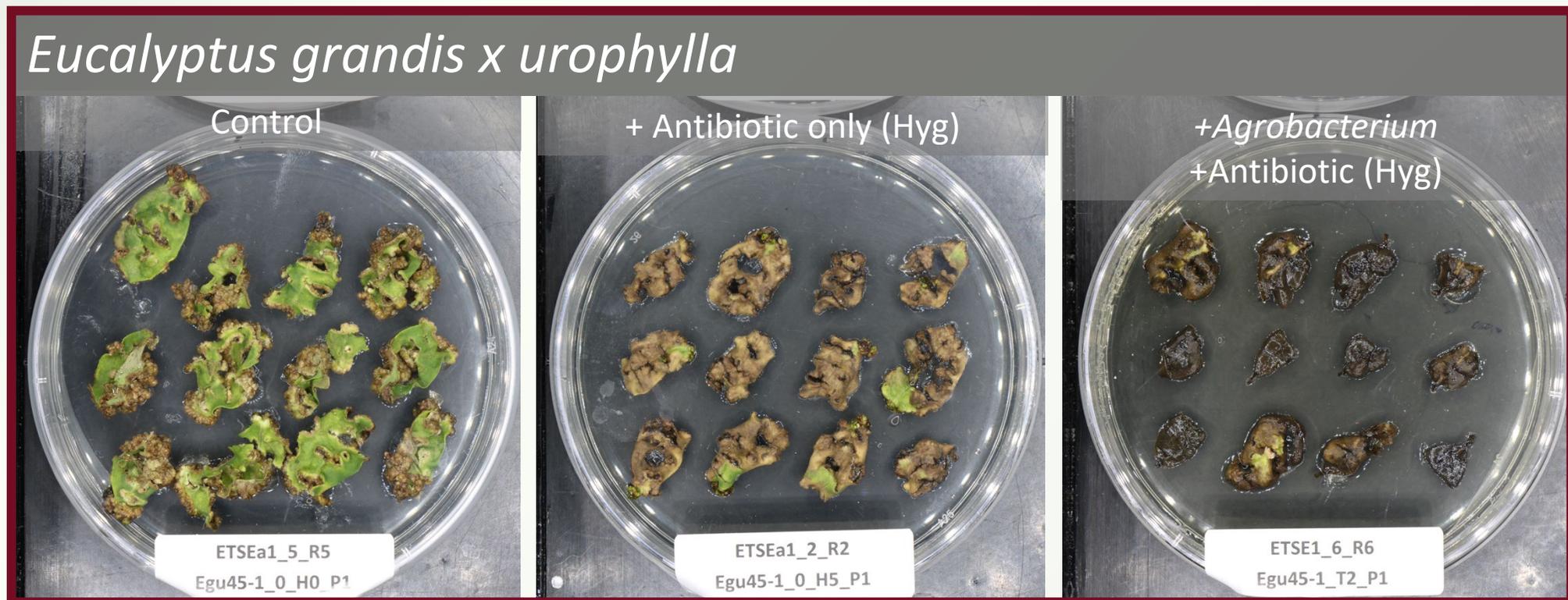


353 catkins

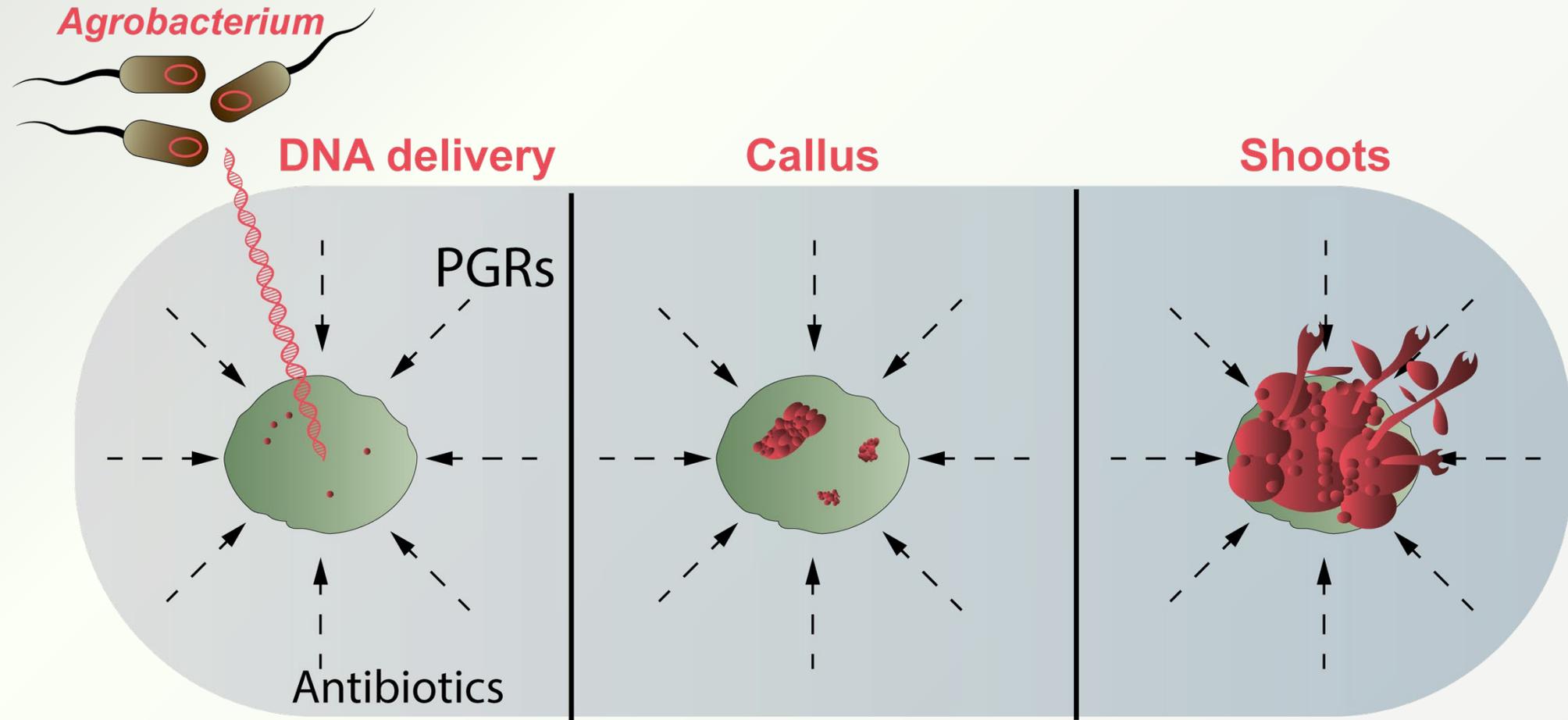


# Many tree species are difficult to transform

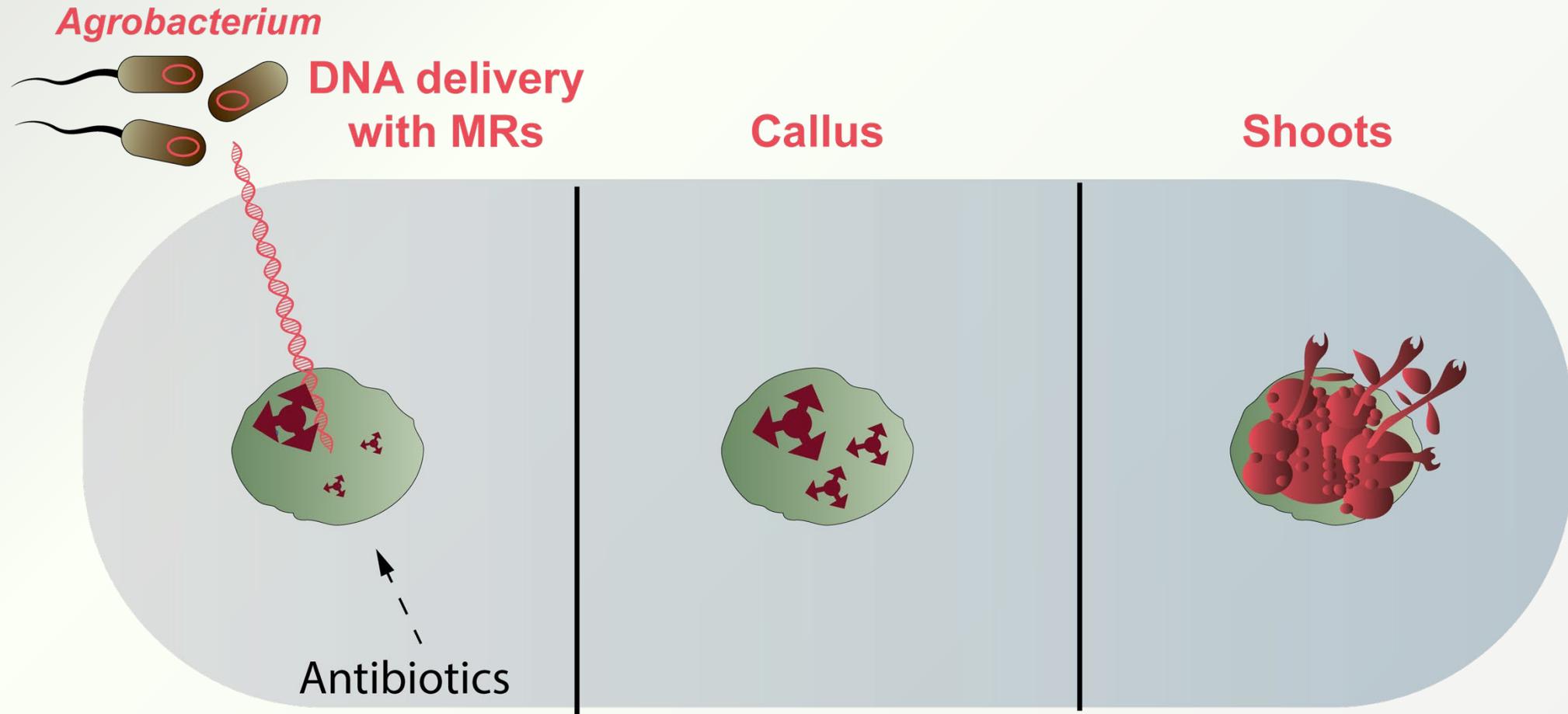
- Elite clones, not seed-derived
- High heterozygosity: each genotype a new adventure *in vitro*
- High physiological diversity and common defense responses



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



Morphogenic regulators 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 HOMEODOMAIN
- WUS – WUSCHEL
- GRF-GIF – GROWTH REGULATOR FACTOR 4 and GRF INTERACTING FACTOR 1
- *Agrobacterium* growth promoting genes
- rol – Hairy root-inducing genes – *Agrobacterium*

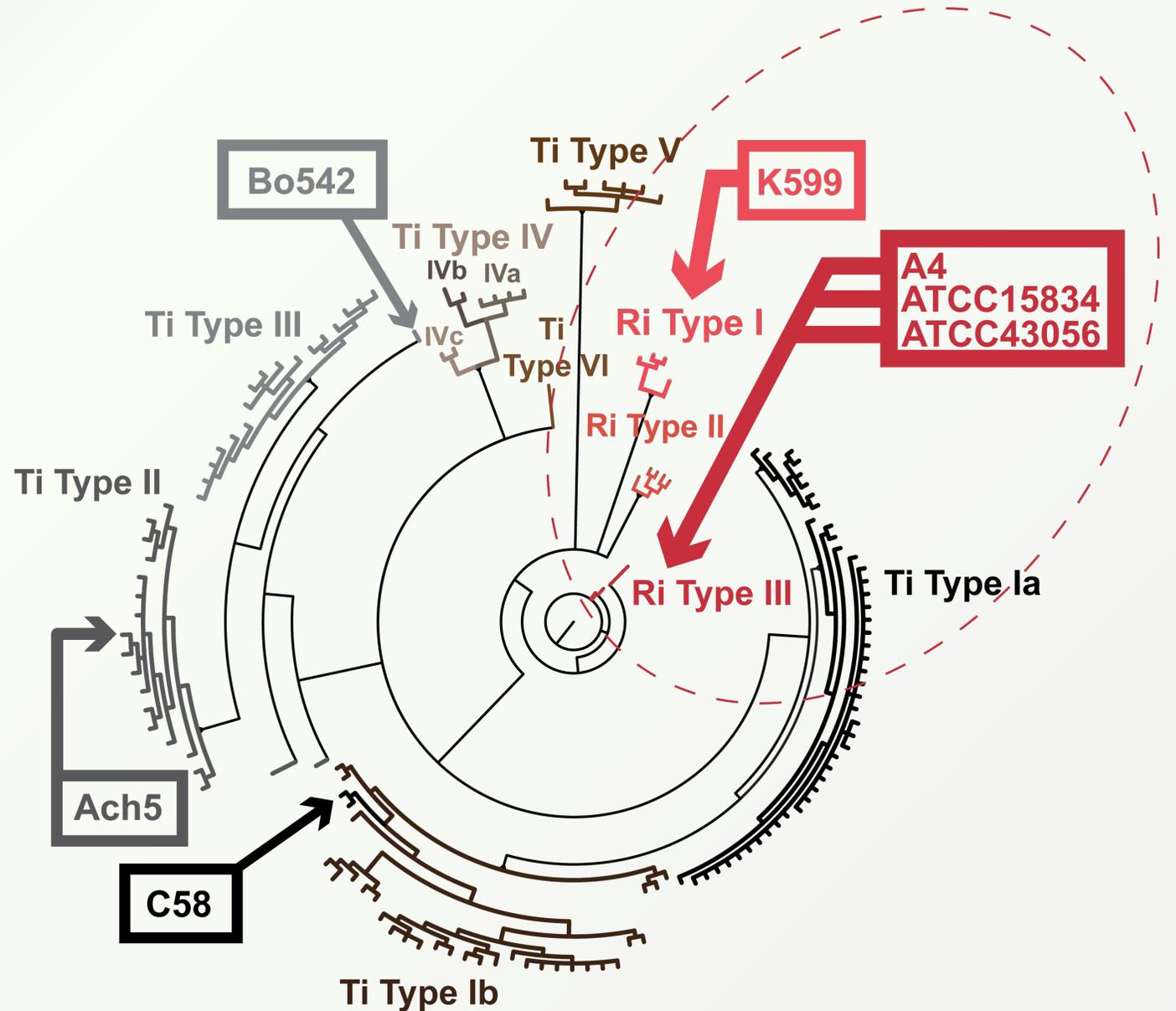
Most have failed with simple overexpression, or given highly genotype-specific enhancement or inhibition

- LEC 1, 2 – LEAFY COTYLEDON
- EBB1 - EARLY BUD BREAK 1 (ESR family)
- BBM – BABY BOOM
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- *Agrobacterium* growth promoting genes
- ***rol* – Hairy root-inducing genes – *Agrobacterium***

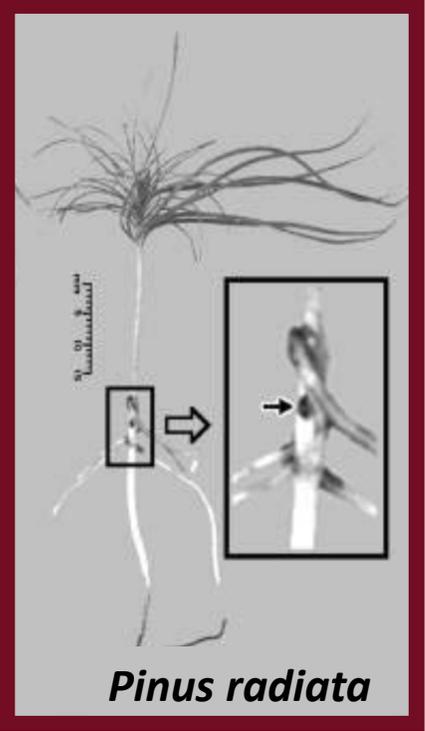
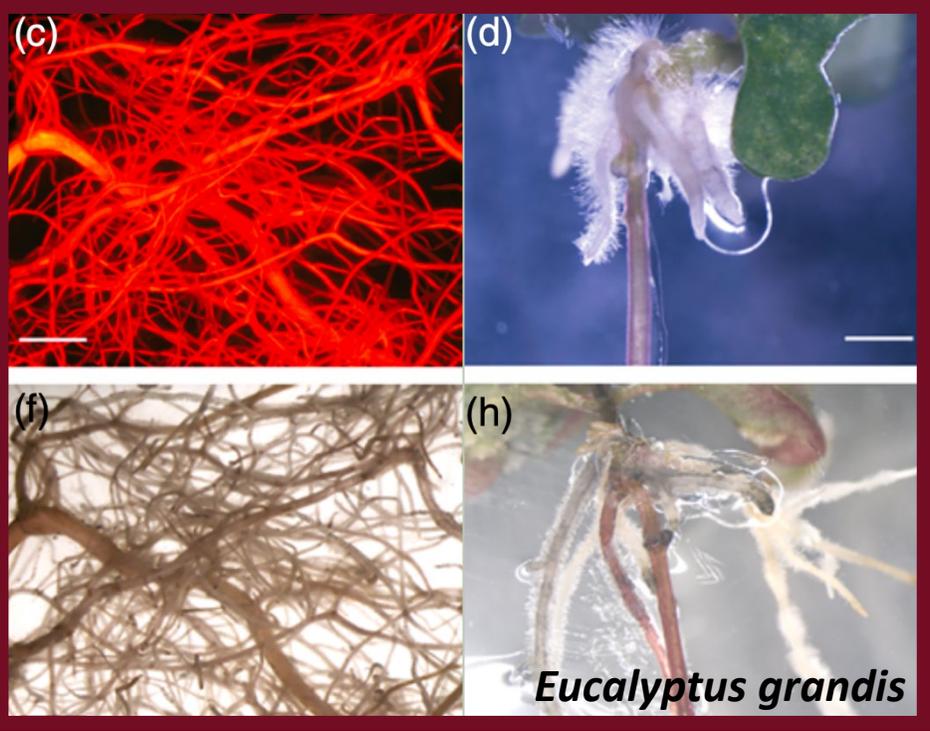
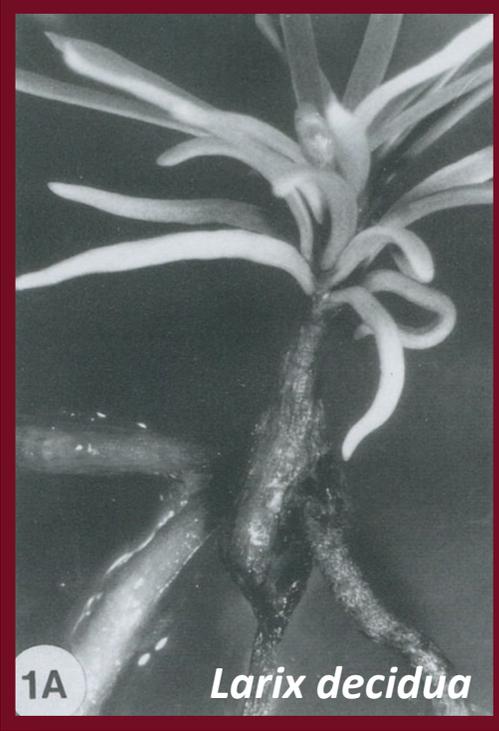
Hairy root disease is caused by unique T-DNA genes contained in Ri plasmids



Hairy roots in hydroponically grown tomato



# 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



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

doi: 10.1111/pbi.14096

## Brief Communication

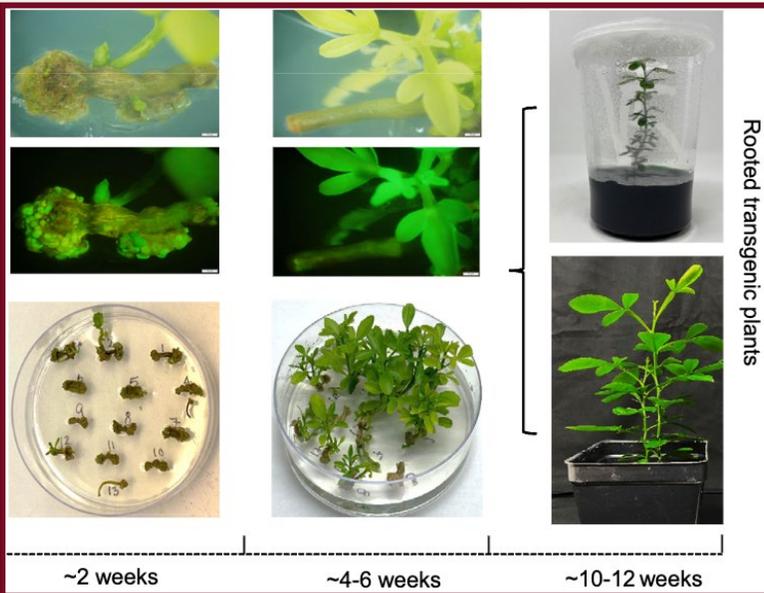
### *Rhizobium rhizogenes*-mediated hairy root induction and plant regeneration for bioengineering citrus

Manikandan Ramasamy<sup>1</sup>, Michelle M. Dominguez<sup>1</sup>, Sonia Irigoyen<sup>1</sup>, Carmen S. Padilla<sup>1</sup> and Kranthi K. Mandadi<sup>1,2,3,\*</sup>

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<sup>2</sup>Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX, USA

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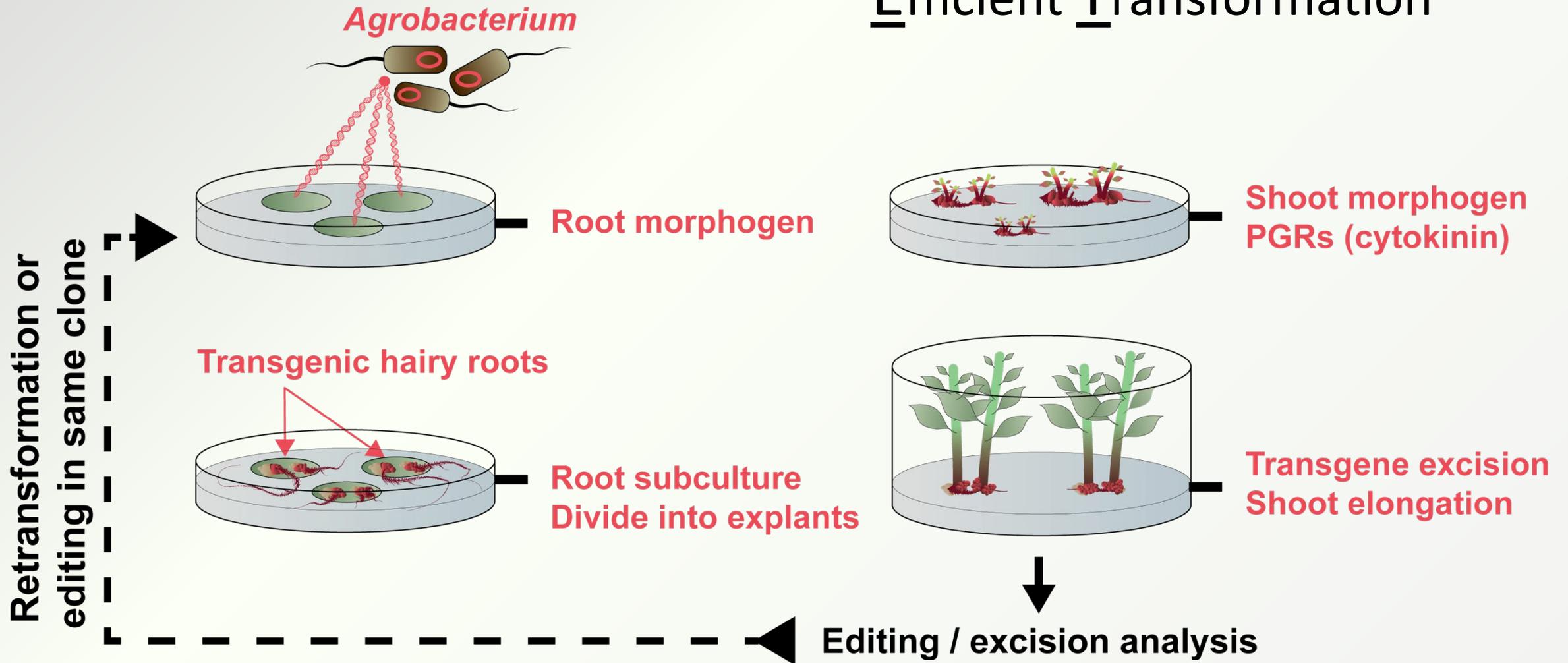


*Osteospermum fruticosum* (cape daisy)



*rol* transgenics often show dwarfism and changed floral timing and architecture

# A concept for Root Excision System for Efficient Transformation



# Root to shoot regeneration is a synthesis of older ideas

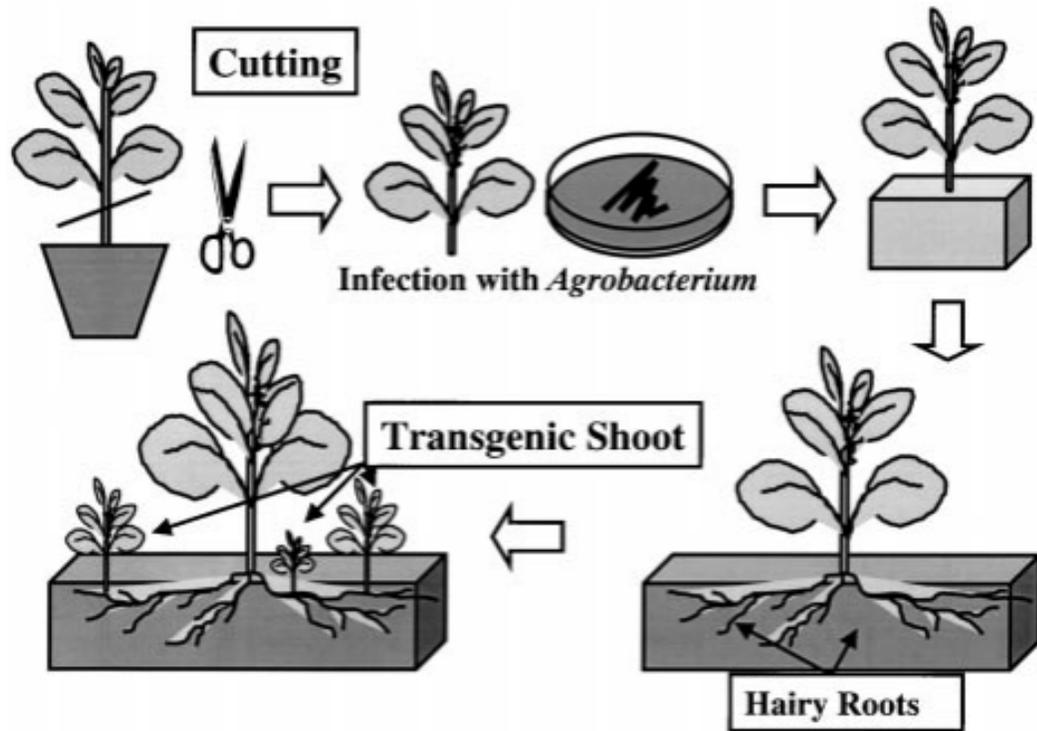
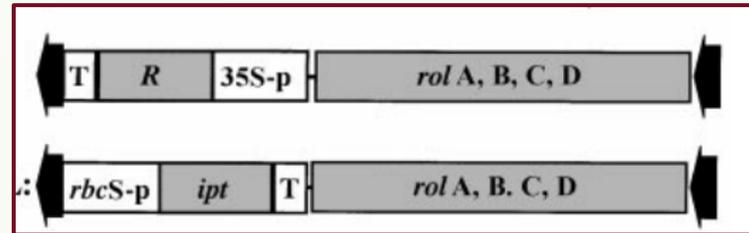


FIG. 8. Outline of *in vivo* transformation using cuttings. Cuttings are infected with *Agrobacterium* containing the *rol*-type MAT vector. After their rooting, marker-free transgenic shoots are induced from hairy roots by the light.



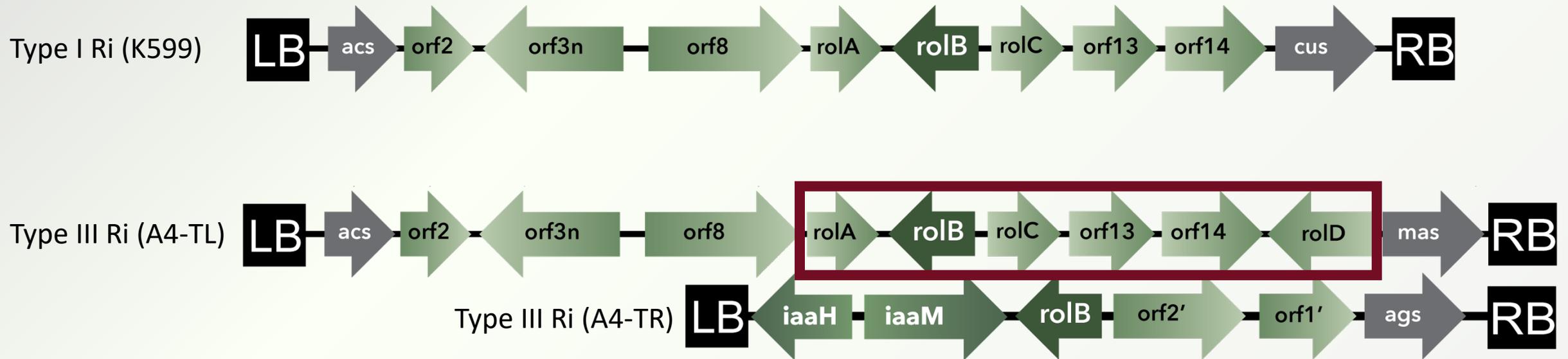
from Ebinuma and Komamine,  
2001 *In vitro cell and developmental biology -Plant*



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

# 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

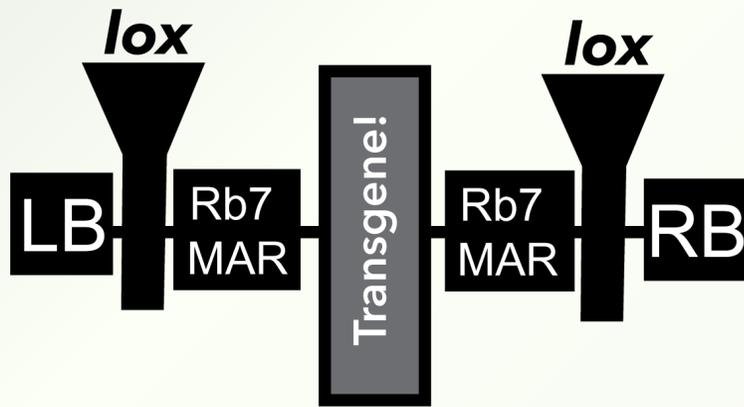


- *rolB* is required for hairy root formation
- Others including *rolA, B, C, D*, *orf13* and *orf14* quantitatively increase hairy root formation in many species

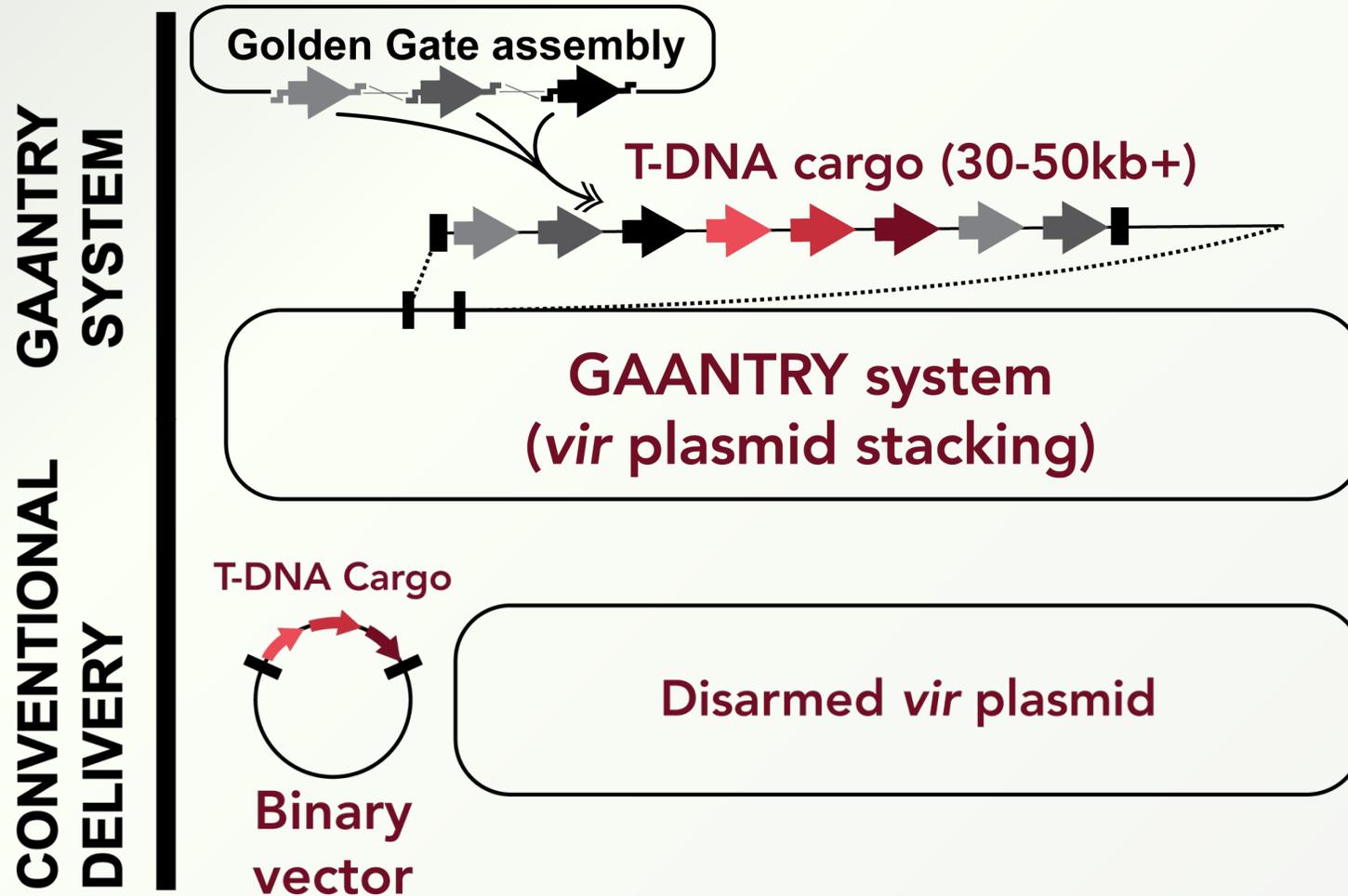
We selected **WUSCHEL** and **ipt** as shoot morphogens, **Cre-lox** for excision and chose heat shock as our induction system



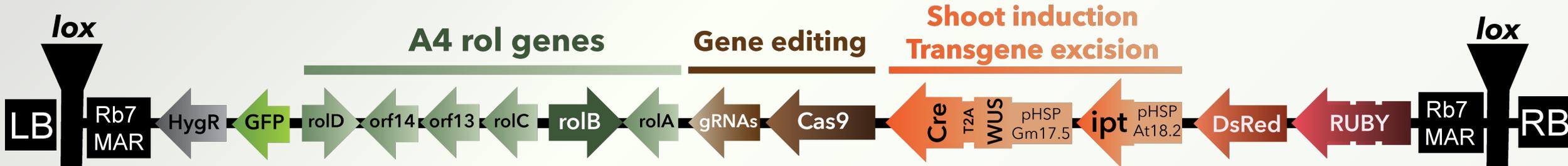
- *WUS* shown to induce shoot trans-differentiation from roots
- *ipt*, an *Agrobacterium* T-DNA gene which produces cytokinins and works well in our experimental system
- Heat shock induction is leaky, but we have tested all promoters in poplar
- Cre recombinase used to induce transgene excision, construct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



# Assembly would be next to impossible without GAANTRY

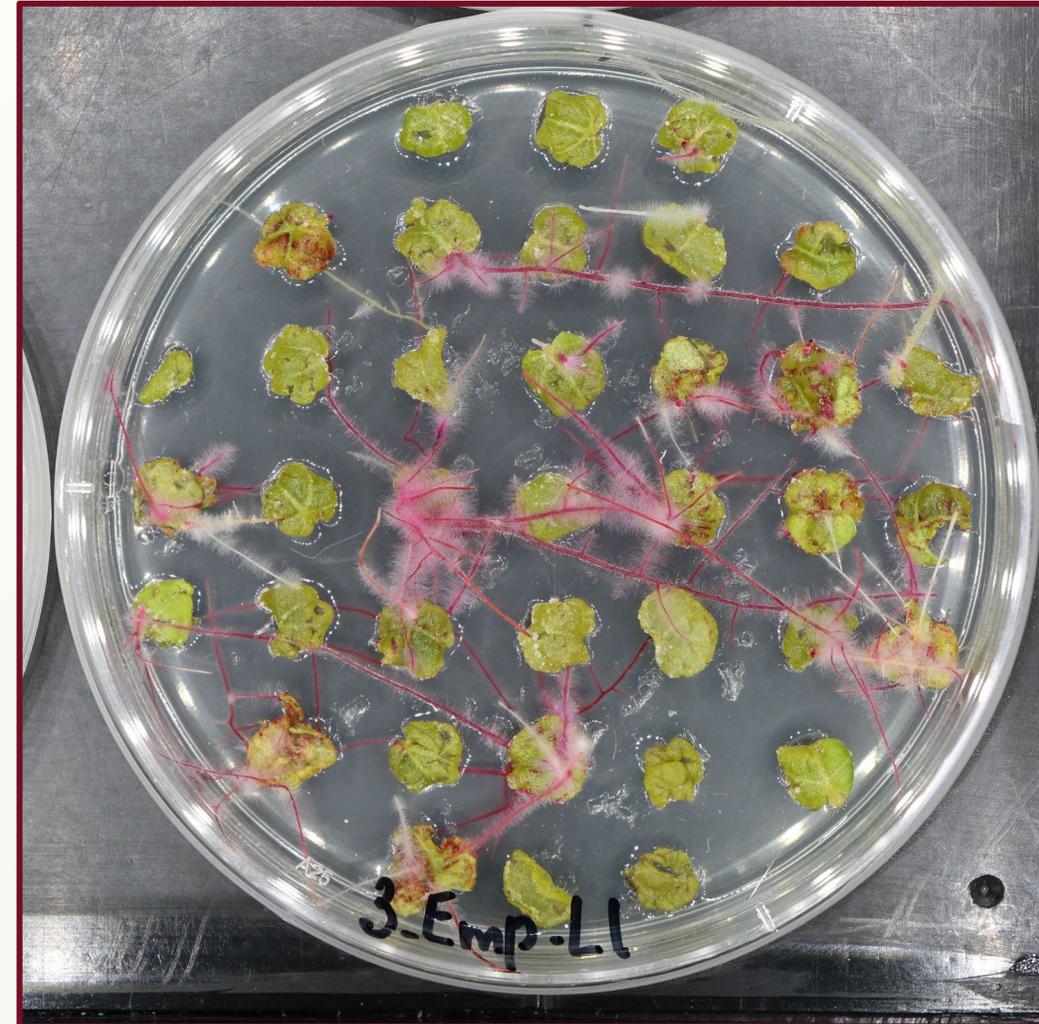
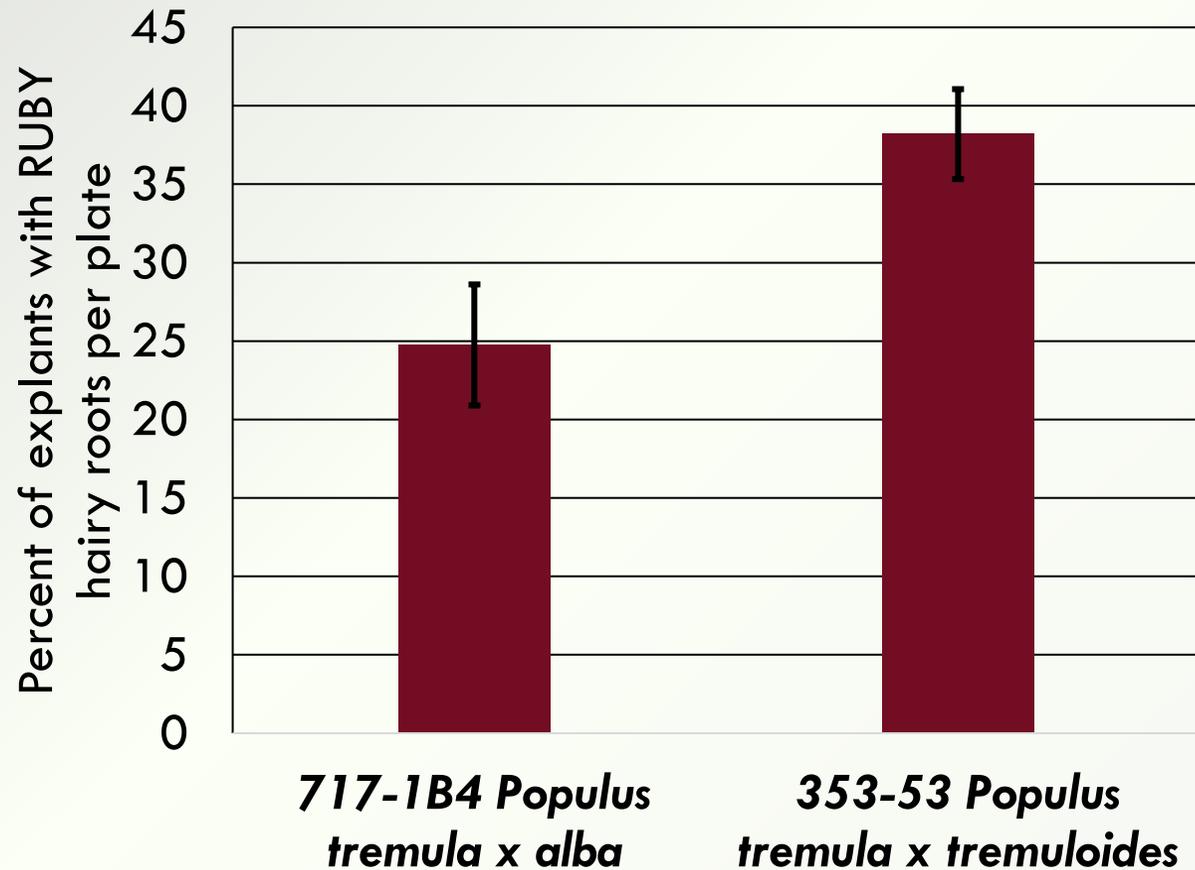


# The “Kitchen sink” RESET construct

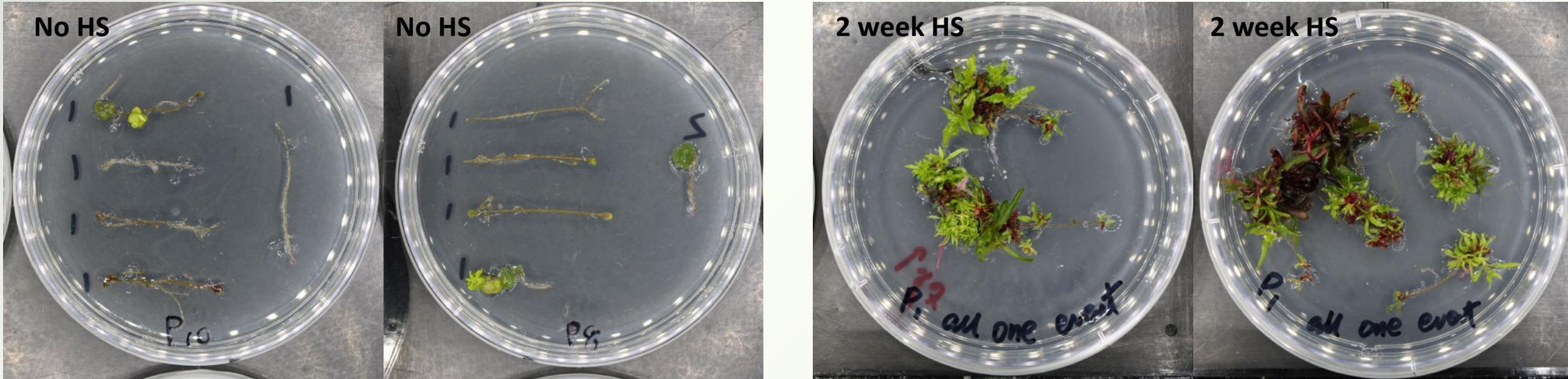


- 39kbp in size in this configuration
- 14 transcriptional units
- 16 independent peptides
- Included 3 marker genes to identify transgene insertion (GFP, DsRed, and RUBY)
- Includes gene editing through CRISPR-Cas9 (tRNA-arrays targeting *RGA1* gene)
- Hygromycin selection gene present but no selection was used in these experiments

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

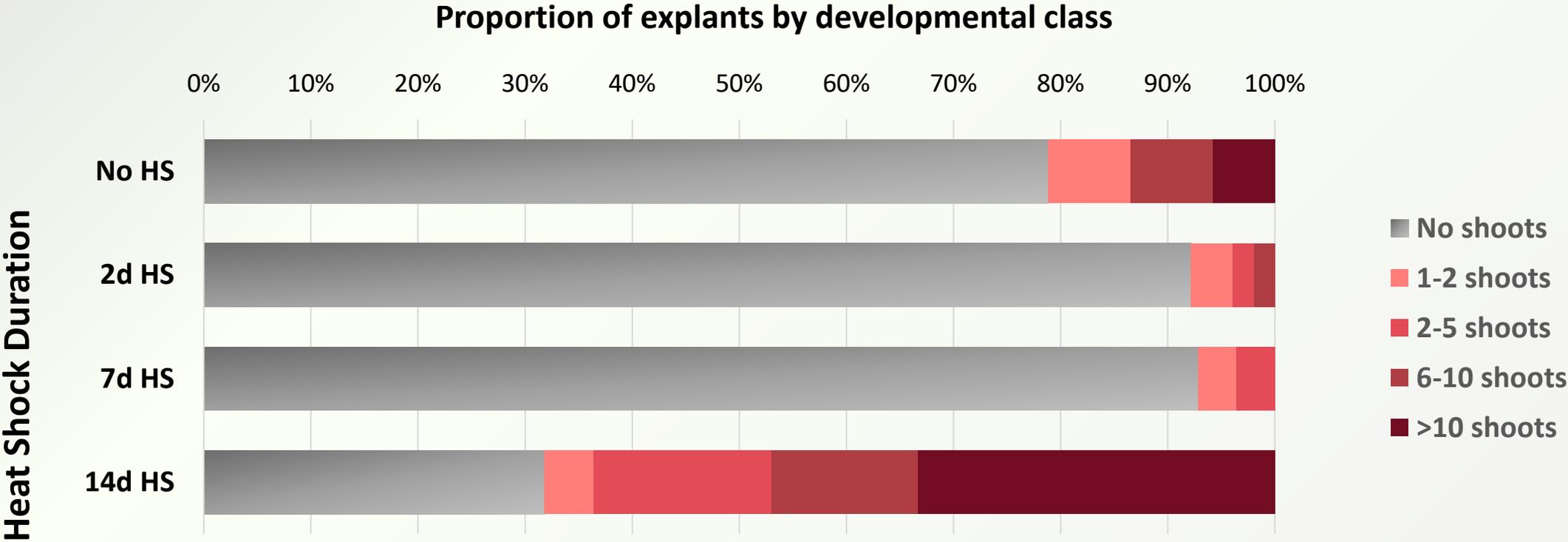


# Two weeks of pulsed heat shock resulted in efficient shoot regeneration and excision from hairy roots



- Four hours heat shock at 39 degrees Celsius, different treatment durations
- RUBY vs. non-ruby shoots can be easily identified for propagation, then other reporters can be closer 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

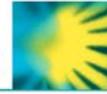


We tracked individual hairy root insertion events through the transformation and excision process

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

Full Paper

New  
Phytologist

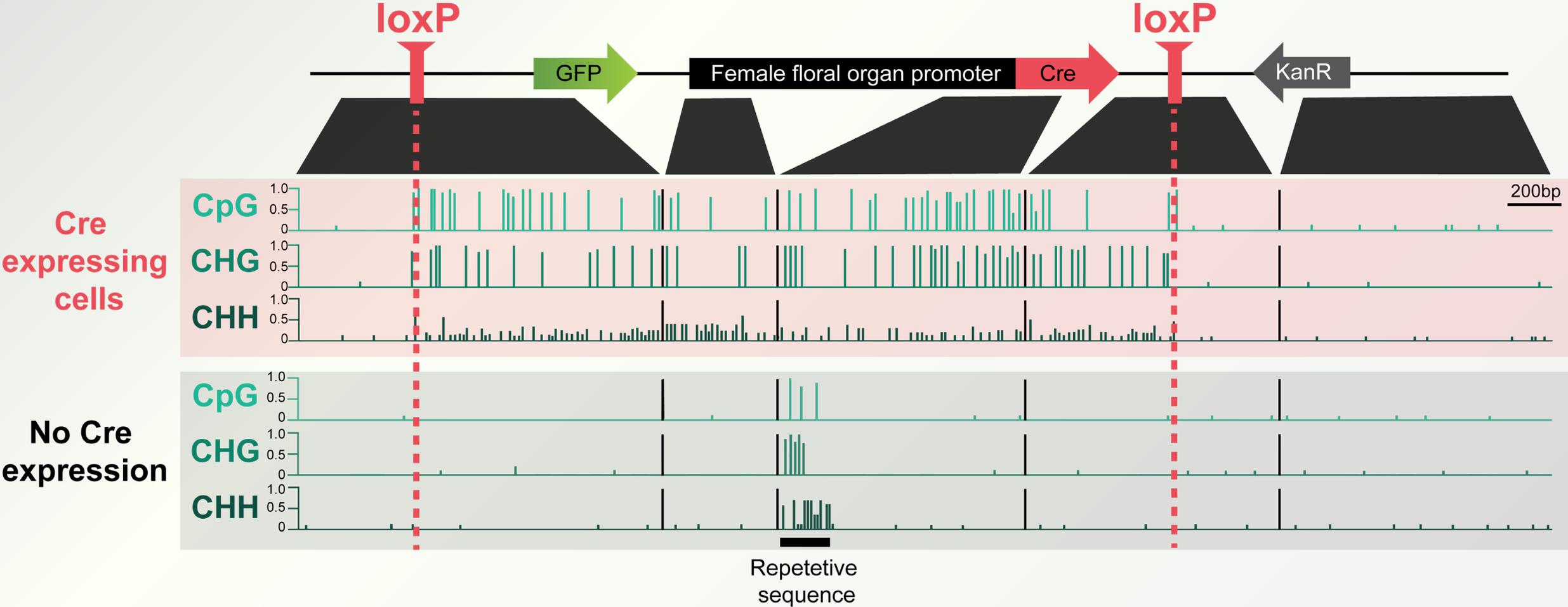


## DNA methylation occurring in Cre-expressing cells inhibits loxP recombination and silences loxP-sandwiched genes

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

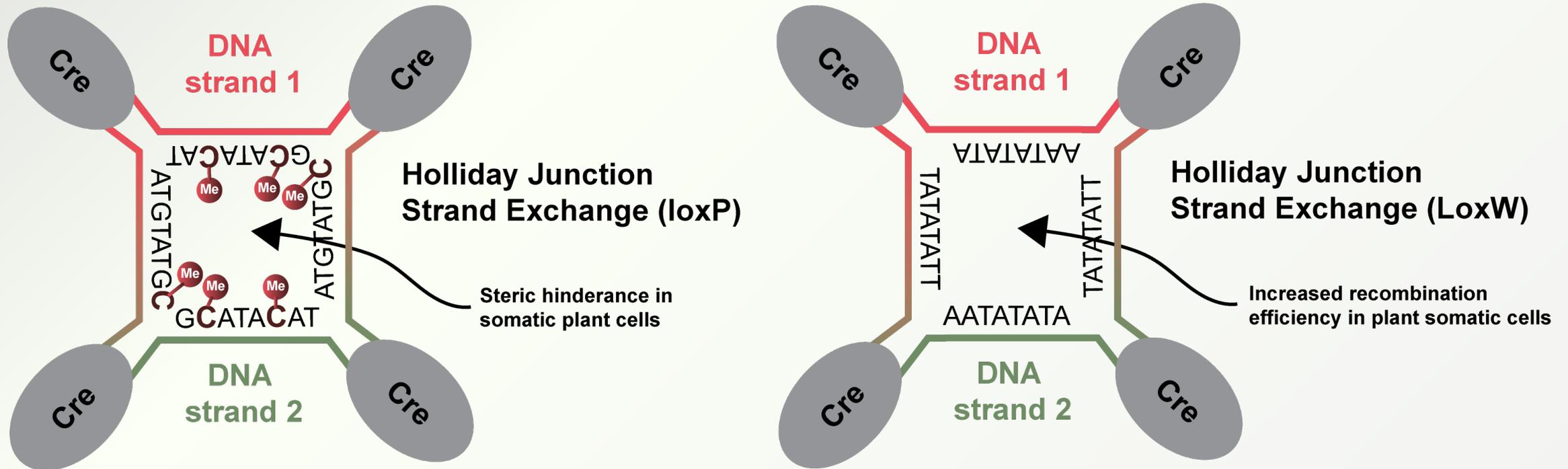
Chongqing Key Laboratory of Application and Safety Control of Genetically Modified Crops; Biotechnology Research Center, Southwest University, No. 2 Tiansheng Road Beibei, Chongqing 400715, China

# Cre can methylate transgene regions flanked by its recognition site loxP

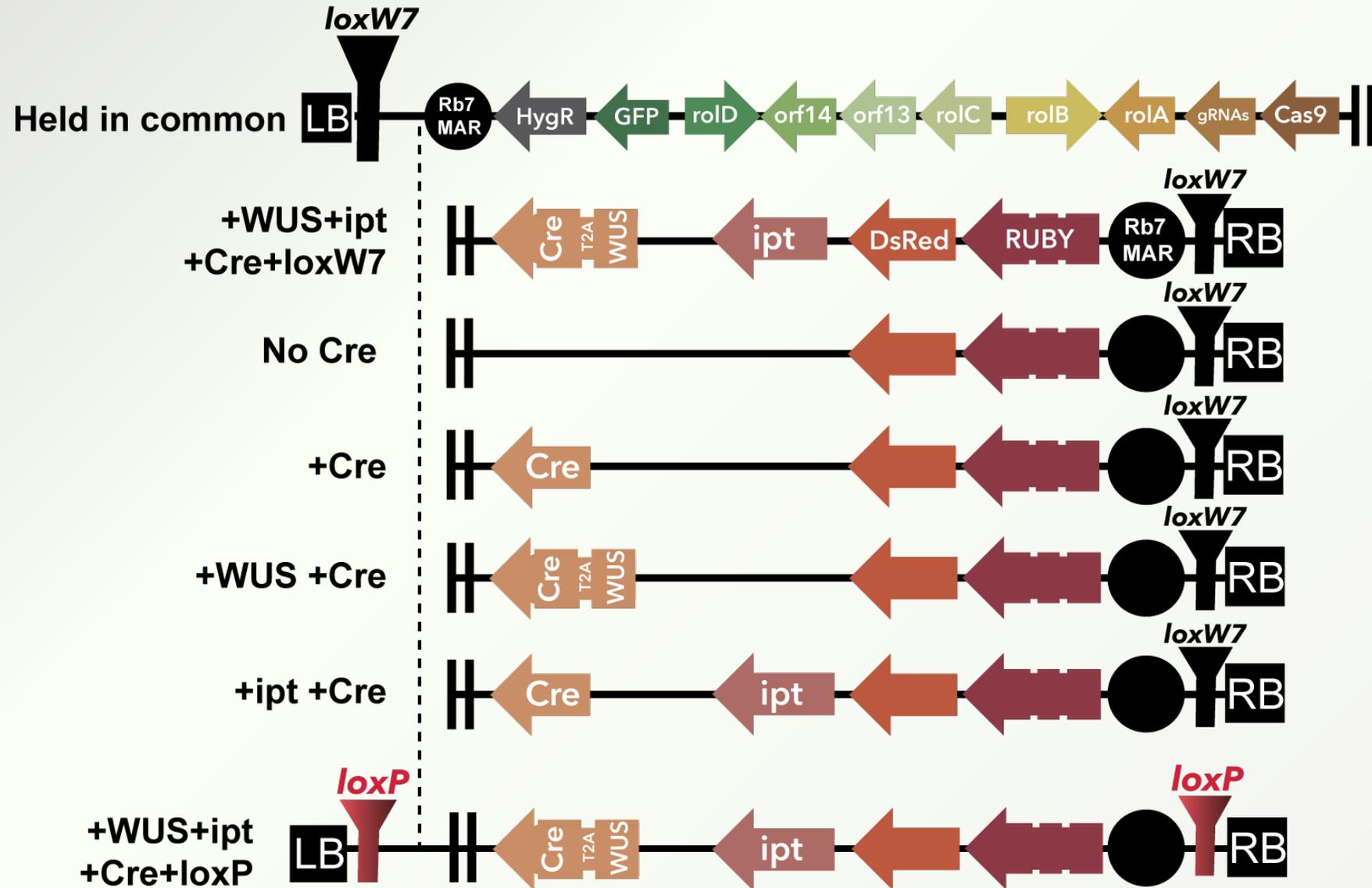


Modified from Liu et al. 2021 *New Phytologist*

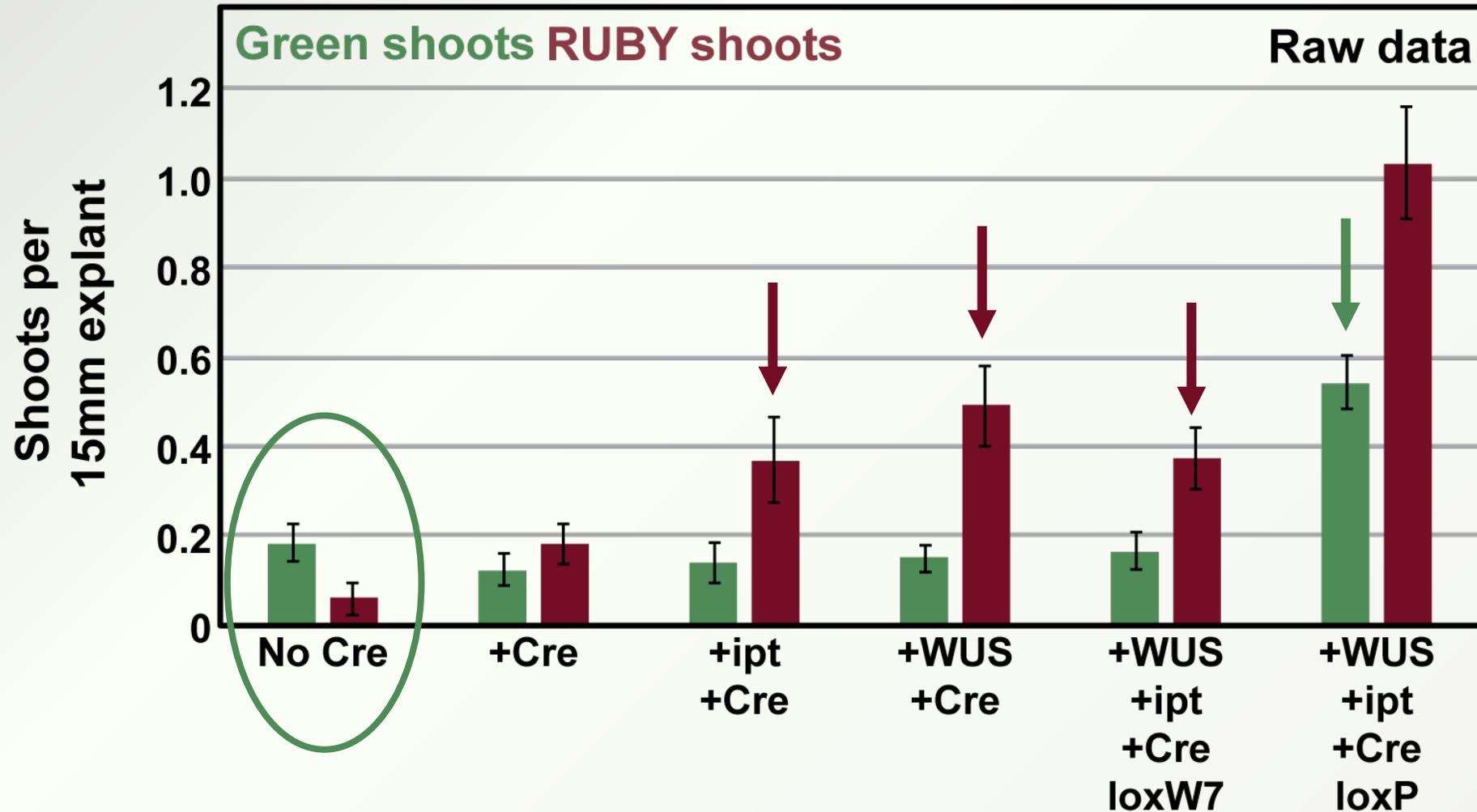
We made several variations of RESET constructs, including changes to flanking lox sites designed to be resistant to DNA methylation



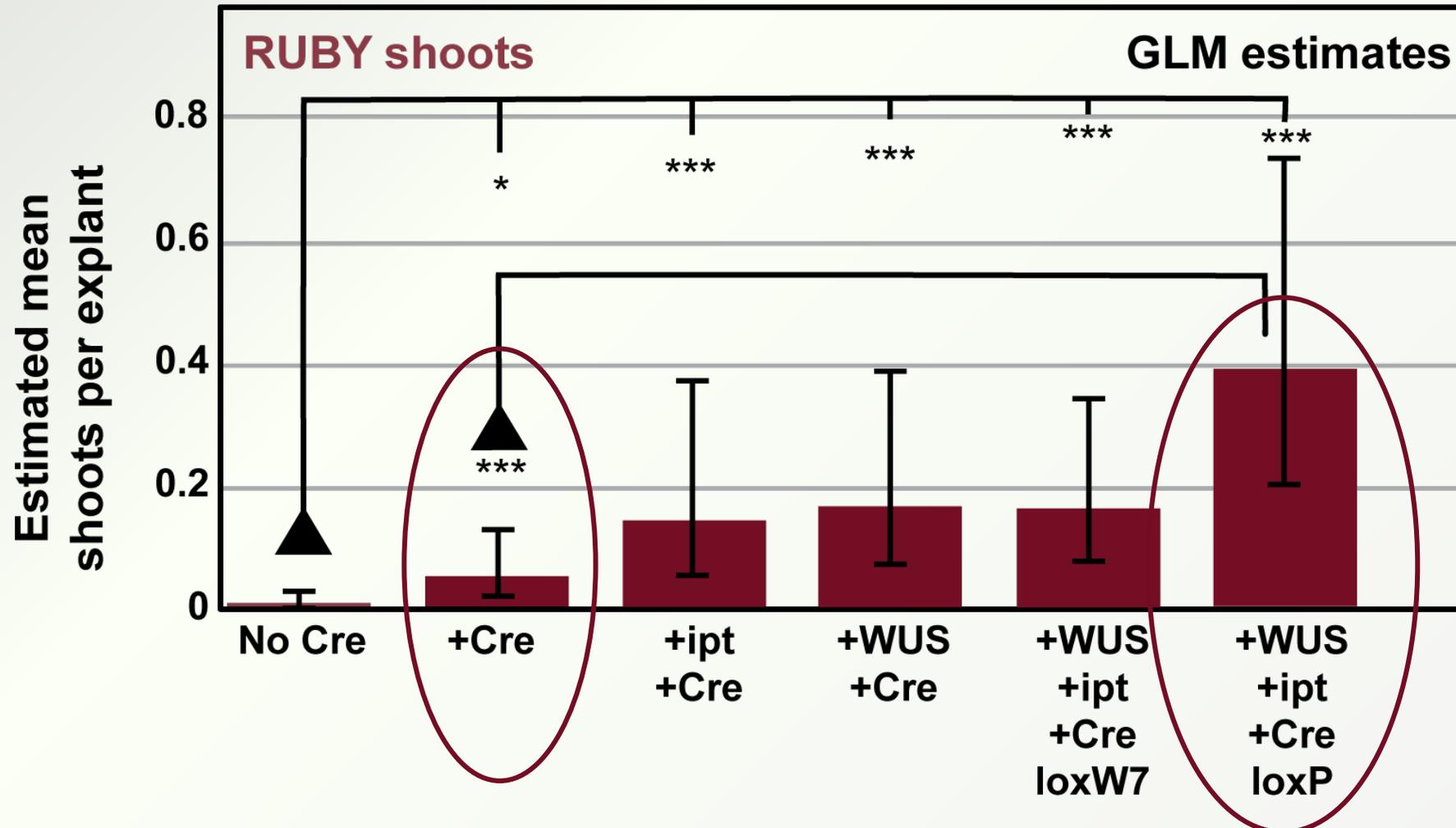
We also compared the presence of *WUS* and *ipt* morphogens



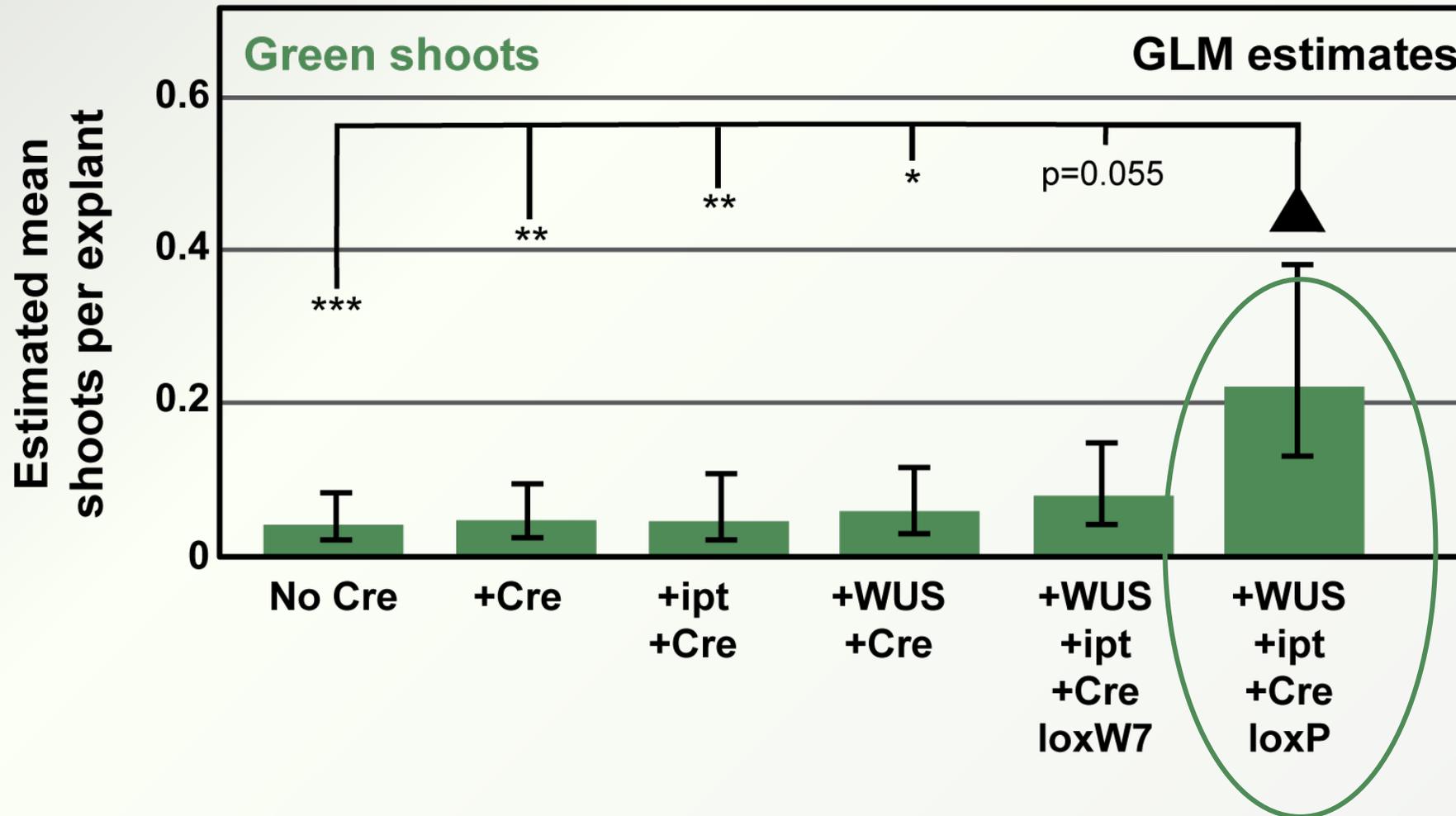
loxP-flanked RESET constructs gave the highest shoot regeneration after heat shock induction of transgenic roots



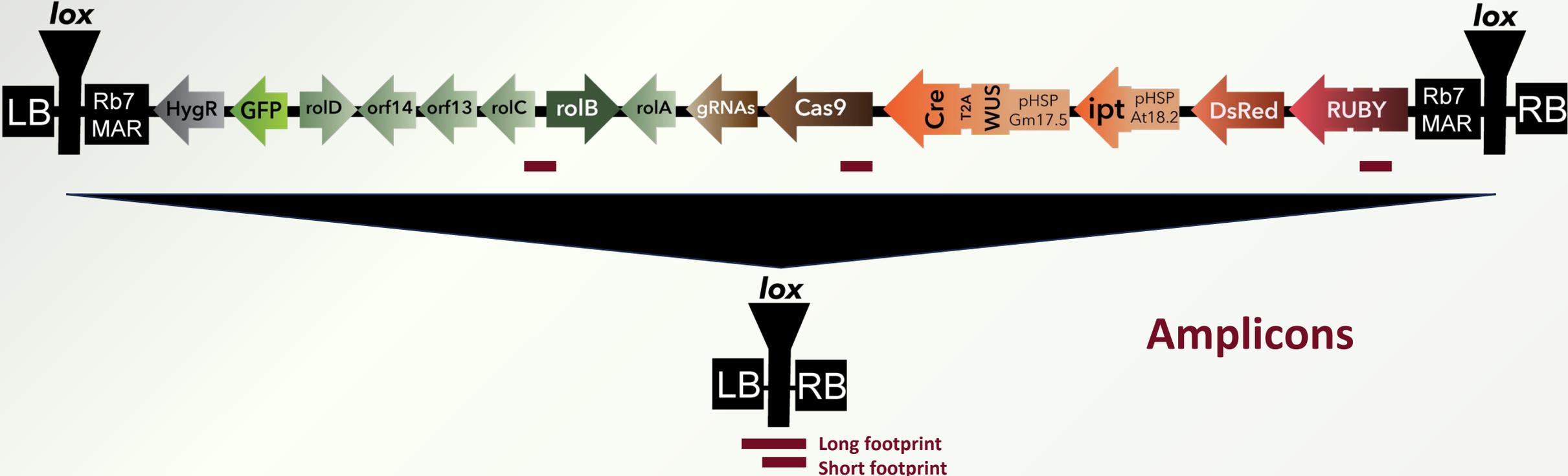
loxP-flanked RESET constructs had a significant effect on transgenic shoot regeneration



loxP-flanked RESET constructs had increased numbers of excised shoots versus other variants



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



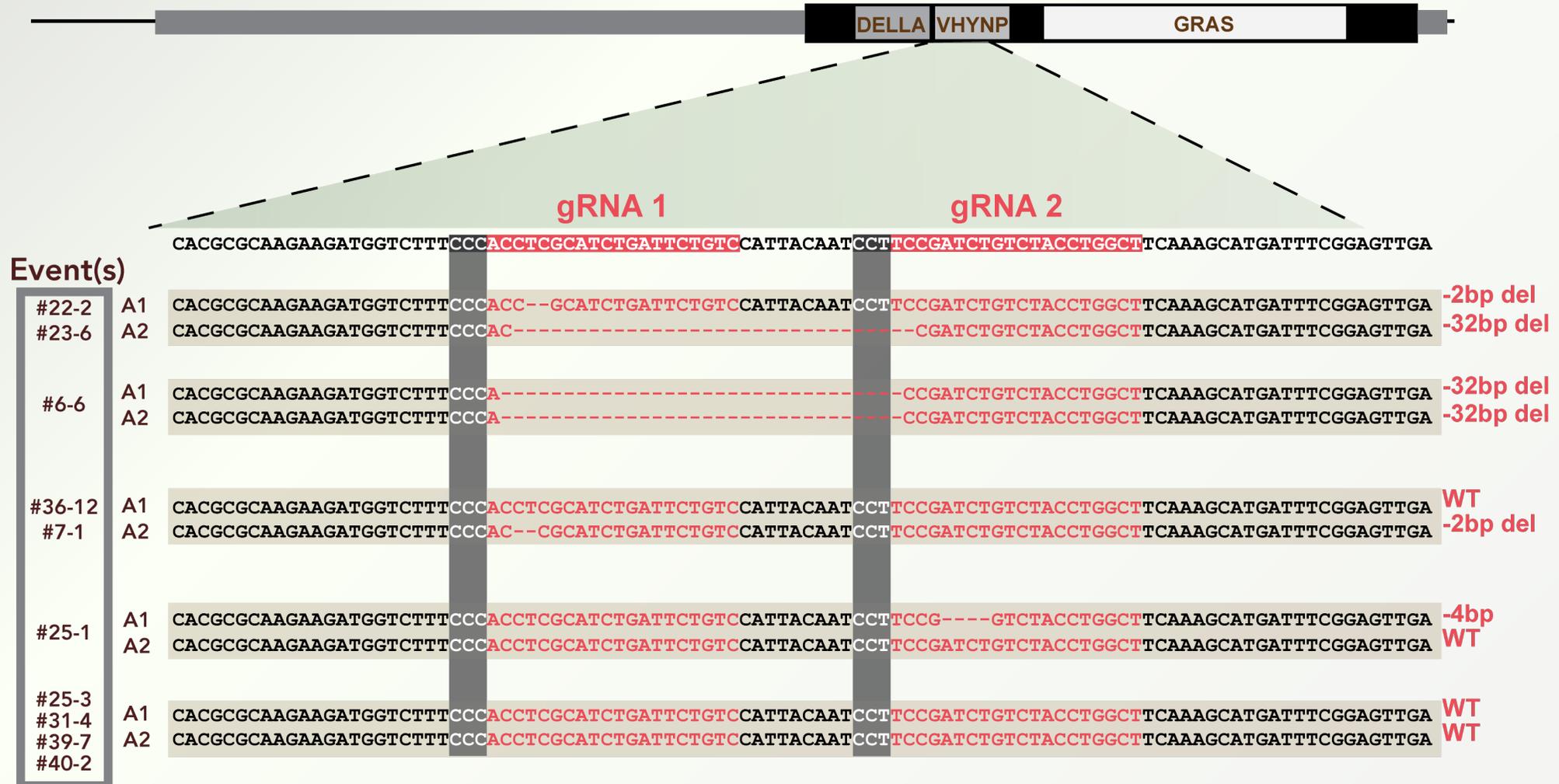
- The presence of any transgene band eliminated a shoot as being categorized fully excised (partial chimera)
- Two footprint amplicons were included in case of truncation of the T-DNA left border

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

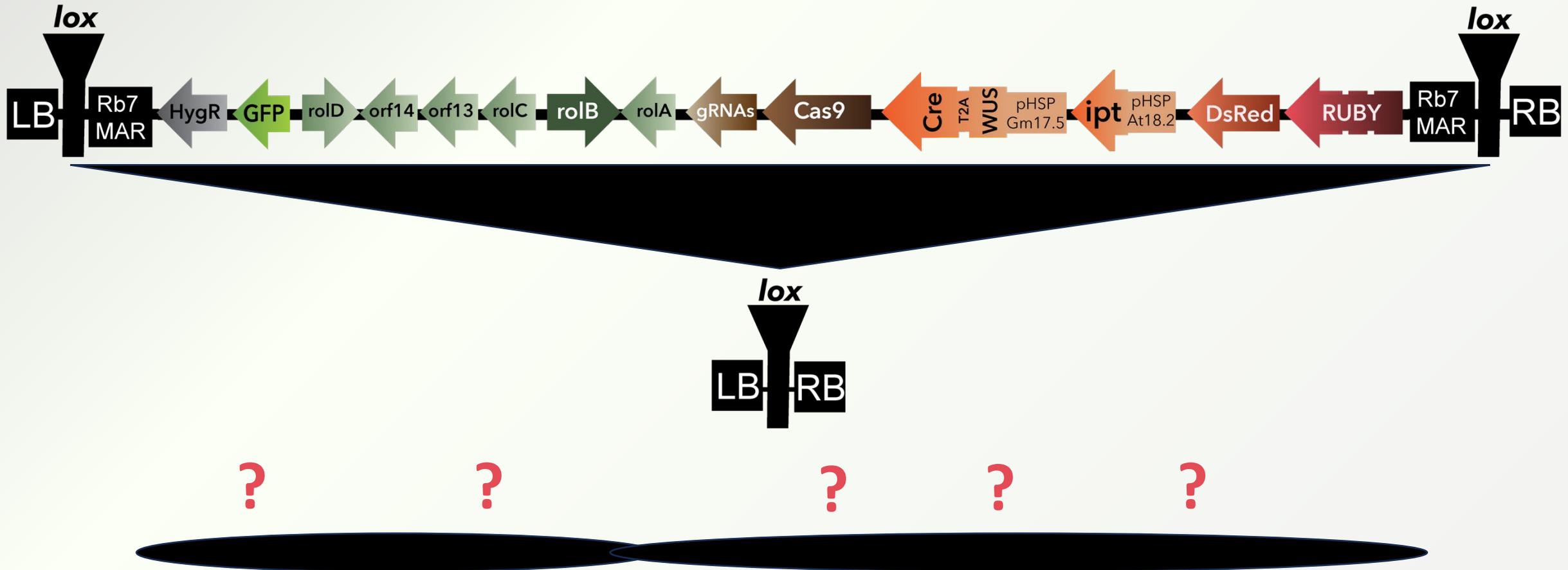


We found typical rates of editing among excised RESET shoots

**PtXaAlbH.08G111700 (REPRESSOR OF GA1/ RGA1)**

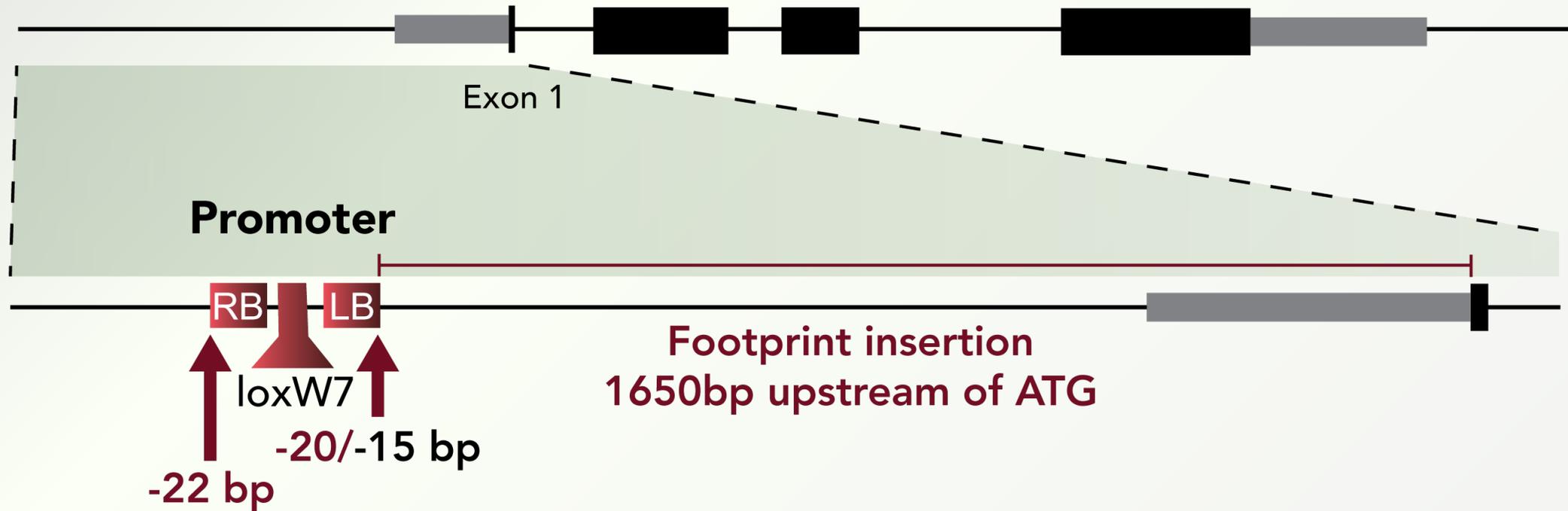


Where did our transgenes insert? How did the excision process resolve the footprints into final configuration?



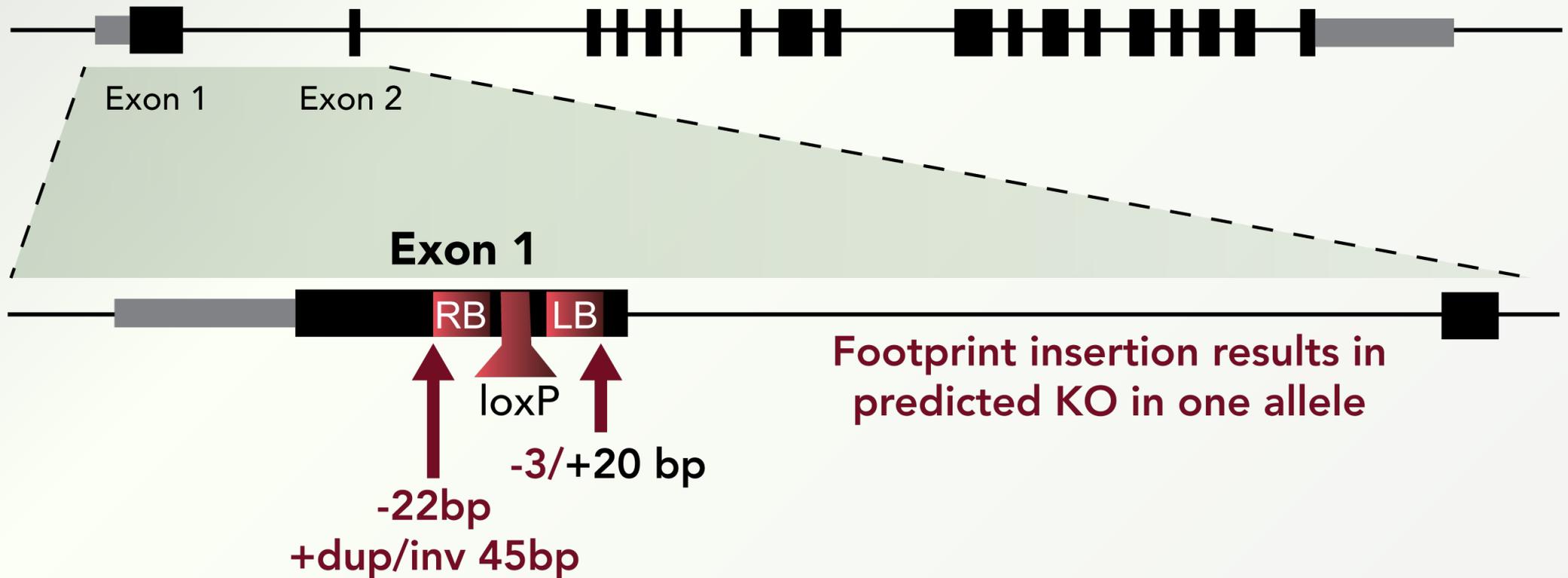
We used TAIL-PCR and WGS to find where transgenes landed and resolved into smaller footprints

### PtXaAlbH.06G051600 (PLATZ TRANSCRIPTION FACTOR)

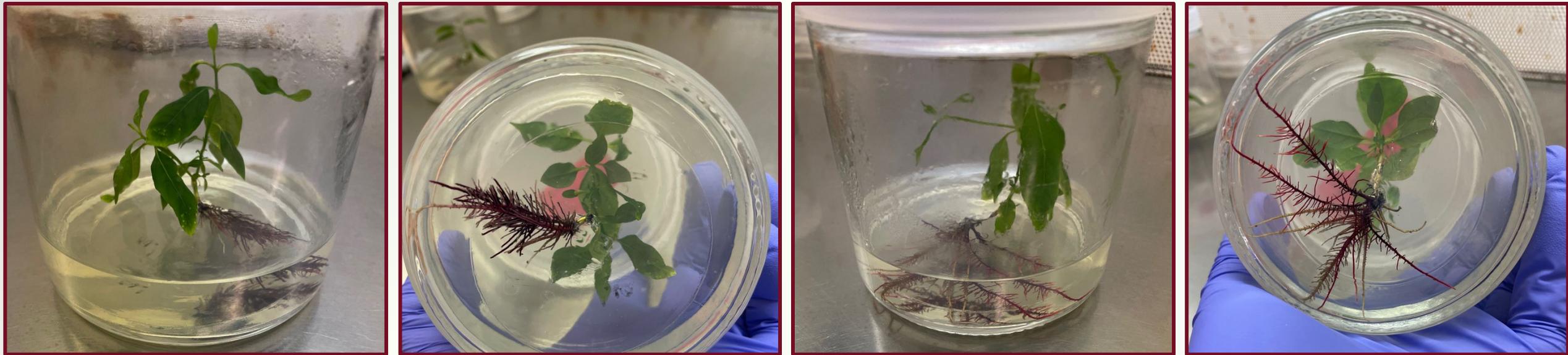


We did find some events where the footprint inserted into an exon of one allele

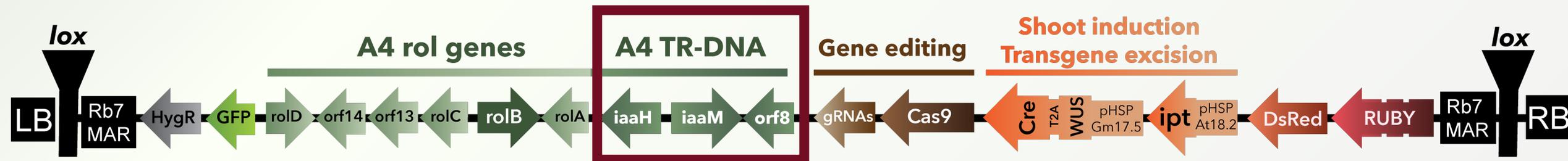
**PtXaTreH.05G116800** (NADPH--CYTOCHROME P450 REDUCTASE 1)



Does this work in other, more recalcitrant species?

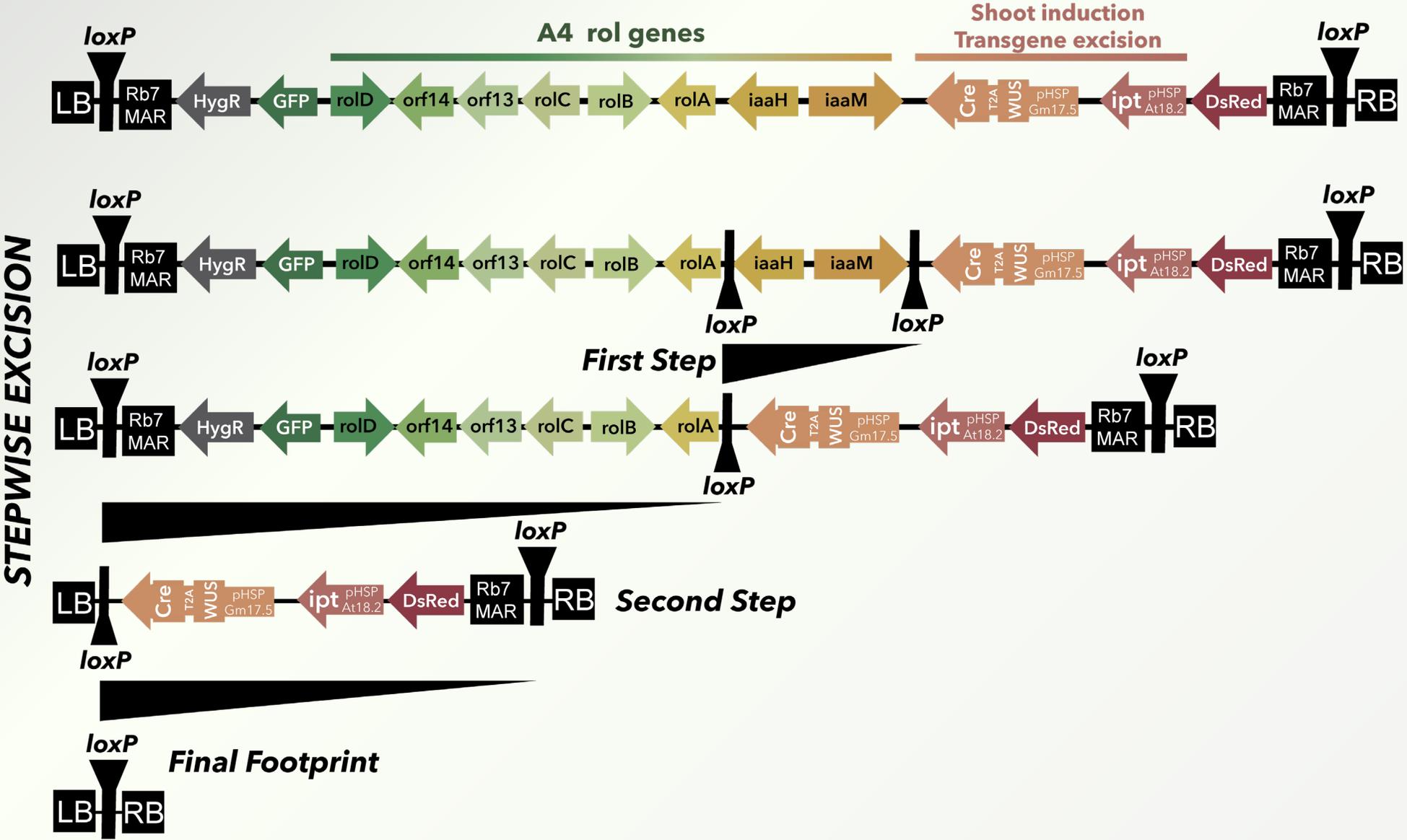


*Eucalyptus grandis* x *urophylla* RESET composite plants



So far, adding the iaaH/M genes seems to interfere with shoot development in *Eucalyptus*

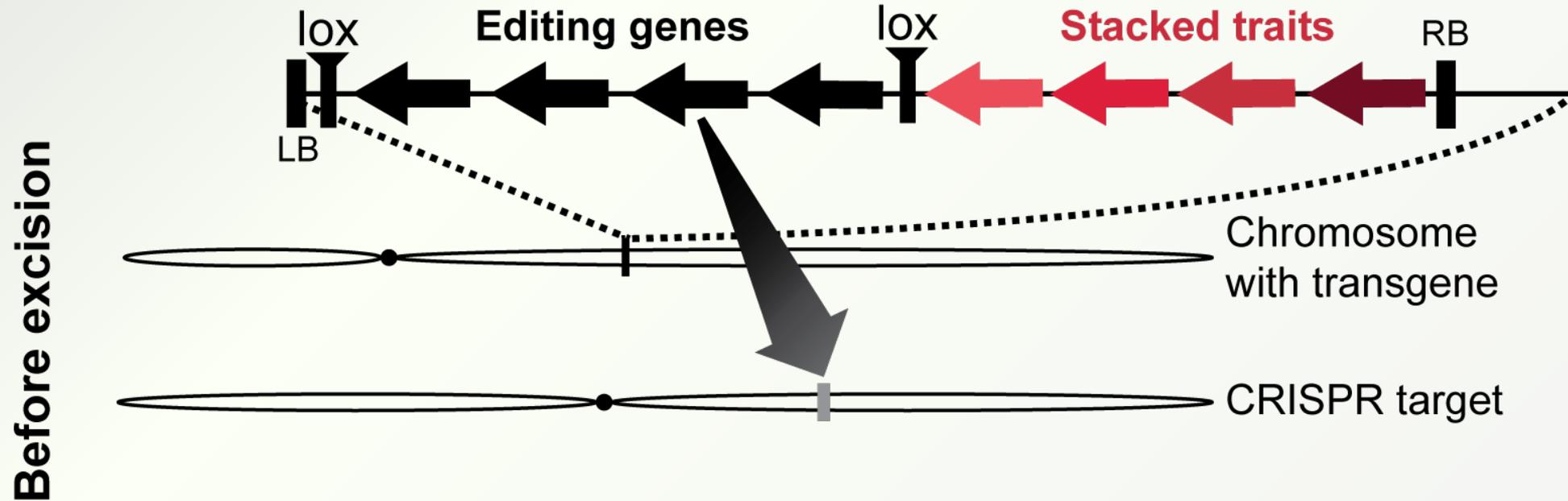
# We are focusing on excision improvement in new variants



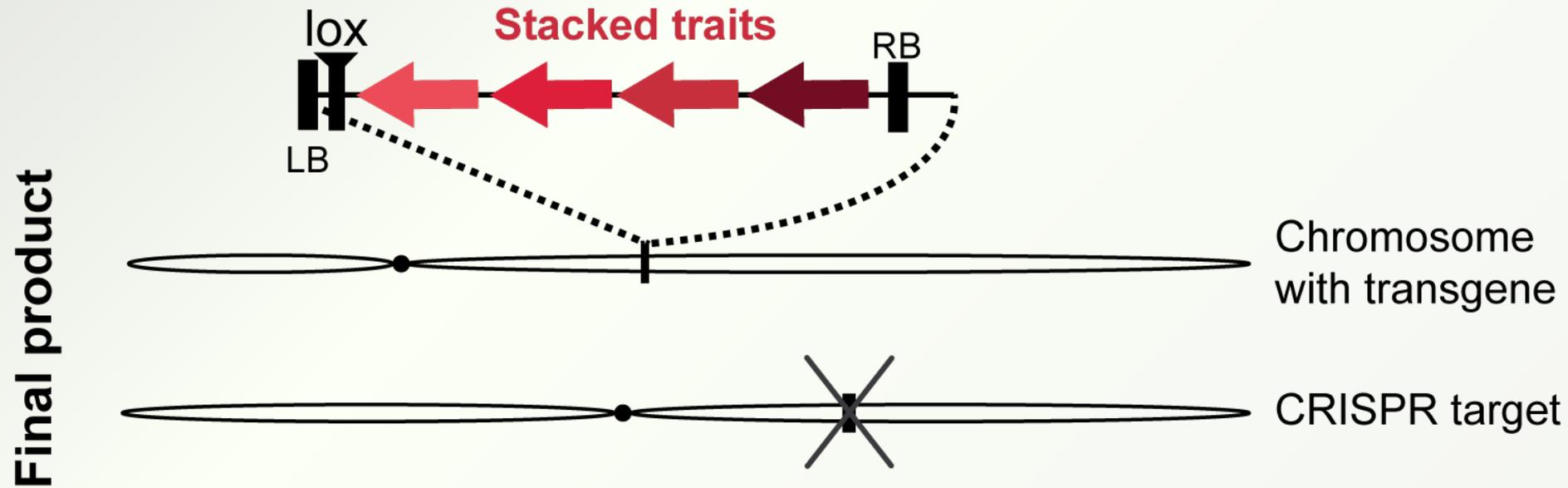
# Hairy root to shoot methods are functional for editing and transformation in clonally propagated plants

- RESET system gave high rates of hairy root transformation, and especially with loxP-flanked borders gave efficient heat induced regeneration and transgene excision from root tissue
- High rates of editing are seen so far in excised shoots
- Significant numbers of escape shoots found which should be easily eliminated through adding selection during hairy root subculture
- We hope this system will function broadly in clonal woody plant species

We envision this system being used simultaneous editing and transgene insertion, enabling synthetic biology approaches



....resulting in products with traits that could make downstream processes simpler, all in one operation



# Acknowledgements: People



**Victoria  
Conrad**  
Undergraduate



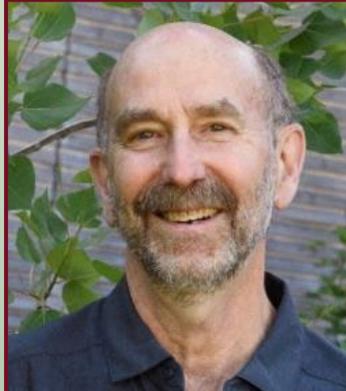
**Sydney Gould**  
Undergraduate



**Cathleen Ma**  
Tissue culture and  
transformation



**Kate  
Peremyslova**  
Tissue culture and  
transformation



**Steve Strauss**  
Professor FES



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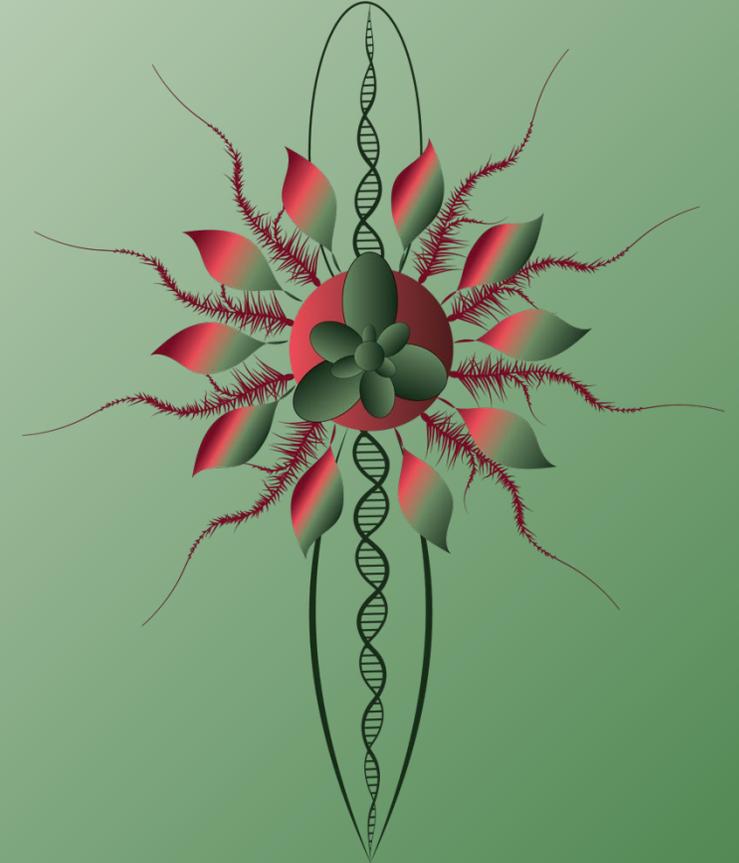
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**Thanks for listening!**