RESET*:* An *Agrobacterium rhizogenes*-based System for Smart Editing in Clonally Propagated Crops

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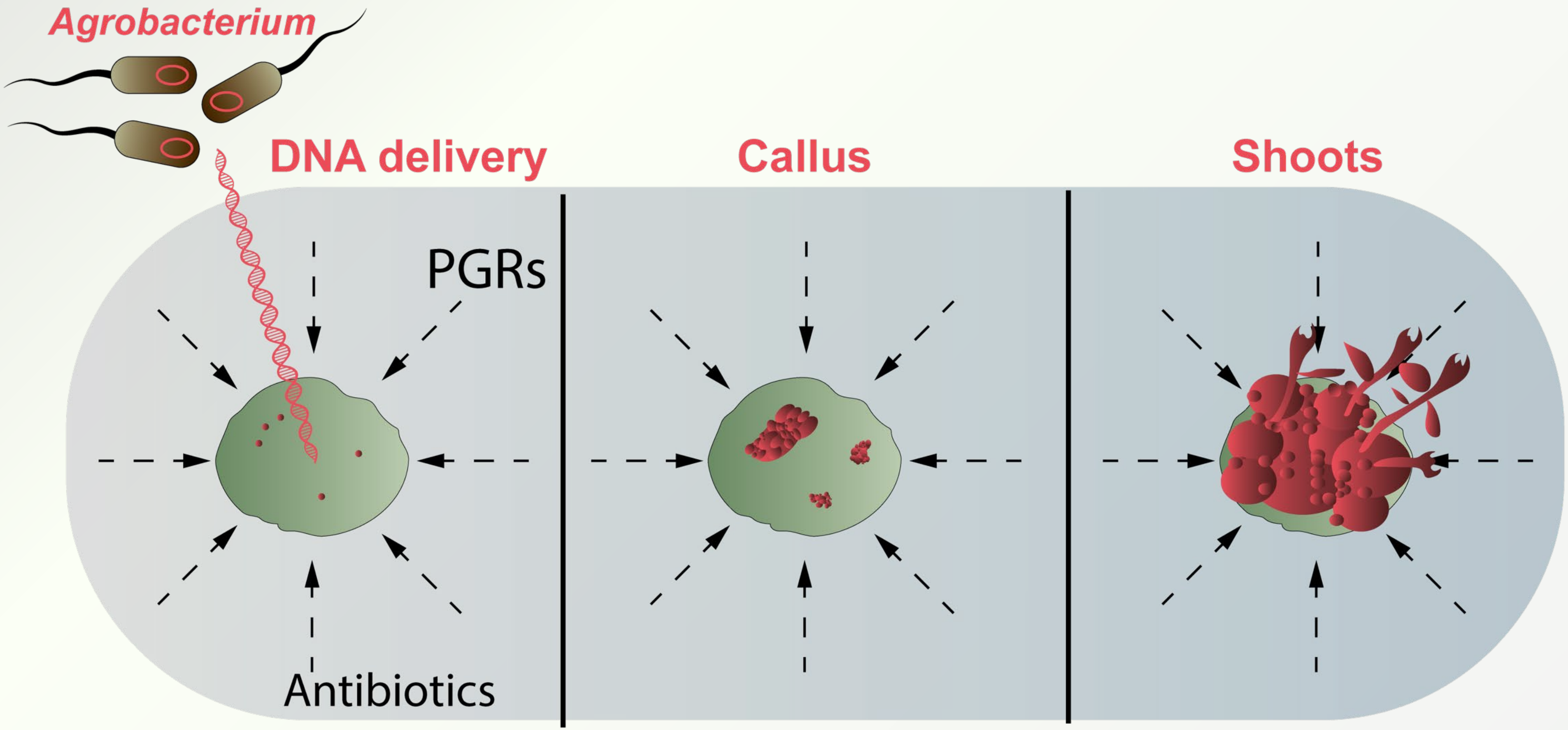
Forest Ecosystems and Society

Many tree species are difficult to transform

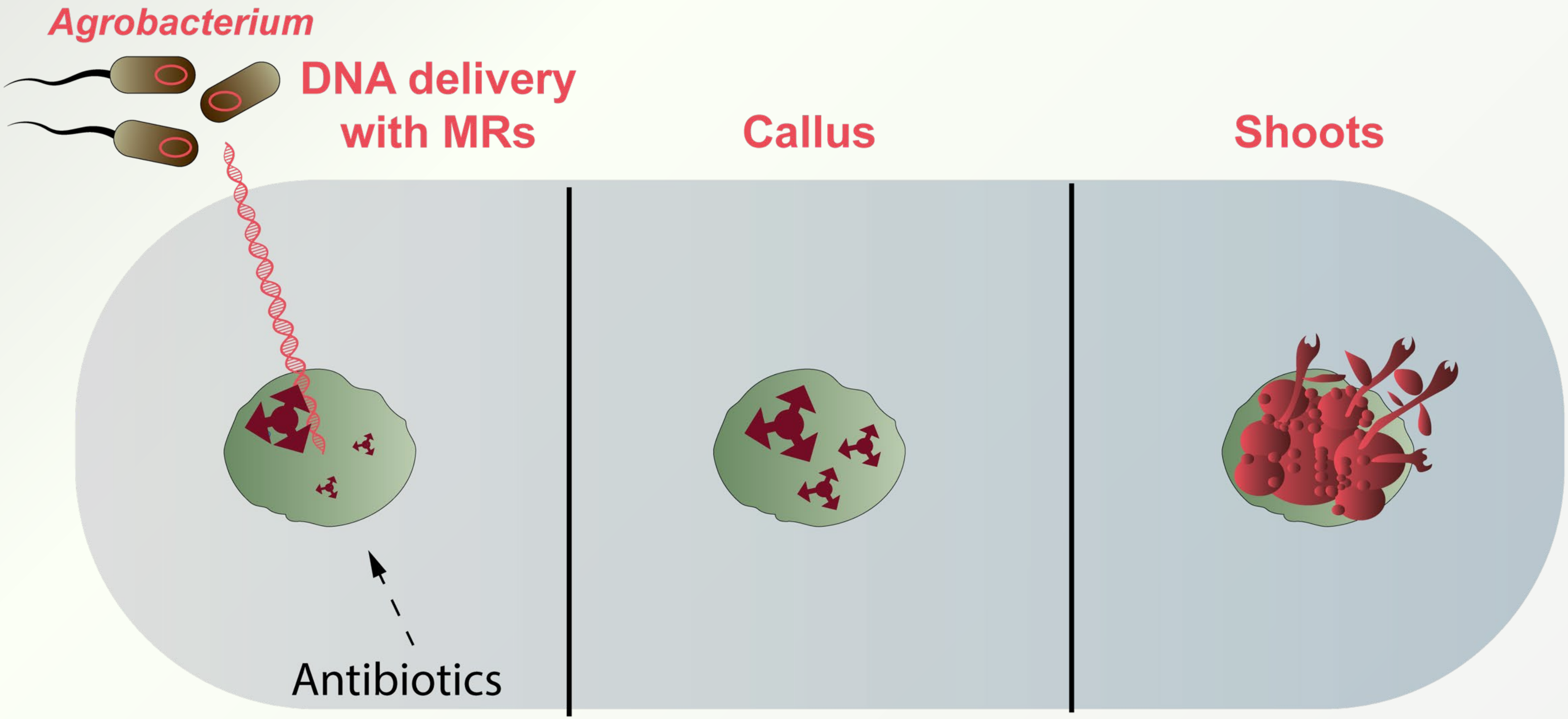
- Elite clones, not seed-derived
- High heterozygosity: each genotype a new adventure *in vitro*
- High physiological and epigenetic diversity
- Strong defense responses common

Eucalyptus grandis x urophylla

Conventional transformation methods rely on exogenous plant growth regulators (PGRs) supplied in the culture medium



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

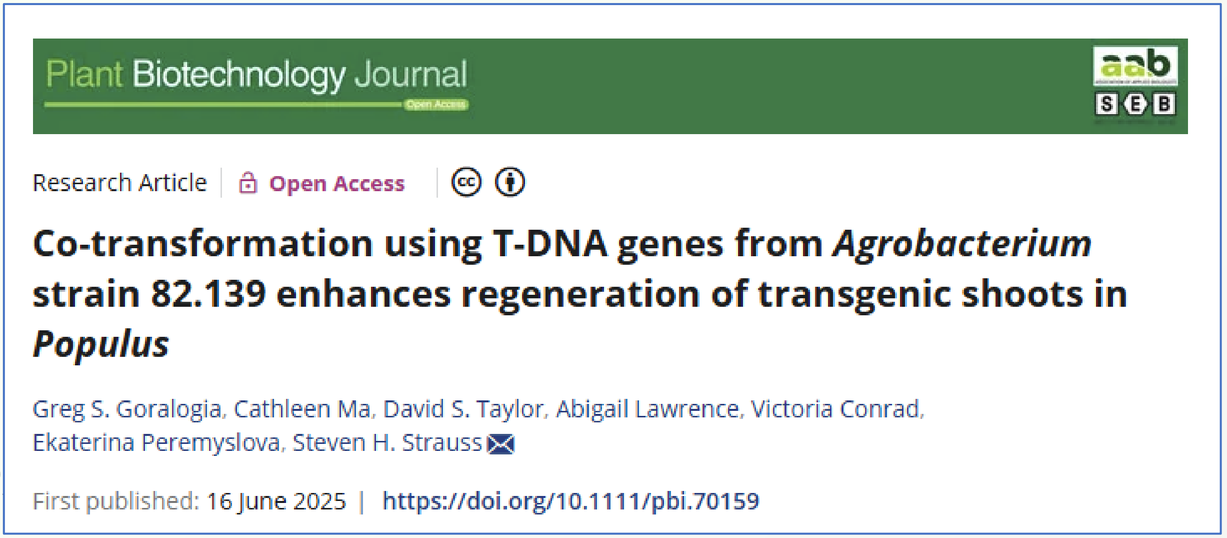


We have tested many types of MR genes in poplars or eucalypts – many both *in vitro* and *in planta*

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

- *Agrobacterium* tumefaciens growth promoting genes

- **rol – Hairy root-inducing genes – from *Agrobacterium rhizogenes***



Plant Biotechnology Journal

Research Article | Open Access

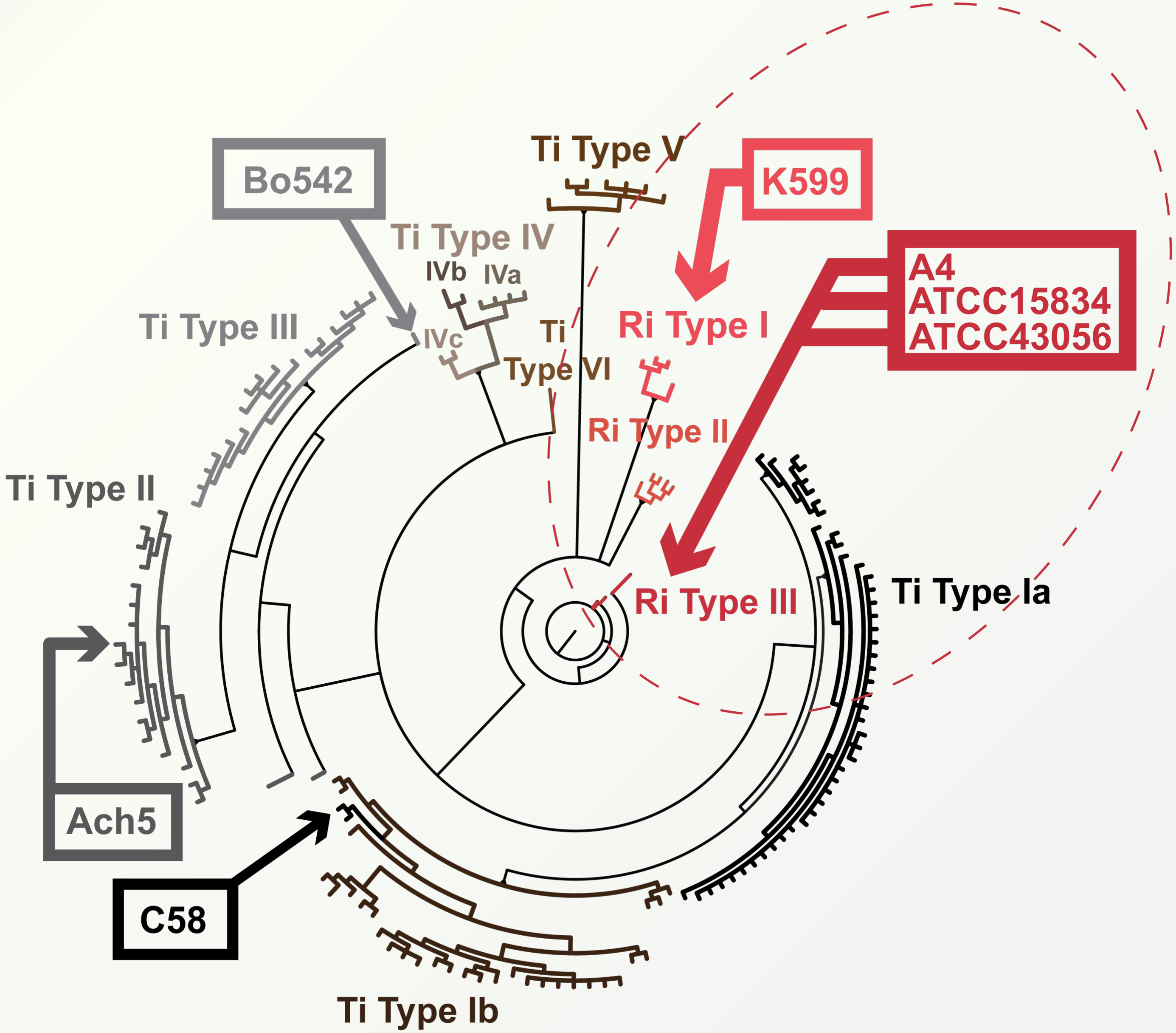
Co-transformation using T-DNA genes from *Agrobacterium* strain 82.139 enhances regeneration of transgenic shoots in *Populus*

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

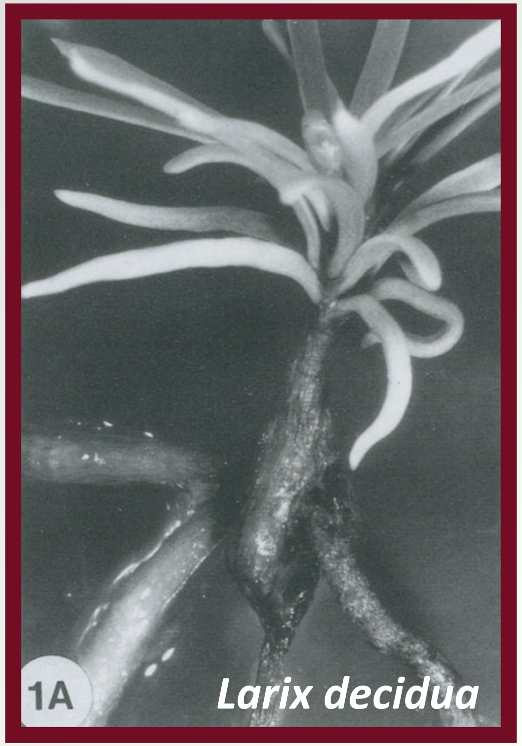
First published: 16 June 2025 | https://doi.org/10.1111/pbi.70159

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

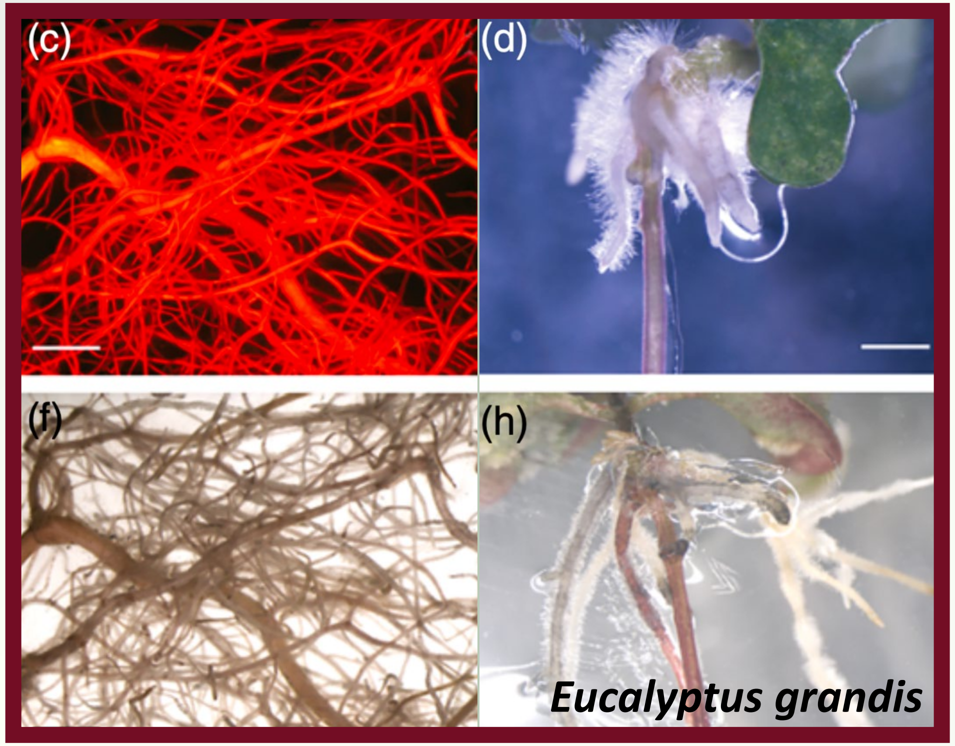
Hairy roots in hydroponically grown tomato



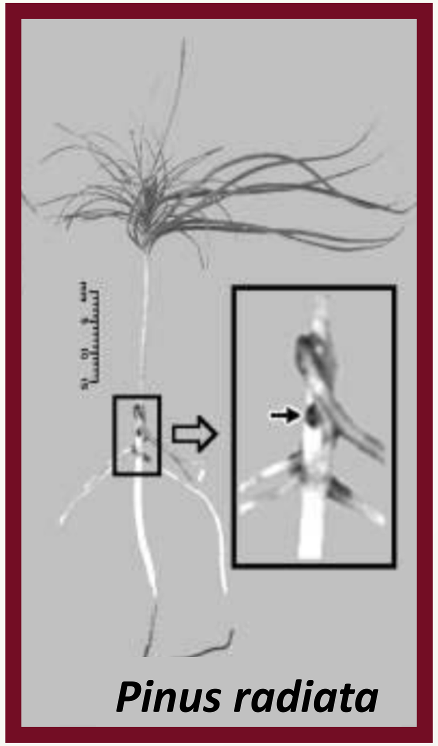
Hairy root *rol* genes are an effective way to generate transgenic tissues across diverse tree species



Larix decidua



Eucalyptus grandis



Pinus radiata

Ginkgo biloba

Salix purpea

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 also effective in many clonal, woody, food / flavoring crops

Mentha peperita

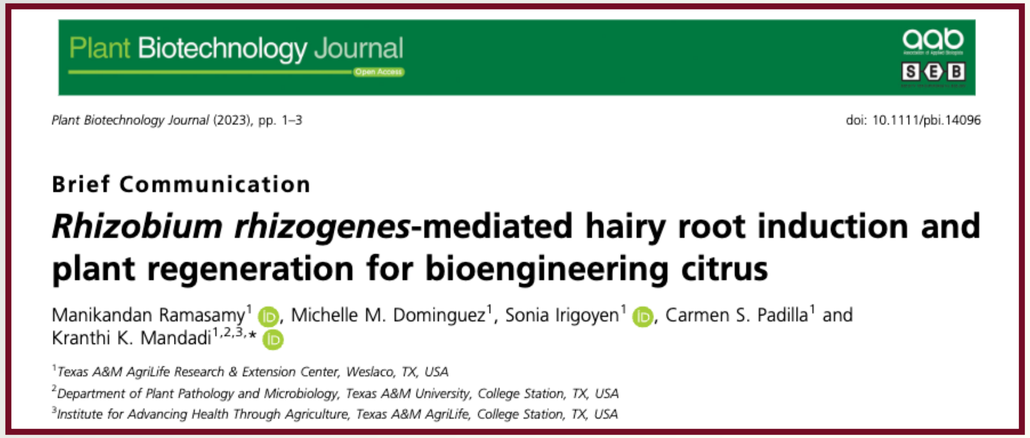
Corylus avellana

Prunus (cherry)

Vitis vinifera cv. Syrah

Inoue et al. 2002 *Plant Biotech*, Parizi et al. 2020 *IVCDB-Plant*, Bosselut et al. 2011. *Plant Cell Rep.*, Meteier et al. 2019 *FIPS*.

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,*]

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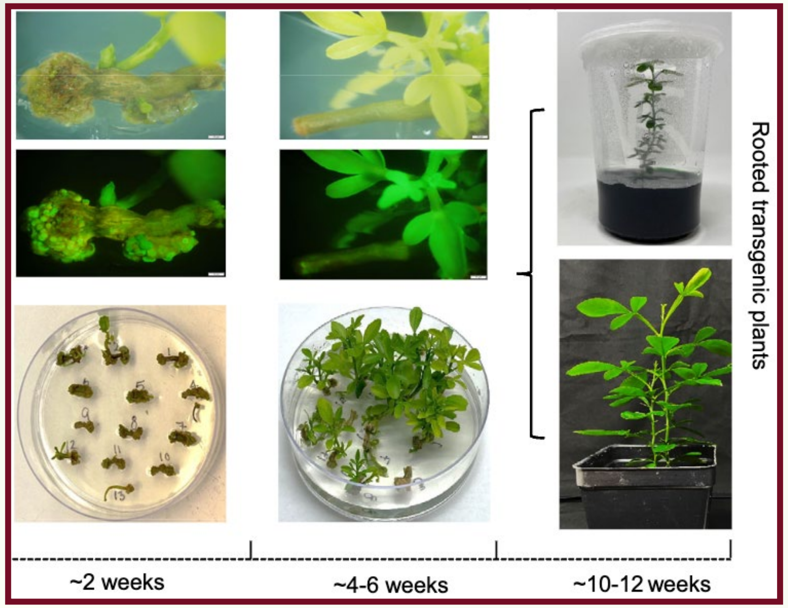
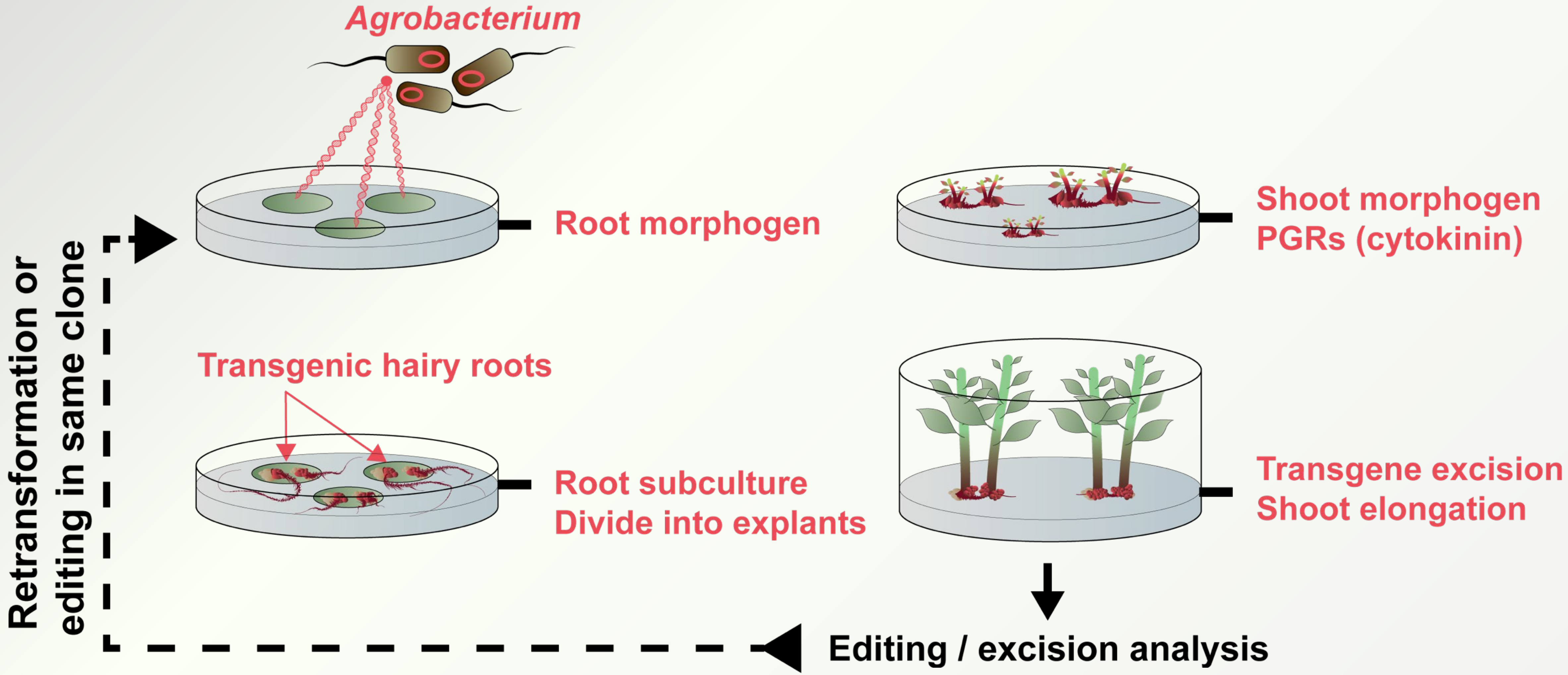


Osteospermum fruticosum (cape daisy)

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

Desmet et al. 2021 *Plant Cell Rep.*

The concept for Root Excision System for Efficient Transformation



Agrobacterium

Root morphogen

Transgenic hairy roots

Root subculture
Divide into explants

Shoot morphogen
PGRs (cytokinin)

Transgene excision
Shoot elongation

Editing / excision analysis

Retransformation or
editing in same clone

Root to shoot regeneration is a synthesis of older ideas

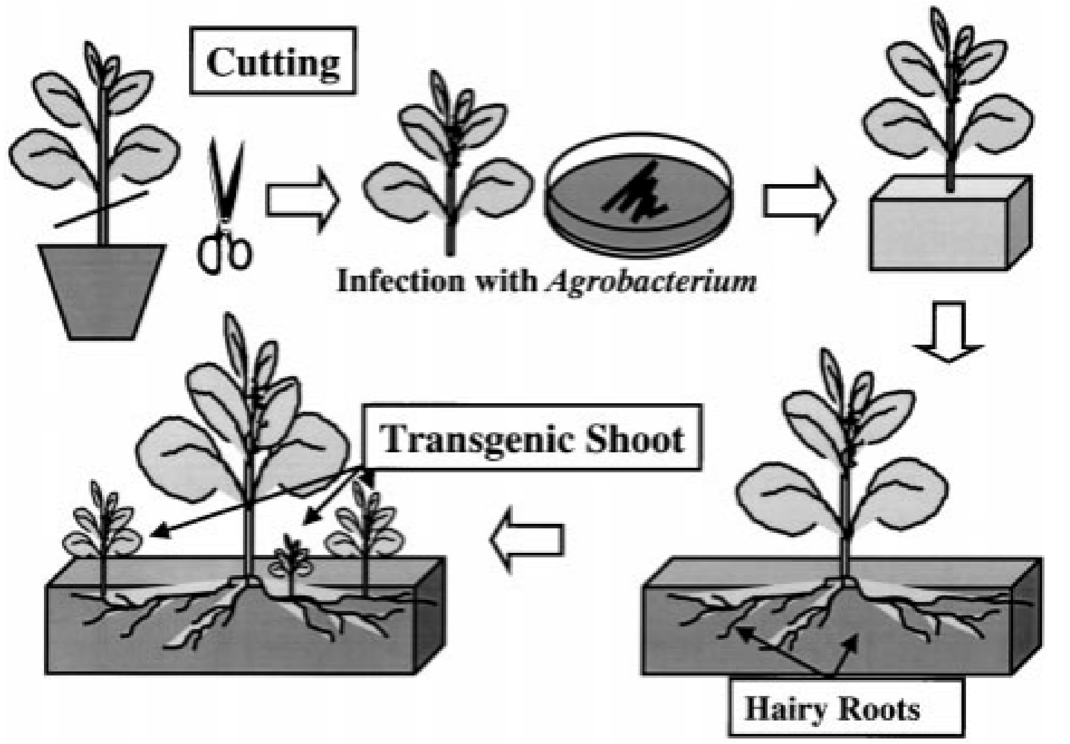
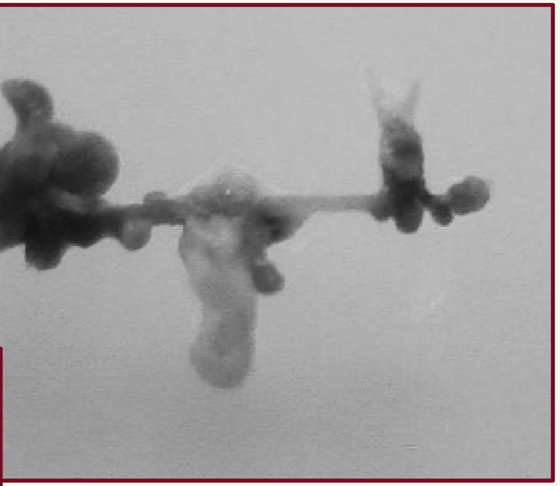


FIG. 8. Outline of *in vivo* transformation using cuttings. Cuttings are infected with *Agrobacterium* containing the *rol*-type MAT vector. After their rooting, marker-free transgenic shoots are induced from hairy roots by the light.

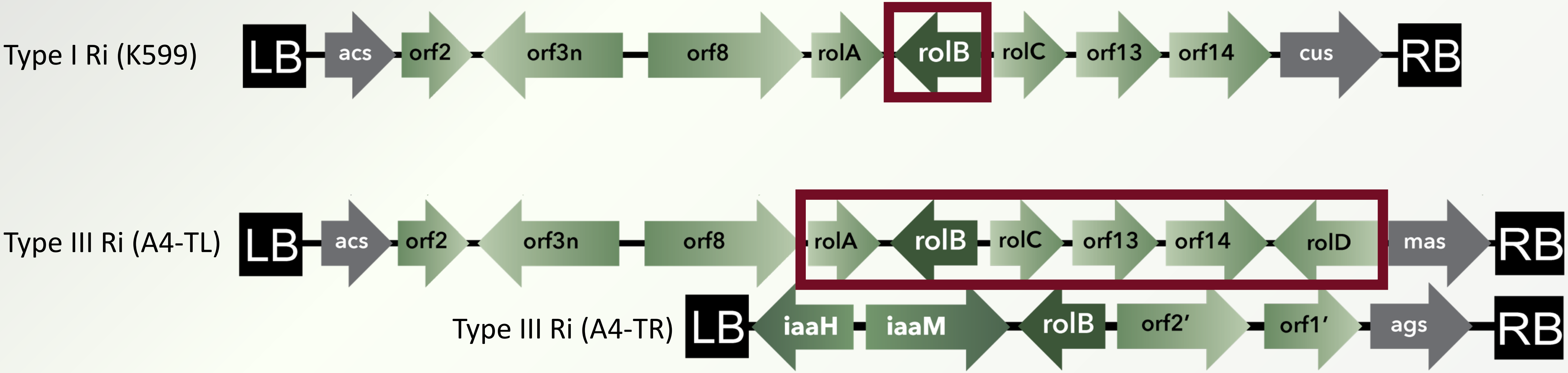


Ebinuma and Komamine,
2001 In vitro cell and developmental biology -Plant

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

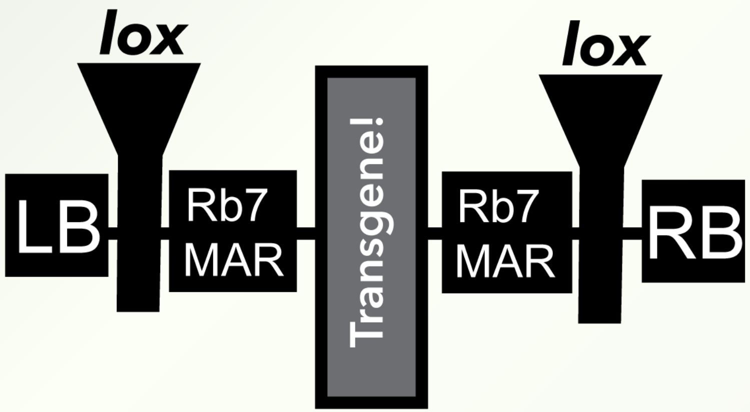
Let's go shopping for parts!

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



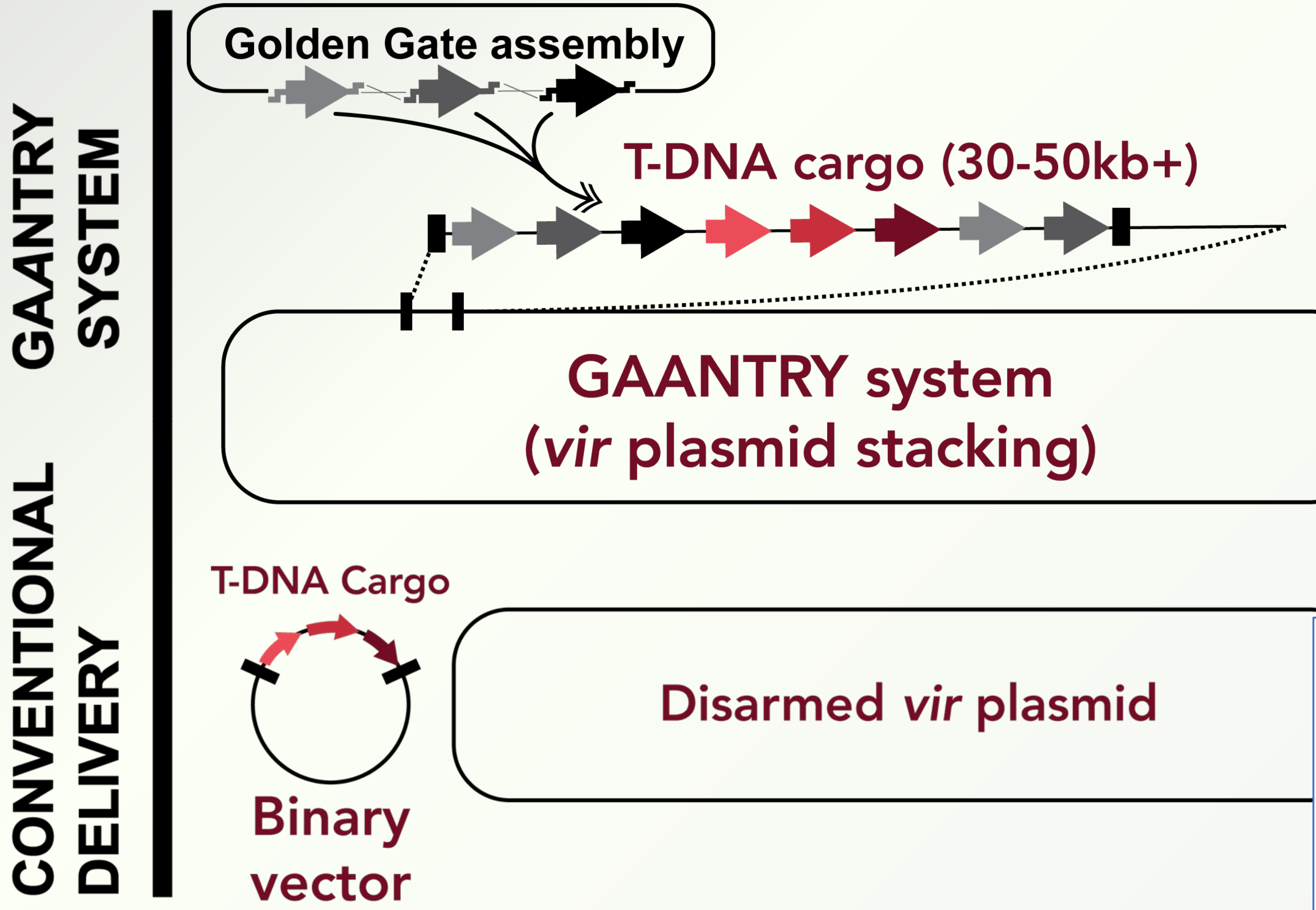
- *rolB* is required for hairy root formation
- Others including *rol A, B, C, D, orf13,* and *orf14* quantitatively increase hairy root formation in many species

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



- *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 functional 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



GAANTRY SYSTEM

Golden Gate assembly

T-DNA cargo (30-50kb+)

GAANTRY system
(*vir* plasmid stacking)

CONVENTIONAL DELIVERY

T-DNA Cargo

Binary vector

Disarmed *vir* plasmid

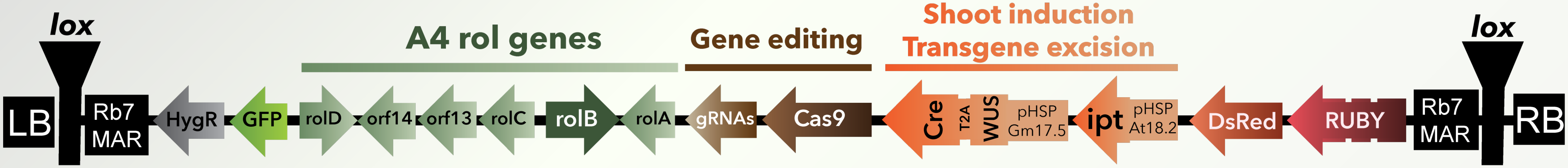
Technical Advance | Open Access

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

Ray Collier, James G. Thomson, Roger Thilmony

First published: 14 June 2018 | https://doi.org/10.1111/tpj.13992 | Citations: 56

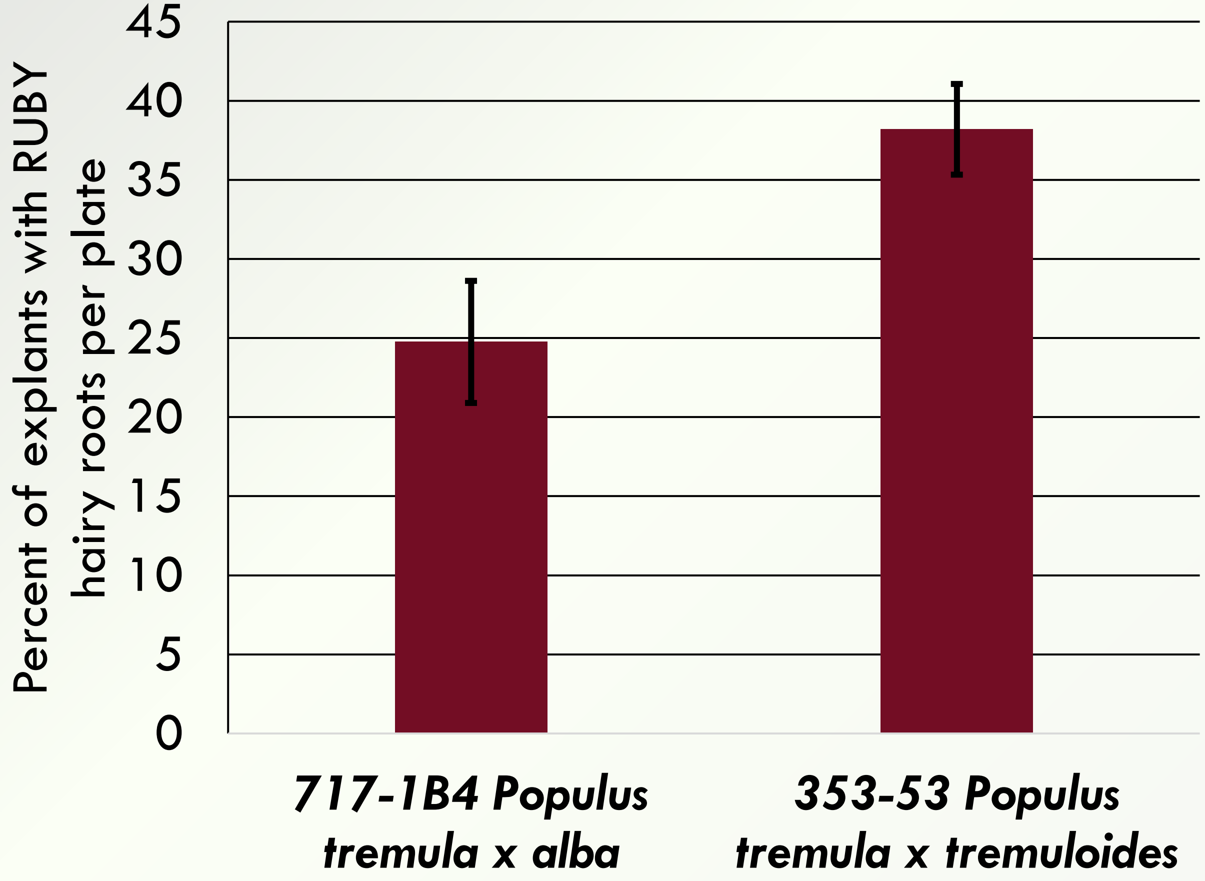
The “kitchen sink” RESET construct we tested

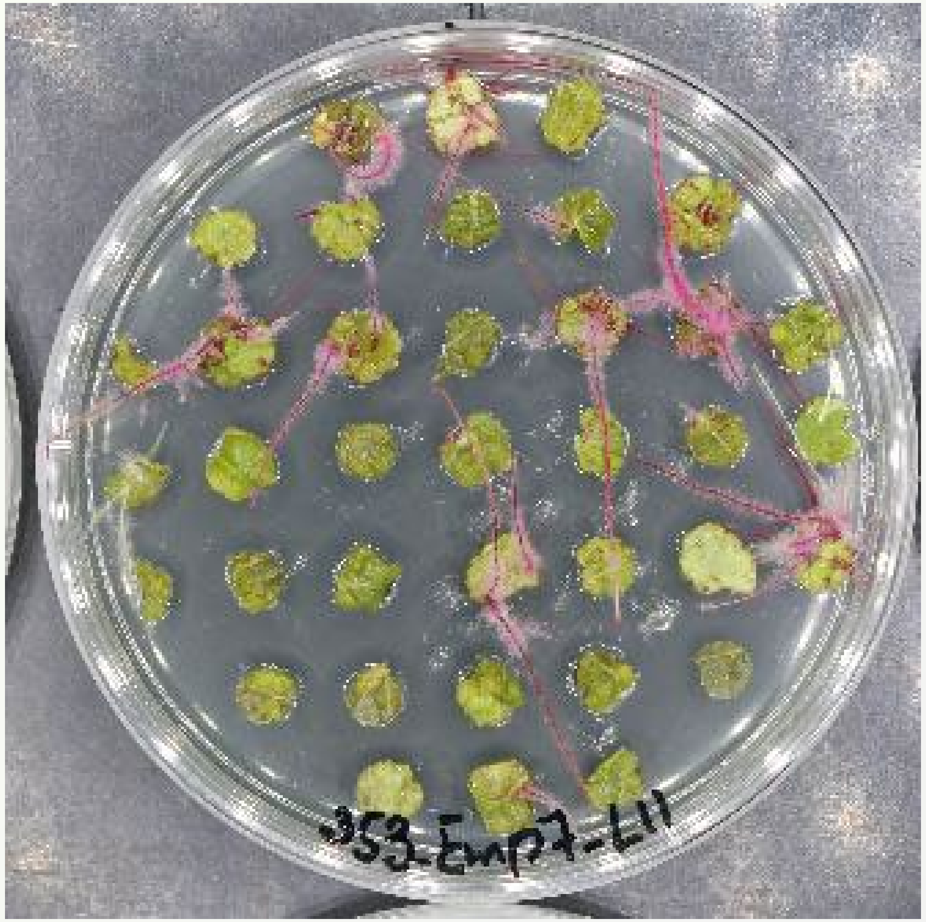


- 39 kb
- 14 transcriptional units
- 16 independent peptides

- 3 marker genes (GFP, DsRed, and RUBY)
- Gene editing through CRISPR-Cas9 (tRNA-arrays targeting *RGA1* gene)
- Hygromycin gene (but no selection was used in these experiments)

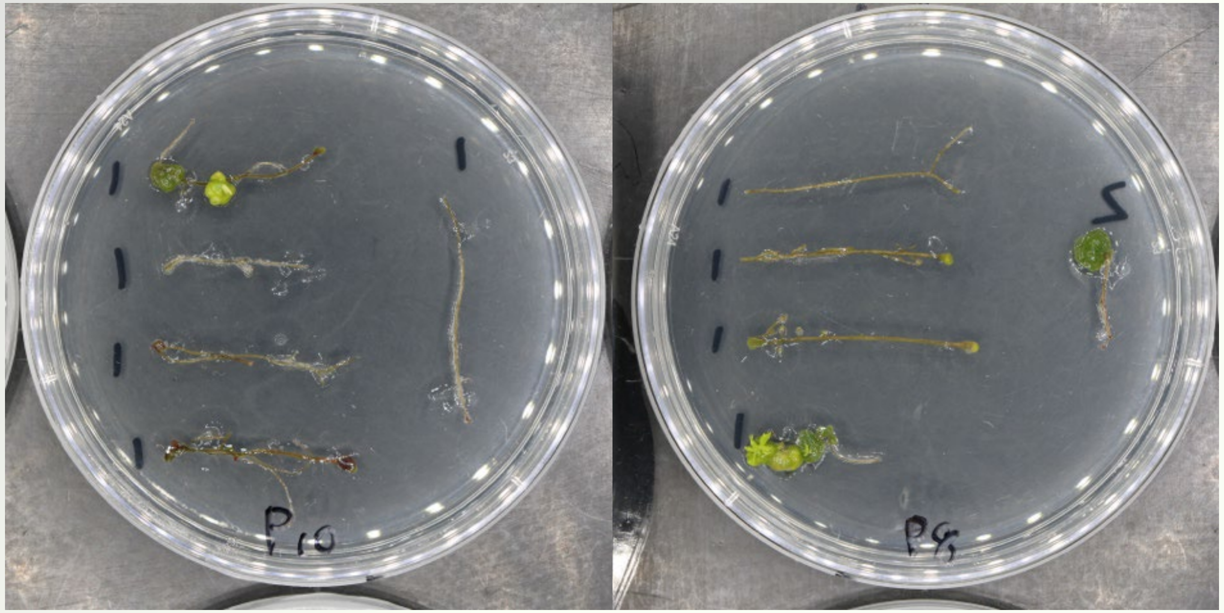
We efficiently generated transgenic hairy roots in two poplar genotypes



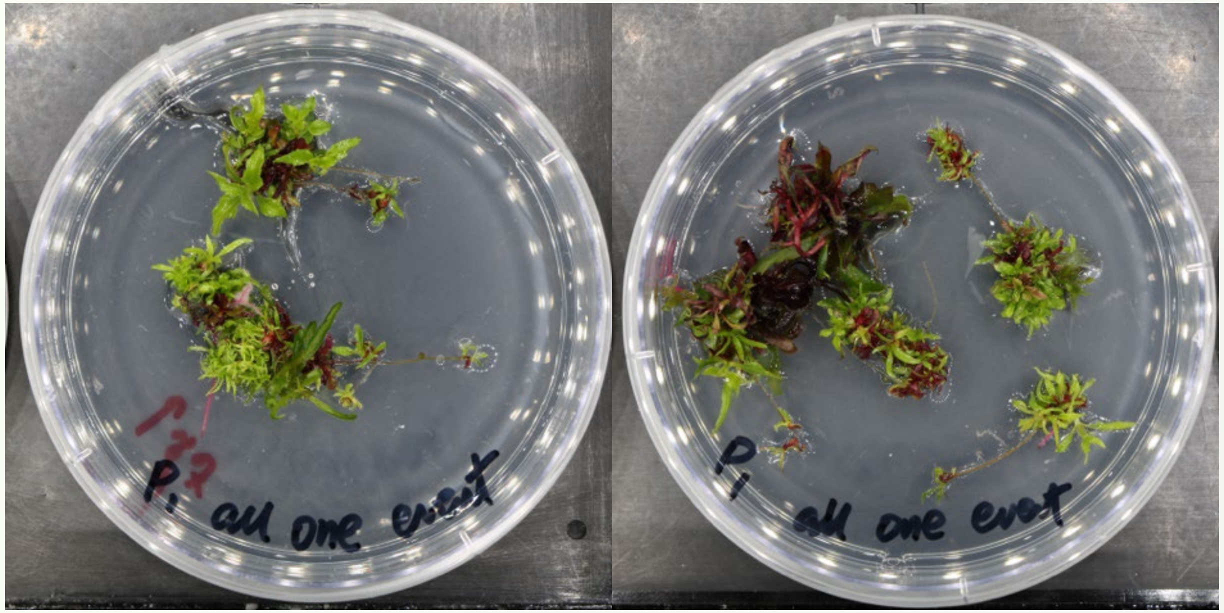


Two weeks of pulsed heat shock (HS) resulted in efficient shoot regeneration and excision from hairy roots

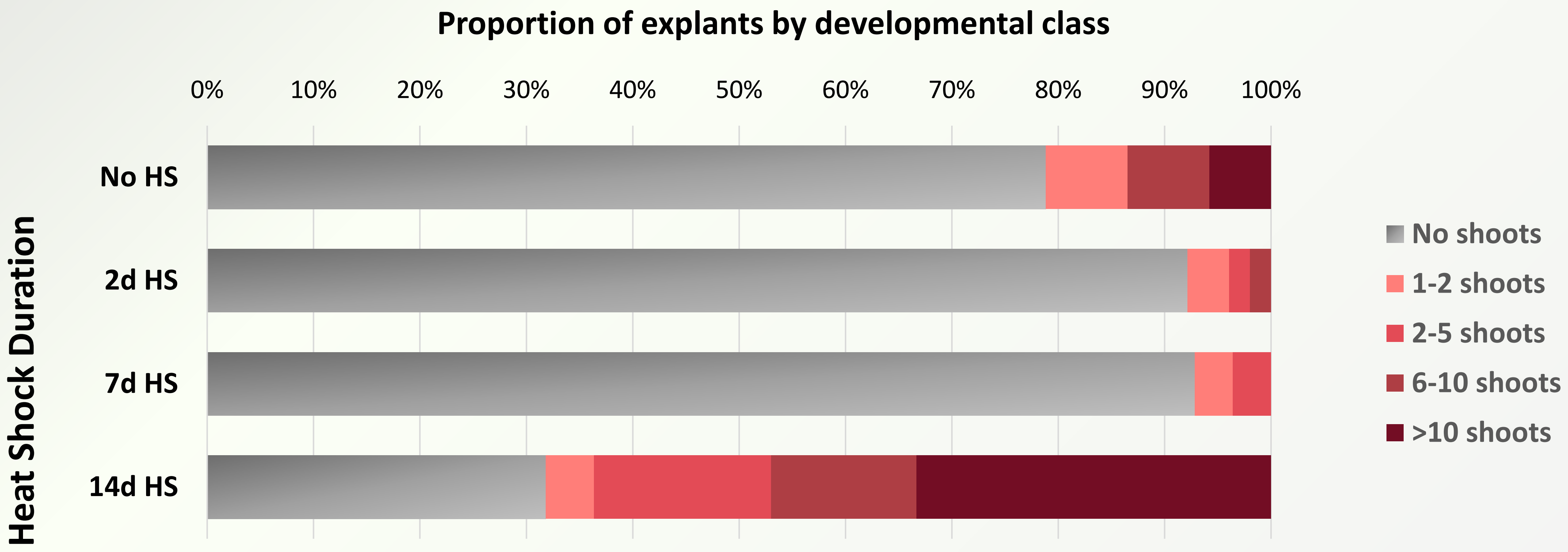
No HS



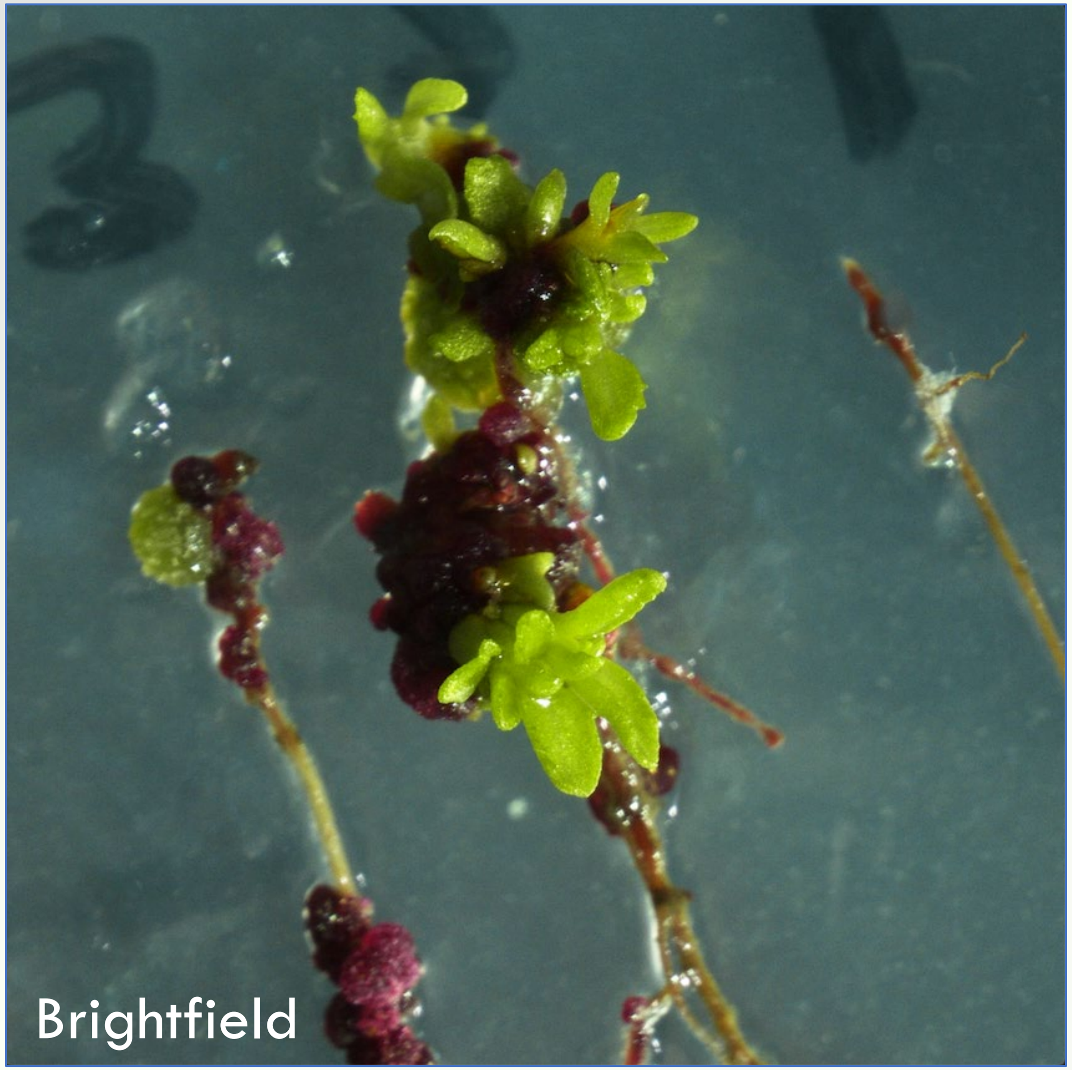
2 week HS, 4 hrs/day, 39 C

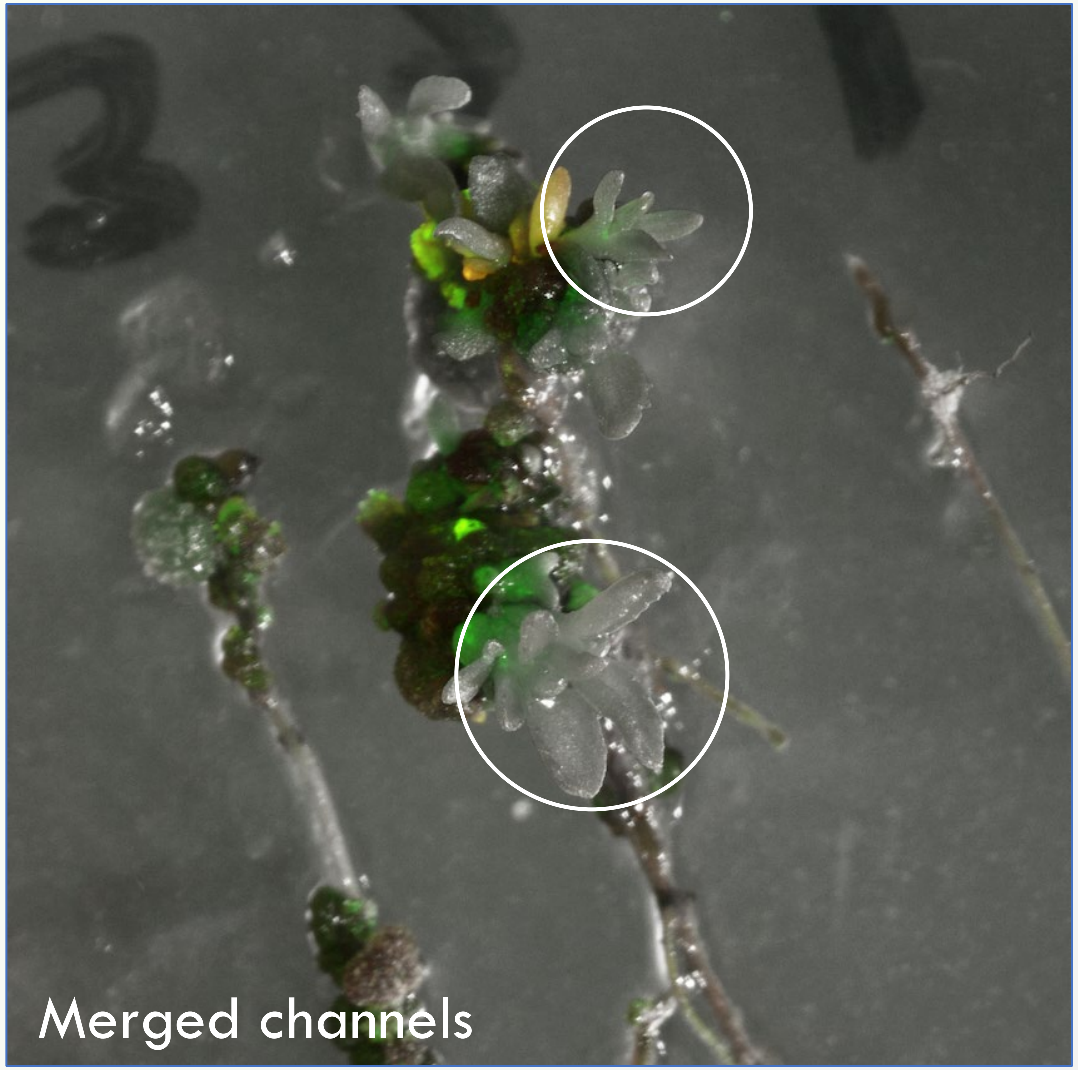


Longer heat-shock duration improved shoot regeneration rates per explant (segments of hairy roots)

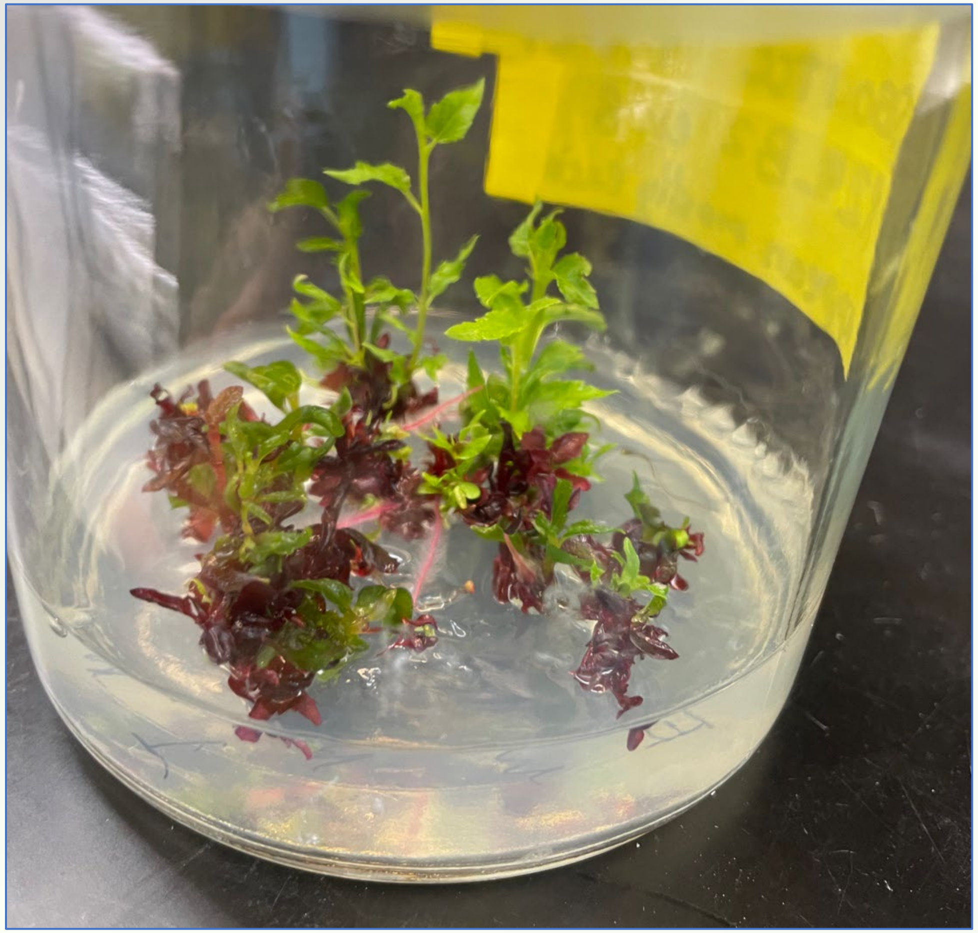


Using multiple reporters (GFP, DsRed and RUBY), we can find shoots with excised transgenes





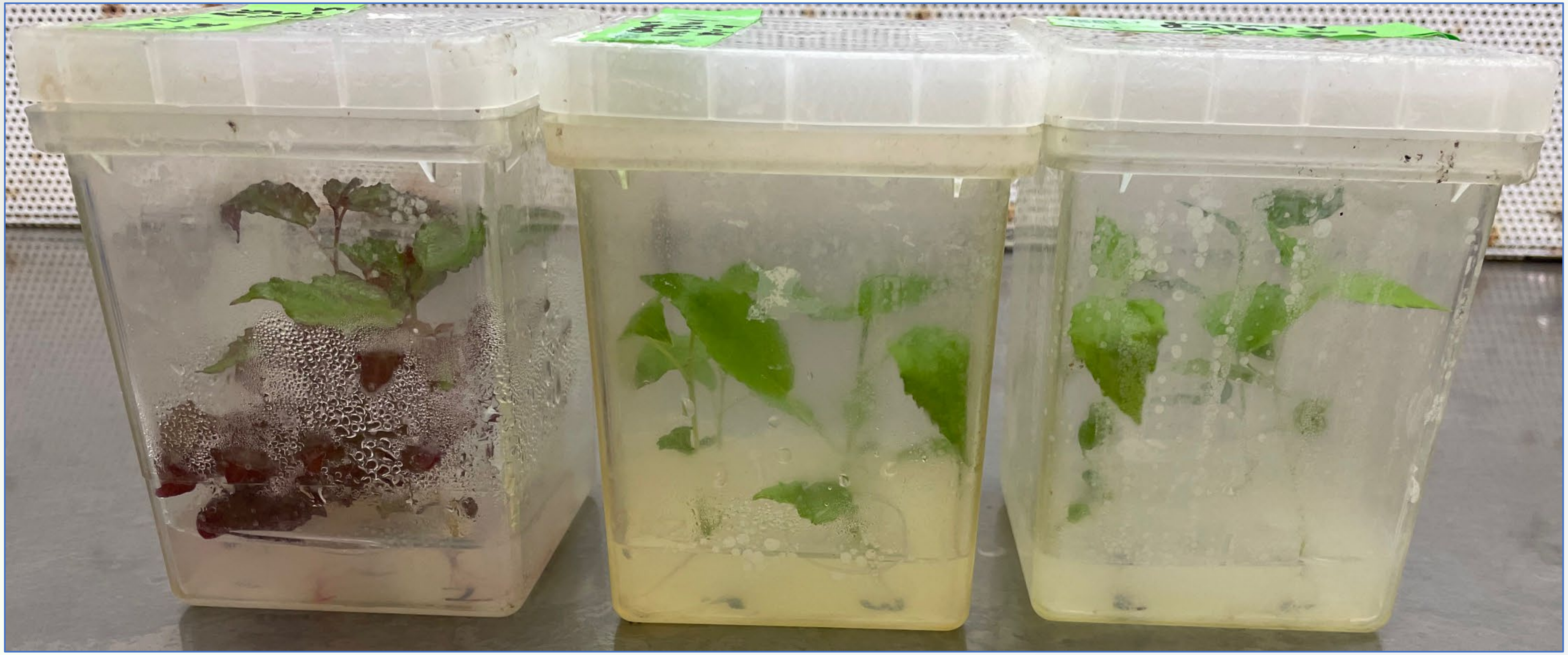
After transfer of regenerating root explants, elongated shoots without marker genes can be identified



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

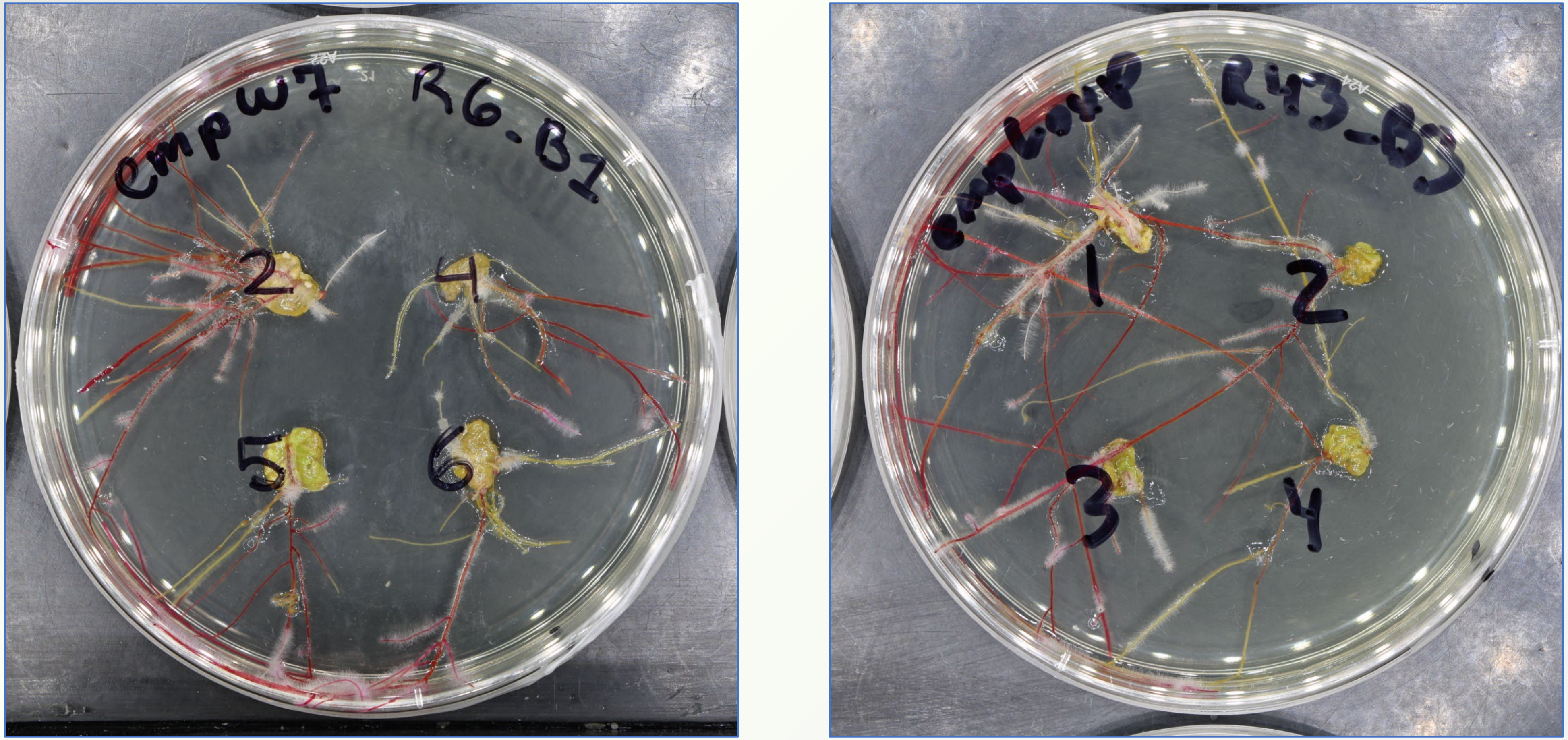
Populations of putative excised RESET shoots for further molecular analysis

RESET excised shoots had normal phenotypes with continued *in vitro* culture and propagation



Unexcised transgenic Putative excised event Putative excised event

Quantifying system performance: We performed large numbers of transformations to estimate frequency and excision rates



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

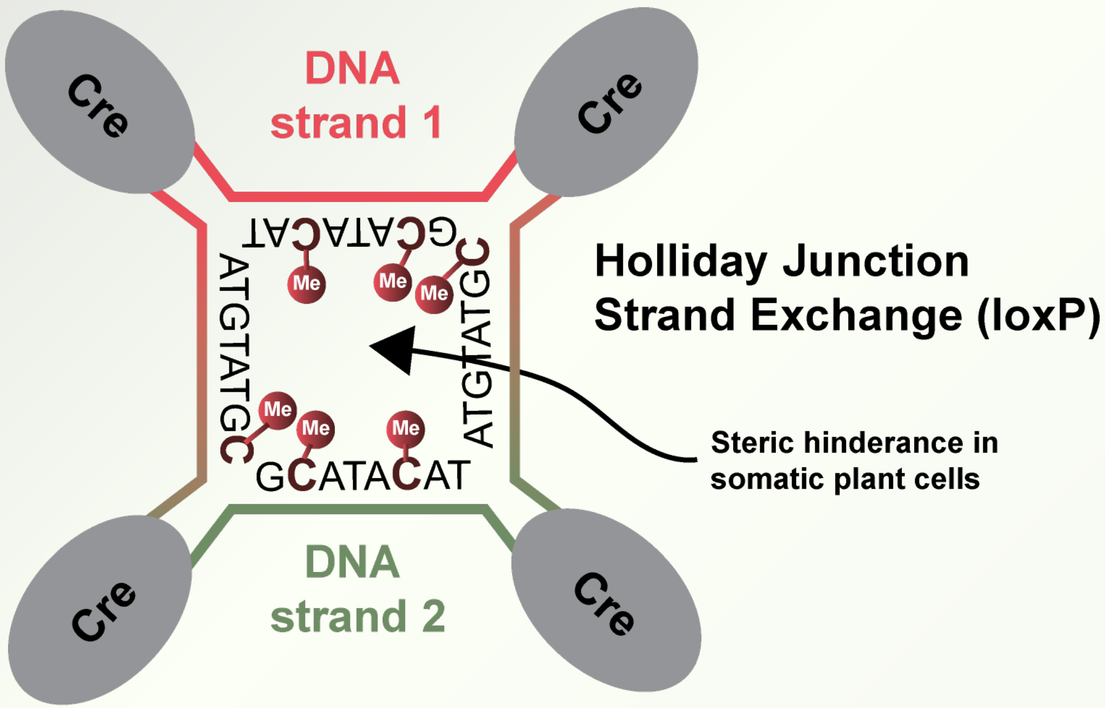
New Phytologist

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

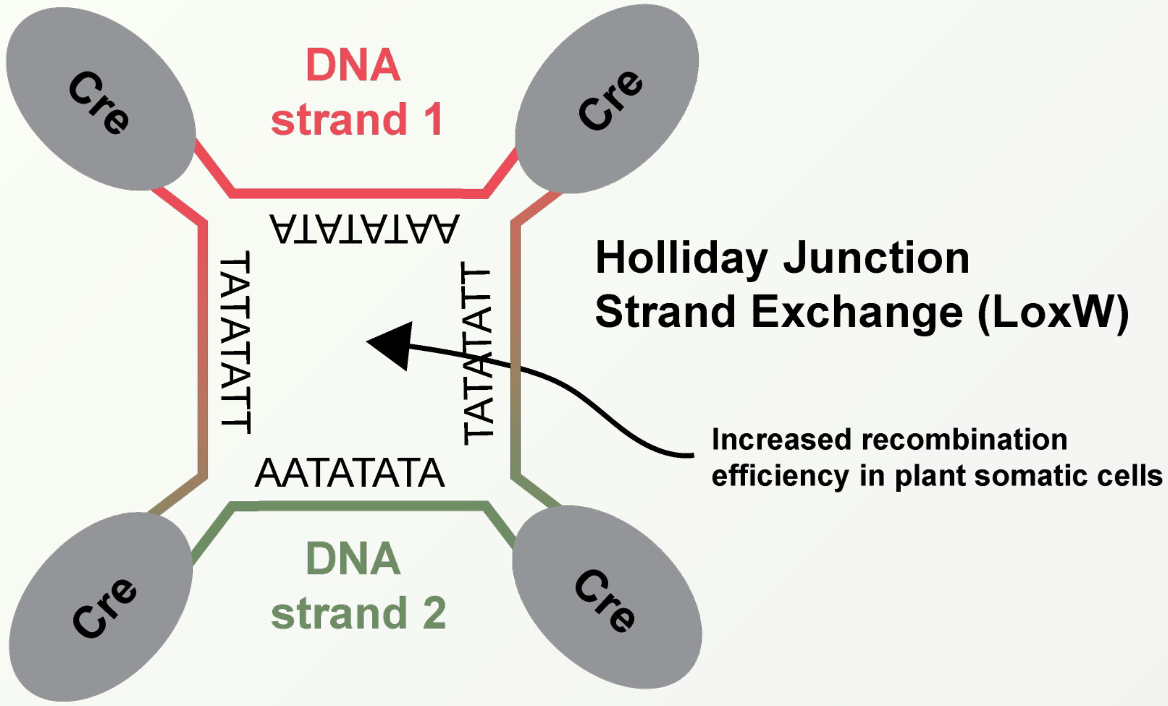
Chongqing Key Laboratory of Application and Safety Control of Genetically Modified Crops; Biotechnology Research Center, Southwest University, No. 2 Tiansheng Road Beibei, Chongqing 400715, China

We made several variations of RESET constructs, including flanking lox sites lacking cytosines that may be resistant to DNA methylation



Holliday Junction Strand Exchange (loxP)

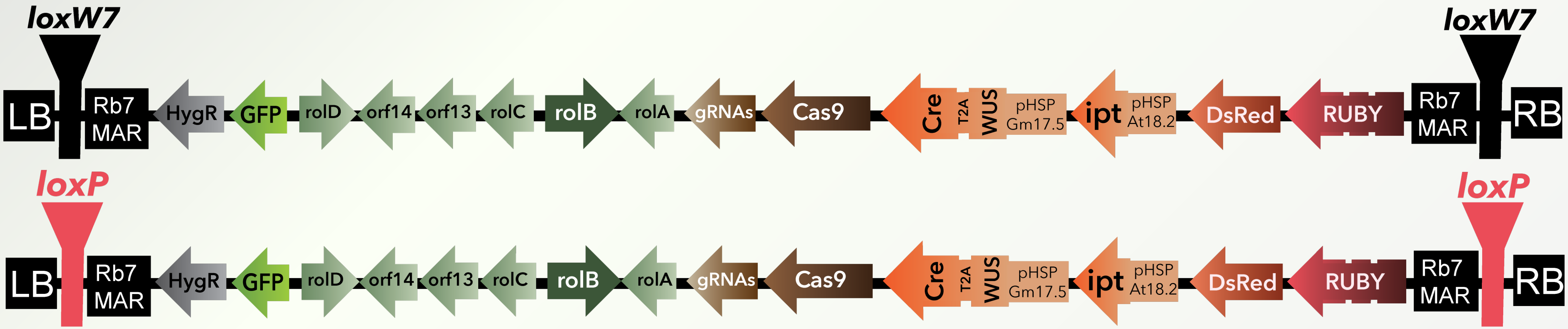
Steric hinderance in somatic plant cells



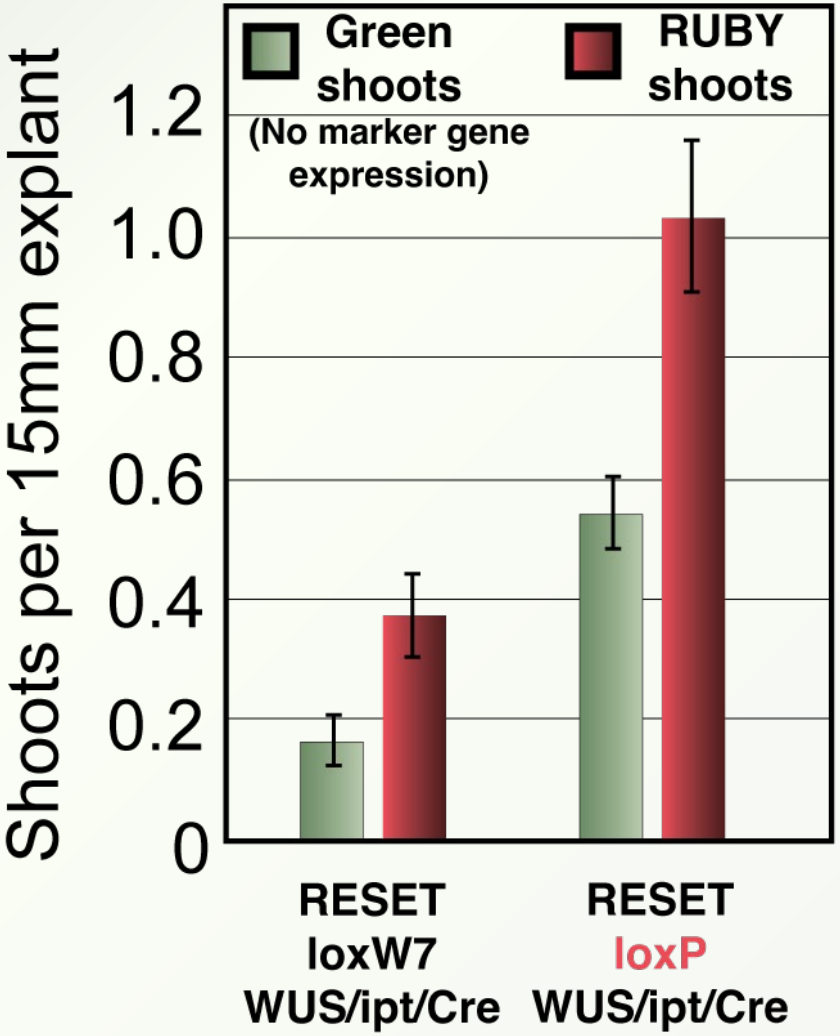
Holliday Junction Strand Exchange (LoxW)

Increased recombination efficiency in plant somatic cells

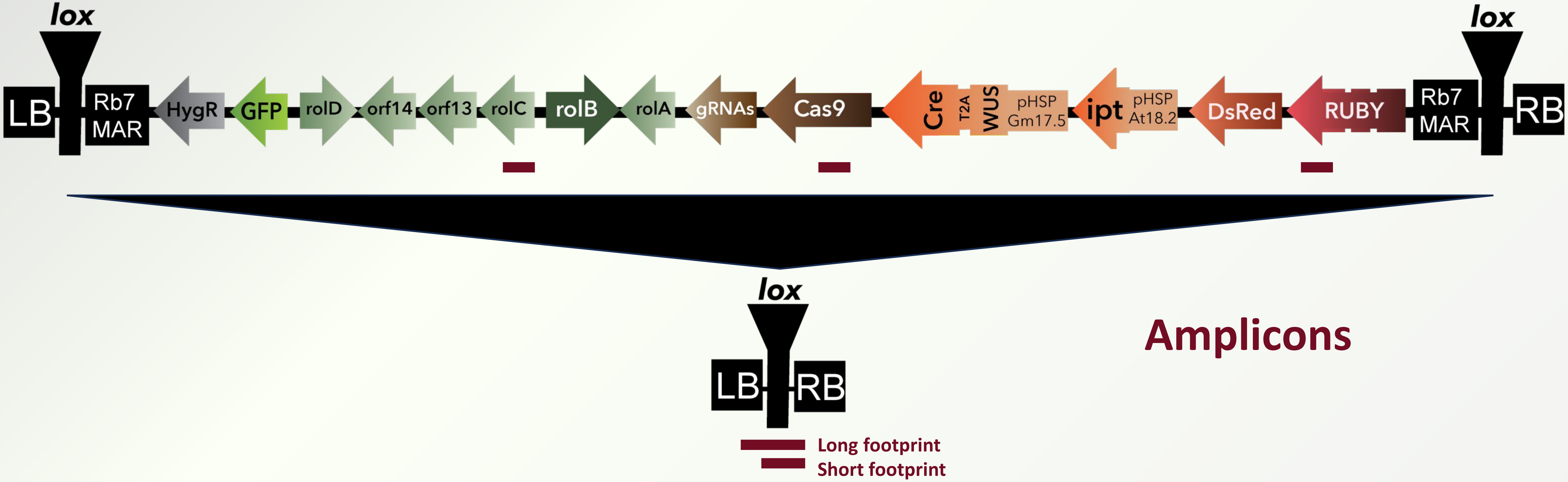
We compared rates of excision of the two lox sites



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

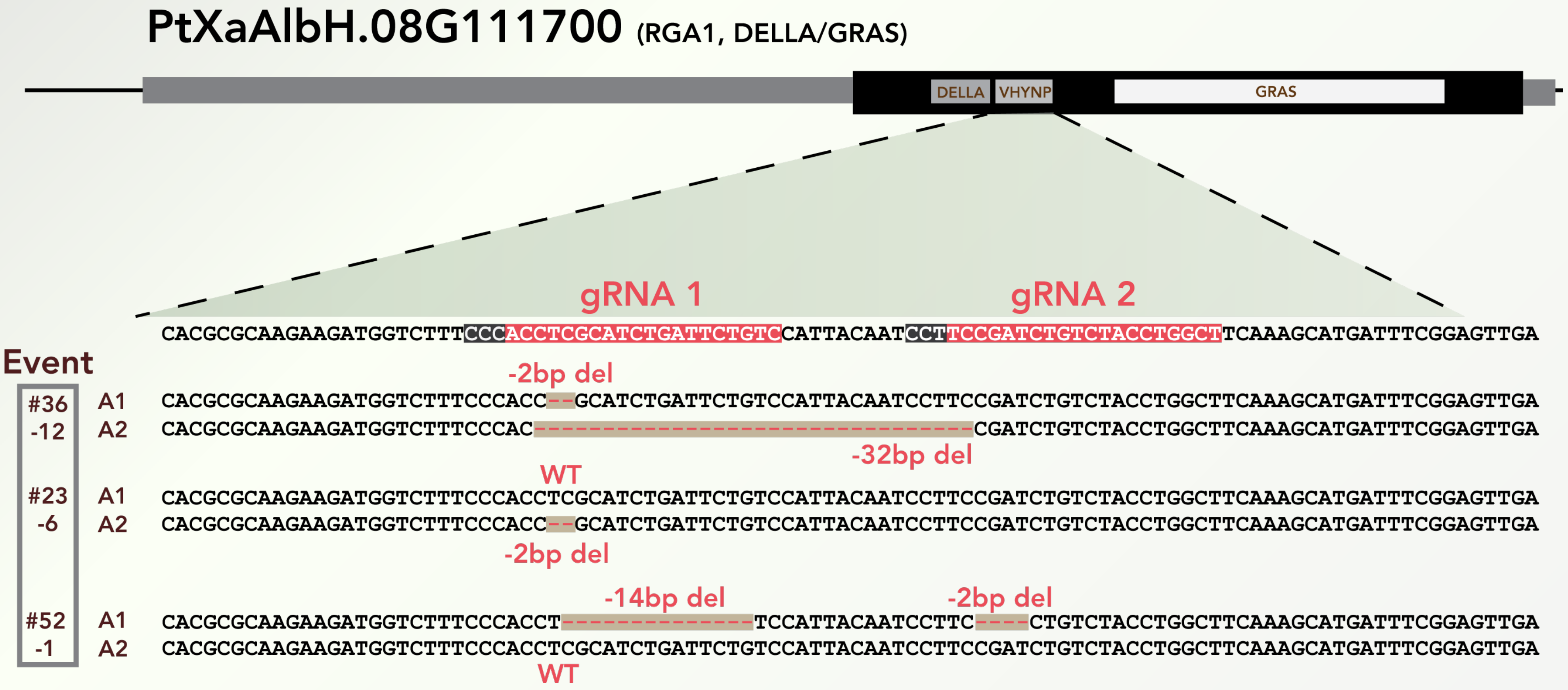


To figure out if the shoots without RUBY, GFP, and DsRed were completely excised, we used a panel of PCR amplicons



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

We also found high rates of editing among excised RESET shoots



PtXaAlbH.08G111700 (RGA1, DELLA/GRAS)

gRNA 1 gRNA 2

`CACGCGCAAGAAGATGGTCTTTCCCACCTCGCATCTGATTCTGTCCATTACAATCCTTCCGATCTGTCTACCTGGCTTCAAAGCATGATTTCGGAGTTGA`

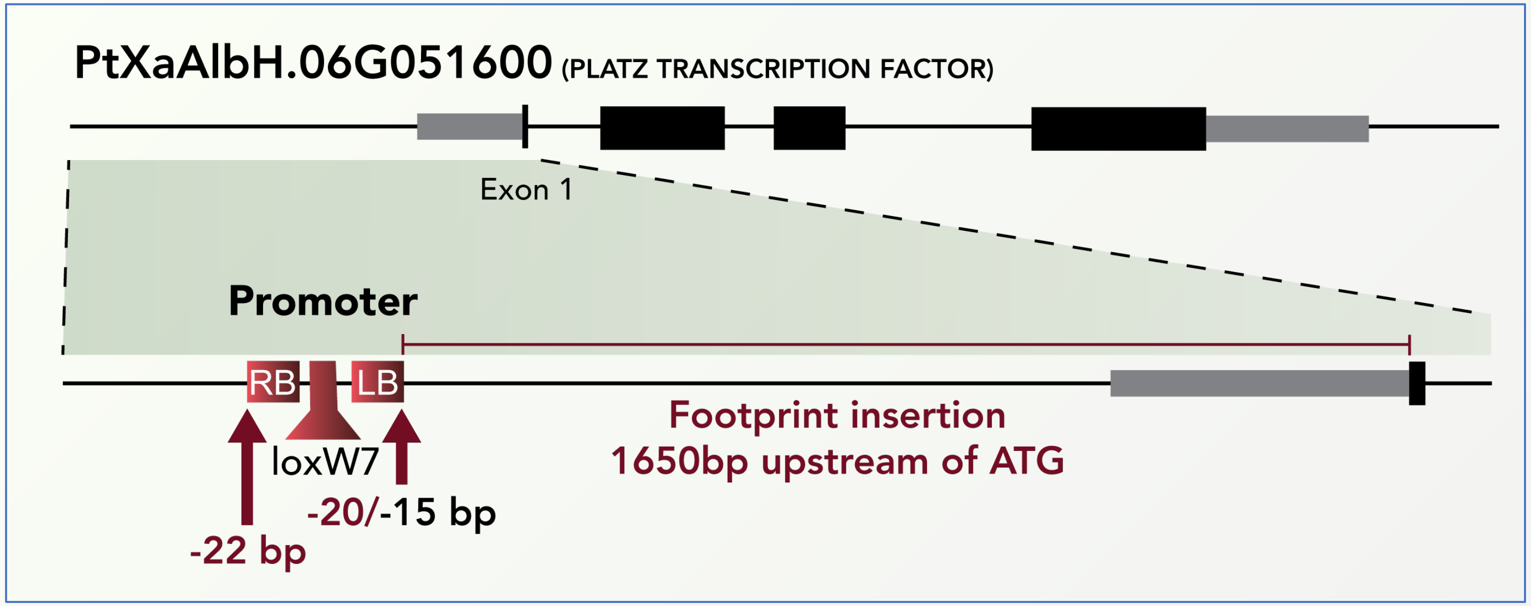
Event

#36 -12	A1	-2bp del `CACGCGCAAGAAGATGGTCTTTCCCACC--GCATCTGATTCTGTCCATTACAATCCTTCCGATCTGTCTACCTGGCTTCAAAGCATGATTTCGGAGTTGA`
	A2	`CACGCGCAAGAAGATGGTCTTTCCCAC--------------------------------CGATCTGTCTACCTGGCTTCAAAGCATGATTTCGGAGTTGA` -32bp del
#23 -6	A1	WT `CACGCGCAAGAAGATGGTCTTTCCCACCTCGCATCTGATTCTGTCCATTACAATCCTTCCGATCTGTCTACCTGGCTTCAAAGCATGATTTCGGAGTTGA`
	A2	`CACGCGCAAGAAGATGGTCTTTCCCACC--GCATCTGATTCTGTCCATTACAATCCTTCCGATCTGTCTACCTGGCTTCAAAGCATGATTTCGGAGTTGA` -2bp del
#52 -1	A1	-14bp del -2bp del `CACGCGCAAGAAGATGGTCTTTCCCACCT--------------TCCATTACAATCCTTC----CTGTCTACCTGGCTTCAAAGCATGATTTCGGAGTTGA`
	A2	`CACGCGCAAGAAGATGGTCTTTCCCACCTCGCATCTGATTCTGTCCATTACAATCCTTCCGATCTGTCTACCTGGCTTCAAAGCATGATTTCGGAGTTGA` WT

Analysis is ongoing but so far 6/10 events (60%) were edited to fixation in at least one allele

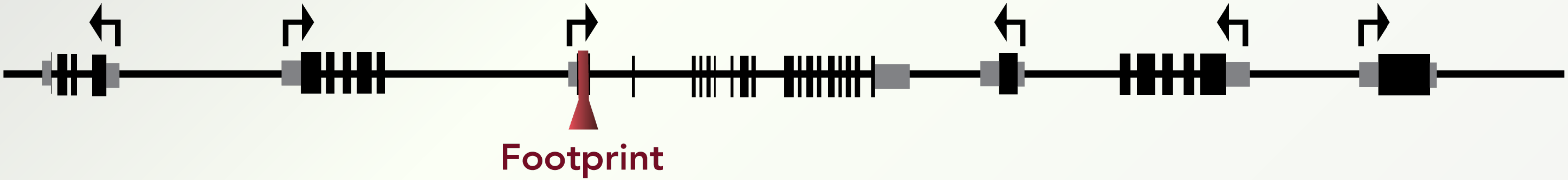
We used TAIL-PCR and whole genome sequencing to find where transgenes landed, and how they resolved after excision

- Usual genome/border truncations at site of insertion
- Complete excision in two single-copy insertion events sequenced to date



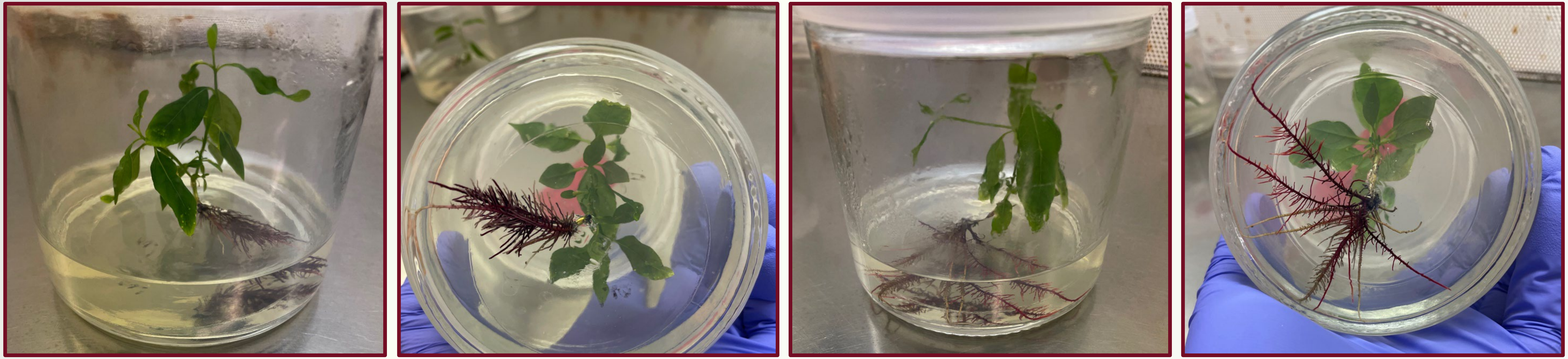
Could these one day be considered “clean” edits for regulatory purposes?

Are footprints inert, are expression/epigenetic effects on surrounding genes different from cisgenic inserts or conventional breeding/mutation?

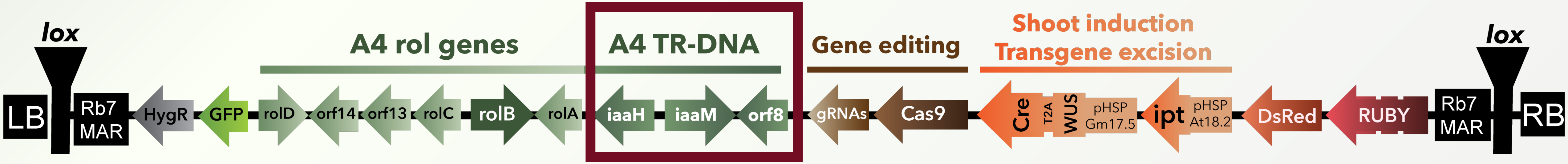


- More whole genome sequencing
- Gene expression analysis of surrounding genes
- Local epigenetic/methylation impacts

Does this work in other recalcitrant species?

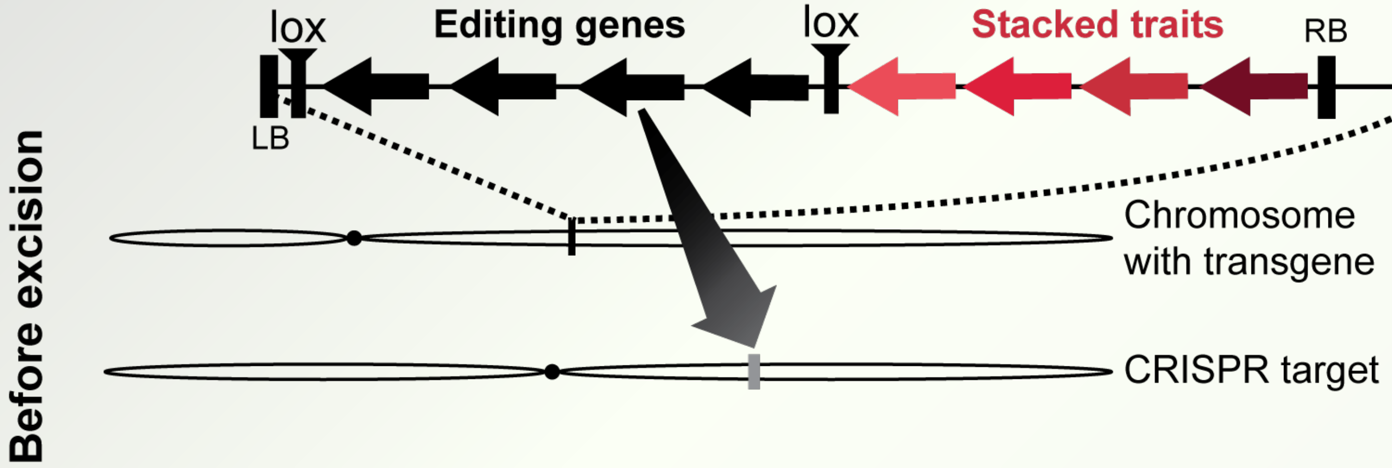


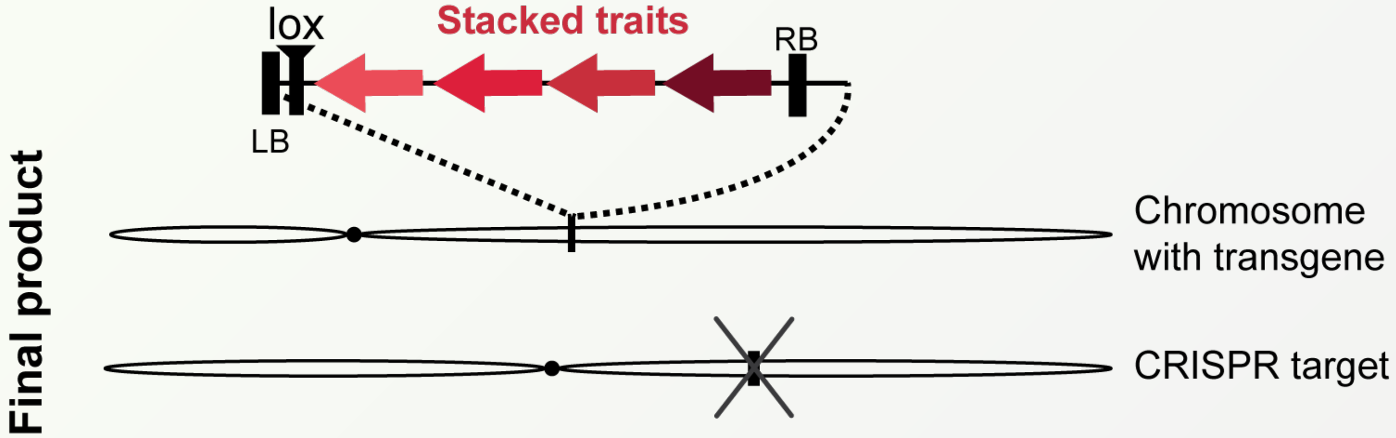
Eucalyptus grandis x urophylla RESET composite plants



Auxin biosynthetic circuit required for high rates of hairy-root induction in Eucalyptus

We envision this system being used for simultaneous editing and/or transgene insertion, and enabling sequential addition of genes and edits





SUMMARY: The RESET system appears efficient and functional

- High rates of hairy root transformation in two poplar genotypes
- Efficient heat-induced regeneration and transgene excision, especially with loxP
- High rates of editing in excised shoots
- Clean footprints after excision
- Significant numbers of escape shoots (which should be easily eliminated through adding selection during hairy root subculture)
- Though complex, we hope this system will function broadly in clonal woody plant species
- We hope that system will come to be recognized as “clean” by regulatory agencies

Acknowledgements: People

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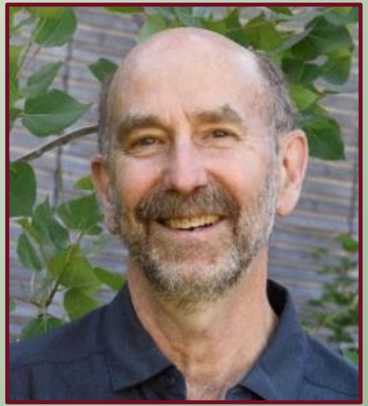
Tissue culture and transformation

Kate Peremyslova
Tissue culture and transformation



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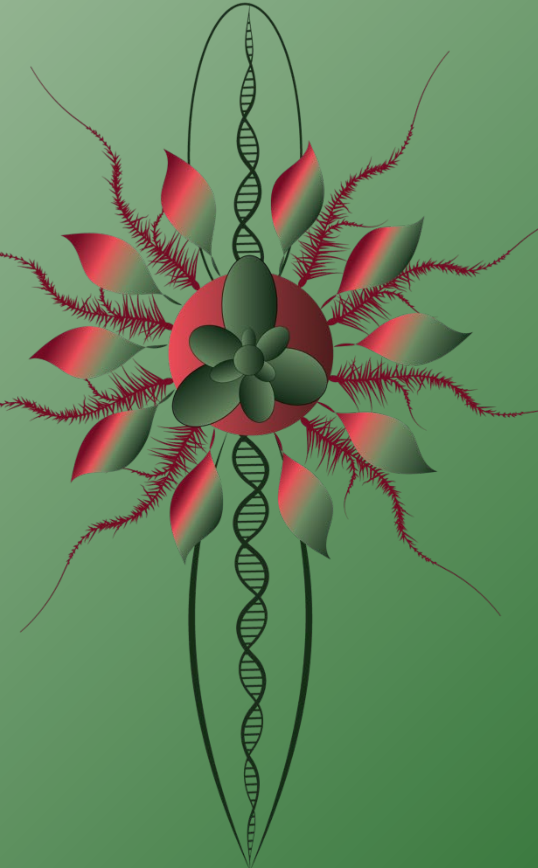

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Thanks for listening!