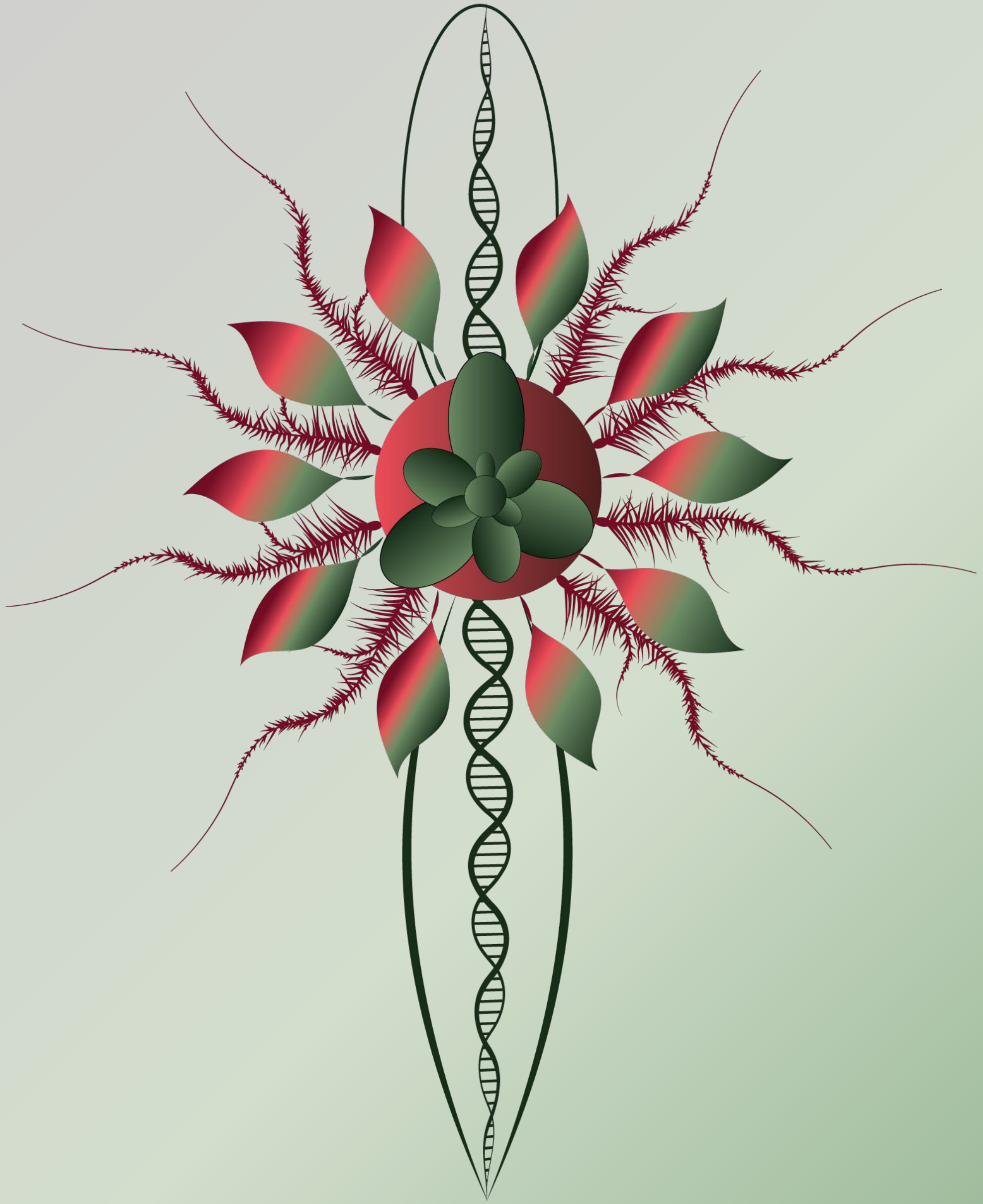


RESET

A hairy root-to-shoot transgene excision system for improved transformation and clean editing in clonally propagated plants

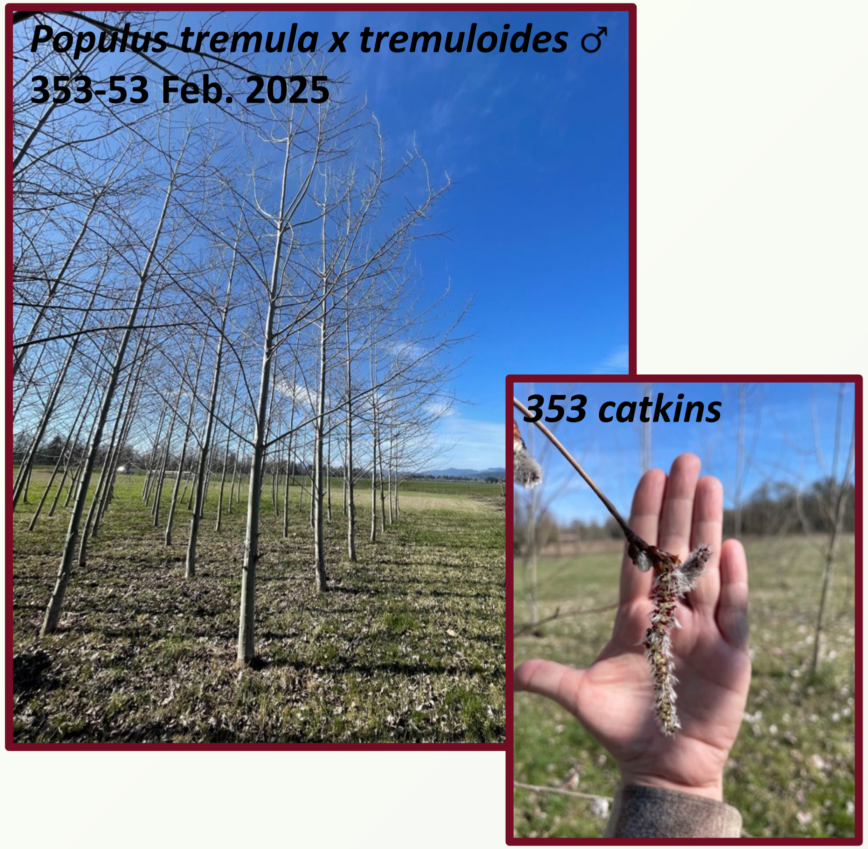
Greg Goralogia
Laboratory of Steve Strauss

Oregon State University
College of Forestry
Forest Ecosystems and Society

We work in tree species which have unique challenges for biotechnology

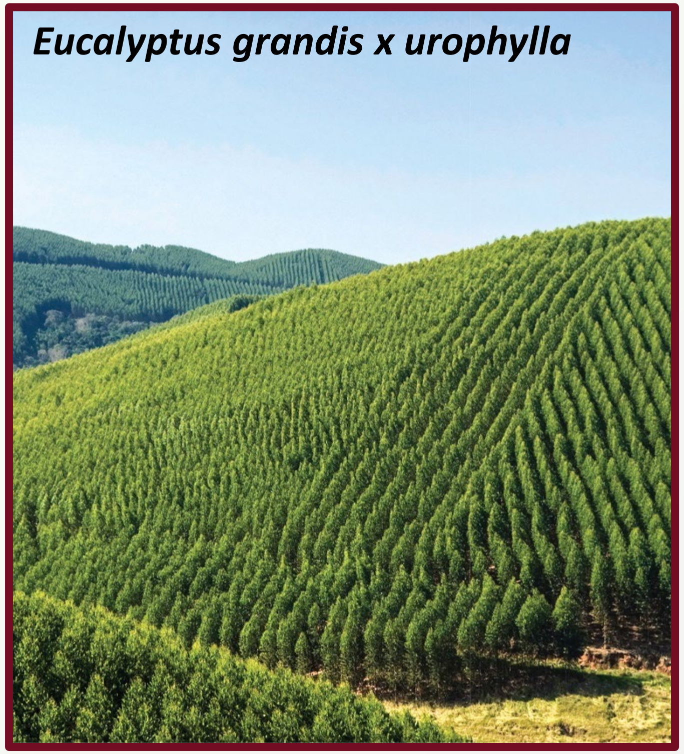

Populus tremula x alba 717-1B4 ♀

CRISPR/Cas9 edited trees –8th year in field


Populus tremula x tremuloides ♂

353-53 Feb. 2025

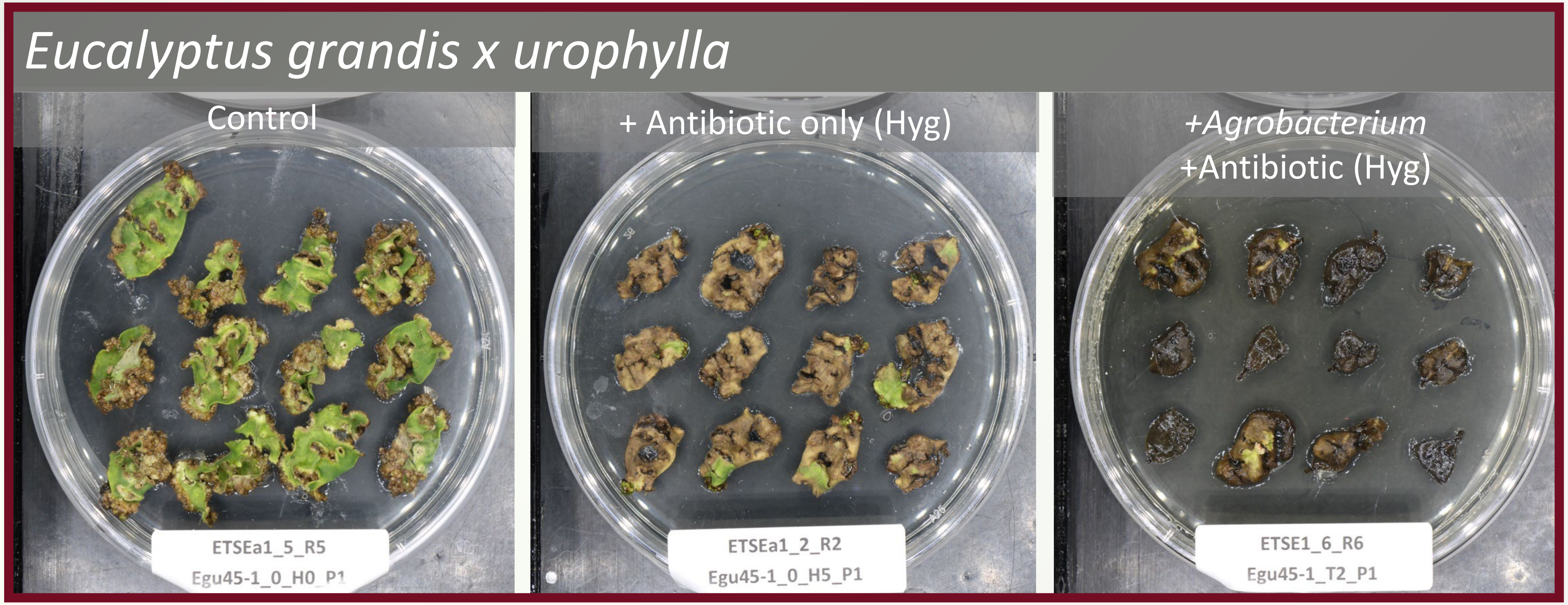
353 catkins


Eucalyptus grandis x urophylla

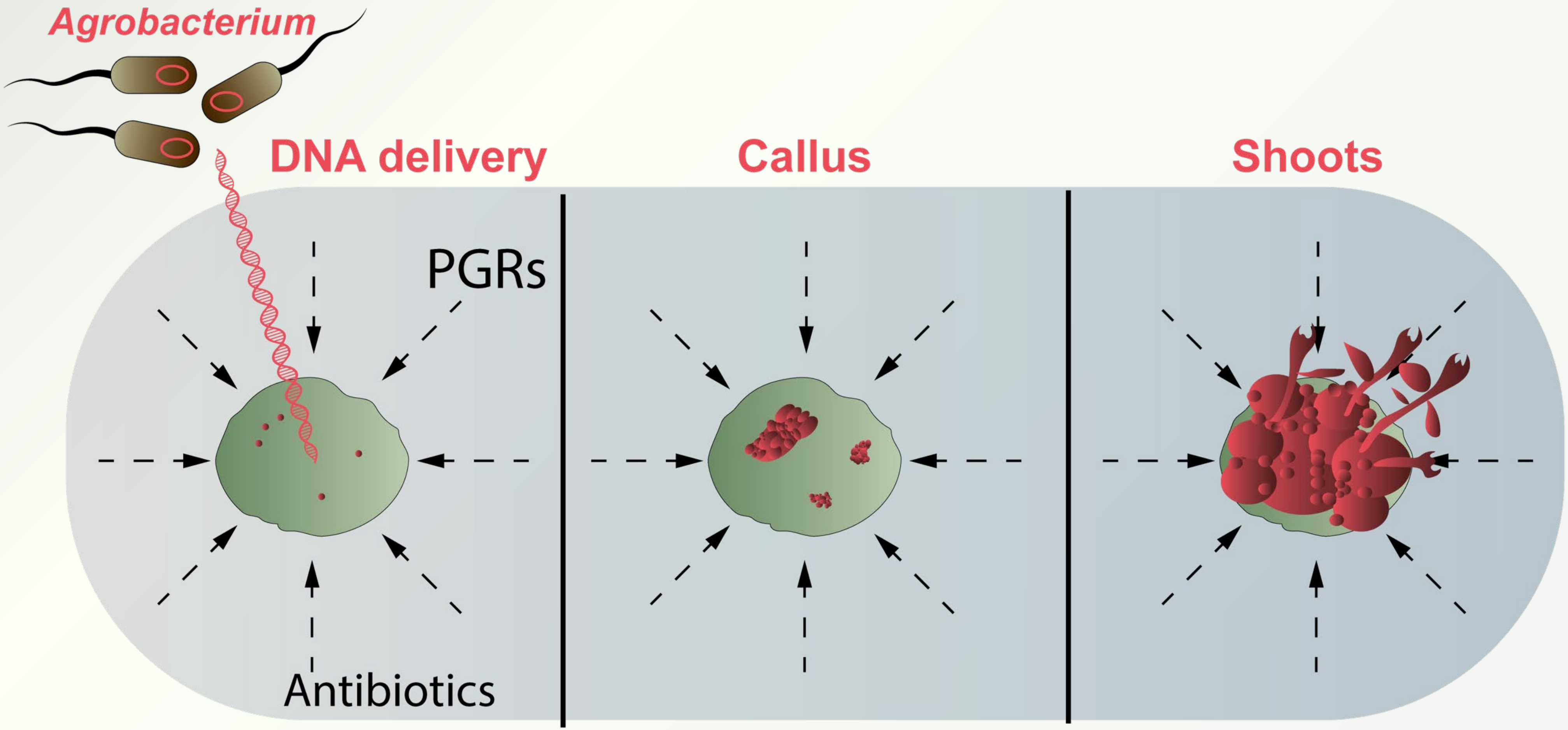
- Long testing cycles
- Regulatory challenges
- Clones are wide interspecific crosses, products of long breeding programs

Many tree species are difficult to transform

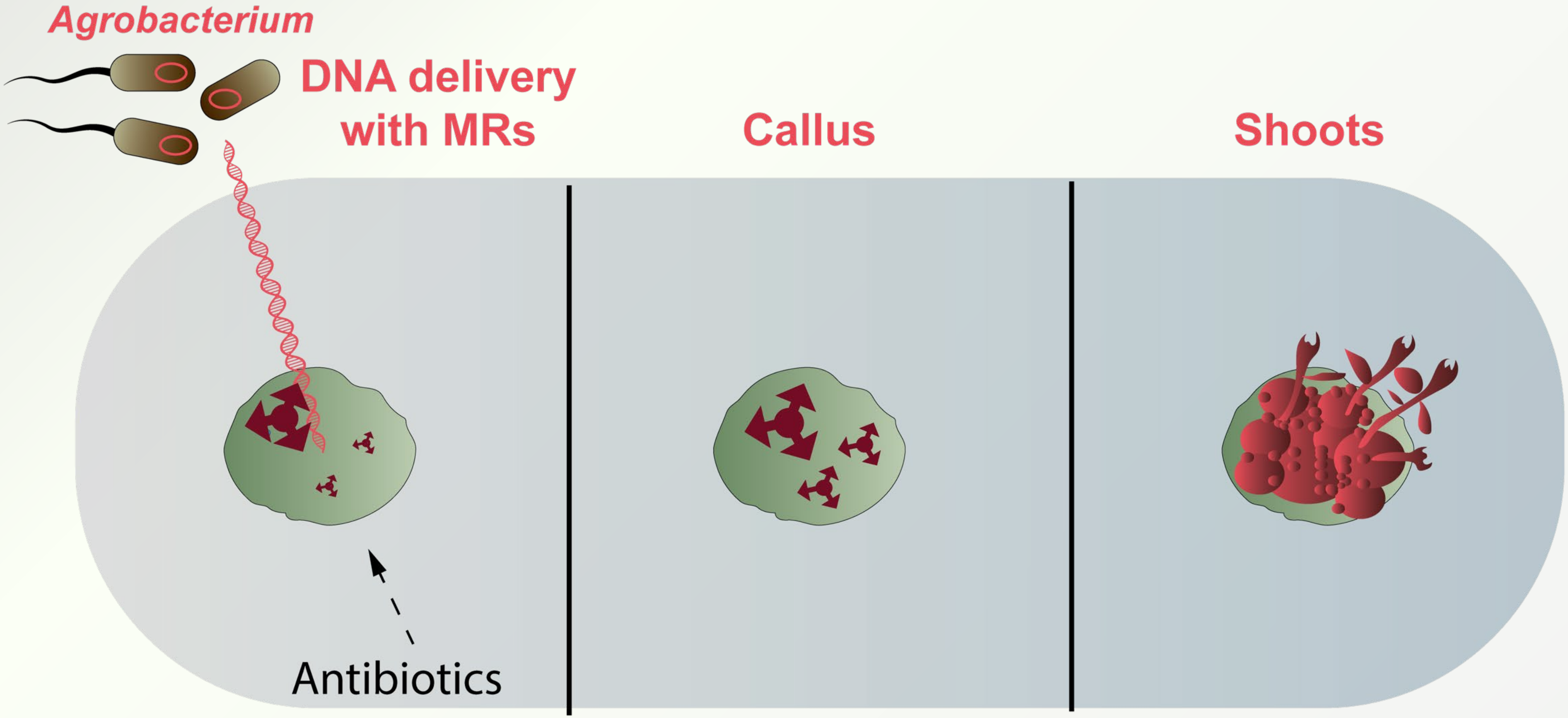
- Elite clones, not seed-derived
- High heterozygosity: each genotype a new adventure *in vitro*
- High physiological diversity and common defense responses



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



Morphogenic regulators spur developmental reprogramming via delivere 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*

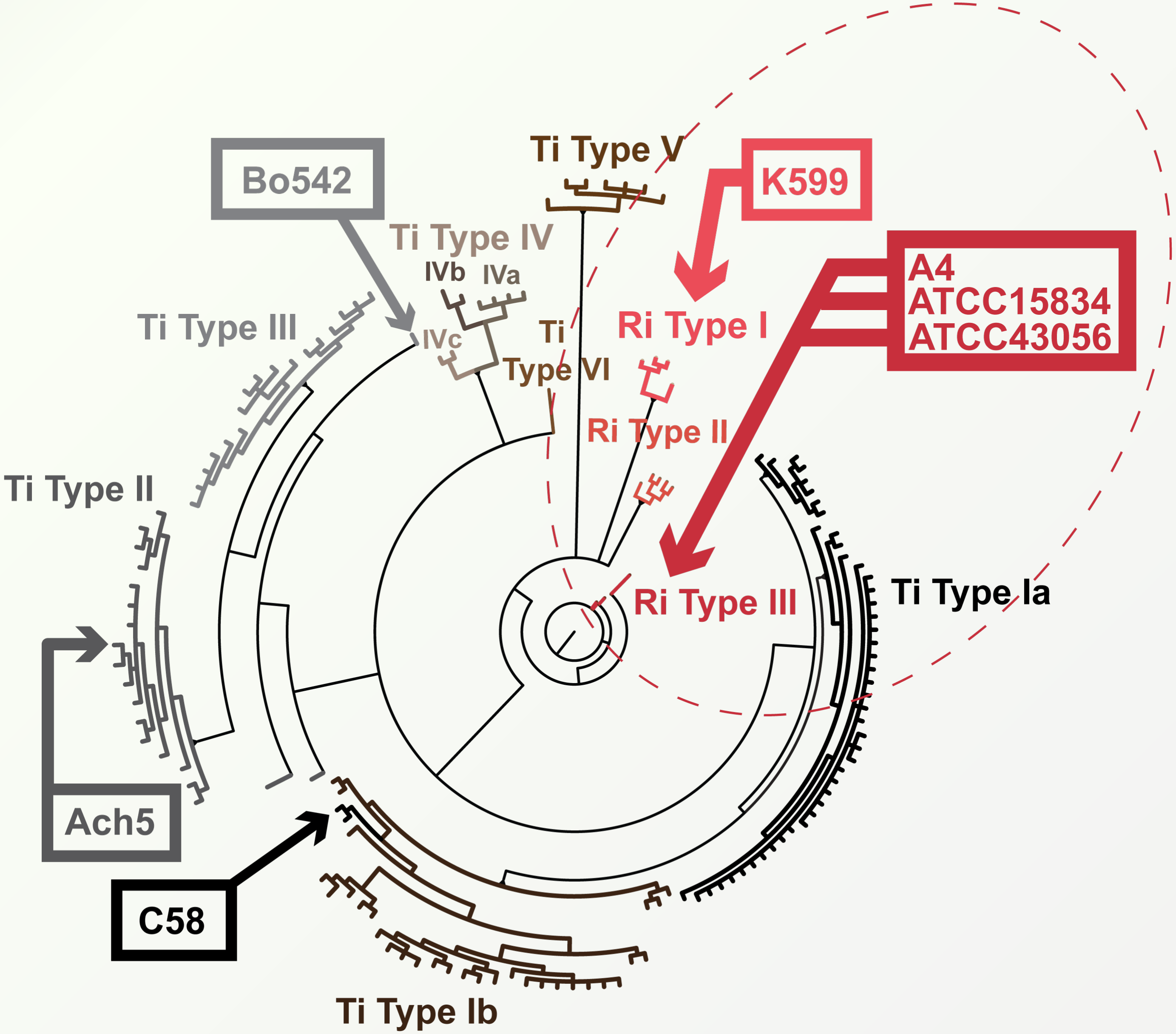
- LEC 1, 2 – LEAFY COTYLEDON
- EBB1 - EARLY BUD BREAK 1 (ESR family)
- BBM – BABY BOOM
- WOX 5, 11 -- WUSCHEL RELATED HOMEOBOX
- WUS – WUSCHEL
- GRF-GIF – GROWTH REGULATOR FACTOR 4 and GRF INTERACTING FACTOR 1
- *Agrobacterium* growth promoting genes
- rol – Hairy root-inducing genes – *Agrobacterium*

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

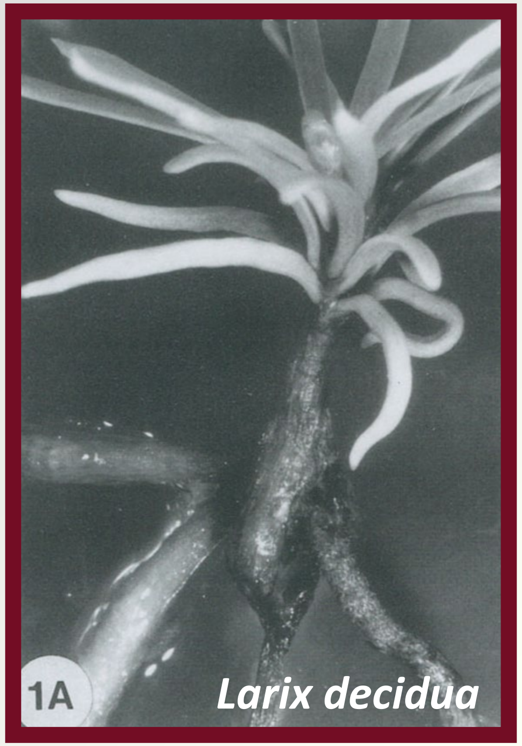
- LEC 1, 2 – LEAFY COTYLEDON
- EBB1 - EARLY BUD BREAK 1 (ESR family)
- BBM – BABY BOOM
- WOX 5, 11 -- WUSCHEL RELATED HOMEOBOX
- WUS – WUSCHEL
- GRF-GIF – GROWTH REGULATOR FACTOR 4 and GRF INTERACTING FACTOR 1
- *Agrobacterium* growth promoting genes
- ***rol* – Hairy root-inducing genes – *Agrobacterium***

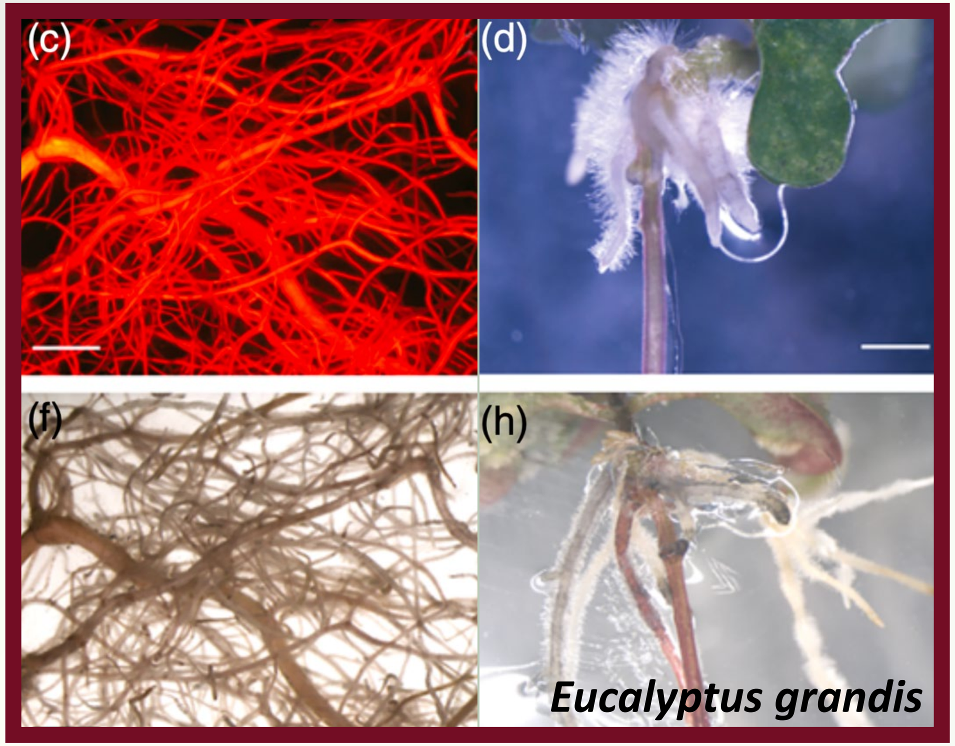
Hairy root disease is caused by unique T-DNA genes contained in Ri plasmids

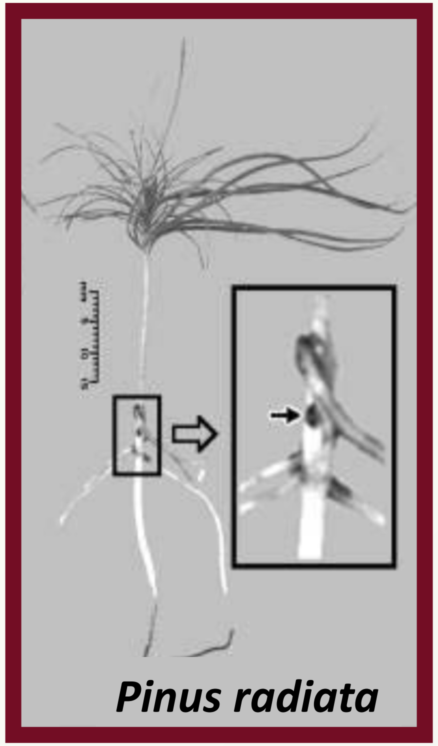
Hairy roots in hydroponically grown tomato



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

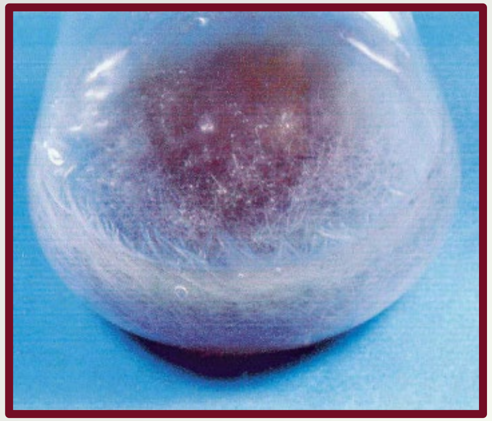






Huang et al. 1991 *IVCDB-Plant*, Placencia et al. 2016. *Plant Biotech J.*, Li et al. 2003 *EJ Biotech,* Gomes et al. 2019 *FIPS,* Du et al. 2025 *PNAS*.

Hairy root transformation is effective in many recalcitrant clonal specialty crops



Mentha peperita

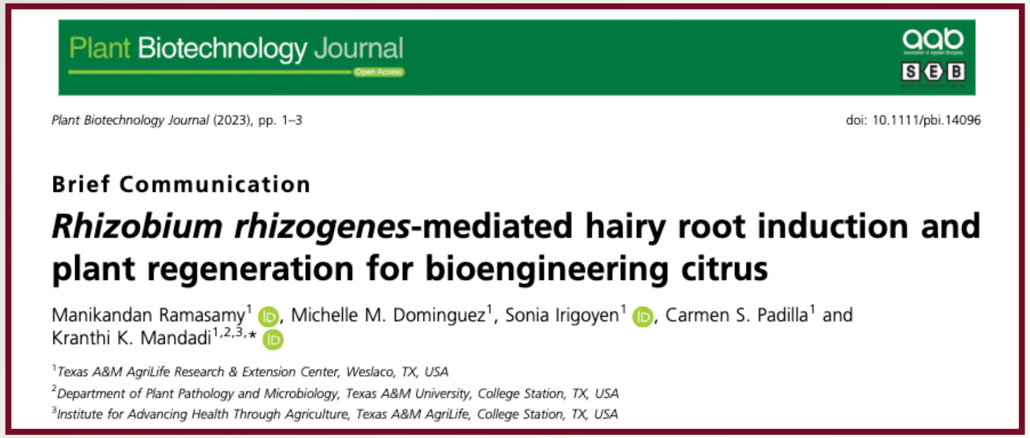
Corylus avellana

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

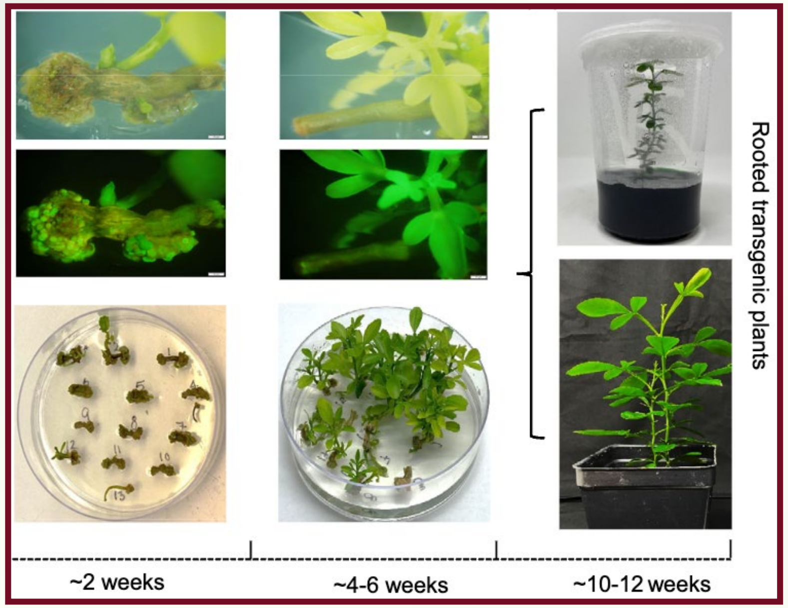
Plant Biotechnology Journal (2023), pp. 1–3 doi: 10.1111/pbi.14096

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

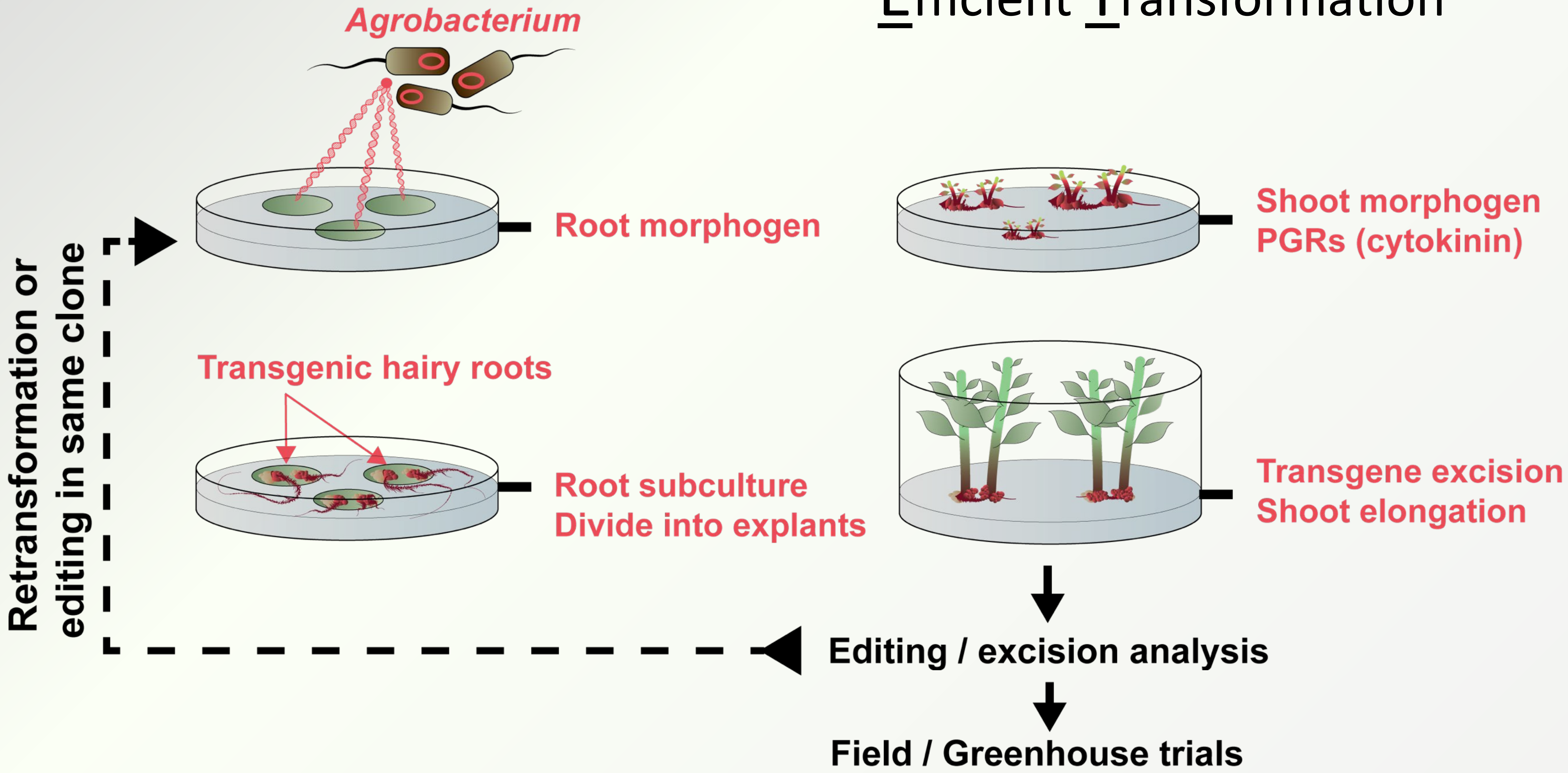


Osteospermum fruticosum (cape daisy)

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



Agrobacterium

Root morphogen

Transgenic hairy roots

Root subculture
Divide into explants

Shoot morphogen
PGRs (cytokinin)

Transgene excision
Shoot elongation

Editing / excision analysis

Field / Greenhouse trials

Retransformation or editing in same clone

Root to shoot regeneration is a synthesis of older ideas

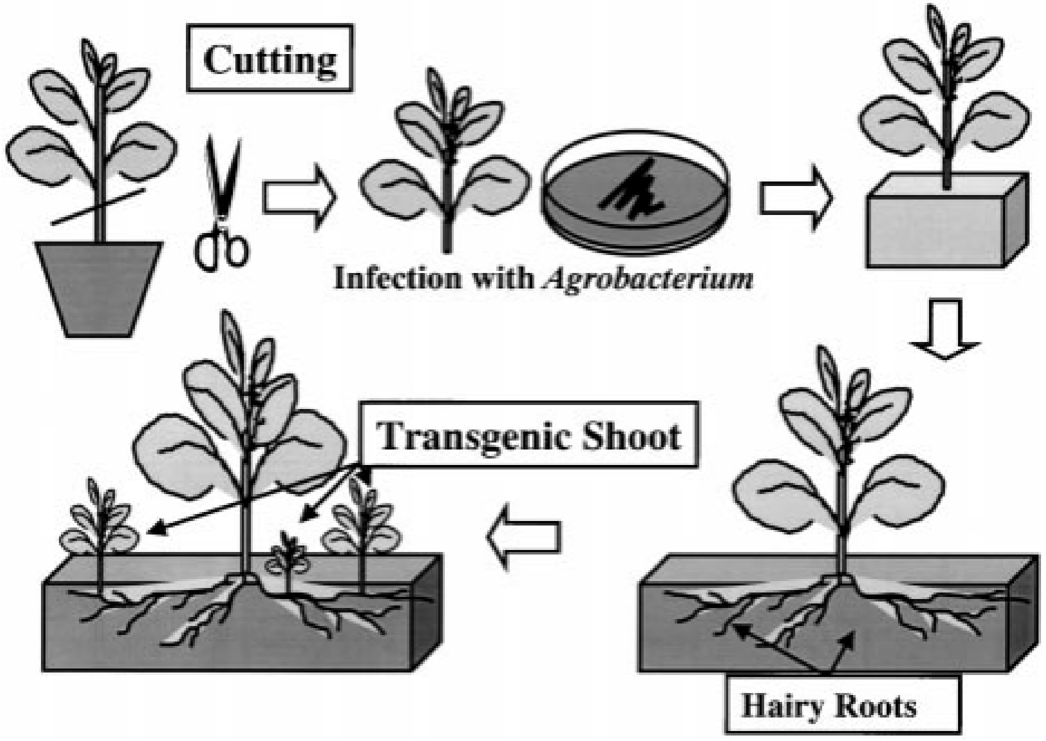
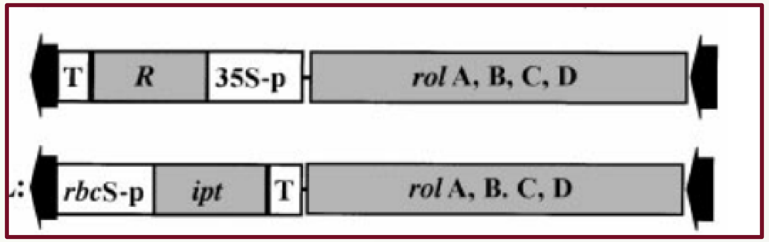


FIG. 8. Outline of *in vivo* transformation using cuttings. Cuttings are infected with *Agrobacterium* containing the *rol*-type MAT vector. After their rooting, marker-free transgenic shoots are induced from hairy roots by the light.

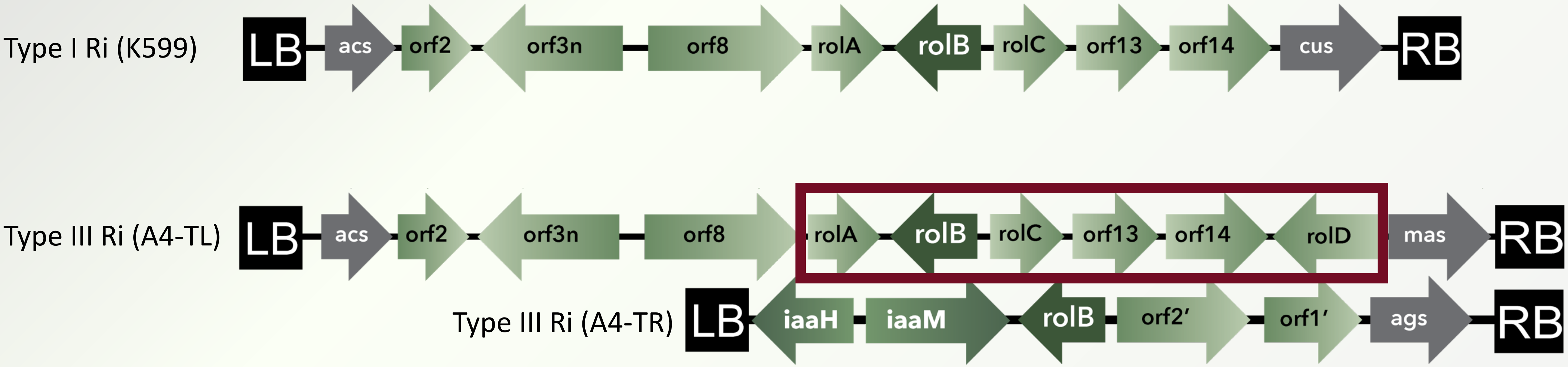


from Ebinuma and Komamine,
2001 In vitro cell and developmental biology -Plant

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

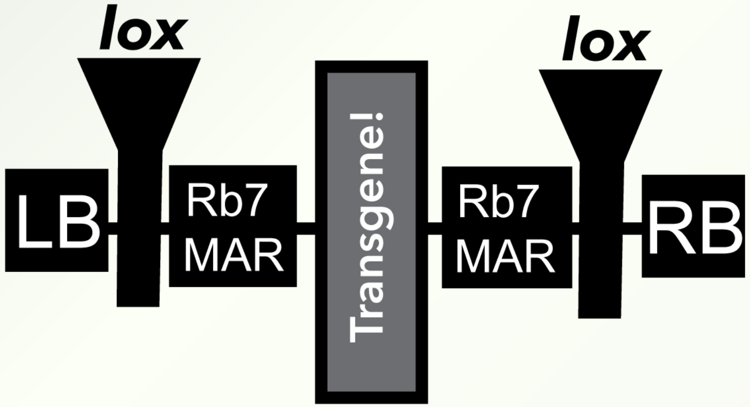
Let's go shopping for parts!

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



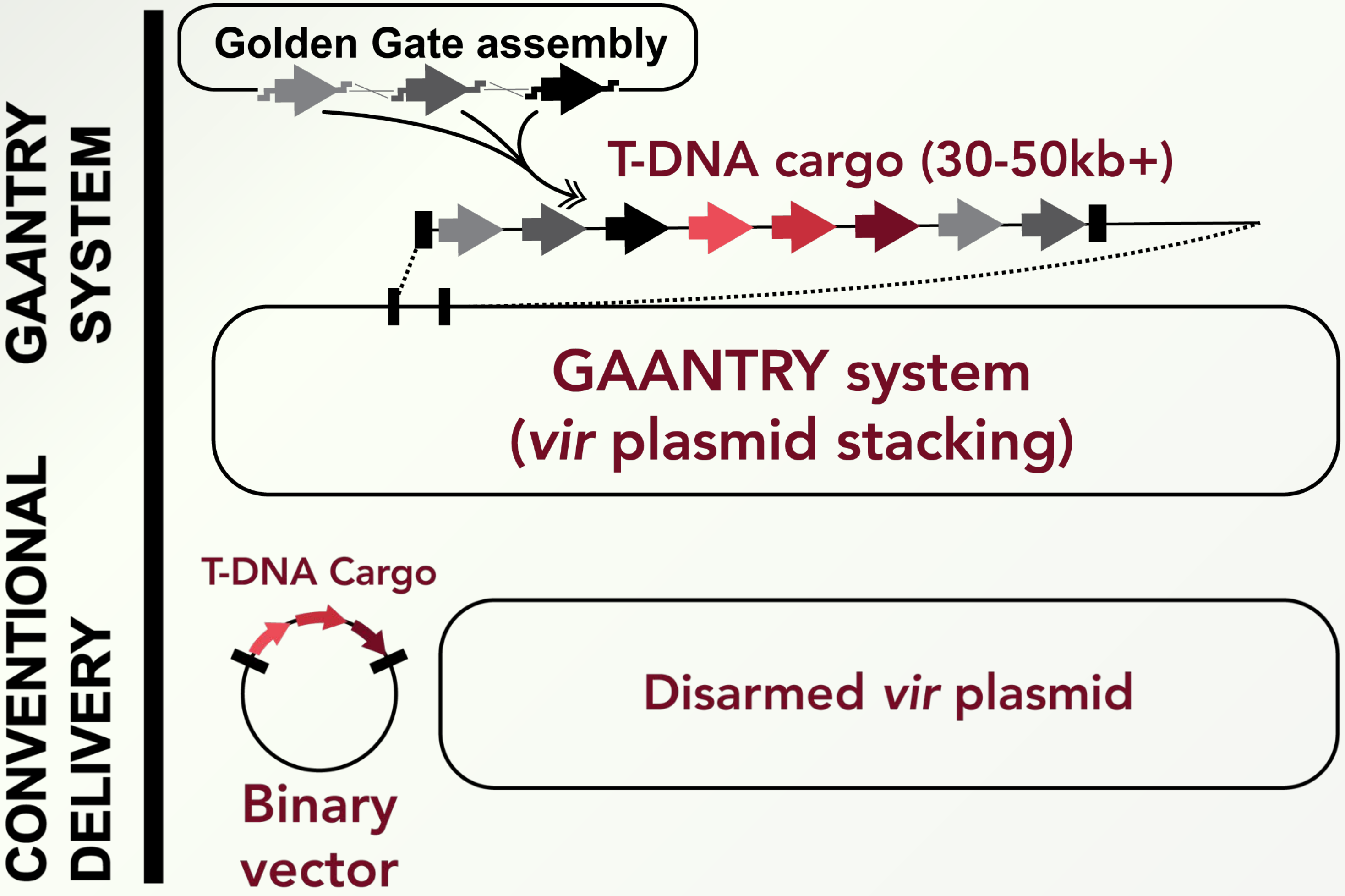
- *rolB* is required for hairy root formation
- Others including *rolA,B,C,D, orf13* and *orf14* quantitatively increase hairy root formation in many species

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

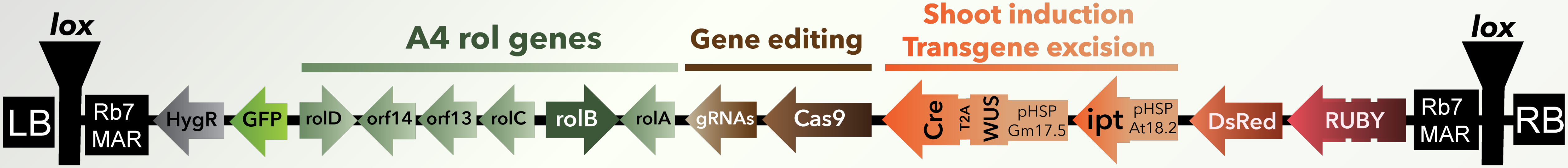


- *WUS* shown to induce shoot trans-differentiation from roots
- *ipt*, an *Agrobacterium* T-DNA gene which produces cytokinins and works well in our experimental system
- Heat shock induction is leaky, but we have tested all promoters in poplar
- Cre recombinase used to induce transgene excision, construct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

Assembly would be next to impossible without GA*A*NTRY



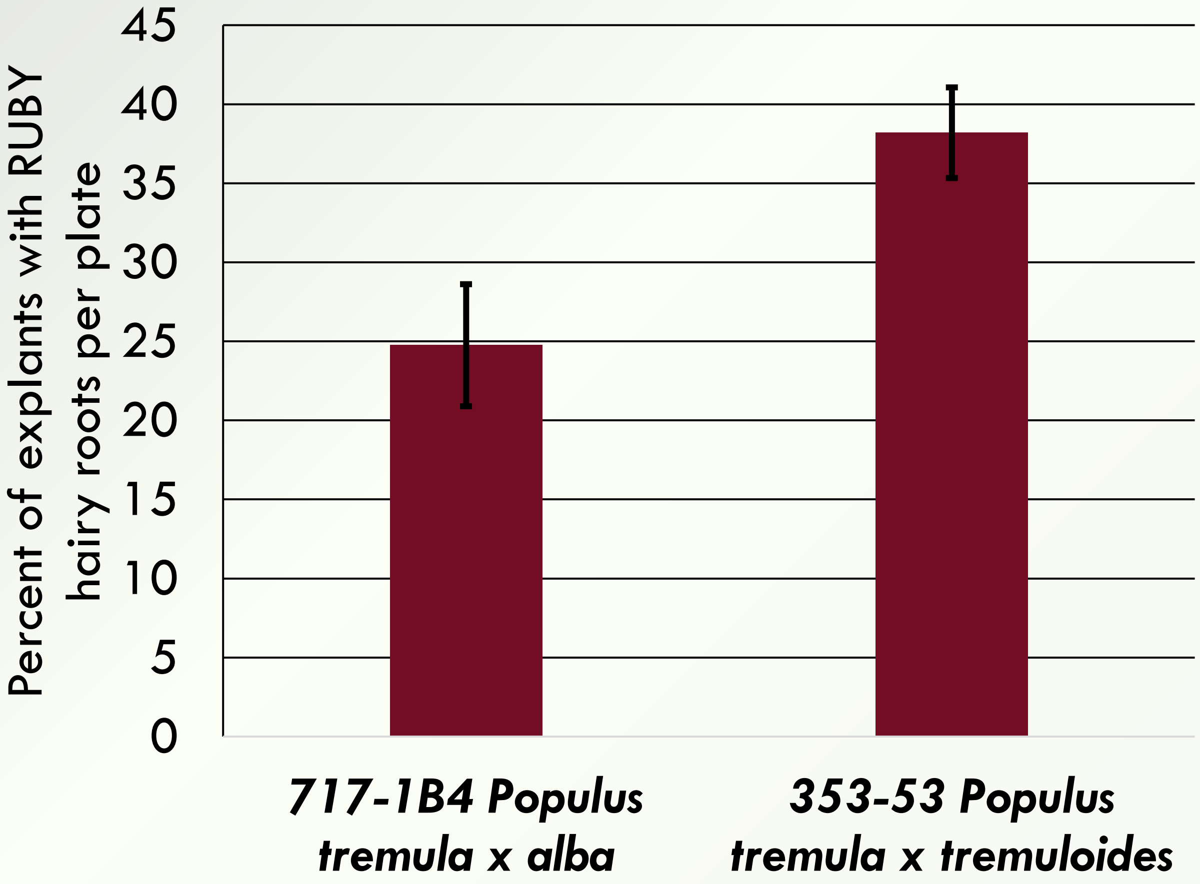
The “Kitchen sink” RESET construct

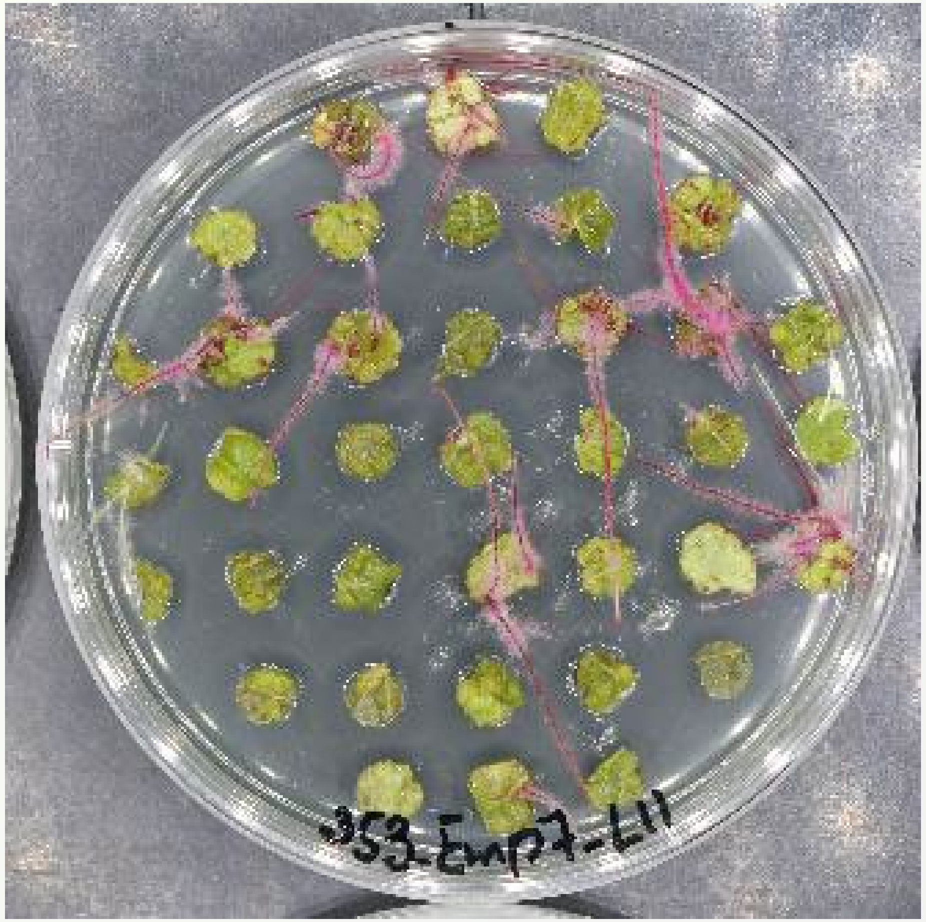


- 39kbp in size in this configuration
- 14 transcriptional units
- 16 independent peptides

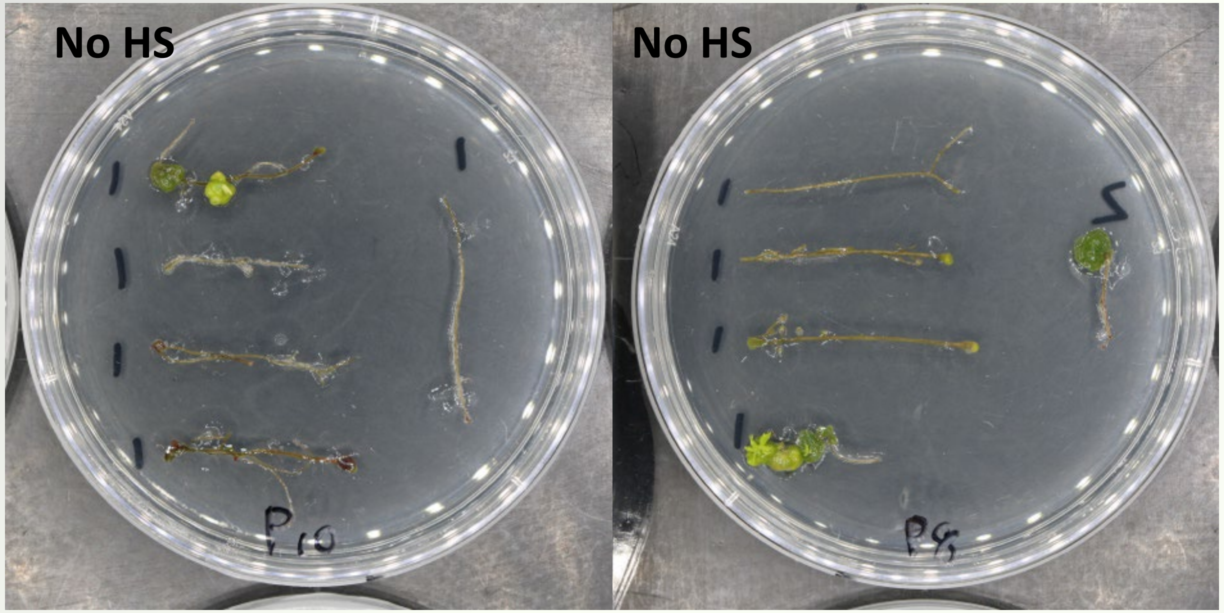
- Included 3 marker genes to identify transgene insertion (GFP, DsRed, and RUBY)
- Includes gene editing through CRISPR-Cas9 (tRNA-arrays targeting *RGA1* gene)
- Hygromycin selection gene present but no selection was used in these experiments

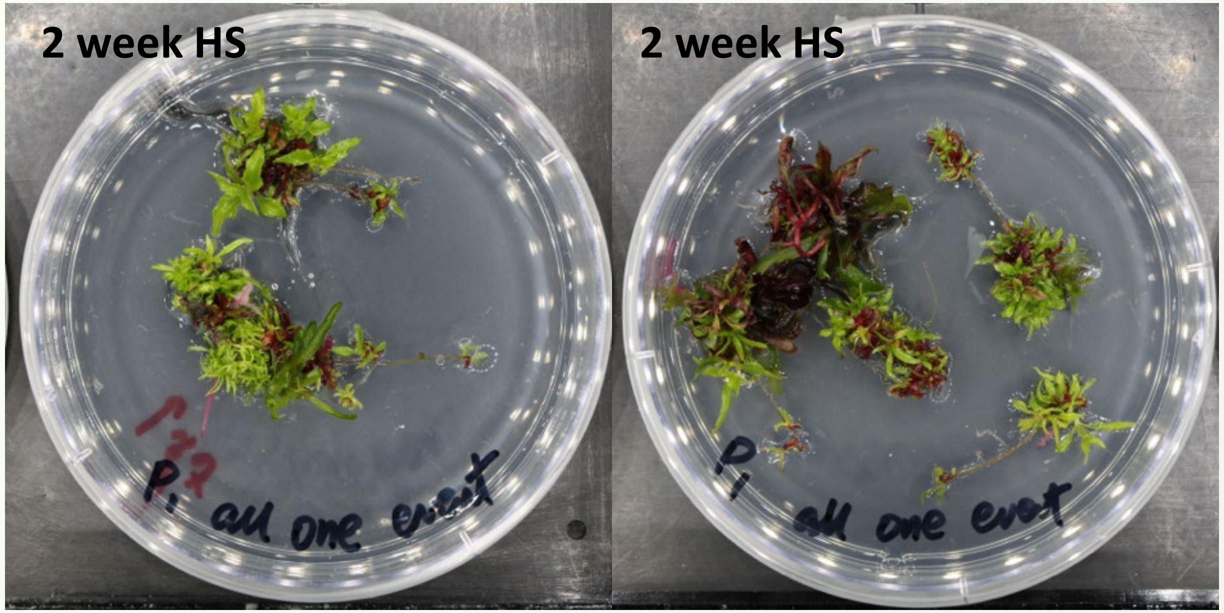
We efficiently generated transgenic hairy roots in two poplar genotypes for regeneration analysis





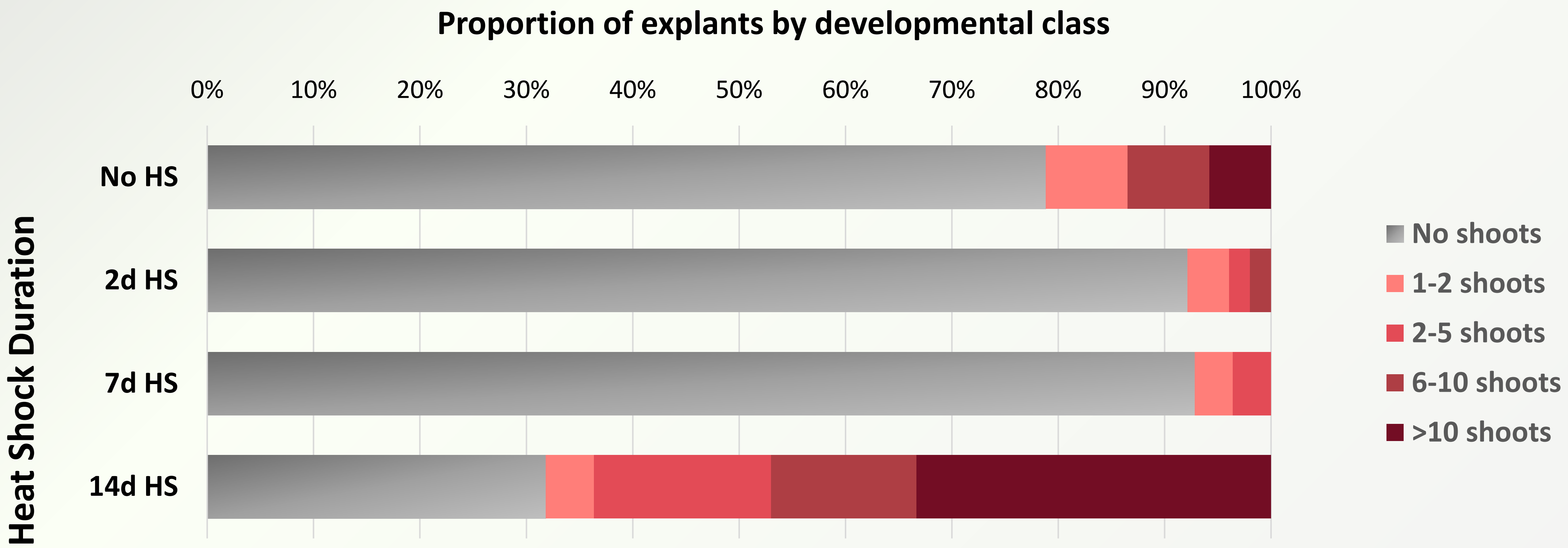
Two weeks of pulsed heat shock resulted in efficient shoot regeneration and excision from hairy roots



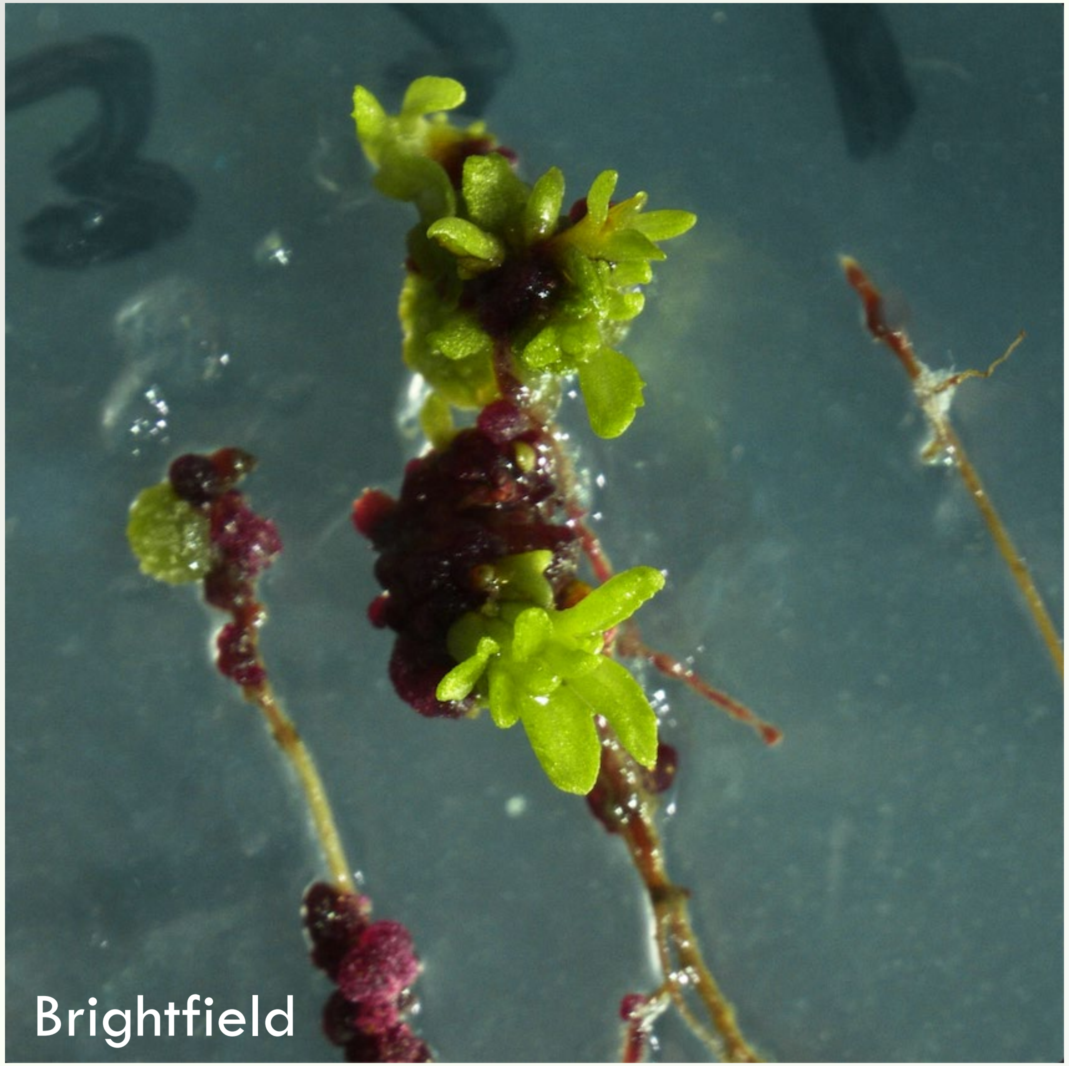


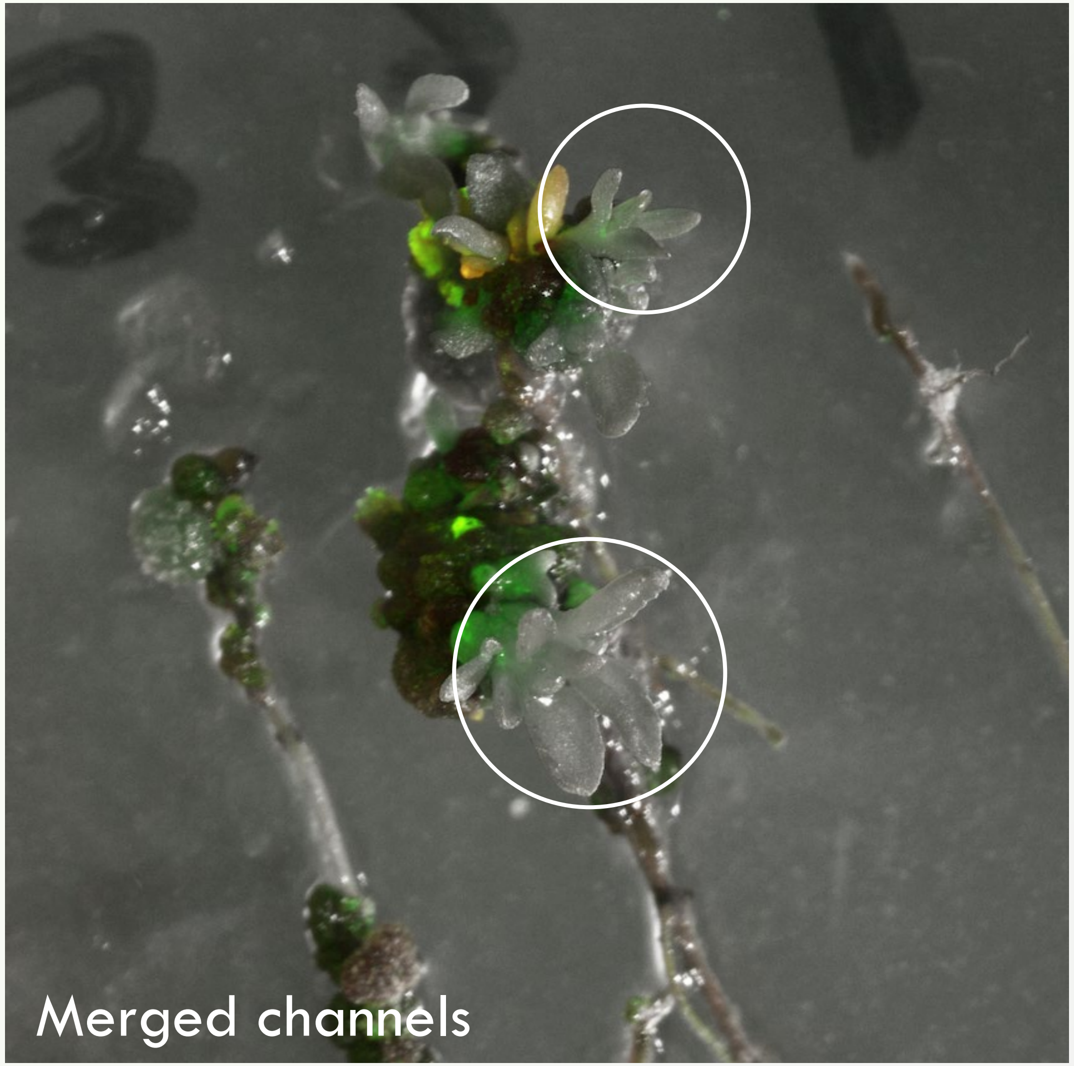
- Four hours heat shock at 39 degrees Celsius, different treatment durations
- RUBY vs. non-ruby shoots can be easily identified for propagation, then other reporters can be closer examined by fluorescent microscopy after isolation

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

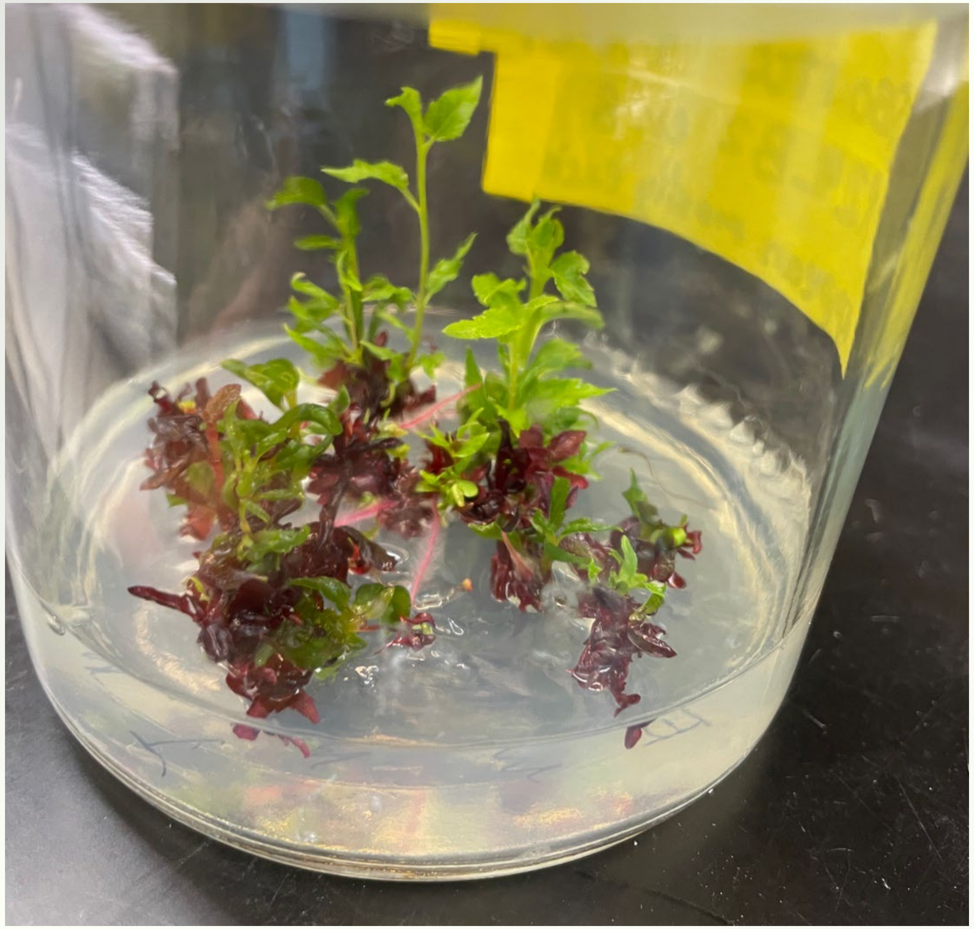


Using multiple reporters (GFP, DsRed and RUBY), we can find shoots with excised transgenes





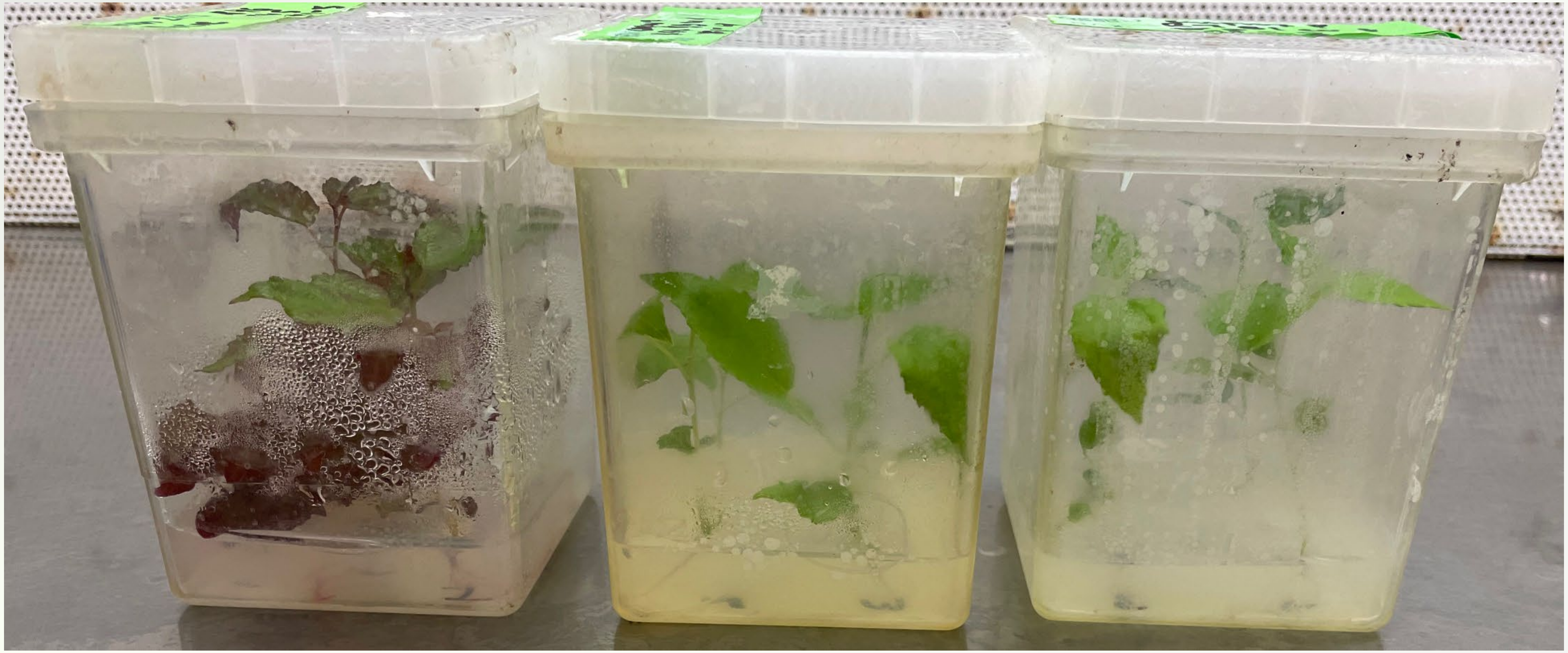
After transfer of regenerating root explants, elongated shoots without marker genes can be identified



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

Populations of putative excised RESET shoots for further molecular analysis

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

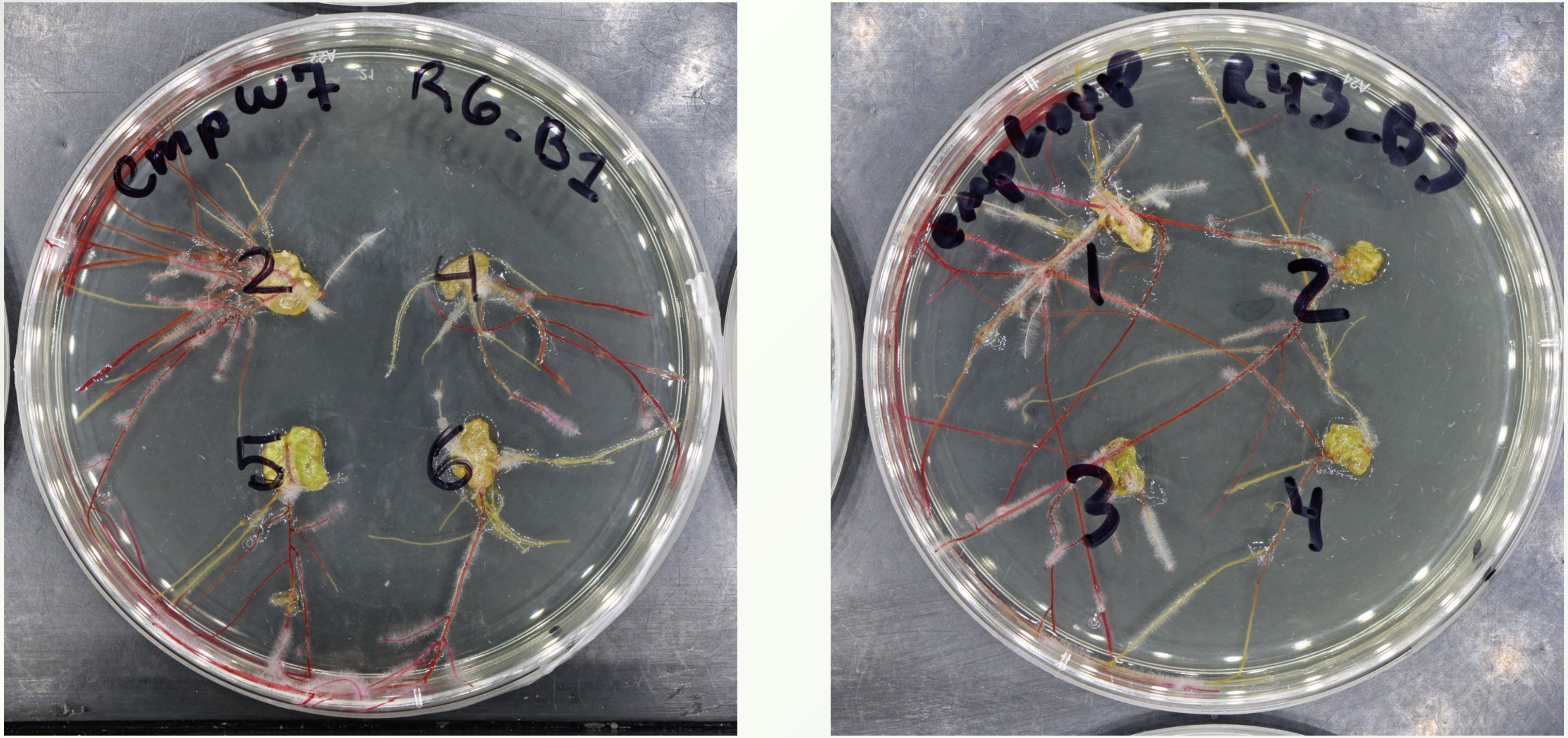


Unexcised transgenic

Putative excised event

Putative excised event

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

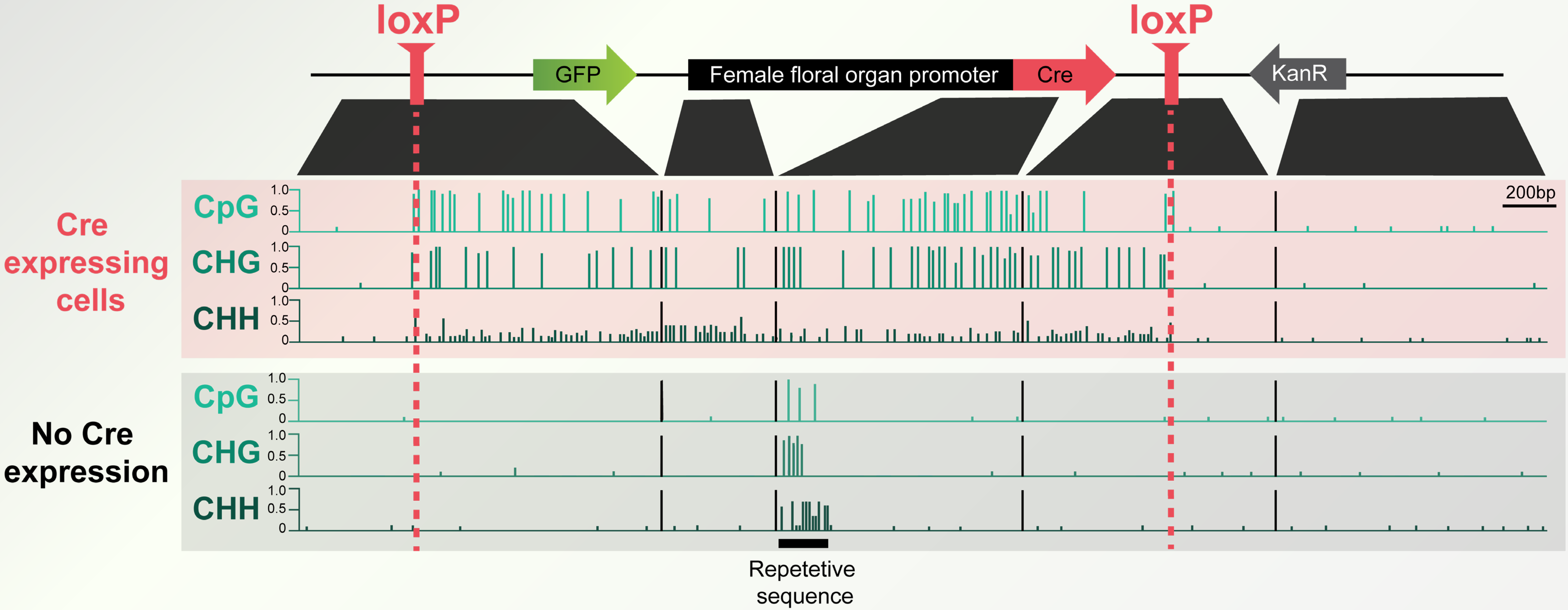
New Phytologist

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

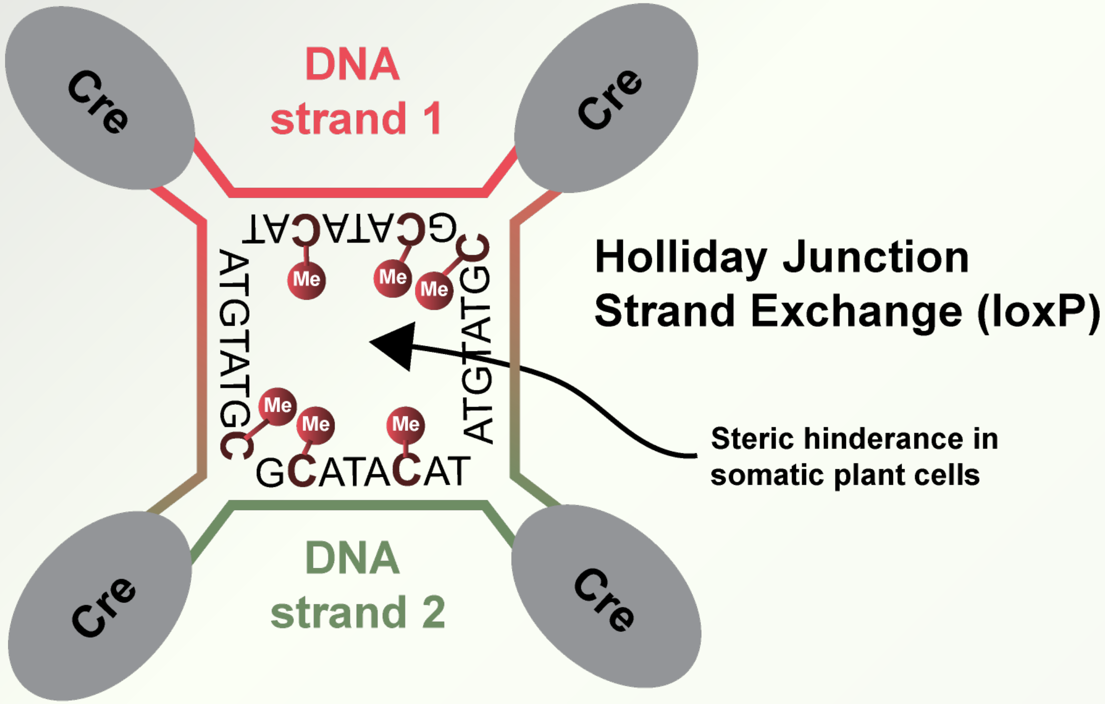
Chongqing Key Laboratory of Application and Safety Control of Genetically Modified Crops; Biotechnology Research Center, Southwest University, No. 2 Tiansheng Road Beibei, Chongqing 400715, China

Cre can methylate transgene regions flanked by its recognition site loxP



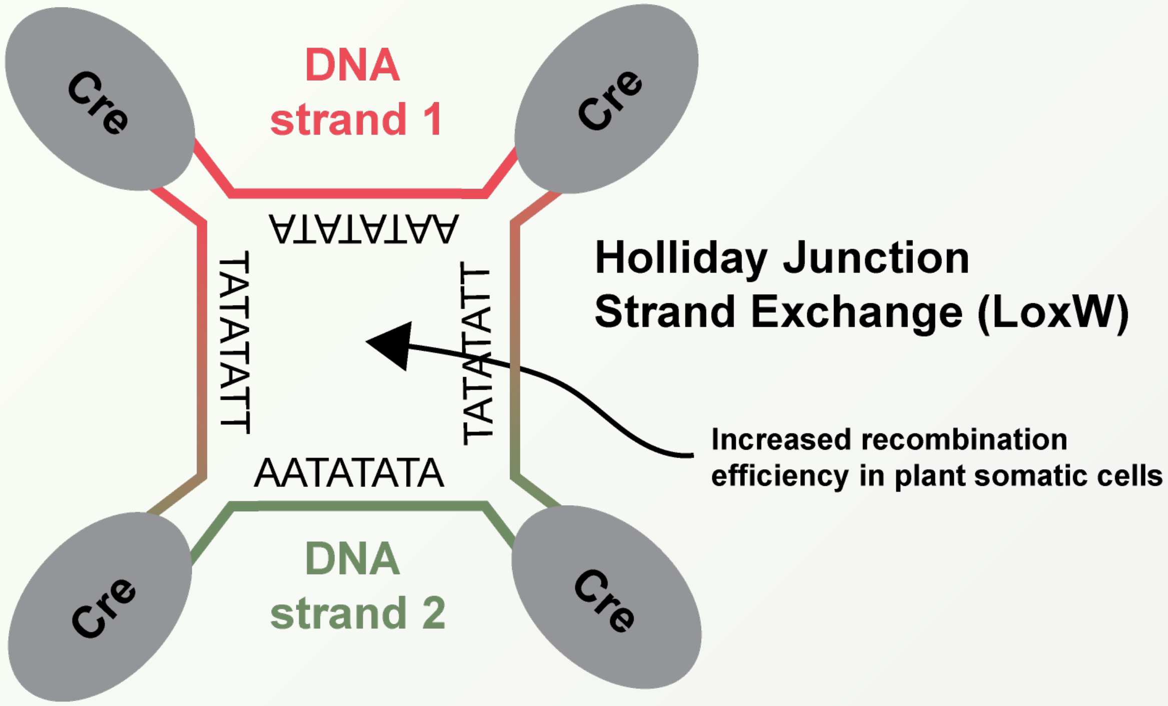
Modified from Liu et al. 2021 *New Phytologist*

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



Holliday Junction Strand Exchange (loxP)

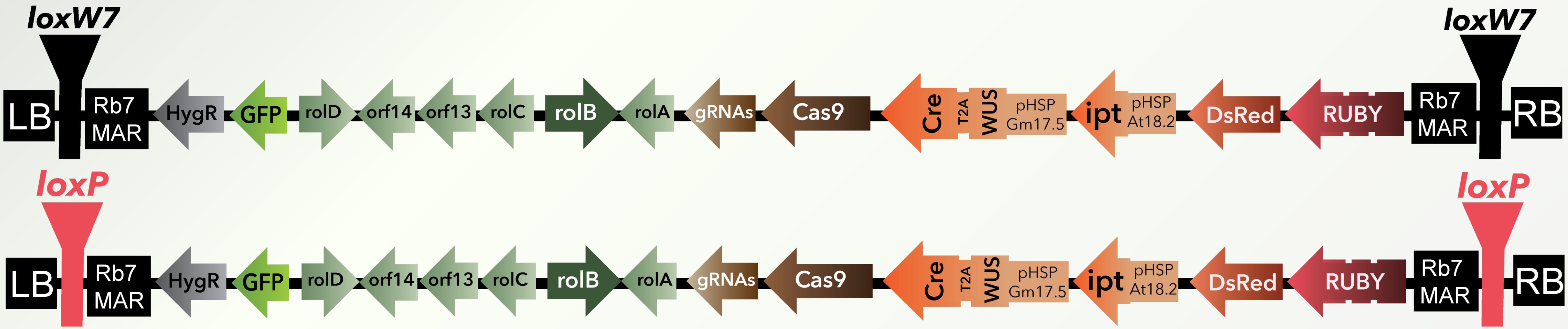
Steric hinderance in somatic plant cells



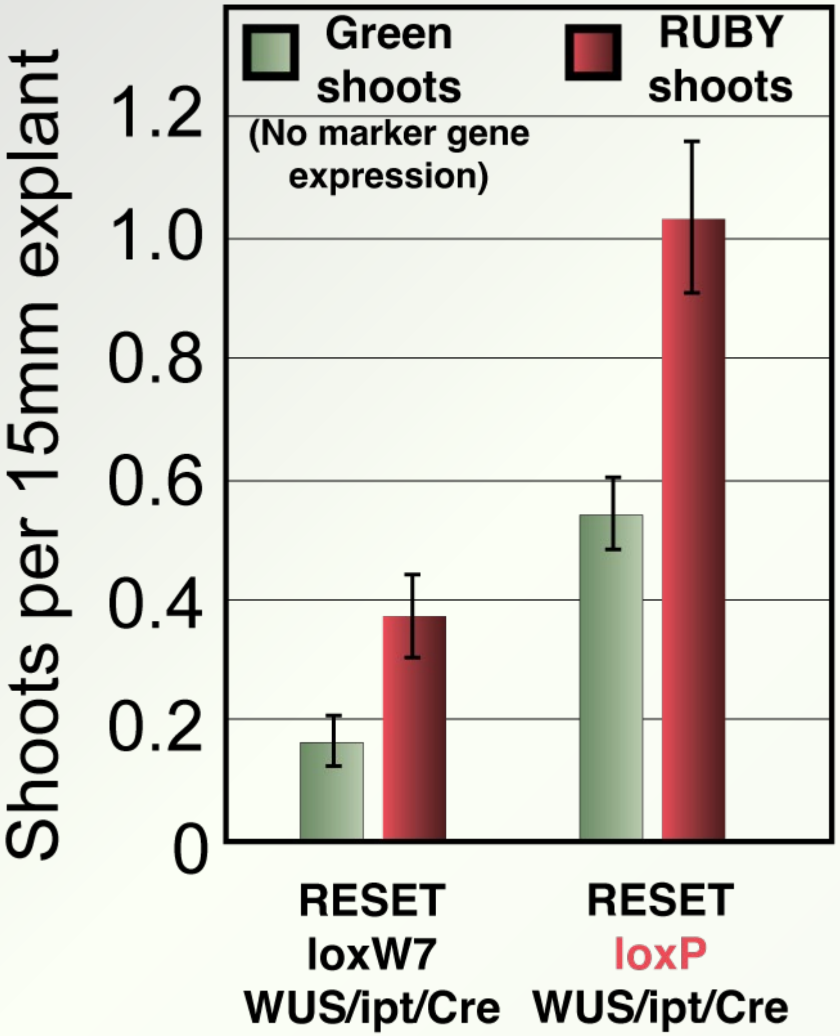
Holliday Junction Strand Exchange (LoxW)

Increased recombination efficiency in plant somatic cells

We compared rates of excision of the two lox sites

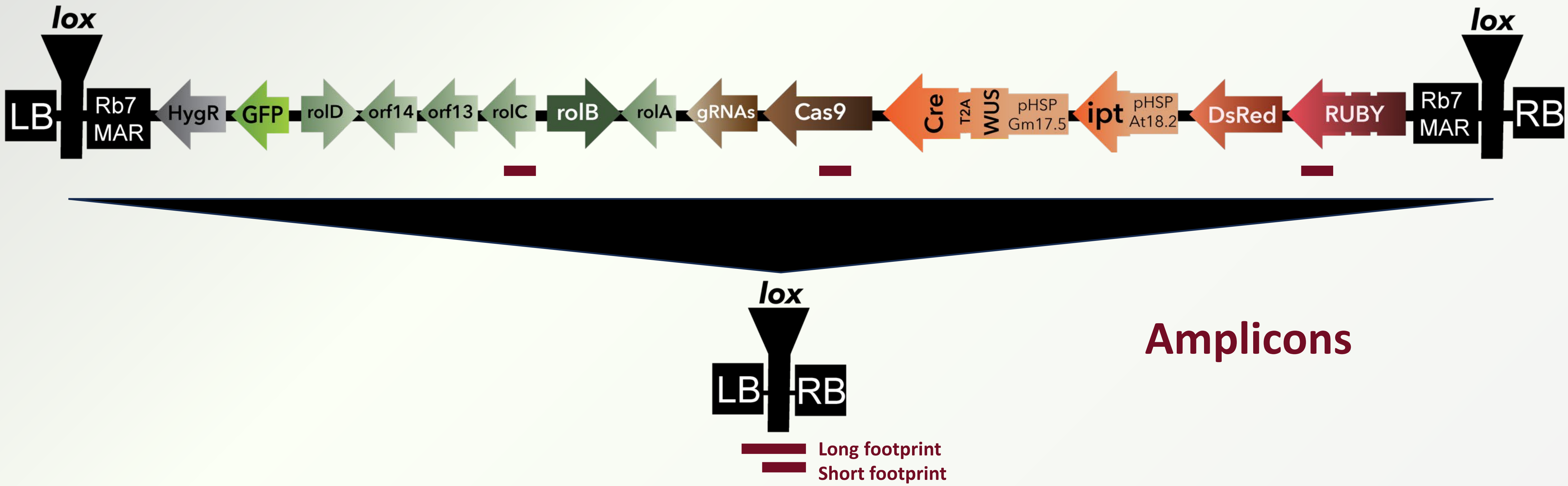


loxP-flanked RESET constructs gave the highest shoot regeneration after heat shock induction of transgenic roots



- 3.3x higher green shoot induction in loxP vs. loxW7
- 2.8x higher ruby shoot induction in loxP vs. loxW7
- Hairy root explant position (root tip, middle sections, or end) was not significant factor in regeneration outcome
- We kept multiple shoots originating from the same “mother” hairy root insertion event for detailed molecular analysis

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



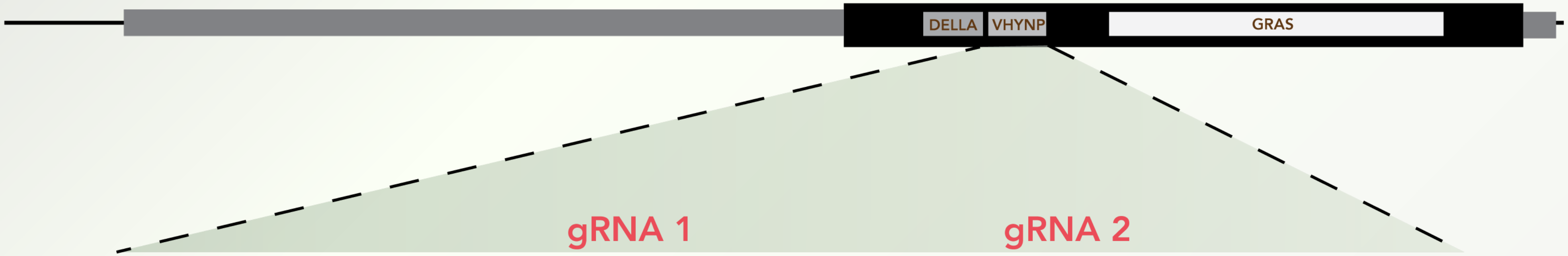
- The presence of any transgene band eliminated a shoot as being categorized fully excised (partial chimera)
- Two footprint amplicons were included in case of truncation of the T-DNA left border

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

Are they edited?

-We found high rates of editing among excised RESET shoots

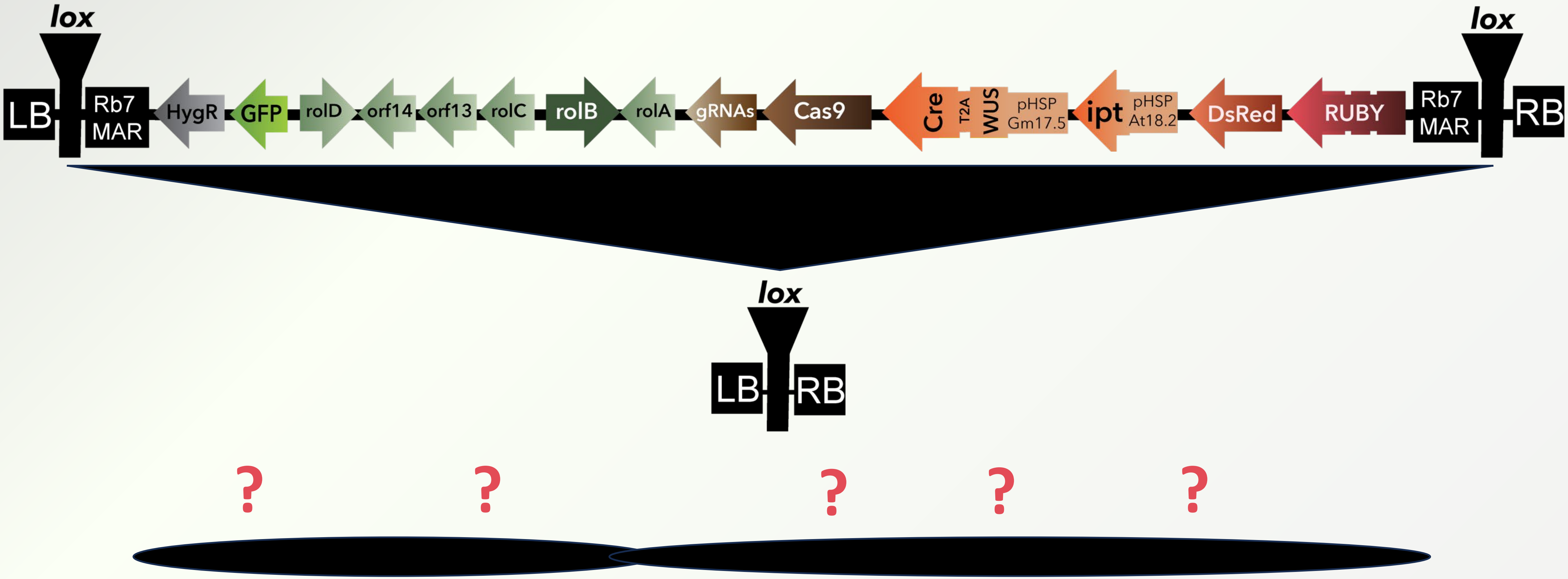
PtXaAlbH.08G111700 (RGA1, DELLA/GRAS)



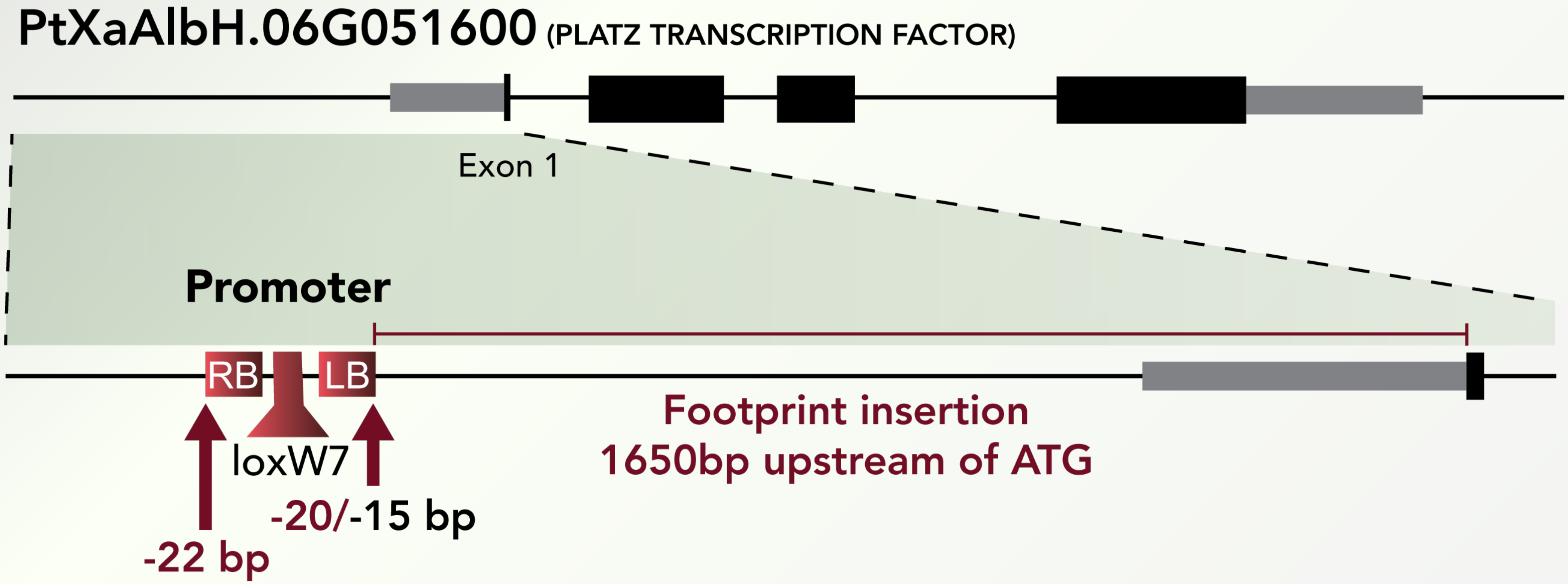
```
                                        gRNA 1                          gRNA 2
          CACGCGCAAGAAGATGGTCTTTCCCACCTCGCATCTGATTCTGTCCATTACAATCCTTCCGATCTGTCTACCTGGCTTCAAAGCATGATTTCGGAGTTGA
Event
                                   -2bp del
#36  A1   CACGCGCAAGAAGATGGTCTTTCCCACC--GCATCTGATTCTGTCCATTACAATCCTTCCGATCTGTCTACCTGGCTTCAAAGCATGATTTCGGAGTTGA
-12  A2   CACGCGCAAGAAGATGGTCTTTCCCAC--------------------------------CGATCTGTCTACCTGGCTTCAAAGCATGATTTCGGAGTTGA
                                                                  -32bp del
                                      WT
#23  A1   CACGCGCAAGAAGATGGTCTTTCCCACCTCGCATCTGATTCTGTCCATTACAATCCTTCCGATCTGTCTACCTGGCTTCAAAGCATGATTTCGGAGTTGA
-6   A2   CACGCGCAAGAAGATGGTCTTTCCCACC--GCATCTGATTCTGTCCATTACAATCCTTCCGATCTGTCTACCTGGCTTCAAAGCATGATTTCGGAGTTGA
                                   -2bp del

                                              -14bp del               -2bp del
#52  A1   CACGCGCAAGAAGATGGTCTTTCCCACCT--------------TCCATTACAATCCTTC----CTGTCTACCTGGCTTCAAAGCATGATTTCGGAGTTGA
-1   A2   CACGCGCAAGAAGATGGTCTTTCCCACCTCGCATCTGATTCTGTCCATTACAATCCTTCCGATCTGTCTACCTGGCTTCAAAGCATGATTTCGGAGTTGA
                                      WT
```

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

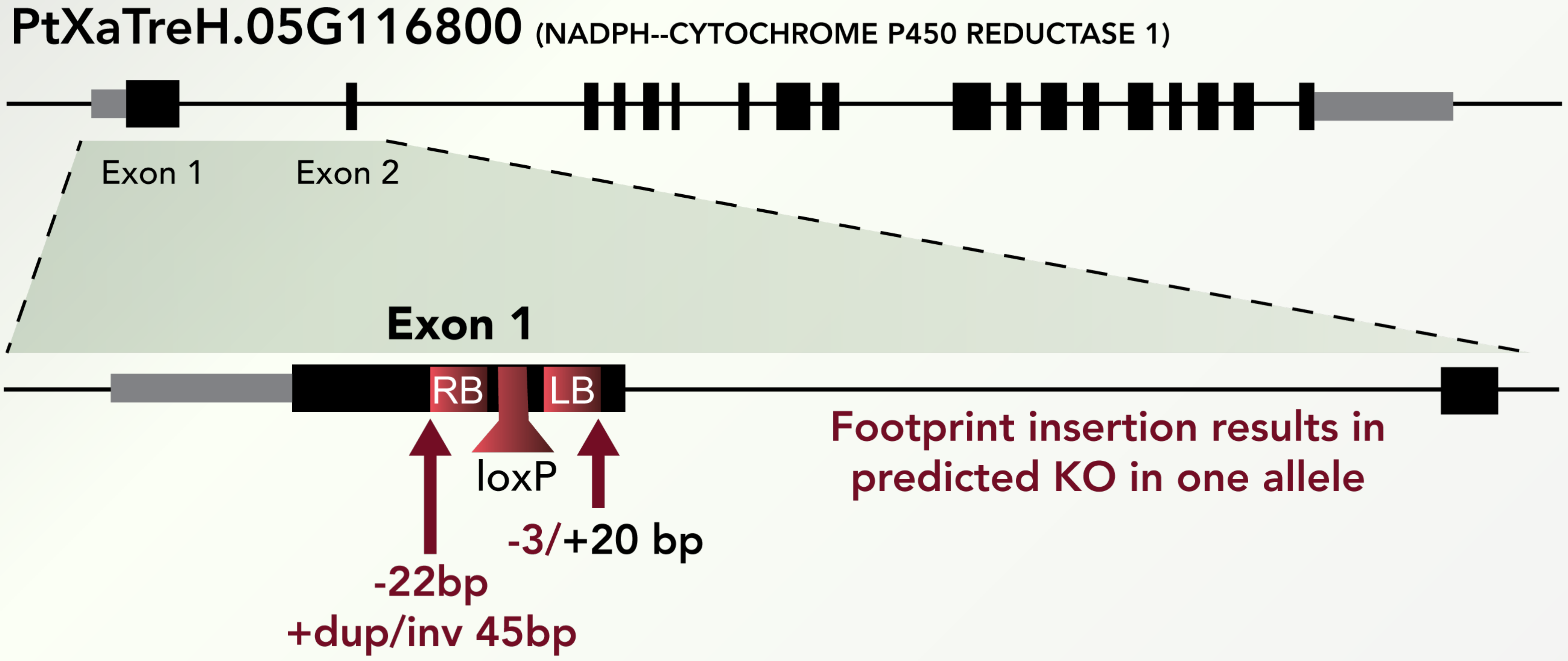
Where did our transgenes insert? How did the excision process resolve the footprints into final configuration?



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



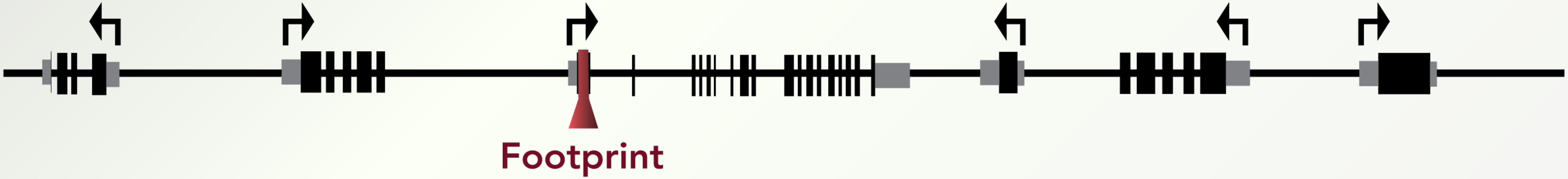
We did find some events where the footprint inserted into an exon of one allele



Footprint insertion results in predicted KO in one allele

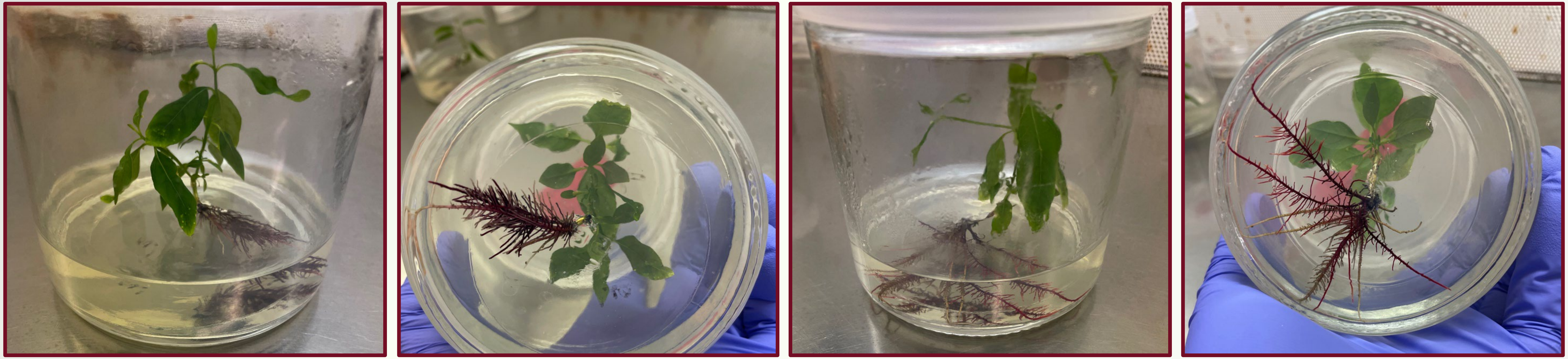
Could these one day be considered clean edits for regulatory purposes?

Are surrounding genes impacted?

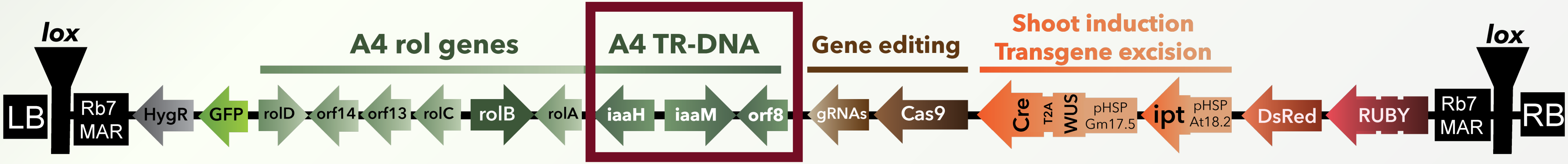


- Gene expression analysis of surrounding genes in characterized events to better understand local genetic impacts
- ddPCR to understand low abundance copy number variation of any residual transgenic cells
- Whole genome sequencing to confirm TAIL-PCR results and characterize excision resolution of footprints

Does this work in other, more recalcitrant species?



Eucalyptus grandis x urophylla RESET composite plants



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

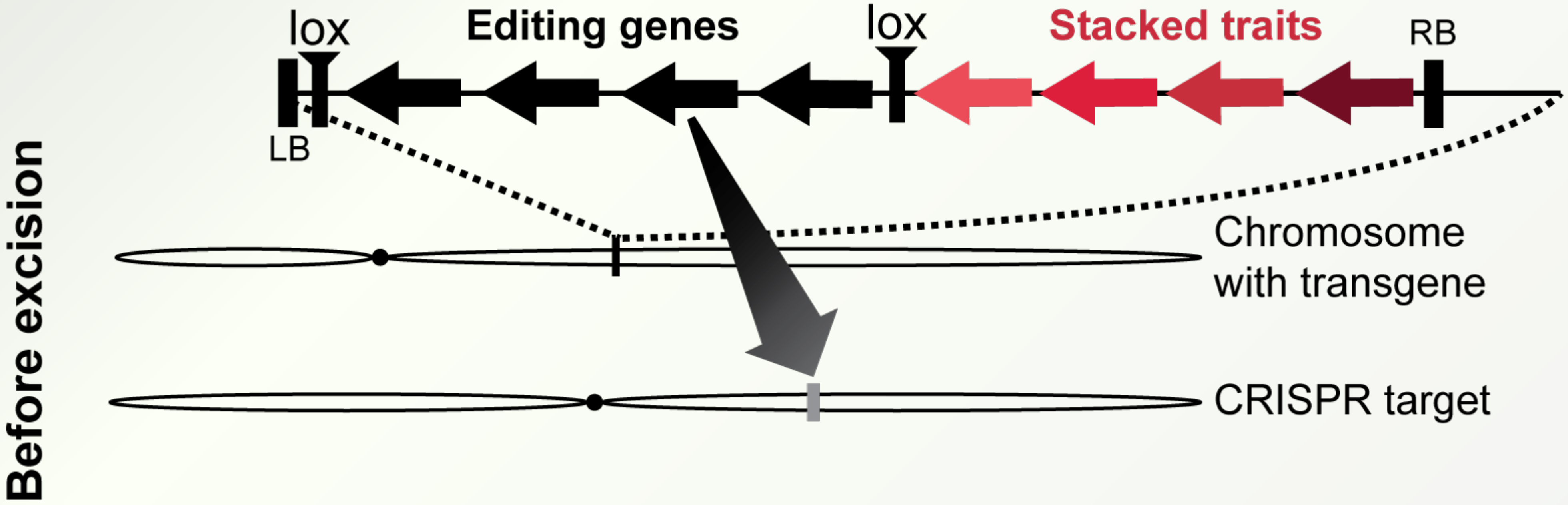
- RESET system gave high rates of hairy root transformation, and especially with loxP-flanked borders gave efficient heat induced regeneration and transgene excision from root tissue

- High rates of editing are seen so far in excised shoots

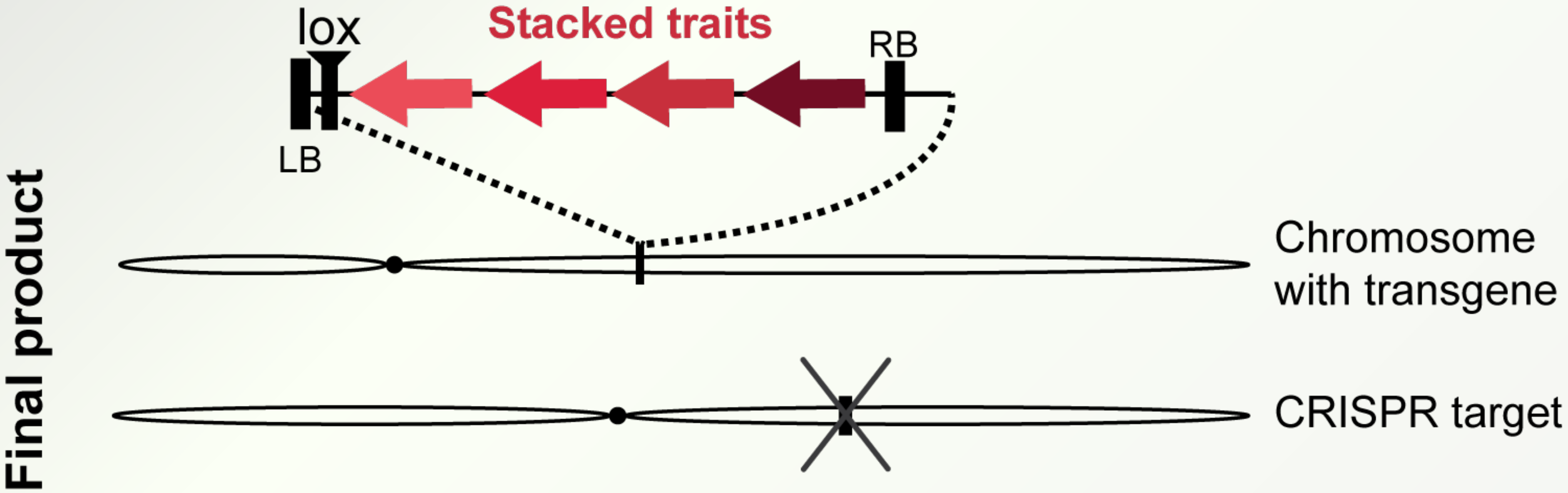
- Significant numbers of escape shoots found which should be easily eliminated through adding selection during hairy root subculture

- Though complex, 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



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Undergraduate

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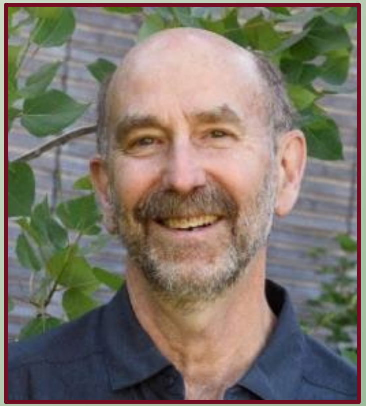
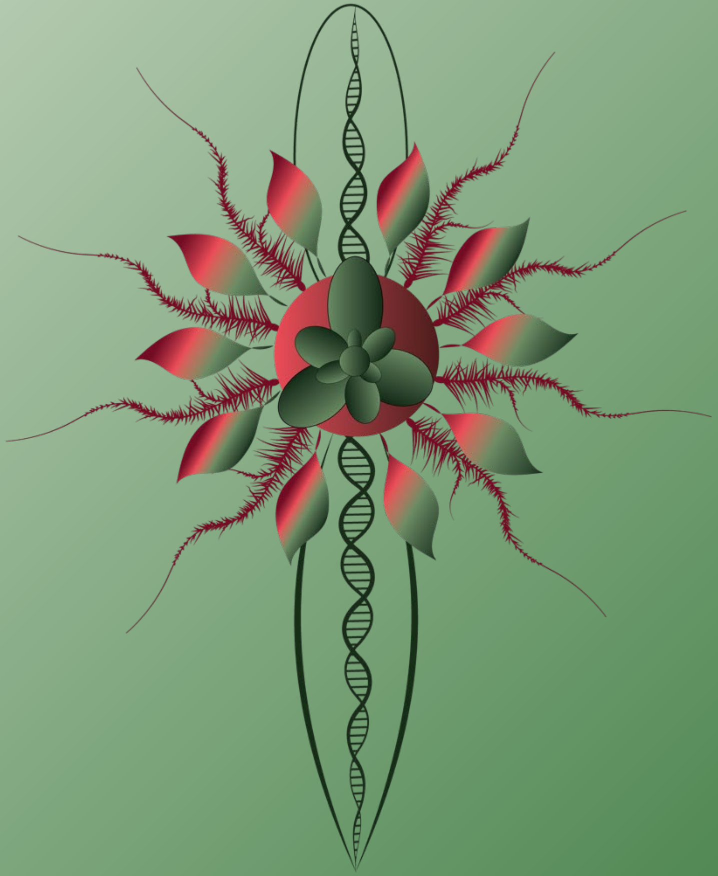
Tissue culture and transformation

Kate Peremyslova
Tissue culture and transformation



Steve Strauss
Professor FES



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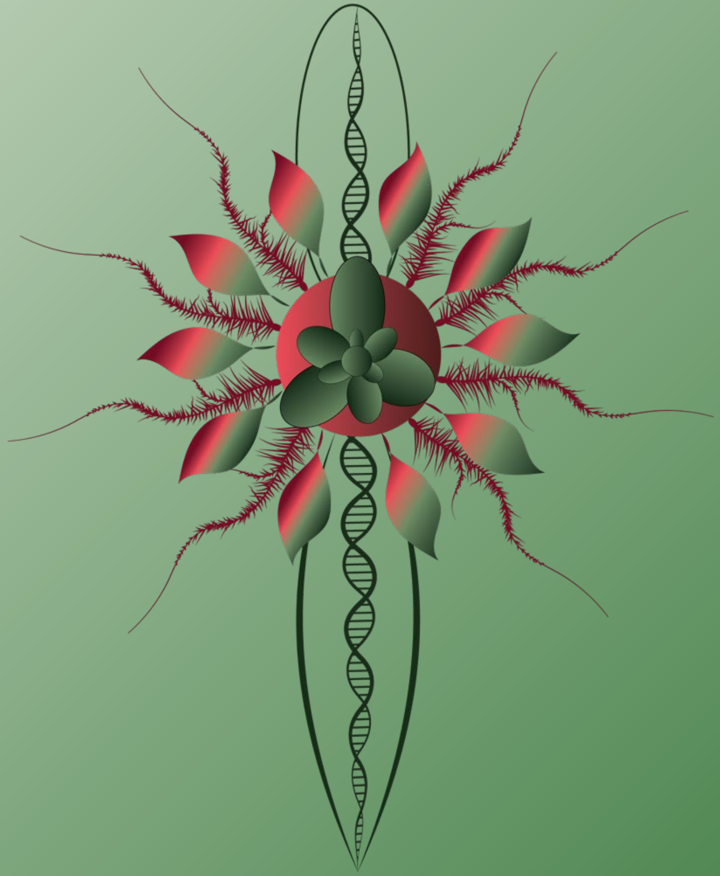

Thanks for listening!