Greg Goralogia
Laboratory of Steve Strauss
Oregon State University
Forest Ecosystems and Society

BACK TO THE FUTURE:

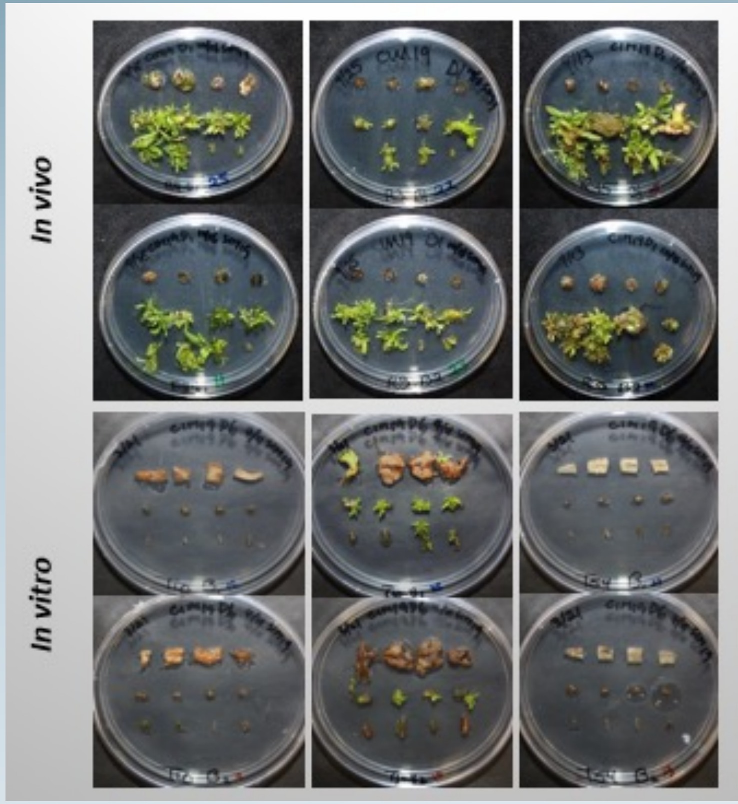
A co-transformation approach with *Agrobacterium* T-DNA genes promotes regeneration of transgenic woody plants

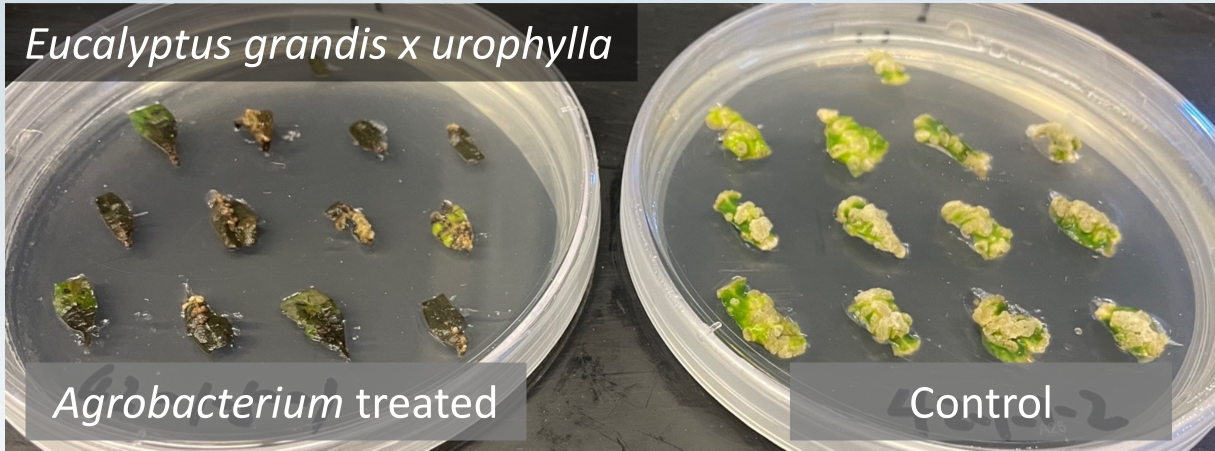
Regeneration and transformation continue to be major limiting factors for gene editing and engineering in plants

- Species and genotypic differences often dramatic
- Slow, complex customization efforts usually needed
- Costly reagents and skill-intensive labor often required

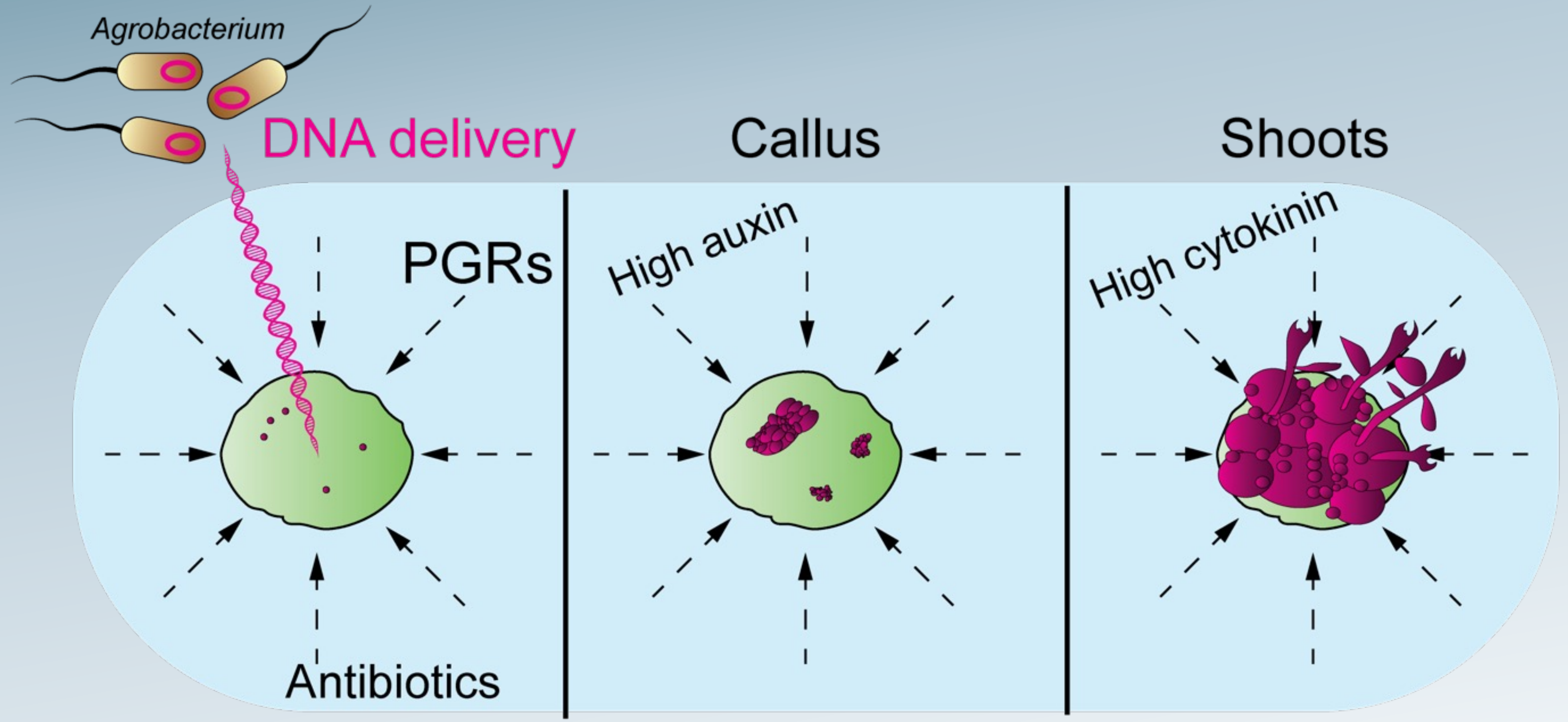
Many tree species are difficult to transform

- Woody (forest) trees – slow, tough biochemistry
- Elite clones, mature propagules, not seed-derived
- High physiological diversity
  - Growth environment, age, explant type and source
- Common necrotic responses
- Very high genetic diversity of forest trees

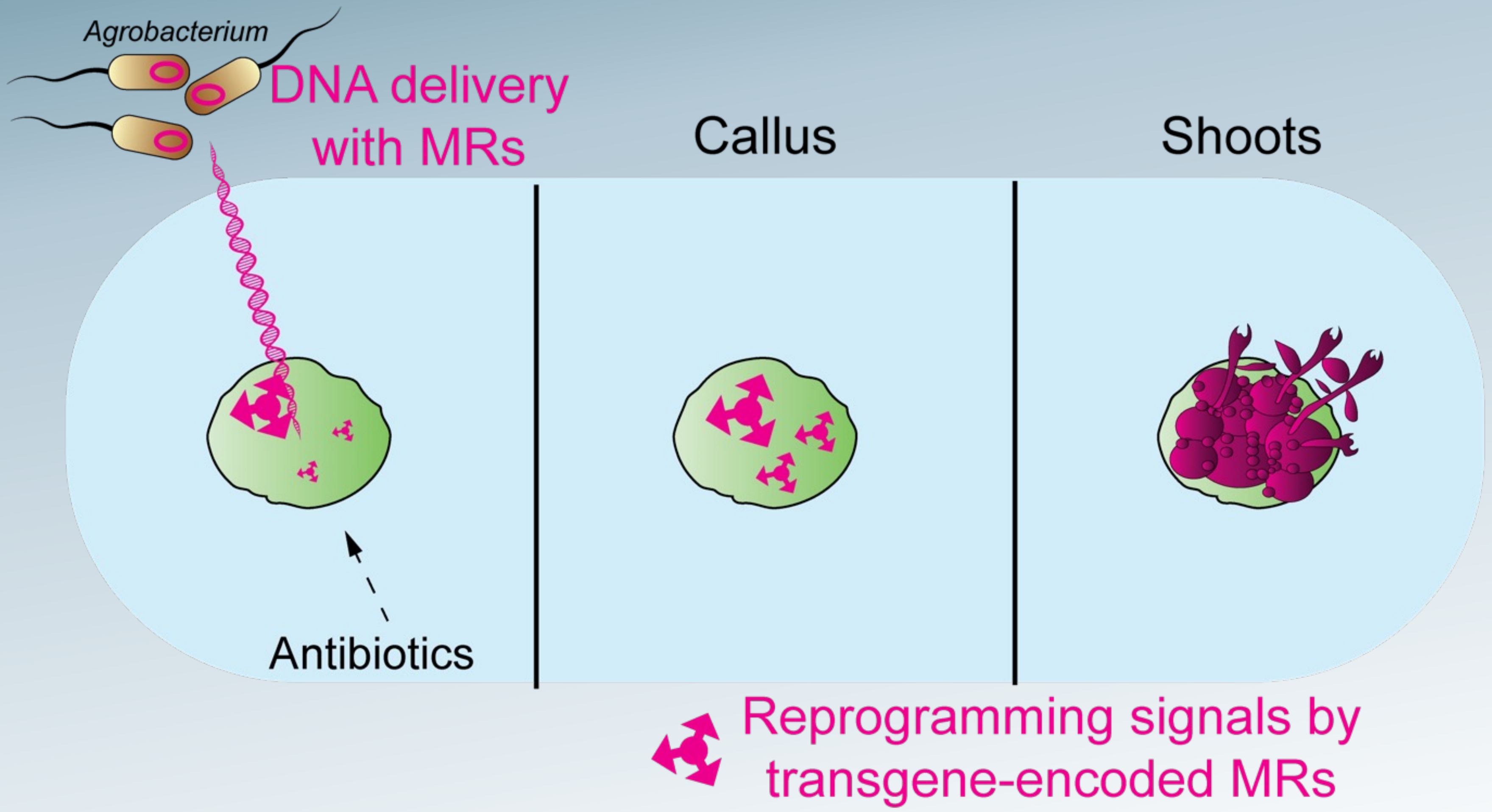




Conventional transformation methods rely on exogenous phytohormones supplied in the culture medium



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



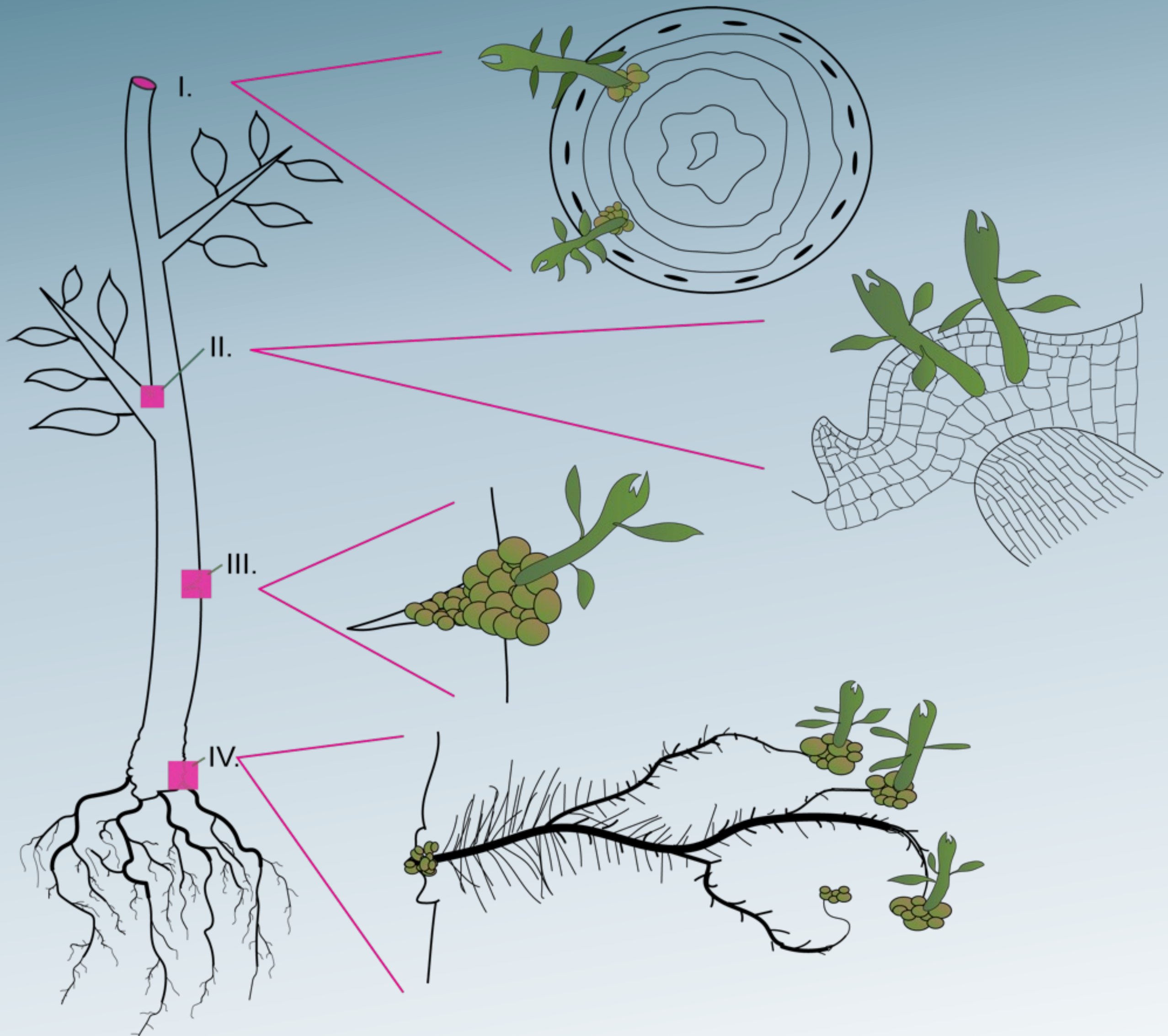
Types of MR genes we have studied in poplars or eucalypts – many both *in vitro* and *in planta*

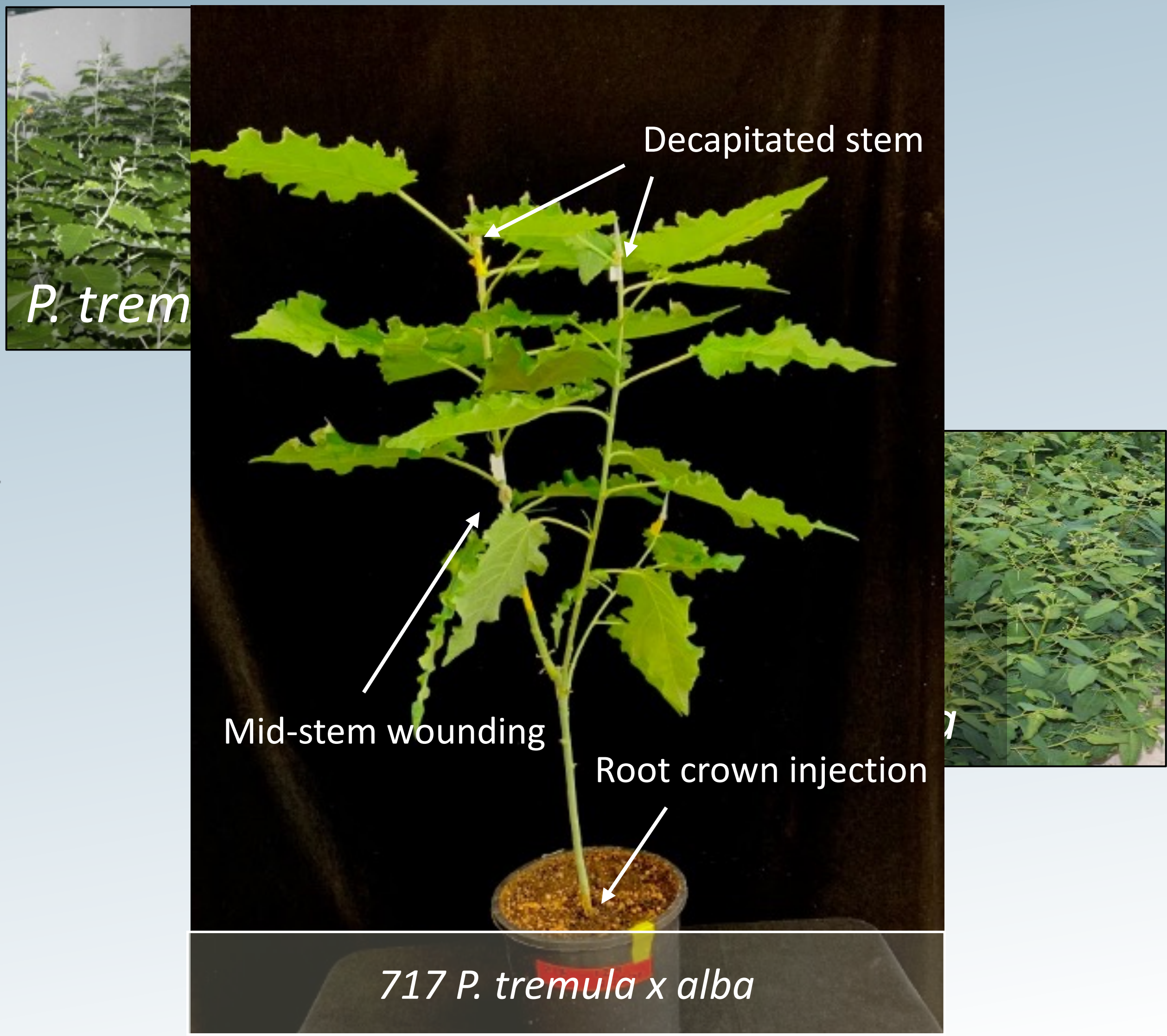
- LEC 1, 2 – LEAFY COTYLEDON
- EBB1 - EARLY BUD BREAK 1 (ESR family)
- BBM – BABY BOOM
- WOX 5, 11 -- WUSCHEL RELATED HOMEOBOX
- WUS – WUSCHEL
- GRF-GIF – GROWTH REGULATOR FACTOR 4 and GRF INTERACTING FACTOR 1
- *Agrobacterium* growth promoting genes
- rol – Hairy root-inducing genes – *Agrobacterium*

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

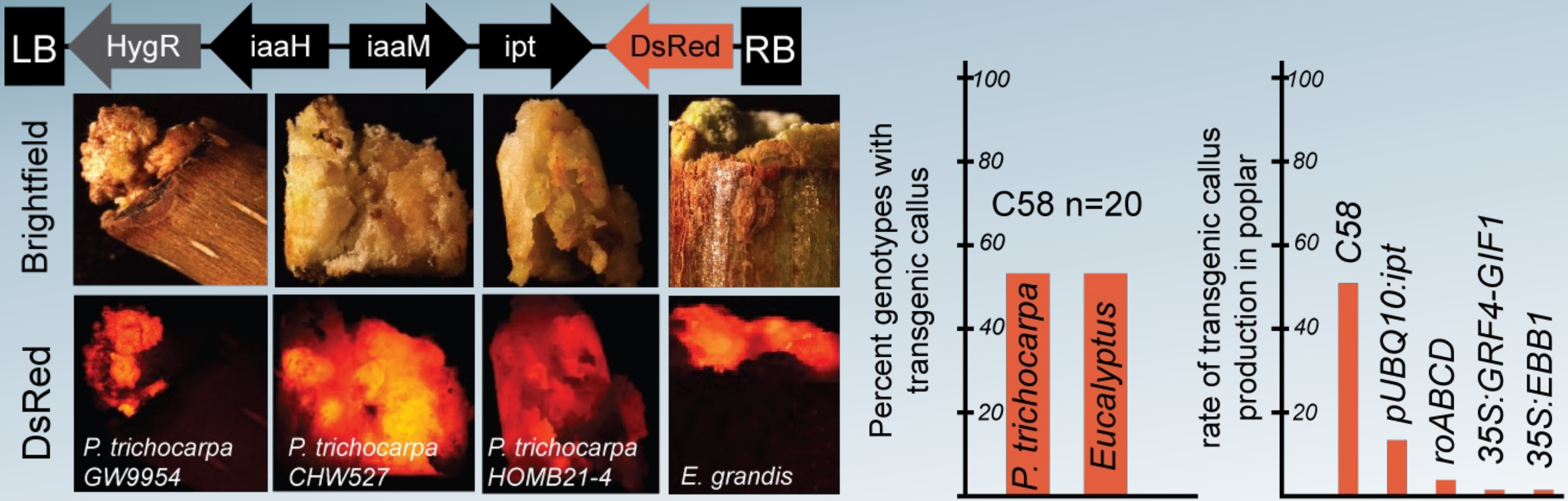
- LEC 1, 2 – LEAFY COTYLEDON
- EBB1 - EARLY BUD BREAK 1 (ESR family)
- BBM – BABY BOOM
- WOX 5, 11 -- WUSCHEL RELATED HOMEOBOX
- WUS – WUSCHEL
- GRF-GIF – GROWTH REGULATOR FACTOR 4 and GRF INTERACTING FACTOR 1
- ***Agrobacterium* growth promoting genes**
- ***rol* – Hairy root-inducing genes – *Agrobacterium***

To try to sidestep tissue culture barriers, we experimented with *in planta* transformation on greenhouse plants



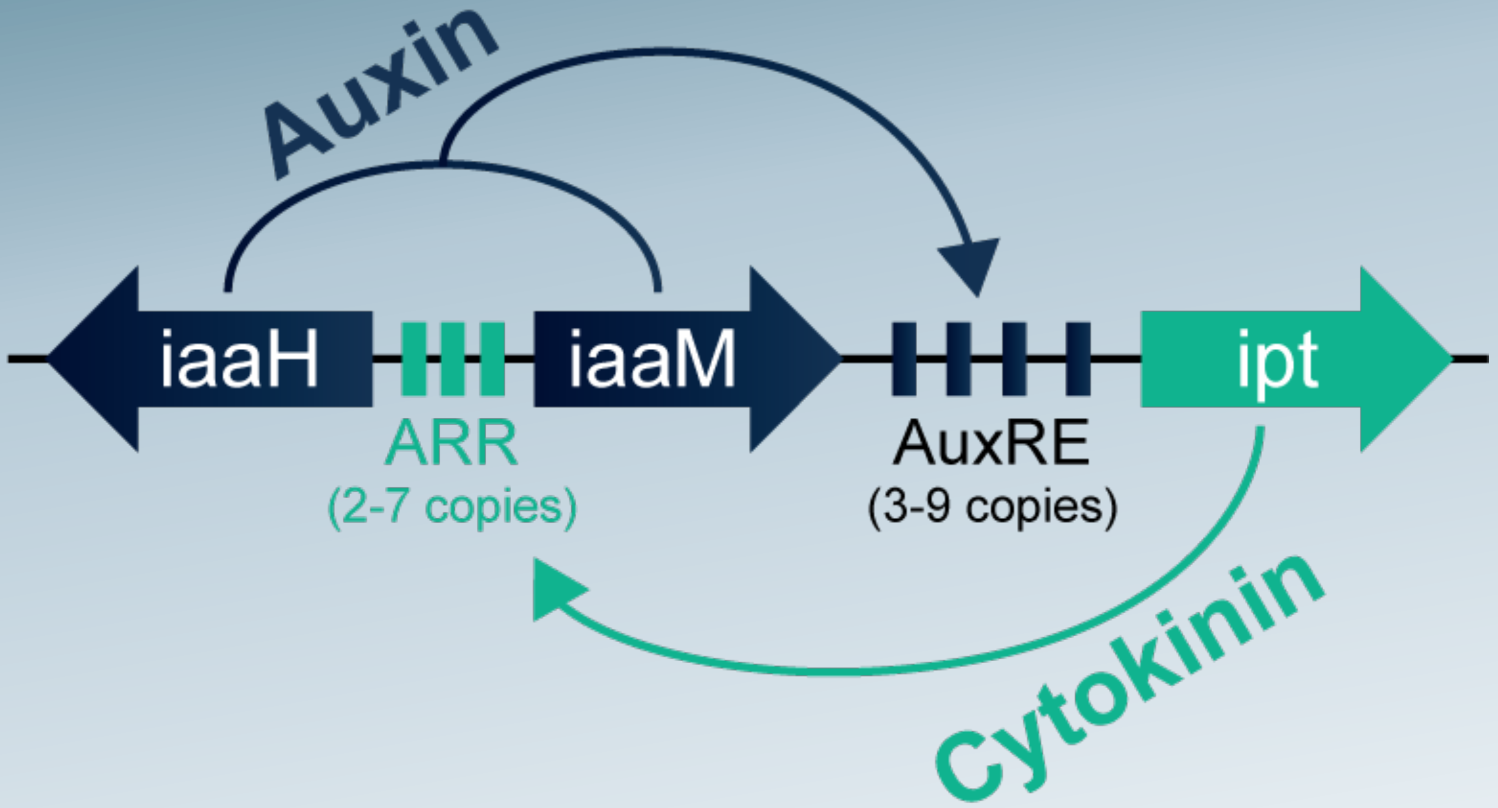


iaaH/M and *ipt* genes from *Agrobacterium* were effective inducers of transgenic callus in diverse poplar and eucalypt genotypes



Despite a variety of configurations and trials we were never able to use these to produce transgenic shoots

Agrobacterium iaa and *ipt* genes create a self-reinforcing feedback loop to induce undifferentiated growth



- *iaaH/iaaM* and *ipt* indirectly produce auxin and cytokinin
- Feedback loop maintains high levels of hormone production during gall development

Can we find more useful, developmentally flexible systems? Jouanin group (INRA-France) characterized a shooty Agro strain, and leveraged it for *in planta* regeneration in the 1990s

Plant Molecular Biology 17: 441–452, 1991.

An alternative approach for gene transfer in trees using wild-type *Agrobacterium* strains†

Ana Cristina Miranda Brasileiro[1], Jean-Charles Leplé[2], Joris Muzzin[2,3], Dalila Ounnoughi[2], Marie-France Michel[2†] and Lise Jouanin[1*]
[1] *Laboratoire de Biologie Cellulaire, INRA, route de Saint-Cyr, F-78026 Versailles Cedex, France (*author for correspondence);* [2] *Station d'Amélioration des Arbres Forestiers, INRA, Ardon, F-45160 Olivet, France;* [3] present address: *Piccoplant Mikrovermehrungen, Brockhauser Weg 75, D-2900 Oldenburg, Germany*

Received 3 January 1991; accepted in revised form 24 May 1991

Key words: Agrobacterium, crown gall, poplar, tree transformation, wild cherry

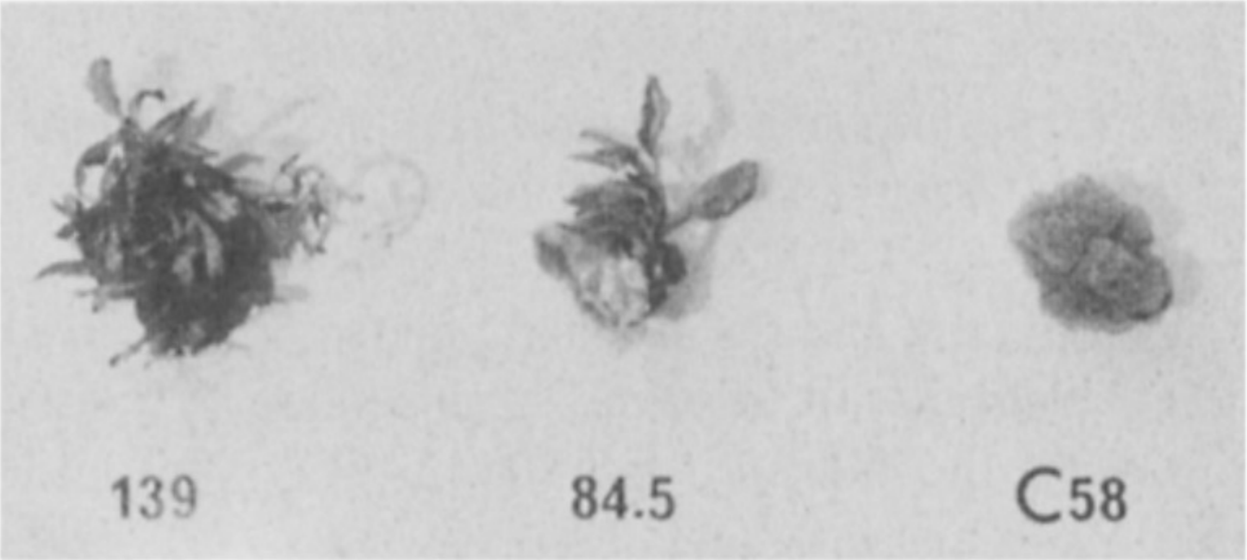
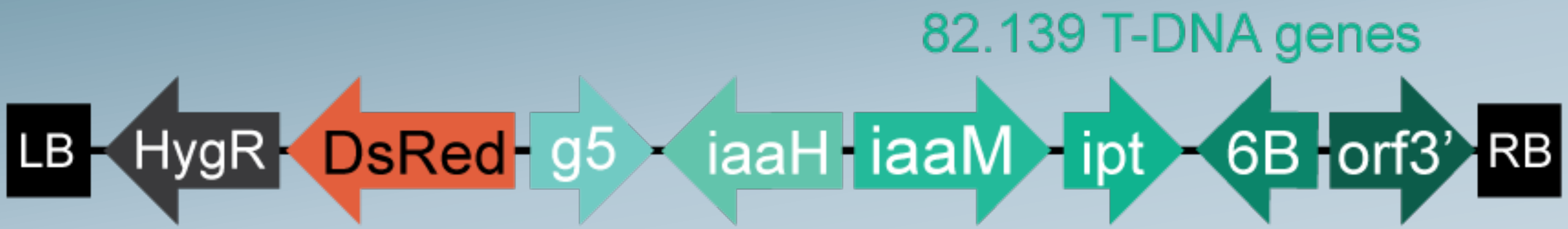


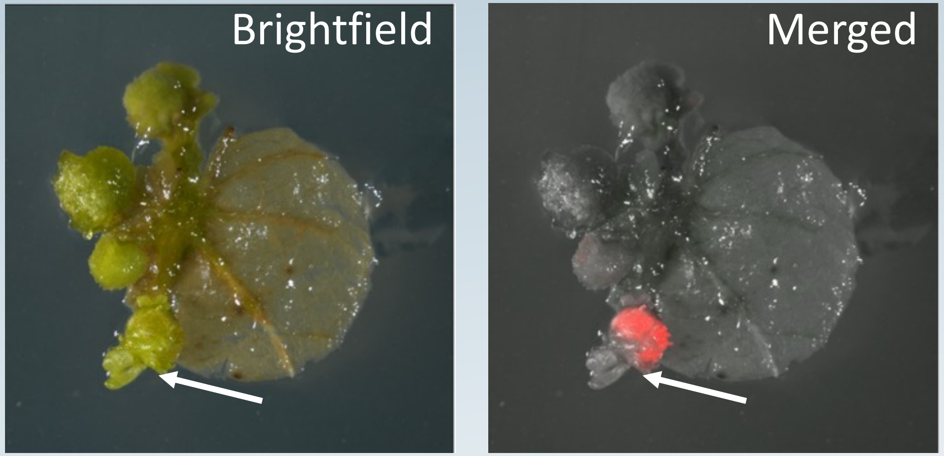
Fig. 1. Tumors and shoot differentiation from poplar tumors induced by *A. tumefaciens* strains 82.139, 84.5 and C58 and cultivated on MS medium, 6 weeks after inoculation.

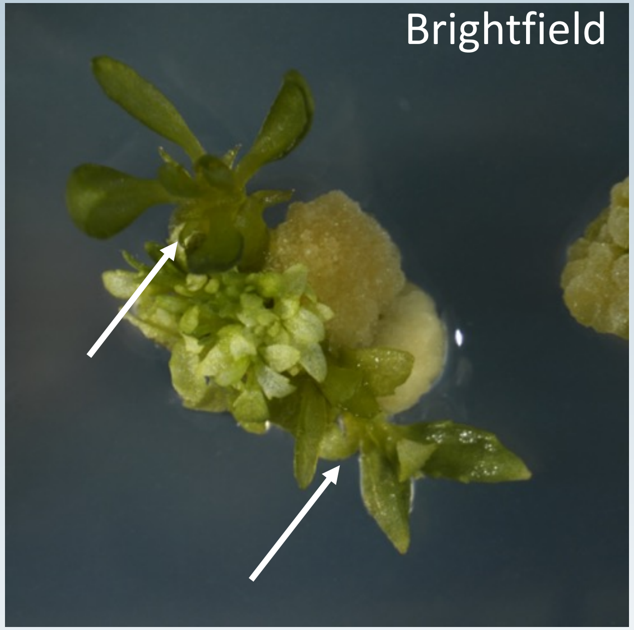
Though very promising, this work essentially came to a halt – due to GMO pushback in Europe – and due to the challenges of dealing with the large constructs prior modern sequencing and gene cloning systems

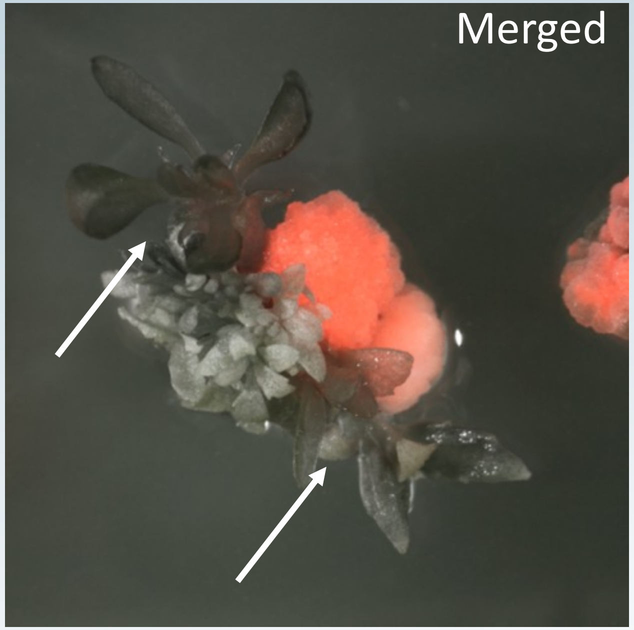
We cloned out the growth-promoting genes from our resurrected clone, and added modern amenities like DsRed (called "S82")



S82 callus promoted regeneration of non-transgenic shoots



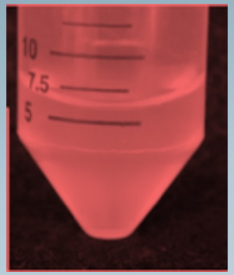




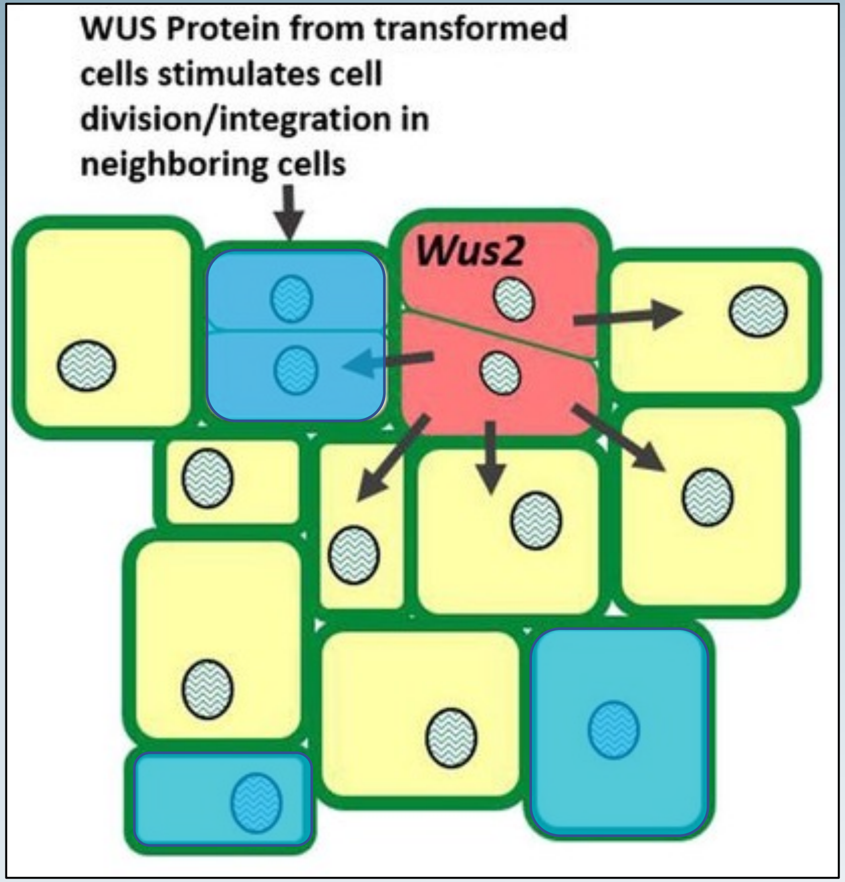
“Altruistic” transformation approach – strain mixtures

Mixed Agro in different ratios

MR gene Agro
(like WUS)



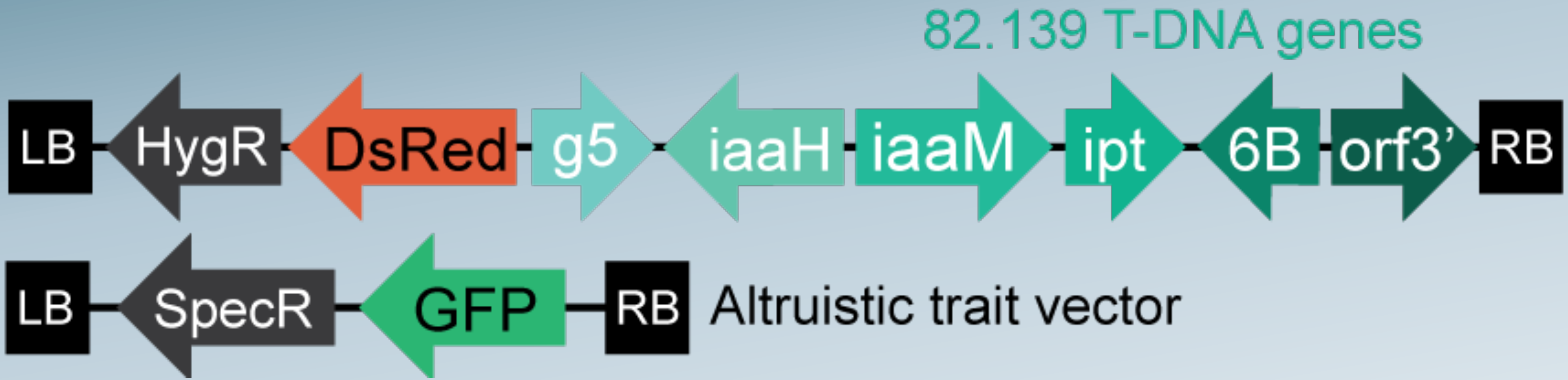
Trait gene Agro



Plant with trait gene
and no MR genes

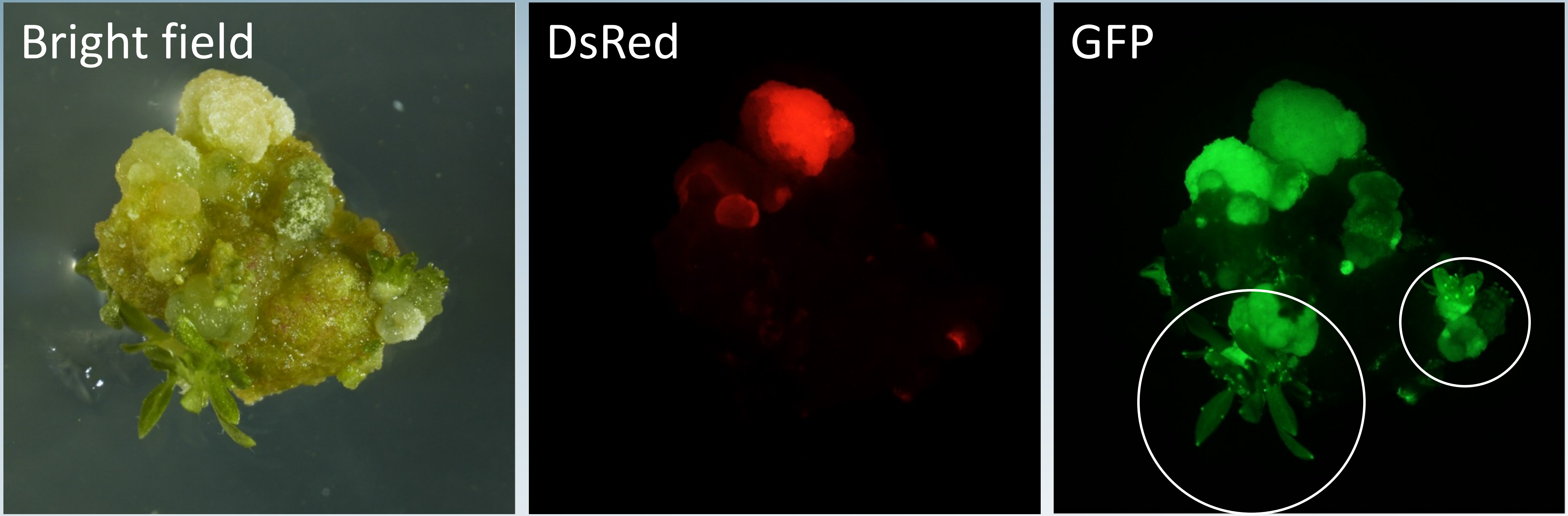
Figures used from Hoerster et al. 2021 *In vitro cell and developmental biology -Plant*

We set up a similar experimental system for use in hybrid poplar clones "717-1B4" and "353-53"

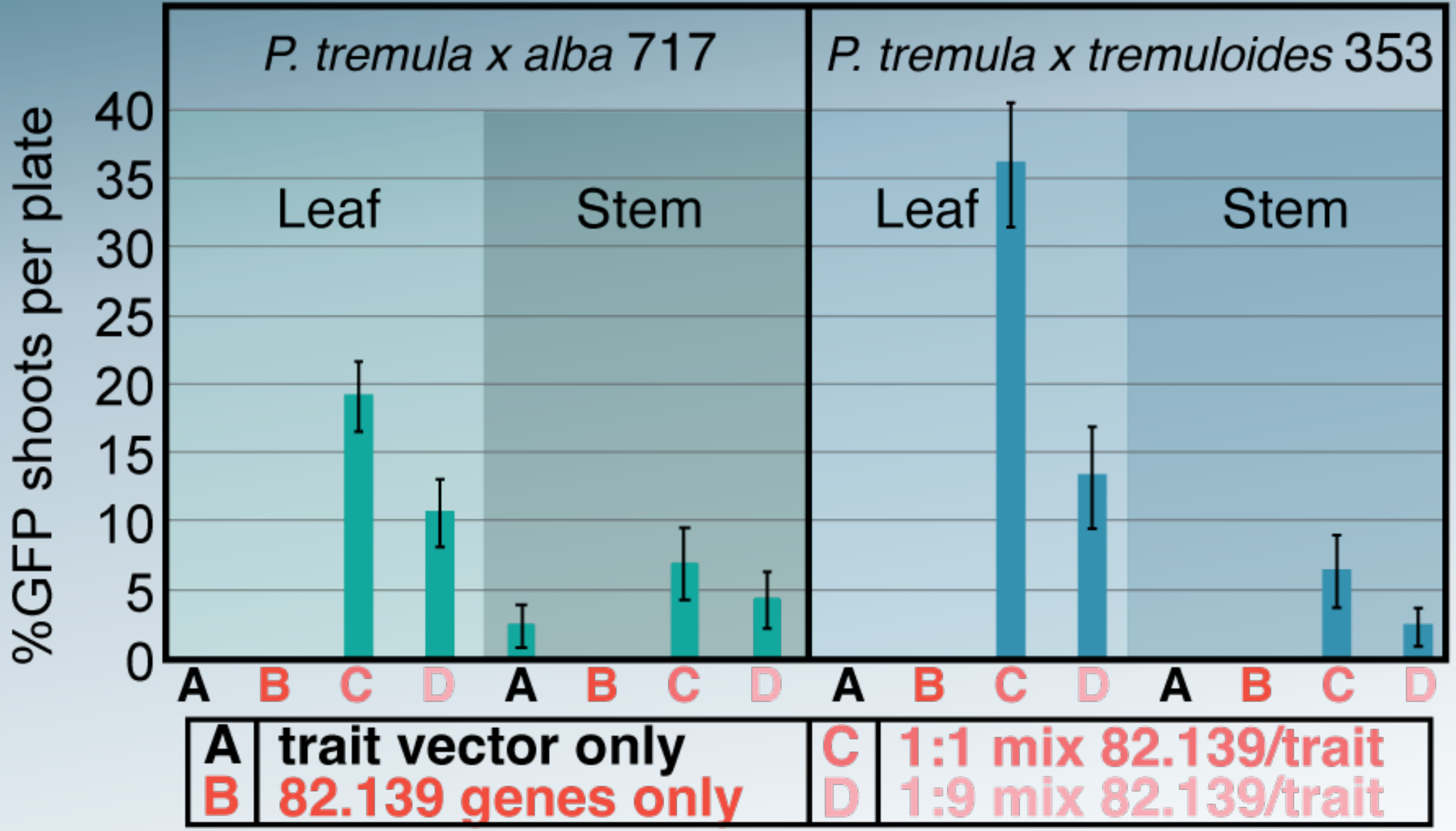


- We mixed these in equal ratios
- Selected using spectinomycin on hormone-free media

Under the microscope: cells distal to those transformed with 82.139 regenerate into transgenic trait-vector only shoots

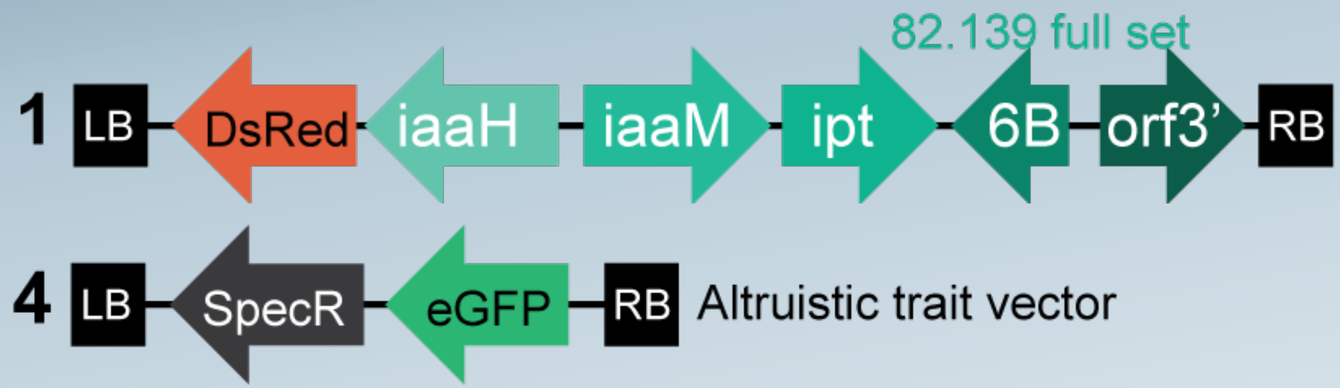


Altruistic 82.139 transformation was efficient in two independent hybrid poplar genotypes

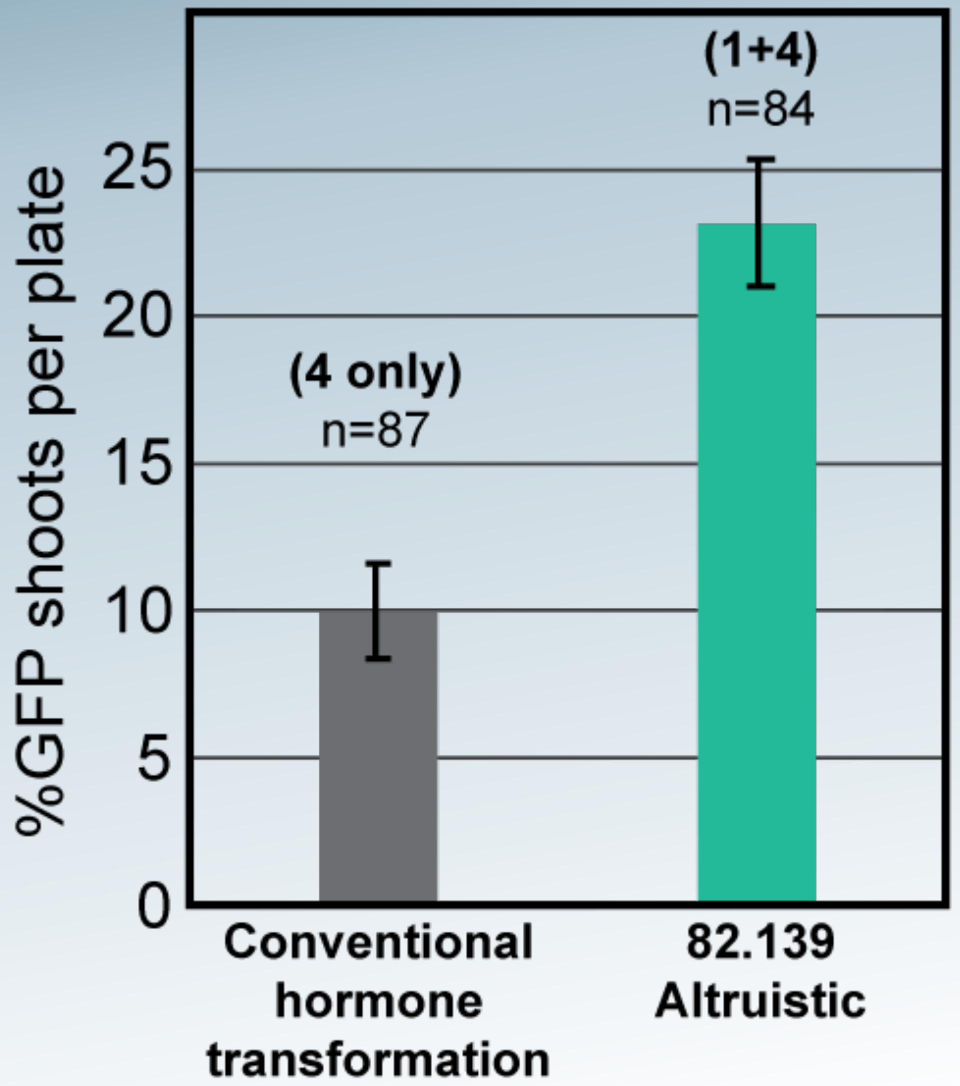


82.139 altruistic transformation was superior to routine hormone-based transformation

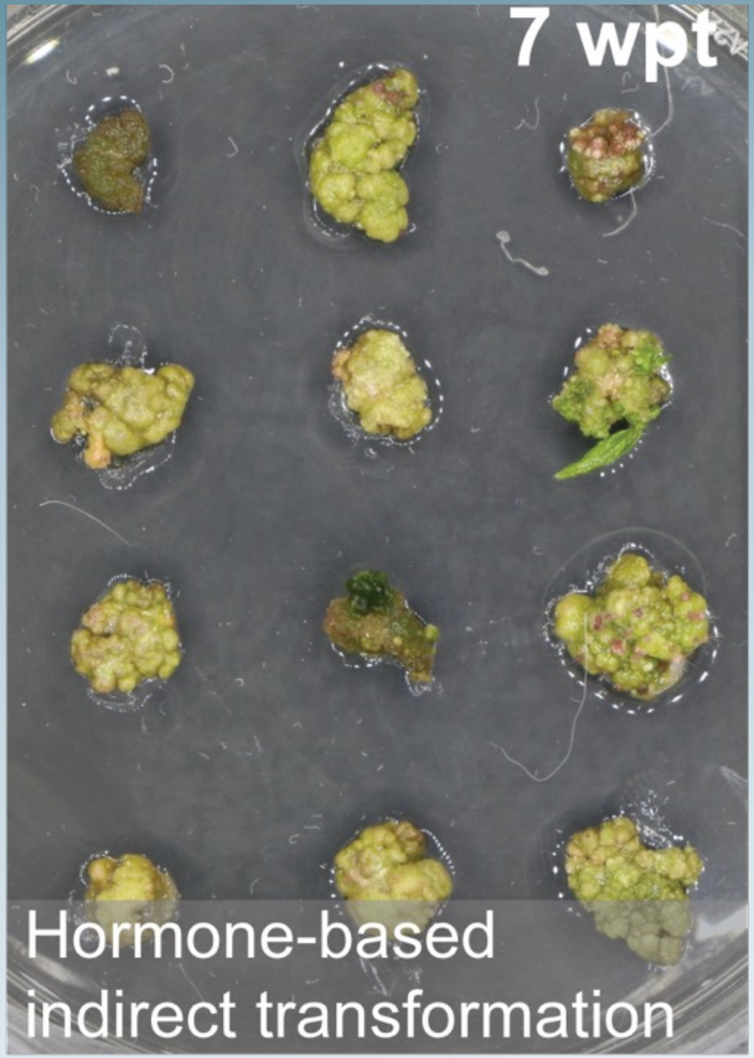
Vector types used

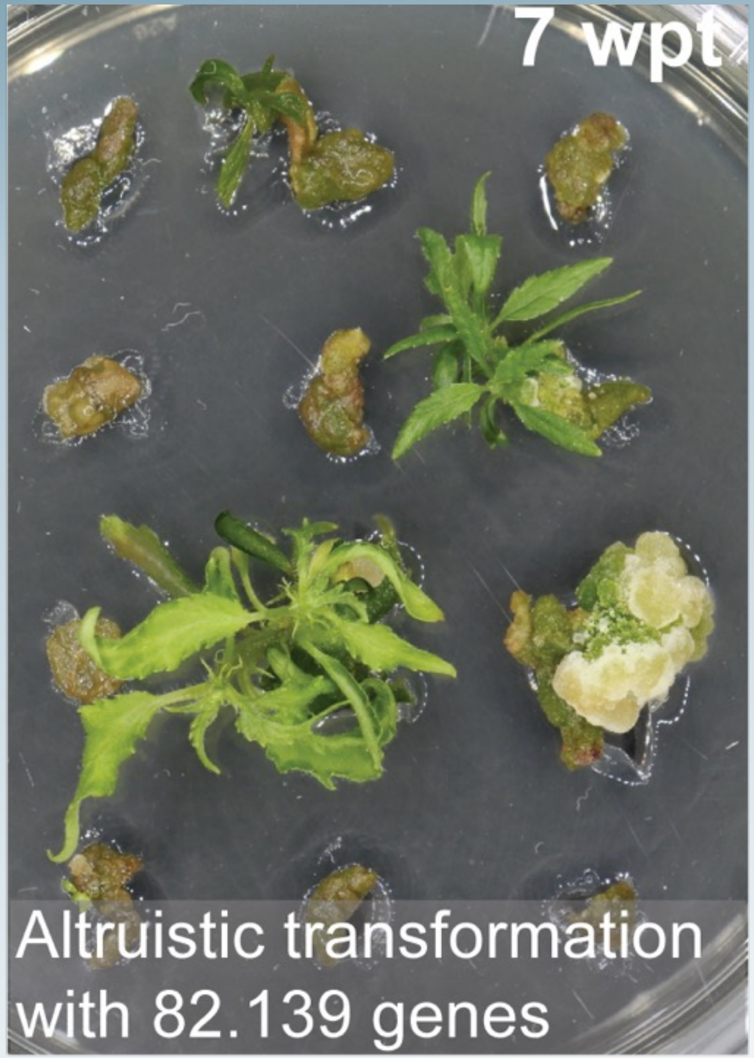


n= number of plates of 12 explants each



The 82.139 altruistic method was also significantly faster, shortening time to propagation by half





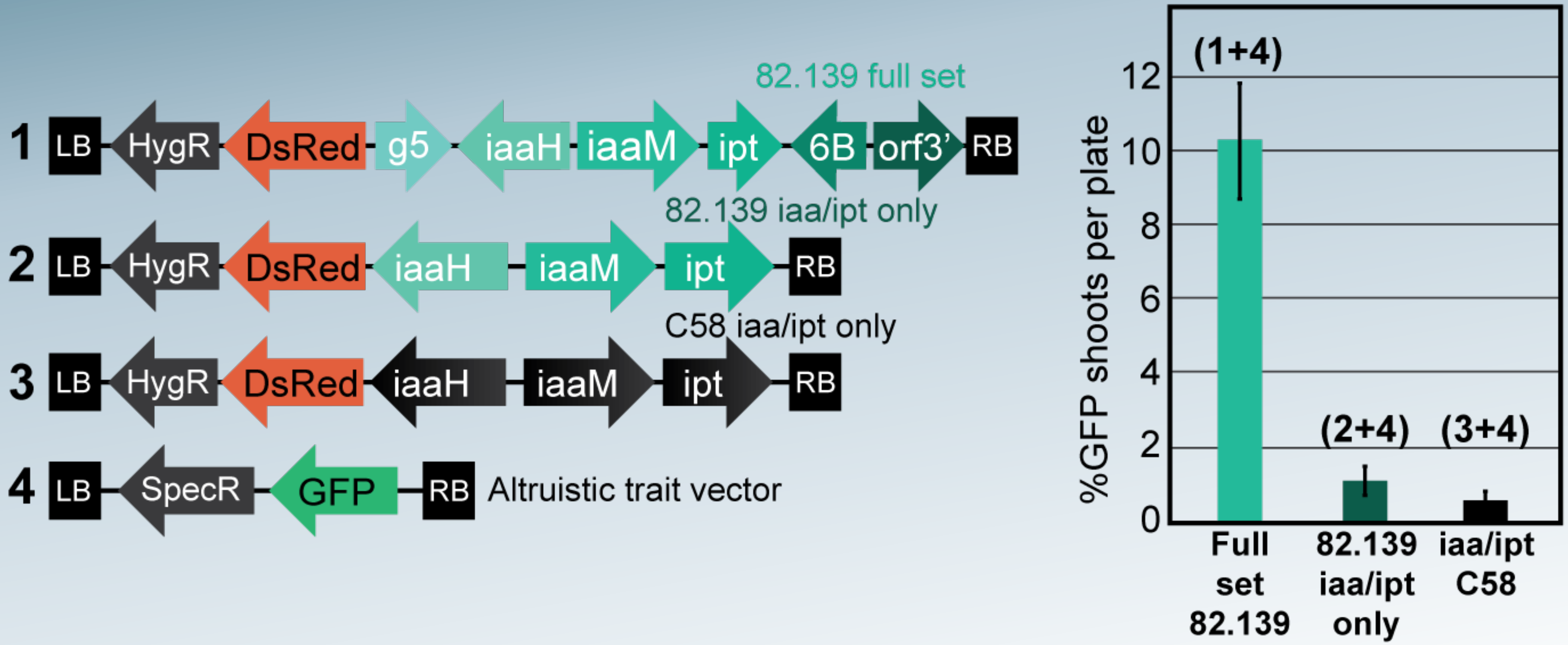
- Currently launched via GAANTRY strain ARport1 (Contact us if you want the strain, so far we have sent to ~15 lab groups)
- Developed binary strains but gene orientation matters
- Delivery ratio between binary and *vir*-launched DNA matters for shoot regeneration

Which genes are most important for non-cell autonomous shoot promotion?

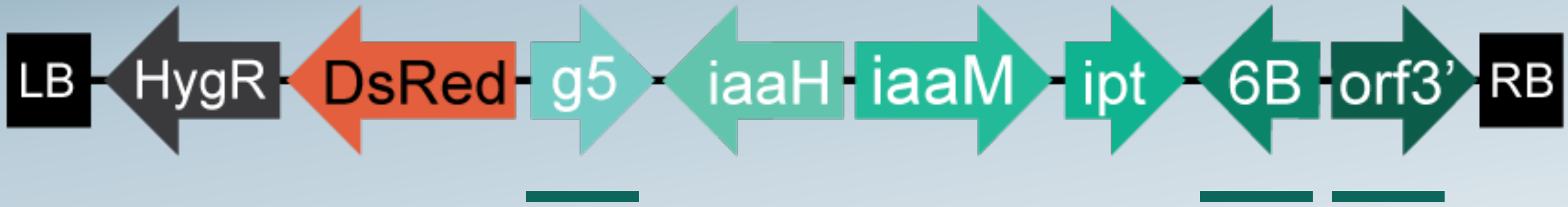
Is there novel *iaa/ipt* expression in this strain?

Or are the unique genes there most important?

82.139 hormone producing genes (*iaa/ipt*) were not capable of inducing altruistic shoot production

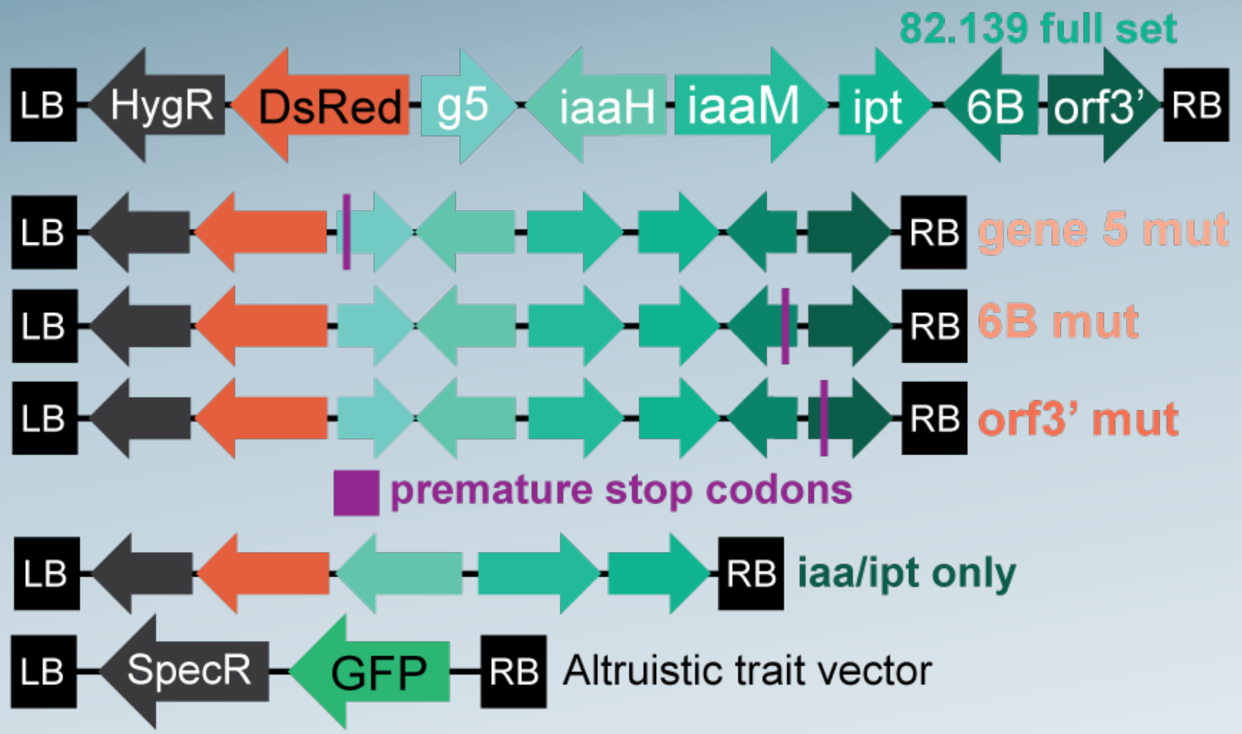


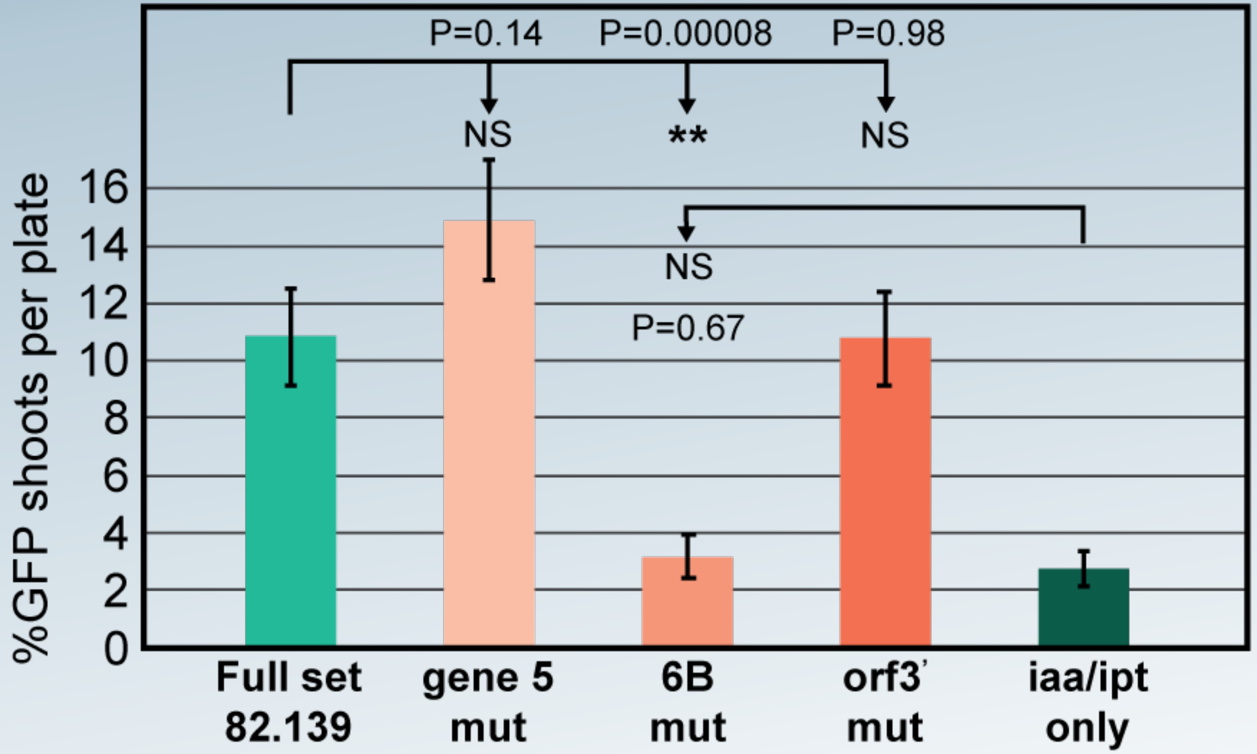
Which genes are most important for non-cell autonomous shoot promotion?



Which of the other T-DNA genes are most important?

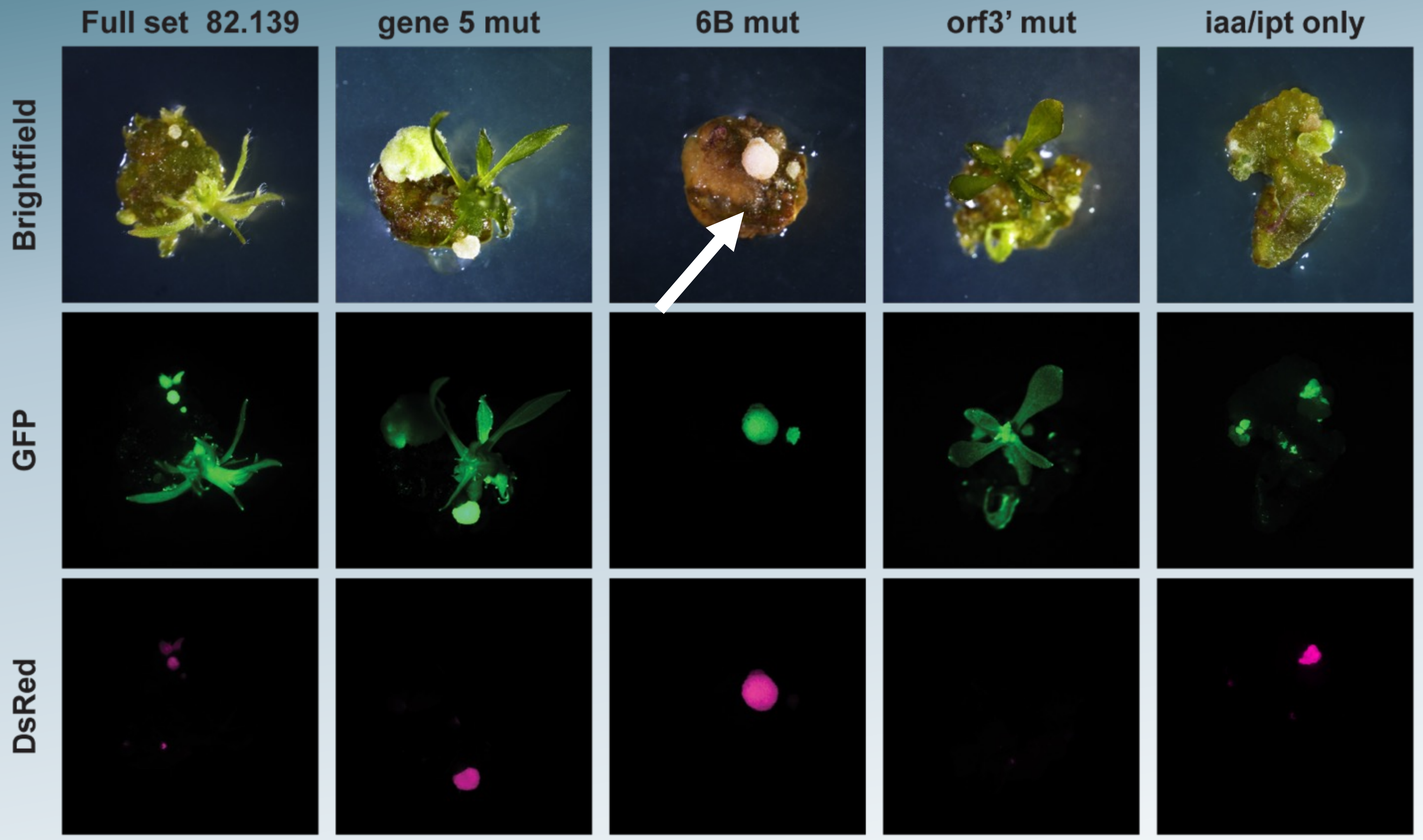
We introduced premature stop codons in each gene to assess contribution to shoot phenotype





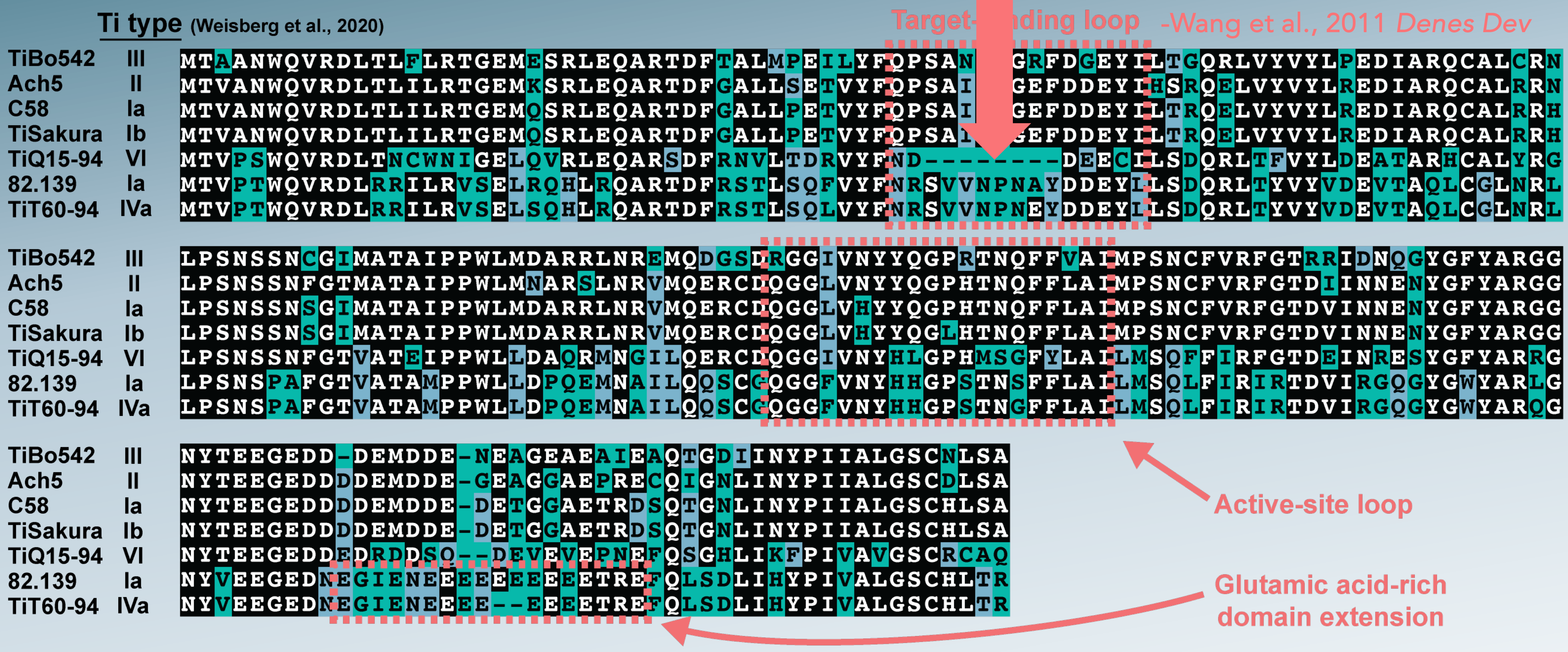
To our surprise there were no additive genetic effects, 6B is the main important gene for alt. shoot formation

Mutations in 6B prevent altruistic shoot formation



Representative images of explants from each construct

What is unique about 6B?



Protein alignment of different Ti plasmid groups from sequenced wild collections

Potential mechanisms of action for 6B include interference with miRNA biogenesis in plants

Molecular insights into plant cell proliferation disturbance by *Agrobacterium* protein 6b

Meimei Wang,[1,2] Takashi Soyano,[3] Satoru Machida,[1,2] Jun-Yi Yang,[3] Choonkyun Jung,[3] Nam-Hai Chua,[3] and Y. Adam Yuan[1,2,4]

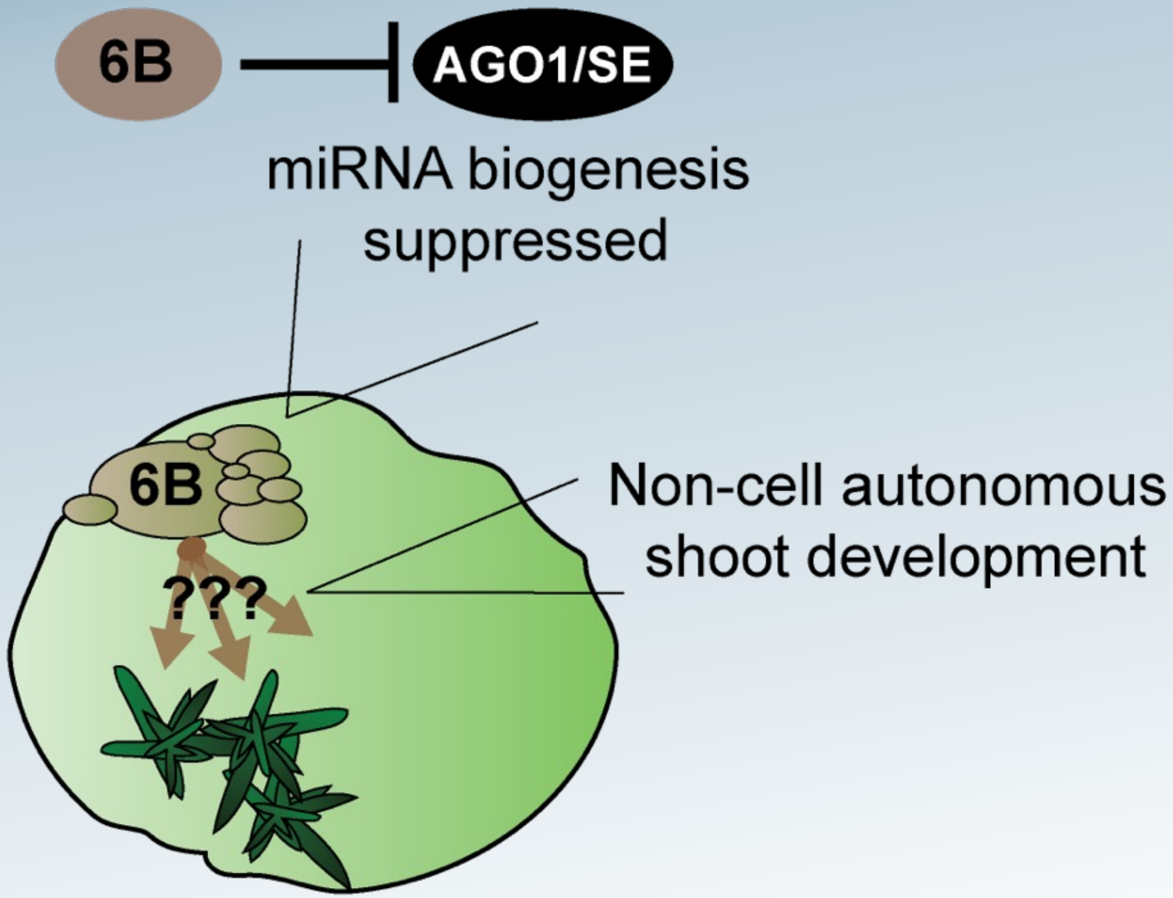
[1]Department of Biological Sciences, National University of Singapore, Singapore 117543, Singapore; [2]Temasek Life Sciences Laboratory, National University of Singapore, Singapore 117604, Singapore; [3]Laboratory of Plant Molecular Biology, The Rockefeller University, New York, New York 10065, USA

Plant Physiol. (1996) 112: 939–951

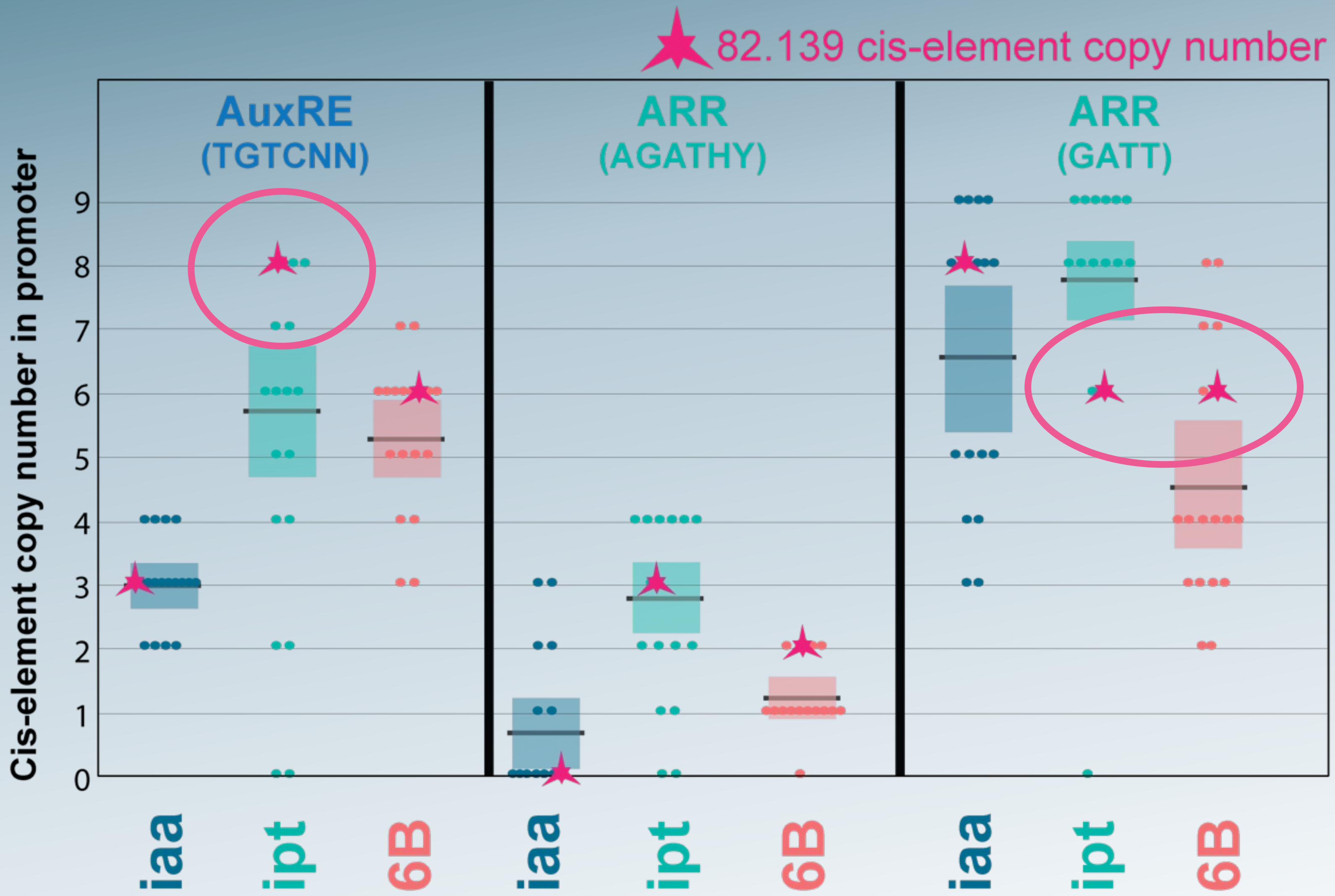
Exogenous Phytohormone-Independent Growth and Regeneration of Tobacco Plants Transgenic for the *6b* Gene of *Agrobacterium tumefaciens* AKE10[1]

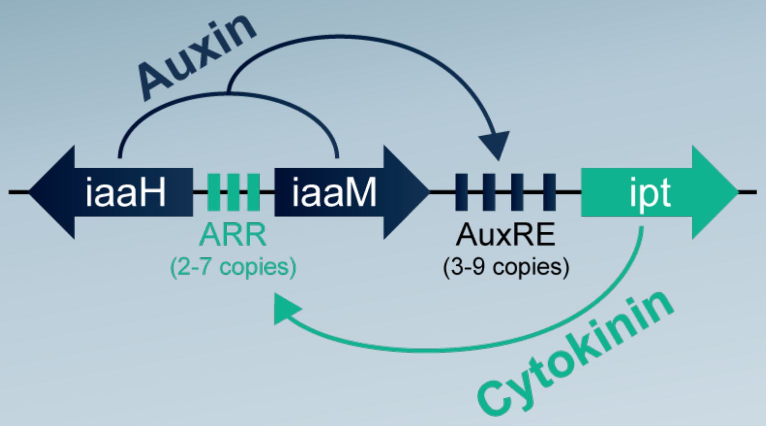
Hiroetsu Wabiko* and Masayo Minemura

Biotechnology Institute, Akita Prefectural College of Agriculture, 2–2 Minami, Ohgata, Akita 010–04, Japan



High levels of diversity of expression elements in *Agrobacterium* T-DNA genes



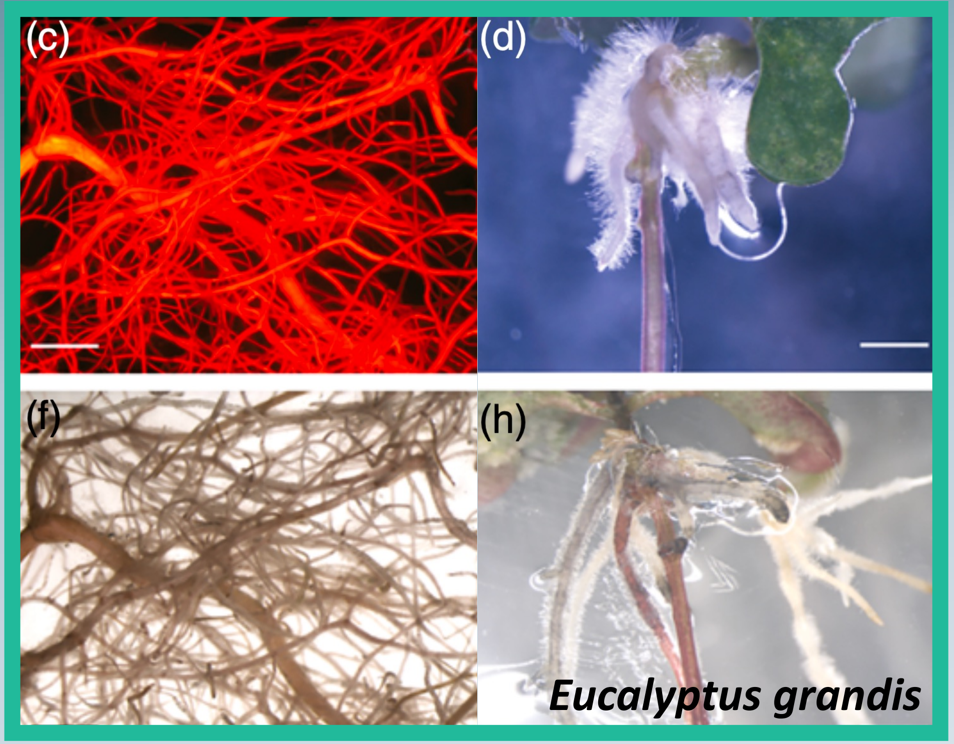


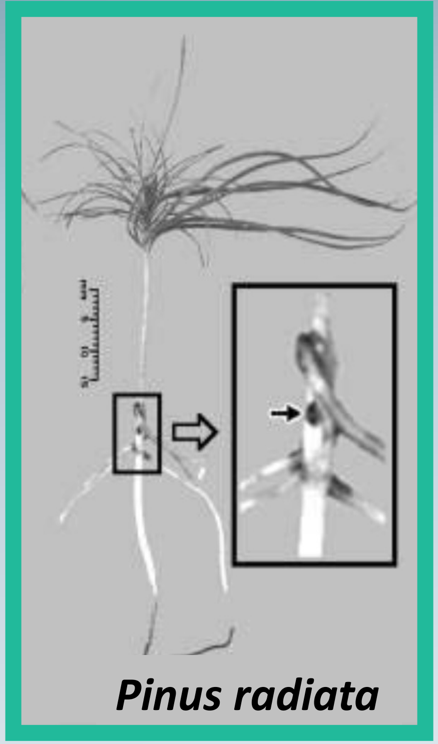
Pairing expression elements not found in nature with "shooty" 6B genes -or synthetically modified ones, is of interest to us

Are there other useful *Agrobacterium* genes we can leverage for transformation?

What about hairy root *rol* genes?

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





And many more such as…

- Poplars
- Yew
- Ginkgo
- *Prunus* sp.
- Apple
- Citrus
- Grape

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

Root-then-shoot transformation is a synthesis of older ideas

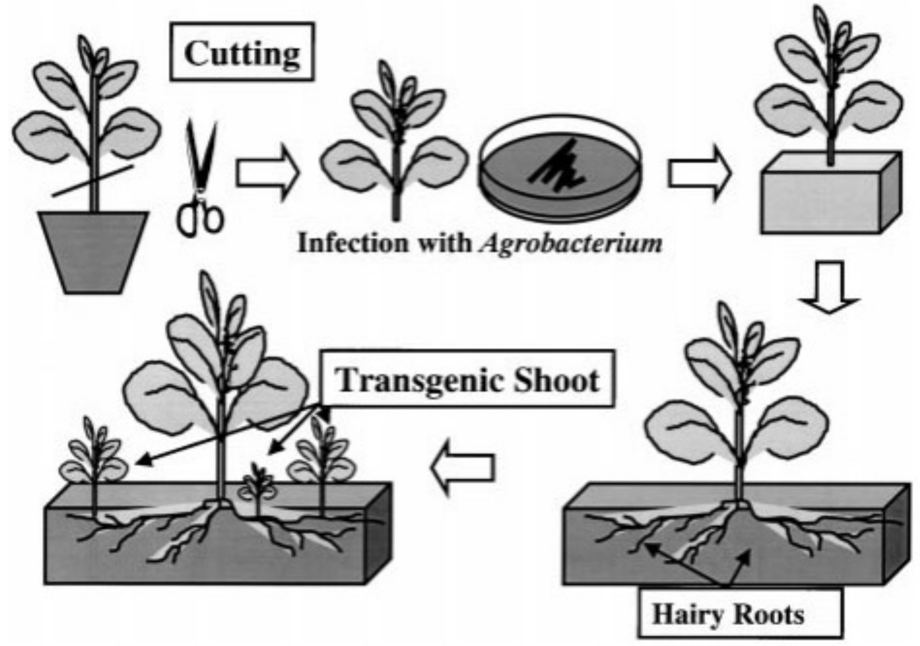
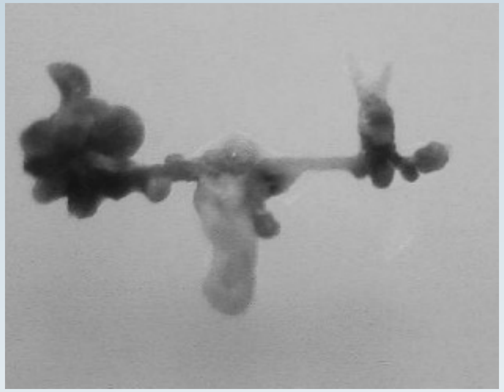


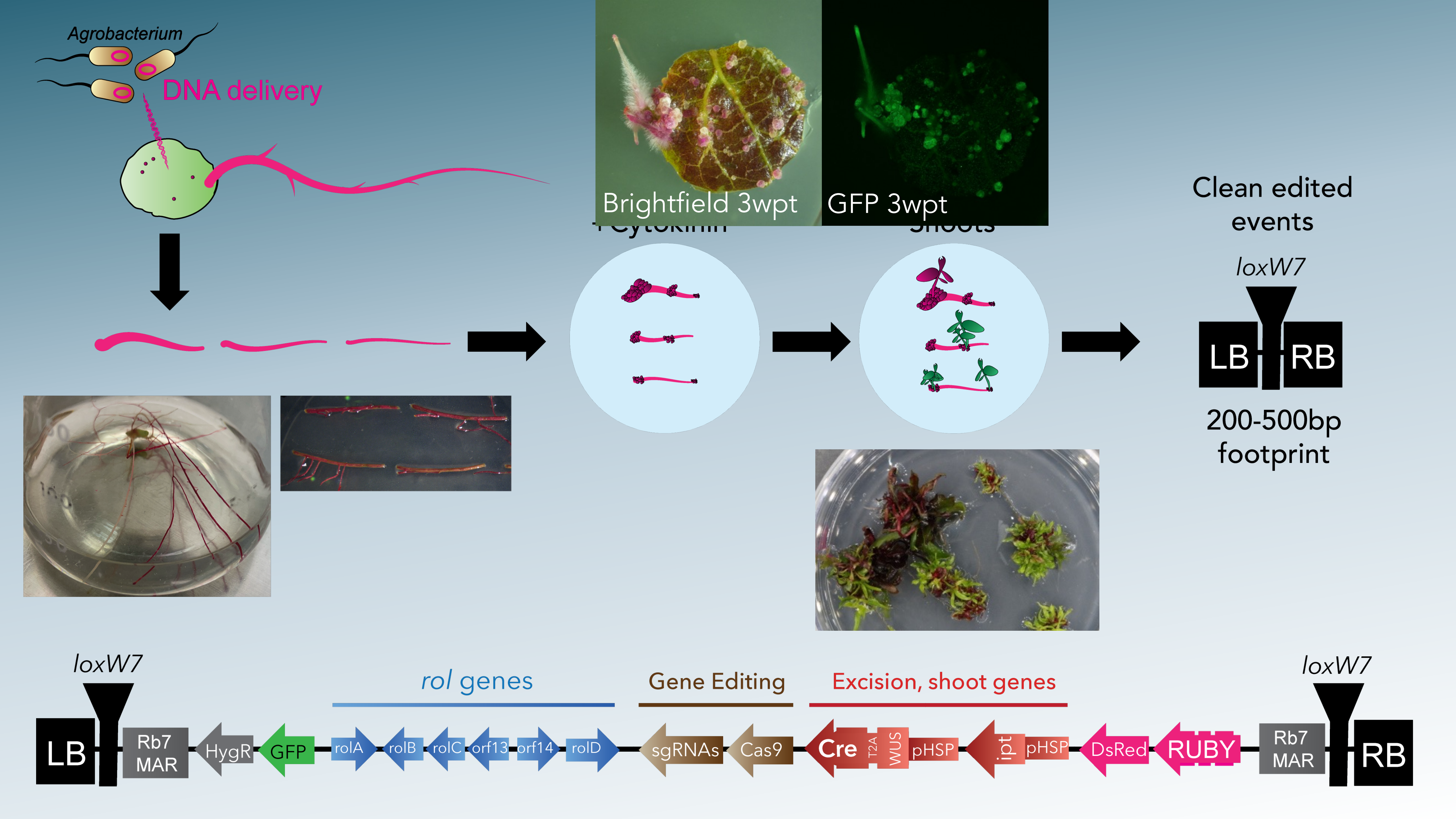
FIG. 8. Outline of *in vivo* transformation using cuttings. Cuttings are infected with *Agrobacterium* containing the *rol*-type MAT vector. After their rooting, marker-free transgenic shoots are induced from hairy roots by the light.

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

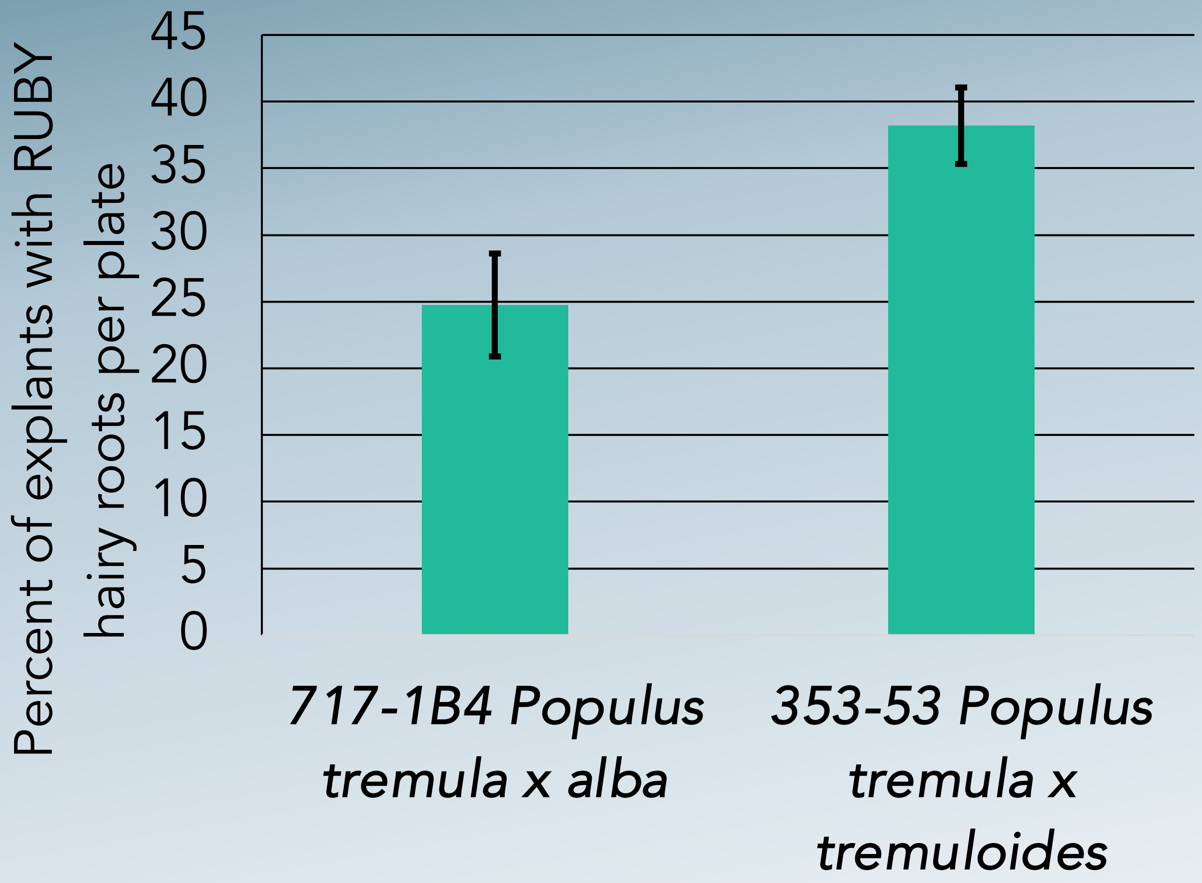


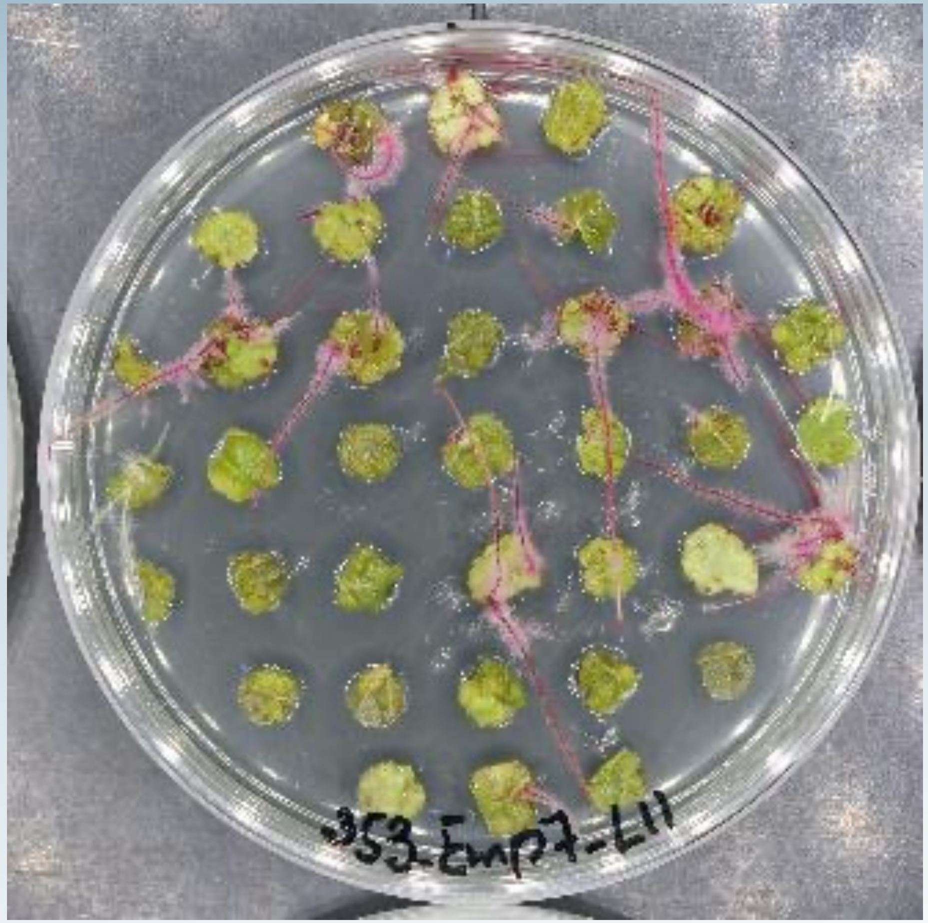
Hiroyasu Ebinuma
(Shinsu U. em.)

Walt Ream
(Microbiology em.)

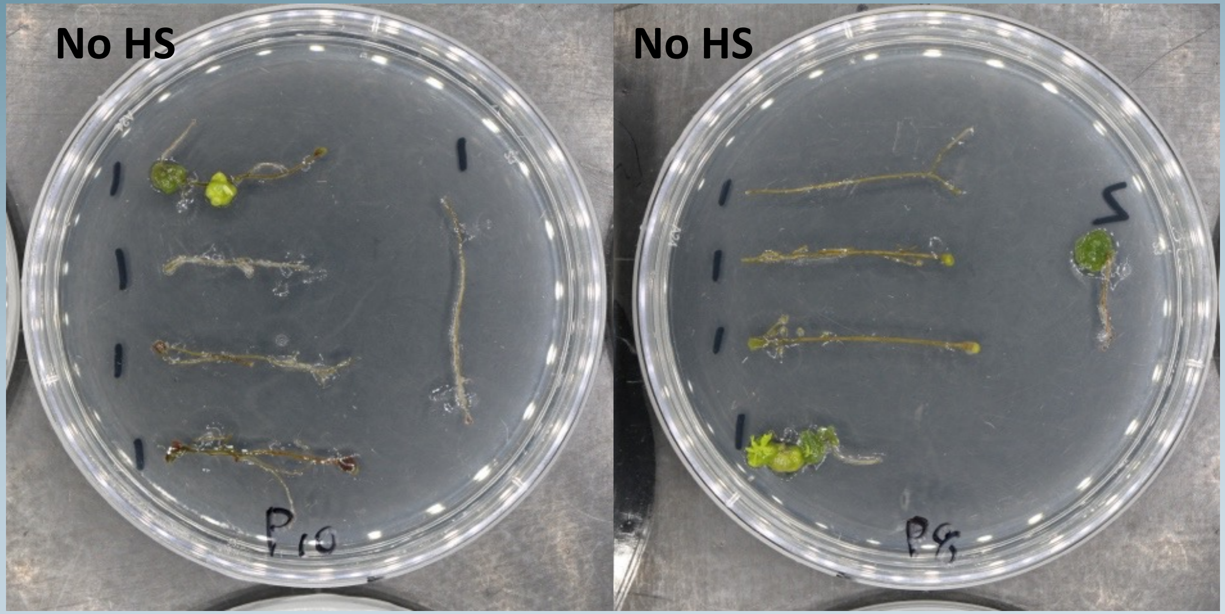

Agrobacterium
DNA delivery
Brightfield 3wpt
GFP 3wpt
Clean edited events
loxW7
LB
RB
200-500bp footprint
loxW7
rol genes
Gene Editing
Excision, shoot genes
loxW7
LB
Rb7 MAR
HygR
GFP
rolA
rolB
rolC
orf13
orf14
rolD
sgRNAs
Cas9
Cre
T2A
WUS
pHSP
ipt
pHSP
DsRed
RUBY
Rb7 MAR
RB

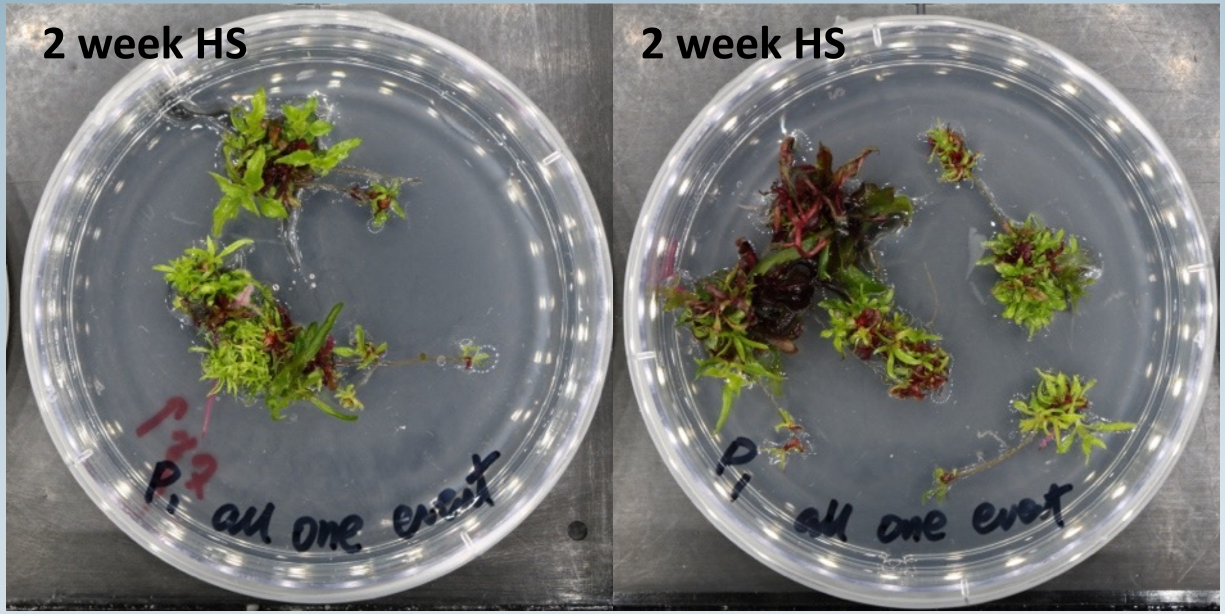
We efficiently generated transgenic hairy roots in two poplar genotypes for regeneration / excision analysis





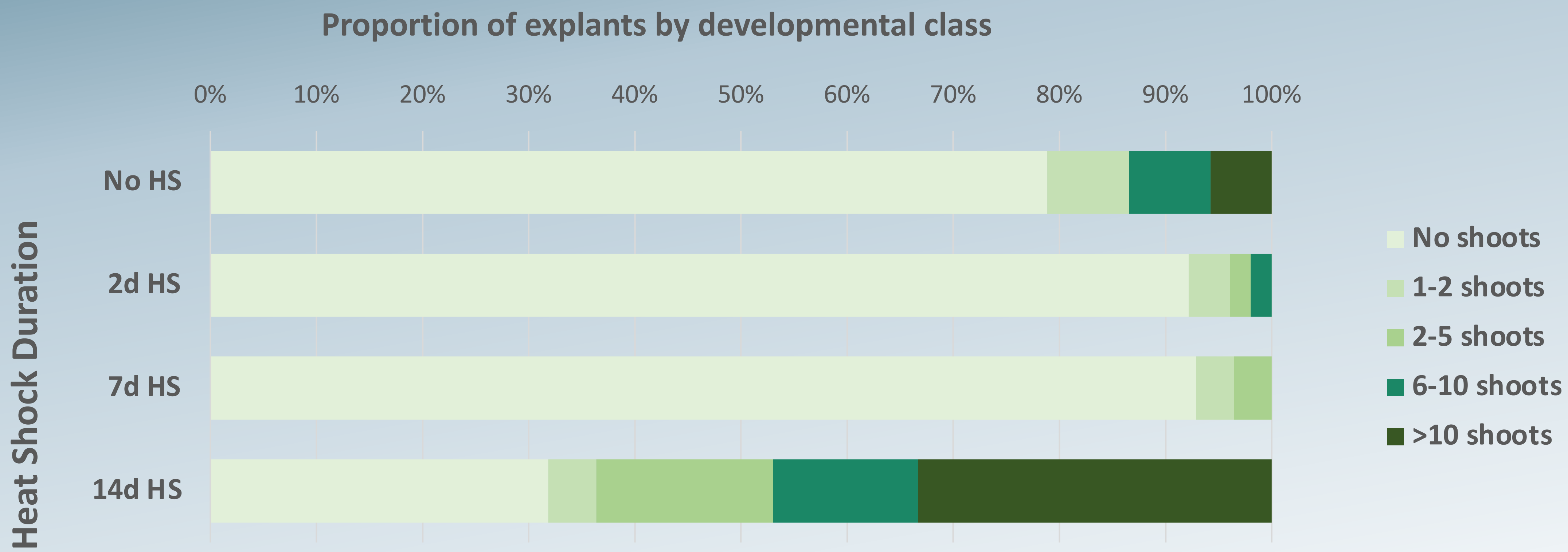
Two weeks of pulsed heat shock resulted in efficient shoot regeneration and excision from hairy roots



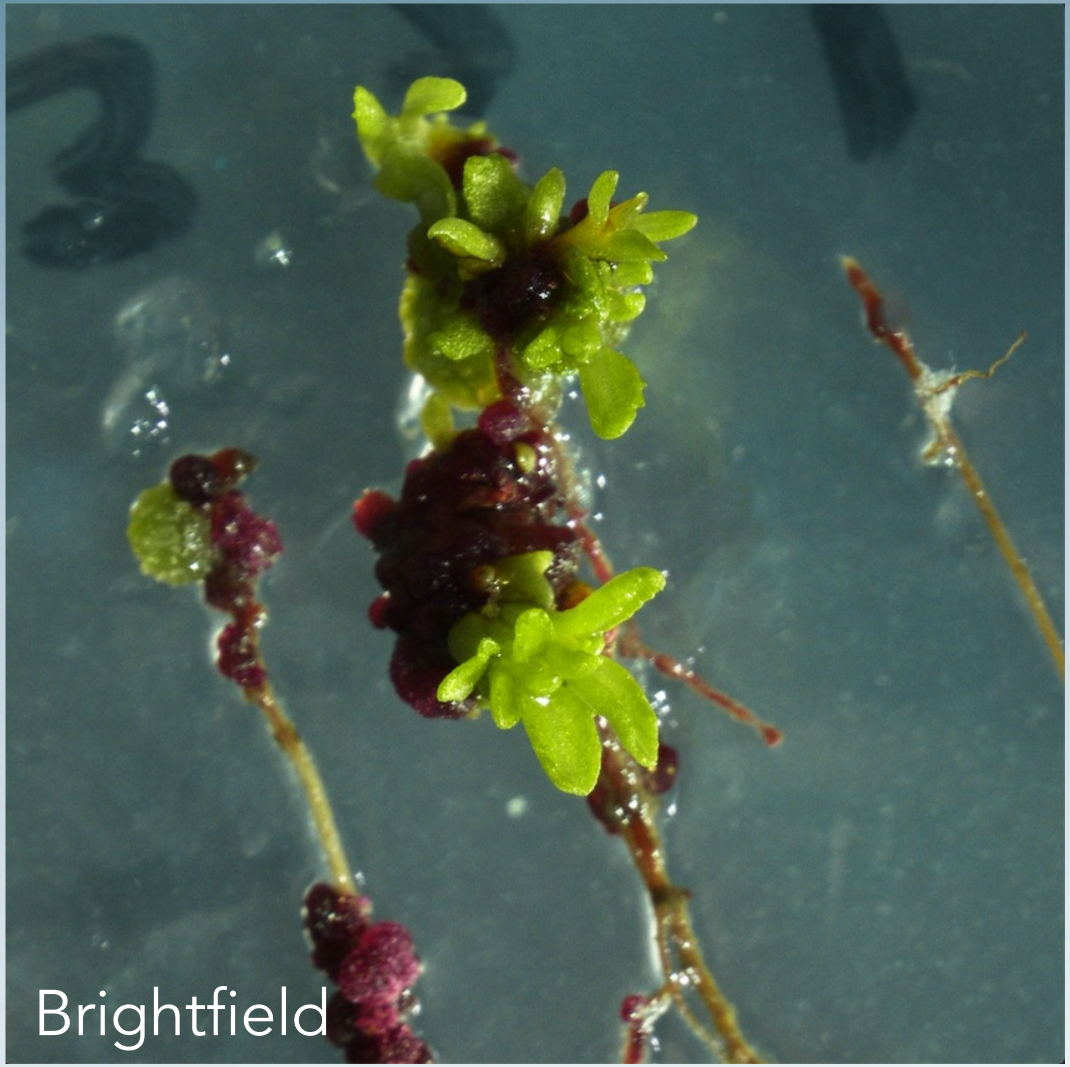


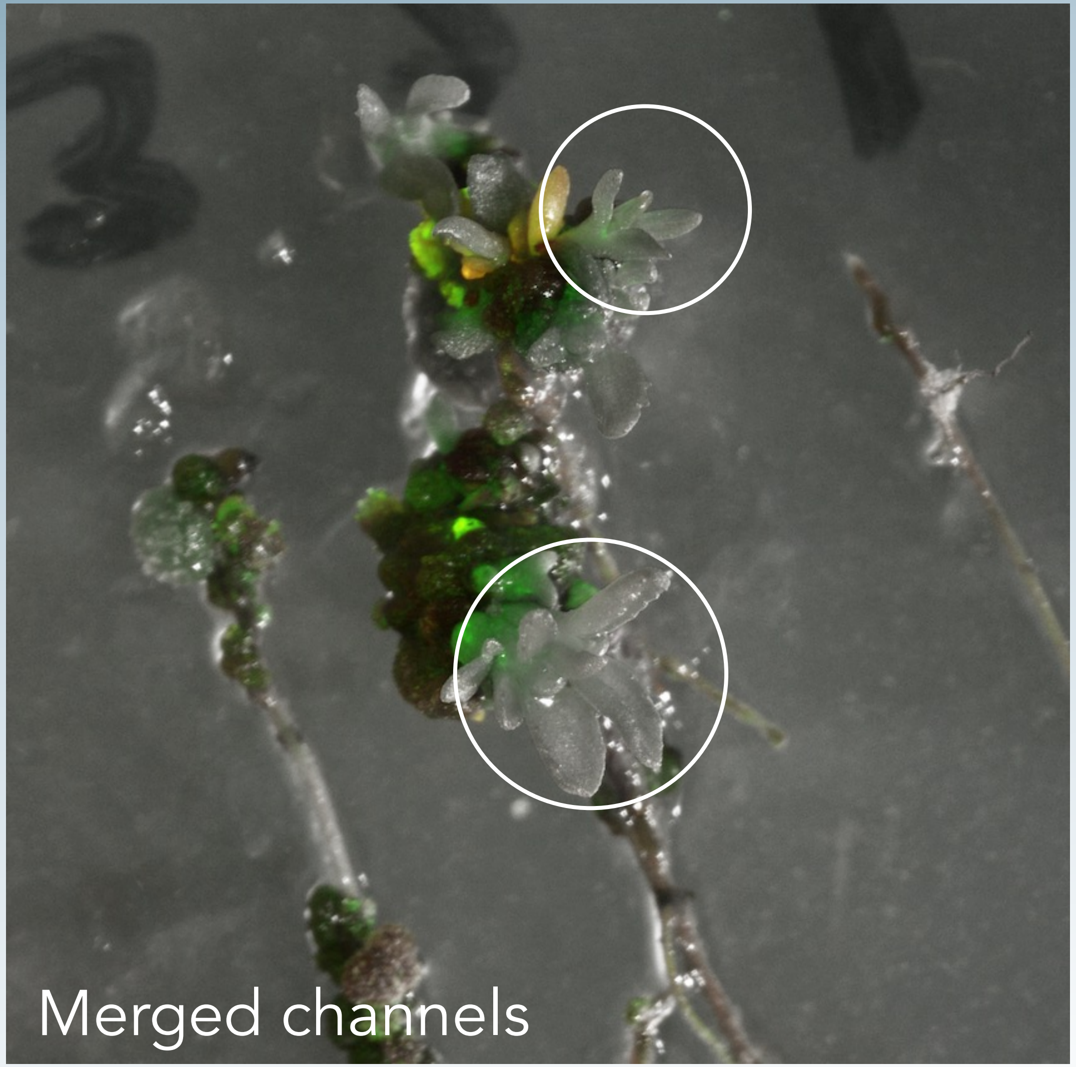
- Four hours heat shock at 38 degrees Celsius, different treatment durations
- RUBY vs. non-ruby shoots can be easily identified for propagation, then other reporters can be closer examined by fluorescent microscopy after isolation

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





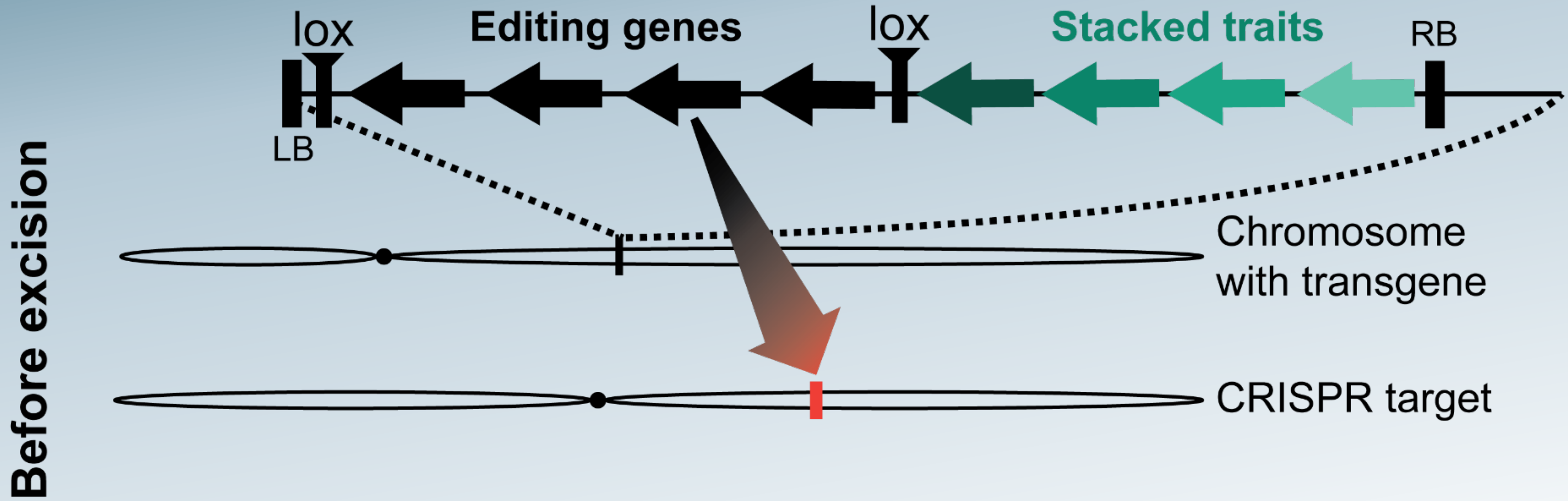
So far high rates of excision and editing have been achieved using this system

Scoring class of recovered shoots	Fully excised	Escapes	Transgenic but no reporter signal
717 (n=25)	32%	28%	40%
353 (n=27)	37%	19%	44%

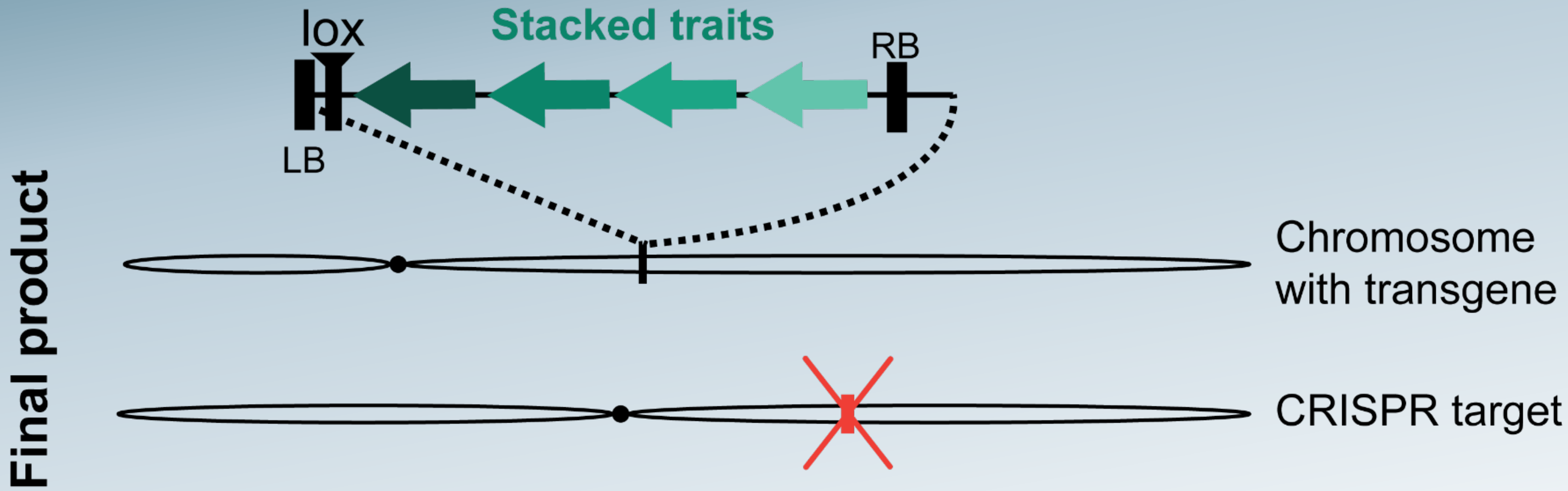
Editing rate (>1 allele edited)	Fully excised (n=3)	Escapes (n=3)	Transgenic but no reporter signal (n=3)
717 (n=9)	100%	33%	100%

- Adjusting to a low level of hygromycin selection to reduce escape rates (currently selection free)
- Determining contributing effects for shoot inducing response (*ipt* vs. *WUS*)

Though not completely transgene-free when gene editing alone, root-then-shoot excision allows for simultaneous incorporation of multiple traits suited to long test cycles in trees



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



Are there other useful growth promoting genes?

Agrobacterium diversity has hardly been studied for use in transformation tools

RESEARCH ARTICLE SUMMARY

PLASMID EVOLUTION

Unexpected conservation and global transmission of agrobacterial virulence plasmids

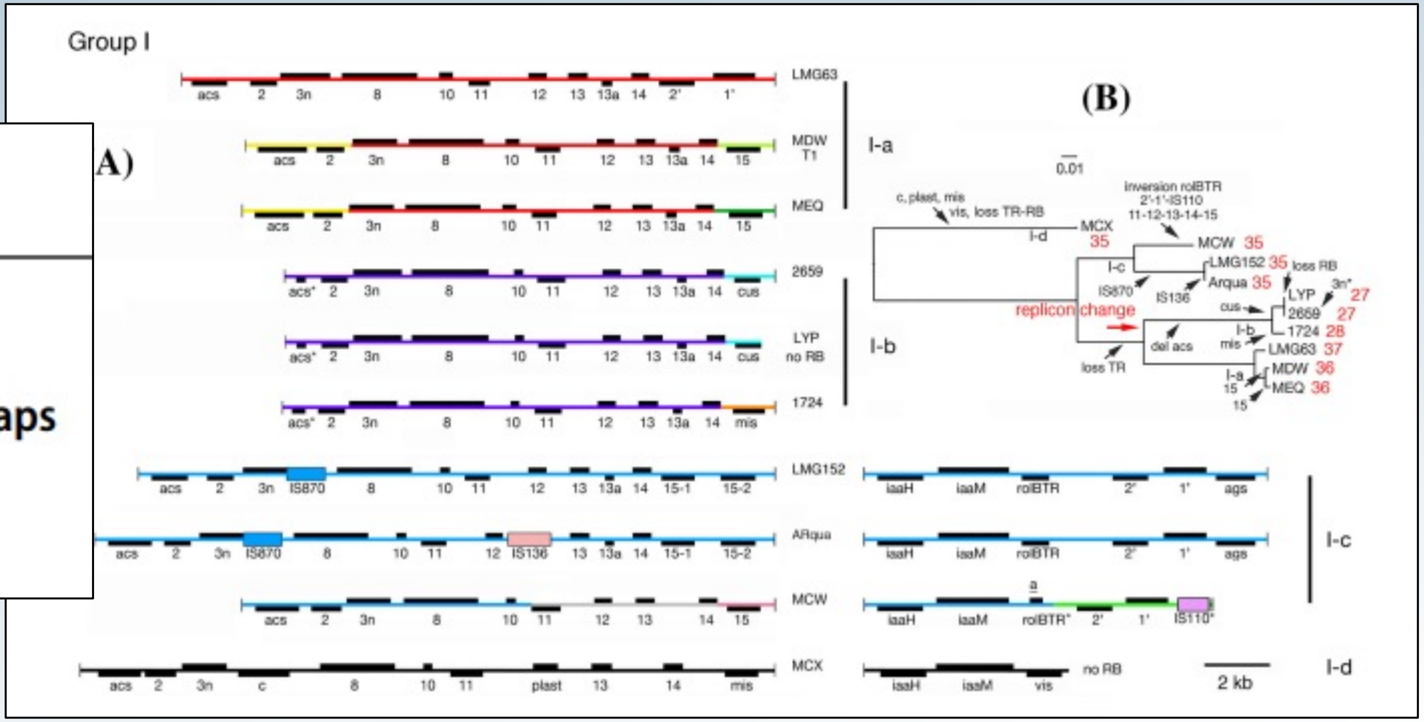
Alexandra J. Weisberg, Edward W. Davis II, Javier Tabima, Michael S. Belcher, Marilyn Miller, Chih-Horng Kuo, Joyce E. Loper, Niklaus J. Grünwald, Melodie L. Putnam, Jeff H. Chang*

Bayer | Grants 4Ag

Plant Molecular Biology (2021) 106:239–258
https://doi.org/10.1007/s11103-021-01140-0

T-DNA regions from 350 *Agrobacterium* genomes: maps and phylogeny

Léon Otten[1]



Summary:
Agrobacterium T-DNA genes are useful for woody plant transformation

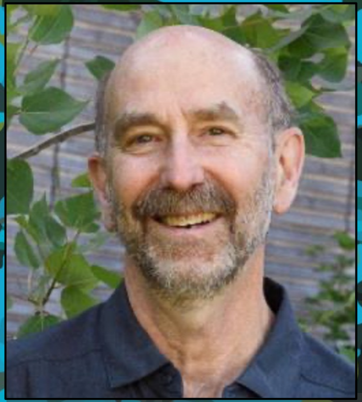
- Genes from shooty *Agrobacterium* strain 82.139 can induce transgenic shoots altruistically in poplar resulting in more efficient and faster transformation

- Gene 6B is the main factor for non-cell autonomous shoot formation

–we will test if it can function alone, or if it works better when paired with iaa/ipt genes from other strains

- Hairy root then shoot excision systems are promising tools for recalcitrant species to produce clean edited plants

Acknowledgements: People



Steve Strauss
Professor FES

Cathleen Ma
Tissue culture and transformation

Kate Peremyslova
Tissue culture and transformation

Victoria Conrad
URSA/honor's college

David Taylor
Technician

Abby Lawrence
Undergrad technician

Work on the project

Victoria Conrad (Honor's College,Hairy root system)
David Taylor (Technician, S82 system)
Abby Lawrence (Undergraduate, S82 system)
Henson Tran (Undergraduate, *in planta*)
Teaghan Knox (Undergraduate, *in planta*)
Katyayani Karlapati ((Undergraduate, *in planta*)

Scientific assistance

Jeff Anderson (BPP,OSU)
Bill Gordon-Kamm (Corteva)
Todd Jones (Corteva)
Jim Thomson (ARS Albany)
Roger Thilmony (ARS Albany)

Acknowledgements: Funding sources

Federal grants
NIFA-Biotechnology Risk Assessment Grant (BRAG)
NSF-Plant Genome Research Program (PGRP)

GREAT TREES Consortium
Suzano, SAPPI, Arauco, Klabin, SweTree, Corteva Agriscience

The National Science Foundation


Thanks for listening!