Back to the future: Innovations to overcome transformation and gene editing bottlenecks

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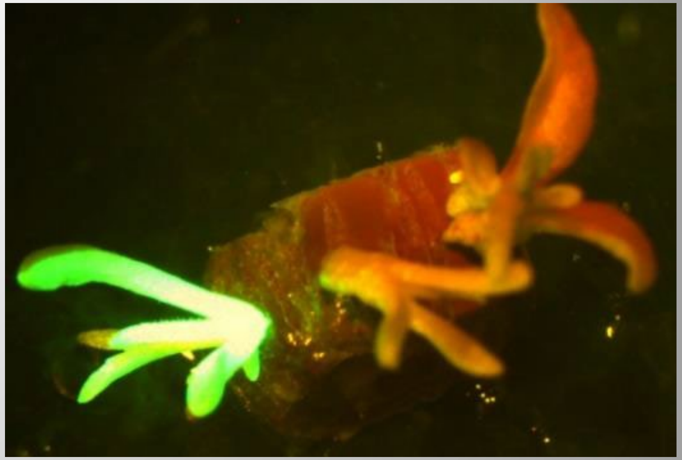
Agenda



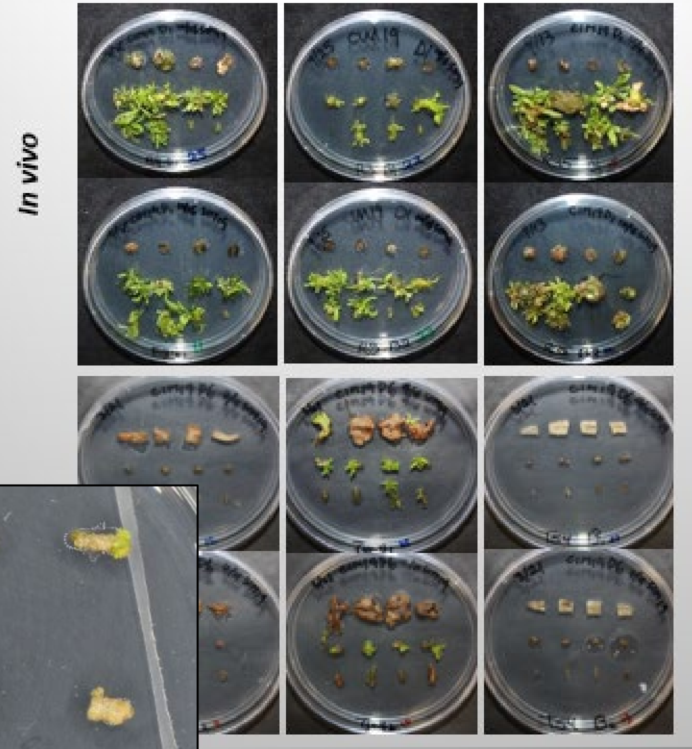
- Perspectives & experimental system
- Brief summary of experiences with some of the DEV genes we have tried, mostly unhappily
- Some stuff we are excited about
  - “Shooty” developmental regulator genes from Agrobacterium

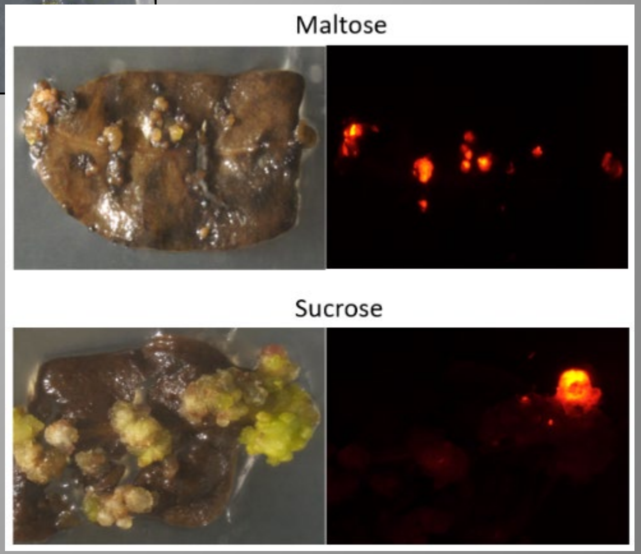
Transformation & regeneration (TR) continue to be major limiting factors for gene editing & engineering in plants, and especially trees

- Species, genotypic, physiological variation often dramatic
- Slow, costly, complex customization efforts usually needed
- On top of often large social/regulatory constraints, often a “deal breaker”

Our experimental system features

- Woody (forest) trees – slow, tough biochemisty
- Elite clones, mature propagules, not seed-derived
- High physiological diversity
  - Growth environment, age, explant type and source
- Great tissue sample heterogeneity in response
- Common necrotic responses
- Very high genetic diversity of forest trees
- Large interactions among all of the above





“DEV” genes can help, are they the miracles we hope for?

plants

Review

Using Morphogenic Genes to Improve Recovery and Regeneration of Transgenic Plants

Bill Gordon-Kamm *, Nagesh Sardesai, Maren Arling, Keith Lowe, George Hoerster, Scott Betts and Todd Jones

Table 1. The effects of *WUS, BBM, GRFs*, and *GRFs–GRFs* on plant development and genetic transformation.

Gene*	Promoter	Explants	Effects	Ref.
AtWUS	Estrogen-inducible	*A. thaliana* root	High somatic embryo formation frequency	[15]
	Estrogen-inducible	*Nicotiana tabacum* leaf	Shoot formation from root tip	[20]
	35S	*Gossypium hirsutum* hypocotyl	Shoot formation from root tip	[16]
	vsp1	*Medicago truncatula* seedling radicle	47.75% increase in embryogenic callus formation	[18]
ZmWUS2	*ZmPLTP*	*Zea mays* immature embryo	Enhanced callogenesis and embryogenesis	[66]
	Nos	*A. thaliana* (seedling), *Solanum lycopersicum* (seedling), *N. tabacum* (seedling/mature plant), *Solanum tuberosum* (mature plant), *Vitis. vinifera* (mature plant)	*de novo* meristem induction	[38]
AtWUS-GR, AtSTM-GR	*35S*	*A. thaliana* (floral dip)	Triggered ectopic organogenesis	[18]
AtWUS, CHAP3A (PmLEC1)	Estrogen-inducible	*Picea glauca* immature embryo	Did not induce somatic embryogenesis	[59]
eGFP-GhWUS1a, eGFP-GhWUS1b	Estrogen-inducible	*G. hirsutum* hypocotyl	Inhibited embryogenic callus formation	[60]
AtBBM, BnBBM	*35S*, inducible	*N. tabacum* leaf	Enhance the regeneration capacity	[24]
BcBBM	*35S*	*Populus tomentosa* calli	Plant regeneration through somatic embryogenesis	[25]
BnBBM	*35S, HnUbB1*	*A. thaliana* (floral dip) *B. napus* haploid embryo	Spontaneous formation of somatic embryos and cotyledon-like structures	[22]
BnBBM	*35S*	*Capsicum. annuum* cotyledon	Made recalcitrant pepper transformable	[23]
EgAP2-1 (BBM)	*35S*	*A. thaliana* (floral dip)	Enhanced regeneration capacity	[63]
GmBBM1	*35S*	*A. thaliana* (floral dip)	Induced somatic embryos on vegetative organs	[64]
TcBBM	*35S*	*A. thaliana* (floral dip)	Enhanced/hormone-independent somatic	[65]
AtBBM-GR	*35S*	*A. thaliana* (floral dip)	Improved plant regeneration for extended periods of time in tissue culture	[62]
HvWUS, HvBBM	*ZmAxig1, ZmPLPT*	*Hordeum vulgare*	Co-expression increased transformation efficiency by 3 times	[61]
ZmBBM+ZmWUS2	*ZmUbi, Nos*	*Z. mays* immature embryo, mature embryo, seedling leaf segment; *Oryza sativa* calli; *Sorghum bicolor* immature embryo; *Saccharum officianrum* calli	Enabled transformation of recalcitrant varieties and/or increased transformation efficiency	[26–28]
	ZmAxig1, ZmPLTP	*Z. mays* immature embryo	Established rapid callus-free transformation	[29]
	ZmPLTP	*S. bicolor* immature embryo	Reduced genotype dependence, accelerated regeneration, increased transformation efficiency	[67]
AtGRF5/BvGRF5-L	*2×35S*	*Beta. vulgaris* cotyledon, hypocotyl	Enabled transformation of recalcitrant varieties. Increased transformation efficiency	[33]
AtGRF5/HaGRF5-L	*2×35S*	*Helianthus annuus* cotyledon	Improved transgenic shoot formation	
GmGRF5-L	*PcUbi4-2*	*Glycine. max* primary node	Improved transgenic shoot formation	
BnGRM5-L	*PcUbi4-2*	*B. napus* hypocotyl	Promoted callus production	
ZmGRF5-L1/2	*BdEF1*	*Z. mays* immature embryo)	Increased transformation efficiency ~3 times	
TaGRF4-GIF1	*ZmUbi*	*Triticum aestivum* immature embryo	Increased regeneration efficiency 7.8 times; shortened protocol	[34]
		O. sativa calli from seeds	Increased regeneration efficiency 2.1 times	
ClGRF4[1]-GIF1/VvGRF4-GIF1	*35S*	*Citrus limon* etiolated epicotyl	Increased regeneration efficiency ~4.7 times	
ClGRF4[2]-GIF1	*35S*	*Citrullus lanatus* cotyledon	Increased transformation efficiency ~9 times	[68]

At, A. thaliana; Zm, Z. mays; Pm, Picea mariana; Gh, G. hirsutum; Bn, B. napus; Bc, B. campestris; Eg, Elaeis guineensis; Gm, G. max; Tc, Theobroma cacao; Hv, H. vulgare; Bv, B. vulgaris; Ta, T. aestivum; Cl, [1]C. limon, [2]C. lanatus; Vv, V. vinifera.

Ornamental Plant Research

https://doi.org/10.48130/OPR-2022-0004

Ornamental Plant Research **2022**, 2: 4

New opportunities for using *WUS/BBM* and *GRF-GIF* genes to enhance genetic transformation of ornamental plants

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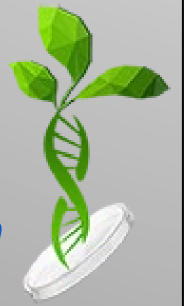
Focus of GREAT TREES Coop:

“Developmental genes as methods to enhance gene editing and transformation in eucalypts”



What are DEV genes?

- Many names in literature – including "morphogenetic genes"
- **DEV gene** = any gene whose expression is useful in promoting the transformation or regeneration (TR) of transgenic or gene-edited tissues
- Derived from basic studies of plant development and pathology – but use in TR deviates from natural roles due to the radical interventions that are part of TR
  - Redifferentiation from terminally differentiated somatic tissues
  - Wounding and pathogen attack (Agrobacterium)
  - Complexity of natural meristem / embryo / organ regeneration pathways

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

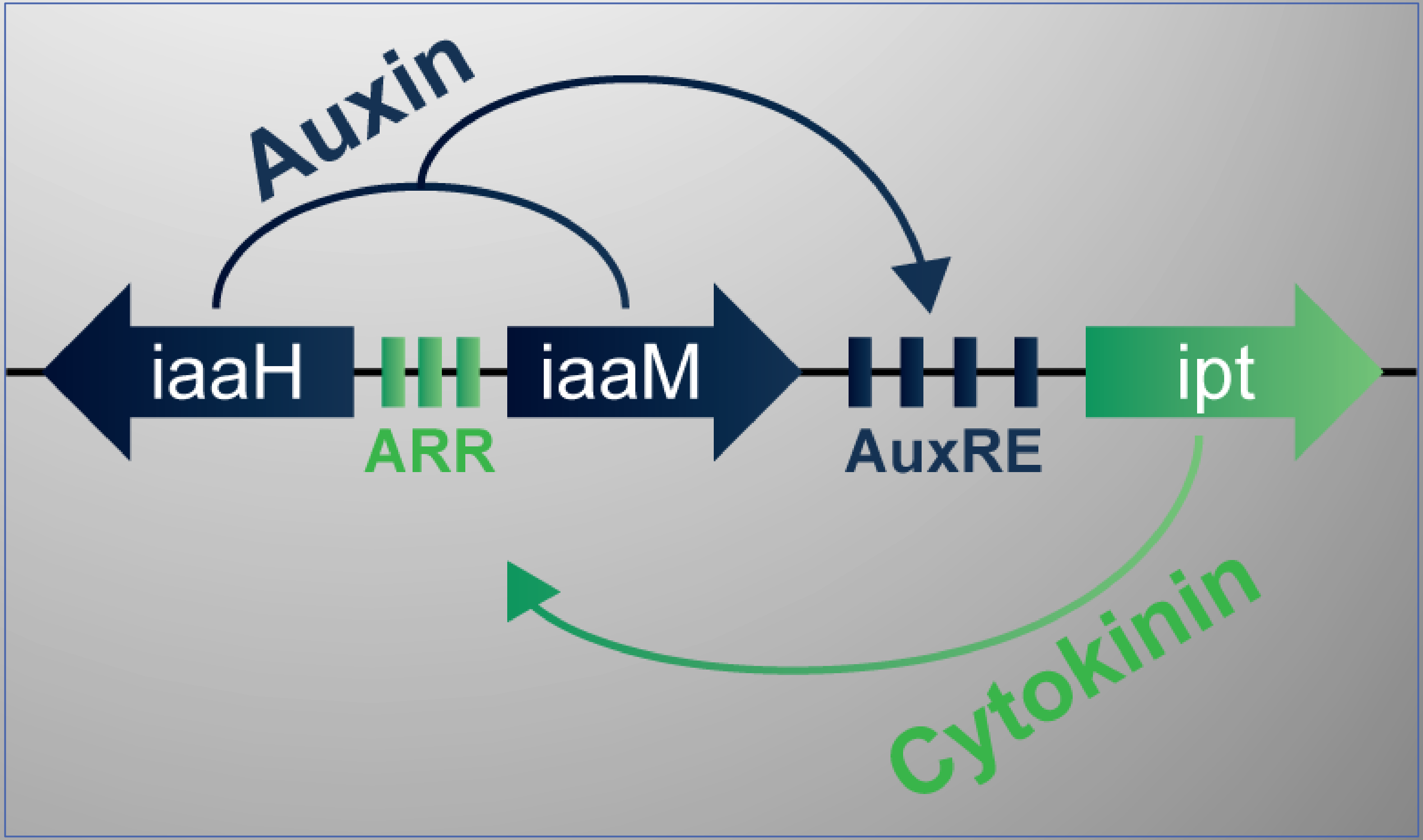
- *LEC* 1, 2 – *LEAFY COTYLEDON*
- *EBB1 - EARLY BUD BREAK 1* (ESR family)
- *BBM – BABY BOOM*
- *WOX* 5, 11 -- *WUSCHEL RELATED HOMEOBOX*
- *WUS – WUSCHEL*
- *GRF-GIF – GROWTH REGULATOR FACTOR 4* and *GRF INTERACTING FACTOR* 1
- *IPT – ISOPENTYL TRANSFERASE* (cytokinin) – Agrobacterium
- Sets of Agrobacterium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

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
- *IPT – ISOPENTYL TRANSFERASE* (cytokinin) – Agrobacterium
- **Sets of Agrobacterium developmental regulator genes**

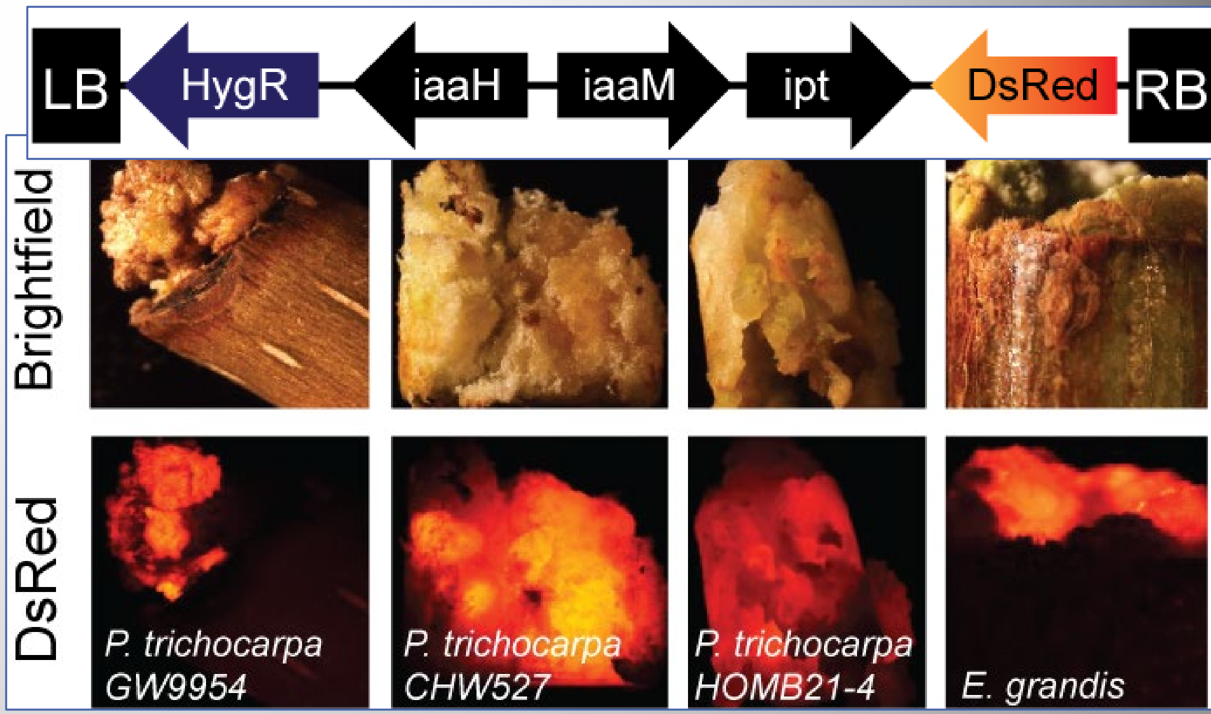
Back to the future: *A. tumefaciens* developmental regulator genes revisited with new techniques?

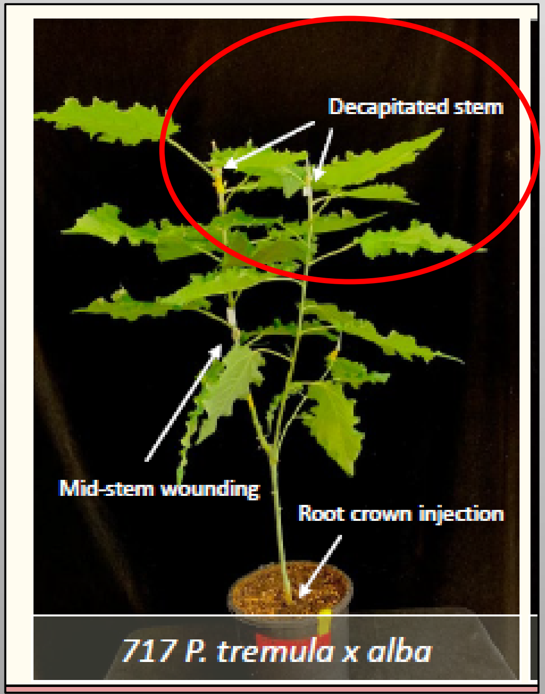
iaa/ipt genes form a positive feedback loop to induce and maintain gall development

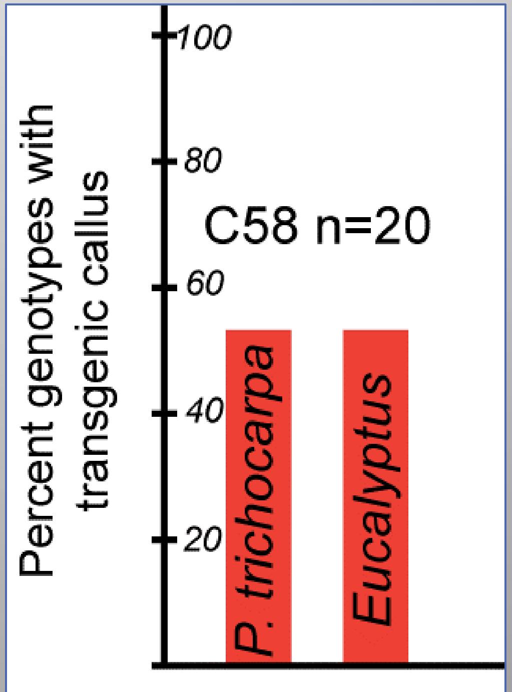


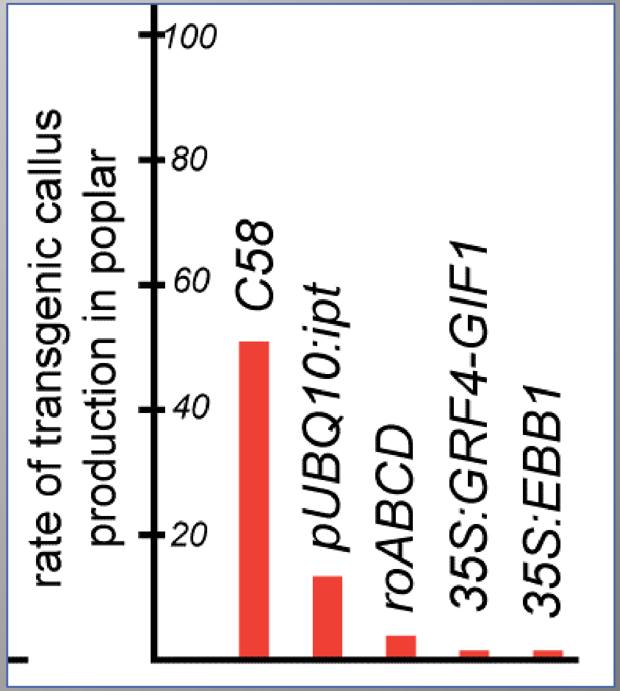
iaaH/M and ipt genes from *Agrobacterium* (C58) were effective *in planta* inducers of transgenic galls in diverse poplar and eucalypt genotypes

But shoots could not be regenerated from transgenic galls









Can we find more useful, developmentally flexible galls?

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

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

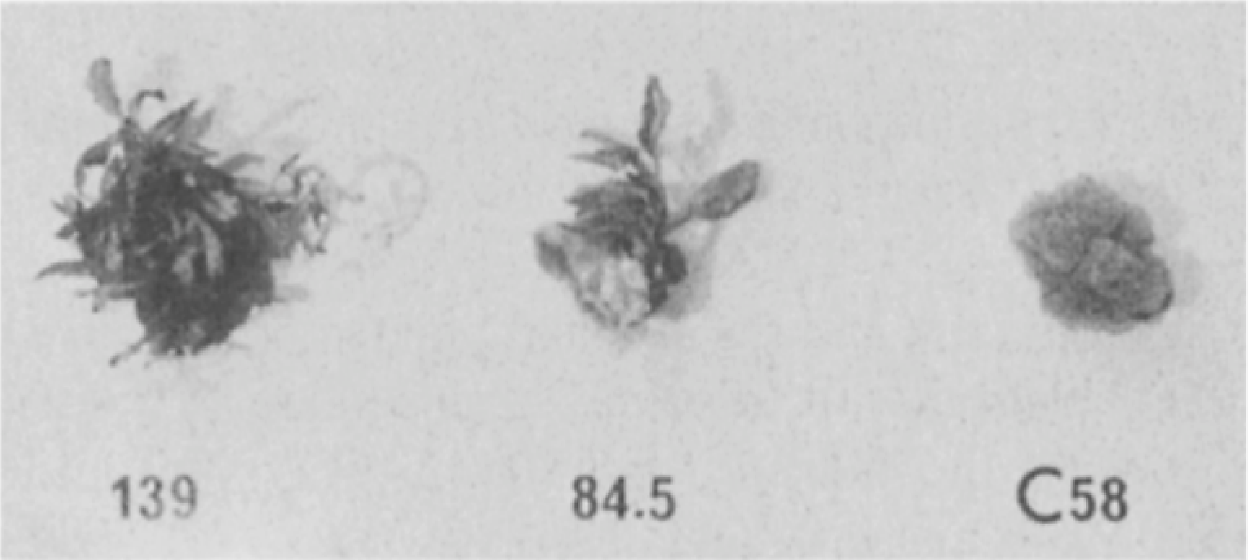

Fig. 1. Tumors and shoot differentiation from poplar tumors induced by *A. tumefaciens* strains 82.139, 84.5 and C58 and cultivated on MS medium, 6 weeks after inoculation.

The method also reportedly worked in *Eucalyptus*, less well in birch, using the wild strain

Agrobacterium strain specificity and shooty tumour formation in eucalypt (_Eucalyptus grandis_ × _E. urophylla_)

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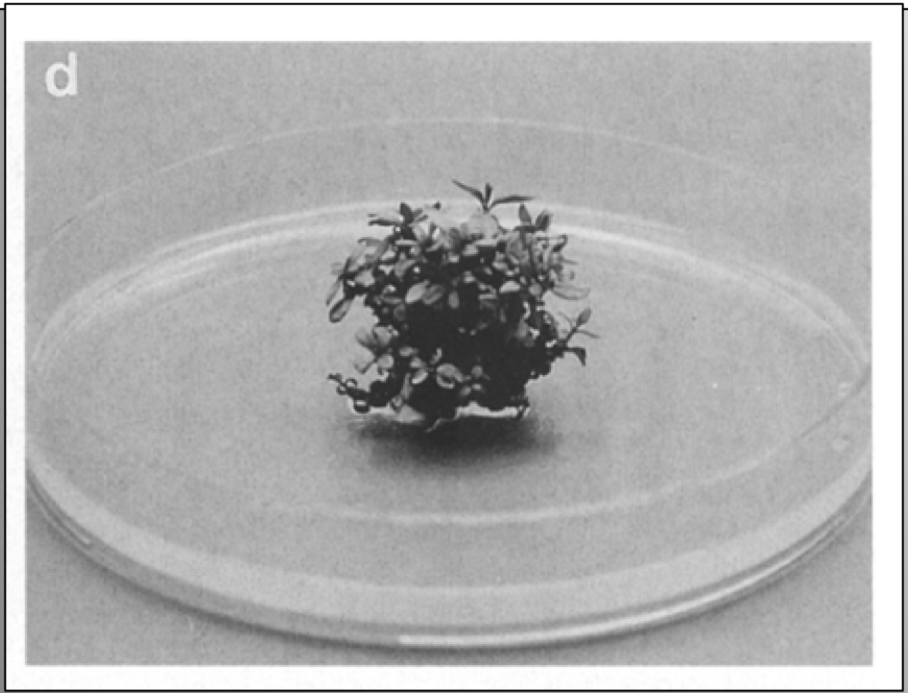
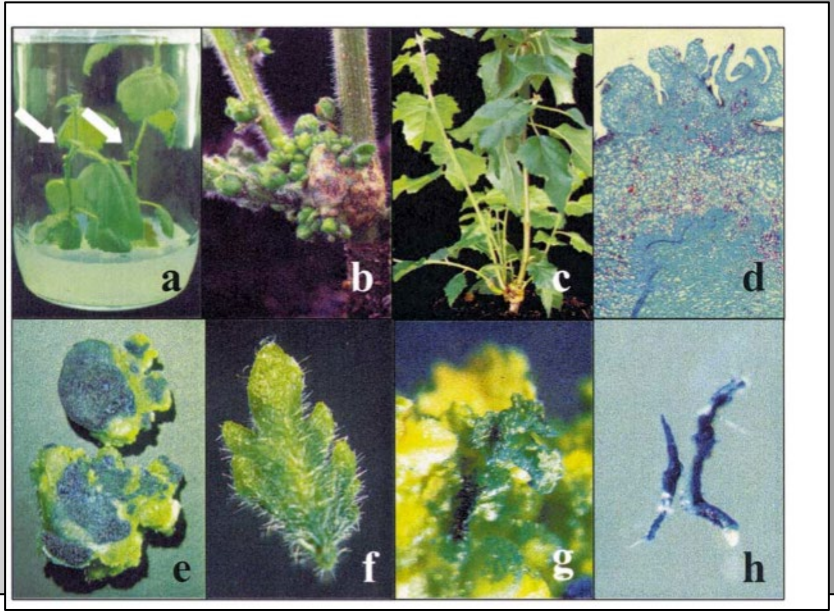
[2] Aracruz Celulose S. A. Rua Prof. Lobo, 1128, 29.190-000 Aracruz – ES, Brazil

Received 27 November 1995/Revised version received 2 July 1996 – Communicated by M. R. Davey





Plant Cell, Tissue and Organ Culture **70:** 147–154, 2002.

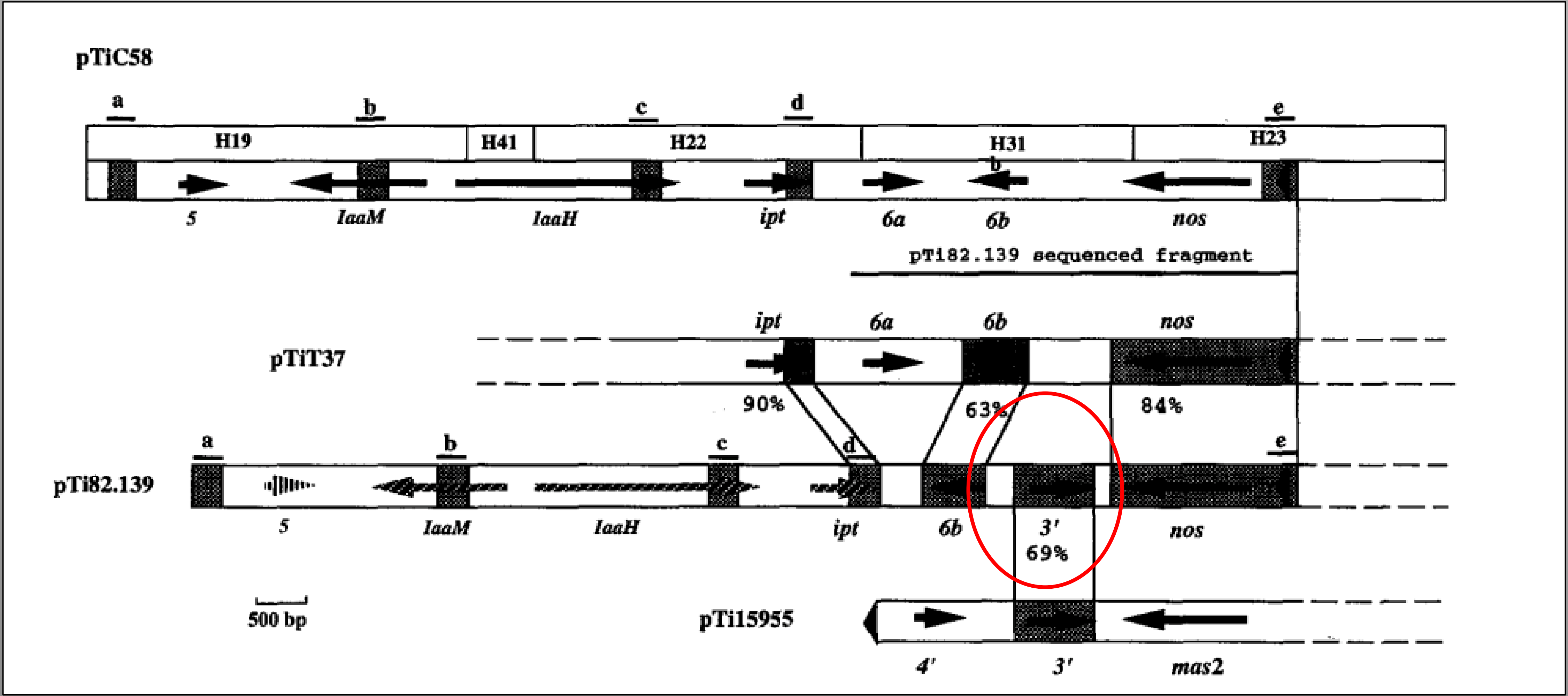
Applicability of the co-inoculation technique using _Agrobacterium tumefaciens_ shooty-tumour strain 82.139 in silver birch

Tuija S. Aronen[1], Juhani H. Häggman[1] & Hely M. Häggman[1,2,*]
[1] *Finnish Forest Research Institute, Punkaharju Research Station, Finlandiantie 18, FIN-58450 Punkaharju, Finland;* [2] *University of Oulu, Department of Biology, PO Box 3000, FIN-90014 Oulu, Finland (*requests for offprints; Fax: +358-08-5531061; E-mail: hely.haggman@oulu.fi)*

Received 19 December 2000; accepted in revised form 2 November 2001

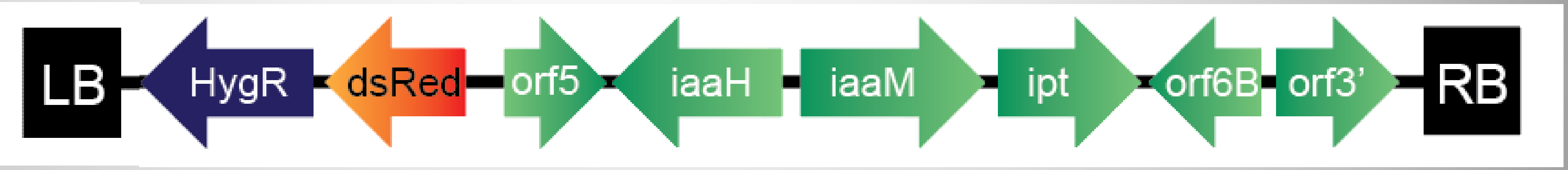
Key words: Betula pendula, genetic transformation, *in planta*, *in vitro*, oncogenic agrobacteria, pGUSINT

This strain has several genes added compared to C58 due to a recombination event, although expression of *iaa/ipt* could also be different

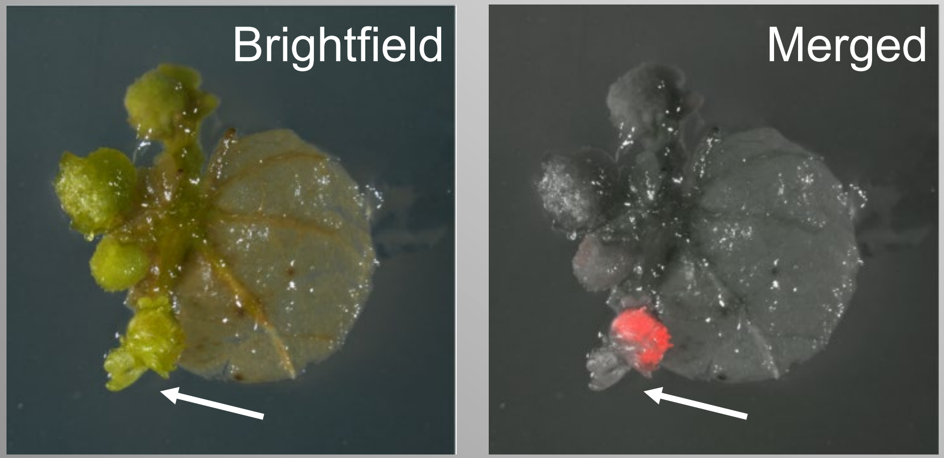


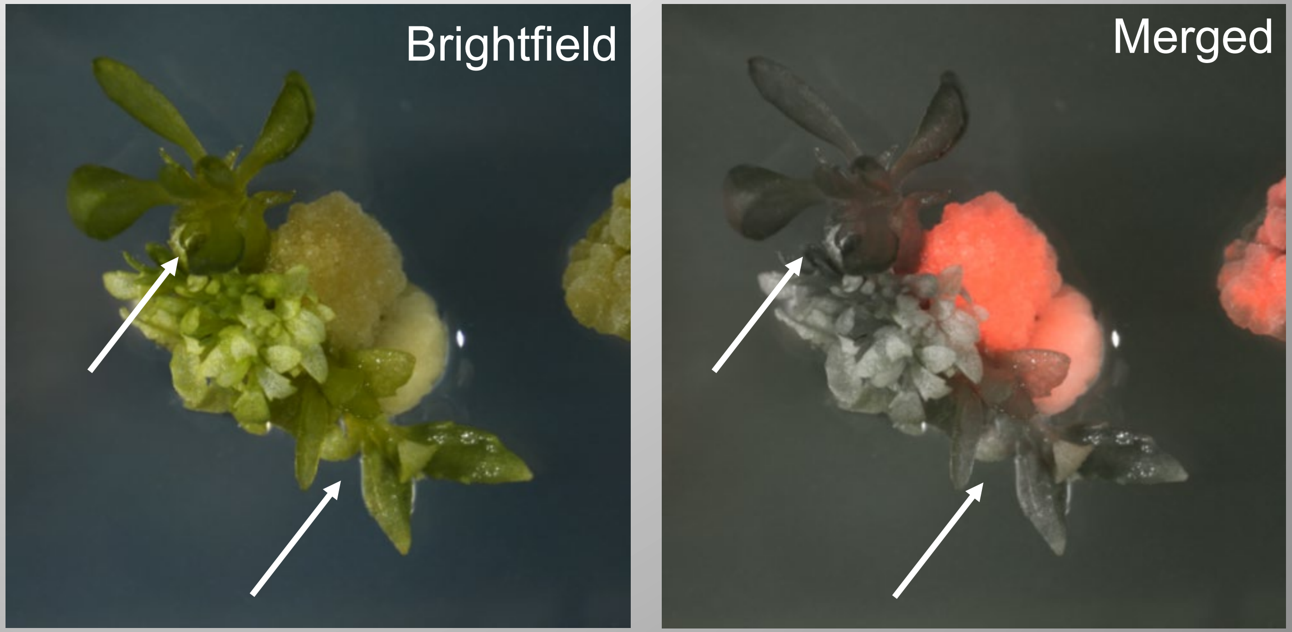
Though very promising, this work essentially came to a halt – due to GMO pushback in Europe – and due to the challenges of dealing with the large Ti plasmids and its many *vir* and development genes prior to high throughput sequencing and advanced gene cloning systems

We cloned out the genes from our resurrected clone in deep freeze, and added modern amenities like DsRed (called “S82”)

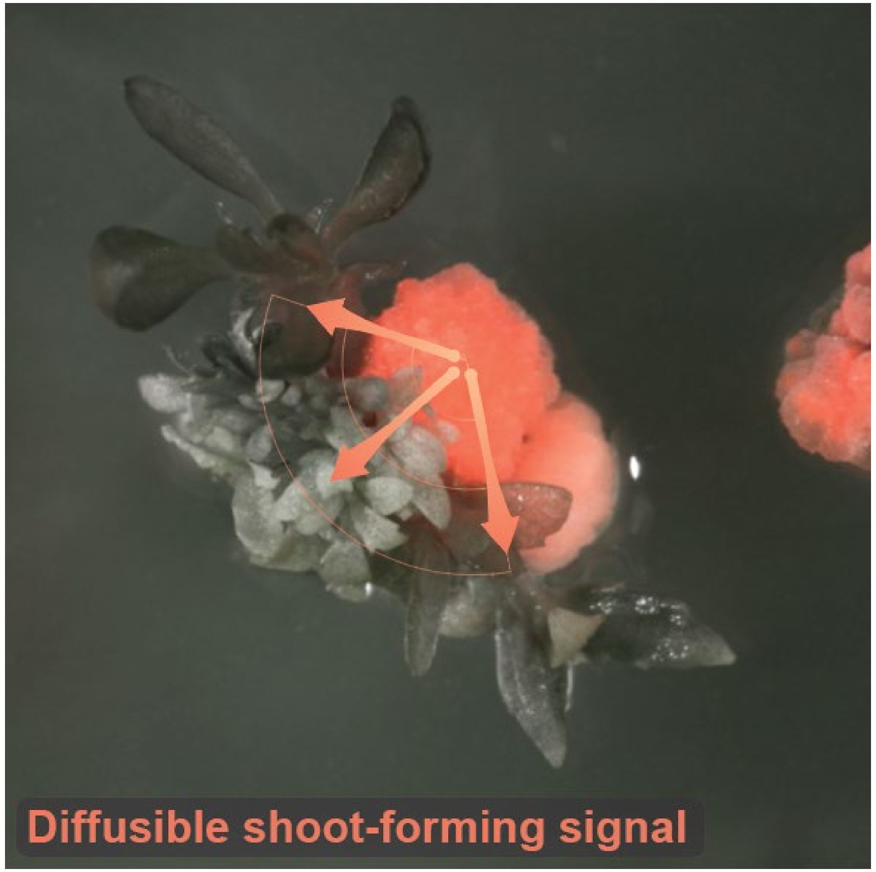


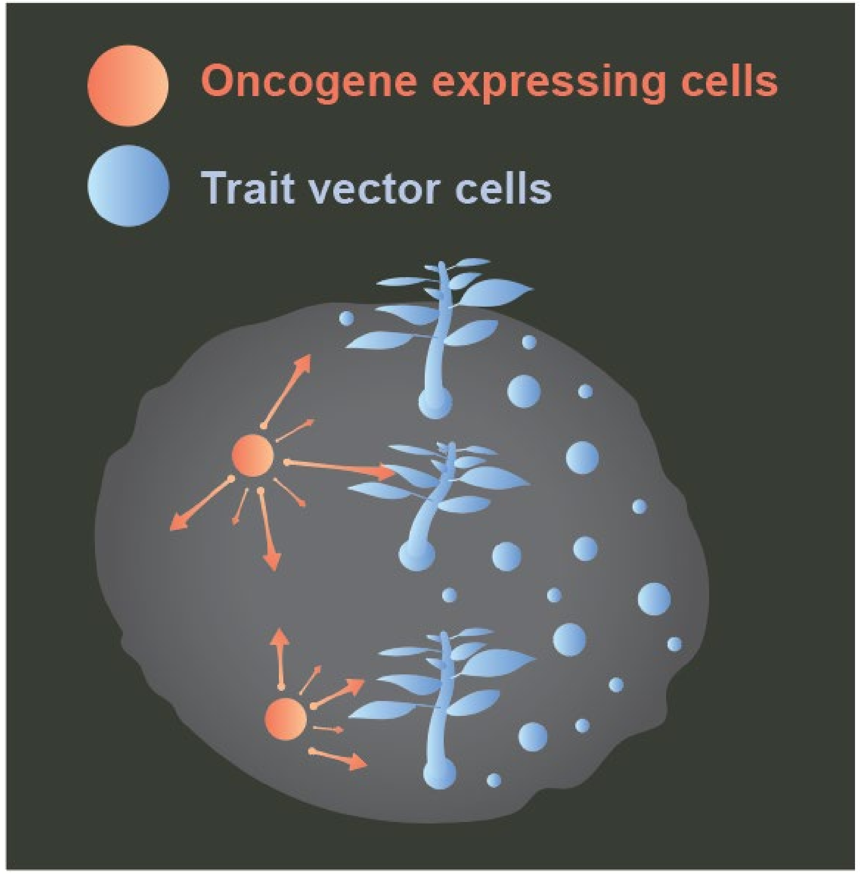
Transgenic galls promoted regeneration of galls and shoots





After pilot studies we thought these genes were well suited for “altruistic” transformation

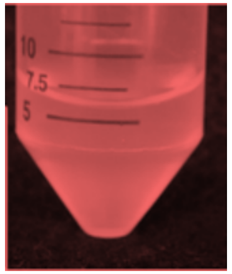




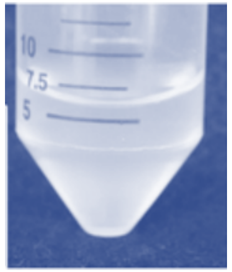
“Altruistic” transformation approach – strain mixtures

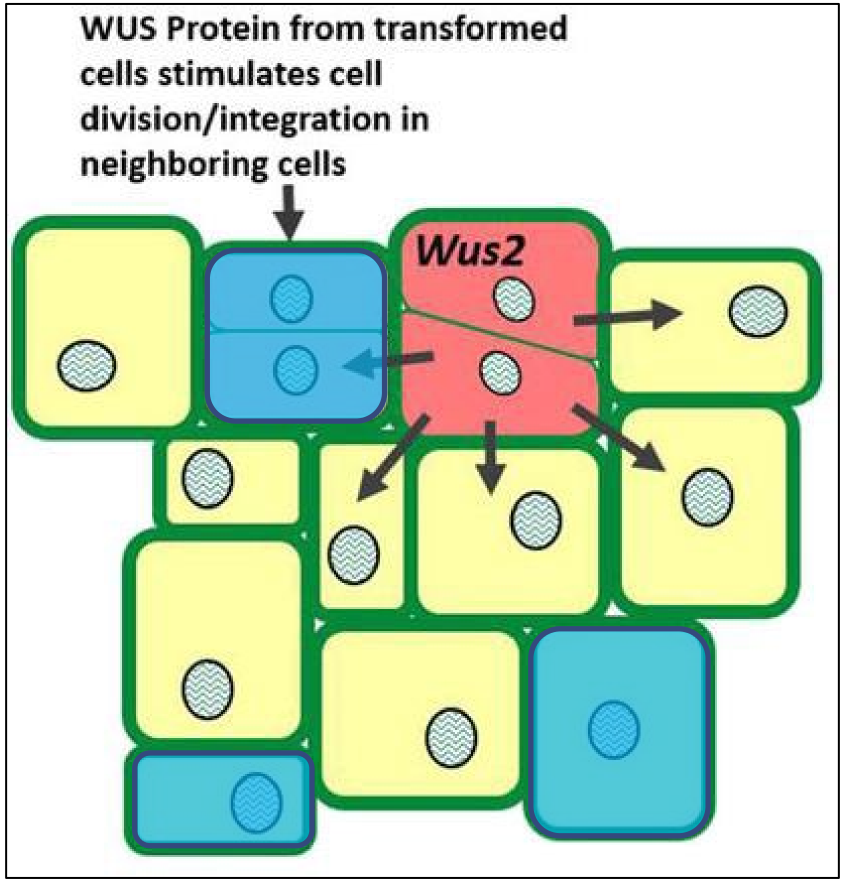
Mixed Agro in different ratios

DEV gene Agro
(like *WUS*)



Trait gene Agro

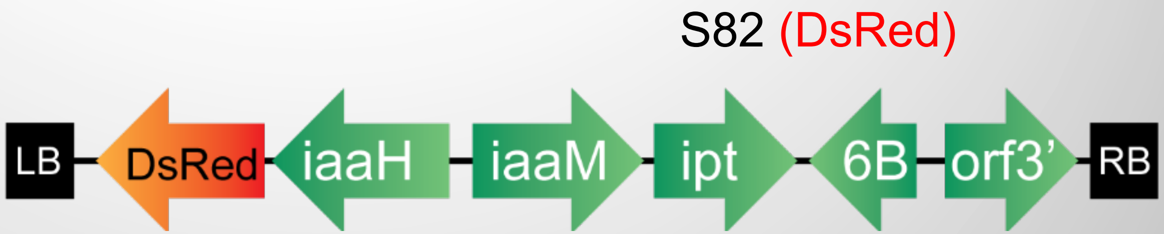




Plant with trait gene
and no DEV genes

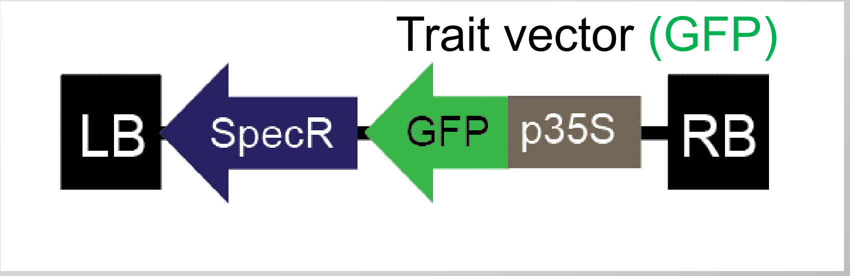
Gordon-Kamm et al. 2021

Altruitsic “S82” transformation in hybrid poplar



<u>4 transformations</u>

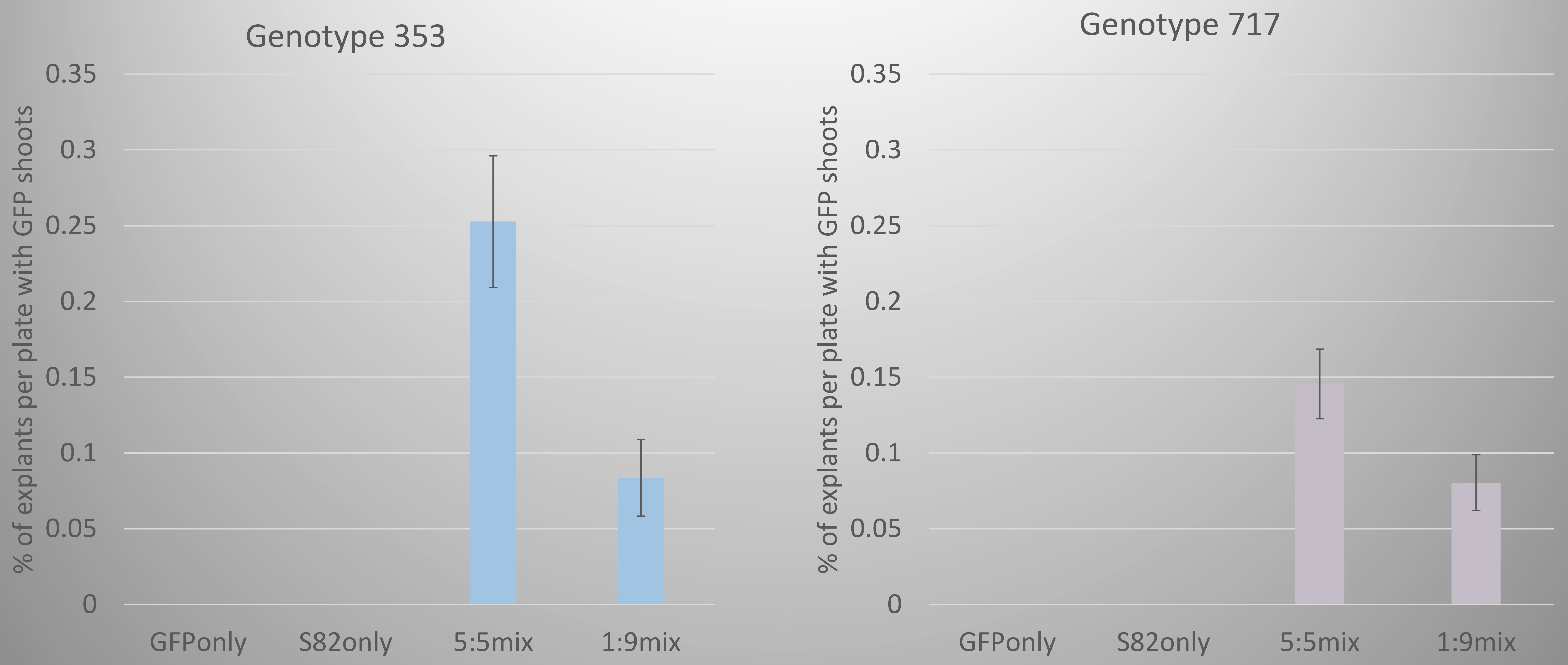
- 100% S82
- 50% S82 / 50% Trait-GFP
- 10% S82 / 90% Trait-GFP
- 100% Trait-GFP



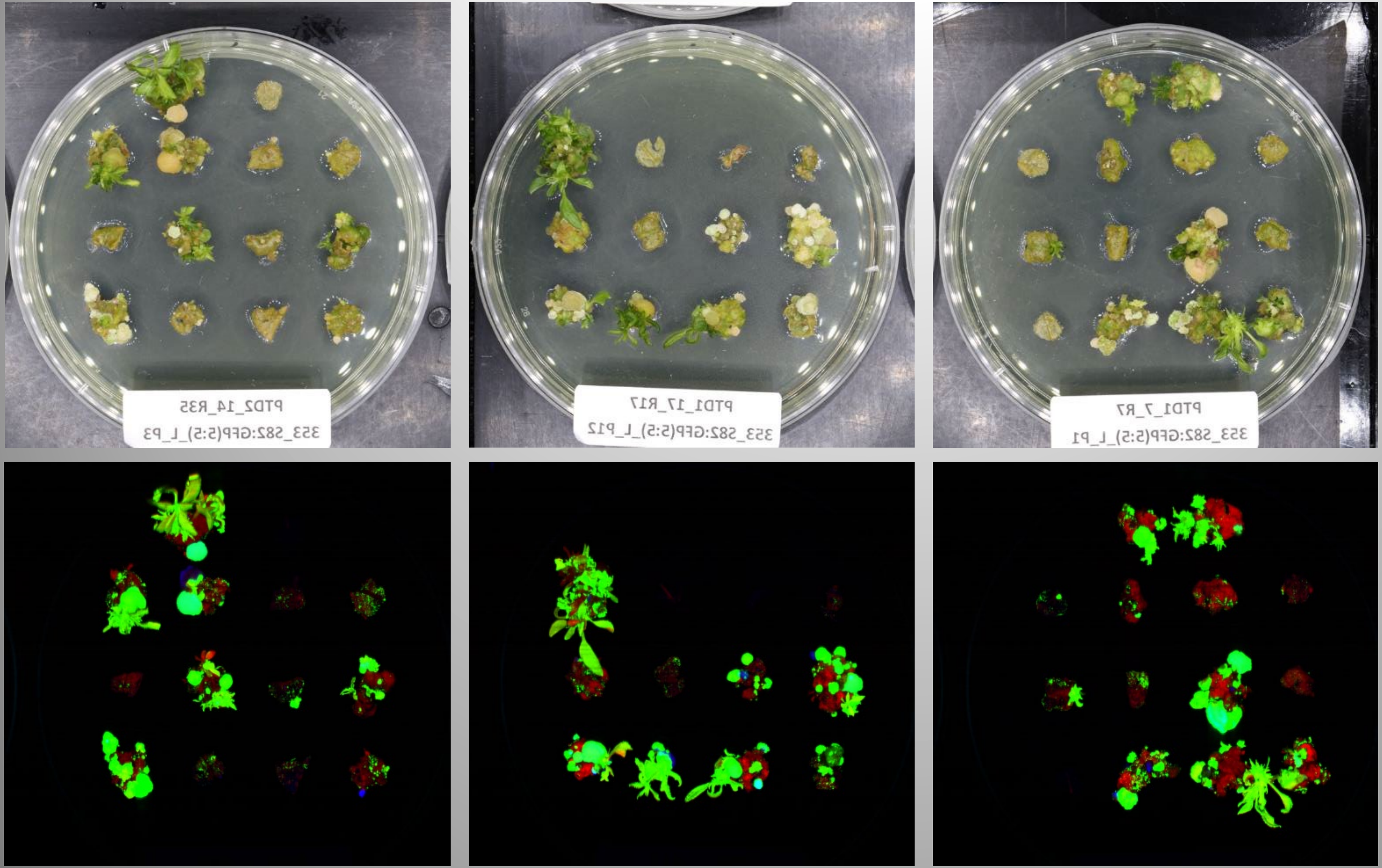
No hormones to induce regeneration

Only spec selection

5:5 mixes of the two strains worked best in two poplar genotypes



Hyperspectral imaging showed altruistic shoot regeneration

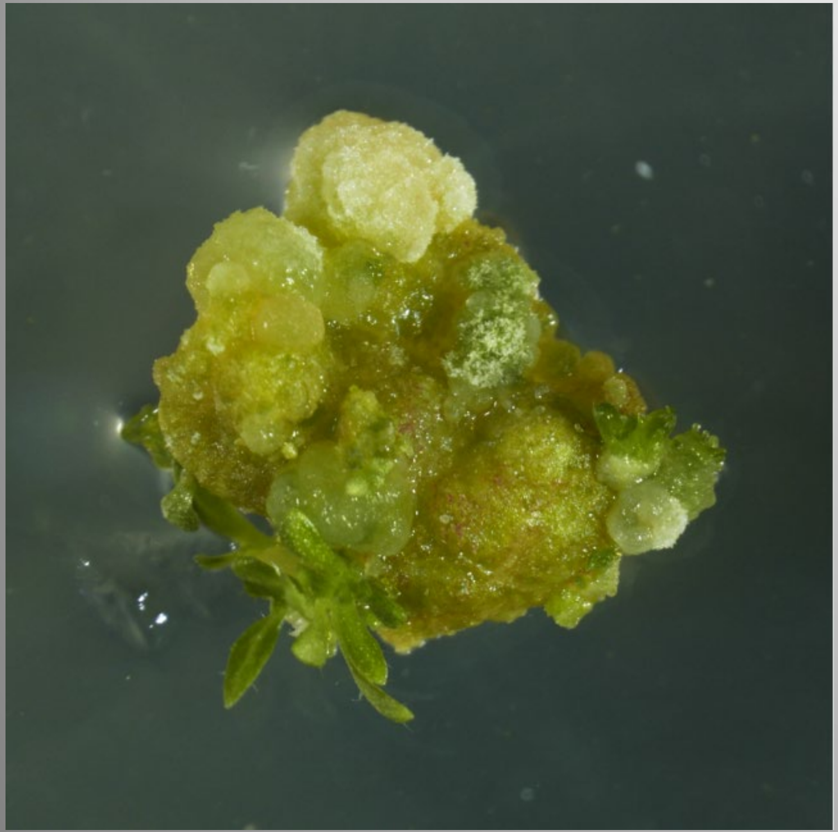


Green = GFP

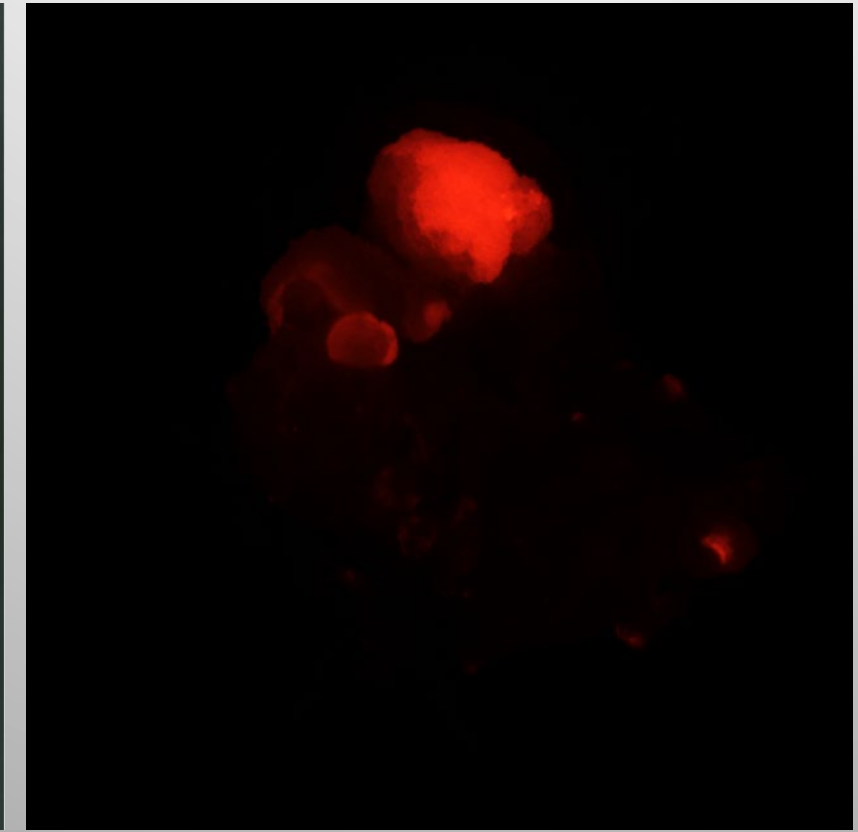
Red = Chlorophyll

A closer look: 5:5 mix at week 6

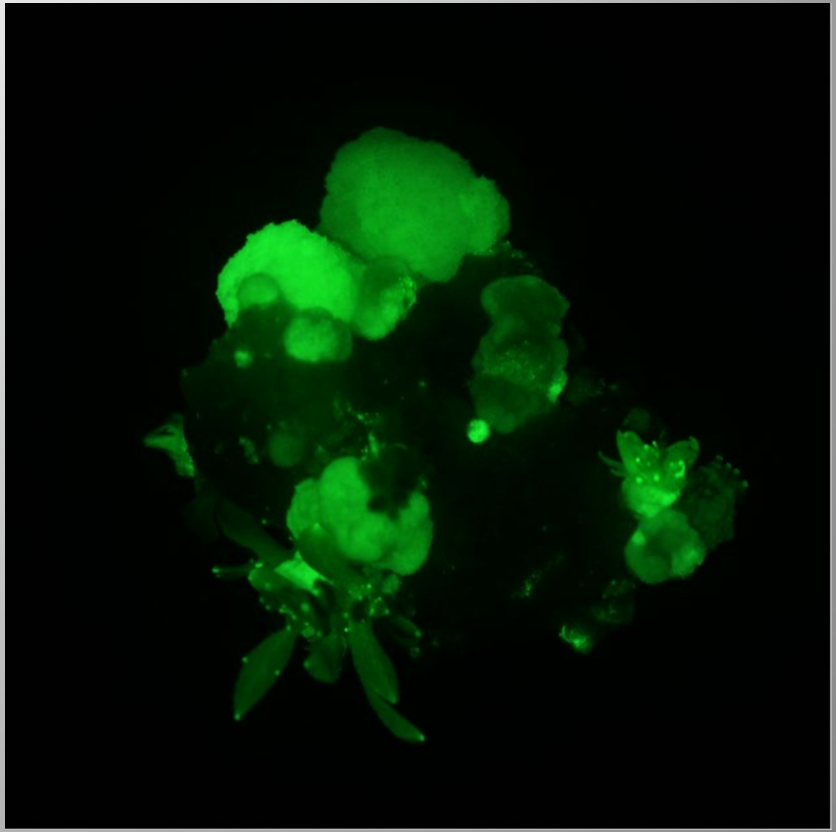
Bright-field

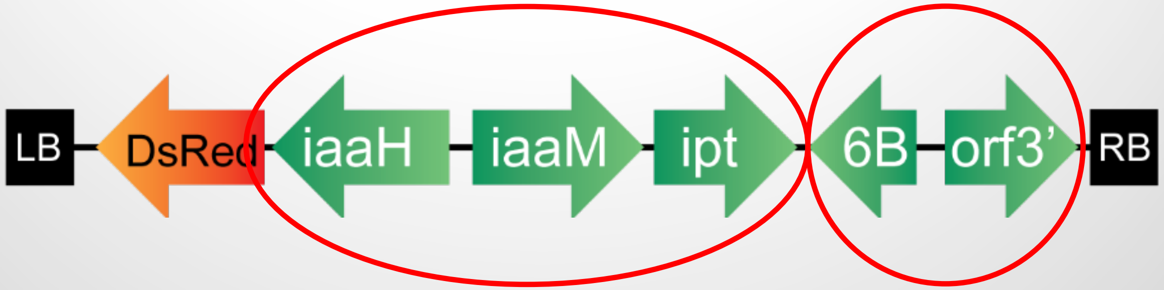


DsRed



GFP





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

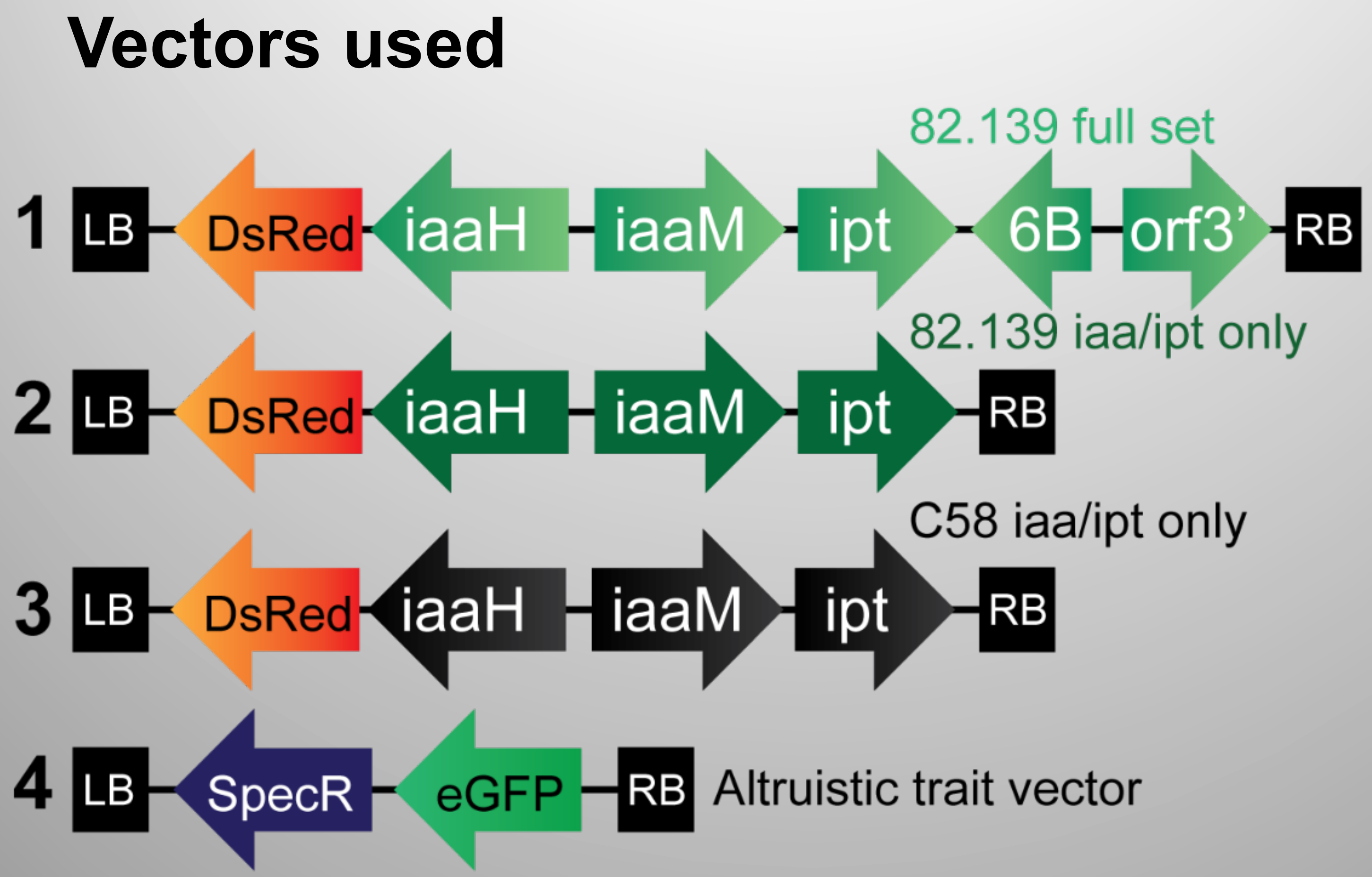
Is there novel *iaa/ipt* expression in this strain?

Or are the novel genes there most important?

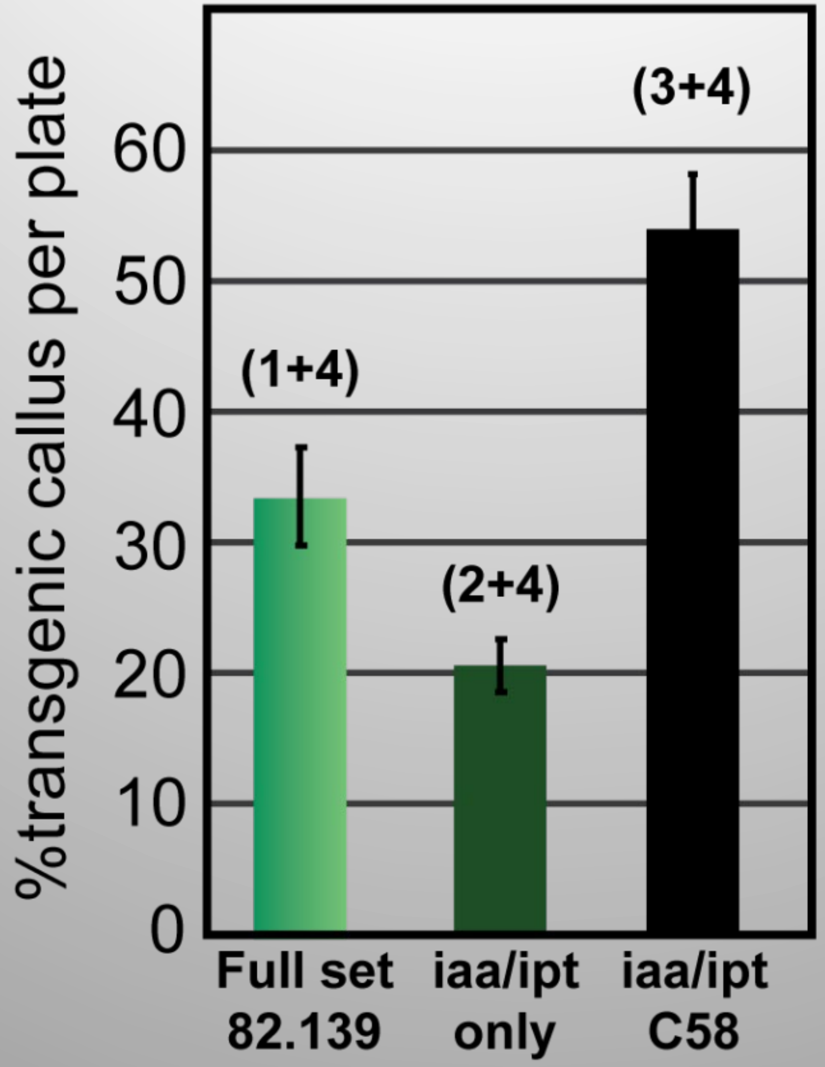
Experimental setup

- 3 constructs
  - – C58 (just *iaa* and *ipt* genes)
  - – S82 (all six cloned genes)
  - – S82 (just its *iaa* and *ipt* genes)
- 5:5 mixture with SpecR GFP binary vector, no hormones

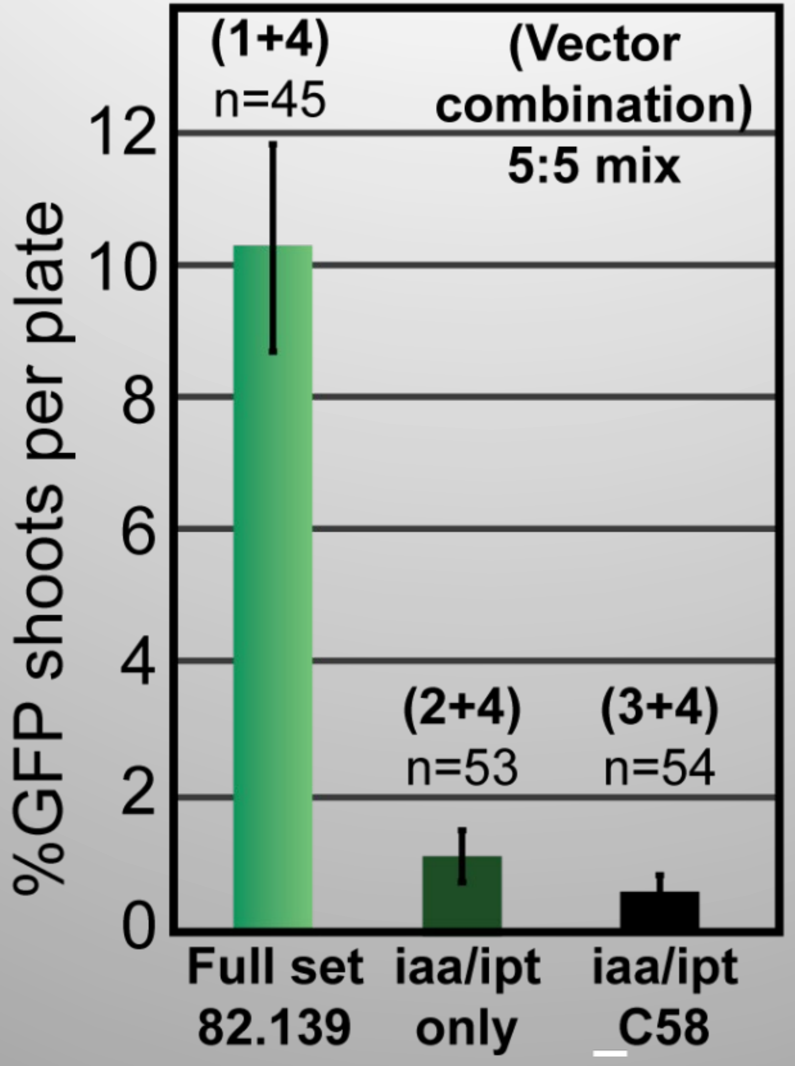
Four vectors used in combination: 1-3 + 4



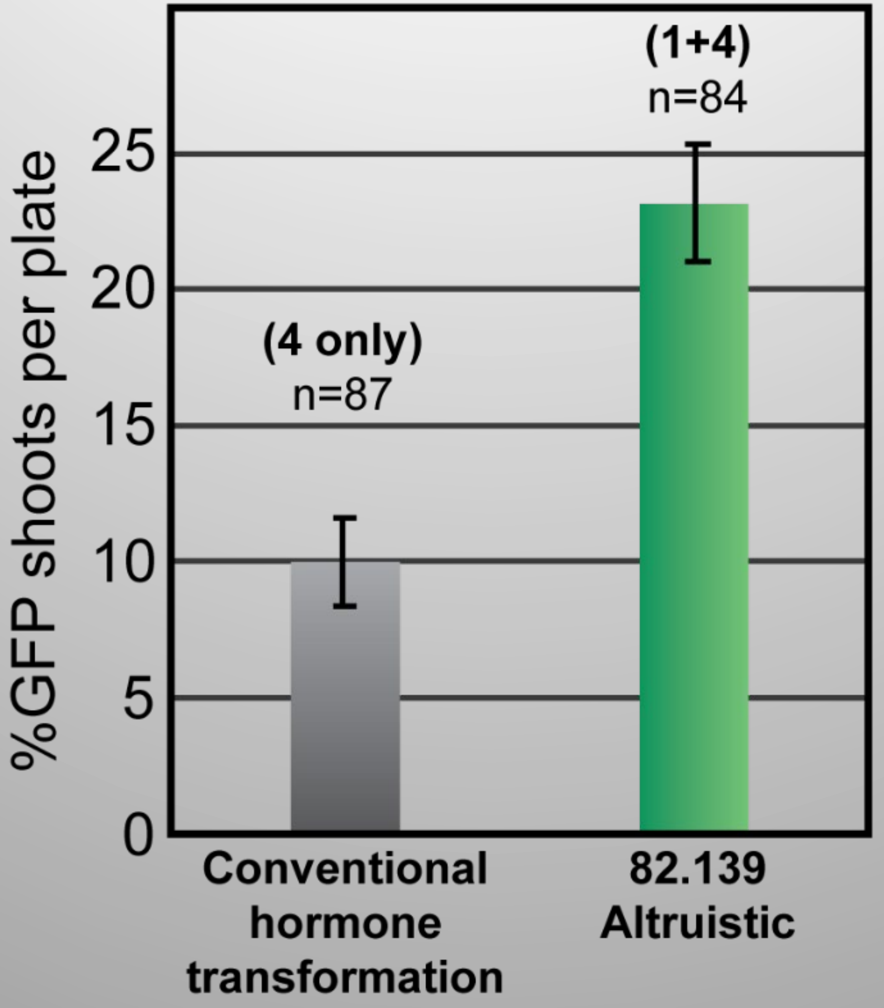
C58 *iaa/ipt* genes were best at inducing transgenic callus



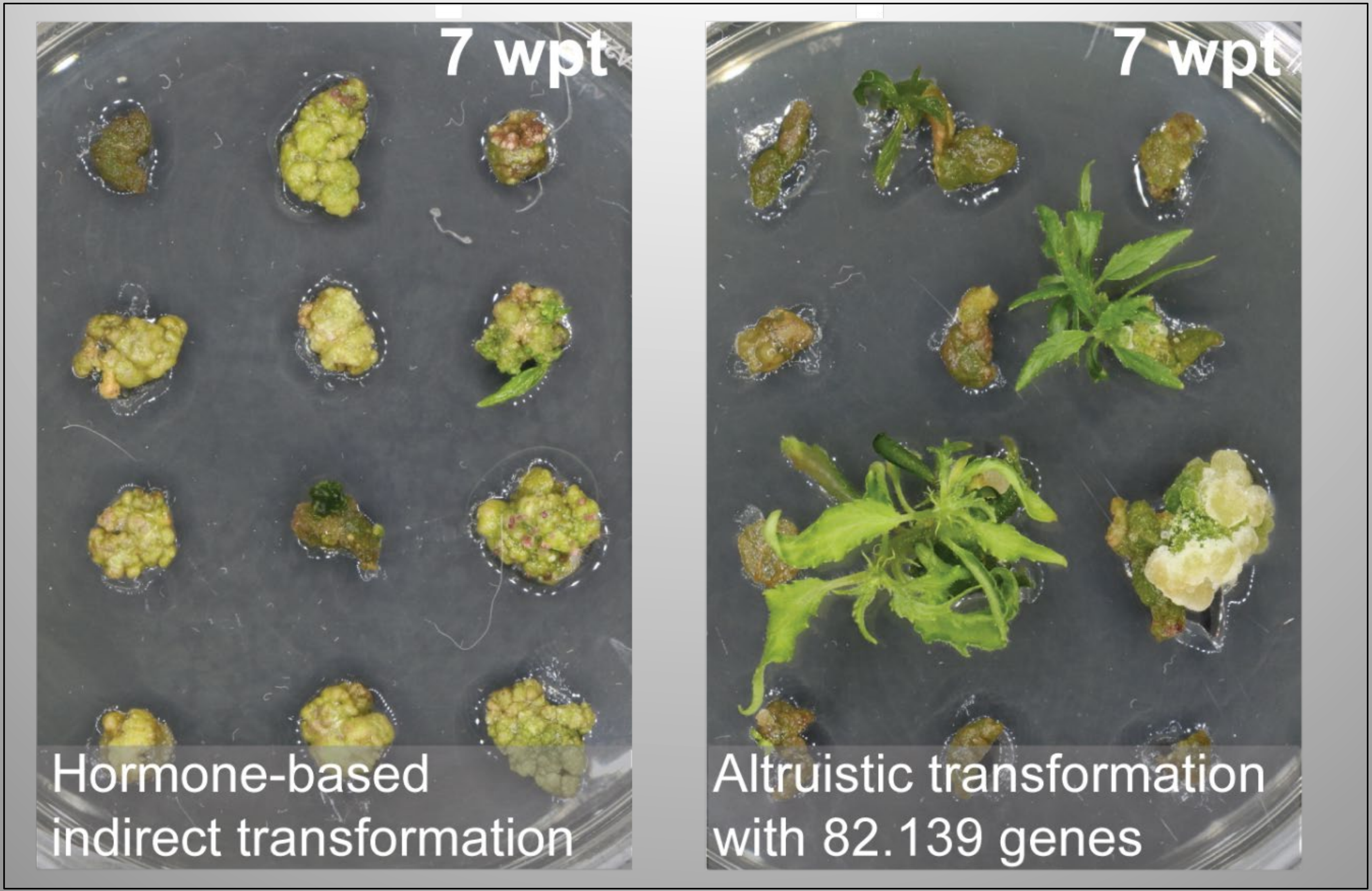
82.139 *iaa/ipt* genes alone did not support high rates of altruistic shoot induction



82.139 altruistic transformation was superior to routine hormone-based indirect transformation



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

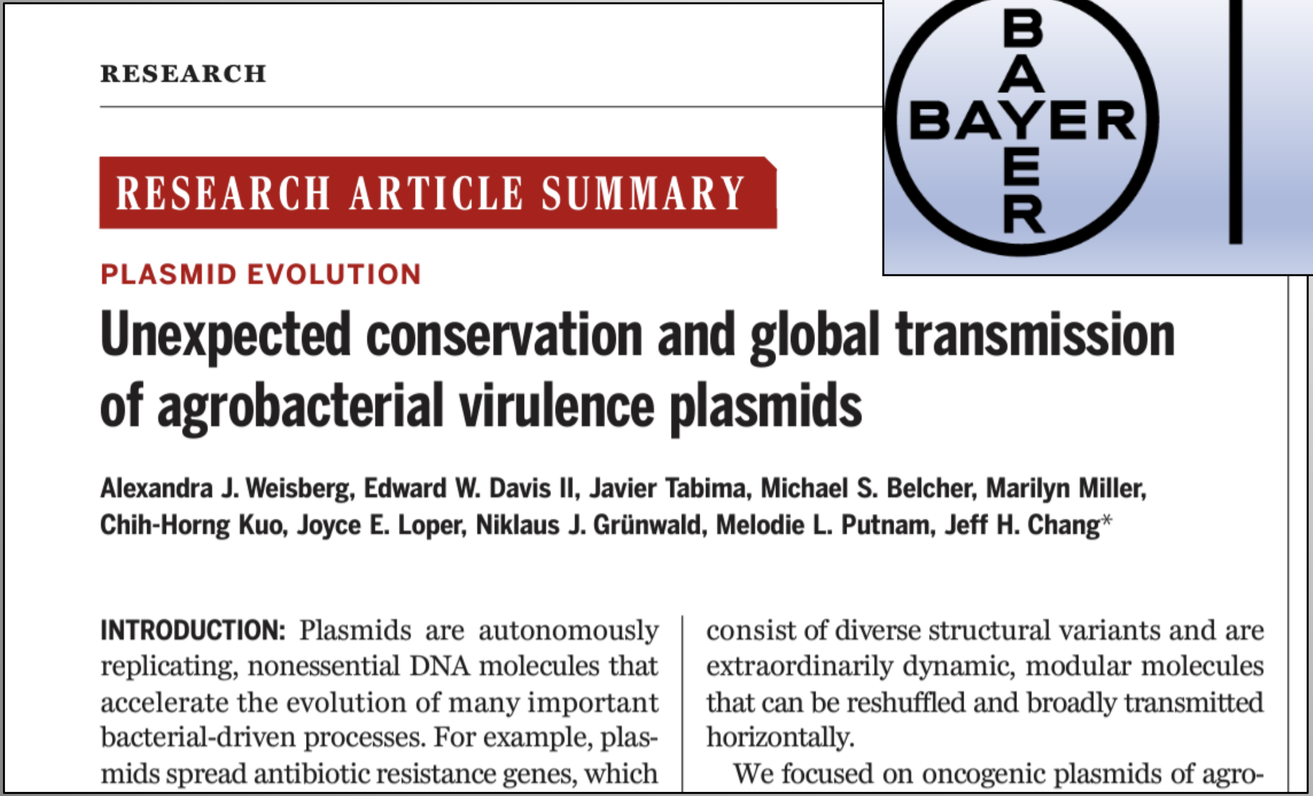


Next steps for making altruistic transformation with 82.139 a useful tool

- Delivery of the 82.139 genes is presently in our vir-plasmid-based GAANTRY strain (ARS Albany, J. Thomson)
  - This strain is aggressive and not an auxotroph
  - We will move into auxotrophic strains for ease of use
- We have mobilized the genes into binary-compatible vectors
  - Different altruistic ratios appear to be needed
- We have begun further testing to identify which genes are most critical for non-autonomous shoot induction
- We are testing in a wide variety of genotypes and species

Other useful developmental regulatory genes? Agro diversity hardly studied

Starting to test ~300 fully sequenced wild Agrobacterium strains to look for increased virulence & shooty phenotypes in altruistic modes



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*

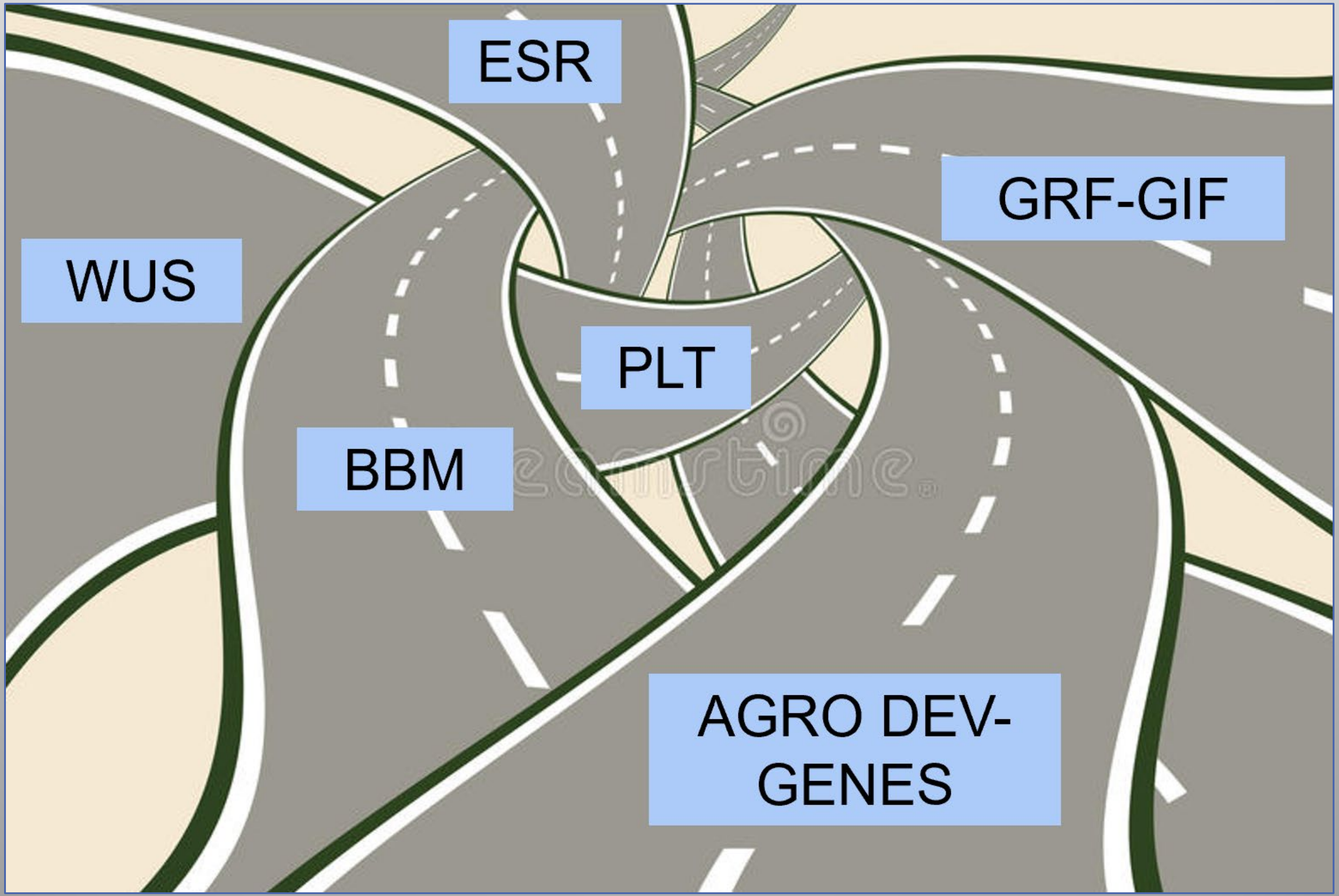
INTRODUCTION: Plasmids are autonomously replicating, nonessential DNA molecules that accelerate the evolution of many important bacterial-driven processes. For example, plasmids spread antibiotic resistance genes, which consist of diverse structural variants and are extraordinarily dynamic, modular molecules that can be reshuffled and broadly transmitted horizontally.

We focused on oncogenic plasmids of agro-

Bayer | Grants 4Ag

What I imagined.....easy and rapid DEV gene-assisted transformation..

What we got, a messy and winding road, leading us back and propelling us forward



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<u>GREAT TREES Consortium</u>

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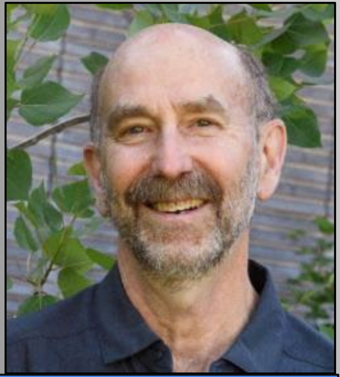

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