The bumpy road of DEV-gene-assisted transformation of trees

Steve Strauss

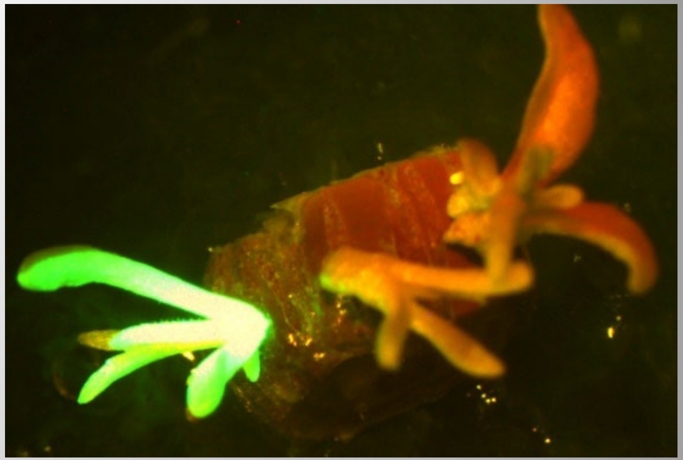
Oregon State University

Greg Goralogia, postdoc

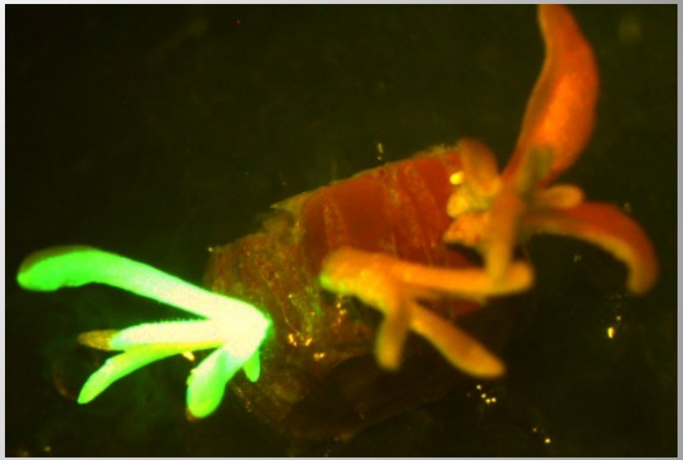
Presented to Baer Crop Science / Plant Biotechnology Academy / 5 May 2023

Agenda

- Perspectives & experimental system
- Experiences from some of the genes we have tried, mostly unhappily
  - Focus on GRF-GIF
- Some stuff we are excited about
  - "Shooty" oncogenes from Agrobacterium



Agenda



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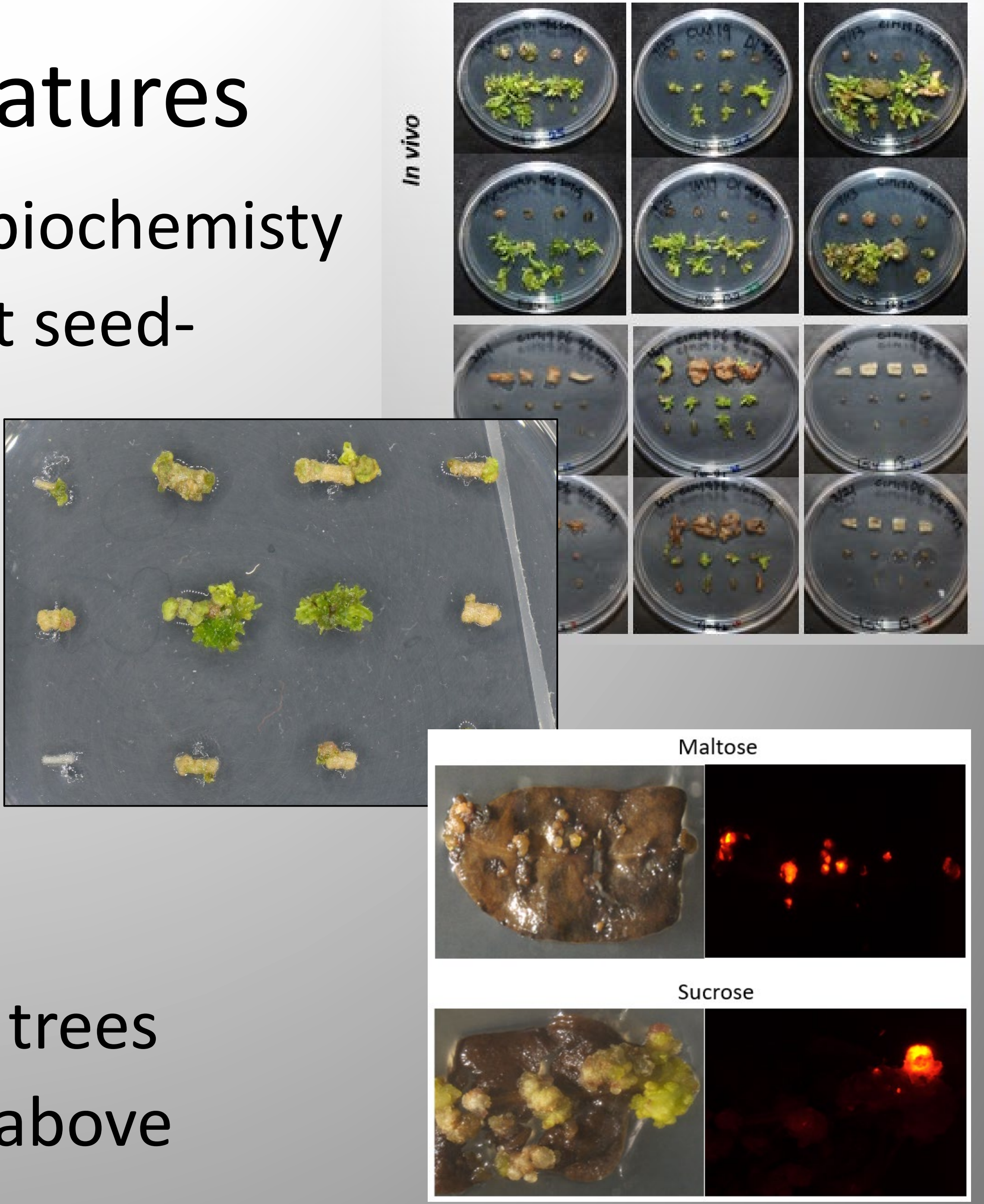
Regeneration & transformation continue to be major limiting factors for gene editing & engineering in plants, and especially trees

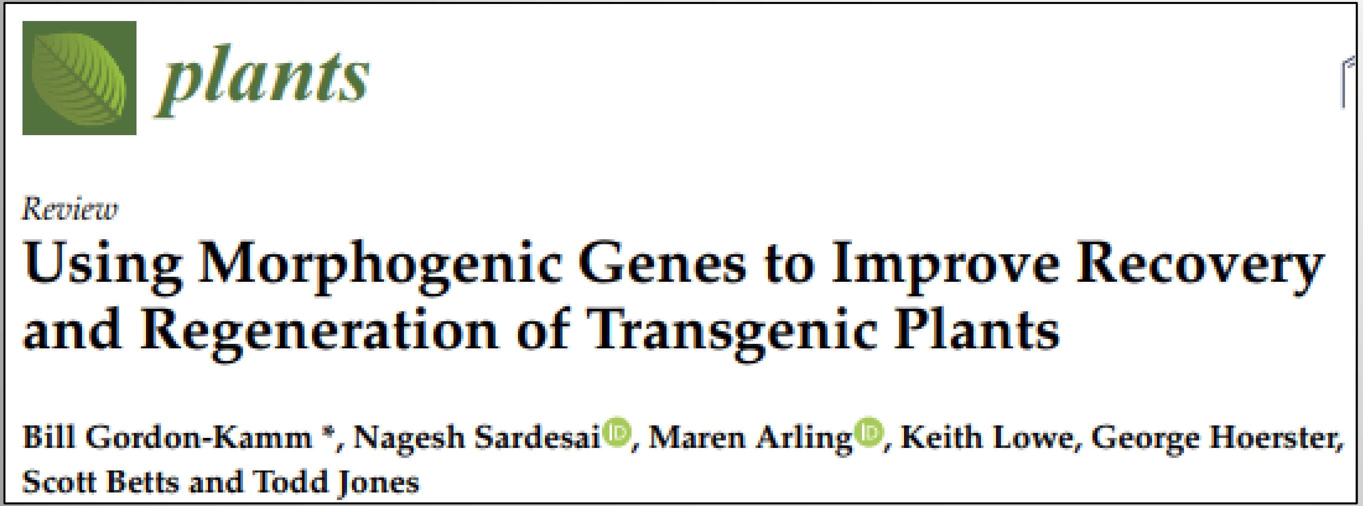
- Species and genotypic differences often dramatic
- Slow, costly, complex customization efforts usually needed
- On top of often large social/regulatory constraints, often a “deal breaker”

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 work, 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

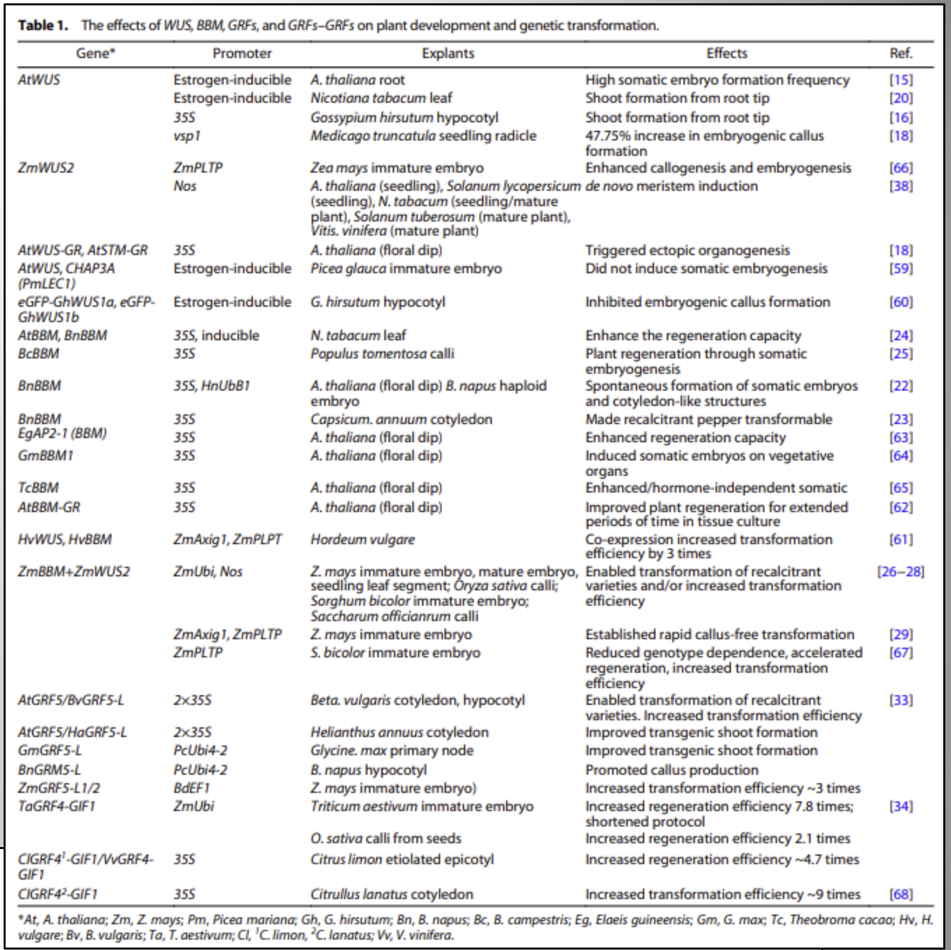


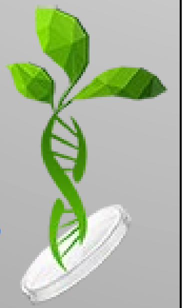
Table 1. The effects of *WUS, BBM, GRFs*, and *GRFs–GRFs* on plant development and genetic transformation.

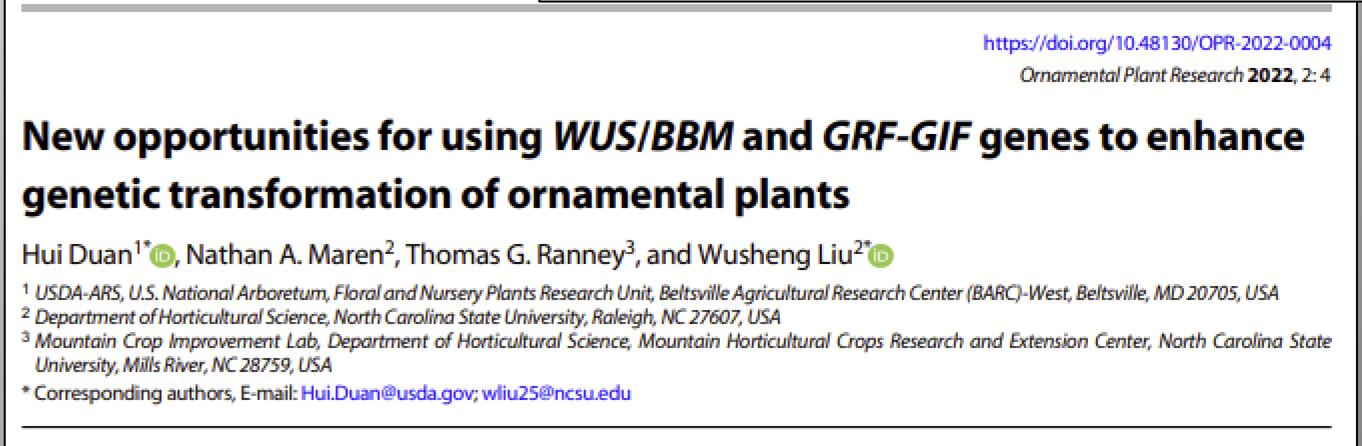
Gene*	Promoter	Explants	Effects	Ref.
AtWUS	Estrogen-inducible	*A. thaliana* root	High somatic embryo formation frequency	[15]
	Estrogen-inducible	*Nicotiana tabacum* leaf	Shoot formation from root tip	[20]
	35S	*Gossypium hirsutum* hypocotyl	Shoot formation from root tip	[16]
	vsp1	*Medicago truncatula* 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.*

Focus of GREAT TREES Coop:

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





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

Hui Duan[1*], Nathan A. Maren[2], Thomas G. Ranney[3], and Wusheng Liu[2*]

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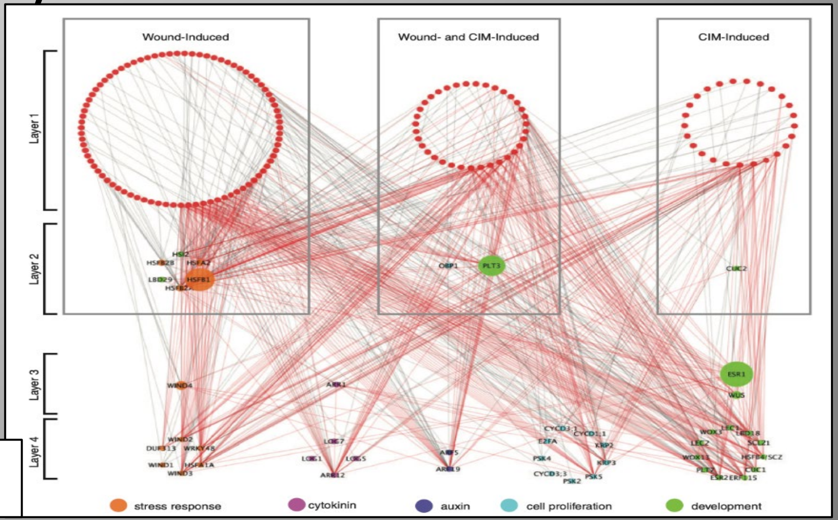
[2] *Department of Horticultural Science, North Carolina State University, Raleigh, NC 27607, USA*
[3] *Mountain Crop Improvement Lab, Department of Horticultural Science, Mountain Horticultural Crops Research and Extension Center, North Carolina State University, Mills River, NC 28759, USA*
* Corresponding authors, E-mail: Hui.Duan@usda.gov; wliu25@ncsu.edu

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
- New tools that complements and interacts with – is not a replacement for – the many tools and chemical reagents in everyday use during *in vitro* or *in vivo* transformation procedures
  - Macro and micronutrients, hormones, buffers, light/dark treatments, plant donor tissues, gene insertion vectors/treatments

What are DEV genes?

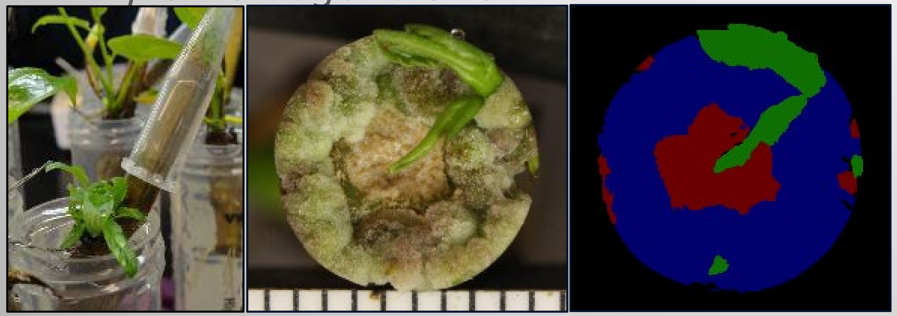
- The genes are derived from basic studies of plant development and pathology – but whose use in TR often deviate substantially from their natural roles due to the radical interventions that are often part of TR
  - Redifferentiation from terminally differentiated somatic tissues
  - Wounding and pathogen attack (Agrobacterium)
  - Complexity of natural meristem / embryo / organ regeneration pathways



Ikeuchi et al. 2018

NSF-funded GWAS to discover developmental genes in poplar*:* Four studies, machine vision phenomic system

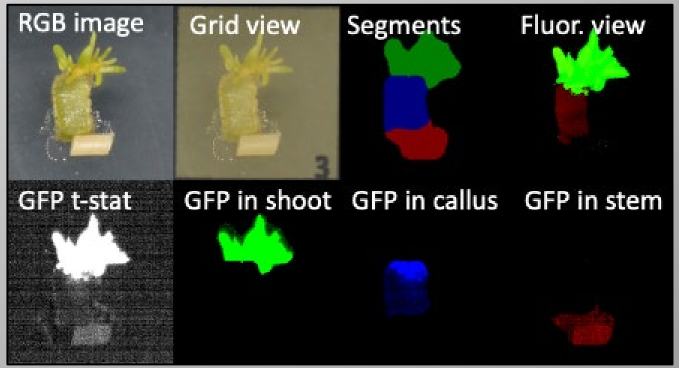
1. *In planta* regeneration



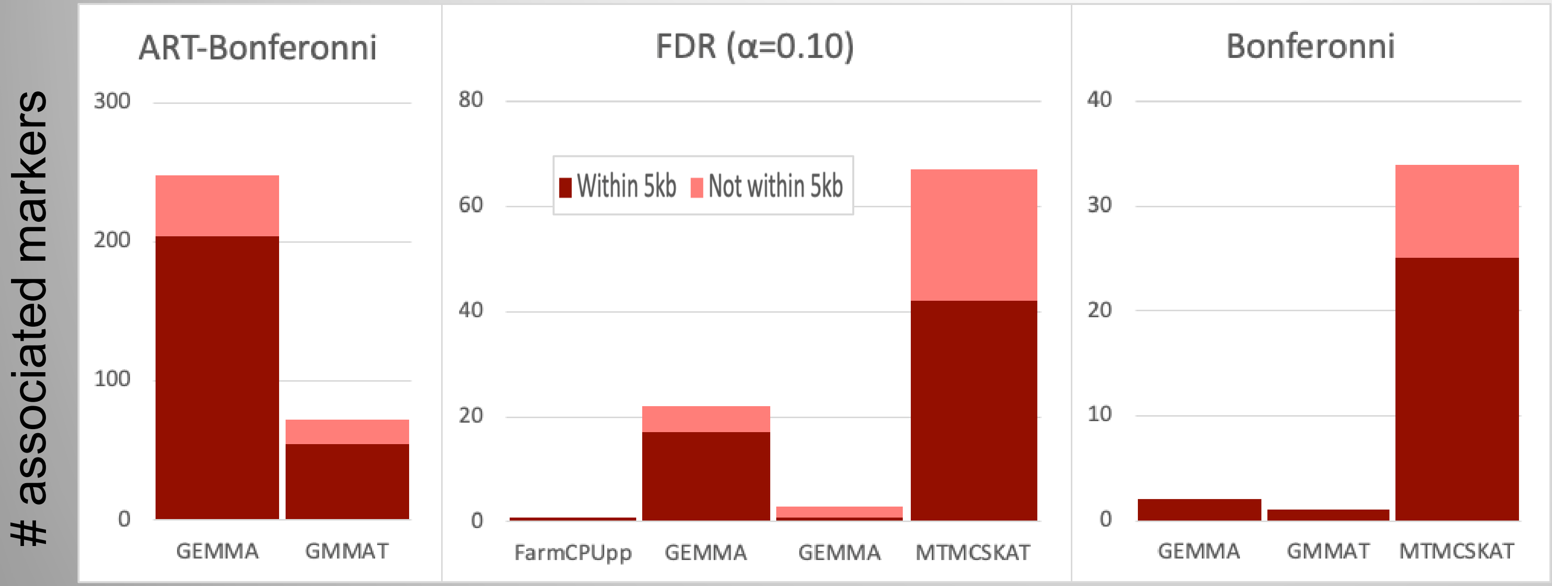
2. *In planta* rooting

3. *In vitro* regeneration

4. *In vitro* regeneration + transformation



Hundreds of GWAS hits with various statistical pipelines – highly polygenic traits



- None of the hits include any of the common DEV genes
- Little overlap between the genes we identified and similar studies in other poplars or plant species
- Pathway analysis suggests extensive wound/stress hormone cross-talk with growth and differentiation pathway genes
- **The biotech genes of today are only a first step down this new path**

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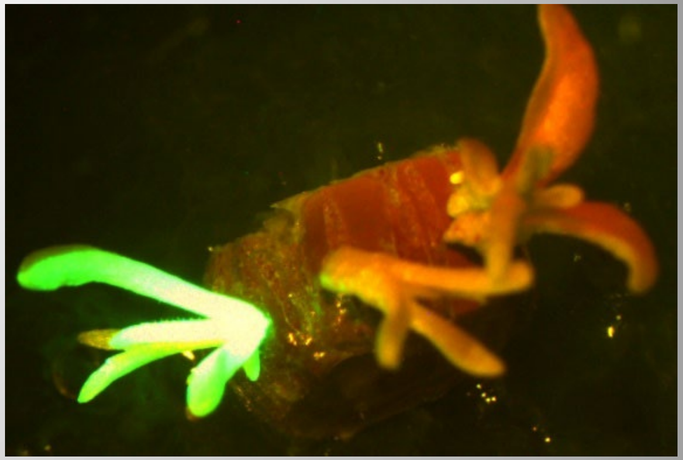

Our DEV-gene related areas of focus in the lab

Anti-Silencing Genes

Transgene Removal

Woody Plant Universal GE / Editing Systems

CRISPR Activation

Vector Tools and Systems

Shooty Agrobacterium Oncogenes

Developmental Genes For Transformation

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*
- *IPT – ISOPENTYL TRANSFERASE* (cytokinin) – Agrobacterium
- Agrobacterium oncogenes
- *ROL* – Hairy root-inducing genes – Agrobacterium
- *WUS – WUSCHEL*
- *GRF-GIF – GROWTH REGULATOR FACTOR 4* and *GRF INTERACTING FACTOR* 1

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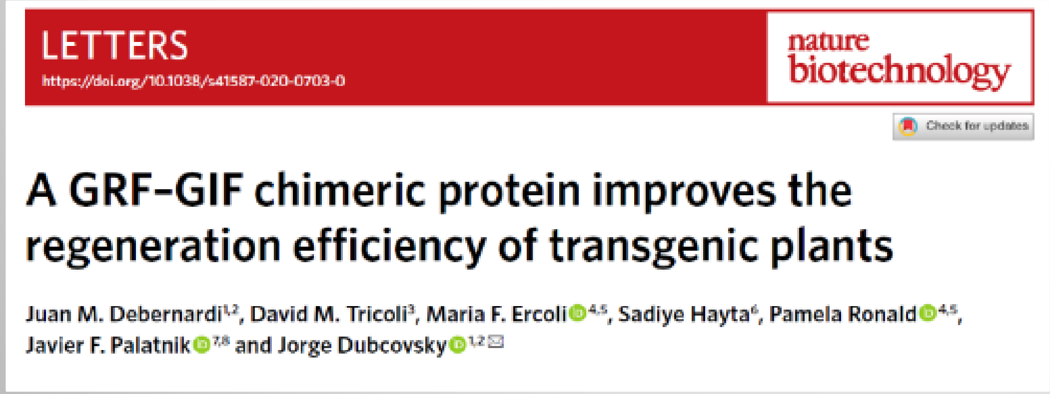
- **Agrobacterium oncogenes**
- *ROL* – Hairy root-inducing genes – Agrobacterium
- *WUS – WUSCHEL*
- ***GRF-GIF – GROWTH REGULATOR FACTOR 4*** **and** ***GRF INTERACTING FACTOR*** **1**

GRF-GIF with much encouraging results in recent years



LETTERS

https://doi.org/10.1038/s41587-020-0703-0

nature biotechnology

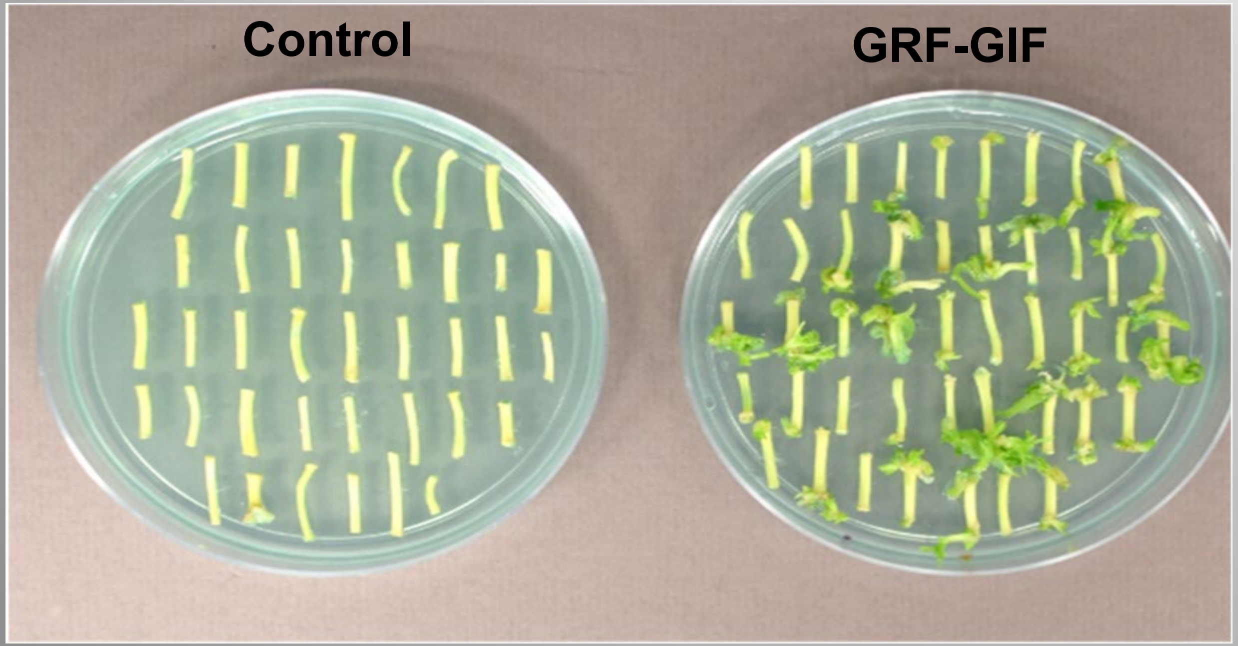
Check for updates

A GRF–GIF chimeric protein improves the regeneration efficiency of transgenic plants

Juan M. Debernardi[1,2], David M. Tricoli[3], Maria F. Ercoli[4,5], Sadiye Hayta[6], Pamela Ronald[4,5], Javier F. Palatnik[7,8] and Jorge Dubcovsky[1,2]

A chimeric protein comprised of *GROWTH-REGULATING FACTOR (GRF)* and *GRF-INTERACTING FACTOR (GIF)*

GRF & GIF interact with **chromatin remodeling** machinery and regulate transcription of meristem development genes

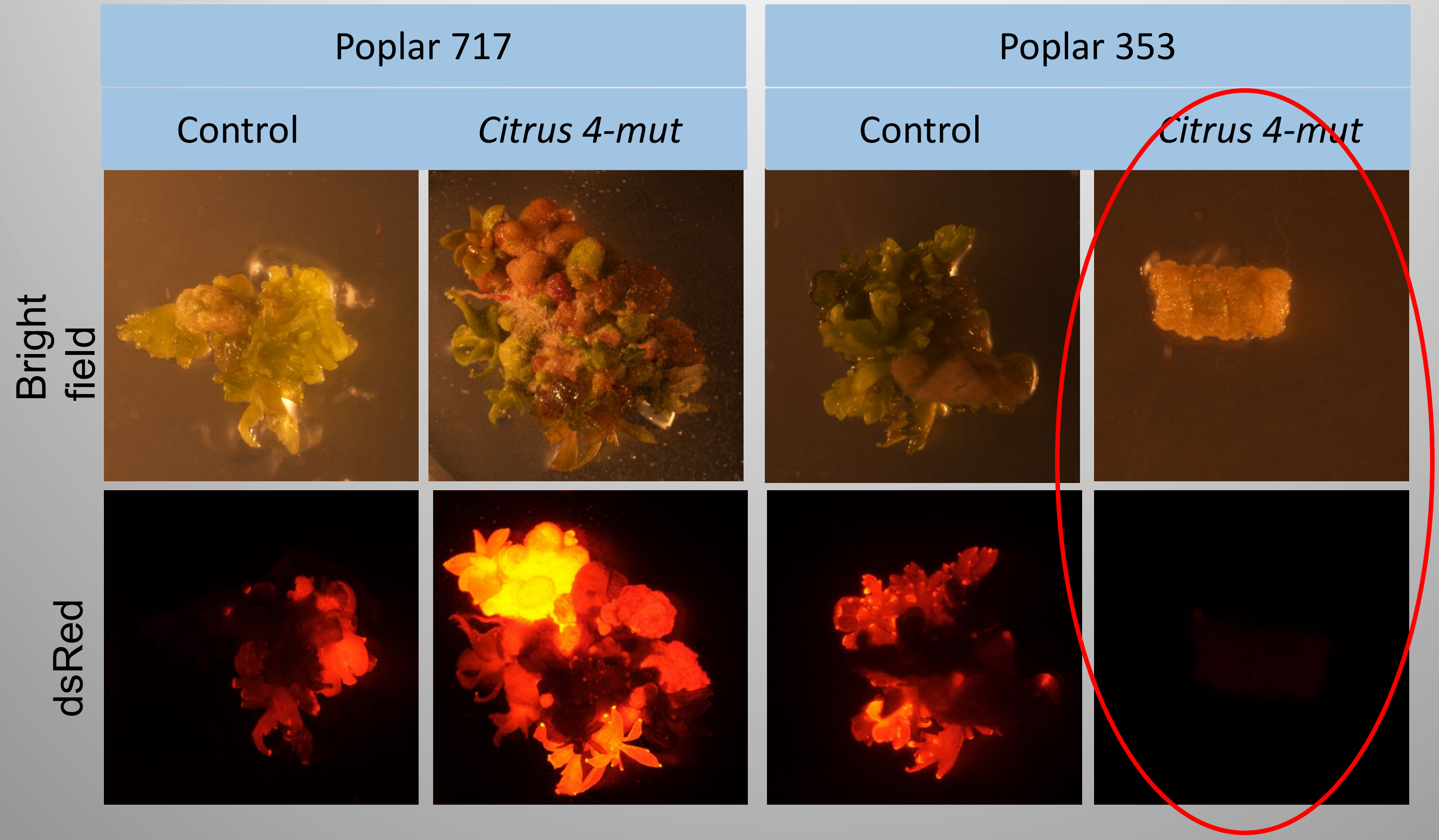


Citrus epicotyl explants; Debernardi et al., 2020

Studied a wide variety of GRF-GIF homologs & sources, promoters, and miRNA sensitivities (MS thesis 2022)

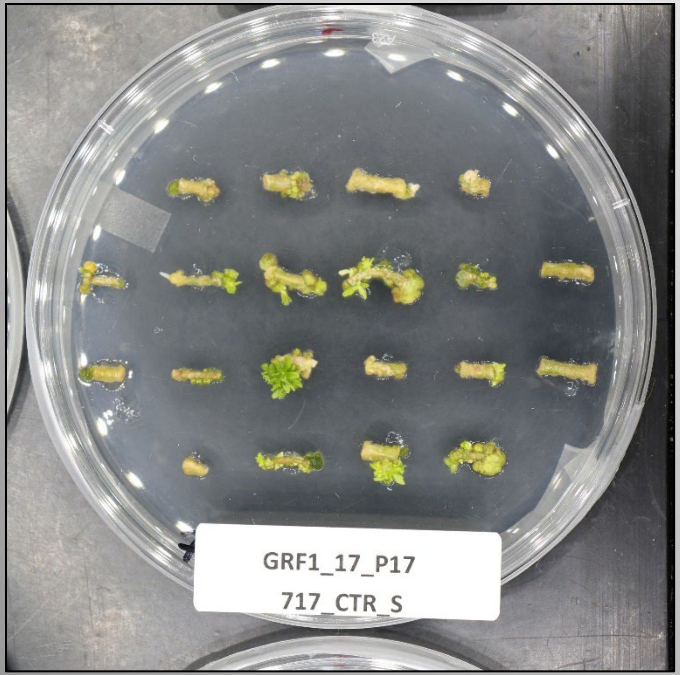
Two poplar genotypes displayed very different callus responses to *Citrus 4-mut* GRF-GIF overexpression

717=*Populus tremula x alba* / 353 = *P. tremula x tremuloides*

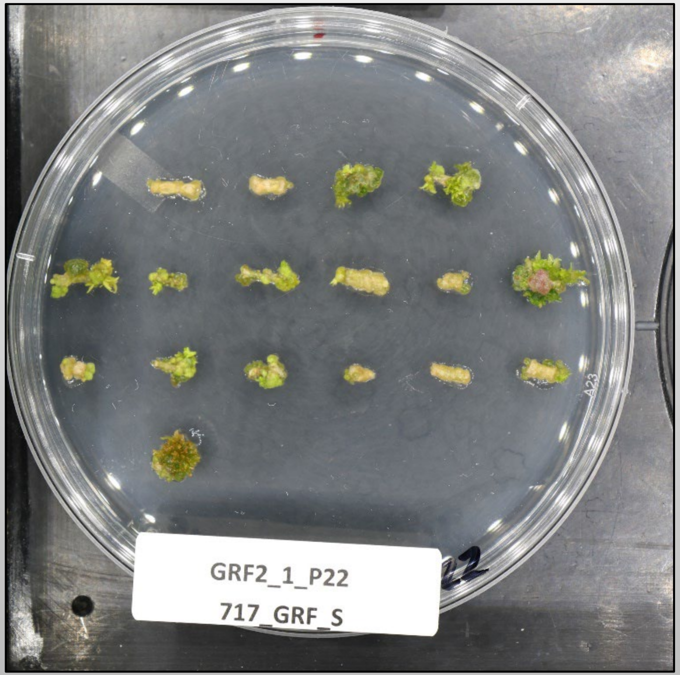


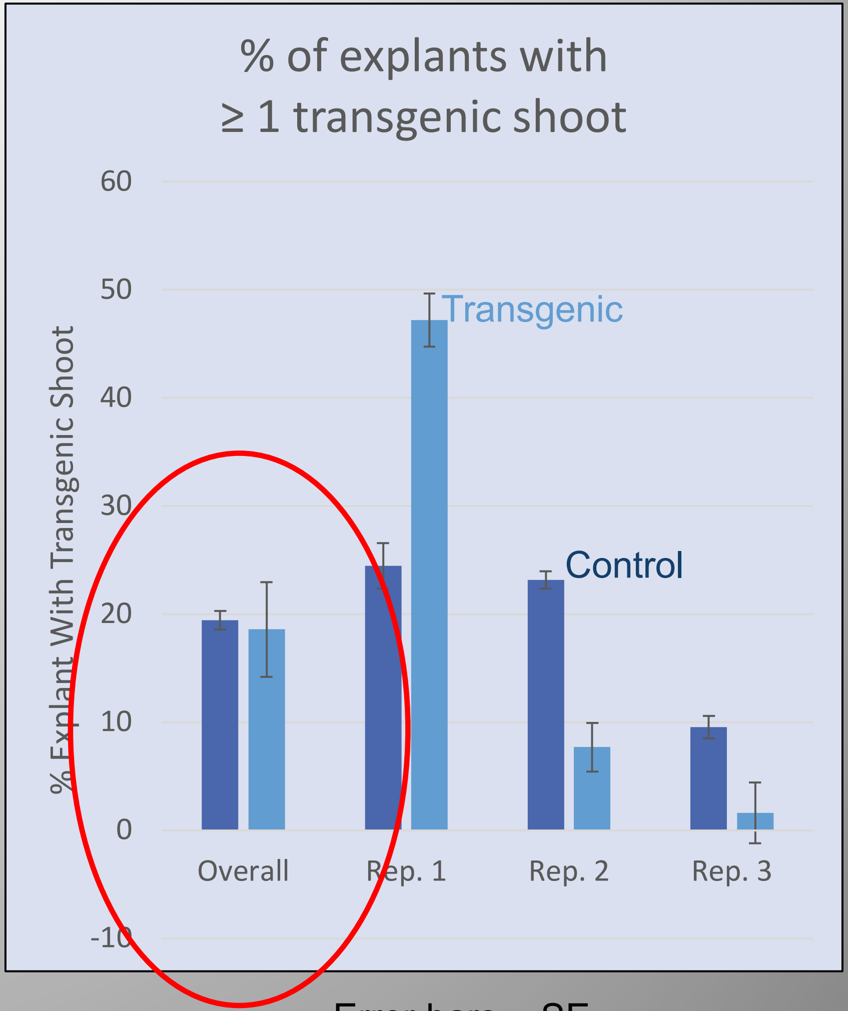
Citrus 4-mut GRF-GIF had little overall effect on shoot formation in poplar clone 717

Control



GRF-GIF





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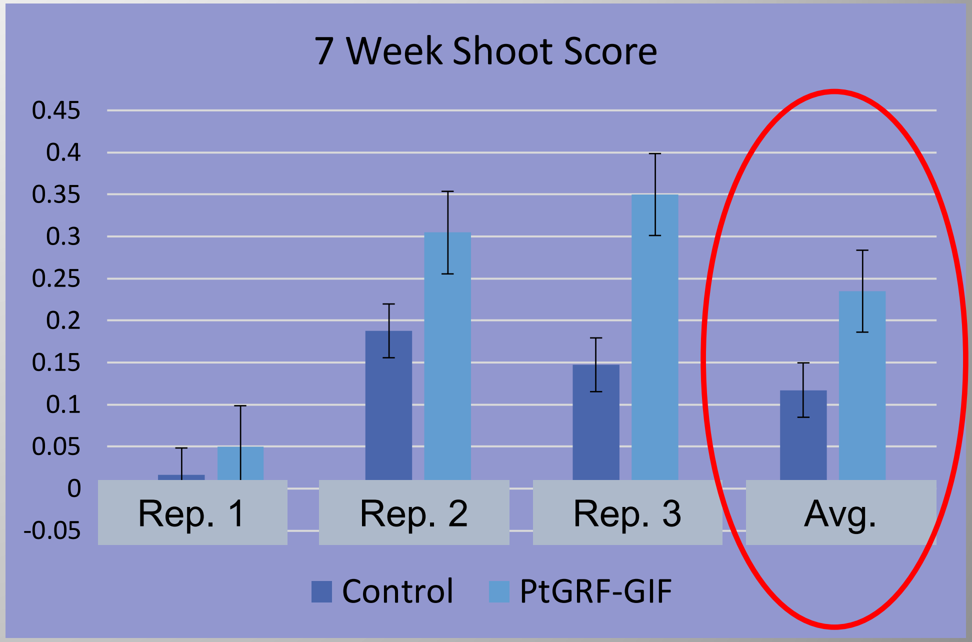
An ortholog of GRF-GIF from *Populus* doubled shoot regeneration in poplar 717 (single 35S promoter)

Control

1x35S::GRF4-GIF1

= dsRed positive shoot



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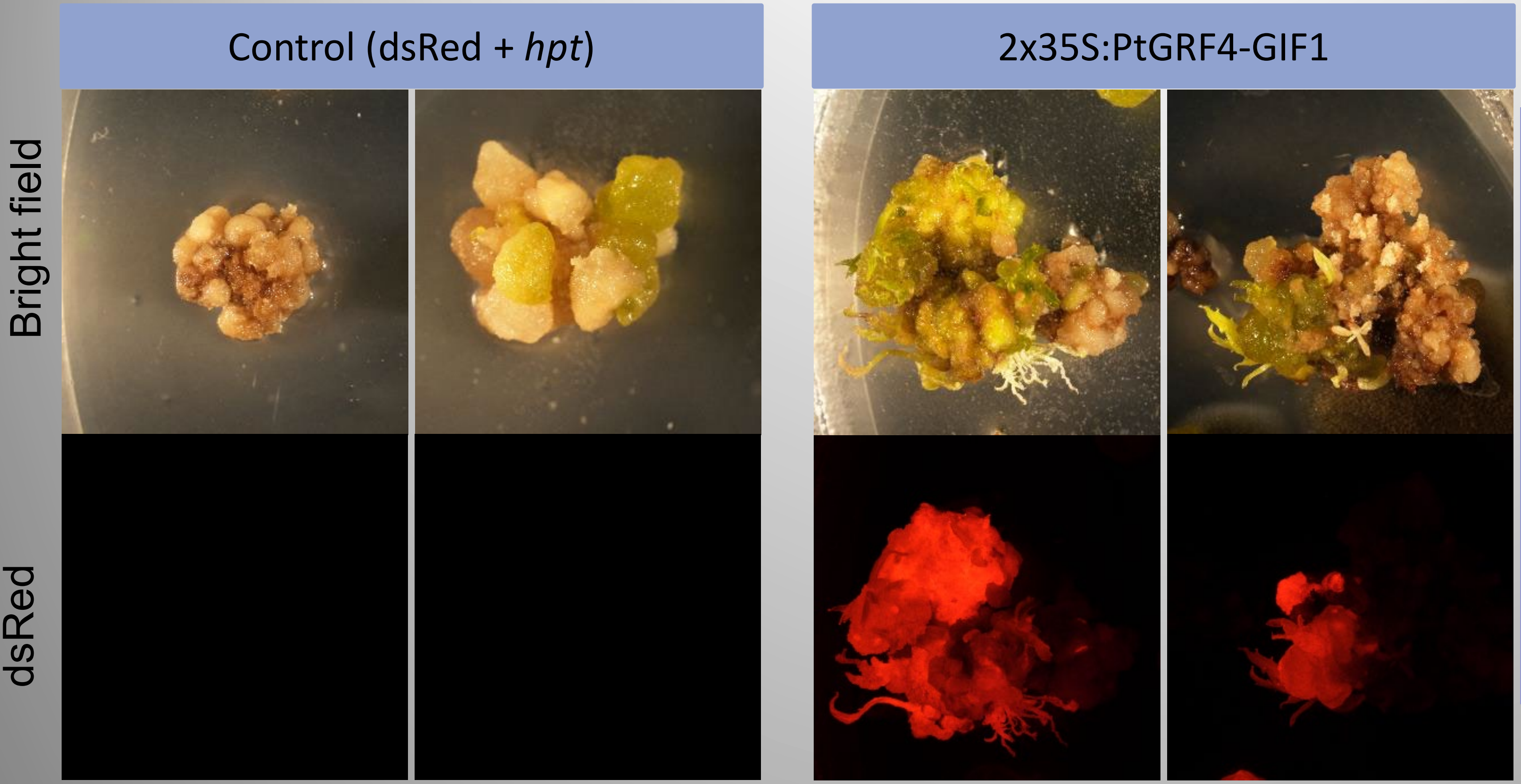
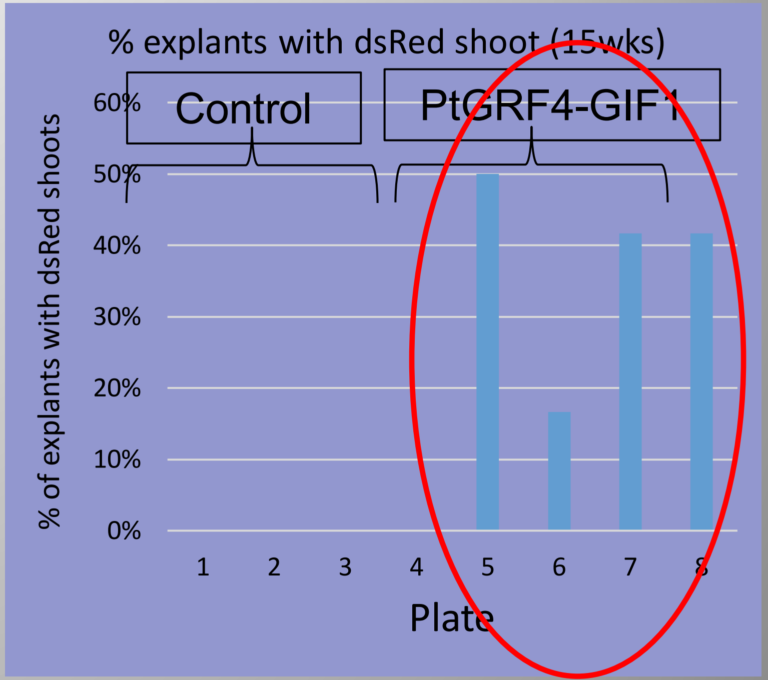
Populus GRF-GIF also promoted shoot regeneration in recalcitrant *P. alba* clone ‘6K10’

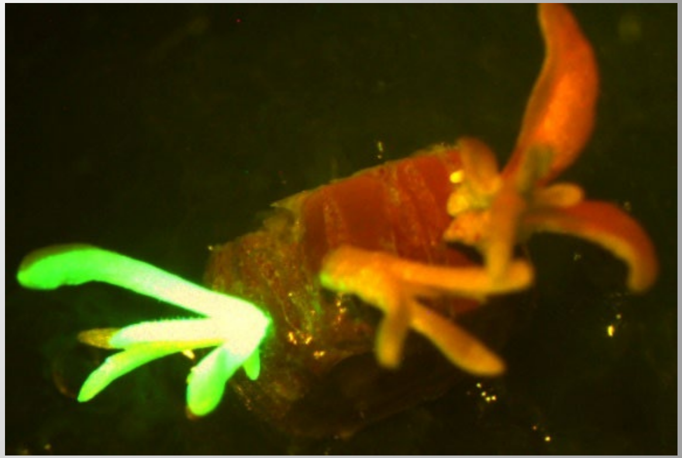




GRF-GIF experience to date

- Gene source matters
- Degree of miRNA sensitivity matters
- Promoter matters
- Plant genotype matters
- Induction of expression seems wise, did not solve the genotype problem
- So far no general solutions to how to use it in poplar (or eucalypts) – today like one more medium/hormone customization tool

Agenda



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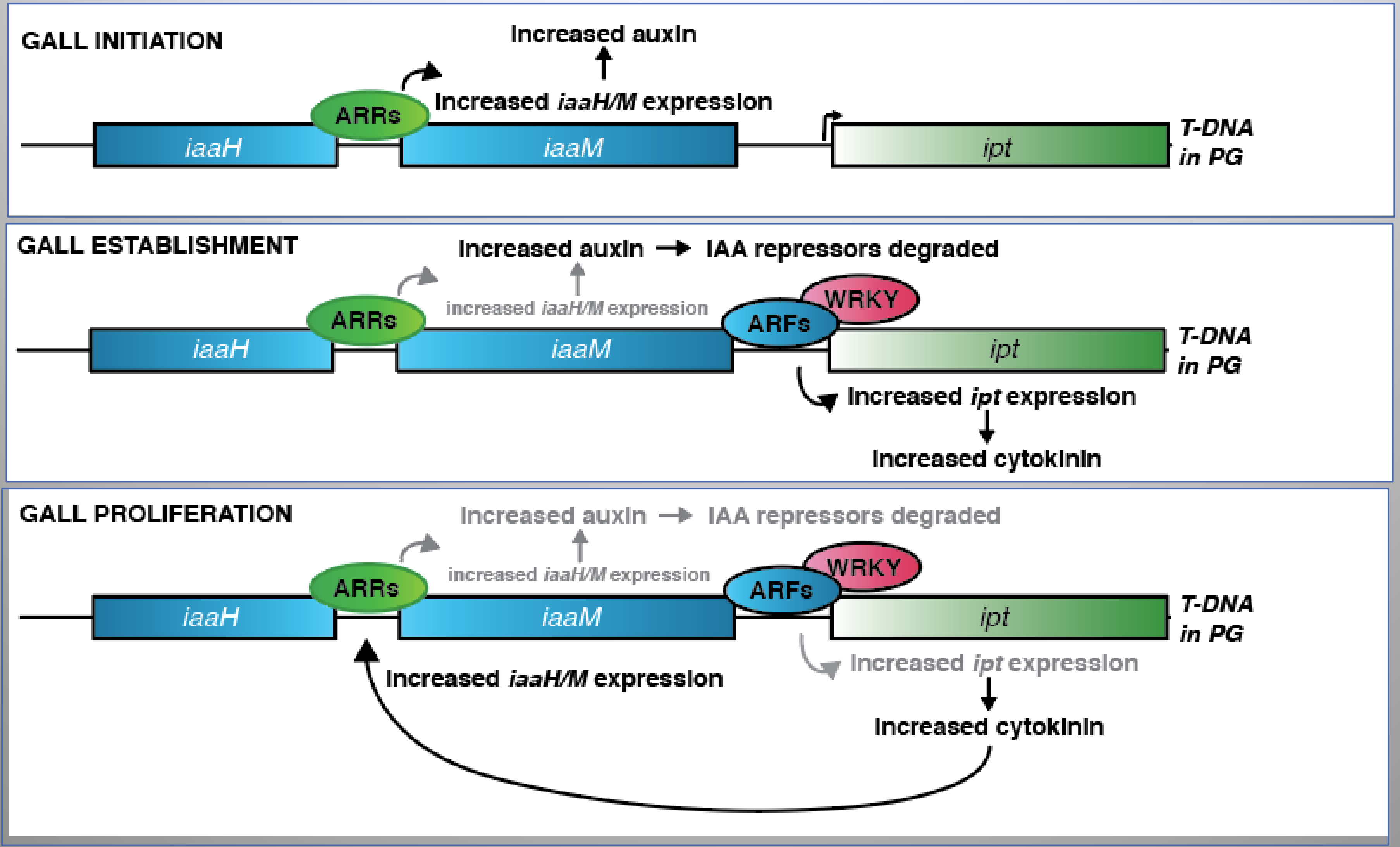
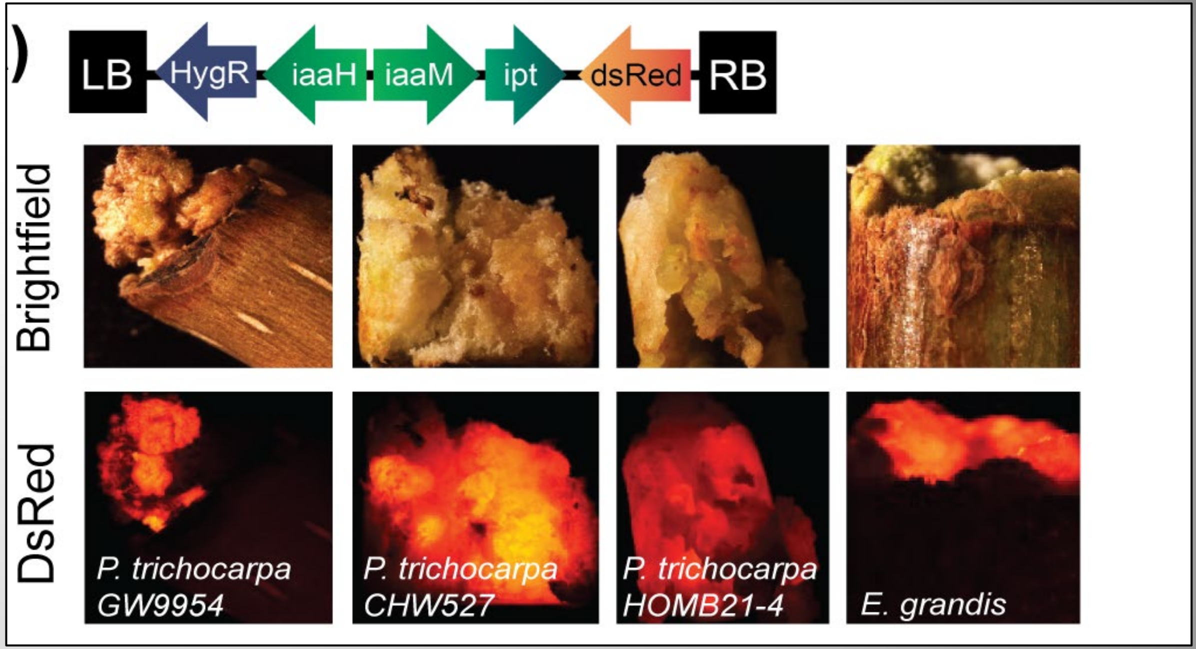
Back to the future: *A. tumefaciens* oncogenes revisited with new techniques? Useful for *in planta* transformation?

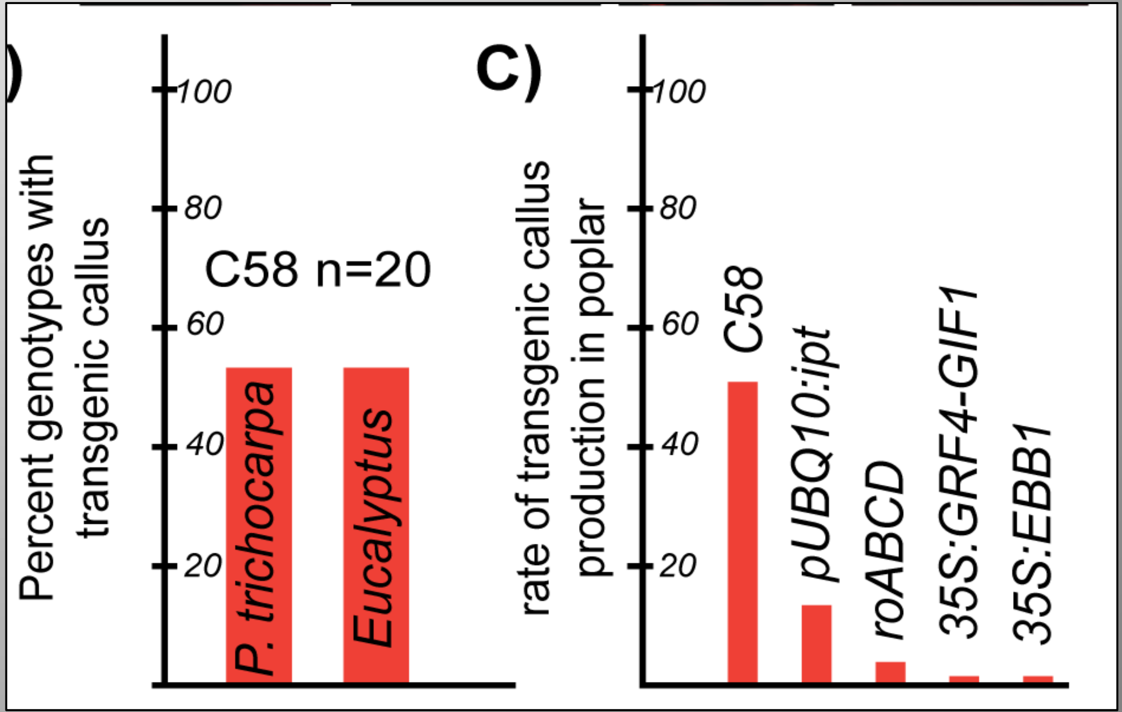
iaa/ipt genes form a positive feedback loop to induce and gall development

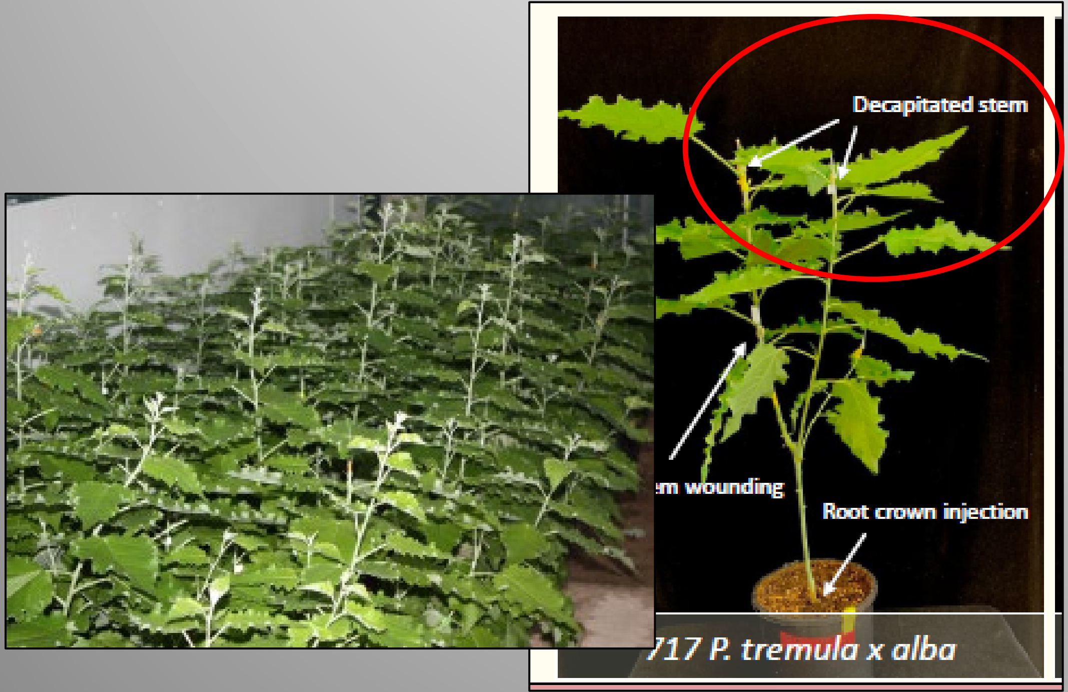


iaaH/M and ipt genes (C58 oncogenes) from *Agrobacterium* were effective *in planta* inducers of transgenic galls in diverse poplar and eucalypt genotypes

But shoots could not be regenerated from transgenic galls

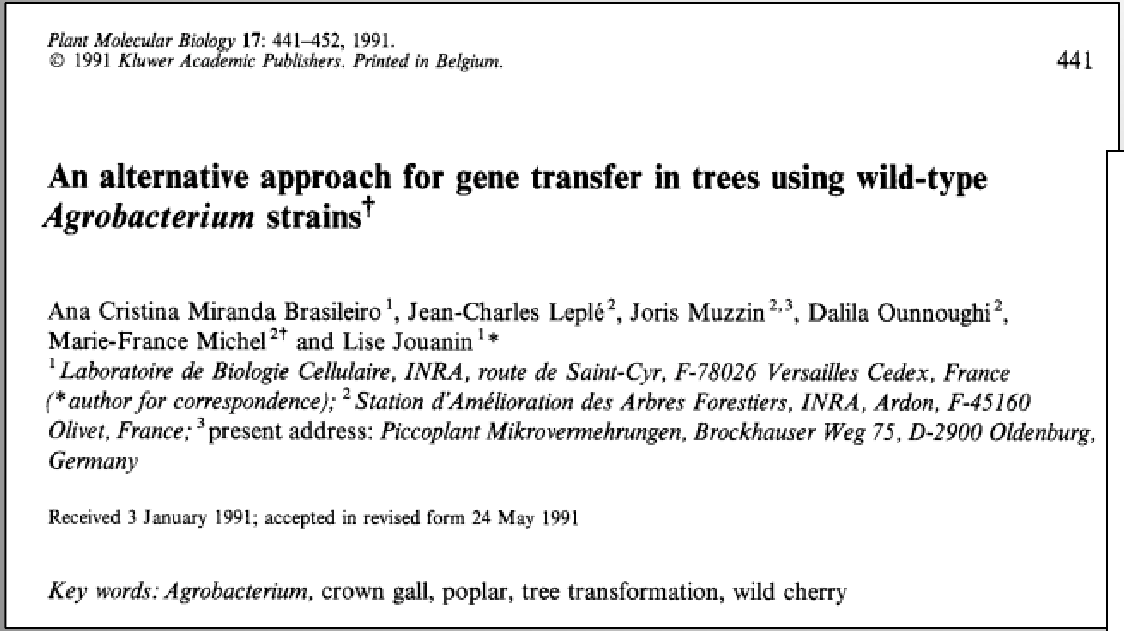






Can we find more useful, developmentally flexible galls?

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.
© 1991 *Kluwer Academic Publishers. Printed in Belgium.* 441

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

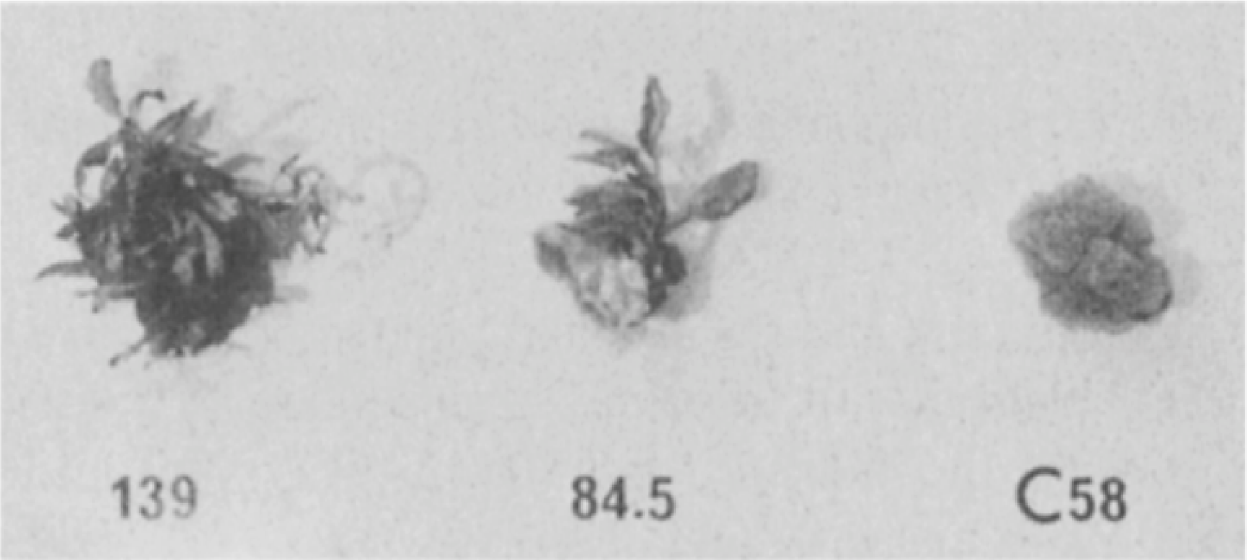
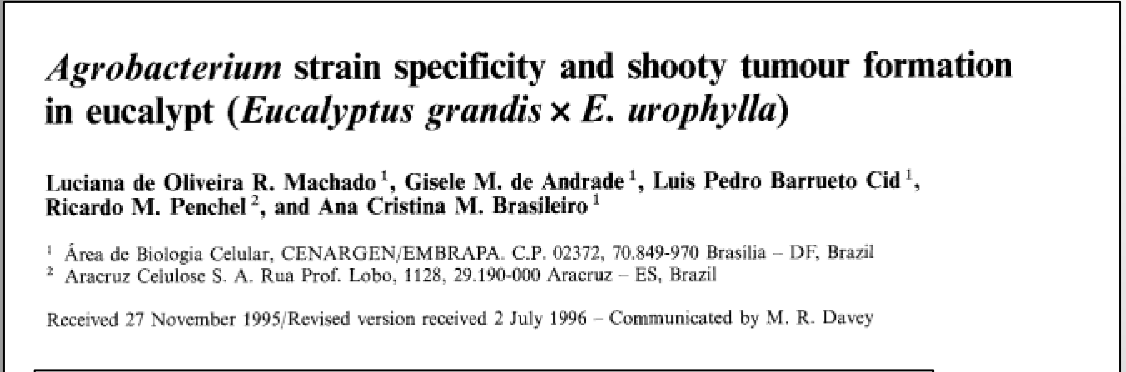


Fig. 1. Tumors and shoot differentiation from poplar tumors induced by *A. tumefaciens* strains 82.139, 84.5 and C58 and cultivated on MS medium, 6 weeks after inoculation.

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*)

Luciana de Oliveira R. Machado[1], Gisele M. de Andrade[1], Luis Pedro Barrueto Cid[1], Ricardo M. Penchel[2], and Ana Cristina M. Brasileiro[1]

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

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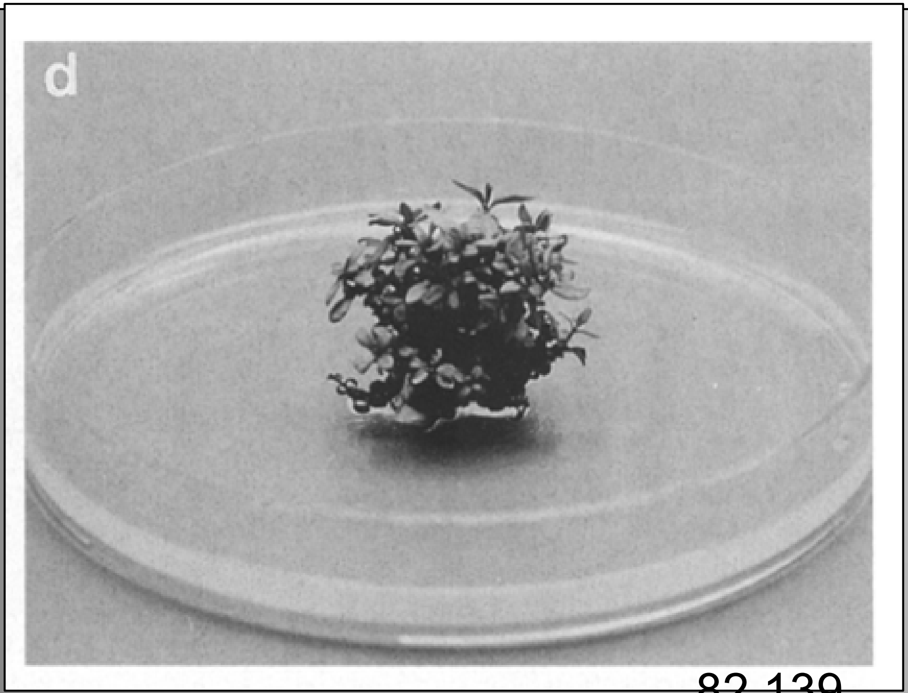
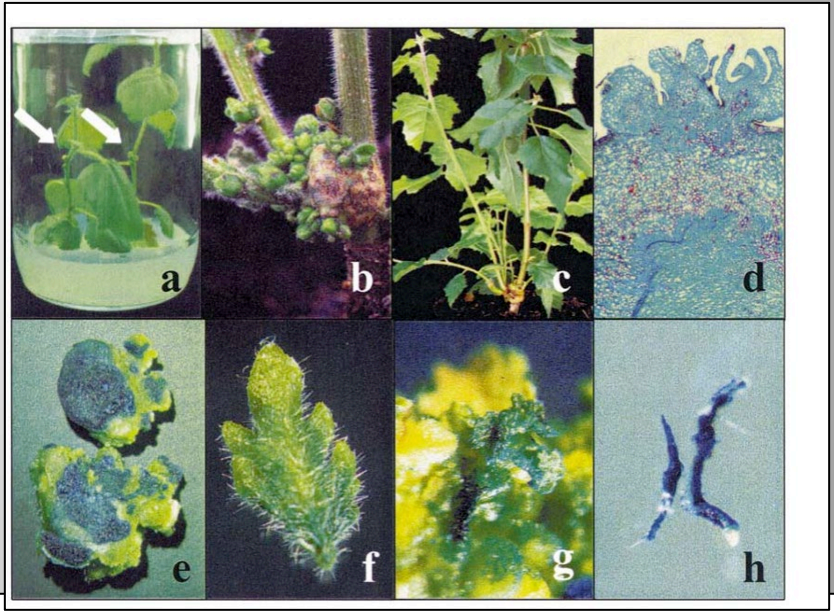
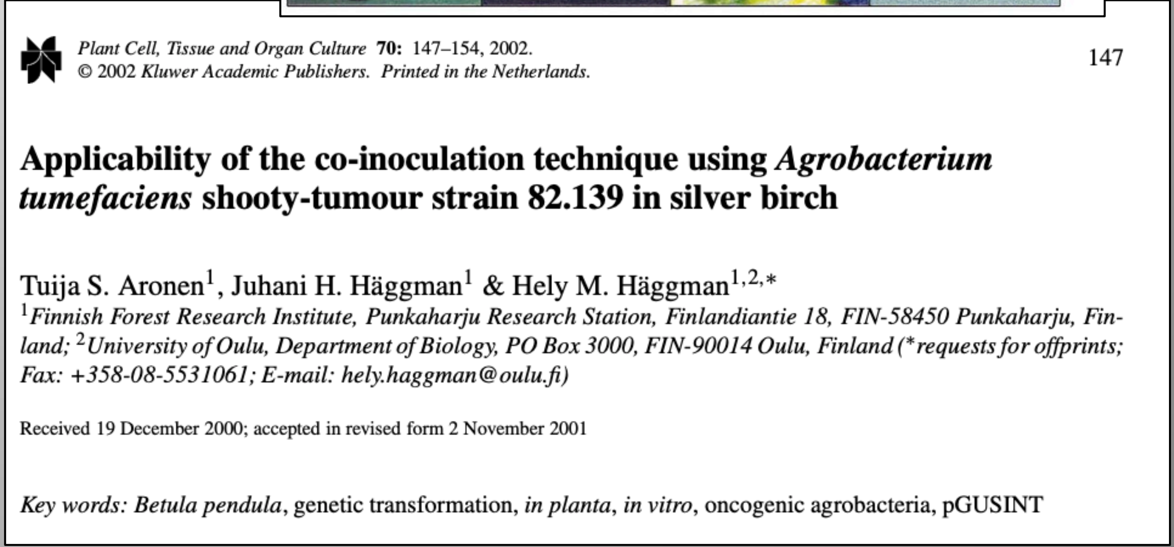


82.139





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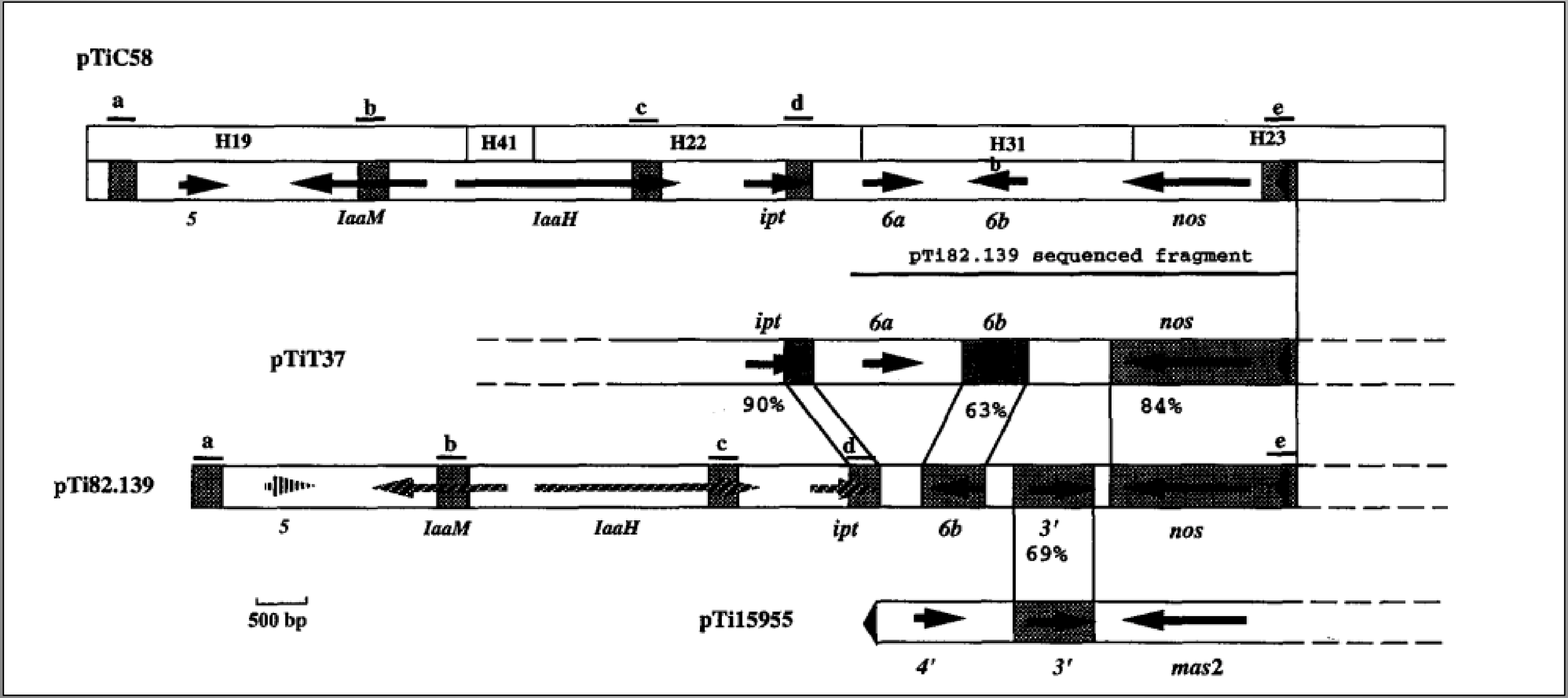
Applicability of the co-inoculation technique using *Agrobacterium tumefaciens* shooty-tumour strain 82.139 in silver birch

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

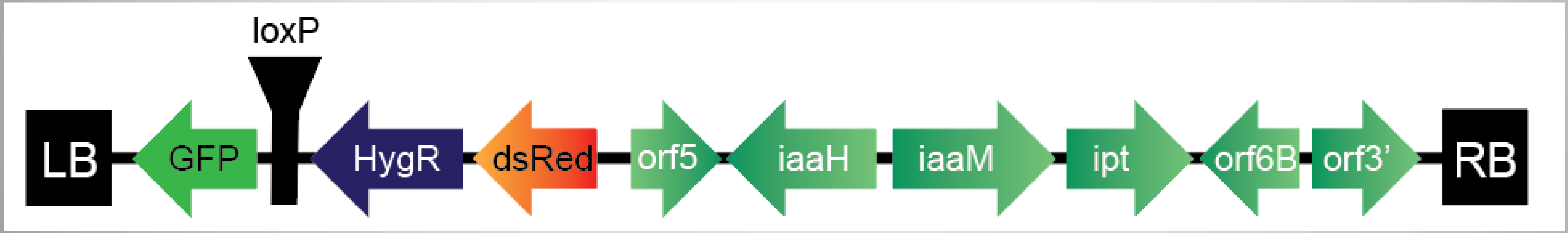
Key words: Betula pendula, genetic transformation, *in planta*, *in vitro*, oncogenic agrobacteria, pGUSINT

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

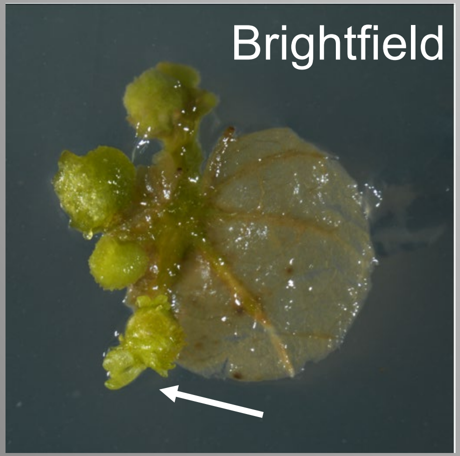


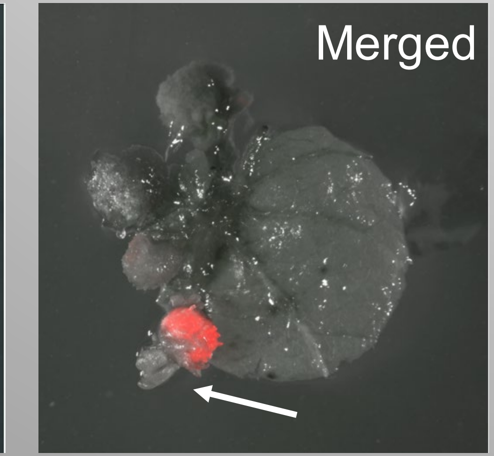
Though very promising, this work essentially came to a halt – due to GMOitis in Europe – and due to the challenges of dealing with the large Ti plasmids and its many vir and oncogenes prior to high throughput sequencing and advanced gene cloning systems

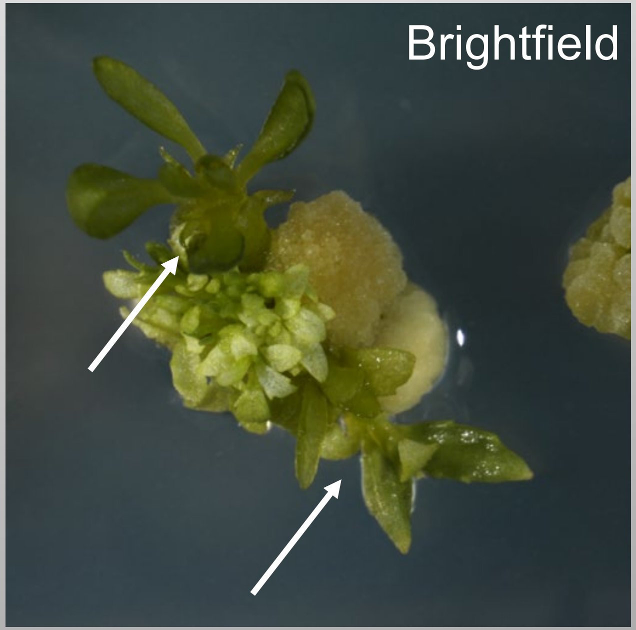
We cloned out the oncogenes from our resurrected clone in deep freeze, and added modern amenities like DsRed (called "S82")

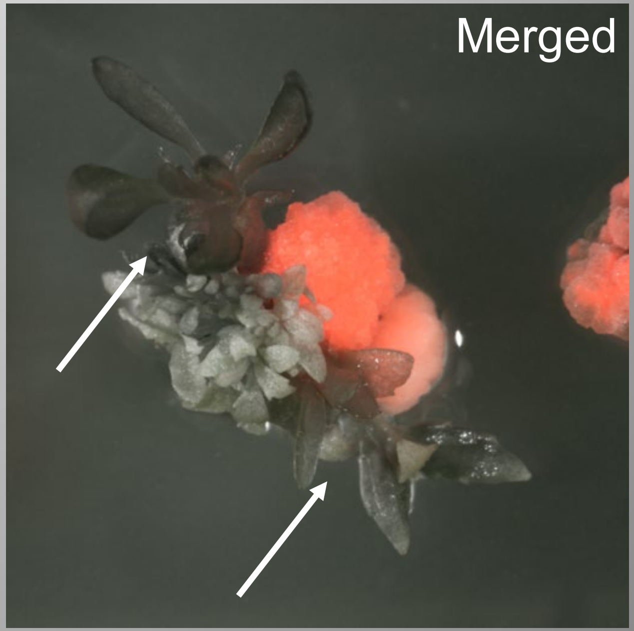


Transgenic galls promoted regeneration of galls and shoots



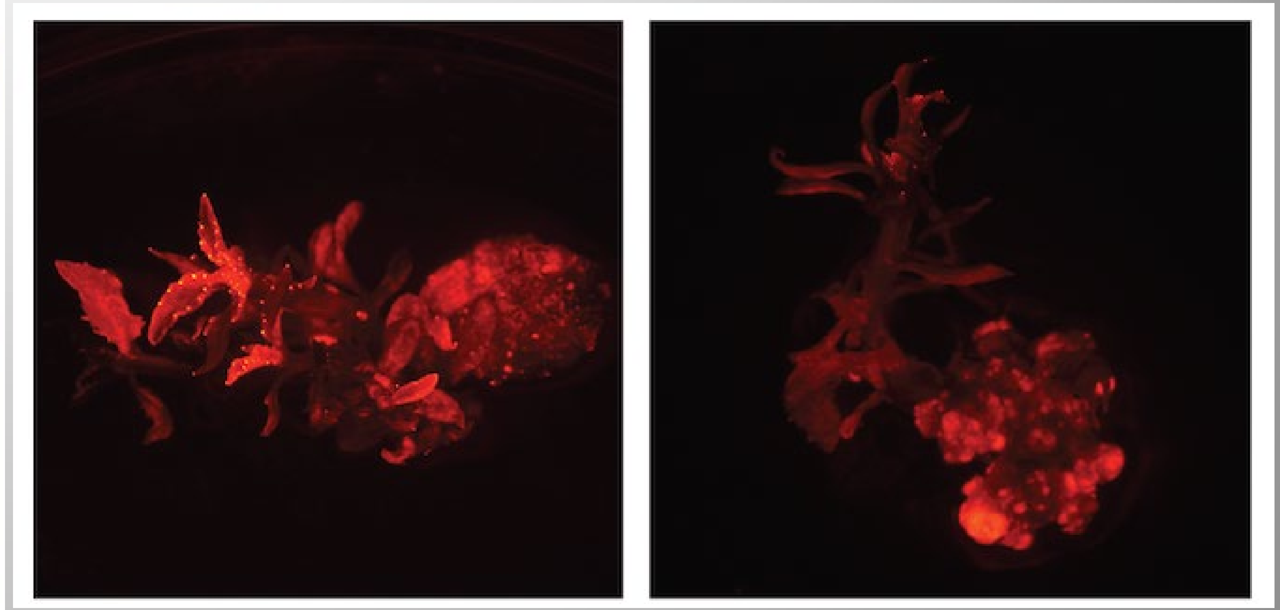






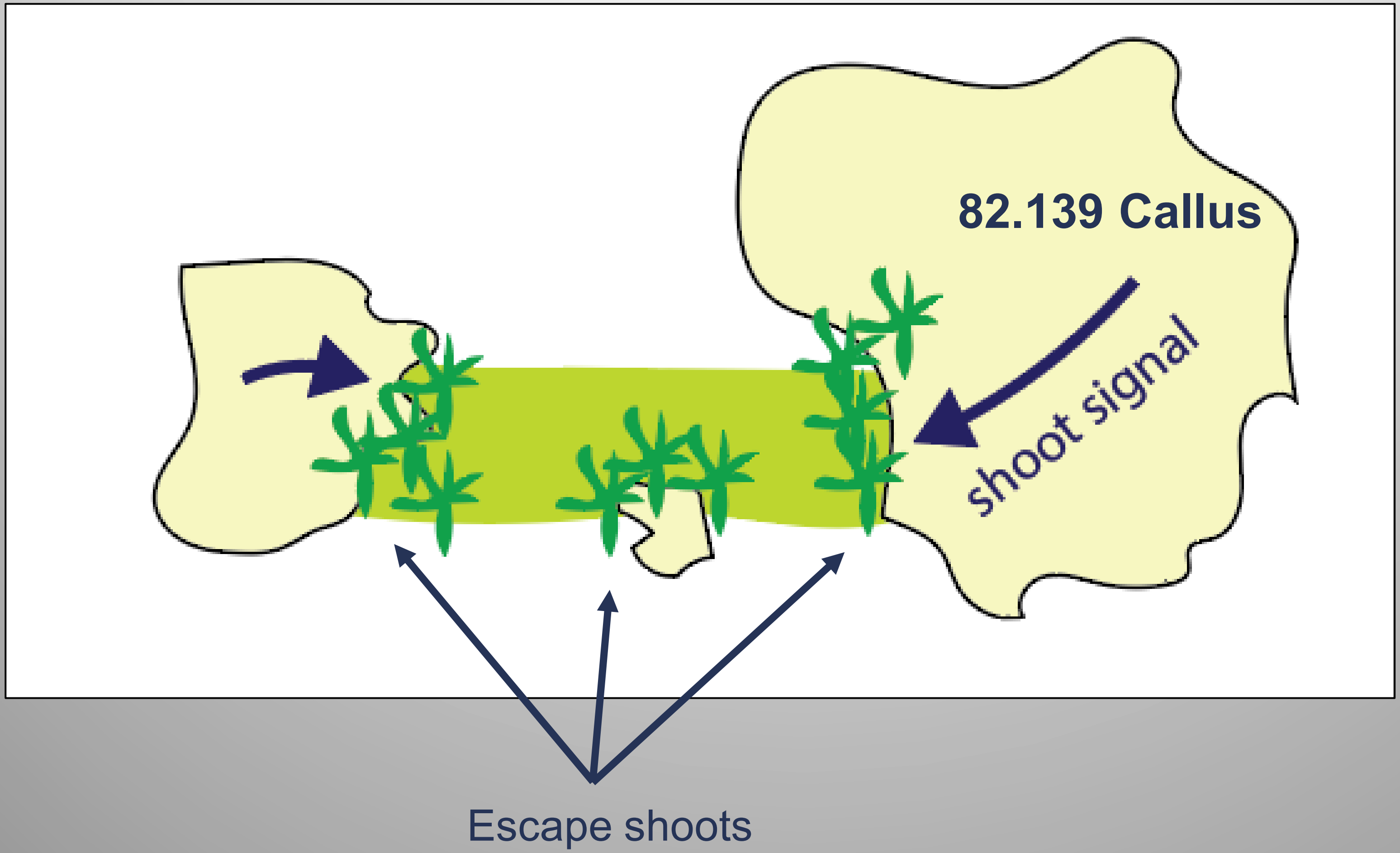
We also recoverd rare transgenic shoots using the 82.139 strain, but they had highly abnormal morphology

Brightfield



DsRed

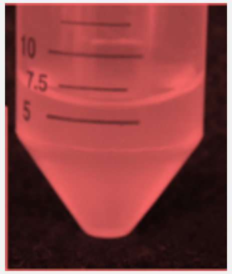
82.139 oncogenes appear to cause non-cell autonomous shoot inducing signals, useful for transformation systems?



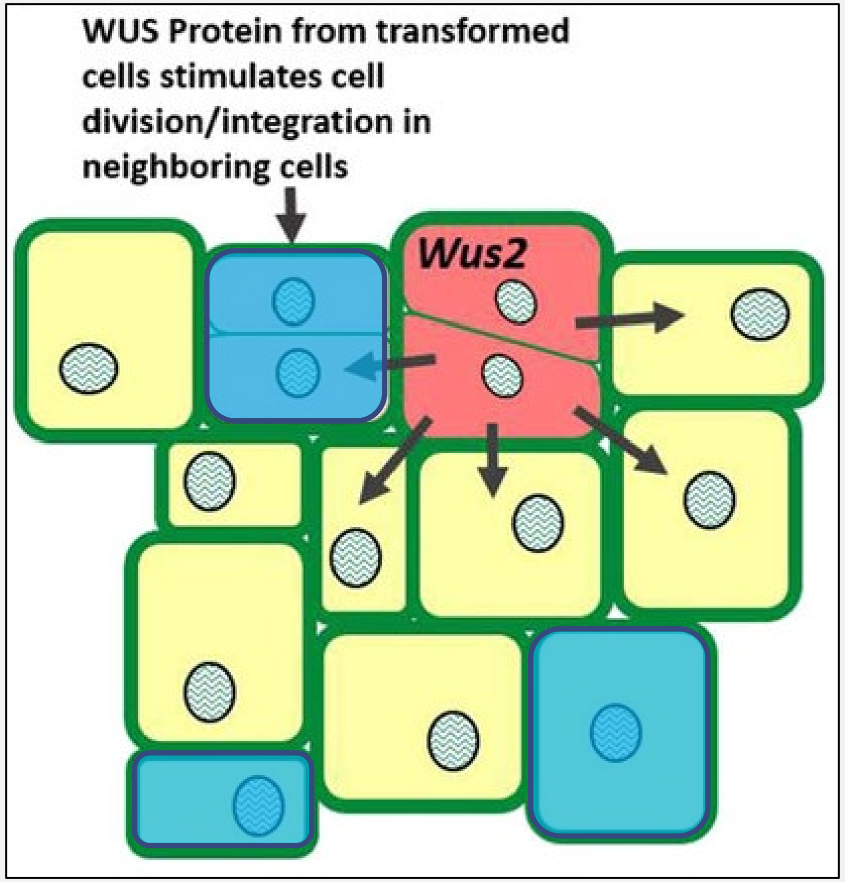
“Altruistic” transformation approach – strain mixtures

Mixed Agro in different ratios

DEV gene Agro
(like *WUS*)

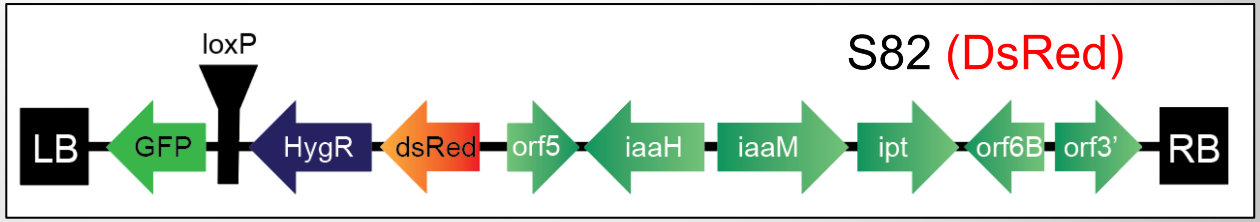


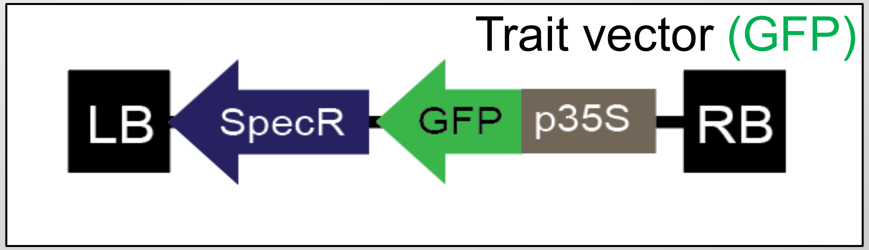
Trait gene Agro


WUS Protein from transformed cells stimulates cell division/integration in neighboring cells

Plant with trait gene
and no DEV genes

Altruitsic “S82” transformation – pilot study

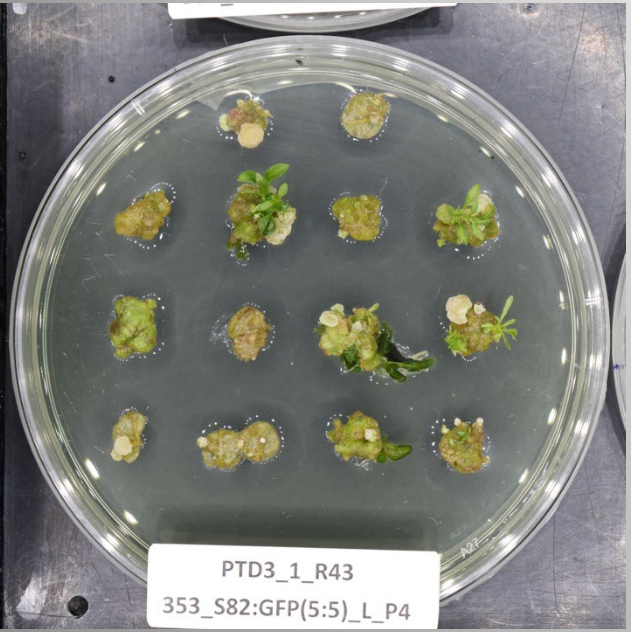




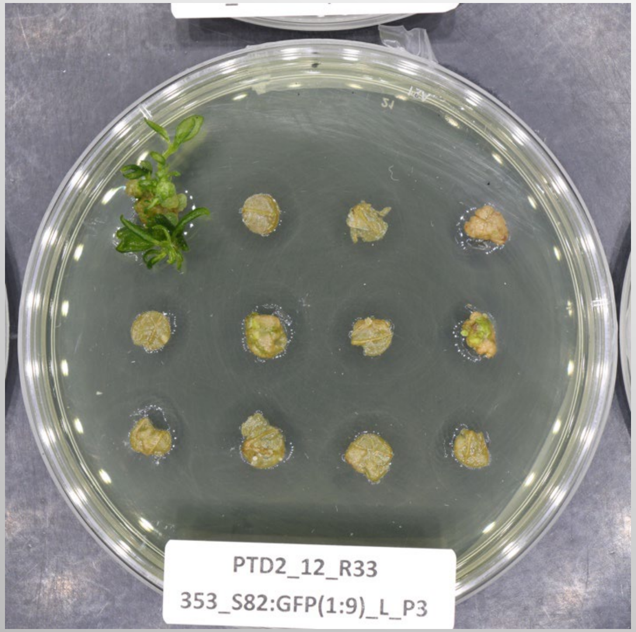
- 4 transformations
  - 100% S82
  - 50% S82 / 50% Trait-GFP
  - 10% S82 / 90% Trait-GFP
  - 100% Trait-GFP

1. Co-culture 2 days (dark)
2. Transfer to 1xMS with Rif to counterselect Agro for 1 week (resting phase, dark)
3. Transfer to 1xMS + Rif + Spec to select for Trait-GFP (Dark 2 weeks)
4. Transfer to light, and subculture at 1 month onto same media

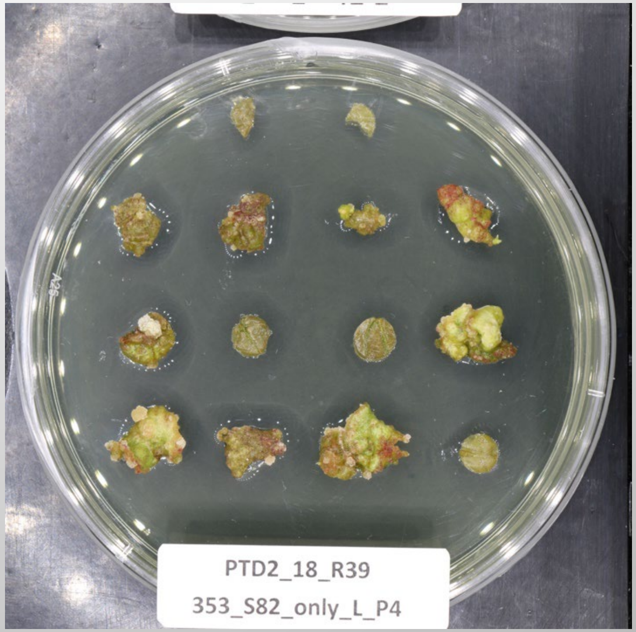
Single constructs did not regenerate transgenic shoots as predicted, 5:5 mix ratio was best



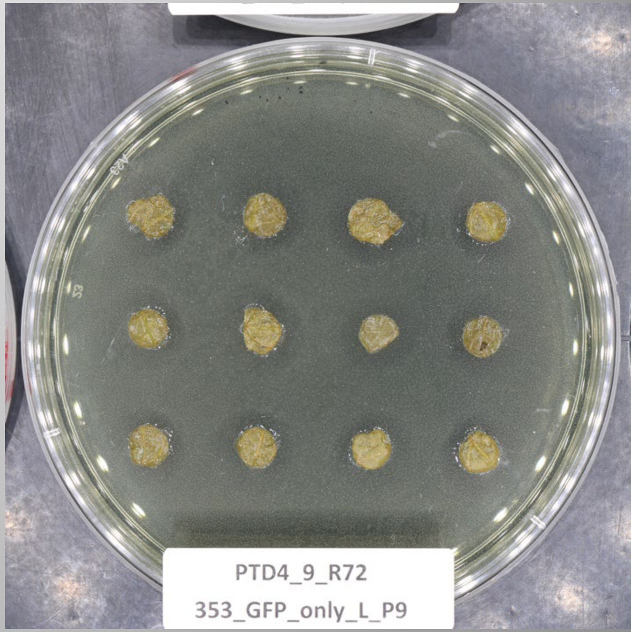
5:5 mix



1:9 mix

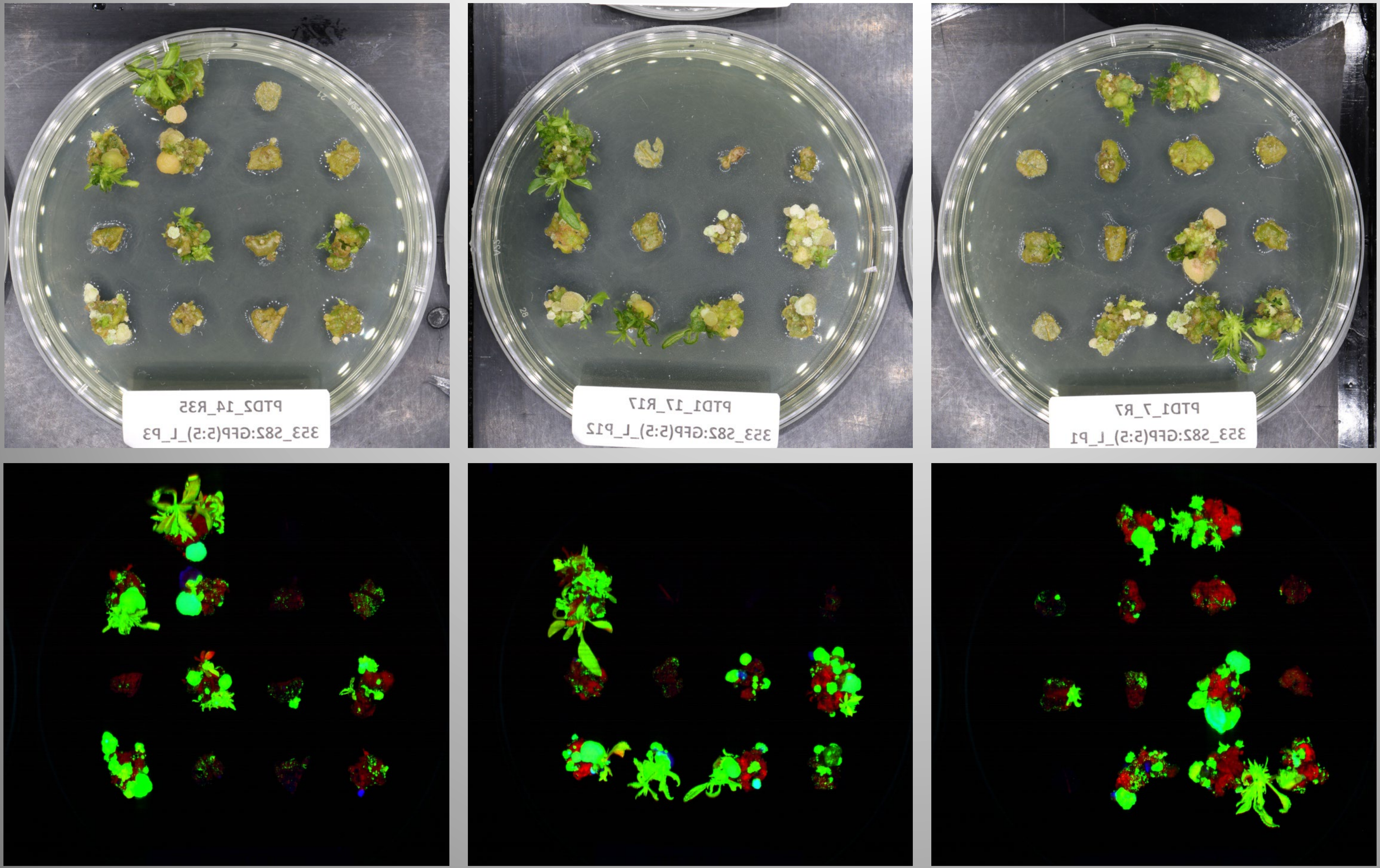


Oncogenes only



GFP only

Hyperspectral imaging showed altruistic shoot regeneration

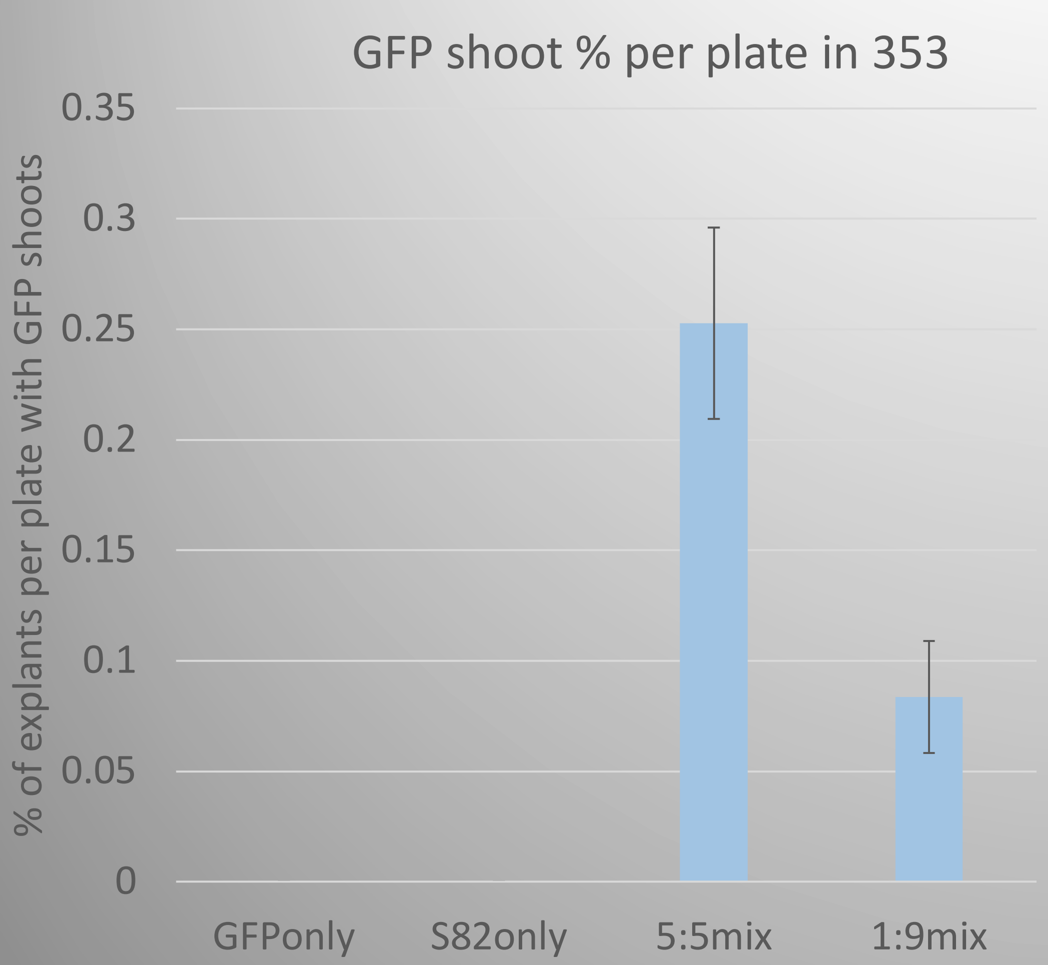


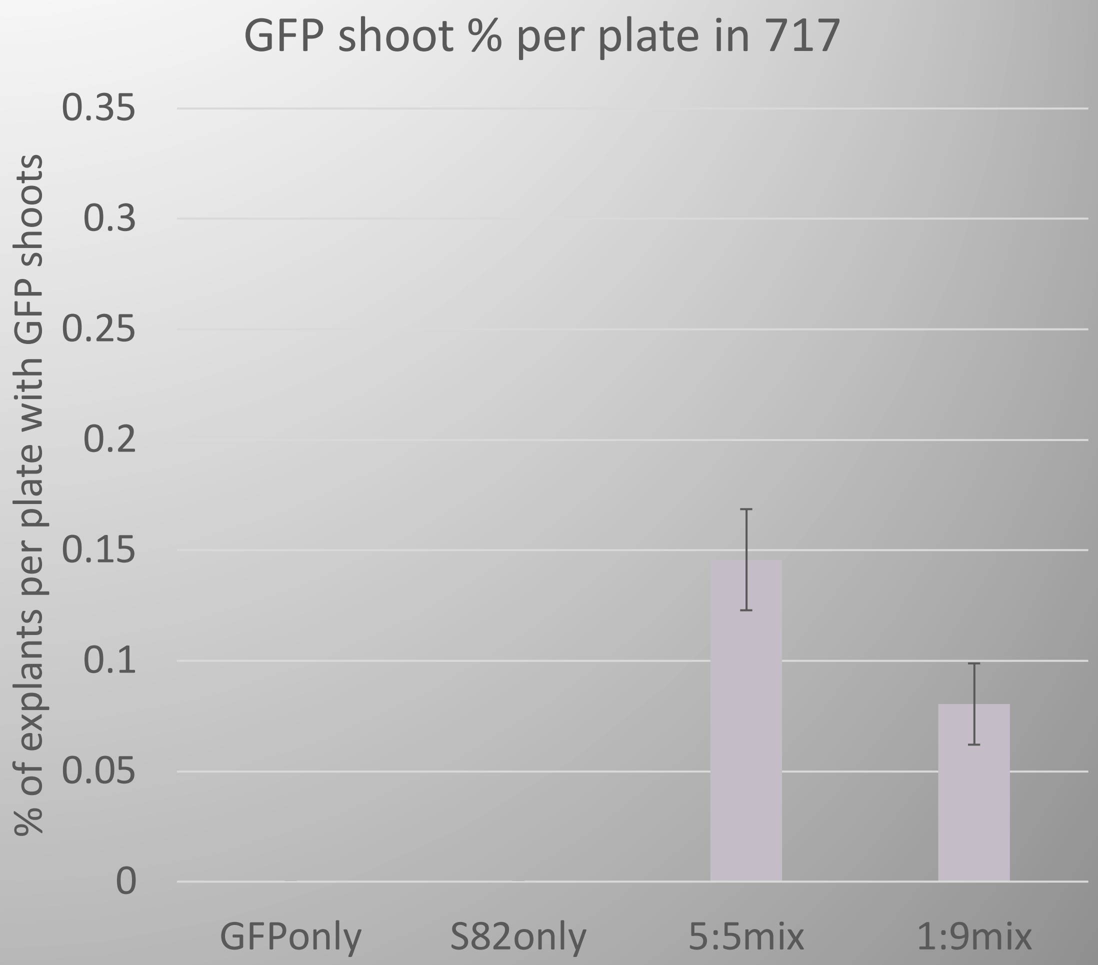
Green = GFP

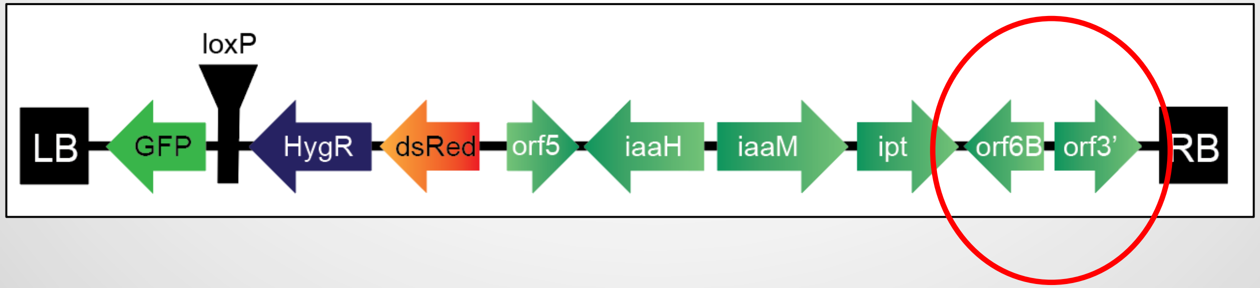
Red = dsRED

A closer look: 5:5 mix at week 6

5:5 mix treatments worked best in two poplar genotypes







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?

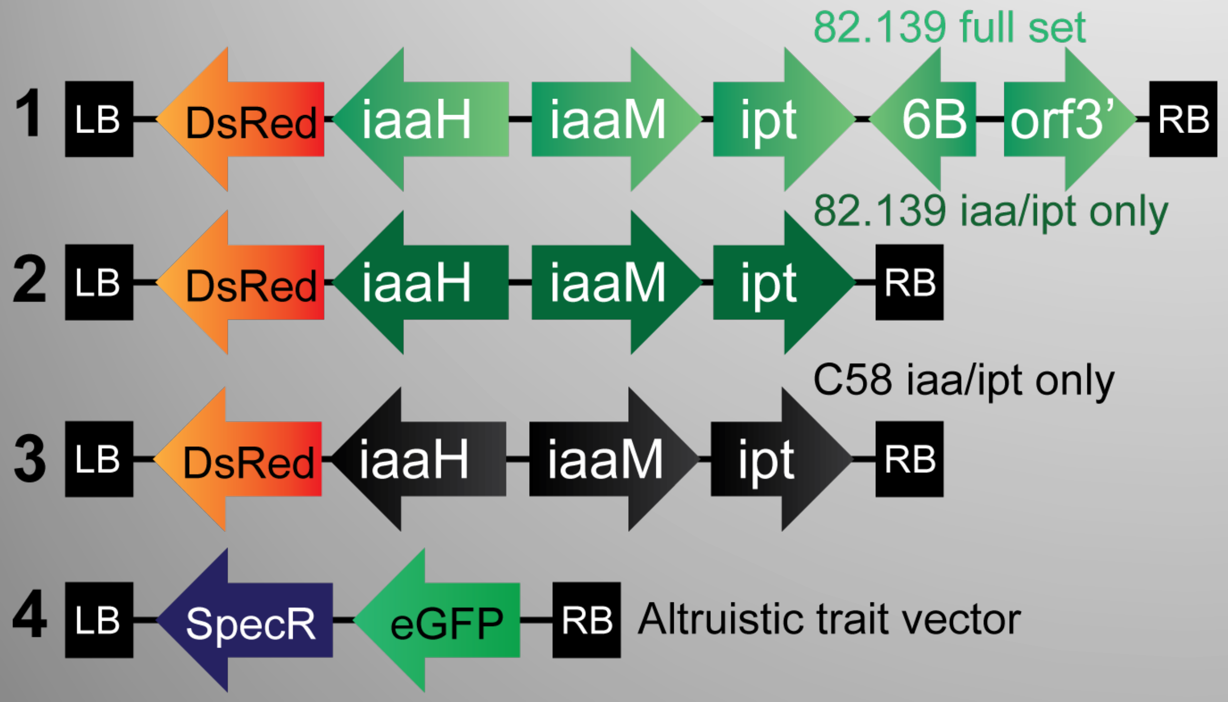
Experimental setup

- 3 constructs
  - C58 (just *iaa* and *ipt* genes)
  - S82 (all six cloned genes)
  - S82 (just iaa and ipt genes)

- All constructs mixed 5:5 with SpecR GFP binary vector
- 1 week rest after co-culture without spectinomycin, 6 weeks on MS media without hormones but with spectinomycin

Callus: C58 iaa/ipt genes were best at inducing transgenic callus, but all three versions worked well

Vector types used


82.139 full set

1 LB DsRed iaaH iaaM ipt 6B orf3’ RB

82.139 iaa/ipt only

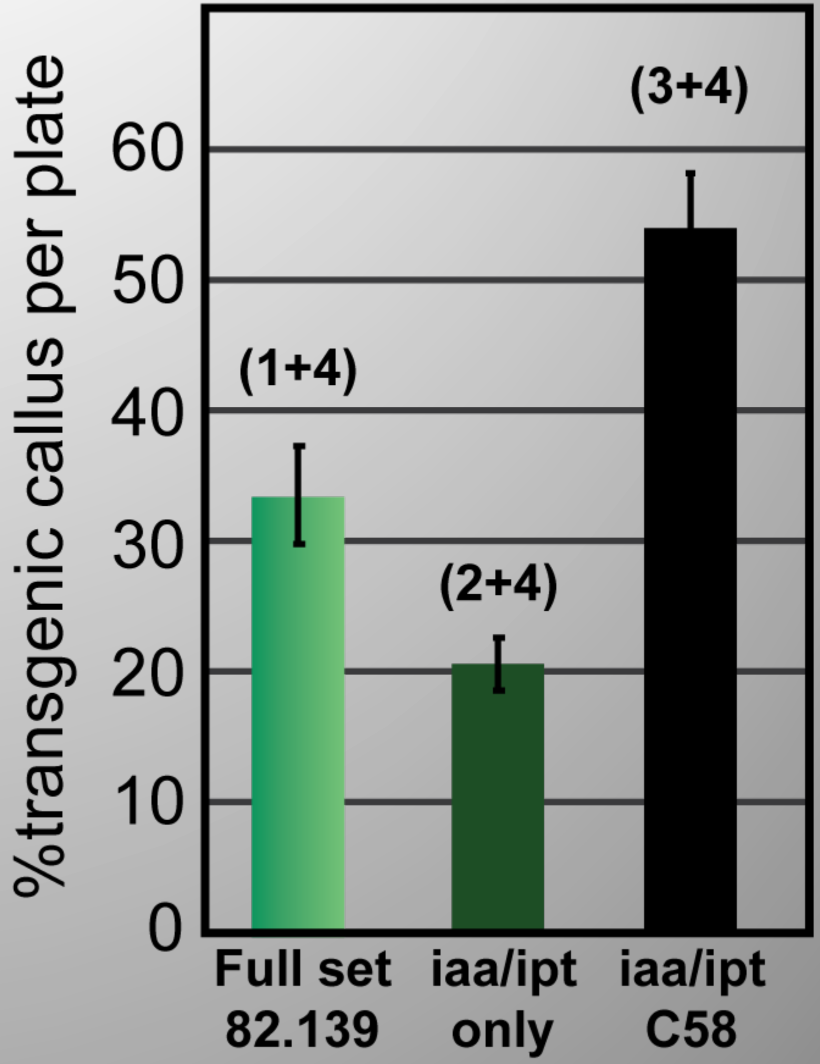
2 LB DsRed iaaH iaaM ipt RB

C58 iaa/ipt only

3 LB DsRed iaaH iaaM ipt RB

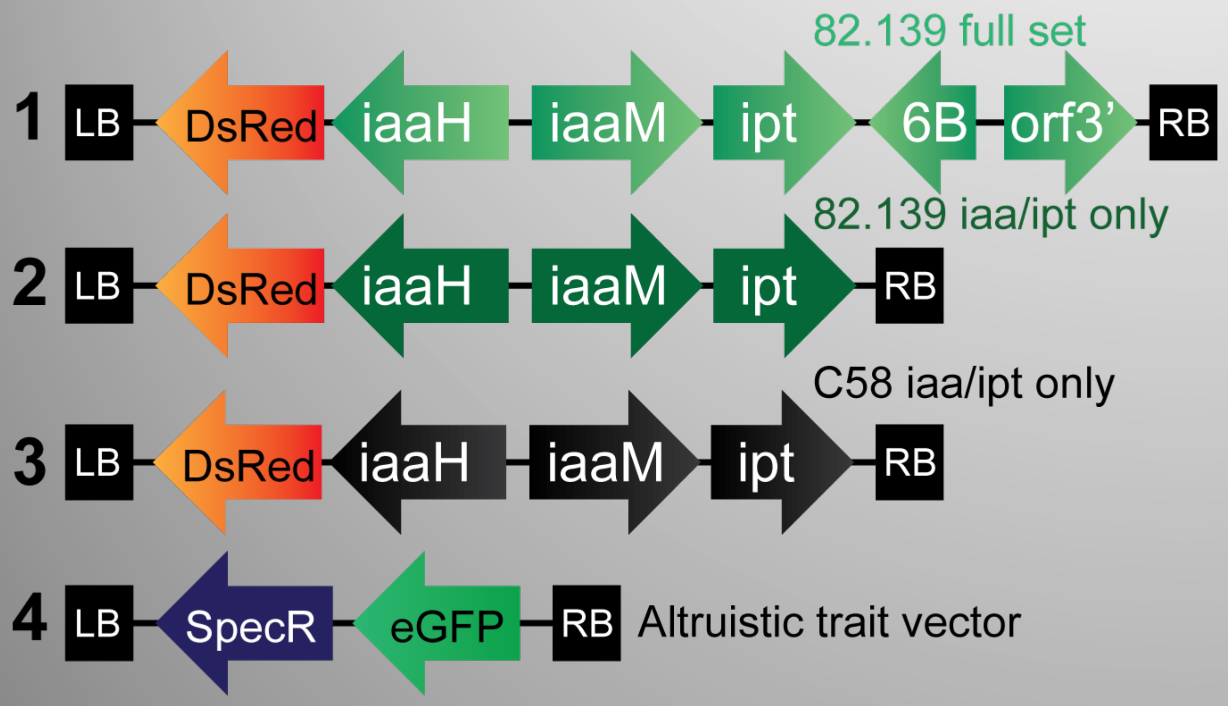
4 LB SpecR eGFP RB Altruistic trait vector

n= number of plates of 12 explants each



<u>Shoots</u>: 82.139 *iaa/ipt* genes alone are insufficient for altruistic shoot induction

Vector types used



82.139 full set

1 LB DsRed iaaH iaaM ipt 6B orf3' RB

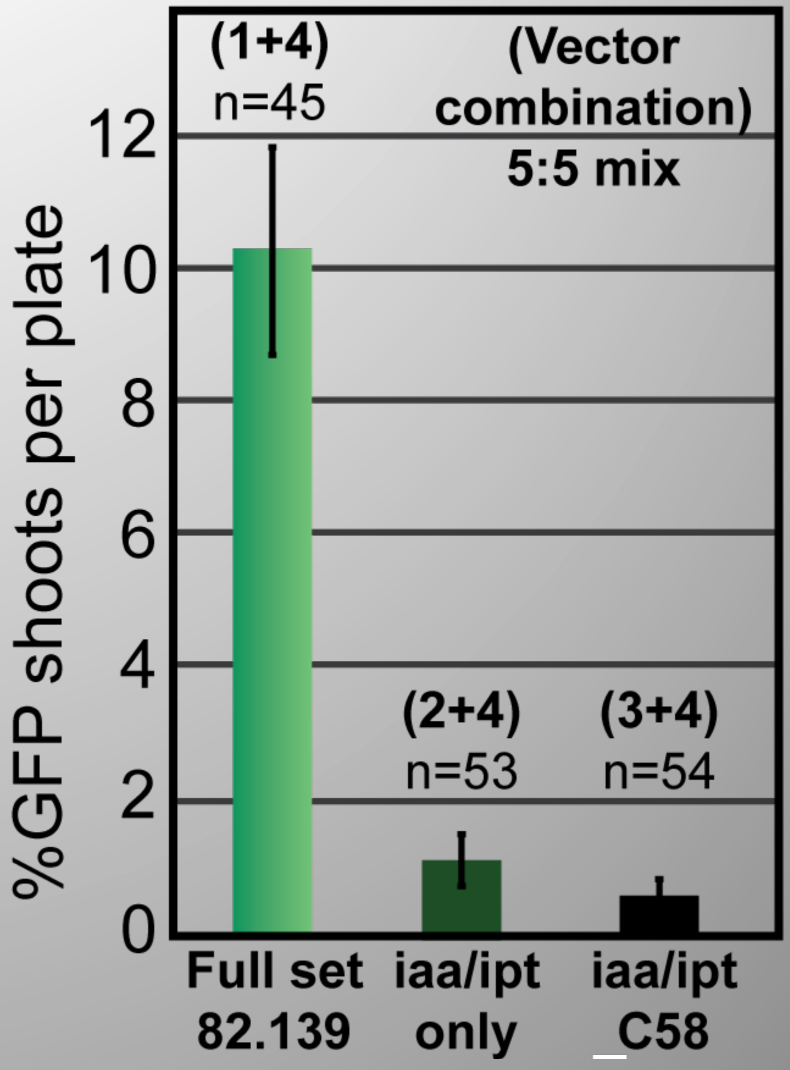
82.139 iaa/ipt only

2 LB DsRed iaaH iaaM ipt RB

C58 iaa/ipt only

3 LB DsRed iaaH iaaM ipt RB

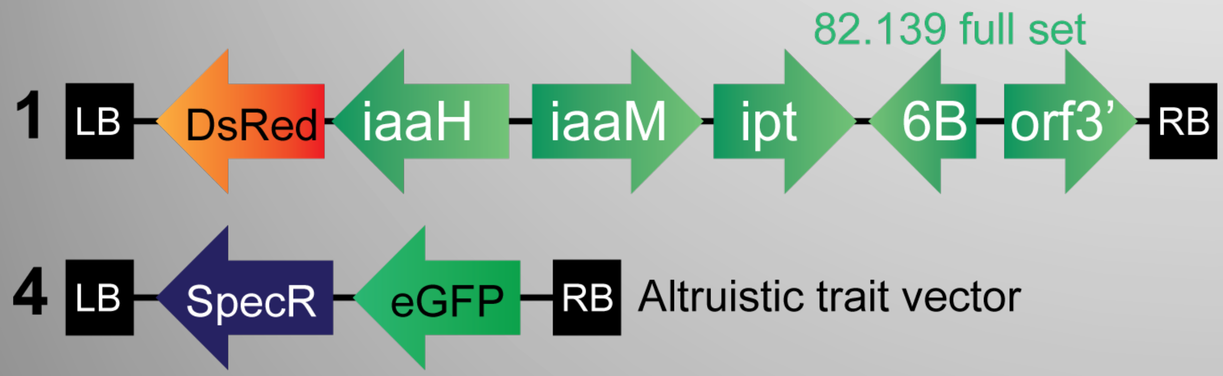
4 LB SpecR eGFP RB Altruistic trait vector



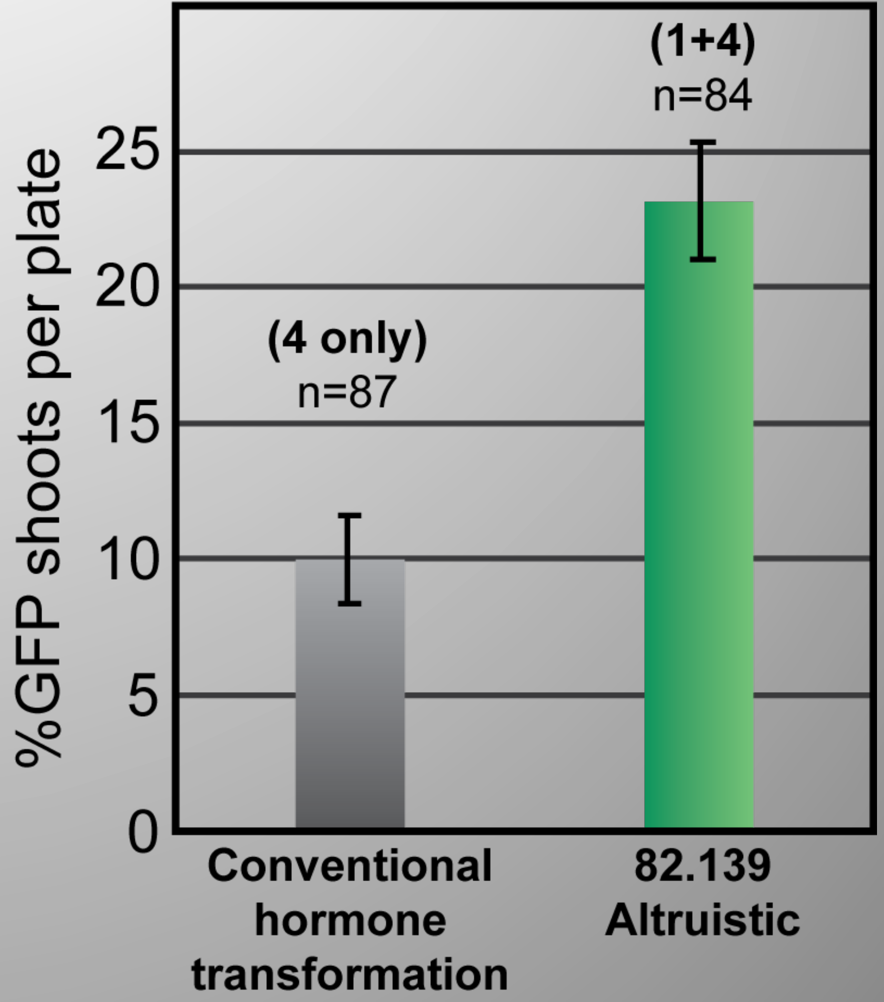
n= number of plates of 12 explants each

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

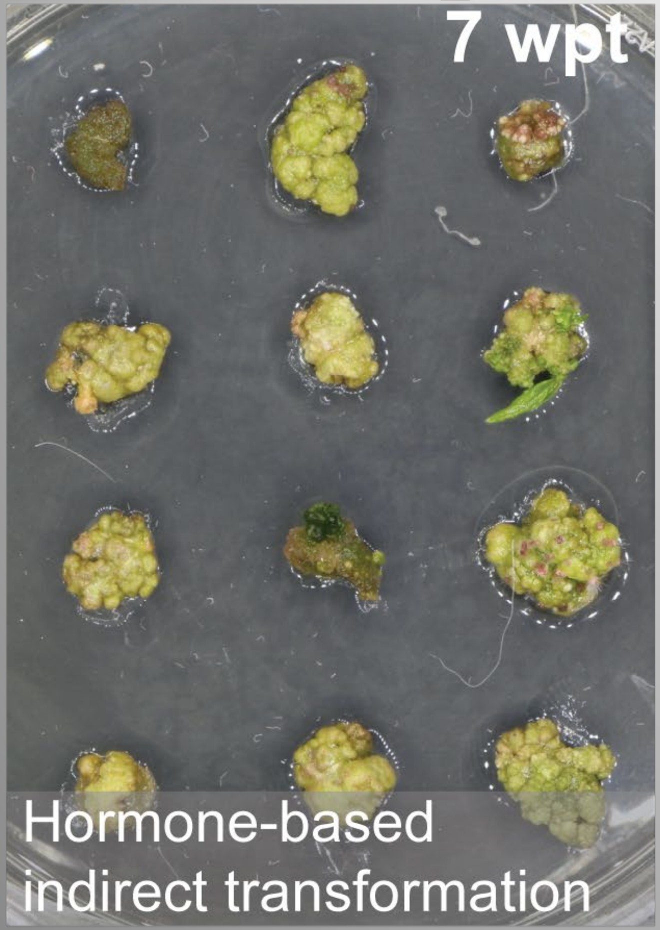
Vector types used



n= number of plates of 12 explants each



The 82.139 altruistic method was also significantly <u>faster</u>, shortening time to propagation by half



Hormone-based indirect transformation

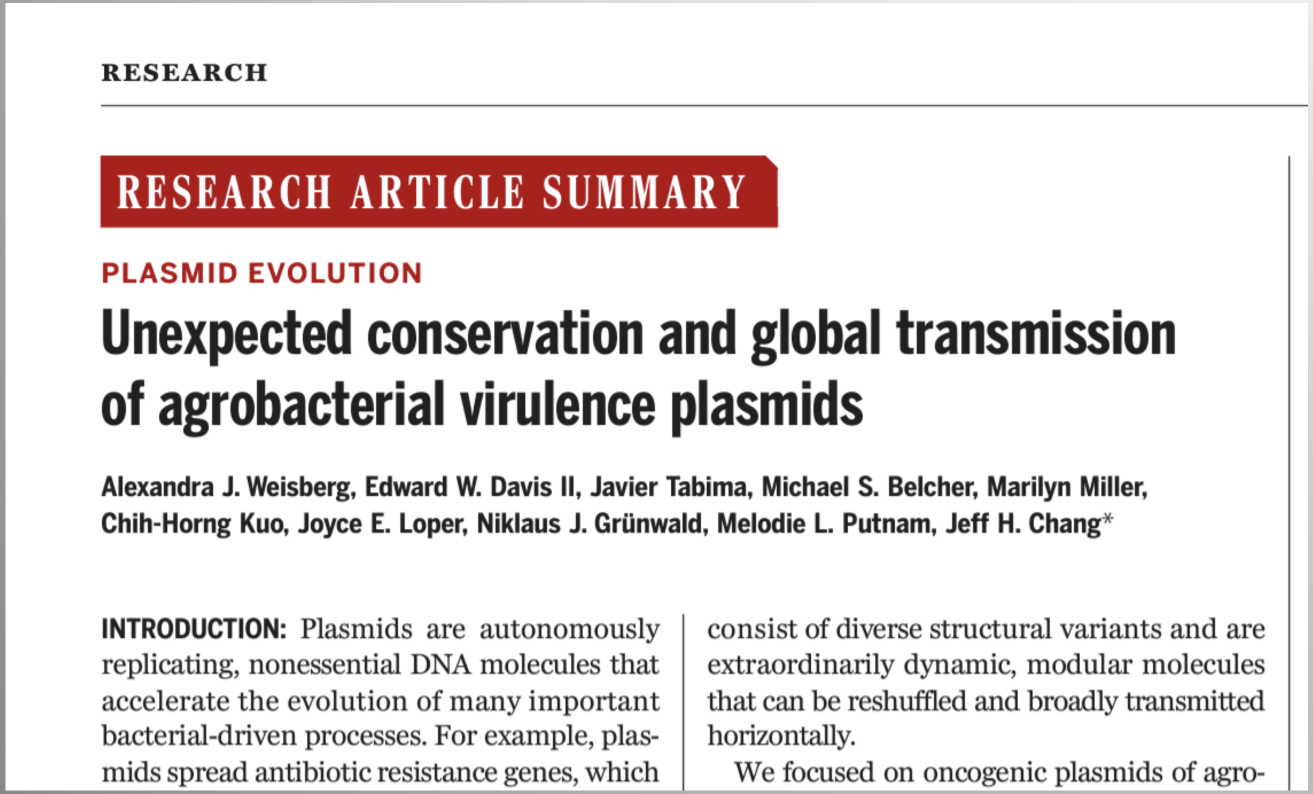
Altruistic transformation with 82.139 genes

Making this a useful tool

- **Delivery of the 82.139 oncogenes is presently in our vir-delivered GAANTRY strain (ARS Albany, J. Thomson), this strain is aggressive, not an auxotroph**
- **We have mobilized the genes into binary-compatible vectors**
- **We aim to test in auxotrophic strains for general ease of use**
- **We have begun further testing to identify which oncogenes are most critical for non-autonomous shoot induction**

Are there other useful oncogenes? Agro diversity hardly studied

We are starting to test ~300 fully sequenced wild Agrobacterium strains from the Chang lab at Oregon State, to look for shooty phenotypes



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*

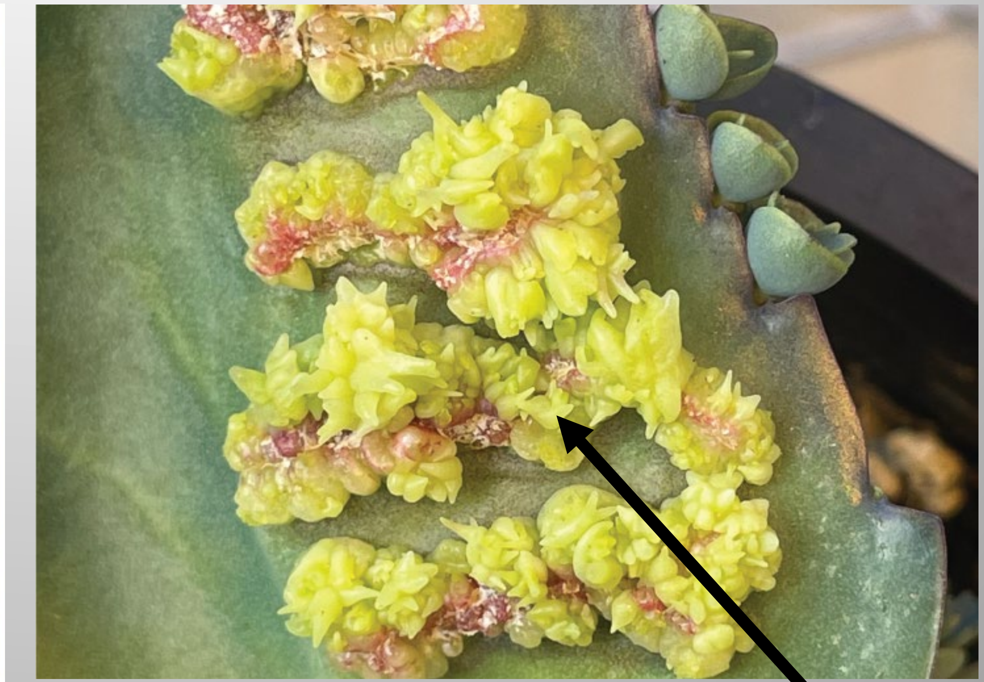
INTRODUCTION: Plasmids are autonomously replicating, nonessential DNA molecules that accelerate the evolution of many important bacterial-driven processes. For example, plasmids spread antibiotic resistance genes, which consist of diverse structural variants and are extraordinarily dynamic, modular molecules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

We focused on oncogenic plasmids of agro-

BAYER | Grants 4Ag

Transformation of Kalanchoe in progress, then oncogenes will be cloned out from the best strains

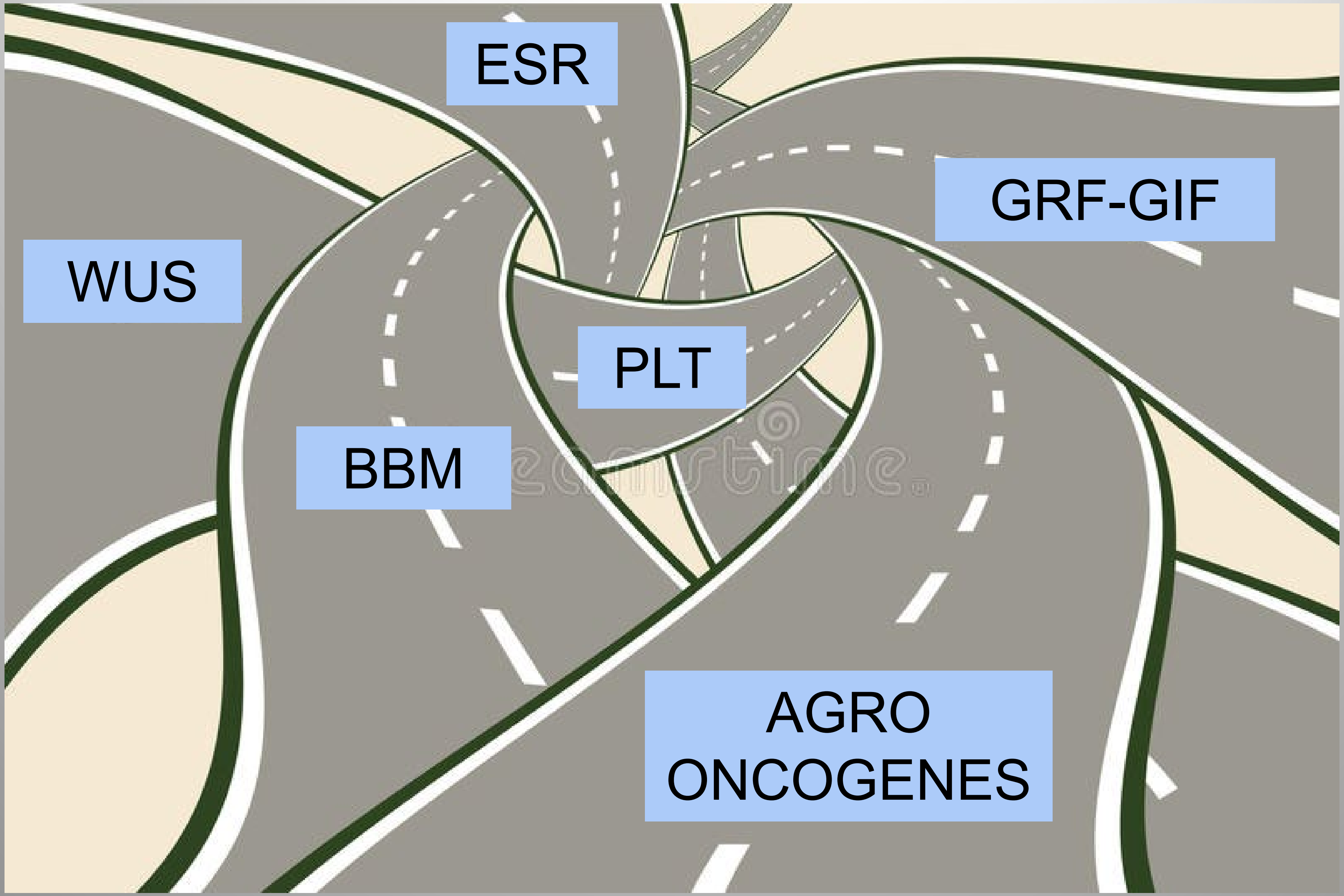
Vector only inoculation



Wild strain inoculation

What I imagined.....

What we are experiencing.....



Going forward

- Woody species, mature-clonal tissues, are tough and slow – poplars exceptional
- As our GRF-GIF results show, there is *major league* genetic diversity in response to most everything we try – media amendments and DEV genes
- Not shown today: Promising transformation rate enhancement with DEV gene activation used CRISPRa – *WOX11*
  - Yiping Qi vectors / U. Maryland
- Not shown today: Promising developments in recombinase excision via methylation control mechanisms
  - New USDA grant to study and develop into useful tools
- <u>The main take-home</u>: Shooty Agro oncogenes, delivered altruistically, very promising transformation approach – now also being tested *in planta*

Thanks to our funders and collaborators

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The National Science Foundation

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Steve Strauss
Director TBGRC,
Professor

Cathleen Ma
Transformation &
Greenhouse
Experiments

Amanda Goddard
Program & Field
Manager

Greg Goralogia
Postdoc,
Flowering &
Gene Editing

Kate Peremyslova
GWAS,
Transformation
Experiments

Michael Nagle
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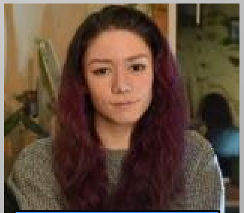
Chris
Post-doc
Hops
transformation

Nathan Ryan
Masters
GRF genes

Alexa Nino de RIvera
Tissue culture &
greenhouse technician



Megan McEldowney
Tissue culture &
GWAS technician