# Of media and miracles:

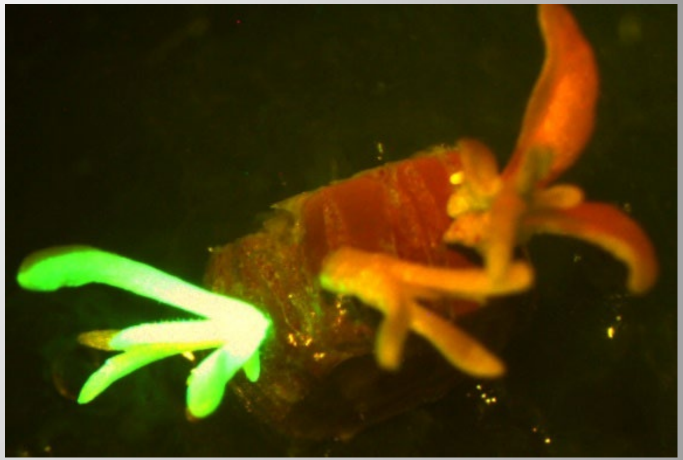
## Successes and frustrations in the search for efficient regeneration and transformation methods for trees and crops

*Steve Strauss*

*Oregon State University*

**Society for in Vitro Biology Annual Meeting / recorded 30 April 2022**

# Agenda



- Perspectives - experimental system
- Culture conditions
  - Lipoic acid example
- Morphogenic regulator genes (“DEV genes”)
  - GRF-GIF example
- Highlights from regeneration GWAS

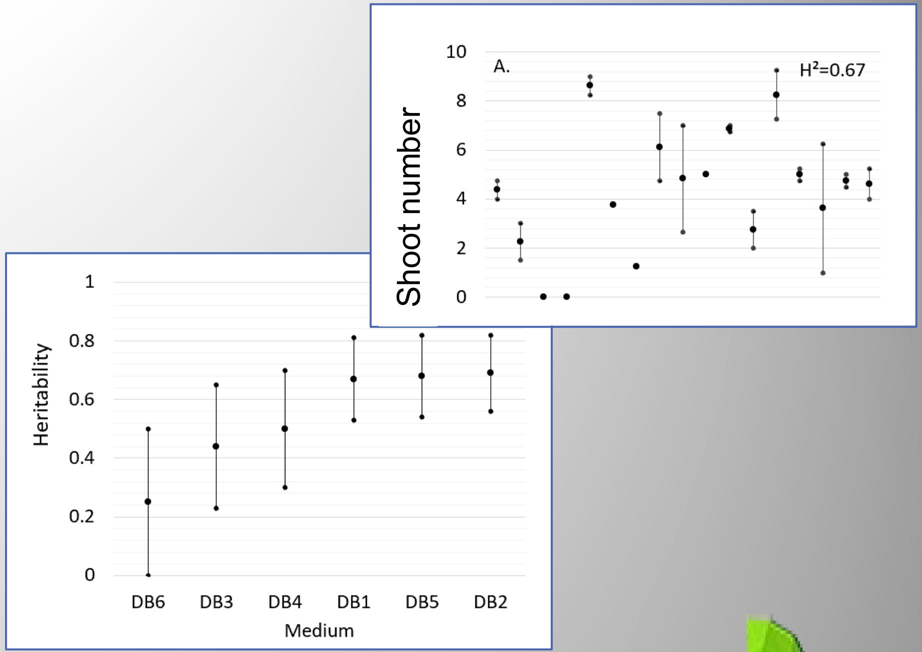
# Regeneration & transformation continue to be major limiting factors for gene editing & engineering in plants

- Species and genotypic differences often dramatic
- Minor and woody crop species perhaps most problematic
  - Tough biology, research investment limited
- Slow, costly, complex customization efforts usually needed
- On top of often large social/regulatory constraints, often a “deal breaker”

# Experimental system features

- Woody (forest) trees
- 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 and more – *which leads to low reproducibility*

# Today: A few lessons from…

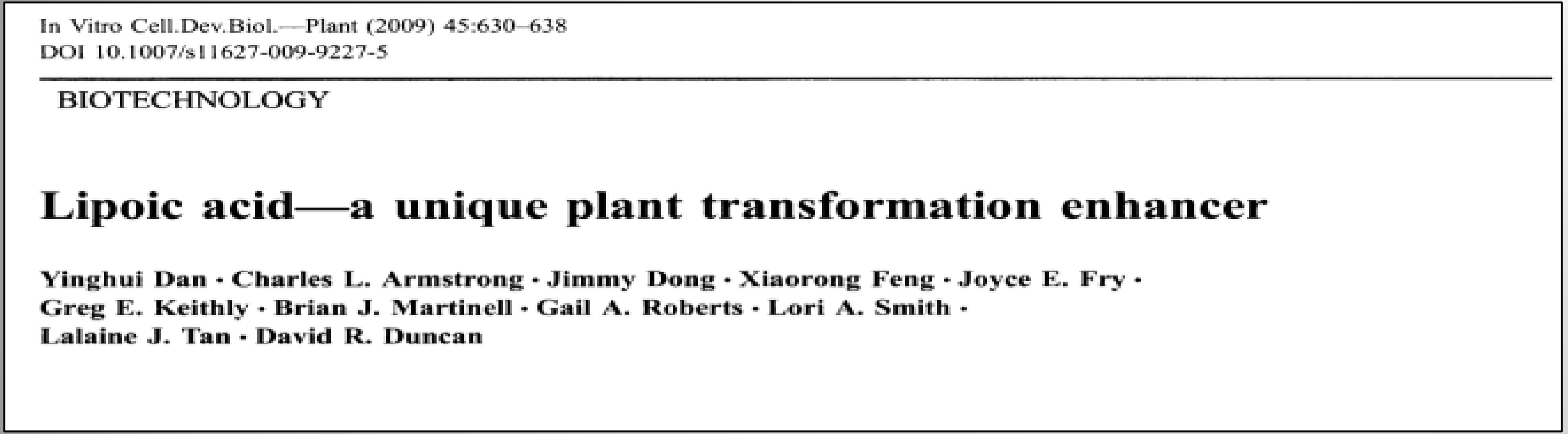


- An NSF-funded study to enhance $h^2$ of regeneration and transformation in prep for GWAS, and results thereof
  - About 10-20 genotypes per treatment
  - Several hundred treatments x genotypes studied
- Industry consortium-funded studies of DEV genes for eucalypt transformation
  - “GREAT TREES”
- Masters thesis research on GRF-GIF by Nathan Ryan

# Many culture conditions studied, mostly with little or no general benefit for regeneration or transformation

- Basal media
- Light intensity & quality
- Explant sterilization
- Vitamin C
- Activated carbon
- Melatonin/serotonin
- Silver nitrate
- Proline
- Major sugars/concentrations
- **Lipoic acid**

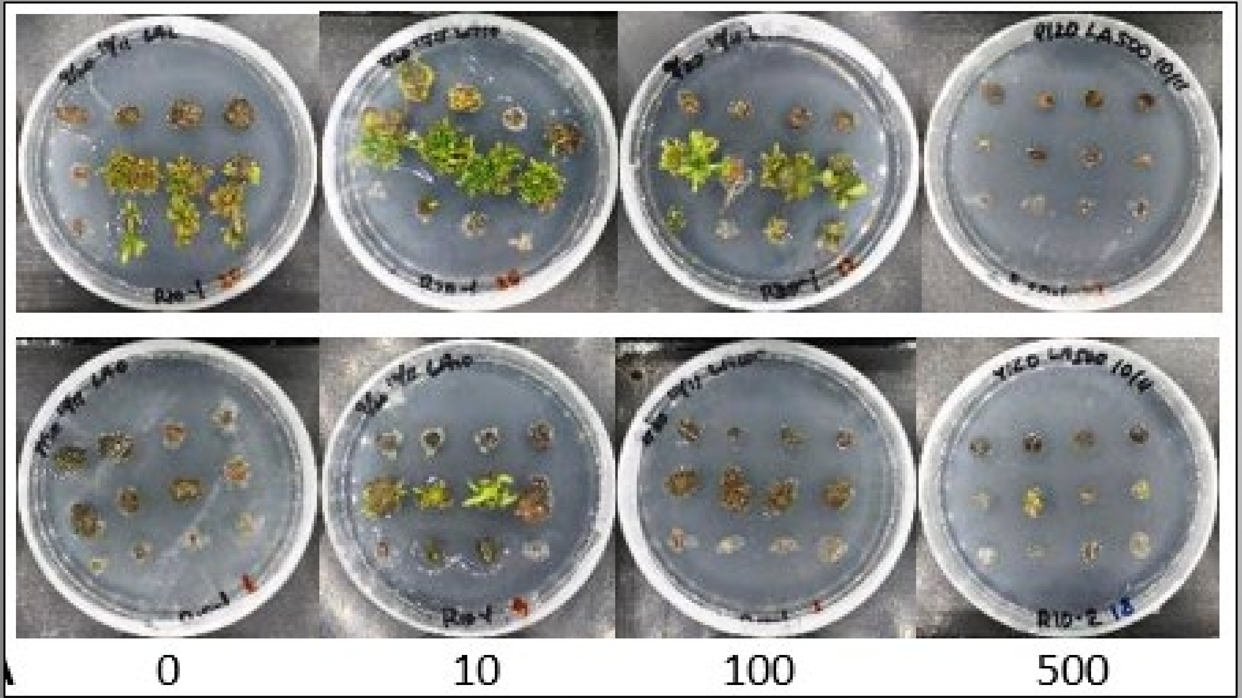
# Lipoic acid (LA) a “vunder” antioxidant ?


In Vitro Cell.Dev.Biol.—Plant (2009) 45:630–638
DOI 10.1007/s11627-009-9227-5

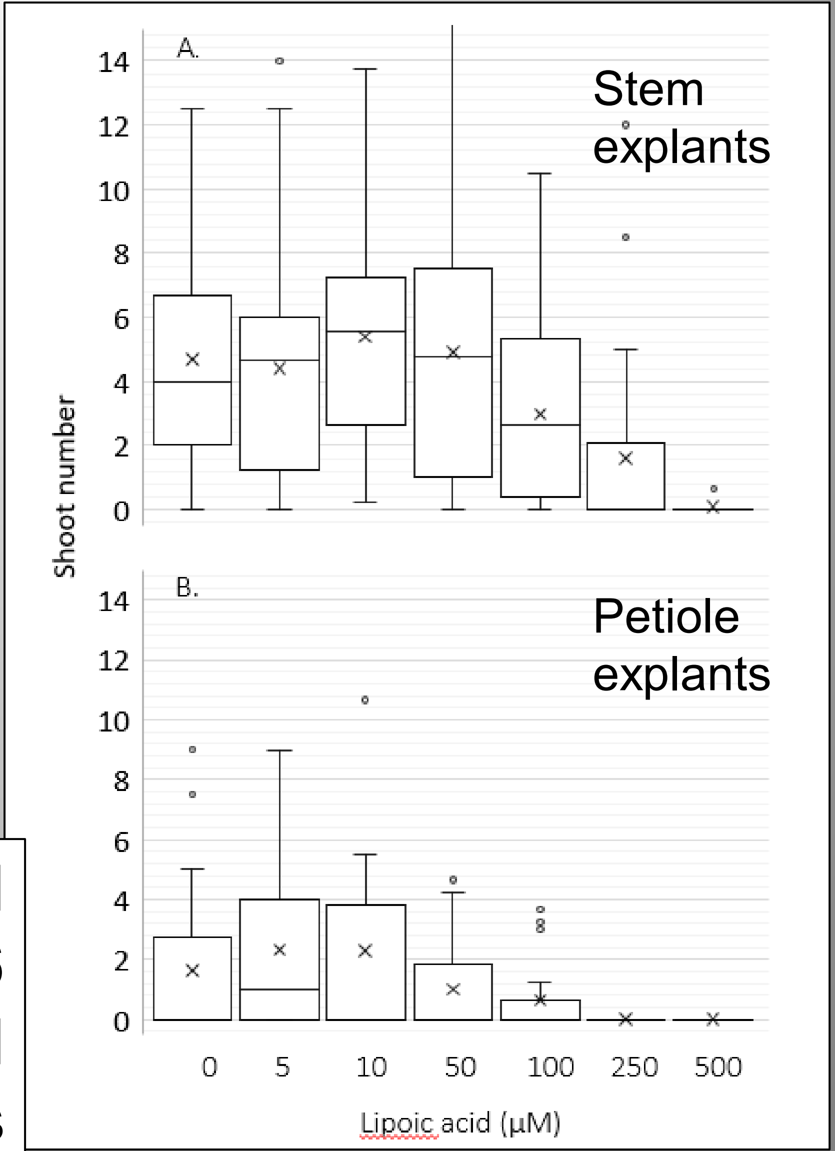
BIOTECHNOLOGY

**Lipoic acid—a unique plant transformation enhancer**

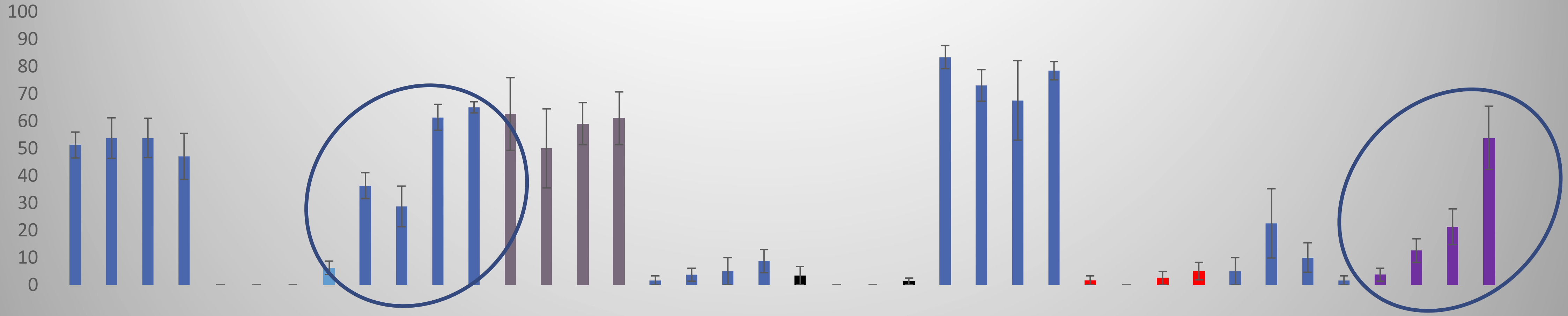
Yinghui Dan · Charles L. Armstrong · Jimmy Dong · Xiaorong Feng · Joyce E. Fry ·
Greg E. Keithly · Brian J. Martinell · Gail A. Roberts · Lori A. Smith ·
Lalaine J. Tan · David R. Duncan



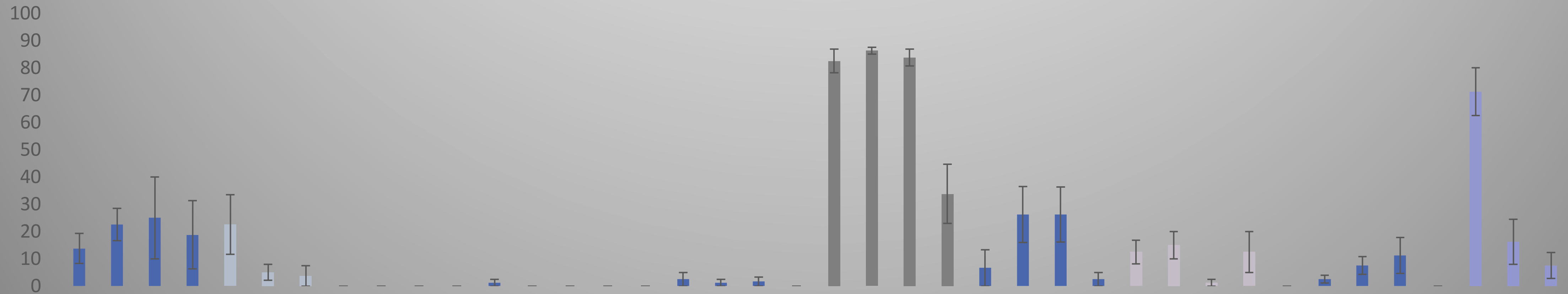
Averaged over 16 wild genotypes



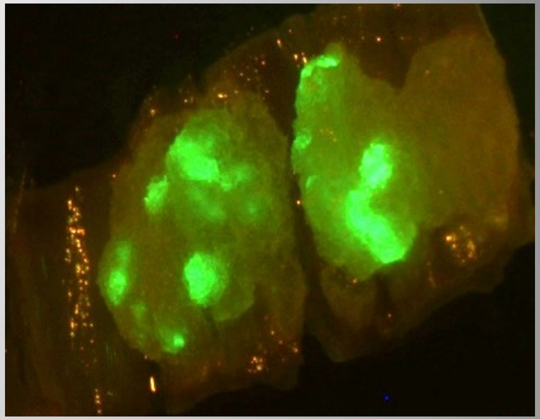
# LA promoted shoot formation in minority of 20 genotypes (0, 5, 10, 50 uM)

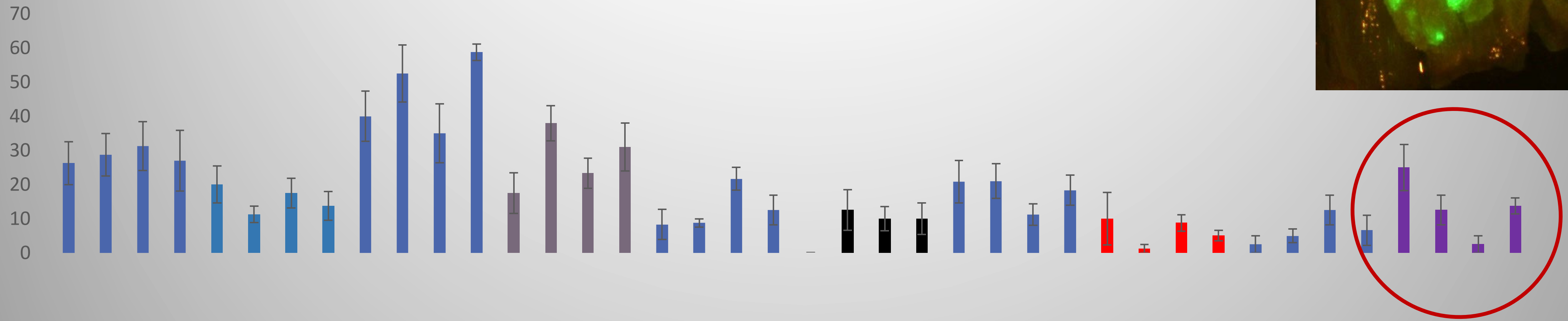


% explants forming shoots

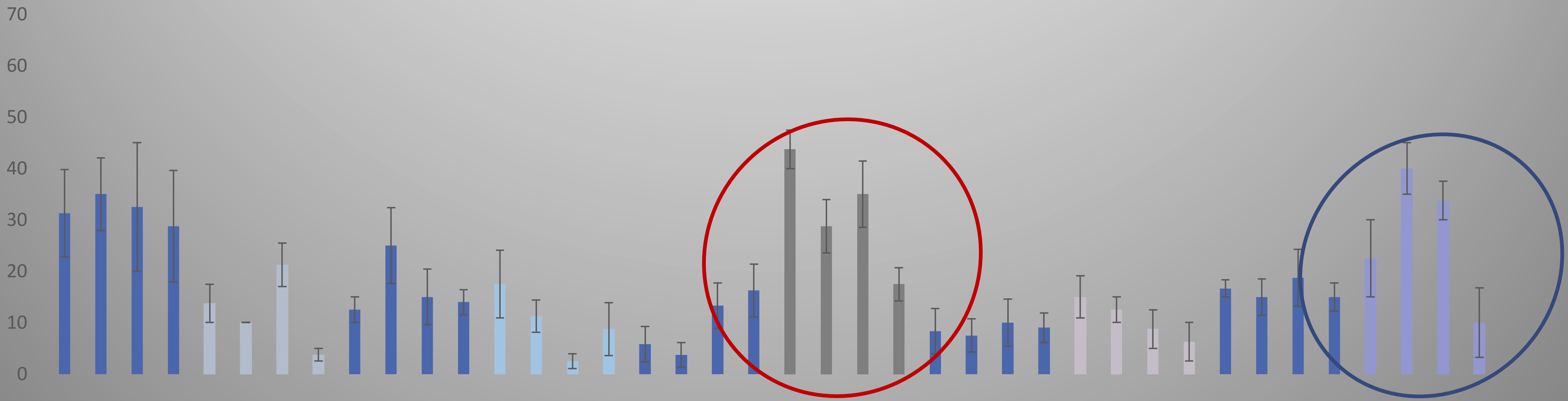


# LA effect on transformation more complex, also varied widely among genotypes



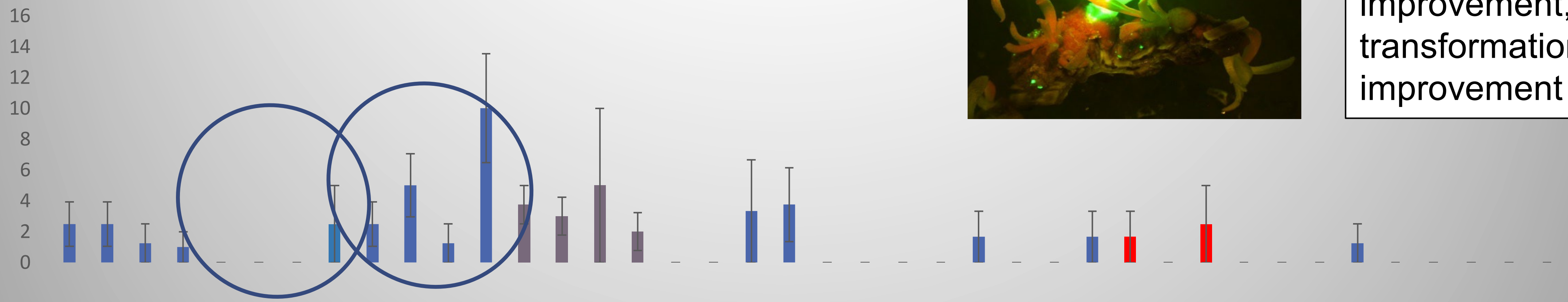


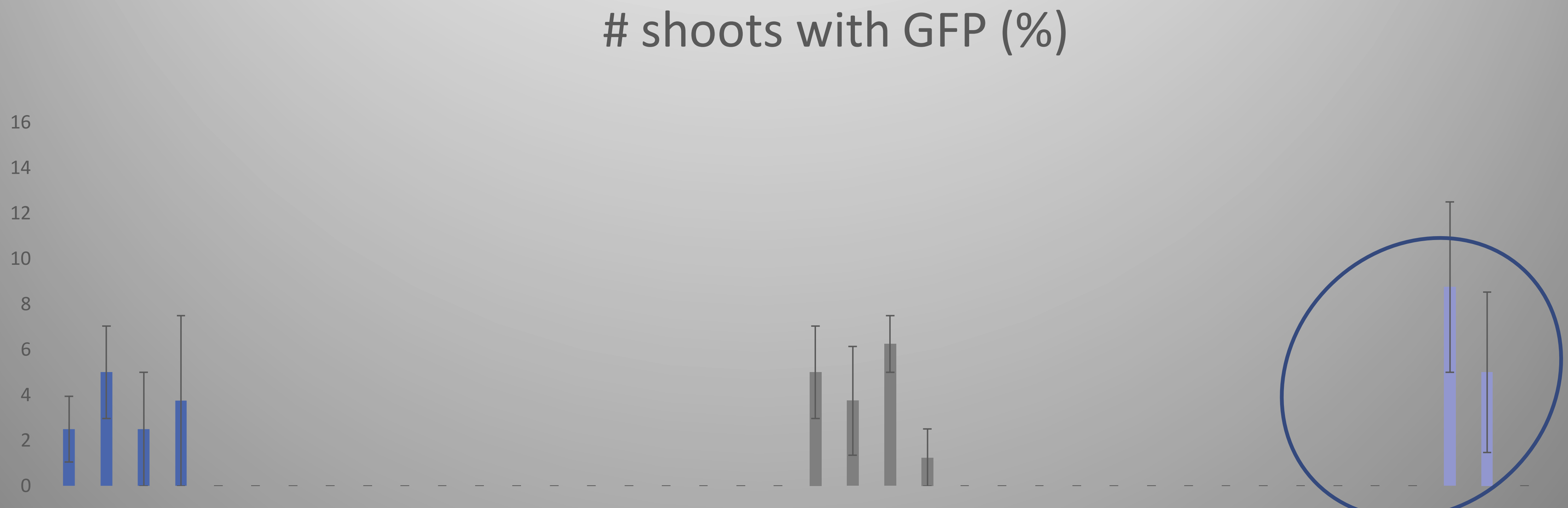
% explants with GFP



# Transgenic shoot rate much lower, LA effect complex

<u>In the 8 best responding genotypes</u>: 5 uM LA gave >420% regen improvement, >79% transformation improvement

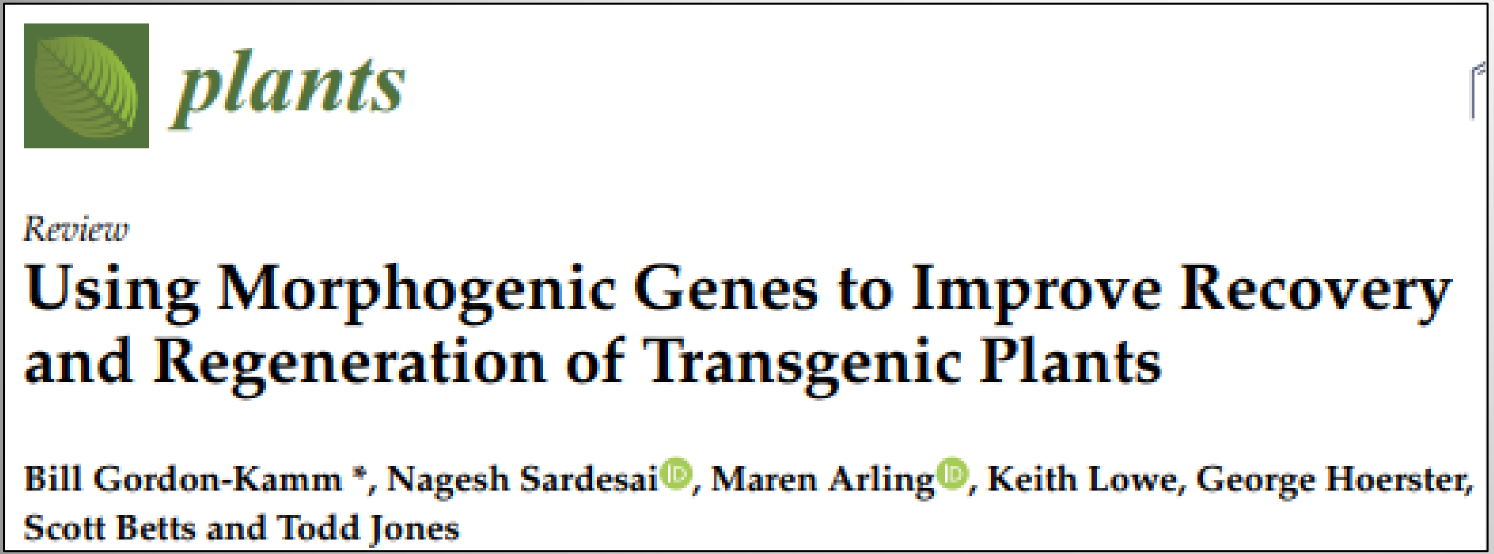




# Overall, LA of small, variable benefit

- We use 5 uM in all our cottonwood transformations
- Low enough to not hurt, and for a minority it helps
- But not much of a tool to promote genotype-independent regeneration or transformation

# 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

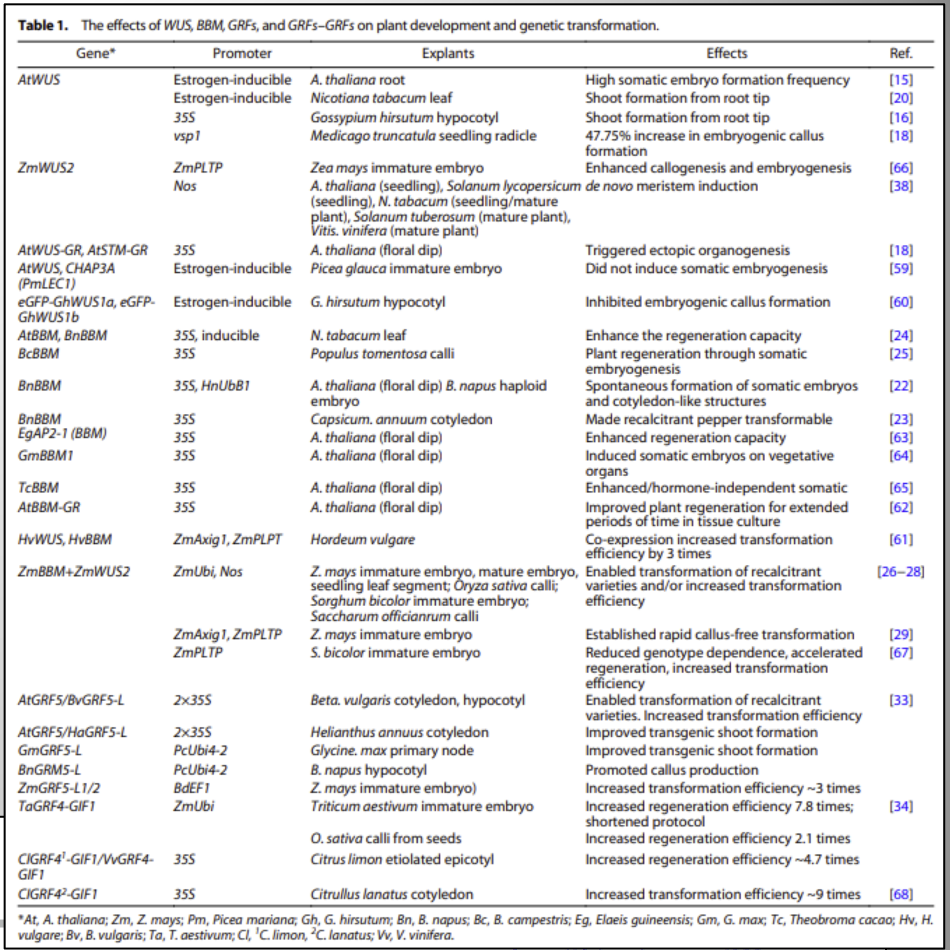
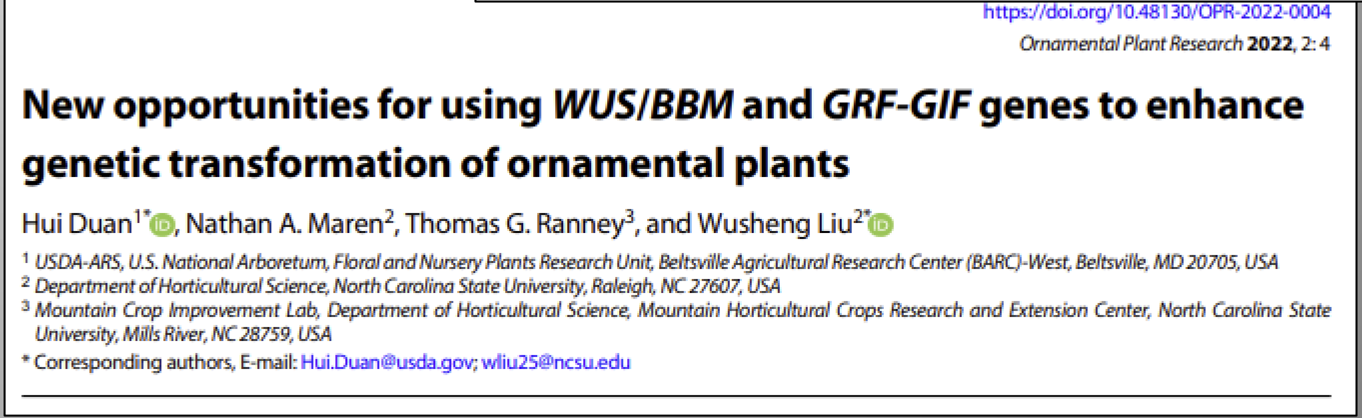


**Table 1.** The effects of *WUS*, *BBM*, *GRFs*, and *GRFs–GRFs* on plant development and genetic transformation.

| 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.*



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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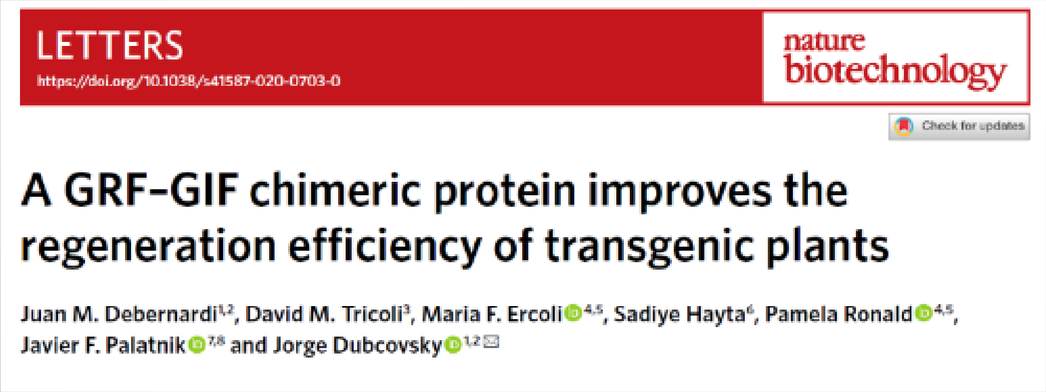
# Types of DEV genes we have studied in poplars or eucalypts

- *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
- *iaaH/iaaM* (auxin) – Agrobacterium
- *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

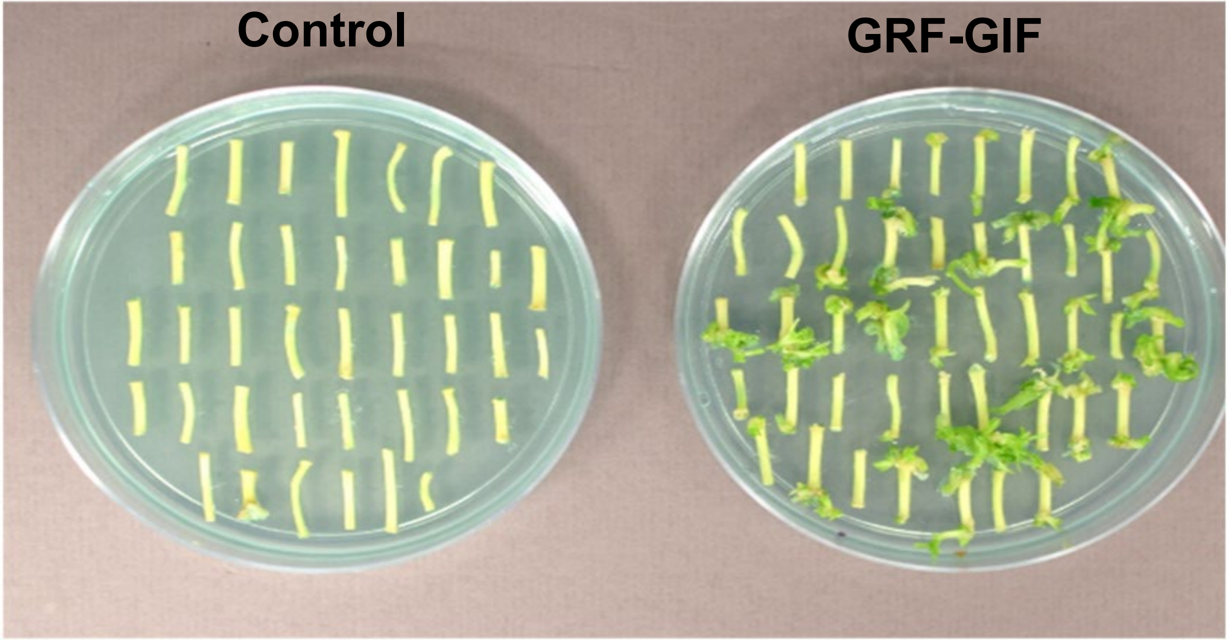
https://doi.org/10.1038/s41587-020-0703-0

nature biotechnology

Check for updates

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

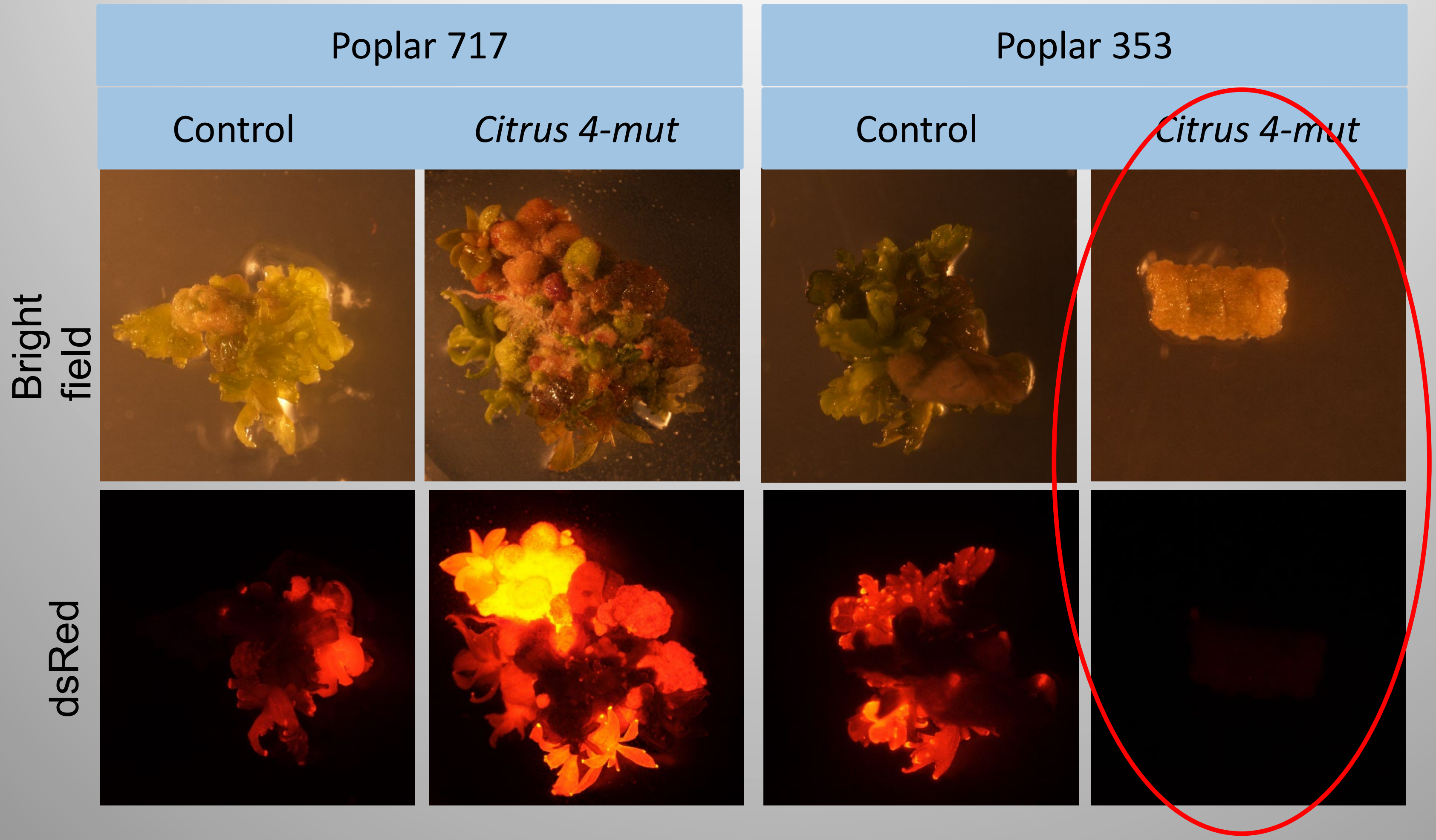
Juan M. Debernardi[1,2], David M. Tricoli[3], Maria F. Ercoli[4,5], Sadiye Hayta[6], Pamela Ronald[4,5], Javier F. Palatnik[7,8] and Jorge Dubcovsky[1,2] ✉



Citrus epicotyl explants; Debernardi et al., 2020

**Studying a wide variety of GRF-GIF homologs & sources, promoters, and miRNA sensitivities**

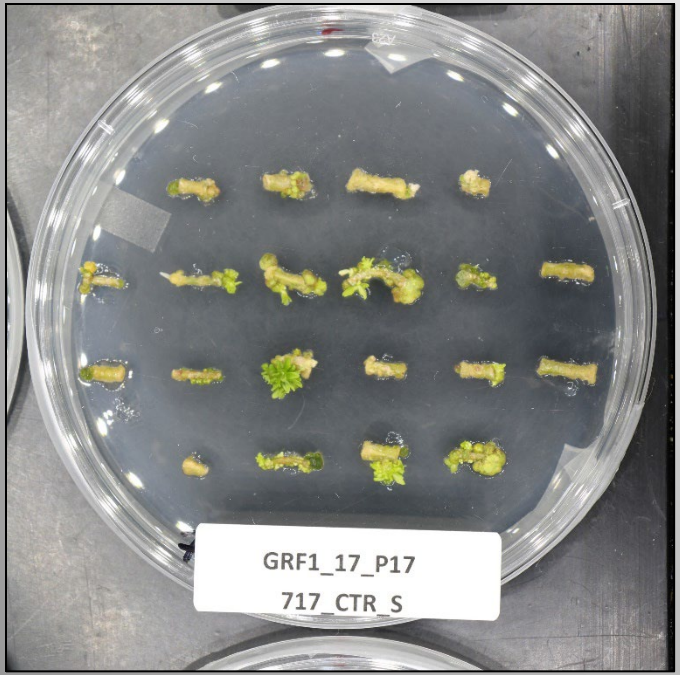
# Two poplar genotypes displayed very different callus responses to *Citrus 4-mut* GRF-GIF overexpression



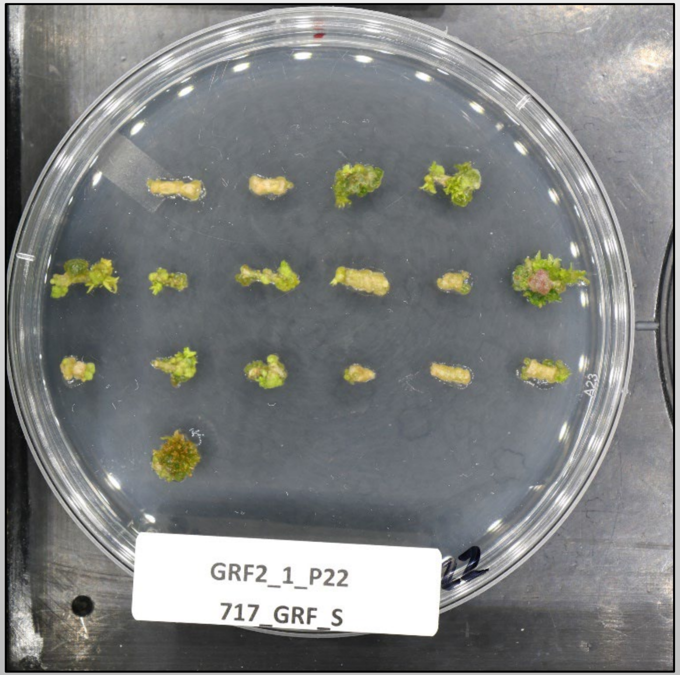
**717=Populus tremula x alba / 353 = P. tremula x tremuloides**

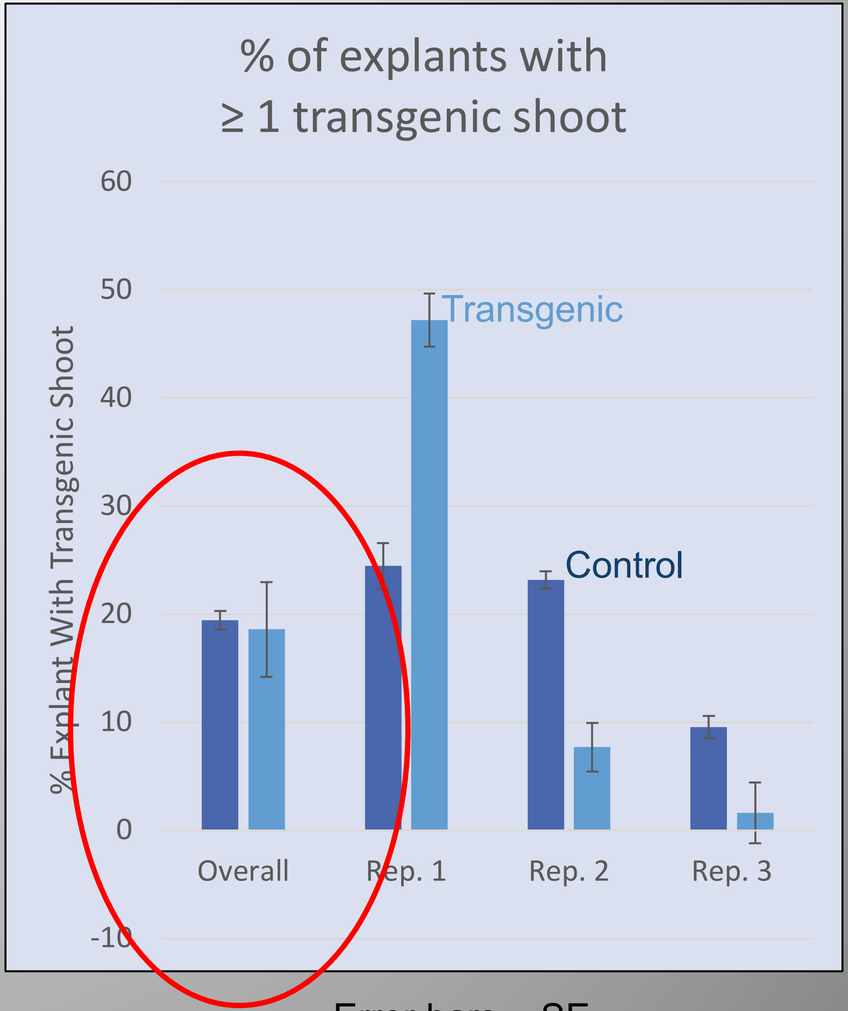
# *Citrus 4-mut* GRF-GIF had little overall effect on shoot formation in poplar clone 717

**Control**



**GRF-GIF**





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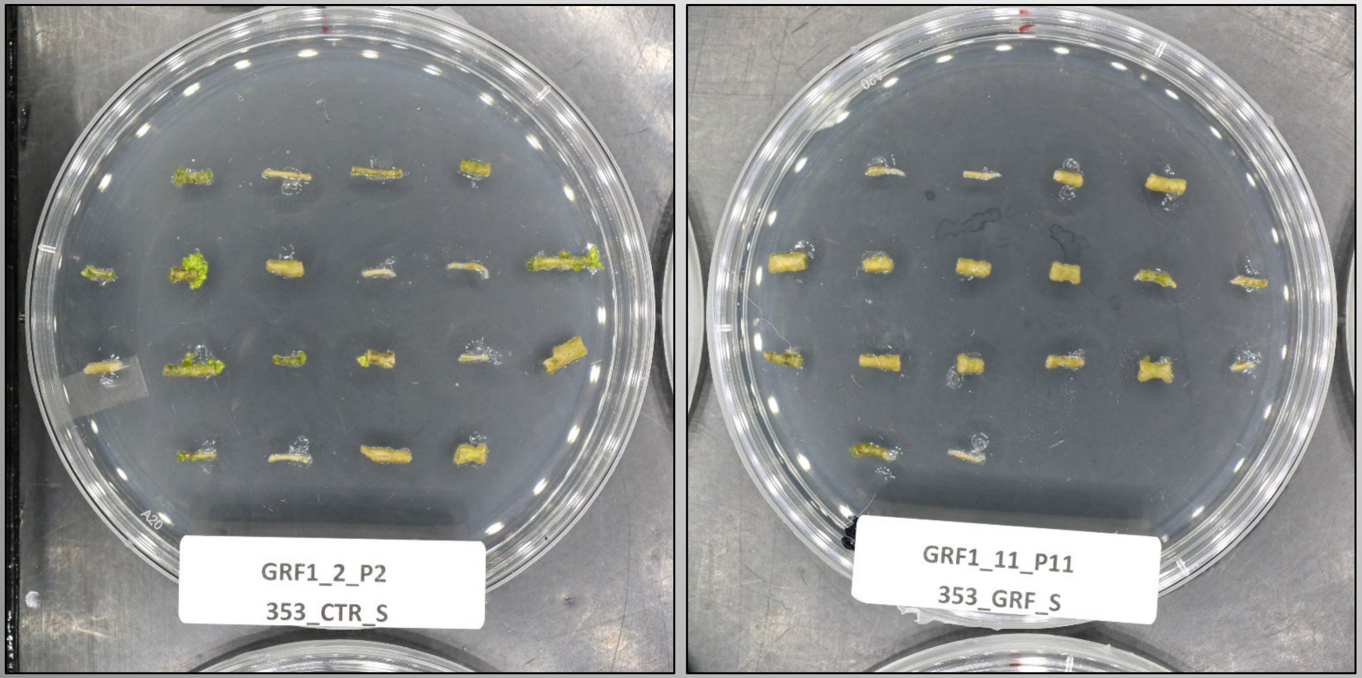
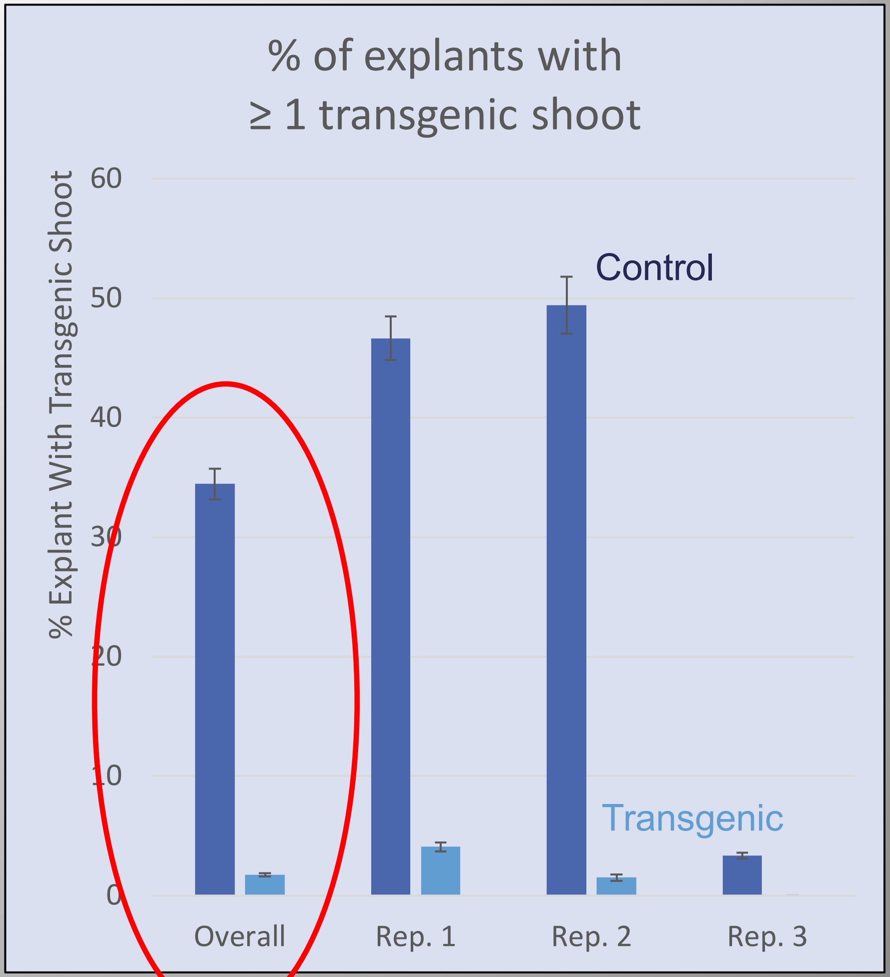
# But *Citrus 4-mut* GRF-GIF strongly inhibited shoot formation in poplar clone 353



**5 weeks post-transformation**

**353 Control**

**353 GRF-GIF**



% of explants with ≥ 1 transgenic shoot

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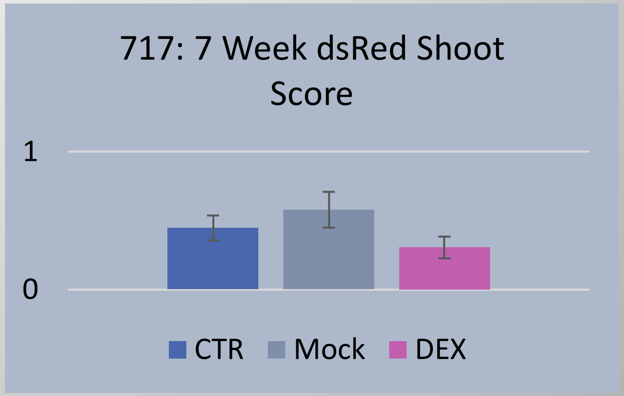
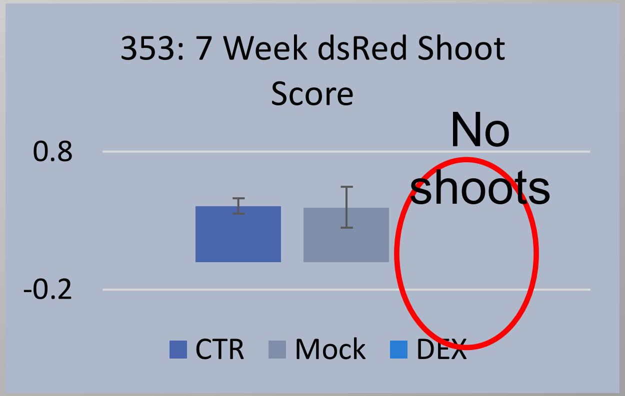
# Dexamethasone-inducible GRF-GIF also worsened shoot regeneration in 353 poplar

Construct also provided by Juan Debernardi / Jorge Dubcovsky of UC Davis

= dsRed positive shoot





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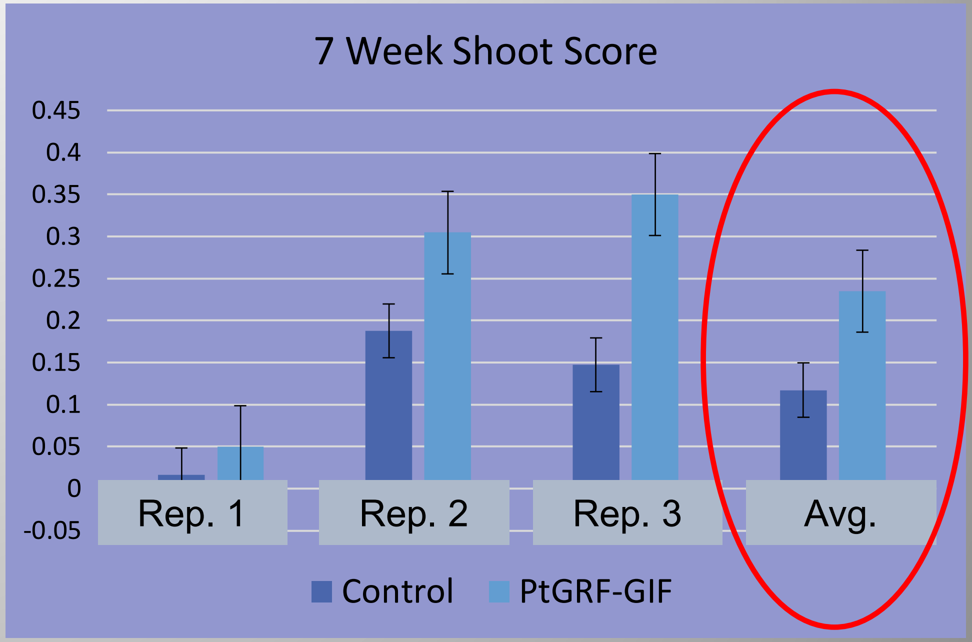
# An ortholog of GRF-GIF from *Populus* doubled shoot regeneration in poplar 717

**Control**

**1x35S::GRF4-GIF1**

○ = dsRed positive shoot



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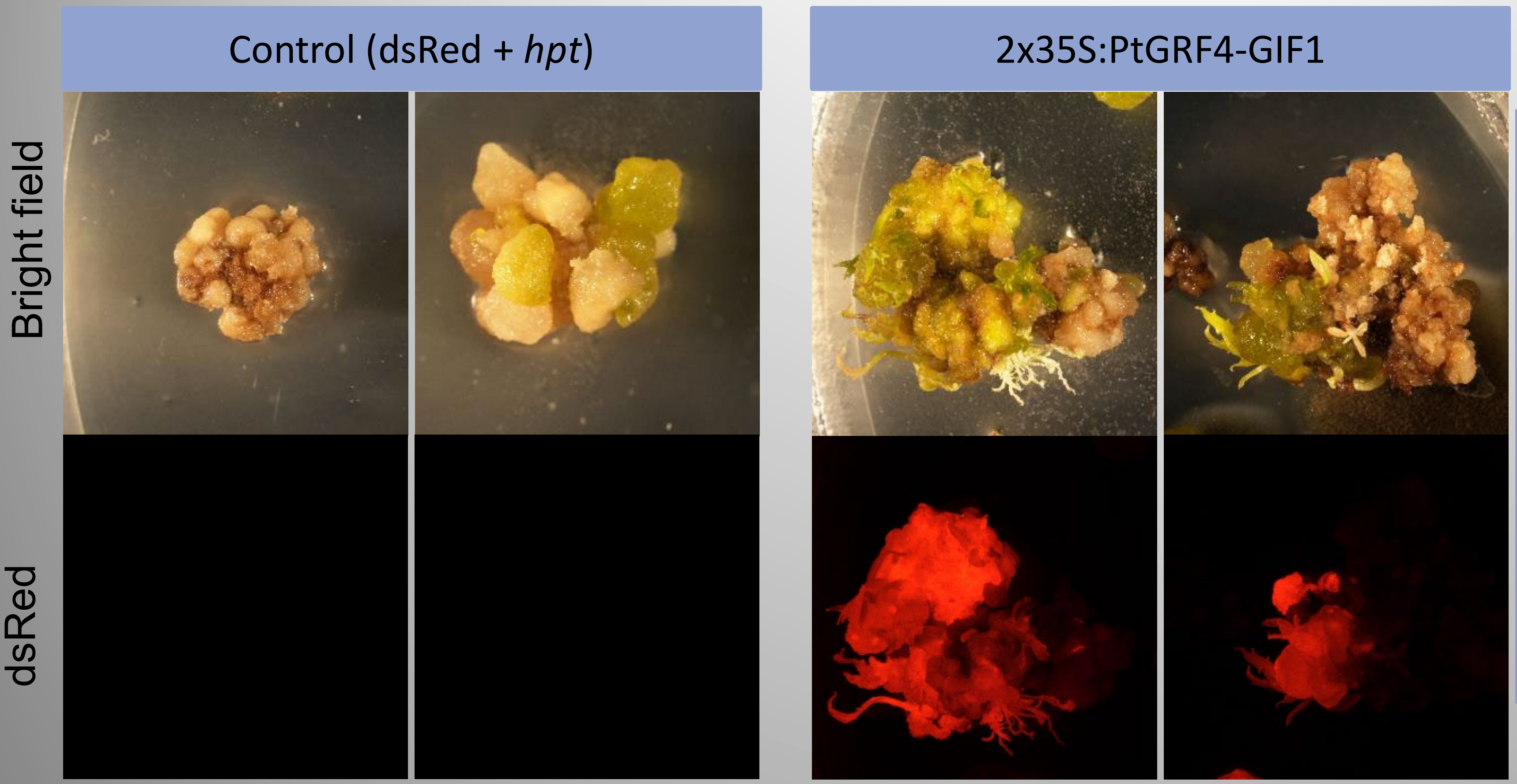
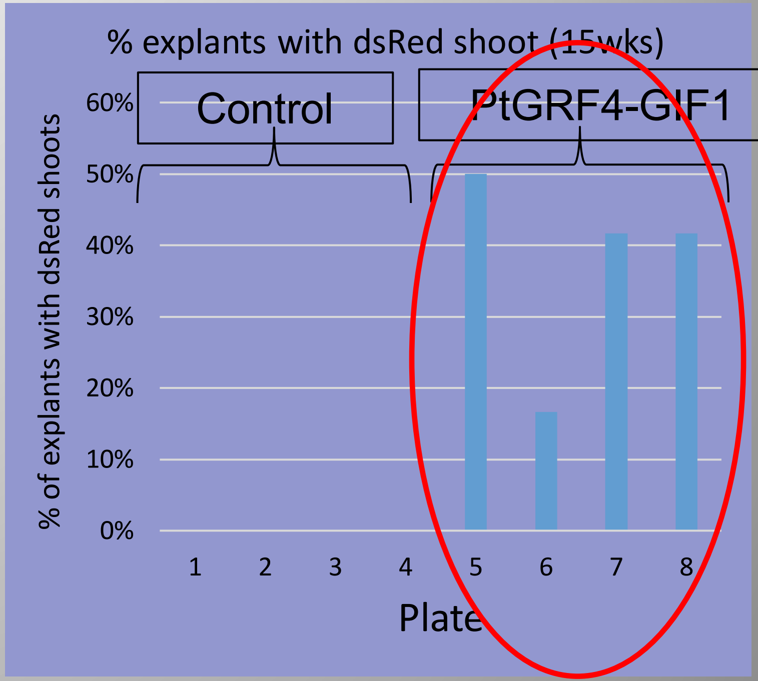
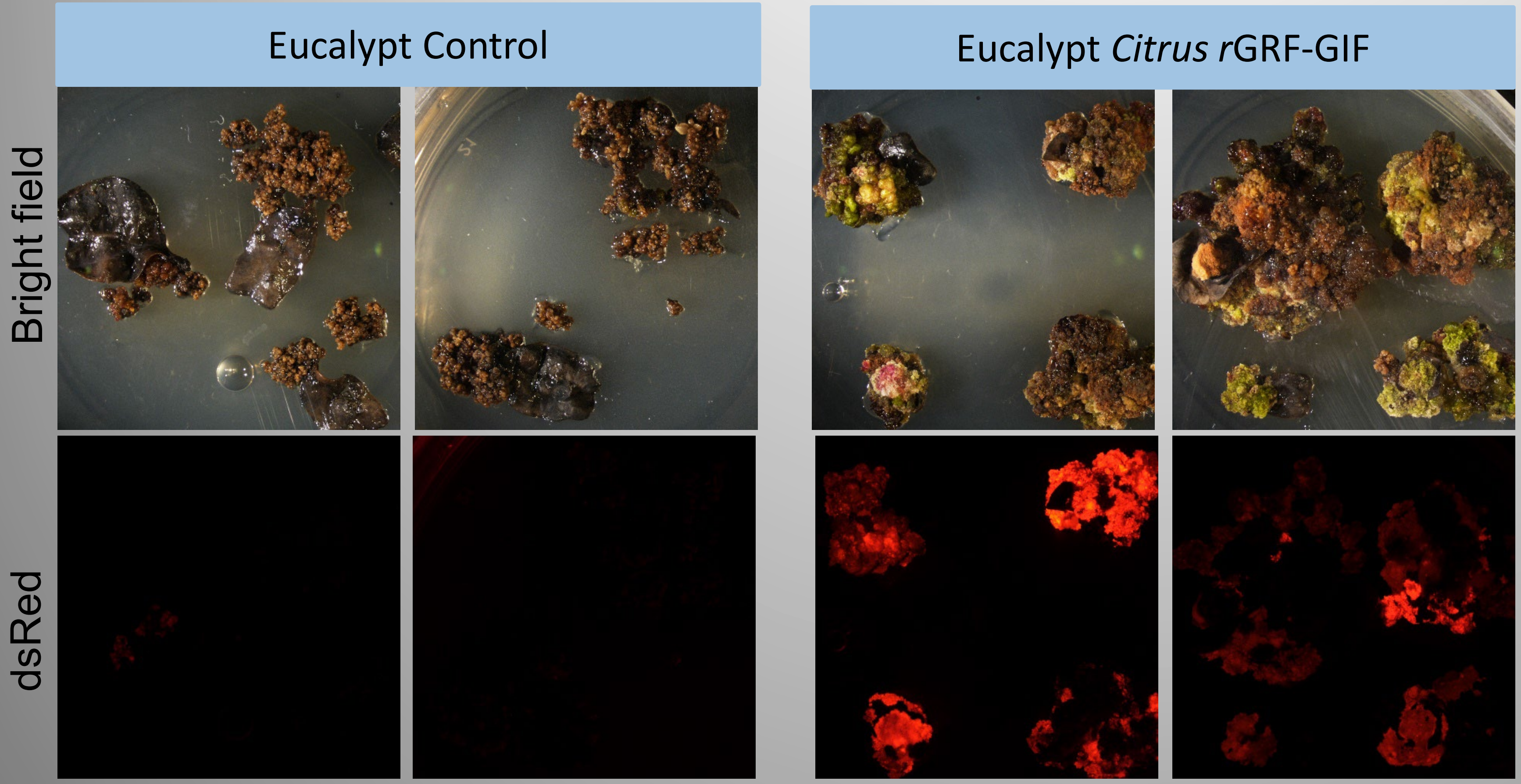
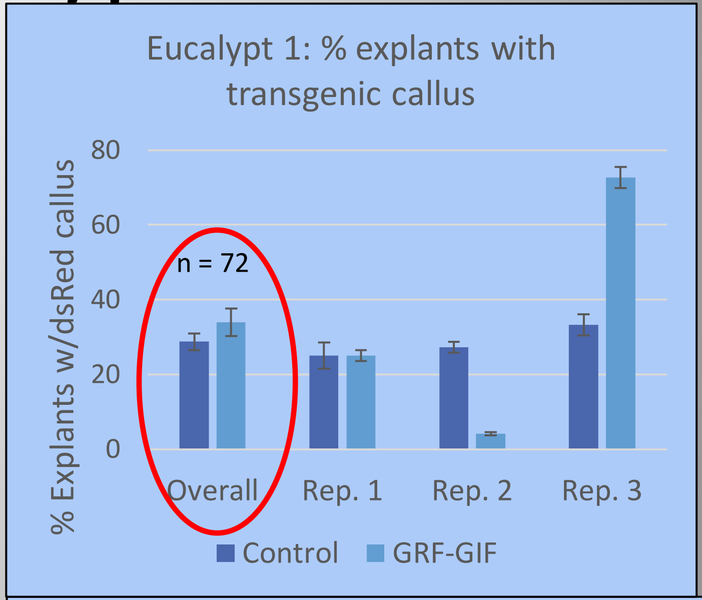
# *Populus* GRF-GIF also promoted shoot regeneration in recalcitrant *P. alba* clone ‘6K10’

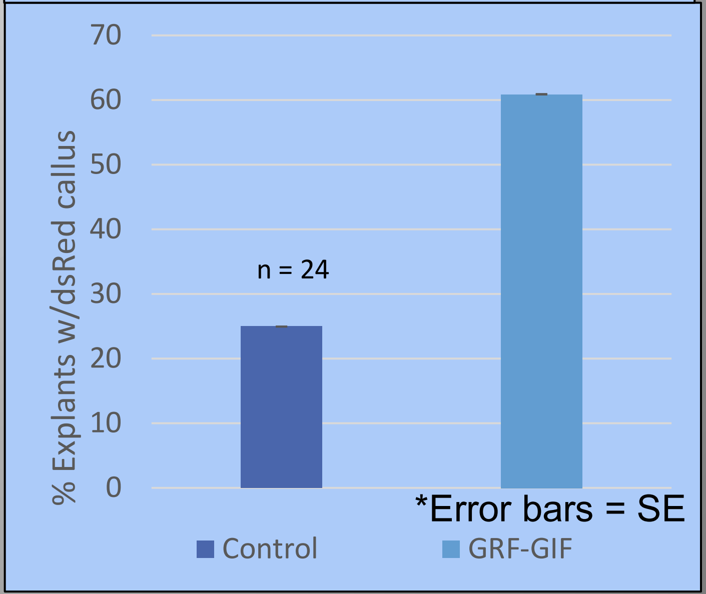




# *Citrus* miRNA insensitive GRF-GIF increased transgenic callus occurrence in some eucalypt genotypes







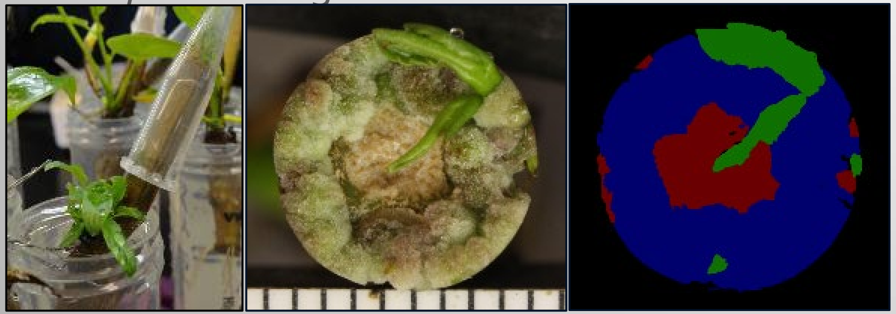
*Error bars = SE

# DEV genes to date

- Studied several, searching for what might work consistently
- Most without effect or suppress regeneration
- GRF-GIF encouraging, but with extremely high genotype x gene interaction
  - Native forms show most promise so far
- Everything needs more replication

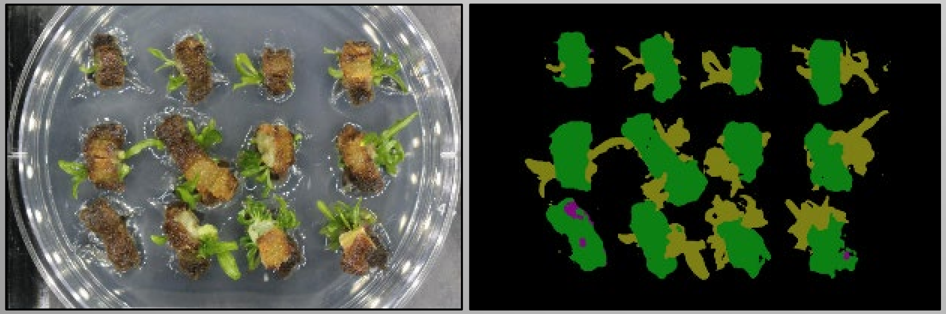
# GWAS to discover developmental genes in *P. trichocarpa:* Four studies, machine vision system

*1. In planta* regeneration

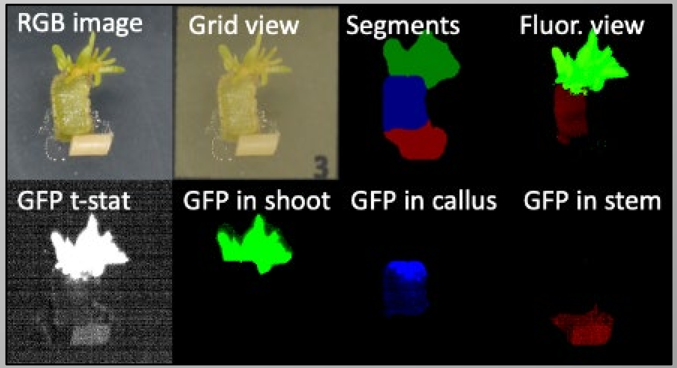


*2. In planta* rooting

*3. In vitro* regeneration

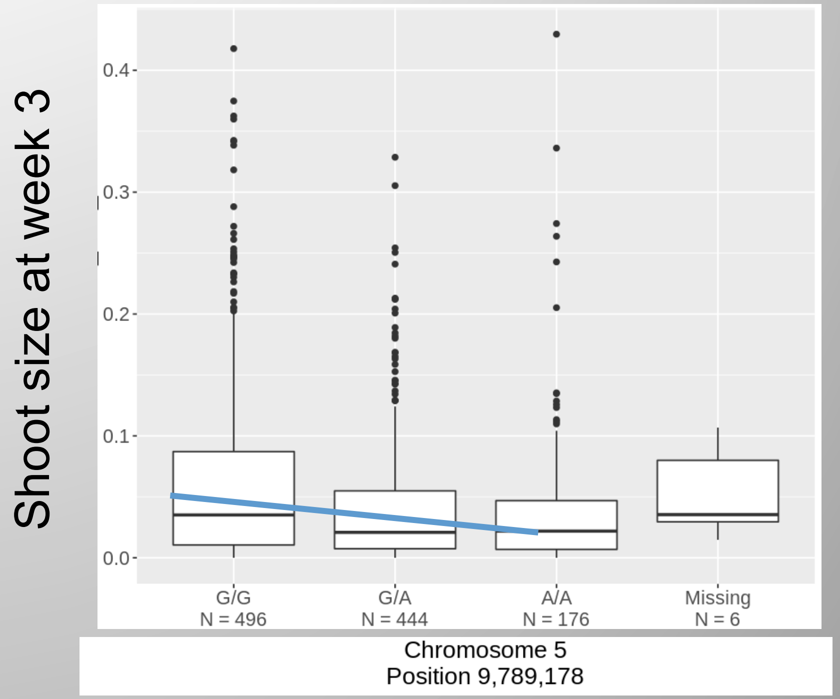


*4. In vitro* regeneration + transformation

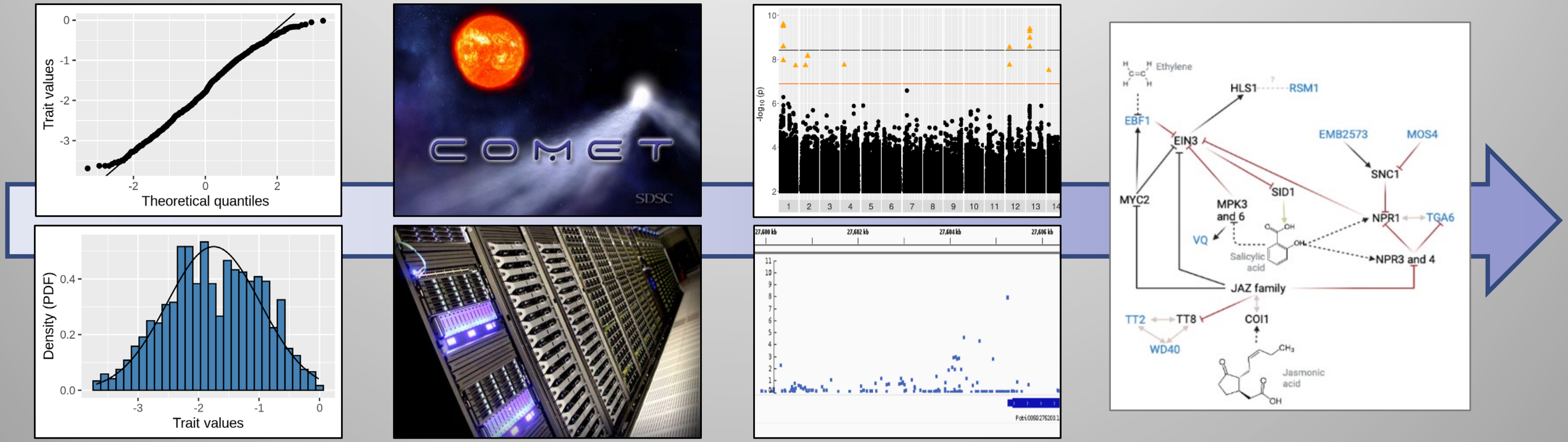


# Genome-Wide Association Studies (GWAS) uncover links between genetic markers and traits

- Genetic variation represented by single-nucleotide polymorphisms (SNPs) in and around genes
- Poplar GWAS features ~1,300 clones with over 30 million SNPs, most with low linkage disequilibrium
- GWAS uses statistical models to find significant correlations between SNPs and traits of interest



# GWAS workflow intensive: From trait data to gene candidates



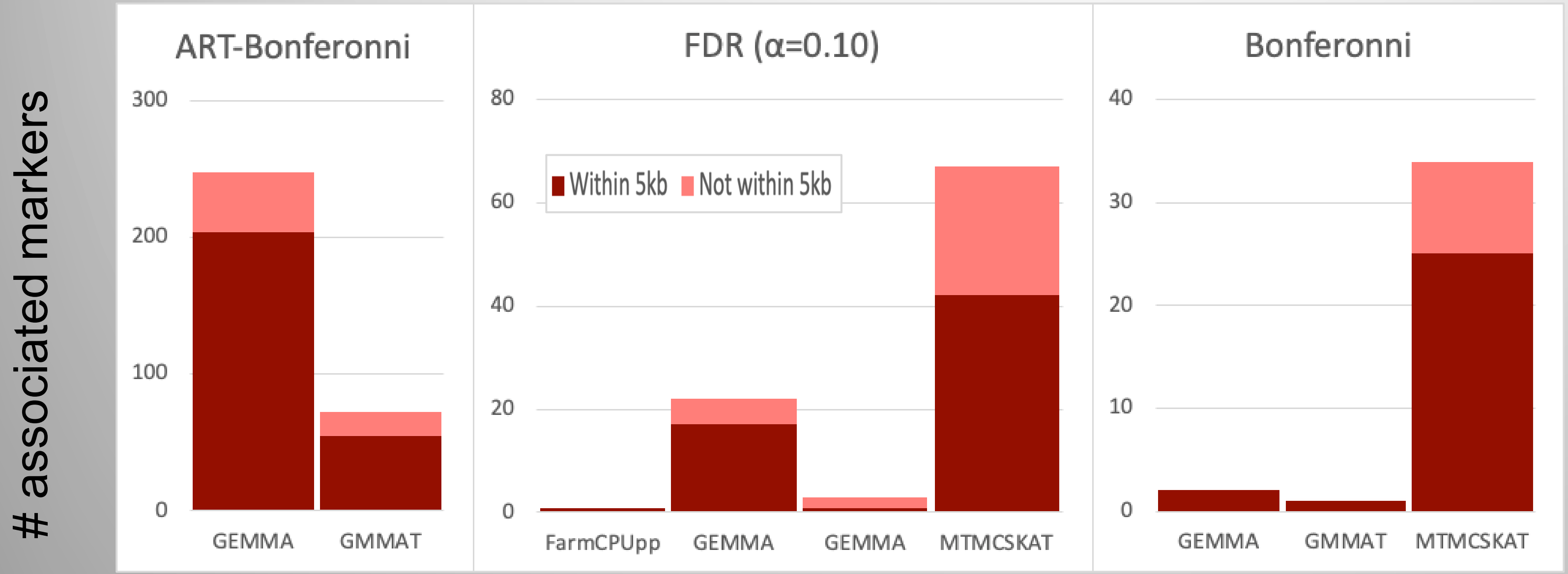
**Trait evaluation and transformations**

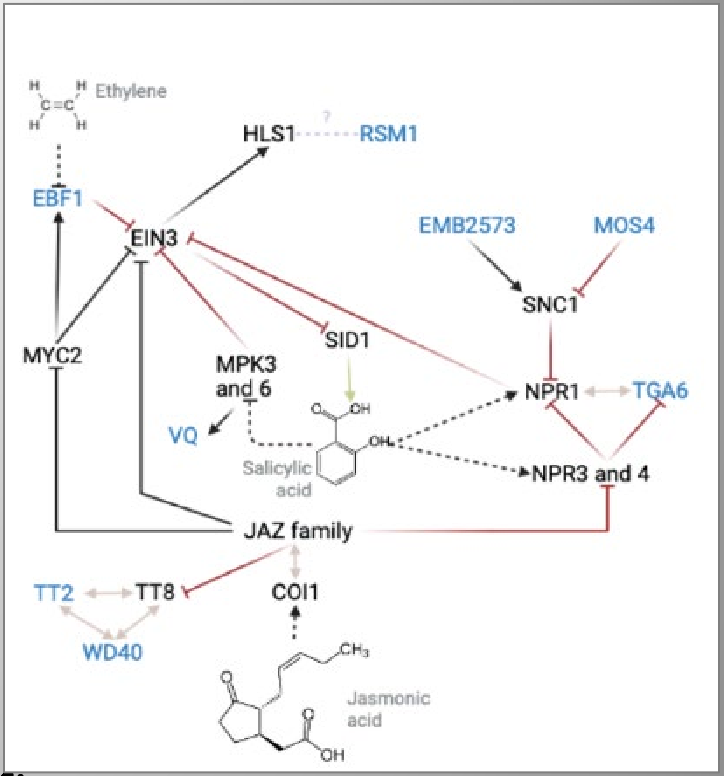
**Statistical modeling**

**Graphics, interpretation**

**Interpreting biological pathways**

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

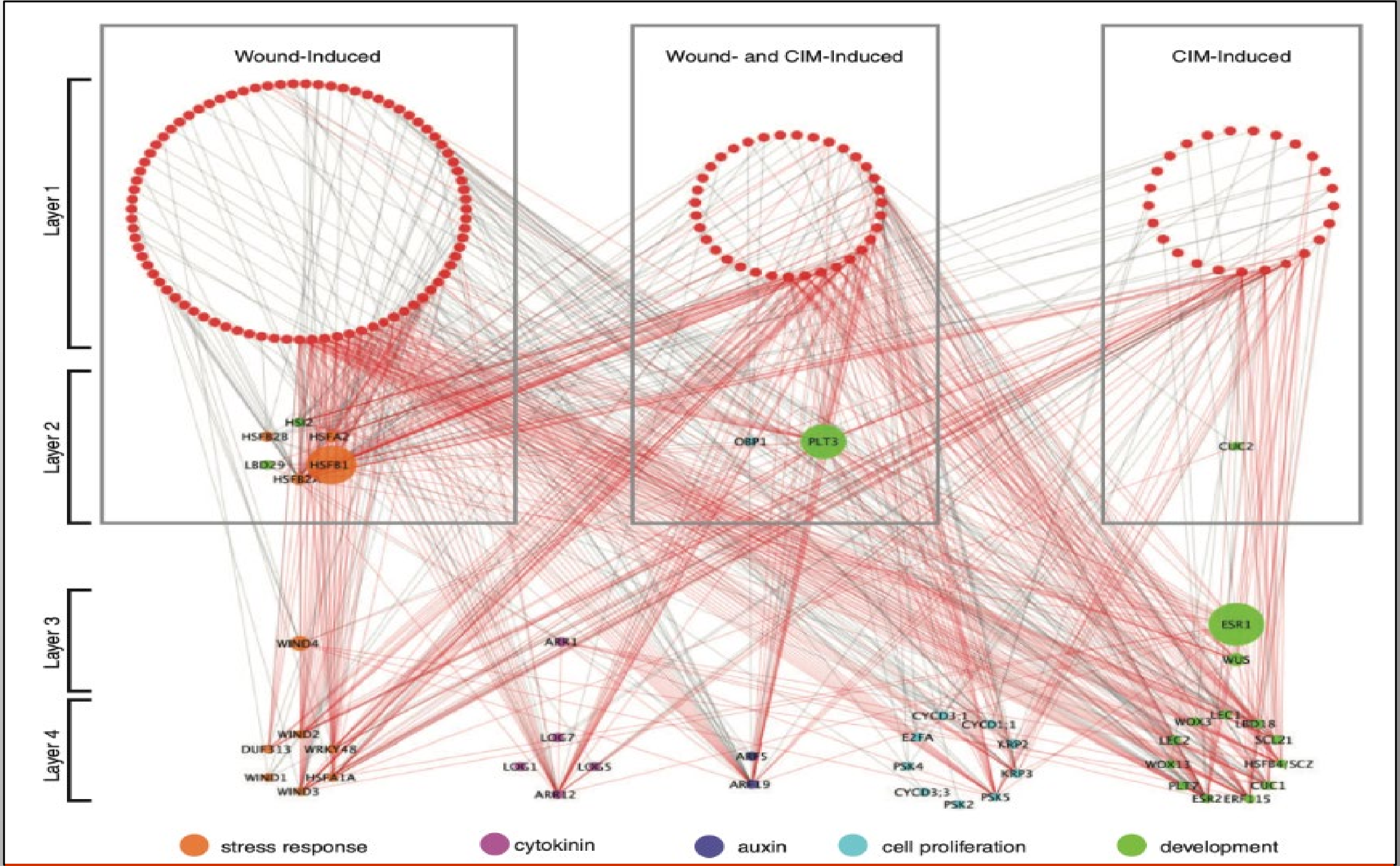




- Four statistical approaches used, three methods used to define significance
- Majority of associated markers within or near genes – in regulatory vs. coding regions
- None of the hits include any of the common DEV genes
- Genes marked by SNPs identified important pathways

# GWAS message

- Many genes and options yet to be explored for transformation enhancement ?
- Highly diverse taxa, genotypes, and regeneration systems



A Gene Regulatory Network for Cellular Reprogramming in Plant Regeneration” – Ikeuchi et al. 2012

# Summary

- Woody species, mature-clonal tissues, are tough
- There is *major league* genetic diversity in response to most everything we try – media amendments and DEV genes
- DEV genes require subtlety in which forms, how expressed, how controlled, and for what genotypes – so far not a step toward a genotype-independent method
- Genotype independent transformation systems?
  *Is the future instead to use genomic analysis to guide and abbreviate the customization process?*

# Thanks to our funders and collaborators

USDA
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Agriculture

NSF
The National
Science Foundation

<u>GREAT TREES Consortium</u>
Suzano, SAPPI, Arauco, Klabin, SweTree,
Corteva Agriscience

Juan Debernardi and Jorge Dubcovsky
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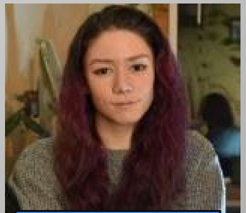
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