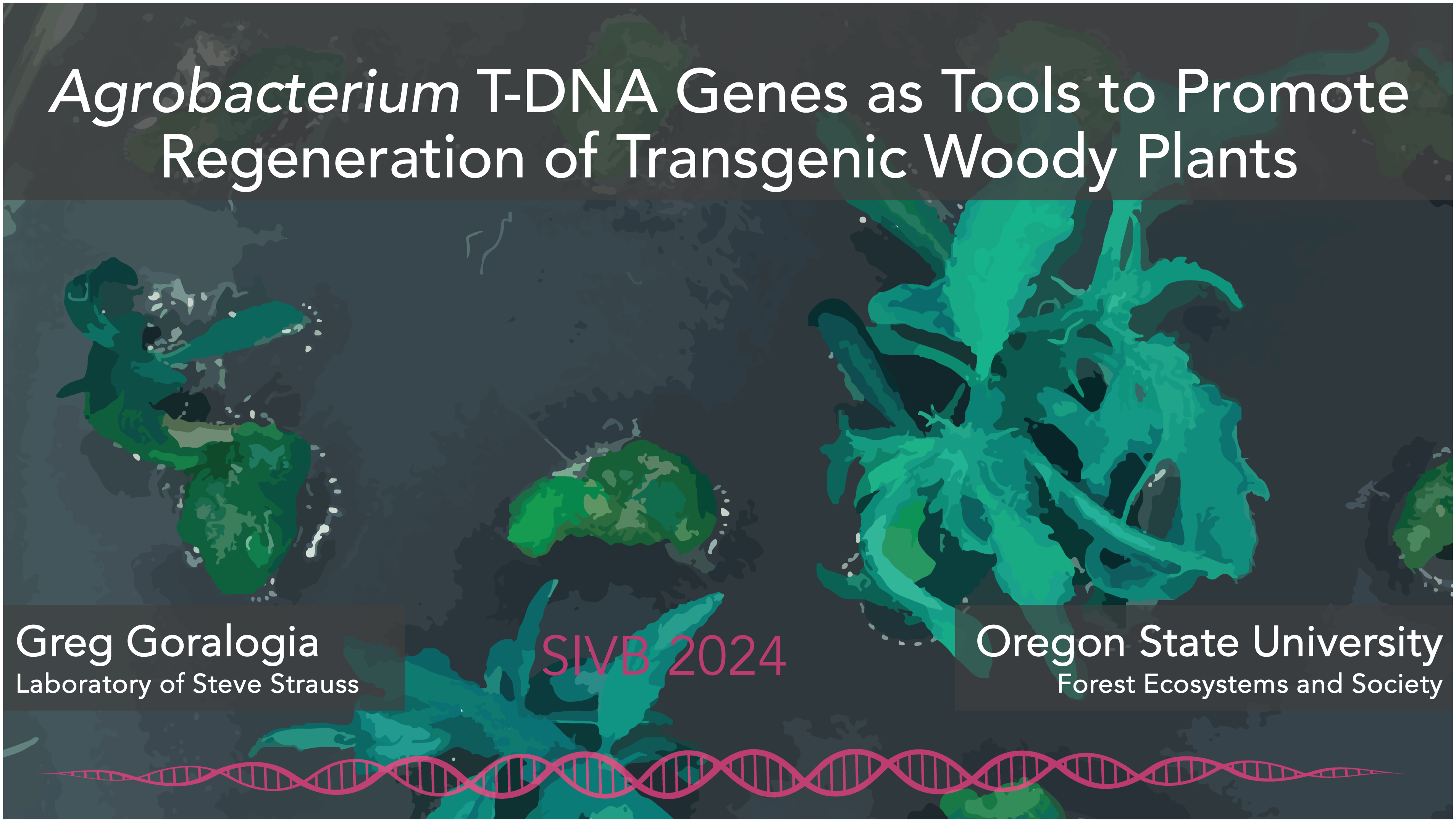


Agrobacterium T-DNA Genes as Tools to Promote Regeneration of Transgenic Woody Plants

Greg Goralogia
Laboratory of Steve Strauss

SIVB 2024

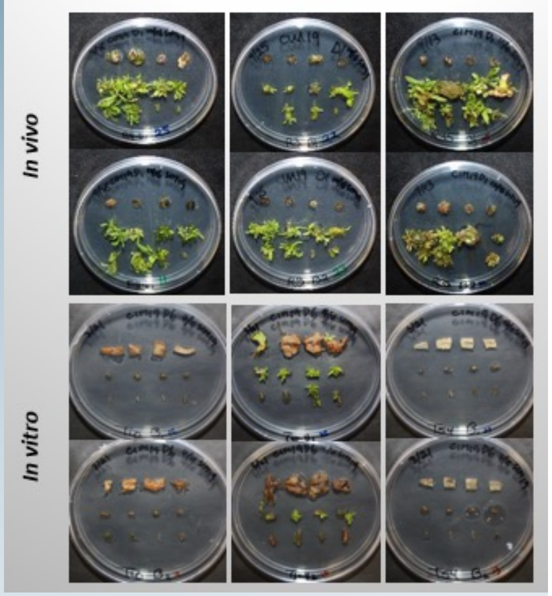
Oregon State University
Forest Ecosystems and Society

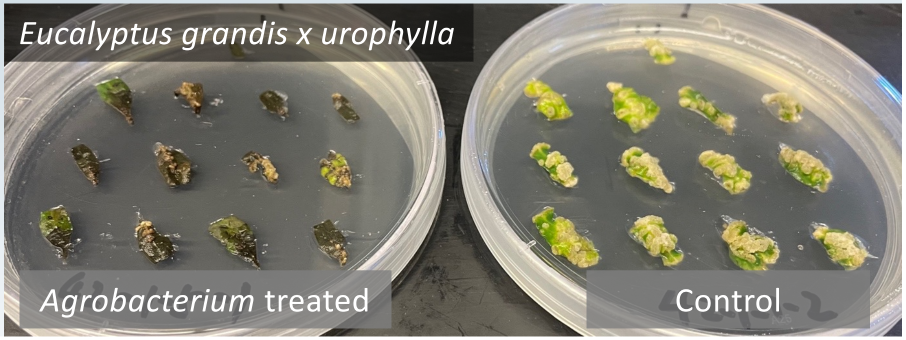
Regeneration and transformation continue to be major limiting factors for gene editing and engineering in plants

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

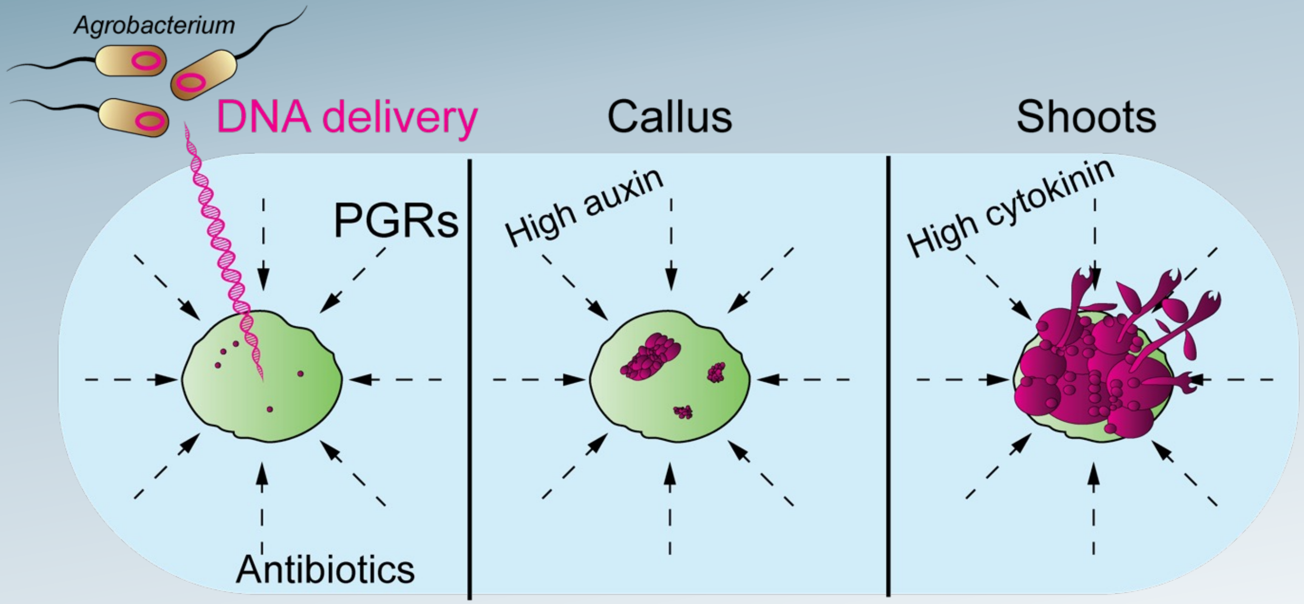
Many tree species are difficult to transform

- 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

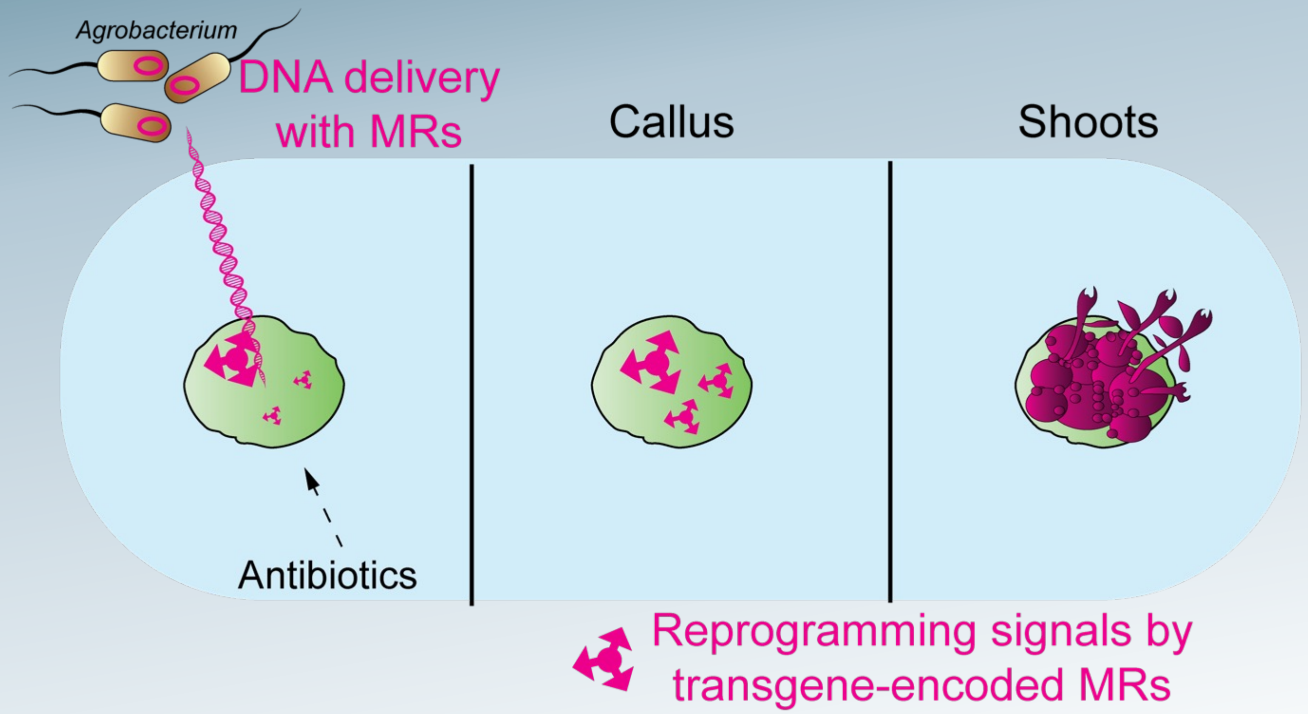




Conventional transformation methods rely on exogenous phytohormones supplied in the culture medium



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



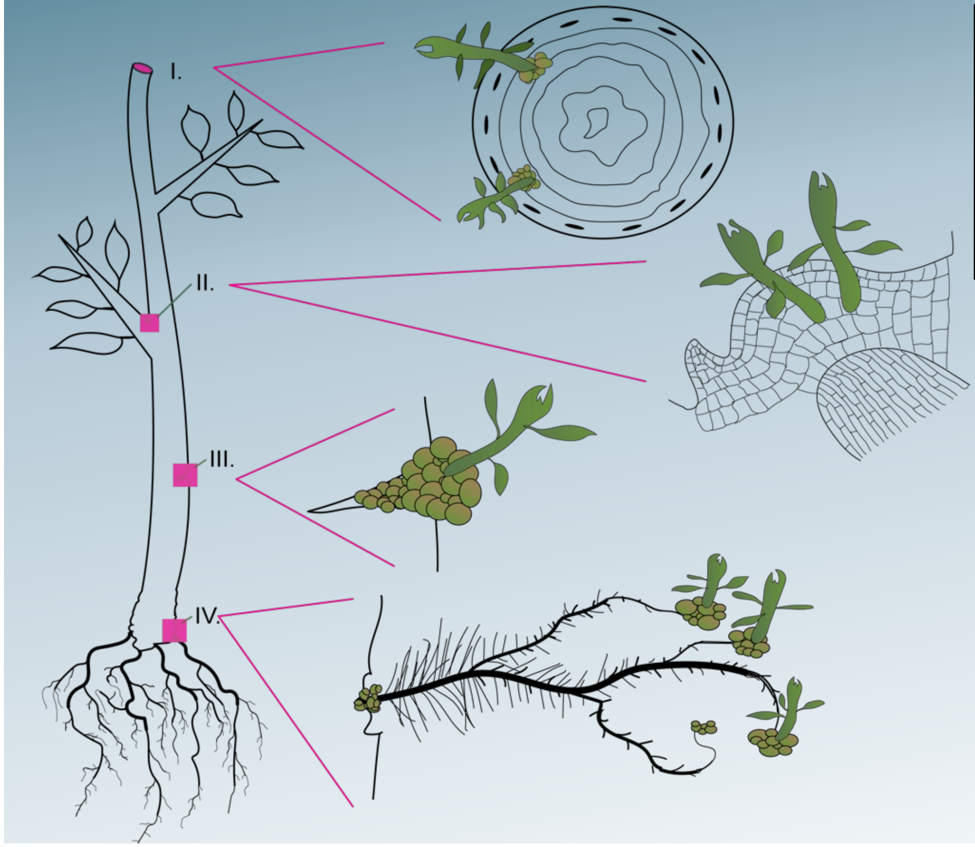
Types of MR genes we have studied in poplars or eucalypts – many both *in vitro* and *in planta*

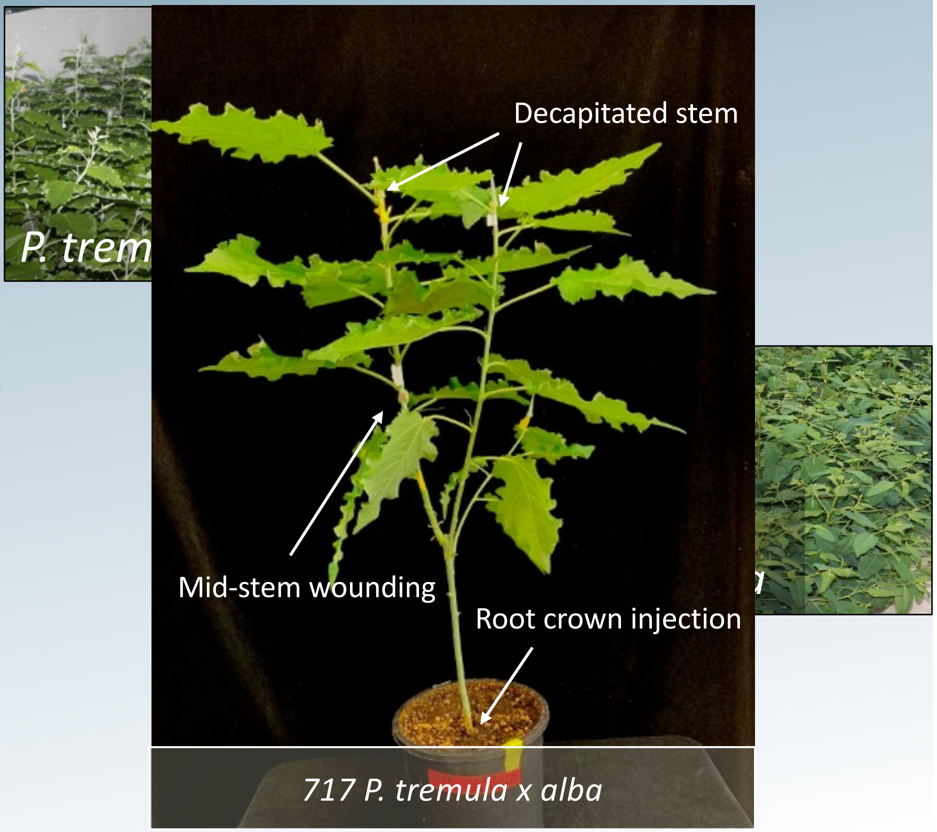
- LEC 1, 2 – LEAFY COTYLEDON
- EBB1 - EARLY BUD BREAK 1 (ESR family)
- BBM – BABY BOOM
- WOX 5, 11 -- WUSCHEL RELATED HOMEOBOX
- WUS – WUSCHEL
- GRF-GIF – GROWTH REGULATOR FACTOR 4 and GRF INTERACTING FACTOR 1
- *Agrobacterium* growth promoting genes
- rol – Hairy root-inducing genes – *Agrobacterium*

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_**

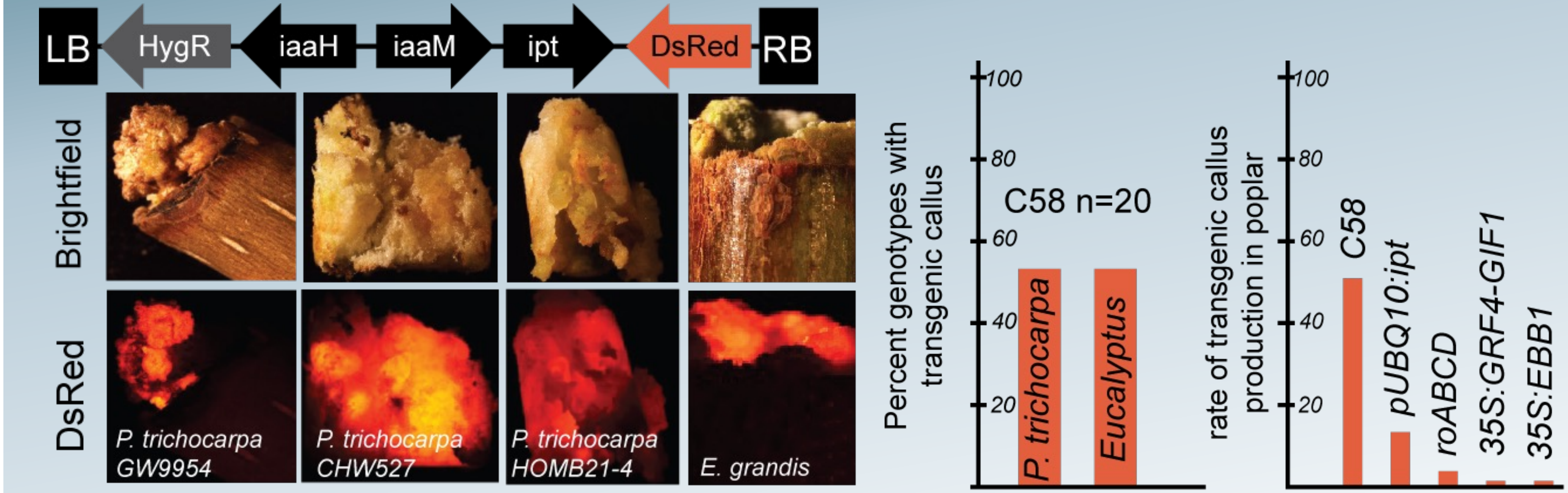
To try to sidestep tissue culture barriers, we experimented with i*n planta* transformation on greenhouse plants





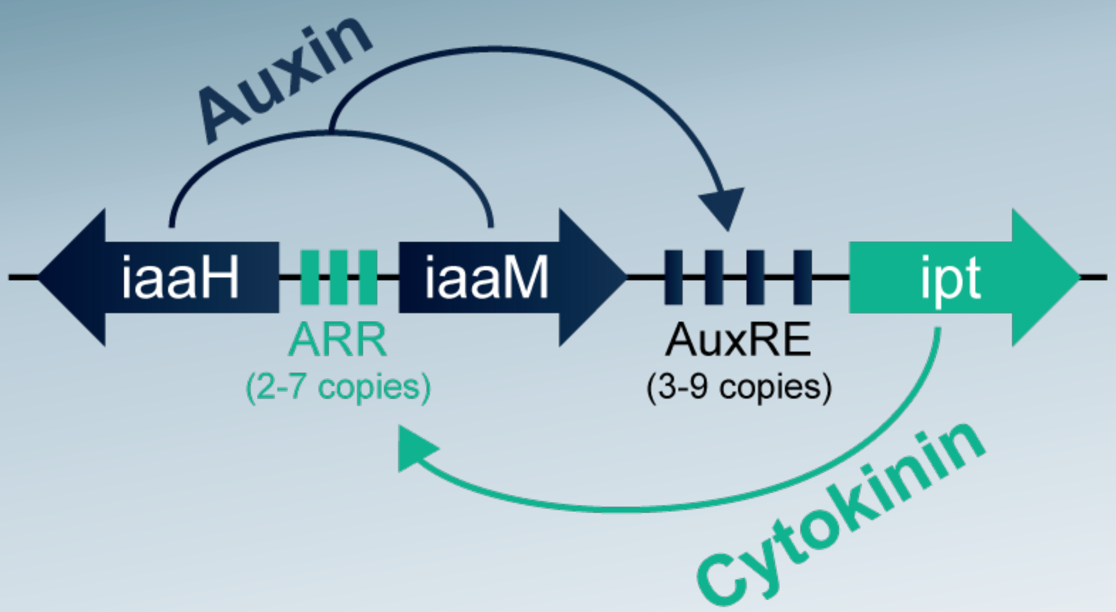
717 P. tremula x alba

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

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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

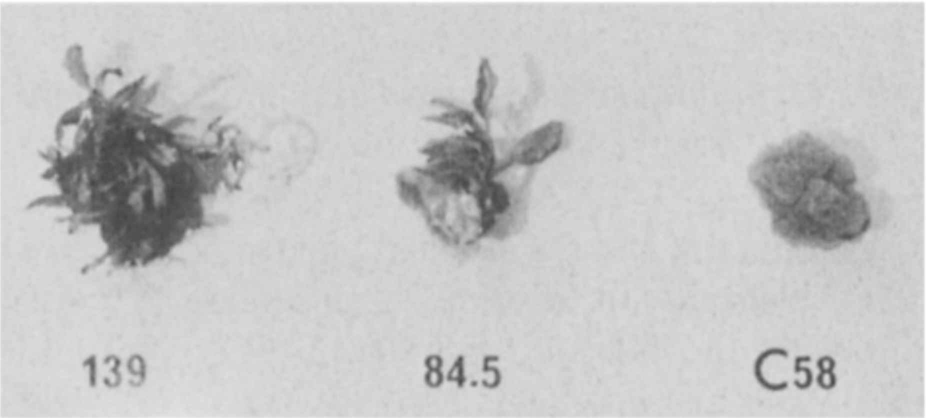
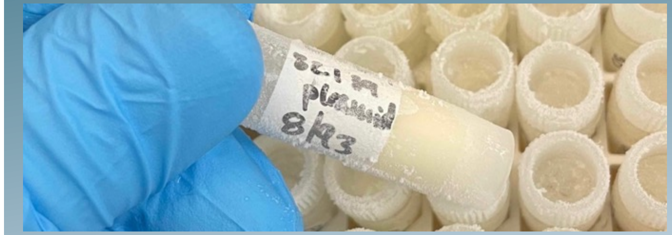


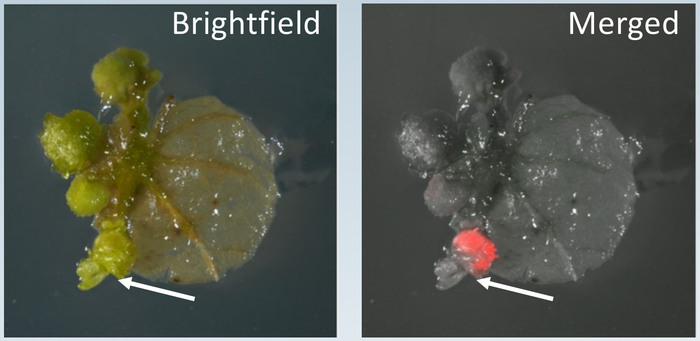
Fig. 1. Tumors and shoot differentiation from poplar tumors induced by *A. tumefaciens* strains 82.139, 84.5 and C58 and cultivated on MS medium, 6 weeks after inoculation.

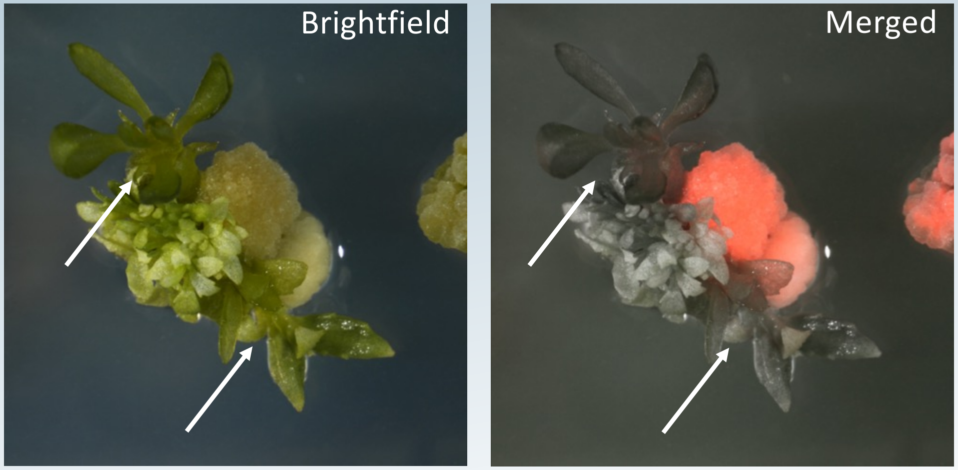
Though very promising, this work essentially came to a halt – due to GMO pushback in Europe – and due to the challenges of dealing with the large constructs prior modern sequencing and gene cloning systems

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

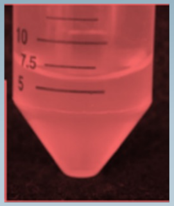




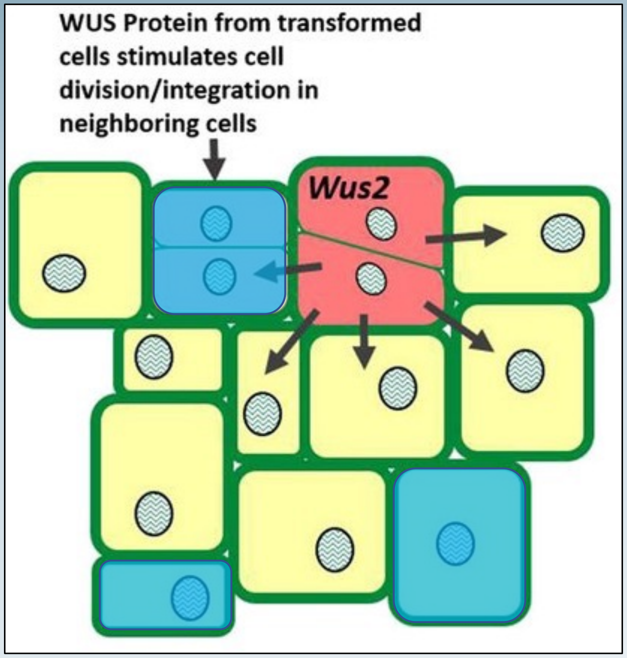
“Altruistic” transformation approach – strain mixtures

Mixed Agro in different ratios

MR gene Agro
(like WUS)



Trait gene Agro



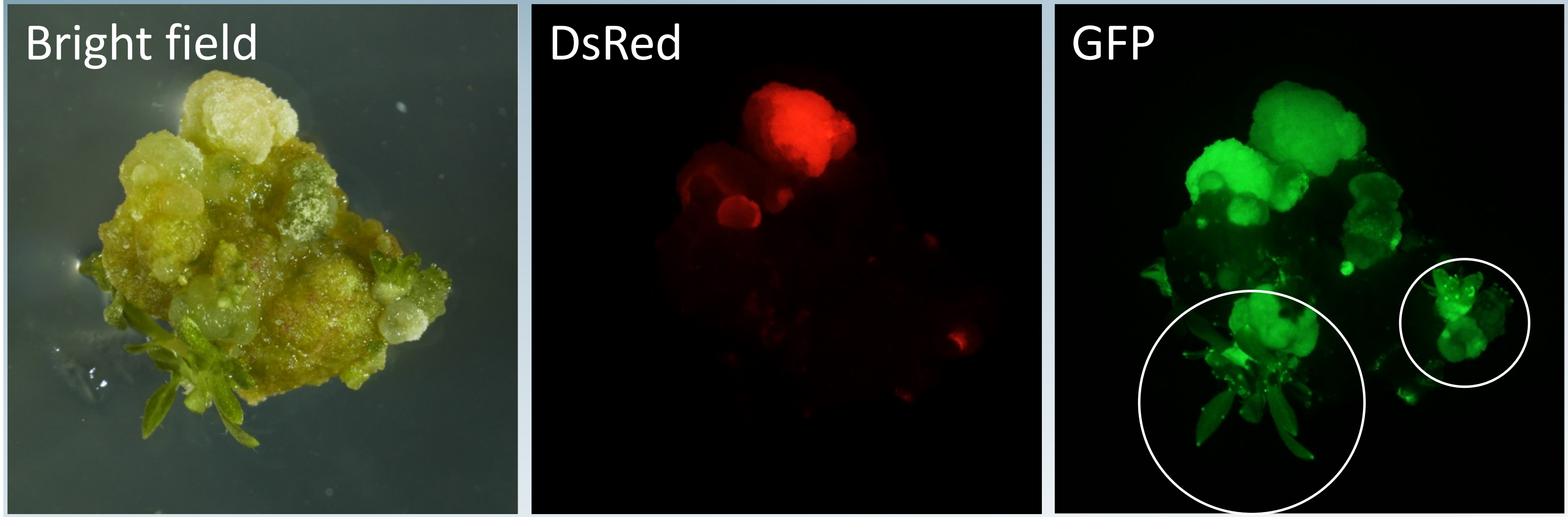
Plant with trait gene
and no MR genes

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

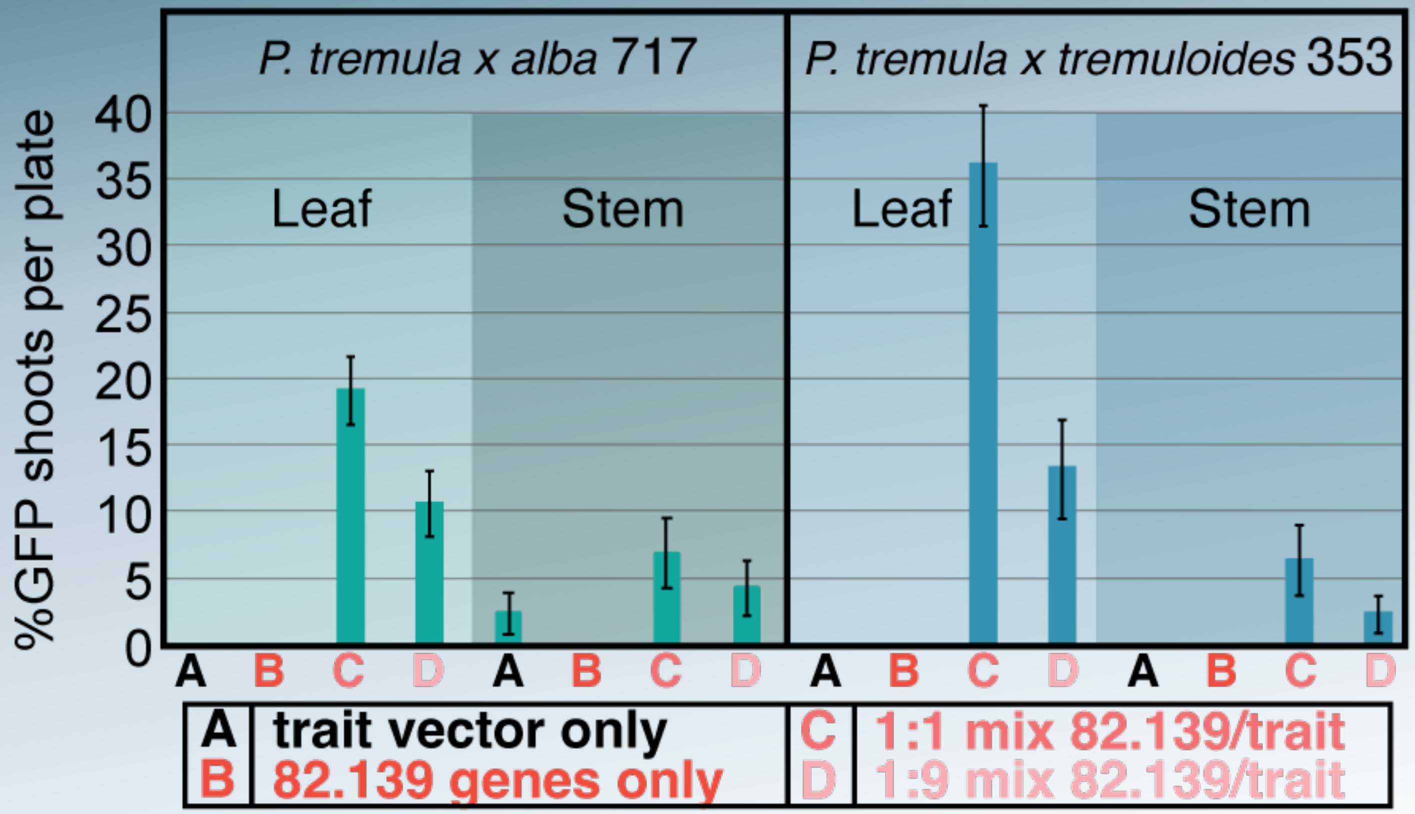


Bright field

DsRed

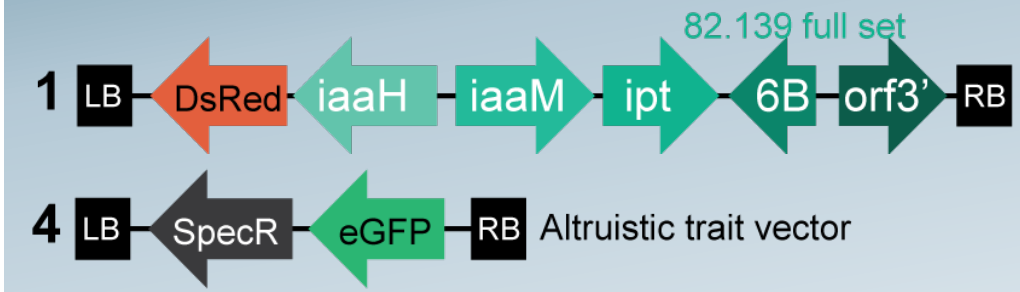
GFP

Altruistic 82.139 transformation was efficient in two independent hybrid poplar genotypes

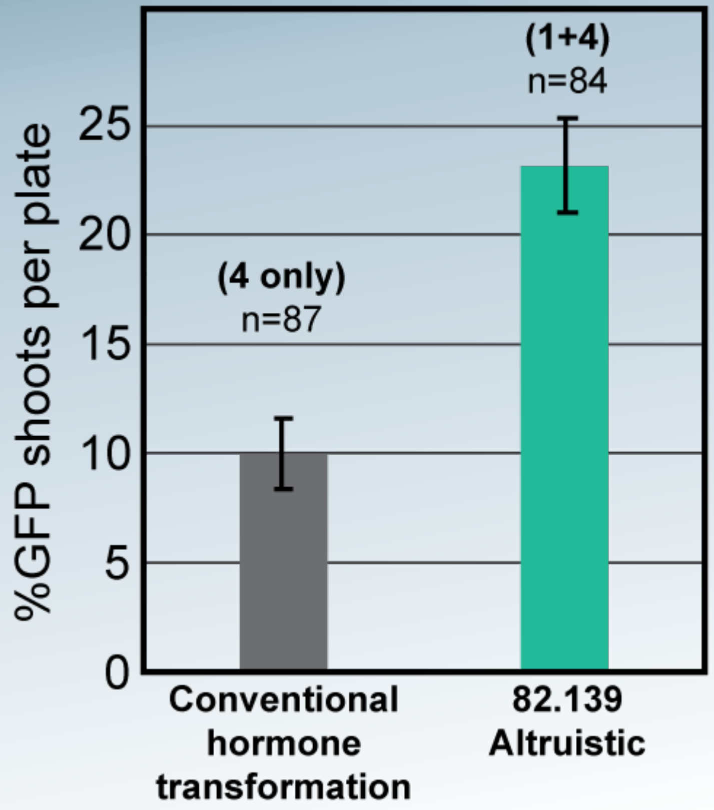


82.139 altruistic transformation was superior to routine hormone-based transformation

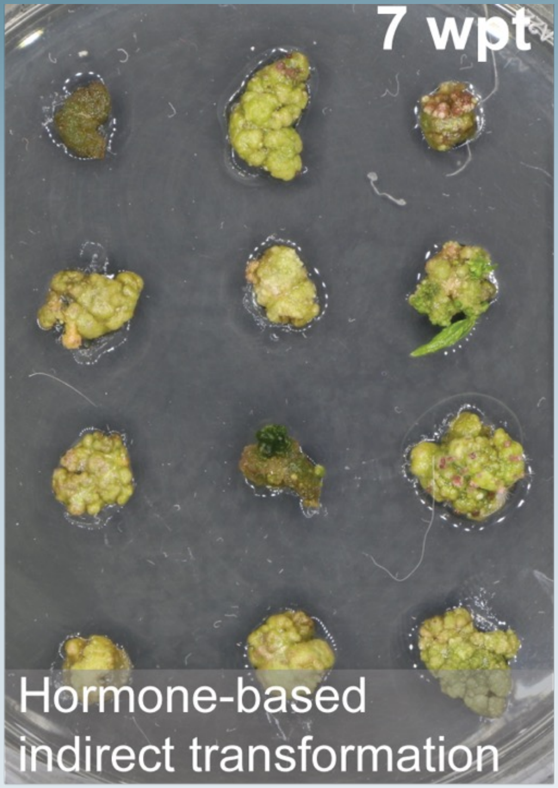
Vector types used



n= number of plates of 12 explants each



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

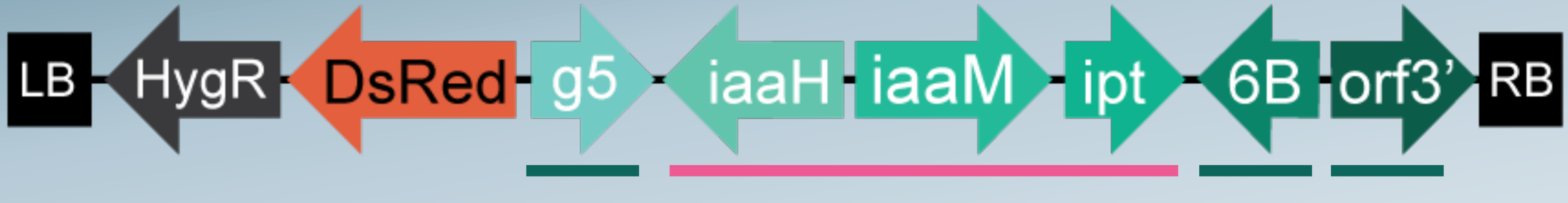


Hormone-based indirect transformation

Altruistic transformation with 82.139 genes

- Currently launched via GAANTRY strain ARport1
- 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?

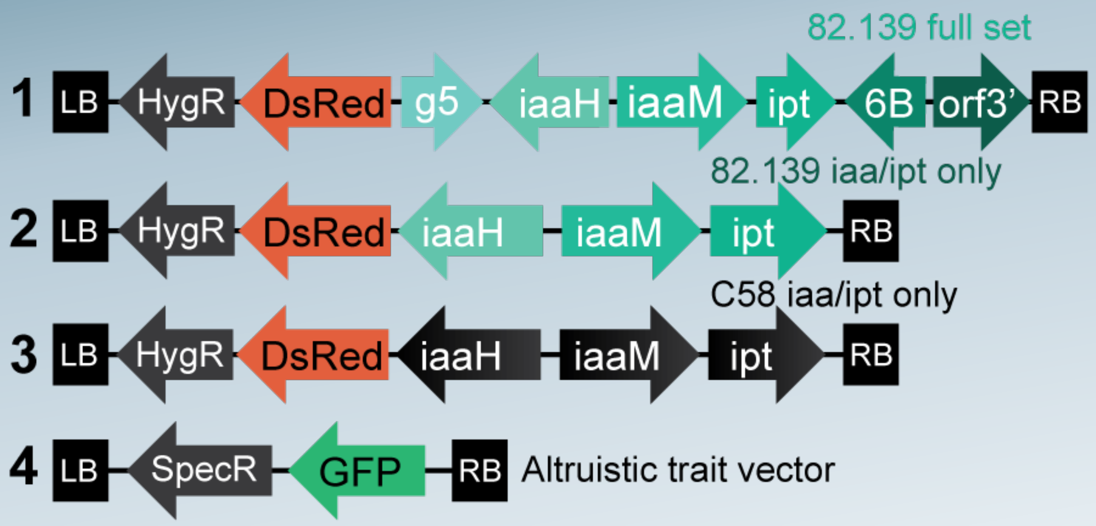


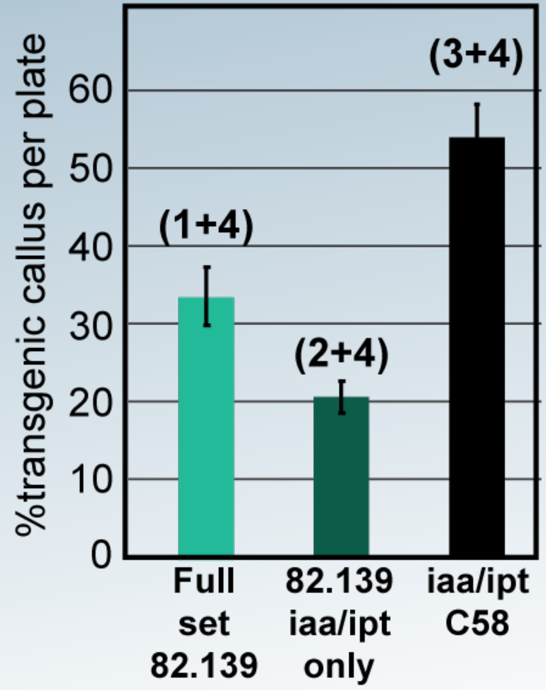
Is there novel *iaa/ipt* expression in this strain?

Or are the unique genes there most important?

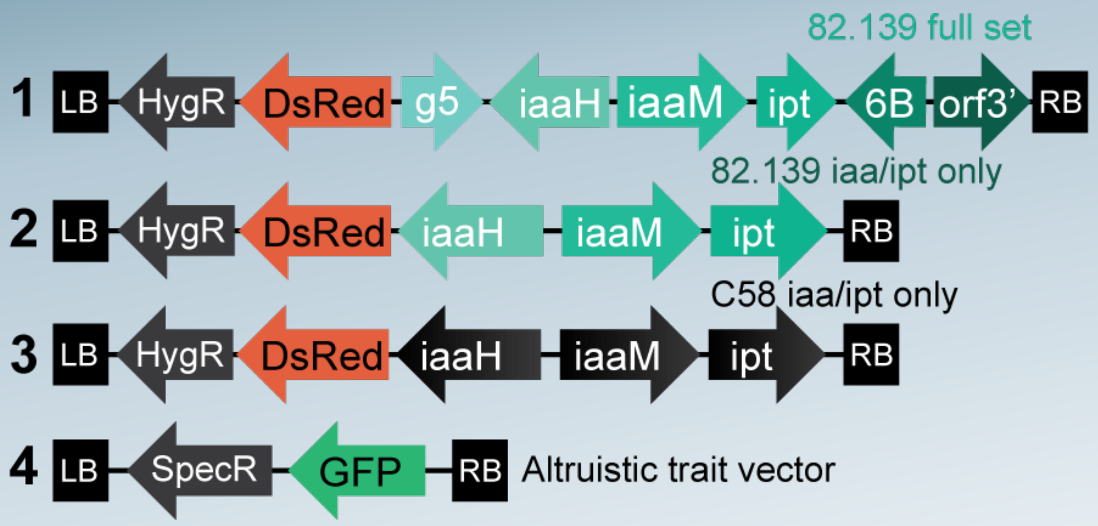
We tested the hormone genes alone or from strain C58 against the full-length 82.139 gene set

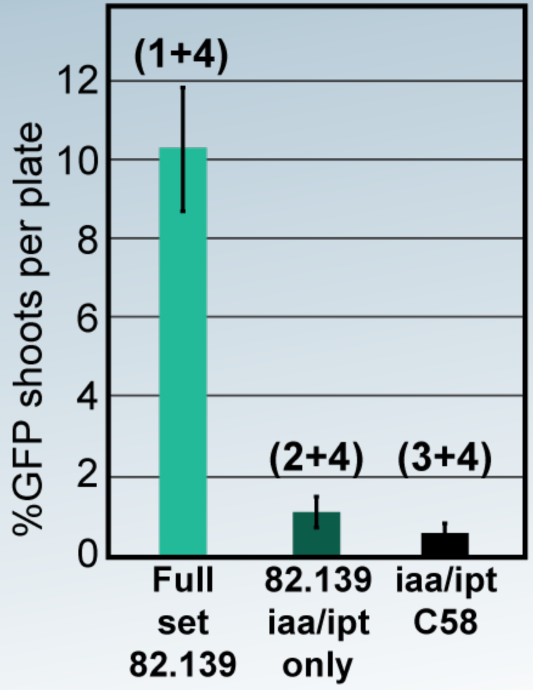
–C58 was best at forming transgenic callus



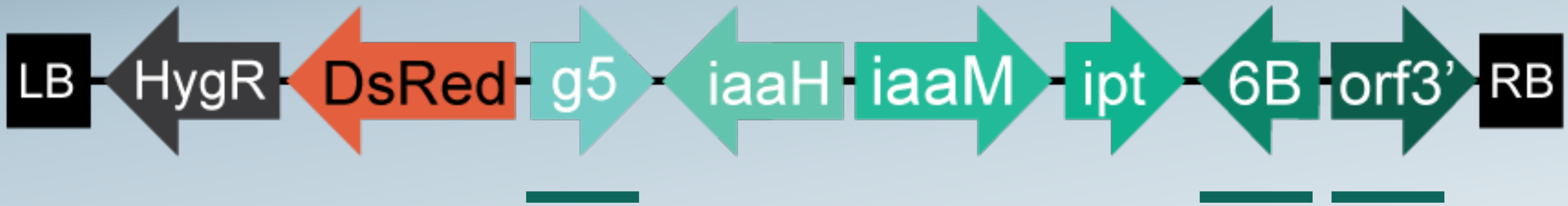


82.139 hormone producing genes (*iaa/ipt*) were not capable of inducing altruistic shoot production



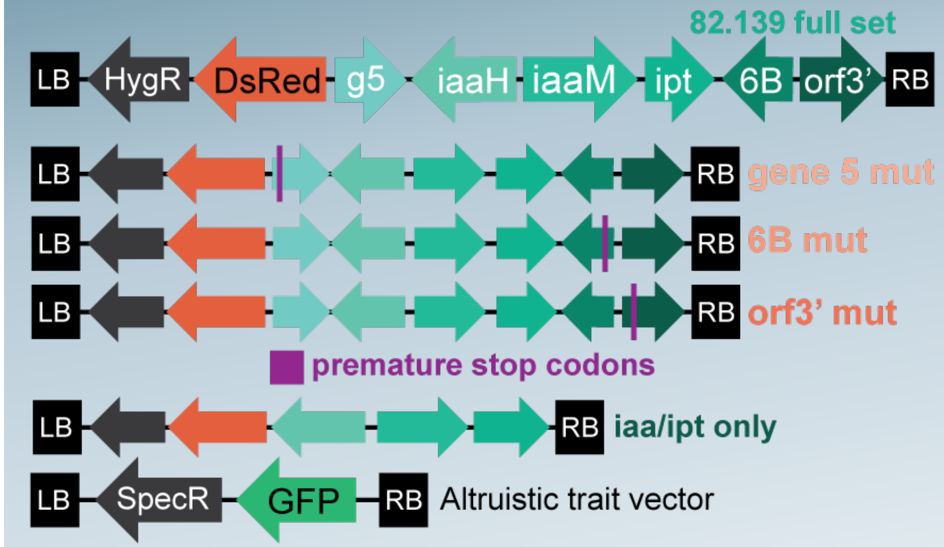


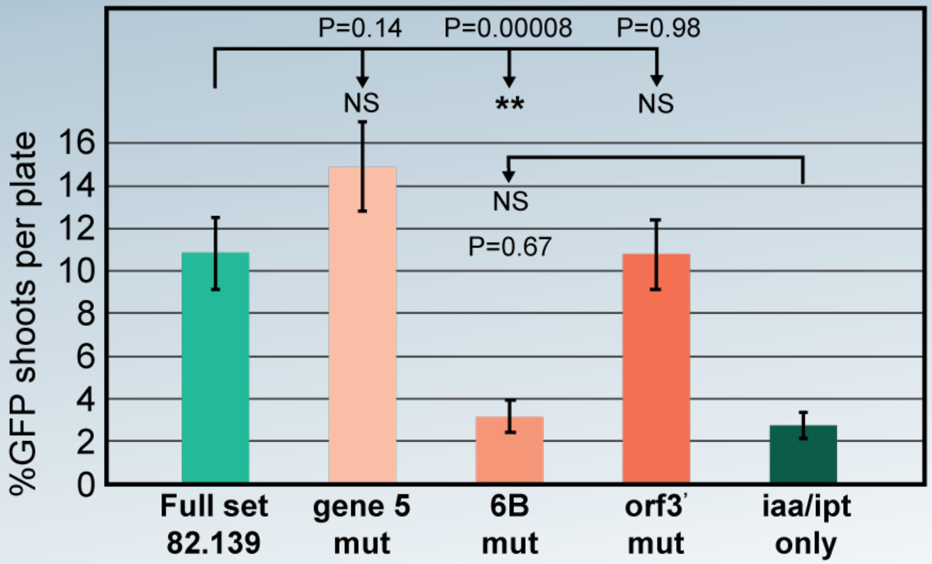
Which genes are most important for non-cell autonomous shoot promotion?



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

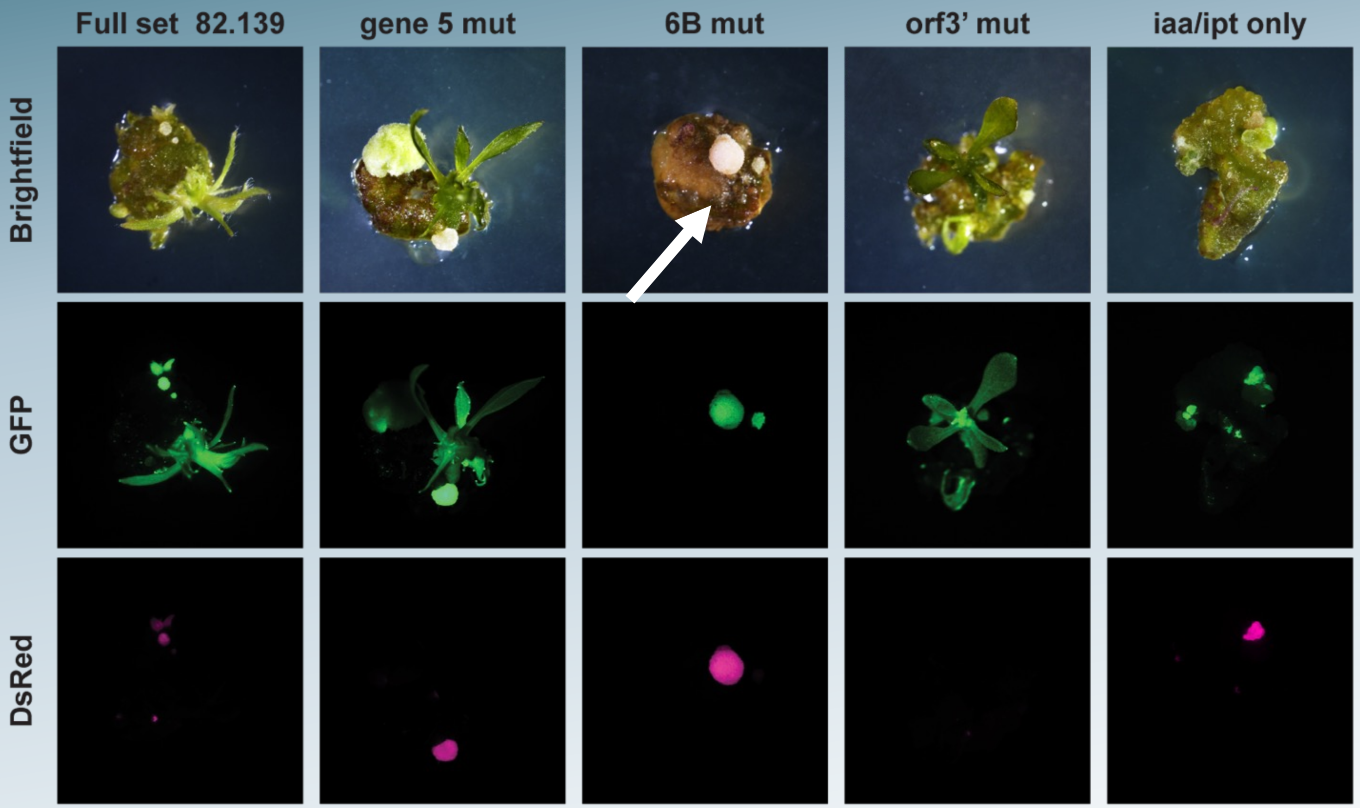
We introduced premature stop codons in each gene to assess contribution to shoot phenotype





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

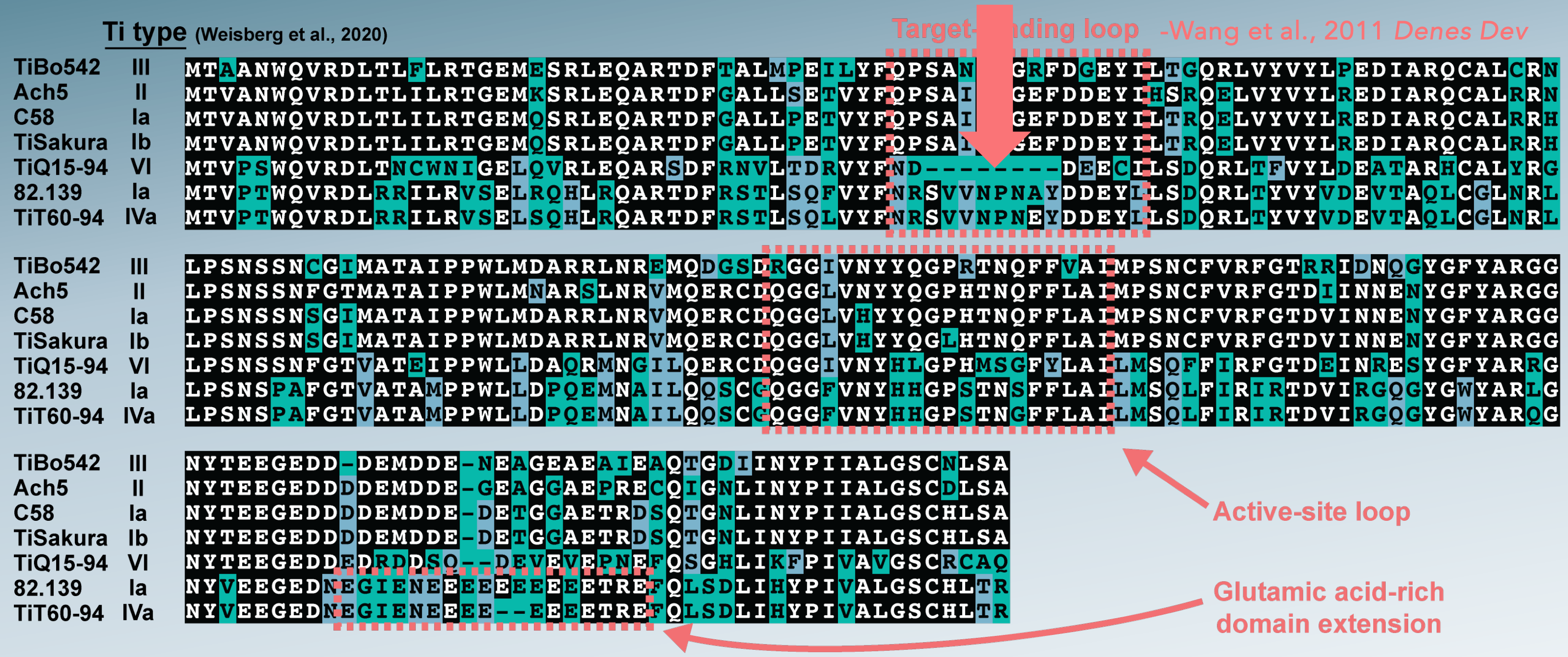
Mutations in 6B prevent altruistic shoot formation



Representative images of explants from each construct

What is unique about 6B?

Novel
N-terminal domain



Ti type (Weisberg et al., 2020)

Target-binding loop -Wang et al., 2011 *Denes Dev*

```
TiBo542   III  MTAANWQVRDLTLFLRTGEMESRLEQARTDFTALMPEILYFQPSAN[illegible]GRFDGEYILTGQRLVYVYLPEDIARQCALCRN
Ach5      II   MTVANWQVRDLTLILRTGEMKSRLEQARTDFGALLSETVYFQPSAI[illegible]GEFDDEYIHSRQELVYVYLREDIARQCALRRN
C58       Ia   MTVANWQVRDLTLILRTGEMQSRLEQARTDFGALLPETVYFQPSAI[illegible]GEFDDEYILTRQELVYVYLREDIARQCALRRH
TiSakura  Ib   MTVANWQVRDLTLILRTGEMQSRLEQARTDFGALLPETVYFQPSAI[illegible]GEFDDEYILTRQELVYVYLREDIARQCALRRH
TiQ15-94  VI   MTVPSWQVRDLTNCWNIGELQVRLEQARSDFRNVLTDRVYFND--------DEECILSDQRLTFVYLDEATARHCALYRG
82.139    Ia   MTVPTWQVRDLRRILRVSELRQHLRQARTDFRSTLSQFVYFNRSVVNPNAYDDEYLLSDQRLTYVYVDEVTAQLCGLNRL
TiT60-94  IVa  MTVPTWQVRDLRRILRVSELSQHLRQARTDFRSTLSQLVYFNRSVVNPNEYDDEYLLSDQRLTYVYVDEVTAQLCGLNRL

TiBo542   III  LPSNSSNCGIMATAIPPWLMDARRLNREMQDGSDRGGIVNYYQGPRTNQFFVAIMPSNCFVRFGTRRIDNQGYGFYARGG
Ach5      II   LPSNSSNFGTMATAIPPWLMNARSLNRVMQERCDQGGLVNYYQGPHTNQFFLAIMPSNCFVRFGTDIINNENYGFYARGG
C58       Ia   LPSNSSNSGIMATAIPPWLMDARRLNRVMQERCDQGGLVHYYQGPHTNQFFLAIMPSNCFVRFGTDVINNENYGFYARGG
TiSakura  Ib   LPSNSSNSGIMATAIPPWLMDARRLNRVMQERCDQGGLVHYYQGLHTNQFFLAIMPSNCFVRFGTDVINNENYGFYARGG
TiQ15-94  VI   LPSNSSNFGTVATEIPPWLLDAQRMNGILQERCDQGGIVNYHLGPHMSGFYLAILMSQFFIRFGTDEINRESYGFYARRG
82.139    Ia   LPSNSPAFGTVATAMPPWLLDPQEMNAILQQSCGQGGFVNYHHGPSTNSFFLAILMSQLFIRIRTDVIRGQGYGWYARLG
TiT60-94  IVa  LPSNSPAFGTVATAMPPWLLDPQEMNAILQQSCGQGGFVNYHHGPSTNGFFLAILMSQLFIRIRTDVIRGQGYGWYARQG

TiBo542   III  NYTEEGEDD-DEMDDE-NEAGEAEAIEAQTGDIINYPIIALGSCNLSA
Ach5      II   NYTEEGEDDDDEMDDE-GEAGGAEPRECQIGNLINYPIIALGSCDLSA
C58       Ia   NYTEEGEDDDDEMDDE-DETGGAETRDSQTGNLINYPIIALGSCHLSA
TiSakura  Ib   NYTEEGEDDDDEMDDE-DETGGAETRDSQTGNLINYPIIALGSCHLSA
TiQ15-94  VI   NYTEEGEDDEDRDDSQ--DEVEVEPNEFQSGHLIKFPIVAVGSCRCAQ
82.139    Ia   NYVEEGEDNEGIENEEEEEEEEEETREFQLSDLIHYPIVALGSCHLTR
TiT60-94  IVa  NYVEEGEDNEGIENEEEE--EEEETREFQLSDLIHYPIVALGSCHLTR
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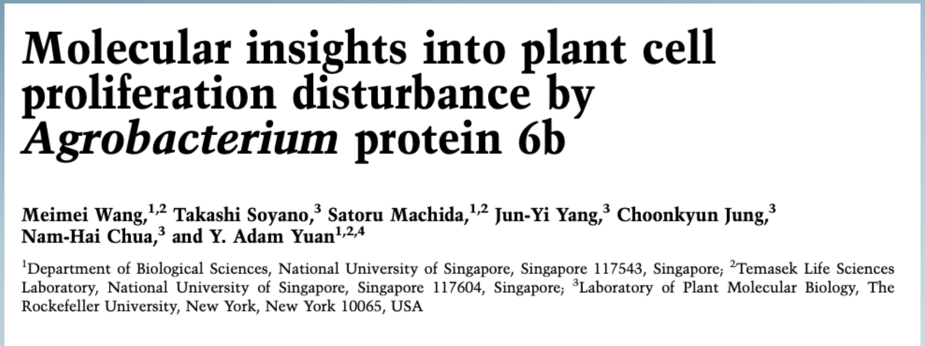
Active-site loop

Glutamic acid-rich
domain extension

Protein alignment of different Ti plasmid groups from sequenced wild collections

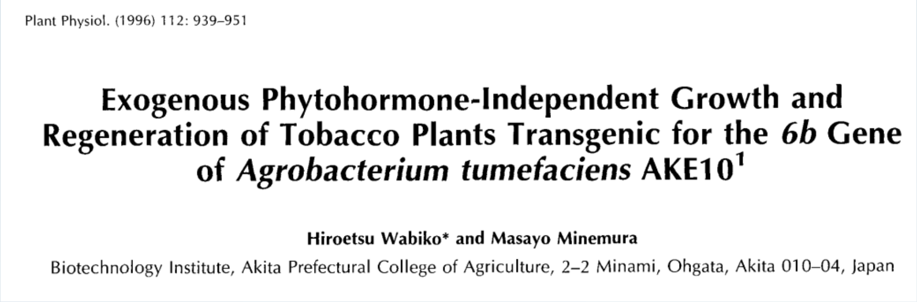
Potential mechanisms of action for 6B include interference with miRNA biogenesis in plants



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

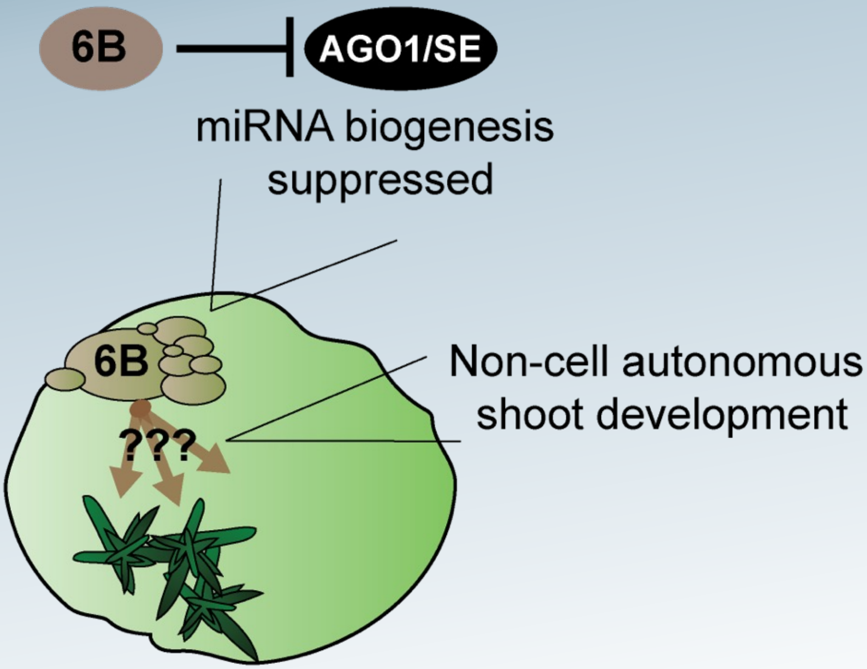


Plant Physiol. (1996) 112: 939–951

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

Hiroetsu Wabiko* and Masayo Minemura

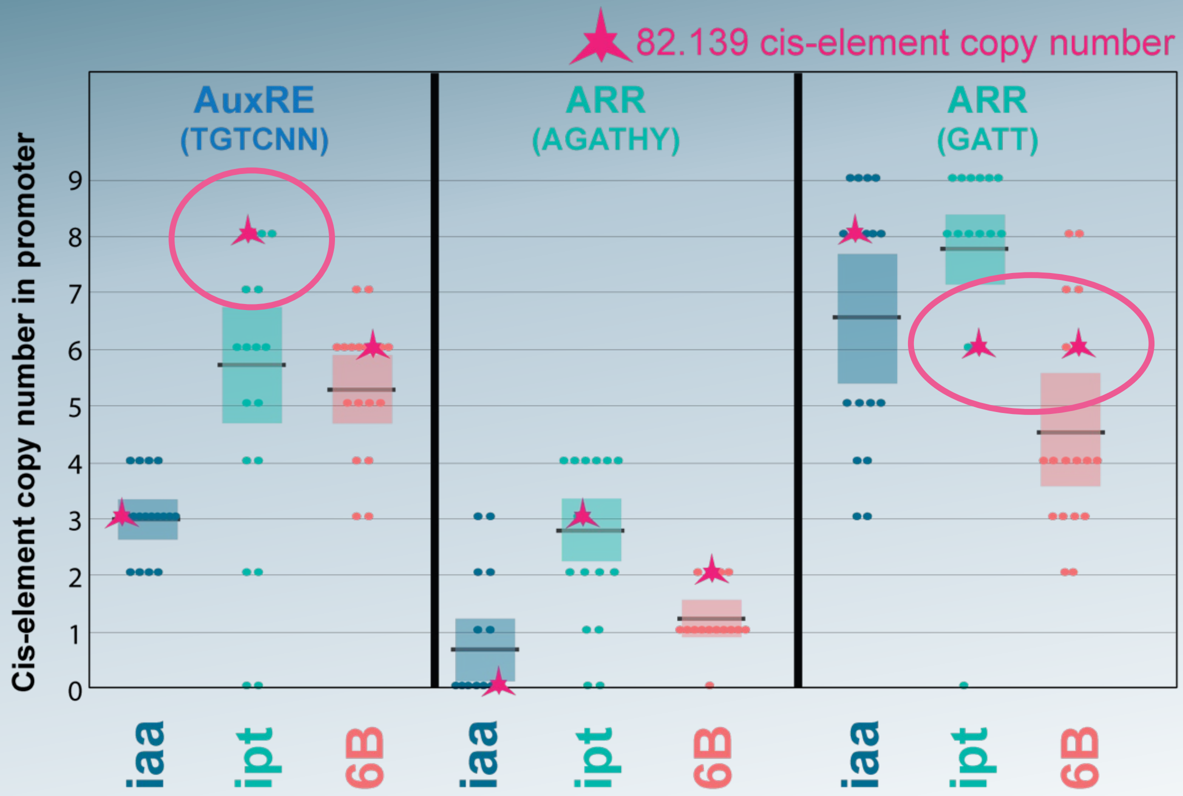
Biotechnology Institute, Akita Prefectural College of Agriculture, 2-2 Minami, Ohgata, Akita 010–04, Japan

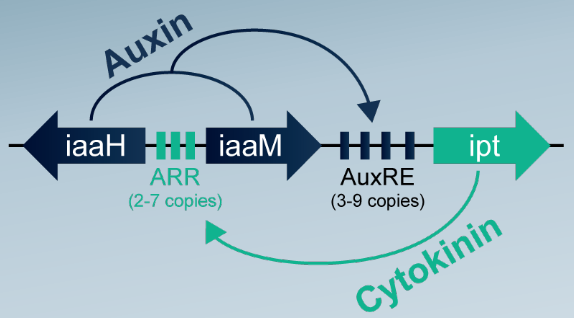


miRNA biogenesis suppressed

Non-cell autonomous shoot development

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

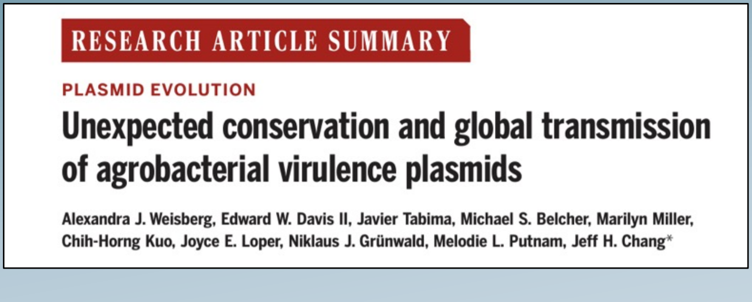




Pairing expression elements not found in nature with "shooty" 6B genes -or synthetically modified ones, is of interest to us

Are there other useful growth promoting genes?

Agrobacterium diversity has hardly been studied for use in transformation tools

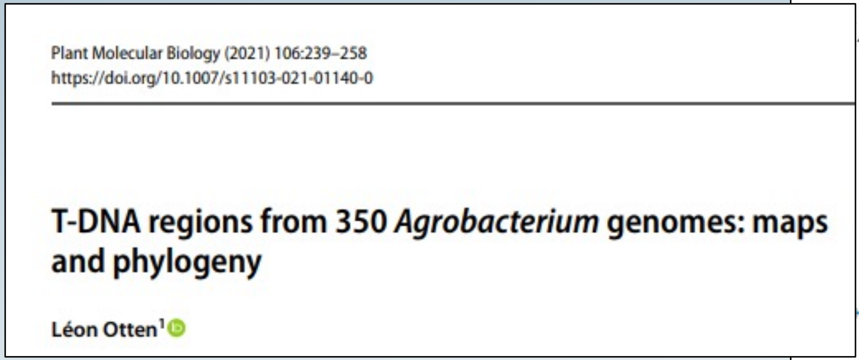


RESEARCH ARTICLE SUMMARY

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*

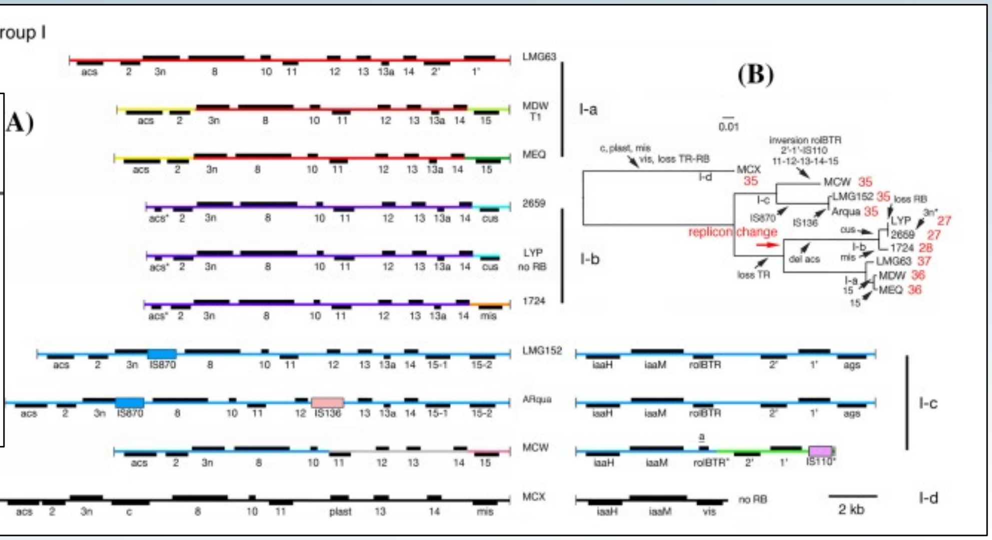


Plant Molecular Biology (2021) 106:239–258
https://doi.org/10.1007/s11103-021-01140-0

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

Léon Otten[1]

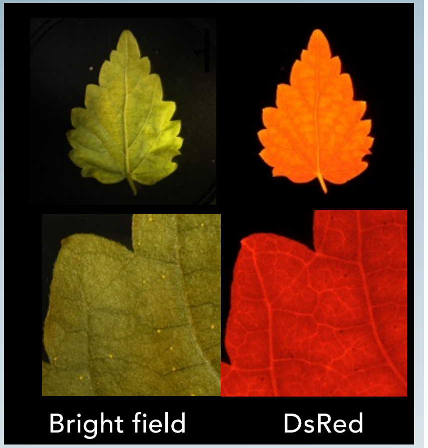
BAYER | Grants 4Ag



We are hoping to see if this is a generalizable tool for woody dicots such as hops

-We have sent to ~10 labs around the US

- "Shooty" Agro genes are the only system that has worked after two years of transformation testing
- First transformation of US hop cultivar
- **If you want to try it please feel free to contact us!**

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Strauss Lab OSU


Michele Wiseman
BPP Gent Lab


David Gent
ARS Corvallis

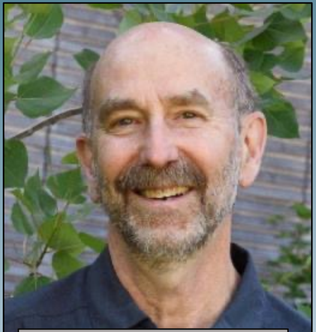
Summary and next steps

- 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

- We hope through testing in multiple species we can get a better understanding how generalizable this tool will be for the community
- We are testing diverse wild Agro strains for their utility in plant transformation

Acknowledgements



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 (URSA/honor's college)
- 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*)

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- Alex Weisberg (BPP ,OSU)
- Jeff Chang (BPP ,OSU)
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NSF-Plant Genome Research Program (PGRP)

<u>GREAT TREES Consortium</u>
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Thanks for listening!