Genetic transformation of perennial ryegrass (*Lolium perenne*) using ectopic expression of morphogenic regulators.

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“EditGrass4Food»

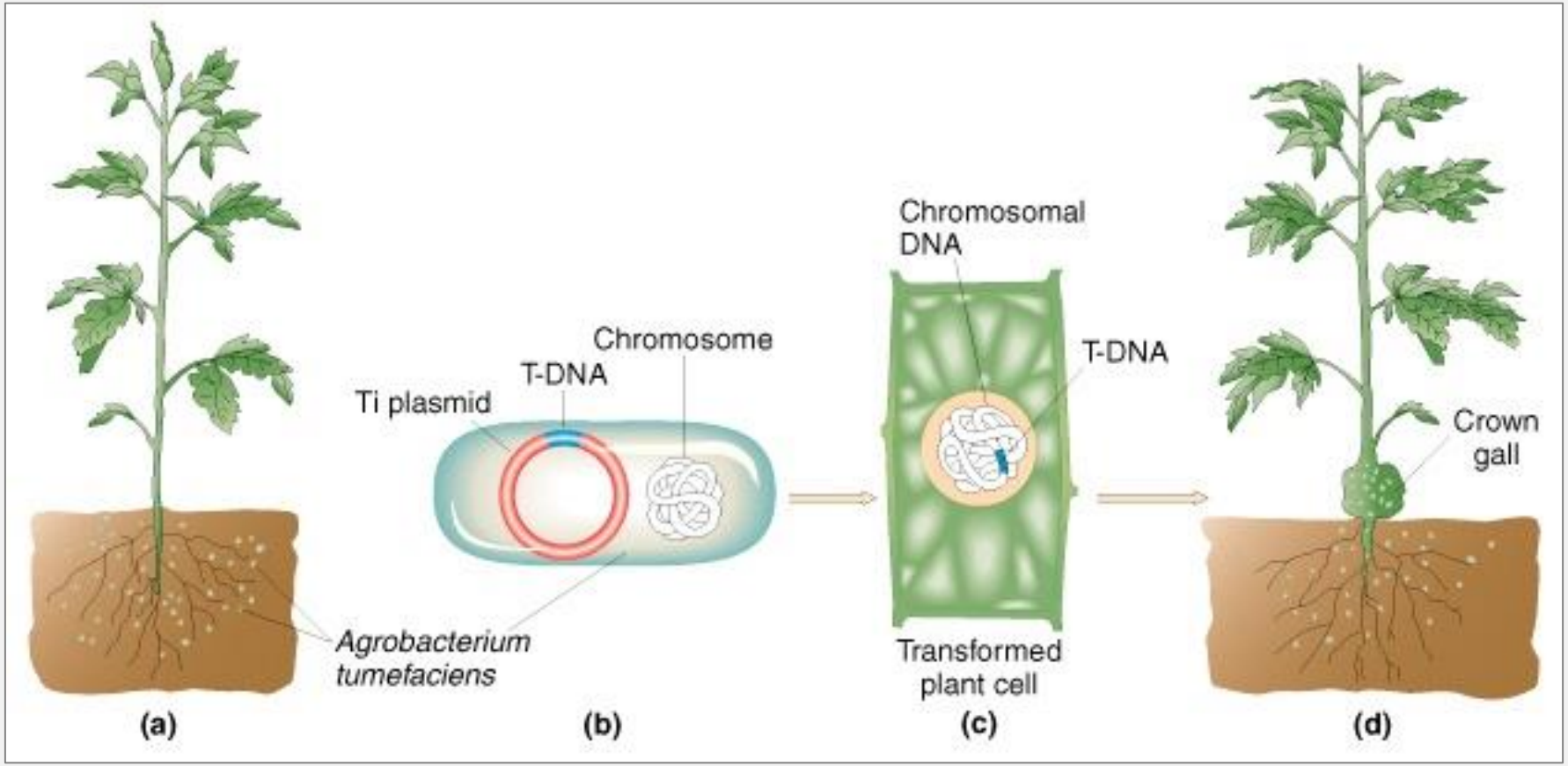
is financially supported by European Economic Area (EEA) grants

Why genetic transformation & ryegrass?

- Discovery, validation and taking advantage of the phenotype-genotype causality linkage
- Genetic improvement of the major forage crop for the better performance and higher biomass quality in diverse and changing agroclimatic conditions
- Mechanistic understanding of the S/Z self-incompatibility in pollen-pistil recognition

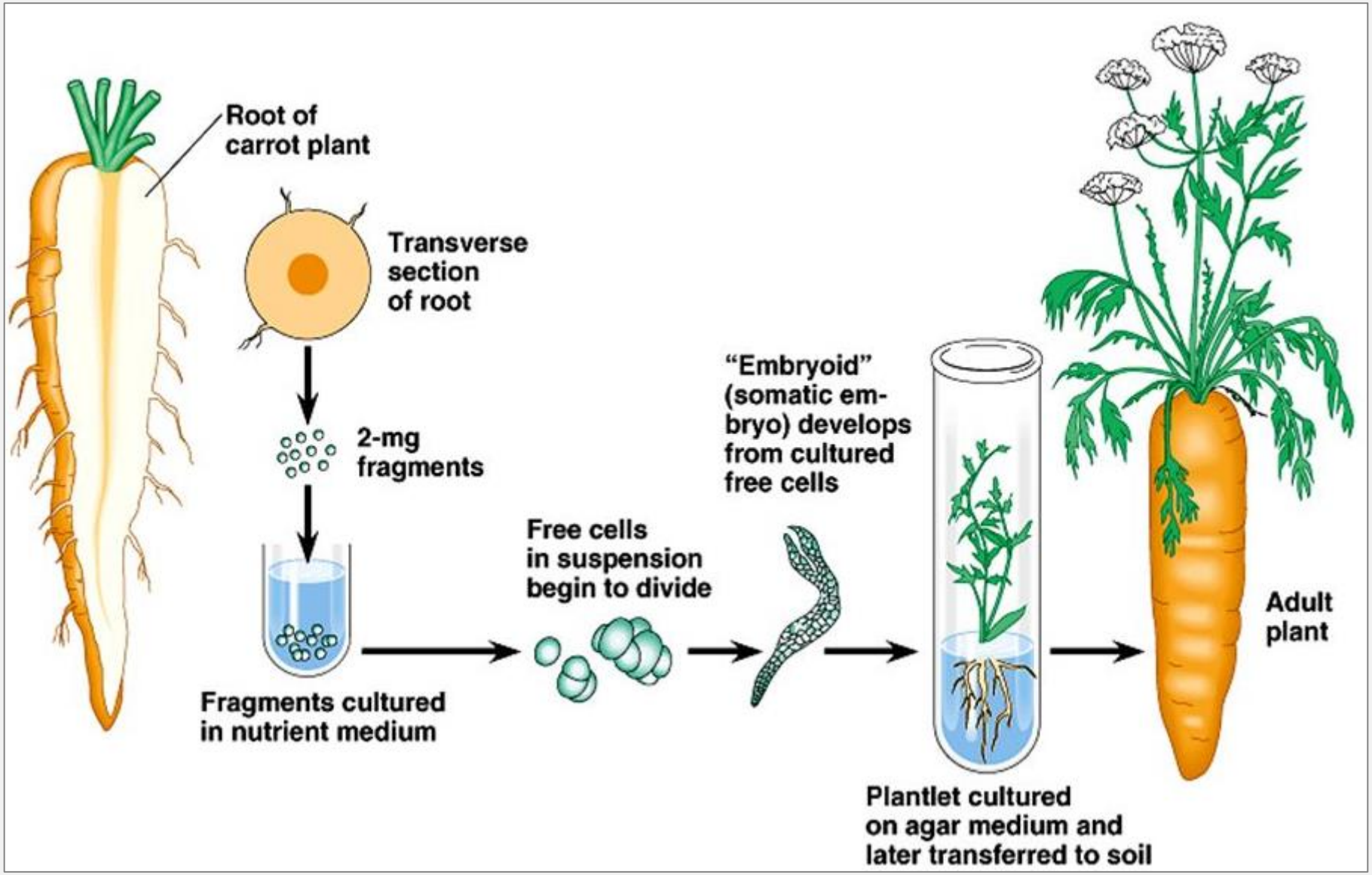
Plant genetic transformation technology was born in 1983 exploiting, (A) trans-kingdom horizontal gene transfer, and (B) …

Herrera-Estrella, L., Depicker, A., Van Montagu, M. and Schell, J., 1983. Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector. *Nature*, *303*(5914), pp.209-213.



https://www.mun.ca/biology/scarr/Gr13-14.htm

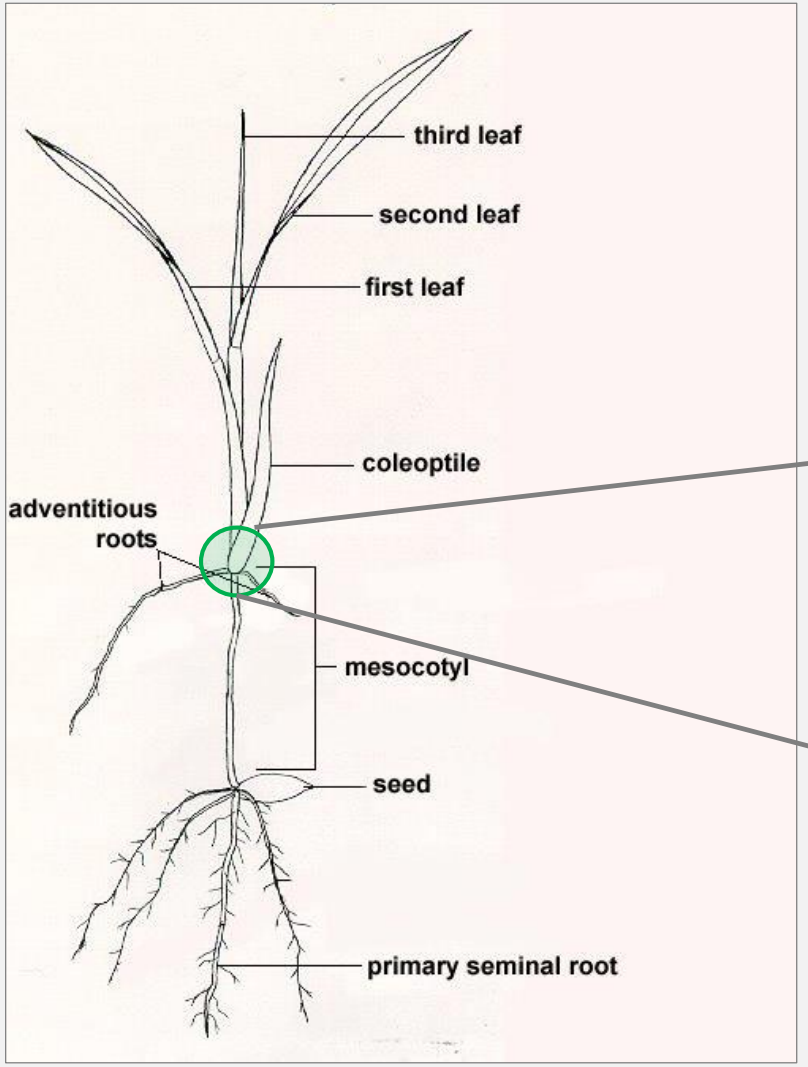
… the differentiated plant cell totipotency and developmental plasticity

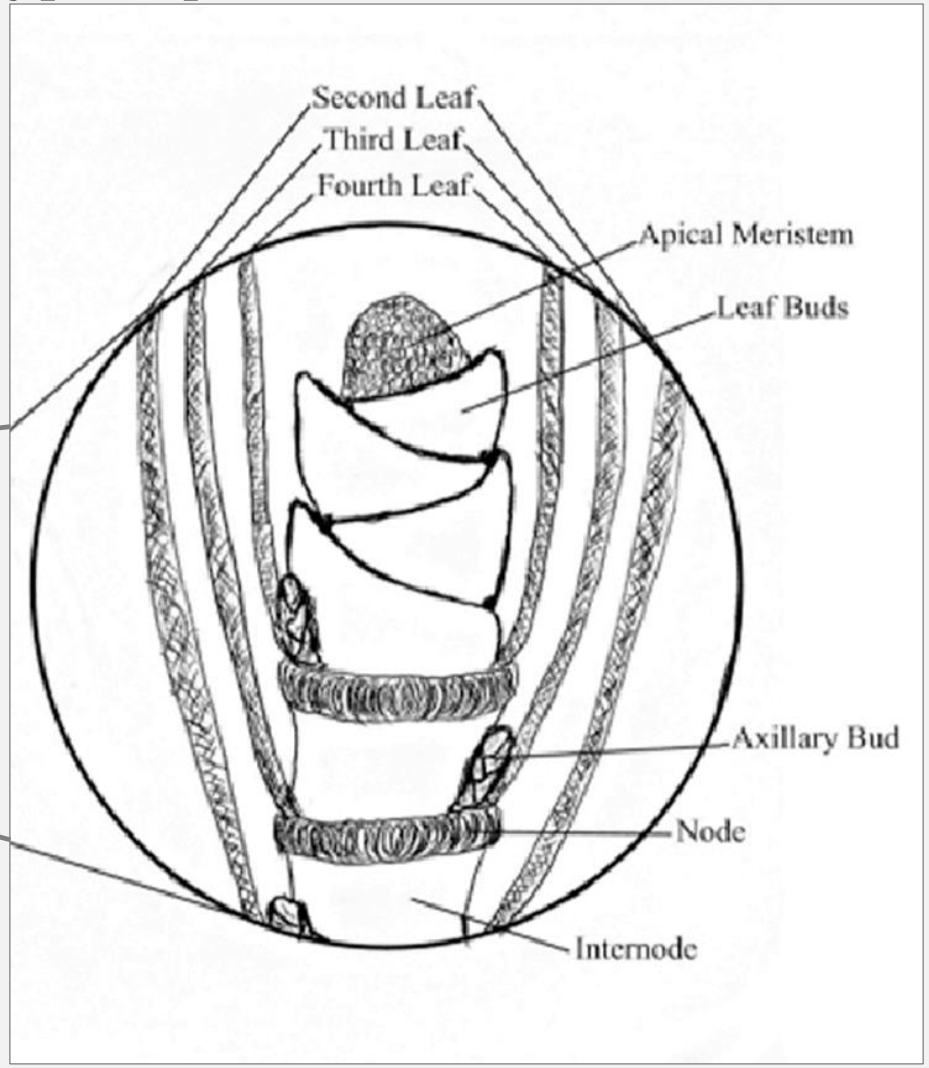


https://plantlet.org/cellular-totipotency-from-a-single-cell-to-a-fully-formed-organism/#google_vignette

Grass family (*Poaceae*) constrains genetic transformation freedom

Restricted somatic cell totipotency, a part of genotype-dependent tissue culture recalcitrance





In perennial ryegrass, only SAM and 2 mm of the leaf bases respond with cell division in *in vitro* culture on synthetic media

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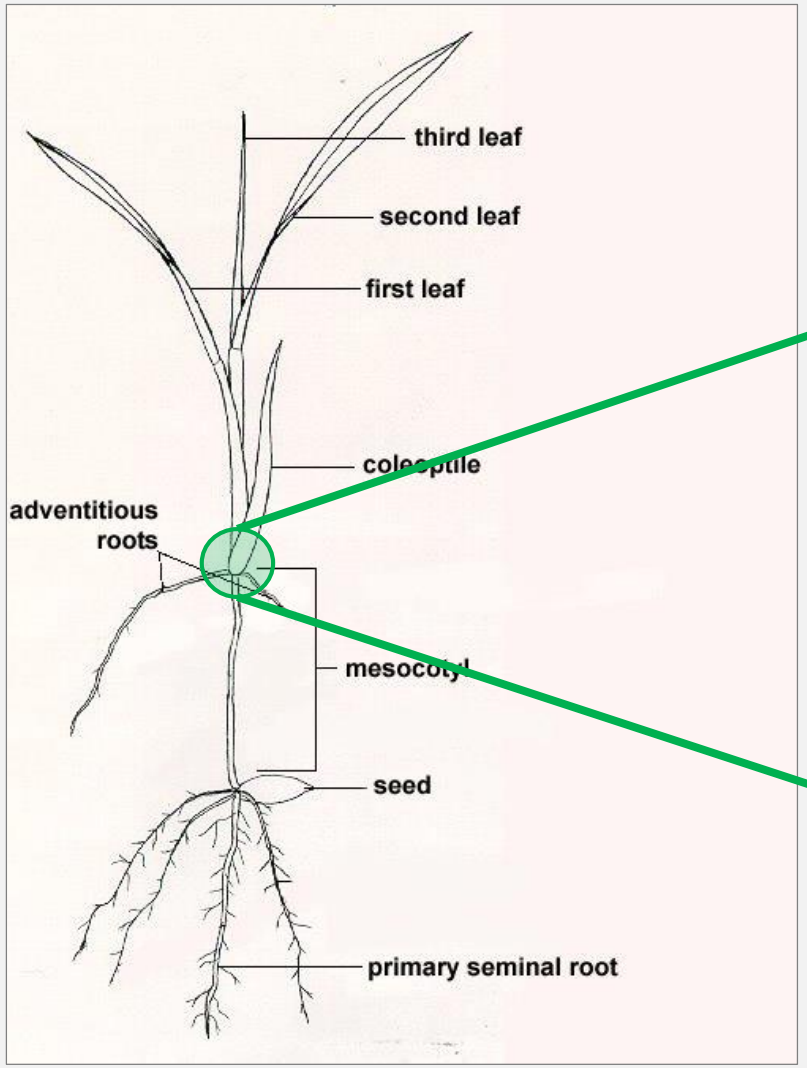
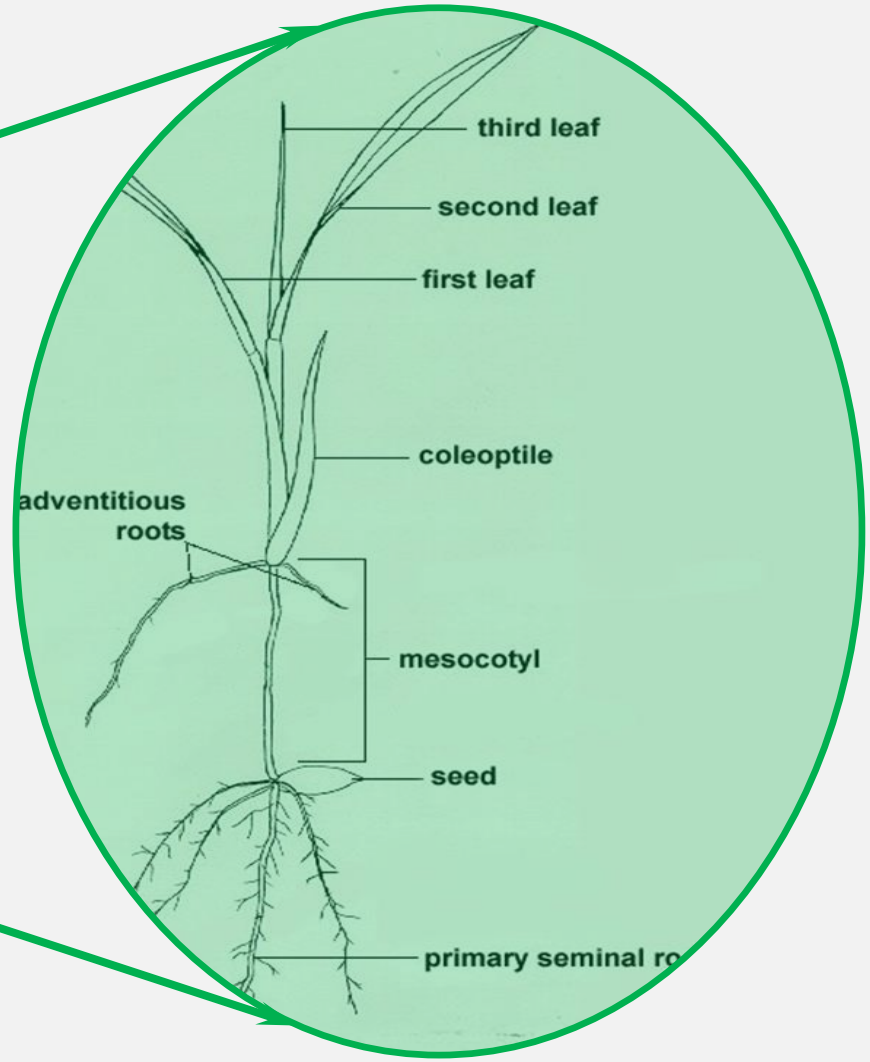
Our study question.

Can we change the somatic cell totipotency pattern in a ryegrass plant?





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Ectopic expression of morphogenic regulators can overcome genotype-dependent tissue culture recalcitrance

WUS, WUSCHEL and some WUS-like (WOX) homeo-box transcription factors promote **stem cell fate**

BBM, BABY BOOM AP2/EREB family transcription factor stimulates **embryogenesis**

GRF-GIF, GROWTH-REGULATING FACTOR 4 (GRF4) fused to its cofactor GRF-INTERACTING FACTOR 1 (GIF1) enhances **plant cell proliferation** in a species-specific manner

IPT, cell autonomous cytokinin biosynthesis implicated in **shoot apical meristem establishment**

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Hayta, S., et al. (2023). *Nature Plants*, *9*(2), pp.197-198.
Debernardi, J.M., et al. (2020) *Nature Biotechnology*, *38*(11), pp.1274-1279.
Cody, J.P., et al. (2023) *Nature Protocols*, *18*(1), pp.81-107.

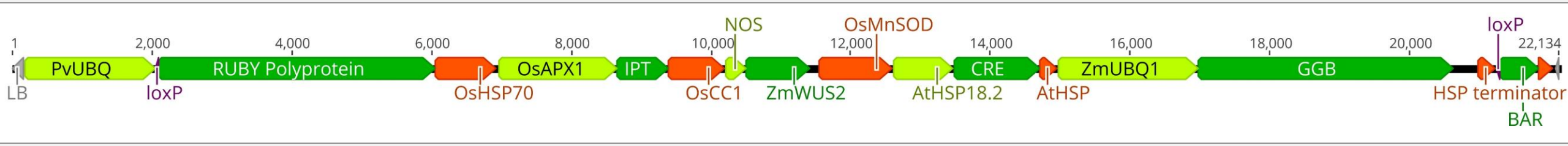
Prototype T-DNA in a fourteen plasmid vectors series

Red color visual marker
RUBY, Betalain biosynthesis

Cytokinin biosynthesis
IPT, Isopentyl Transferase

Stem cell fate
WUS2, WUSCHEL-like

Cell proliferation & Embryogenesis
GGB, GRF-GIF-BBM polyprotein



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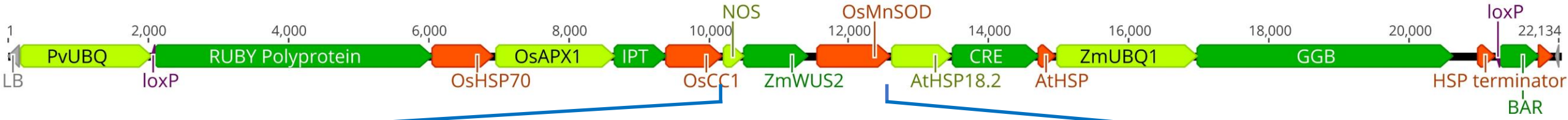
Binary plasmid isoforms



Classic *ZmWUS2*

Auxin inducible promoter

WUS2 clade

WOX4 clade

Embryo-specific *WOX*

Expansion of lateral organs

Zm - *Zea mays*, corn
Hv - *Hordeum vulgare*, barley
Ta - *Triticum aestivum*, wheat

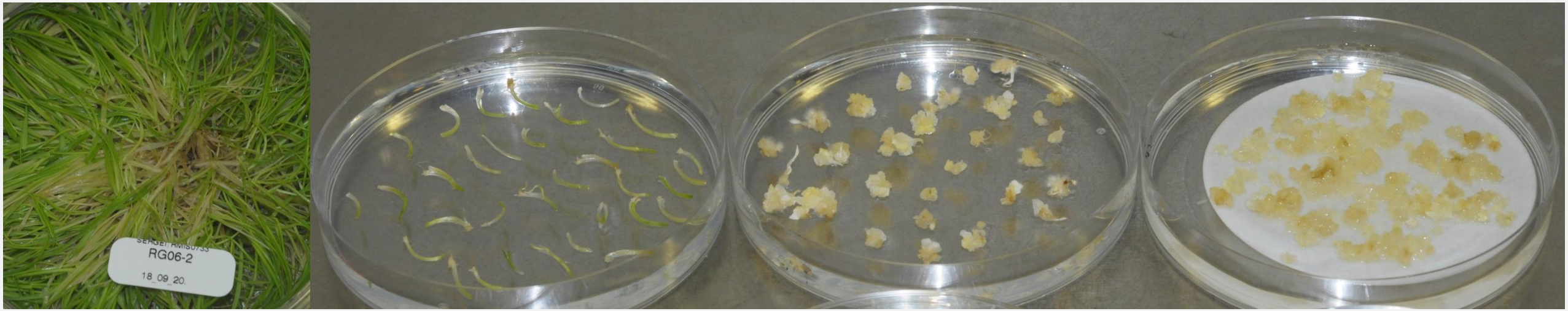
WOX - WUSCHEL-related homeobox
NAL & TAB - barley & wheat WOX

AMT, Agrobacterium-Mediated Transformation workflow

Generation of ryegrass cells suitable for genetic transformation

Isolate meristematic zones → 3-4 weeks → 3-4 weeks → 5-7 days → + Agrobacterium

AMT day 1 (d1)



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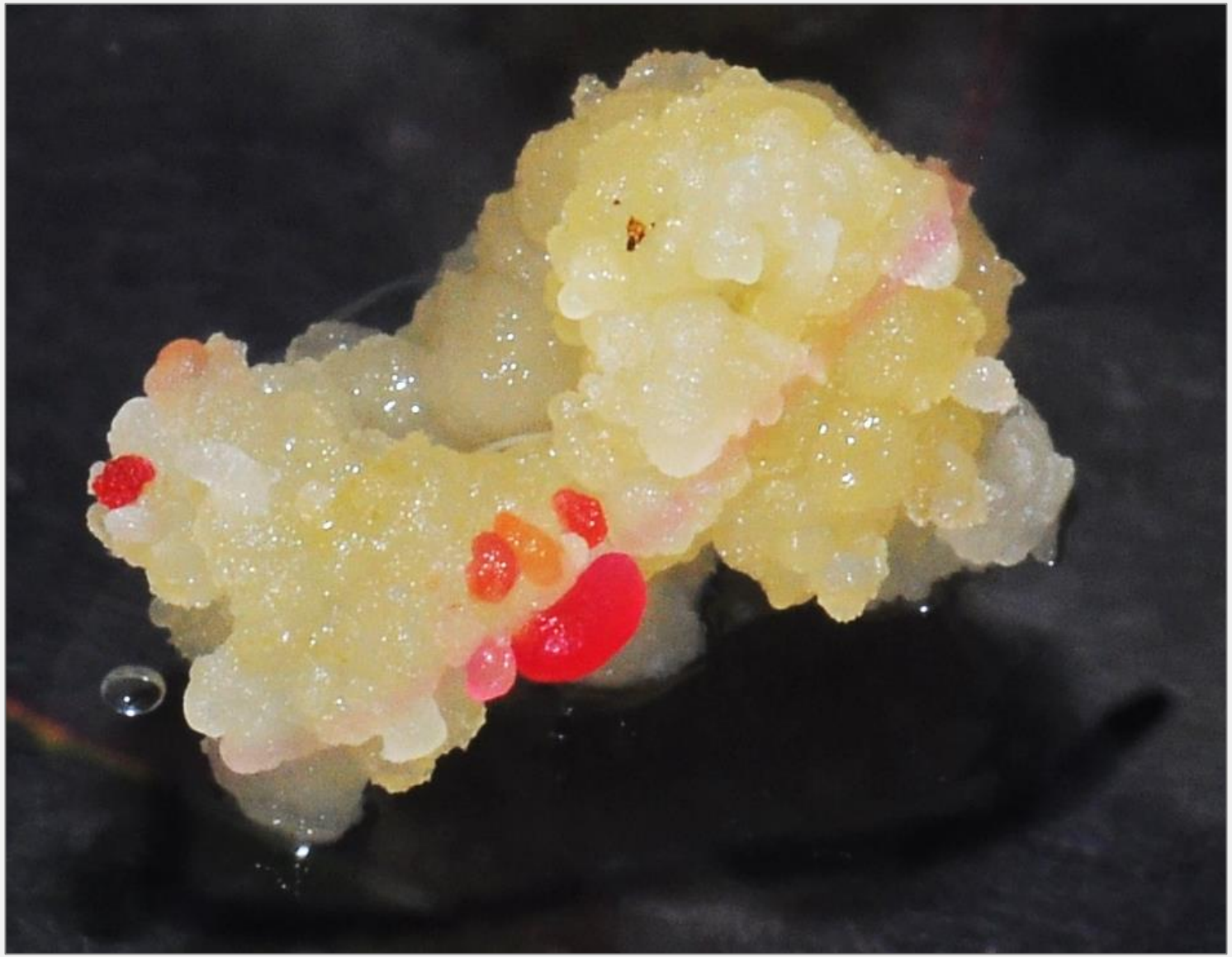
Visual selection and tracking of transgenic events

RUBY-positive clones develop organized cellular structures in two-three weeks



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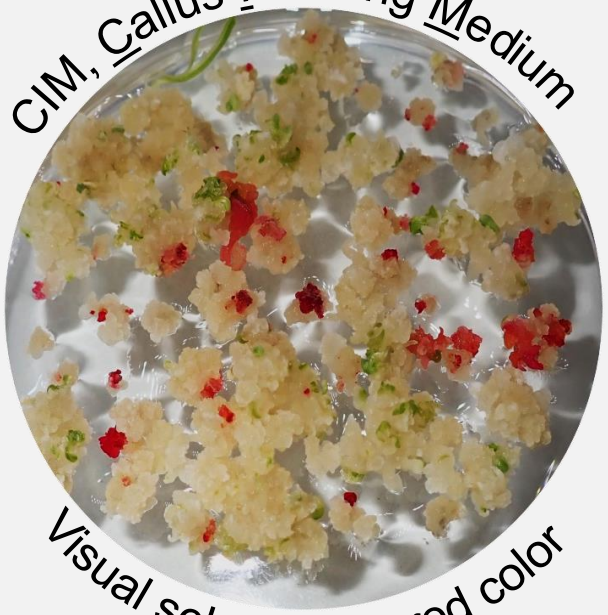
Next AMT workflow steps leading to plants

CIM, Callus Inducing Medium



Visual selection by red color

→

Regeneration media

→

Hormone-free medium to stimulate shoot elongation

→

Trimmed individual shoots

E clones, n=110

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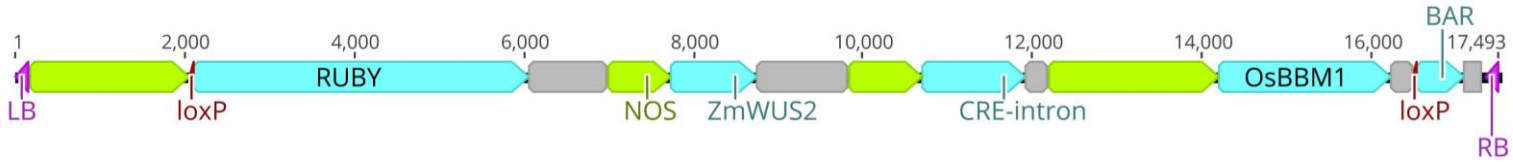
A set of eight plasmids assembled in December 2023 to compare GRF-GIF-BBM (GGB) versus BBM only

Plasmids have been used in an ongoing AMT experiment started on December 29

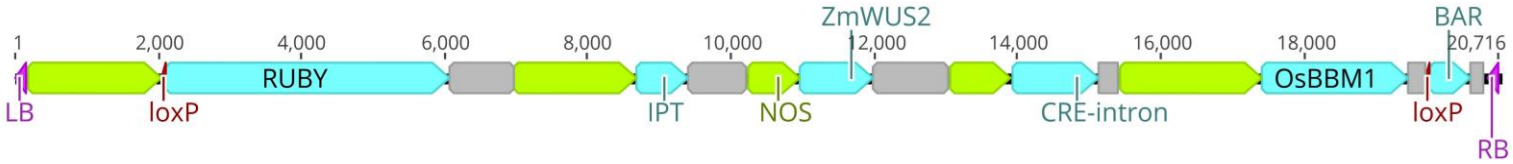
Previous (Teagasc) combinations WUS2/BBM/± IPT

BBM#1



pL2rBWUS2

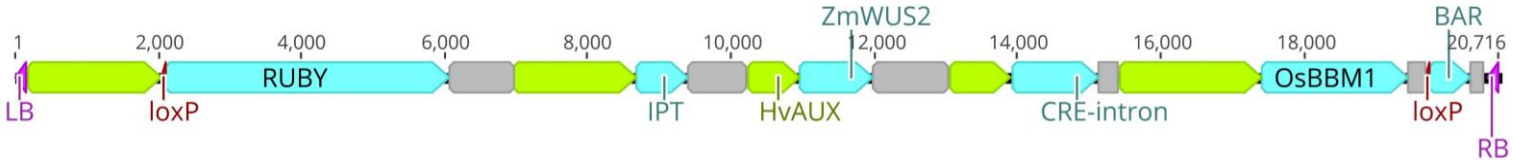
BBM#2



pL2rBW2TMR

Comparing promoters NOS::WUS2 vs AUX::WUS2

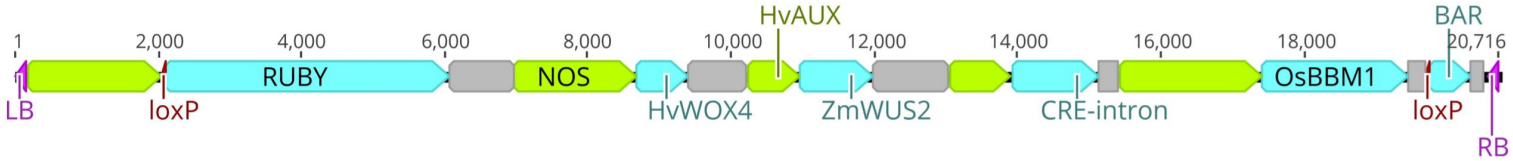
BBM#3



pL2rBAUX

WOX interclade synergy NOS::WOX4 + AUX::WUS2

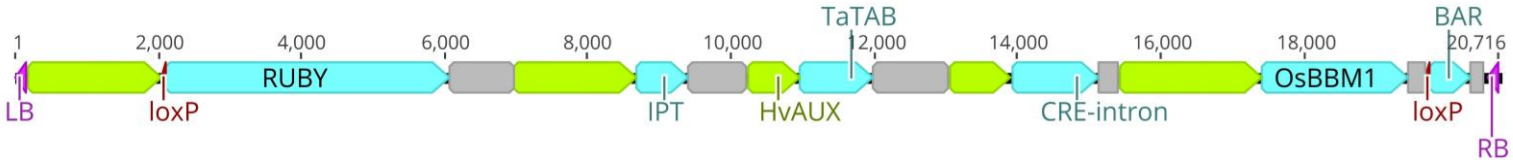
BBM#4



pL2W2WOX4

Species-specificity within the WUS2 clade Maize vs wheat AUX::WUS2 vs AUX::TAB

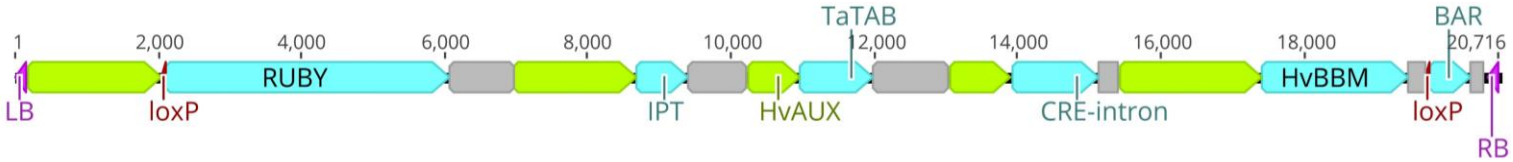
BBM#5



pL2rBBMTAB

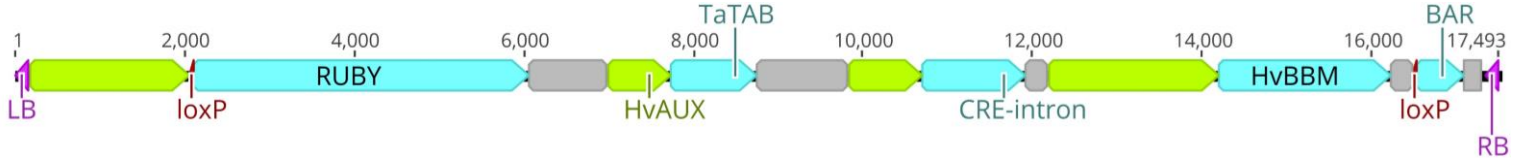
Species-specificity within the BBM clade, i.e., Rice BBM1 vs barley BBM

BBM#6



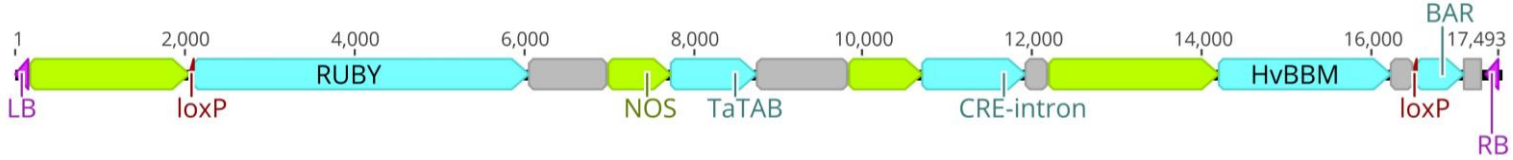
pL2hBTT

BBM#7



pL2hBAUXTAB

BBM#8



pL2hBNOSTAB

T-DNA evaluation metric “time from a cell to a plant”

January 9, 2024
Ryegrass cells + Agrobacteria

→

February 16, 2024
Shown images

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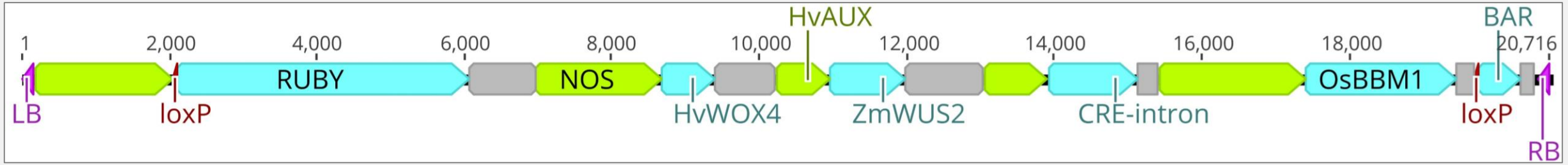
From a single cell to a shoot in 38 days



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The Design-Test-Repeat cycle
https://www.strategyzer.com/library/design-test-repeat-cycle-visual

Root meristems show cell proliferation response on CIM medium (d30)

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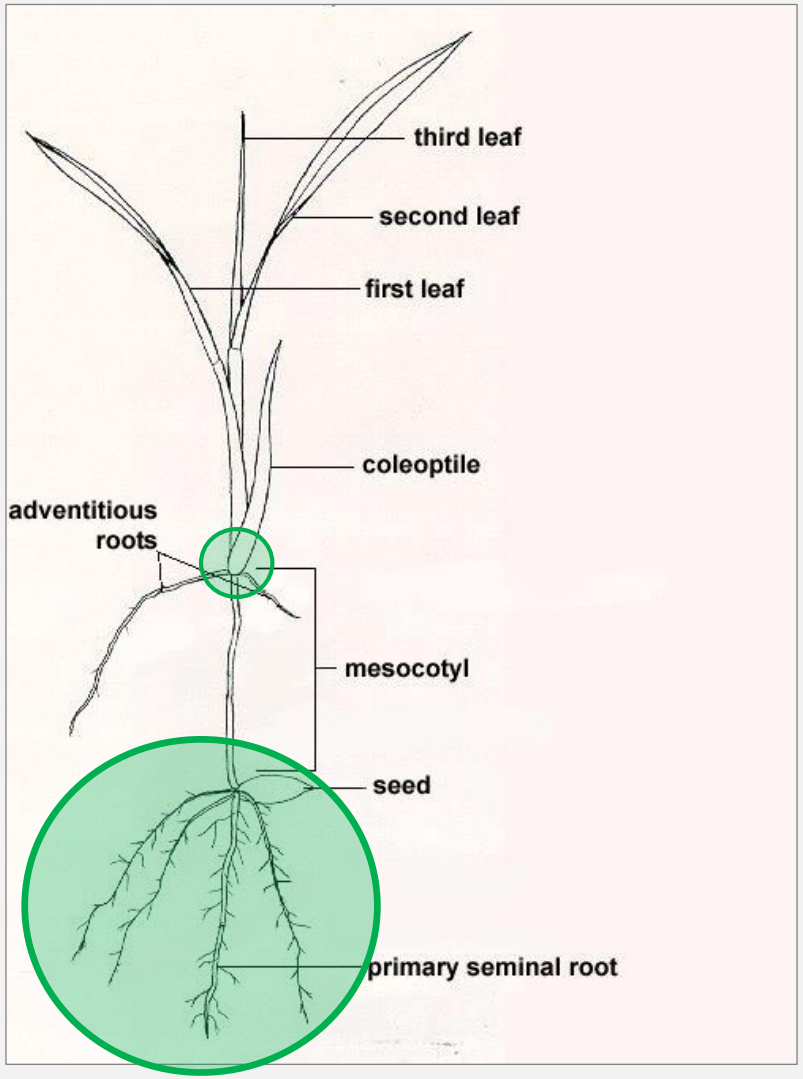
Our transgenic plants have expanded somatic cell totipotency pattern



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Outlook

1. Can we now perform AMT using roots as an explant?

2. Will protoplasts from roots divide?

3. Will the innovation Design-Test-Repeat cycle further
   → expand somatic cell totipotency to the leaves
   → shorten time “from a cell to a plant”
   → enable tissue-culture-free workflow?

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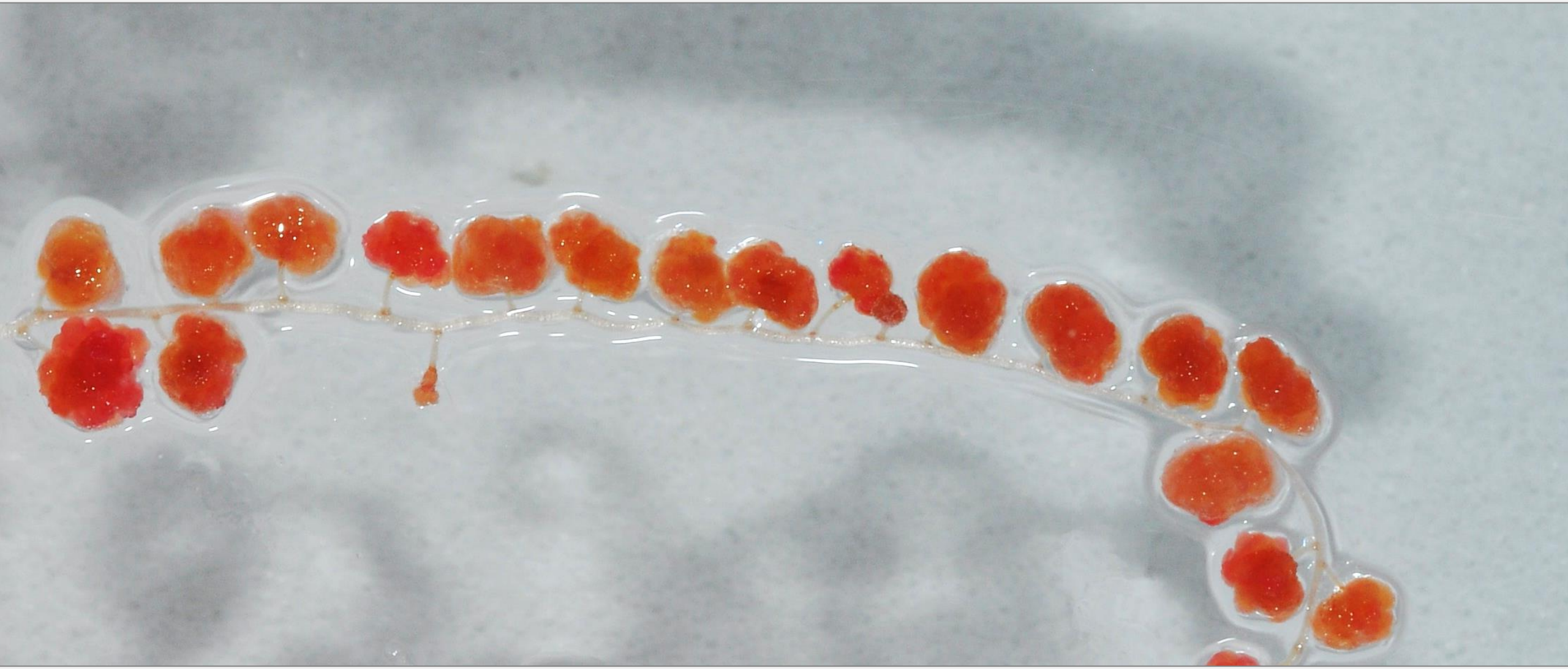
Thank You for Your Time!



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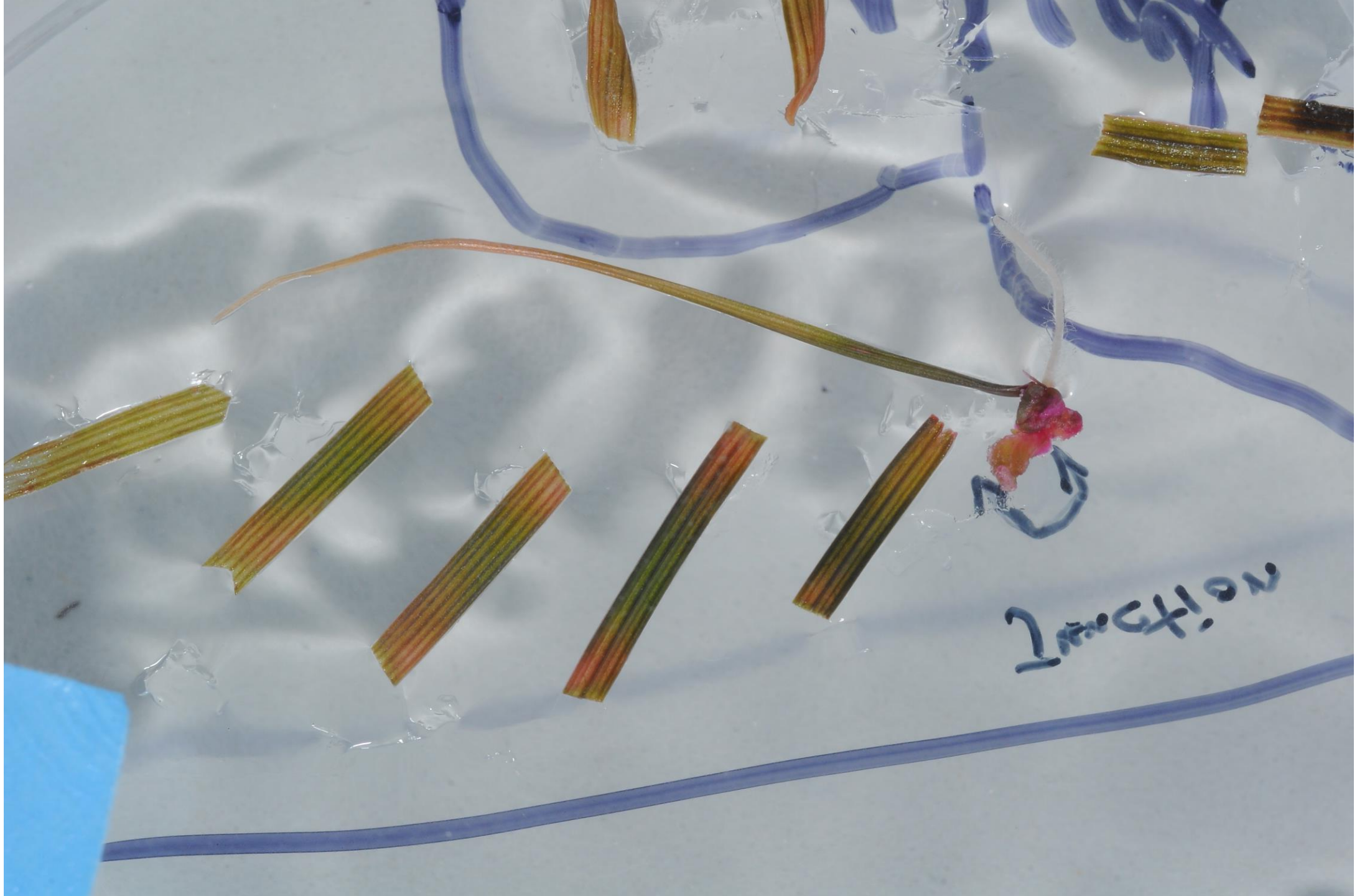
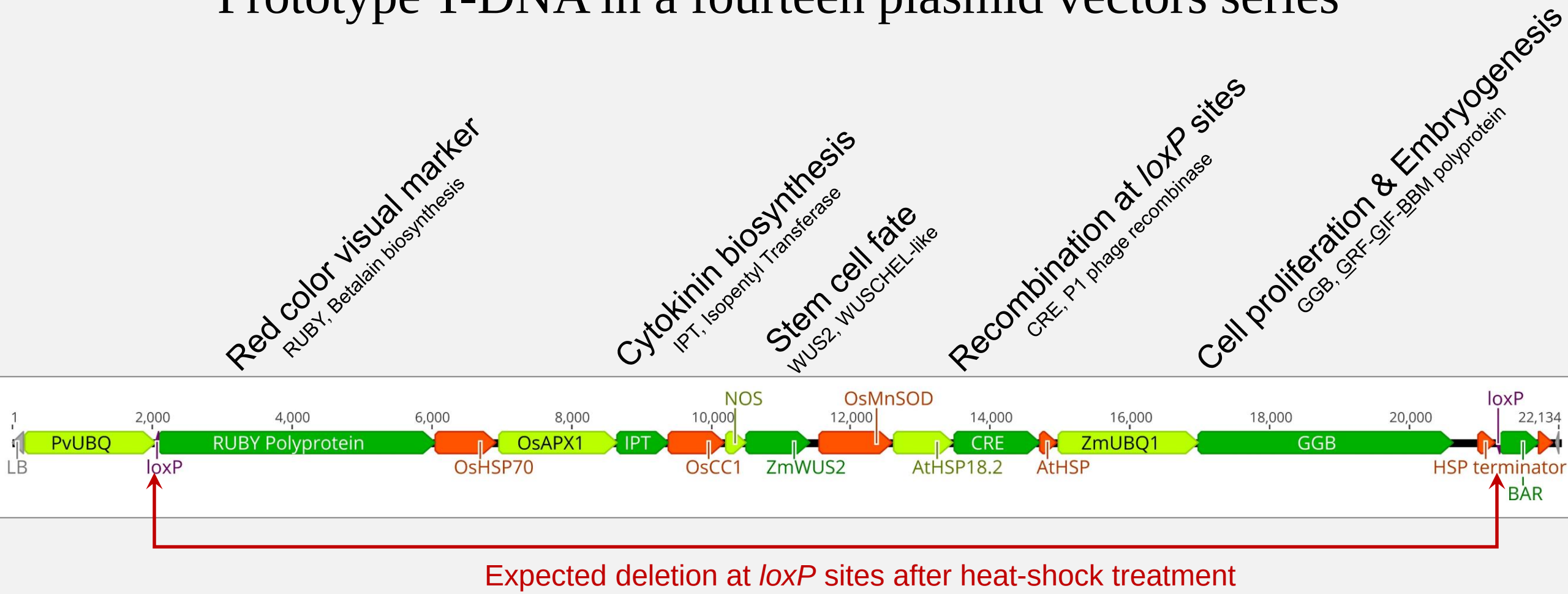

JUNCTION

Prototype T-DNA in a fourteen plasmid vectors series



The Design-Test-Repeat cycle
https://www.strategyzer.com/library/design-test-repeat-cycle-visual

Species-specific constrains of genetic transformation freedom

We work with single-seed-descend clones

1. A standard ten (often twenty) weeks of vernalization (4 °C) to induce flowering

2. Flowering asynchrony

3. One-year-long seed-to-seed cycle

4. Active two loci S/Z self-incompatibility

5. High natural genetic variation in cultivated varieties and wild accession

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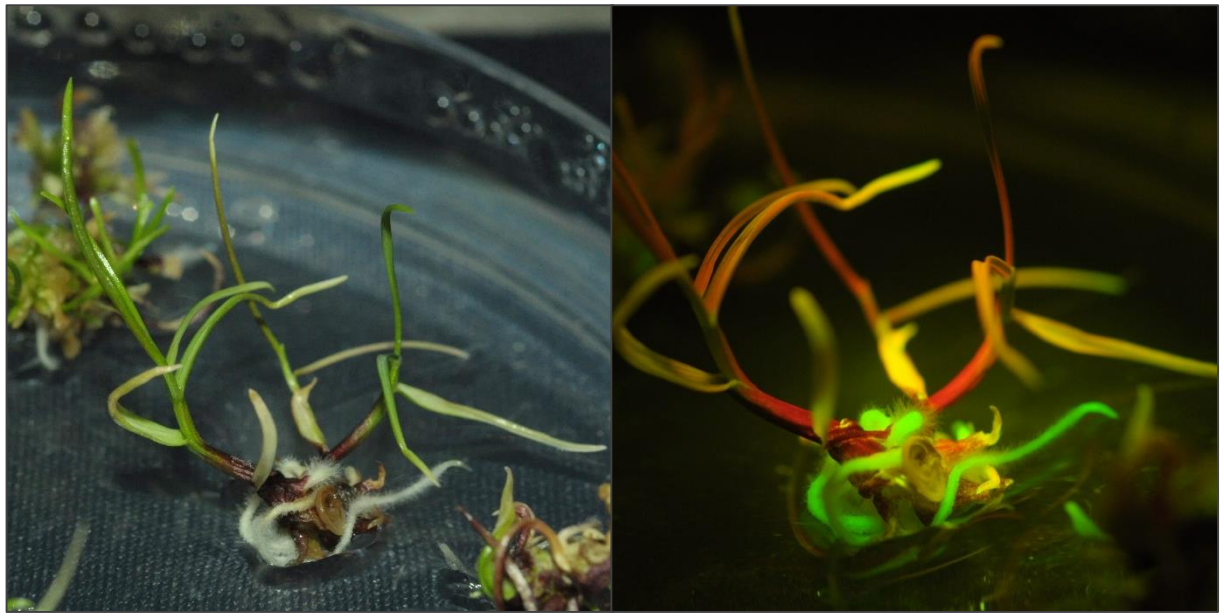
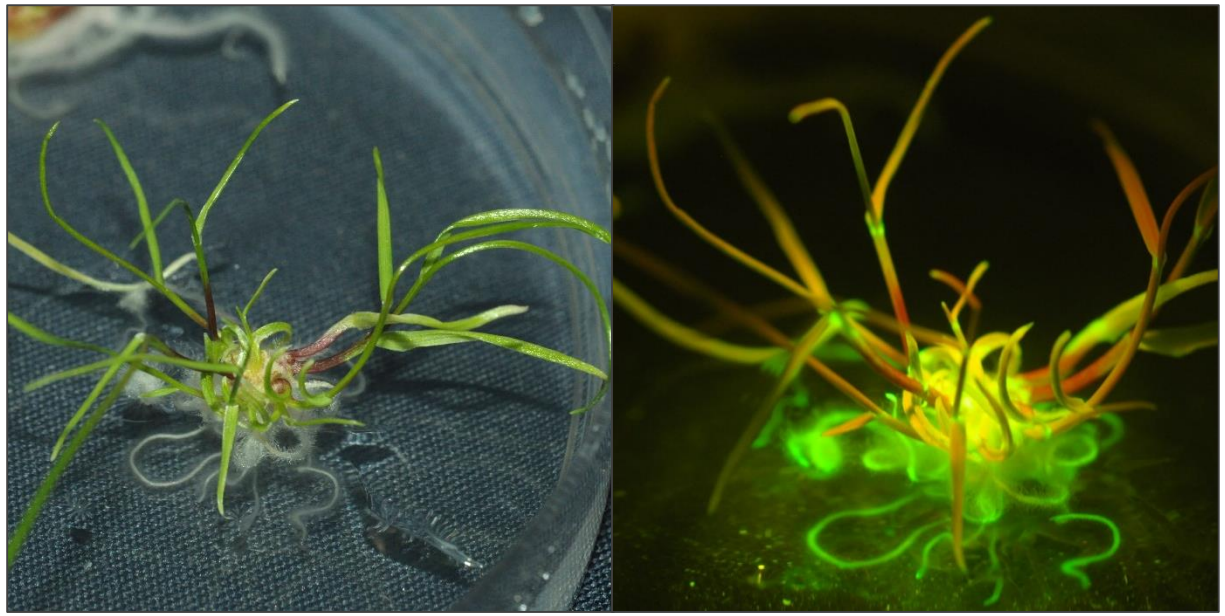
Towards genotype independent ryegrass genetic transformation



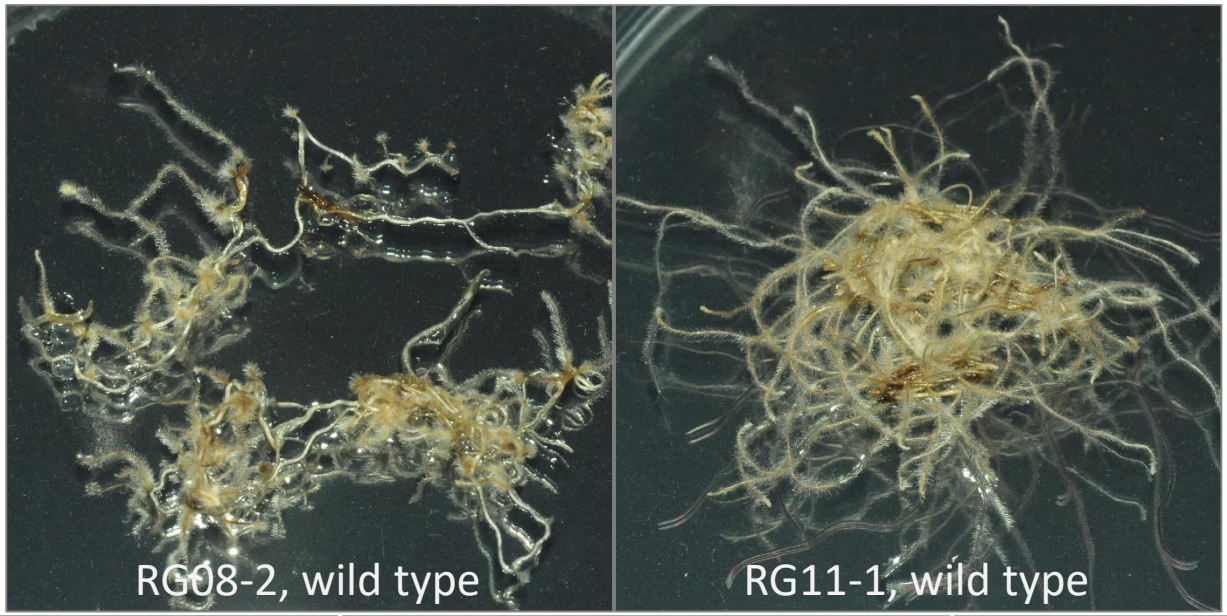
BBM → WUS

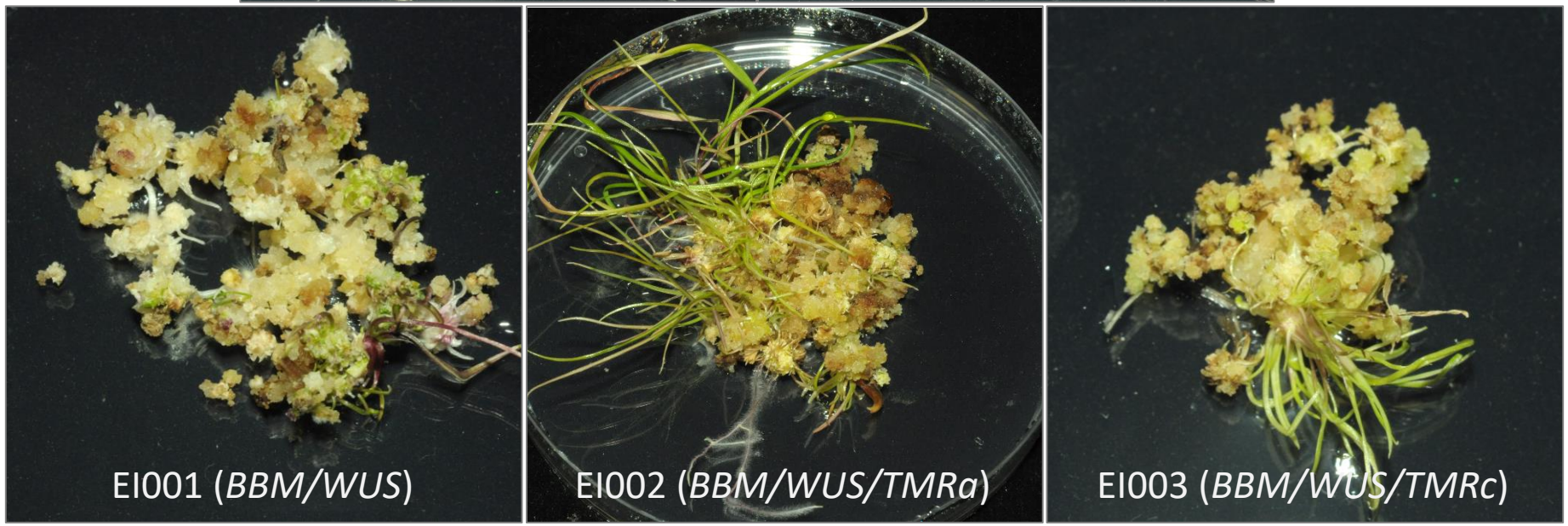


BBM → WUS → TMR

BABY BOOM (*BBM*), *WUSCHEL* (*WUS*), *CYTOKININ BIOSYNTHESIS* (*TMR*) – tissue culture recalcitrance suppression genes
Images to the right are under NightSea blue light, showing ZsGreen protein fluorescence

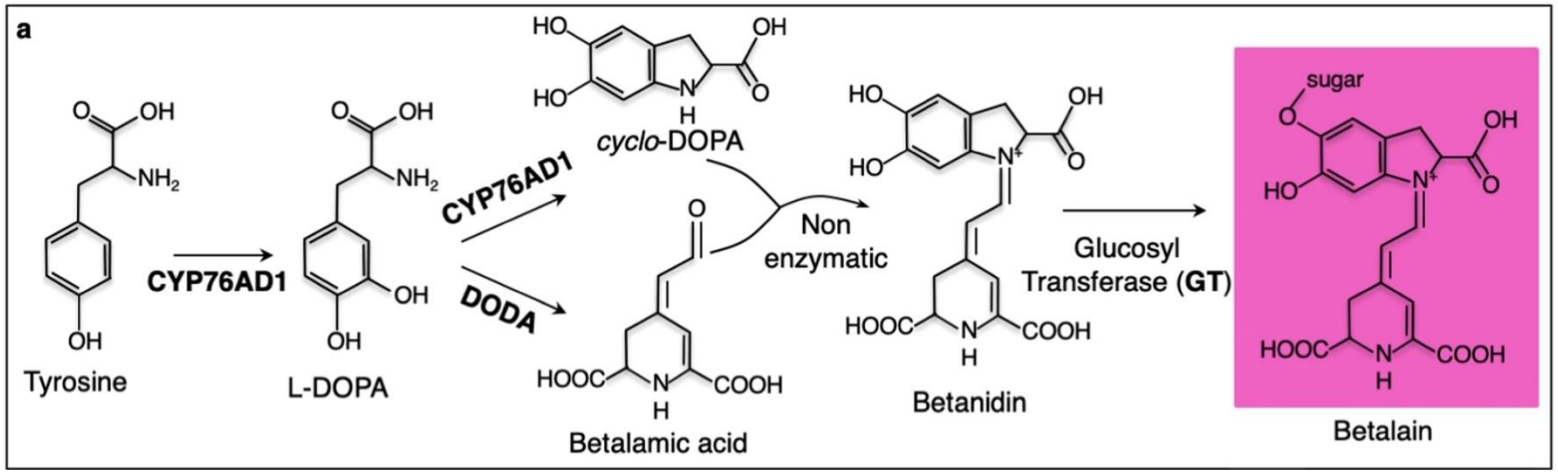
Tissue culture recalcitrance suppression in ryegrass roots





Roots of wild types and transgenic lines were cultured on the media for 4 weeks

RUBY polyprotein fusion as visual reporter



CYP76AD1	P2A	DODA1	P2A	cDOPA5GT

CYP76AD1 and DODA1 red beet proteins, and *Mirabilis jalapa* cDOPA5GT are interspersed with a self-cleaving P2A peptide are expressed as a single polyprotein known as RUBY developed by a team of Chinese researchers.

Root explant *Agrobacterium*-mediated transformation with RUBY containing T-DNA