Enhancing the Efficiency of Somatic Embryogenesis and Genetic Transformation in *Theobroma cacao* L.

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Public Dissertation Defense
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Theobroma (Greek) – “food of the gods”

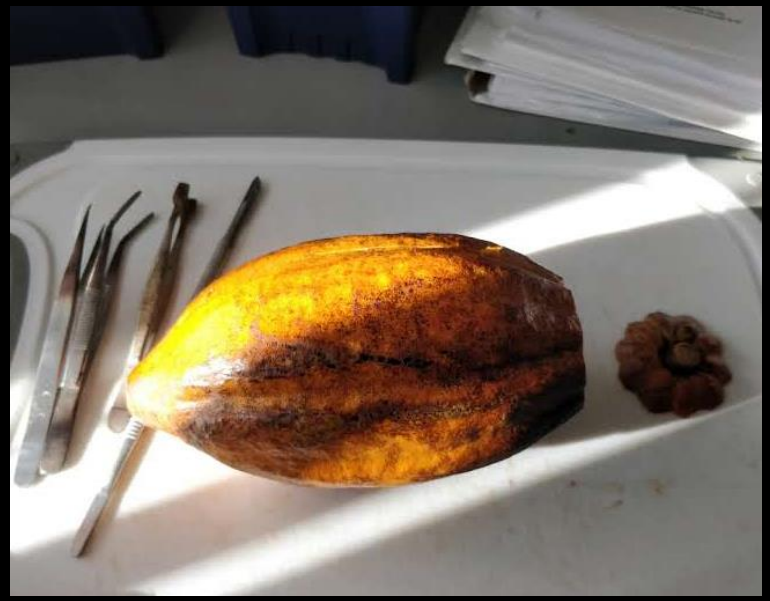
Young Carl Linnaeus (1792)

https://s3-us-west-2.amazonaws.com/ntbgmeettheplants/images/600h/1571.jpg
https://www.aoc.gov/sites/default/files/styles/scaled_lg/public/crosssection_of_theobroma_cacao_chocolate_tree.jpg.webp?itok=Q_TweFjv

https://earthlingnature.wordpress.com/wp-content/uploads/2018/05/429px-carolus_linnaeus_by_hendrik_hollander_1853.jpg

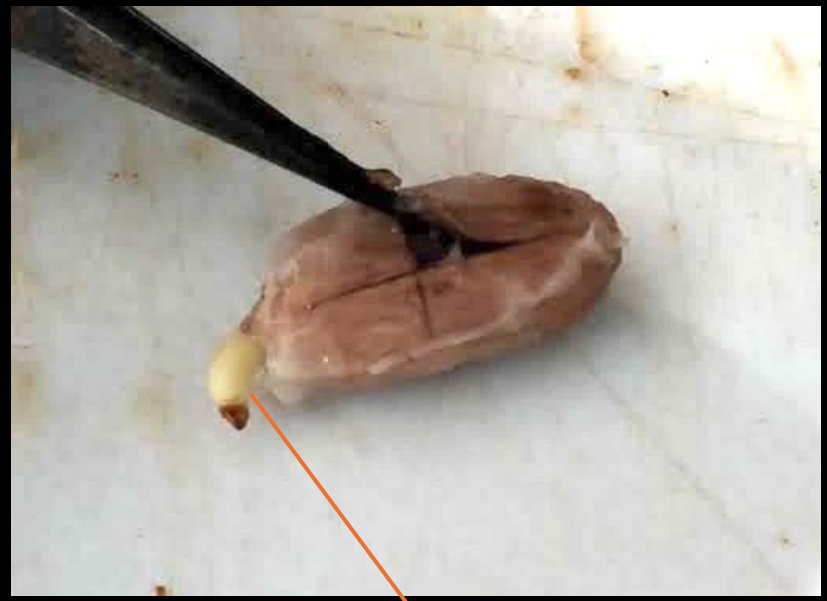
unopened immature flower &
open mature flower

https://www.jungleblunts.com/wp-content/uploads/2022/01/cacao-tree-1.jpg



cacao pod

zygotic embryos



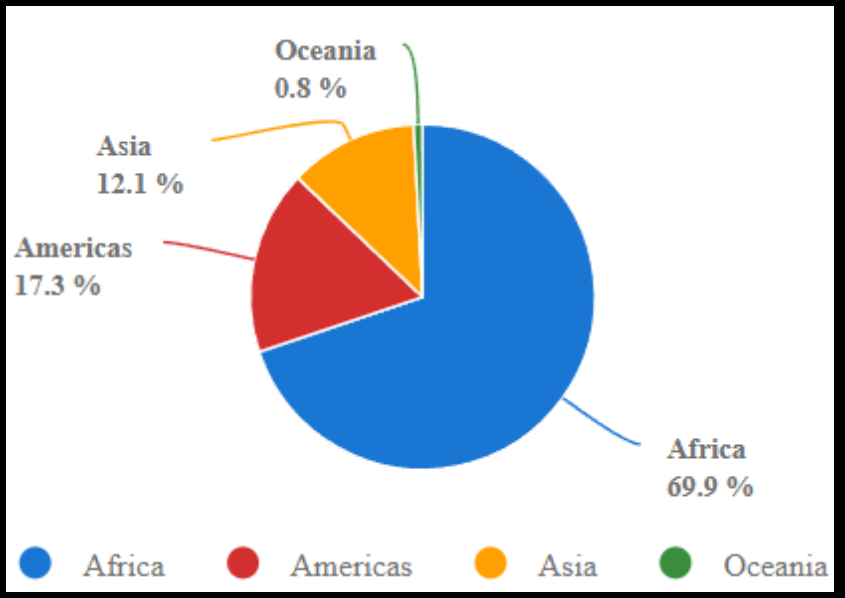
emerging root

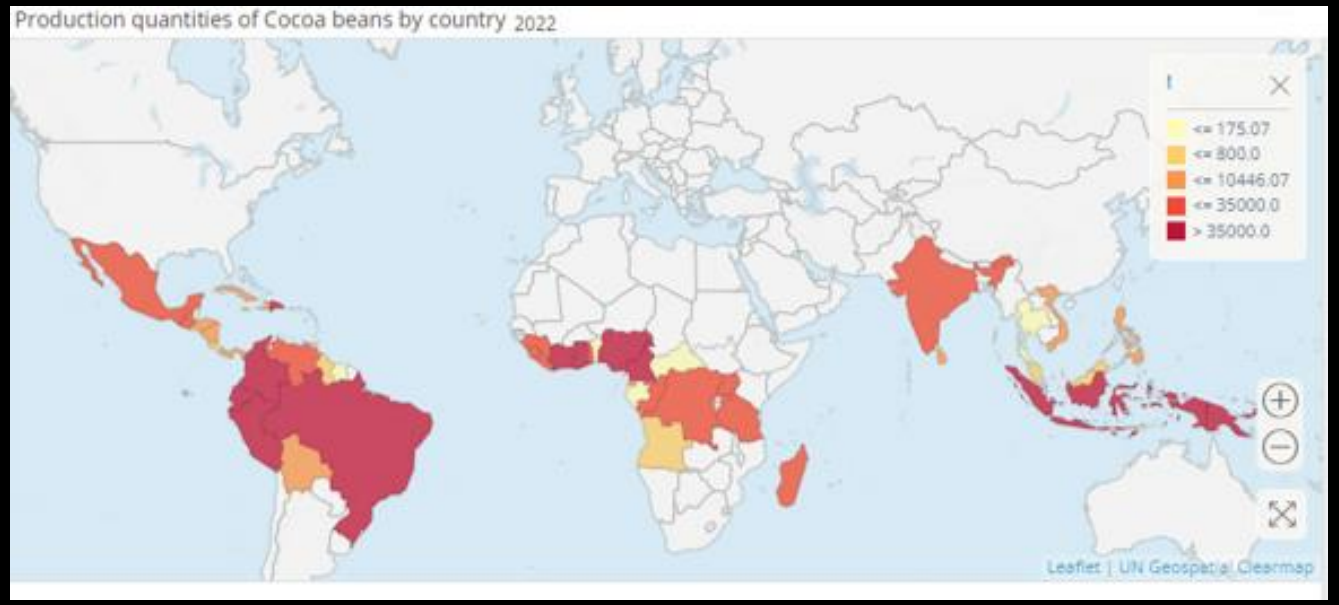
https://www.cacao-barry.com/sites/default/files/styles/paragraph_video_fallback/public/2023-05/Episode-8---Fermentation.jpg?itok=Y8_UWJ0w

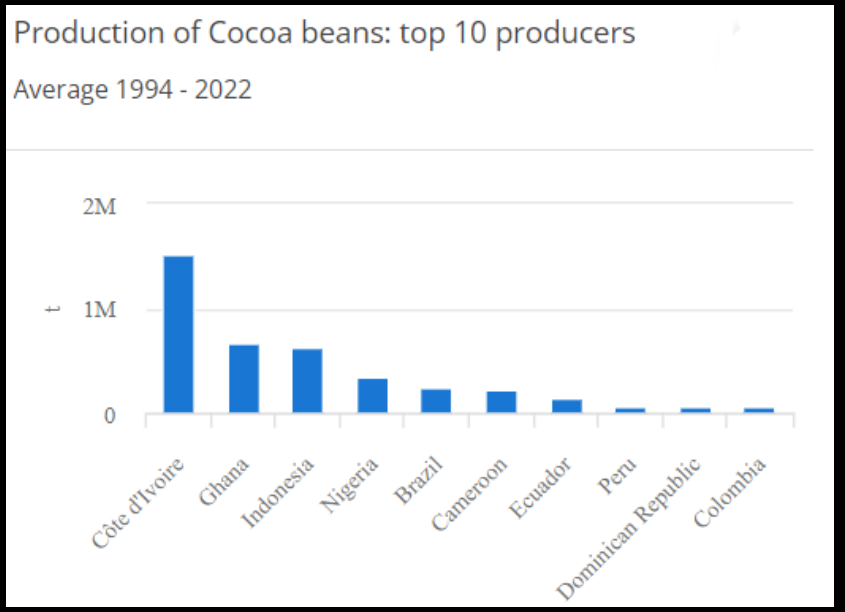
Lanaro, Matthew & Desselle, Mathilde & Woodruff, Maria. (2019). 3D Printing Chocolate. 10.1016/B978-0-12-814564-7.00006-7.

Cacaos' Economic Importance

- UN Food and Agriculture statistics (FAOSTAT) estimates ~5,874,582 million tons of cacao beans (2022)
- Gross production value ~ $8.33 billion
- Retail market expected to grow to ~$189.89 billion in 2026







https://www.fao.org/faostat/en/#data/QCL/visualize

Cacao Diseases

- Cumulative losses of productivity from pests and diseases – **20-38% globally**
- **Three diseases** responsible for **~87%** of global annual **losses**
- Biologically superior varieties are the preferred strategy
- **Annual** global **replanting requirement** of **~1 billion plants/year**

Black Pod Rot

https://bugwoodcloud.org/images/768x512/1317030.jpg

Witches' Broom Disease

https://www.nybg.org/blogs/science-talk/content/uploads/2015/04/0415-pink-mushrooms296x460.jpg

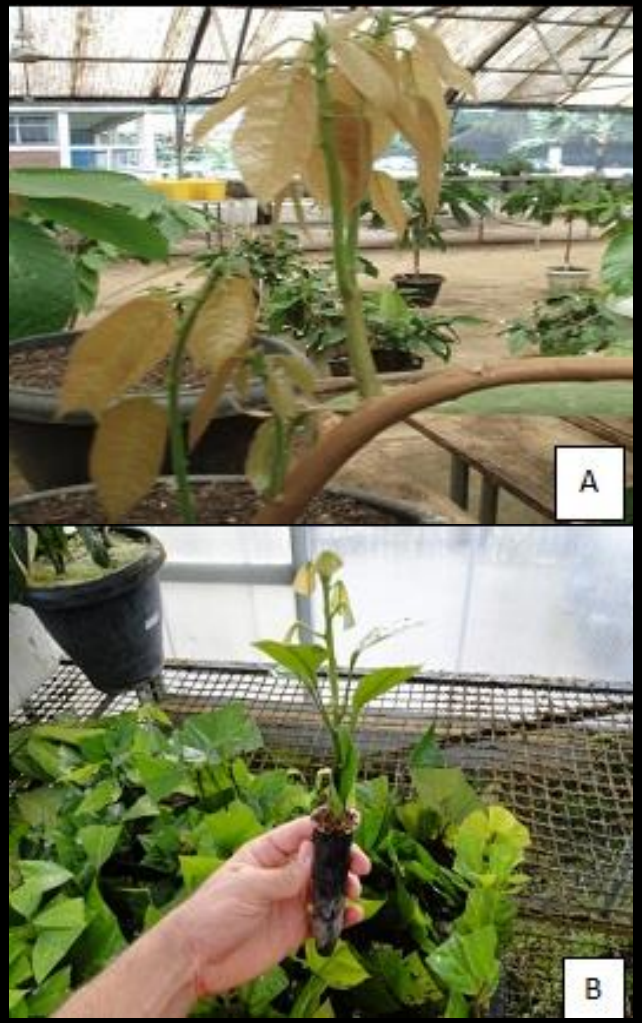
Cacao Swollen Shoot Virus

https://upload.wikimedia.org/wikipedia/commons/e/ef/Cocoa_Swollen_Shoot_Stem_symptom.jpg

Cacao Propagation Strategies

- Seedlings are most commonly used
- Low labor input
- High % of healthy plantlets
- Variation in traits
- Loss of breeding improvements

Cacao Asexual Propagation Strategies



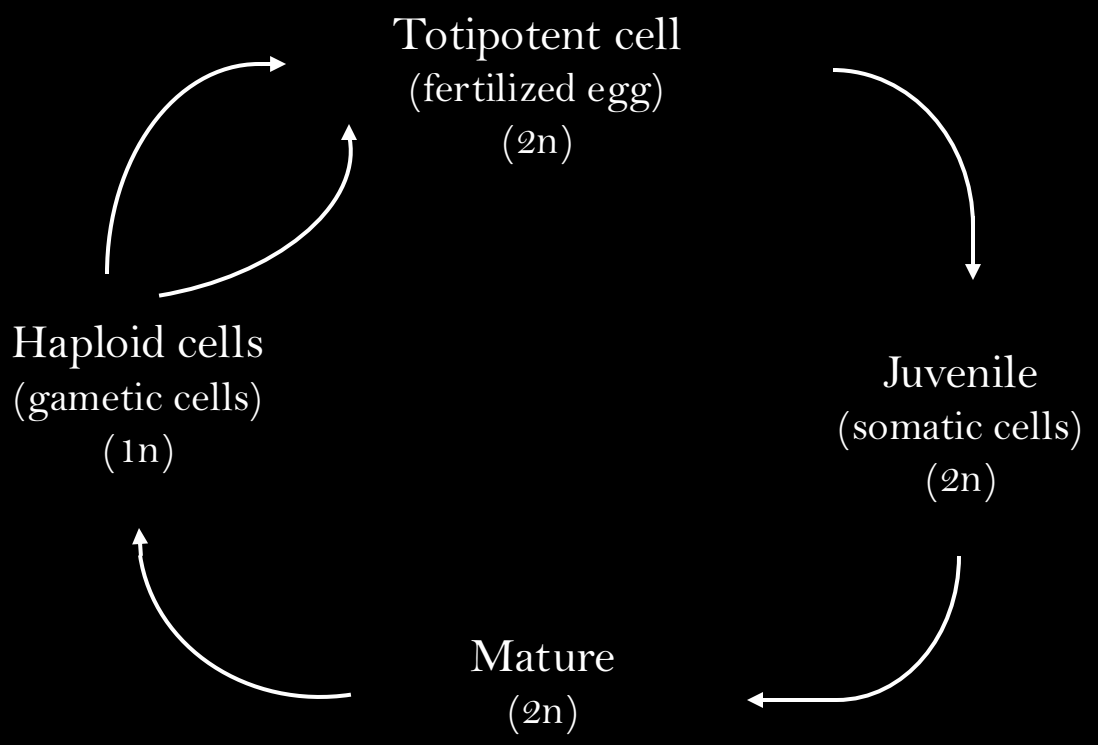
Cuttings

Air Layering

Grafting

https://www.youtube.com/watch?v=wpW4sNWJ6vA
https://minio.scielo.br/documentstore/1806-9967/rnWHGgHNV6npxWQjwbCyDSh/2df14055dbfd37facd49f816ea037a018b41e44d.jpg
https://www.growables.org/information/TropicalFruit/documents/CacaoCTAHR.pdf
https://agronosotros.com/grafting-our-cacao-saplings/

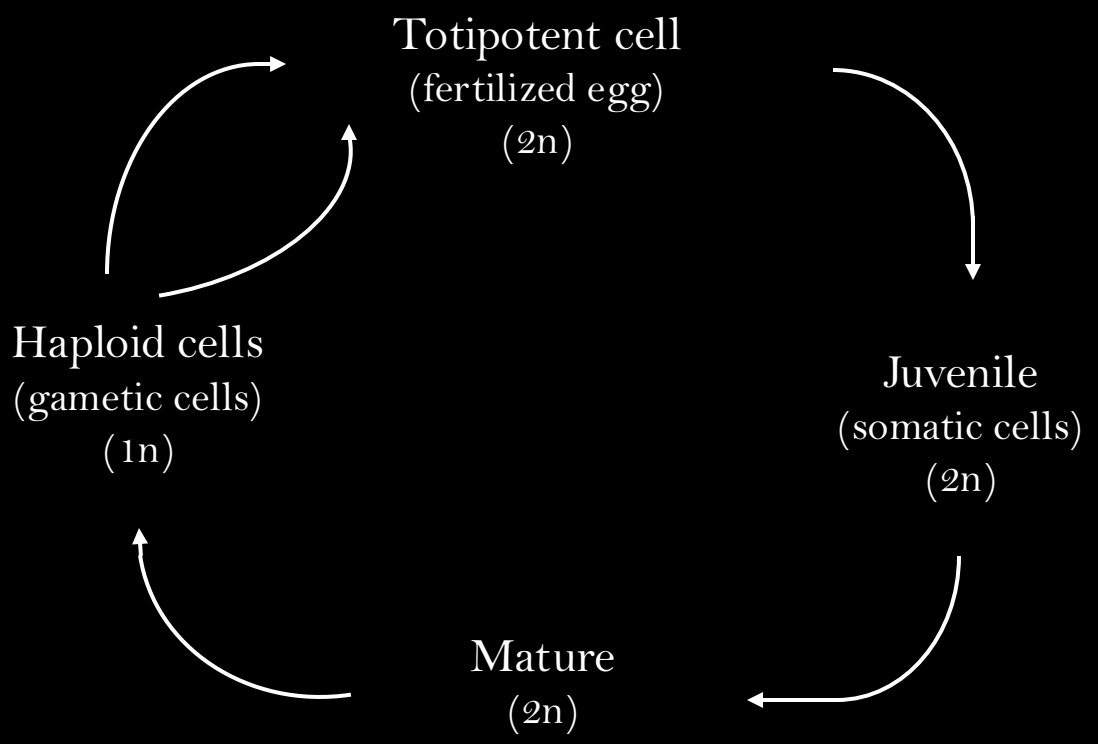
Lifecycle of a sexually reproducing organism



Totipotency ~ the ability to give rise to **all** the different cell-types and tissues to fully regenerate an organism in a permissive environment

Pluripotency ~ the ability to give rise to **multiple** different cell-types and tissues of an organism in a permissive environment

Lifecycle of a sexually reproducing organism



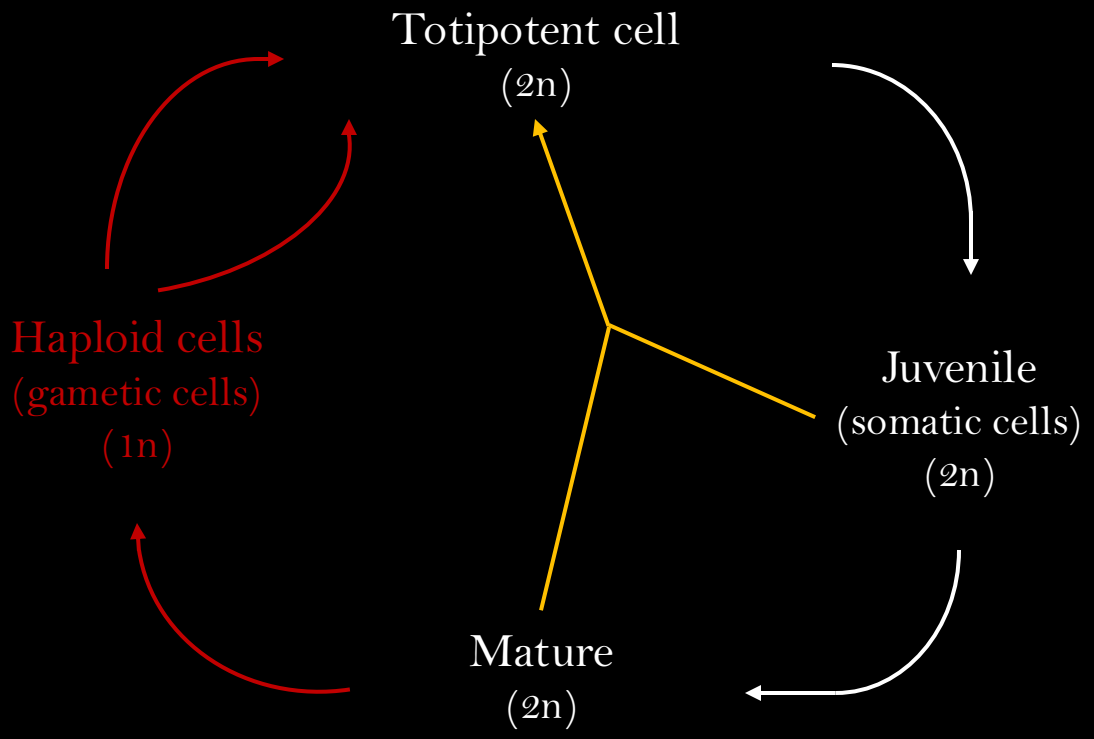
Differentiation ~ the process by which unspecialized cells transform into specialized cells with defined structures, functions, and roles

Somatic cell ~ any cell of an organism apart from its' reproductive cells

Somatic Embryogenesis

The process of induced totipotency where embryos are formed from somatic cells rather than zygotic germline cells

Kalanchoe sp.

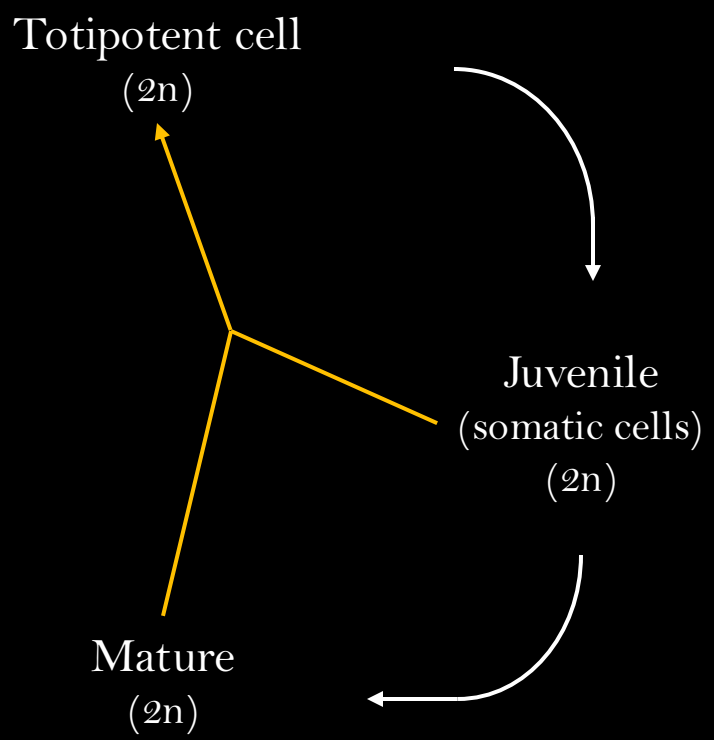


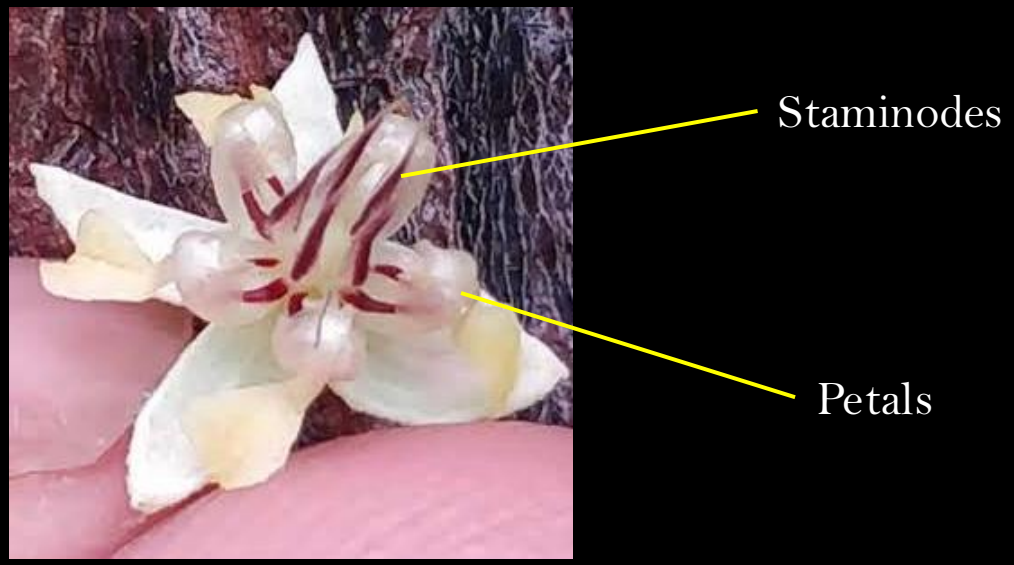
- Occurs in over 500 species of higher plants
- Can be induced *in-vitro*
- Uses genetic & developmental pathways of seed development

Cacao Somatic Embryogenesis Induction

Competent cell ~ capable of de-differentiating towards totipotency

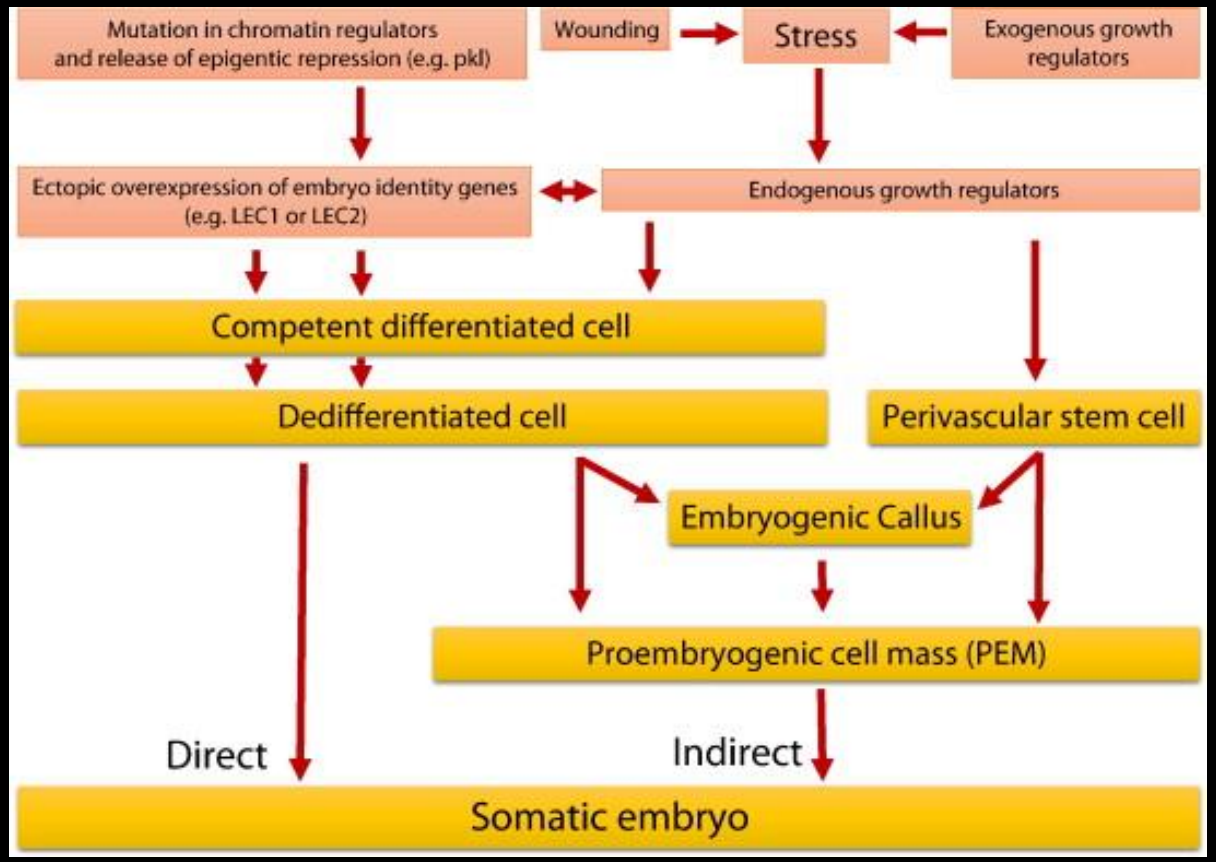
In-vitro recalcitrance ~ when plants fail to induce somatic embryogenesis in tissue culture despite extensive optimization of conditions





Cacao Somatic Embryogenesis Induction

- Induction occurs through stress
- Most tissue culture systems use plant growth regulating hormones like 2,4-dichlorophenoxyacetic acid (auxin) & thidiazuron (TDZ) (cytokinin)
- Results in endogenous auxin production
- Expression of totipotency-promoting and embryo-identity genes
- Removal of stress allows for somatic embryo development



(Feher *et al.*, 2015)

Cacao Somatic Embryogenesis (SE)

PCG
Two-week TDZ

SCG
Two-week 2,4-D

EDN

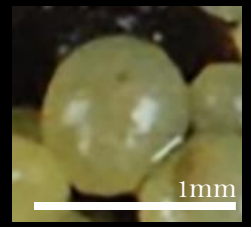
Petals &
Staminodes

Somatic Cell → De-differentiation → Acquisition of totipotency → Differentiation

Indirect SE

Callus proliferation

Proembryogenic mass



Globular Embryo

Heart-stage

Torpedo-stage

Cotyledonary stage

Plantlet conversion

4 weeks after culture initiation (ACI)

8-16 weeks ACI

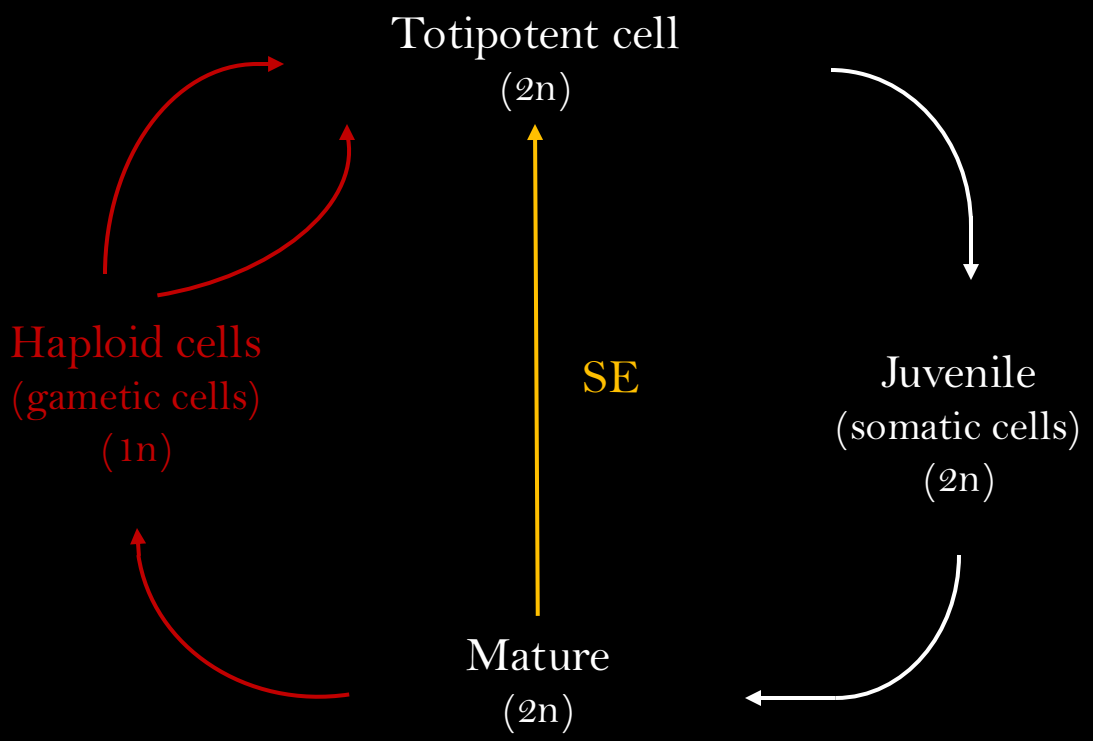
Callus ~ unorganized mass of cells in various states of differentiation

Proembryogenic masses (PEMs) ~ Cluster of proembryogenic cells from which embryos emerge

https://cdn.shopify.com/s/files/1/0223/5181/products/Cacao_Flower-1024x1024_1200x.jpg?v=1639537311

https://plantscience.psu.edu/research/labs/guiltinan/resources/protocols/photographs-of-cacao-tissue-culture-protocols/stages-of-cacao-somatic-embryogenesis

Propagation via Somatic Embryogenesis



Advantages:

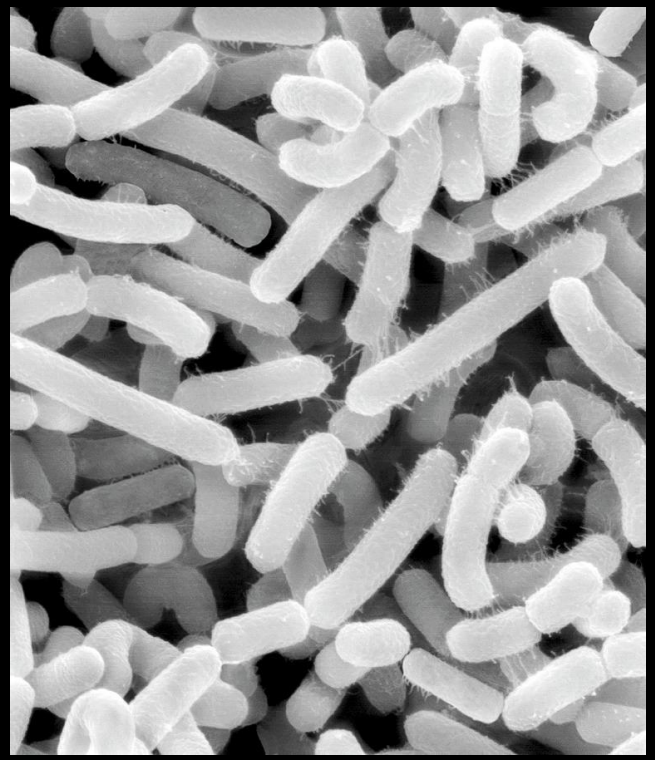
- Highest multiplication ratio for asexual propagation
- Maintain agronomic characteristics of the parent
- Virus and disease-free
- SE-derived plants are just as healthy as seed-derived

Disadvantages:

- Highly skilled labor
- Expensive infrastructure
- Optimization of tissue culture protocol
- Genotype dependent
- Requires mature trees to initiate SE (2-6 years)
- Abnormalities of somatic embryos

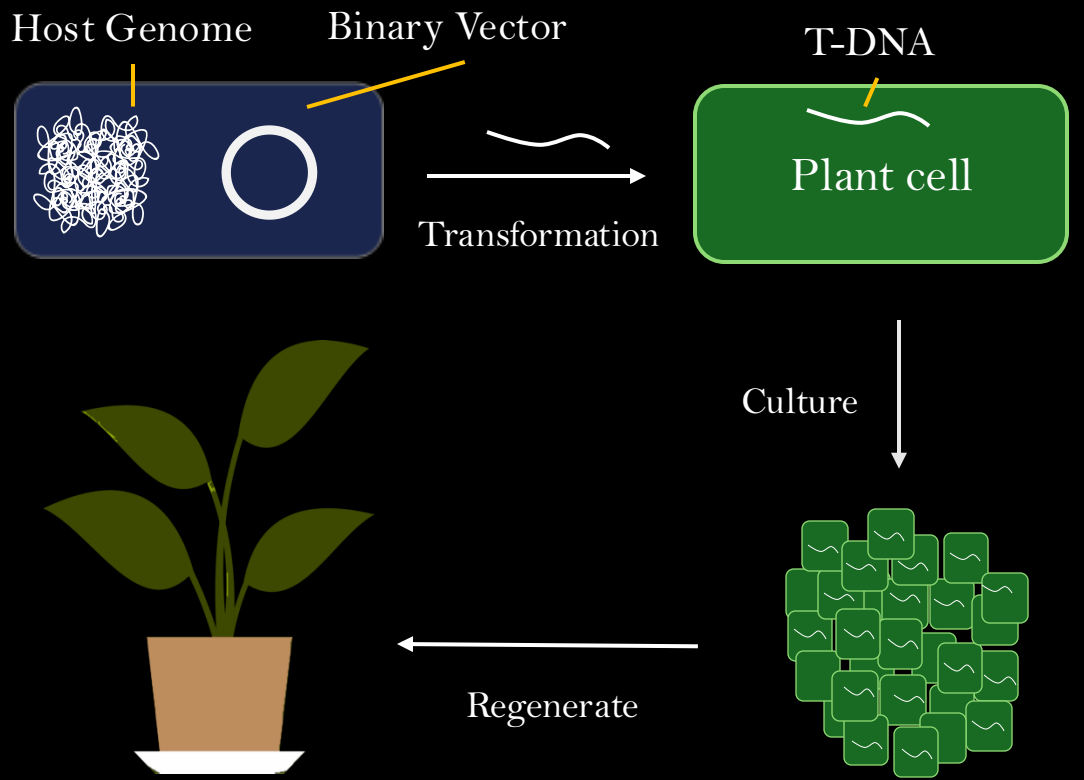
Genetic Transformation

The process of altering an organisms' DNA by adding, removing or changing sequences for the purposes of affecting changes within the host cell



Agrobacterium tumefaciens

Crown-gall disease



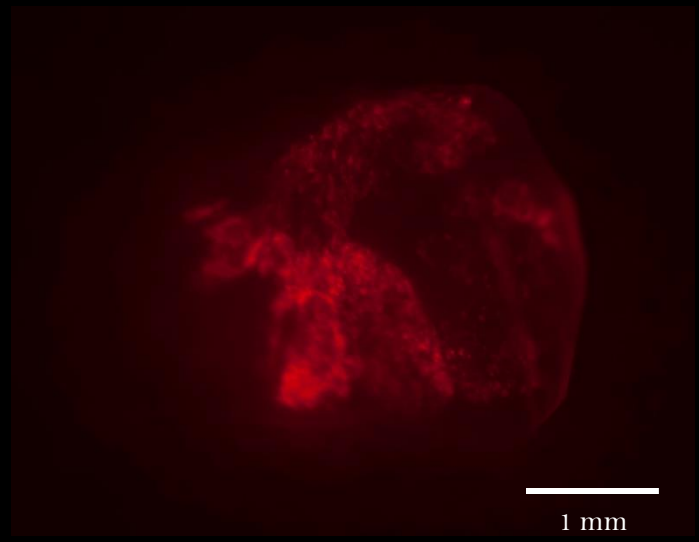
'biologically superior' variety

https://images.fineartamerica.com/images-medium-large-5/7-agrobacterium-tumefaciens-dennis-kunkel-microscopyscience-photo-library.jpg https://www.shutterstock.com/image-photo/giant-tree-burl-caused-by-600nw-2140647801.jpg

Cacao Genetic Transformation

Somatic embryo <u>cotyledons</u> are the only tissues **amenable** to stable *Agrobacterium* transformation and subsequent regeneration

Agrobacterium mediated transformation



cacao SE cotyledon transformed with mScarlet fluorescent gene

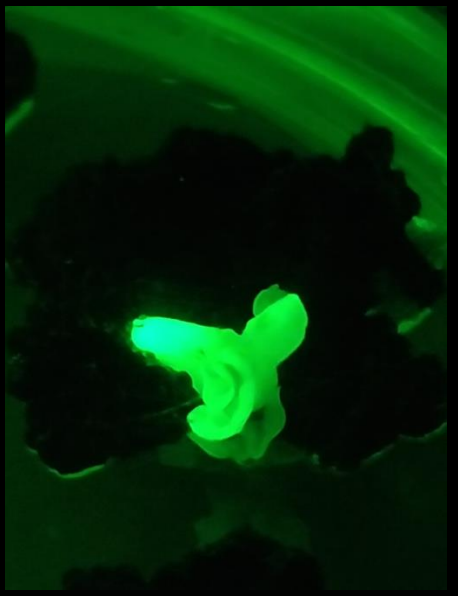
Cacao Genetic Transformation

Cacao somatic embryos

SE cotyledon explants

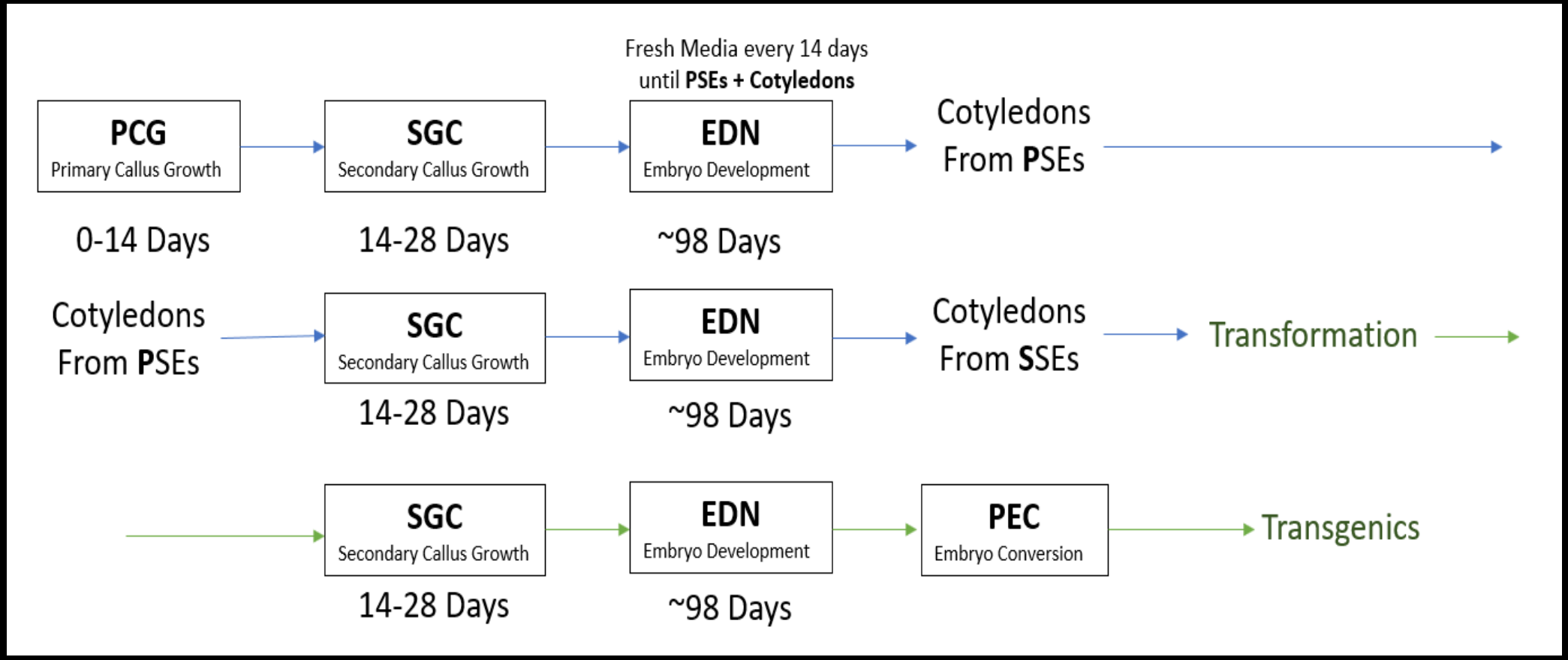
Agro-mediated transformation

Culture and regenerate SEs



Transgenic somatic embryo expressing EGFP (enhanced green fluorescent protein)

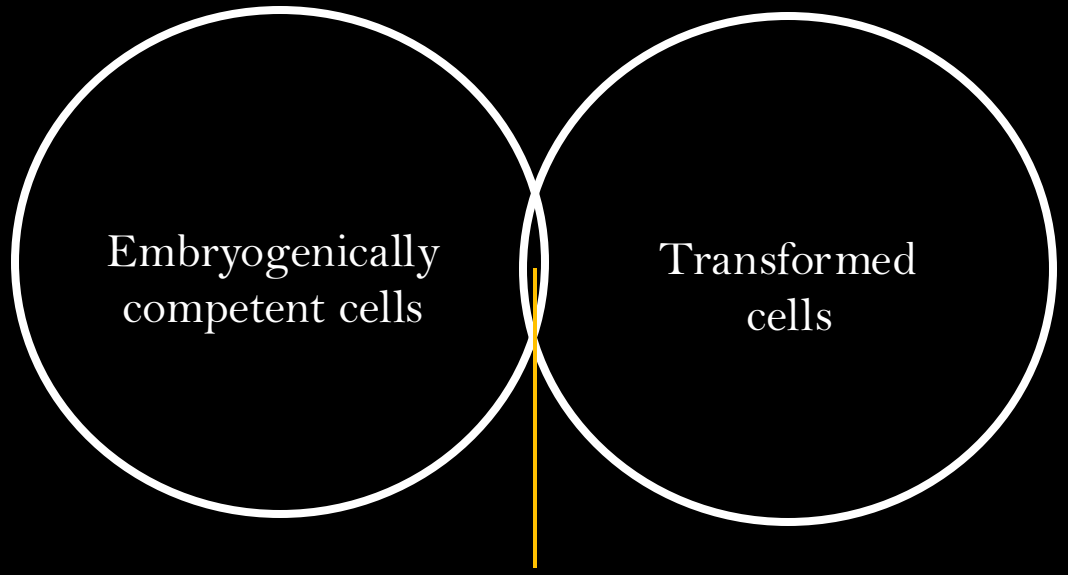
Cacao Genetic Transformation



Cacao Transformation Efficiency

Transformation Recalcitrance ~ Inability to incorporate foreign DNA into the host plants' genome

- Transient ~ The T-DNA is not reaching and expressing within the host cells' nucleus
- Stable ~ T-DNA is not integrating within the hosts' nucleus and regenerating transgenic SEs
    - Transgenic embryo regeneration efficiency ~0-4%



Two Major Bottlenecks

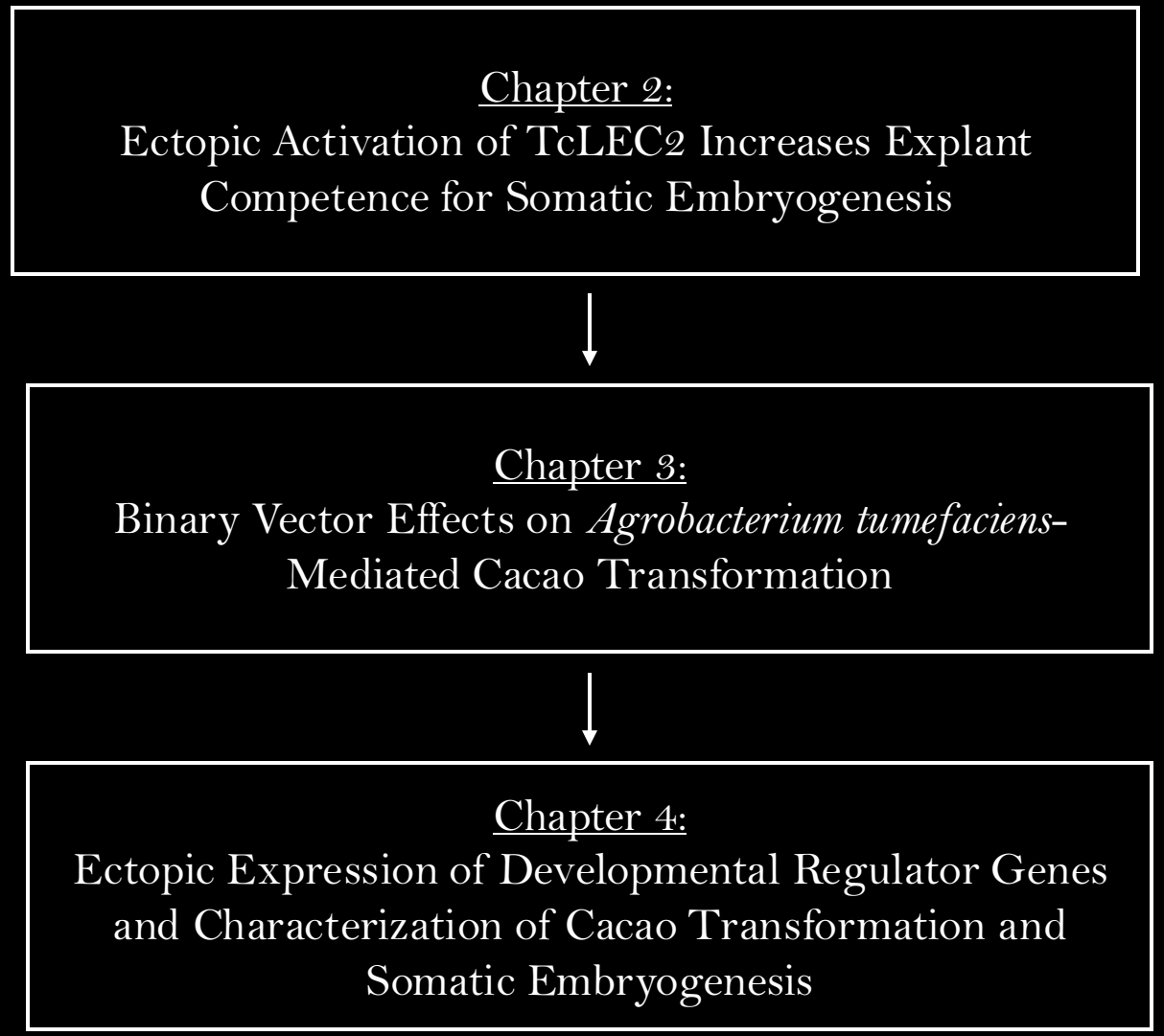
In-vitro recalcitrance:

- Most tissues of cacao trees are not capable of regenerating SEs. This limits all SE work to mature, flowering, trees

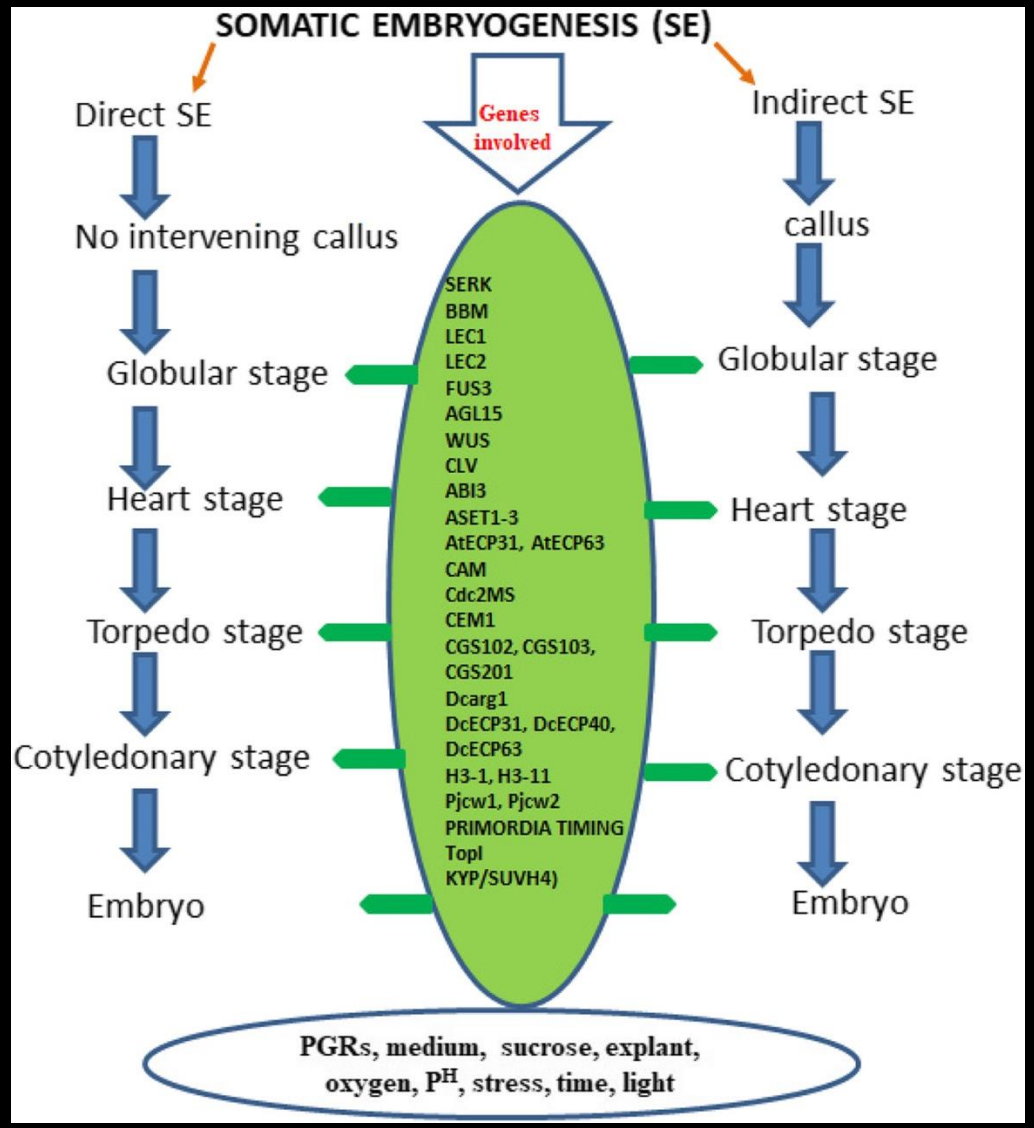
Transformation recalcitrance:

- *Agro*-mediated transformation efficiency is insufficient to increase the number of stable transgenics regenerated

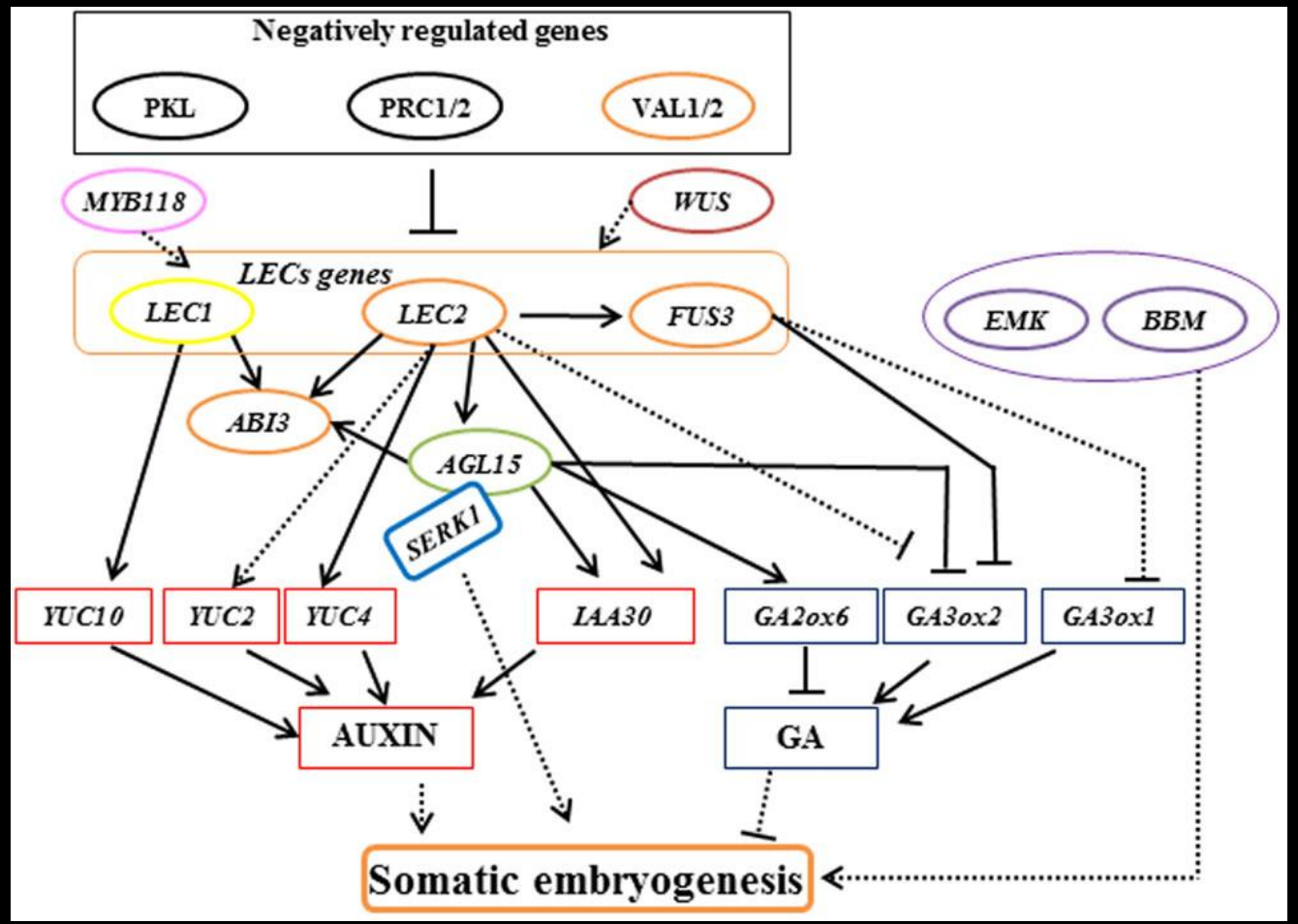
Dissertation Chapters Overview



Chapter 2:
Ectopic Activation of TcLEC2 Increases Explant Competence for Somatic Embryogenesis



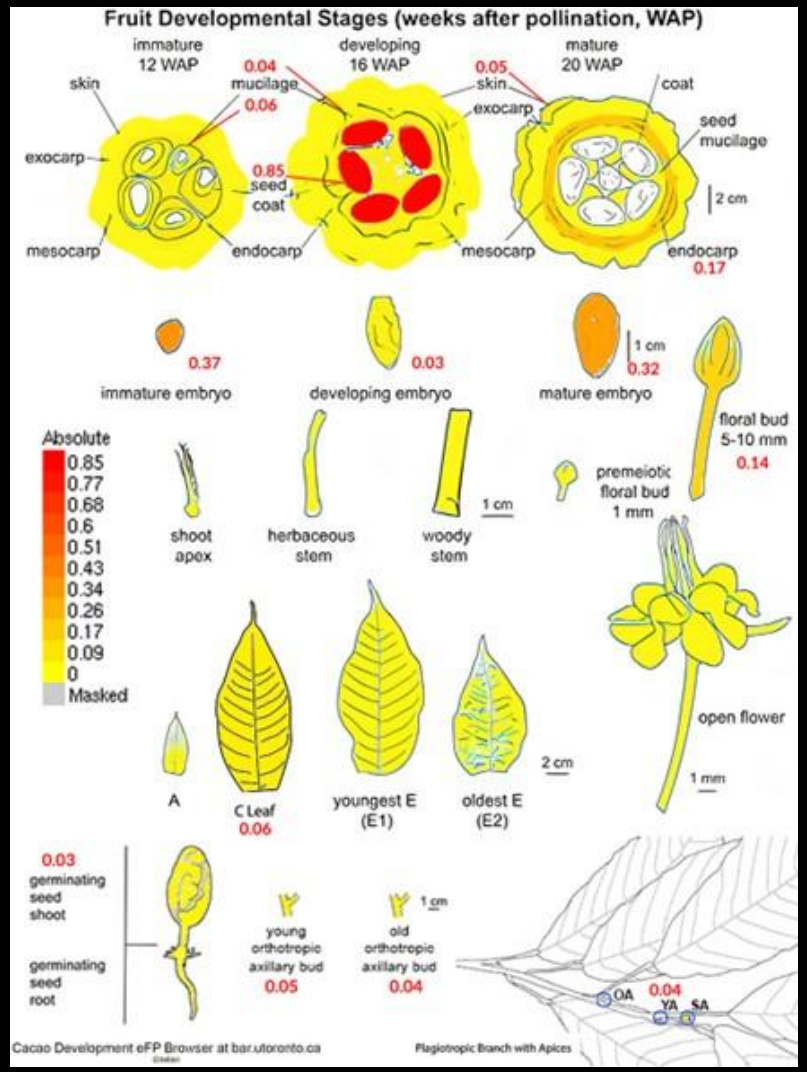
(Gulzar *et al.*, 2020)



(Guan *et al.*, 2016)

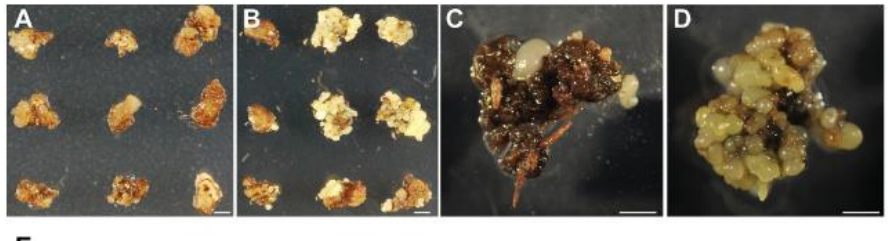
Cacao *Leafy Cotyledon 2* (*TcLEC2*) Transcription Factor

- Master regulator of somatic embryogenesis
- Expressed early in embryonic development and asexual tissues
- Establishes the ideal cellular environment for zygotic embryo development



(Kulesza *et al.*, 2024)

Cacao *Leafy Cotyledon 2* (*TcLEC2*) Transcription Factor



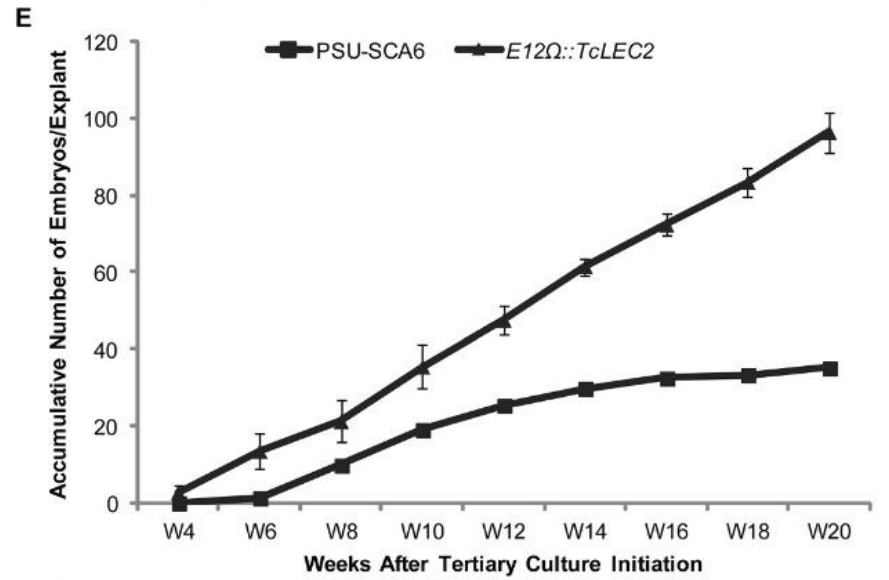
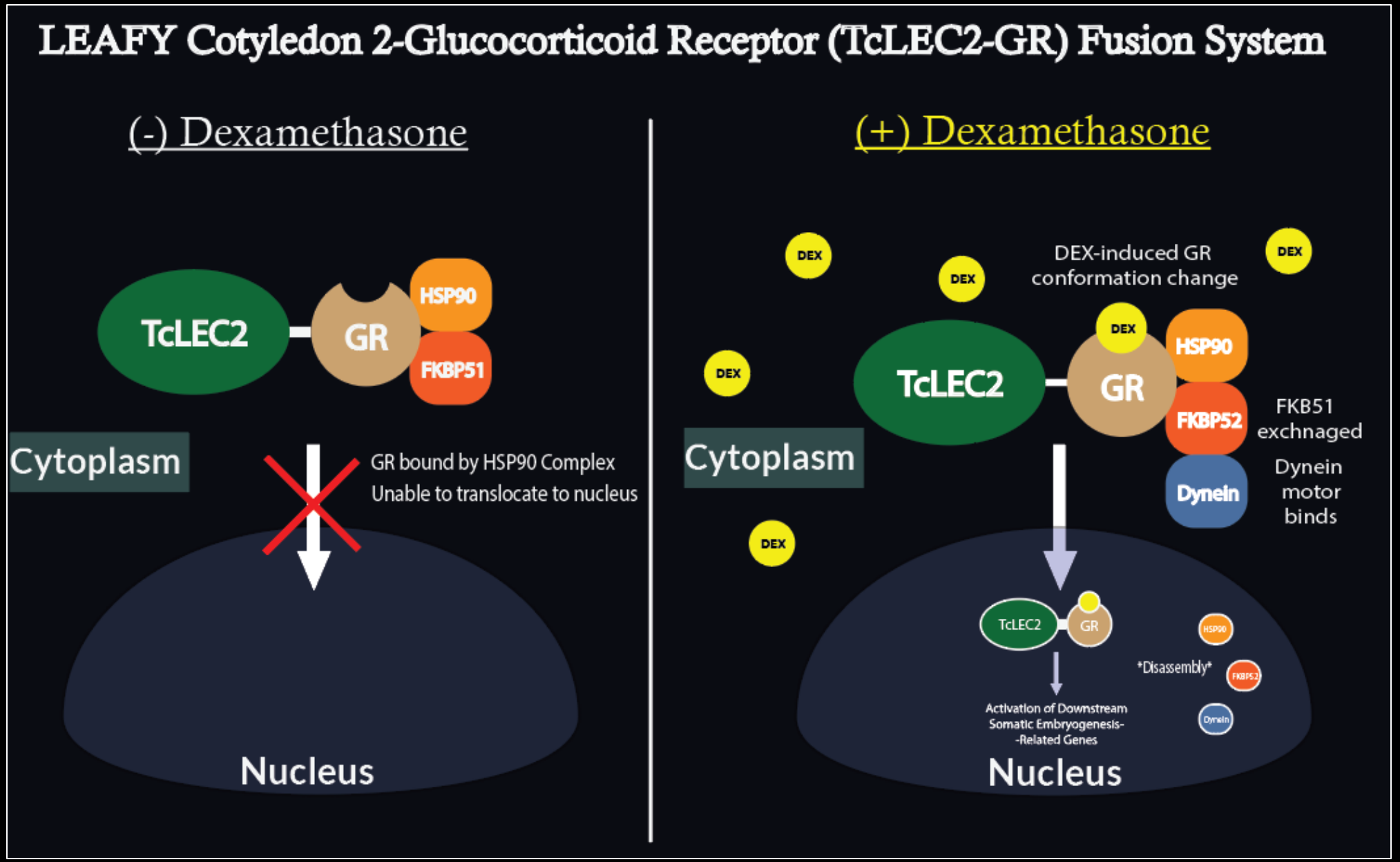


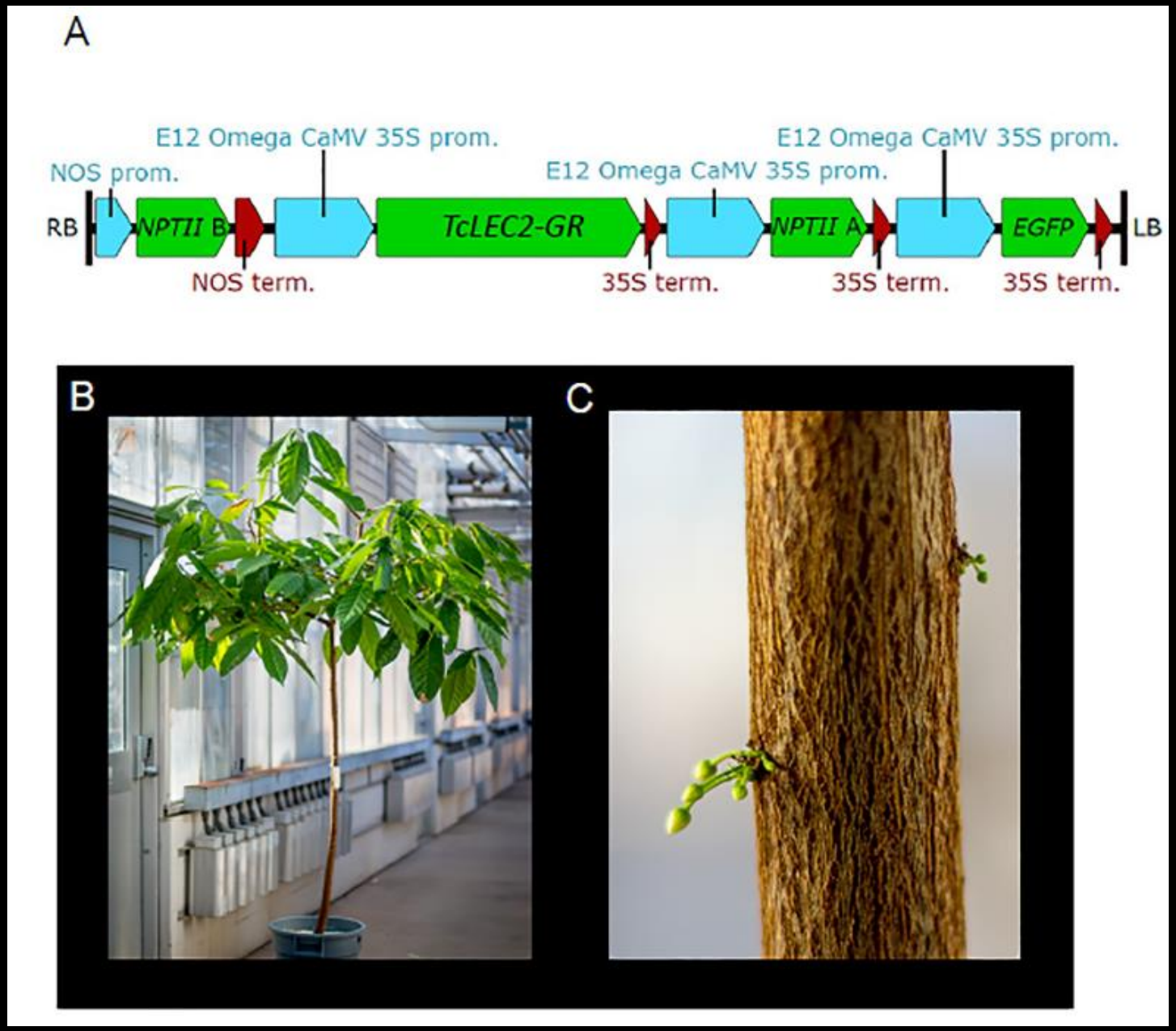
Figure 6 Overexpression of *TcLEC2* increases tertiary somatic embryogenesis efficiency. A. Tertiary *PSU-SCA6* culture on hormone free medium at 4 weeks ACI. **B**. Tertiary stable transgenic E12Ω::TcLEC2 culture on hormone free medium at 4 weeks ACI. **C**. Tertiary *PSU-SCA6* culture on hormone free medium 20 weeks after culture initiation. **D**. Tertiary stable transgenic E12Ω::TcLEC2 culture on hormone free medium at 20 weeks ACI. **E**. Average number of tertiary embryos produced per explant from *PSU-SCA6* and stable transgenic E12Ω::TcLEC2 explants (n = 4, mean ± SE) (Bars = 2 mm).

- Constitutive expression results in cyclic embryogenesis
- Transgenic embryos are unable to develop properly

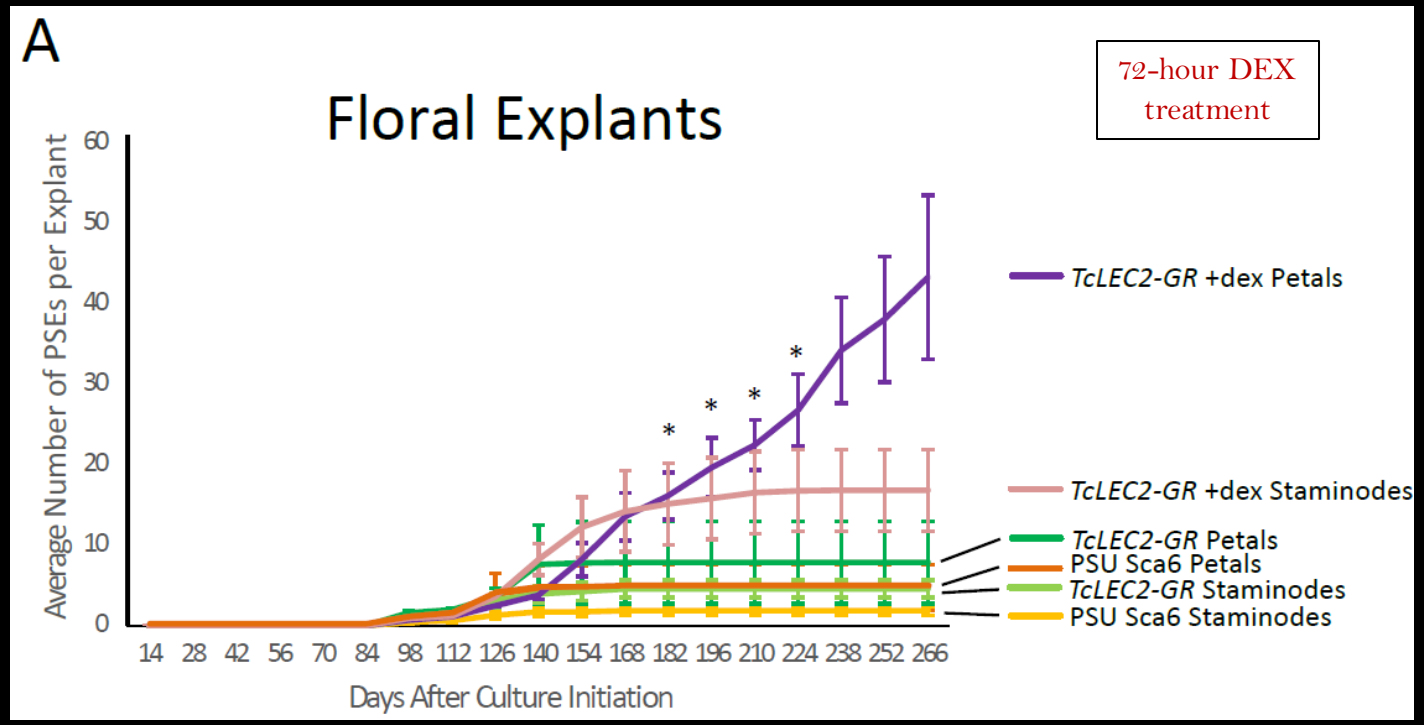
(Zhang *et al.*, 2014)

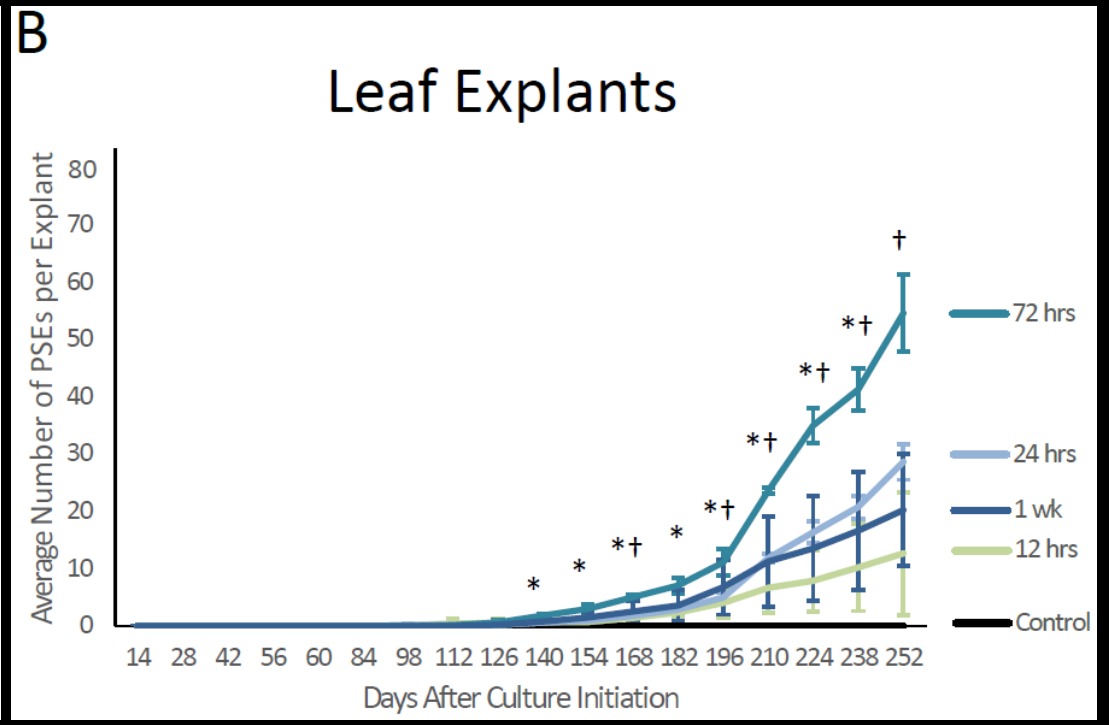


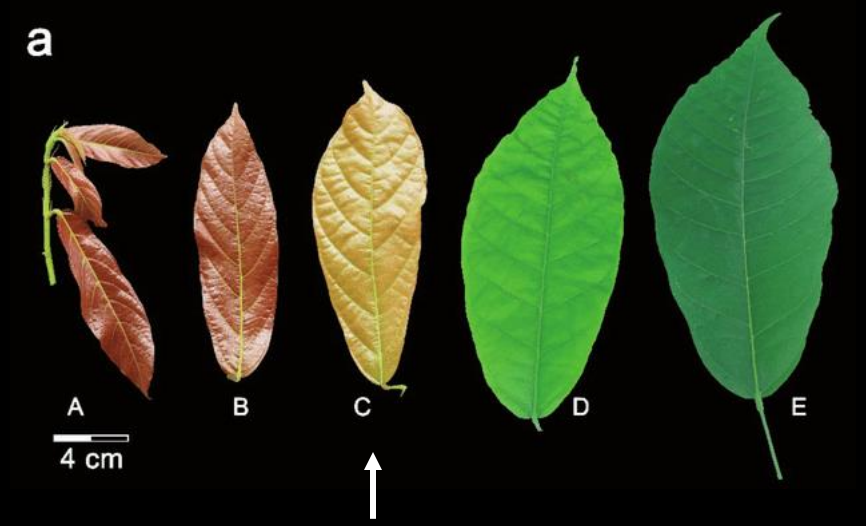
(+) DEX = ACTIVATED

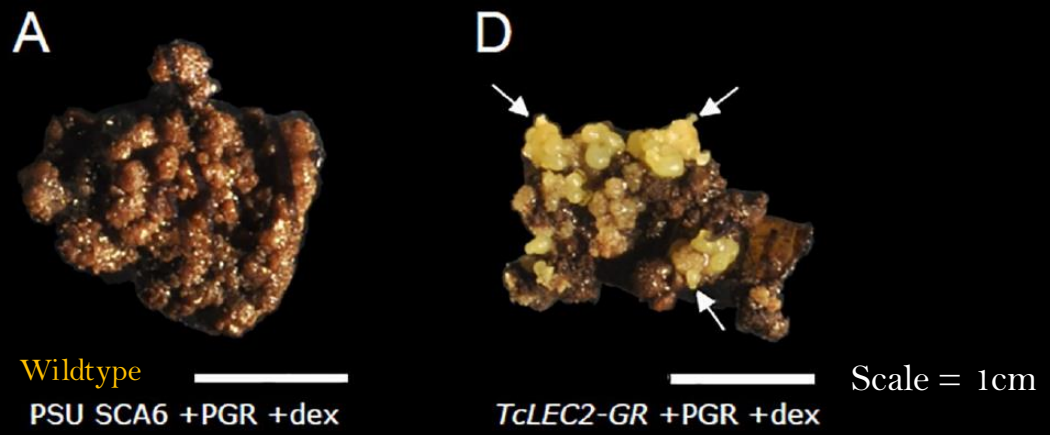


(*Fister et al., 2018*)









(*Fister et al., 2018*)

What other cacao tissues might be amenable to somatic embryogenesis via *LEC2* activation?

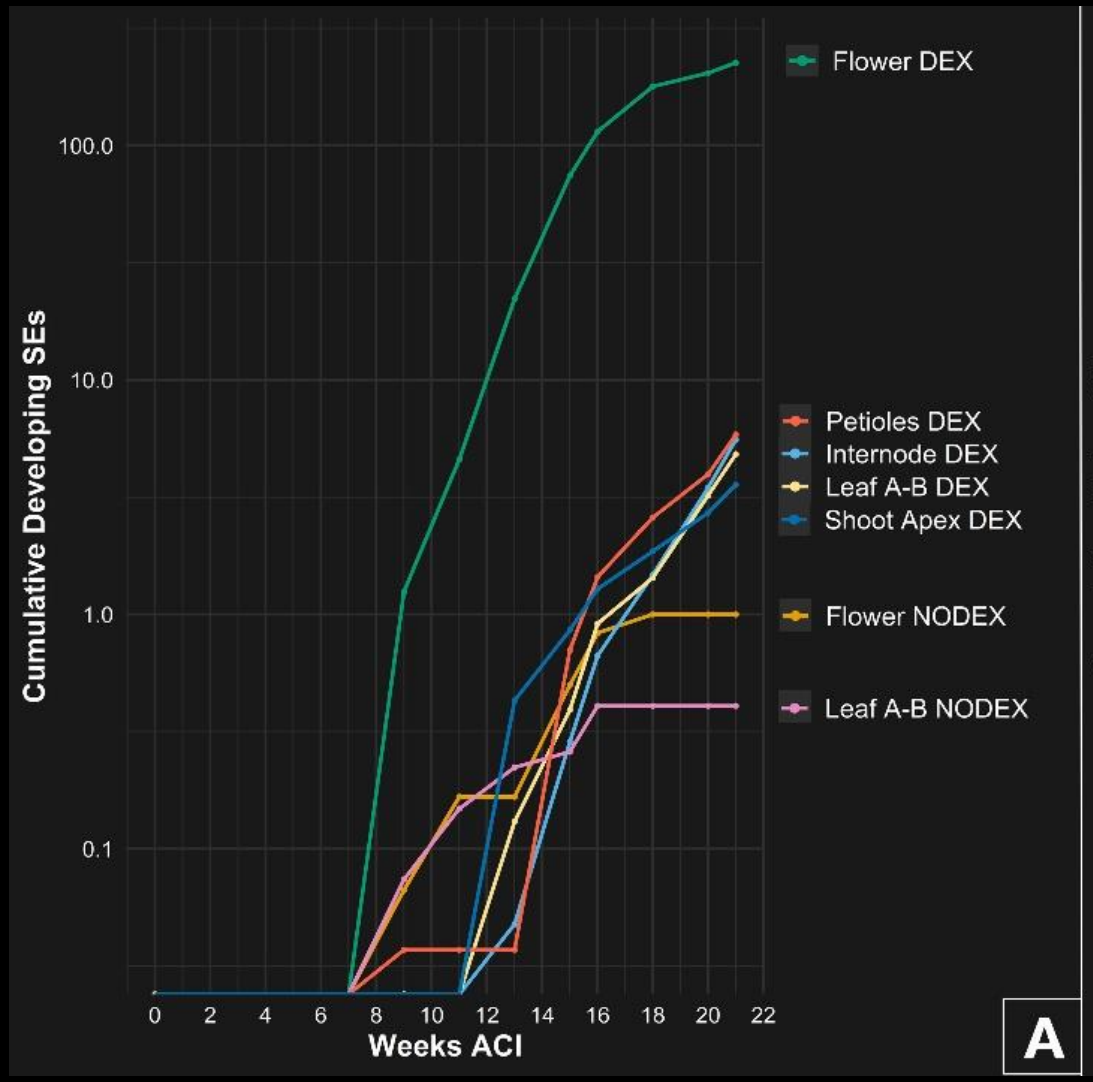
Pilot Experiment

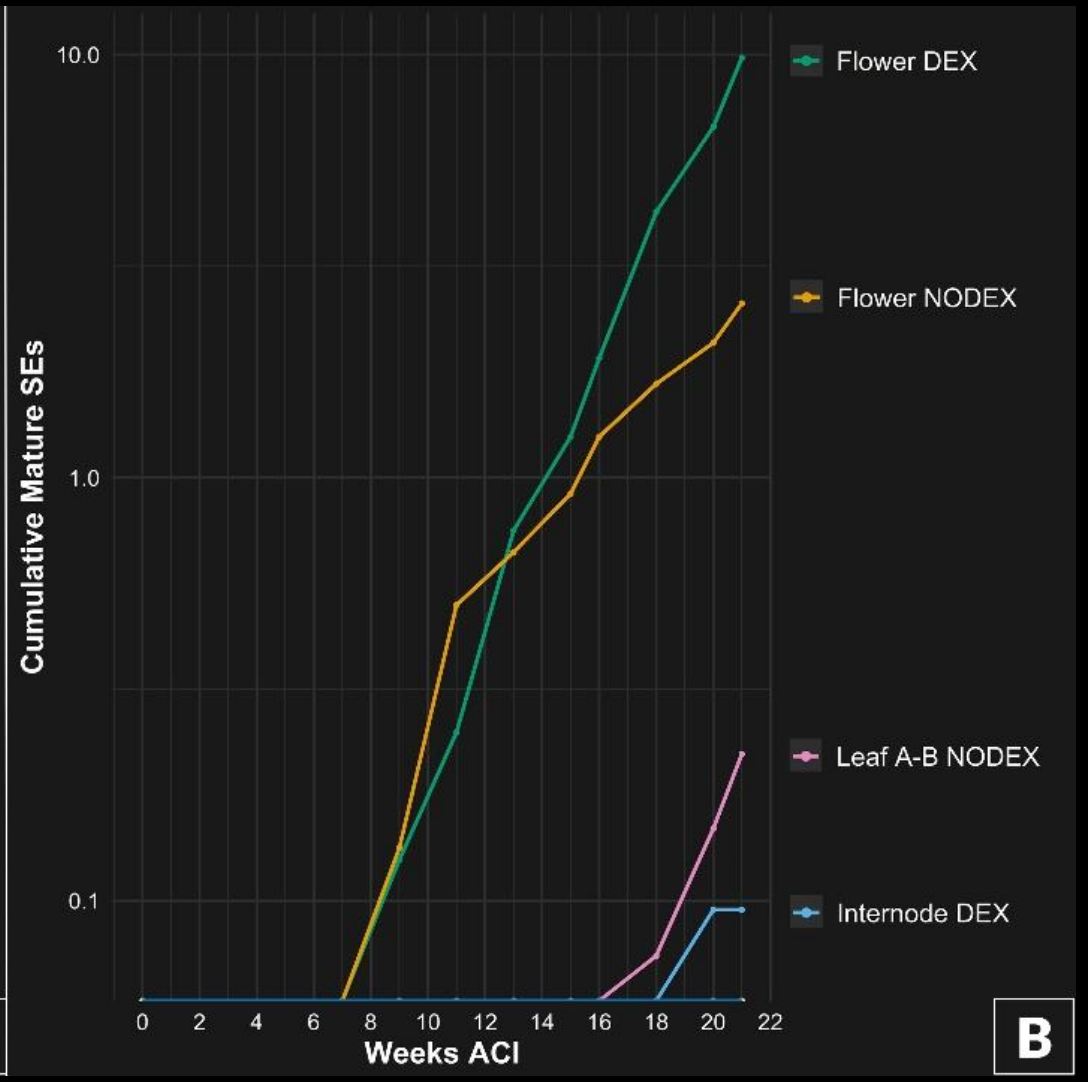
Two Genotypes: PSU Sca6 (wild-type) & TcLEC2-GR

Whole Flowers

Internodes Petioles Leaf Stage A-B Shoot Apex

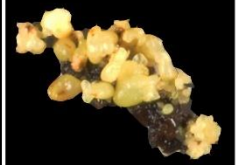
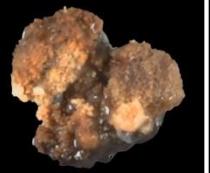
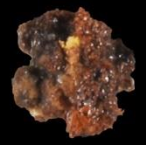
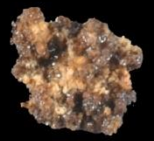
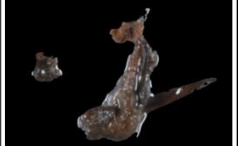
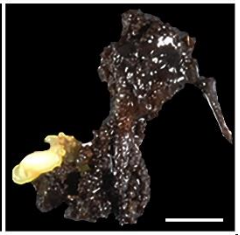
LEC2 Tissues





Developing ~ globular –> torpedo stage

Mature ~ cotyledonary stage

	PSU SCA 6		*TcLEC2-GR*	
	(-) DEX	(+) DEX	(-) DEX	(+) DEX
Flower				
Internode				
Petiole				
Leaf A-B				
Shoot Apex				

Result:
LEC2 activation allows juvenile somatic tissues to regenerate SEs

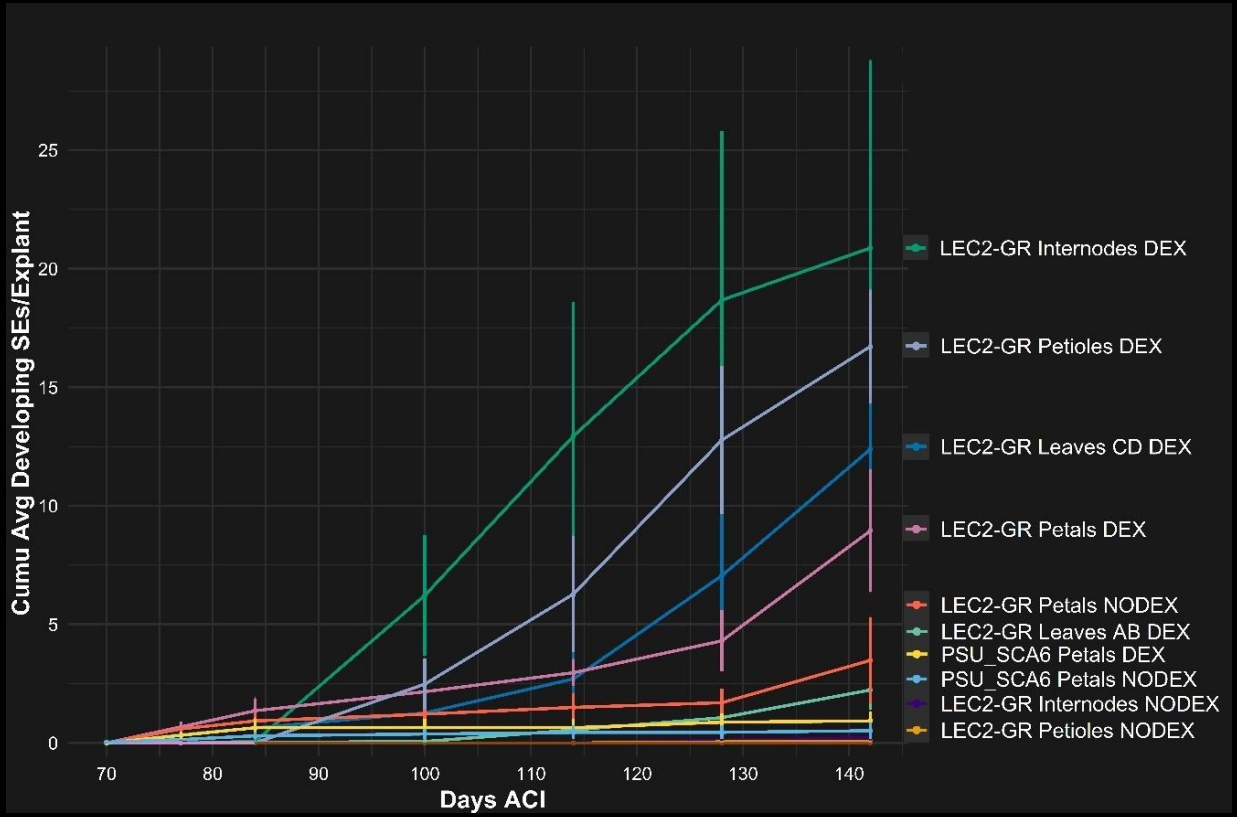
Large Scale Experiment – replicated twice

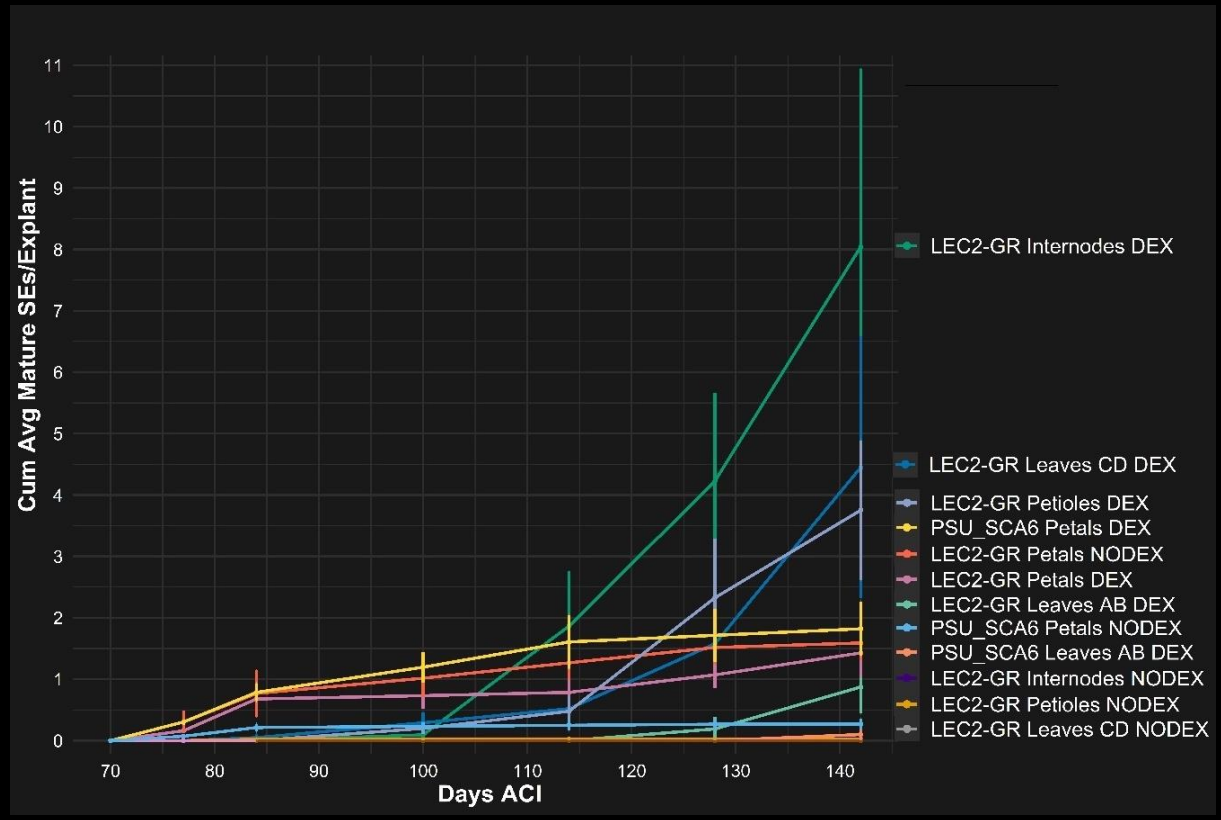
Petals

Leaf Stage E | Internodes | Petioles | Leaf Stage C-D | Leaf Stage A-B

<u>Two Genotypes:</u>
- PSU Sca6 (wild-type)
- TcLEC2-GR

<u>Explant Numbers:</u>
-5-9 plates/treatment
-56-90 explants/treatment





Results:

Petals:

- PSU SCA6 & L2-GR regenerated similar numbers of mature SEs (+/- DEX)
- L2GR (+/- DEX) had a significantly higher number of developing SEs

L2GR Internodes (+DEX):

- Regenerated the most SEs

L2GR Petioles (+DEX):

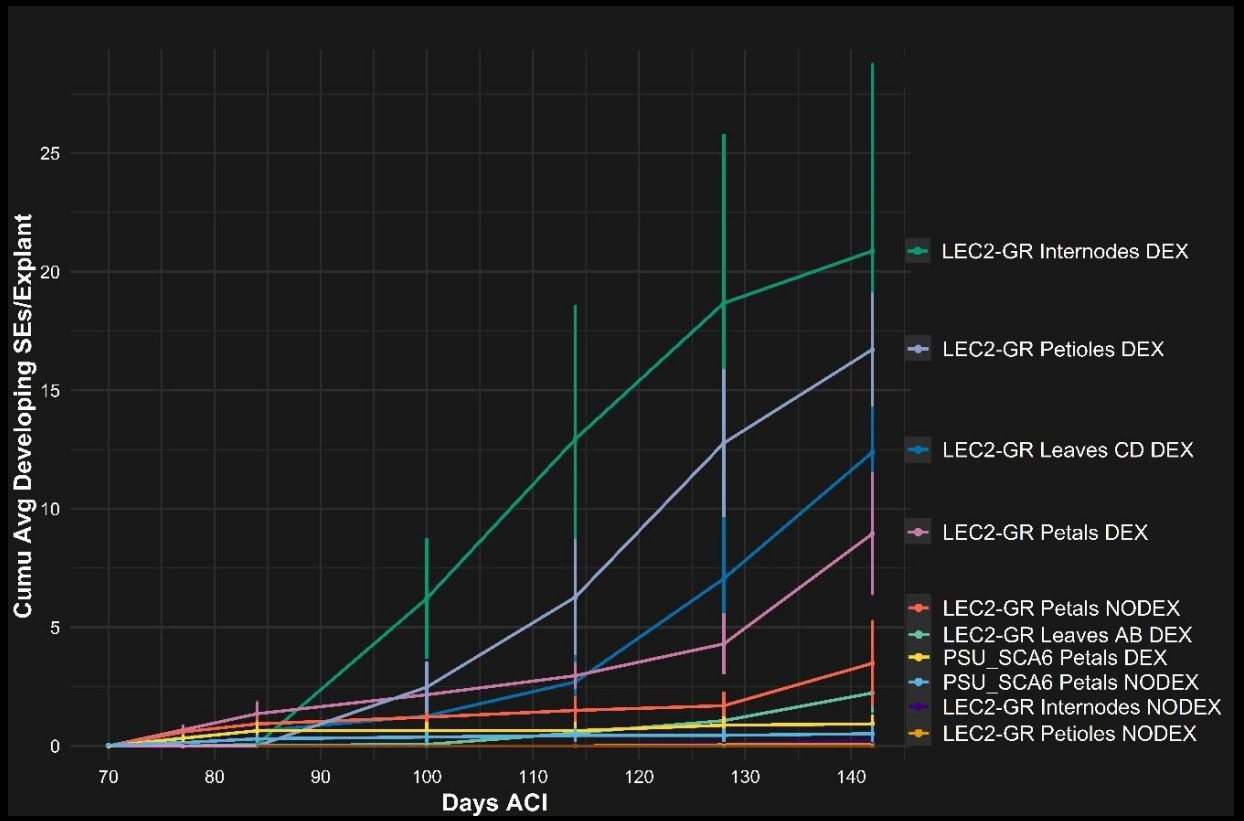
- Tied second best at SE regeneration

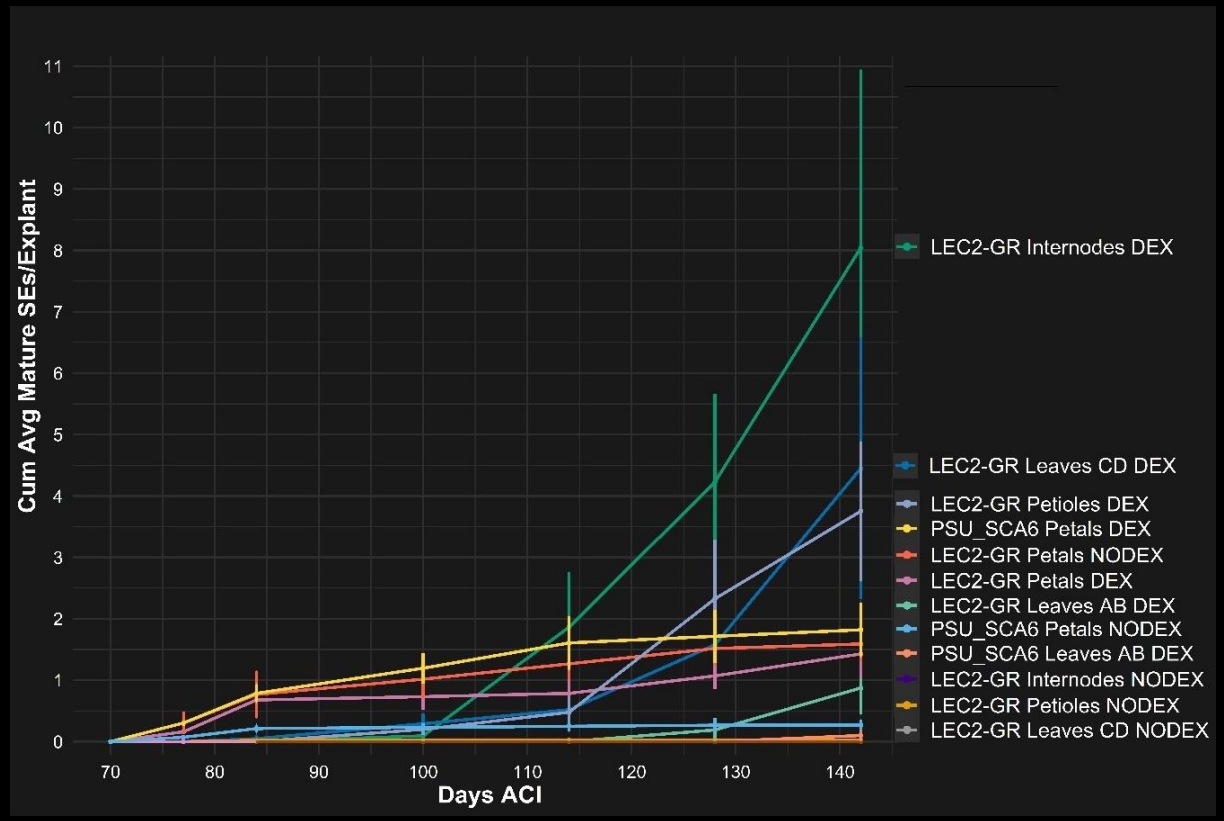
L2GR C-D Stage Leaves (+DEX):

- Tied second best at SE regeneration

L2GR A-B Stage Leaves(+DEX):

- Regenerated the fewest compared to other juvenile somatic tissues





L2GR E Stage Leaves:

- Regenerated no SEs (+/- DEX) treatment

PSU SCA6 juvenile tissues

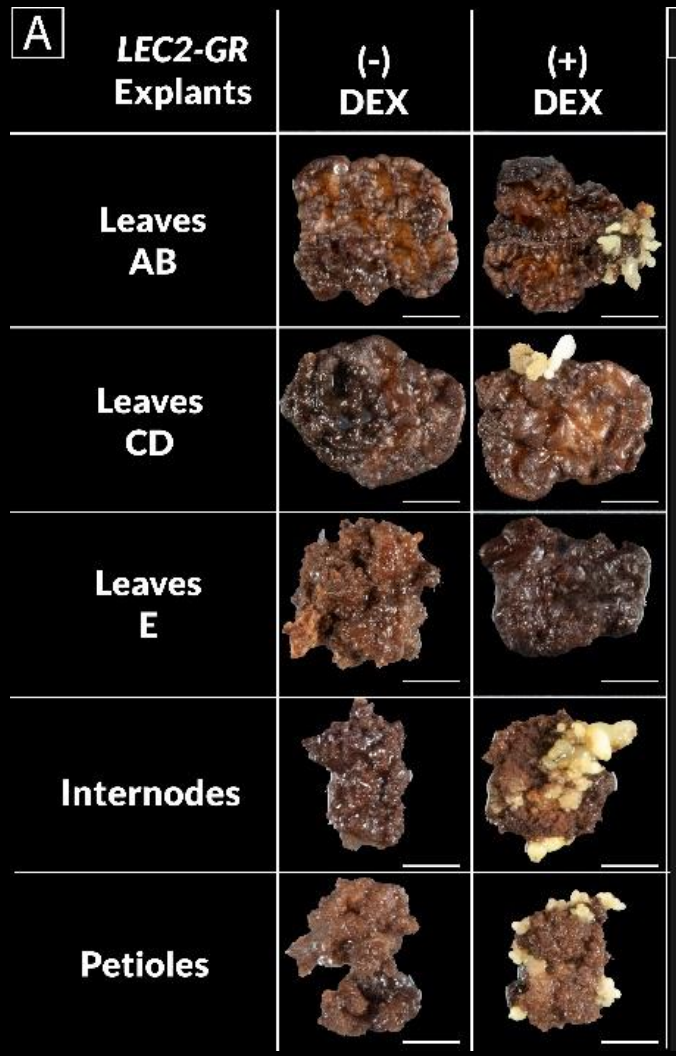
- Regenerated no SEs

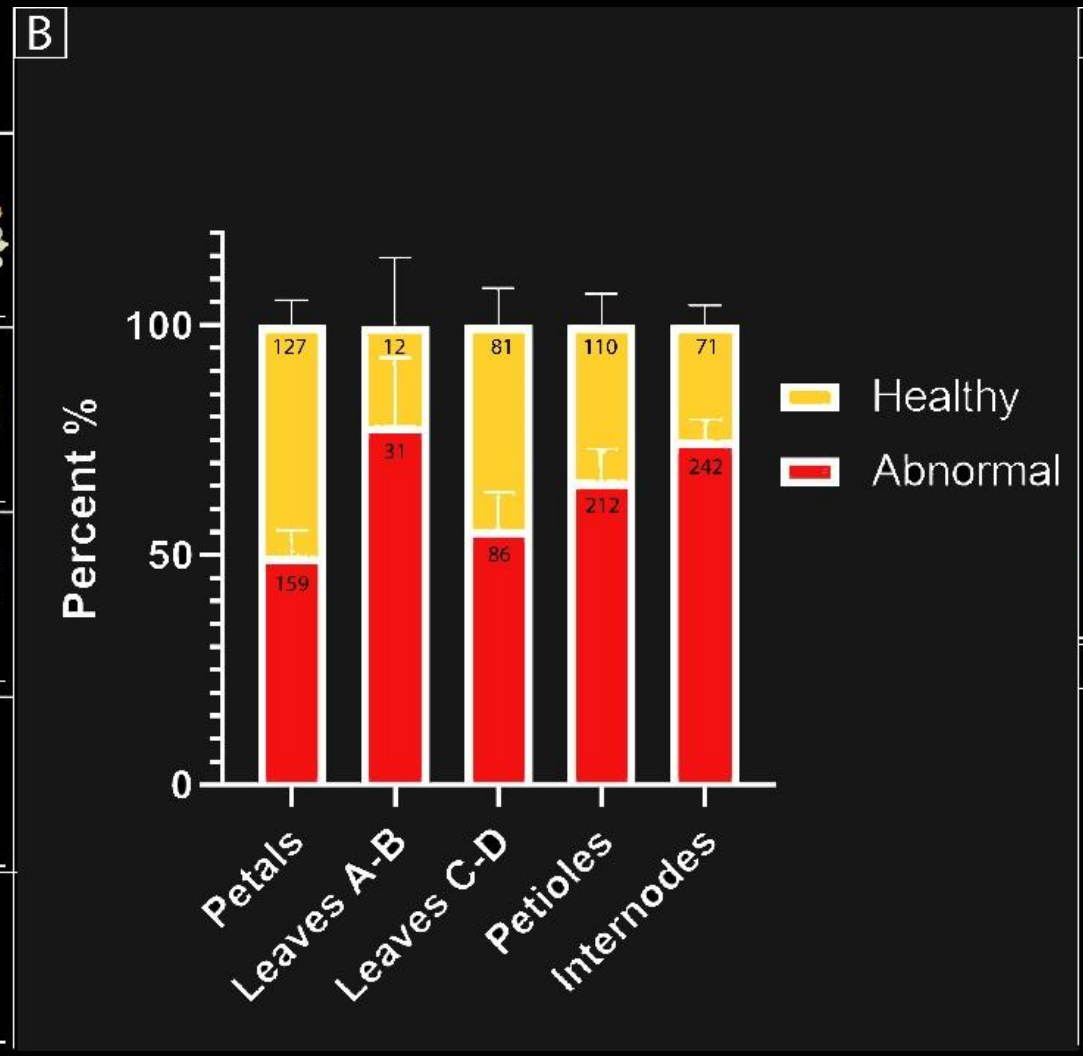
Outliers:

- PSU Sca6 Leaves AB
- L2GR Internodes (-DEX)
- L2GR petioles (-DEX)
- L2GR Leaf Stage C-D (-DEX)

L2GR Leakiness

Developing ~0.60%
Mature ~0.30%





Health of L2-GR Explants

- Petals ~ 49%
- C-D leaves ~ 39%
- Petioles ~ 30%
- Internodes ~ 25%
- A-B leaves ~ 8%

Discussion:

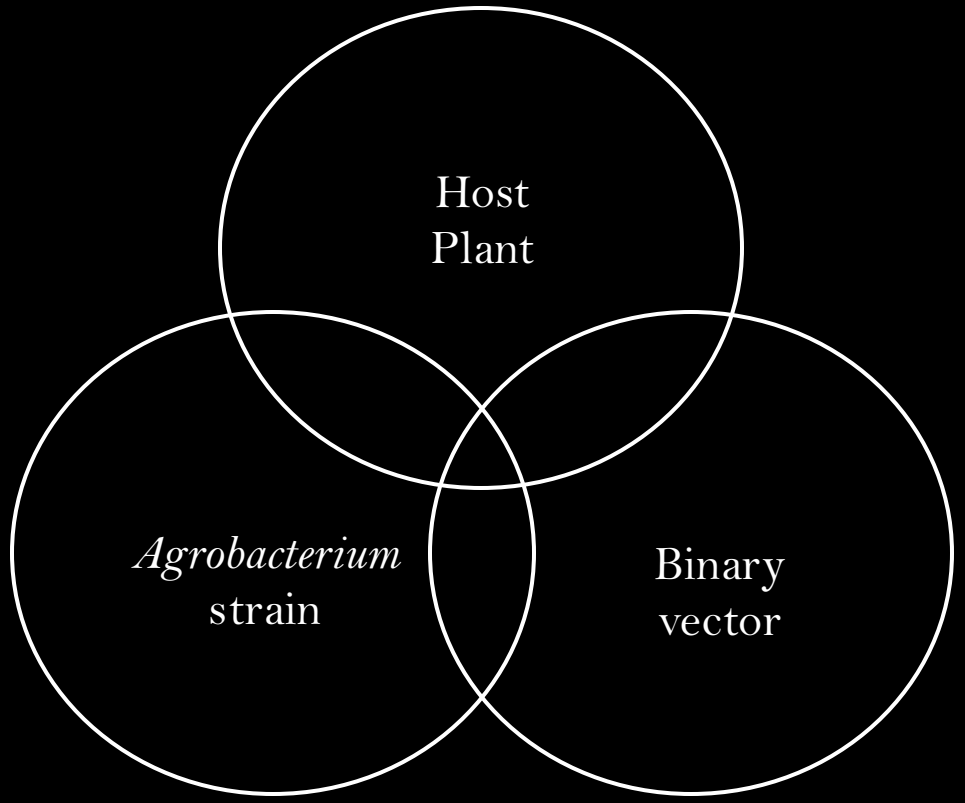
- Activation of TcLEC2 in cacao tissues allows internodes, petioles, leaves staged A-D, shoot apices, and whole flowers to regenerate SEs
- Developmental regulating genes such as LEC2 can help overcome *in-vitro* recalcitrance of highly recalcitrant tissues
- Pre-competent/competent cells exist throughout the tree

Future Directions:

- Identification of pre-competent and competent cell-types in cacao
- Transformation of juvenile tissues to recover stable transgenics

Chapter 3:
Binary Vector Effects on *Agrobacterium tumefaciens*-Mediated Cacao Transformation

Successful *Agrobacterium*-mediated Transformation

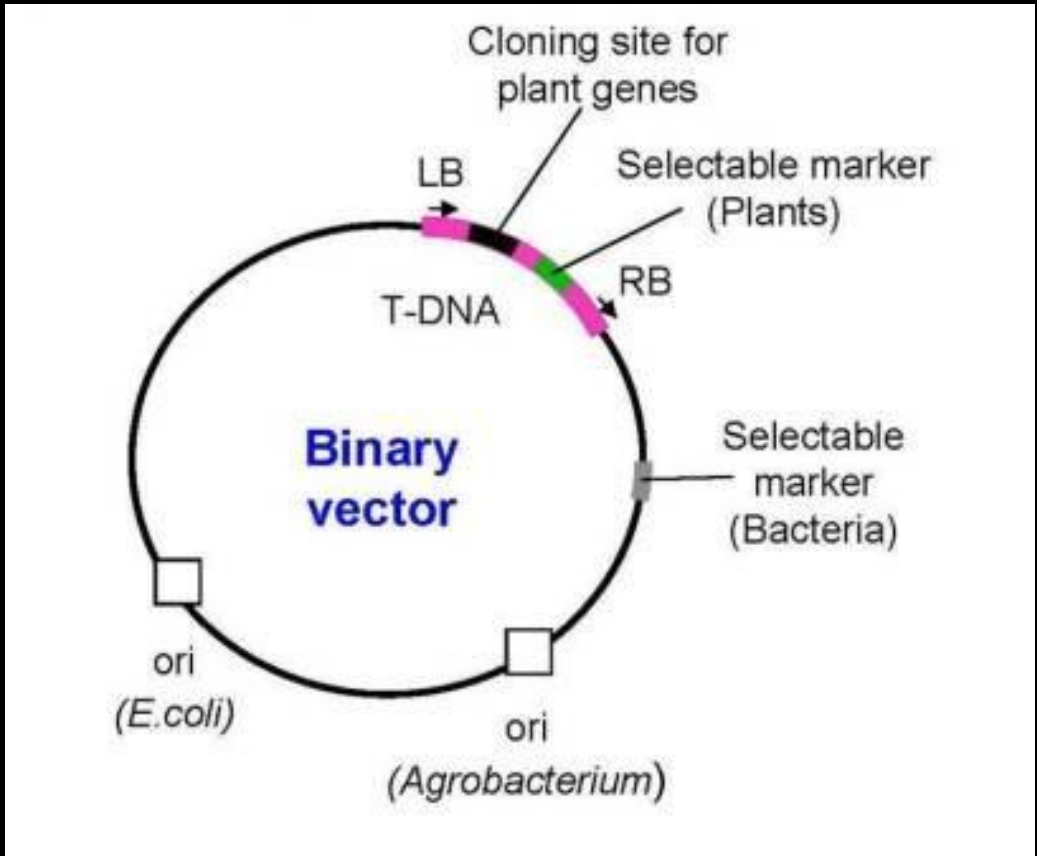


Host:
Cacao (PSU Scavina 6)

Agrobacterium strain:
-AGL1 (hyper-virulent)

Binary vector:
-pCambia based backbone

Components of a binary vector



T-DNA region

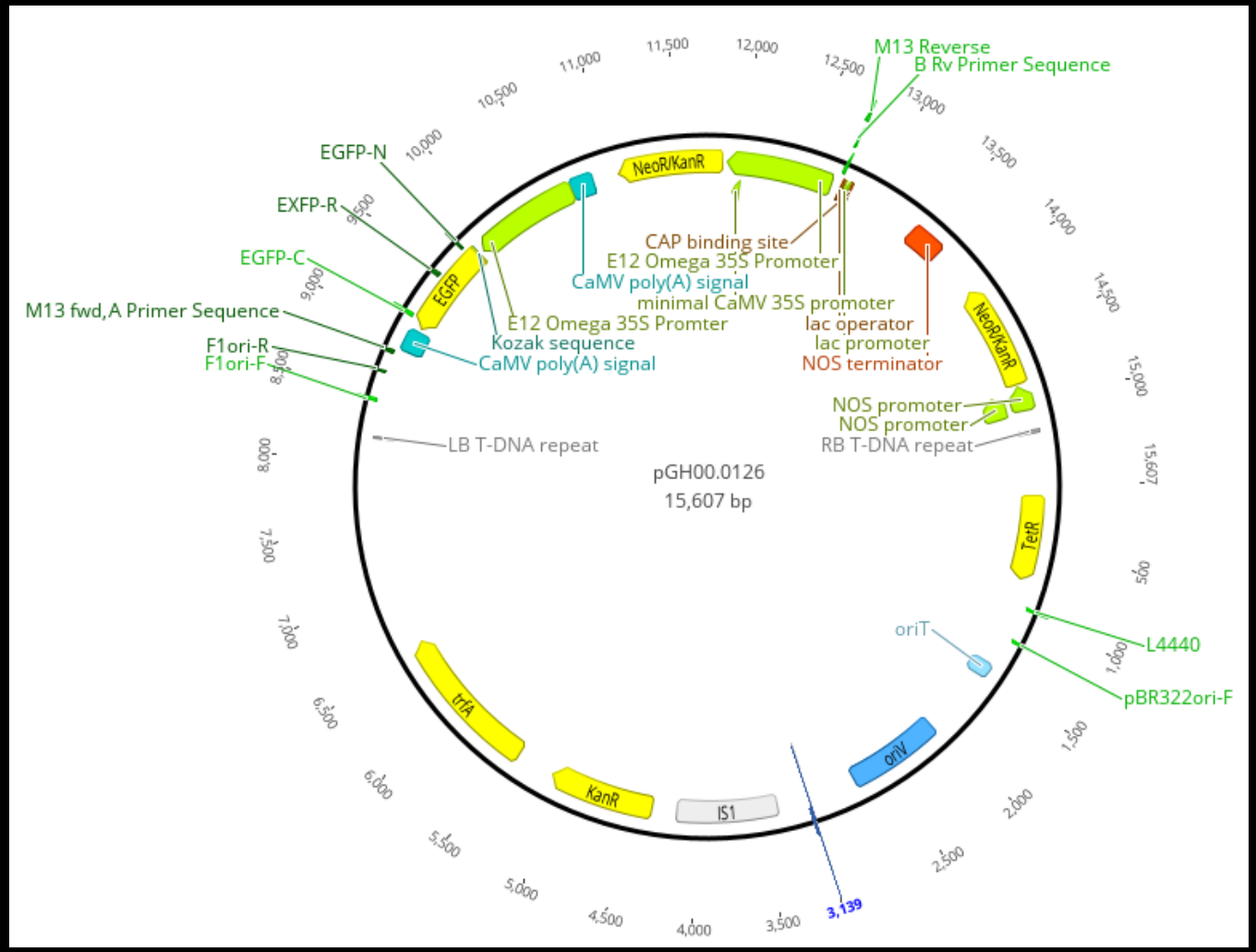
- Defined by left border and right border sequences
- Plant selectable marker
- Gene of interest/payload region
- Multiple cloning site

Backbone region

- Bacterial selectable marker
- *E.coli* origin of replication
- *Agrobacterium* origin of replication

https://blogger.googleusercontent.com/img/b/R29vZ2x1/AVvXsEiW3oWsg5WdPSsbXN1kYhHh3nKjrlGZ6O0V8g0h5hgv1VbKa78-f8PB6gVb3_1PTZOuwXLn_T0BmZWjShbhllsQDCTRIsFBCr0gjepD2_y1YFI8DjAokozfyYA8d0LA0XBxhKF3Xa9a1W8/?imgmax=800

What binary vector is used for cacao transformation?



pGH00.0126 ('p126')

Backbone – pCambia line

E. coli ori - ColE1

Agrobacterium ori – pVS1

Backbone ~6.2 kbp

Total size ~ 15.6 kbp

Two small binary vectors identified from the literature

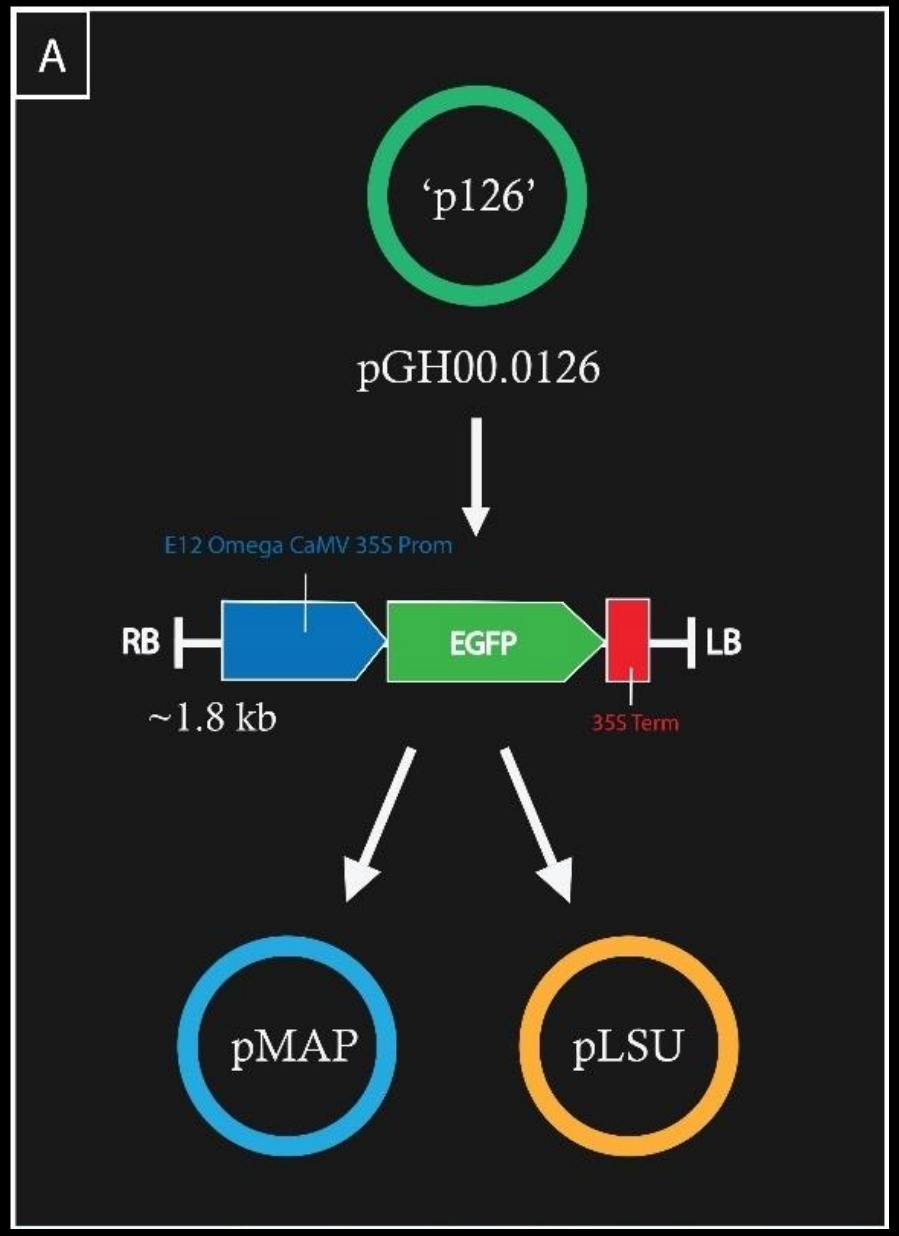
Vector	Backbone	Origin of Replication	Backbone Size
pGH00.0126	pCAMBIA	pVS1+ ColE1	6.2 kb
pMAP EGFP	pLX	WKS1-pUC19	4.3 kb
pLSU-1 EGFP	pLSU	Truncated pVS1+ ColE1	4.6 kb

‘p126’

(Andreou *et al.*, 2021)

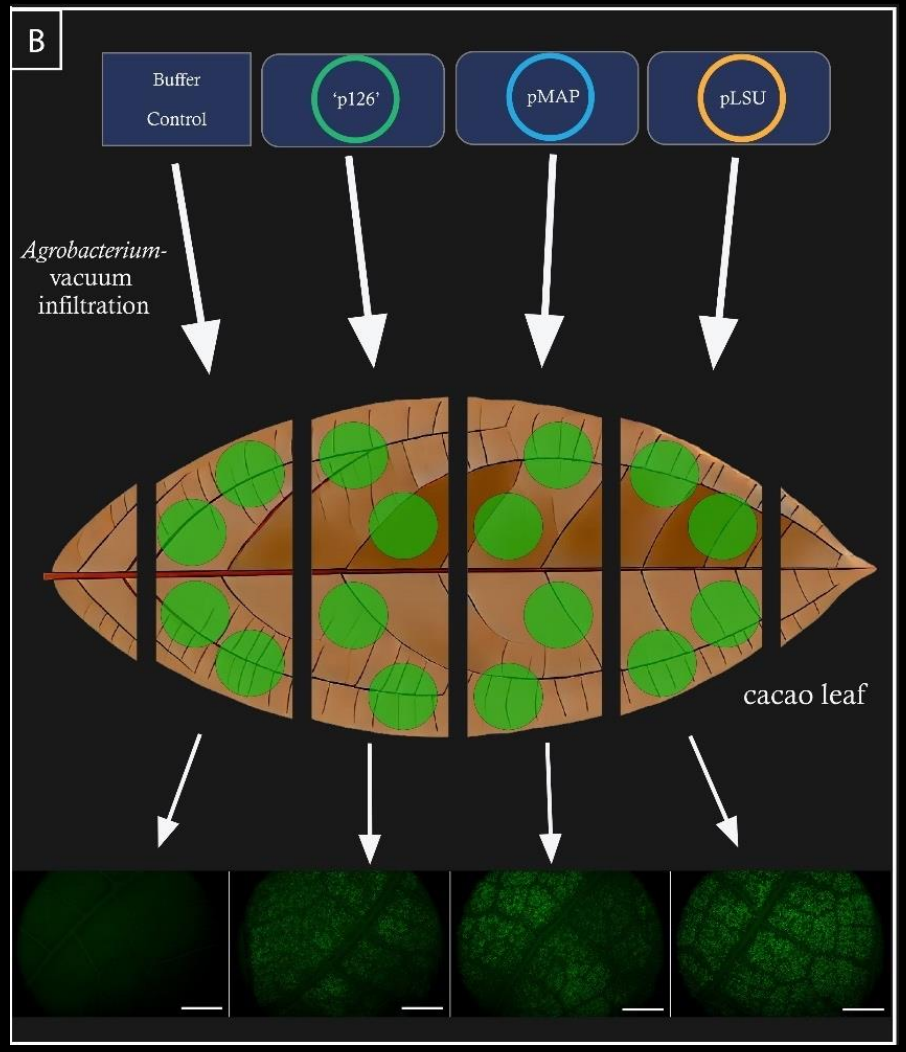
(Lee et al., 2011)

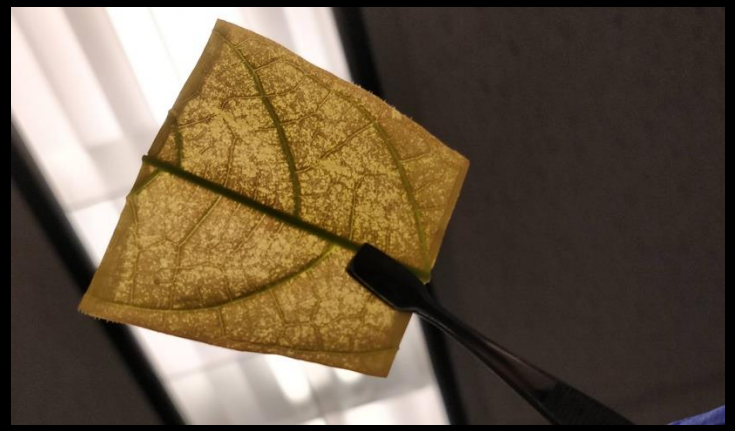
Can changing vector backbone influence *Agrobacterium*-mediated transformation efficiency?

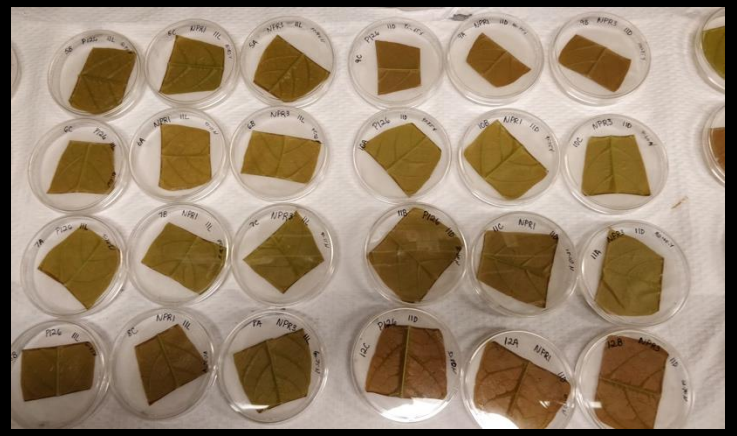


Clone the EGFP cassette from the 'p126' vector and insert it into the two empty small binary vectors

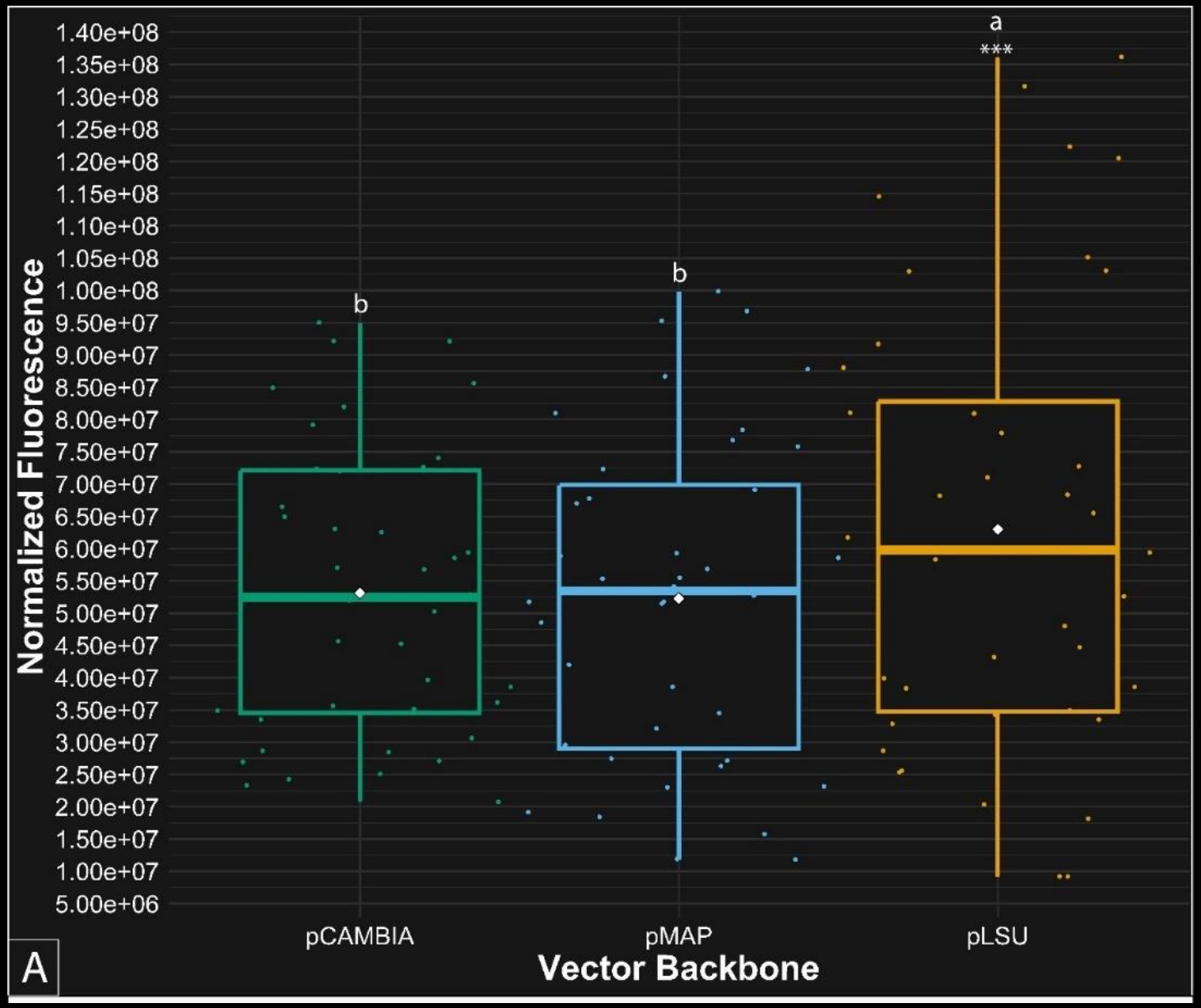
Detached Cacao Leaf Transient Expression Assay







- Insert binary vectors with EGFP cassettes into *Agrobacterium*
- Infiltrate 11 leaf sections/vector
- Allow infection for 48 hours
- Photograph leaves under a fluorescence macro-scope
- Use ImageJ (software) to quantify fluorescence



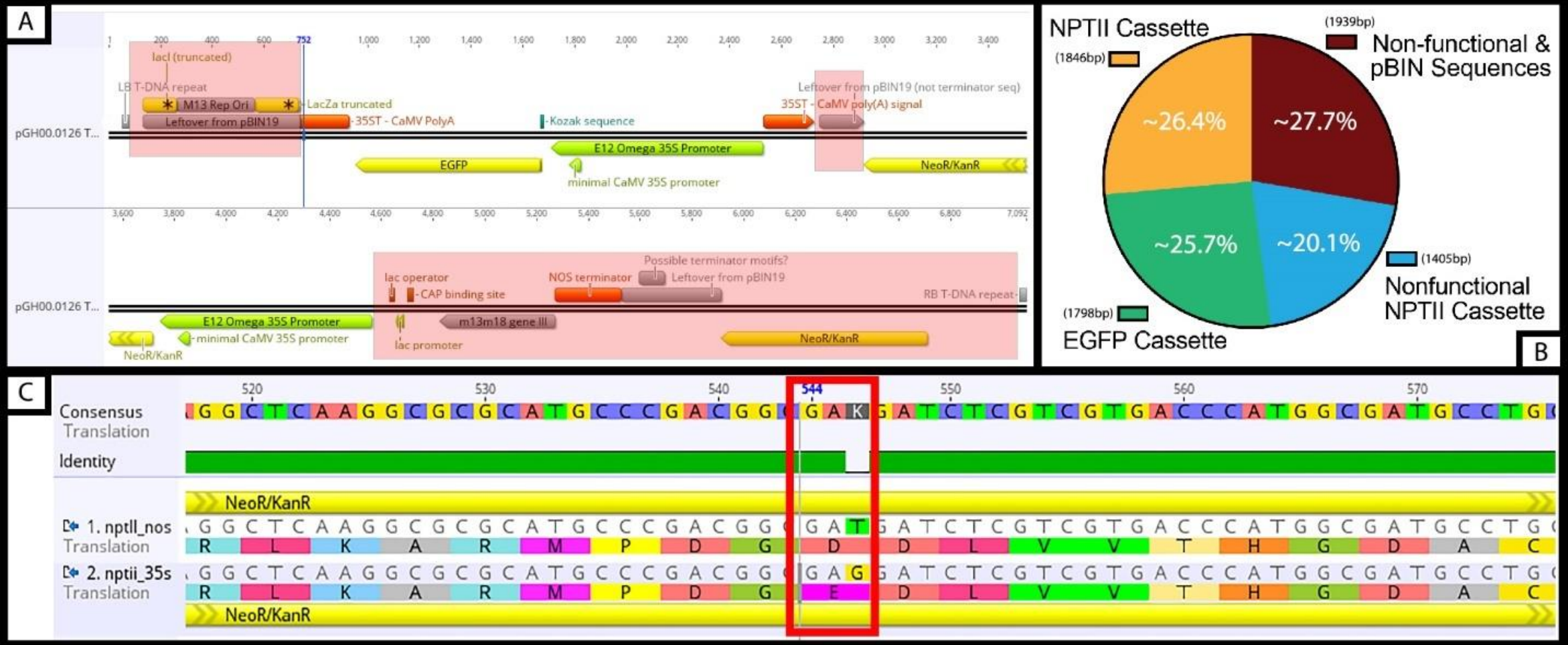
*** p-value <0.0001, one-way ANOVA

Result:

pLSU normalized fluorescence was
~18% higher than pCambia (p126)
~20% higher than pMAP

What does normalized fluorescence measure?

- Indirect measurement of the number of EGFP protein molecules expressed in the cacao leaf
- Transient expression of T-DNA molecules
- ~18% more T-DNA molecules from *Agro*-mediated transformation of the pLSU vector entered the nucleus of cacao leaf cells and were transcribed and tranlated



Analyzing p126 T-DNA Region

- 47.8% of sequences – leftover/non-functional/superfluous
- NPTII – mutation (Yenofsky *et al.*, 1990)
- Very few unique restriction sites
- Redundant genetic parts (promoters, terminators)

T-DNA region design criteria

Required cassettes:

- Reporter gene
- Plant selectable marker
- Easy ‘drop-in’ multiple cloning site

Rules:

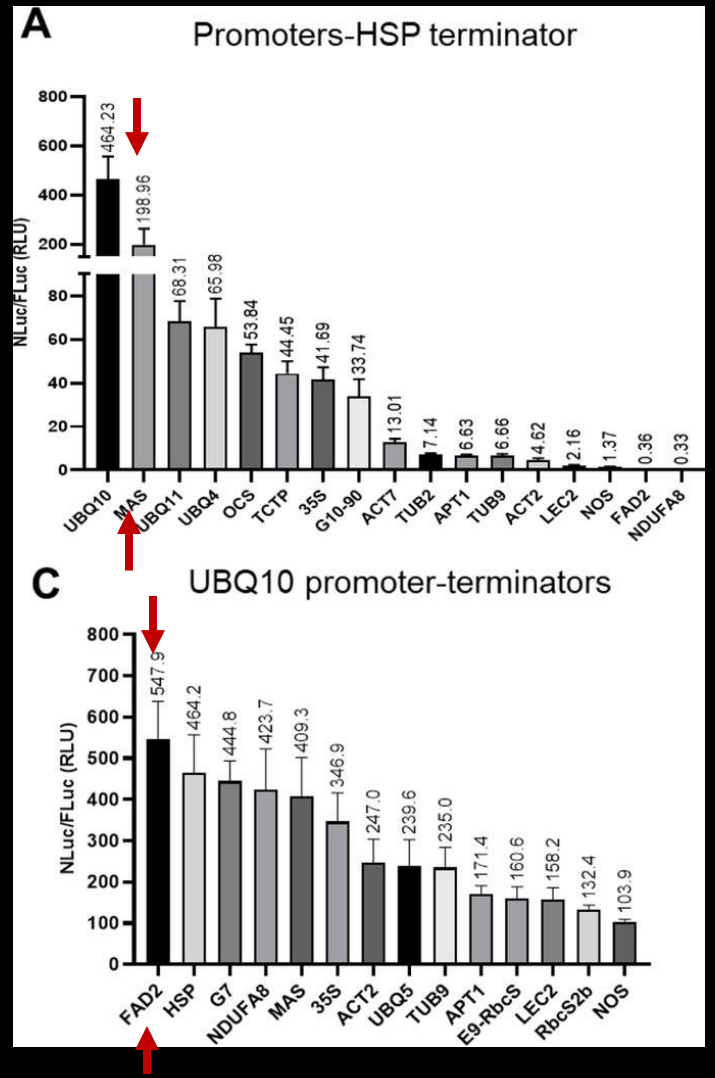
1. No redundant genetic parts
2. No superfluous DNA
3. Every genetic component flanked by a unique restriction site
4. Restriction enzymes must be of the highest efficiency available

1) No redundant genetic parts

1) **Reporter cassette:** E12 35S Prom : EGFP/reporter : 35S Term
- Functions well, being tested in cacao for ~2 decades

2) **Plant selection cassette:** Nos Prom : NPTII : Nos Term
- Agrobacterium promoter and terminator – used since 1985
- Tested in a wide variety of plant species

3) **'Drop-in' cassette**
MAS Prom : (Drop-in site) : AtFAD2 Term
- Promoter and terminator pair functions well in *Arabidopsis thaliana*
- Golden-gate cloning 'drop-in' site.



(Andreas *et al.*, 2021)

2) No superfluous DNA

NCBI BLAST every genetic piece & identify minimal correct sequence

E12 Omega 35S Promoter	**EGFP**	**CaMV 35S Terminator**
NOS Promoter	**NPTII (*no mut*)**	**NOS Terminator**
MAS Promoter	**GG Drop-in site**	**AtFAD2 Terminator**

3) Every genetic component flanked by a unique restriction site

4) Restriction enzymes must be of the highest efficiency available

Enzyme		Sequence	Supplied NEBuffer	% Activity in NEBuffer				Heat Inac.	Incu. Temp.	Dilute	Dam	Dcm	CpG	Unit Substrate	Notes
				r1.1	r2.1	r3.1	rCutSma								
AatII	RR e CpG	GACGT/C	rCutSmart™ Buffer	<10	50*	50	100	80°C	37°C	B	●	●	■	λ DNA	
Acc65I	RR e dcm CpG	G/GTACC	NEBuffer™ r3.1	10	75*	100	25	65°C	37°C	A	●	□ scol	□ scol	pBC4 DNA	
AccI	RR e CpG	GT/MKAC	rCutSmart™ Buffer	50	50	10	100	80°C	37°C	A	●	●	□ ol	λ DNA	
AciI	RR e CpG	CCGC(-3/-1)	rCutSmart™ Buffer	<10	25	100	100	65°C	37°C	A	●	●	■	λ DNA	
AclI	RR e CpG	AA/CGTT	rCutSmart™ Buffer	<10	<10	<10	100	No	37°C	B	●	●	■	λ DNA	
AcuI	RR e	CTGAAG(16/14)	rCutSmart™ Buffer	50	100	50	100	65°C	37°C	B	●	●	●	λ DNA	1, b, d
AfeI	RR CpG	AGC/GCT	rCutSmart™ Buffer	25	100	25	100	65°C	37°C	B	●	●	■	pXba DNA	
AflII	RR e	C/TTAAG	rCutSmart™ Buffer	50	100	10	100	65°C	37°C	A	●	●	●	ΦX174 RF I DNA	
AflIII	RR e	A/CRYGT	NEBuffer™ r3.1	10	50	100	50	80°C	37°C	B	●	●	●	λ DNA	
AgeI-HF®	RR e e CpG	A/CCGGT	rCutSmart™ Buffer	100	50	10	100	65°C	37°C	A	●	●	■	λ DNA	
AhdI	RR e CpG	GACNNN/NNGTC	rCutSmart™ Buffer	25	25	10	100	65°C	37°C	A	●	●	◇ scol	λ DNA	a
AleI-v2	RR e CpG	CACNN/NNGTG	rCutSmart™ Buffer	<10	<10	<10	100	65°C	37°C	B	●	●	◇ ol	λ DNA	
AluI	RR e	AG/CT	rCutSmart™ Buffer	25	100	50	100	80°C	37°C	B	●	●	●	λ DNA	b
AlwI	RR dam	GGATC(4/5)	rCutSmart™ Buffer	50	50	10	100	No	37°C	A	■	●	●	λ DNA (dam-)	1, b, d
AlwNI	RR e dcm	CAGNNN/CTG	rCutSmart™ Buffer	10	100	50	100	80°C	37°C	A	●	□ ol	●	λ DNA	
ApaI	RR e dcm CpG	GGGCC/C	rCutSmart™ Buffer	25	25	<10	100	65°C	37°C	A	●	□ ol	□ ol	pXba DNA	

210 Restriction Enzymes on NEB

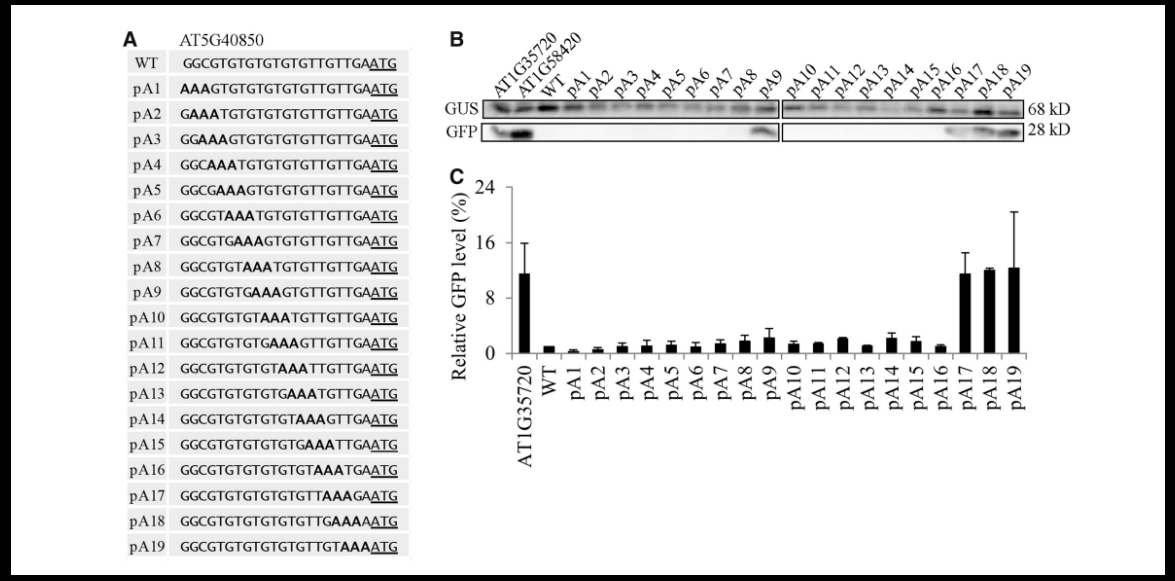
- Not present in 9 genetic pieces
- Activity % in rCutsmart must be 100%
- Incubation temperature at 37C
- No Methylation sensitivity
- Low - minimal star activity

Legend

●	Not Sensitive
■	Blocked
□ ol	Blocked by Overlapping
□ scol	Blocked by Some Combinations of Overlapping
◆	Impaired
◇ ol	Impaired by Overlapping
◇ scol	Impaired by Some Combinations of Overlapping

The immediate upstream region of the 5′-UTR from the AUG start codon has a pronounced effect on the translational efficiency in *Arabidopsis thaliana*

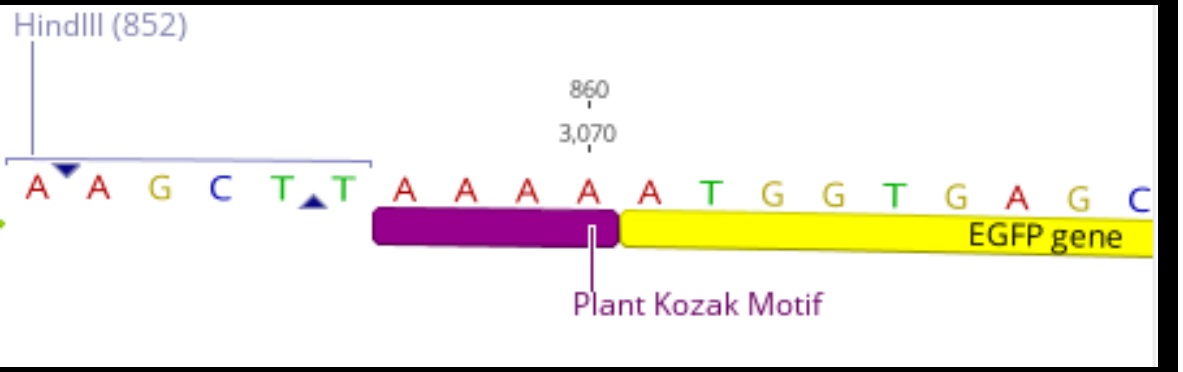
Younghyun Kim[1], Goeun Lee[2], Eunhyun Jeon[1], Eun ju Sohn[3], Yongjik Lee[3], Hyangju Kang[1], Dong wook Lee[3], Dae Heon Kim[1] and Inhwan Hwang[1,2,3,*]

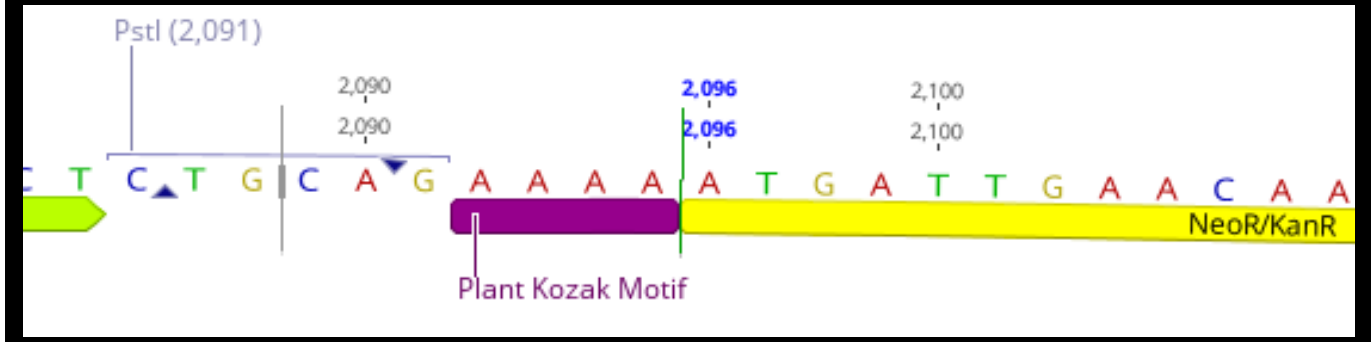


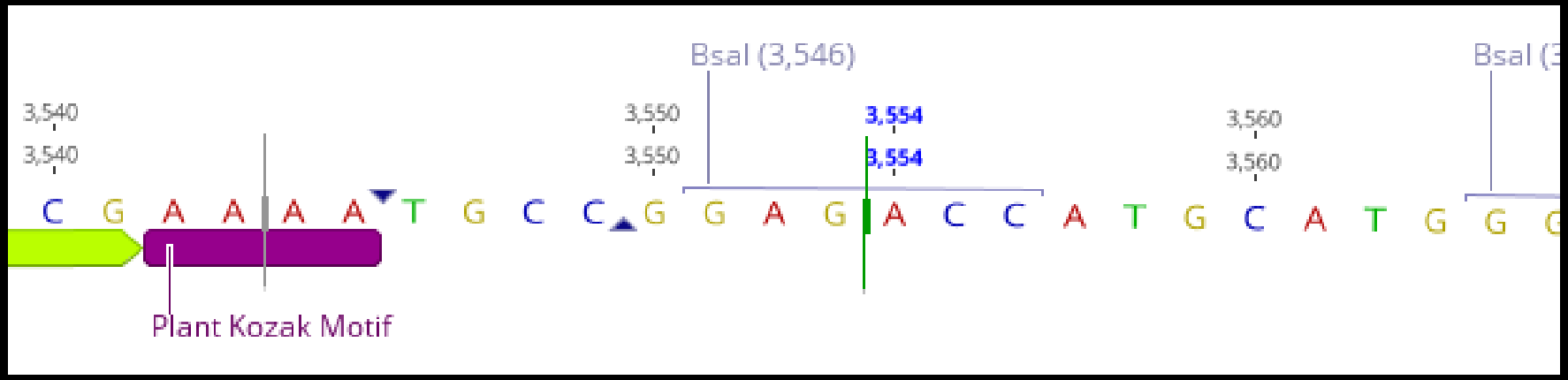
systems (45). In fact, in plants, the A residue at positions −1 to −4 is most favourable in the translational efficiency

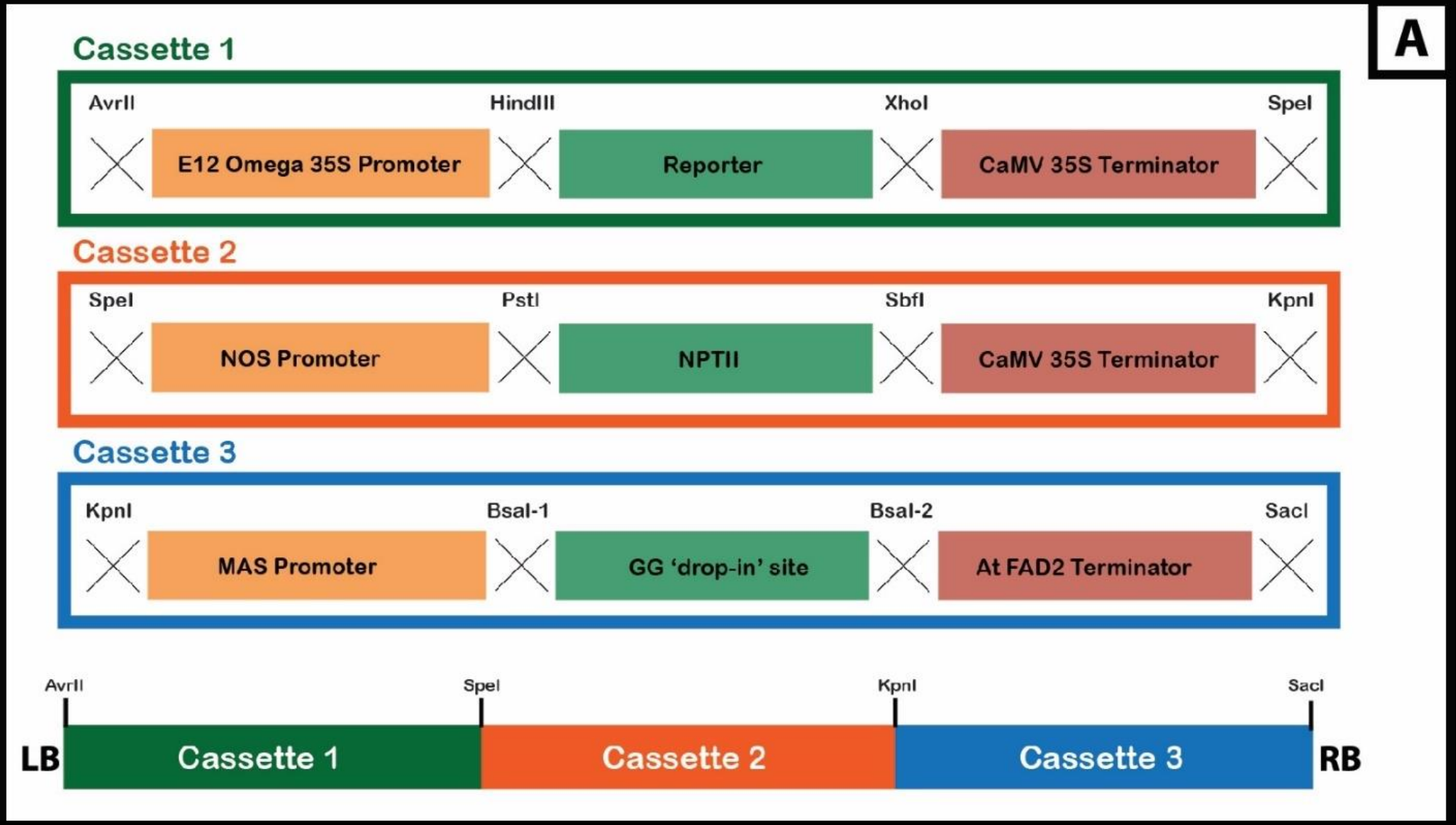
- "AAAAAAA(A/C)AAUGGCU for Dicots (derived from 3643 genes)"
- "AAAAAAAUG and GCCGCCAUG are the most and the second most over-represented patterns"
- "We then found that such mixed sequences (e.g. GAAACCAUG or ACAGACAUG) are significantly suppressed in genes ($P < 0.01$)"

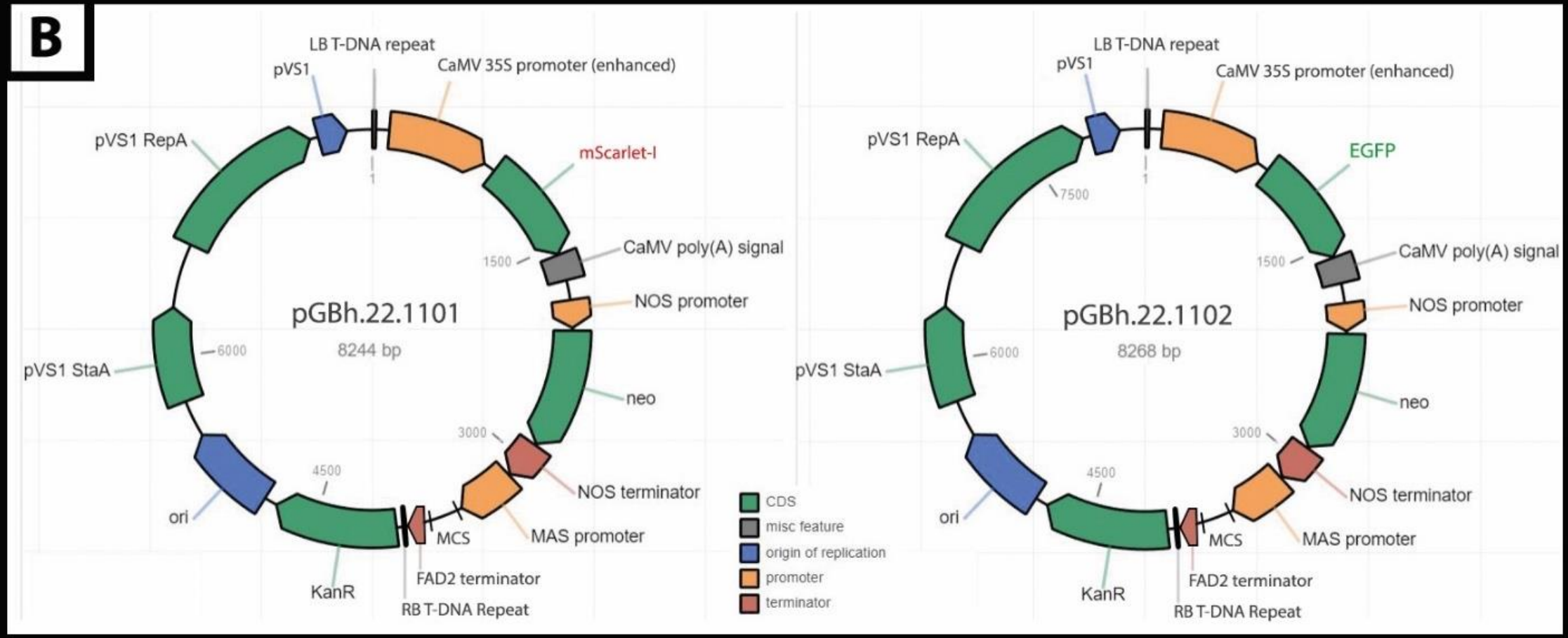
Addition of efficient kozak sequences in front of each gene








A
Cassette 1
AvrII
HindIII
XhoI
SpeI
E12 Omega 35S Promoter
Reporter
CaMV 35S Terminator
Cassette 2
SpeI
PstI
SbfI
KpnI
NOS Promoter
NPTII
CaMV 35S Terminator
Cassette 3
KpnI
BsaI-1
BsaI-2
SacI
MAS Promoter
GG 'drop-in' site
At FAD2 Terminator
AvrII
SpeI
KpnI
SacI
LB
Cassette 1
Cassette 2
Cassette 3
RB



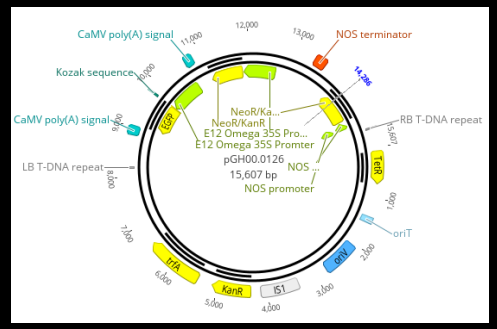
Binary vector with:

1. No redundant genetic parts
2. No superfluous DNA
3. Every genetic component flanked by a unique restriction site
4. Restriction enzymes must be of the highest efficiency available
5. Higher translational efficiency

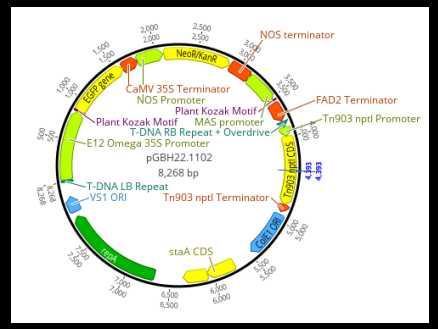
3 cassettes within T-DNA region
Only ~8.2kbp!

Can the binary vector influence stable transformation efficiency?

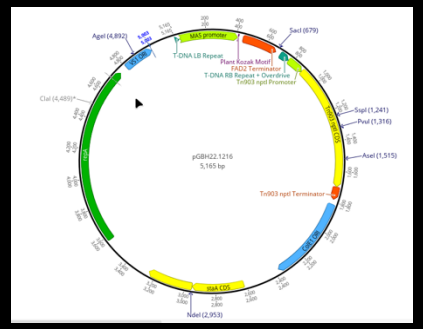
Cacao Stable Transformation Experiments



‘p126’
EGFP



‘p1102’
EGFP

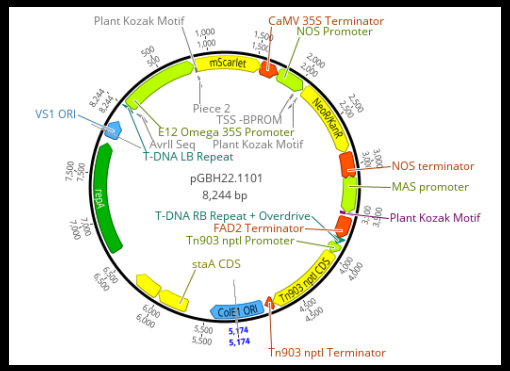


‘p1216’
Empty
Vector

Standard transformation experiment

75 PSU Sca6 secondary somatic embryo cotyledons/vector

Cultured 24 weeks



‘p1101’
mScarlet

Standard transformation experiment

~120 LEC2-GR secondary somatic embryo cotyledons

Cultured 24 weeks

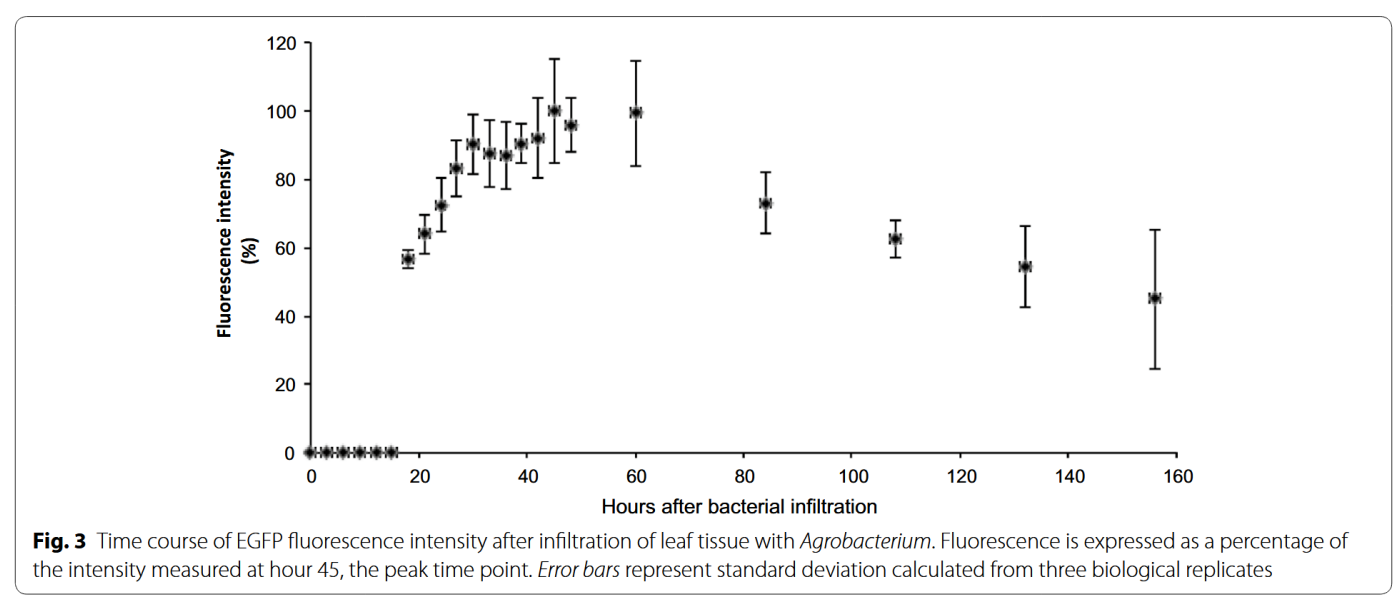


Fig. 3 Time course of EGFP fluorescence intensity after infiltration of leaf tissue with *Agrobacterium*. Fluorescence is expressed as a percentage of the intensity measured at hour 45, the peak time point. *Error bars* represent standard deviation calculated from three biological replicates

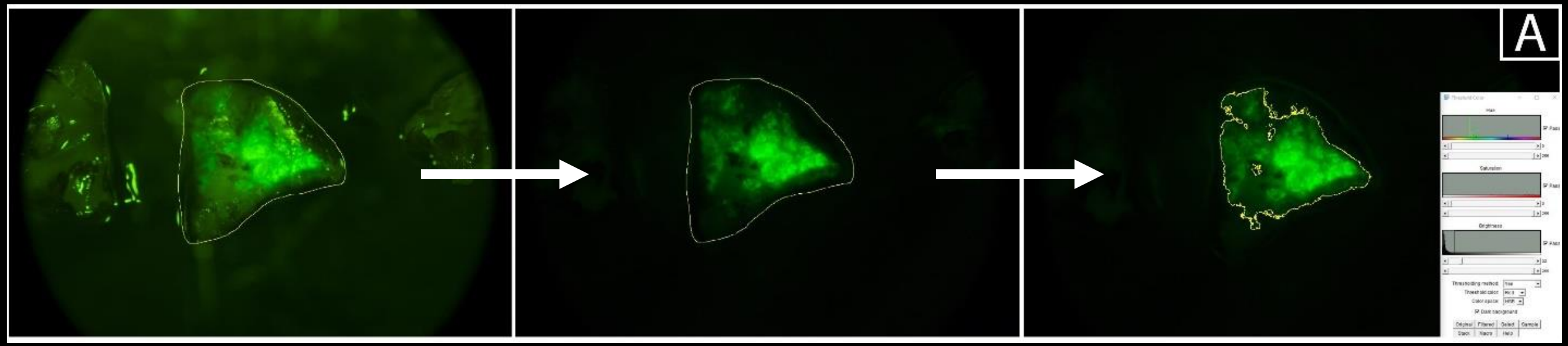
Transient expression 'spike' stabilizes after 6 days

Metrics collected:

1) Stable normalized fluorescence
2) # Recovered stable transgenic SEs

(Fister *et al.*, 2016)

Tissues Imaged at 6 days ACI



Each explant manually traced

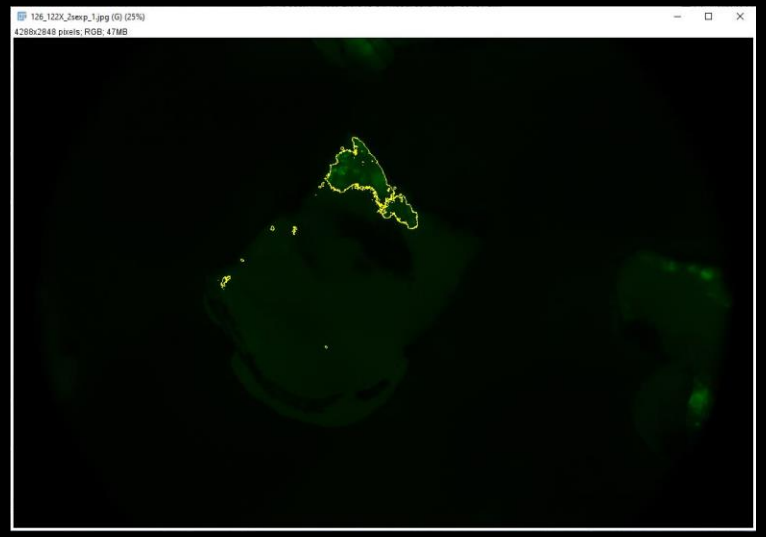
Explant Area

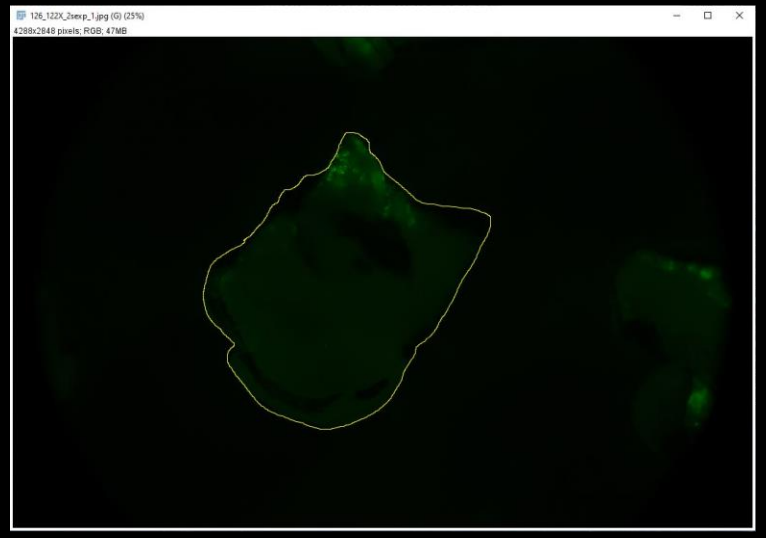
Fluorescence Area

Measurements taken for
transformed tissue (Area ,IntDen,etc.)

Threshold value set by p1216 EV Control
Method = YEN, Brightness minimum = #32

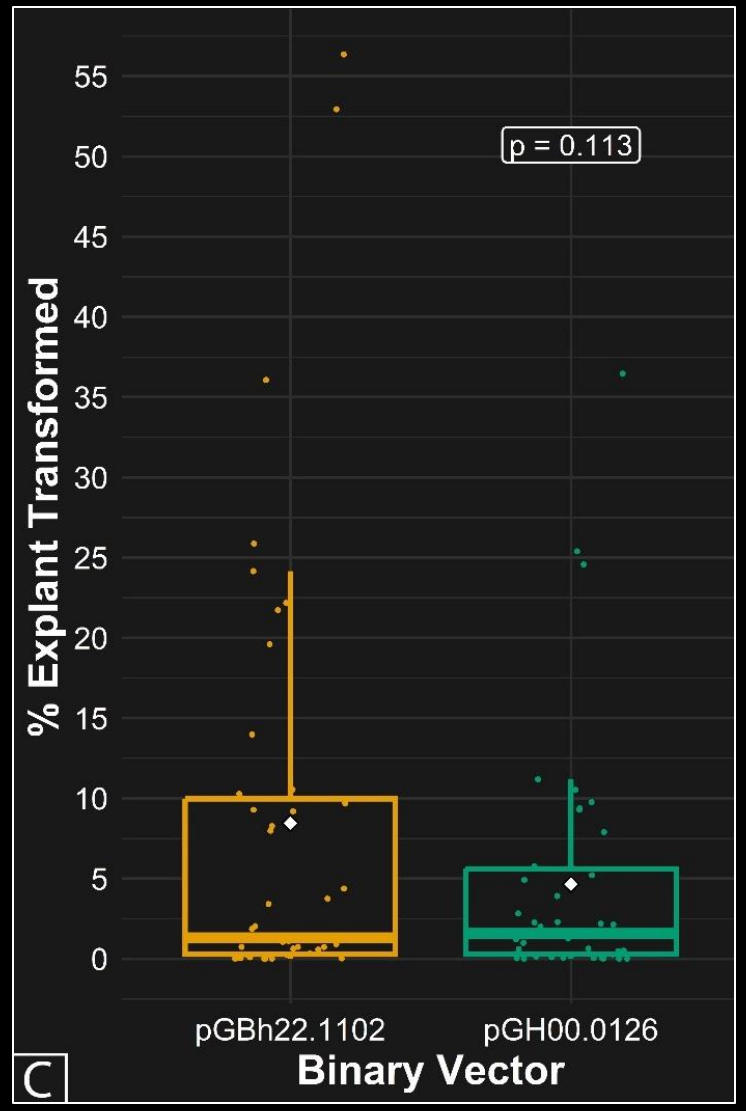
What % area of explants was transformed?





$$\frac{\text{Area of tissue transformed}}{\text{Total Area of Explant}} \times 100$$

% Area of Explants Transformed

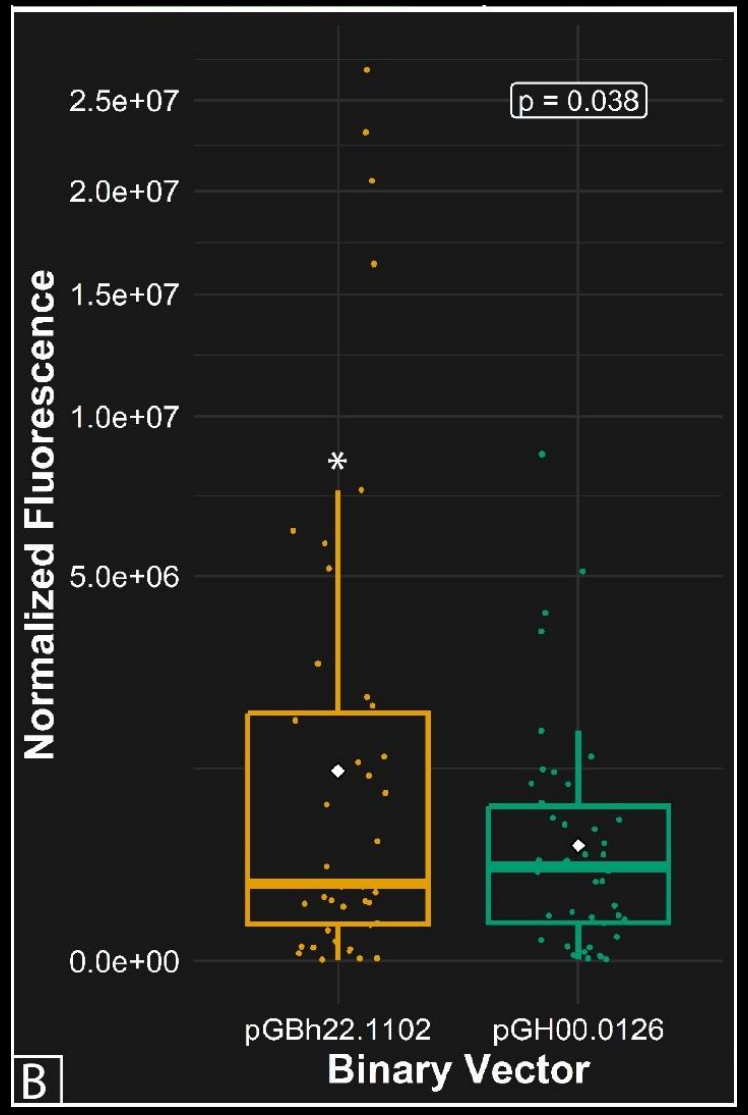


Average Area of Explant Transformed:
p1102 ~6.99% of explant transformed
p126 ~3.35% of explant transformed

ANOVA, *Alpha = 0.05*
Accept null hypothesis - p1102 and p126
The two means not significantly different

There is no significant difference in the area of tissues transformed due to binary vector

Stable Normalized Fluorescence



ANOVA, *Alpha = 0.05*
Reject null hypothesis - p1102 and p126
The two means are significantly different

p1102 ~**3.49-fold** greater fluorescence compared to p126

More EGFP molecules are being translated from p1102-transformed cells

- More cells are being transformed
- More cells are integrating the T-DNA
- More T-DNA copies are being integrated
- Higher translational efficiency

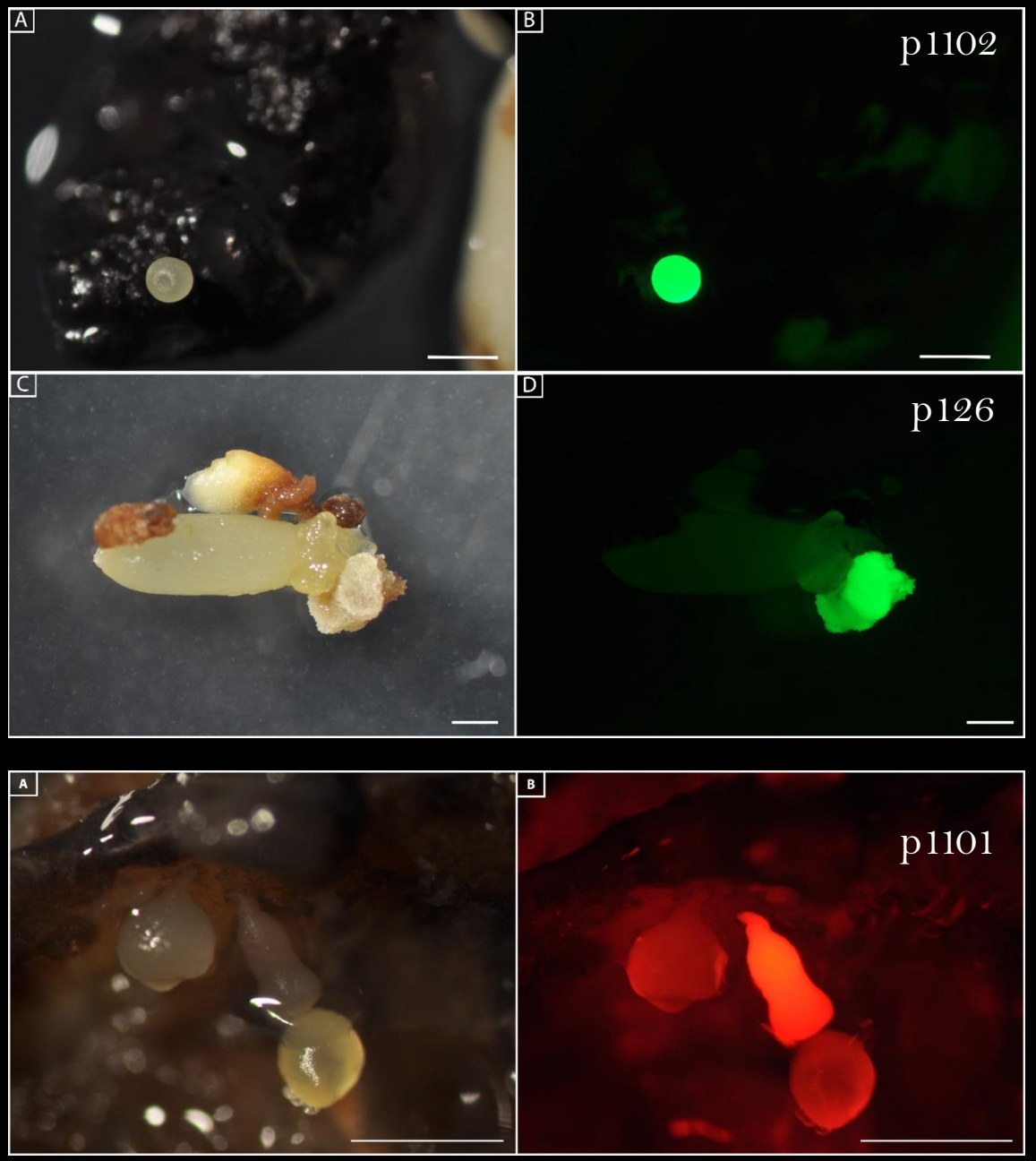
Does increased stable transformation increase the # of stable transgenics?

No increase in the efficiency % for recovering stable transgenics

p1102 ~ 1/75 = 1.3%

p126 ~ no transgenic SEs

p1101 ~ 1/125 = 0.08%



Scale bars represent 1mm

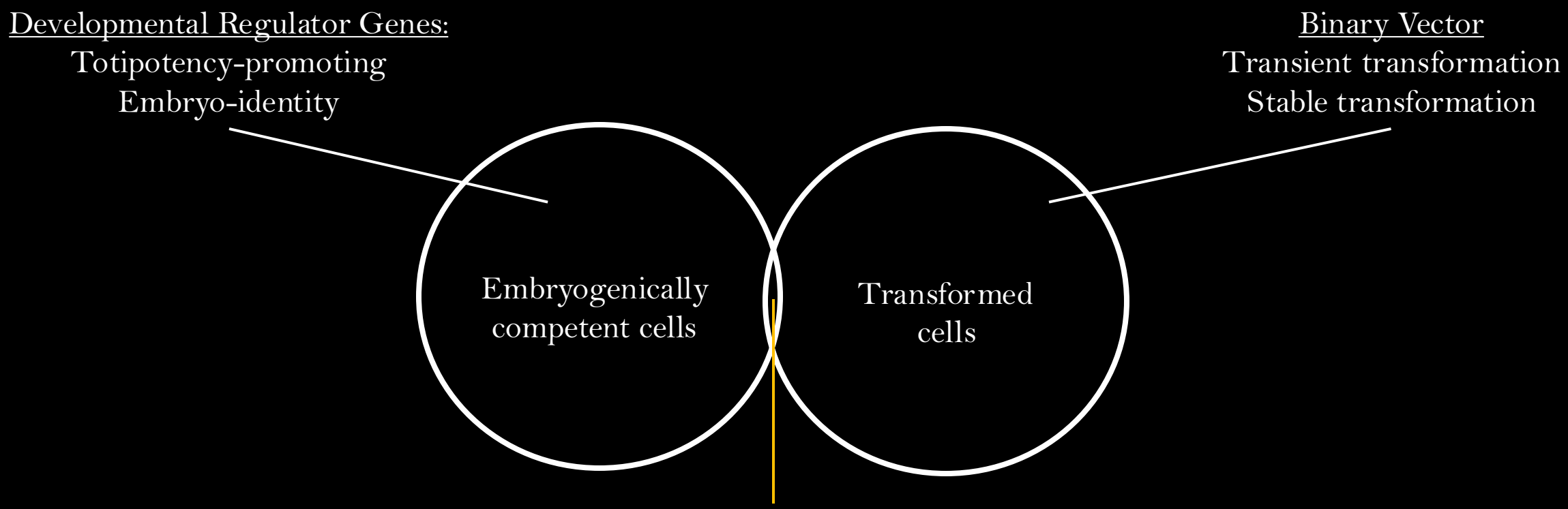
Discussion:

- Binary vector backbone alone improved transient transformation efficiency by ~18% compared to a pCambia based binary vector backbone
- A T-DNA region was designed and constructed to maximize ease of operability in molecular cloning and stable plant transformation work
- Stable transformation of cacao cotyledons was improved by ~3.49-fold using the newly designed T-DNA region within the pLSU-based vector
- Recovery of stable transgenic cacao SEs was not improved

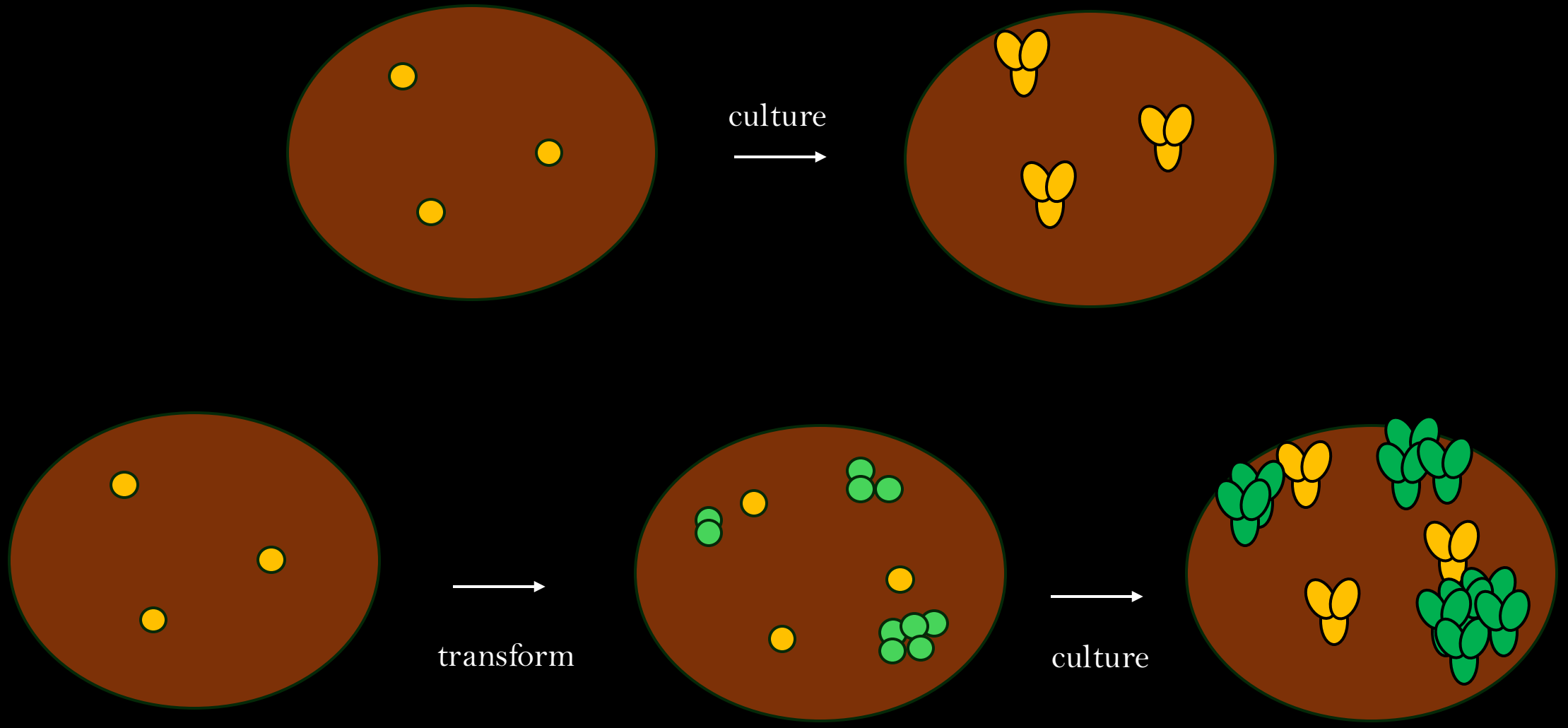
Future Directions:

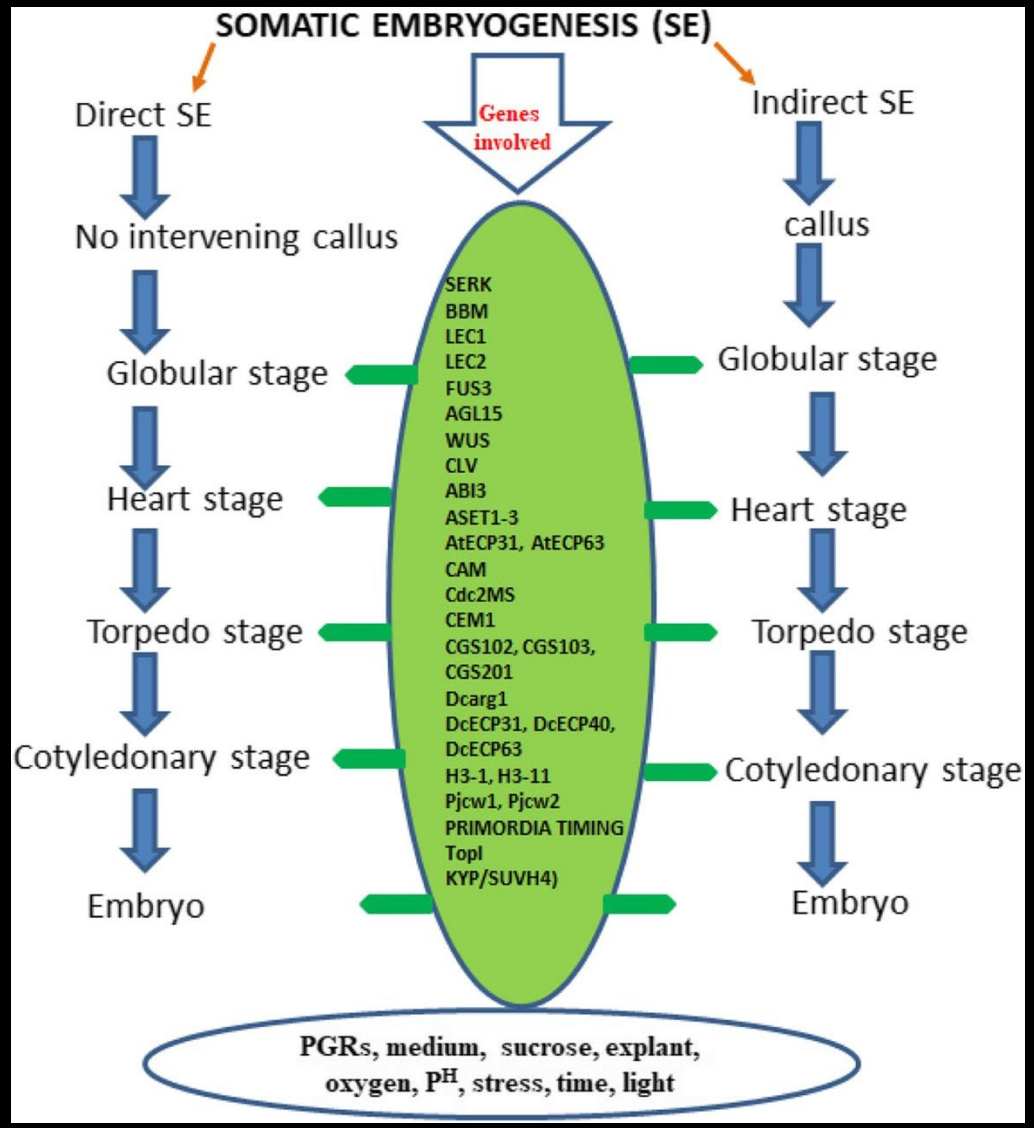
- Test in additional plant species and tissue types
- Systematically determine the source of increased T-DNA transfer

Chapter 4:
Ectopic Expression of Developmental Regulator Genes and Characterization of Cacao Transformation and Somatic Embryogenesis

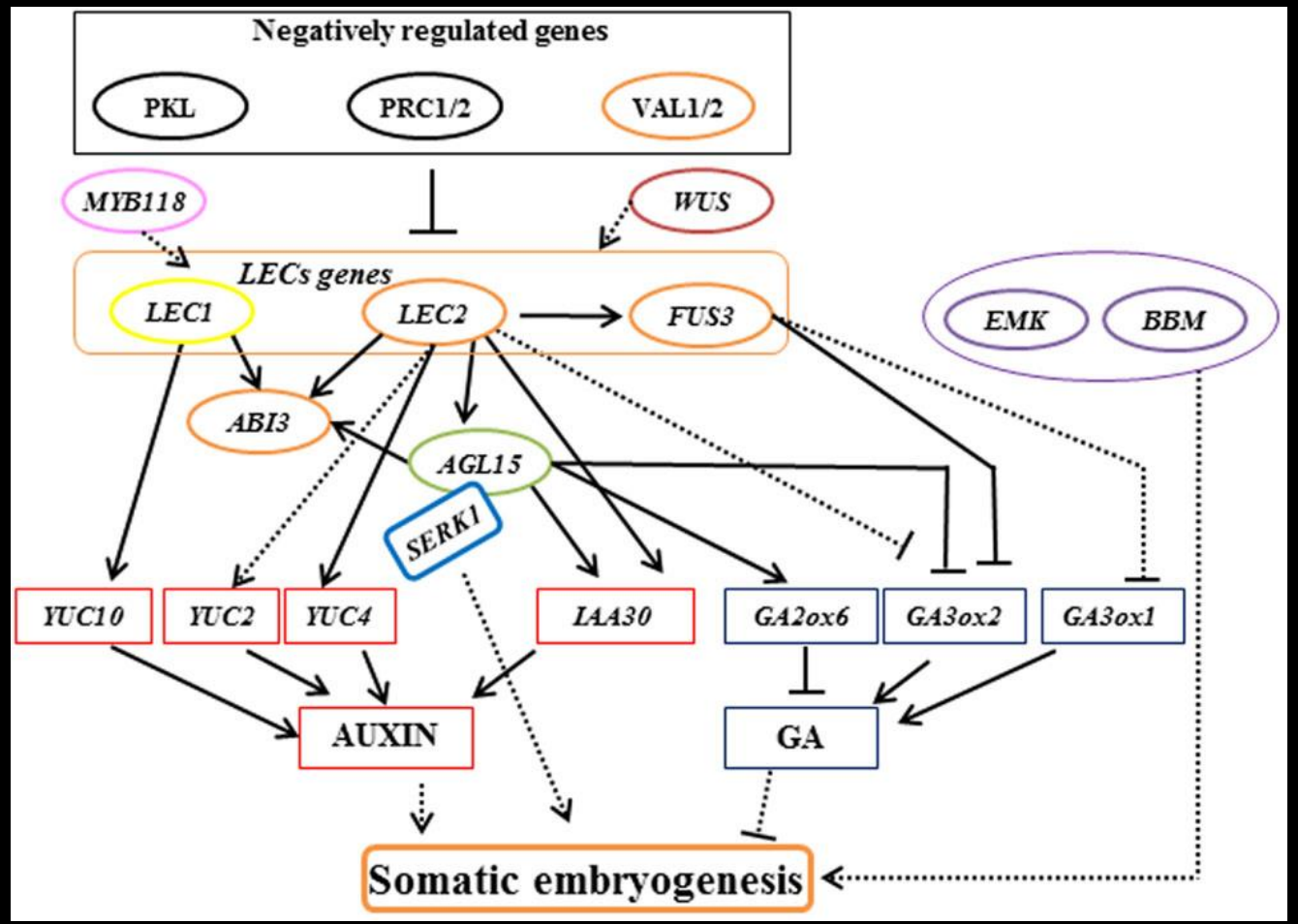

Developmental Regulator Genes:
Totipotency-promoting
Embryo-identity
Binary Vector
Transient transformation
Stable transformation
Embryogenically competent cells
Transformed cells
Recovery of a transgenic cacao SE

Positive Selection Strategy





(Gulzar *et al.*, 2020)



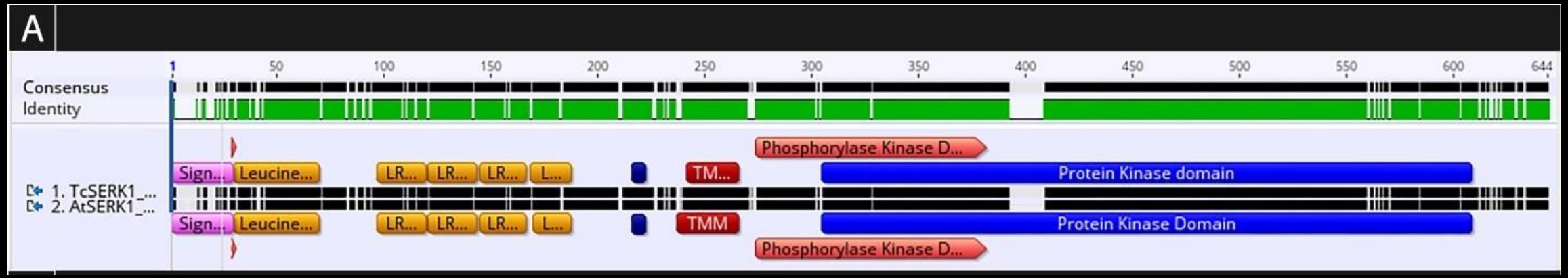
(Guan *et al.*, 2016)

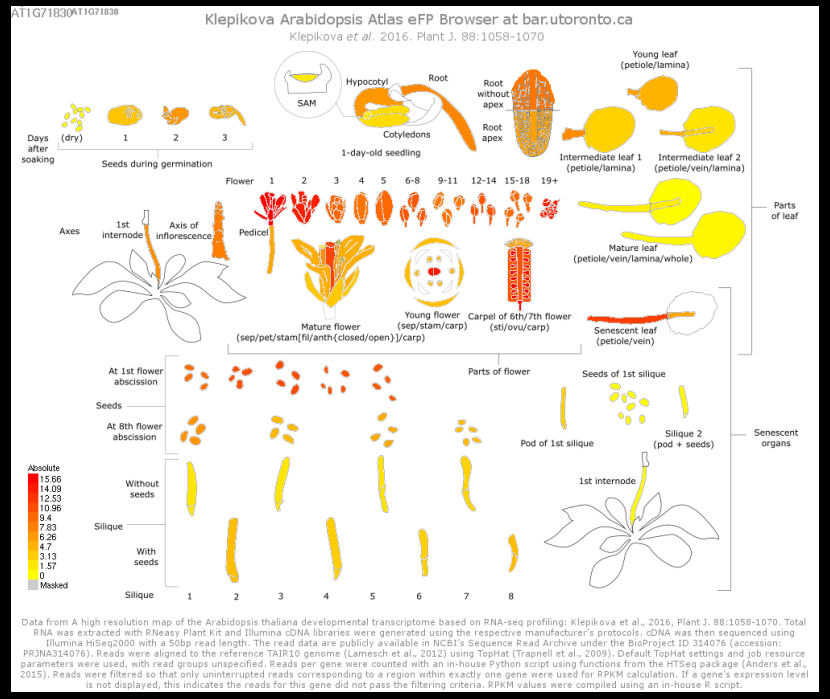
Gene Name	Function	Expression Profile	Ectopic Expression	References
Somatic Embryogenesis Receptor-like Kinase 1 (SERK1)	Auxin perception and biosynthesis induction. Expression of downstream SE-related homeobox genes.	Strongly in globular somatic embryos of cacao and embryogenic clusters. Globular zygotic embryos. Apical meristem. Floral tissues.	Embryonic tissue formation from non-embryonic cells. 3-to-4-fold increase in SE forming calli in A. thaliana.	(Hecht, Vielle-Calzada, Hartog, Ed D. L. Schmidt, et al., 2001; de Oliveira Santos et al., 2005; Hu, Xiong and Yang, 2005; Garcia et al., 2019b, 2019a)
Plethora 5 / Aintegumenta 5/ Embryomaker (PLT5)	Confers embryonic identity to cells. Suppression of cell differentiation. Maintenance of stem cells in roots.	Strongly in developing embryos. Root apical meristem. During wounding and callus formation.	Increased embryogenic calli formation by 3 -to 4-fold. SE regeneration on A. thaliana cotyledons.	(Tsuwamoto, Yokoi and Takahata, 2010; Ikeuchi et al., 2017; Radhakrishnan et al., 2020; Kerstens et al., 2022; Lian et al., 2022; Luo et al., 2023)
Wound Inducing Dedifferentiation 1 (WIND1)	Cell reprogramming towards pluri/toti-potency. Promotes cytokinin signaling and shoot formation.	Expressed throughout vegetative tissues. Induced strongly at wound sites especially in margins in de-differentiating cells.	Hormone-free callus growth and somatic embryo regeneration. Shoot formation.	(Iwase, Ohme-Takagi and Sugimoto, 2011, p. 201; Iwase et al., 2015b, 2021; Lup et al., 2016)
Wuschel (WUS)	Promotes stem cell proliferation. Activator in floral patterning. Activates cytokinin signaling.	Small number of cells in the lower part of the central zone of the shoot and floral apical meristem.	Increased SEs regenerated from embryogenic calli. Hormone-free induction of SEs in multiple species.	(Klaus F. X. Mayer et al., 1998; Zuo et al., 2002b; Rashid, Yamaji and Kyo, 2007; Arroyo-Herrera et al., 2008; Bouchabké-Coussa et al., 2013)
Wuschel-related homeobox 9 (WOX9)	Establish embryo apical-basal polarity. Establish meristematic tissue. Promote cell proliferation.	Early SE in pro-embryogenic structures. Two-cell stage of zygotic embryogenesis. Dividing cells. Basal region of meristems.	Stimulates development of SEs. Callus formation. Stimulates regeneration.	(Haecker et al., 2004; van der Graaff, Laux and Stefan A Rensing, 2009; Fambrini, Usai and Pugliesi, 2022; Krasnoperova et al., 2023; Long et al., 2023)

Cacao ortholog identification

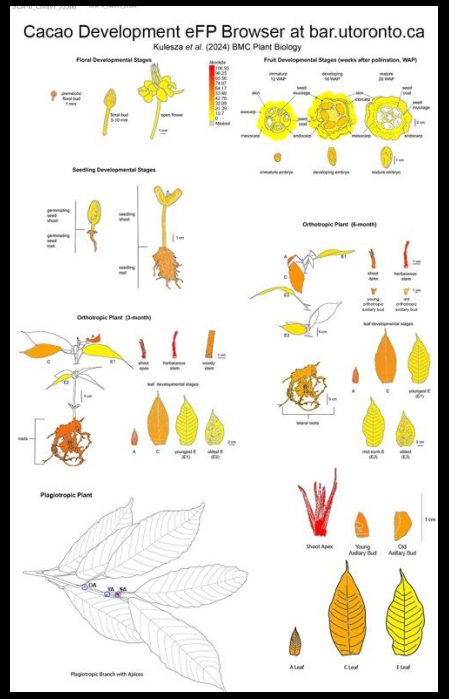
- Alignments to functionally identified gene from *Arabidopsis thaliana*
- Phylogenetic tree
- Gene expression profile from *Arabidopsis* and cacao atlases

TcSERK1

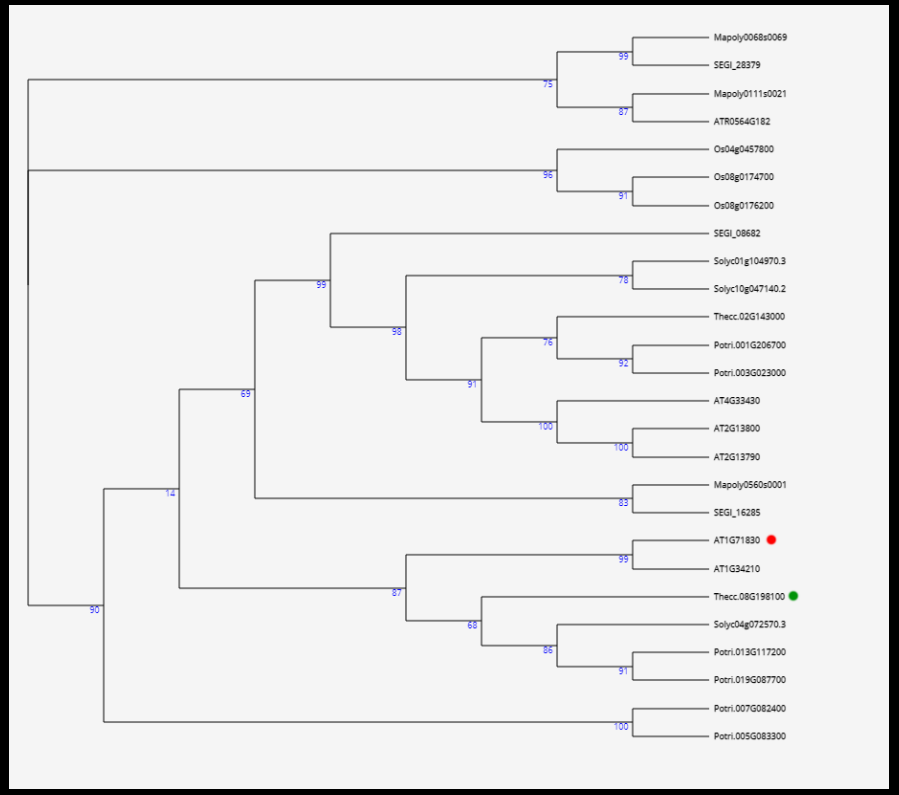




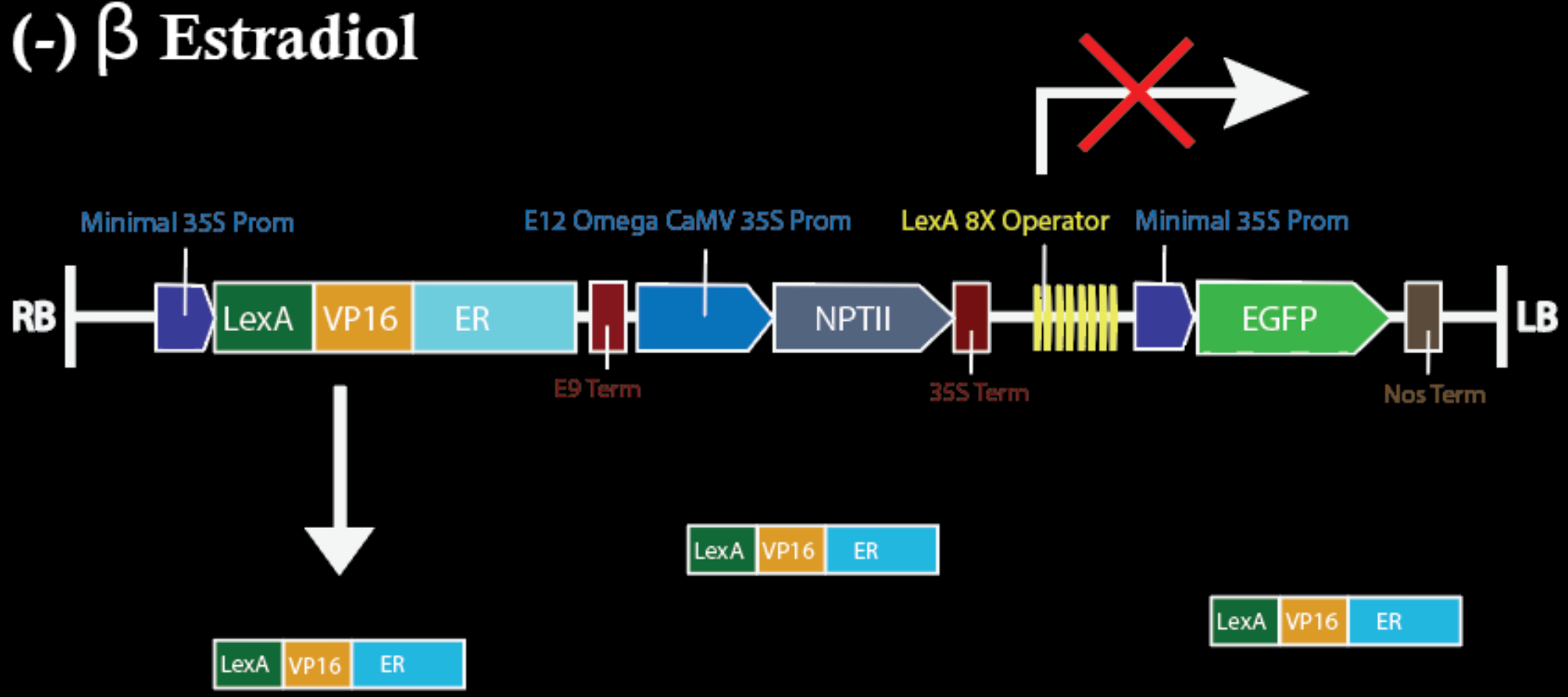
(Klepikova *et al.*, 2016)



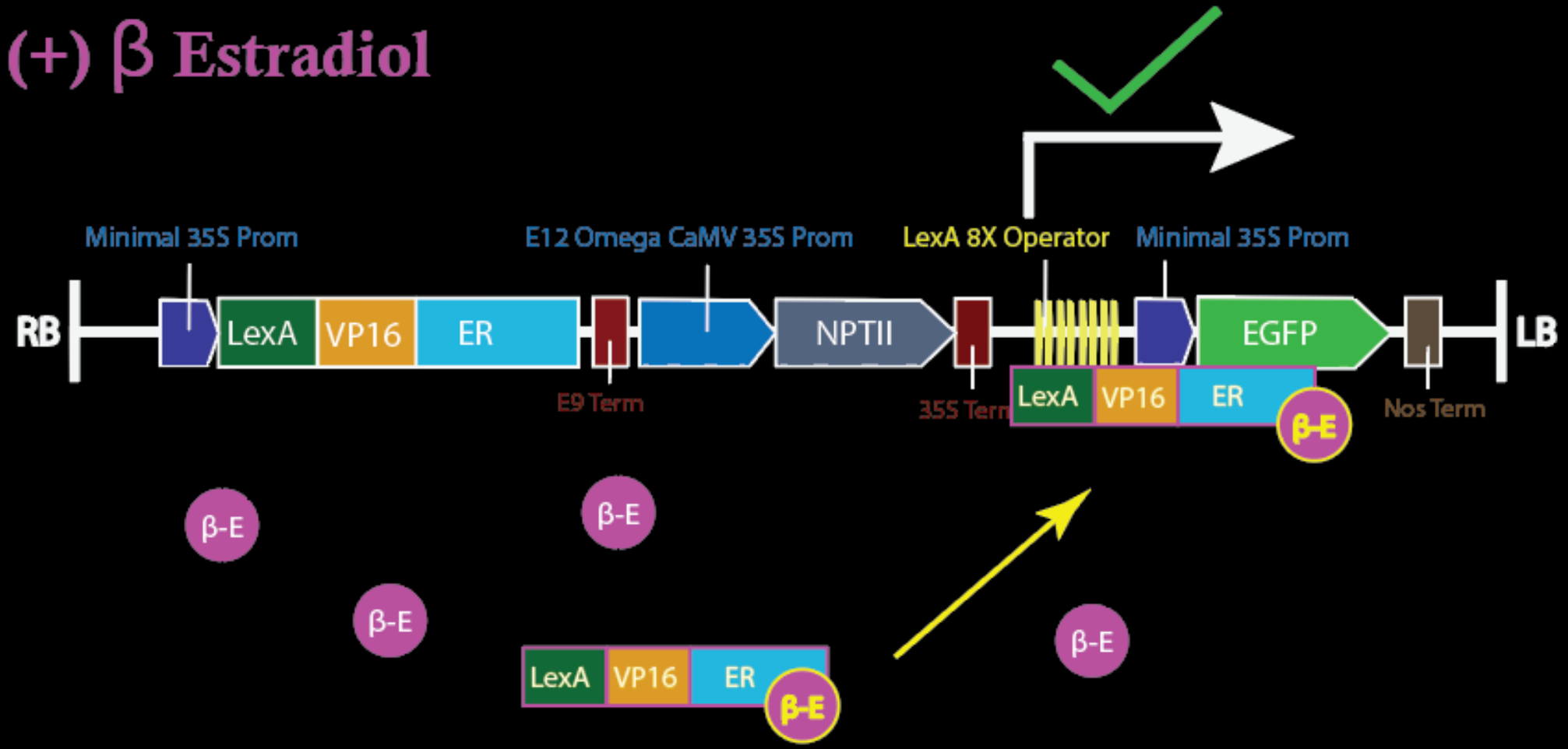
(Kulesza *et al.*, 2024)



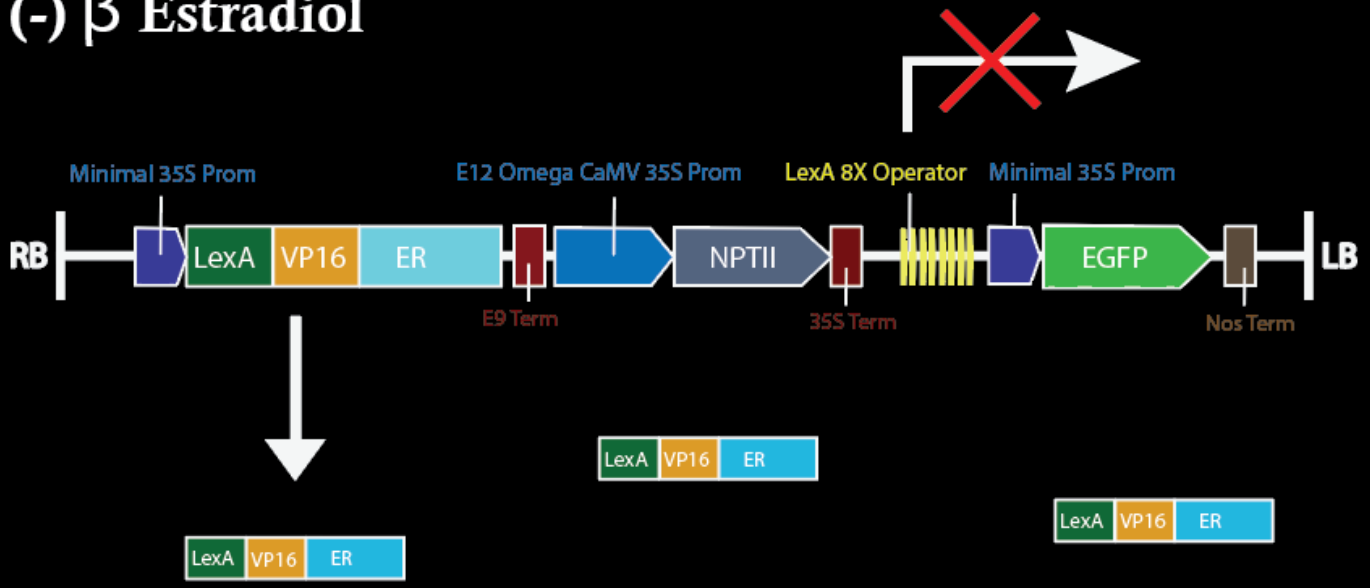
XVE β-Estradiol Inducible System



XVE β-Estradiol Inducible System

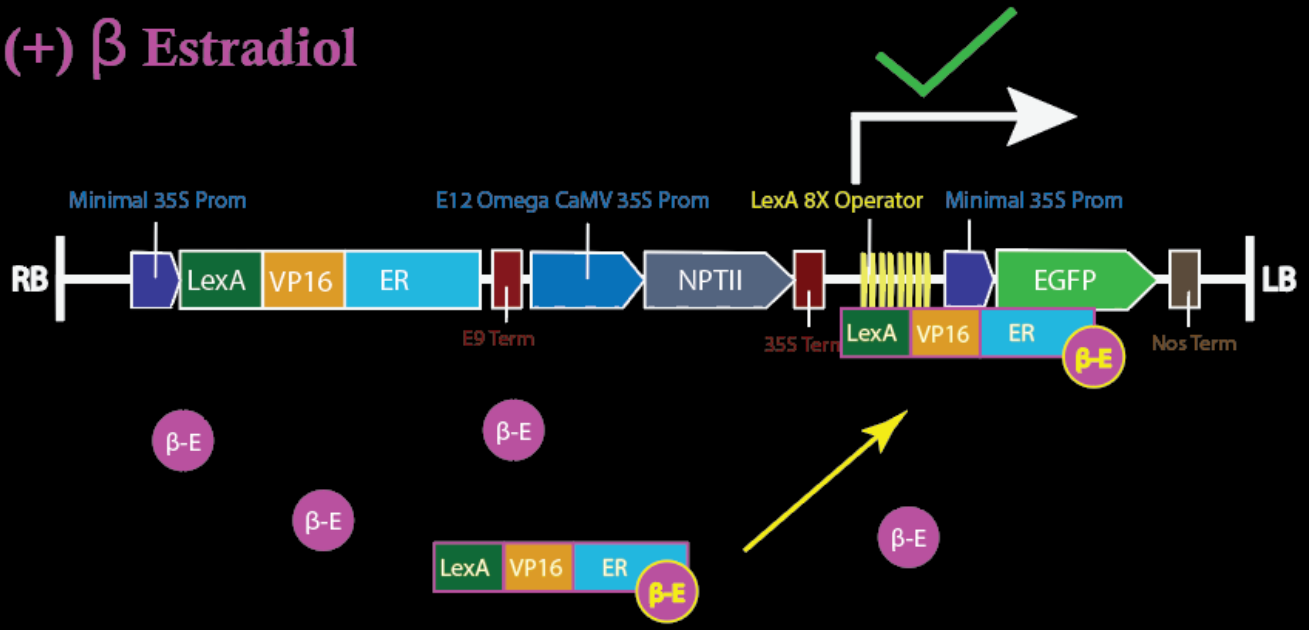


(-) β Estradiol



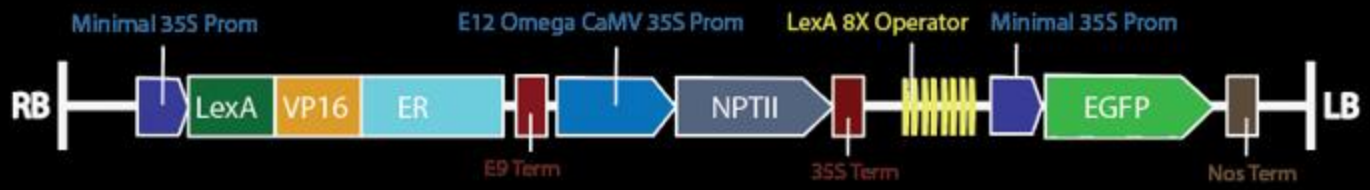
No β-Estradiol
=
no expression

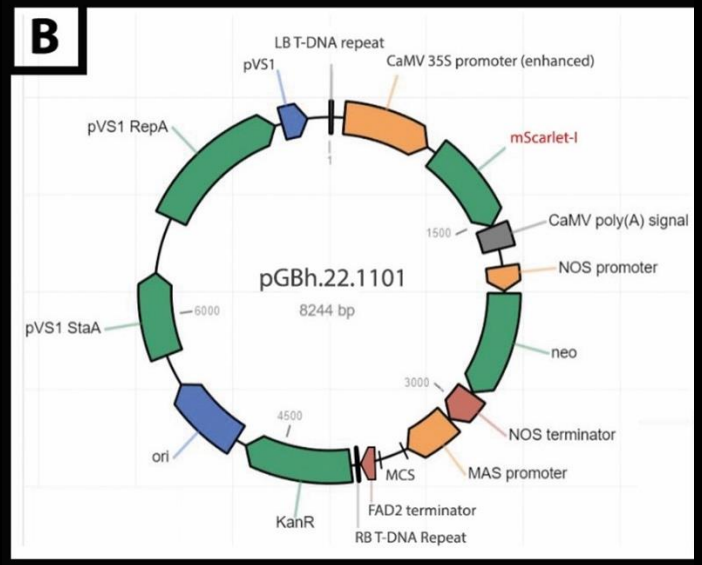
(+) β Estradiol



Yes β-Estradiol
=
gene expressed

XVE System Inserted into new vector





'pXVE-EGFP'

EGFP under the control of XVE-inducible system

Functionally testing the XVE-system: Tobacco *Agro*-mediated Transient Expression Assay

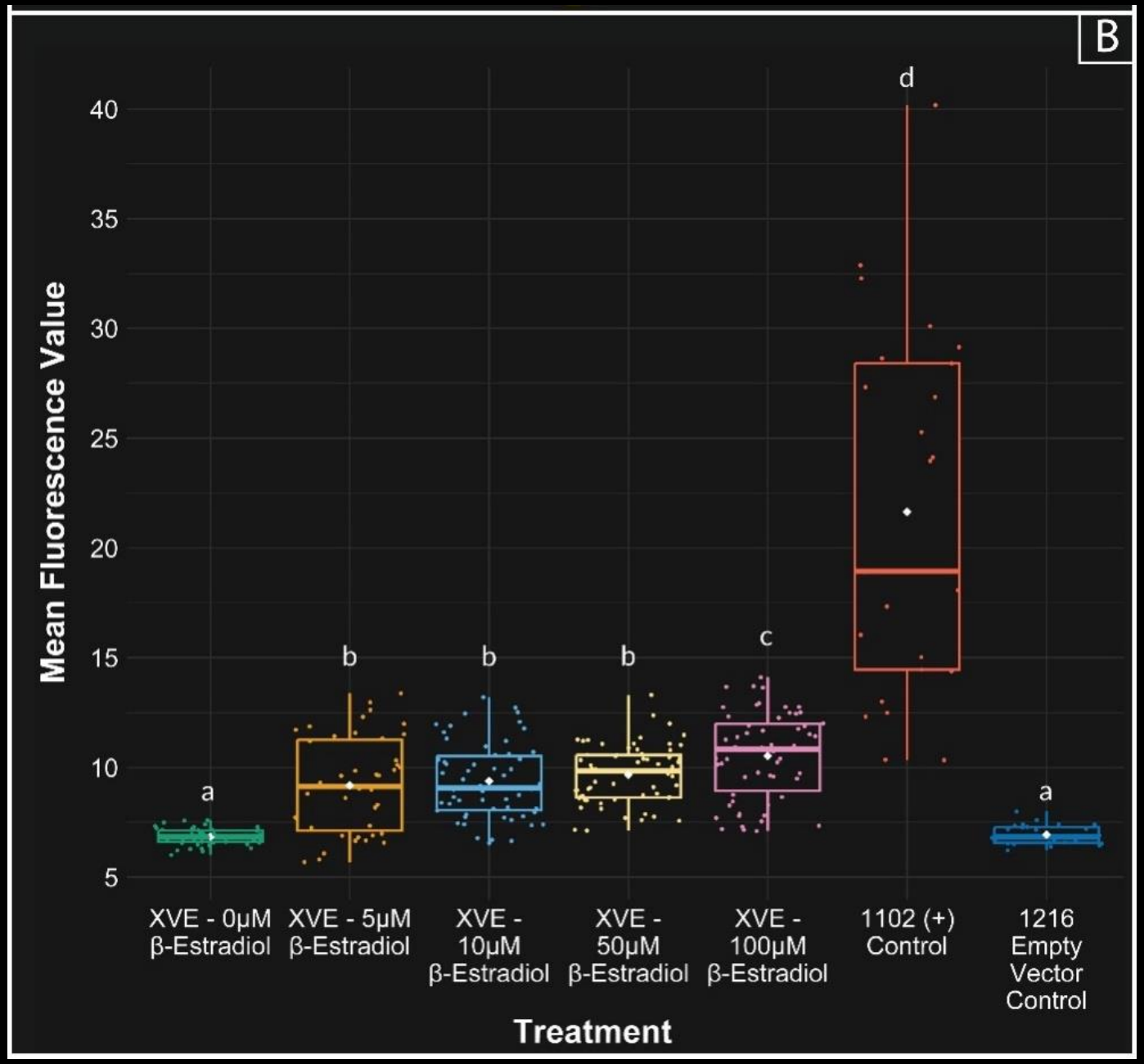
β-Estradiol concentrations: (0μM, 5μM, 10μM, 50μM, 100μM)

Vectors Tested:
pXVE-EGFP, p1102 (constitutive positive control), p1216 (empty vector control)

Infiltrated leaf

3 plants infiltrated / treatment

Imaging after 72 hours



Expression saturates at ~5µM

Mild increase in mean fluorescence at 100µM

0µM ~ p1216 empty vector
‘Non-Leaky’

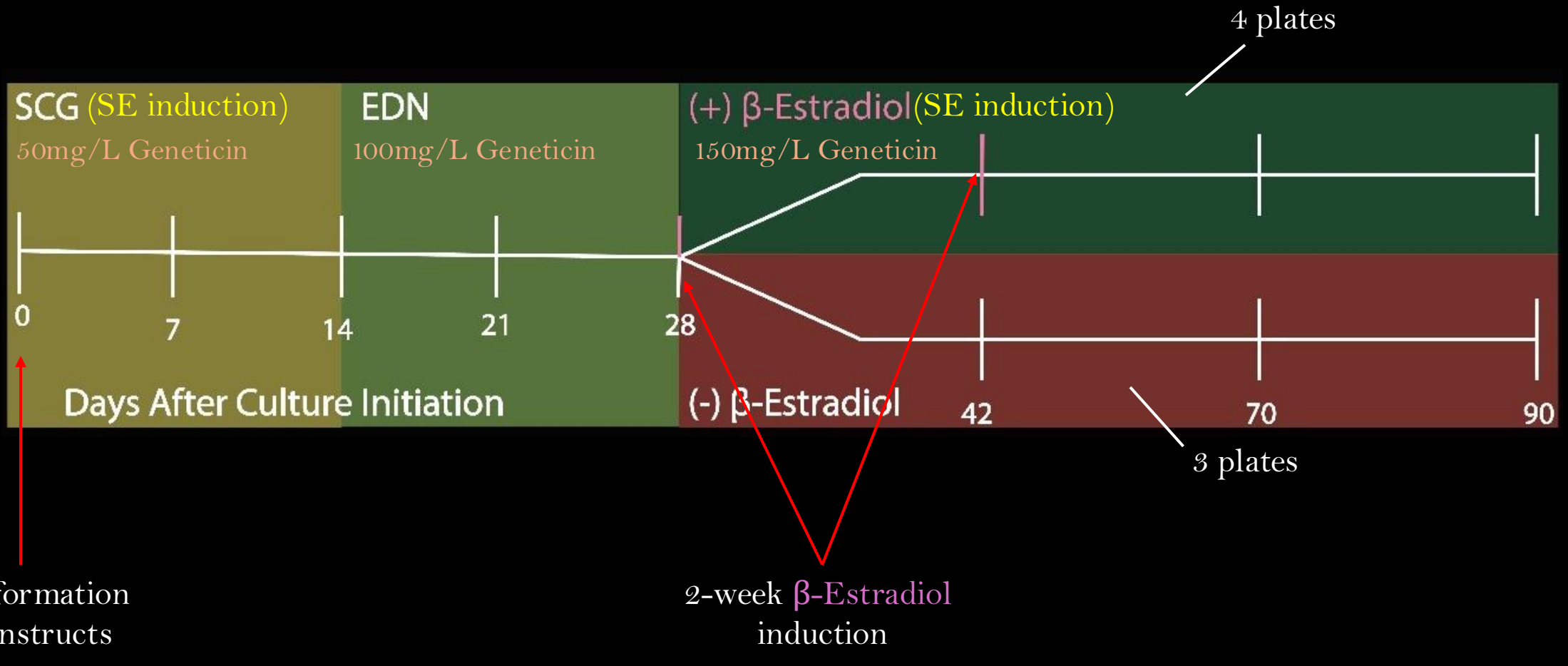
Expression saturates at ~5µM

Cacao Secondary SE Cotyledon Transformation Experiment

Vector being transformed:

Five XVE-DRG Constructs	-7 replicate plates (10 expl/plate)
p1101 (control vector)	-6 replicate plates (10 expl/plate)
p1216 (fluorescence normalization)	- 3 replicate plates (10 expl/plate)

Timeline:


4 plates
SCG (SE induction)
50mg/L Geneticin
EDN
100mg/L Geneticin
(+) β-Estradiol(SE induction)
150mg/L Geneticin
0
7
14
21
28
42
70
90
Days After Culture Initiation
(-) β-Estradiol
3 plates
Transformation of constructs
2-week β-Estradiol induction

Measurements Taken

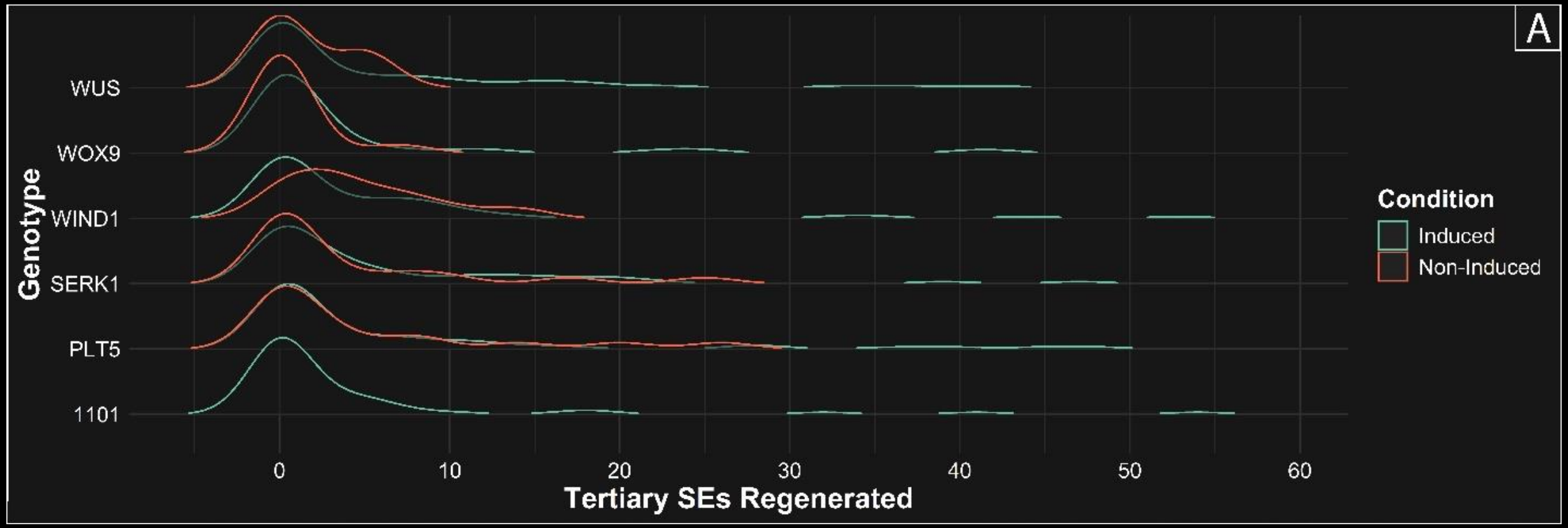
- Brightfield and fluorescence image taken of every explant (~400)
- 7 timepoints over 3 months

ImageJ:

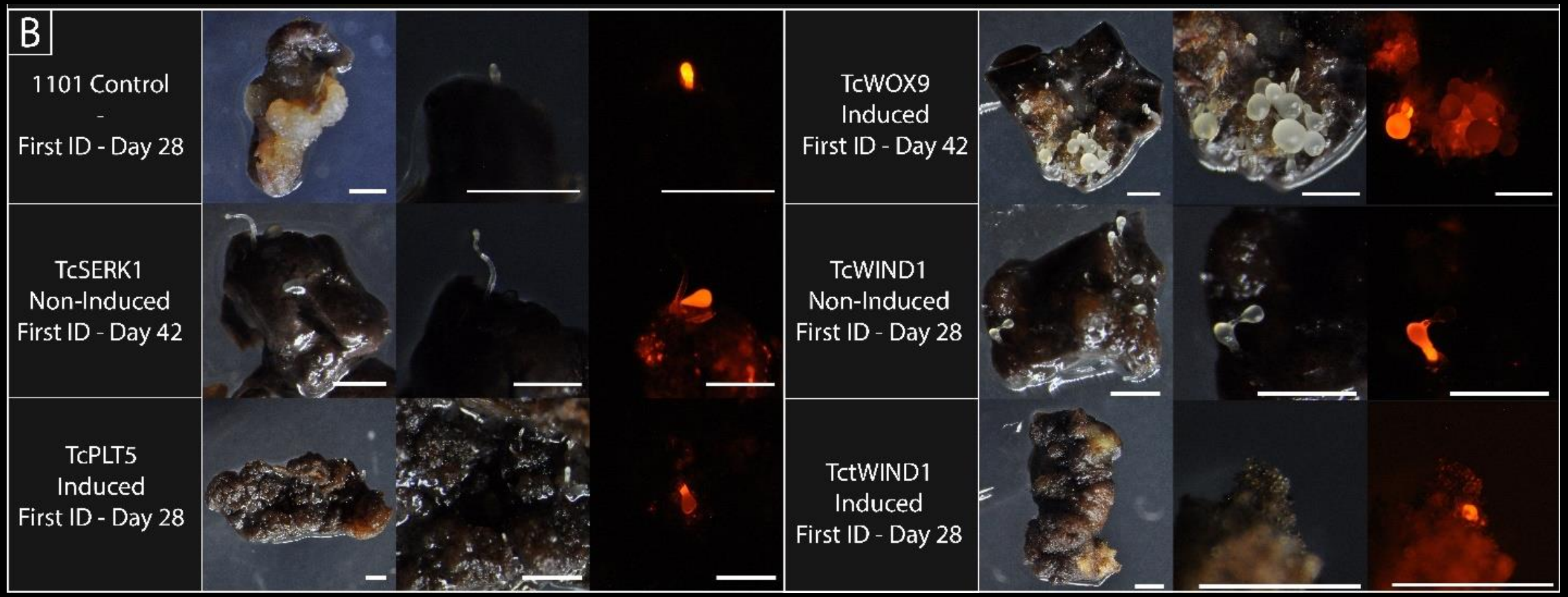
- Explant area
- Explant normalized fluorescence

Metrics calculated:

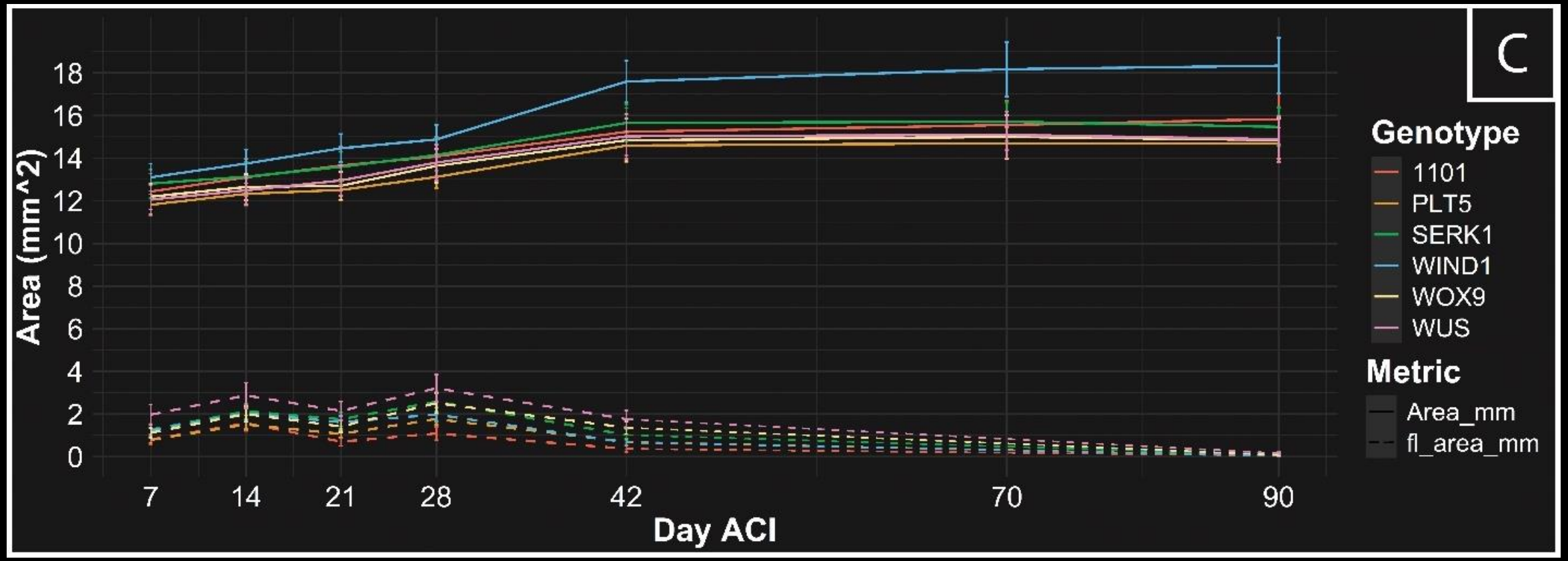
- Normalized fluorescence
- % Explant transformed (coverage)
- Total Growth of tissue
- **Tertiary SEs regenerated**



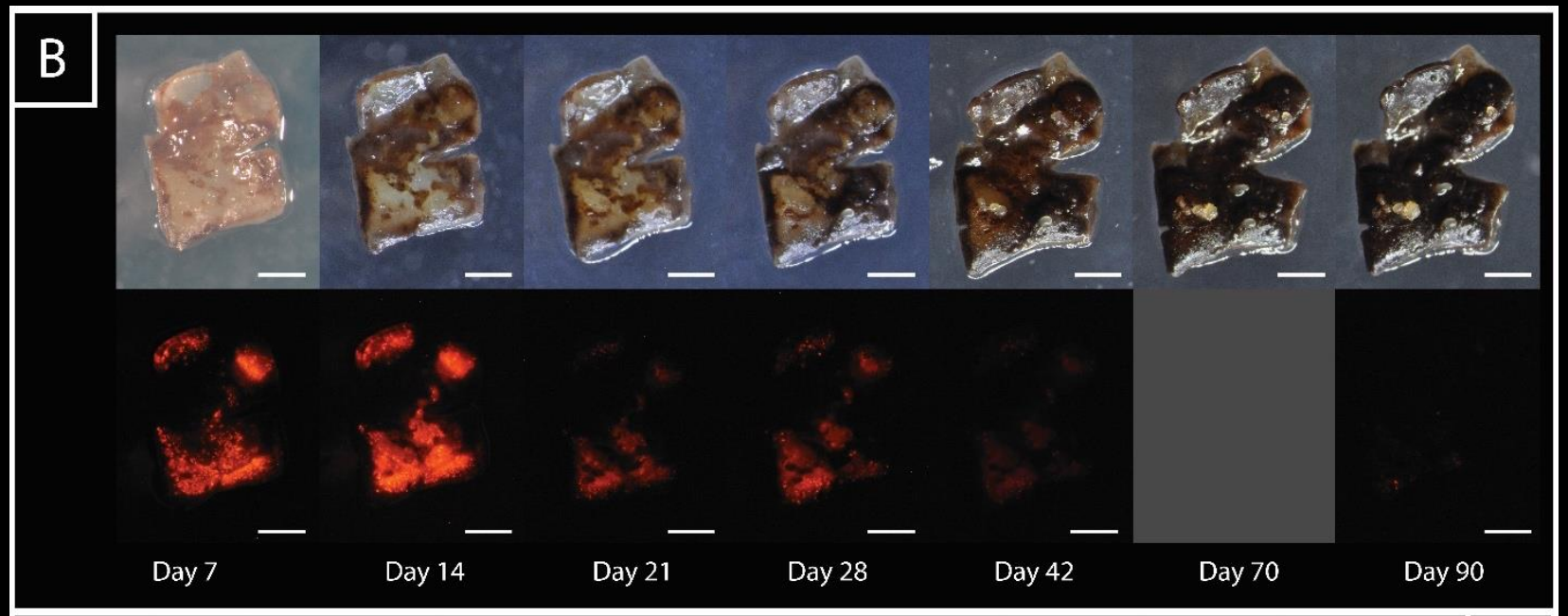
No statistical difference in # of TSEs regenerated

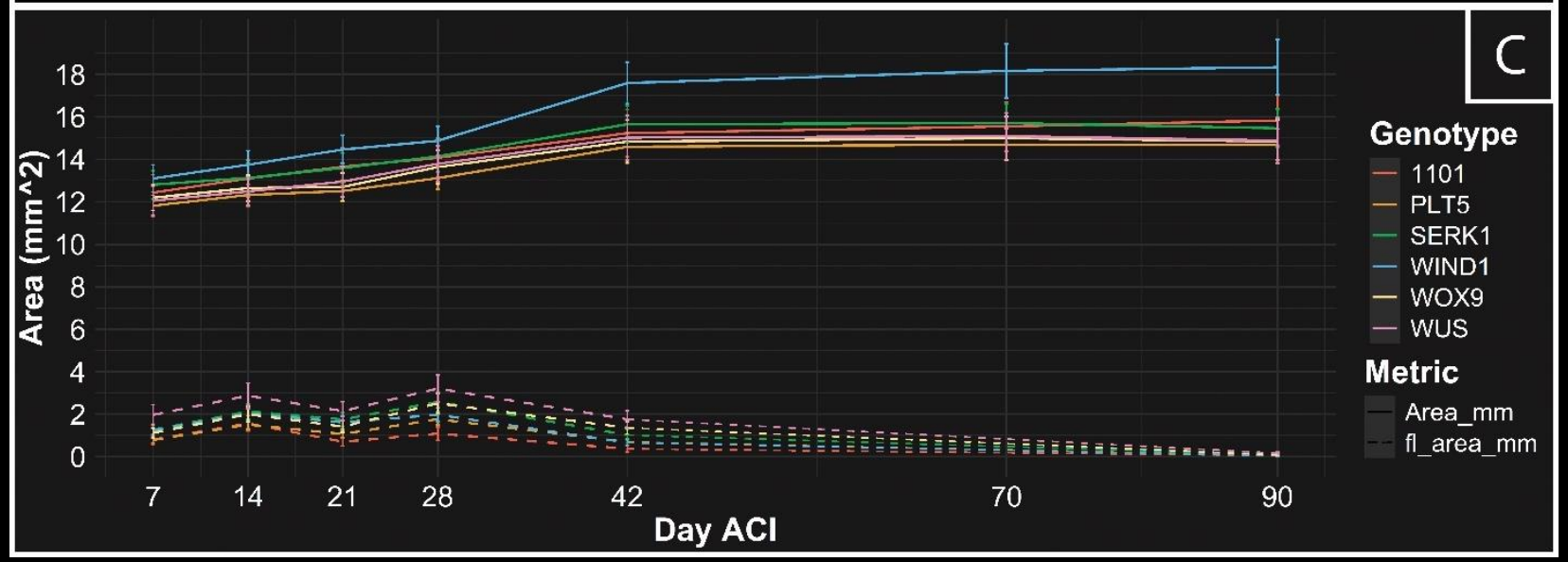


No increase in # transgenic TSEs regenerated



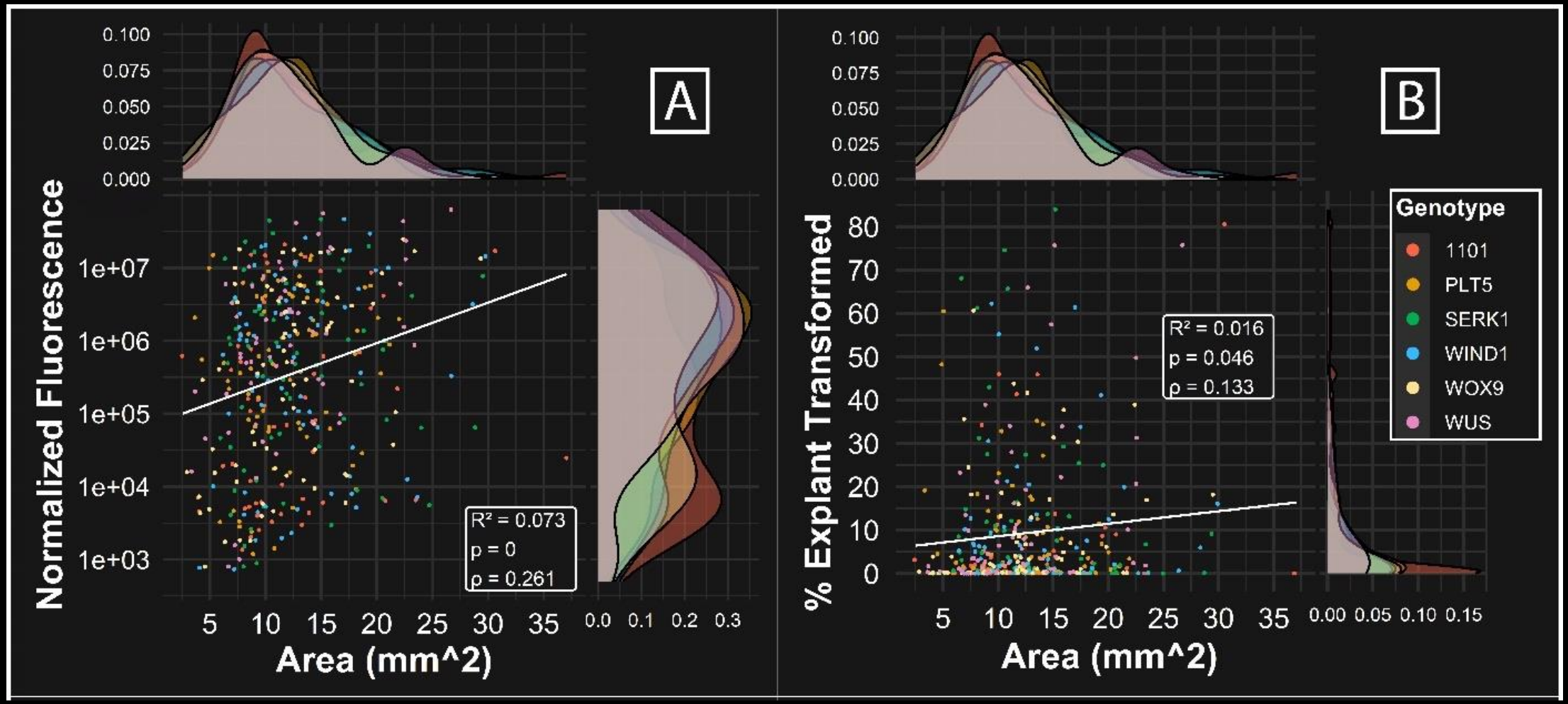
Tissues grow ~4.4% /week until the geneticin selection concentration of 150mg/L





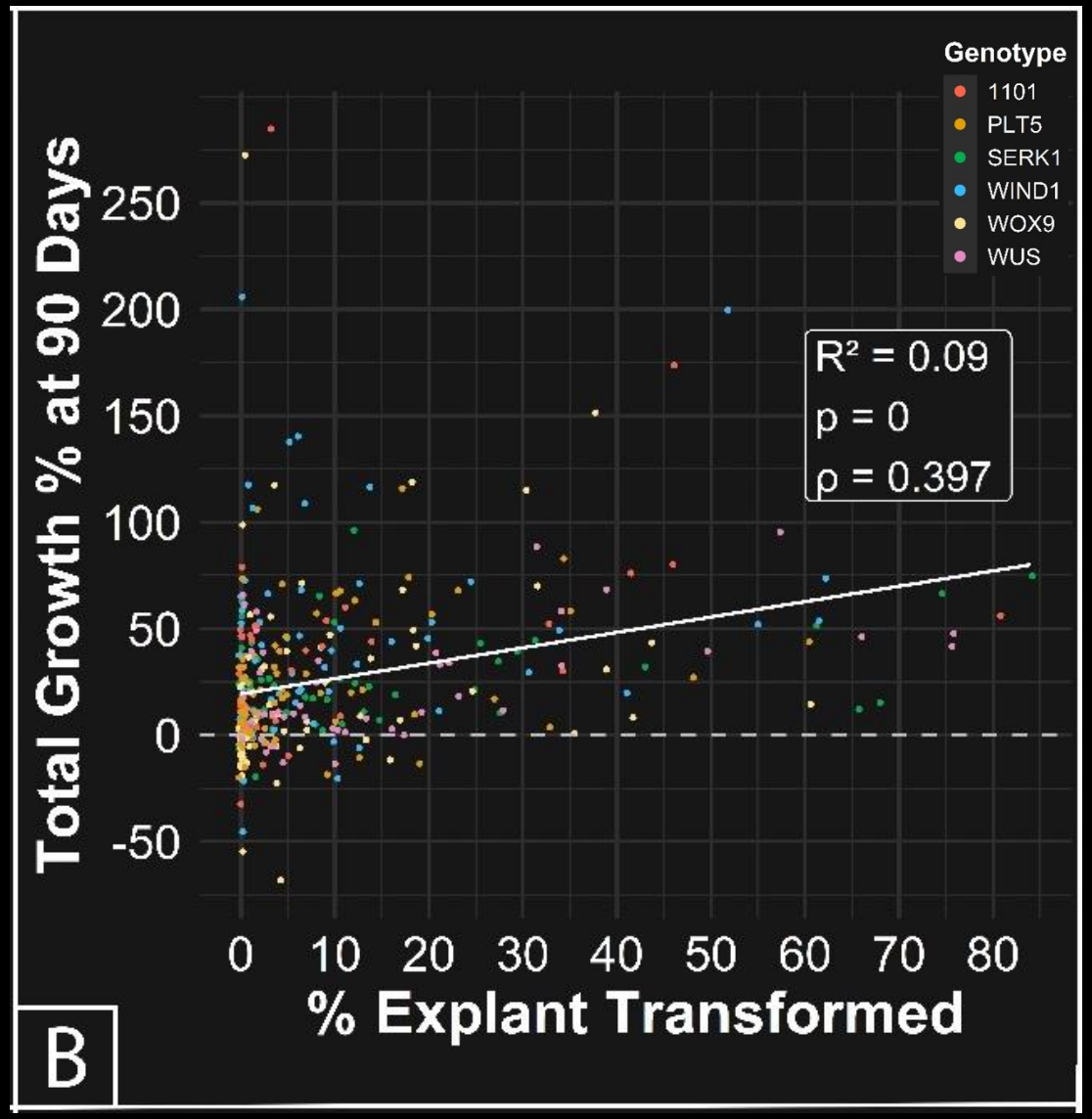
Fluorescence coverage averages ~ 9.5% of total explant area

Fluorescence expression begins declining after 28 days



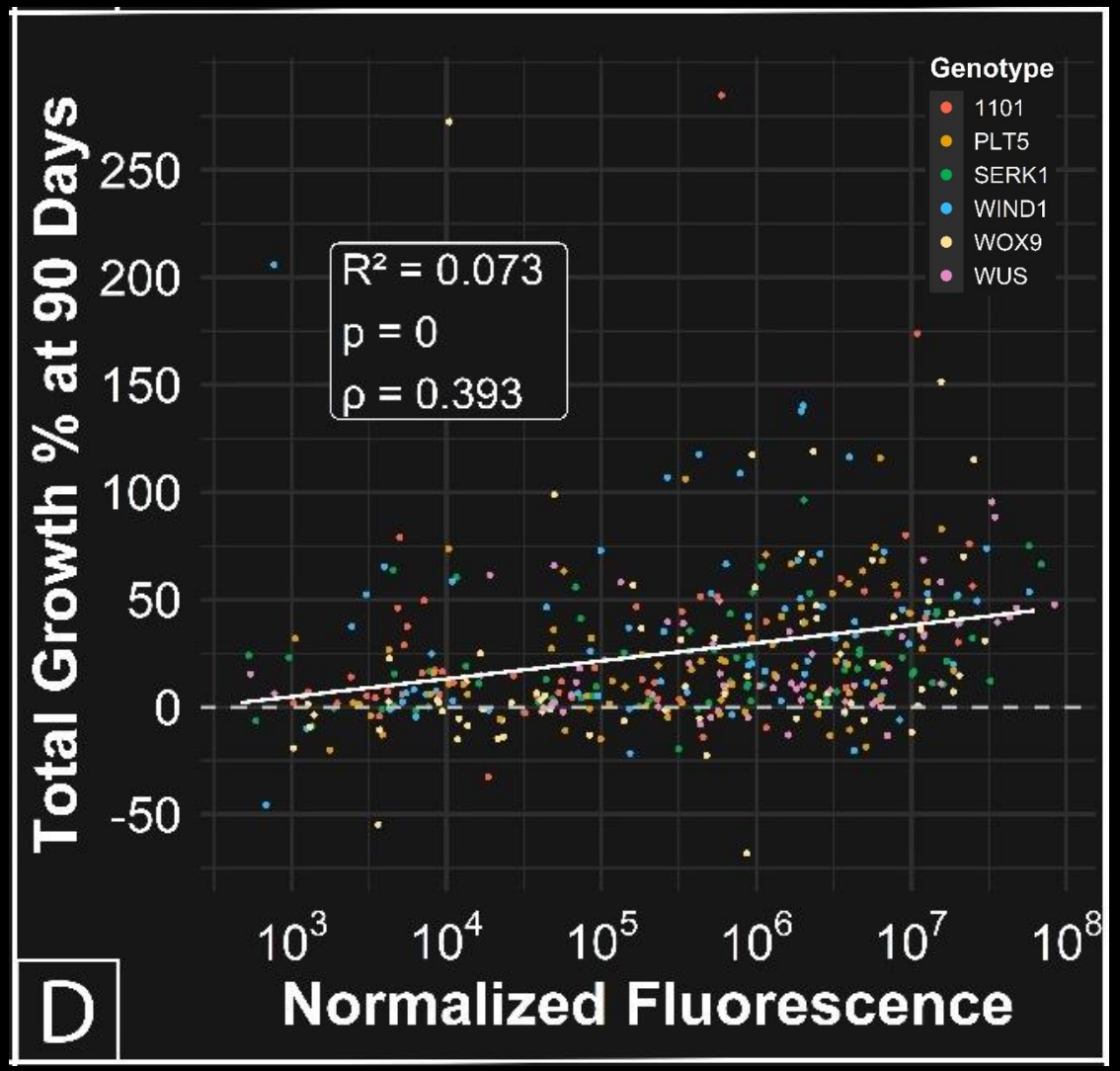
Slight positive monotonic relationship between the initial size of an explant and its' normalized fluorescence

Low positive monotonic relationship between explant size & its' transformation coverage



Positive monotonic relationship between transformation coverage & an explants' total growth

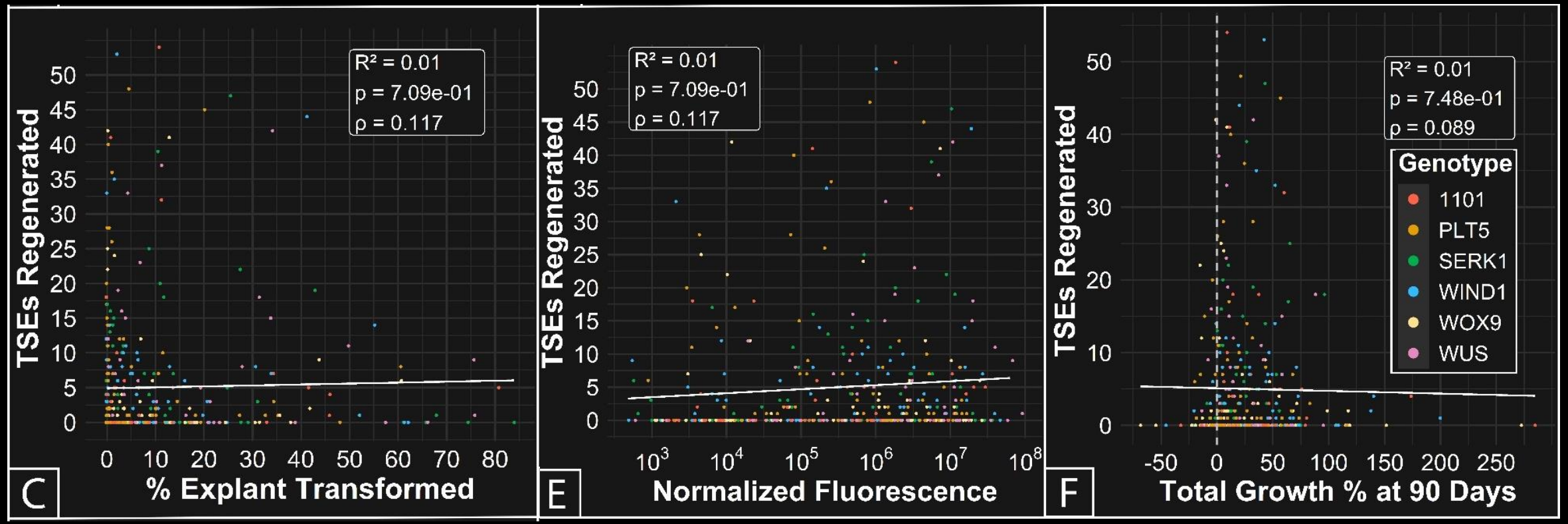
Higher transformation coverage = more cells transformed with selectable marker resistance gene



Positive monotonic relationship between normalized fluorescence & an explants' total growth

Higher expression of T-DNA = more molecules of selectable marker resistance protein

Selection cassette functioning



Transformation coverage, normalized fluorescence, total growth have no impact on the number of TSEs regenerated

Discussion:

- 5 developmental regulator genes were bioinformatically identified, and cloned into an improved stable transformation vector containing an XVE inducible system
- The XVE-EGFP construct was functionally tested and deemed 'non-leaky' in a tobacco leaf transient expression assay
- A DRG pulse 1-month ACI was insufficient to affect regeneration of TSEs and transgenic TSEs in a cacao stable transformation experiment
- Correlating transformation coverage/normalized fluorescence against tissue growth provides an indication that the NPTII resistance cassette is functioning
- An upper threshold of geneticin selection was identified to be 150mg/L for stable cacao cotyledon transformation
- Explant size has no impact on its transformation coverage
- Further optimization to the transformation and regeneration are needed to improve the regeneration of transgenic SEs

Future Directions:

- Further studies to determine to test the functionality of XVE-DRG constructs
- Optimization of DRG expression times for positive selection
- Combinations of ectopic expression of DRGs for positive selection

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Questions?