National Science Foundation
Plant Genome

Plant Transformation Workshop

University of Missouri

NSF Cereal Transformation Workshop

University of Missouri Plant Biotechnology Innovation Laboratory

And

Plant Transformation Core Facility

July 30 to August 1, 2018

University of Missouri-Columbia

A Key to the Transgenic Research

007B, Ernie & Lottie Sears Plant Growth Facility

University of Missouri, Columbia, MO 65201, U.S.A.

Workshop Organization

Organizer: Plant Biotechnology Innovation Laboratory

Zhanyuan J. Zhang	Ph.D. and Director
Muruganantham Mookkan	Ph.D. and Research Scientist
Christopher J. Willig	Ph.D. student
Alexander Wells	Undergraduate student

Contributor: Plant Transformation Core Facility

Hyeyoung Lee	Ph.D. and Senior Research Specialist
Yanjiao Zou	Ph.D. and Senior Research Specialist
Neng Wan	B.S. and Senior Research Lab Technician
Hien Bui	B.S. and Research Specialist

To find out more about our Plant Biotechnology Innovation Laboratory, please visit our web:

https://plantsciences.missouri.edu/plantbiotechlab/

We will post stepwise protocols of cereal species online soon.

Agenda

Monday, July 30, 2018 107 Bond Life Science Center

AM

8:00 – 8:30	Check in
8:30 - 9:00	Welcome and introduction
9:00 -10:00	Lecture: Mechanisms of somatic embryogenesis
10:00-11:00	Lecture: Best practice in cereal transformation
11:00-12:00	Lecture: Cereal transformation protocol overview

Welcome lunch Mizzou Reynolds Alumni Center

PM 007B, Sears Plant Growth Facility

1:30 - 2:00	Tour Plant Biotechnology Innovation Laboratory & Transformation Core Facility
2:00 - 3:30	Maize inbred transformation - embryo isolation and *Agrobacterium* infection
3:30 - 4:00	Coffee break
4:00 - 5:30	Sorghum transformation - embryo isolation and *Agrobacterium* infection
Adjourn	Dinner on your own

Internet access in Life Science Center:

Network: TigerWifi-Guest

Password: ask workshop staff to enter

Agenda

Tuesday, July 31, 2018 007B, Sears Plant Growth Facility

AM

8:30 - 10:00 Maize inbred culture transfer

10:00 – 10:30 Coffee break

10:30 - 12:00 Sorghum culture transfer

Lunch Mizzou Reynolds Alumni Center

PM 007B, Sears Plant Growth Facility

1:30 - 3:30 Isolation of *Brachypodium* immature embryos for callus initiation

3:30 - 4:00 Coffee break

4:00 - 5:30 *Brachypodium* infection and culture transfer

Adjourn Dinner on your own

Internet access in Sears Plant Growth Facility:

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Agenda

Wednesday, August 1, 2018 107 Bond Life Science Center

AM

8:30 - 10:30 Discussion forum and workshop wrap up

Internet access in Sears Plant Growth Facility:

Network: TigerWifi-Guest

Password: ask workshop staff to enter

Molecular processes behind somatic embryogenesis

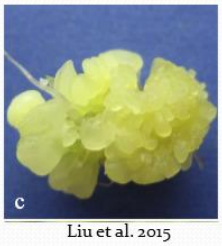
Presented by Chris Willig

07/30/2018

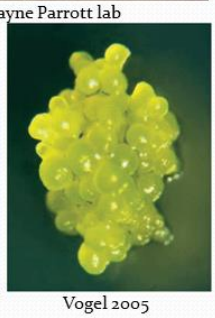
Somatic embryos: tiny plant clones

- Somatic embryos are structures formed from vegetative cells (single or a small group) that undergo development to become independent mature plants
- Unlike normal (zygotic) embryos that are formed from the fusion of two gamete cells, somatic embryos are genetic clones of the parent tissue.

Image credit: Wayne Parrott lab



Liu et al. 2015



Vogel 2005

Uses of somatic embryos in science and industry

- The study of the development of zygotic embryos
  - Synchronized development
  - Easier access to embryo tissue

Image credit: Dutch Passion Seed Company

- Mass propagation of genetically identical plants
  - Synthetic seeds
- Regeneration of transgenic plants following genetic modification

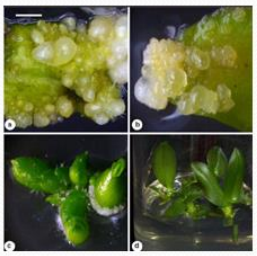


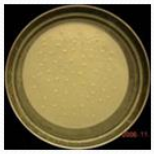
Image credit: Gow et al., 2010

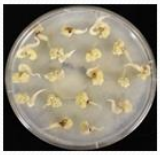
Image credit: Karami & Saidi, 2010

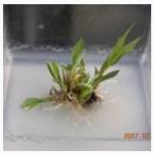
Production of transgenic plants

Explant

Tissue derived from wild-type plant







Transformation

Agrobacterium-mediated, particle bombardment, etc

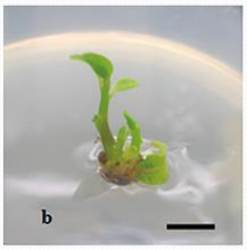
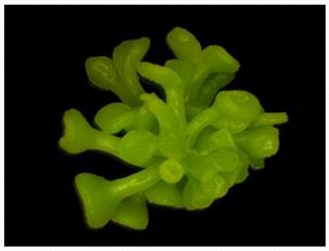
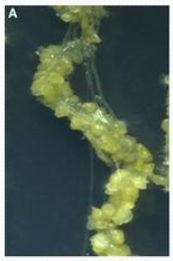
Regeneration

Somatic embryogenesis, organogenesis

Modified plant

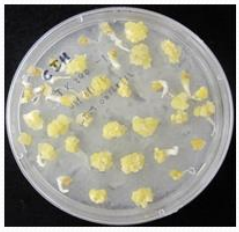
Tissue culture

Types of plant regeneration

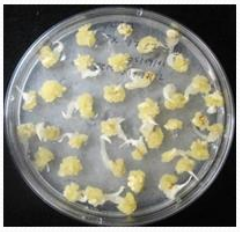
	Organogenesis	Somatic embryogenesis
Direct		
Indirect		

Genotype screen for regeneration capacity

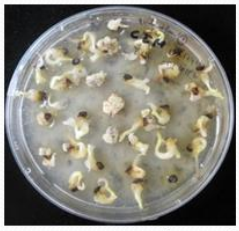
BTx623 (<10%)



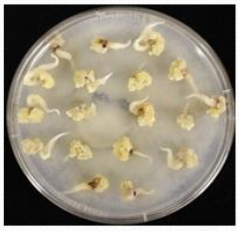
Tx430 (>80%)



Tx 2737 (>80%)



Wheatland1(<10%)



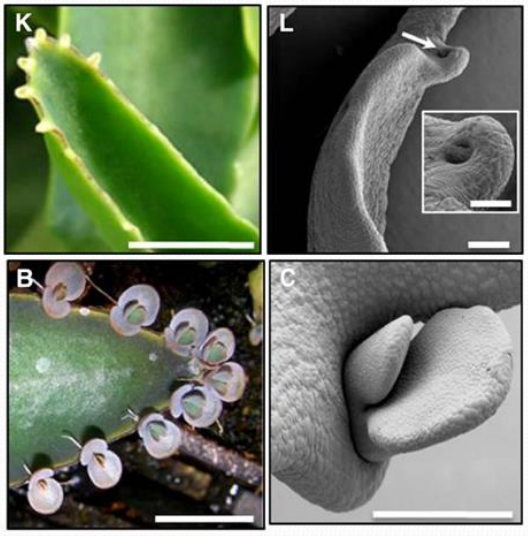
PI 898012 (>80%)

Do et al. 2016

Somatic embryogenesis in biotechnology

- Somatic embryogenesis is a process that is mainly induced in vitro by stress treatment and/or hormone exposure in tissue culture media
- Most of the transformation protocols used for monocot crop plants utilize somatic embryogenesis to regenerate transformants
- Deriving mature plants from a single cell (or small group of cells) increases the likelihood that all cells in the plant carry the desired modification

Kalanchöe, "mother of thousands": a case of naturally occurring somatic embryogenesis



Stages of somatic embryo development

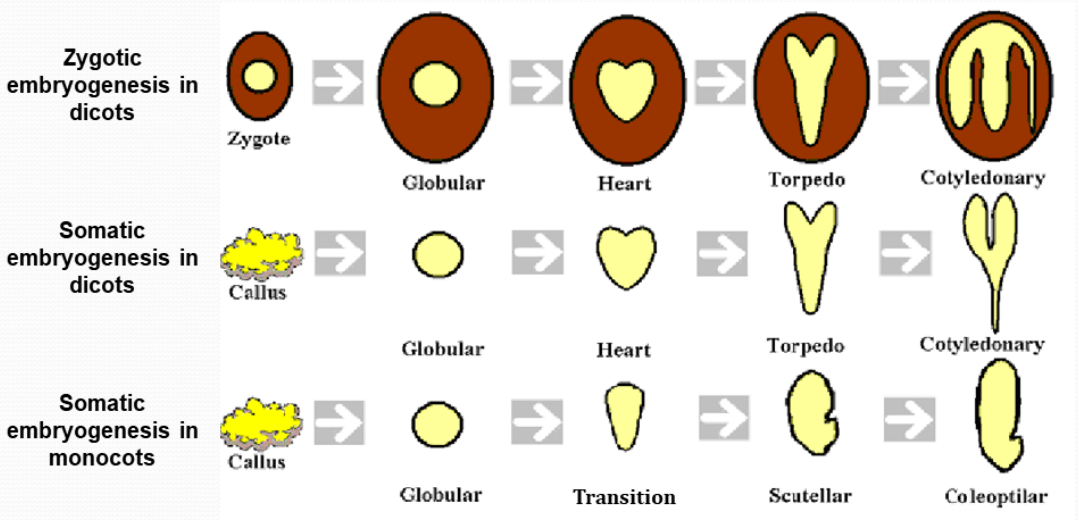


Image credit: Greer, 2008

Broad-scale cellular changes required for somatic embryogenesis

- De-differentiation
  - Somatic cells that have undergone some degree of developmental specialization are re-programmed in order to become stem cell-like.
- Acquisition of totipotency
  - Cells must be competent to receive signals that cause it's gene expression profile to mirror that of a zygote.
- Commitment to embryogenesis
  - Cells undergo wide-ranging expression changes to processes such as cell cycle, signal transduction, and meristem formation and maintenance.

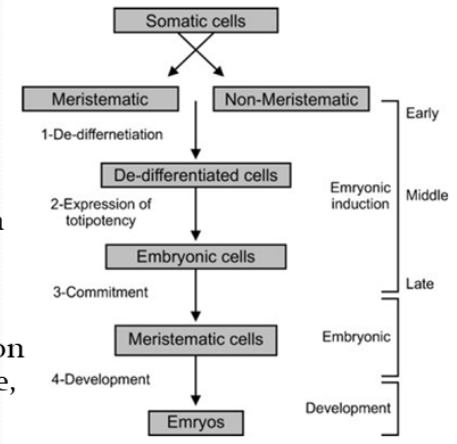
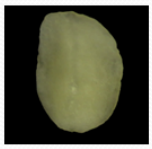


Image credit: Elhiti et al. 2013

Tissue source for somatic embryos

- In general, less developed tissue is more amenable to de-differentiation and somatic embryo production



- Some common cell types that embryogenic callus is derived from include root pericycle, pro-vasculature, and apical/axillary meristem

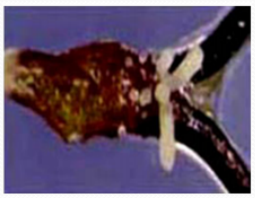


Image credit: Karami & Saidi, 2010

- Explant tissue that has been used includes cotyledon, leaf disk, immature/mature zygotic embryos, and even other somatic embryos (secondary SE).

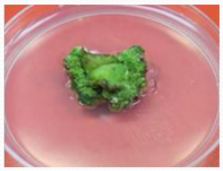


Image credit: Heidari et al., 2012

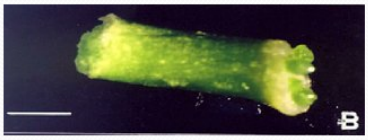


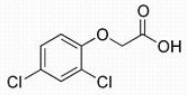
Image credit: Kaul et al., 2014

Factors that can induce somatic embryogenesis

- Stress
  - Somatic embryogenesis has been induced by exposure to heavy metal ions, osmotic pressure, tissue dehydration, explant wounding, and heat shock in various plants.
  - The induction of abiotic stress responses (i.e. ROS production) during SE suggests that stress is important in initiation.
- Hormones
  - Mainly auxin and cytokinin. These plant growth regulators are commonly added to tissue culture media to promote callus formation or embryogenesis.

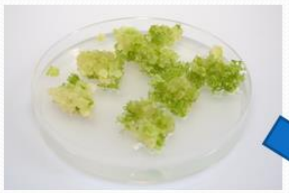
Use of plant hormones in tissue culture

Manipulation of relative auxin and cytokinin levels in tissue culture media can induce different stages of plant regeneration



2,4-D, a potent artificial auxin compound

High auxin : low cytokinin



Callus induction

High cytokinin : low auxin

Shoot induction

High auxin : low cytokinin

Root induction

Effects of other hormones on somatic embryogenesis

- Gibberellins (GA) are thought to generally inhibit somatic embryogenesis. Transcripts of GA metabolizing enzyme GA20-oxidase are increased during SE in arabidopsis, maize, and sorghum.
- Nevertheless, GA3 is used in media to induce SE in chickpea, geranium, and *Medicago sativa*.
- Ethylene also has an ambiguous effect on SE. It has been shown to be inhibitory in Arabidopsis (Bai et al., 2013) and necessary for SE proliferation in *Medicago* (Kępczyńska et al., 2009).
- Abscisic acid (ABA) generally has a positive effect on SE. This may be explained by its role in abiotic stress response.

Why study the mechanism of somatic embryogenesis?

- Insight into the evolutionary origin of zygotic embryogenesis
- Enhanced understanding of what hormone/media treatments will promote plant regeneration
- Utilization of genetic factors to induce somatic embryogenesis in plant species or genotypes that are recalcitrant to this process

Molecular regulation of somatic embryogenesis

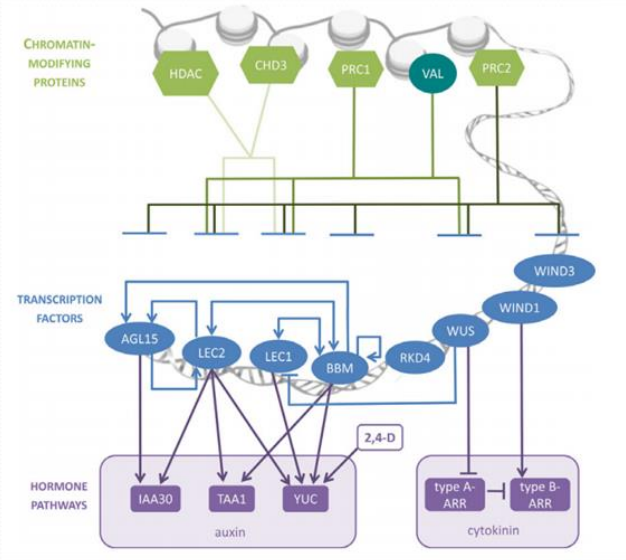


Image credit: Horstman et al. 2017, Figure 2

De-differentiation

- Plant cells developmentally reverting to a less specialized state involves wide-scale epigenetic re-organization.
- The formation of callus is characterized by cellular de-differentiation.
- Callus formation occurs in nature in response to wounding or other stressors such as pathogen attack.

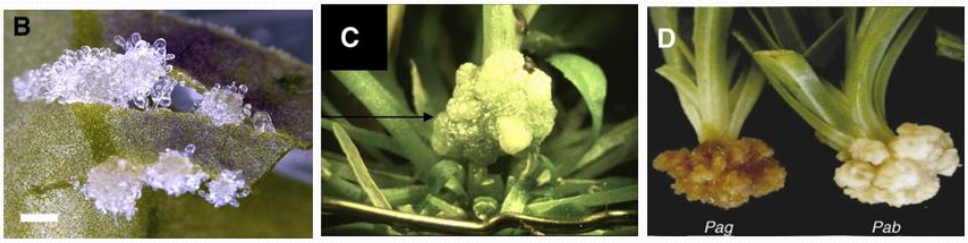


Image credit: Ikeuchi et al. 2013

De-differentiation

- Chromatin modification genes are essential for re-programming of somatic cell fate.
- A complex of proteins known as the POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) is responsible for histone methylation which represses gene expression.

The protein PICKLE (PKL) functions in chromatin remodeling. It plays an antagonistic role to PRC2 and may work to prevent the repression of important genes during de-differentiation.

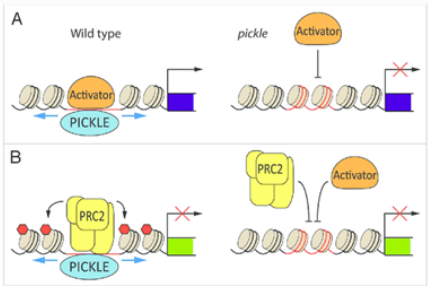


Image credit: Köhler et al., 2010

De-differentiation

- WIND1 is an AP2/ERF family transcription factor that has been shown to be important for controlling de-differentiation in Arabidopsis.
- Transcripts of this gene are accumulate rapidly near a wound just prior to callus formation.
- Ectopic expression of WIND1 maintains cells in an undifferentiated state in tissue culture without hormone application.
- Arabidopsis WIND1 promotes callus formation when expressed in tomato, tobacco and rapeseed.

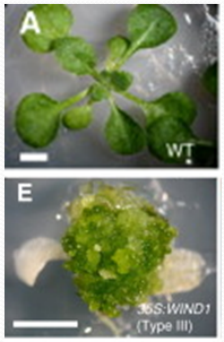


Image credit: Iwase et al. 2011

De-differentiation

- WIND1 expression induces cytokinin signaling genes (i.e. ARR1, ARR12).
- Double mutant plants of ARR1 and ARR12 are compromised in WIND1-induced callus formation.
- This cytokinin response is also important in promoting shoot organogenesis.
- ARR signaling induces a wide array of genes involved in more specialized processes, which probably also contribute to successful de-differentiation and callus formation.

Acquisition of totipotency

- There is a high degree of overlap between genes involved in early zygotic embryogenesis and those important for expressing totipotency in somatic cells.
- The transcription factor BABY BOOM (BBM) functions in both processes.
- It was first isolated in *Brassica napus* and was shown to induce the formation of somatic embryos without hormone induction when ectopically expressed in *B. napus* and Arabidopsis.

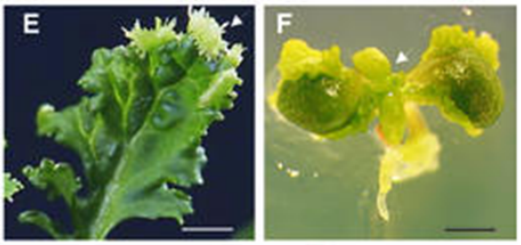


Image credit Boutilier et al., 2002

Acquisition of totipotency

- LEAFY COTYLEDON 1 & 2 (LEC1/2) are also among the earliest genes found to enhance somatic embryo formation.
- Although mutants of these genes give the same phenotype, their molecular functions are different.
- LEC1 is a subunit of a nuclear factor complex Y, while LEC2 encodes a B3 family transcription factor.
- These two genes, along with the regulators ABI3 and FUS3 make up what has been termed the "LAFL" network, which control embryo identity of development.

Acquisition of totipotency

- BBM was recently shown to directly regulate the LAFL network genes by binding the promoter regions of all four of them in Arabidopsis.
- Other AP2-like transcription factors closely related to BBM (PLT2, AIL5, AIL6) are at least partially functionally redundant to BBM.
- The effectiveness the BBM and related genes inducing totipotency is dose- and tissue context-dependent.
- LEC1, LEC2, and BBM also induce auxin biosynthetic genes.

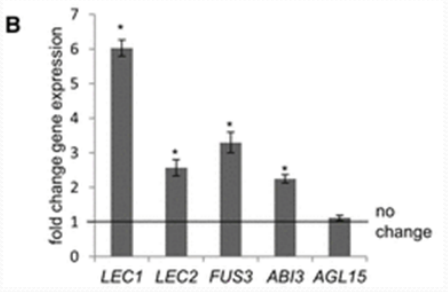


Image credit: Horstman et al, 2017

Acquisition of totipotency

- LEC2 induces the expression of AGAMOUS-like 15 (AGL15).
- This MADS-domain transcription factor enhances somatic embryogenesis from immature zygotic embryo explants, but does not cause spontaneous formation of embryos from seedlings as LEC1/2 and BBM do.
- Both LEC2 and AGL15 activate expression of the auxin signaling protein IAA30. AGL15, in turn, positively regulates expression of the LAFL genes.

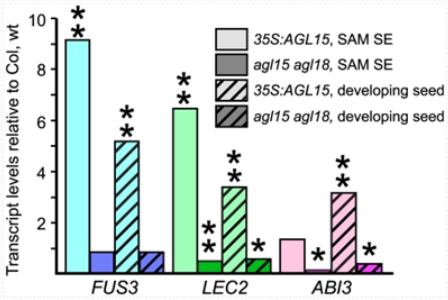


Image credit: Zheng et al., 2009

Acquisition of totipotency

- The developmental gene lateral organ boundaries-domain 29 (LBD29) is induced during 2,4-D mediated somatic embryogenesis by the auxin response factors ARF7 and ARF19.
- LBD29 mutants are less sensitive to auxin in tissue cuture media.
- LBD29 is also involved in the developmental process of lateral root formation. Because of its expression in callus tissue, callus shares several molecular markers with lateral root primordia.

Acquisition of totipotency

- The functionally redundant B3 transcription factors VAL1 and VAL2 recruit histone deacetylase (HDAC) proteins to repress the expression of LAFL genes.
- Normally this facilitates the developmental transition from embryonic to vegetative growth.
- *val1*/*val2* double mutant plants have higher rates of embryogenesis.
- PKL also represses expression of the LAFL genes

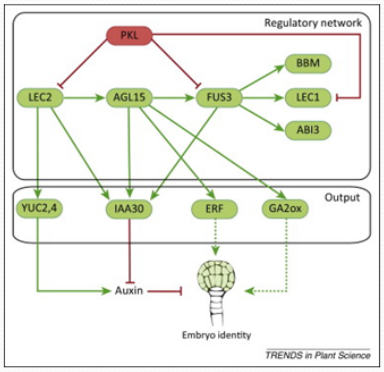


Image credit: Radoeva & Weijers, 2014

Embryogenic commitment

- The homeobox transcription factor protein WUSCHEL (WUS) contributes to the maintenance of the shoot apical meristem in plants.
- It is also important in embryogenic development as overexpression of WUS in several Arabidopsis tissues leads to the formation of somatic embryos.
- WUS expression can be regulated by auxin or cytokinin depending on the plant species.

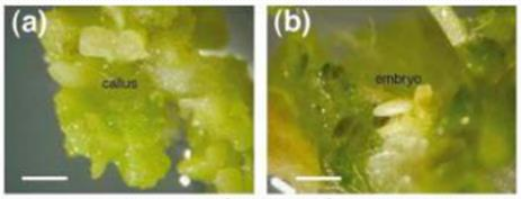


Image credit: Zuo et al., 2002

Embryogenic commitment

- The transcription factor SHOOT MERISTEMLESS (STM), increases WUS expression levels in Arabidopsis.
- It also up-regulates auxin production in immature zygotic embryo explants.
- In the shoot apical meristem, the peptide CLAVATA1 (CLV1) negatively regulates WUS and confines its expression to a few cells in the organizing center.
- Overexpression of CLV1 in Arabidopsis, represses somatic embryo formation.

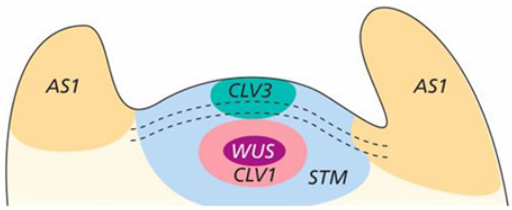


Image credit: Slideshare

Hormone pathways in somatic embryogenesis induction

- As 2,4-D is commonly used in tissue culture to induce somatic embryogenesis, many genes that respond to auxin play a part in regeneration.
- During embryogenesis, endogenous auxin in the form of IAA is produced creating a positive feedback loop.
- WIND1 and WUS seem to participate in a signaling program mediated by cytokinin rather than auxin. This may represent a separate developmental pathway that works in concert with auxin-mediated signaling.

Other genes involved in somatic embryo initiation

- Somatic embryogenesis receptor kinase (SERK) genes are highly expressed during embryo development. They also play a roles in other processes such as floral development.
- Stress-related genes that are active in embryogenesis
  - Peroxidases
  - Glutathione S-Transferase (GST)
  - Germin-like proteins (GLP)
  - Heat shock proteins
  - Non-symbiotic hemoglobins

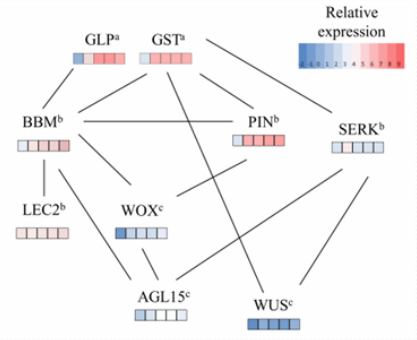


Image credit: Salvo et al., 2004

Small RNA regulation

- Some recent research has investigated the role of small RNA in controlling somatic embryogenesis.
- One study (Szyrajew et al., 2017) found that 64% of the Arabidopsis genes giving rise to primary miRNA transcripts were differentially regulated during embryogenesis.
- Another (Wójcik et al., 2017) showed that miR160 and miR166/165 help control embryogenesis through the post-transcriptional regulation of ARF genes and HD-ZIPIII transcription factors PHB and PHV, respectively.
- Lin et al. (2015) determined that trans-acting short interfering RNA (tasiRNAs) derived from the TAS3 transcript regulates the expression of ARF3 and ARF4 during embryogenesis in *Dimocarpus longan*.

Global gene expression studies

- As whole transcriptome sequencing has become more practical and widely utilized, it has been used to study large-scale gene expression changes during somatic embryogenesis.
- RNA-seq studies of this kind have been carried out in Arabidopsis, cotton, maize, and banana among others.
- Genes such as WUS, LEC1/2, BBM, and AGL15 can be found among those differentially expressed in these studies.
- The datasets that are generated can be useful for identifying genes that have yet to be characterized for their role in somatic embryogenesis.

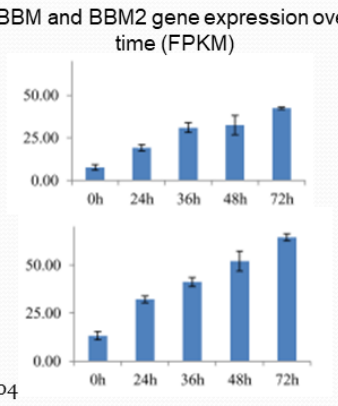


Image credit: Salvo et al., 2004

Verifying potential embryogenic genes

- Genes identified from differential expression experiments, mutant screens, or promoter binding assays need to be validated for their effect on somatic embryogenesis.
- Genetic engineering can be used to produce plants that overexpress these genes or to silence their expression.
- Tissue culture experiments can help determine whether modifying the expression levels of a given gene alters the regeneration capacity of a plant.

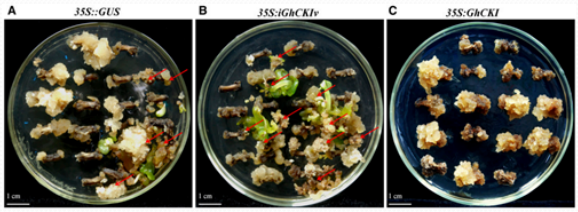
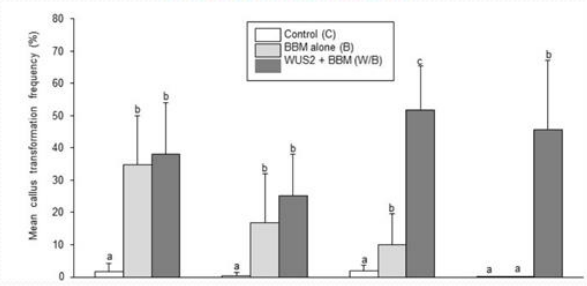
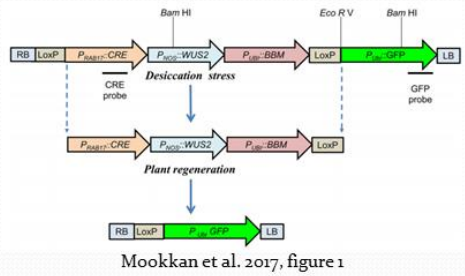


Image credit: Min et al., 2015

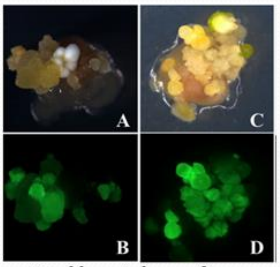
Overcoming the regeneration bottleneck in genetic modification



Lowe et al. 2016, figure 2



Mookkan et al. 2017, figure 1



Mookkan et al. 2017, figure 3

Best Practice in Cereal Transformation

Zhanyuan J. Zhang

Presentation Outline

Plant Transformation Basics

Lab Set Up

Sources of Explant Materials

Culture Conditions

Training

Plant Transformation Basics

Terms and Concepts
Plant Tissue Culture Media
Plant Growth Regulators
Plant Regeneration Systems
Transgene Delivery Systems
Agrobacterium biology

Terms and Concepts

In vitro	performed outside a living organism
In vivo	performed in a living organism
In planta	performed on an intact plant
Totipotency	ability of a single cell to divide and produce all of the differentiated cells in an organism
Explant	a plant part used for *in vitro* study
Regeneration	regrowth of cell, tissue or organ

Plant Tissue Culture Medium Components

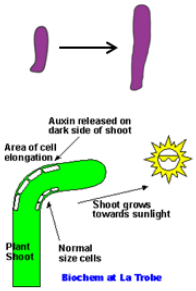
- **Components of media and their functions:**
  - **Water**: solvent of medium
  - **Inorganic salts**: mineral nutrients
  - **Organic substances**: vitamins (serving as co-enzymes), amino acids
  - **Carbohydrate**: sucrose, glucose (energy sources)
  - **Gelling agents**: support tissues, provide nutrients, and allow observations
  - **Growth regulators**:
    - Auxin (cell expansion and elongation): roots, callus
    - Cytokinins (cell division): shoots, prevent root formation
- **Medium types**:
  - Murashige & Skoog (1962): MS
  - Gamborg et al. (1968): B_5
  - Chu (1975): N_6
  - Linsmaier and Skoog (1964): LS
  - Schenk and Hildebrandt (1972): SH

MS Basal Salt Mixture

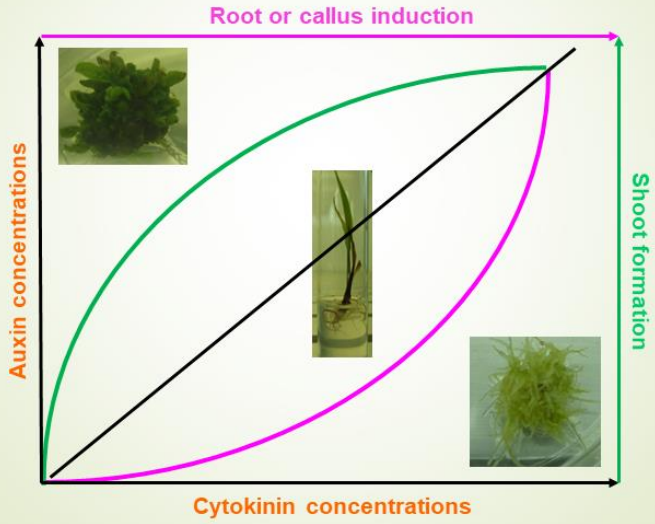
Macronutrients (Major salts)	**(mg/L)**
NH_4NO_3, Ammonium nitrate	1650
KNO_3, Potassium nitrate	1900
$CaCl_2H_2O$, Calcium chloride	440
$MgSO_47H_2O$, Magnesium sulfate	370
KH_2PO_4, Potassium phosphate	170
Micronutrients (Minor salts)	
H_3BO_3, Boric acid	6.2
$MnSO_4H_2O$, Manganous sulfate	169
$ZnSO_47H_2O$, Zinc sulfate	8.6
KI, Potassium iodide	0.83
$Na_2MoO_42H_2O$, Sodium molybdate	0.25
$CuSO_45H_2O$, Cupric sulfate	0.025
$CoCl_26H_2O$, Cobalt chloride	0.025
Ion-EDTA	
$FeSO_47H_2O$, Ferrous sulfate	28
NaEDTA, Ethylenediamine teraacticacid, disodium salt	37.5

Plant Growth Regulators

- **Cytokinin**: BAP, Zeatin, kinetin, TDZ, 2iP
  - DNA synthesis/mitoses/cell division
  - Differentiation in bud/shoot meristem, tissues other than vascular
  - Repress apical dominance
- **Auxin**: IAA, IBA, 2,4-D, etc.
  - Elongation/extension/meiosis
  - Differentiation in roots and vascular tissues
  - (Promote) apical dominance
  - Phototropism and Polarity
- **Gibberellic acid (GA3)**: internode elongation and seed germination
- **Abscisic acid (ABA)**: stress response, stomata closing, leaf abscission
- **Ethylene**: fruit ripening, regulate auxin synthesis



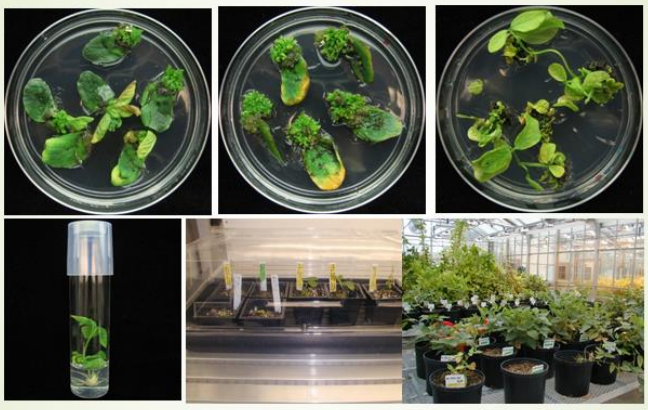
In vitro Responses to Plant Growth Regulators



Plant Regeneration Systems

- Organogenesis (direct and indirect organogenesis)
- Somatic embryogenesis
- Cell suspension culture
- Protoplast culture
- Anther culture

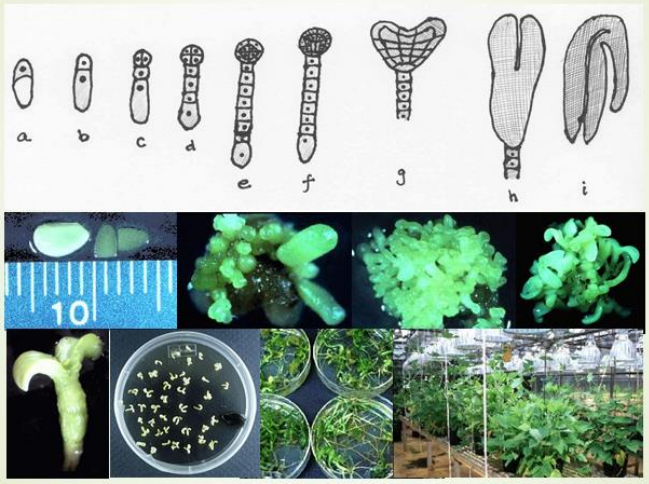
Short Organogenesis



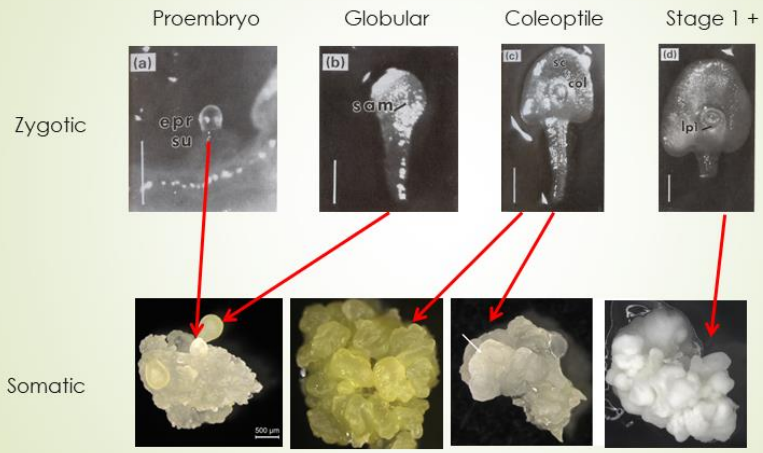
- Simple and short time frame
- Less somaclonal variation
- High fertility
- Most commonly employed

Somatic Embryogenesis - Dicot

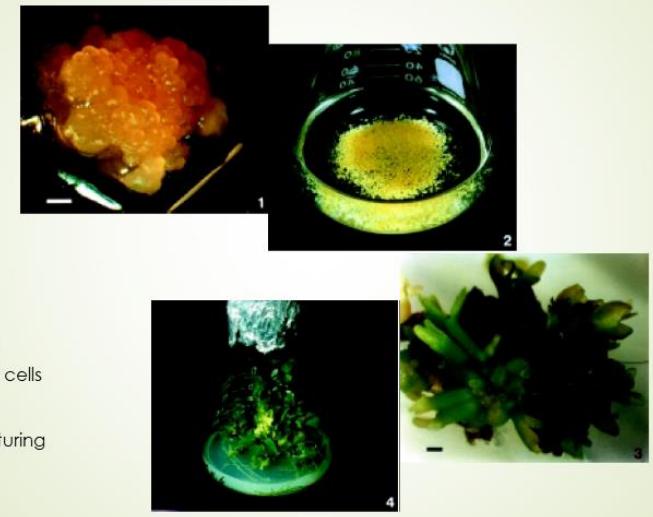
- High yield
- Low fertility
- Somaclonal variation



Somatic Embryogenesis - Monocot

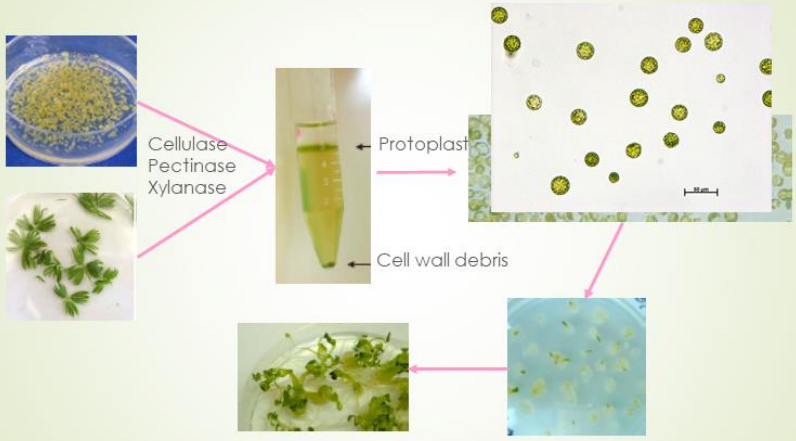


Cell Suspension Culture



- Fast growing
- Synchronized cells
- High yielding
- Extensive culturing
- Bioreactor

Protoplast Culture



- Polyethylene Glycol (PEG)-Mediated Transformation
- Suitable for transient assays

Anther Culture

(1n)

- Unique for haploid plant production
- Test recessive gene functions

Gene Delivery Systems

- *Agrobacterium*-mediated T-DNA transfer
  - Tissue culture-based approach
  - Non-tissue culture approach
  - *A. rhizogenes*: hairy root system
- Direct DNA delivery
  - Biolistic-mediated DNA delivery (Gene-gun)
  - Electroporation
  - Polyethylene glycol (PEG)
  - Silicon carbide whiskers
- Terminology: transient and stable transformation

Ti-Plasmid

Ti-plasmid

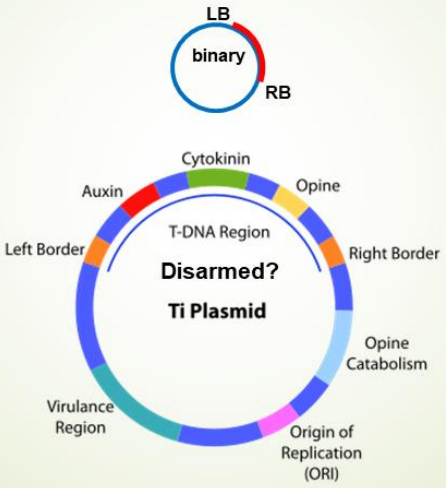
200 kb
196 genes
195 proteins
Extremely low copy

Chromosomal DNA

3000kb circular
2100kb linear

Cryptic plasmid

450kb



Ti-plasmid types

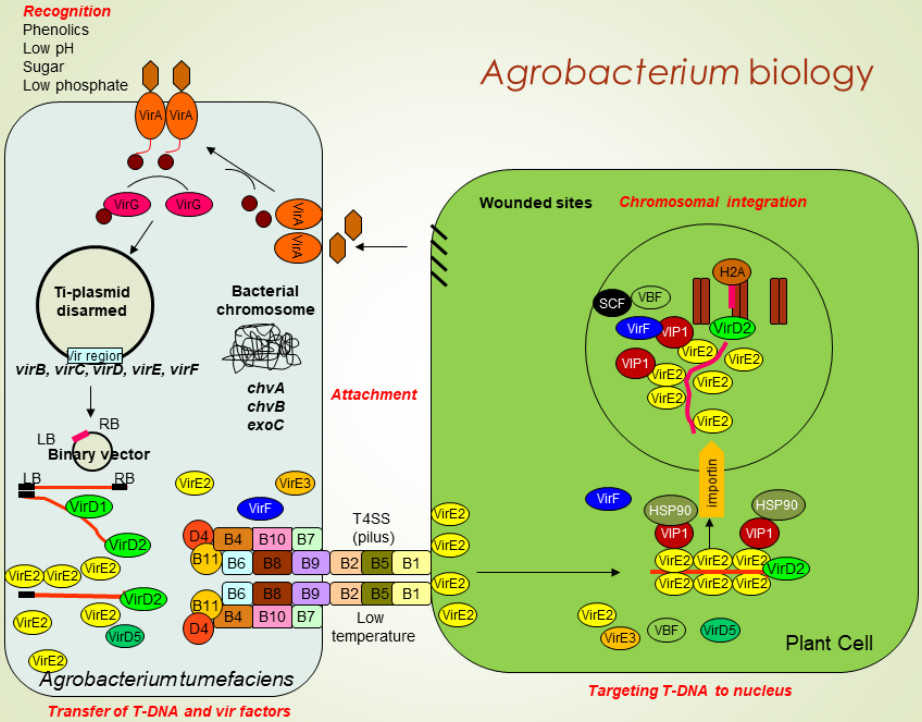
pTi, pRi

Octopine
Nopaline
Succinamopine
Leucinopine

Host range

Plants
Bacteria
Fungal
Mammalian

Agrobacterium biology



Plant Selectable Markers

- **Herbicide resistance genes**:
  - *bar* - *bia*laphos *r*esistance (resistance to glufosinate ammonia)
  - *PAT* - *p*hosphinothricin *a*cetyl*t*ransferase (to glufosinate ammonia)
  - *EPSPS* (5-*e*nol*p*yruvyl*s*hikimate-3-*p*hosphate *s*ynthase) (to glyphosate)
- **Antibiotic resistance genes**:
  - *npt* II – *n*eomycin *p*hospho*t*ransferase (to kanamycin, paramomycin, G418)
  - *hpt* II – *h*ygromycin *p*hospho*t*ransferase (to hygromycin)
- **Positive selectable marker**:
  - *pmi* - *p*hospho*m*annose *i*somerase (fructose production)

Lab Set Up

Greenhouse or growth chamber set up vs. field

Ultra freezer for *bacterium* strain storage

-20°C freezer and refrigerator for chemical storage

Culture chamber or incubator

Shaker incubator

Centrifuge

Water bath

Culture hood

Sources of Explant Materials

Immature embryos

Maize
Sorghum
Wheat

Seed-derived callus

Brachyposium
Switchgrass
Setaria

Culture Conditions

***Agrobacterium* culture initiation**: Integrity, Fresh glycerol, 28˚C, 2-3d
Infection conditions: Fresh explant & size, avoid damage, At density
Resting stage: Adjust duration based on embryo vigor
Callus induction and selection: morphogenesis selection
Maturation: timing and duration
Regeneration: sooner of shoot harvest is more beneficial
Rooting: Sooner of transplanting to soil is more beneficial
Acclimatization: multiple clones, if possible, as back up
Greenhouse care: drought training helpful

Training

Reference search

Thorough implementing protocol

High performance standard

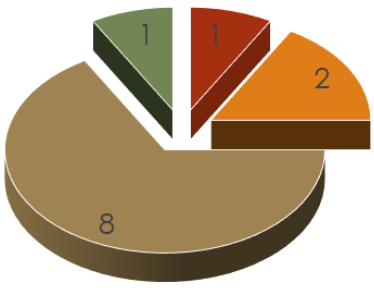
Trouble shooting

Communication

Examples

Training

Staff



- TC background B.S.
- TC background M.S.
- No TC background B.S.
- No TC background M.S.

- **Learning Curves**
  - Contamination
  - Making correct stocks and media
  - Explant preparation
  - Handling *Agrobacterium*
  - Culture transfer – Explant manipulations
  - Scheduling
  - Heavy workload under the pressure
  - Inconsistent transformation result
  - Inconsistent empty vector control result
  - Consistent pipeline

6-8 months of training for pipeline

1-1.5 year of training for consistency

NSF PLANT TRANSFORMATION WORKSHOP PROTOCOLS

Muruganantham Mookkan

Maize (*Zea mays* L.)

Sorghum
[*Sorghum bicolor* (L.) Moench]

Grass
Brachypodium distachyon (L.) P.Beauv.

Morphogenic regulator-mediated transformation protocol for Maize inbred B73

Maize (*Zea mays* L.) is the most important food, feed, and biofuel crop in the world. Plant genetic engineering is a unique and advanced approach for crop improvement because gene transformation takes advantage of totipotent plant cells by introducing a desired gene of interest into such cells and further regenerating to plants. B73 is of particular interest as it provides reference maize genome and is an important genetic resource for breeding and genomic studies. The following protocol describes the production of transgenic maize B73 lines using morphogenic regulator genes of maize *BBM* and *WUS2* via *Agrobacterium tumefaciens*. This protocol is fast and efficient, allowing the transgenic B73 plants to produce within 8-10 weeks.

Agrobacterium construct (*BBM/WUS* gene construct PHP78891) and preparation

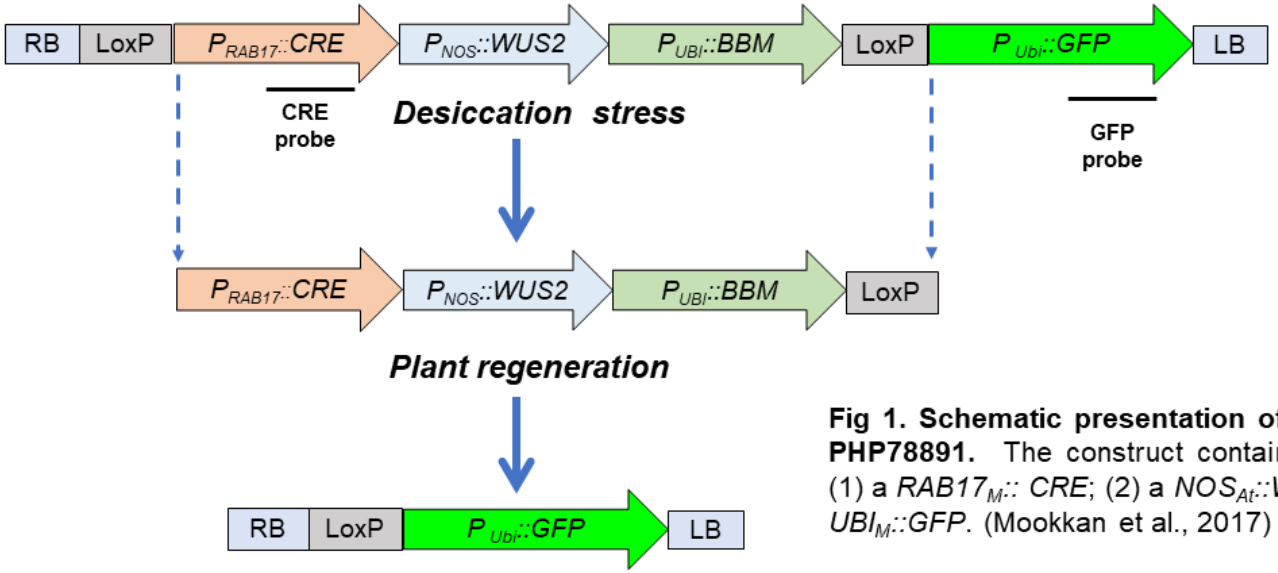


Fig 1. Schematic presentation of T-DNA region of construct PHP78891. The construct contains four expression cassettes: (1) a *RAB17$_M$:: CRE*; (2) a *NOS$_{At}$::WUS2*; (3) *UBI$_M$::BBM*; and (4) *UBI$_M$::GFP*. (Mookkan et al., 2017)

Streak the *Agrobacterium tumefaciens* strain AGL1 carrying PHP78891 vector and super binary vector from −80°C glycerol stock on YEP agar plate with 100mg/l spectinomycin, 30mg/l rifampicin, 2.5mg/l tetracycline antibiotics, as the "master" plate. Seal the master plate with Parafilm and incubate 3 days at 28°C dark. (*The "master" plate can be used for up to 1 month when stored a 4°C).*

A single colony is picked from the "master" plate and streaked onto a fresh YEP agar plate containing 100mg/l spectinomycin, 30mg/l rifampicin, 2.5mg/l tetracycline antibiotics. Seal the Parafilm and incubated in the dark for 2 days at 28°C.

Agrobacterium culture preparation

- Add 5 ml of Zm-1 (infection medium) to a 15-ml Falcon tube.
- Take two full loops of *Agrobacterium* culture from the YEP plate into the tube. Shake the tube well to suspend bacterial cells thoroughly.
- Pipette a 1.5 ml of this suspension and place in a spectrophotometer cuvette to check the optical density at 550nm (OD550). Adjust the suspension culture to 0.35 at OD550 (0.5x109 cfu/ml) at 24 ° C by either diluting the culture with more Zm-1 or adding more *Agrobacterium* cells.
- Shake the culture in a tabletop shaker at 100rpm for 3-4 hrs at 24° C.
- Pipette a 1ml of Zm-1 infection medium into 2-ml sterile microcentrifuge tube.

Plant Material (*Zea mays* L.) cv. B73

Maize plants are grown in greenhouse with day/night temperatures of 28/21°C, photoperiod of light/dark of 16h/8h, and 3-gallon pots containing Promix soil supplemented by Osmocote (19-6-12)

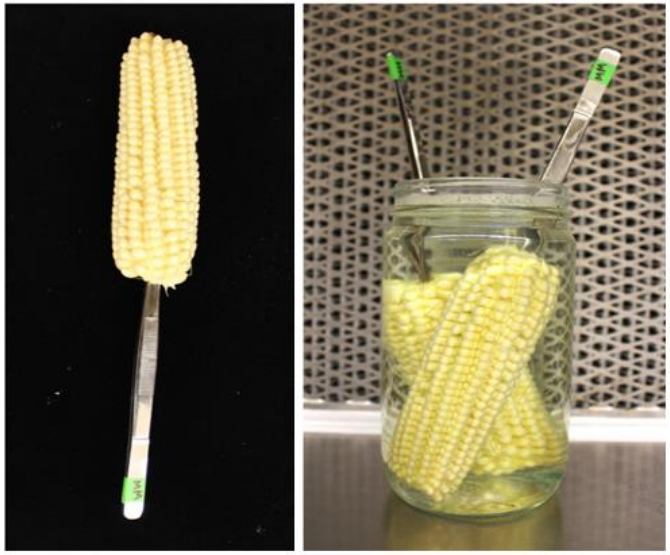
Immature embryos (10-12 days after pollination) are used as explants for maize B73 transformation.

Ears are harvested for transformation when immature embryos are between 1.5-2.0 mm in size.

Medium formulations for Maize B73 transformation

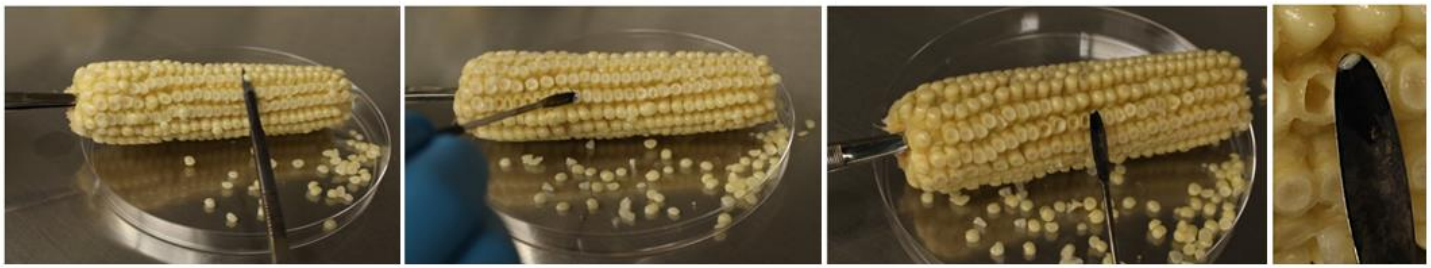
Medium Components	Infection Zm-1	Co-cultivation Zm-2	Resting Zm-3	Somatic embryo development Zm-4	Desiccation Zm-5	Regeneration Zm-6	Rooting Zm-7
N6 salts	4.0 g	4.0 g	4.0 g	-	-	-	-
MS salts	-	-	-	4.0 g	-	4.3 g	4.3 g
N6 salts macro (10x)	-	-	-	6 ml	-		
B5 salts micro (10x)	-	-	-	6 ml	-	-	-
Sucrose	68.5 g	30 g	30 g	20 g	-	40 g	30 g
Glucose	36.0 g	-	-	10 g	-	-	-
L-Proline	0.7 g	0.7 g	0.7 g	0.7 g	-	-	-
MES	0.5 g	0.5 g	0.5 g	0.5 g	-	-	-
2,4-D	1.5 mg	1.5 mg	1.5 mg	0.5 mg	-	-	-
pH	5.2	5.8	5.8	5.8	-	5.8	5.8
Whatman filter paper	-	-	-	-	2 layers	-	-
Solidifying agent	-	Gelrite 3 g	Gelrite 3 g	Gelrite 3 g	-	Agar 8 g	Agar 8
Autoclave	Filter	20 min	20 min	20 min	60 min	20 min	20 min
Eriksson's Vitamins (100x)	1 ml	1 ml	1 ml	1 ml	-	-	-
Thiamine HCL (1mg/L)	1 ml	-	1 ml	1 ml	-	-	-
SH vitamins (100x)	-	-	1 ml	1 ml	-	-	-
Myo-inositol	-	100 mg	100 mg	100 mg	-	100 mg	100 mg
MS vitamins (1000x)	-	-	-	-	-	5 ml	5 ml
Acetosyringone 100 μM	1 ml	1 ml	-	-	-	-	-
Cefotaxime	-	-	250 mg	250 mg	-	250 mg	250 mg

Surface Sterilization of Ears



Insert metal forceps into bottom end of the ear to act as a handle. Prepare 0.5-0.8 litters of 30% commercial bleach with a few drops of Tween 20 in a sterile 1-liter wide-mouth glass bottle. Put B73 ear into the bottle and the ear should be fully covered by the bleach solution for 20 min. Then, rinse the ears three to four times with sterile distilled water.

Immature Embryo Isolation

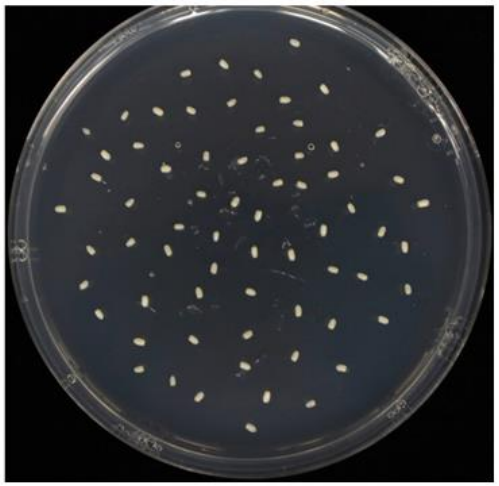


Place the ear on a sterile 150 x 15 mm petri plate.
Remove the top half of the kernels with a sterile # 11 razor blade on a blade holder. Isolate immature 1.5-2.0 mm immature embryos from the sterile ear with a micro-spatula

Immature Embryo Infection

- Embryos are transferred to 2ml microcentrifuge tube, which is filled with Zm-1 liquid medium. Wash embryos with 1ml Zm-1 liquid medium three times to remove debris and starch.
- Add immediately 1.5ml of *Agrobacterium* suspension to the 2ml microcentrifuge tube containing the embryos, and allow the tube to stand 5 min.

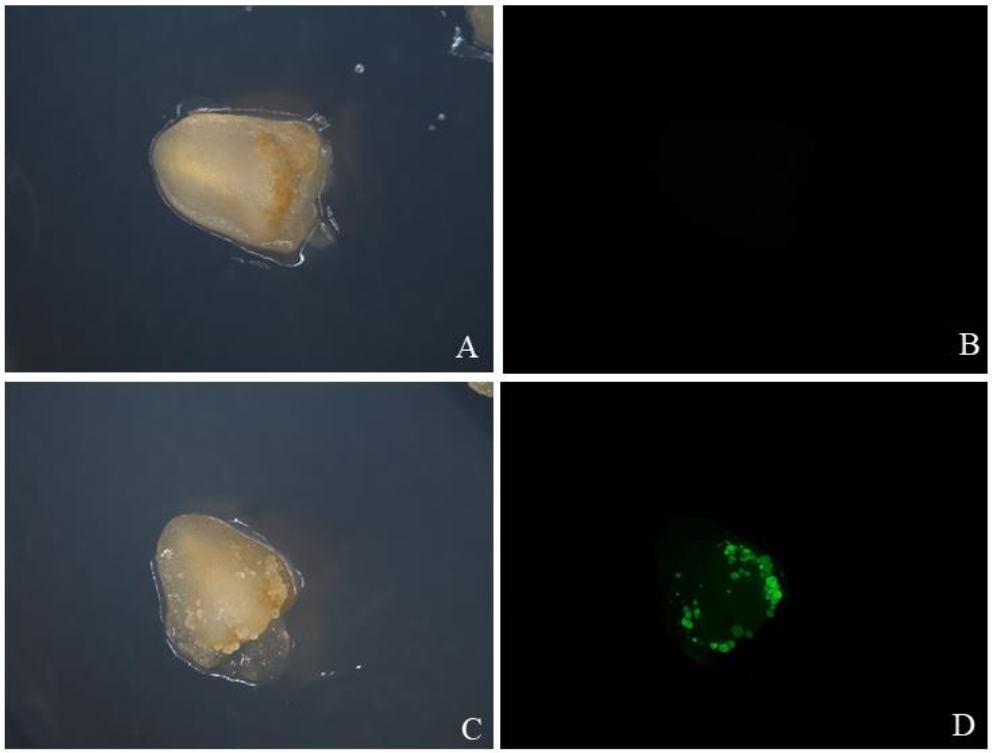
Co-cultivation



- Pour entire tube solution with immature embryos onto Zm-2 medium (co-cultivation medium).
- Use pipette with 1ml tip to suck out all *Agrobacterium* suspension from the petri plate, then place embryos with scutellum face up in the same petri plate
- Seal the plate with parafilm and incubate in the dark at 20°C for 3 days.

Resting

- After 3 days, transfer embryos to resting medium Zm-2 with # 15 razor blade on a blade holder. (Gently lift the bottom side of the embryo, without any damage).
- Seal the plate with Parafilm and incubate in the dark at 28°C for 7 days.
- After 7 days, remove the radicle then transfer to the somatic embryo development medium in dark condition at 28°C for 2 weeks.



Somatic embryo formation and GFP confirmation

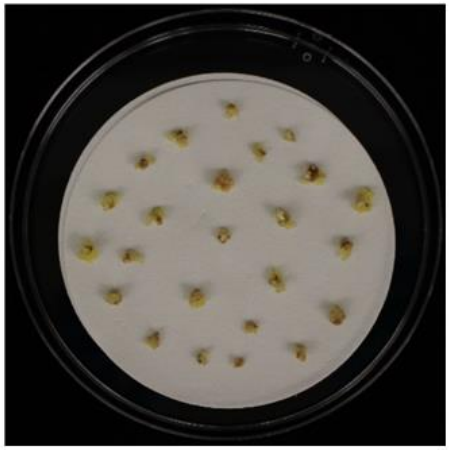
A. Infected AGL1 empty vector control (0% somatic embryo on the scutulum) bright field image

B. GFP florescence image

C. Infected with morphogenic maize *BBM* and *WUS2* genes and early stage somatic embryo formation bright field image

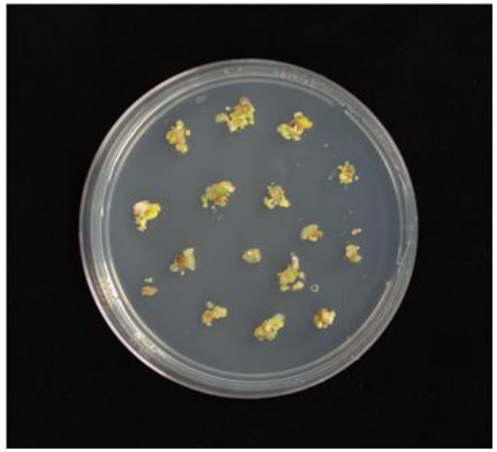
D. Confirmed with *GFP* expression GFP florescence image

Desiccation



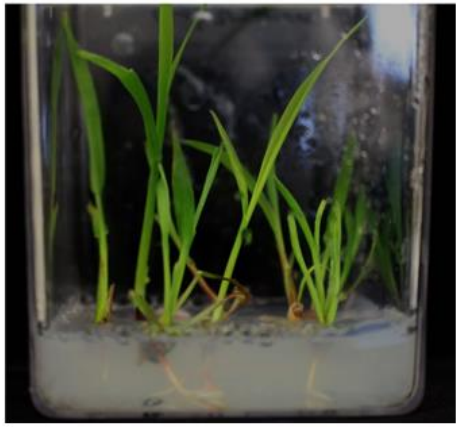
- Place 3 layer of Whatman sterile filter paper into the empty sterile 15x100mm petri dish, then transfer somatic embryos carefully onto the filter paper.
- Seal the plate and incubate in the dark at 25°C for 3 days.

Regeneration



- After 3 days, transfer desiccated embryos to regeneration medium Zm-4 in the light with 18hr photoperiod at 25°C for 20-25 days with 2 subcultures to the fresh medium.
- Shoot bud will emerge within 4-7 days from the tiny somatic embryos.

Rooting



- Transfer regenerated shoots to rooting medium Zm-5 in Magenta box.
- Allow shoots stay 10-14 days for rooting.

Hardening

- Identify well-rooted and healthy plants, remove gently from agar media and wash the roots with room temperature tap water, and make sure to remove the entire agar from the roots. (*Do not damage the roots or shoots during these steps)*.
- Make a hole on the center of the soil in the pot, then insert the roots into the hole then cover the hole with soil. Completely wet the soil.
- Use suitable polythene bag to cover the plantlet for one week
- Remove the polythene bag and let plantlets stay in soil another 1-2 weeks, during these days watch the plantlets every day and apply water as needed.

Key References

Lowe K, Wu E, Wang N, et al. (2016) Morphogenic regulators Baby boom and Wuschel improve monocot transformation. Plant Cell 28:1998–2015.

Mookkan M, Nelson-Vasilchik K, Hague J, Zhang ZJ, Kausch AP (2017) Selectable marker independent transformation of recalcitrant maize inbred B73 and sorghum P898012 mediated by morphogenic regulators *BABY BOOM* and *WUSCHEL2*. Plant Cell Rep 36:1477–1491.

Agrobacterium-mediated transformation protocol for *Sorghum bicolor*

Agrobacterium culture initiation

Streak *Agrobacterium* culture from −80°C glycerol stock on YEP agar plate with appropriate antibiotics, as the "master" plate. Seal the master plate with Parafilm and incubate 3 days at 28°C dark. (*The "master" plate can be used for up to 1 month when stored a 4°C).*

A single colony is picked up from the "master" plate and streaked onto a fresh YEP agar plate containing 30mg/l rifampicin and 50mg/l Kanamycin antibiotics. Seal the plate with Parafilm and incubate it in the dark for 2 days at 28°C.

Agrobacterium culture preparation

- Add 5 ml of infection medium to a 15-ml Falcon tube.
- Take two full loops of *Agrobacterium* culture from the YEP plate into the tube. Shake the tube well to suspend bacterial cells thoroughly.
- Pipette a 1.5 ml of this suspension and place in a spectrophotometer cuvette to check the optical density at 550nm (OD550). Adjust the suspension culture to 0.35 at OD550 (0.5x109 cfu/ml) at 24°C by either diluting the culture with more infection medium or adding more *Agrobacterium* cells.
- Shake the culture in a tabletop shaker at 100rpm for 3-4 hrs at 24°C.
- Pipette a 1ml of infection medium into 2-ml sterile microcentrifuge tube.

Plant Material (*Sorghum bicolor)* cv. P898012

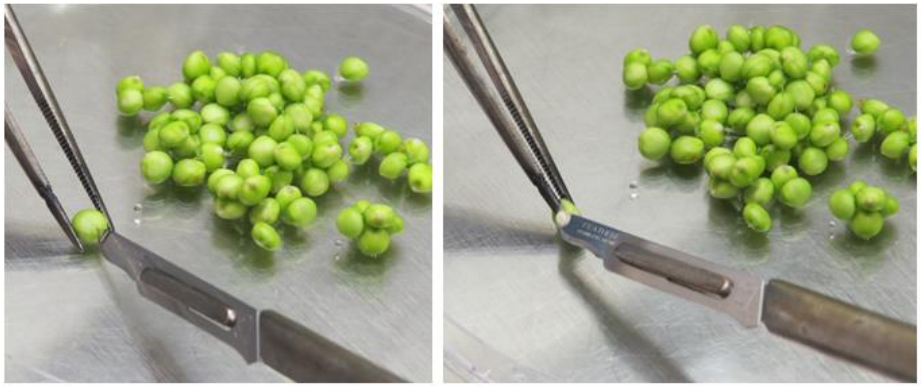
Sorghum plants are grown in greenhouse with day/night temperatures of 28/21 °C, a photoperiod of 16h light/ 8h dark, and in 3-gallon pots containing Promix soil supplemented by Osmocote
(14-14-14)

Immature embryos (10-14 days after pollination) are used as the the explant for *Sorghum* transformation.

Surface sterilization of the immature caryopsis

- Immature seeds (caryopsis) are removed by hand 10-12 days after pollination and placed into the 100 ml sterile conical flask
- Add 50% bleach with few drops of Tween 20 followed by gentle agitation for 20 min.
- After 20min. rinse the caryopsis three to four times with sterile distilled water.
- After the final rinse, remove all the water
- Place the caryopses on a sterile petri plate.

Immature embryo isolation



- Use fine forceps and sterile #15 razor blade on a blade holder to cut the caryopses carefully and
- isolate the immature embryo (The immature embryo is opposite the divot in the caryopsis).
- Isolate immature 1.5-2.0 mm and transfer 50 embryos/2ml microcentrifuge tube filled with liquid
- infection medium.
- Wash the embryos with 1ml with liquid infection medium three times to remove debris and starch.

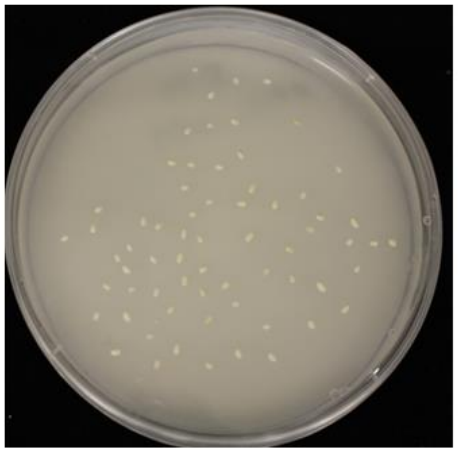
Medium formulations for *Sorghum bicolor* cv. P898012

Medium Components	Infection	Co-cultivation	Resting	Callus Induction	Regeneration	Rooting
MS salts	4.3 g	4.3 g	4.3 g	4.3 g	4.3 g	4.3 g
MES	0.5 g	0.5 g	0.5 g	0.5g	0.5 g	0.5 g
L-proline	-	0.7g	1.0 g	1.0 g	-	-
Sucrose	68.5 g	20 g	30g	30 g	30 g	30 g
Glucose	36 g	10 g	-	-	-	-
2,4-D 1mg/ml	1.5ml	1.5ml	1.5ml	1.5ml	-	-
KH2PO4	-	-	1.0 g	1.0 g	-	-
pH	5.2	5.8	5.8	5.8	5.6	5.6
Agar	-	8.0 g	8.0 g	8.0 g	8.0 g	8.0 g
PVPP	-	10.0 g	10.0 g	10.0 g	10.0 g	10.0 g
Autoclave	filtered	20 min	20 min	20 min	20 min	20 min
B5 vitamin, 100X	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml
Asparagine	-	-	1.0 g	1.0 g	-	-
Acetosyringone	100 µM	1ml µM	-	-	-	-
Ascorbic acid	-	10 mg	-	-	-	-
BAP	-	-	-	-	1 mg	-
IAA	-	-	-	-	1 mg	-
IBA	-	-	-	-	-	1 mg
CuSo4	-	-	0.16 mg	0.16 mg	0.16 mg	0.16 mg
Cefotaxime	-	-	400 mg	300 mg	300 mg	300 mg
Glufosinate	-	-	-	2.5 mg	2.5 mg	-

Immature embryo heat treatment and *Agrobacterium* infection

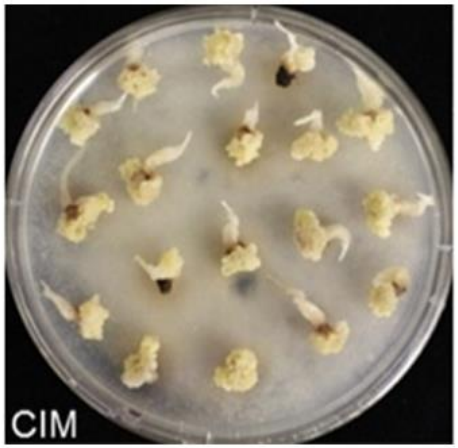
- After wash, remove the liquid medium from the 2 ml tube and keep 50-100 µl only to cover the immature embryos
- Then, incubate the tubes in 43ºC water bath for 3 min.
- Then, transfer the tubes to 25ºC for 2 min to cool the immature embryos, finally remove all the liquid infection medium, add 1 ml of *Agrobacterium* infection medium in to the tubes and allow the tubes to stand to 5 min

Co-cultivation



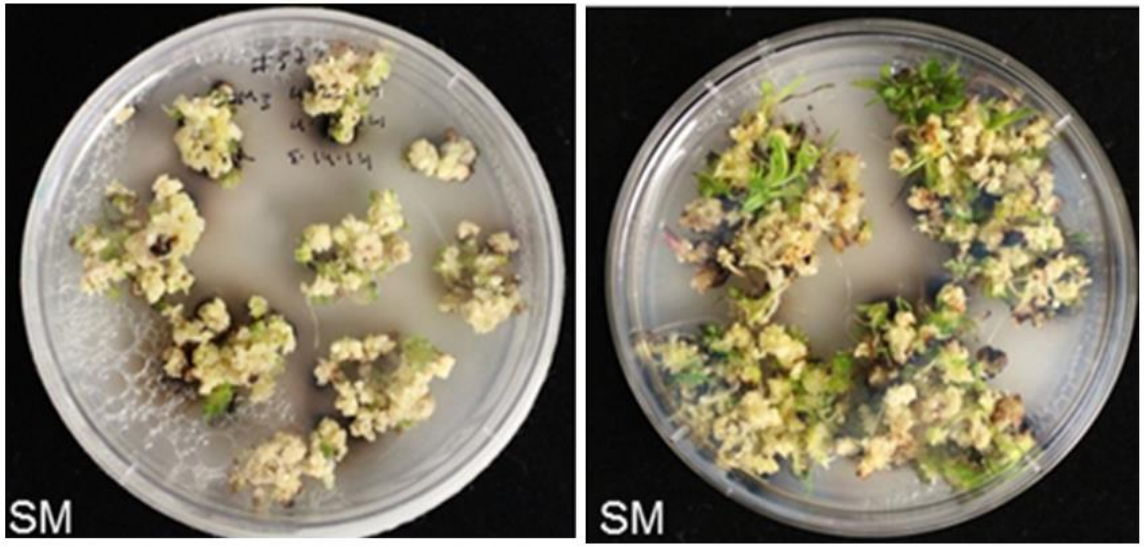
- Then, pour the entire tube solution with immature embryos onto co-cultivation medium.
- Use pipette with 1ml tip to suck out all the *Agrobacterium* suspension from the petri plate, then place the embryos with scutellum face up in the same petri plate
- Seal the plate with parafilm and incubate in the dark at 25°C for 3 days.

Callus induction



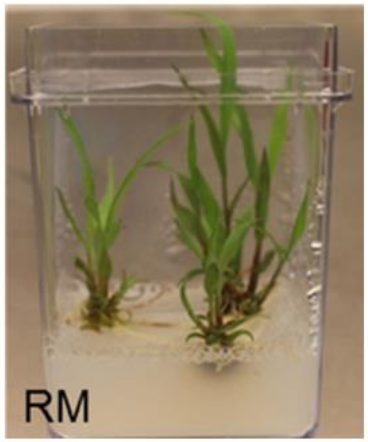
After the resting, remove the radicle then transfer to the callus induction medium in dark condition at 25°C for 10 days.

Shoot regeneration



Transfer embryogenic calli to shoot regeneration medium in the light with 18hr photoperiod at 25°C for 6-10 weeks with every 2 weeks subcultures to the fresh medium.

Rooting



Transfer regenerated shoots to rooting medium in Magenta box. Allow shoot stay 2-3 weeks for rooting.

Hardening

Regenerated shoots with healthy roots are transferred to Promix soil in a growth chamber for 2-4 weeks.

Key Reference

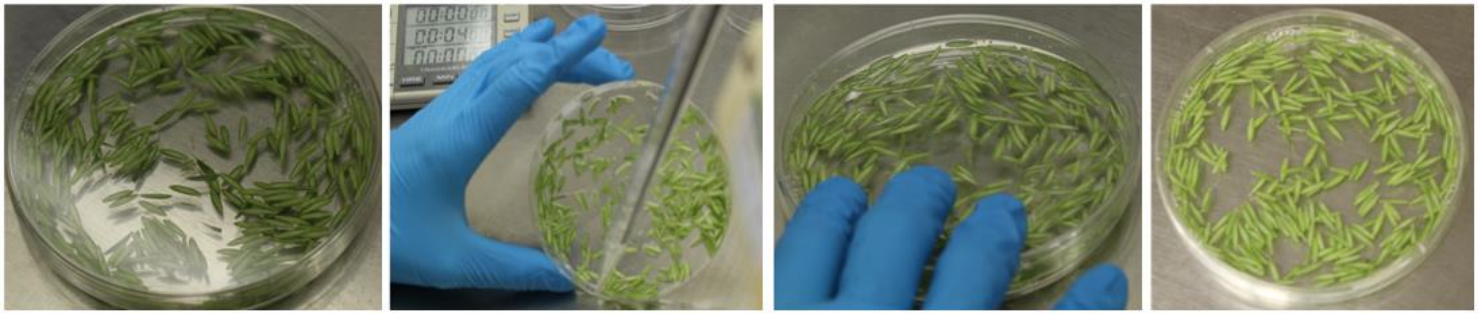
Do P, Lee HY, Mookkan M, Folk WR, Zhang Z (2016) Rapid and efficient *Agrobacterium-mediated* transformation of sorghum (*Sorghum bicolor*) employing standard binary vectors and bar gene as a selectable marker. Plant Cell Rep 35:2065–2076

Agrobacterium-mediated transformation protocol for *Brachypodium distachyon* (L.) P.Beauv.

Plant material

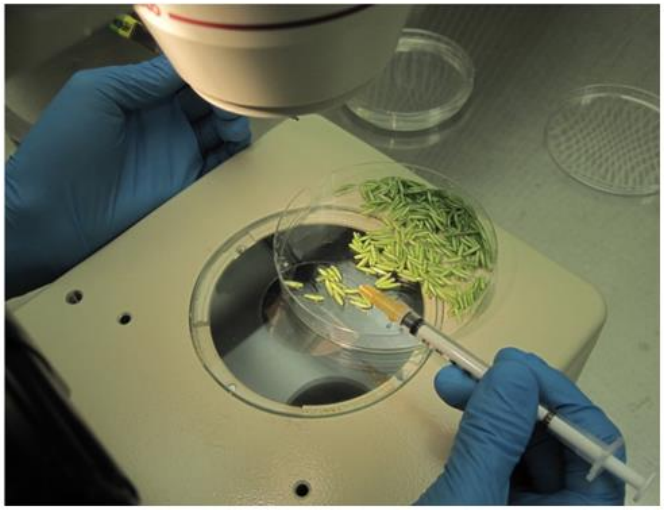
Harvest the tillers from 7-9 week old *Brachypodium* plants.

Surface sterilization of the immature caryopsis



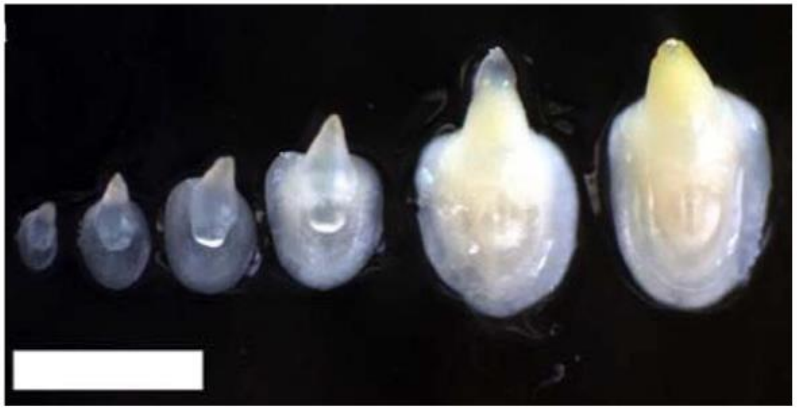
- Select immature seeds with a soft endosperm and remove top glume (lemma) by hand carefully and transfer into a sterile petri plate containing sterile water
- Once enough seeds are collected, remove all water from the petri plate
- Add 20 ml of 70% ethanol in a sterile petri plate and covered the lid for 30 second
- Remove the ethanol and rinse the seeds with sterile water 3 times.
- Add 20 ml 22% of commercial bleach solution and gently agitate the seeds for 4 min.
- Remove the bleach solution and rinse the seeds 3 times with sterile water.

Immature embryo isolation



Using a sterile needle or razor blade #11 to isolate immature embryos under the dissecting light microscope

Immature embryos



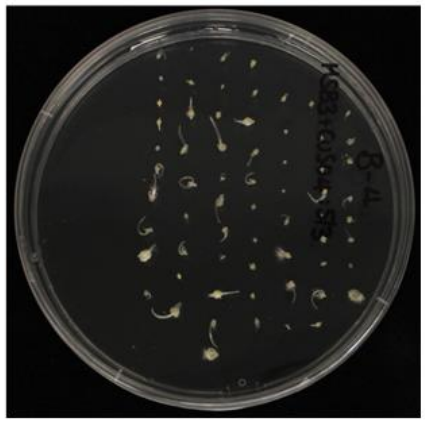
Isolate immature 0.3 mm and transfer to the callus proliferation medium

Plant Cell Rep (2008) 27:471–478

Medium formulations for *Brachypodium* transformation

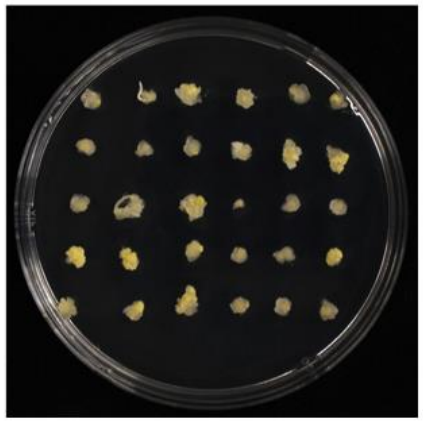
Medium components or stock/Liter	MSB3+CuSo4 (embryogenic callus proliferation)	MSB3+AS45 (Inoculation)	MSB3+AS60 (Co-cultivation)	CS (Selection medium)	MSR26 (Regeneration)	MSR63 (Rooting)
MS Macronutrients (10x)	100	100	100	100	100	40
MS micronutrients (100x)	10	10	10	10	10	40
Fe-EDTA (100x)	10	10	10	10	10	10
Sucrose (g)	30	30	30	30	30	10
Glucose (g)	-	30	-	-	-	-
CuSO4 (1mg/ml)	0.6	-	-	0.6	0.6	-
MS Vitamin (100x)	10	10	10	10	10	-
B5 Vitamin (100x)	-	-	-	-	-	10
2,4-D (1mg/ml)	2.5		2.5	2.5	-	-
Phytagel (g)	2	-	2	2	2	2
Agar (g)	-	-	-	-	-	6
Charcoal (g)	-	-	-	-	-	4
pH	5.8	5.5	5.8	5.8	5.8	5.8
Acetosyringone (30mg/ml)	-	1.5	2	-	-	-
Kinetin (0.1mg/ml)	-	-	-	-	0.2	-
Timentin (mg)	-	-	-	225	225	112
Hygromycin B (mg)	-	-	-	40	20	15

Callus proliferation



Culture immature embryos with scutellum facing up onto MSB3+ Cu0.6 solid medium. After 3-5 days remove the radicle/shoot part and further maintain the culture for 6 weeks at 25°C in the dark with every 2 weeks subculture.

Embryogenic callus proliferation



After 3 weeks, split compact embryogenic callus (CECs) with a creamy color and pearly surface into one to three pieces. Discard all non-CEC tissue and transfer pieces of CEC onto fresh MSB3+Cu0.6 solid medium for additional 2 weeks at 25°C in the dark. After the 5 week, again divide CECs with a creamy color and pearly surface into four to six pieces. Discard all non-CEC tissue and transfer pieces of CEC onto fresh MSB3 + Cu0.6 solid medium for an additional week at 25°C in the dark.

Agrobacterium culture initiation and preparation

Pipette 5 μl glycerol stock of *Agrobacterium* culture from -80C into 1 ml liquid YEP medium with appropriate antibiotics.

Allow to grow overnight in the incubator shaker at 28C with 200 rpm.

Pipette a 200 μl of Agrobacterial overnight culture onto solid YEP (containing 10g mannitol, 2.32 g L-glutamic acid sodium salt, 0.5 g KH_2PO_4, and 0.2 g $MgSO_4 \cdot 7H_2O$ and biotin 2μl from 1mg/ml stock with appropriate antibiotics) plate and spared with sterile spreader.

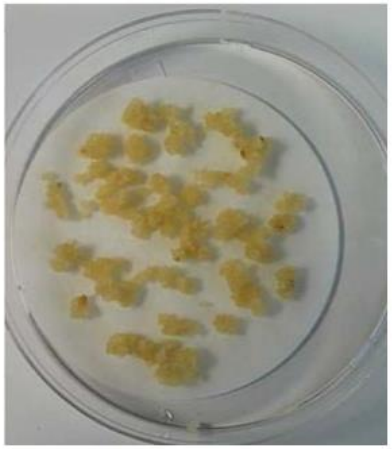
Allow to grow for 2 days at 28°C in the dark.

Take 2-4 full loops of *Agrobacterium* culture from the plate and suspend sterile 50 ml Falcon tube containing 10ml of inoculation medium.

Shake the tube into the incubator shaker at 220 rpm for 1 min in 28°C.

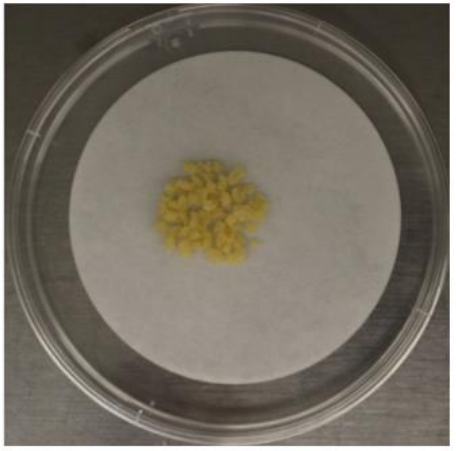
Pipette a 1.5 ml of this suspension and place in a spectrophotometer cuvette to check the optical density at 550nm (OD600). Adjust the suspension culture to 1.0 at OD600 (0.5x109 cfu/ml) at 24°C by either diluting the culture with more infection medium or adding more *Agrobacterium* cells.

Agrobacterium infection



Place the 50 calli into the sterile petri dish containing gently pipetting 13 mL *Agrobacterium* suspension ($O.D._{600}$ = 1) onto the plates and gently agitate. Inoculate for 5 min at room temperature in a laminar flow hood.

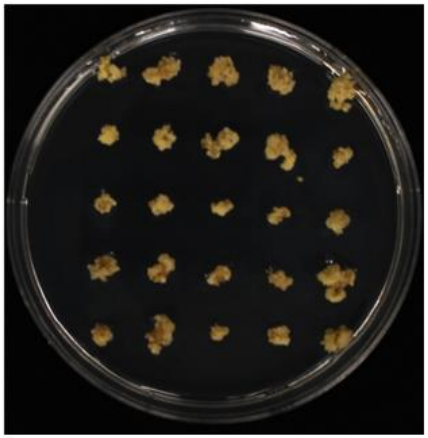
Co-cultivation



Remove the *Agrobacterium* suspension culture from the plates with a pipette. Transfer each callus piece without a drying step and place onto the sterile filter paper wetted with 500 mL MSC+AS45 liquid medium. To prevent excessive drying of the calli, seal the Petri dish immediately with cling film and co-culture CECs for 2 days at 25°C in the dark.

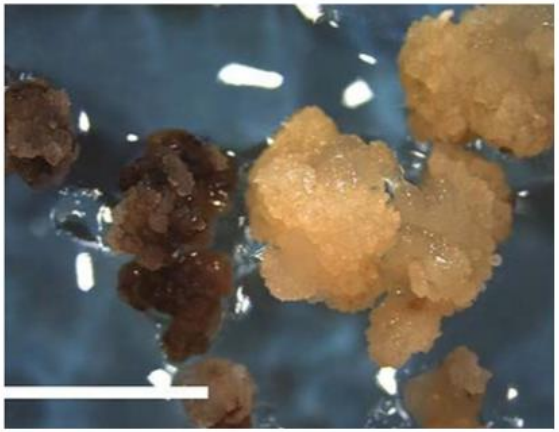
Plant Cell Rep (2008) 27:471–478

Selection



After the co-cultivation, transfer the calli onto MSB3+Cu0.6+H40+T225 solid medium and maintain for 2.5 weeks at 25°C in the dark.

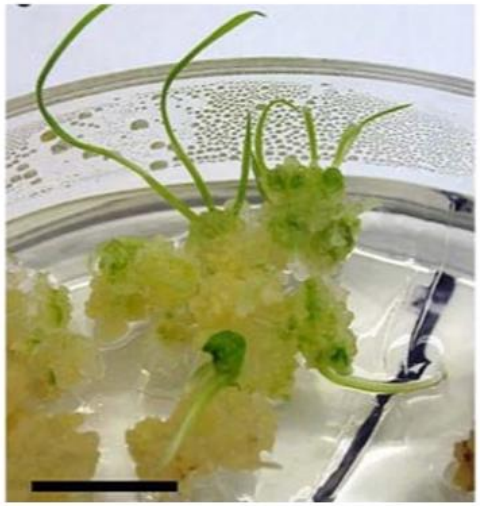
Selection



Dissect compact embryogenic sectors from each originally inoculated CEC and discard brown and/or soft non-embryogenic callus. Transfer pieces of CEC onto fresh MSB3+ Cu0.6+H40+T225 solid medium for an additional 2.5 weeks at 25°C in the dark.

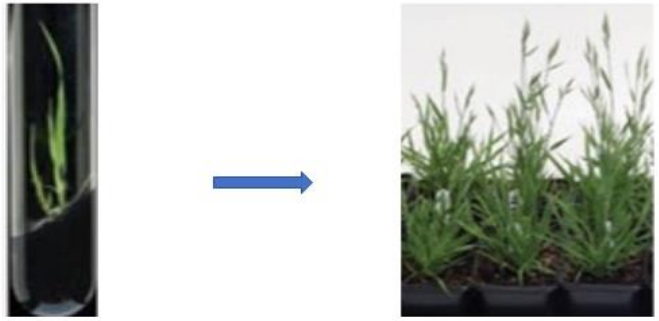
Plant Cell Rep (2008) 27:471–478

Regeneration



After 5 weeks, transfer hygromycin-resistant calli onto regeneration medium MSR26+H20+T225 for 2–3 weeks at 25°C under 16-h photoperiod.

Rooting and Hardening



Transfer shoots to rooting medium in tubes (containing MSR63 + H15 + Ch4 + T112) for 2–3 weeks at 25°C under 16-h photoperiod.

Regenerated shoots with healthy roots are transferred to Promix soil in a growth chamber for 2-4 weeks.

Plant Cell Rep (2008) 27:471–478

Key References

Thole V, Vain P (2012) In: Dunwell JM, Wetten AC (eds.), *Transgenic Plants: Methods and Protocols*, Methods in Molecular Biology, vol. 847, DOI 10.1007/978-1-61779-558-9_12, Springer, New York, pp497, ill.

Vogel J, Hill T (2008) High-efficiency *Agrobacterium*-mediated transformation of *Brachypodium distachyon* inbred line Bd21-3. Plant Cell Rep 27:471-478.

Plant Biotechnology Innovation Laboratory

The Plant Biotechnology Innovation Laboratory is established as a result of separation from Plant Transformation Core Facility, the latter of which is being transitioned from the administration of Division of Plant Sciences to the University of Missouri Core Facility system and will provide service only.

Mission

The mission of our Plant Biotechnology Innovation Laboratory is to advance plant transgenic technologies, which include, but are not limited to, development of new, efficient or improved plant regeneration and transformation systems to meet the needs of gene discovery and genome editing as well as crop trait engineering. The Laboratory is very interested in development of transformation systems or other transgenic approaches for public research and industrial laboratories to meet their specific needs.

The Laboratory also aims to enhance multi-disciplinary collaborations and grant applications for both public and industrial researchers.

As a part of educational role, the Laboratory provides technical training for graduate and undergraduate student, post-doctoral associates and other scholars in plant tissue culture and transformation and molecular biology.

Collaborations

In addition to its independent research programs, our Plant Biotechnology Innovation Laboratory is particularly interested in the following types of collaborations:

- Establish, develop, or improve regeneration and transformation systems for desired plant species for either public and industrial collaborators
- Design and construct plant transformation constructs and engineer desired plant species with the constructs to achieve collaborator's research goal
- Collaborate in grant applications to accomplish specific research goals
- Employ state-of-the-art transgene analysis approaches to meet the needs of both public and industrial collaborators.

Contact

To explore collaboration opportunity with us, contact Zhanyuan J. Zhang, Ph.D. and Director

Plant Biotechnology Innovation Laboratory

3-22D Agriculture Building (office); 1-33 Agriculture Building (mailing)

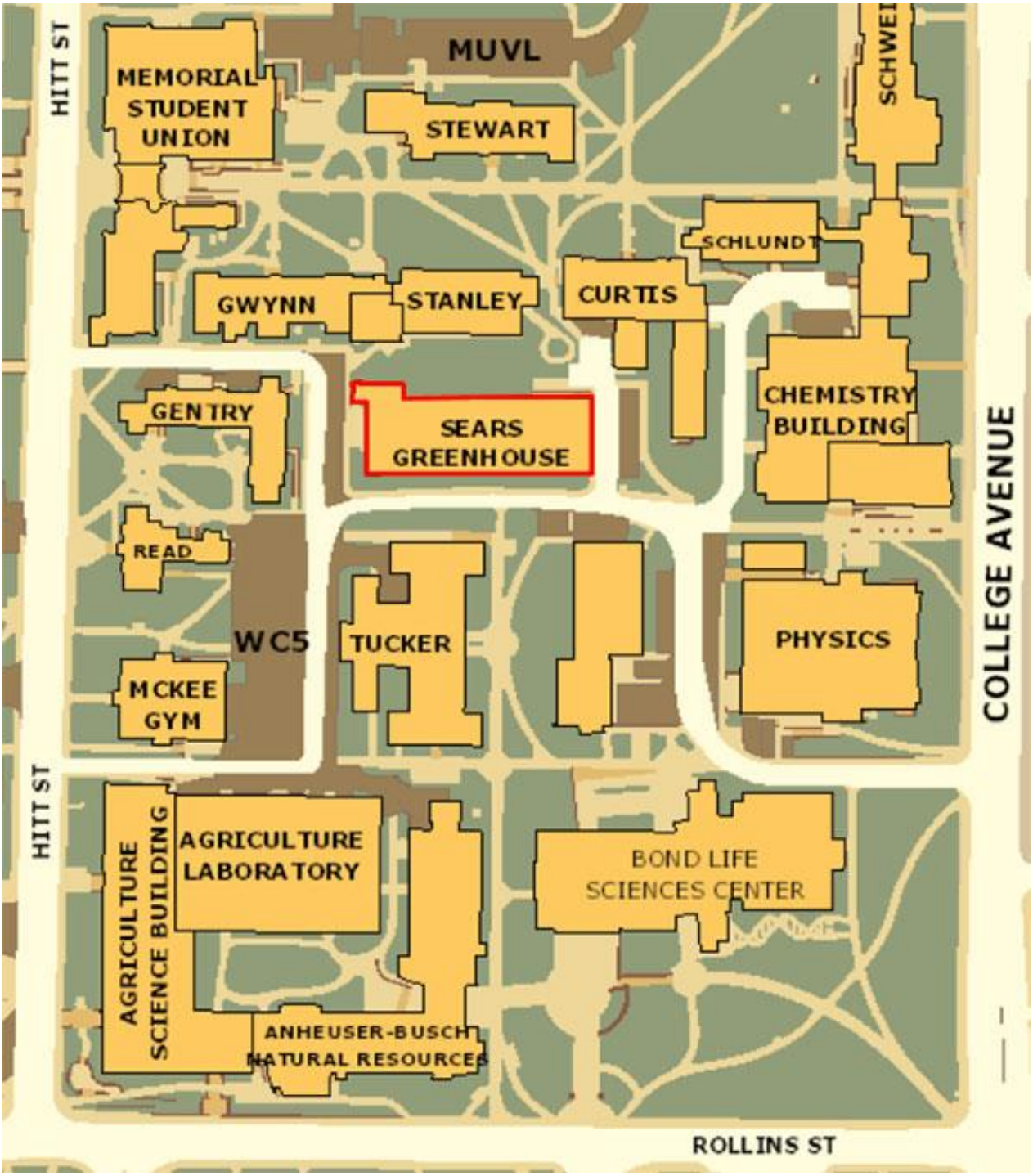
Division of Plant Sciences, University of Missouri, Columbia, MO 65201

Email: zhangzh@missouri.edu. Tel: 573-882-6922

https://plantsciences.missouri.edu/plantbiotechlab/

NSF workshop location

North



Ring the doorbell at 007B door of the west entrance of Sears greenhouse. Welcome and reception room is located in 107 Bond Life Science Center, Mizzou. There are restaurants nearby around the Mizzou campus.