THE UNIVERSITY OF RHODE ISLAND

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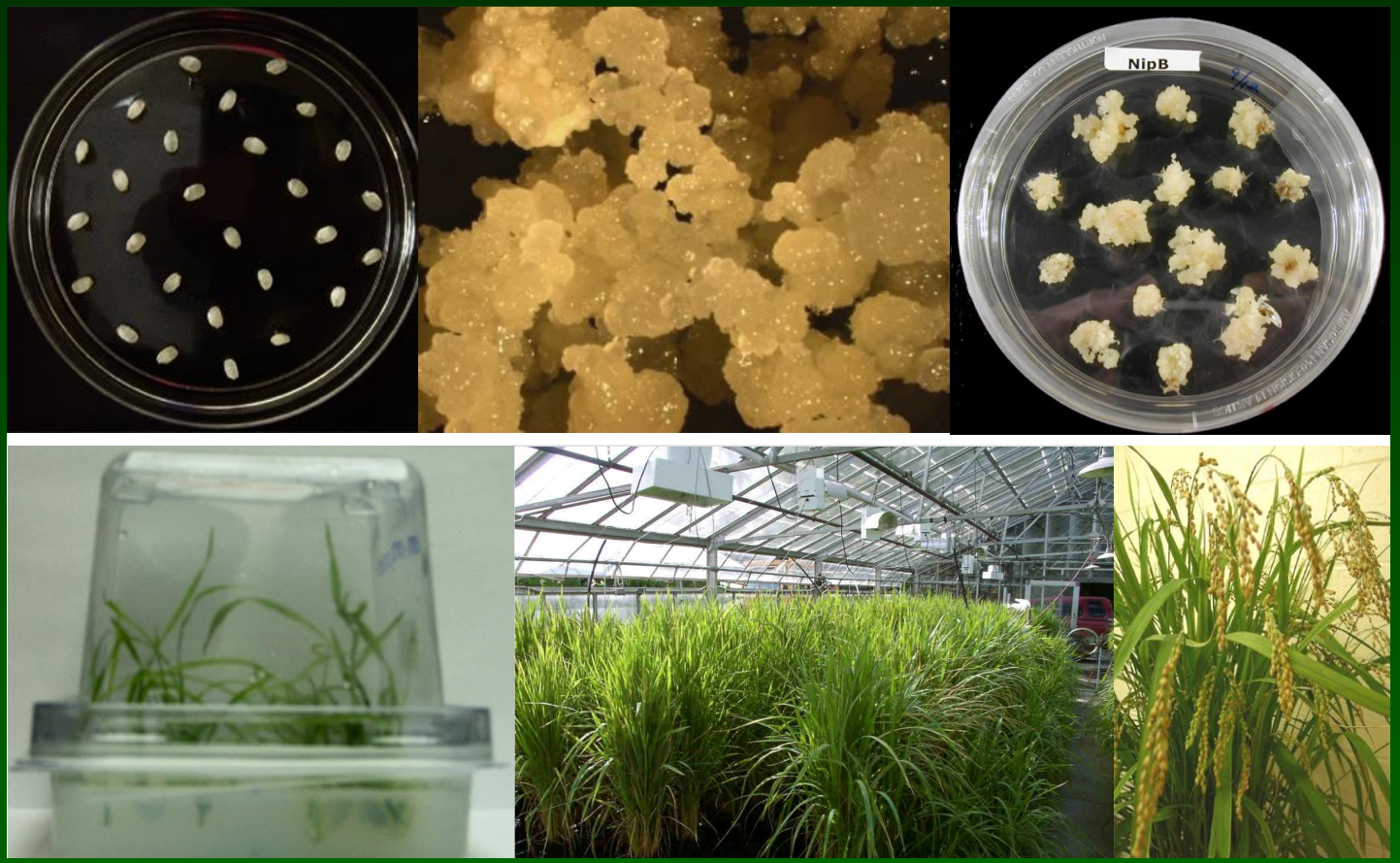
Agrobacterium-mediated transformation of rice (*Oryza sativa* cv. Nipponbare) from mature seeds: A stalwart protocol of cereal transformation

Albert P Kausch

An Efficient Protocol for Rice Transformation



Rice transformation can be initiated from mature seeds to produce a very ’early’ embryogenic callus which is highly transformable

Rice Transformation

Plant Material

Mature rice seeds *Oryza sativa ssp. japonica,* cv. Nipponbare were obtained from USDA Agricultural Research Service; Dale Bumpers National Rice Research Center www.ars.usda.gov/sea/dbnrrc/gsor are used.

On the Importance of Nipponbare

Hundreds of plant genome sequences have now been published. The map-based sequence of *Oryza sativa* ssp. *japonica* cv. Nipponbare was one of the early few monocot genomes that had been sequenced to a high-quality level. It has therefore become a reference for sequencing of other cereal crops with much larger genome sizes such as maize (Schnable et al. 2009), sorghum (Paterson et al. 2009), soybean (Schmutz et al. 2010), barley (International Barley Genome Sequencing Consortium 2012), and wheat (International Wheat Genome Sequencing Consortium 2014). Robust *Agrobacterium*-mediated transformation protocols for rice were established in the early 2000's and were applied to cv. Nipponbare because of its status as an early refence genome.

Mature Seed Explant Preparation

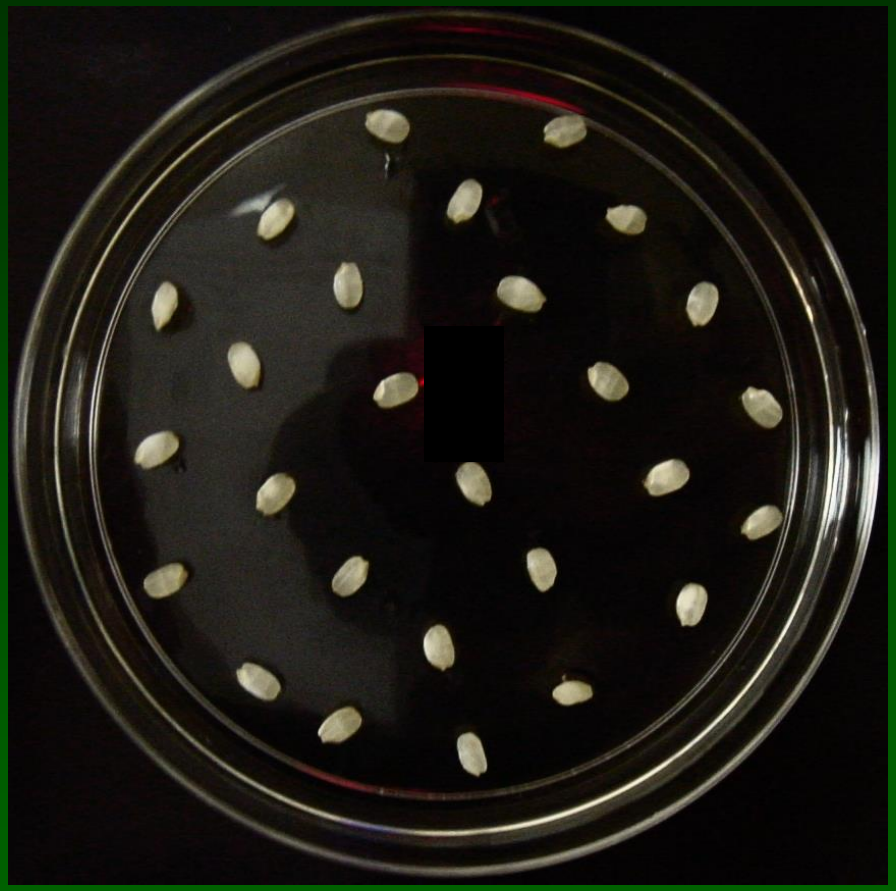
Use mature seeds, stored in seed storage room under proper conditions.
Seeds are manually dehusked using fine grit sand paper.

Mature Seed Explant Preparation

Place 300 dehusked seeds in 50 ml Falcon tube. Seeds are manually dehusked using fine grade sand paper being careful not to damage seeds. Select only intact seeds for the sterilization and tissue culture. Sterilize seeds in 50% bleach 30 min. 200 rpm 28 C

Embryogenic Callus Induction

Mature seeds on embryogenic callus induction media



Place 20 seeds into Petri dish containing callus induction medium. Seeds are evenly spaced out to allow for growth. Seal the Petri plates with parafilm and incubate at 28°C in dark.

Rice Transformation Media

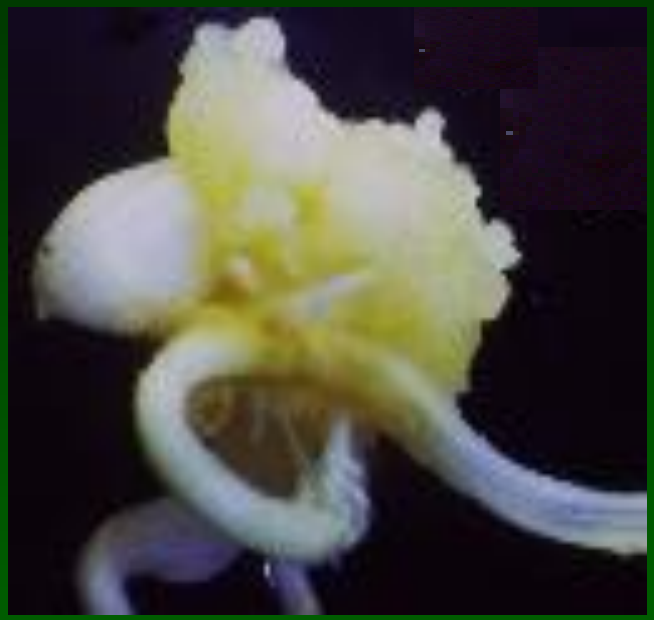
Component	Callus Induction	Infection	Co-cultivation	Resting	Selection I	Regeneration I	Regeneration II
MS salts						4.33 g	4.33g
N6 Salts	4.0 g	4.0g	4.0 g	4.0 g	4.0 g		
L-proline	2.8 g	700mg	2.8 g	2.8 g	2.8 g		
Myo-inositol							100mg
Sucrose	30.0 g	68.4g	30.0 g	30.0 g	30.0 g	30g	30g
Glucose		36g	10.0 g				
Sorbitol						30g	
2,4-D 1mg/ml	2 ml	1.5ml	2 ml	2 ml	2 ml		
Gelrite	4.0 g		4.0 g	4.0 g	4.0 g	4g	3g
pH	5.8	5.2	5.8	5.8	5.8	5.8	5.8
Autoclave	yes	No/filter	yes	yes	yes	yes	Yes
N6 Vitamins (1000x)	1 ml	1ml	1 ml	1 ml	1 ml		
MS Vitamins (1000x)						1ml	1 ml
Acetosyringone, 100mM		1ml fresh	1 ml fresh				
Casamino acids	300 mg		300 mg	300 mg	300 mg	2g	
Carbenicillin				200 mg	200 mg	200mg	
Timentin				150 mg	150 mg	150mg	
PPT					*10 mg*	*10mg*	*10mg*
Hygromycin					*50 mg*		
Kinetin, 1mg/ml						2 ml	
NAA, 1mg/ml						20 ul	

N6 Salts; N6 vitamins, proline; 2 mg 2,4 D; Gelrite

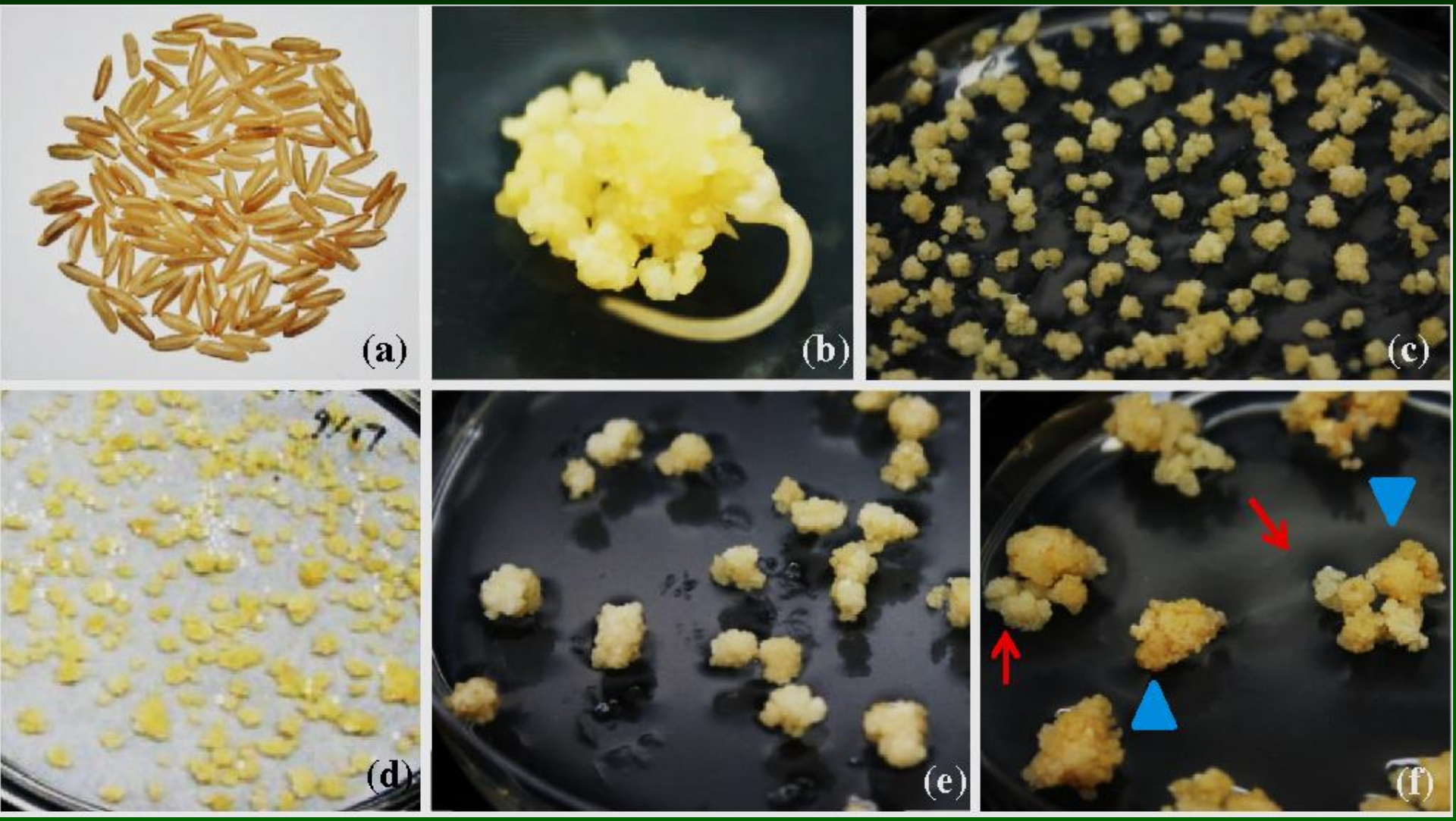
Embryogenic Callus Induction

Mature seeds on embryogenic callus induction media after 1 week incubation at 28 C in darkness

After 2 weeks, the germinated roots and shoots are removed to allow for embryogenic callus growth. Seal the Petri plates with parafilm and incubate at 28°C in dark.



Embryogenic Callus Induction

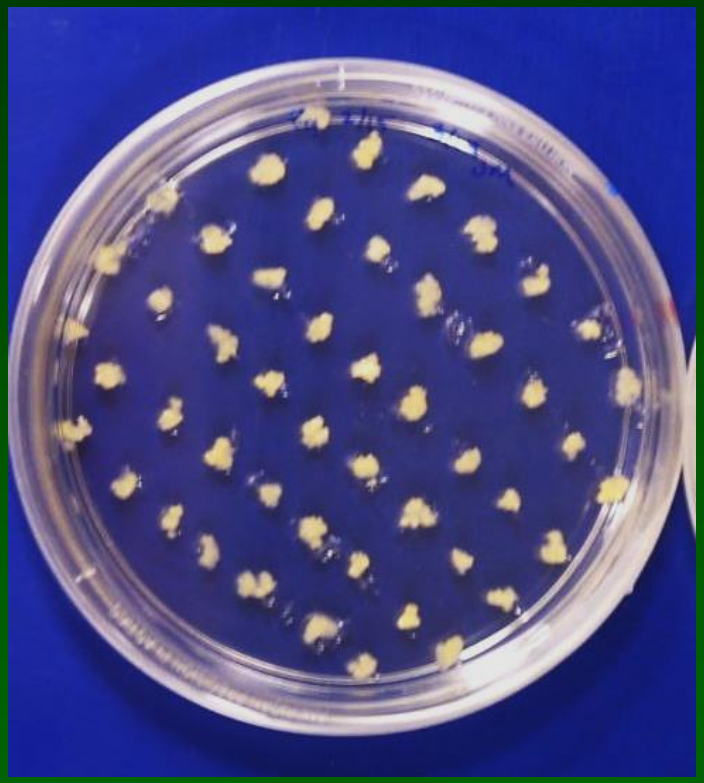


Embryogenic Calli for Transformation

After 2-3 weeks, growth of embrogenic calli should be visible using a dissecting scope. Embryogenic callus is subcultured using the dissecting microscope onto fresh callus induction media every 2 weeks. Seal the Petri plates with parafilm and incubate at 28°C in dark. ***Note: Somaclonal variation increases with the amount of time callus is in culture. For this reason, fresh cultures should be started every 3-4 months.***

Rice Embryogenic Calli Explant Preparation

One week prior to infection: **To make sure that callus is actively growing and dividing, embryogenic callus is subcultured into small pieces (~1 -2 mm.) onto fresh callus induction medium about 50 pieces/plate. Incubate at 25°C in the dark.**



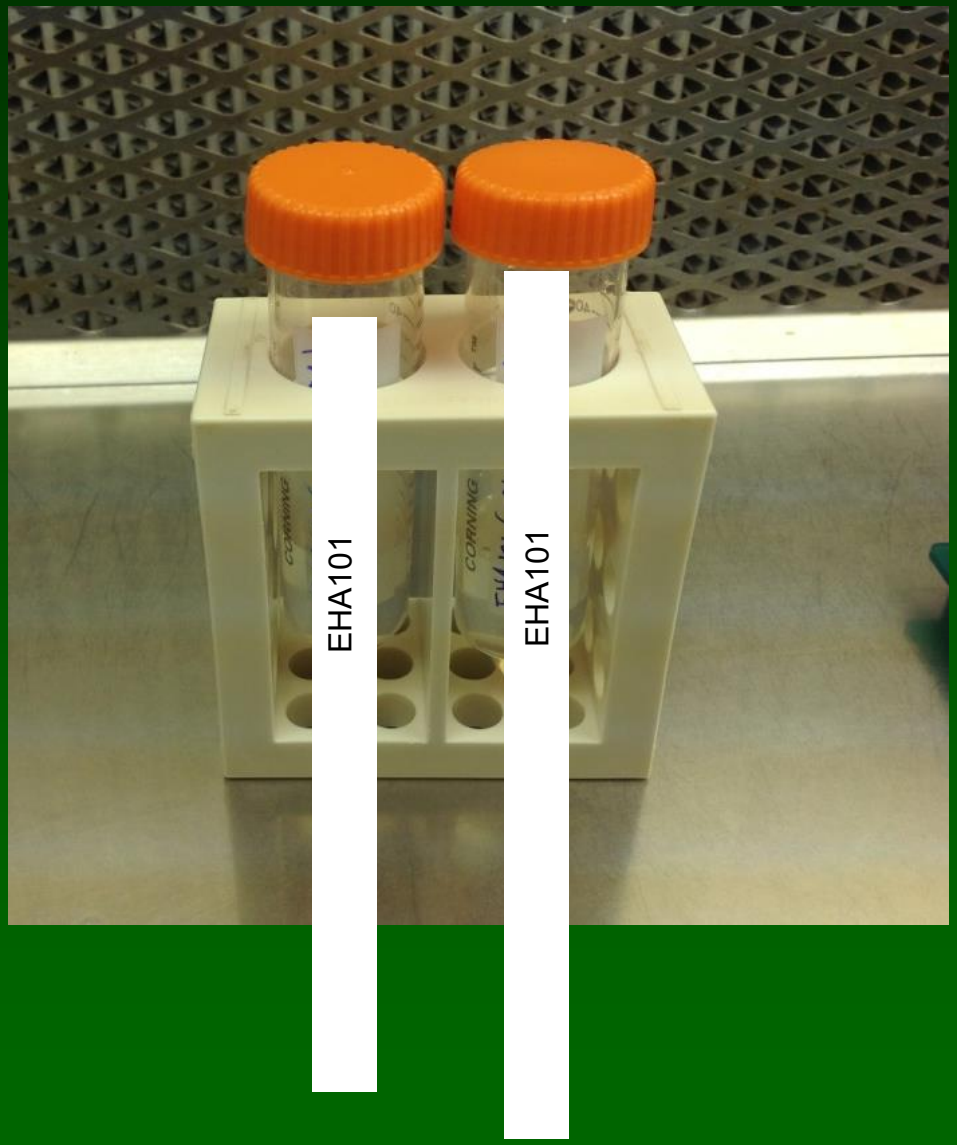
On the day before infection: **Transfer the small pieces to co-cultivation media containing acetosyringone. Incubate overnight at 25°C in the dark.**

Rice Transformation Media

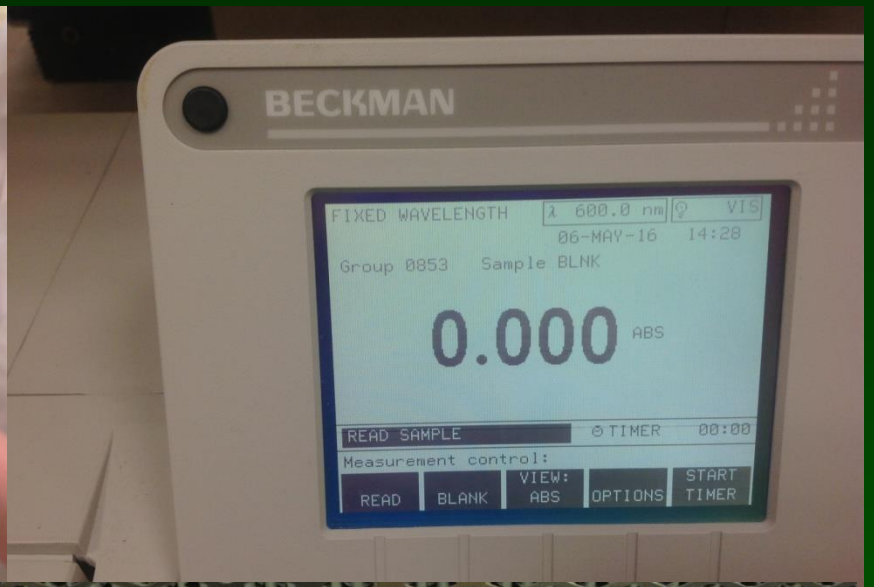
Component	Callus Induction	Infection	Co-cultivation	Resting	Selection I	Regeneration I	Regeneration II
MS salts						4.33 g	4.33g
N6 Salts	4.0 g	4.0g	4.0 g	4.0 g	4.0 g		
L-proline	2.8 g	700mg	2.8 g	2.8 g	2.8 g		
Myo-inositol							100mg
Sucrose	30.0 g	68.4g	30.0 g	30.0 g	30.0 g	30g	30g
Glucose		36g	10.0 g				
Sorbitol						30g	
2,4-D 1mg/ml	2 ml	1.5ml	2 ml	2 ml	2 ml		
Gelrite	4.0 g		4.0 g	4.0 g	4.0 g	4g	3g
pH	5.8	5.2	5.8	5.8	5.8	5.8	5.8
Autoclave	yes	No/filter	yes	yes	yes	yes	Yes
N6 Vitamins (1000x)	1 ml	1ml	1 ml	1 ml	1 ml		
MS Vitamins (1000x)						1ml	1 ml
Acetosyringone, 100mM		1ml fresh	1 ml fresh				
Casamino acids	300 mg		300 mg	300 mg	300 mg	2g	
Carbenicillin				200 mg	200 mg	200mg	
Timentin				150 mg	150 mg	150mg	
PPT					*10 mg*	*10mg*	*10mg*
Hygromycin					*50 mg*		
Kinetin, 1mg/ml						2 ml	
NAA, 1mg/ml						20 ul	

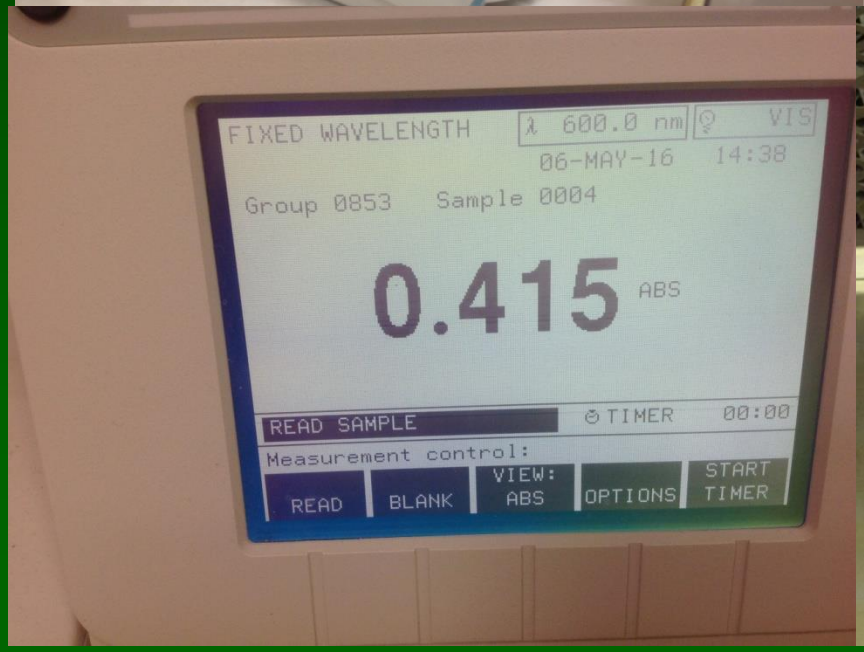
N6 salts; less proline; high sucrose plus glucose;
And acetosyringone; filter sterilized

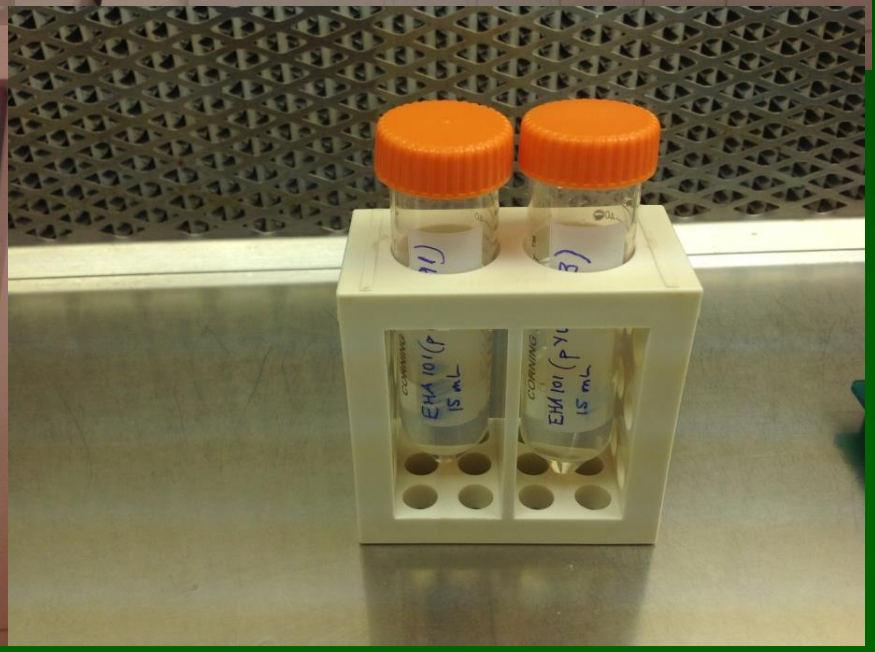
Preparation of *Agrobacterium* Infection Medium



The final suspension should be adjusted to OD_{600}. = 0.30-0.45.







Note: Agro concentration and strain may affect co-cultivation incubation times

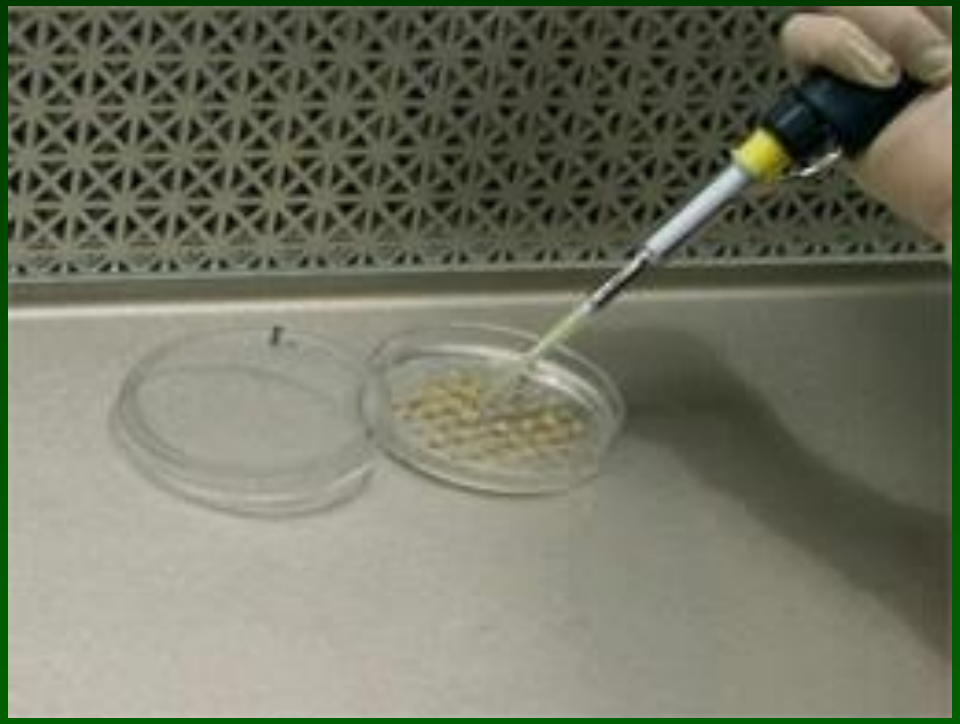
Rice Transformation Media

Component	Callus Induction	Infection	Co-cultivation	Resting	Selection I	Regeneration I	Regeneration II
MS salts						4.33 g	4.33g
N6 Salts	4.0 g	4.0g	4.0 g	4.0 g	4.0 g		
L-proline	2.8 g	700mg	2.8 g	2.8 g	2.8 g		
Myo-inositol							100mg
Sucrose	30.0 g	68.4g	30.0 g	30.0 g	30.0 g	30g	30g
Glucose		36g	10.0 g				
Sorbitol						30g	
2,4-D 1mg/ml	2 ml	1.5ml	2 ml	2 ml	2 ml		
Gelrite	4.0 g		4.0 g	4.0 g	4.0 g	4g	3g
pH	5.8	5.2	5.8	5.8	5.8	5.8	5.8
Autoclave	yes	No/filter	yes	yes	yes	yes	Yes
N6 Vitamins (1000x)	1 ml	1ml	1 ml	1 ml	1 ml		
MS Vitamins (1000x)						1ml	1 ml
Acetosyringone, 100mM		1ml fresh	1 ml fresh				
Casamino acids	300 mg		300 mg	300 mg	300 mg	2g	
Carbenicillin				200 mg	200 mg	200mg	
Timentin				150 mg	150 mg	150mg	
PPT					*10 mg*	*10mg*	*10mg*
Hygromycin					*50 mg*		
Kinetin, 1mg/ml						2 ml	
NAA, 1mg/ml						2 ml	

N6 salts; N6 vitamines; proline; back to 30g sucrose but plus 10g glucose;
And acetosyringone; filter sterilized

Callus Infection

On day of infection: **Dispense 1 drop (~5 uL) of *Agrobacterium* suspension onto each piece of callus making sure to fully immerse each piece with the suspension. Make sure that every piece is treated.**



A drop (5 uL) of infection media with *Agrobacterium* is spotted to each

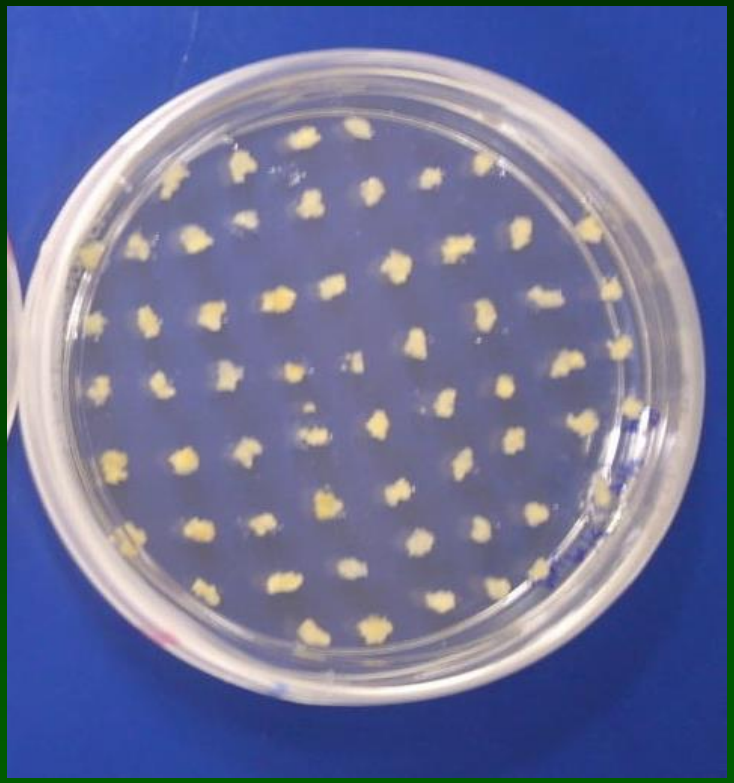
Callus Infection

A repeater micropipet works well for this step

Allow pieces to air dry in the hood ~20 minutes and wrap the plate with parafilm and incubate at 25°C in dark for 3 days for the co-cultivation period.

Note: Make sure that pieces are fully dry before wrapping plates and incubating.

Co-Cultivation of Infected Calli



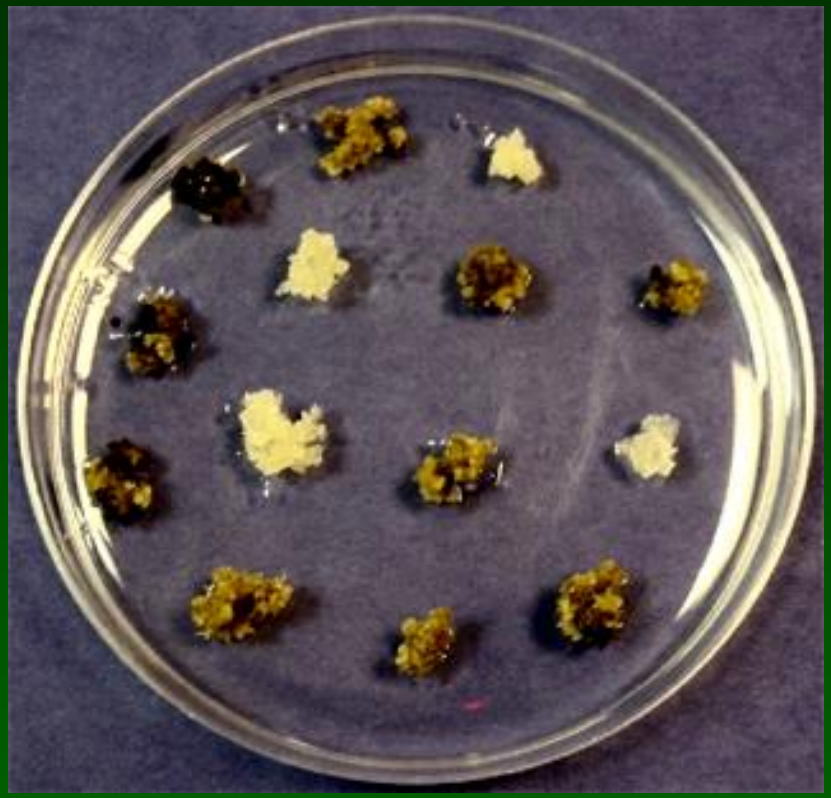
Following the 3 day co-cultivation period, callus is transferred to Resting Media containing 200 mg/L Carbenicillin and 150 mg/L Timentin. *Note the antibiotics used are specific to the Agrobacterium strain, check the literature*). Cultures remain on Resting Medium for 7 days to begin killing the *Agrobacterium. Care is taken to make sure to not break the pieces apart. The number of pieces transferred to resting media is counted and recorded. This will be the initial number of callus infected for calculating transformation efficiency later.* Incubate calli at 25°C in the dark for

Rice Transformation Media

Component	Callus Induction	Infection	Co-cultivation	Resting	Selection I	Regeneration I	Regeneration II
MS salts						4.33 g	4.33g
N6 Salts	4.0 g	4.0g	4.0 g	4.0 g	4.0 g		
L-proline	2.8 g	700mg	2.8 g	2.8 g	2.8 g		
Myo-inositol							100mg
Sucrose	30.0 g	68.4g	30.0 g	30.0 g	30.0 g	30g	30g
Glucose		36g	10.0 g				
Sorbitol						30g	
2,4-D 1mg/ml	2 ml	1.5ml	2 ml	2 ml	2 ml		
Gelrite	4.0 g		4.0 g	4.0 g	4.0 g	4g	3g
pH	5.8	5.2	5.8	5.8	5.8	5.8	5.8
Autoclave	yes	No/filter	yes	yes	yes	yes	Yes
N6 Vitamins (1000x)	1 ml	1ml	1 ml	1 ml	1 ml		
MS Vitamins (1000x)						1ml	1 ml
Acetosyringone, 100mM		1ml fresh	1 ml fresh				
Casamino acids	300 mg		300 mg	300 mg	300 mg	2g	
Carbenicillin				200 mg	200 mg	200mg	
Timentin				150 mg	150 mg	150mg	
PPT					*10 mg*	*10mg*	*10mg*
Hygromycin					*50 mg*		
Kinetin, 1mg/ml						2 ml	
NAA, 1mg/ml						20 ul	

"Resting" medium (so-called)
Callus induction medium with carbenicillin and timentin

Selection I



Selection of transformants can be done using Hygromycin or PPT

Selection for hpt expression

Following the resting phase, each piece of callus is transferred to Selection I medium and incubated at 25°C in dark for 4-6 weeks sub-culturing bi-weekly. *Each piece of callus is kept together and not broken apart.*

Note: These transformants (above) are selected using 50 mg/L Hygromycin in Selection I

Rice Transformation Media

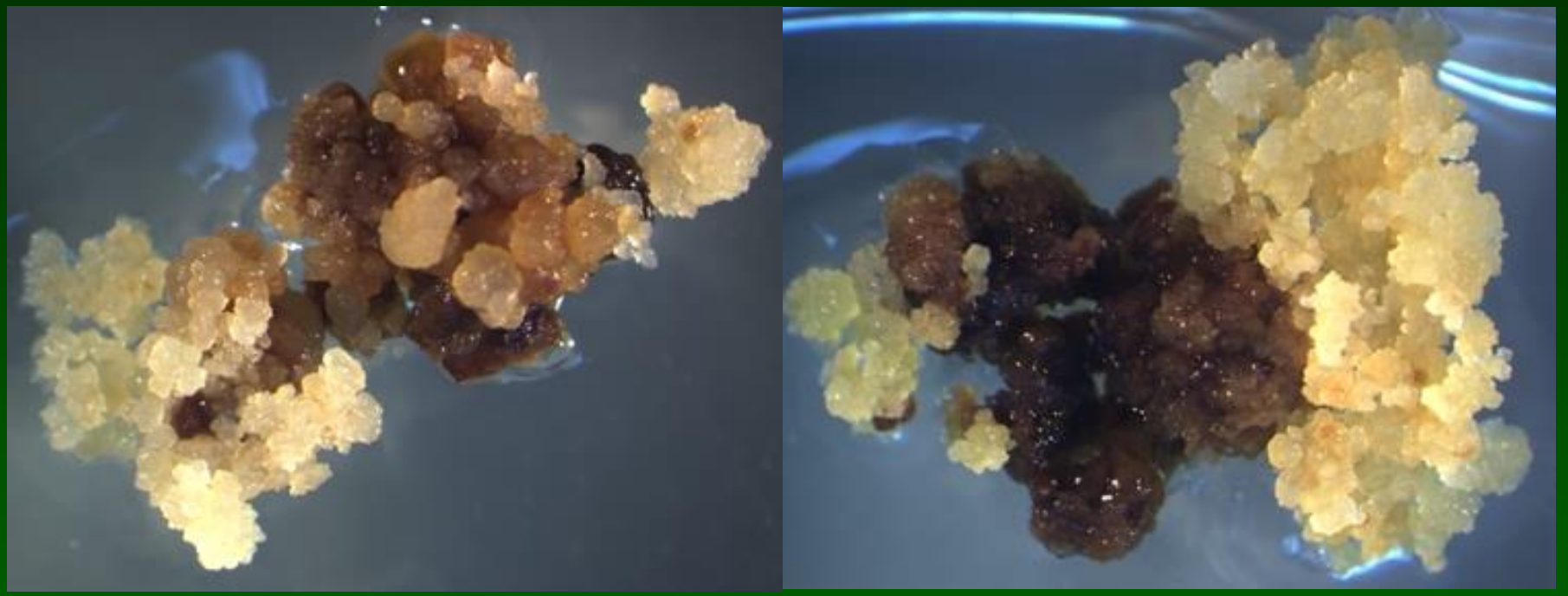
Component	Callus Induction	Infection	Co-cultivation	Resting	Selection I	Regeneration I	Regeneration II
MS salts						4.33 g	4.33g
N6 Salts	4.0 g	4.0g	4.0 g	4.0 g	4.0 g		
L-proline	2.8 g	700mg	2.8 g	2.8 g	2.8 g		
Myo-inositol							100mg
Sucrose	30.0 g	68.4g	30.0 g	30.0 g	30.0 g	30g	30g
Glucose		36g	10.0 g				
Sorbitol						30g	
2,4-D 1mg/ml	2 ml	1.5ml	2 ml	2 ml	2 ml		
Gelrite	4.0 g		4.0 g	4.0 g	4.0 g	4g	3g
pH	5.8	5.2	5.8	5.8	5.8	5.8	5.8
Autoclave	yes	No/filter	yes	yes	yes	yes	Yes
N6 Vitamins (1000x)	1 ml	1ml	1 ml	1 ml	1 ml		
MS Vitamins (1000x)						1ml	1 ml
Acetosyringone, 100mM		1ml fresh	1 ml fresh				
Casamino acids	300 mg		300 mg	300 mg	300 mg	2g	
Carbenicillin				200 mg	200 mg	200mg	
Timentin				150 mg	150 mg	150mg	
PPT					*10 mg*	*10mg*	*10mg*
Hygromycin					*50 mg*		
Kinetin, 1mg/ml						2 ml	
NAA, 1mg/ml						2 ml	

either/or

First phase of selection: Selection I
Callus induction medium with carbenicillin and timentin
And selection agent (10mg PPT for bar or 50 mg hygromycin)

Selection I

Selection for bar expression



Putative transformants (events) after 8 weeks on Selection I medium. ***The transformants should be a pale yellow color, friable, and growing.*** **They should look exactly the same as non-transformed callus. If the colonies do not look the same as non-transformed callus, they are probably not transformed or non-regenerable.**

Note: These transformants (above) are selected using 10 mg/L PPT in Selection I

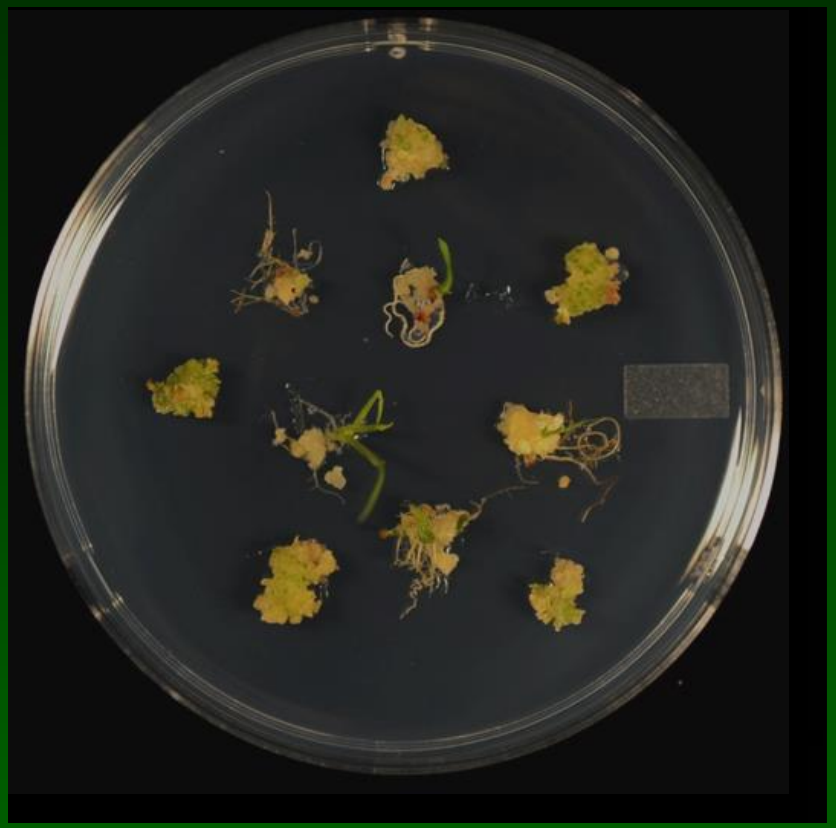
rice

Rice Transformation Media

Component	Callus Induction	Infection	Co-cultivation	Resting	Selection I	Regeneration I	Regeneration II
MS salts						4.33 g	4.33g
N6 Salts	4.0 g	4.0g	4.0 g	4.0 g	4.0 g		
L-proline	2.8 g	700mg	2.8 g	2.8 g	2.8 g		
Myo-inositol							100mg
Sucrose	30.0 g	68.4g	30.0 g	30.0 g	30.0 g	30g	30g
Glucose		36g	10.0 g				
Sorbitol						30g	
2,4-D 1mg/ml	2 ml	1.5ml	2 ml	2 ml	2 ml		
Gelrite	4.0 g		4.0 g	4.0 g	4.0 g	4g	3g
pH	5.8	5.2	5.8	5.8	5.8	5.8	5.8
Autoclave	yes	No/filter	yes	yes	yes	yes	Yes
N6 Vitamins (1000x)	1 ml	1ml	1 ml	1 ml	1 ml		
MS Vitamins (1000x)						1ml	1 ml
Acetosyringone, 100mM		1ml fresh	1 ml fresh				
Casamino acids	300 mg		300 mg	300 mg	300 mg	2g	
Carbenicillin				200 mg	200 mg	200mg	
Timentin				150 mg	150 mg	150mg	
PPT					*10 mg*	*10mg*	*10mg*
Hygromycin					*50 mg*		
Kinetin, 1mg/ml						2 ml	
NAA, 1mg/ml						20 ul	

First phase of Regeneration:
Shifts to MS salts and vitamins, drops proline, adds sorbitol
Drops 2,4 D; adds NAA
Keeps carbenicillin and timentin
And keeps selection agent (10mg PPT for bar or 50 mg hygromycin

Regeneration

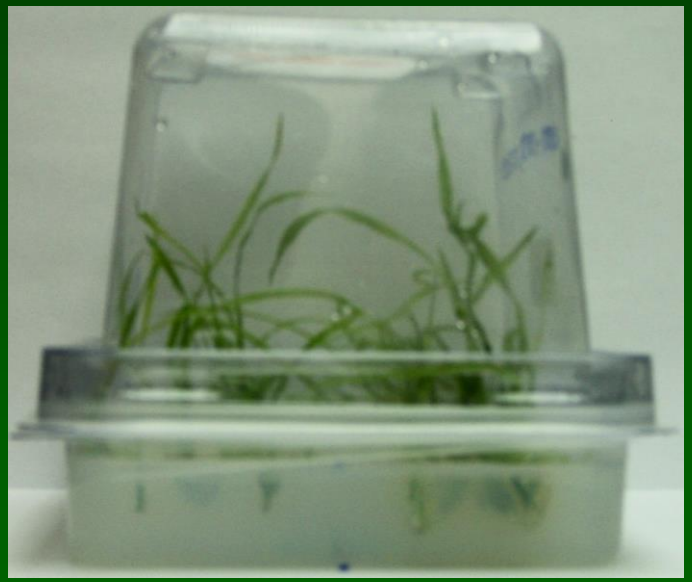


The putative transformants (events) are sub-cultured onto Regeneration I medium to initiate shoot formation. The transformants should be incubated for one week in the dark at 25°C and then transferred to low light (16 hour light/8 hour dark) for 2 weeks. Shoots should begin to appear after 2-3 weeks. If shoots are not well formed, the transformants can be transferred to fresh Regeneration I media for 2 additional weeks. *Each event is kept separate.*

rice

Plantlet Regeneration

Once shoots are well formed (~2cm), they are transferred individually to Regeneration II medium for root formation. The Regeneration II media should be poured thick into petri dishes. Plantlets are transferred to these thick poured petri dishes and covered with an empty, sterile petri dish bottom. These plates are sealed with parafilm and incubated at 25°C in the light (16 hour light/8 hour dark) for 2 weeks.



Once plantlet shoots are elongated and roots are beginning to form, they are transferred to Regeneration II media in plantcons for further growth. The plantcons are incubated at 25°C in the light (16 hour light/8 hour dark) for 2 weeks.

rice

Rice Transformation Media

Component	Callus Induction	Infection	Co-cultivation	Resting	Selection I	Regeneration I	Regeneration II
MS salts						4.33 g	4.33g
N6 Salts	4.0 g	4.0g	4.0 g	4.0 g	4.0 g		
L-proline	2.8 g	700mg	2.8 g	2.8 g	2.8 g		
Myo-inositol							100mg
Sucrose	30.0 g	68.4g	30.0 g	30.0 g	30.0 g	30g	30g
Glucose		36g	10.0 g				
Sorbitol						30g	
2,4-D 1mg/ml	2 ml	1.5ml	2 ml	2 ml	2 ml		
Gelrite	4.0 g		4.0 g	4.0 g	4.0 g	4g	3g
pH	5.8	5.2	5.8	5.8	5.8	5.8	5.8
Autoclave	yes	No/filter	yes	yes	yes	yes	Yes
N6 Vitamins (1000x)	1 ml	1ml	1 ml	1 ml	1 ml		
MS Vitamins (1000x)						1ml	1 ml
Acetosyringone, 100mM		1ml fresh	1 ml fresh				
Casamino acids	300 mg		300 mg	300 mg	300 mg	2g	
Carbenicillin				200 mg	200 mg	200mg	
Timentin				150 mg	150 mg	150mg	
PPT					*10 mg*	*10mg*	*10mg*
Hygromycin					*50 mg*		
Kinetin, 1mg/ml						2 ml	
NAA, 1mg/ml						20 ul	

Second phase of Regeneration:
Keeps MS salts and vitamins, adds myo-inositol; drops sorbitol
And keeps selection agent if using 10mg PPT for bar

Rice Transformation

Individual plants are planted into well moistened Metromix 510 in 5 gallon pots. Excess media is carefully washed away by dipping roots into room temperature water to minimize fungal growth. Plants are immediately cover with an empty plantcon top to ensure the high humidity that the plants are accustomed to is maintained. *Plants have a very thin cuticle after tissue culture and care must be taken to prevent desiccation.* **rice**

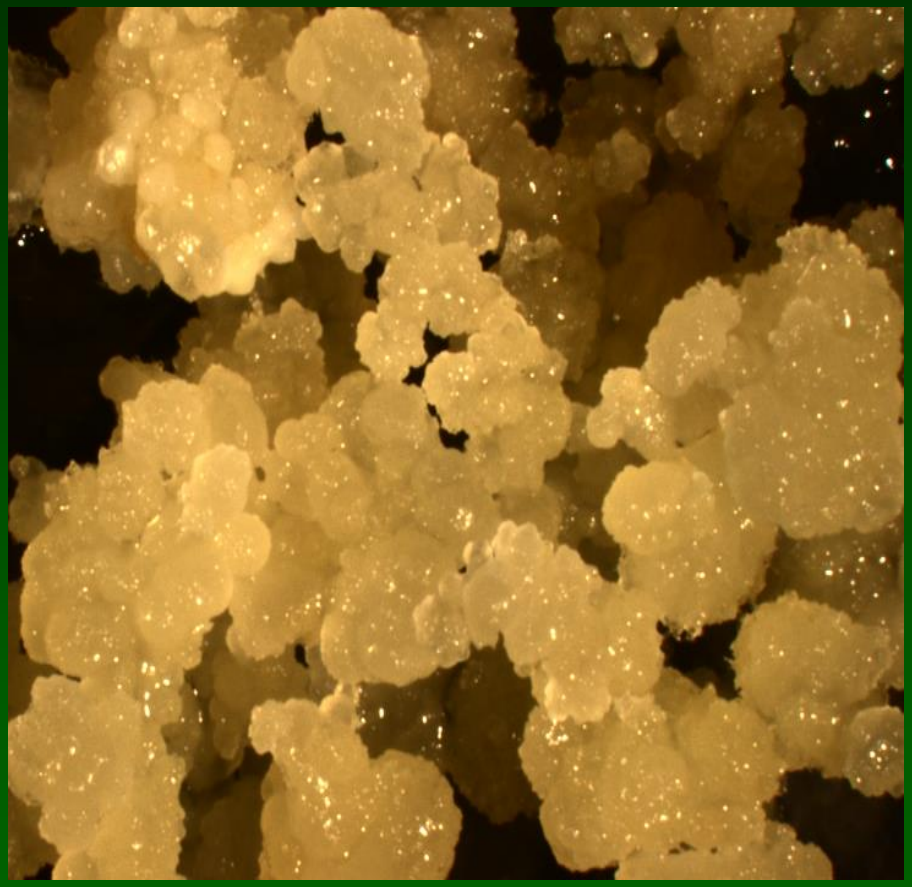
T0 Transgenic Rice in the Greenhouse

Plants in pots are also maintained in trays, watered thoroughly and placed in the growth chamber under shade cloth at 27°C under sodium halide lighting. Transgenic rice can also be grown in a greenhouse with supplemental lighting. Rice is slowly allowed to acclimate to the higher light. *Also, rice uses a lot of water and care must be taken to prevent drought conditions.*
Plants are grown to maturity and selfed.

T0 Transgenic Rice in the Greenhouse

How does this Work?

Embryogenic Callus Induction in Rice



Early embryogenic ice callus as observed under a dissecting light microscope. Note: the absence of later stages of somatic embryogenesis. Rather the somatic embryos appear as early stages as spherical structure. This callus is highly friable.

The Centrality of Somatic Embryogenesis for Monocot Transformation

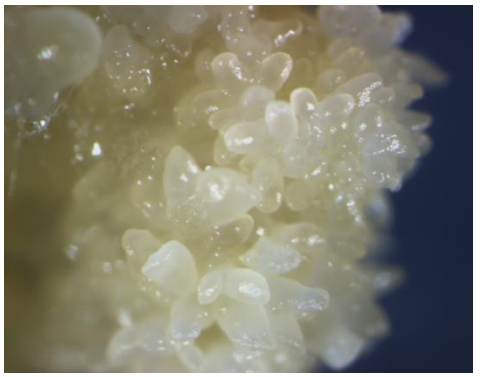
Somatic embryogenic cultures in monocots must contain cells at stages in development similar to a six-day old zygotic embryo

Selection can be achieved when a single transformed cell or small group of cells can survive to recapitulate a transgenic embryogenic culture that can be regenerated

"You are not making 'new' cells in tissue culture, you are controlling the developmental programs which are already in place"

Ian Sussex
Personal communication 1996

The Selectablity of Early Somatic embryos in culture



Comparative developmental morphology of embryogenesis in maize. (A) Zygotic embryogenesis.(A) Zygotic embryo in maize *Zea mays*. (a) Three-celled embryo showing first division of terminal cell (b) Six day embryo showing embryo proper and suspensor. (c) Seven day embryo showing delimitation of the protoderm. (d) nine day embryo show meristem differentiation (APK inspired by Randolf 1936). (B) Somatic embryogenesis from immature embryos (B), leaf mesophyll (C), and late vacuolate stage microspores (APK inspired)