National Science Foundation Plant Genome

Cereal Plant Transformation Workshop

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Rice Transformation

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Agrobacterium-mediated Rice Transformation From Mature Seeds

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Rice Transformation

Plant Material

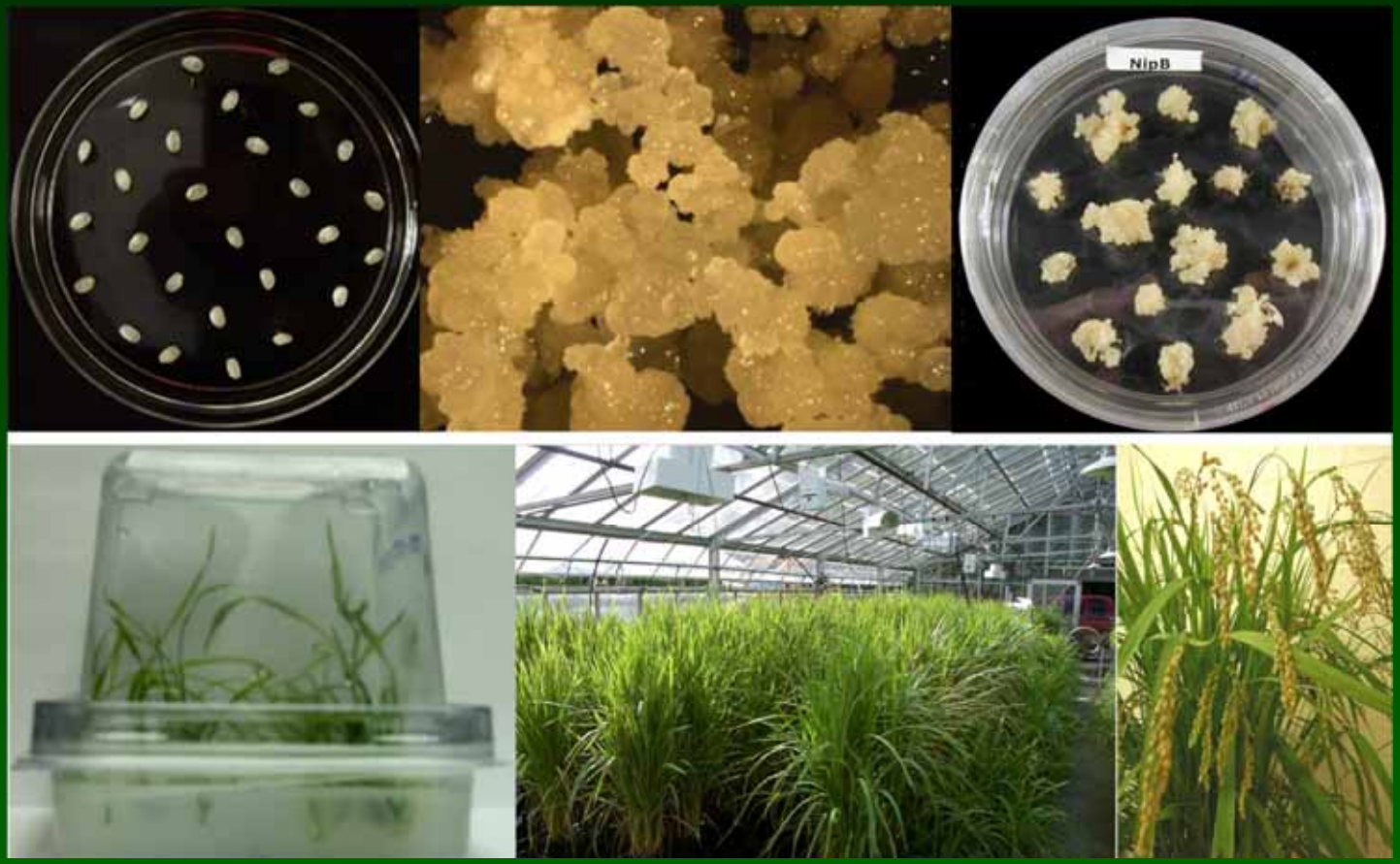
Mature rice seeds *Oryza sativa* cv. Nipponbare were obtained from USDA Agricultural Research Service; Dale Bumpers National Rice Research Center www.ars.usda.gov/sea/dbnrrc/gsor are used.

Rice Transformation

Nipponbarre

- Rice is a staple crop for a vast population in the world, making a better rice plant means making better food.
- Since it is an economically and agriculturally important crop there is good reason for a commercial endeavor into it's betterment.
- The rice genome is almost totally complete, making a functional genomics experiment in it easier and more valid.
- Rice is an easily accessible and reasonably inexpensive plant to use for the comparison of vectors and tissue culture protocols.
- Rice is fast becoming the arabidopsis of monocotyledonous plants, its genome can be used as a model, and protocols developed for rice can easily be modified for other monocots.
- Rice generates high numbers of progeny, giving excellent data of the transgenic plants.
- There are innumerous past experiments that yield valuable information to make these experiments much easier to perform.

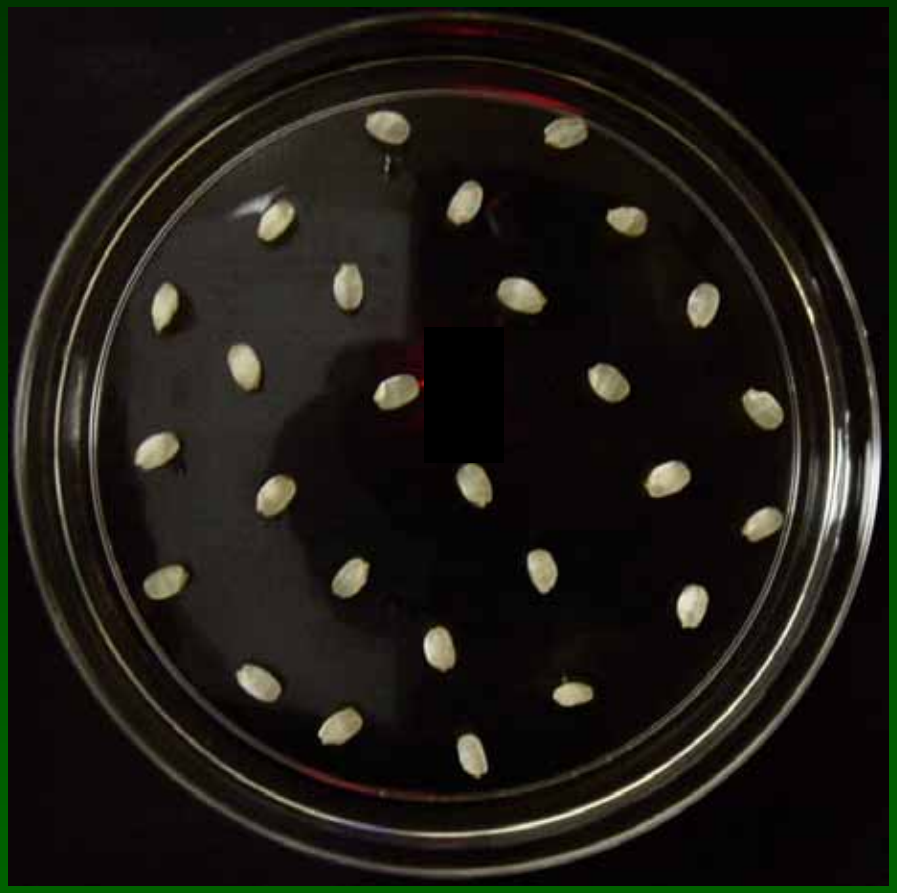
Rice Transformation



Rice transformation typically is initiated from mature seeds to produce a very 'early' embryogenic callus which is highly transformable

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	

Embryogenic Callus Induction

Mature seeds on embryogenic callus induction media after 1 week incubation at 25 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.

Mature Seed Explant Preparation

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

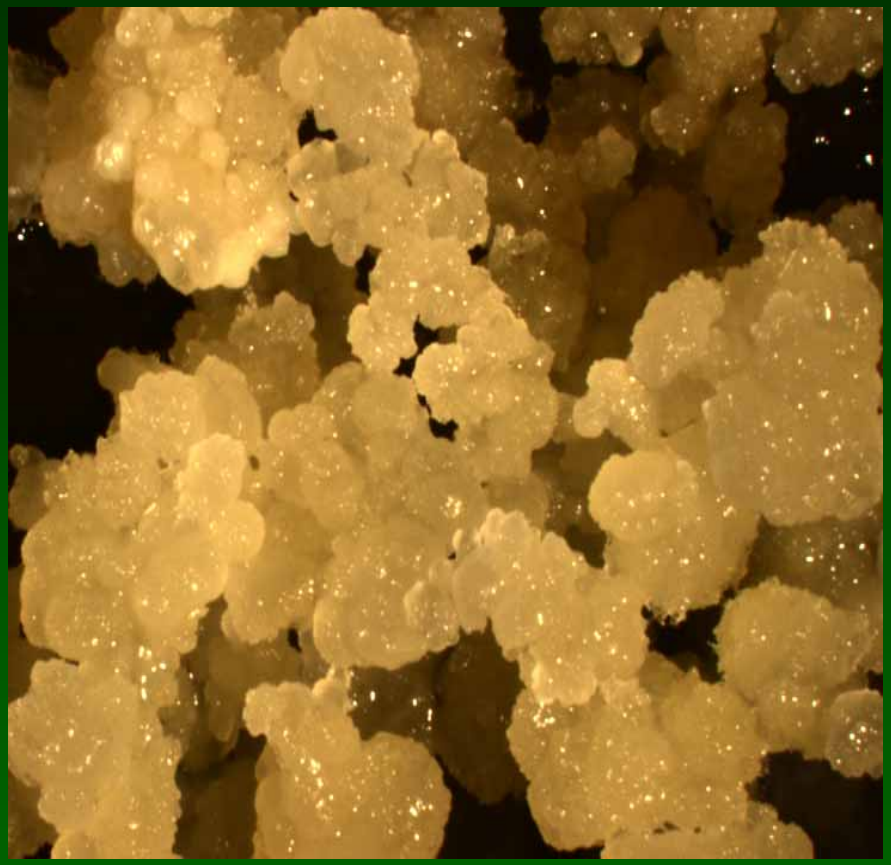
Mature Seed Explant Preparation

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.

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.*

Embryogenic Callus Induction



Early embrogenic 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.

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	

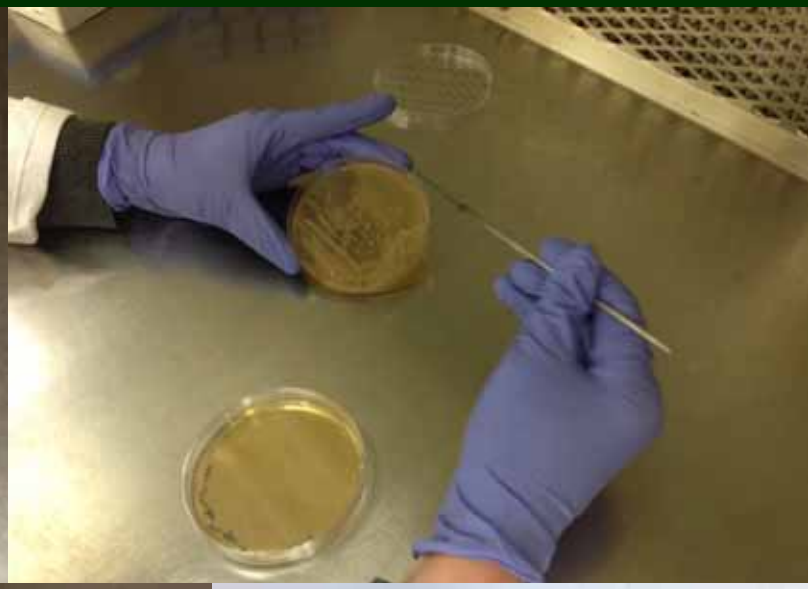
Preparation of *Agrobacterium*

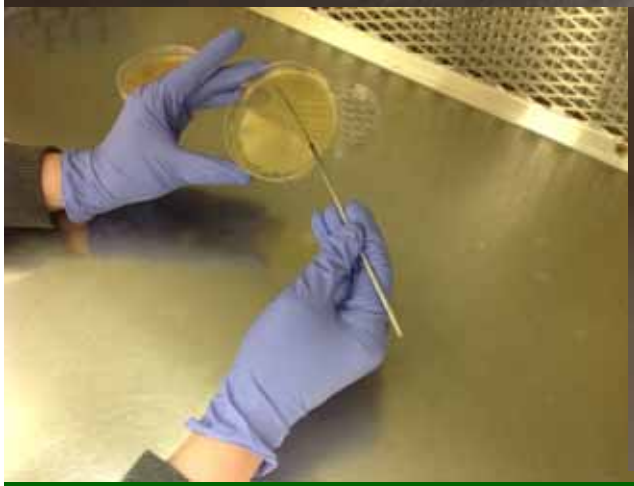
Initiation of *Agrobacterium tumefaciens* from stock culture:

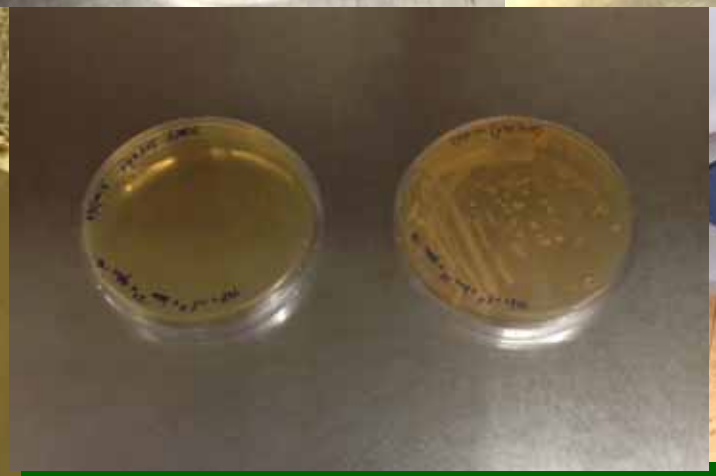
Streak the *Agrobacterium* culture for colony isolation from an -80°C glycerol stock onto a YEP medium plate containing appropriate antibiotics. Incubate the plate at 28°C for 3 days. This plate is the "master" plate, and should be stored at 4°C and can be reused for 30 days. A new master plate needs to be initiated after this period.

To begin a sorghum transformation experiment, streak a single colony onto a fresh YEP plate containing the appropriate antibiotics. Incubate this plate for 3 days at 20°C or 2 days at 25°C.

Preparation of *Agrobacterium*





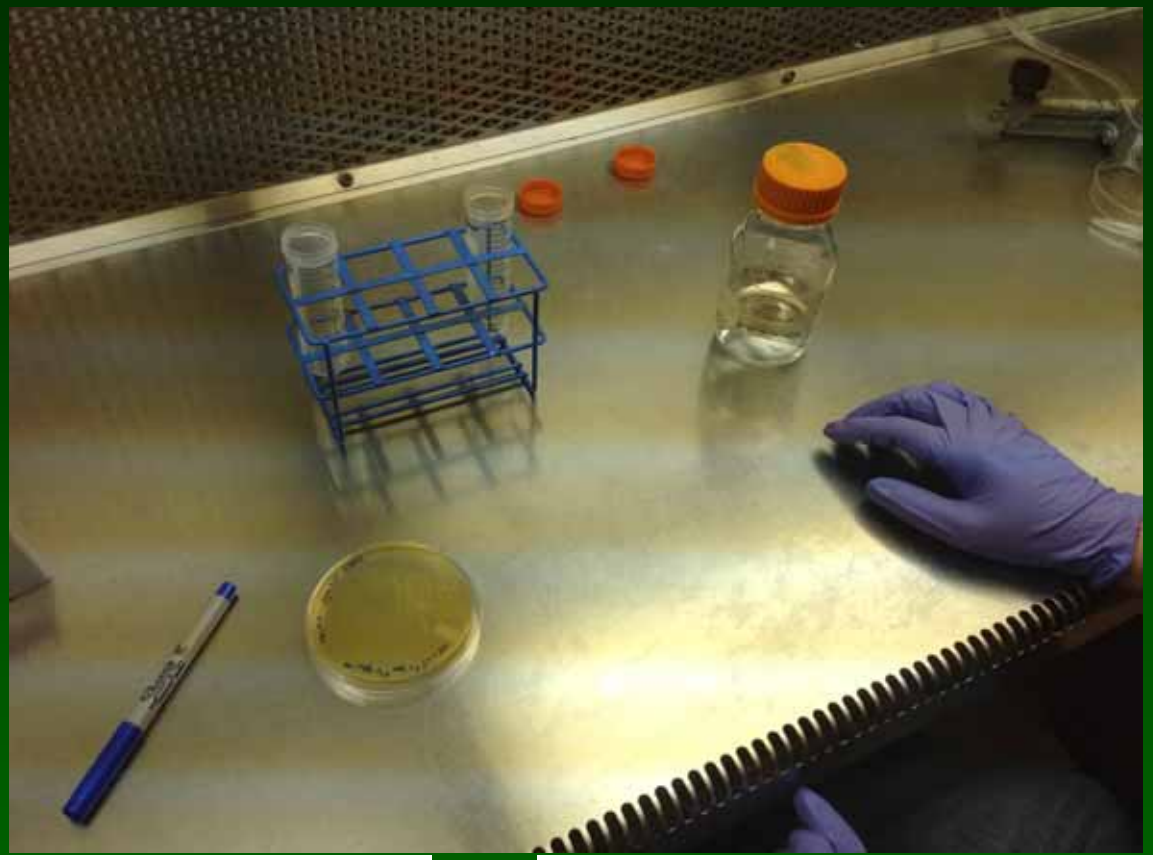


Initiation of *Agrobacterium tumefaciens* from stock culture: To begin a sorghum transformation experiment, streak a single colony onto a fresh YEP plate containing the appropriate antibiotics. Incubate this plate for 3 days at 20°C , 2 days at 25°C, or 28°C Overnight (caution *Agro* plasmids may be cured over 28°C)

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	

Preparation of *Agrobacterium* Infection Medium

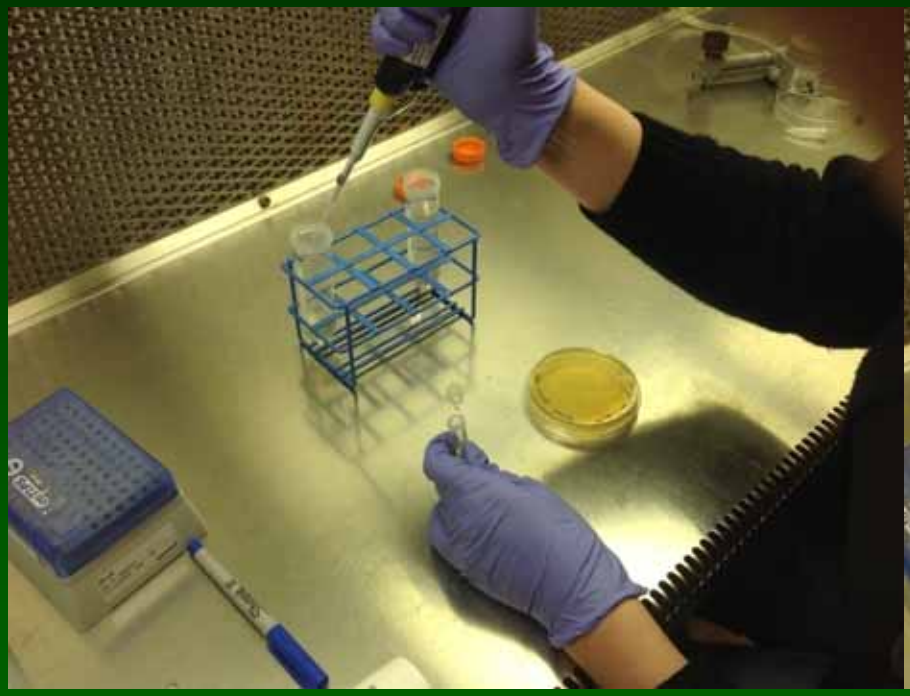


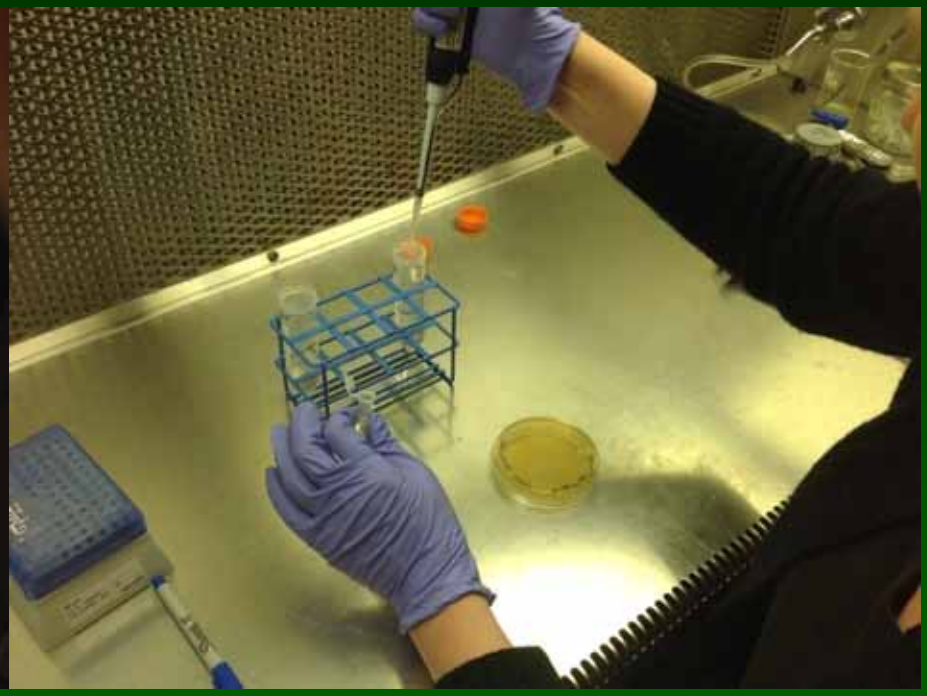
Infection medium + acetosyringone with Agro

Infection medium + acetosyrigone without Agro

Prepare two sterile 50 mL Falcon tubes (depending on the size of the experiment) . One tube will be used to make the co-cultivation infection with the *Agrobacterium*, the other, will be used to adjust the concentration of bacteria after the OD is read.

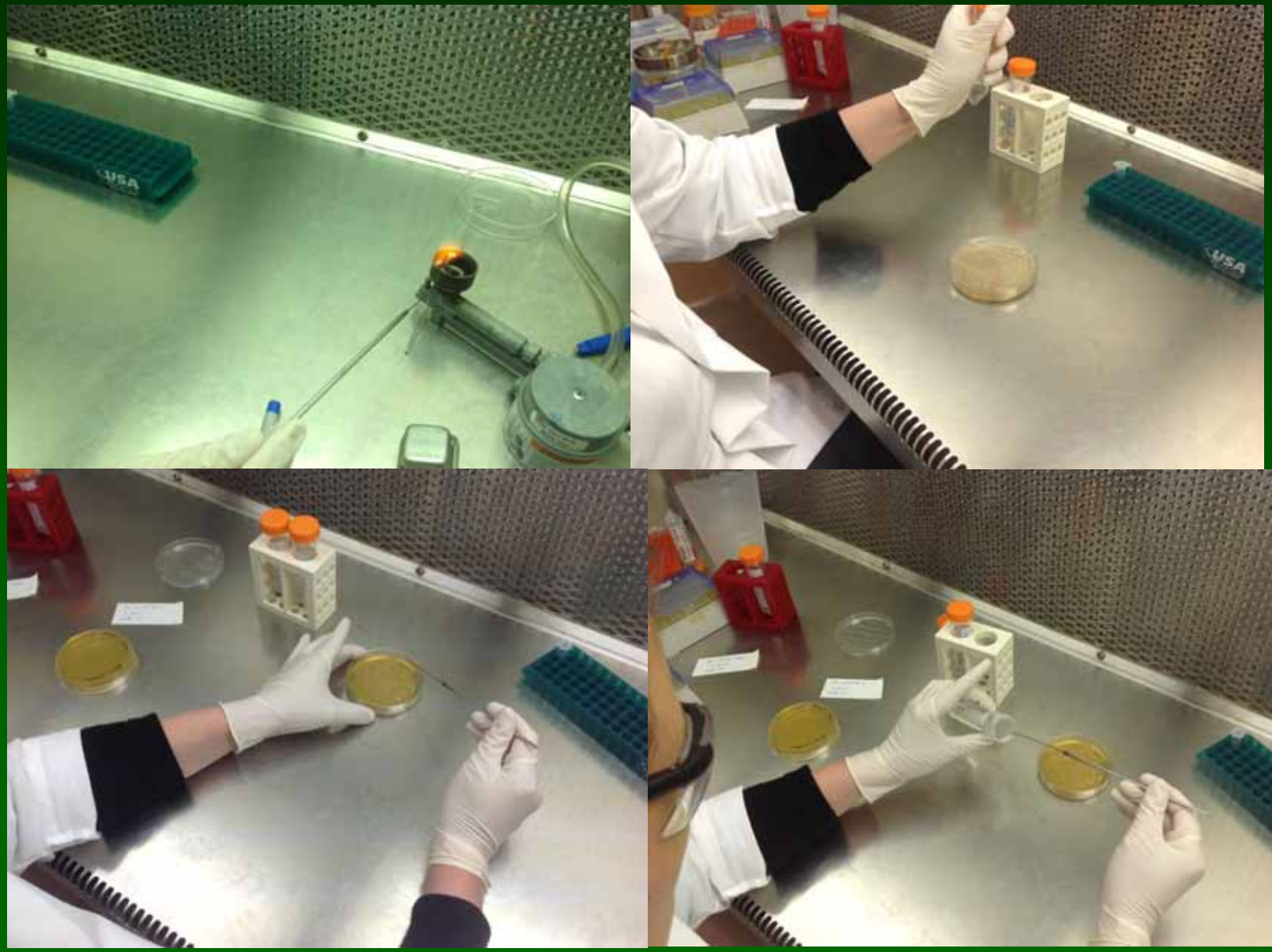
Preparation of *Agrobacterium* Infection Medium



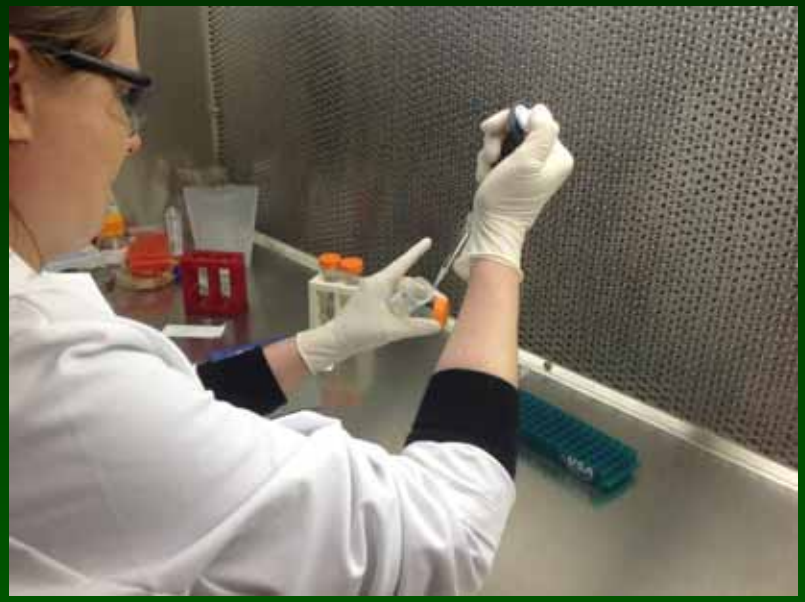


Add acetosyringone (100 mM/mL stock solution) to the each of the tubes containing infection media for a final concentration of 100 µM/mL acetosyringone.

Preparation of *Agrobacterium* Infection Media



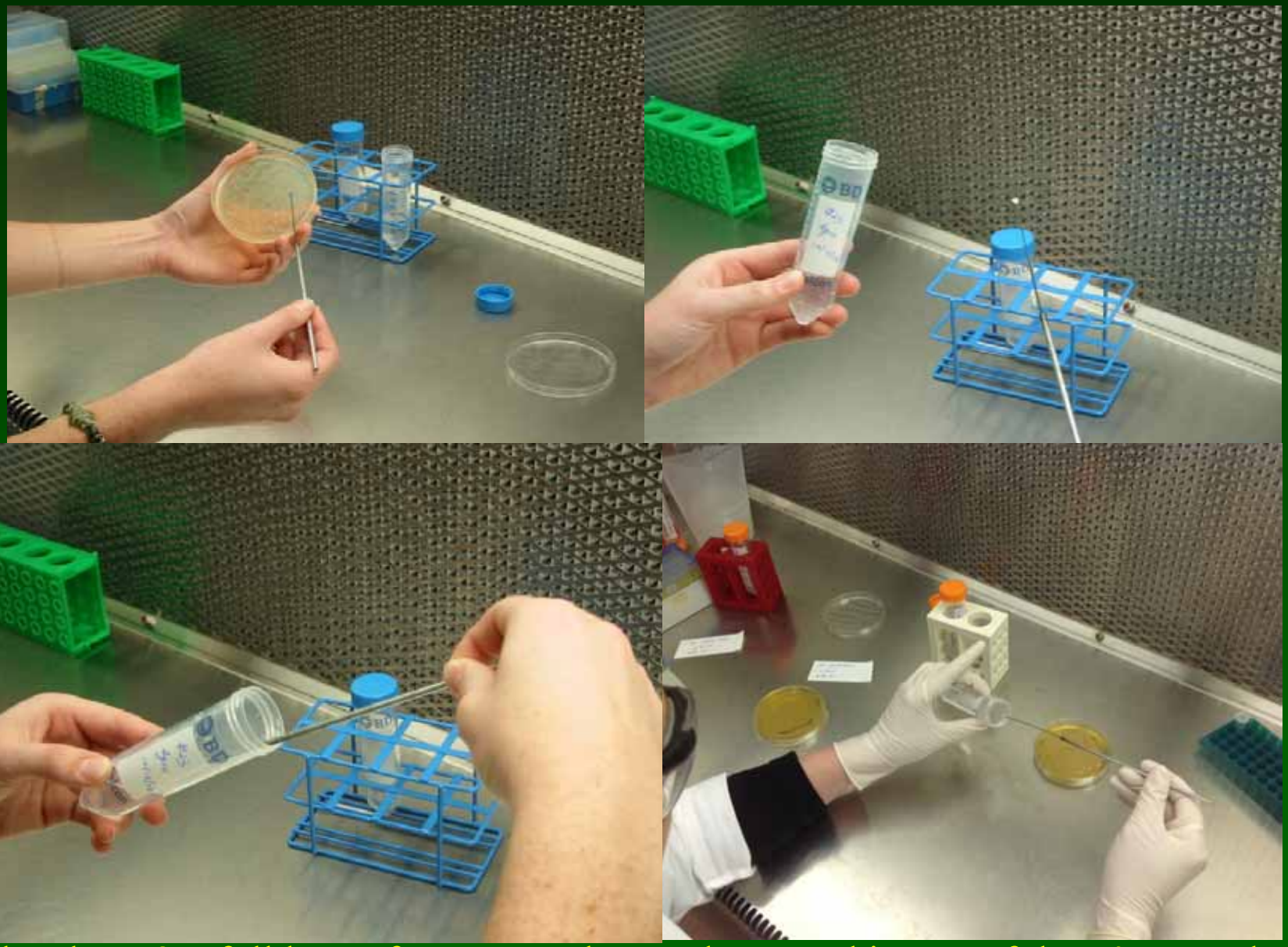
Preparation of *Agrobacterium* Infection Medium



Add 15-25 mL (depending on the size of the experiment) each of sterile co-cultivation infection medium into two sterile 50 mL Falcon tubes. One tube will be used to make the co-cultivation infection with the *Agrobacterium*, the other, will be used to adjust the concentration of bacteria after the OD is read.

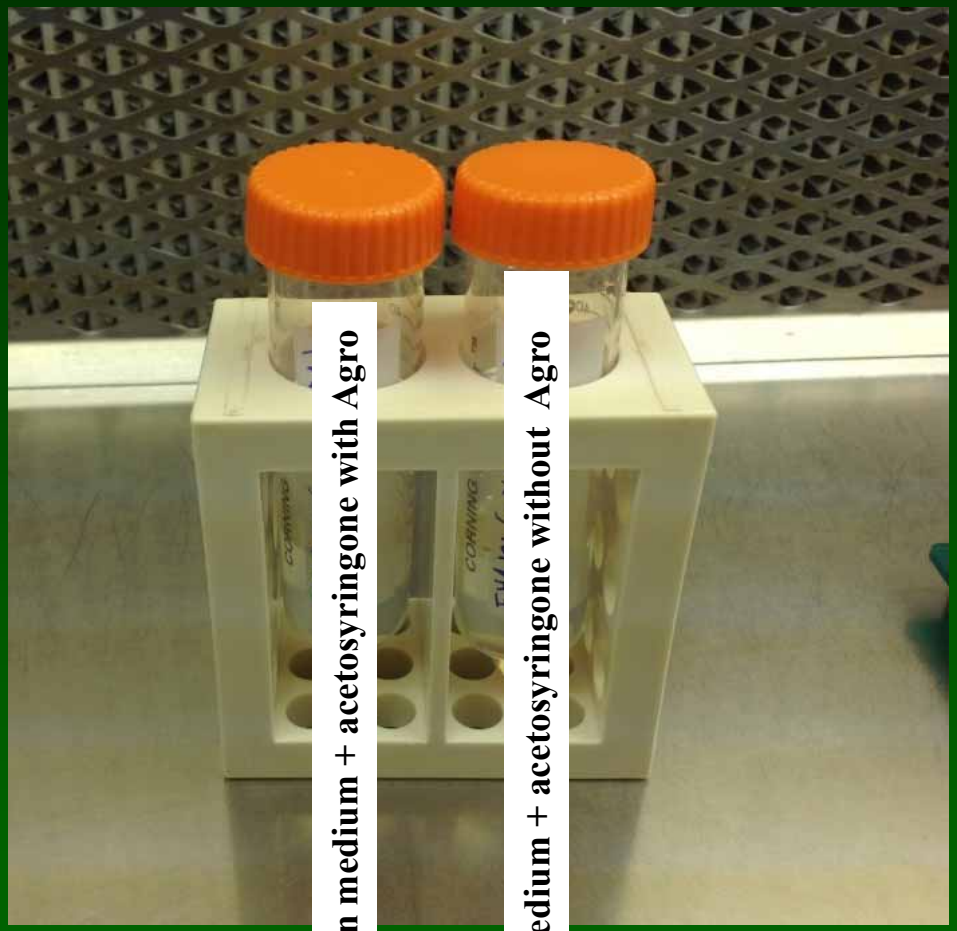
Add acetosyringone (100 mM/mL stock solution) to the each of the tubes containing infection media for a final concentration of 100 µM/mL acetosyringone.

Preparation of *Agrobacterium* Infection Medium



Take about 2.5 full loops from YEP plate and suspend in one of the 50 mL Falcon tubes (about 1 full loopful/10 mL of infection medium + 100 μM acetosyringone

Preparation of *Agrobacterium* Infection Medium



Preparation of Infection Medium Containing the *Agrobacterium*

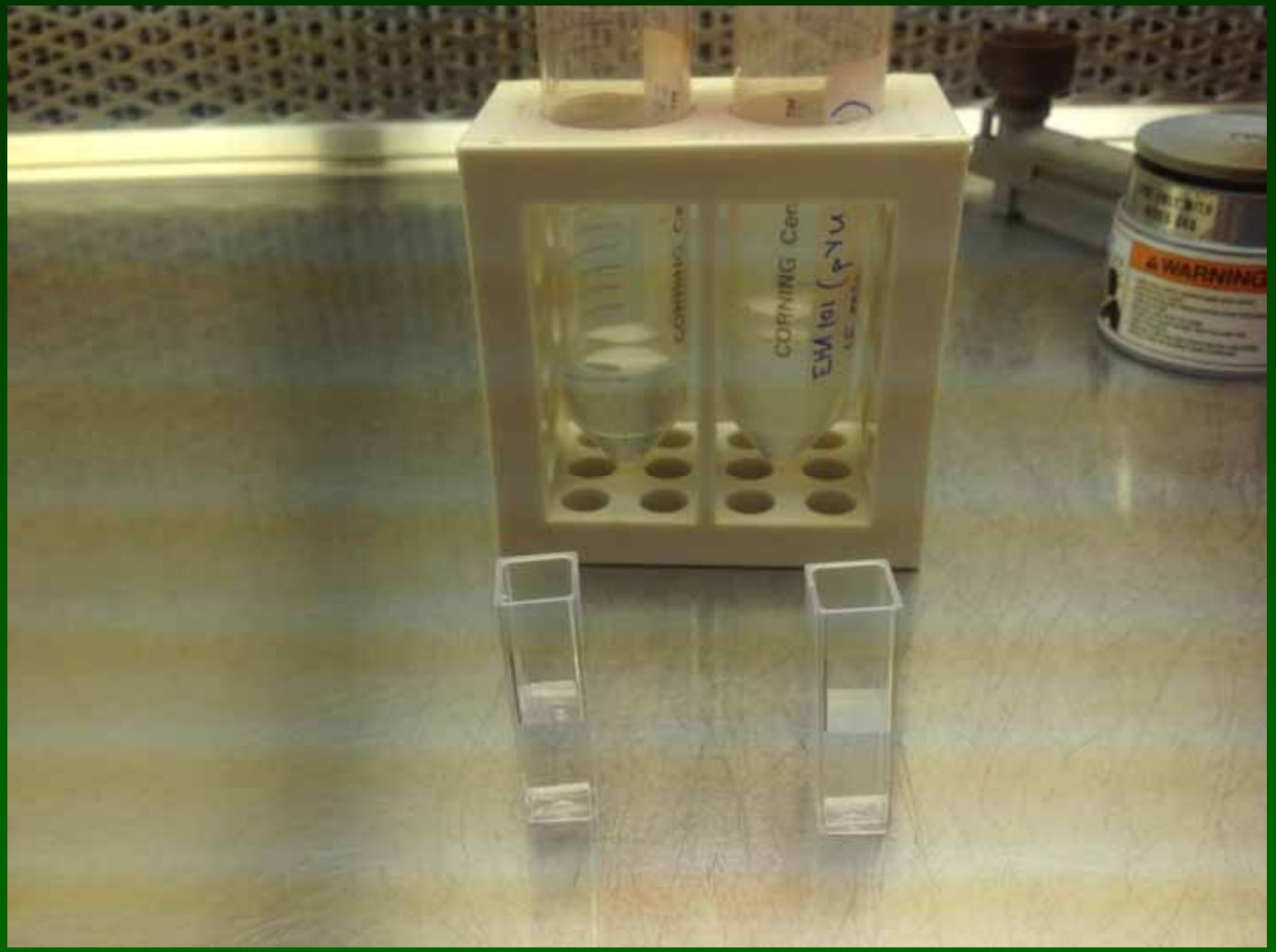
Shake the *Agrobacterium* suspension at 25-27°C at 150-175 rpm for 1 hour to re-suspend fully.

Re-suspension of *Agrobacterium* in Infection Medium

Shake the *Agrobacterium* suspension at 25°C at 150-175 rpm for 1 hour to re-suspend fully.

Determination of Agro Concentration at OD_{600}

Determination of Agro Concentration at OD_{600}



Use Infection medium + 100 µM acetosyngone to blank the spectrophotometer

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

After re-suspension for 1 hr, transfer an appropriate portion (2 ml) of the *Agrobacterium* suspension to a spectrophotometer curette and measure the OD_{600}. The final suspension should be adjusted to = 0.30-0.50.

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

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

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

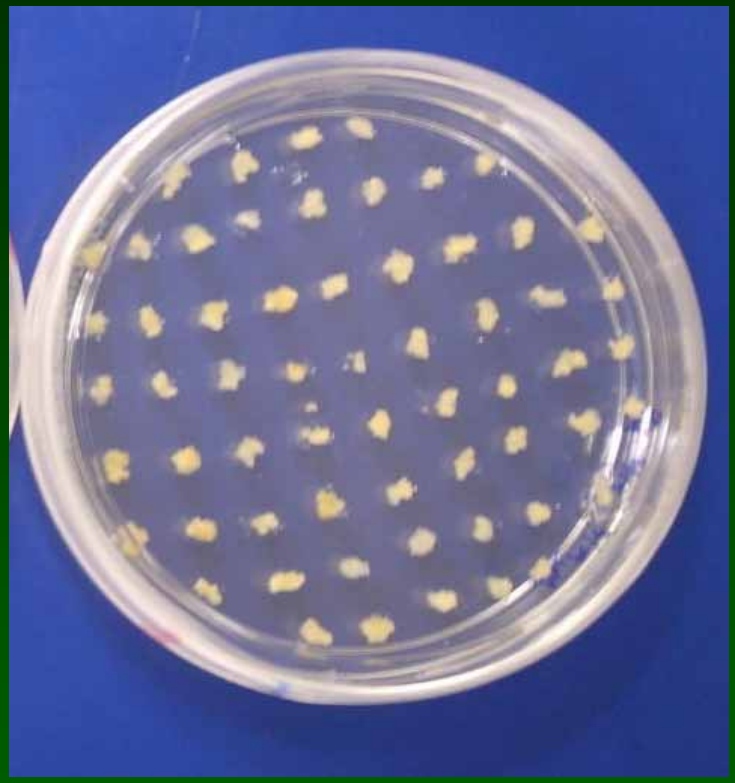
Callus Infection

A repeater micropipet works well for this step

Allow pieces to 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 one week.

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	

Selection I

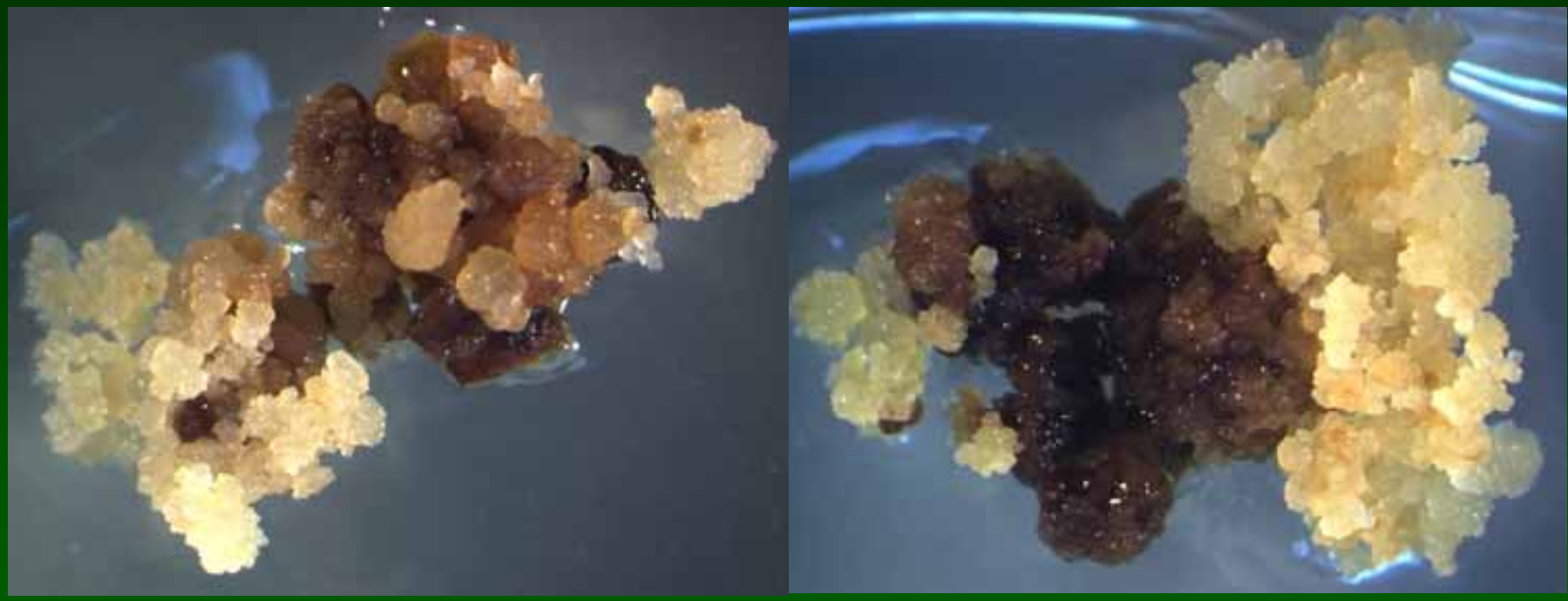
Following the resting phase, each piece of callus is transferred to Selection I medium and incubated at 25°C in dark for 4-6 weeks sub-culturing bi-weekly. *Each piece of callus is kept together and not broken apart.*

Note: These transformants are selected using 50 mg/L Hygromycin selecting for constructs containing the hpt gene

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	

Selection I



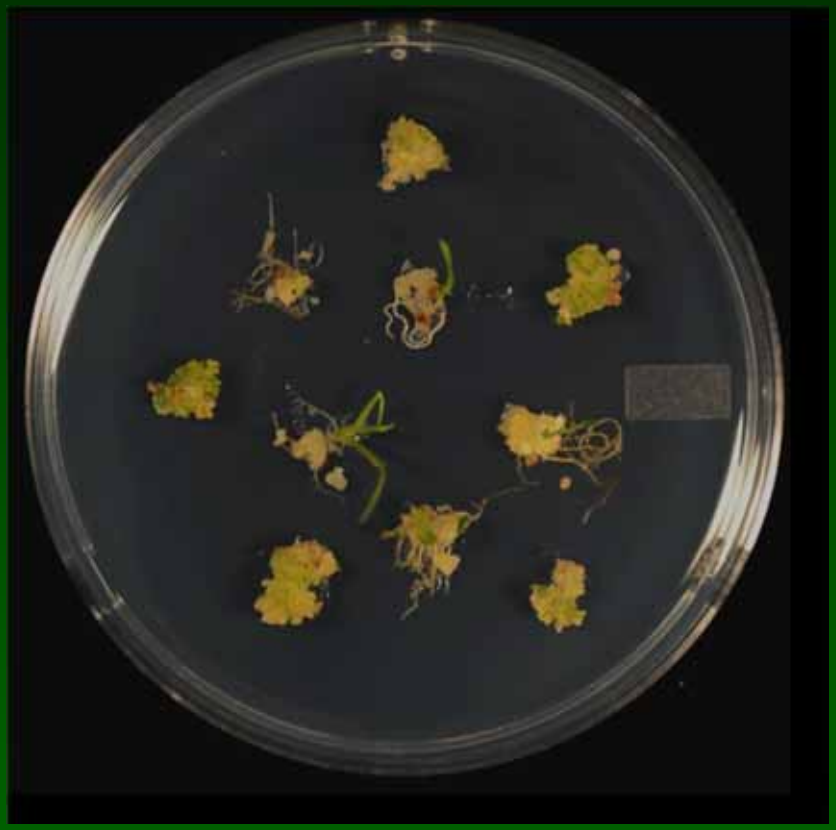
Putative transformants (events) can be selected after 8 weeks on selection I medium. *The transformants should be a pale yellow color, friable, and growing.* They should look exactly the same as non-transformed callus. If the colonies do not look the same as non-transformed callus, they are probably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

Note: These transformants are selected using 10 mg/L PPT or 3 mg/L Bialaphos selecting for constructs containing the bar gene

Transformants are easily distinguished from non transformed cells

rice

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

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	

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	

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.

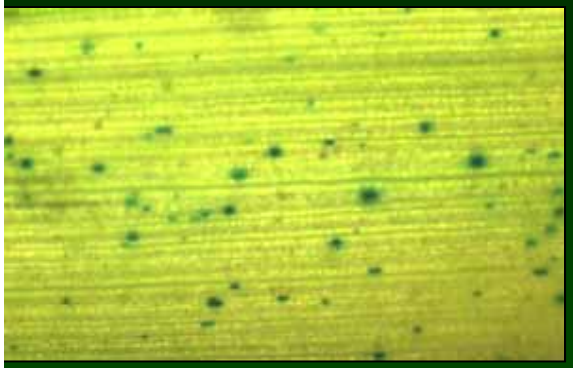
T0 Transgenic Rice in the Greenhouse

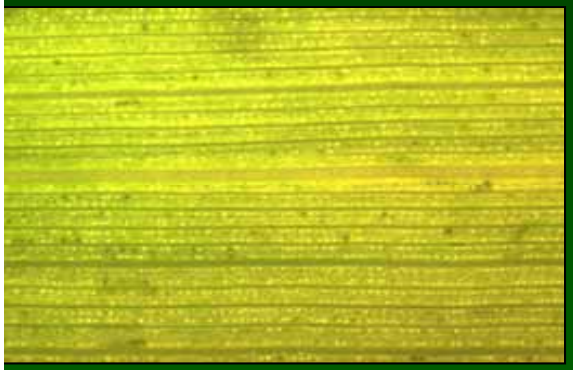
Transient and Stable expression Analysis in Transgenic Rice

Microprojectile Bombardment

Thank You

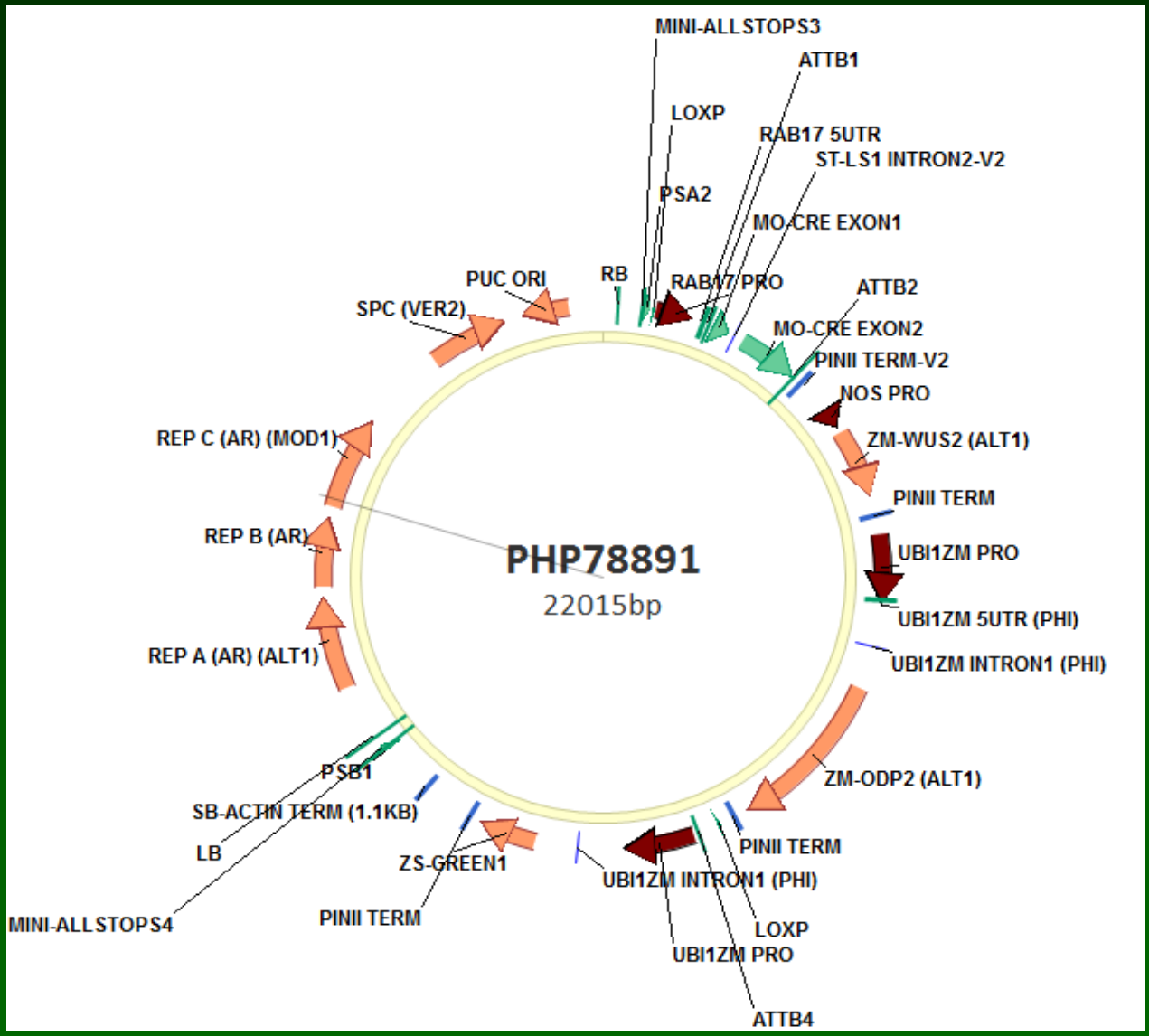
T0 Transgenic Rice in the Greenhouse





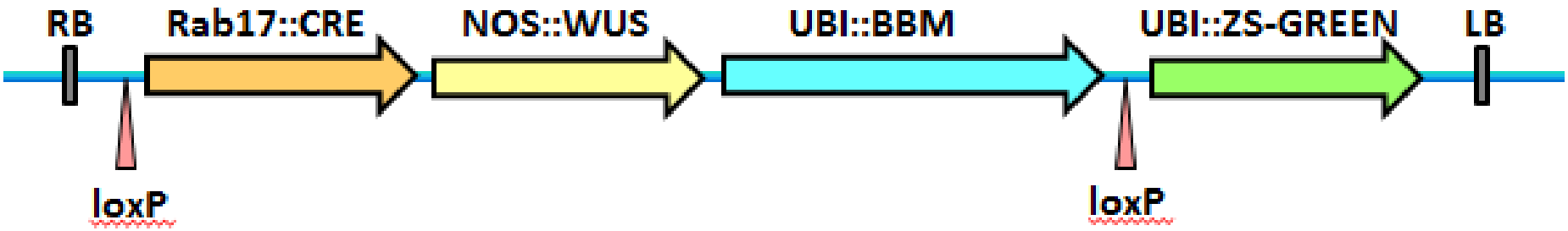
Sterilize seeds with 50% bleach in a shaking incubator at 200 rpm for 30 minutes at room temperature. Seeds are then rinsed 3 times with sterile ddH_2O or until there is no odor of bleach. Seeds are carefully transferred to an empty sterile Petri dish.

Recalcitrant varieties



A Revolutionary New Approach -Pioneered by Pioneer

Recalcitrant varieties



A Revolutionary New Approach -Pioneered by Pioneer

The use of Transcription factors Baby Boom (BBM) and Wuschel (WUS) overexpressed causes somatic embryogenesis in many cell types. A drought inducible promoter (RAB17 driving CRE a (a site specific recombinase) is activated by placing calli on dry filter paper. Activation of CRE causes excision at recombination site (loxP) removing BBM and WUS allowing for regeneration of transformant. This approach appears to be genotype independent. A newer version reported at the SIVB meeting June 2016 is even more robust.