National Science Foundation Plant Genome

Cereal Plant Transformation Workshop

Albert Kausch

University of Rhode Island

National Science Foundation Plant Genome

Cereal Plant Transformation Workshop

Albert Kausch

University of Rhode Island

Sorghum Transformation

NSF Plant Transformation Workshop

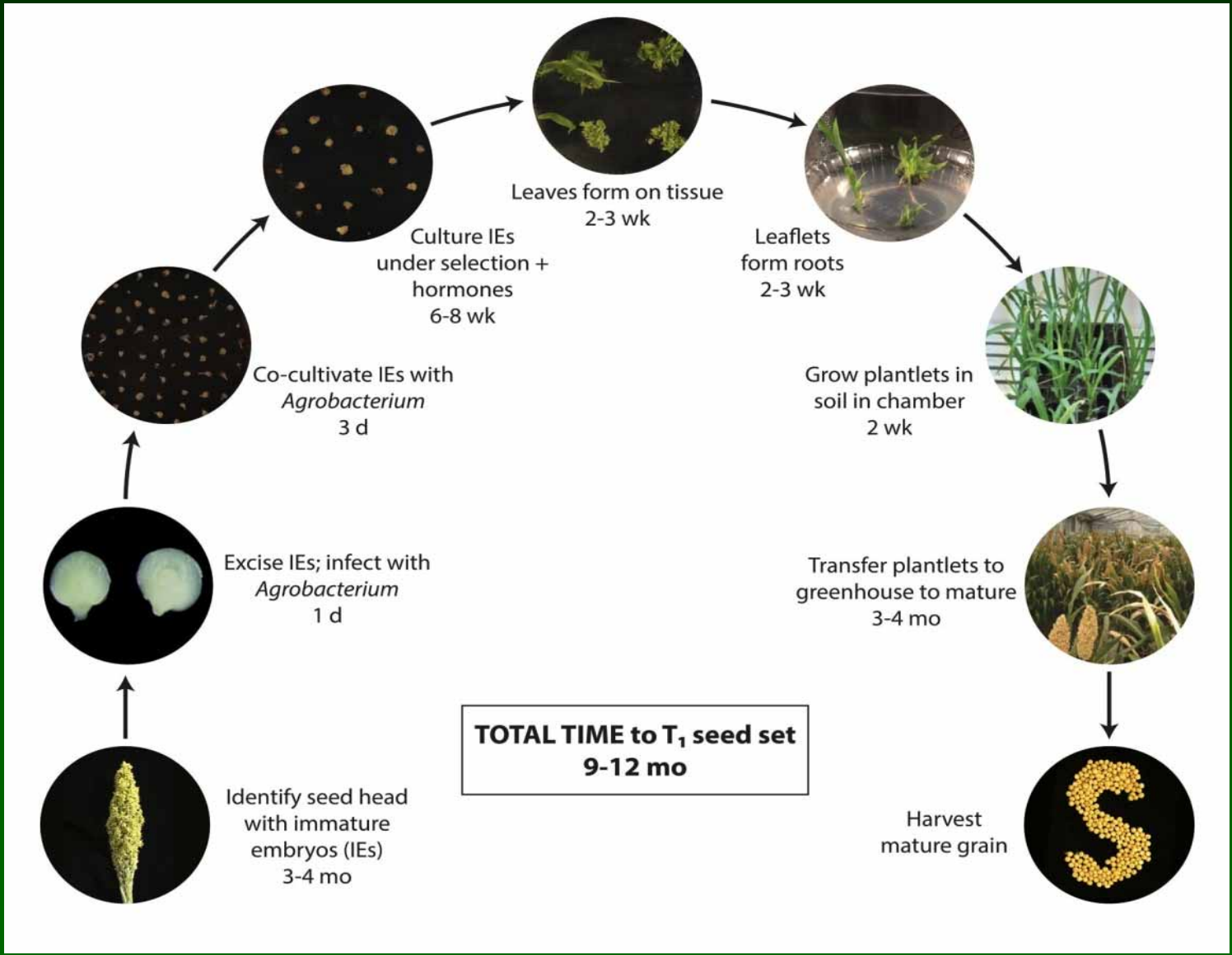
Albert Kausch

University of Rhode Island

Agrobacterium-mediated SorghumTransformation From Immature Embryos

Kimberly Nelson-Vasilchik, Joel Hague, and Albert Kausch
University of Rhode Island

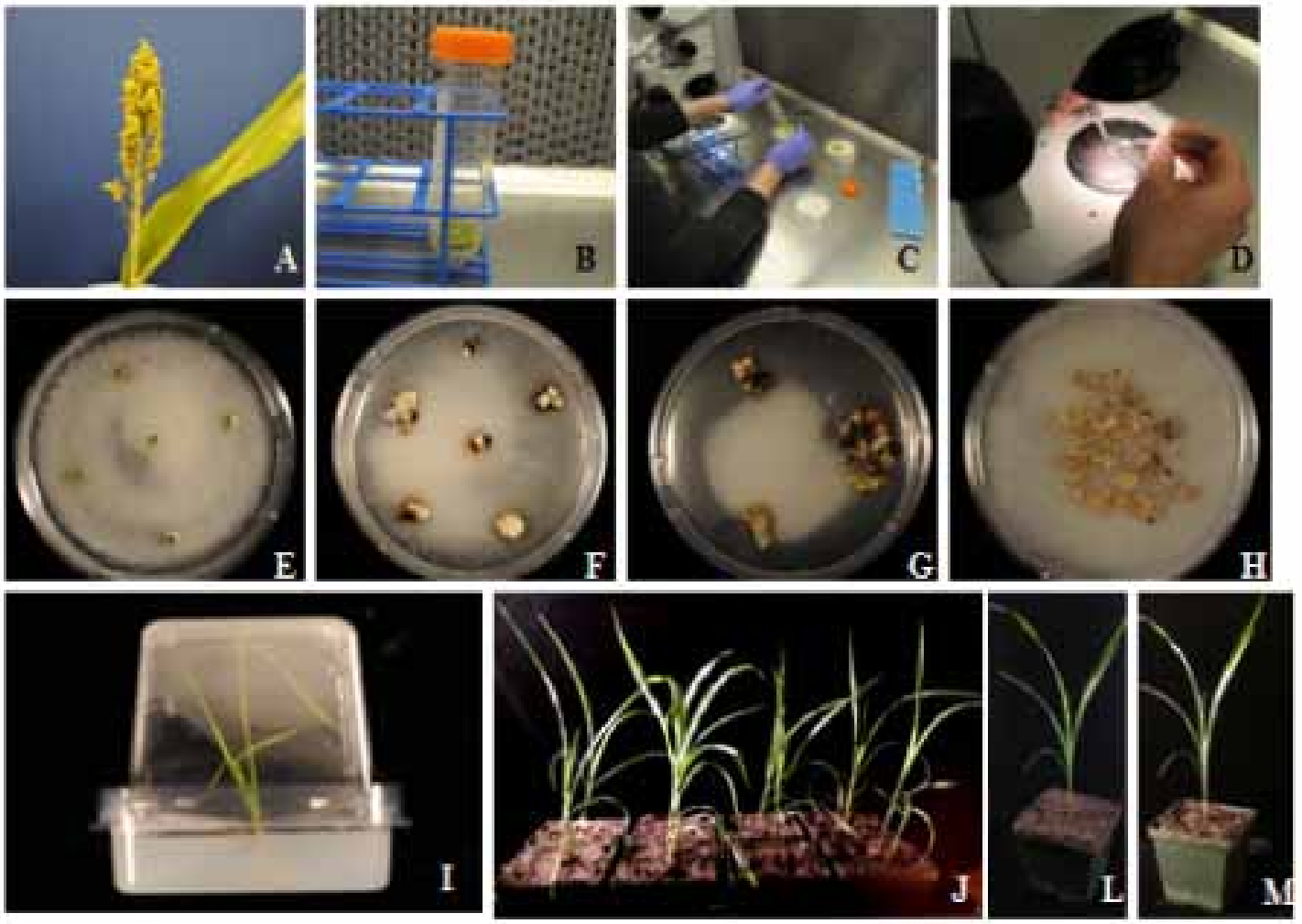
Sorghum Transformation



Altpeter et al. 2016 The Plant Cell, in press

sorghum

Sorghum Transformation



sorghum

Sorghum Transformation:
Autoclave all materials (beakers, tools, dd water, etc.) ahead of time

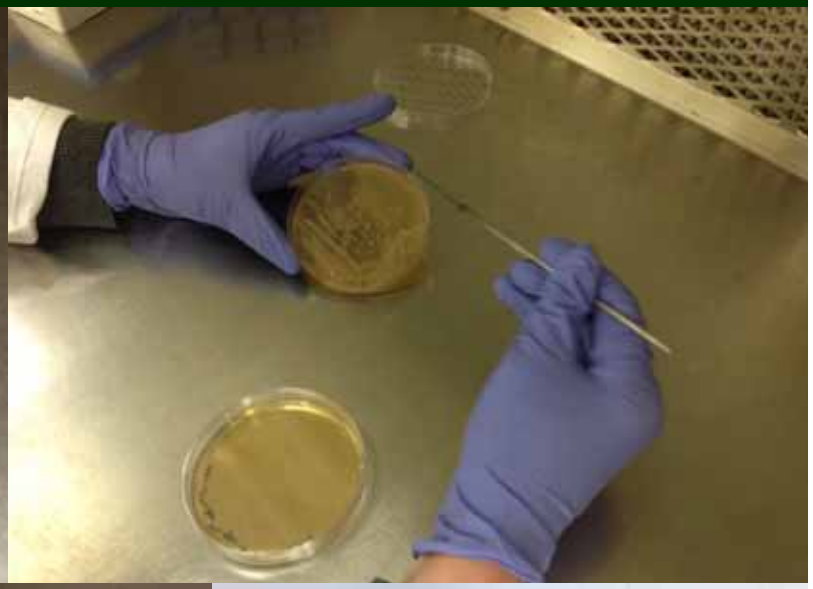
Preparation of *Agrobacterium*

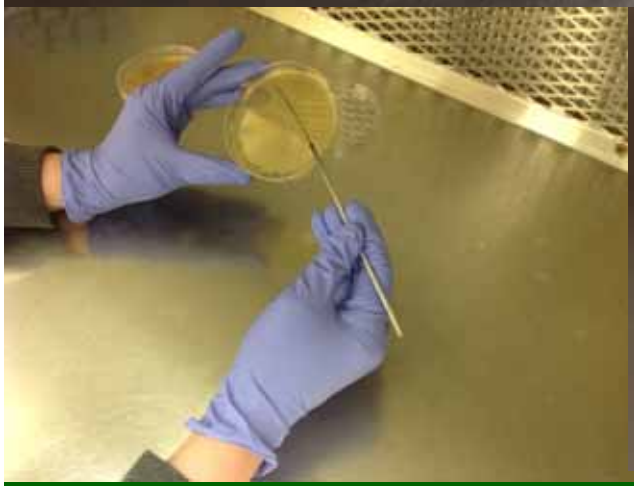
Initiation of *Agrobacterium tumefaciens* from stock culture:

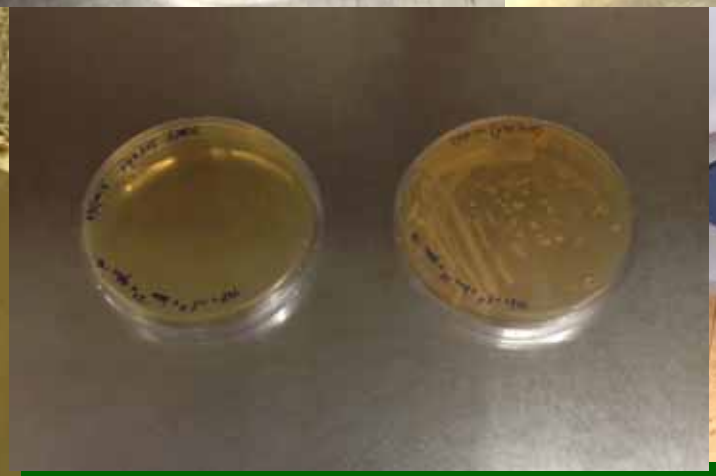
Streak the *Agrobacterium* culture for colony isolation from an -80°C glycerol stock onto a YEP medium plate containing appropriate antibiotics. Incubate the plate at 28°C for 3 days. This plate is the "master" plate, and should be stored at 4°C and can be reused for 30 days. A new master plate needs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

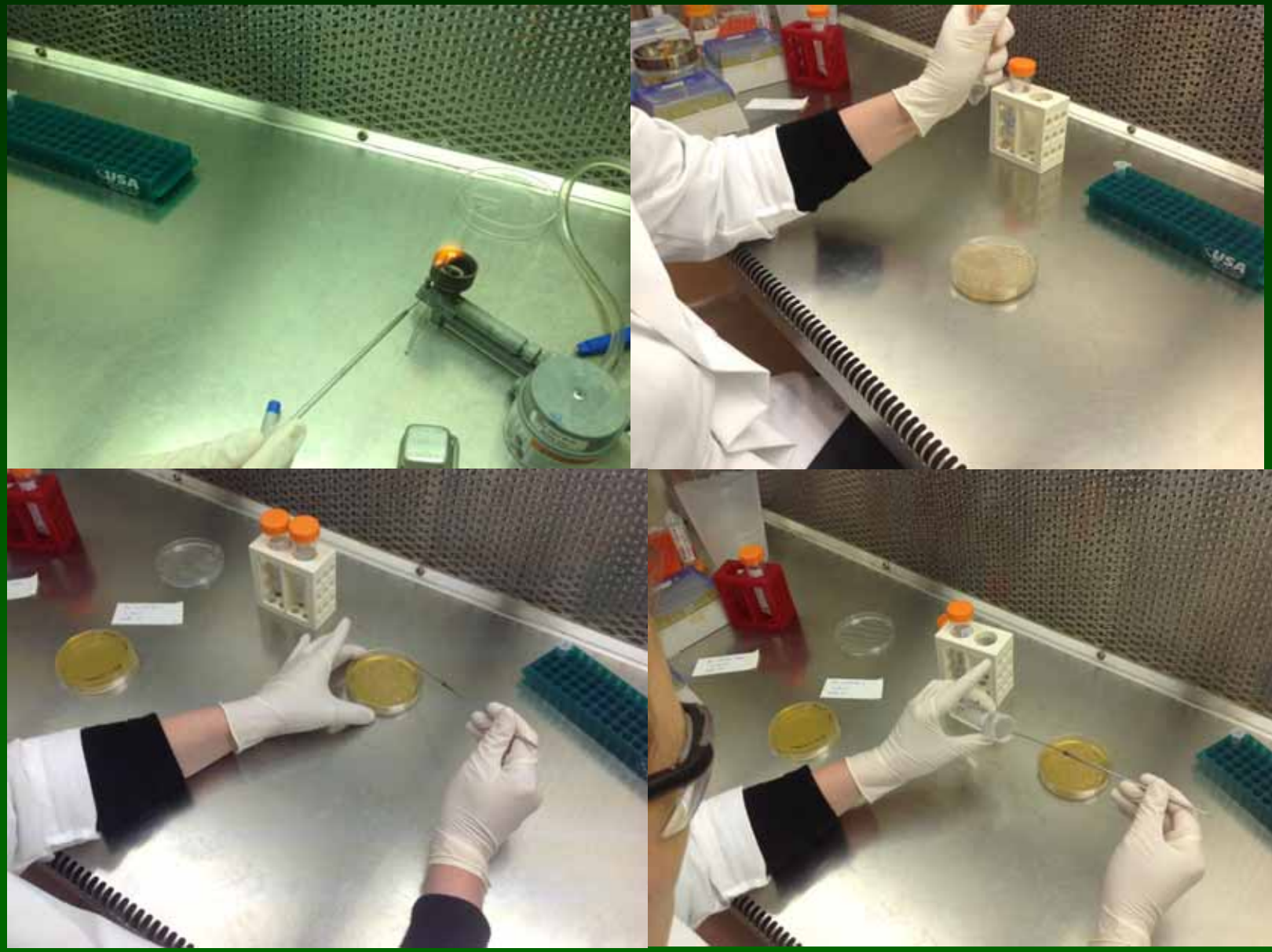






Initiation of *Agrobacterium tumefaciens* from stock culture: To begin a sorghum transformation experiment, streak a single colony onto a fresh YEP plate containing the appropriate antibiotics. Incubate this plate for 3 days at 20°C , 2 days at 25°C, or 28°C Overnight (caution Agro plasmids may be cured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

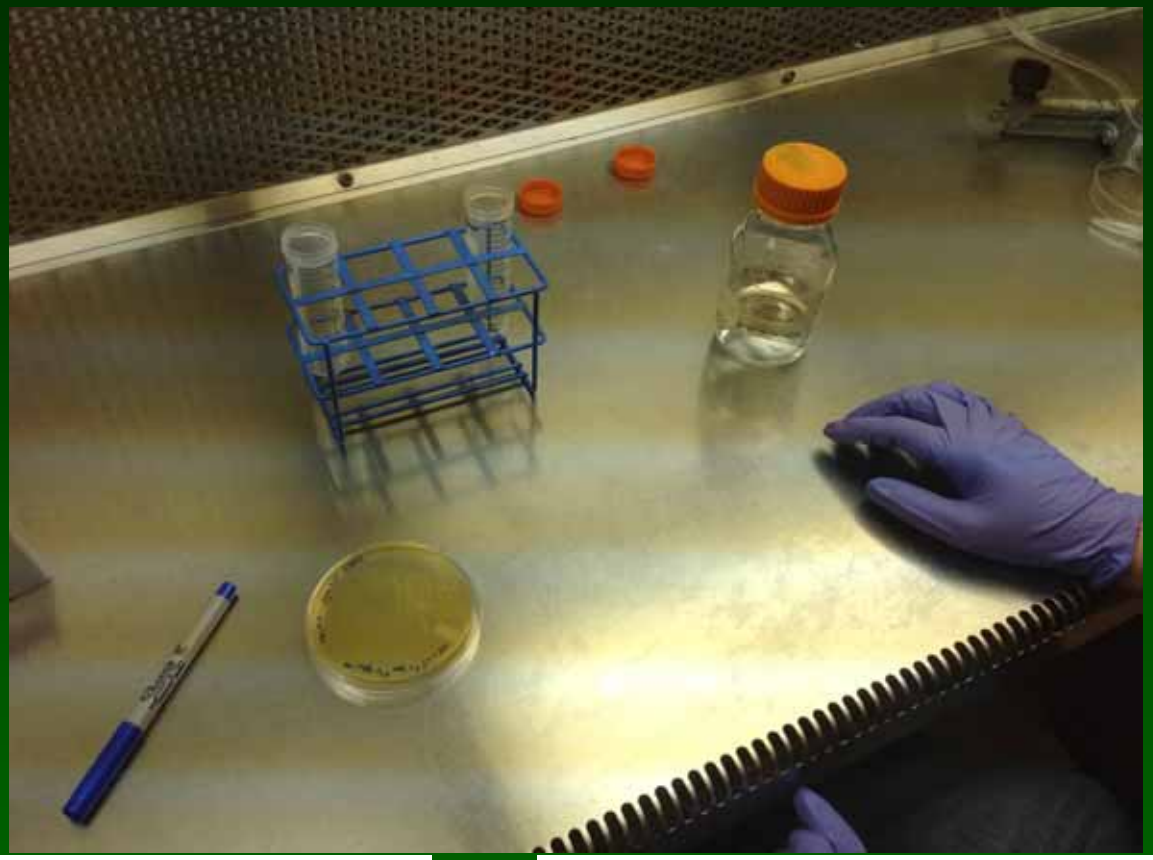
Preparation of *Agrobacterium* Infection Media



Sorghum Medium Recipes (1L)

Component	Infection	Co-cultivation	Resting	Selection I	Selection II	Regeneration I	Regeneration II
MS salts	2.15g	2.15g	4.3g	4.3g	4.3g	4.3g	2.15g
MES		0.5g	0.5g	0.5g	0.5g	0.5g	
L-proline		0.7g				0.7g	
Sucrose	68.5g	20g	30g	30g	30g	60g	30g
Glucose	36g	10g					
2,4-D 1mg/ml	1.5ml	2ml	2ml	1.5ml	1.5ml		
Agar		8g				8g	
Phytagel			2.5g	2.5g	2.5g		2.5g
pH	5.2	5.8	5.8	5.8	5.8	5.6	5.6
Autoclave	filtered	20min	20min	20min	20min	20min	20min
B5 vitamin stock, 100X	10ml	10ml	10ml	10ml	10ml		
Acetosyringone, 100mM	1ml	1ml					
Ascorbic acid		10mg	10mg				
Casamino acids	1g						
Asparagine			0.15g				
Coconut water			100ml				
Carbenicillin			0.2g	0.2g	0.2g	0.2g	0.2g
PPT, 20mg/ml				0.25ml	0.5ml	0.5ml	
Kinetin, 1mg/ml					0.5ml		
Zeatin, 1mg/ml						0.5ml	
IAA, 1mg/ml						1ml	
ABA, 0.025mg/ml						1ml	
TDZ, 0.5mg/ml						0.2ml	
IBA, 1mg/ml							0.25ml
NAA, 1mg/ml							0.25ml
MS vitamin, 1000X						1ml	1ml
PVPP (1% final)		10g	10g	10g	10g	10g	5g

Preparation of *Agrobacterium* Infection Medium

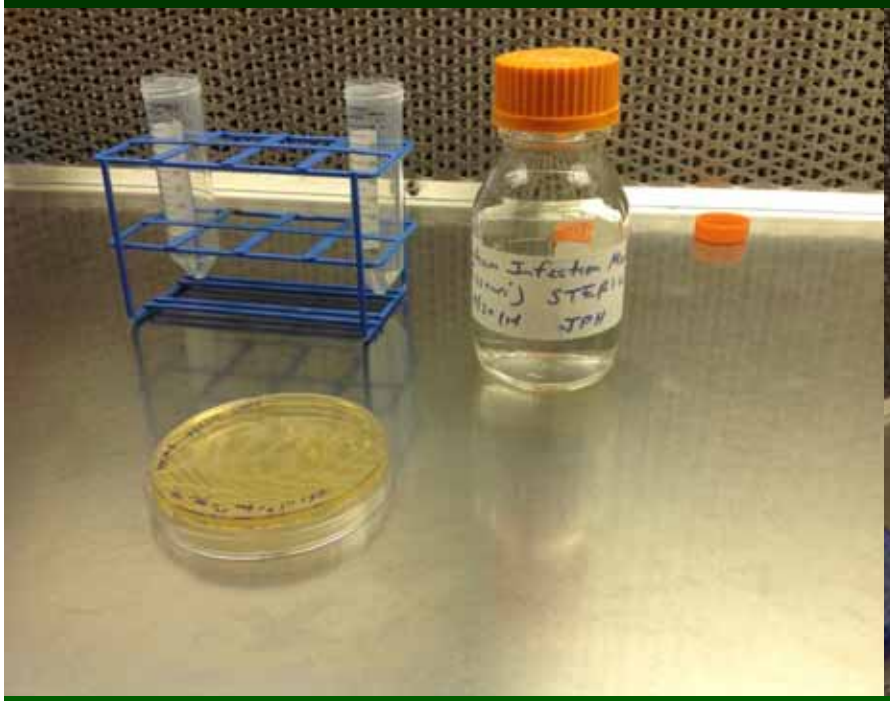


Infection medium + acetosyringone with Agro

Infection medium + acetosyrigone without Agro

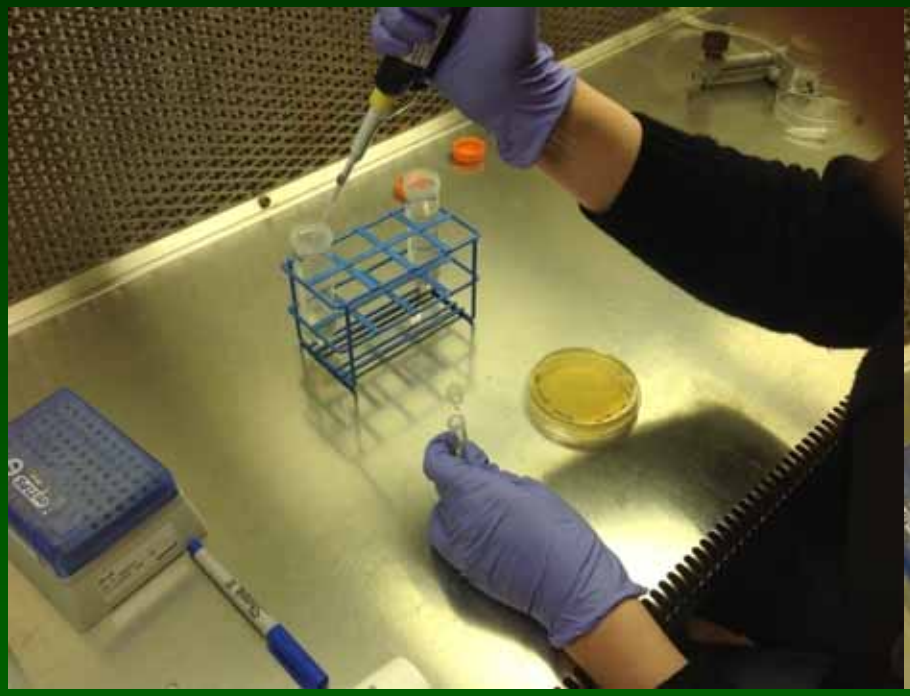
Prepare two sterile 50 mL Falcon tubes (depending on the size of the experiment) . One tube will be used to make the co-cultivation infection with the *Agrobacterium*, the other, will be used to adjust the concentration of bacteria after the OD is read and make tubes used in the embryo isolation prior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

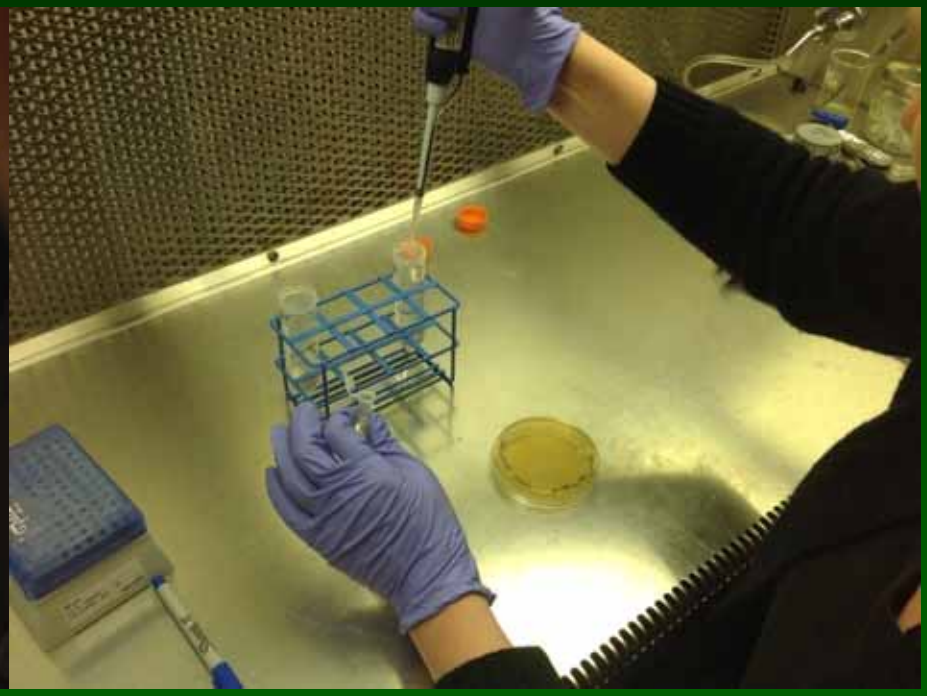
Preparation of *Agrobacterium* Infection Medium



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.

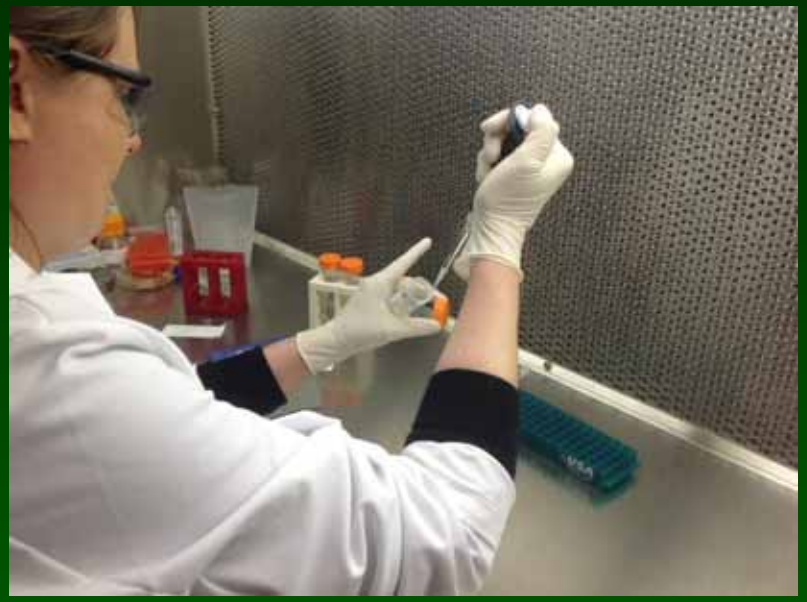
Preparation of *Agrobacterium* Infection Medium





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 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 and make tubes used in the embryo isolation prior to infection.

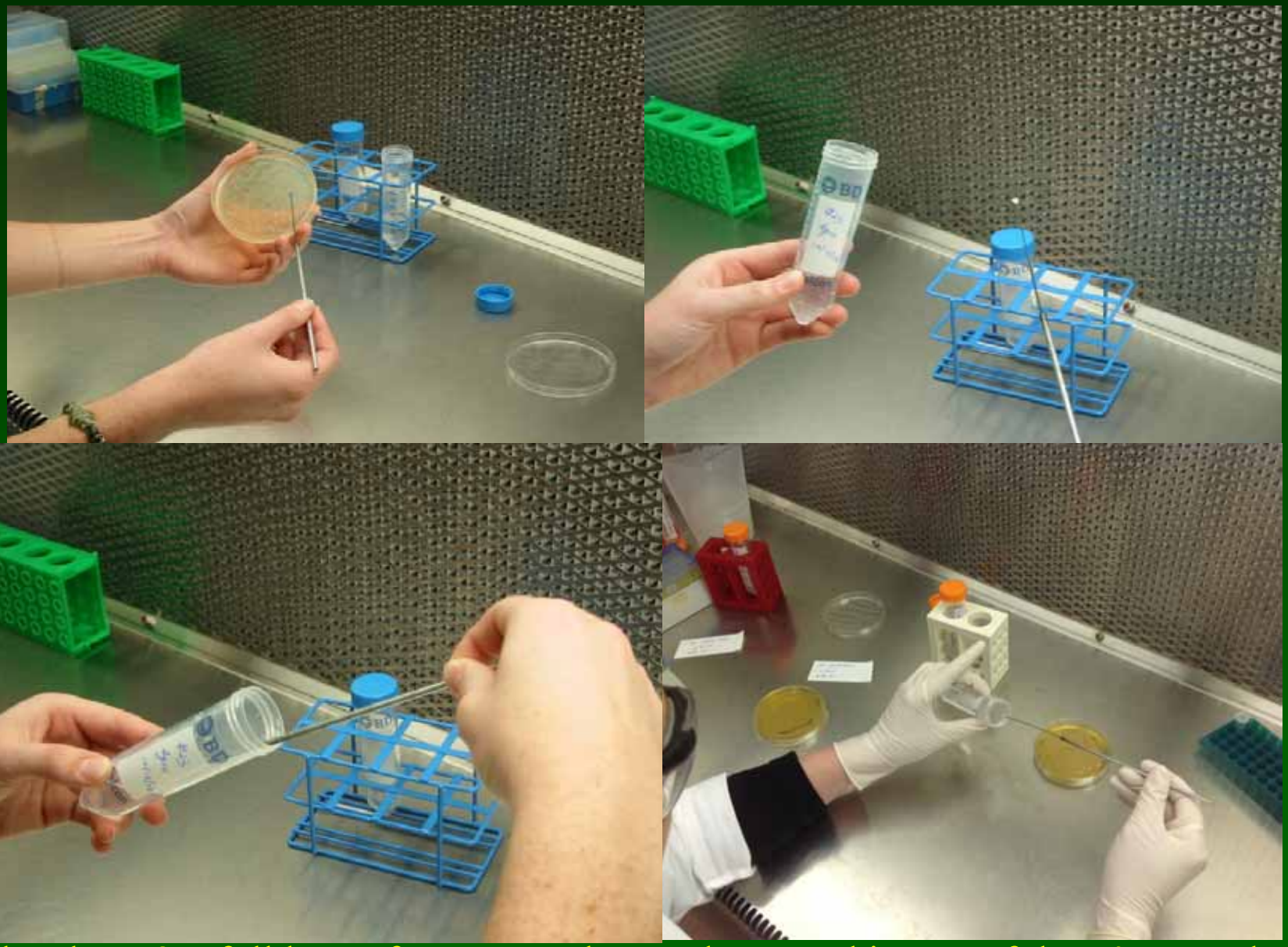
Add acetosyringone (100 mM/mL stock solution) to the each of the tubes containing infection media for a final concentration of 100 μM/mL acetosyringone.

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 co-cultivation infection medium.

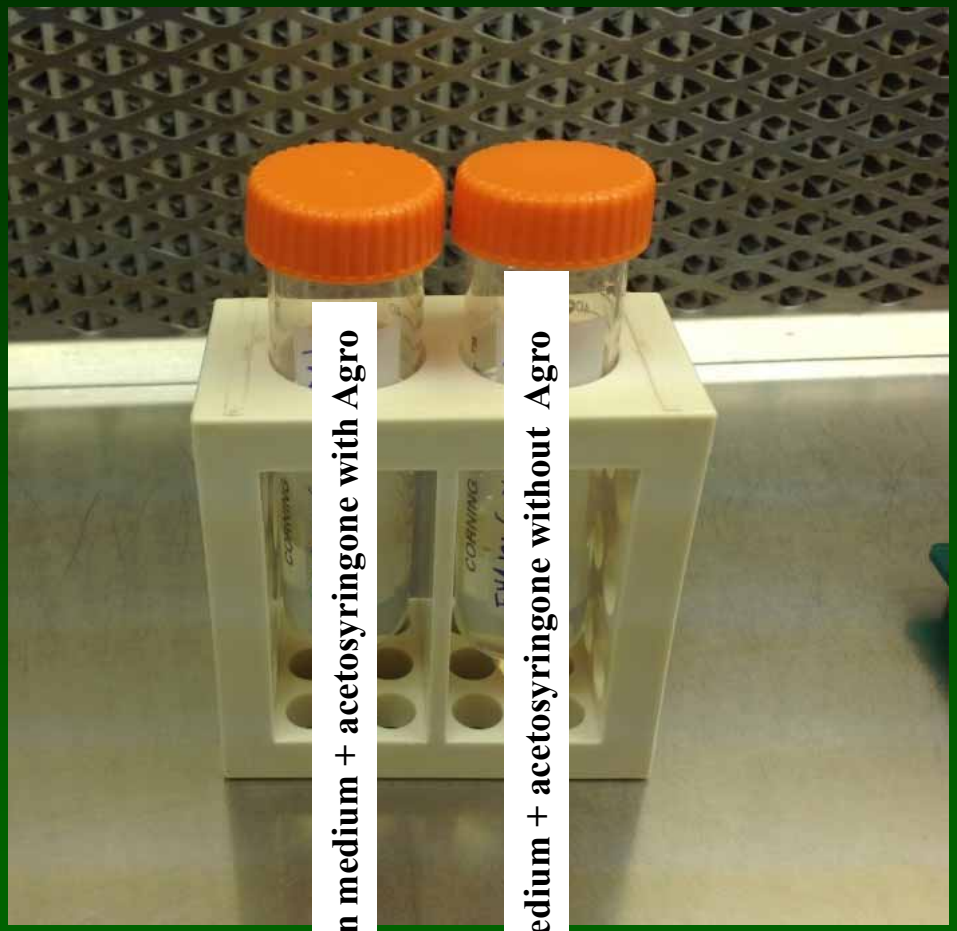
Using the other 50 ml Falcon tube containing the infection media + acteosyringone, using a sterile tip transfer ~ 1.8 mL of the infection medium + acetosyringone (without *Agrobacterium*) to 10 sterile 2 mL microcentrifuge tubes. These will be used to transfer the isolated embryos prior to co-cultivation. This can be done while the *Agrobacterium* suspension is shaking .

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



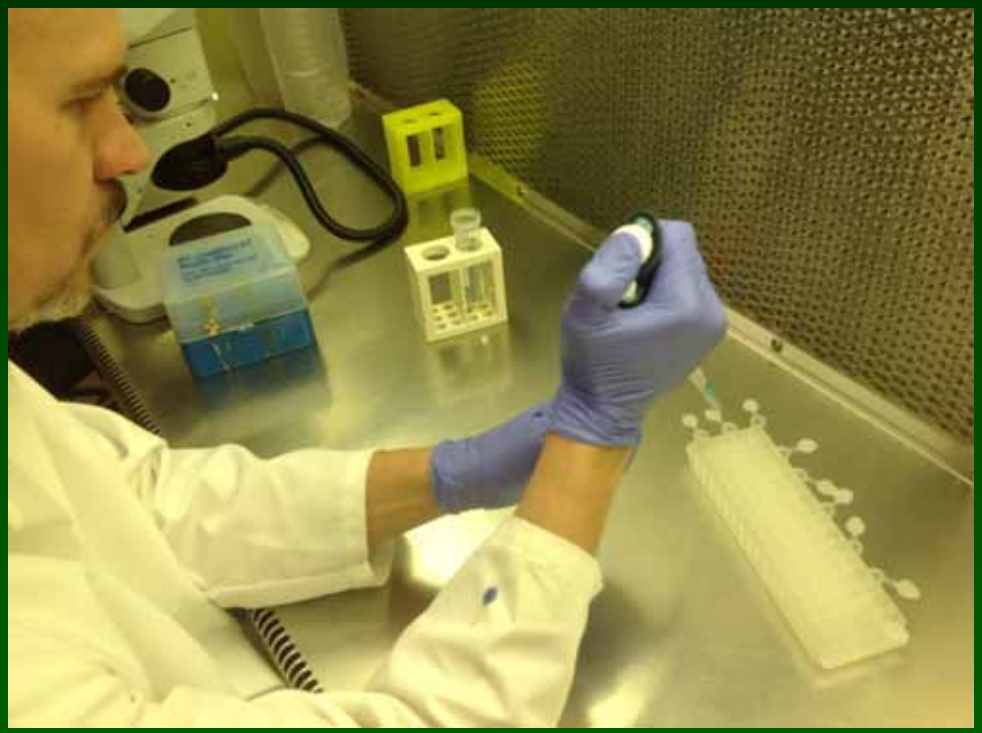
Preparation of Infection Medium Containing the *Agrobacterium*

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.

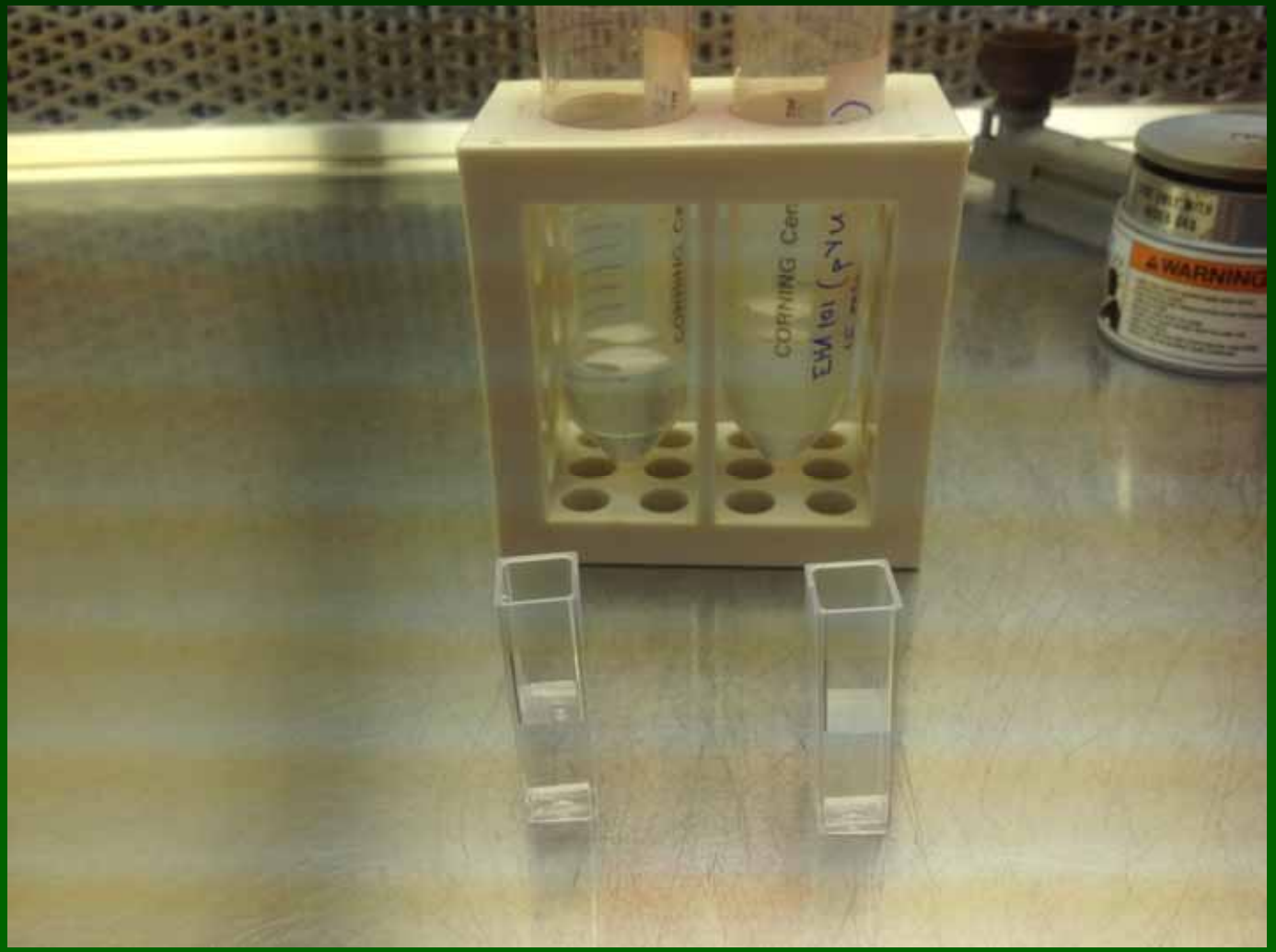
Preparation of microfuge tubes containing infection medium without *Agrobacterium* for embryo isolation



While the *Agrobacterium* suspension is shaking using the other 50 ml Falcon tube containing the infection media + 100 µM acteosyringone, and using a sterile tip transfer ~ 1.8 mL of the infection medium + 100 µM acetosyringone (without the *Agrobacterium*) to 10 sterile 2 mL microcentrifuge tubes. These will be used to transfer the isolated embryos prior to the infection step

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 = 0.30-0.45.

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

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

Donor Plant Material

Plant Material Genotype Specificity

Genotypes: Sorghum bicolor cv. P898012
others are possible at low frequencies

Donor Plant Material

Immature embryos from Sorghum bicolor cv. P898012 are used for transformation. Plants are grown under standard greenhouse condition until flowering. Sorghum plants are self-pollinating and are bagged with a pollination bag as soon as the flower inflorescence is fully emerged.

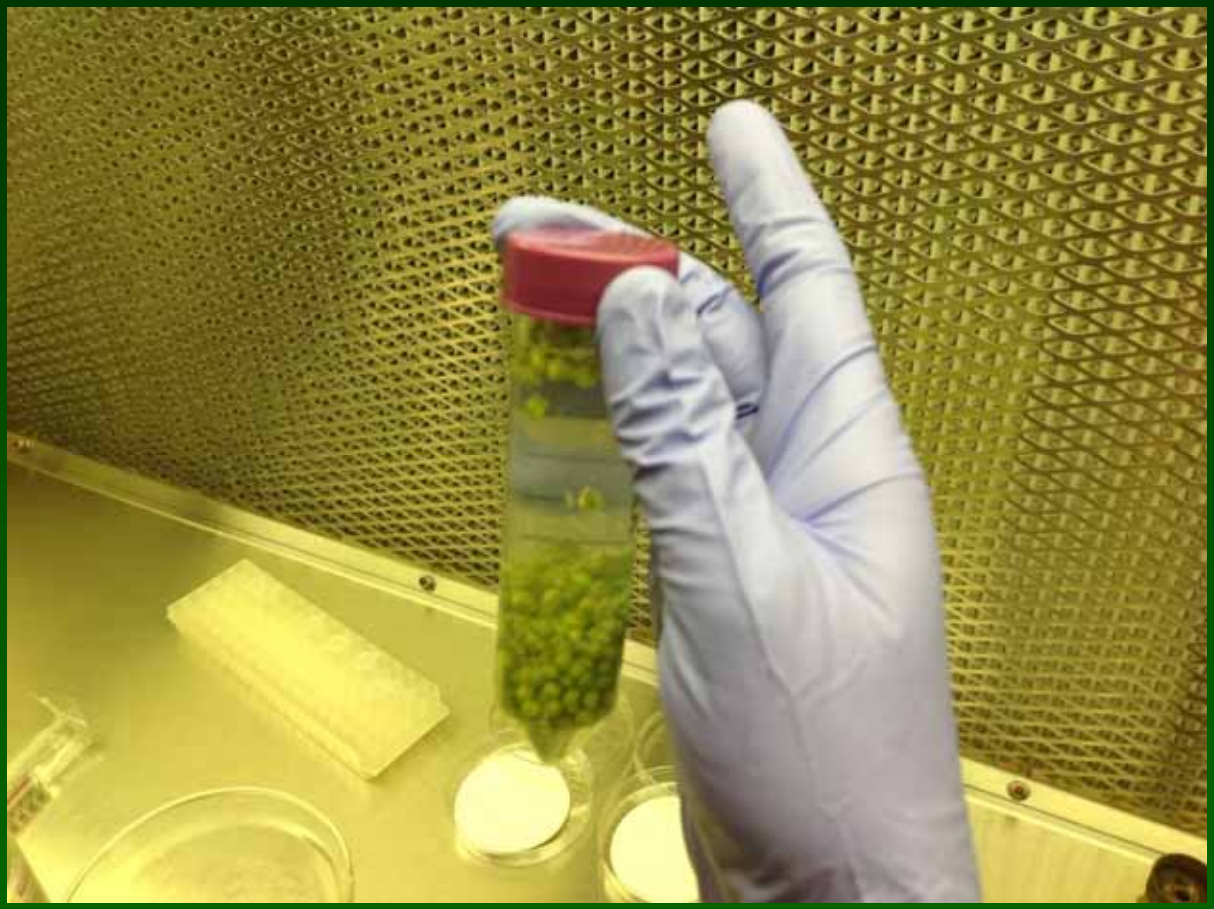
Donor Plant Material

Sample several caryopses from the inflorescence prior to harvest to determine the size of the immature embryos about 13 days past anthesis. The embryos should be 1.4-1.8mm. Embryo size will vary depending on their location on the inflorescence.

Removing the Developing Seeds from the Inflorescence

Immature caryopses are removed by hand at about 13 days past anthesis. (1.4-1.8mm) and placed into a 50 ml Falcon tube.

Surface Sterilization of the Caryopses



Surface sterilize the sorghum caryopses with 50% bleach (some protocols add a few drops of Tween as a wetting agent) followed by shaking at 200 rpm at room temperature for 30 min.

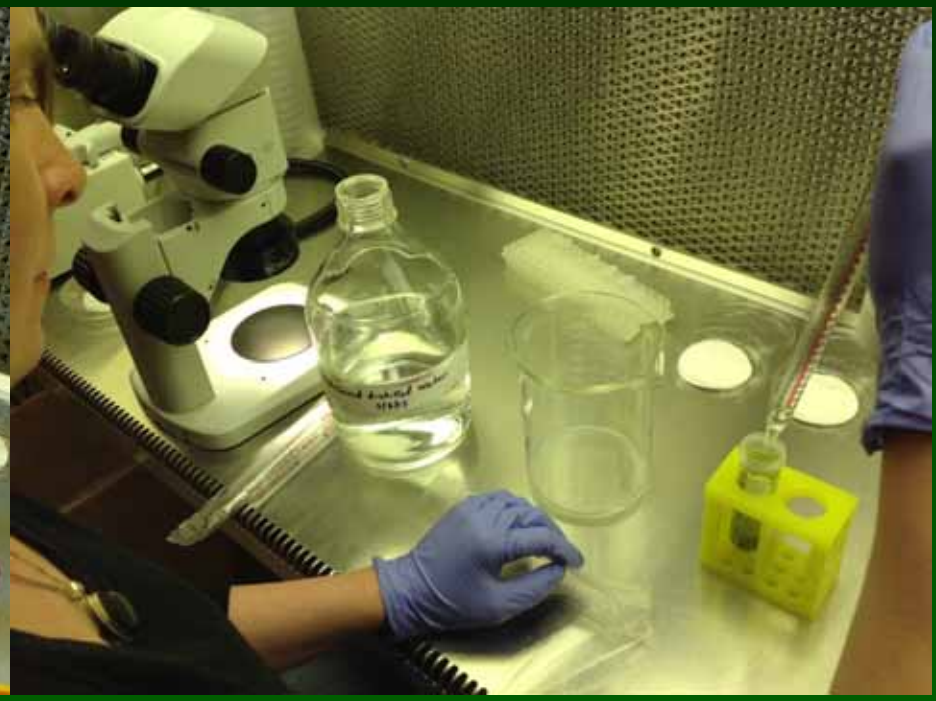
Surface Sterilization of the Caryopses

Agitate the isolated caryopses in 50% bleach for 200 rpm at room temperature for 30 min.

Surface Sterilization of the Caryopses

Set timer for 30 min.

Surface Sterilization of the Caryopses



Rinse the caryopses with sterile ddH_2O 3 times or until there is no odor of bleach..

Surface Sterilization of the Caryopses

Rinse the caryopses with sterile ddH_2O 3 times or until there is no odor of bleach.

After the final rinse remove all of the water.

Surface Sterilization of the Caryopses

Transfer the sterilized caryopses from Falcon tube (a sterile spatula or forceps may be used) and place into sterile, empty Petri dish containing sterile Whatman filter paper. Moisten filter paper with a few drops of sterile distilled water.

Surface Sterilization of the Caryopses

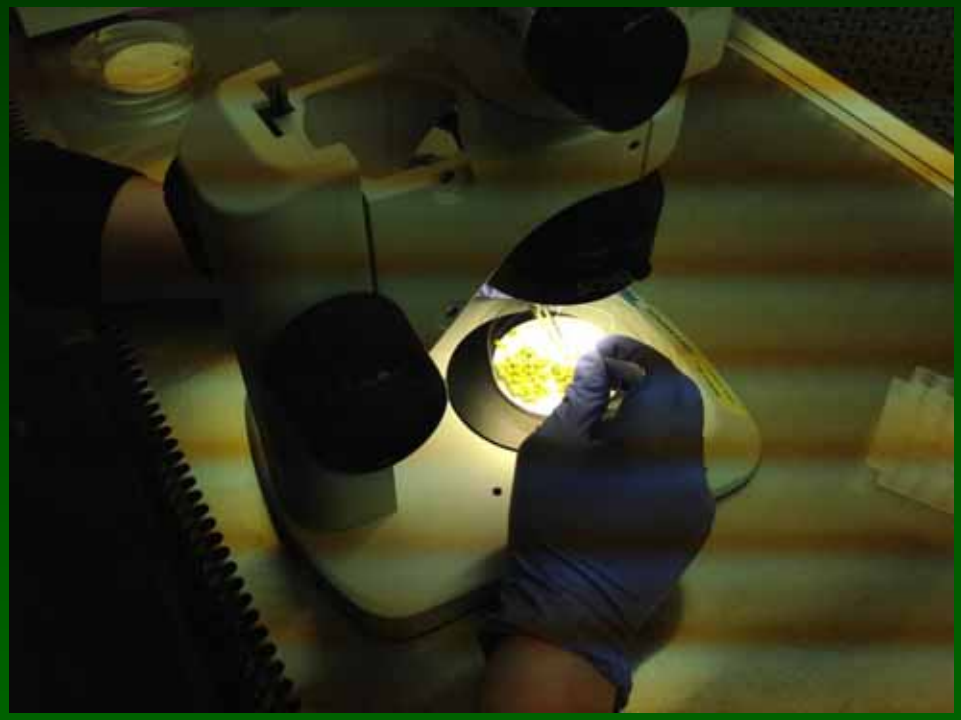
Depending on the size of the experiment, aliquot surface sterilized caryopses into several Petri dishes with the moistened Whatman filters . Aliquots will prevent desiccation of any one sample during embryo isolation.

Surface Sterilization of the Caryopses

Following transfer of the surface sterilized caryopses into several Petri dishes place covers onto the Petri dishes to prevent desiccation during embryo isolation.

Embryo isolation and infection

Note: This is best conducted by a 2 person team, with one person doing the embryo isolations and another person rinsing, infecting and plating the embryos



Working in the sterile Petri dish and under a dissecting microscope, use 2 fine jewelers forceps to slit the caryposis and carefully remove the immature embryo. The immature embryo is opposite the divot in the caryopsis. *If the embryos are too large, they will be sticky and hard to remove. The embryo size should be 1.4-1.6mm.*

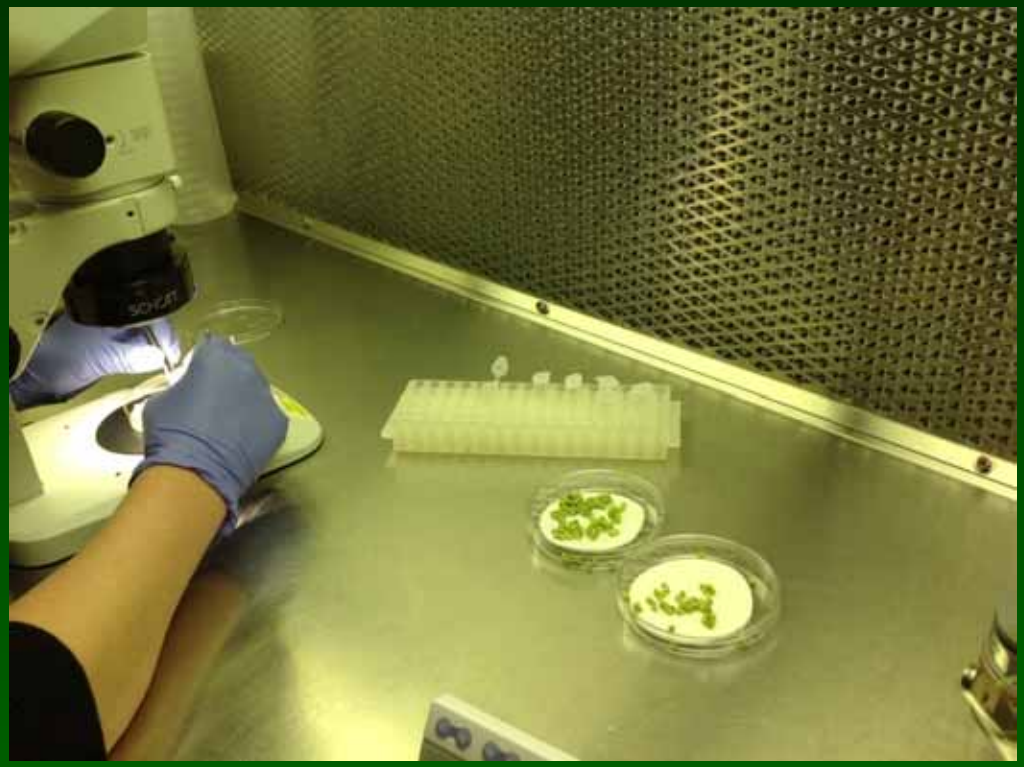
Embryo isolation and infection

This will require some practice and patience. Using the 2 fine jewelers forceps first slit the caryposis. The immature embryo is opposite the divot in the caryopsis. Carefully remove the immature embryo and place it into the microfuge tube containing the infection medium. Be careful to maintain aseptic technique, as one contaminated embryo will ruin the entire sample

It must be emphasized, that *the embryo size should be 1.4-1.6mm. If the embryos are too large, they will be sticky and hard to remove.*

Embryo isolation and infection

Embryo isolation and infection

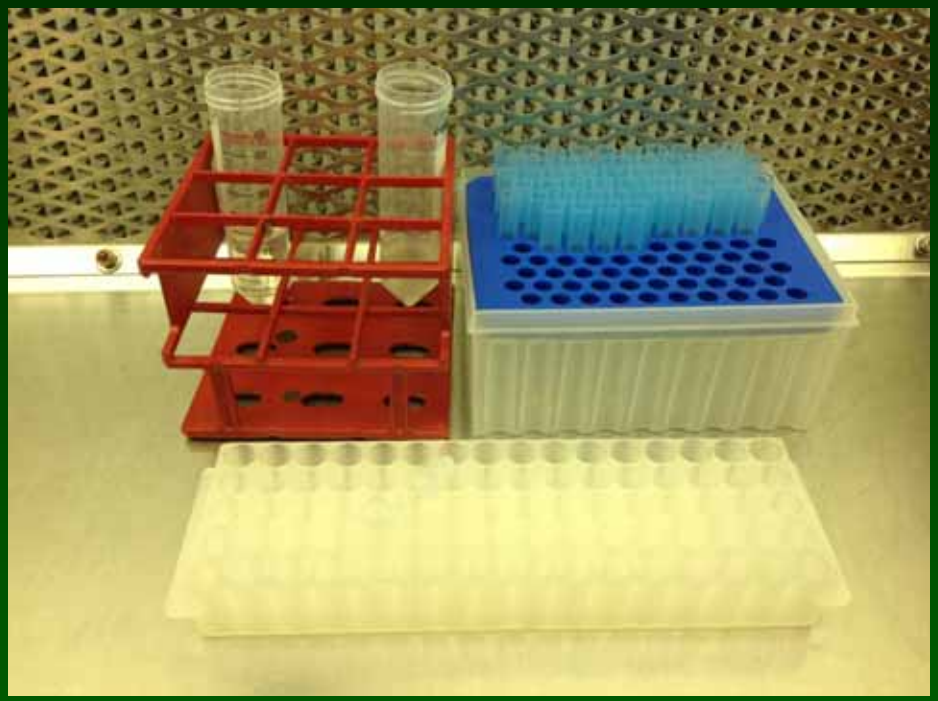


Place embryos into the sterile 2.0ml micro-centrifuge tube containing infection) medium+ acetrosyringone (100uM) that was previously prepared . Continue to collect embryos into the micro-centrifuge tube for no longer than 20 minutes to prevent hypoxia of the embryos. *Note: It is important to not let the embryos stay in the liquid media too long. Set a timer to help.*

Embryo isolation and infection

Working to avoid hypoxia of the embyos in the infection medium, set a time for twenty minutes and only isolate as many embryos as possible during this time. An experienced researcher can isolate 20-40 embryos during this time.

Embryo isolation and infection



As mentioned previously this procedure is best accomplished with two people, to minimize the time the embryos are submerged in the infection medium. After 20 minutes, transfer the capped tube containing the isolated embryos in the infection media to the partner. The person working on the embryo isolation can start on the next tube.

The partner should then remove the infection medium from the micro-centrifuge tube and replace with the infection medium containing the *Agrobacterium* suspension. Let stand for 5 minutes for inoculation.

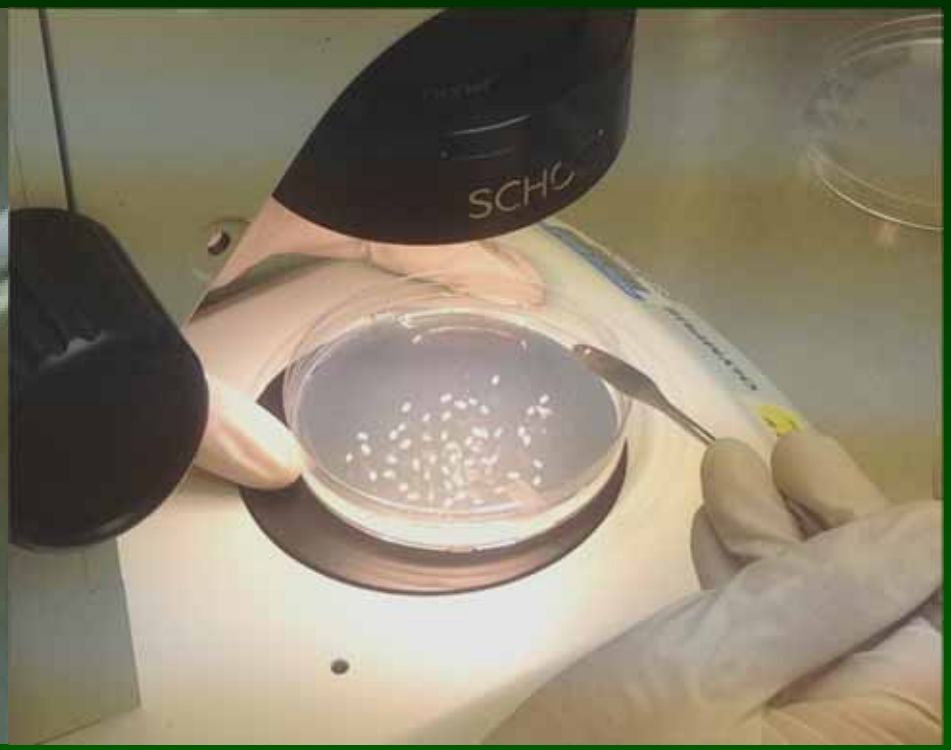
Embryo isolation and infection

The partner should then transfer embryos to a plate containing Co-Cultivation medium.

Sorghum Medium Recipes (1L)

Component	Infection	Co-cultivation	Resting	Selection I	Selection II	Regeneration I	Regeneration II
MS salts	2.15g	2.15g	4.3g	4.3g	4.3g	4.3g	2.15g
MES		0.5g	0.5g	0.5g	0.5g	0.5g	
L-proline		0.7g				0.7g	
Sucrose	68.5g	20g	30g	30g	30g	60g	30g
Glucose	36g	10g					
2,4-D 1mg/ml	1.5ml	2ml	2ml	1.5ml	1.5ml		
Agar		8g				8g	
Phytagel			2.5g	2.5g	2.5g		2.5g
pH	5.2	5.8	5.8	5.8	5.8	5.6	5.6
Autoclave	filtered	20min	20min	20min	20min	20min	20min
B5 vitamin stock, 100X	10ml	10ml	10ml	10ml	10ml		
Acetosyringone, 100mM	1ml	1ml					
Ascorbic acid		10mg	10mg				
Casamino acids	1g						
Asparagine			0.15g				
Coconut water			100ml				
Carbenicillin			0.2g	0.2g	0.2g	0.2g	0.2g
PPT, 20mg/ml				0.25ml	0.5ml	0.5ml	
Kinetin, 1mg/ml					0.5ml		
Zeatin, 1mg/ml						0.5ml	
IAA, 1mg/ml						1ml	
ABA, 0.025mg/ml						1ml	
TDZ, 0.5mg/ml						0.2ml	
IBA, 1mg/ml							0.25ml
NAA, 1mg/ml							0.25ml
MS vitamin, 1000X						1ml	1ml
PVPP (1% final)		10g	10g	10g	10g	10g	5g

Embryo isolation and infection



Remove the excess suspension and orient the embryos scutellum side up. Allow the plates to sit ajar with no lid for up to 30 min in the laminar flow hood. To dry any excess remaining infection medium.(there should not be any liquid surrounding the embryo prior to wrapping the plates, but do not over dry). Wrap the plate with parafilm or Micropore tape and place them in the incubator at 25°C in dark for 3 days.

Embryo Co-Cultivation

Co-Cultivation is in a tissue culture incubator at 25°C in dark for 3 days.

This may vary depending on the concentration of *Agrobacterium* in the inoculation and the *Agrobacterium* strain.

Resting

After the 3 day co-cultivation period, transfer embryos to Resting medium and incubate for 7 days at 28°C in dark. The embryos should be slightly yellow, swollen and firm. If the embryos are flaccid and white, do not transfer. Count the number of embryos. This will be the initial number of embryos infected when calculating transformation efficiency.

Resting

Sorghum Medium Recipes (1L)

Component	Infection	Co-cultivation	Resting	Selection I	Selection II	Regeneration I	Regeneration II
MS salts	2.15g	2.15g	4.3g	4.3g	4.3g	4.3g	2.15g
MES		0.5g	0.5g	0.5g	0.5g	0.5g	
L-proline		0.7g				0.7g	
Sucrose	68.5g	20g	30g	30g	30g	60g	30g
Glucose	36g	10g					
2,4-D 1mg/ml	1.5ml	2ml	2ml	1.5ml	1.5ml		
Agar		8g				8g	
Phytagel			2.5g	2.5g	2.5g		2.5g
pH	5.2	5.8	5.8	5.8	5.8	5.6	5.6
Autoclave	filtered	20min	20min	20min	20min	20min	20min
B5 vitamin stock, 100X	10ml	10ml	10ml	10ml	10ml		
Acetosyringone, 100mM	1ml	1ml					
Ascorbic acid		10mg	10mg				
Casamino acids	1g						
Asparagine			0.15g				
Coconut water			100ml				
Carbenicillin			0.2g	0.2g	0.2g	0.2g	0.2g
PPT, 20mg/ml				0.25ml	0.5ml	0.5ml	
Kinetin, 1mg/ml					0.5ml		
Zeatin, 1mg/ml						0.5ml	
IAA, 1mg/ml						1ml	
ABA, 0.025mg/ml						1ml	
TDZ, 0.5mg/ml						0.2ml	
IBA, 1mg/ml							0.25ml
NAA, 1mg/ml							0.25ml
MS vitamin, 1000X						1ml	1ml
PVPP (1% final)		10g	10g	10g	10g	10g	5g

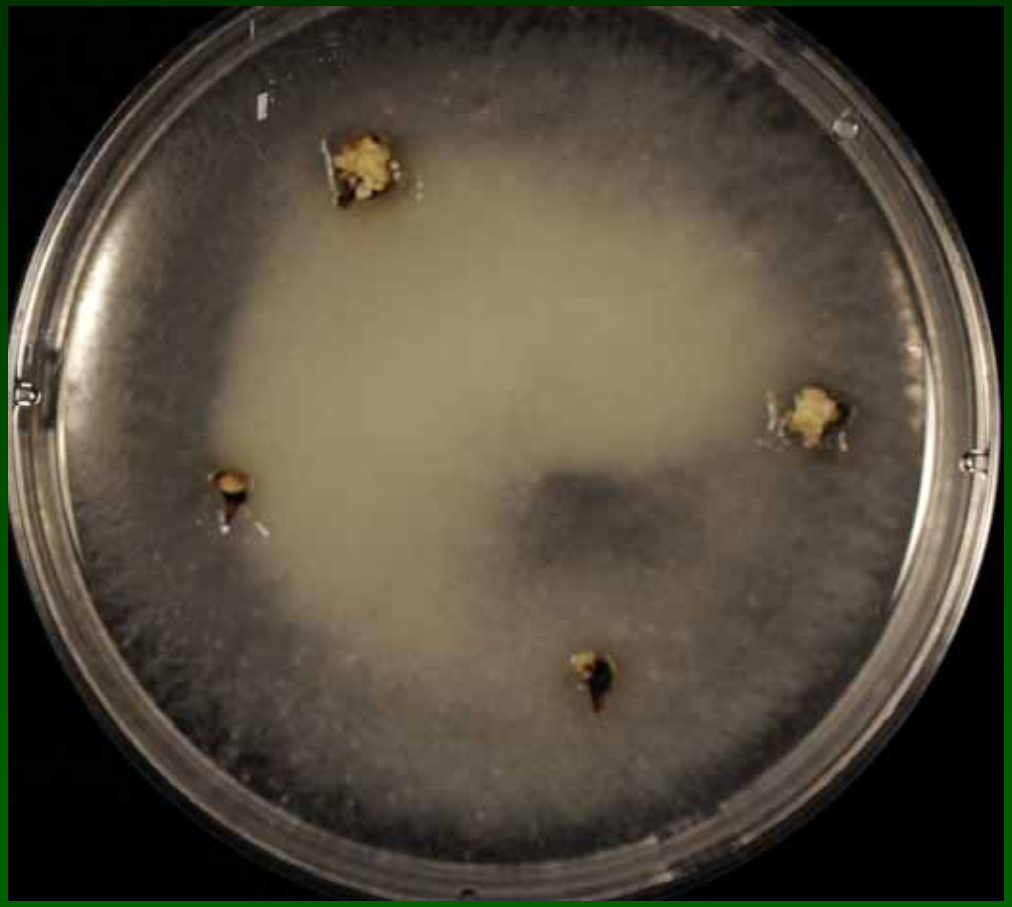
Selection of Transgenic Events

Following the Resting phase, embryos are transferred to Selection I medium. Seal the plates with parafilm and incubate them at 28°C in dark . Due to high phenolic accumulation in the medium, the embryos should be sub-cultured every 5-7 days. Putative transformants should be visible after 6-8 weeks. *Note: It is important to not allow the phenolic zone around the embryo to grow too large. Frequent subcultures are very, very important.*

Sorghum Medium Recipes (1L)

Component	Infection	Co-cultivation	Resting	Selection I	Selection II	Regeneration I	Regeneration II
MS salts	2.15g	2.15g	4.3g	4.3g	4.3g	4.3g	2.15g
MES		0.5g	0.5g	0.5g	0.5g	0.5g	
L-proline		0.7g				0.7g	
Sucrose	68.5g	20g	30g	30g	30g	60g	30g
Glucose	36g	10g					
2,4-D 1mg/ml	1.5ml	2ml	2ml	1.5ml	1.5ml		
Agar		8g				8g	
Phytagel			2.5g	2.5g	2.5g		2.5g
pH	5.2	5.8	5.8	5.8	5.8	5.6	5.6
Autoclave	filtered	20min	20min	20min	20min	20min	20min
B5 vitamin stock, 100X	10ml	10ml	10ml	10ml	10ml		
Acetosyringone, 100mM	1ml	1ml					
Ascorbic acid		10mg	10mg				
Casamino acids	1g						
Asparagine			0.15g				
Coconut water			100ml				
Carbenicillin			0.2g	0.2g	0.2g	0.2g	0.2g
PPT, 20mg/ml				0.25ml	0.5ml	0.5ml	
Kinetin, 1mg/ml					0.5ml		
Zeatin, 1mg/ml						0.5ml	
IAA, 1mg/ml						1ml	
ABA, 0.025mg/ml						1ml	
TDZ, 0.5mg/ml						0.2ml	
IBA, 1mg/ml							0.25ml
NAA, 1mg/ml							0.25ml
MS vitamin, 1000X						1ml	1ml
PVPP (1% final)		10g	10g	10g	10g	10g	5g

Selection of Transgenic Events



Putative transformants should be visible after 6-8 weeks. *Note: It is important to not allow the phenolic zone around the embryo to grow too large. Frequent subcultures are very, very important. Also, do not overcrowd the plates with cultures, give each culture ample space.*

Selection of Transgenic Events

Once putative transformants/herbicide resistant calli can be identified, they are transferred to Selection II medium and incubated at 28°C in dark for 2-3 weeks to develop somatic embryos transferring weekly. Note: Frequent subcultures are necessary to preserve callus health.

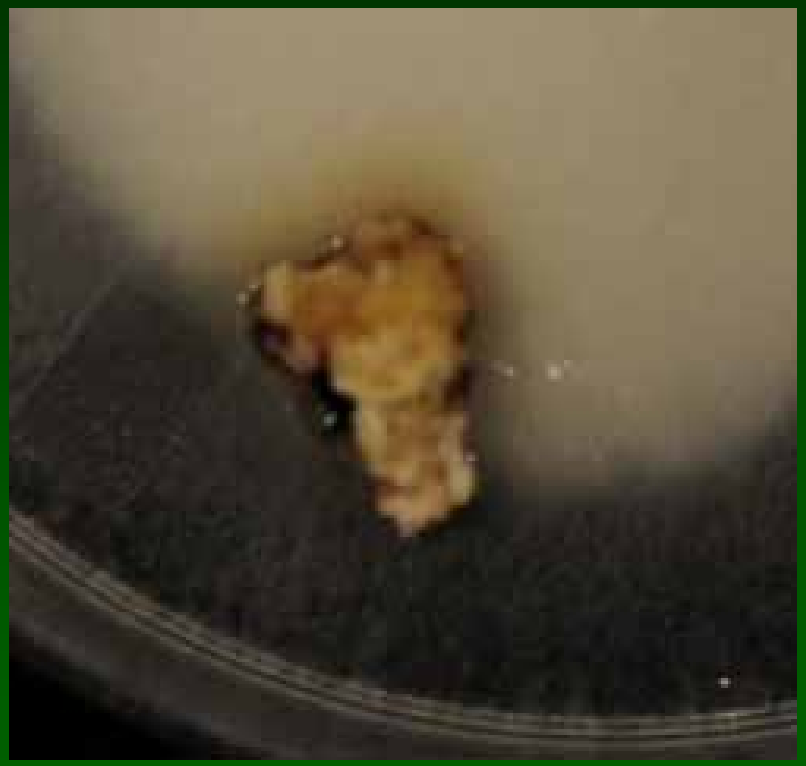
Sorghum Medium Recipes (1L)

Component	Infection	Co-cultivation	Resting	Selection I	Selection II	Regeneration I	Regeneration II
MS salts	2.15g	2.15g	4.3g	4.3g	4.3g	4.3g	2.15g
MES		0.5g	0.5g	0.5g	0.5g	0.5g	
L-proline		0.7g				0.7g	
Sucrose	68.5g	20g	30g	30g	30g	60g	30g
Glucose	36g	10g					
2,4-D 1mg/ml	1.5ml	2ml	2ml	1.5ml	1.5ml		
Agar		8g				8g	
Phytagel			2.5g	2.5g	2.5g		2.5g
pH	5.2	5.8	5.8	5.8	5.8	5.6	5.6
Autoclave	filtered	20min	20min	20min	20min	20min	20min
B5 vitamin stock, 100X	10ml	10ml	10ml	10ml	10ml		
Acetosyringone, 100mM	1ml	1ml					
Ascorbic acid		10mg	10mg				
Casamino acids	1g						
Asparagine			0.15g				
Coconut water			100ml				
Carbenicillin			0.2g	0.2g	0.2g	0.2g	0.2g
PPT, 20mg/ml				0.25ml	0.5ml	0.5ml	
Kinetin, 1mg/ml					0.5ml		
Zeatin, 1mg/ml						0.5ml	
IAA, 1mg/ml						1ml	
ABA, 0.025mg/ml						1ml	
TDZ, 0.5mg/ml						0.2ml	
IBA, 1mg/ml							0.25ml
NAA, 1mg/ml							0.25ml
MS vitamin, 1000X						1ml	1ml
PVPP (1% final)		10g	10g	10g	10g	10g	5g

Selection of Transgenic Events

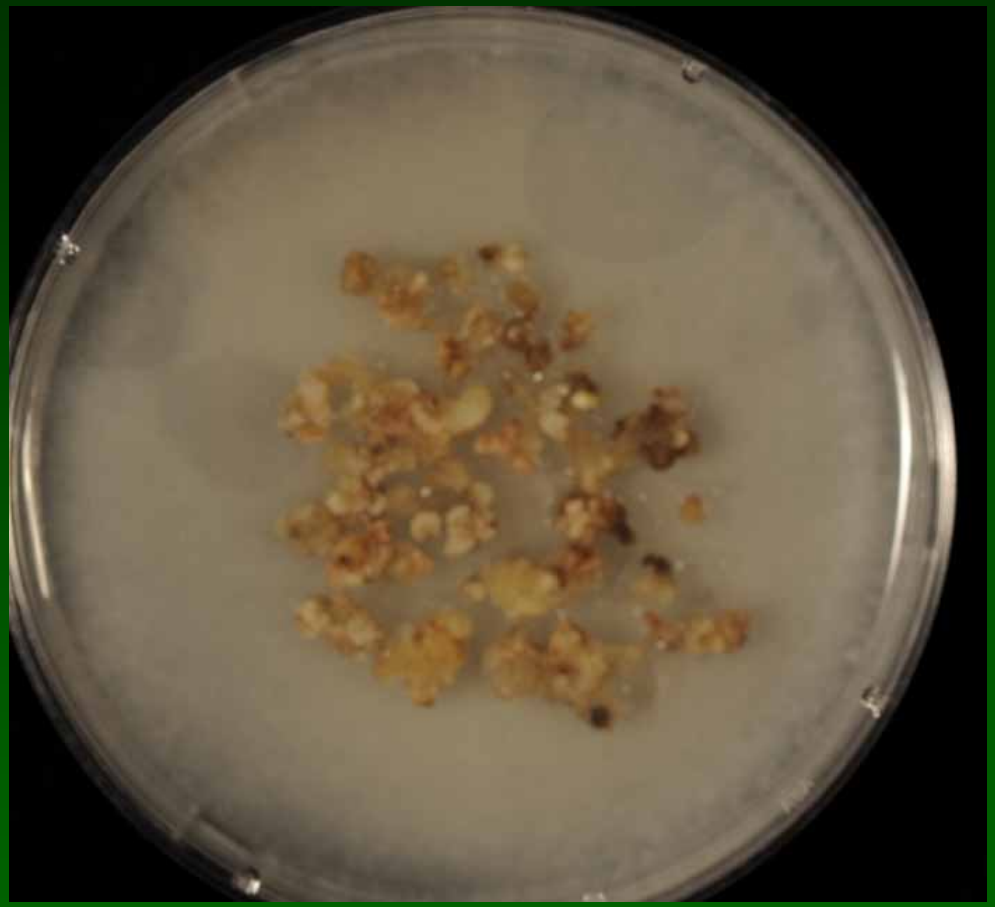
Selection I on the third subculture

Selection of Transgenic Events



Colonies Selection I on the third subculture

Selection of Transgenic Events



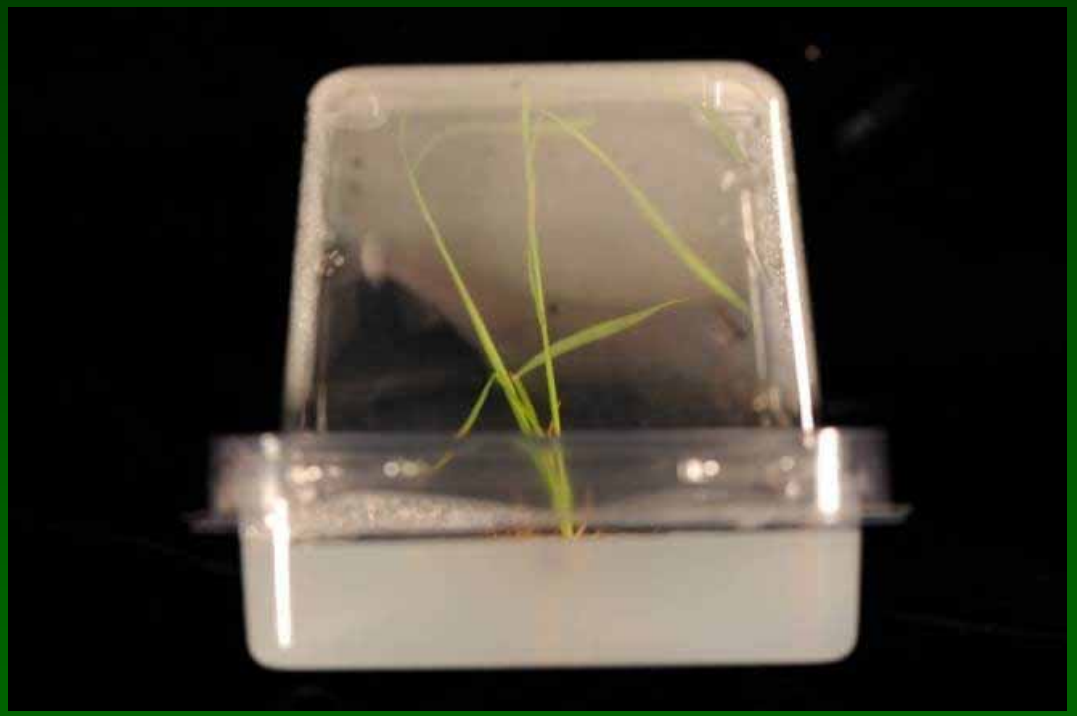
A single colony expanded Selection II prior to regeneration

Sorghum Medium Recipes (1L)

Component	Infection	Co-cultivation	Resting	Selection I	Selection II	Regeneration I	Regeneration II
MS salts	2.15g	2.15g	4.3g	4.3g	4.3g	4.3g	2.15g
MES		0.5g	0.5g	0.5g	0.5g	0.5g	
L-proline		0.7g				0.7g	
Sucrose	68.5g	20g	30g	30g	30g	60g	30g
Glucose	36g	10g					
2,4-D 1mg/ml	1.5ml	2ml	2ml	1.5ml	1.5ml		
Agar		8g				8g	
Phytagel			2.5g	2.5g	2.5g		2.5g
pH	5.2	5.8	5.8	5.8	5.8	5.6	5.6
Autoclave	filtered	20min	20min	20min	20min	20min	20min
B5 vitamin stock, 100X	10ml	10ml	10ml	10ml	10ml		
Acetosyringone, 100mM	1ml	1ml					
Ascorbic acid		10mg	10mg				
Casamino acids	1g						
Asparagine			0.15g				
Coconut water			100ml				
Carbenicillin			0.2g	0.2g	0.2g	0.2g	0.2g
PPT, 20mg/ml				0.25ml	0.5ml	0.5ml	
Kinetin, 1mg/ml					0.5ml		
Zeatin, 1mg/ml						0.5ml	
IAA, 1mg/ml						1ml	
ABA, 0.025mg/ml						1ml	
TDZ, 0.5mg/ml						0.2ml	
IBA, 1mg/ml							0.25ml
NAA, 1mg/ml							0.25ml
MS vitamin, 1000X						1ml	1ml
PVPP (1% final)		10g	10g	10g	10g	10g	5g

Plant Regeneration Transition to Soil

Remove the small shoots of 3-5 cm tall and cut apart with a scalpel to individualize plants. If plants are not separated here, it will be very hard to transplant into soil. Transfer shoots to Regeneration II medium in Plantcons. Incubate at 28°C under 16 hour light/8 hour dark (low intensity light).



When plants reach 8-10cm tall with large, healthy roots systems they can be acclimated to soil.

Plant Regeneration Transition to Soil

Once plants are well formed with shoots and roots, they are acclimated to soil. Up to 10 clones from each event are planted to ensure that seed is obtained.

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.*

Plant Regeneration Transition to Soil

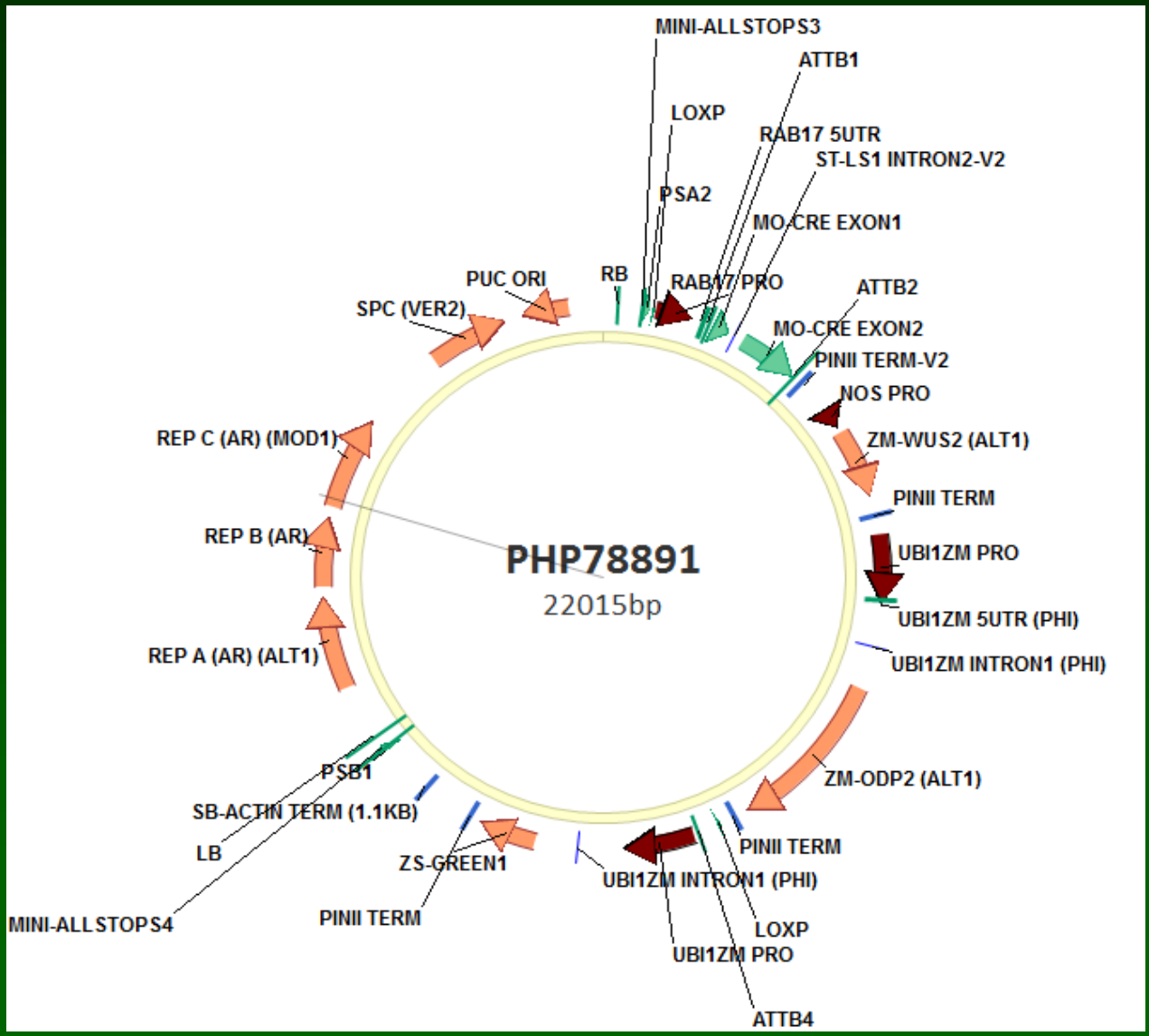
Plants are place in trays, watered thoroughly and placed in the growth chamber under shade cloth at 27°C under sodium halide lighting. Sorghum is slowly allowed to acclimate to the higher light. After 4-5 days, Plantcon tops are propped up slightly to allow plants to begin to develop a thicker cuticle. Plantcon tops can typically be removed after 10 days.

Mature Sorghum Inflorescence

Plants are grown to maturity and selfed. T1 seed is collected at maturity.

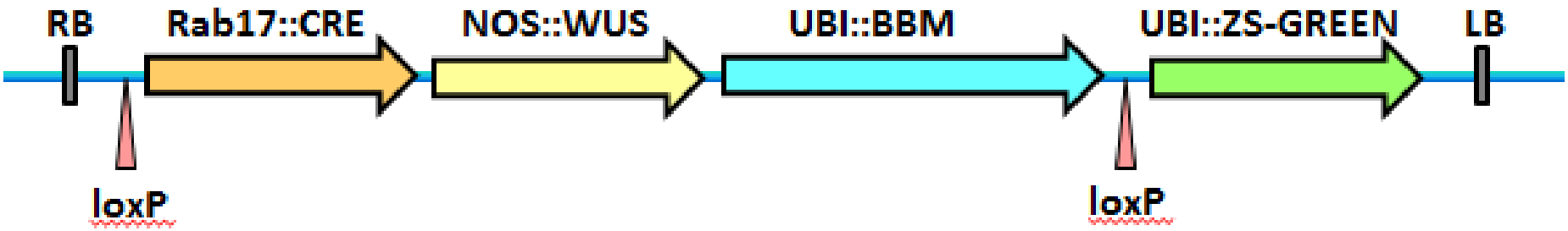
Thank You

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.