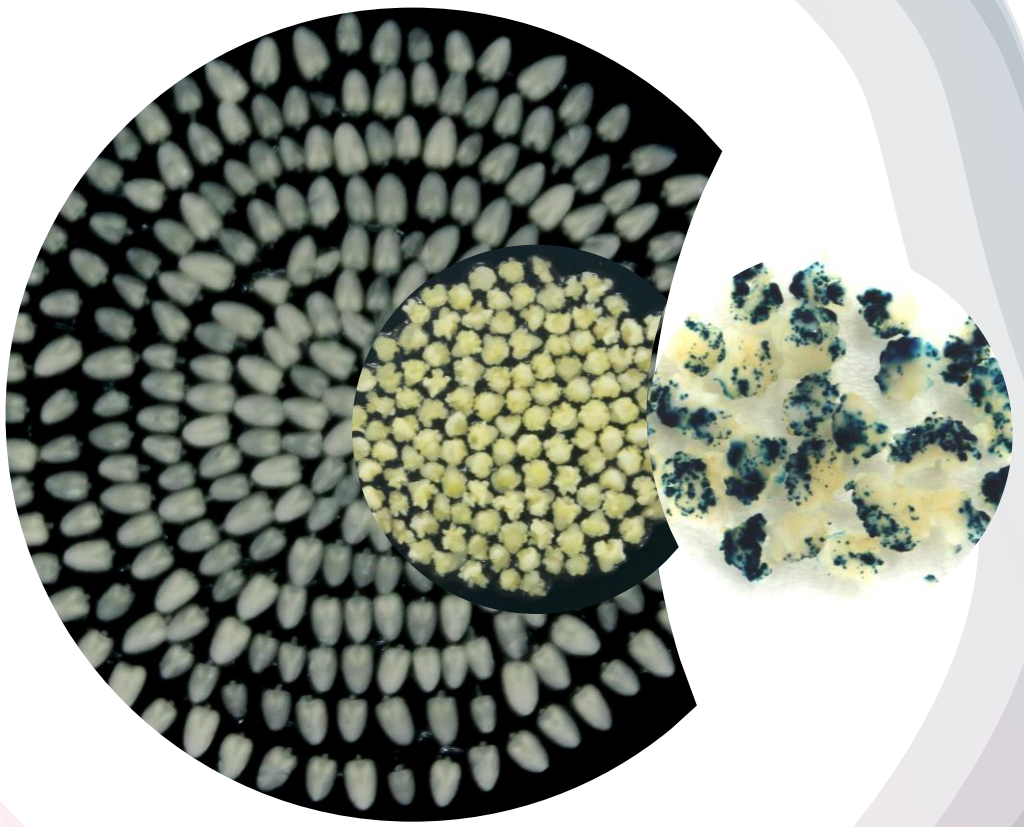
Corn Immature Embryo Transformation: Protocol Training

Bayer Russia Biotechnology Conference

July 2023

The training is focused on:



- Isolation of immature embryos and preparation of embryos for callus induction
- Callus induction from corn immature embryos (cv. LH244)
- *Agrobacterium*-mediated transformation of immature embryos
- *Agrobacterium*-mediated transformation of seedling-derived callus

PURPOSE

- This training describes the corn transformation protocol using glyphosate or NPTII selection.

- *Agrobacterium*-mediated transformation of freshly isolated immature embryos is the most preferred method for delivery of transgenes into a wide range of plant species including maize.

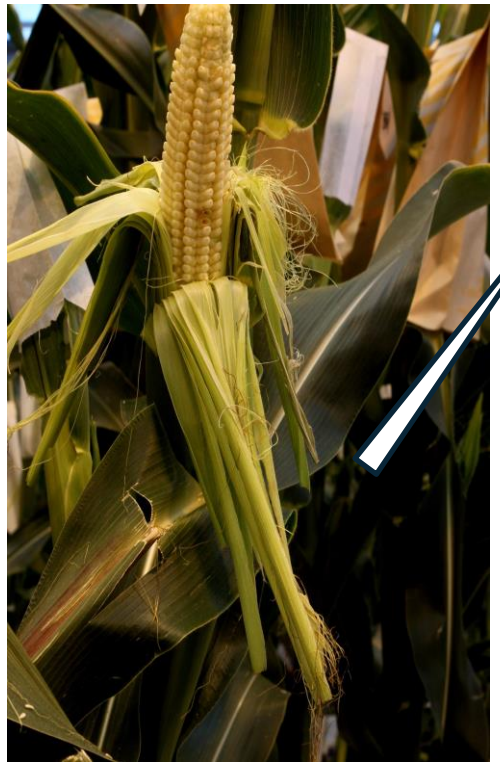
- Embryogenic callus derived from plant seedlings has also been found suitable for the transformation of a wide variety of corn genotypes including commercial inbred lines. ***This system does not require donor plant materials for immature embryo isolation***

- *Agrobacterium* harboring a binary vector containing *nptII* (neomycin phosphotransferase) or *cp4* (glyphosate resistant gene) as selectable marker gene with GUS gene (*uidA) or* GFP *gene (gfp)* as a visual marker genes have been used in corn transformation. ***GUS and GFP are a visual screening markers which allows tracking of transformation during different selection and regeneration steps.***

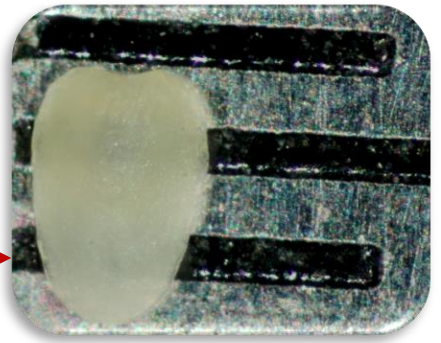
The described protocols can be routinely used to produce a large numbers of transgenic corn plants.

Isolation of immature embryos at the appropriate developmental age is critical for callus induction and transformation of corn immature embryos

Greenhouse-grown embryo donor ears are harvested 10–12 days after pollination when immature zygotic embryos are 1.5–2.0 mm long.



1.9 mm



Embryo sizes can be measured by a small ruler. Typically, ears can be harvested between 10 and 14 days depending on the temperature of the greenhouse

Donor plants in the green house **Pollination of ear**

Record the date of pollination

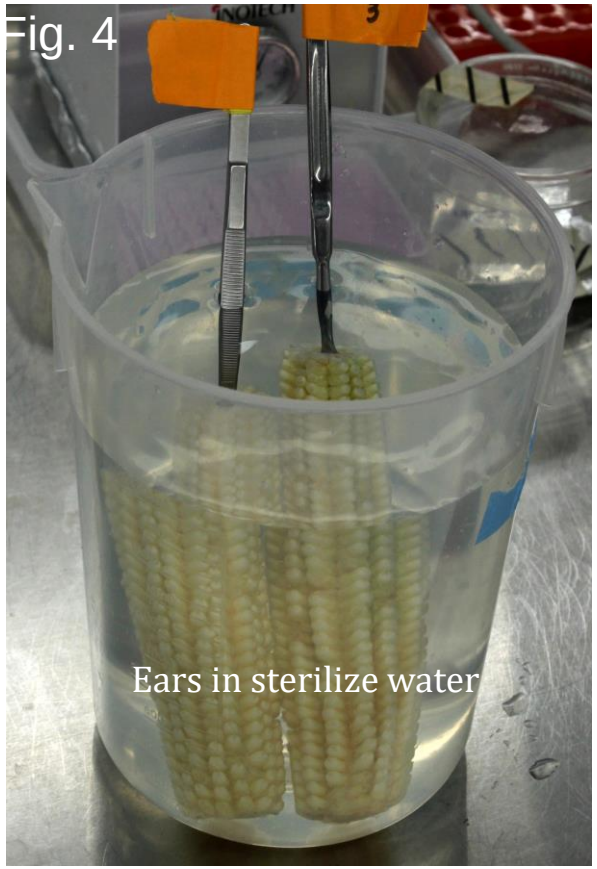
To obtain the embryos of the appropriate sizes, ears should be checked for embryo development while they are still on the plant around 9-10 days after the pollination

Dehusking and sterilization of ear for immature embryo isolation

Harvest ears with appropriate size of immature embryos

dehusk

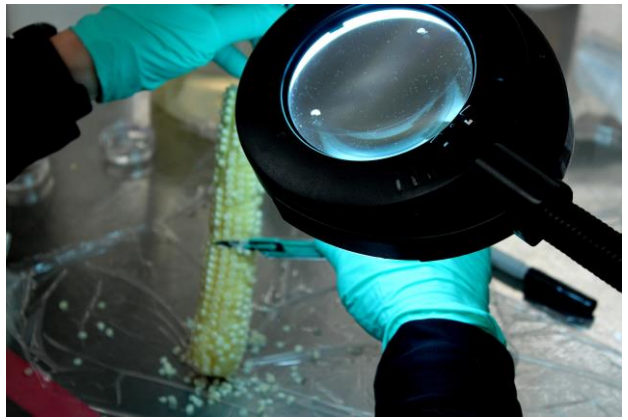
Sterilize in 2L of 20% commercial bleach (8.25% sodium hypochlorite) for 30 min, stir every 10 min to ensure all ear surfaces are in contact with the solution



wash ears 5-7 times using sterilize water

For callus induction: Isolation of undamaged immature embryos at the right stage of development is one of the critical factors

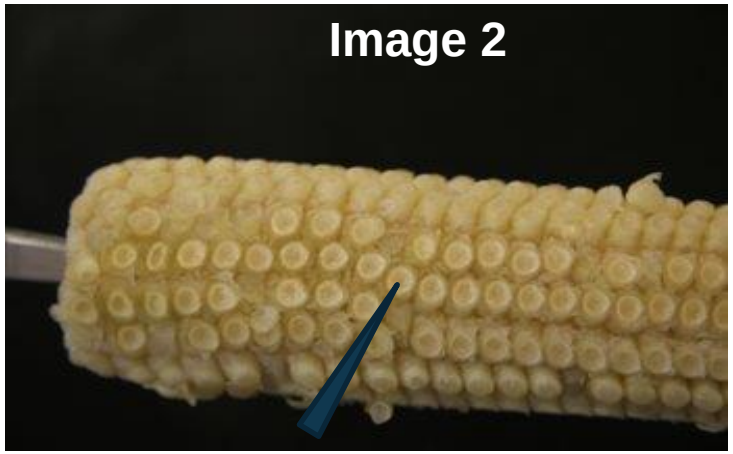
Preparation of corn ear is critical to isolate undamaged corn immature embryos



Use a sharp, sterile scalpel to carefully remove the opaque kernel top, 2-3 mm thickness at once until the clear endosperm is exposed (Image-2). Cutting too deep into the kernel may accidentally damage the embryos (Image-3)

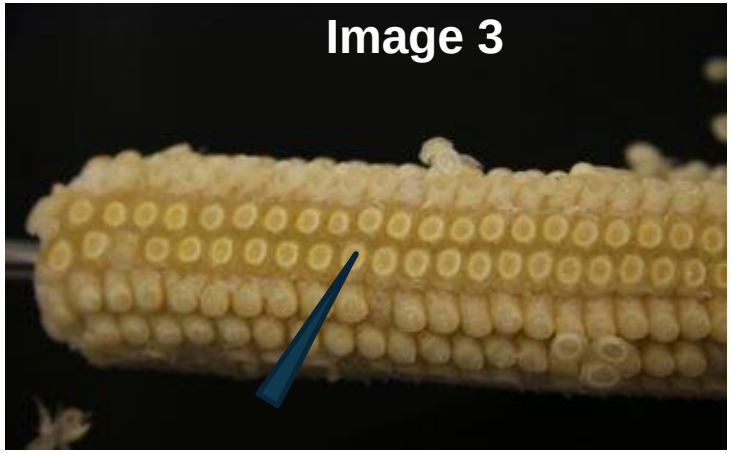
Immature embryos harvested at 1.6-2 mm in size produce high frequency of embryogenic callus and are suitable target for transformation

Uncut ear



Just right slice of kernel

Isolation will lead un-damaged embryos in very high quantity

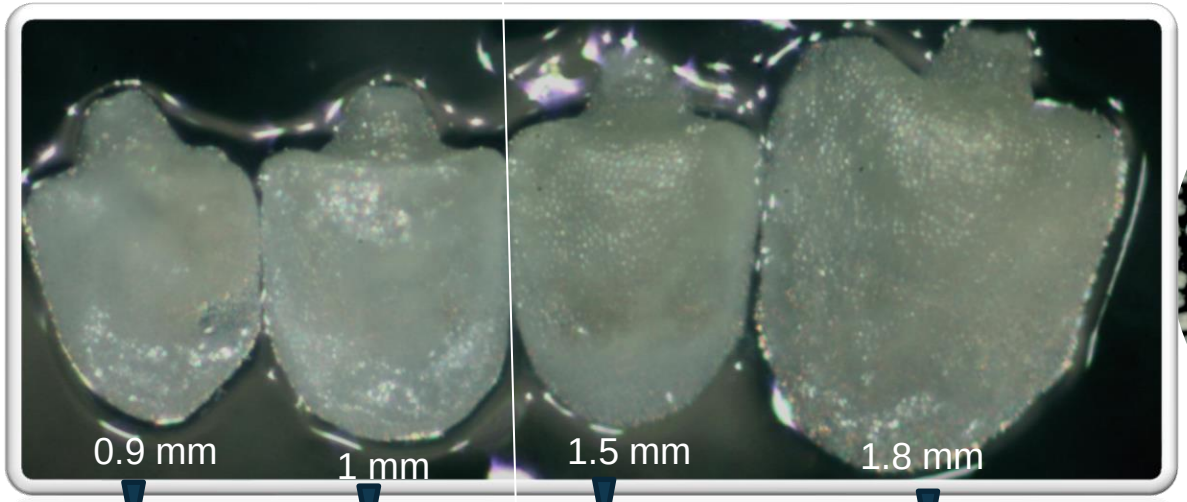


Too deep slice of kernel

Isolation will lead damaged embryos in very low quantity

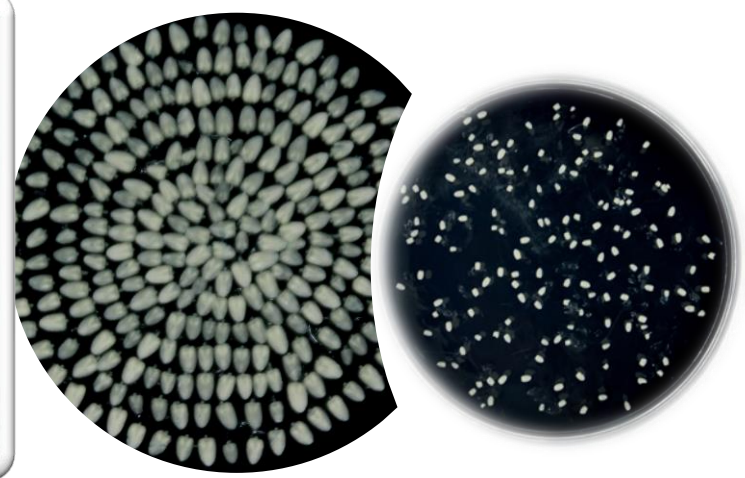
Isolation of immature embryos at an appropriate size is critical for callus induction and transformation

Immature embryos of **1.5–2.0 mm in size** are ideal for maize transformation.



Too small to transform

Appropriate size to transform

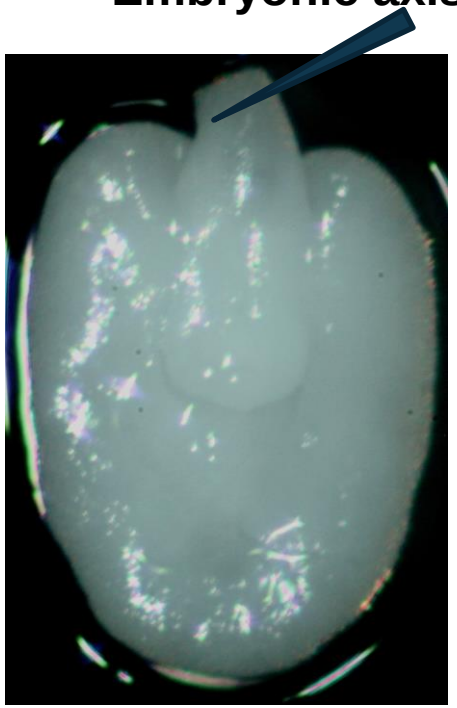


Culture of embryos with scutellum side up on the medium

To isolate embryos:

- Insert a sterile micro-spatula perpendicular to the ear axis at the bottom of the kernel then squeeze gently toward the ear-tip side.
- The embryo is located at the bottom, ear-tip side of the kernel. The embryo will emerge between the pericarp and endosperm.

Correct orientation of embryos on the medium is critical for callus induction and transformation of corn immature embryos



Freshly isolated Embryo

Embryos placed on the medium with embryonic axis facing up will not make callus and therefore, transgenic plants cannot be regenerated

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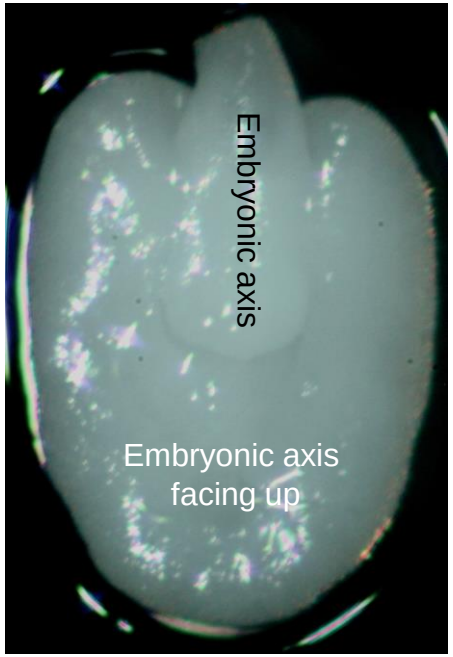
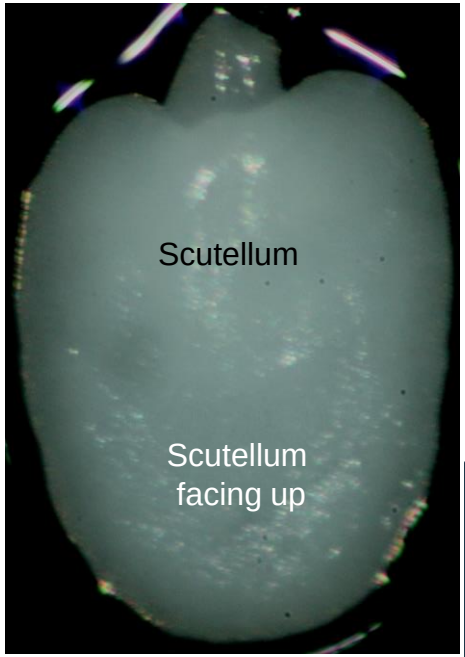


Unoriented immature embryos on medium

Embryonic axis facing up



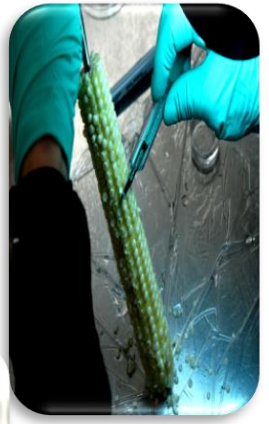
Oriented embryos on medium

Scutellum facing up

For successful transformation, correct orientation of immature embryos is very important. Scutellum of freshly isolated embryo has actively dividing cells and therefore, it is the target tissue for transformation

Procedure for isolation of immature embryos

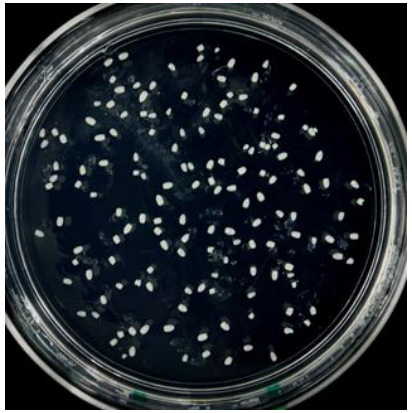
- Carefully remove the husks and silk from ears which are harvested 10–13 days post-pollination. Insert a blunt tip holder (can be forceps) at the basal end of ear
- Surface-sterilize ears for 20 min with a 20% dilution (v:v) of a 6.15% (active ingredient) solution of sodium hypochlorite. Occasionally swirl an ear during sterilization and rinse five times with sterile distilled water.
- Grasp the holder in the ear base and transfer the ear to a large sterile plate or another sterile surface. With a fine scalpel remove the upper part of the kernels of an entire ear (remove a flap of pericarp).
- With a blunt spatula pick up an embryo which lies at the basal edge of the endosperm of the immature caryopsis



Development of a protocol for callus induction and plant regeneration from immature embryos is the first step to transform a favorable genotype

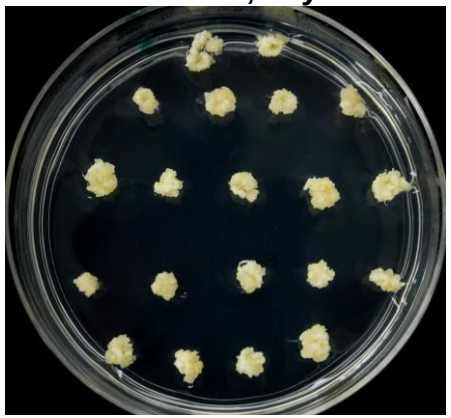
Freshly isolated embryo

Embryos on callus induction Medium, day 1

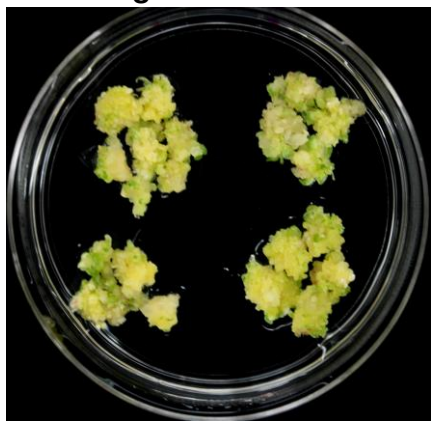


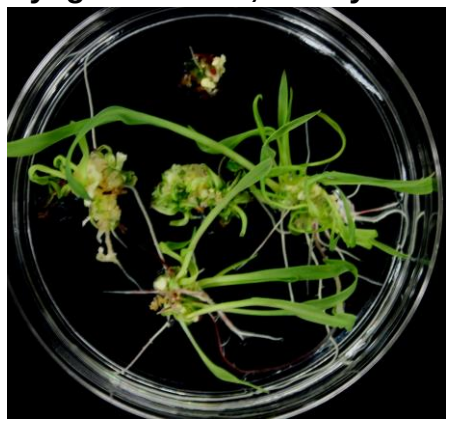
Embryos on callus induction medium, day 7

Embryogenic callus induction medium, day 28



Plant regeneration from embryogenic callus, 56 days



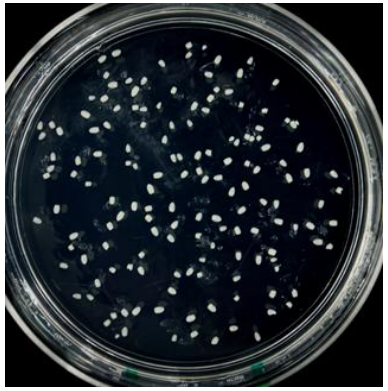


Successful genetic transformation of corn immature embryos depends optimizing parameters for achieving high frequency of embryogenic callus following transformation of targeted totipotent cells such as scutellum in immature embryos

In corn, embryogenic callus induction is strongly influenced by genotype, explant, the tissue culture media used, and the interaction of these factors.

Induction of highly embryogenic callus is very important for high frequency plant regeneration

Embryogenic, dense and compact callus induction from immature embryos – Ideal callus for transformation



Freshly isolated Immature embryos

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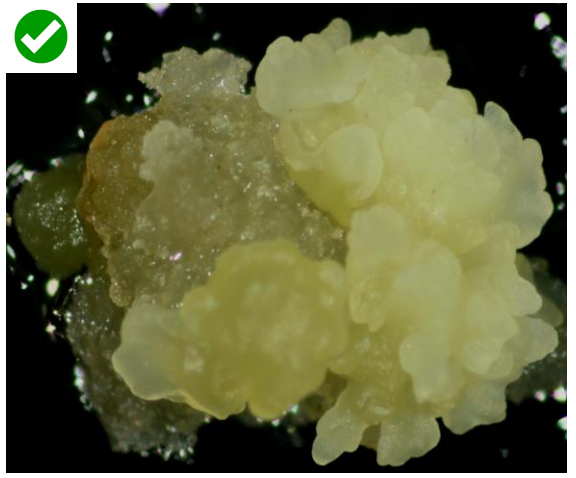

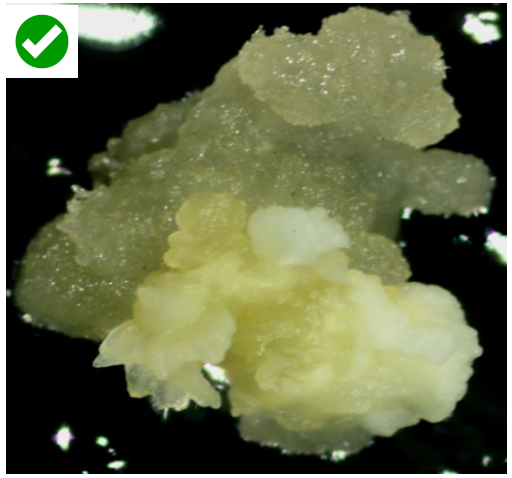

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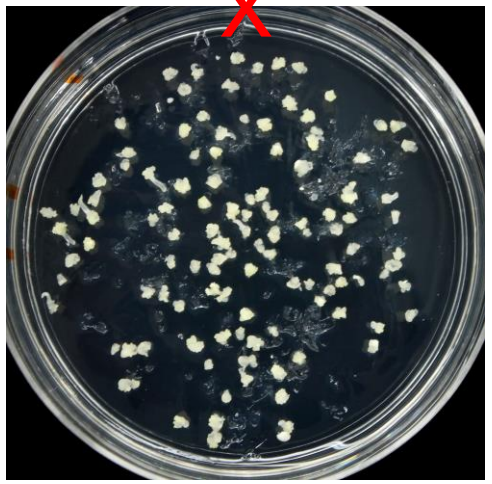

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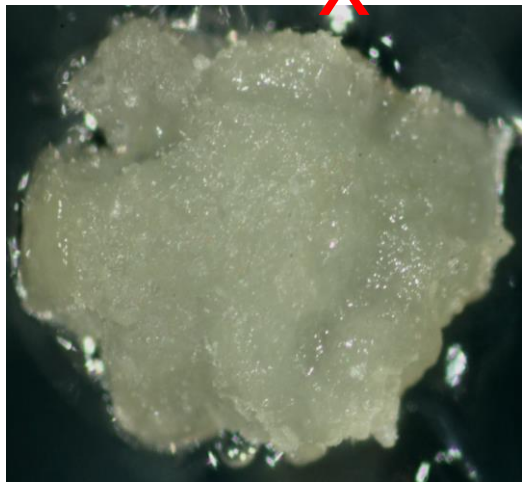

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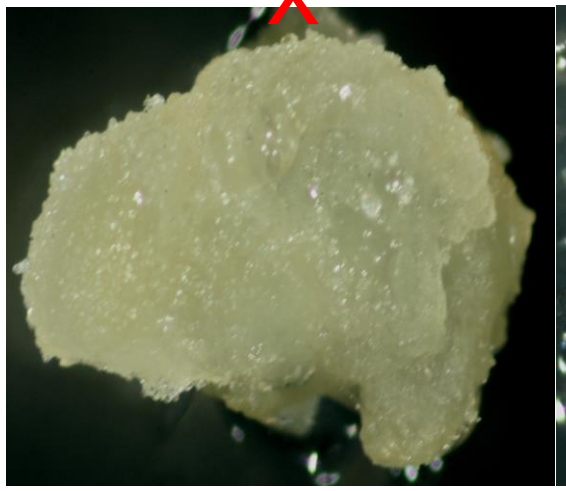

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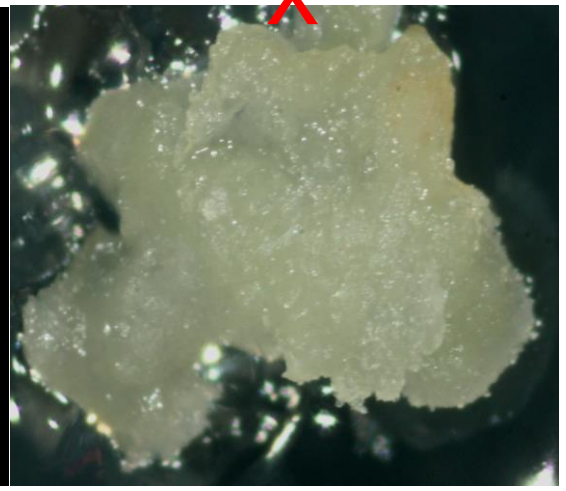
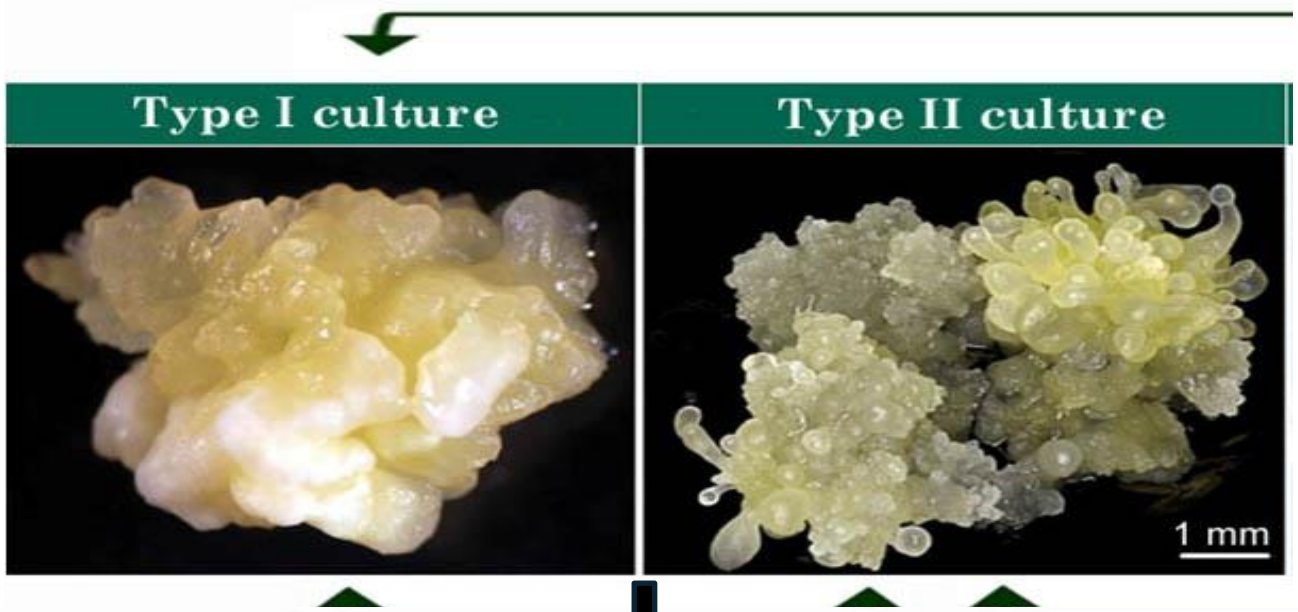


Whitish, friable, lose and watery callus induction from immature embryos – Not suitable for transformation

Types of callus in corn tissue culture



Compact and organogenic

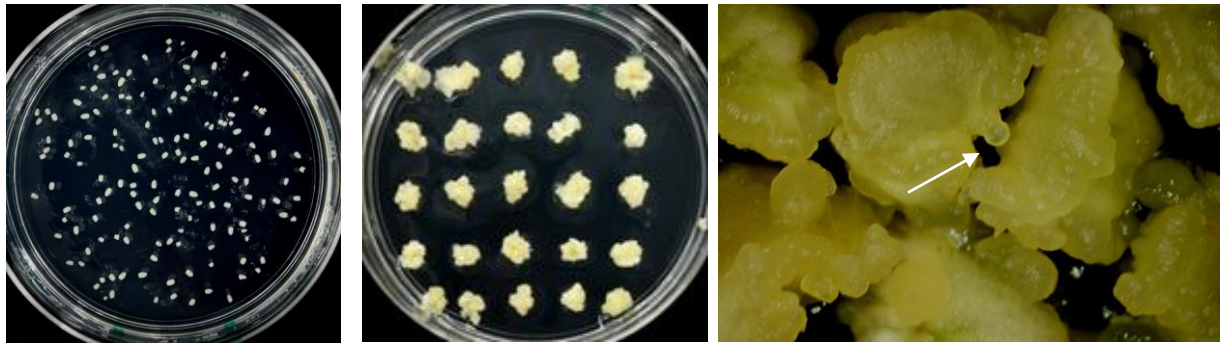
Friable and embryogenic

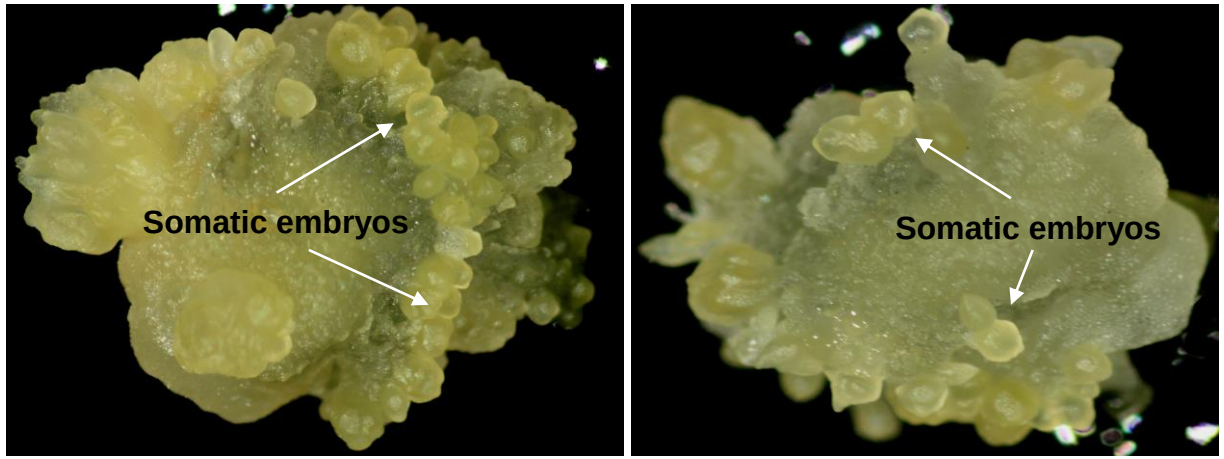
Type I culture	Type II culture
-First report: Green and Phillips, 1975	-First reports: Green, 1982; Armstrong and Green, 1985
-Callus formed from IEs of line A188, 2,4-D	-Callus formed from IEs A188, 2,4-D (N6 medium, 6 mM proline)
-Compact and organized culture	-Highly friable culture
-Regeneration via organogenesis or somatic embryogenesis	-Regeneration via somatic embryogenesis
-Highly transformable	-Highly transformable

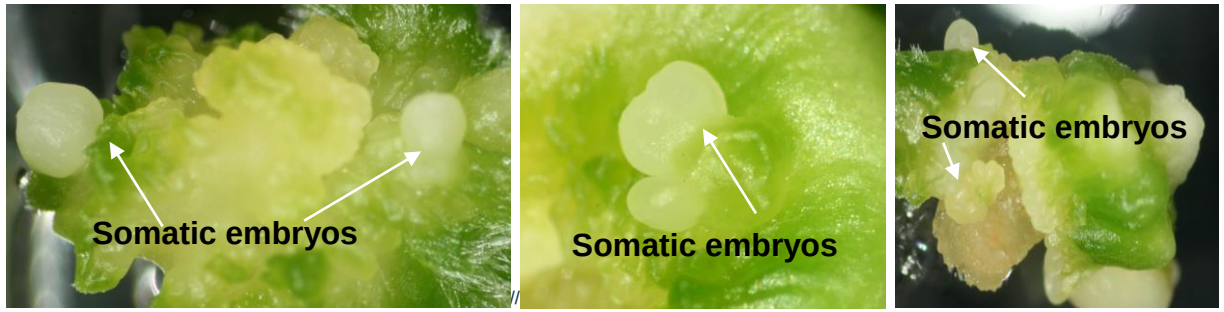
Less genotype-dependent

Highly genotype-dependent

Somatic embryogenesis/embryogenic callus induction frequency is strongly influenced by genotype, the tissue culture medium, and the interaction of these two factors





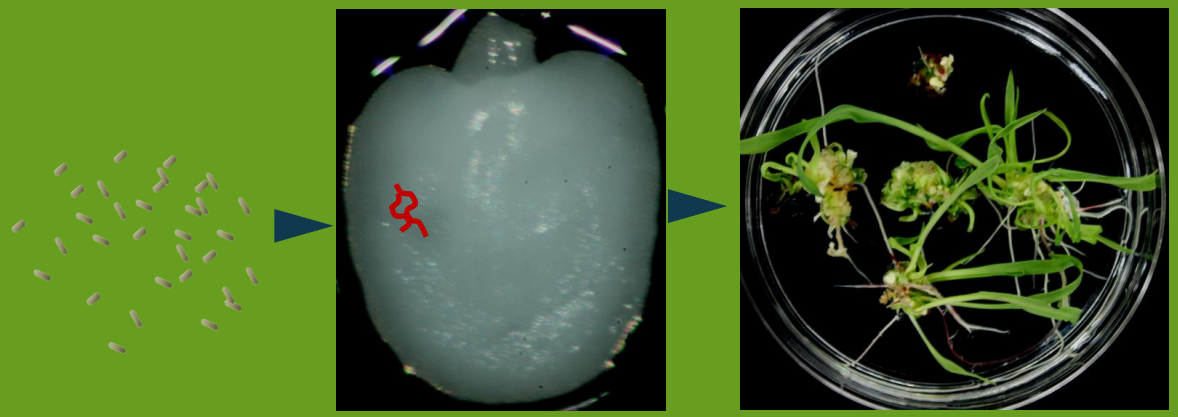


Typical callus induction recipe to induce Embryogenic callus

	Ingredients	Quantity/L
	MS Basal Salts	4.33 g/l
	MS Vitamins (100x)	10 ml/L
Auxin	2,4-D	0.5 – 5 mg/l
Auxin	Picloram (1 mg/ml)	2.2 ml
Cytokinin	BAP	0.0-0.05 mg/l
	Sucrose	30 g/l

Auxin (2,4-D; Picloram, NAA) to Cytokinin (BAP, TDZ, Zeatin) give rise to an unorganized growing and dividing mass of callus cells

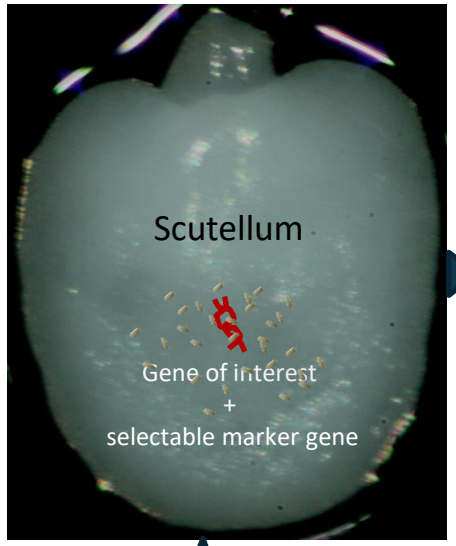
Transformation of Corn and Regeneration of Transgenic Plants



Transformation of Corn and Regeneration of Transgenic Events

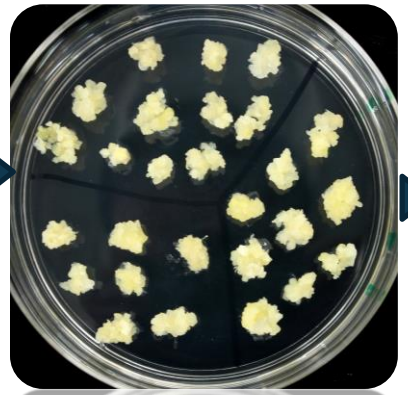
Through tissue culture, the transformed cells can be regenerated into an entire plant with each cell containing the transgene (the gene of interest). The gene becomes a permanent part of the transgenic plant's genome and will be passed on to its progeny.

Immature embryos

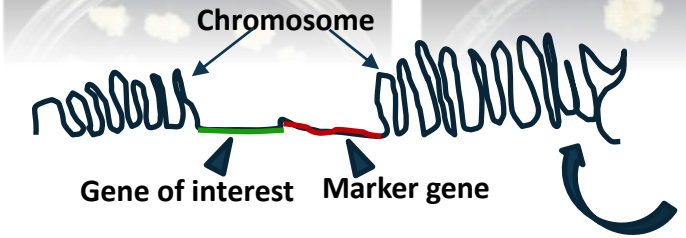


"Scutellum" of immature embryo is the Target tissue for transformation

Embryogenic callus induction

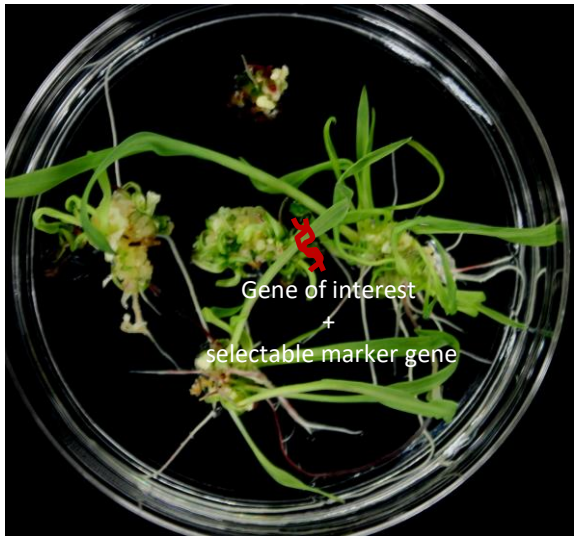


Selection of transformed cells



Shoot buds regeneration

Regeneration and rooting of transgenic plants



In a typical transformation:

- Cells are transformed with a gene of interest and selectable marker gene (such as CP4, BAR or PAT genes).
- Selectable marker gene is integrated in plant genome along with a gene of interest.
- To select out the transgenic cells, cells are grown on a medium containing a selection agent (such as herbicide or antibiotic).
- Only those cells transformed with a selectable marker will survive on a selection medium, while non-transgenic cells around them will die

Agrobacterium tumefaciens-mediated transformation method has been used to transformation corn immature embryos

***Agrobacterium* mediated transformation**

- ***Agrobacterium*** **can carry, transfer, and integrate a gene of interest into the plant genome via transfer of its T-DNA and several effector proteins into host cells**

Agrobacterium-mediated transformation of immature embryos workflow

Agrobacterium Infection and Co-cultivation (Day 1)

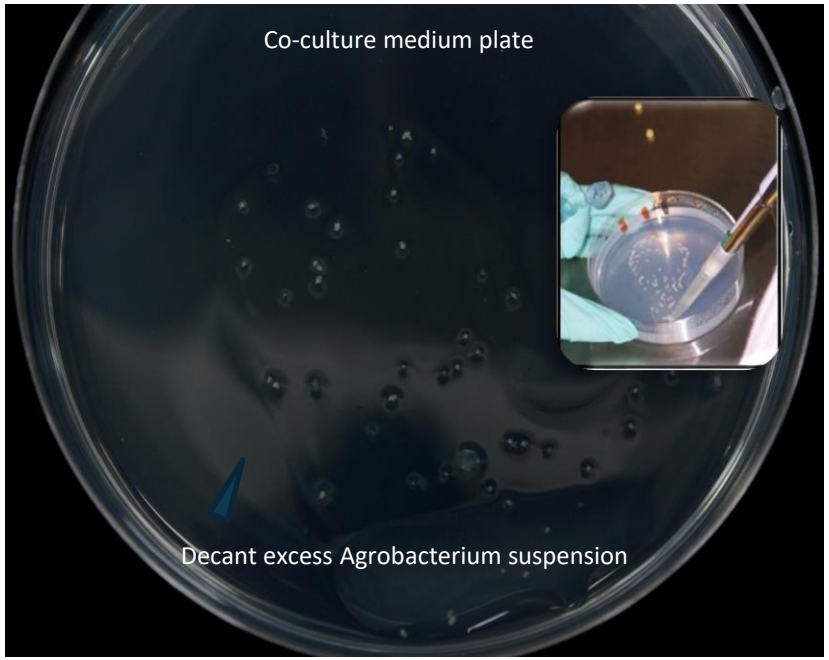
Isolation of embryos

Isolate 80-100 embryos into a 2 mL Eppendorf tube containing embryo isolation medium.

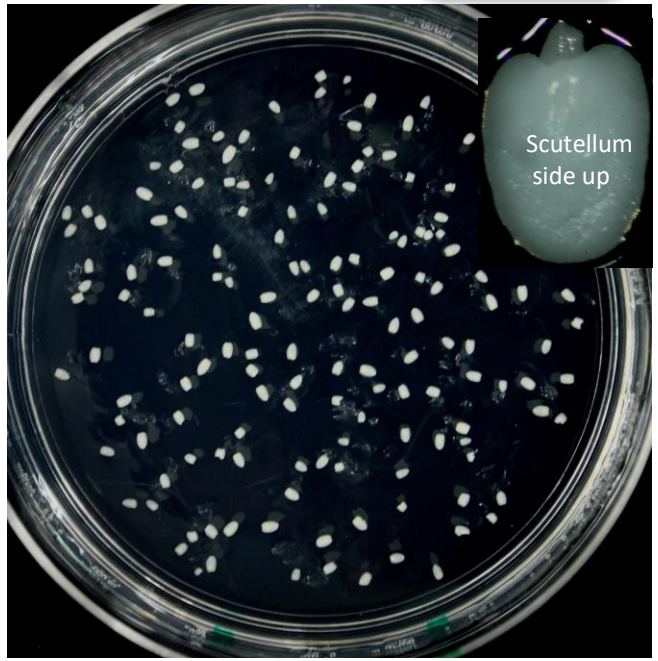
Inoculation of embryos

Decant isolation medium and add add 1 mL of freshly made *Agrobacterium* suspension to the embryos in the tube and incubate the tube at room temperature (22–24°C) for 5-10 min.

Co-culture of embryo



Then pour the bacteria suspension containing the embryos onto a co-cultivation plate. Tilt the plate to collect and remove as much bacterial suspension as possible with a pipette.



Under a dissecting microscope in the laminar flow bench, carefully orient the embryos by placing them scutellum side (smooth and round side) up .

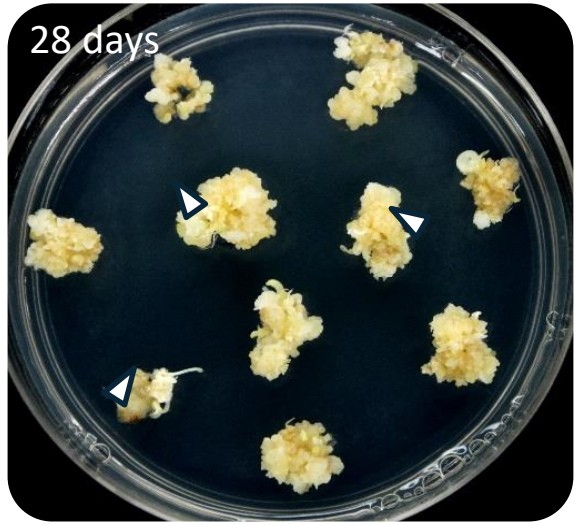
Agrobacterium-mediated transformation of immature embryos workflow

Selection (Day 2)

Selection of callus

Place the co-cultured embryos scutellum side up to a callus induction and selection medium **containing a selection agent** (herbicide or antibiotic) and incubate the plates at 28°C in the dark for 14 days. Transient GUS or GFP expression can be detected at this stage

Callus induction and selection



After 14 days, subculture the callus to same selection medium. At this stage, carefully isolate healthy resistant sectors of the callus and transfer to the **subculture medium**. Transfer 4–5 calli per plate. Incubate the plates at 28°C in the dark for 14 days.

Selection medium has an appropriate selection agent (herbicide or antibiotic)

Regeneration (Day 28)

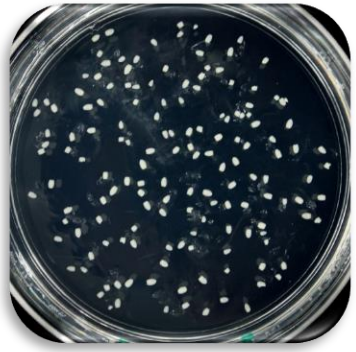
Regeneration

After 28 days of subculture, transfer healthy resistant calli to **regeneration medium containing an appropriate selection agent**. Transfer only 3-4 callus pieces in one plate. Incubate the plates at 28°C in a light chamber, with a photoperiod of 16 h light and 8 h dark

Regeneration medium is hormone free medium with an appropriate selection agent

A visual reporter marker gene such as *uidA* gene can be used to develop transformation protocol using *Agrobacterium*-mediated delivery method

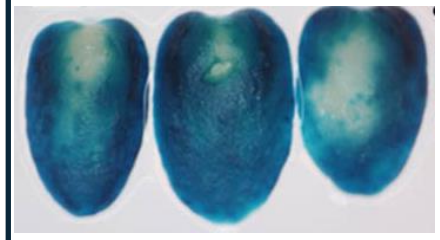
Target tissue



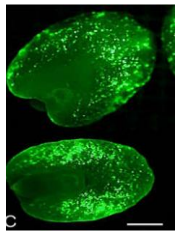
Freshly isolated embryos are used for *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation

uidA or *gfp* visual marker is delivered via *Agrobacterium*-mediated transformation



GUS expression in transformed Immature embryos



GFP expression in transformed Immature embryos

Delivery parameters that are critical:

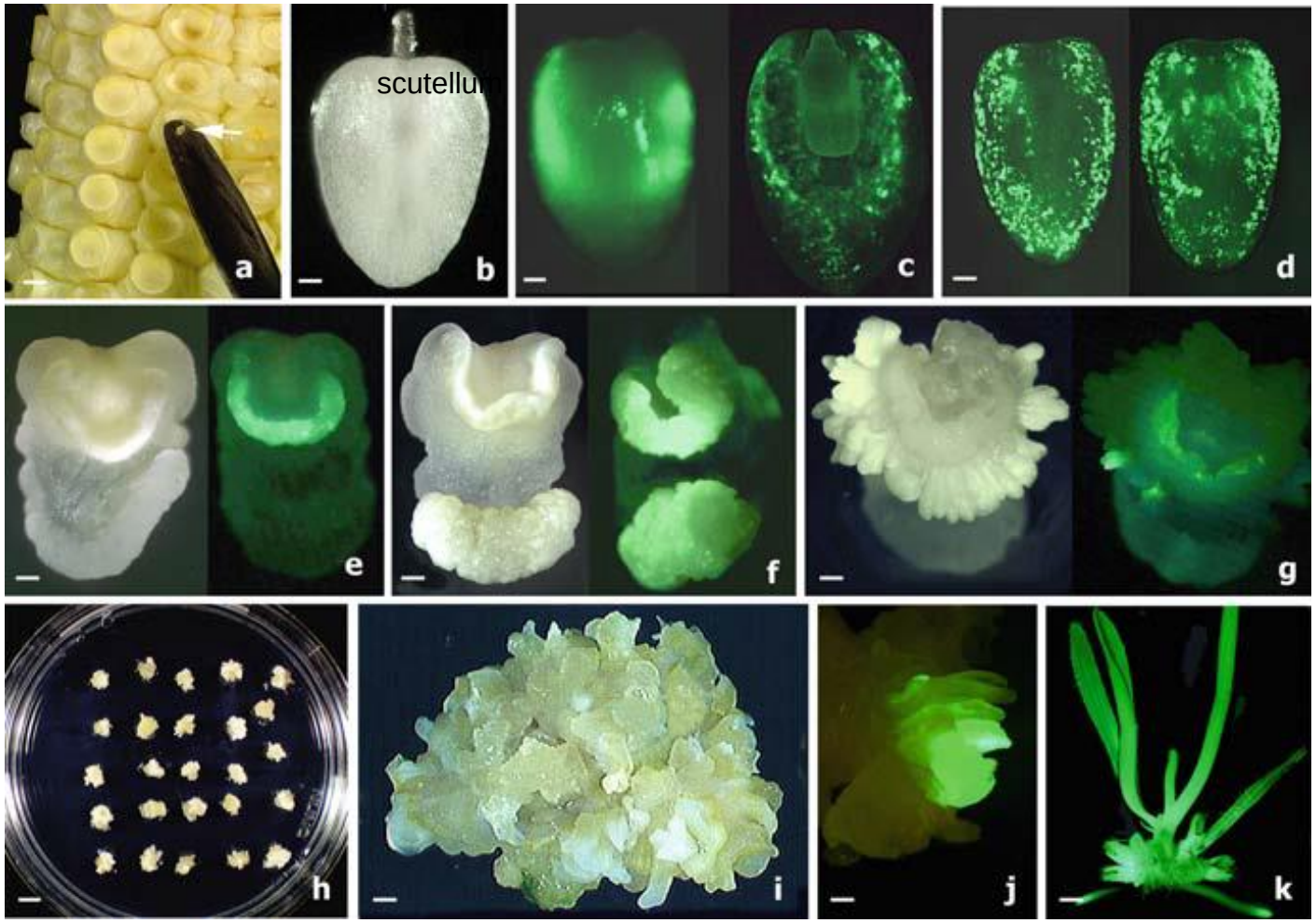
1. *Agrobacterium* strain
2. *Agrobacterium* density (OD)
3. Genotype
4. Target tissue

Quality of immature embryos is the most critical factor

/// Bayer Russia Biotechnology Conference /// July 2023

Agrobacterium-mediated transformation of corn using freshly isolated embryos

gfp gene was used as a visual marker in this study

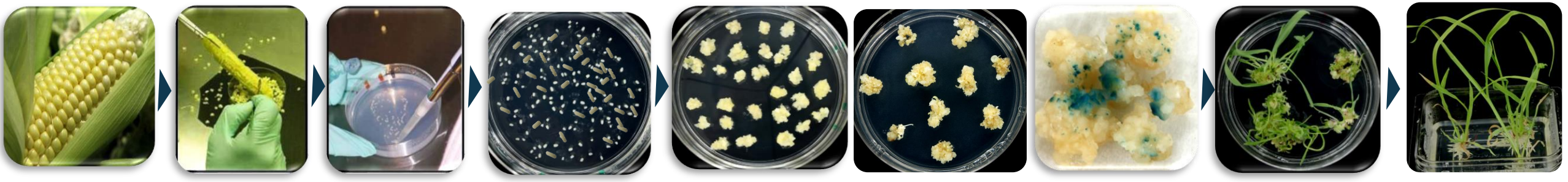


***Agrobacterium*-mediated transformation of corn using freshly isolated immature embryos**

(a) Ear of corn with tops of kernels removed.
(b) Freshly isolated IE with scutellum surface up
(c, d) Transient GFP expression in immature embryos
(e) Immature embryo 7 days on selection. Transient GFP expression is still visible at the area of callus formation
(f) Callus is forming from immature embryo
(g) Immature embryos after 12 days on selection medium. Callus area with stable GFP expression is visible
(h) Individual calli derived from Immature embryos after 1 month of selection
(i) Higher magnification of single callus line
(j) Stable expression of GFP in callus observed after 1 month of selection
(k) GFP expression in regenerated plants obtained 3 months after inoculation

Agrobacterium-mediated transformation of corn immature embryos

uidA gene was used as a visual marker in this study



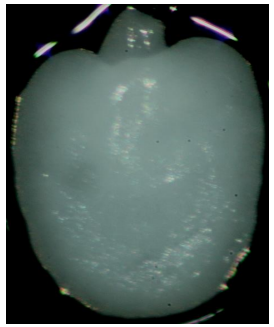
Isolation of immature embryos

***Agrobacterium*-mediated transformation**

Callus induction and selection

Regeneration

Rooting

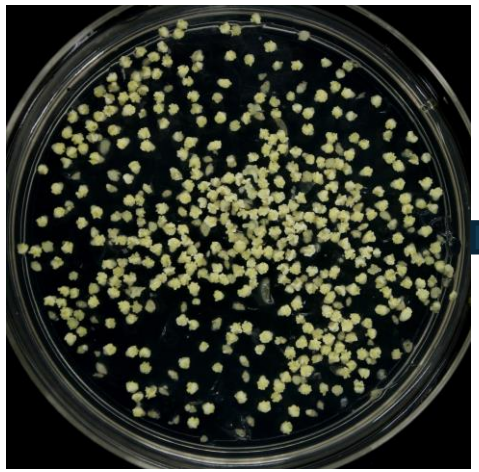


Freshly isolated embryos are transformed

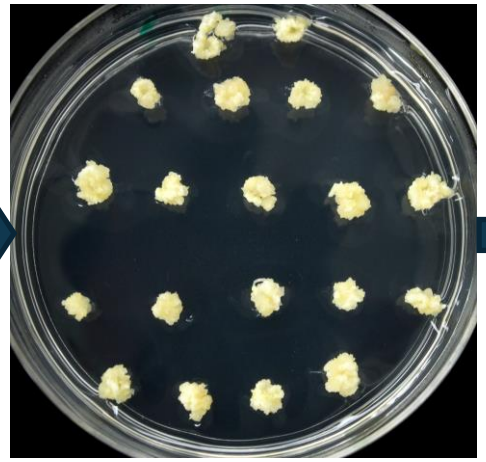
Resistant callus can be checked for stable transformation

Plants can be transferred to soil 10 weeks after transformation

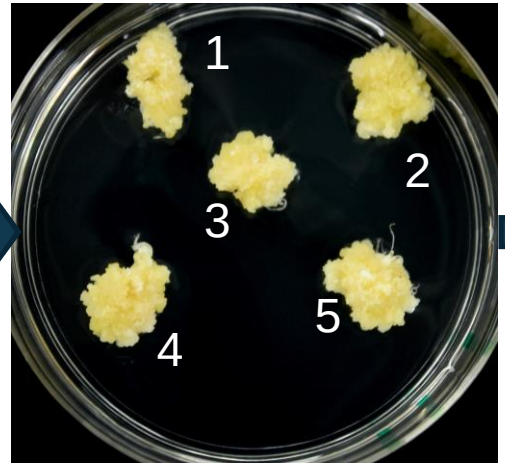
Maintaining identity of mother callus is very important to calculate transformation frequency



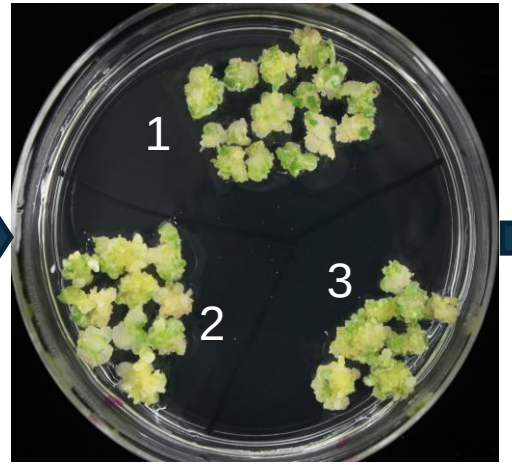
Callus formation from immature embryos, 7 days

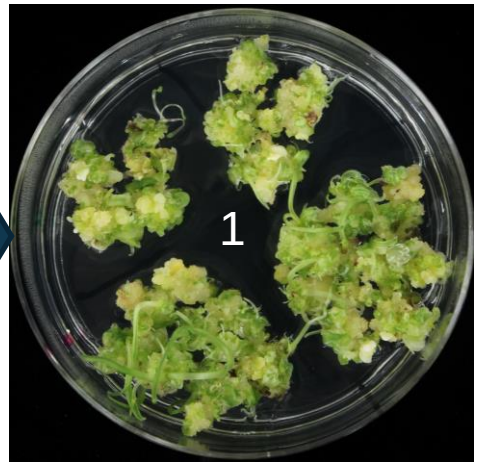


Callus formation from immature embryos, 21 days



Callus formation from immature embryos, 28 days





All four clumps Belong to mother Callus # 1

Agrobacterium Preparation

- Streak out *Agrobacterium* from a glycerol stock on to solid LB medium containing an appropriate antibiotics. Seal the LB plate with Parafilm M ® and place the plate upside down in a 28°C incubator for 2 days.
- Move the plate with *Agrobacterium* to 2–8°C refrigerator.
- In the late afternoon 2 days before inoculating the immature embryo, pick one colony of the *Agrobacterium* from the plate and inoculate 25 ml of liquid LB (LB with appropriate antibiotics) in a 250-ml flask. Place the flask on a shaker at approximately 150 rpm in the dark and 28°C overnight.

- The next morning make a 1:5 dilution of the *Agrobacterium* culture in LB (add 10 ml of the culture into 40 ml fresh LB containing antibiotics).
- In late afternoon of the same day, divide the *Agrobacterium* culture into two 50-ml test tubes and centrifuge at 3,500 rpm for 15 min. Remove the supernatant and resuspend the cells in 10 ml induction broth with 200 uM of acetosyringone (*acetosyringone induce the virulence genes and thus facilitated in transfer of the T-DNA into plant cells*). Check the density of the suspension culture and dilute it to O.D.660 = 0.2 with the induction broth supplemented with acetosyringone and antibiotics.
- The final volume is 50 ml in a 250-ml flask. Place the flask on a shaker at approximately 150 rpm in the dark and 26°C overnight.
- The next morning, centrifuge the *Agrobacterium* cells and resuspend the pellet in 6–10 ml of inoculation medium (1013) with 200 uM acetosyringone. Check the O.D. at 660 and adjust it to an O.D. 660 of 1.0.

Inoculation and Co-culture

- Isolated immature embryos (1.5–2.0 mm) are collected for 15 min in an *Agrobacterium* cell suspension in 1.5-ml microcentrifuge tubes. After 15 min of embryo isolation the microfuge tube is set aside for 5 min.
- Remove the *Agrobacterium* suspension using a pipette with fine tip. Transfer the embryos (50-100) to co-culture medium (1898). Flip the embryos so the scutellum is facing up. Keep the coculture plates in a growth chamber set at 24°C and dark for approximately 24 h. Transient expression of GFP in immature embryos after co-culture with *Agrobacterium* can be detected.

Selection

The transformed immature embryos are selected on glyphosate containing medium since a binary vector with the *cp4* gene is transformed into *Agrobacterium* strain.

1. *First selection*. After co-culture, transfer the embryos onto the first selection medium 1278 containing 500 mg/l carbenicillin and 100 uM glyphosate. Each plate (100 × 25 mm) may contain up to 20 embryos. Flip the embryos so the scutellum is facing up. Keep the plates in a growth chamber (28°C, dark) for approximately 2 weeks.
2. *Second selection*. After two weeks, transfer the developing callus onto the second selection medium 1278 containing 500 mg/l carbenicillin and 100 uM glyphosate. Each plate (100 × 25 mm) may contain up to 20 embryos. Flip the embryos so the scutellum is facing up. Keep the plates in a growth chamber (28°C, dark) for approximately 2 weeks. Callus formation and GFP expression during different stages of selection can be detected.

Regeneration

BA pulse. Transfer the callus from the second selection medium onto 1073 BA pulse medium containing 250 mg/l carbenicillin and 100 uM glyphosate. Each plate may contain 5-10 callus pieces. Move the plates to a transparent plastic box in a lighted growth chamber (16-h light, typically 50 par light, 28°C). Leave the cultures on this medium for 7 days.

Regeneration. At the end of BA pulse, transfer the callus onto 1071 medium containing 250 mg/l carbenicillin and 100 uM glyphosate and keep the plates in a growth chamber at 16 h light/150 par light/28°C for 4 weeks. In approximately 2 weeks on the regeneration medium some callus pieces may have regenerated green shoots with or without roots. Those shoots should be healthy looking and easily distinguished from some small shoots, which are no longer growing or have been bleached (dying). After 4 weeks of regeneration, healthy shoots are transferred to rooting medium 1084 in Phytatrays ™ containing 100 uM glyphosate. During transfer, try to remove callus tissue attached to the root area of the shoots.

Transplanting to soil

When plants have reached the lid and developed one or more healthy roots (usually after 1–3 weeks), they are big enough to be moved to soil. Remove plants gently and rinse in room temperature water to remove culture medium from the roots. Transplant T 0 plants into Jiffy ® pots or peat pots containing MetroMix-200. Put plants in a growth chamber at 28°C, 70% humidity and low light intensity for 1–2 week.

Media used for *Agrobacterium*-mediated transformation of corn immature embryos

Inoculation Medium - 1013

	per L
MS Basal Salts	2.2 g
MS Vitamins (100X)	10 mL
Dextrose	36 g
Sucrose	68.5 g
Proline	0.115 g
pH with KOH to 5.8	

Co-culture medium-1898

	per L
MS Basal Salts:	4.33 g
MS Vitamins (100X):	10 mL
Thiamine HCl (0.5mg/mL):	1 mL
2,4-D (1mg/mL)	:0.5 mL
Sucrose	:30 g
Proline	:1.38 g
Casamino Acids	:0.5 g
pH with KOH to 5.8	
Agarose, Low EEO	:5.5 g
Acetosyringone (1M)	:0.2 mL
Silver Nitrate (2mg/mL):	1.7 mL
Carbenicillin (250mg/mL):	0.2 mL

Selection medium-1278

	per L
MS Basal Salts	:4.33 g
MS Vitamins (100X)	:10 mL
Thiamine HCl (0.5mg/mL):	1 mL
2,4-D (1mg/mL)	:0.5 mL
Sucrose	:30 g
Proline	:1.38 g
Casamino Acids	:0.5 g
pH with KOH to 5.8	
Gelzan CM	:3g
Silver Nitrate (2mg/mL):	1.7 mL
Carbenicillin (250mg/mL):	2 mL
BAP (0.5mg/mL)	:0.02 mL
Glyphosate (0.5M)	:0.2 mL

BA Pulse-1073

	per L
MS Basal Salts	:4.33 g
MS Fromm Vitamins (1000X)	:1 mL
BAP (0.5mg/mL)	:7 mL
Sucrose	:30 g
Proline	:1.38 g
Casamino Acids	:0.05 g
pH with KOH to 5.8	
Gelzan CM	:3 g
Carbenicillin (250mg/mL)	:1 mL
Glyphosate (0.5M)	:0.2 mL

Regeneration-1071

	per L
MS Basal Salts	:4.33 g
MS Fromm Vitamins (1000X)	:1 mL
Dextrose	:10 g
Maltose	:20 g
Asparagine Monohydrate	:0.15 g
Myo-Inositol	:0.1 g
pH with KOH	
Gelzan CM	:3 g
Carbenicillin (250mg/mL)	:1 mL
Glyphosate (0.5M)	:0.2 mL

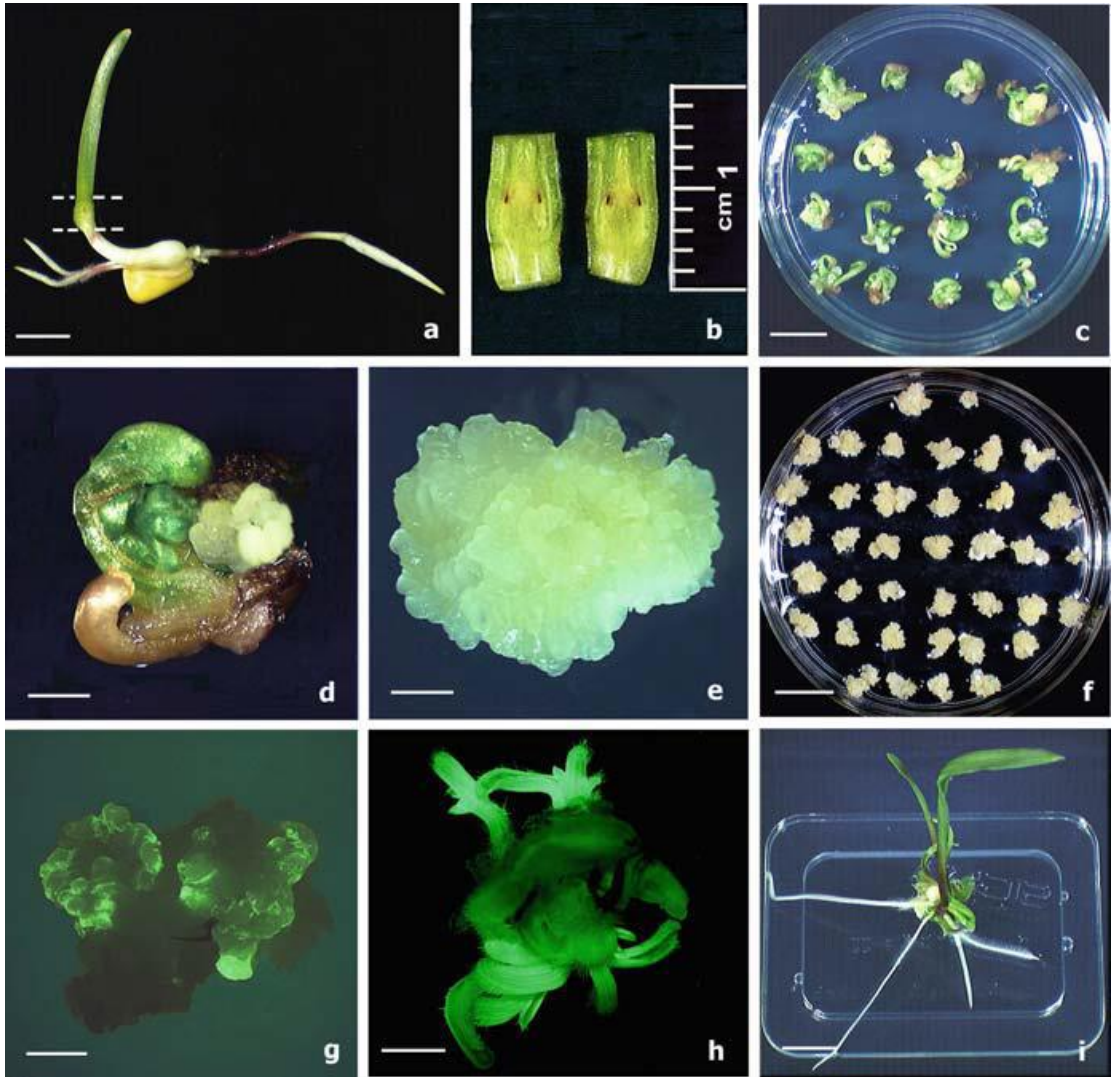
Rooting-1084

	per L
MS Basal Salts	::4.33 g
MS Fromm Vitamins (1000X):	: 1 mL
Sucrose	:20 g
pH with KOH to 5.8	
Gelzan CM	:3 g
IBA (1 mg/ml)	:0.75 mL
NAA (1 mg/ml)	:0.5 mL
Glyphosate (0.5M)	:0.2 mL

Transformation of Seedling-Derived Callus

Transformation of Seedling-Derived Callus

- Embryogenic callus derived from plant seedlings has also been found suitable for the transformation of a wide variety of corn genotypes including commercial inbred lines. ***This system does not require donor plant materials for immature embryo isolation***



Procedure:

Agrobacterium-mediated transformation of seedling-derived callus (cv. LH198 × HiII):
(**a**) Seven days-old corn seedling germinated on MSV34 medium (bar = 10 mm).
(**b**) Nodal sections of seedling used as initial explants for callus induction.
(**c**) Plate with nodal cuttings of seedling cultured for 4 weeks on MSW57 medium (bar = 16 mm).
(**d** , **e**) Formation of embryogenic callus of on MSW57 medium and its morphology after establishment (bar = 4 mm and bar = 1.6 mm, respectively).
(**f**) Petri plate with callus clumps suitable for *Agrobacterium* - mediated
transformation (bar = 16 mm).
(**g**) Transient GFP expression in seedling-derived callus; 2 days after inoculation (bar = 1.7 mm).
(**h**) Regeneration of GFP positive shoots on MSGR – medium (bar = 4 mm).
(**i**) Rooting of plant in Phytatray ™ with MSGR – medium (bar = 16 mm).

Induction of callus from seedling-derived callus

Seed Sterilization and Germination

Fungicide-treated seeds are recommended to use as a starting material. MSVS34 medium for seed germination.

- Put the seeds into sterile Petri plates (1–2 layers of seeds). 3–4 plates with seeds can be placed into one plastic vacuum desiccator.
- Connect vacuum desiccator hose to a vacuum in a fume hood, place 200 ml of undiluted sodium hypochlorite (6.15% active ingredient) in a 200-ml glass beaker and place the beaker in the center of the desiccator. Place plates with seeds into the desiccator and slightly open the lid of each Petri plate. Add 2 ml of concentrated HCl to the beaker.
- Put the cover on the desiccator, turn on the vacuum, and leave on long enough to pull a vacuum. Usually, it takes 1 min. Shut off the vacuum valve and leave the desiccator for 8–12 h.
- When done, release the vacuum, open the desiccator, and close the plates containing the seeds. This is best done in an M2 biosafety hood to minimize chance of re-contaminating the seeds and being exposed to chlorine gas.
- In a culture hood, place 9 or 12 sterilized seeds in Petri dish or Phytatray ™ with MSVS34 germination medium
- Hold 2–3 Petri dishes together with a rubber band and keep the dishes in a transparent plastic box. (The rubber band will keep the Petri dish lid on as the seeds germinate).
- Germinate seeds in a lighted growth chamber (16 h light, 80–100 E, and 27–28°C) for 7 days.

Callus Induction and Propagation

For callus induction, well developed 7-days-old seedlings were used:

- In the culture hood open the plates of germinated seeds, cut the seedlings from the seeds and put approximately eight cut seedlings into an empty Petri dish.
- Hold the seedling with a pair of forceps. Using a scalpel blade cut off the upper part of the seedling approximately 5 mm above and below the apical meristem (nodal area).
- Next, make a longitudinal cut through the entire stem piece
- Place each piece with the wounded surface down on semi-solid callus induction MSW57 medium, 10–16 pieces per plate.
- Incubate plates in a lighted growth chamber (16 h light, 80–100 par light, and 28°C).
- After 2–4 weeks, examine the plates under microscope. Transfer embryogenic callus to fresh MSW57 medium. Nodal cuttings with callus appear after 4 weeks of culture.
- Incubate *in darkness* at 28°C for 2–3 weeks.

Inoculation and Co-cultivation/Desiccation

Agrobacterium preparation is the same as for immature embryo transformation but can be diluted to O.D. 660 0.5.

- Collect in 50-ml sterile centrifuge tubes the callus pieces established and cultured on MSW57, which have been sub-cultured for approximately 1 week. Each tube may contain 10 ml of tissue.
- Add to the tube enough prepared *Agrobacterium* solution to cover the tissue. Set the tube aside for 30 min.
- Remove *Agrobacterium* suspension with a fine tip 5-ml pipette.
- Dump the callus pieces onto two pieces of filter paper (Whatman #1, 8.5 cm in diameter) in a Petri dish (90 × 25 mm). Evenly disperse the callus pieces on the filter paper. Wrap the dishes with rubber bands and place them in a growth chamber (24°C, dark) for 2 days. (This is the coculture stage with a desiccation treatment.)

Selection and Regeneration

This protocol is recommended for selection with paromomycin or kanamycin.

- After the 2-day desiccation/coculture, transfer callus to MSW57 (medium containing 500 mg/l carbenicillin and 100 mg/l paromomycin), about 15–25 callus pieces per plate. Culture for 3 weeks in the dark at 28°C. The transient expression of GFP in callus can be seen at this stage
- Transfer the callus pieces to MSW57 medium and break up each callus into 2–6 pieces. Culture for 3 weeks in the dark at 28°C. Resistant clones start to emerge at the end of this culture. If clones are not large enough for regeneration, leave on the same medium for up to 2 weeks without transferring. Alternately, an additional subculture on MSW57 can be done.
- Transfer the resistant clones to MSBA (250 mg/l carbenicillin and 100 mg/l paromomycin) medium. Culture in the dark at 28°C for 7 days.
- Transfer the tissues to MSGR–medium containing 250 mg/l carbenicillin and 100 mg/l paromomycin medium in Phytatray ™ or deep 90 × 25 mm Petri dishes. Place tissue at a density of 1–6 putative transformants/Phytatray ™ or deep dish. Place in the high light growth chamber (16 h light, 80–100 par light, and 28°C) for approximately 4–6 weeks. Plants may need to be broken up for an additional subculture in 90 × 25 mm Petri dishes or Phytatray ™. GFP expression in regenerating shoots and rooting of transgenic plant can be observed.
- Plantlets can be transferred into soil

Media used for *Agrobacterium*-mediated transformation of seedling derived callus

MSVS34 medium – germination medium

MSVS34, germination medium: 4.33 g/l MS basal salt mixture (Phytotechnology Labs)), 10 ml/l MS vitamins 100x, 40 g/l maltose, 0.5 g/l glutamine, 0.1 g/l casein hydrolysate, 0.75 g/l magnesium chloride, 1.95 g/l MES, 7.0 g/l agar, 6.0 ml/l 6-benzyladenine (0.5 mg/ml), 10.0 ml picloram (1 mg/ml), 2 ml/l ascorbic acid (50 mg/ml).

MSW57 medium – callus induction

Callus induction and maintenance medium: 4.33 g/l MS basal salt mixture (Phytotechnology Labs)), 10 ml/l MS vitamins 100×, 1.25 ml thiamine HCl (0.4 mg/ml), 30 g/l sucrose, 1.38 g/l l -proline, 0.5 g/l casamino acids (Difco ®), 7.0 g/l agar, 0.5 ml/l 2,4- D (1mg/ml), 2.2 ml/l picloram (1 mg/ml), and 1.7 ml/l silver nitrate (2 mg/ml).

MSW57 medium – selection medium

0.1 mM gly/C500 and MSW57/0.25 gly/C500, selection media: MSW57 medium containing with 2 ml/l carbenicillin (250 mg/ml) and 100 mg/l paromomycin

MSBA medium - regeneration

Regeneration medium: 4.33 g/l MS basal salt mixture (Phytotechnology Labs), 1 ml/l Fromm vitamins *(12)*, 1,000× stock, 7.04 ml/l 6-benzyladenine (0.5 mg/ml), 30 g/l sucrose, 1.36 g/l L -proline, 0.05 g/l casamino acids (DifCo), 1 ml/l carbenicillin (250 mg/ml), and 100 mg/l paromomycin.

MSGR medium – rooting

regeneration/rooting medium: 4.33 g/l MS basal salt mixture (Phytotechnology Labs), 10 ml/l Fromm vitamins 1,000×, 10 g/l glucose (Phytotechnology Labs)), 20 g/l maltose (Phytotechnology Labs)), 0.15 g/l asparagine monohydrate, 0.1 g/l myoinositol, 1 ml/l carbenicillin (250 mg/ml stock), and 100 mg/l paromomycin

Summary – Corn Transformation

Plant Tissue culture

In corn, plant regeneration can occur via embryogenesis or organogenesis, which many times depend on the genotypes and target tissues.

The common steps to establish a successful regeneration systems are:

- Identification of a suitable genotype and explant
- Development of suitable medium by optimizing growth regulator is very critical to initiate the process of tissue culture (For example, undifferentiated cells of an explant begin to grow to form callus on suitable medium)
- Immature embryo or immature embryo-derived callus can be used to transform to regenerate transgenic plants

Transformation

- In corn, successful genetic transformation of immature embryos or immature embryo-derived callus depends not only on optimizing transformation parameters, but also on achieving high embryogenic callus induction frequency in a population of targeted explants.

There are several things that must happen correctly for a cell to be successfully transformed:

- The new gene must be delivered into the **nucleus** of a cell and insert into a **chromosome**
- The cells that receive the new gene must grow on a selection medium
- The transformed cell must divide and should regenerate transgenic events on a selection medium

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Any questions?