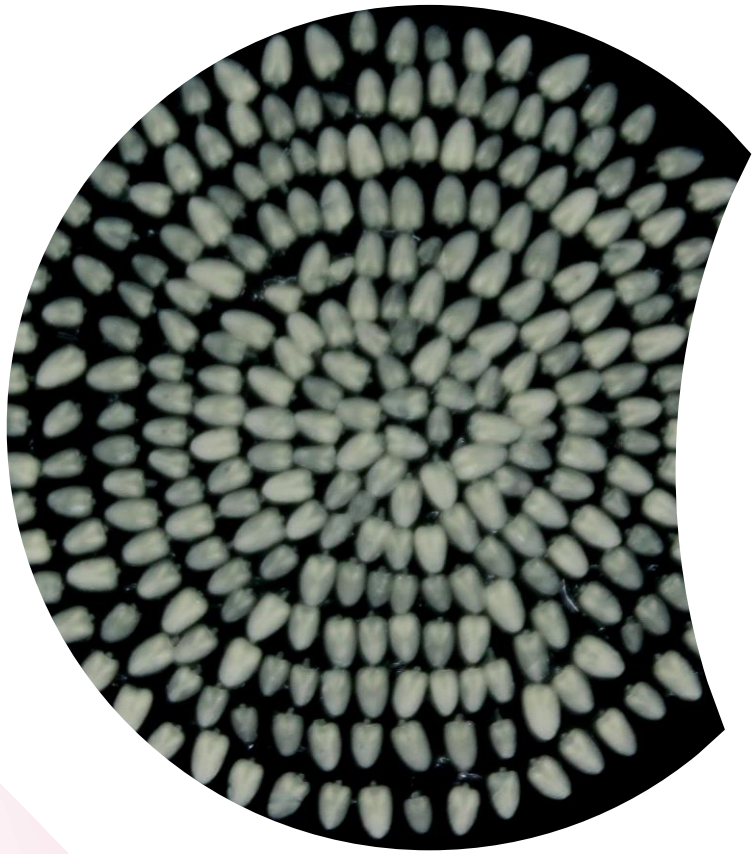
Introduction to Plant Tissue Culture and Transformation

Bayer Russia Biotechnology Conference

July 2023

Introduction to plant tissue culture and transformation



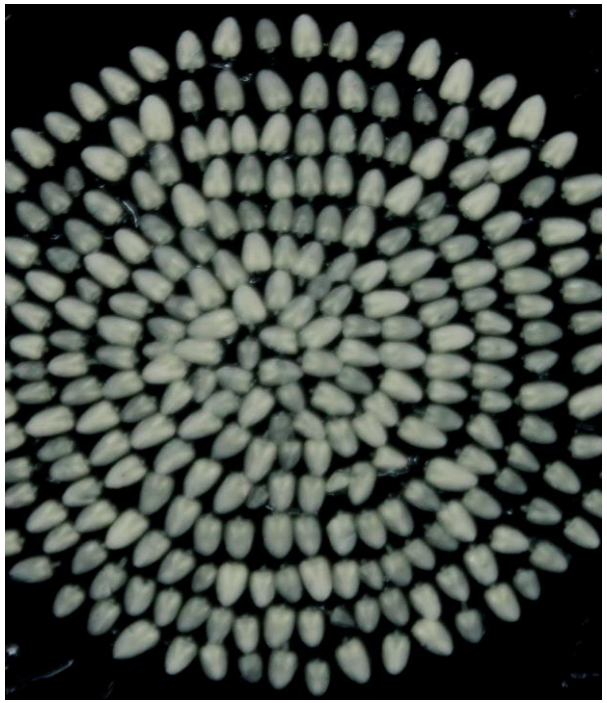
- What is plant tissue culture and regeneration?
- How to establish a successful plant tissue culture system in the lab
- *How can we achieve successful Plant regeneration?*
- *What is plant transformation?*
- *How can we transform plant tissue successfully and get transgenic events*

What is plant tissue culture and regeneration?

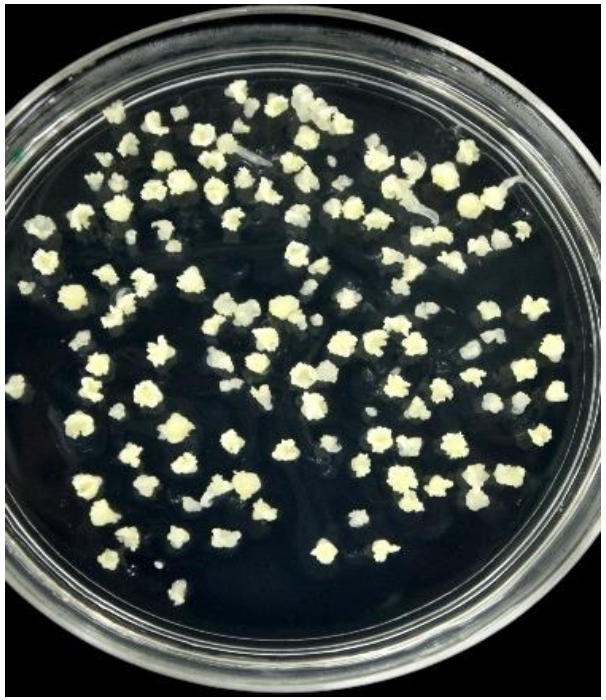
Plant tissue culture is the process of growing plant cells or tissues (called as an **explant**) *in vitro* on **synthetic medium** containing plant growth regulators (hormones)

Regeneration is the process where **totipotent cells or tissues** are differentiated and developed into new plants

→ **Explant: an excised piece or part of a plant used to initiate a tissue culture**

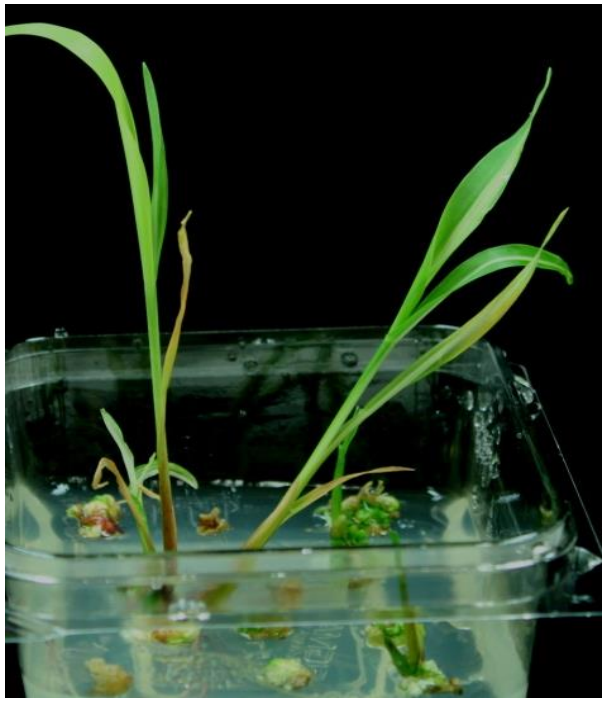


Explant with an actively dividing cells



Differentiation of cells into callus

Proliferation of callus



Regeneration of plants

→ **These explants are cultured *in vitro* on Synthetic Medium**: a solid or liquid nutritive solution used for culturing cells

Plant Tissue Culture and Transformation Terminology

Aseptic condition: sterile condition (working in sterile environment such as in Laminar Flow Hood) to initiate a tissue culture

Callus: Callus is defined as a group of cells derived from competent source tissue that is cultured under *in vitro* conditions to form an undifferentiated mass of cells

Undifferentiated tissue: undifferentiated tissue is referred to cells that do not have specialized structures of functions (e.g. callus)

Differentiated tissue: Differentiated tissue is referred to cells that maintain all or much of the specialized structure and function typical of the cell type *in vivo (callus makes organ)*.

Plant regeneration: is the outcome of plant tissue culture, which is based on the principle of totipotency. Regeneration can be achieved by organogenesis or somatic embryogenesis.

Totipotency: capacity of plant cells to regenerate whole plants when cultured on synthetic medium

Somatic embryogenesis: Somatic embryogenesis is a developmental process where a plant somatic cell may undergo embryogenic pathway to form somatic embryos, which are grown to regenerate whole plants. In comparison, plants can be regenerated via organogenesis. Organogenesis means formation of organs from the cultured explants. The shoot buds or monopolar structures are formed by manipulating the ratio of cytokinin to auxin in the cultures

Organogenesis: Direct organogenesis: direct regeneration without callus intervening. **Indirect organogenesis**: first callus is formed in indirect organogenesis and then regeneration occurred. Organogenesis means formation of organs from the cultured explants. The shoot buds or monopolar structures are formed by manipulating the ratio of cytokinin to auxin in the cultures

Plasmid: A plasmid is a small, circular, double-stranded DNA molecule that is distinct from a cell's chromosomal DNA

Genetic transformation: Transformation is a process by which foreign genetic material is taken up by a cell. The process results in a stable genetic change within the transformed cell.

Developing Plant Tissue Cultures

- **Crop Species**
- **Genotype(s) – screening of genotype to check the tissue culture response**
- **Explants – screening of a suitable explants by investigating the potential totipotent cells**
- **Development of a tissue culture protocol by understanding the balance of plant growth regulators in the synthetic medium**
- **Plant tissue culture conditions – equipment that are required to establish a successful tissue culture lab**

And more than anything, an expert cell biologist/tissue culture and transformation scientist

Plant Tissue Culture Specific Equipment

Laminar flow hoods for tissue culture work

Lighted incubators for early tissue culture work

Green house to grow Regenerated events

REQUIRED MATERIALS, EQUIPMENT, AND DOCUMENTS Standard laboratory safety procedures in addition to any requirements

- Crop species/genotypes/seeds
- Petri dishes
- Phytatray or other culture vessels
- Beakers
- Flasks
- Pipette
- Sodium hypochlorite (6.15% active ingredient)
- Vacuum desiccator
- Parafilm M ®.
- Whatman #1 filter paper
- Translucent plastic box
- Forceps, scalpels, and spatula
- Bead sterilizer
- Regular dissecting microscope (Zeiss or another brand)
- Tabletop centrifuge
- Spectrophotometer
- Rotary shaker 150 rpm
- Dark, 24°C growth culture chamber
- Lighted, 28°C growth culture chamber
- Dark, 28°C growth culture chamber or warm room
- Media

Needs of Plant Tissues

Macronutrients – Nitrogen, Potassium, Phosphorus, Calcium, Magnesium, Sulfur

Micronutrients – Iron, Manganese, Molybdenum, Zinc, Chlorine, Nickel, Copper, Boron, Iodine, Cobalt

Vitamins – Thiamine (B1), Niacin, Riboflavin, Folate, Pyridoxine (B6), Pantothenic acid, Biotin

Carbohydrates –Sucrose, maltose glucose

Gelling Agents – Agar, Gels, Agarose, Gelatin

Buffers (Typical pH range 5.4-5.8)

Plant Tissue Culture Media

PLANT TISSUE CULTURE BASAL MEDIUM	
$Ca(NO_3)_2$	100 mg
KNO_3	80 mg
$MgSO_4$	35 mg
KCl	65 mg
KH_2PO_4	12 mg
NH_4NO_3	400 mg
FeNaSequestrene	25 mg
KI	0.8 mg
$MnSO_4$	4.4 mg
$ZnSO_4$	1.5 mg
H_3BO_3	1.6 mg
Glycine	2.0 mg
Thiamin · HCl	0.1 mg
Niacin	0.5 mg
Pyridoxin · HCl	0.1 mg
Glucose or sucrose	20 grams
Distilled water to	1000 ml

Commercially available media

Optimization of medium is critical to develop a successful plant tissue culture and regeneration System for each crop species and explant

Plant Growth Regulators

Natural or synthetic compounds that are active at low concentrations

Elicitors of plant cells growth and development

The classical hormones:

Auxin (such as 2,4-D, Picloram, NAA)

Cytokinin (such as BAP, Zeatin)

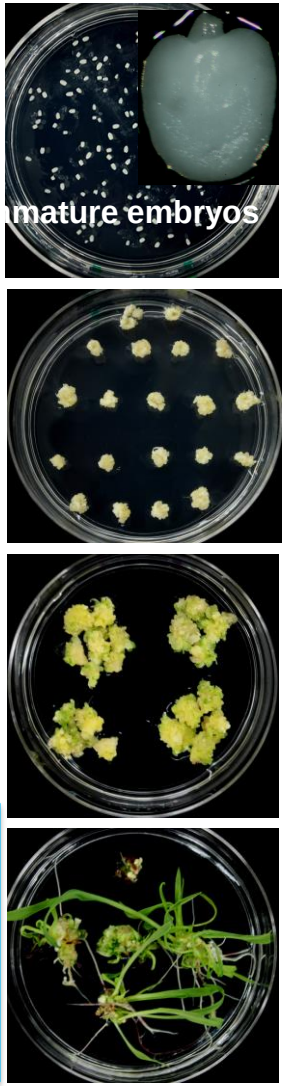
Example of optimized medium for callus induction and plant regeneration from immature embryo of maize

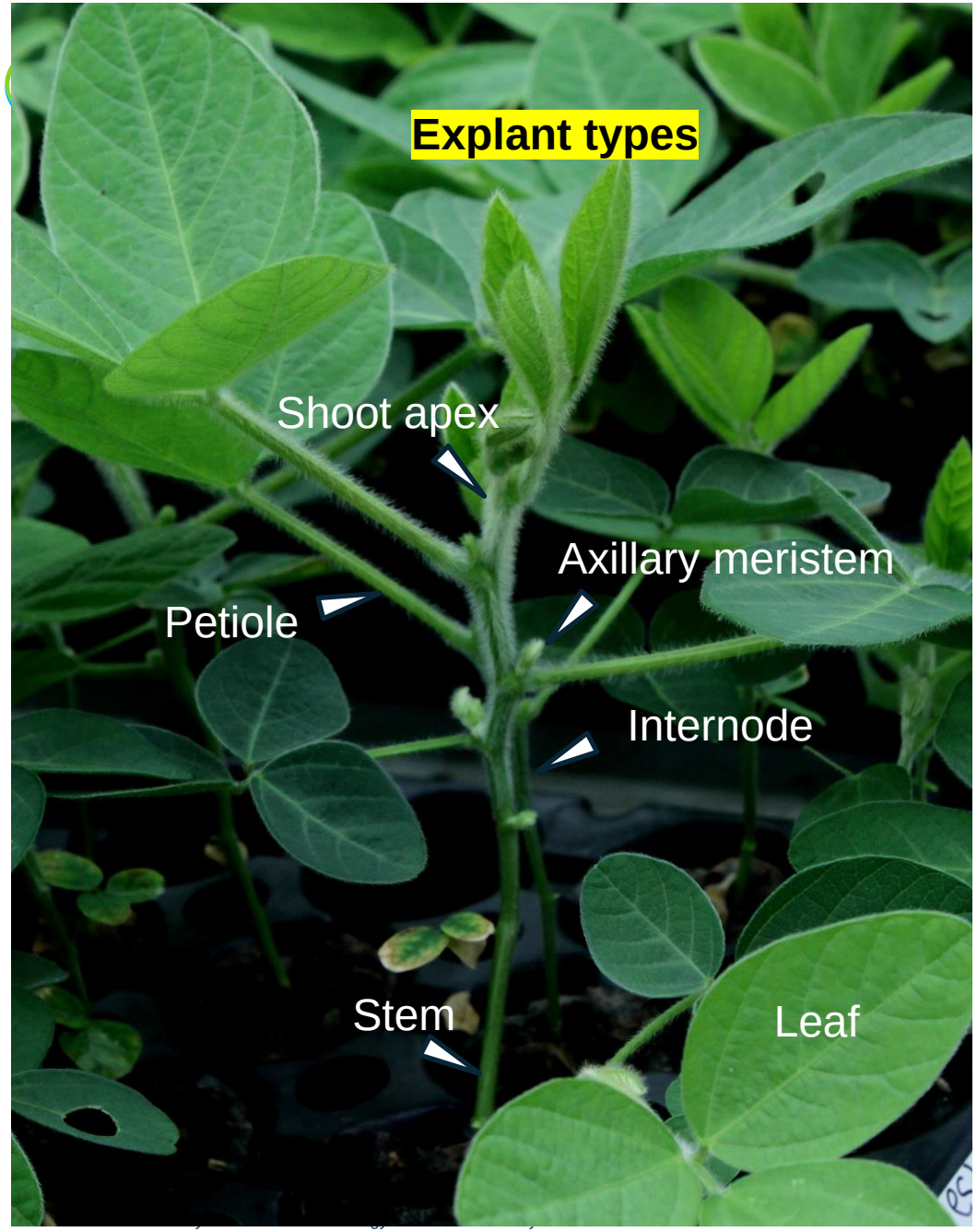
		Callus Induction: Selection MS (mg/L)
Macronutrients	Potassium nitrate	1900
	Ammonium Nitrate	1650
	Calcium Chloride dihydrate	440
	Magnesium Sulfate Heptahydrate	370
	Potassium phosphate monobasic-anhydrous	170
Micronutrients	Boric Acid	6.2
	Sodium Molybdate (VI)-2H2O	0.3
	Zinc Sulfate-7H20	8.6
	Manganese Sulfate-H2O	16.9
	Potassium Iodide	0.8
Iron	Ferrous Sulfate-7H2O	27.8
	Sodium EDTA	37.3
Carbohydrate	Sucrose	30 g/L
	Dextrose	
	Maltose	
Vitamins	Thiamine HCL	0.1
	Nicotinic acid	0.5
	Pyroxidine HCl	0.5
	glycine	2.0
	Calcium Pantothenate	
	myo inositol	100.0
Amino Acids	L-Proline	1380
	Casamino Acids	500
	Asparagine monohydrate	
Gelling Agent	Gelzan	3000
Additives	Silver nitrate	3.4
Hormones- Auxin	2,4-D	500
Hormones-Cytokinin	BAP	0.010
Antibiotics	Carbenicillin	500
Selective Agent	Glyphosate	0.1M

Hormones in the callus induction and selection medium

		Regeneration Mg/L
Macronutrients	Potassium nitrate	1900
	Ammonium Nitrate	1650
	Calcium Chloride dihydrate	440
	Magnesium Sulfate Heptahydrate	370
	Potassium phosphate monobasic-anhydrous	170
Micronutrients	Boric Acid	6.2
	Sodium Molybdate (VI)-2H2O	0.3
	Zinc Sulfate-7H20	8.6
	Manganese Sulfate-H2O	16.9
	Potassium Iodide	0.8
Iron	Ferrous Sulfate-7H2O	27.8
	Sodium EDTA	37.3
Carbohydrate	Sucrose	
	Dextrose	20 g/L
	Maltose	20 g/L
Vitamins	Thiamine HCL	0.25
	Nicotinic acid	1.25
	Pyroxidine HCl	0.25
	glycine	
	Calcium Pantothenate	0.25
	myo inositol	100
Amino Acids	L-Proline	
	Casamino Acids	
	Asparagine monohydrate	150
Gelling Agent	Gelzan	3000
Additives	Silver nitrate	
Hormones-Auxin	2,4-D	
Hormones-Cytokinin	BAP	
Antibiotics	Carbenicillin	250
Selective Agent	Glyphosate	0.1M

No hormones in the regeneration medium





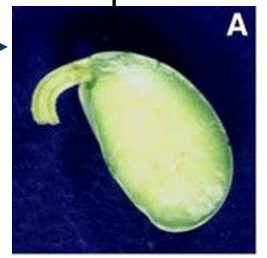
Totipotent Cells (explant)

An explant is a part of the plant by which a whole plant can be produced through plant tissue culture technique

Totipotency is the ability of a single cell to divide and produce all the differentiated cells in an organism

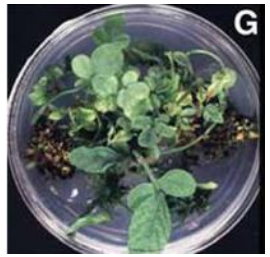
Since plant cells are totipotent, growth hormones can be added to the medium to induce callus or direct shoot formation

Explant



Differentiation

Regeneration



Plant regeneration

Plant regeneration is the process of growing plants using tissue culture techniques. Plant regeneration can be achieved via somatic embryogenesis or organogenesis. In both process, first callus is formed from an explant on a suitable medium

Plant regeneration

Plant regeneration is the process of growing plants using tissue culture techniques. Plant regeneration can be achieved by culturing explants to form organs (organogenesis), or by developing plant embryos (somatic embryogenesis). Both techniques can lead to regenerating entire plants.

Somatic embryogenesis

Embryogenesis is the process of development of embryo from somatic cells. Because of the embryo origin from somatic cells in culture, this process is named somatic embryogenesis (SE).

Direct somatic embryogenesis

Somatic embryos are formed directly from a cell or small group of cells (not common)

the totipotent cells undergo embryogenic pathway to form somatic embryos directly from an explant, which can be isolated and grown to whole plants.

Indirect somatic embryogenesis

Callus is first produced from the explant, and then embryos are formed from the callus tissue or a cell suspension culture (common procedure)

the totipotent cells undergo embryogenic pathway to form somatic embryos in callus and regeneration is achieved from a mixture of cells in callus

Organogenesis

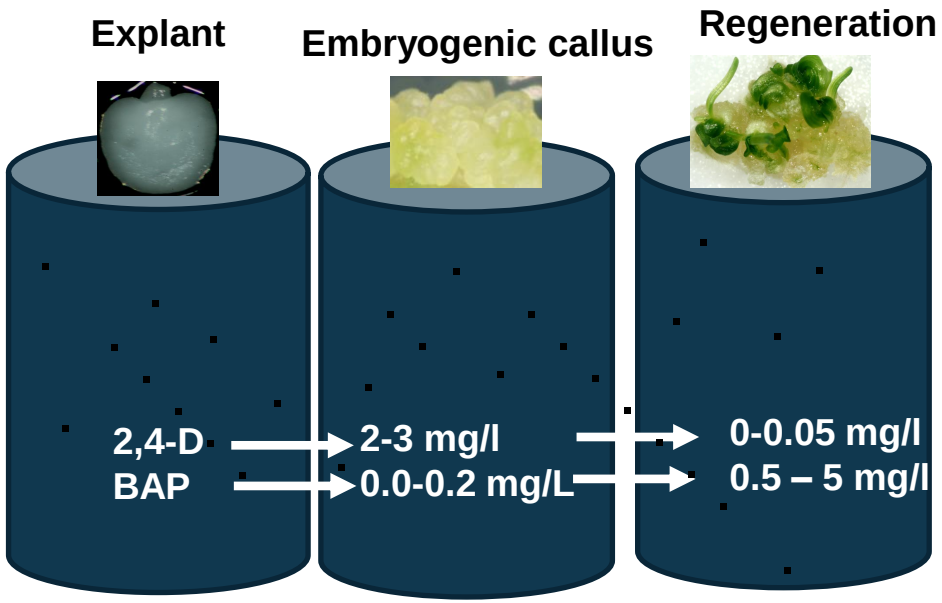
Organogenesis is the process of plant regeneration from callus without somatic embryo formation

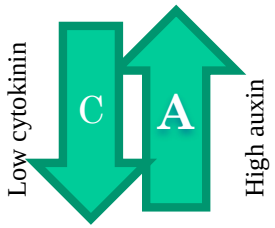
The process in which plant organs are derived from a callus is termed indirect organogenesis.

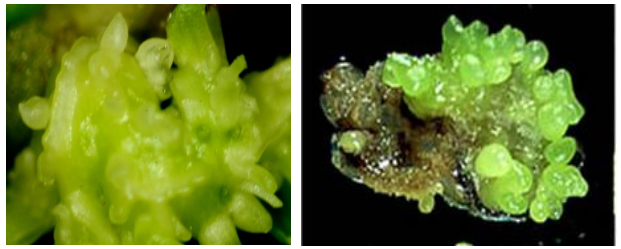
Shoot regeneration

Traditional Model of Hormonal Control in Plant Tissue Culture

Plant regeneration via embryogenic callus induction

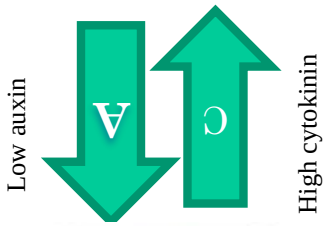






Embryogenesis

- High auxin to cytokinins ratio results in embryogenesis/embryogenic callus induction
- High cytokinin to auxin ratio promotes shoot proliferation/regeneration



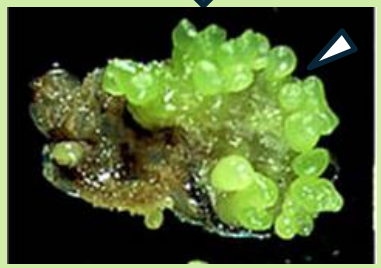
Regeneration

Types of Plant regeneration

Embryogenesis

Types

Direct



There is not callus phase

Indirect

There is a callus phase

Plant regeneration

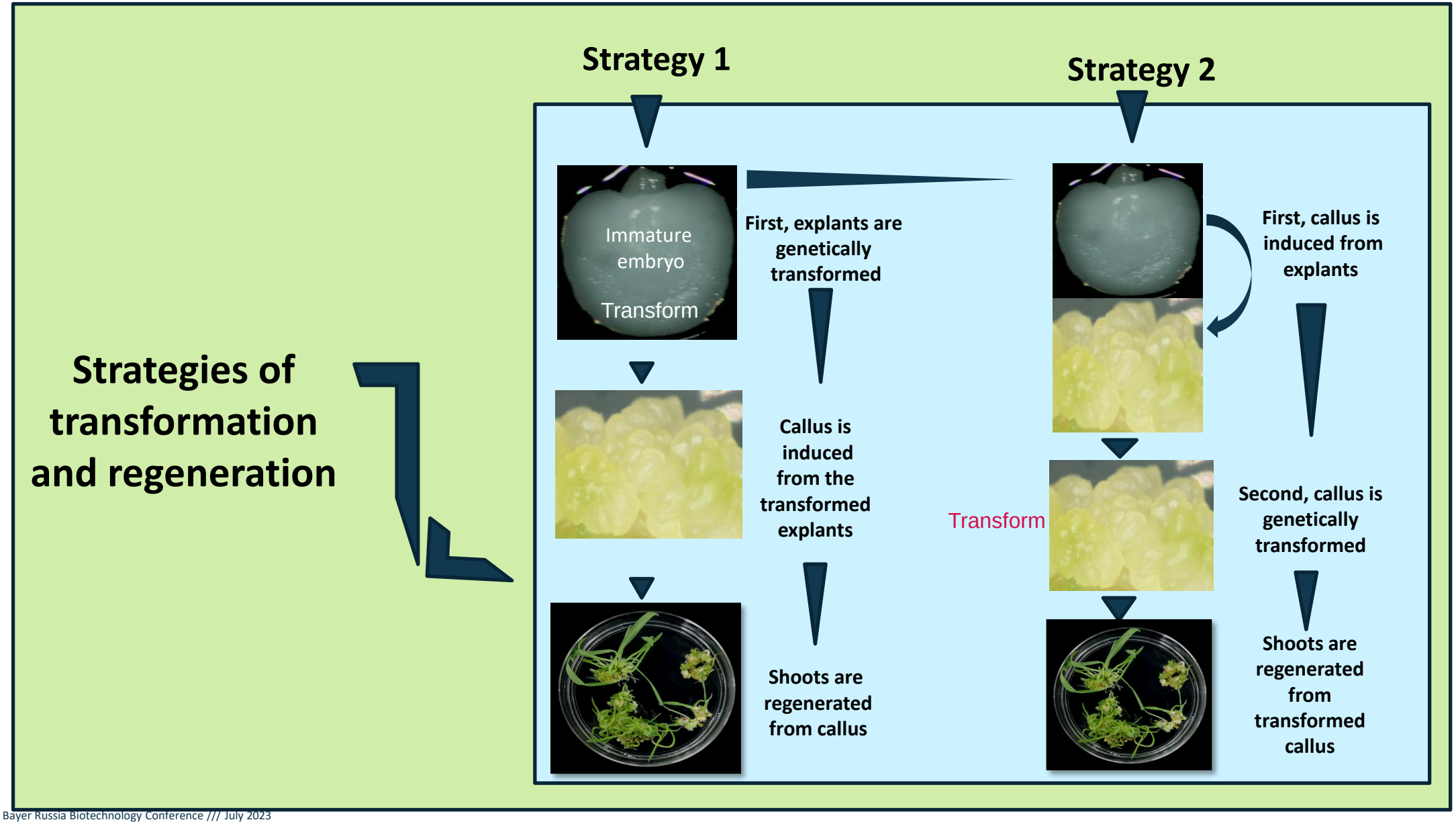
Organogenesis

First, Callus is induced

Second, regeneration

There is a callus phase

Common methods of transformation and regeneration

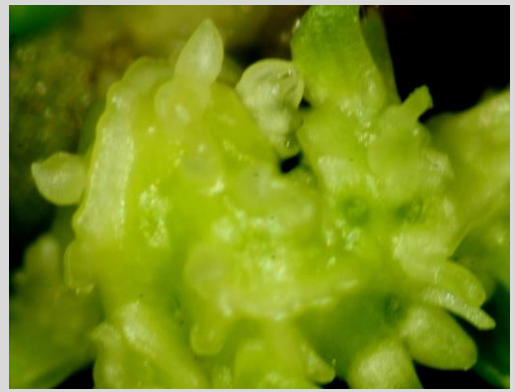


Factors Affecting Organogenesis

- Type of Explant
- Plant Growth Regulators
- Culture Conditions

Factors Affecting Somatic Embryogenesis

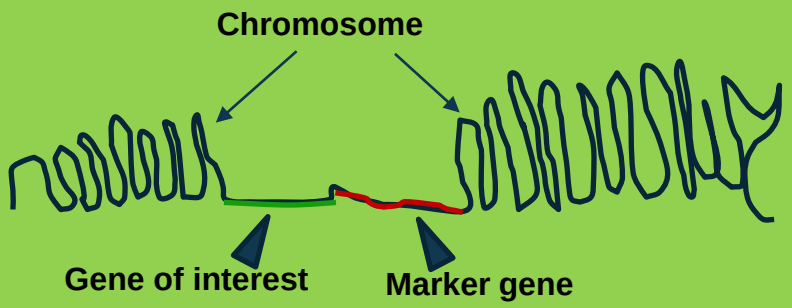
- Type of Explant
- Plant Growth Regulators
- Culture Conditions
- Embryo Maturation



Plant Transformation

Plant transformation is the step in the **genetic engineering** process where a new **gene** (**transgene)** is inserted into a single plant cell

The new gene is delivered into the nucleus of a cell and insert into a chromosome

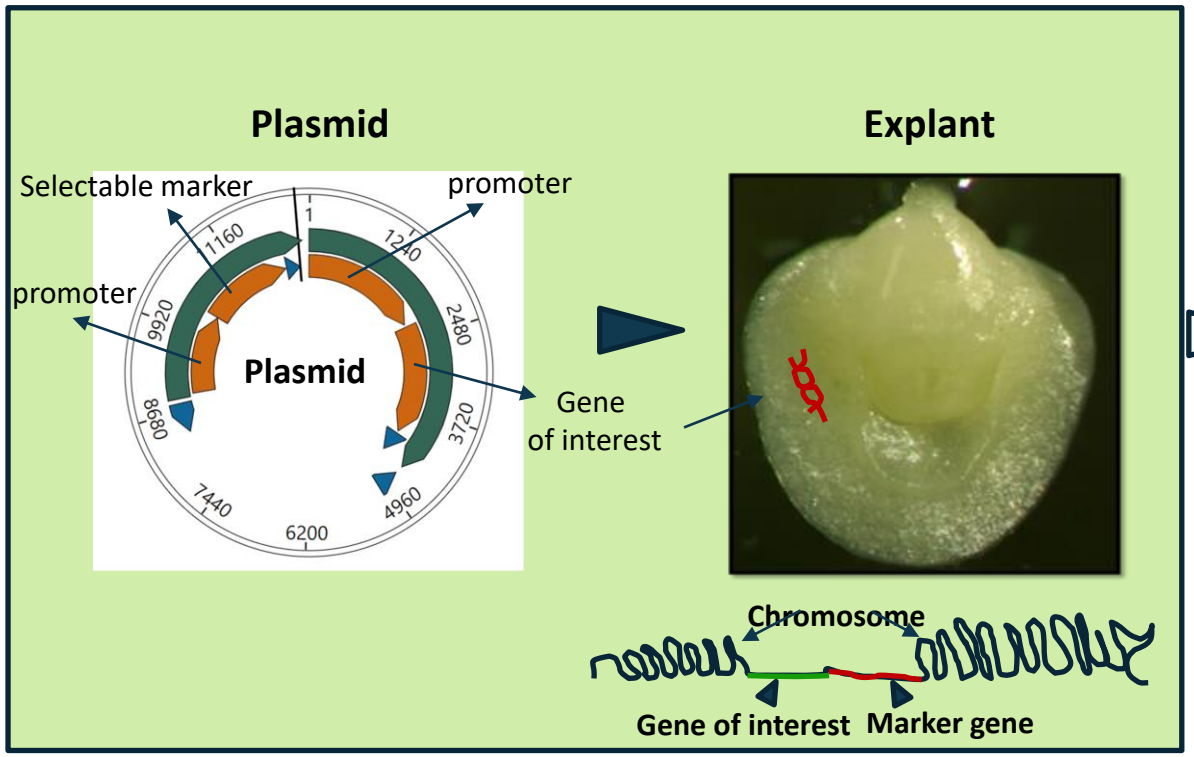


Plant Transformation

Plant transformation is the step in the **genetic engineering** process where a new **gene** (**transgene)** is inserted into a single plant cell

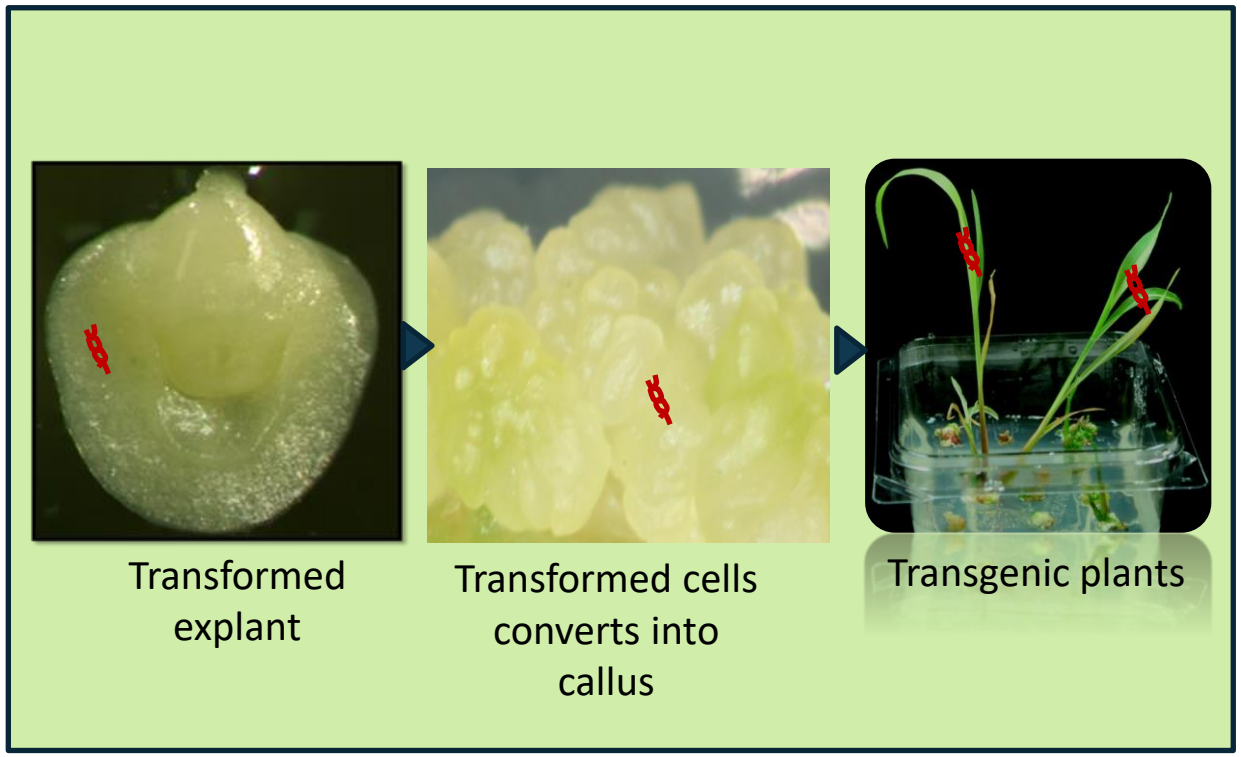
Plant transformation involves two steps: Delivery of the DNA into a single cell, and regeneration into full fertile plants

Delivery of DNA (a gene of interest) into a single cell



In plant transformation, a gene of interest along with a selectable marker is delivered into the single cell of an explant

Regeneration of transgenic plants



Recipient cell convert into callus and callus regenerate into plants containing gene(s) of interest

Two Common Methods Used in Plant Transformation

Biological method of gene delivery

Agrobacterium tumefaciens-Mediated Transformation

***Agrobacterium*: is a soil microbe and nature's genetic engineer**
was identified as the agent causing the plant tumor, crown gall over 100 years ago, and since then, it has been extensively studied and used for genetic transformation of plants.

- ***Agrobacterium*-mediated plant transformation is a highly complex and evolved process involving both the bacterium and the host plant cell.**
- ***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**

Physical method of gene delivery

Biolistic-Mediated Transformation

"Biolistics or Gene Gun", also known as particle-mediated gene transfer, is the method of direct introduction of DNA or RNA into cells.

- In this process, DNA is coated with microparticles such as gold, which are released from a gene gun by high-pressure helium gas and directly penetrate the host cell wall

Advantages and Disadvantages of *Agrobacterium* and Biolistic-mediated transformation methods

Technique	Procedure	Most important parameters involved	Advantages	Drawbacks
Biolistics 	High density carrier particles covered with genes are accelerated through the cells	Kinetic energy of the bombarding particles, temperature, coating of DNA with microparticles, ratio of DNA to microparticles	Simple, allows transformation of different cells, independent of the physiological properties of the cell, allows the use of multiple transgenes.	High cost, parameters must be optimized to each biological target, there is a risk of multiple copies of the introduced genes, DNA and cells can be damaged.
Agrobacterium 	*Agrobacterium* infects plant cells and transfer a defined sequence of their DNA (TDNA) into the plant cell	*Agrobacterium* strain; *Agrobacterium* density, genotype, target tissue	Not expensive method, robust and reproducible transformation method, high-throughput and scalable, produce high frequency of plants with single copy of the introduced gene; less damage to target cells/tissue	Genotype and explant-dependent transformation system; not much versatile system

Agrobacterium-mediated transformation has become a preferred method of plant transformation due to high frequency of plant transformation, simplicity in use, and lost cost

How we transform plants

In most of the cases, there are two types of plant transformation:

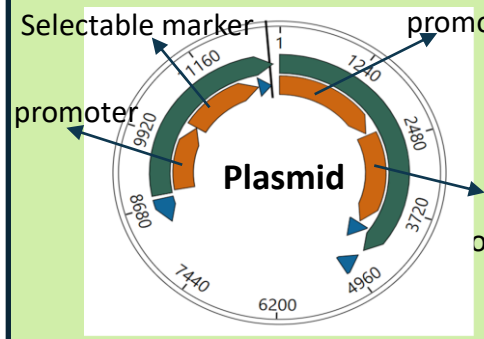
- **Transient transformation**
- **Stable transformation**

Transient Transformation: Transient transformation is used for understanding a gene or protein function. Transient transformation allows temporary introduction of gene(s) to determine their expression. Therefore, the foreign DNA is not integrated into the host cell.

Since a gene is being expressed transiently, this process takes from a few days to weeks

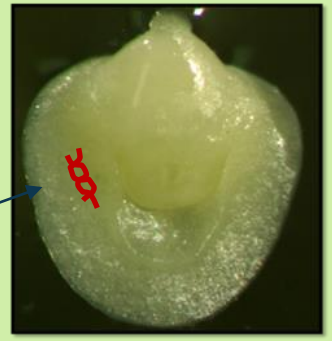
.

Transient Transformation: Delivery of DNA (a gene of interest) into a single cell and study gene expression

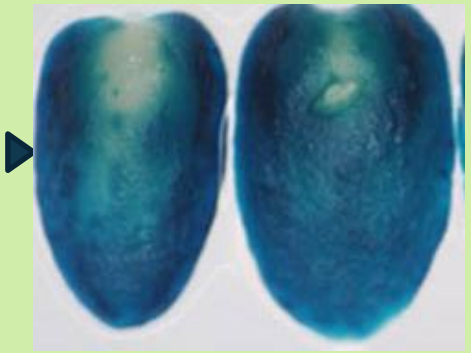


Plasmid

Gene of interest

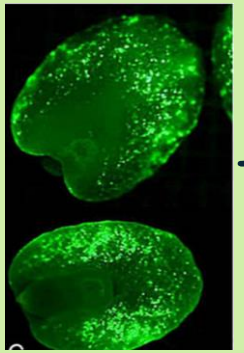


Transformed explant



GUS expression

GUS expression is a destructive assay



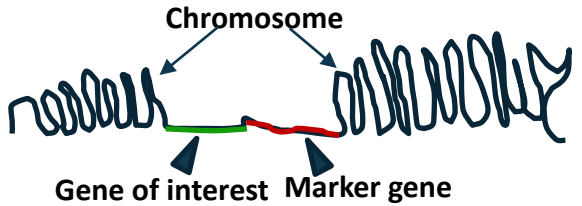
GFP expression

GFP expression is a non-destructive assay

Types of Plant Transformation

In most of the cases, there are two types of plant transformation:

- Stable transformation
- Transient transformation

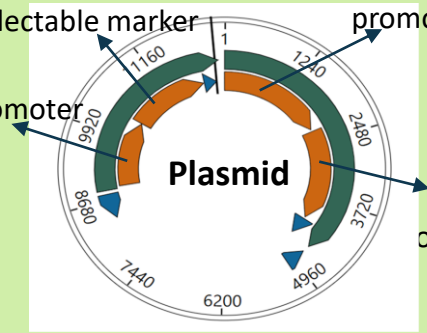


Stable transformation: Stable transformation is used for the stable introduction of a gene into the target tissue. In stable integration, the gene will be fully integrated in the plant genome and expressed in next generations of the plant.

Stable plant transformation is used to produce new plant materials with a desirable trait for agriculture

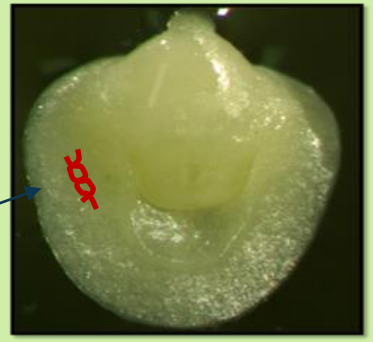
.

Stable Transformation: Delivery of DNA (a gene of interest) into a single cell and regeneration of transgenic plants



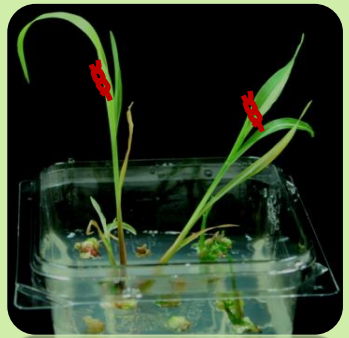
Plasmid

Gene of interest



Transformed explant

Transformed cells converts into callus

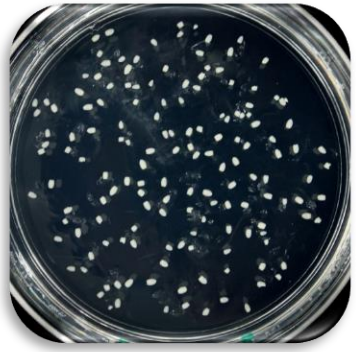


Transgenic plants

Transgenic plants in greenhouse

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

Target tissue



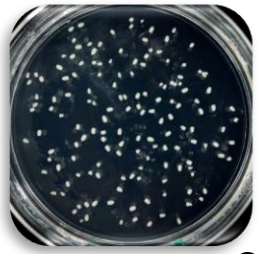
Freshly isolated embryos are used for *Agrobacterium*-mediated transformation

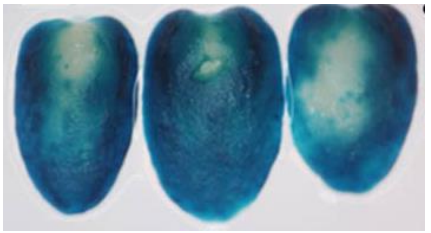
Quality of immature embryos is the most critical factor

Agrobacterium-mediated transformation

uidA gene is delivered via *Agrobacterium*-mediated transformation

Engineered Agrobacteria





GUS expression in transformed Immature embryos

Delivery parameters that are critical:

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

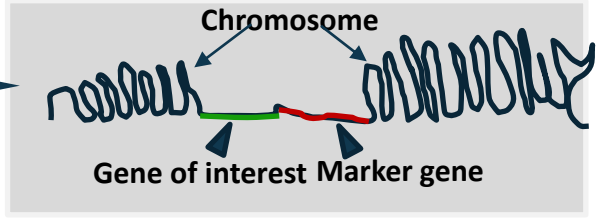
Agrobacterium-mediated transformation system is genotype and explant dependent and therefore, each critical parameters are required to optimize for a given species and target tissue

Selection of cells containing gene(s) 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 gene (such as CP4, BAR or PAT genes) will survive on a selection medium, while non-transgenic cells around them will die.

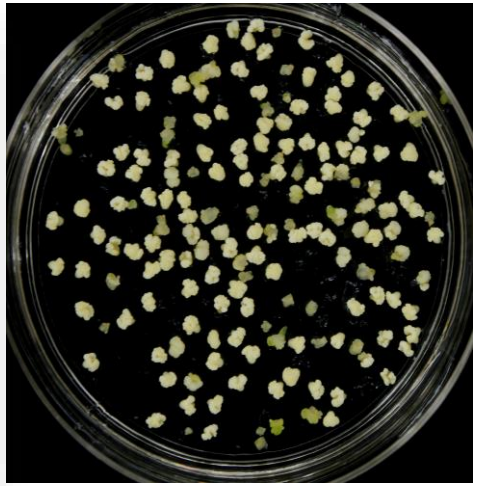
Therefore, growing transformed cells on **selection media** with the herbicide or antibiotic will allow regeneration of transgenic plants

The two most used selectable **marker** genes encode the traits of herbicide and antibiotic **resistance**



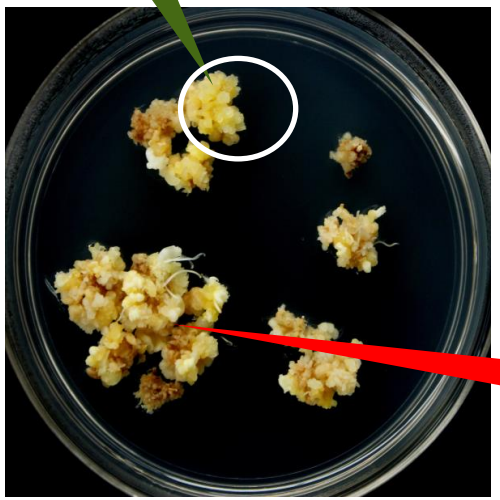
Proliferation of resistance cells

Agrobacterium-mediated transformation of corn embryos with a plasmid containing gene of interest and a selectable marker gene



Callus induction from transformed embryos

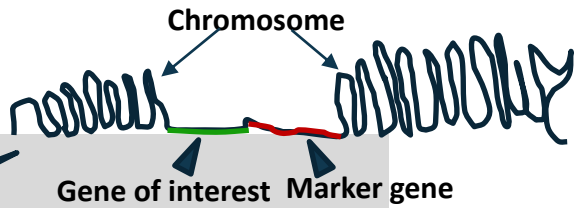
Selection of resistance cells



Proliferation of resistance Cells that containing The marker gene

Non-transformed cells are dying

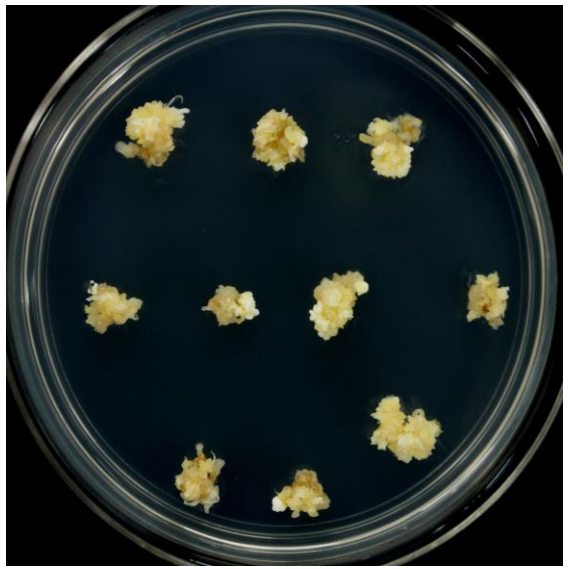
Regeneration of Transgenic Plants



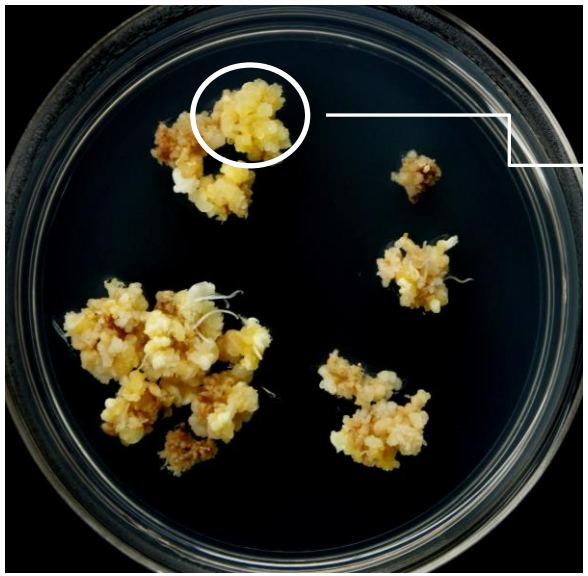
The transformation steps are:

- 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 remain alive
- The cells and plants that contain the new gene must be easily identifiable (by using an appropriate selectable marker).
- The transformed cell must divide and regenerate into transgenic plants

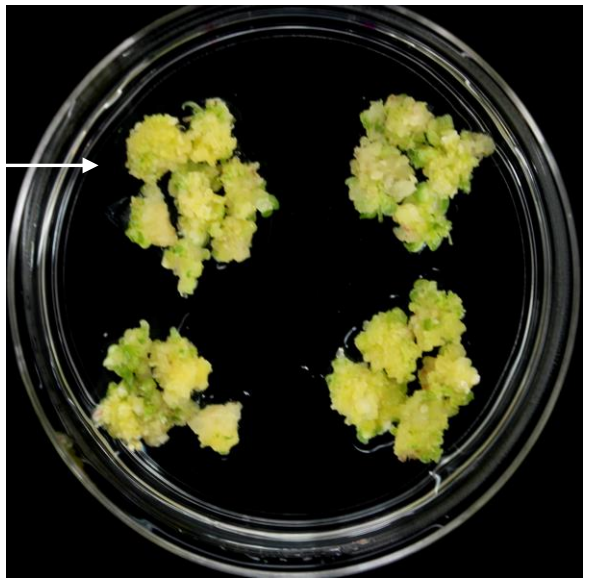
Process of regeneration of transgenic plants



Selection of resistance cells



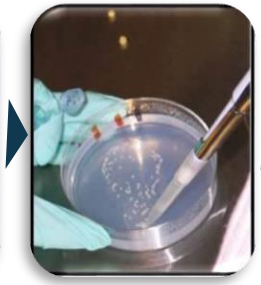
Proliferation of resistance cells



Regeneration of transgenic shoots from resistance cells

An example of a complete workflow for Agrobacterium-mediated transformation of corn immature embryos

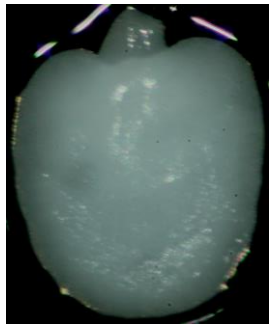
Day 1



Isolation of immature embryos

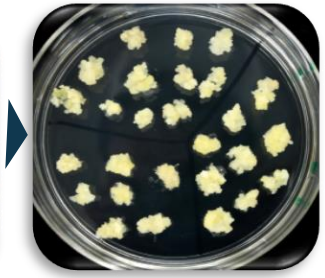
Day 1

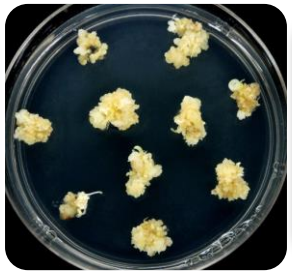
Agrobacterium-mediated transformation



Freshly isolated embryos are transformed

4 weeks

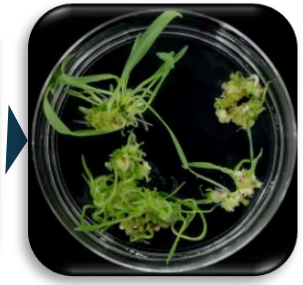




Callus induction and selection

Resistant callus can be checked for stable transformation

4 weeks



Regeneration

2 weeks

Rooting

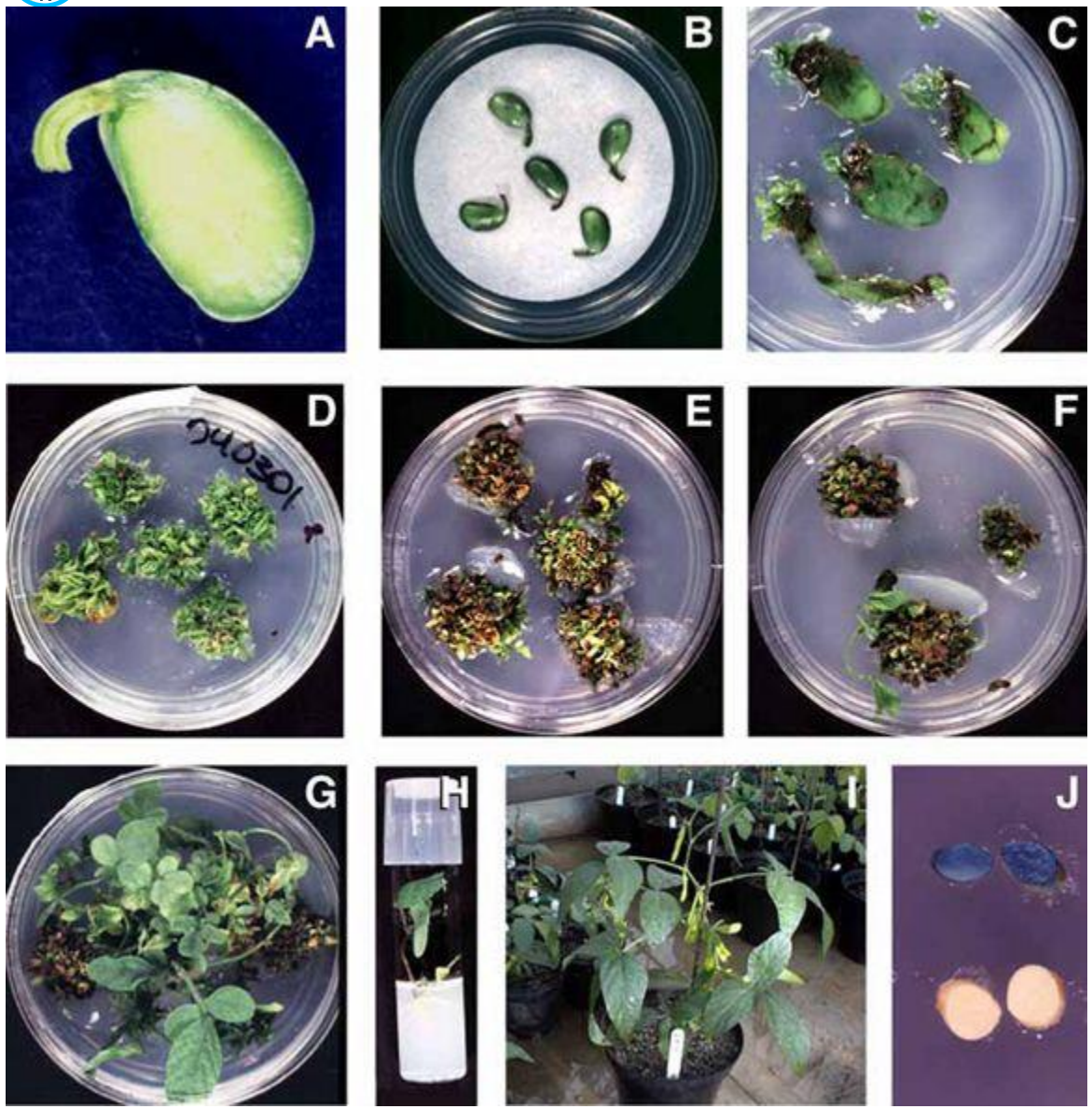
Plants can be transferred to soil 10 weeks after transformation

Transformation of Dicotyledon Plants

Examples of *Agrobacterium*-mediated transformation of soybean

Like monocot transformation such as maize and wheat, successful *Agrobacterium*-mediated transformation in dicots including soybean also depend on targeting T-DNA delivery into regenerable cells, followed by selection for transgenic cell proliferation and shoot formation

An example of Agrobacterium-mediated transformation of the cotyledonary-node of soybean



A) Explants are prepared from 5-d-old seedlings by removing the roots and the majority of the hypocotyl and wounding the axillary meristematic tissue at the cotyledonary-node

B). Inoculation of explants and co-cultivation with *Agrobacterium*

C) After 5 d, the cotyledonary node and hypocotyl of the explants are embedded into solid shoot induction medium to stimulate *de novo* shoot formation from the wounded axillary meristematic tissue

D) Explants cultured on shoot induction medium without hygromycin for 14 days

E) Selection of transformed tissue after 28 days on shoot elongation medium containing 100 mg/L paromomycin

F,G) Two months after cocultivation, explants maintained on shoot induction medium containing 50 mg/L paromomycin regenerated transgenic shoots.

H,I) Shoots elongated to at least 4 cm in length are placed in rooting medium and rooted shoots are directly transferred to a greenhouse and grown to maturity

J) T1 seeds from each T_0 plant showing GUS expression

Summary – Plant Tissue Culture and Transformation

Plant Tissue culture

A successful plant tissue culture initiation can occur via embryogenesis or organogenesis

The common steps in tissue culture 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)
- Either explant or callus can be used to transform to regeneration transgenic events

Transformation

Transformation is the step in the genetic engineering process where a gene of interest is inserted into a single plant cell.

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

DISCLAIMER

THE INFORMATION CONTAINED HEREIN IS EXPERIMENTAL IN NATURE AND IS PROVIDED "AS IS". BAYER MAKES NO REPRESENTATIONS AND EXTENDS NO WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED, AS TO THE MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, OR THAT THE USE OF THE INFORMATION WILL NOT INFRINGE ANY THIRD-PARTY PATENT, COPYRIGHT, TRADEMARK, OR OTHER PROPRIETARY RIGHTS.

Thank you!

Any questions?