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Subject: Genetic engineering

(Lecture 9)

Obtaining transgenic plants. Introduction of genes into plant cells and expression of genetic material.

Obtaining transgenic plants

Here are some common techniques used to obtain transgenic plants:

1. *Agrobacterium-mediated transformation*: This is a commonly used method for obtaining transgenic plants. In this process, the desired DNA sequence is inserted into a plasmid and then introduced into a type of soil bacteria called *Agrobacterium tumefaciens*. The bacteria then infects the plant and transfers the foreign DNA into the plant's genome.

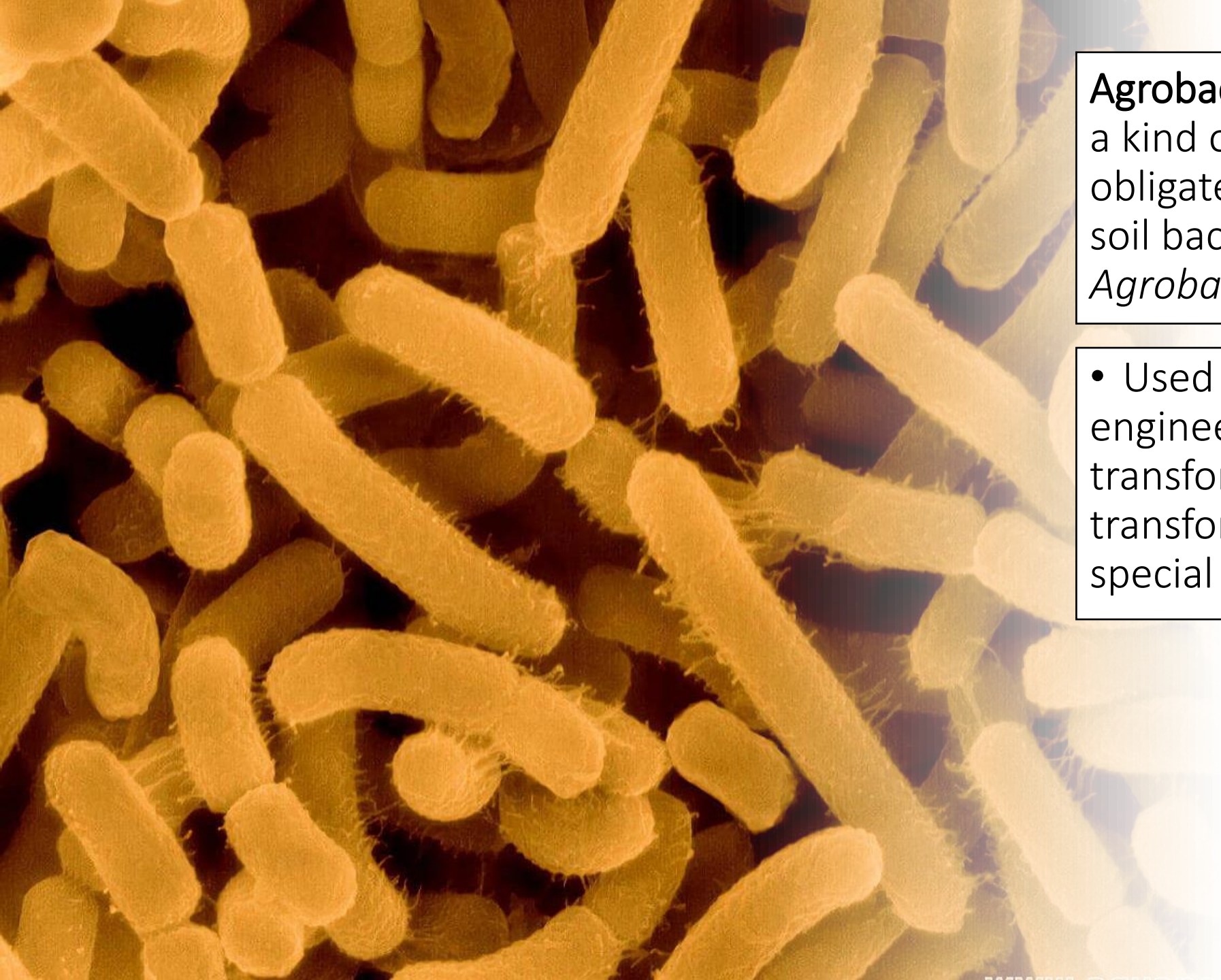
2. *Biolistic transformation*: This method involves using a "gene gun" to shoot DNA-coated particles into the plant's cells. The foreign DNA then integrates into the plant genome.

3. *Protoplast fusion*: This method involves removing the cell walls from two different plant species and fusing the protoplasts (the cells without cell walls) together. The resulting hybrid plant can contain the desired foreign DNA.

4. *Electroporation*: This method involves applying an electrical field to plant cells, which creates small pores in the cell membranes that allow foreign DNA to enter.

Obtaining transgenic plants using A.tumefaciens

Nicotiana tobacco



Agrobacterium tumefaciens - a kind of gram-negative, obligate aerobic rod-shaped soil bacteria of the genus *Agrobacterium*.

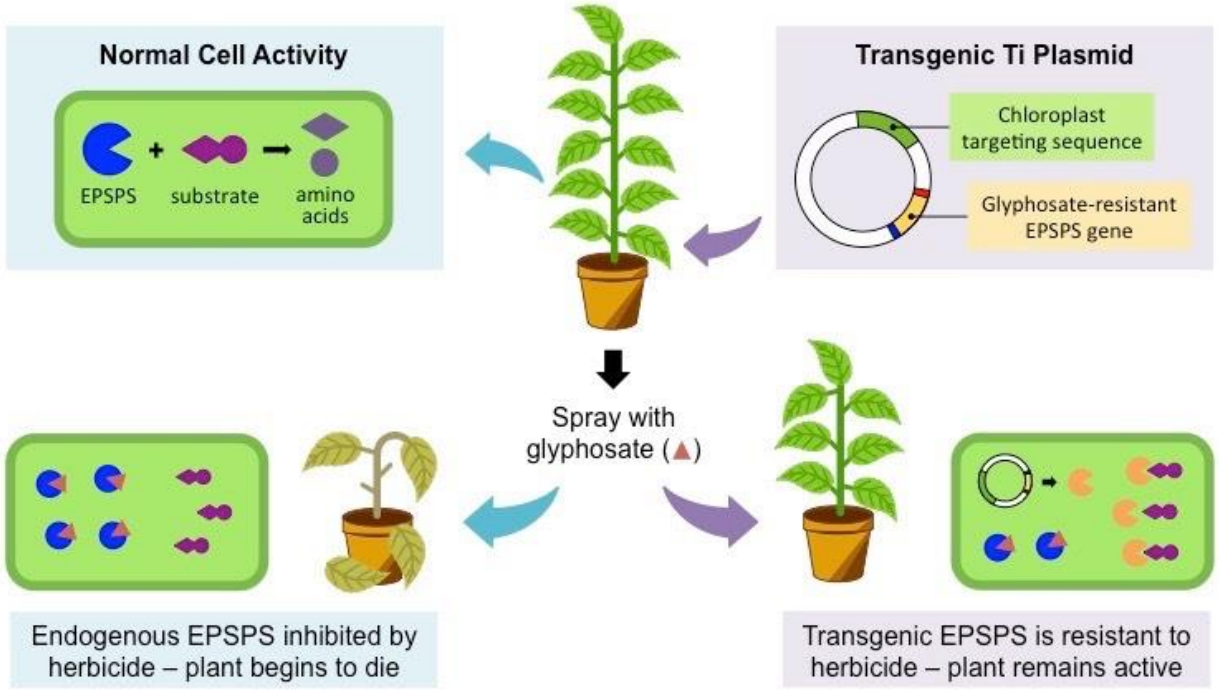
- Used in genetic engineering for plant transformation. Able to transform plant cells using a special plasmid.

Phytopathogen causes the formation of so-called crown galls in plants.

In **1907** the pathogenicity of *A. tumefaciens* was identified by E.Smith and K.Townsend.

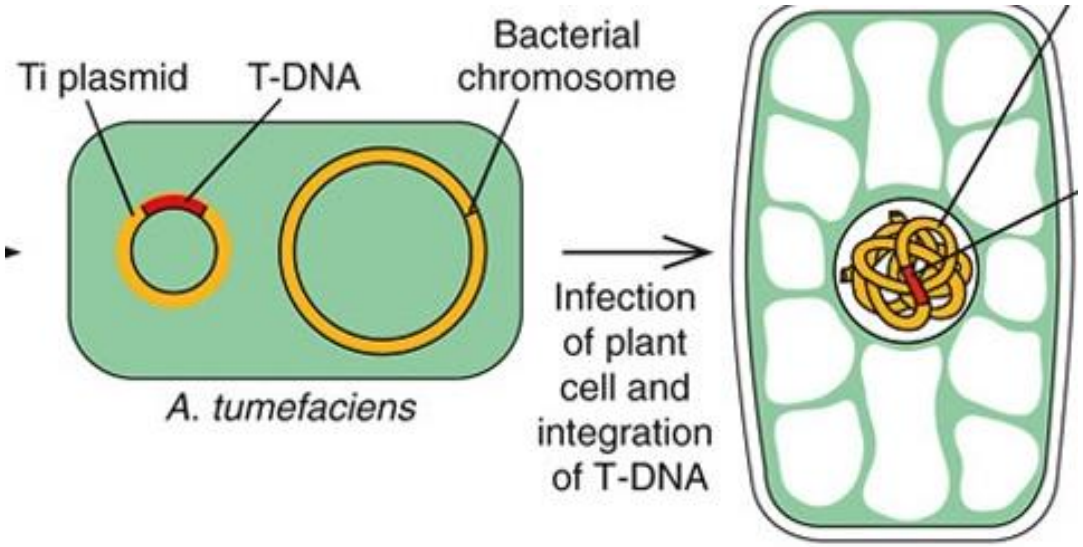
In **1974**, it was established that the tumor agent is Ti (tumor inducing) - a plasmid.

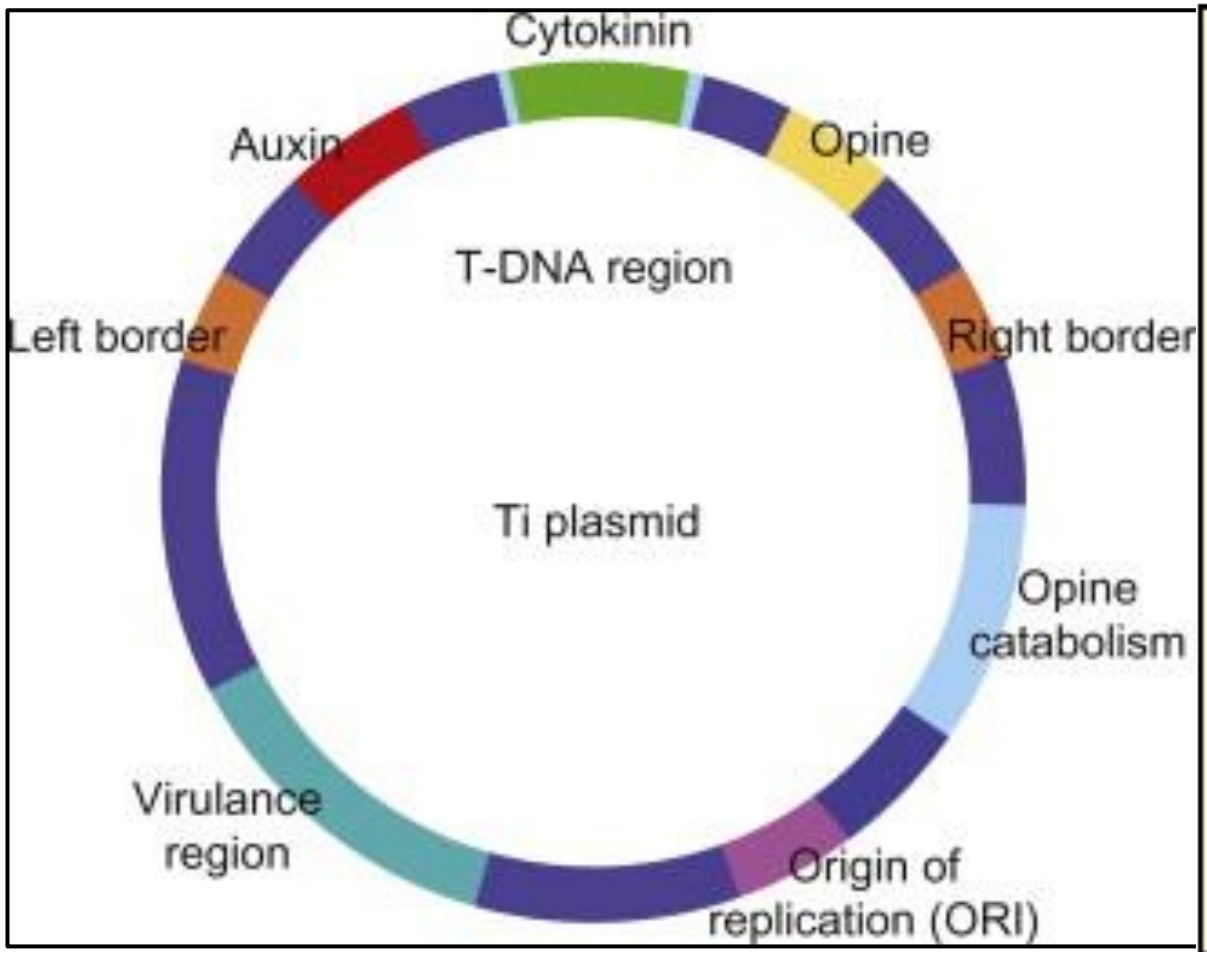
- 200-250 kb.
- T-DNA (transferred DNA) - from 10-30 kb.
- 35 vir genes.
- Direct reruns 25 bp.
- The **vir genes** are activated by sap from the wound of plants with a *pH of 5.0-5.8* and a high content of *phenolic compounds*.

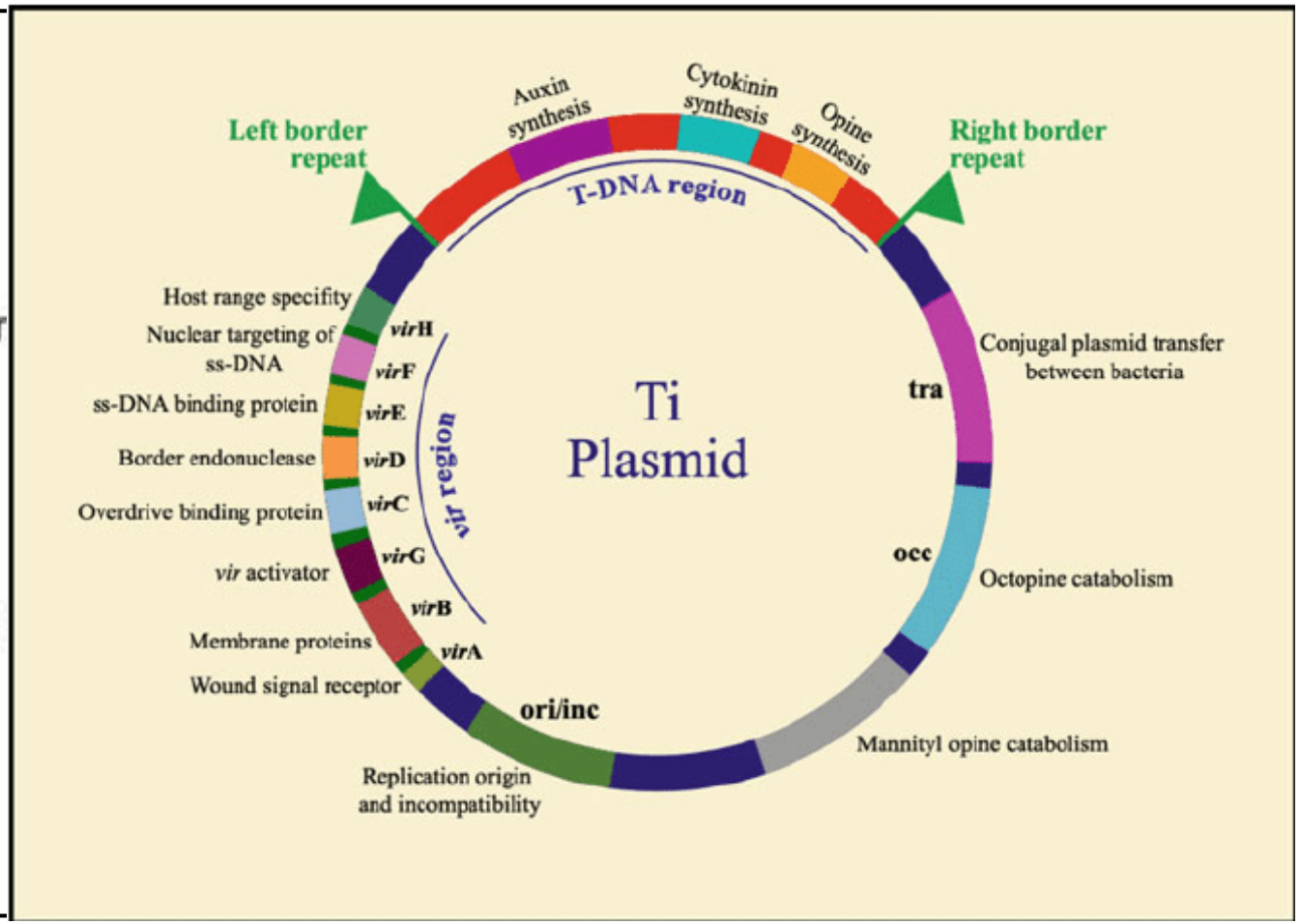


Agrobacterium tumefaciens causes the formation of crown galls (tumors). The tumor-forming agent is the Ti-plasmid, which contains a region of T-DNA (transforming DNA), which integrates into the plant genome.

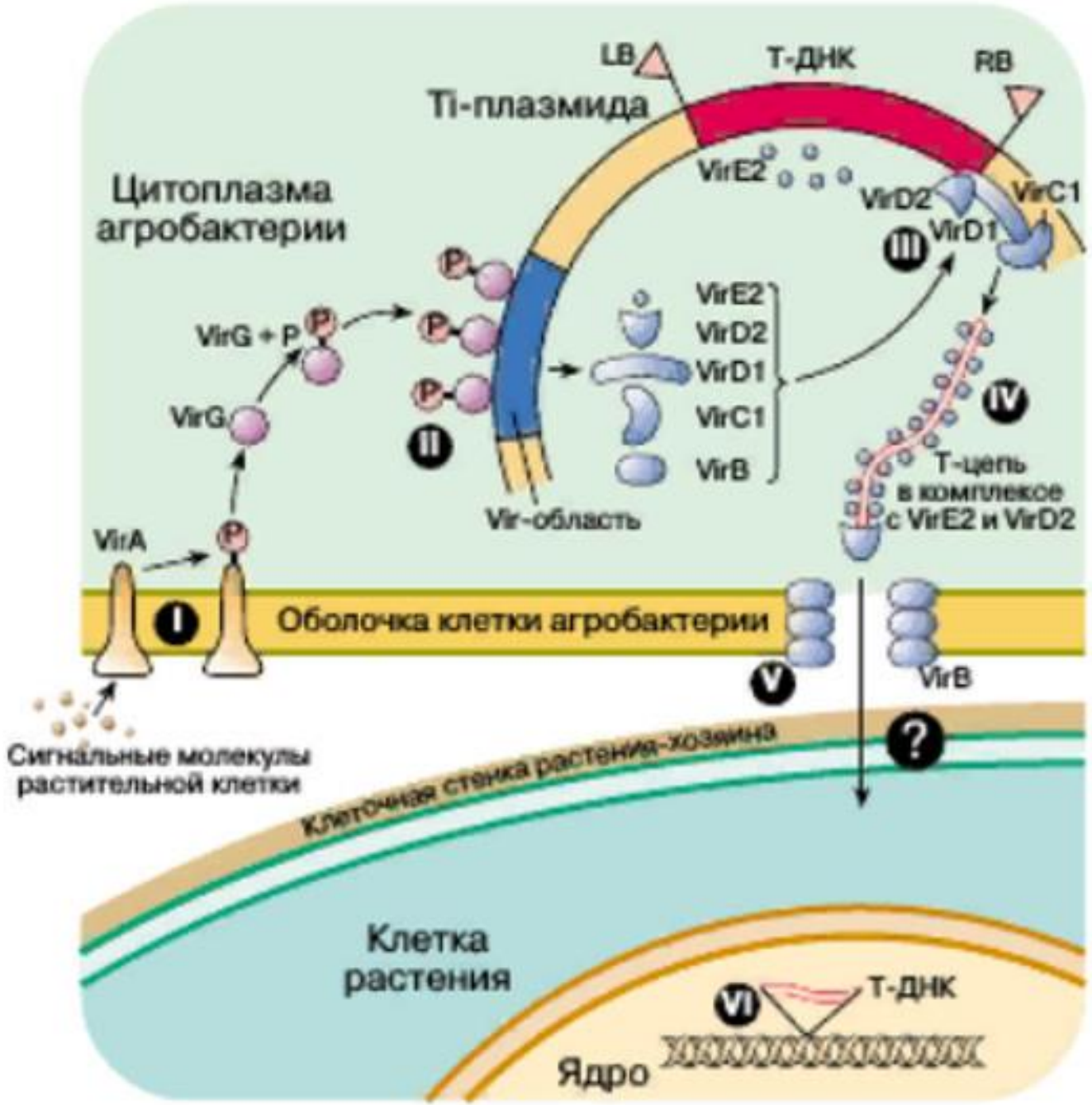
The Ti-plasmid includes: ***vir-region*** — genes whose products ensure the excision and transfer of Ti-DNA into the plant cell. ***region controlling bacterial conjugation*** (tra) — genes responsible for the transfer of genetic material between bacteria. ***ori-region*** — containing genes whose products ensure systemic functions and resistance.







- Several types of opines are known: octopine, nopalin, agropin, manopin, agrocinapine.
- Opines are synthesized in crown galls of plants transformed with different strains of the soil bacterium *Agrobacterium tumefaciens*; serve as a food source for agrobacteria, replace auxins and cytokinins.



The main stages of *Agrobacterium-mediated* transformation:

I — activation of the VirA chemoreceptor by the products of plant cell wall hydrolysis;
II - phosphorylation of VirG;
III — introduction of a single-strand break in the RB region, replication with displacement of the T-chain;
IV — binding of the VirE2 and VirD2 proteins to the T-chain and transport to the pore;
V — passage of the T-chain in a complex with proteins into the plant cell;
VI — integration of the T-chain into the plant chromosome.

1 - plant cell wall;
2 - agrobacterium cell membrane;
3 - plant cell signaling molecules

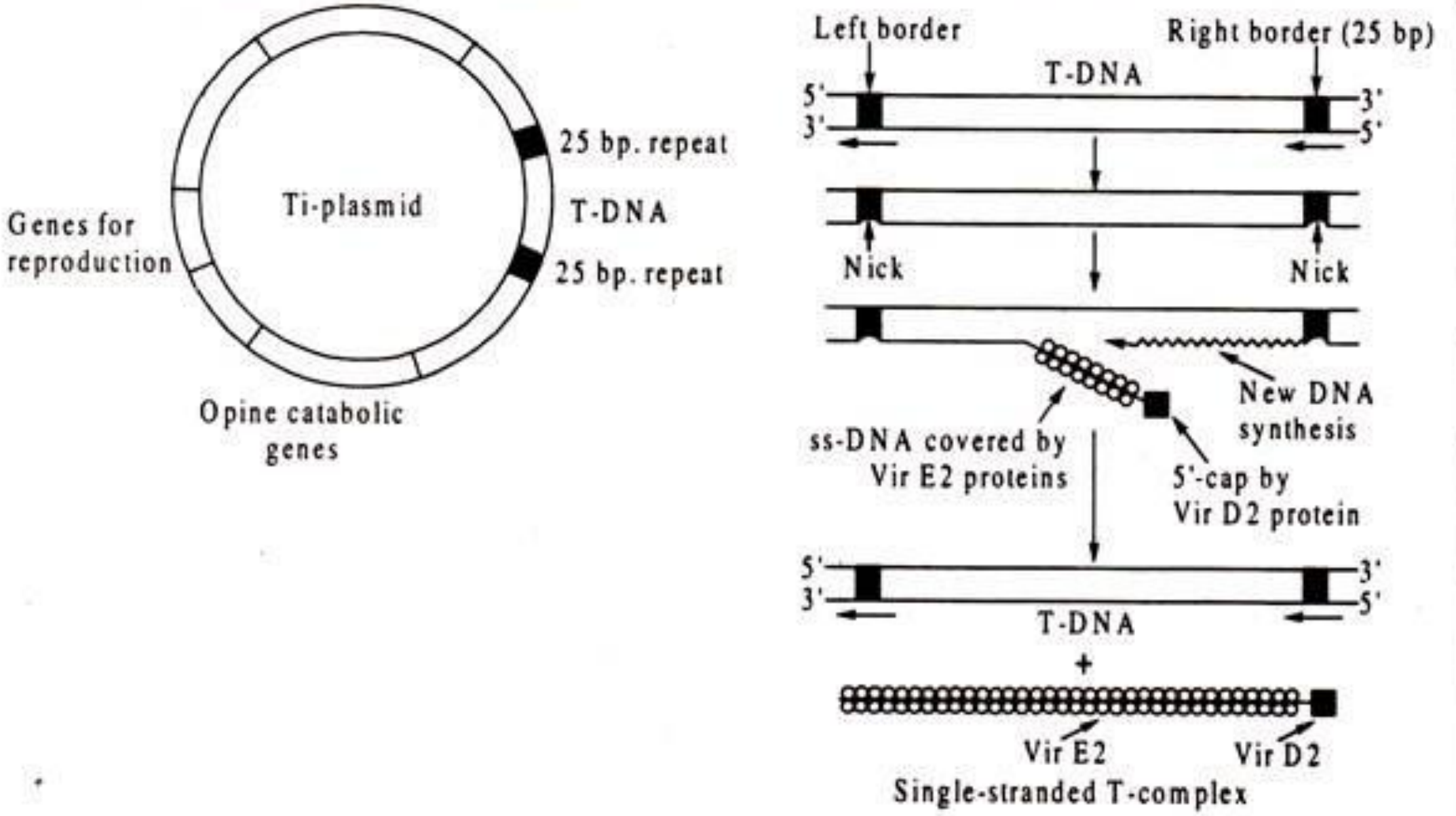


Fig. 9.90 : Ti-plasmid (left) and generation of T-complex (right)

This ***cointegrative vector system***, due to the relative complexity of the analysis of hybrid Ti plasmids obtained *in vivo*, has not found wide application.

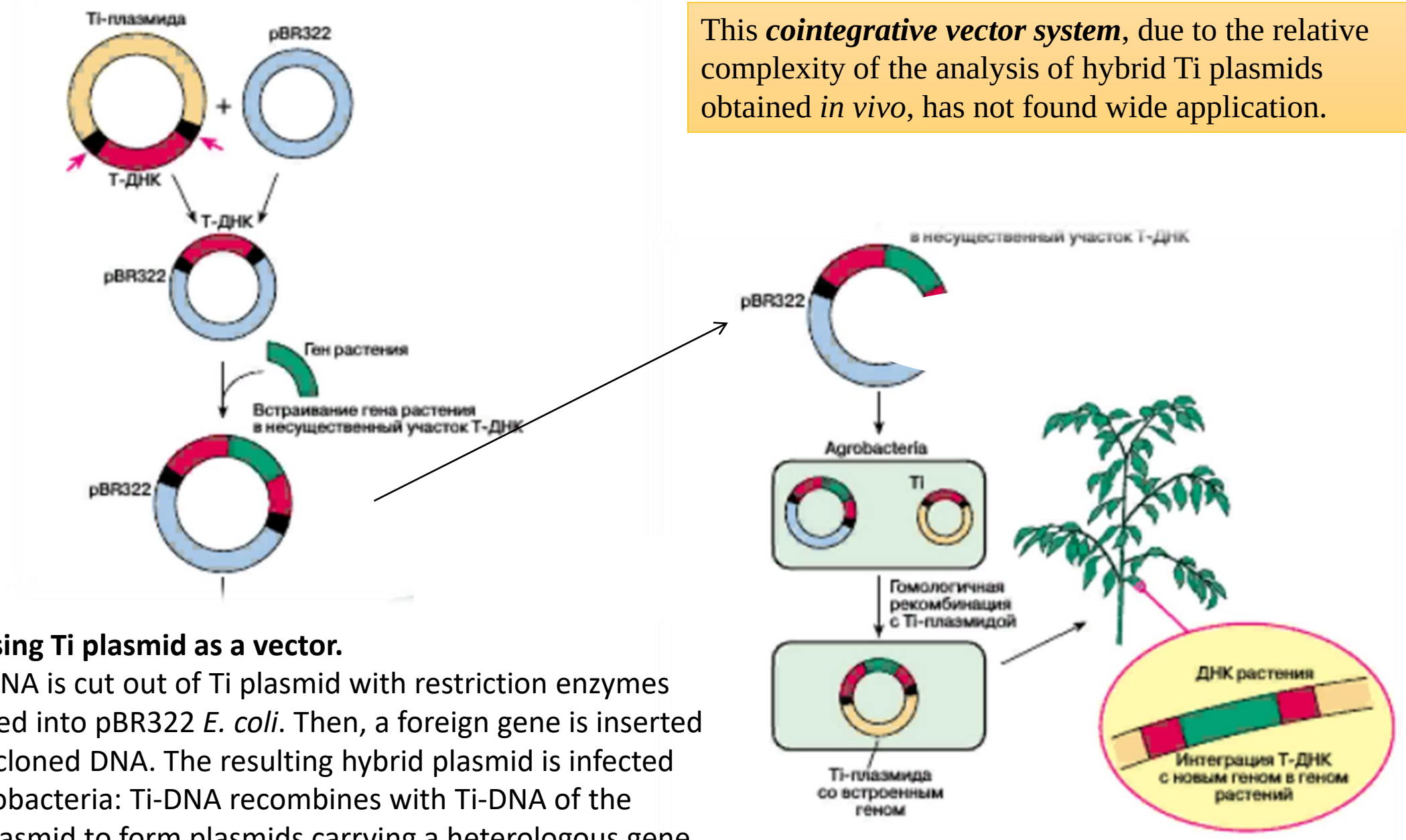
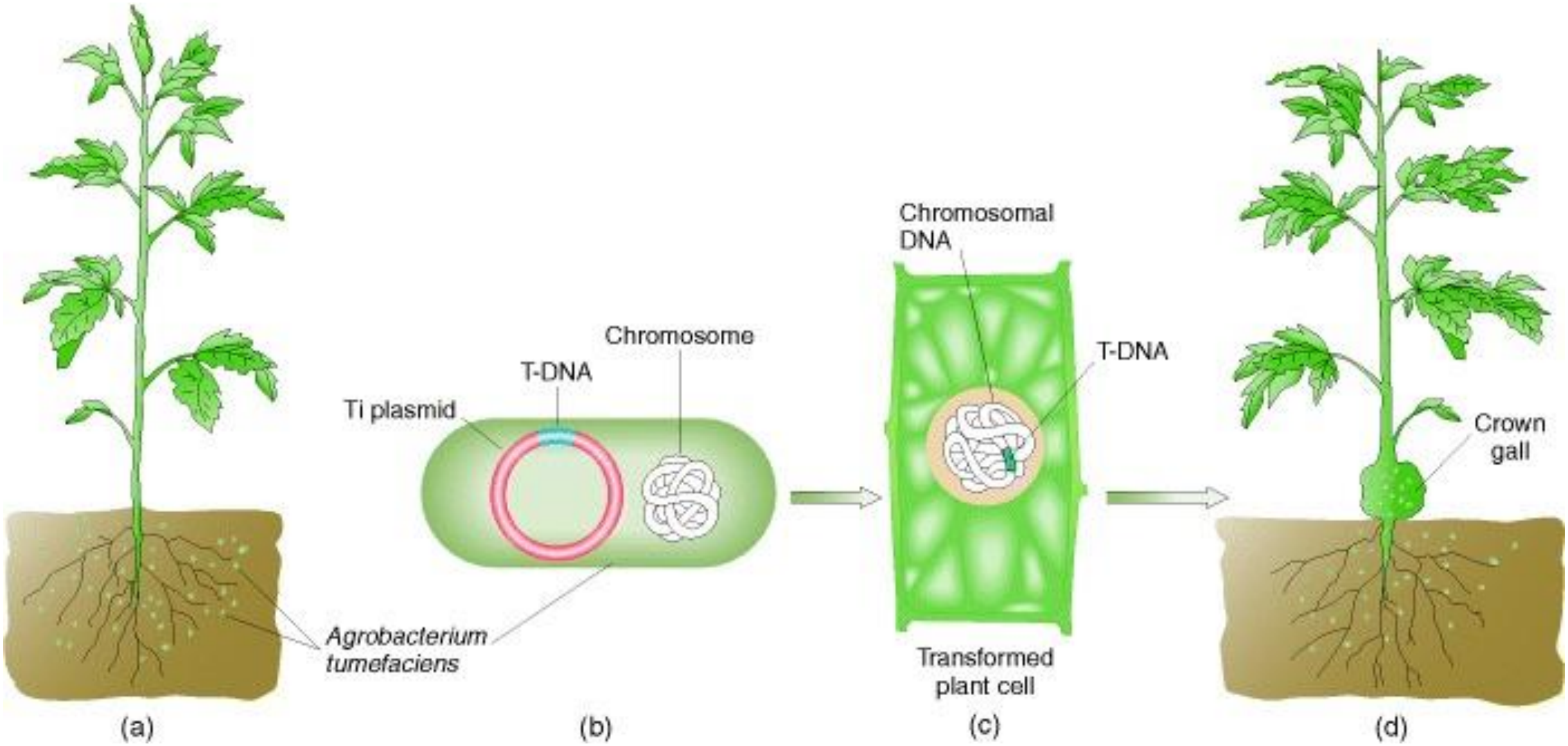
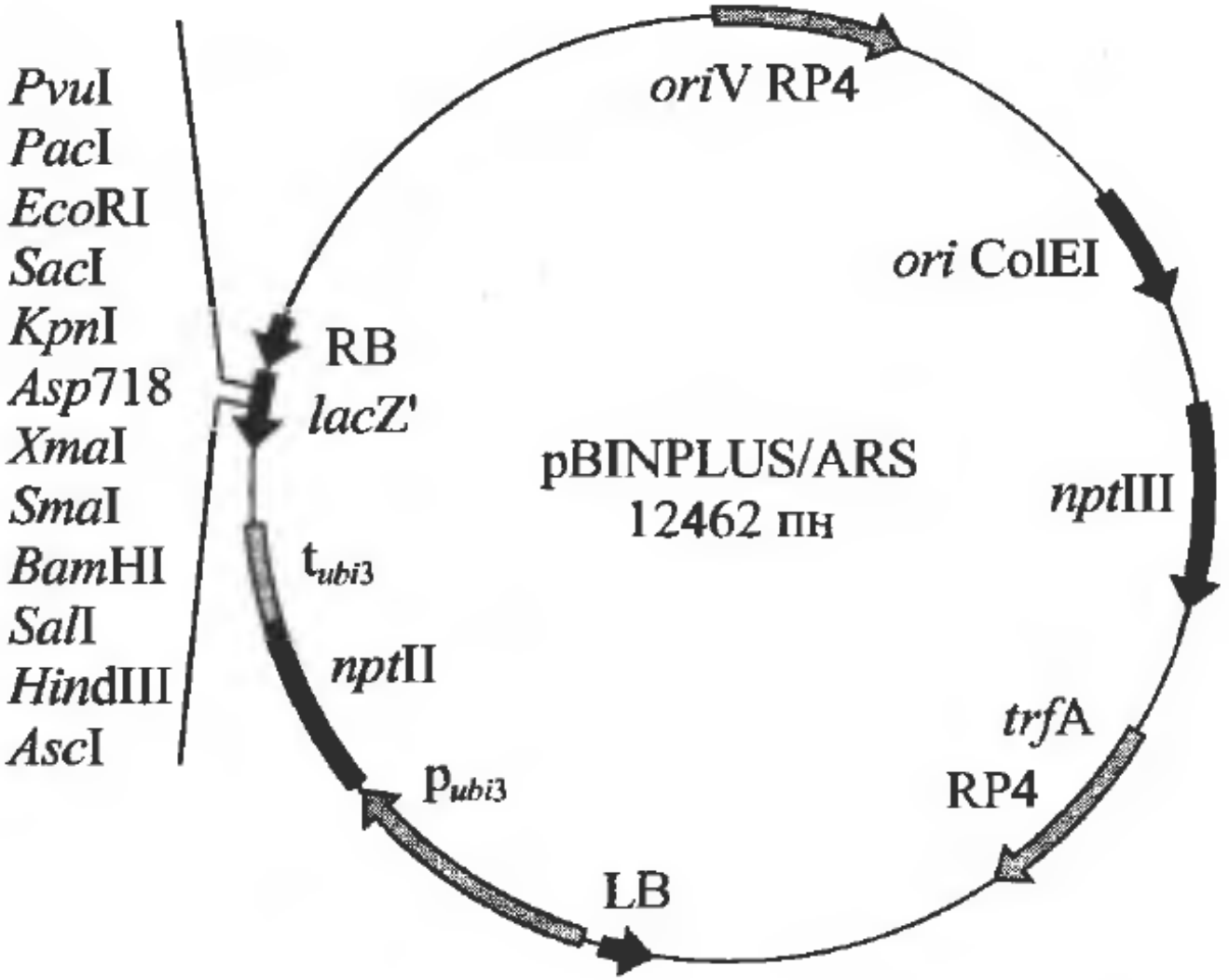


Fig. 4. Using Ti plasmid as a vector.
First, T-DNA is cut out of Ti plasmid with restriction enzymes and cloned into pBR322 *E. coli*. Then, a foreign gene is inserted into the cloned DNA. The resulting hybrid plasmid is infected with agrobacteria: Ti-DNA recombines with Ti-DNA of the hybrid plasmid to form plasmids carrying a heterologous gene. Transgenic plants are obtained using such agrobacteria.


Chromosome
T-DNA
Ti plasmid
Agrobacterium tumefaciens
Chromosomal DNA
T-DNA
Transformed plant cell
Crown gall
(a)
(b)
(c)
(d)

- A. Hoekam et al. (1983) discovered trans positions of T-DNA and vir-genes.



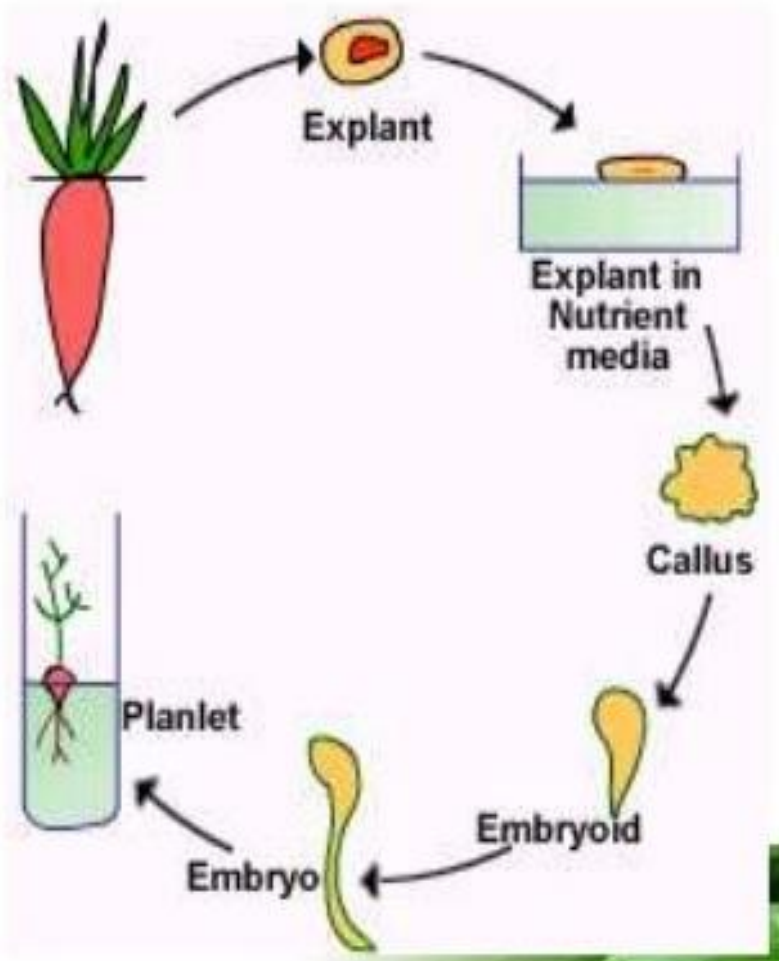
- **Binary vector/+ (helper plasmid)**

RB, LB — terminal repeats of T-DNA of plasmid Ti; *oriV*, *trfA* — genetic elements of plasmid with wide host range RP4, providing its replication; *npt*III — gene of resistance to kanamycin, functioning in bacteria; P_{ubi3}-*npt*II-t_{ubi3} — hybrid gene, expressed in plants and providing resistance to kanamycin; p_{ubi3}, t_{ubi3} — promoter and terminator of gene ubi3, encoding ubiquitin.

TABLE 10.4 Categories of Marker Genes and Selective Agents Used in Plants

Category	Marker Genes	Source of Genes	Selective Agent
Selectable marker genes:			
Antibiotic resistant	nptII, neo, aphII	*Escherichia coli* Tn5 (bacteria)	Kanamycin
	hpt, hph, aphIV	*E. coli* (bacteria)	Hygromycin
Herbicide resistant	bar	*Streptomyces hygroscopicus* (bacteria)	Phosphinothricin
	pat	*S. viridochromogenes* (bacteria)	Phosphinothricin
	CP4 EPSPS	*Agrobacterium sp.* strain CP4 (bacteria)	Glyphosate
Nutritional inhibitor related	manA	*E. coli* (bacteria)	Mannose
	xylA	*S. rubiginosus; Thermoanaerobacterium Thermosulfurogenes* (bacteria)	D-xylose
Hormone related	ipt	*Agrobacterium tumefaciens* (bacteria)	N/A[a]
Ablation	codA	*E. coli* (bacteria)	5-Fluorocytosine
Reporter genes: aka scorable marker genes			
Enzymatic	uidA, gusA	*E. coli* (bacteria)	MUG, X-gluc
	Luc		
Fluorescent proteins	gfp	*Aequorea victoria* (jellyfish)	N/A
	pporRFP	*Porites porites* (hard coral)	N/A
	mOrange	*Discosoma sp.* (soft coral)	N/A

CONCEPT OF TOTIPOTENCY

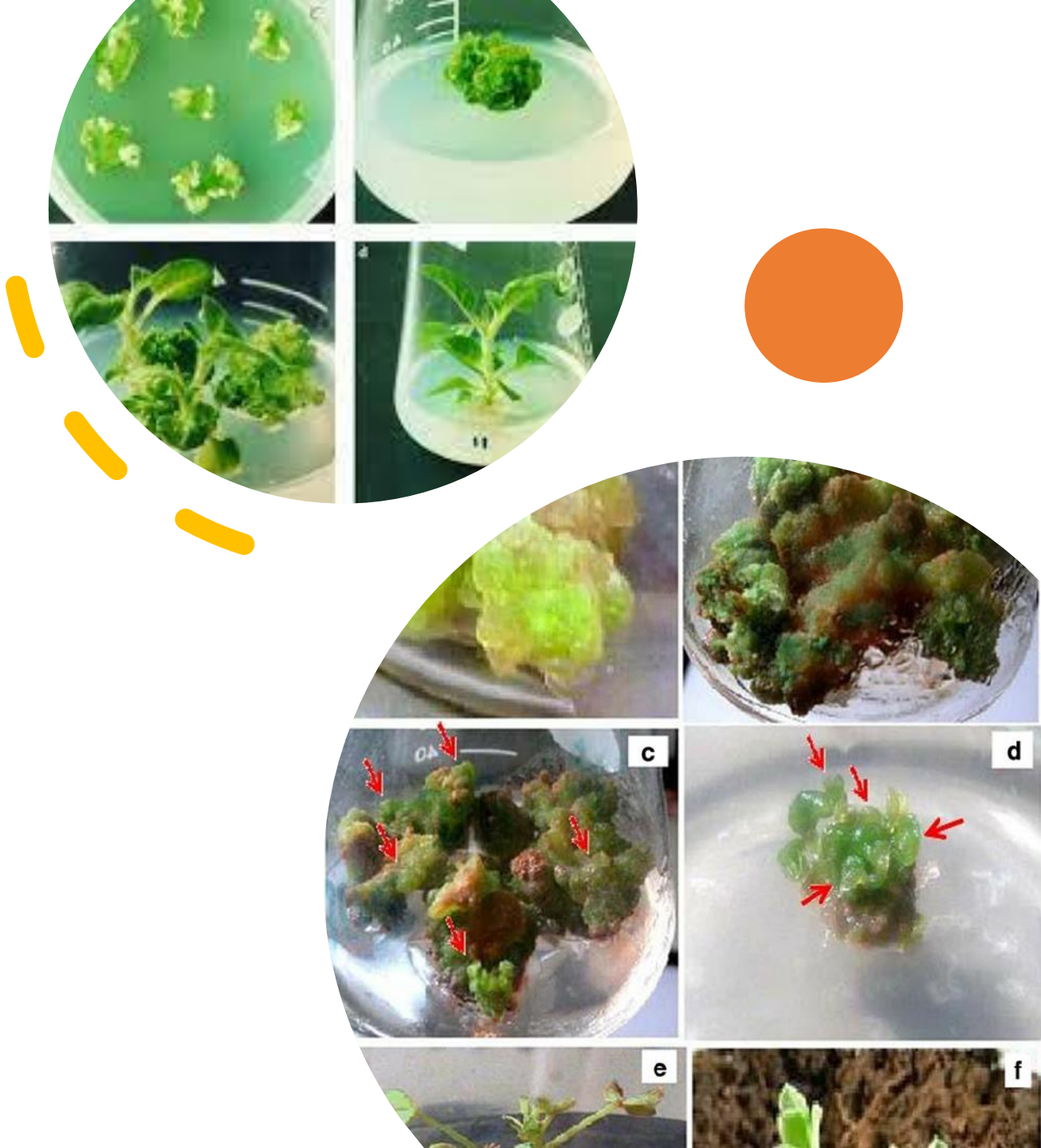


- As cell divide mitotically, they do so eqautionally to produce daughters cells.
- G.Haberlandt's claimed that one day it could be possible to rear plants from isolated would be rarely surviving cells of flowering plants.
- He also sated that out of surviving somatic cells artificial embryos would be reared asexually
- Therefore every cell within the plant has a potential to regenerate into a whole plant.

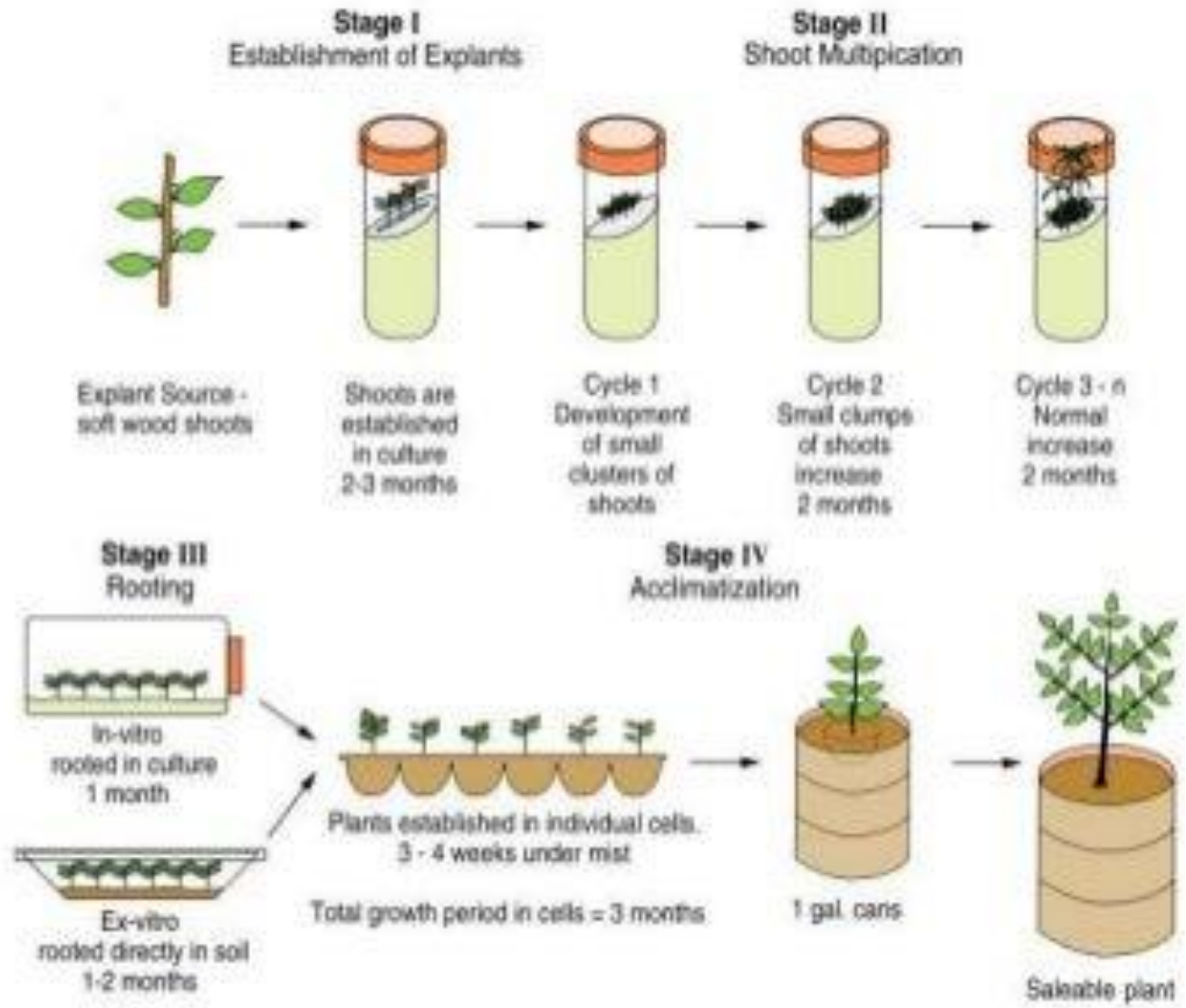
- **An explant** is a group of cells separated from the mother's body. It is used in biological research related to micropropagation of plants.

- In dicotyledonous plants, explants can be, for example, parts of hypocotyls, stems, roots, cotyledons, anthers, etc.

- When explants are cultivated on nutrient media of various compositions can be obser, callusogenesis or organogenesisved (the formation of roots, shoots, etc.)

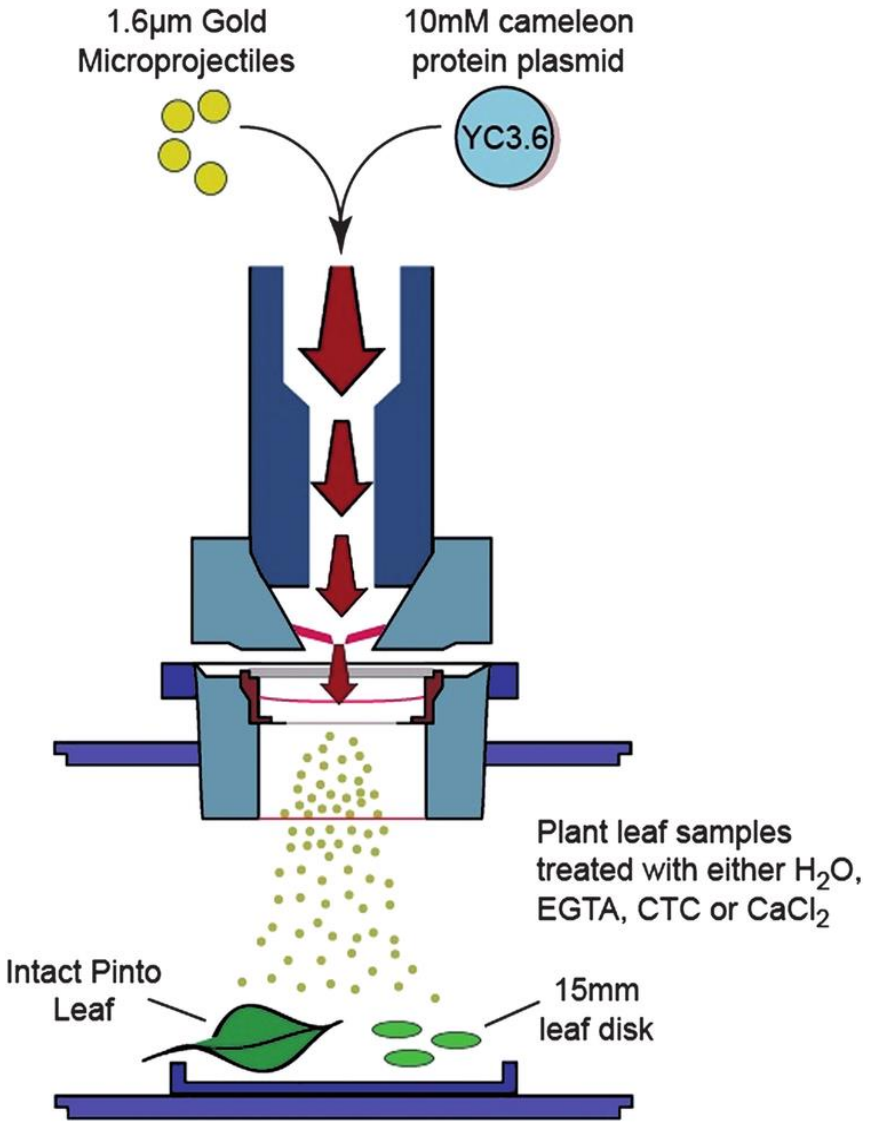


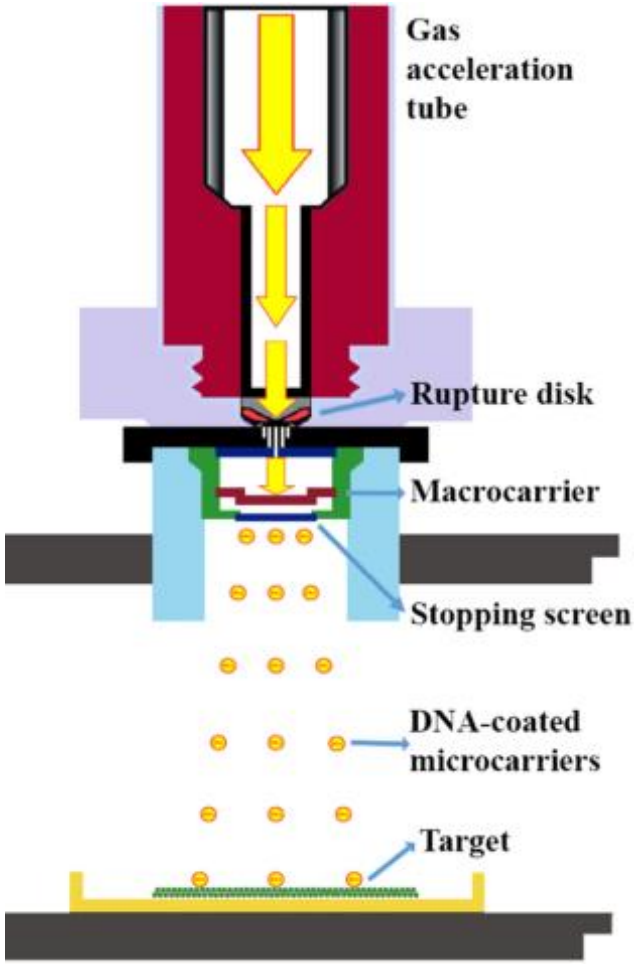
- Plant tissue culture is divided into 2 major method:
- 1 - Types of in-vitro growth – callus and suspensi
- culture .
- 2 - **TYPES OF EXPLANT –**
  - **A) SINGLE CELL CULTURE**
  - **B) SHOOT AND ROOT CULTURE**
  - **C) SOMATIC EMBRYO CULTURE**
  - **D) MERISTEM CULTURE**
  - **E) ANTHR CULTURE**
  - **F) SOMATIC HYBRIDISATION**
  - **G) EMBRYO CULTURE**
  - **H) OVULE CULTURE**
  - **I) OVARY CULTURE**



Biolistic transformation

- Biolistic transformation, also known as **particle bombardment** or gene gun technology, is a genetic engineering technique used to introduce foreign DNA into living cells.
- This technique is often used to create genetically modified organisms (GMOs) for agricultural and medical purposes.
- The biolistic transformation process involves coating **small metal particles**, typically gold or tungsten, with the foreign DNA that is to be introduced into the cell.
- The coated particles are then loaded into a device called a **gene gun**, which accelerates the particles to high velocities using a burst of pressurized helium gas. The particles are propelled towards the target cells, penetrating their cell walls and membranes and delivering the foreign DNA into the cell.





Gene Gun Technology

The gene gun, or biolistic particle delivery system, is a powerful tool used in genetic engineering to introduce foreign DNA into cells. This technique involves the use of microscopic gold or tungsten particles that are coated with DNA. The gene gun propels these particles into target cells using high-velocity bursts of gas.

Products	Proteins	Transgenic plants	References
Blood and plasma proteins	Albumin	Potato, tobacco	[10–12]
	Aprotinin	Maize	[13]
	Collagen I	Tobacco	[14]
	Encephalin	Tobacco	[11]
	Hemoglobin	Tobacco	[15–17]
	Human α1 antitrypsin	Rice	[18,19]
Vaccines	Bet v 1	Tobacco	[20]
	Cholera toxin B subunit	Potato	[21]
	Glycoprotein B from human cytomegalovirus (CMV)	Tobacco	[22]
	Cholera toxin B subunit-insulin fusion protein	Potato	[23]
	D2 peptide of fibronectin binding protein B of *S. aureus*	Black bean	[24]
	VP1	*Medicago sativa*, Black bean	[25–27]
	VP2	*Arabidopsis thaliana*	[28]
	VP4	*Medicago sativa*	[29]
	Hemagglutinin	Tobacco	[30,31]
	Hepatitis antigen	Tobacco and Potato	[32,33–35]
	gp41 glycoprotein	Soybean	[36]
	Enterotoxine B of *E. coli*	Potato, tobacco	[37]
	Cholera toxine B of *V. cholera*	Potato	[38]
	Epitope of *P. falciparum*	Tobacco	[39]
	Norwalk virus capsid	Tobacco, potato	[40,41]
	G protein of rabies virus	Tobacco, spinach, tomato	[42]
	Autoantigène	Potato	[33]
Hormones, cytokins and growth factors	GM-CSF (Granulocyte Macrophage-Colony Stimulating Factor)	Tobacco, candy cane	[43–46]

- http://dx.doi.org/10.1016/S1387-2656(07)13006-4

Major advantages

- is its ability to introduce DNA into a wide variety of cell types and organisms, including those that are difficult to transform using other techniques such as bacterial transformation or electroporation.
- Additionally, biolistic transformation can be used to introduce multiple genes at once, allowing for the creation of genetically modified organisms with multiple desirable traits.

Some potential drawbacks

- The technique can be expensive and time-consuming, and there is a risk of unintended effects or off-target effects on the host organism's genome. Therefore, it is important to carefully consider the potential risks and benefits of using biolistic transformation before applying it in genetic engineering projects.

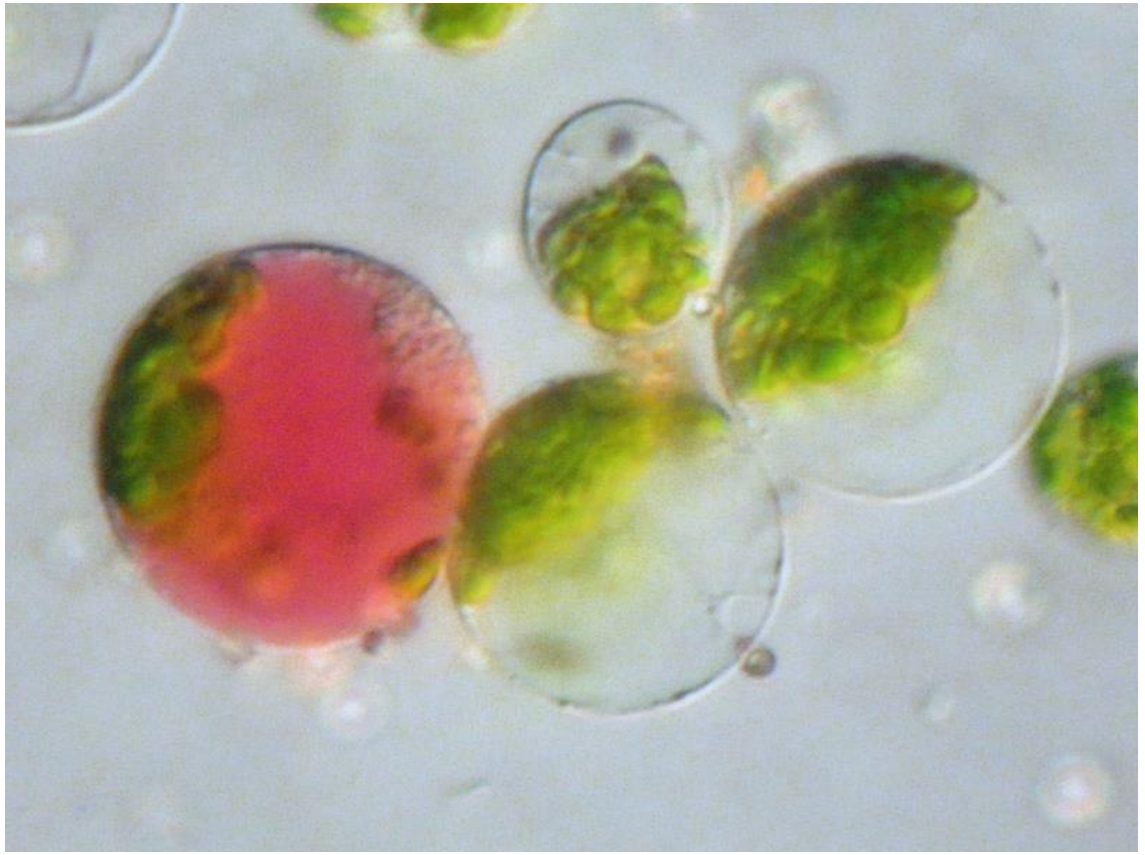
Biolistic transformation

Protoplasts can be used in plant biotechnology to study cell biology, gene expression, and genetic engineering. One important application of protoplasts is in the process of protoplast fusion.

Protoplast fusion is a technique used to create new plant hybrids by fusing protoplasts from two different species or varieties.

The resulting hybrid cells have combined genetic material and can express traits from both parent plants.

This technique has been used to create new varieties of crops with desirable traits, such as disease resistance or higher yields.



Protoplasts are plant cells that have had their cell walls removed, leaving only the plasma membrane and the contents of the cell.

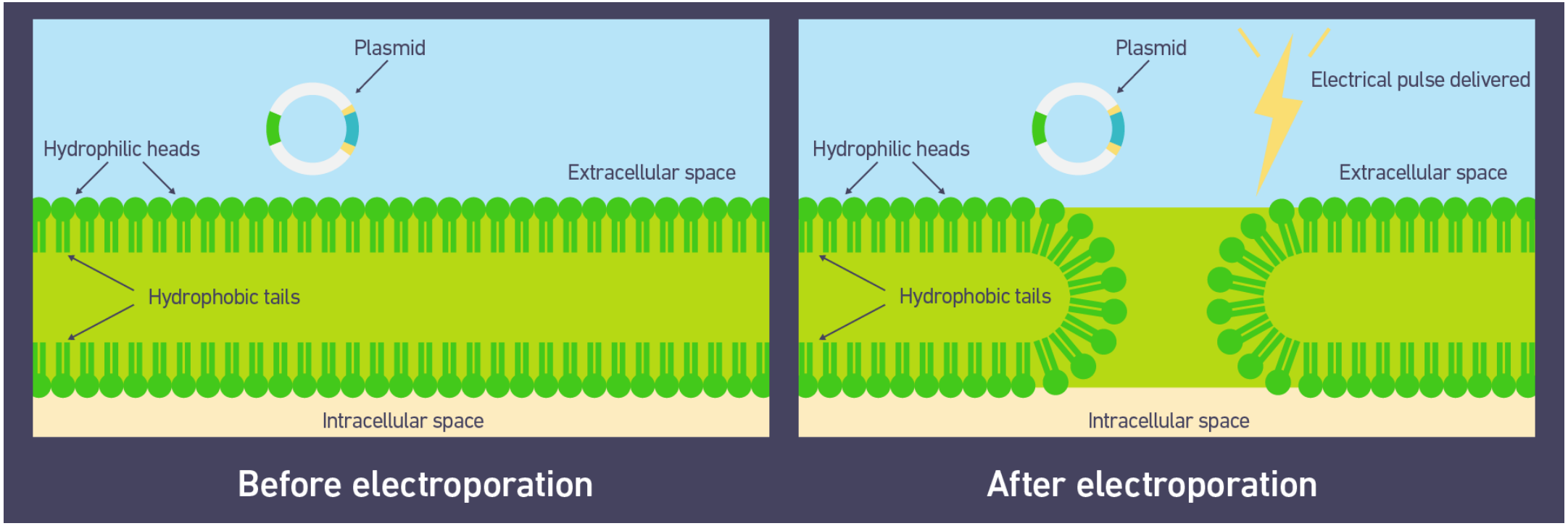
The process of protoplast fusion involves the following steps:

- **Isolation of protoplasts**: Protoplasts can be isolated from plant tissues using enzymes that break down the cell wall. The resulting protoplasts are then purified and washed.
- **Fusion of protoplasts**: Protoplasts from different species or varieties are mixed together in a solution containing a chemical called polyethylene glycol (PEG). PEG causes the protoplasts to fuse together, forming hybrid cells.
- **Regeneration of plants**: The hybrid cells can then be cultured on a nutrient medium to encourage cell division and plant regeneration. The resulting plants will have a combination of traits from the parent plants.

Protoplast fusion has some limitations, such as the need for compatible protoplasts, and the low efficiency of fusion and regeneration. Nevertheless, it remains an important tool in plant biotechnology, and ongoing research is focused on improving the efficiency and reliability of the technique.

Electroporation

Electroporation is a technique used to introduce foreign DNA into plant cells by applying an electric field. The technique involves creating small pores in the plasma membrane of the plant cell, which allows the foreign DNA to enter the cell. Once inside the cell, the foreign DNA can integrate into the plant genome and be expressed.



The electroporation process involves the following steps:

1. **Preparation of plant cells**: Plant cells are first cultured and prepared for electroporation by removing the cell wall and suspending the protoplasts in a solution.
2. **Mixing of DNA with plant cells**: The foreign DNA that is to be introduced into the plant cell is mixed with the protoplast suspension. This can be done using a variety of methods, such as adding the DNA to the suspension and incubating, or electroporating the DNA and protoplasts together.
3. **Electroporation**: The protoplasts and DNA mixture is subjected to a high-voltage electric pulse that creates small pores in the plasma membrane. The electric field disrupts the cell membrane, creating temporary holes through which the foreign DNA can enter the cell.
4. **Regeneration of plants**: The transformed protoplasts can then be cultured on a nutrient medium to encourage cell division and plant regeneration. The resulting plants will have the foreign DNA integrated into their genome.

Electroporator

- Electroporation of plant cells has many advantages over other methods of genetic transformation, such as high efficiency and low cost. However, the technique has some limitations, such as the need for specialized equipment and the fact that not all plant cells are easily transformed by electroporation. Nevertheless, it remains an important tool in plant biotechnology, and ongoing research is focused on improving the efficiency and reliability of the technique.