DEPARTMENT OF BIOTECHNOLOGY

Genetic Engineering

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INTRODUCTION:

- Genetic engineering, also called genetic modification, is the direct manipulation of an organism's genome using biotechnology.
- DNA may be inserted in the host genome by first isolating and copying the genetic material of interest using molecular cloning methods to generate a DNA sequence, or by synthesizing the DNA, and then inserting this construct into the host organism.
- Crops developed through genetic engineering are commonly known as transgenic crops or genetically modified (GM) crops. The gene can be introduced into the cells of the plant being modified through a process called transformation.
- The most common methods used to introduce the gene package into plant cells include biolistic transformation (using a gene gun) or *Agrobacterium*-mediated transformation.
- *Agrobacterium tumefaciens* mediated transformation is the most commonly used method for obtaining transgenic plants.

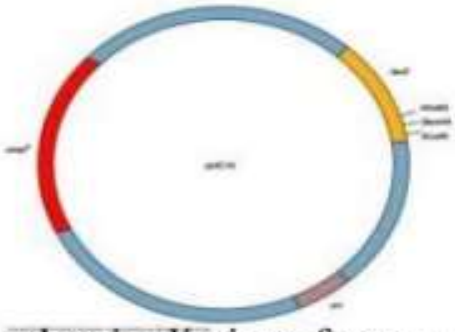
Basics of genetic Engineering

•**R.E**: Restriction enzymes are DNA-cutting enzymes found in bacteria (and harvested from them for use). Because they cut within the molecule, they are often called restriction endonucleases.

•**Vectors**: A vector is a DNA molecule used as a vehicle to artificially carry foreign genetic material into another cell, where it can be replicated and/or expressed.

•**Types of Vectors**:

- •Plasmids (5-10 kb)
- •Bacteriophage (10-15 kb)
- •Cosmids (50 kb)
- •BACs & YACs (300 kb, up to 1,000 kb)



•**Plasmid as a good vector:**

•A plasmid is a small, circular, double-stranded DNA molecule that is distinct from a cell's chromosomal DNA. Plasmids naturally exist in bacterial cells, and they also occur in some eukaryotes.

•**Binary vectors**:

•A T-DNA binary system is a pair of plasmids consisting of a binary plasmid and a helper plasmid.

STEPS IN GENETIC ENGINEERING

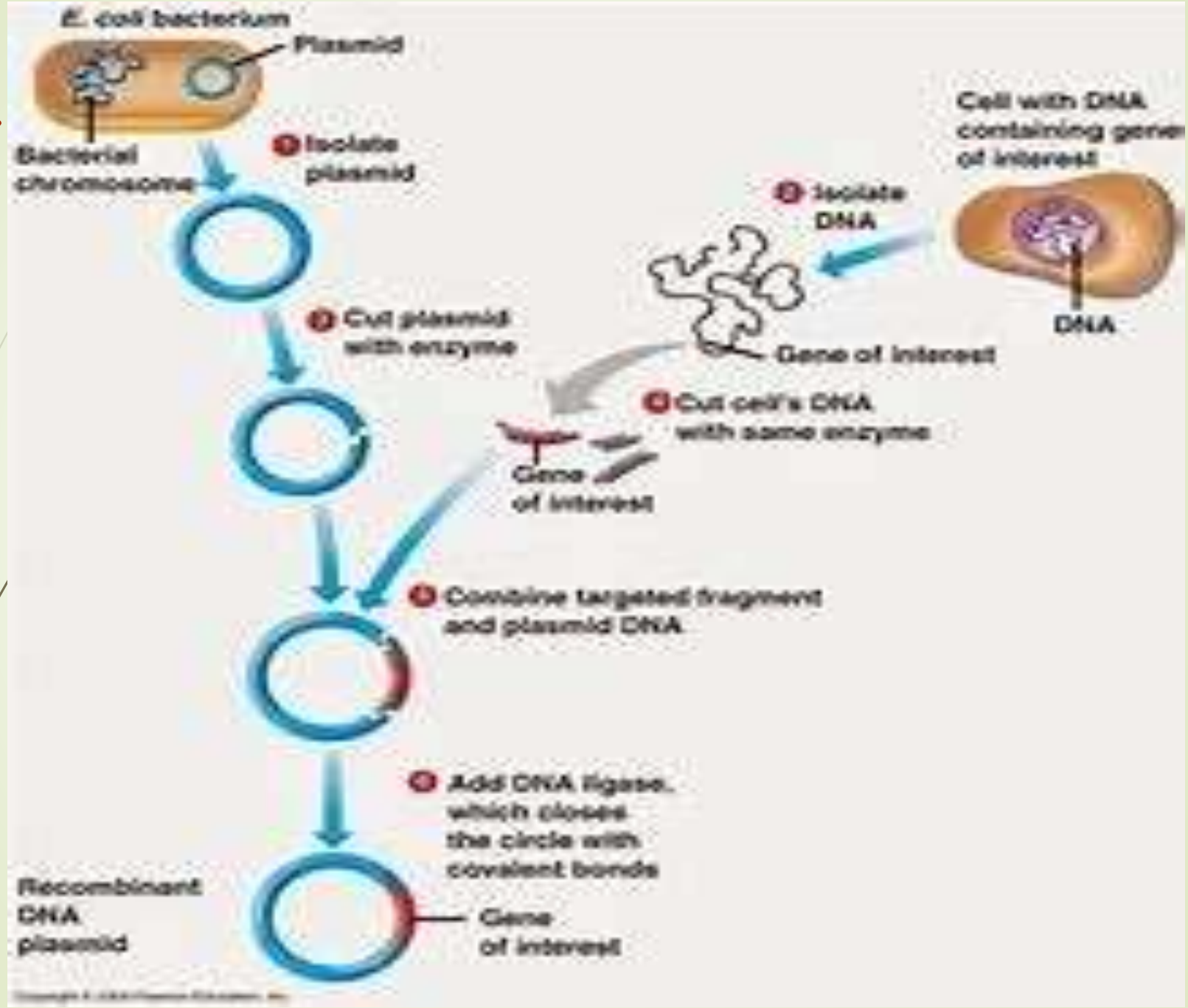
1. DNA is cut with a restriction enzyme

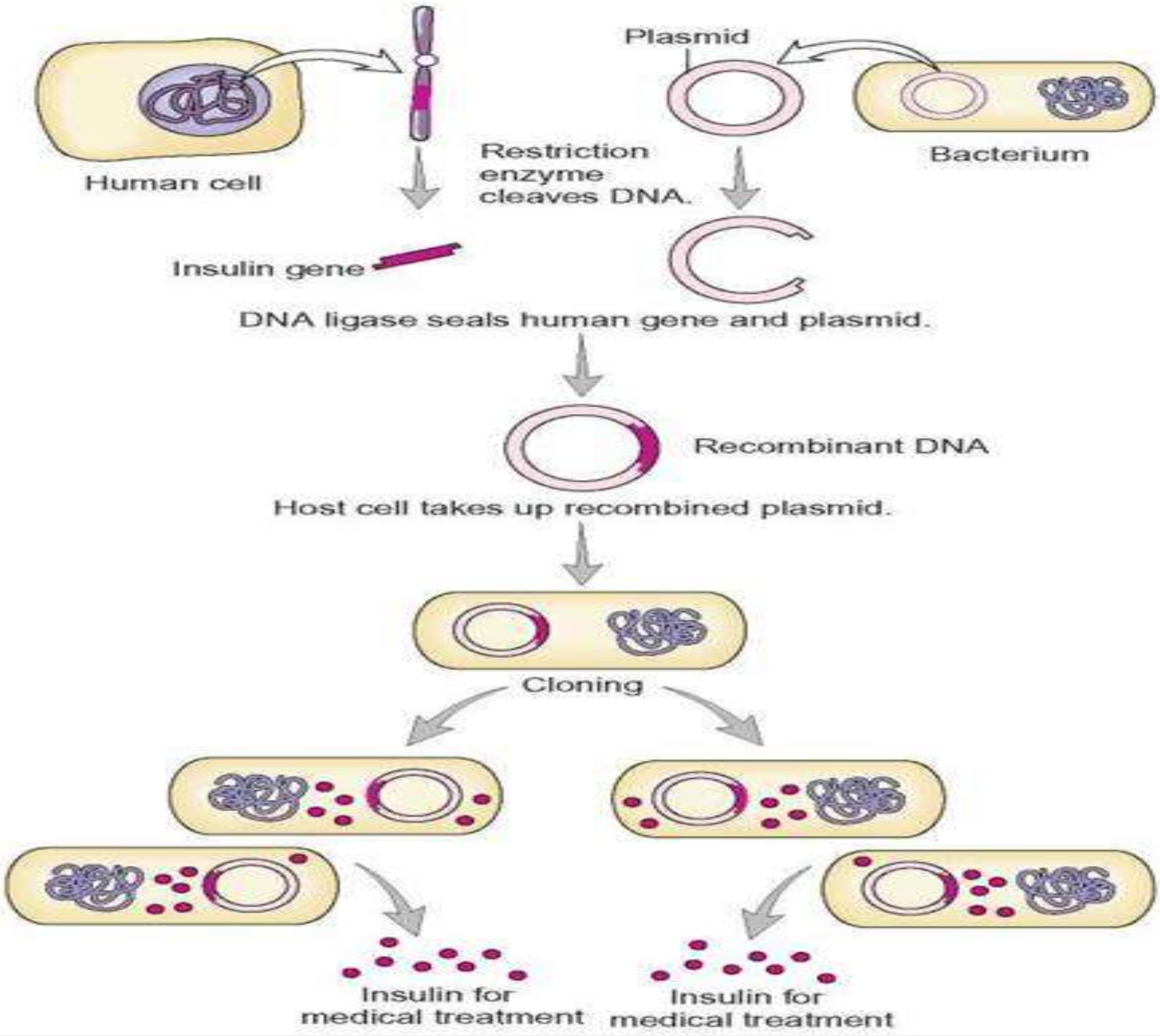
2. Fragments are mixed with vector molecules cut by the same enzyme

   DNA ligase joins recombinant DNA molecules

3. Plasmid vectors with inserted DNA fragments are transferred into bacterial cells

4. Colonies carrying cloned recombinant DNA molecules are identified, collected, and grown


E. coli bacterium
Plasmid
Bacterial chromosome
1 Isolate plasmid
2 Cut plasmid with enzyme
Cell with DNA containing gene of interest
3 Isolate DNA
DNA
Gene of interest
4 Cut cell's DNA with same enzyme
Gene of interest
5 Combine targeted fragment and plasmid DNA
6 Add DNA ligase, which closes the circle with covalent bonds
Recombinant DNA plasmid
Gene of interest


Plasmid
Bacterium
Human cell
Restriction enzyme cleaves DNA.
Insulin gene
DNA ligase seals human gene and plasmid.
Recombinant DNA
Host cell takes up recombined plasmid.
Cloning
Insulin for medical treatment
Insulin for medical treatment

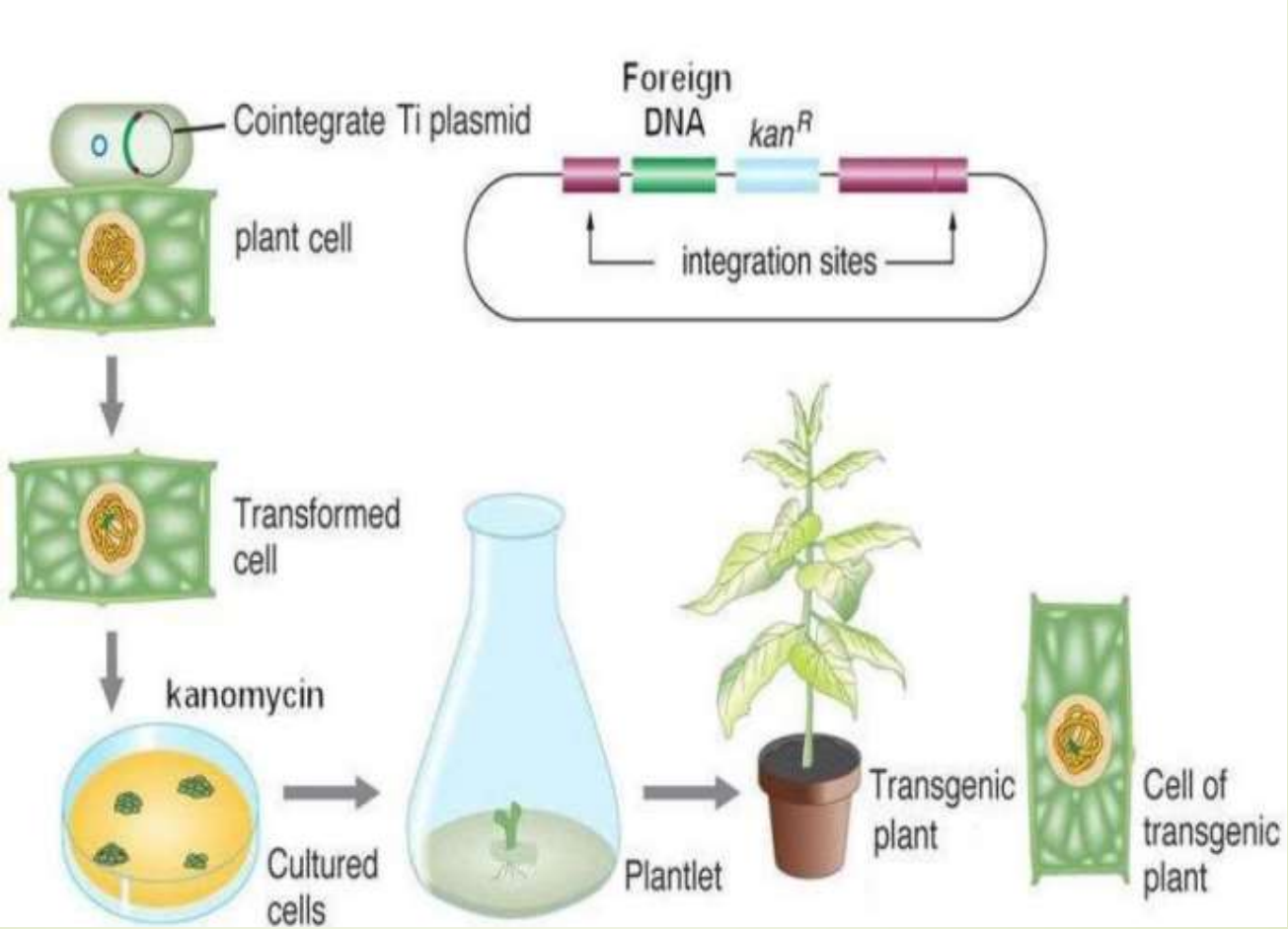
Genetic modification of plant using genetic engineering.

- **Transformation :** The genetic alteration of a cell resulting from the introduction, uptake and expression of foreign genetic material (DNA) in molecular biology.

All stable transformation methods consist of three steps:

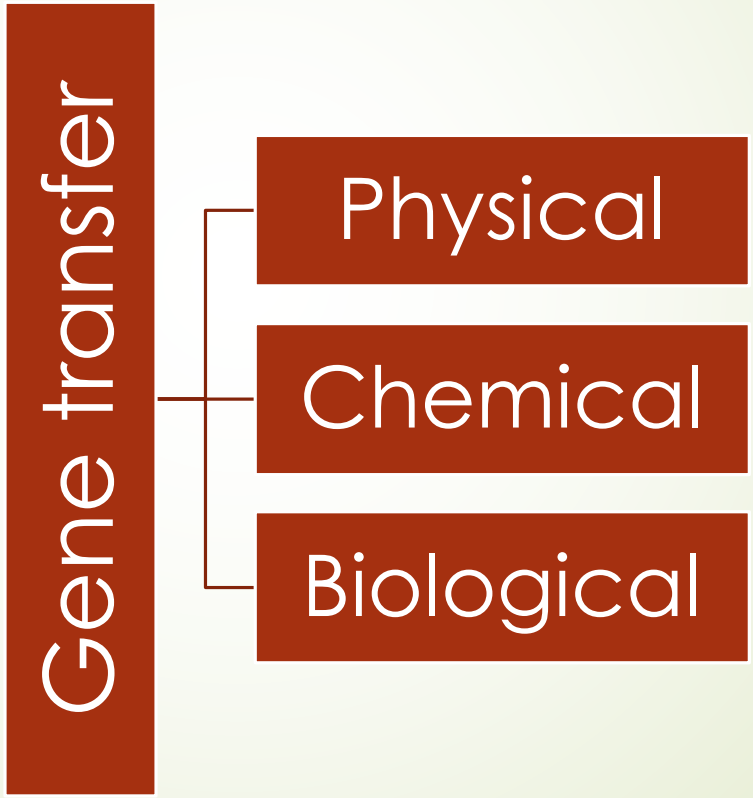
1. Delivery of DNA into a single plant cell.
2. Integration of the DNA into the plant cell genome.
3. Regeneration of the transformed cell into a whole plant.

Process of transformation.



Different Methods of Gene Transfer

Plant Transformation/Methods of Gene transfer



Physical

Microinjection
Pressure
Biolistics - gene gun/ particle bombardment
Electroporation
Silica/carbon fibers
Lazer mediated
SAT

Chemical

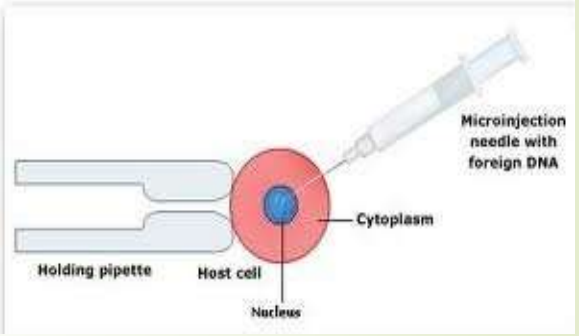
PEG
DEAE-dextran
Calcium phosphate
Artificial lipids
Proteins
Dendrimers

Biological

A. Tumefaciens
A. Rhizogenes
Virus-mediated

Microinjection

- It is the process of transfering foreign DNA into the living cell, through the use of micropipette.
- Glass micropipette is usually 0.5 to 5 micrometer, easily penetrate into the cell membrane and nuclear envelope.



Application:

- Technique is used for producing transgenic animal quickly.
- Applied to inject DNA into plant nuclei.

Limitations: Costly, Require skilled person, method is useful for protoplast.

Particle bombardment/Gene Gun Method

- A Gene gun or a bolistic particle delivery system, is a device for injecting cells with genetic information.
- The gold or tungsten particles are coated with the DNA that is used to transform the plant tissue.
- The particles are propelled at high speed into the target plant material where the DNA is released within the cell & can integrate into the genome.
- Two type of plant tissue are used for paricle bombardement:
- **Primary explant** that are bombardment & then induced to become embryogenic**.**
- **Proliferating embryo culture** that are bombarded & then allowed to proliferate further & subsequently regenerate**.**

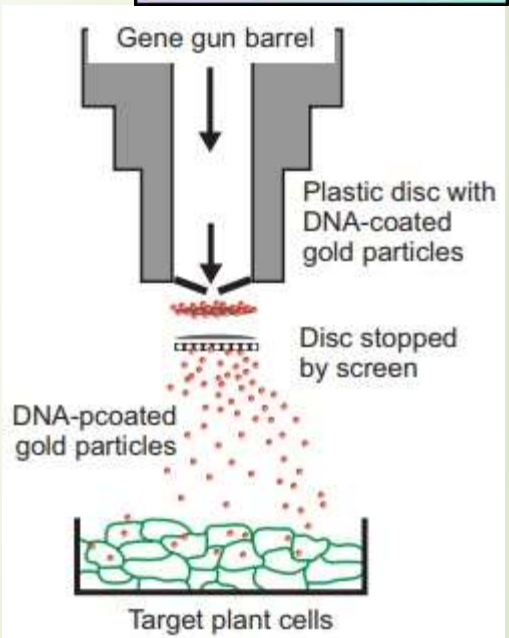
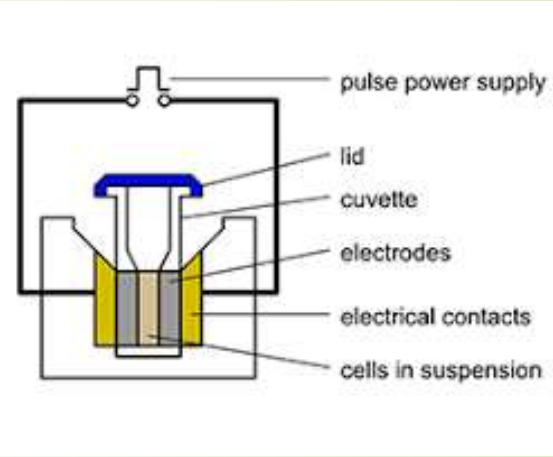


Figure 4.16: Gene gun method of Gene Transfer

Electroporation

- Electroporation is the process of applying electrical field to a living cell for brief duration of time in order to create microscopic pores in the plasma membrane called electropores.
- Also known as **electric permeabilization.**



STEPS:

- Plant material is incubated in buffer solution containing DNA & subjected to high voltage electric pulse.
- The DNA then migrates through high voltage induced por in the plasma membrane & integrates into the genome.
- It can be used to transform all the major cereals particularly rice, wheat, maize.

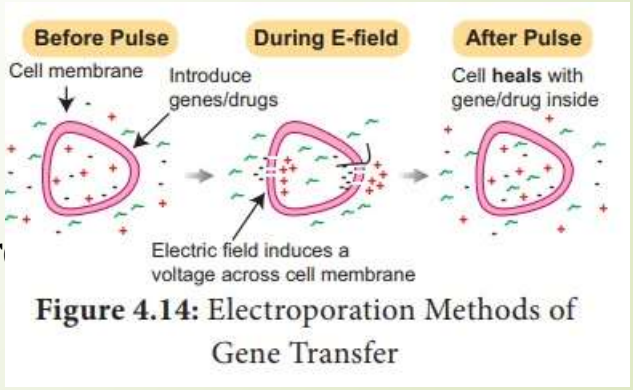
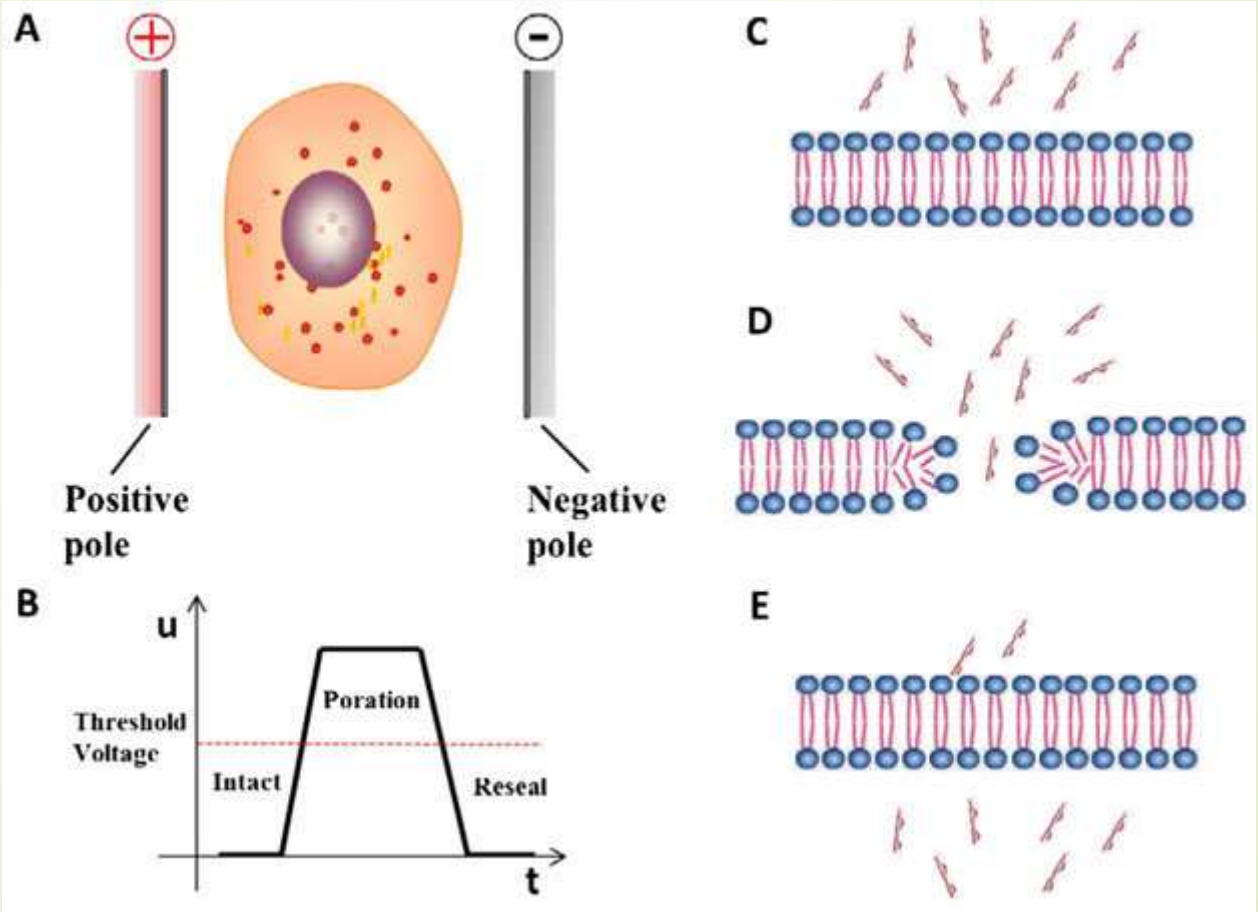
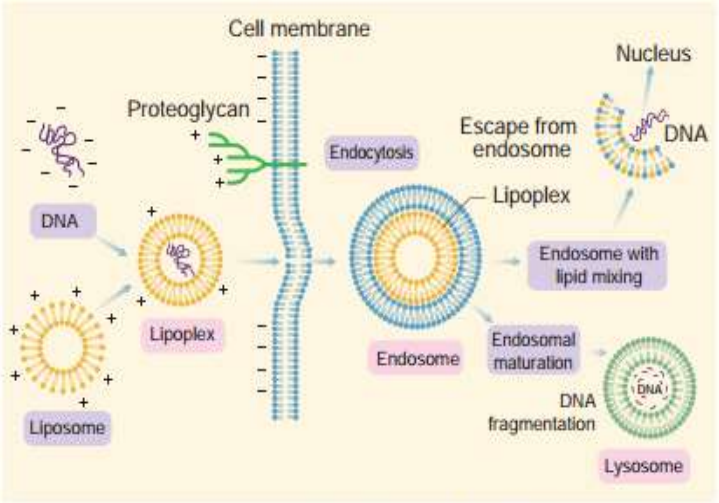


Figure 4.14: Electroporation Methods of Gene Transfer


A
Positive pole
Negative pole
B
u
Threshold Voltage
Intact
Poration
Reseal
t
C
D
E

Liposome Encapsulation (Lipofection)

- Lipofection is a Liposome mediated gene transfer method.
- Liposomes is cationic in nature & made up of phospholipid layer similar as cell membranes.
- Liposomes & target cell adheres and form the aggregates easily because of similar phospholopid bilayer.
- As aggregates of liposomes & cell wall are positively charged it uptake the negatively charged DNA.
- The recombinant DNA enclosed in the liposomes vesicles penetrates into the protoplast of the host cells.



Liposome mediated method of Gene Transfer

Calcium Phosphate mediated gene transfer

- The Process of transfection involves the mixtures of isolated DNA with the solution of calcium chloride & potassium phophate under conditions which allow the precipitates of calcium phosphate to be formed.
- Cells are then incubated with precipiatetd DNA either in solution or in tissue culture dish.
- A Fraction of cells will take up the calcium phosphate DNA precipitates by endocytosis.
- Ca ions interacts with the negatively charged phospholipid heads of the cell membrane, creating an electrostatistically neutral situation.
- Heart shock is necessary which creates

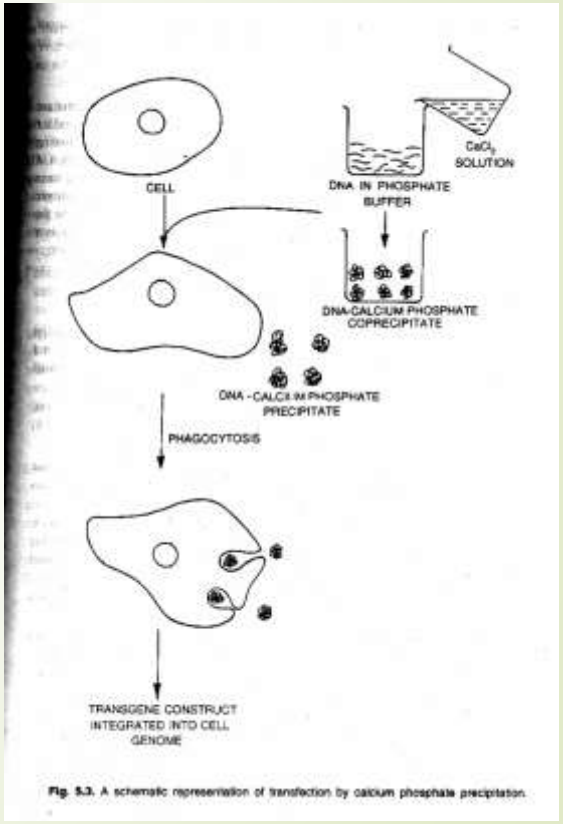


Fig. 5.3. A schematic representation of transfection by calcium phosphate precipitation.

Agrobacterium mediated gene transfer.

- Agrobacterium tumefaciens ,a soil bacterium known as nature's own genetic engineer.
- It is rod shaped ,gram negative , motile bacteria, having 1-6 flagella.
- To be virulent, the bacterium must contain a tumor-inducing plasmid (Ti plasmid or pTi),which contain T DNA & all the genes necessary to transfer it to plants cell.
- It has the natural ability to genetically engineer plants.

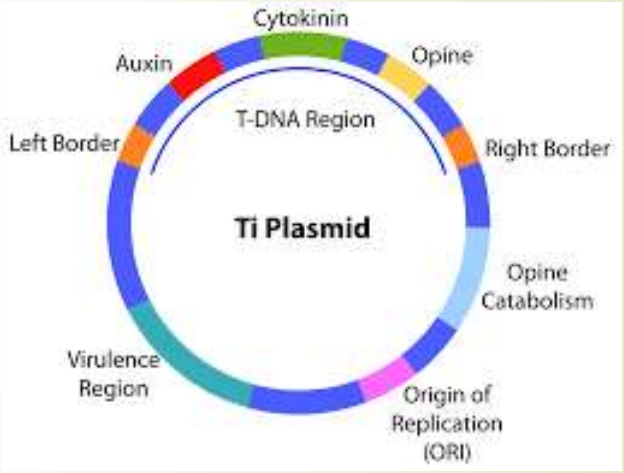
- It carries genetic information from bacteria to plant cells.
- It causes crown gall disease.
- It can be use as vehicle to transfer gene in plants.

Agrobacterium species.

- A. tumefaciens has Ti (tumor inducing) plasmid, & that is responsible for tumor induction (crown gall tumor) in plant.
- A. tumefaciens is also known by the name A. radiobacter/Rhizobium radiobacter.
- A. rhizogenes has Ri (root inducing) plasmid & that is responsible to cause hairy disease in plants. Ri plasmid is analogue to Ti plasmid
- A. rubi cause cane gall in sugarcane plant.
- A. vitis gall in grapes.

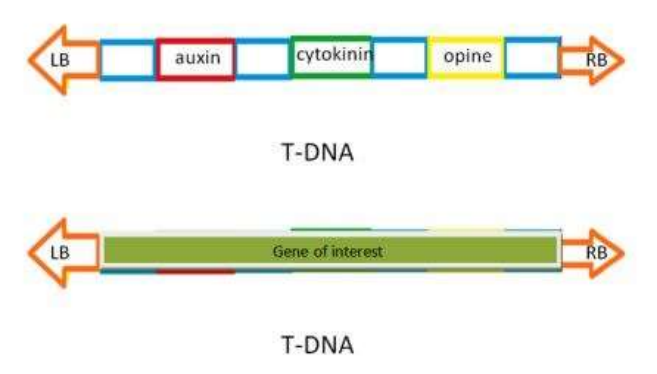
Ti plasmid (Tumor Inducing)

- Large size plasmid of 200 kbp.
- The modification of this plasmid is very important in the creation of transgenic plants.
- Plasmid have T-DNA, right border, left border, vir genes, phtochrome region, origin of replication & opine region.



Organization of T-DNA

- The transfer DNA (T- DNA) is the transferred DNA of the tumor inducing plasmid of some species of bacteria.
- Size of T- DNA is between 15-30 kb.
- It has Left borer & Right border.
- Right border play an important role in transfer & integration of T-DNA. Absence of RB will terminates the T- DNA transfer.
- T- DNA carry genes for phytohormones (Auxin & cytokinin) and opine that are expressed in plant cell.
- Overproduction of these hormones at the site of infection is responsible for proliferaton of wounded cells in tumor.



Organization of T-DNA

- Opines are low molecular weight compounds found in plant's crown gall tumors or hairy root tumor produced by parasitic bacteria of the genus Agrobacterium.
- The opines are used by the bacterium as an important source of nitrogen, carbon and energy.
- Opines are condensation product of amino acids & keto acids.
- Different types of opines amay present : nopaline, octopine& agropin.

Genes required for transfer of T-DNA

Genes	Functions
Vir A	Kinase protein, add phosphate to vir G
Vir A & Vir G	They expressed continuously. Play imp role in responding to phenolics (acetosyringone) which released by wounded plants.
Vir G	Activate other vir G
Vir D1 & Vir D2	Endonuclease
Vir D	Responsible for virulence activity of bacterium.
Vir B/D4	Type 4 secretory system,It is a pore channel formation T- DNA transfer from bacteria to plant cells
Vir E/E2	Protect T-DNA against nuclease & target T- DNA to plant cell. It act sasingle stranded binging protein.

Transformation of cells by A. tumefaciens.

1. Signal induction to Agrobacterium –plant secrets some phenolics compounds acetosyringne& some sugars,induces biochemical changes that help in T-DNA transfer.
2. Attachment of Agrobacterium to plant cells-

Agrobacterium attaches to cell through polysacchrides and cellulose fibres.

3. Production of virulence protein-

Induction causes virulence protein to form vir A, which induces Vir G, this then induces the production of rest of the virulence proteins like VirD1/D2, Vir E, Vir B

4. Production of single stranded T-DNA-

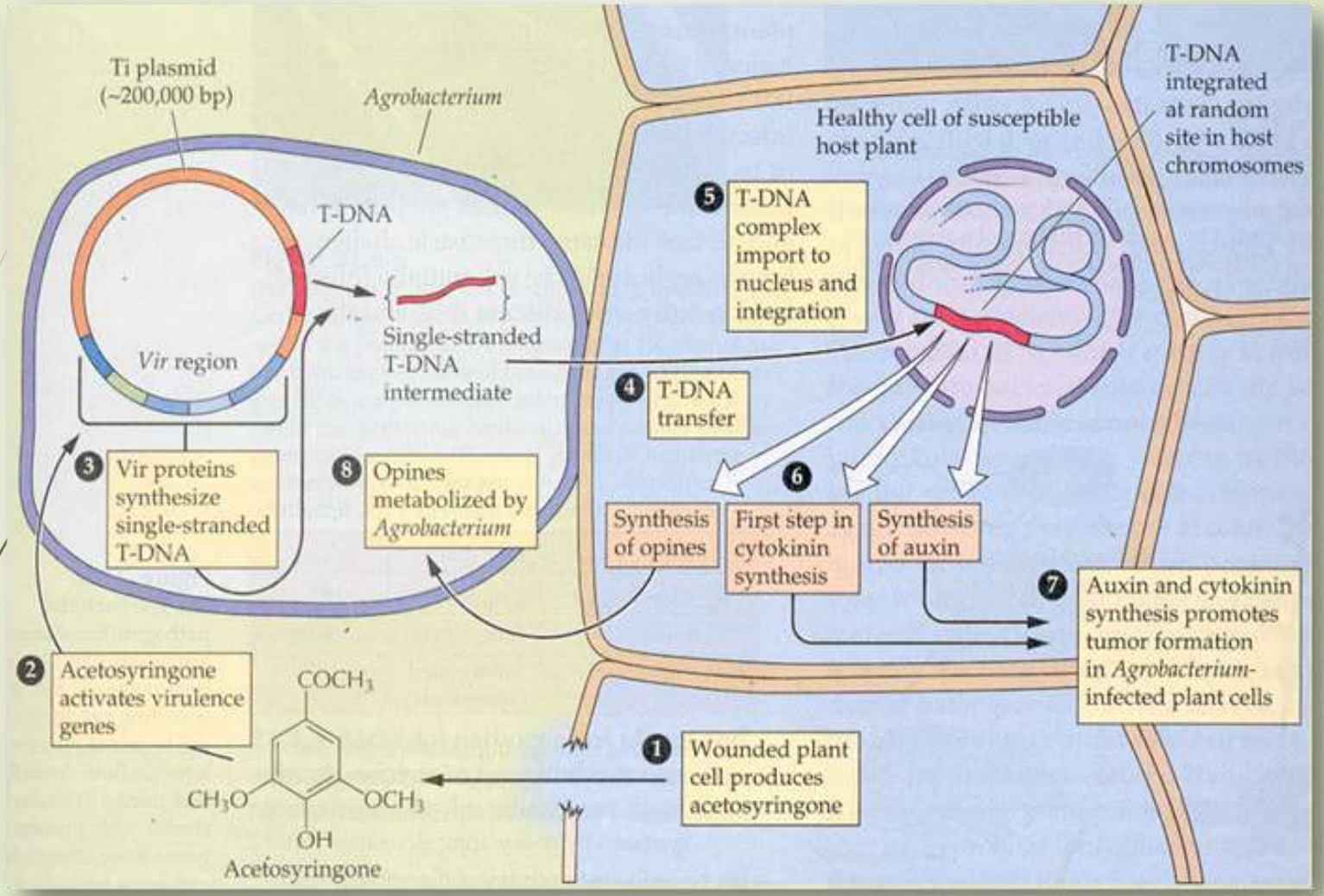
This is recognised by vir D and thus carried forward to the host by the Vir D2

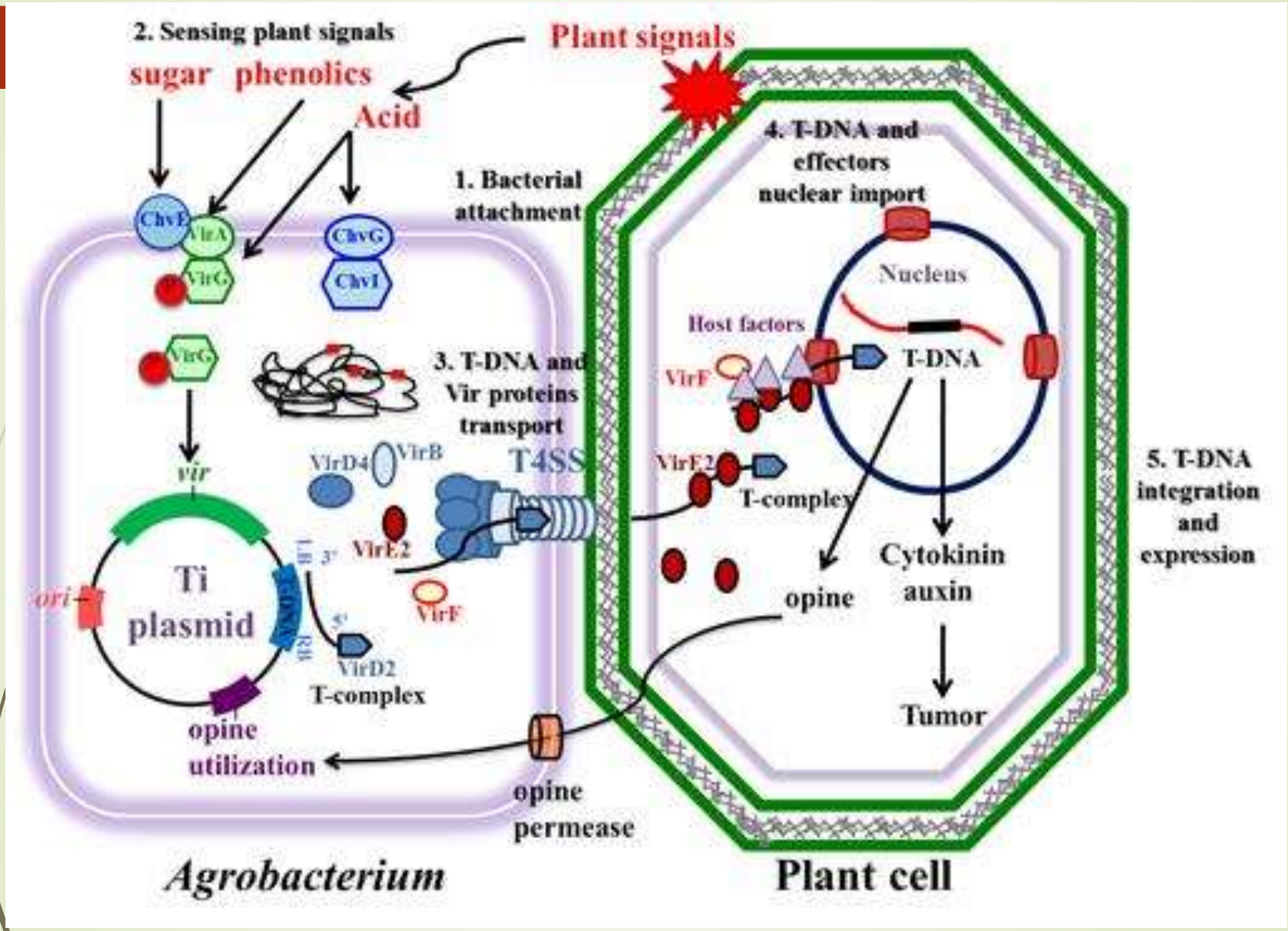
5. Transfer of T- DNA out of Agrobacterium-

The DNA strand is carried out by vir D2 through the channel made by Vir B

6. Transfer of T-DNA into the plant cells & integration-

Integration of he DNA into the host cells is helped by Vir E2, which protect the DNA from degradation by host cell restriction modification system,while Vir D2 helps to navigate the DNA to the nucleus.


Ti plasmid
(~200,000 bp)
Agrobacterium
T-DNA
Vir region
Single-stranded
T-DNA
intermediate
Healthy cell of susceptible
host plant
T-DNA
integrated
at random
site in host
chromosomes
5 T-DNA
complex
import to
nucleus and
integration
4 T-DNA
transfer
6
3 Vir proteins
synthesize
single-stranded
T-DNA
8 Opines
metabolized by
Agrobacterium
Synthesis
of opines
First step in
cytokinin
synthesis
Synthesis
of auxin
7 Auxin and cytokinin
synthesis promotes
tumor formation
in Agrobacterium-
infected plant cells
2 Acetosyringone
activates virulence
genes
COCH3
CH3O
OCH3
OH
Acetosyringone
1 Wounded plant
cell produces
acetosyringone


2. Sensing plant signals
sugar phenolics
Acid
Plant signals
1. Bacterial attachment
ChvE
VirA
VirG
ChvG
ChvI
VirG
3. T-DNA and Vir proteins transport
VirD4
VirB
T4SS
vir
VirE2
VirF
Ti plasmid
ori
T-DNA
RB
LB
3'
5'
VirD2
T-complex
opine utilization
opine permease
Agrobacterium
4. T-DNA and effectors nuclear import
Nucleus
Host factors
VirF
T-DNA
VirE2
T-complex
opine
Cytokinin auxin
Tumor
5. T-DNA integration and expression
Plant cell