GENE TRANSFER METHOD

Totipotency enable plant to regenerate a whole plant from specific cells/tissue by culturing it in suitable media

These cells/tissue are utilized for desired gene transfer in higher plants

Gene transfer method can be broadly classified into two groups:

1. *Agrobacterium* Ti or Ri plasmid mediated gene transfer and

2. Physical delivery or DNA mediated gene transfer (DMGT)

1. *Agrobacterium* mediated gene transfer

Soil bacterium *Agrobacterium tumefaciens* (causing crown gall disease) and *A. rhizogenes* (causing hairy root disease) possess Ti plasmid and Ri plasmid respectively, used for gene transfer in higher plants

Ti plasmid is the most widely used vector for gene transfer

Disease is caused by transfer of T-DNA (a part of Ti/Ri plasmid).

This feature is utilized for gene transfer

A vector is designed

⬇

Insertion of the foreign desired gene to T-DNA

⬇

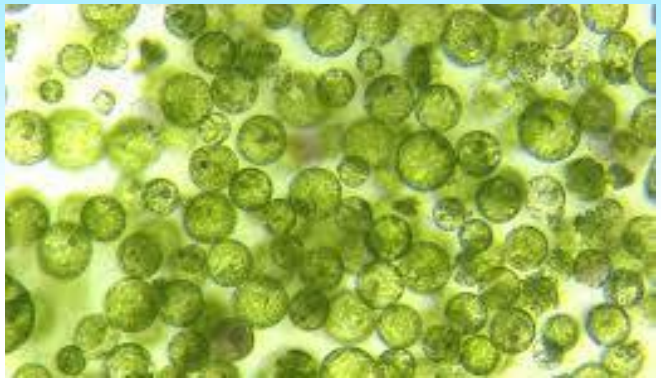
Transferred to *A. tumefaciens* or *A. rhizogenes*

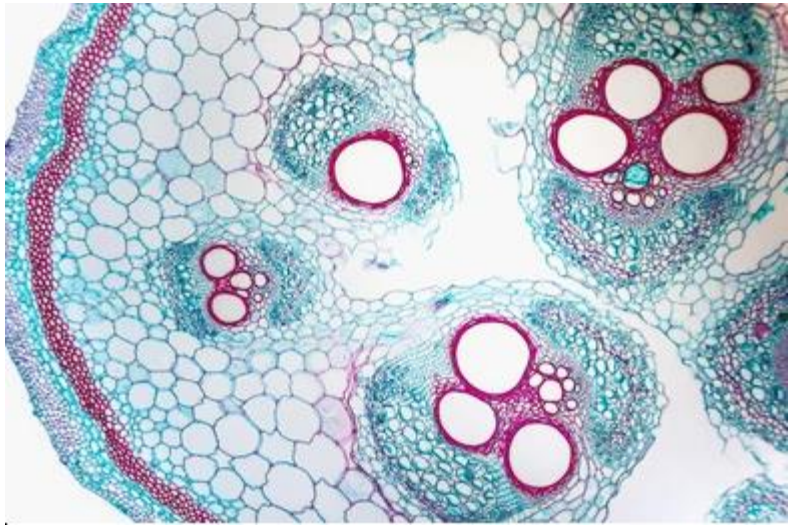
⬇

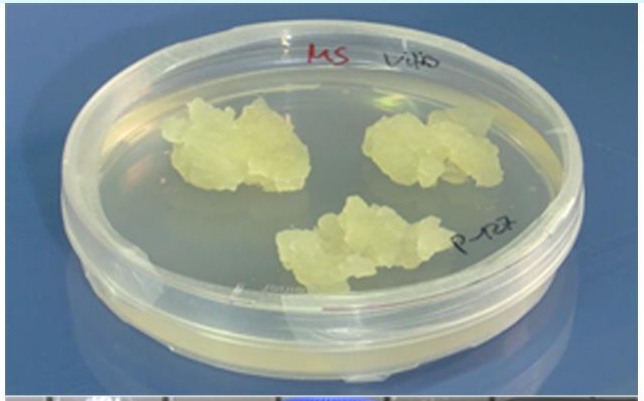
Bacterium used for infecting host cell to which the gene is to be transferred.

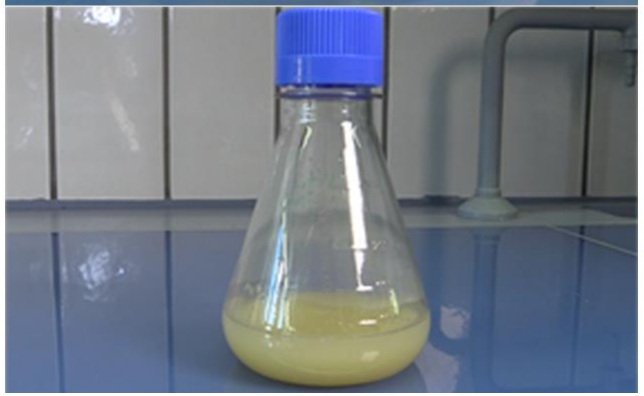
For gene transfer the explants commonly used are :

Protoplast, suspension cultured cells, callus cell clumps, thin layer cells (epidermis), tissue slices, whole organ sections (e.g. leaf discs, section of roots, stems or floral tissues) etc.

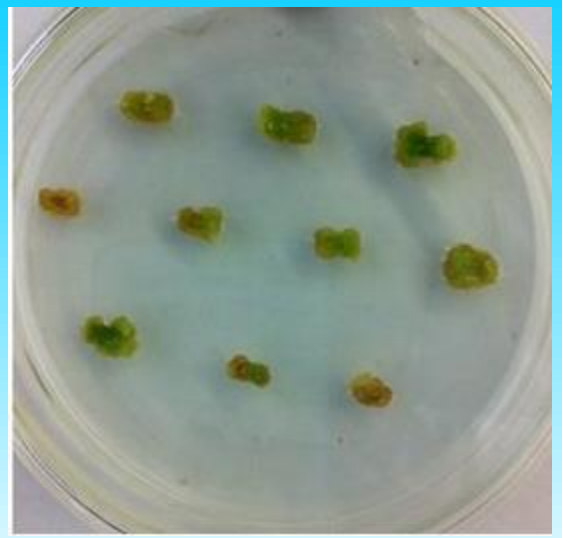






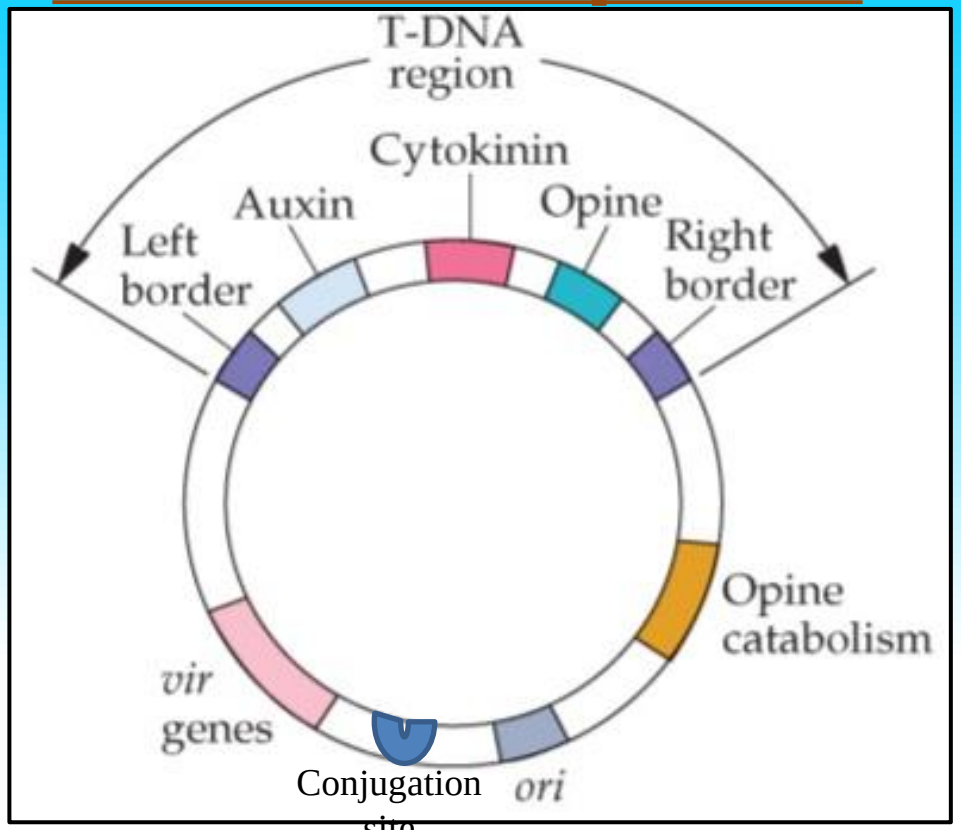


Explants incubated or co-cultivated with *Agrobacterium* carrying the vector with the desired gene. Transformed cells are selected by selectable marker incorporated in vector.



Only transformed cells are utilized for regeneration of whole plant which are expected to be transgenic in nature.

Structure of Ti plasmid



Components of Ti/Ri Plasmid

- T (Transferable) DNA region
- Vir (Virulence) region
- Conjugation Region
- Ori (origin of Replication) region

Ti plasmids are classified into different (about 14) types depending upon the specific opine being synthesized - (octapine/nopaline/agropine)

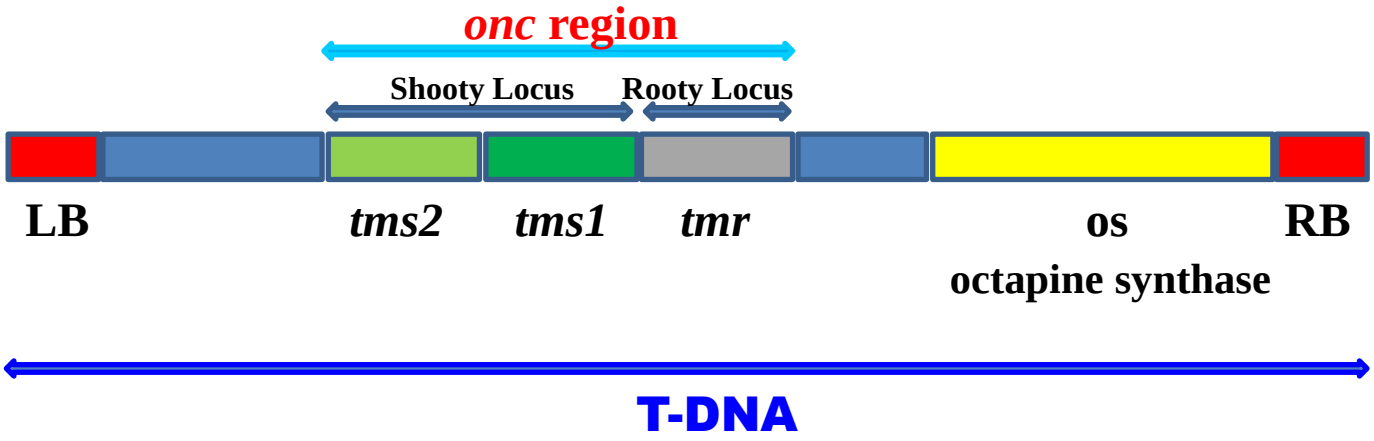
Structure of T-DNA

T-DNA contain following regions:

- *onc* region- containing three genes- *tmsl*, *tms2*, *tmr* – synthesize auxin and cytokinin, responsible for gall formation in host.
- os region – synthesis of opines – an amino acid/sugar derivative

Out side the T-DNA region there is opine (used as source of carbon and nitrogen) catabolizing gene

- T-DNA region is flanked by 25bp direct repeat sequence, which are essential for transfer. Any sequence flanked by this repeat can be transferred to host



vir region is essential for T-DNA transfer

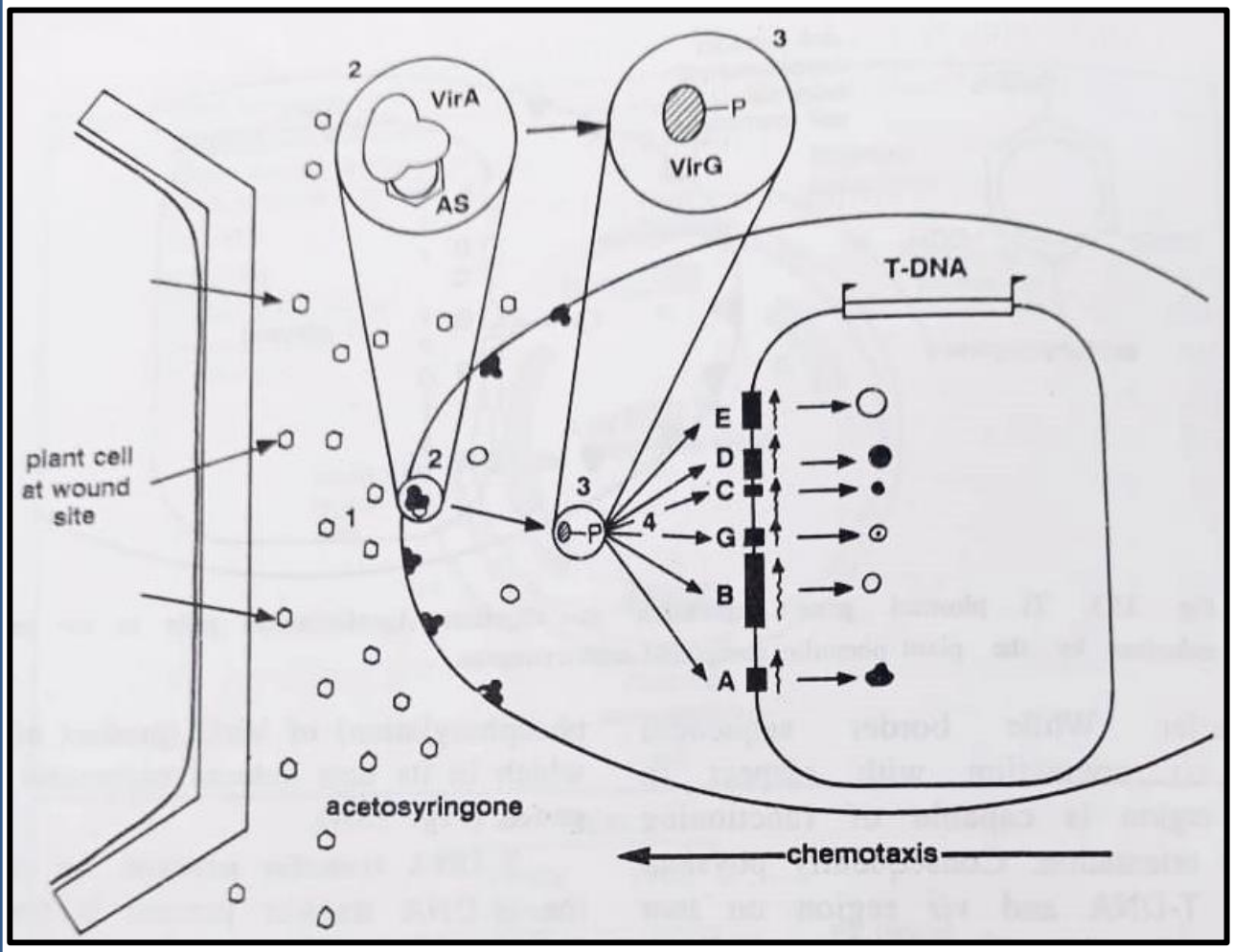
vir region (~ 35 kbp) – organized into 6 operons –

virA*, *virB*, *virC*, *virD*, *virE* and *virG

vir A, B, D and G are required for virulence

vir C and E are required for tumor formation

virA locus expressed constitutively under all conditions, *virG* locus express at low levels, but rapidly induced to higher expression levels by exudates from wounded plant tissue.



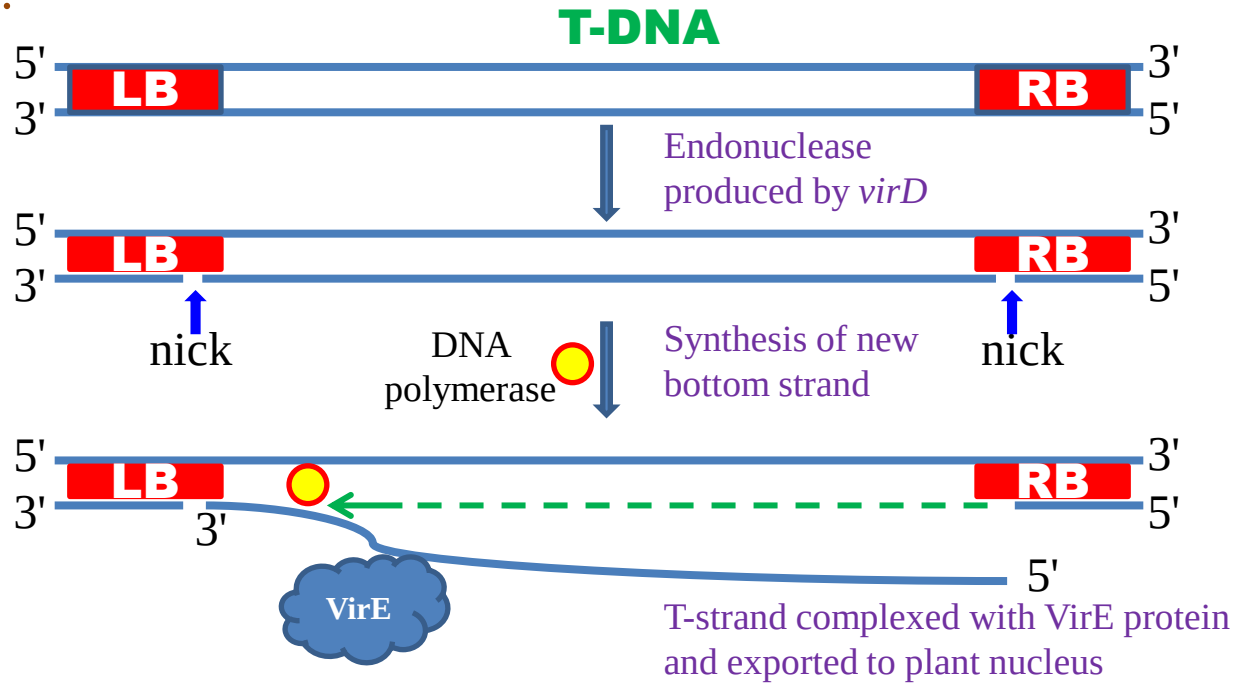
Product of *virA* [is located on the inner membrane of *Agrobacterium* cells and is probably a chemoreceptor, which sense the presence of phenolic compound such as - acetosyringone and hydroxyacetosyringone (found in exudates of wounded plant tissue)] and product of *virG* act to induce the other virulence (*vir*) genes that are carried on the Ti plasmid.

T-DNA transfer process

The vir D operon encodes an endonuclease that produces two nicks, each between the third and fourth base of the bottom strand of each 25bp border repeat

DNA synthesis occur from the nick in right border in 5'→3' direction, thus displacing a single T-DNA strand, which then forms a complex with protein Vir E and get transported to the plant nucleus.

Several gene product of vir B operon may play a role in directing T-DNA transfer extracellularly.



During insertion of T-DNA into plant chromosomal DNA, short deletions of plant DNA are often produced at junction between T-DNA and plant chromosomal DNA.

In addition, while insertion of T-DNA into plant DNA occurs at random sites, T-DNA borders exhibit some homology with plant DNA at site of insertion.

Reconstructed vectors based on Ti and Ri plasmids

Following properties of naturally occoring Ti plasmid did not allow them to be used directly as vectors:

1. Large size
2. Absence of unique restriction sites
3. Tumor induction property

Therefore vectors are reconstructed using only useful attributes of Ti plasmid –

Reconstruction of vector

Disarming is done by replacing tumor inducing gene by selectable marker (like *nptII*) gene.

Auxin, cytokinin synthesizing genes, opine synthesizing genes are removed.

Selectable marker genes (specially different antibiotic/herbicide resistant gene) with promoter and a termination–polyadenylation sequence are added.

[Because the *nptII* gene (neomycin phosphotransferase gene), as well as many of the other marker genes used in plant transformation, is prokaryotic in origin, it is necessary to put it under the control of plant (eukaryotic) transcriptional regulation signals, including both a promoter and a termination–polyadenylation sequence, to ensure that it is efficiently expressed in transformed plant cells. Such promoter may be from opine synthase gene or CaMV35S and CaMV19S (isolated from cauliflower mosaic virus)]

Specific origin of replication regions are added.

A polylinker (multiple cloning site) to facilitate insertion of the cloned gene into the region between T-DNA border sequences – are added.

- T-DNA with border sequence allows manipulation of DNA sequences intended to be transformed. *vir* genes allow infection of host plant.
- Since vir genes can function even in trans configuration, it is not essential that vir genes be present on the same plasmid, which functions as a vector and carries DNA segment to be transferred.
- In view of this, basically two types of *Agrobacterium* vectors are currently in use. These include cointegrative vectors and binary vectors.
- Cointegrative vectors recombine via DNA homology with an intermediate cloning vector. In binary vector vir gene located on a helper Ti plasmid having the whole T-DNA deleted and T-DNA on a separate vector

Prerequisites for transformation

1. Explant must produce acetosyringone or other active compounds to induce vir gene for virulence; alternatively, *Agrobacterium* may be preinduced with synthetic acetosyringone.

2. Agrobacterium cells caring the desired gene should have access to cells that are competent for transformation – cells should be replicating DNA or undergoing mitosis

3. Transformation competent cells and regeneration ability should be combined, which is easily achieved only in some species like tobacco.

***Agrobacterium* mediated gene transfer was preferred over other methods of gene transfer, due to some advantages:**

1. Defined transgene integration
2. Integration of large segments with fewer copies and minimum rearrangements
3. Preferential integration into transcriptionally active region
4. Low-cost

This process is comparatively successful in dicot plants than monocot. Combination of factors may attribute to the success of monocot also –

- Choice of supervirulent *Agrobacterium* strain (e.g. AGLO)
- Use of a suitable target material (e.g. embryo) undergoing active cell divisions and
- Use of better promoters like that from maize ubiquitin gene in plasmids pDM805, pSB11 to drive the expression of the selectable marker gene

2. Physical delivery or DNA mediated gene transfer (DMGT)

- Cereals - initially not amenable to *Agrobacterium* mediated gene transfer method.
- In many crops including cereals and legumes, the tissue culture method for regeneration was not very successful initially.

For these reasons, researchers invented Physical delivery or DNA mediated gene transfer (DMGT) methods – grouped according to the type of target cells

Delivery of DNA to protoplasts only	**Variety of explants (e.g. immature embryos, organ meristems, gametes, zygotes etc. could be used for DNA transfer**
1. Chemically stimulated DNA uptake	**4. Microinjection**
2. Liposome mediated gene transfer	**5. Macroinjection**
3. Electroporation	**6. Shooting with microprojectiles**

Chemically stimulated DNA uptake

15-25% of Polyethylene glycol (PEG) precipitate ionic macromolecules like DNA

Stimulates uptake of DNA by endocytosis without any gross damage to protoplast.

Followed by cell wall formation and initiation of cell division.

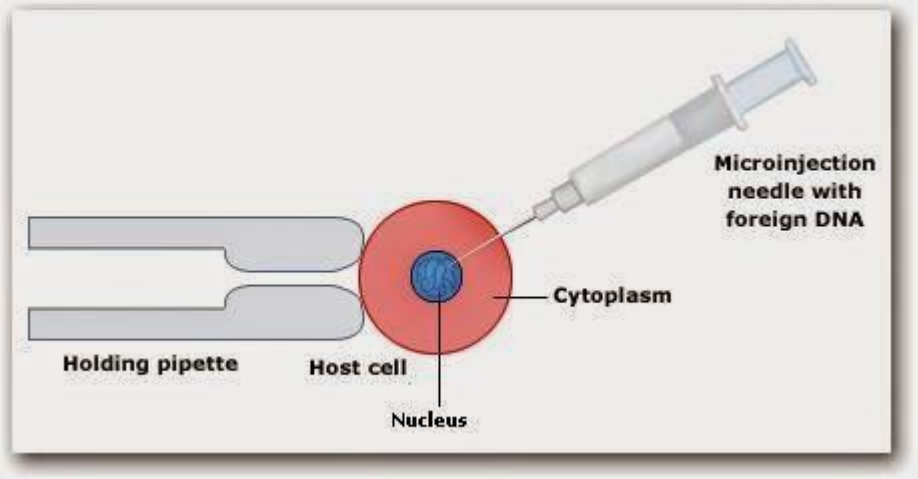
Transformed cells can be selected later.

Microinjection

Glass micropipettes (tip diameter: 0.5-10 µm) are used for transfer of DNA

Recipient cells are immobilized on solid support (cover slip or slide etc.) or artificially bound to substrate or held by pipette under suction.

Often specially designed micromanipulator is employed.



High rate of success, but the process is slow, expensive and requires highly skilled and experienced personnel.

Macroinjection

In rye (*Secale cereal*), a marker gene was macroinjected into stem below immature floral meristem, so as to reach the sporogenous tissue.

Needle diameter greater than cell diameter was used.

Unfortunately, could not be successfully repeated with any other cereal.

Shooting with microprojectiles

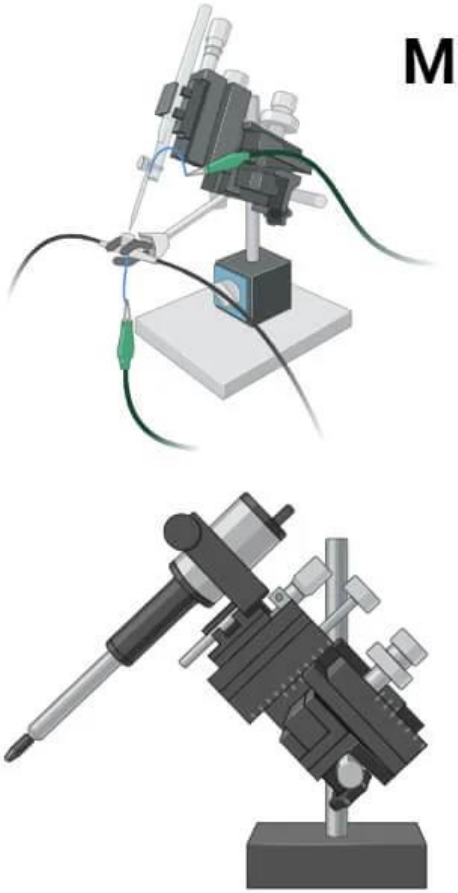
Microprojectiles (heavy microparticles like tungsten or gold coated with DNA, 1-3 µm in diameter) are carried by a macroprojectile (or bullet), and are accelerated into target cells, where they penetrate the cell wall, leaving DNA to be incorporated.

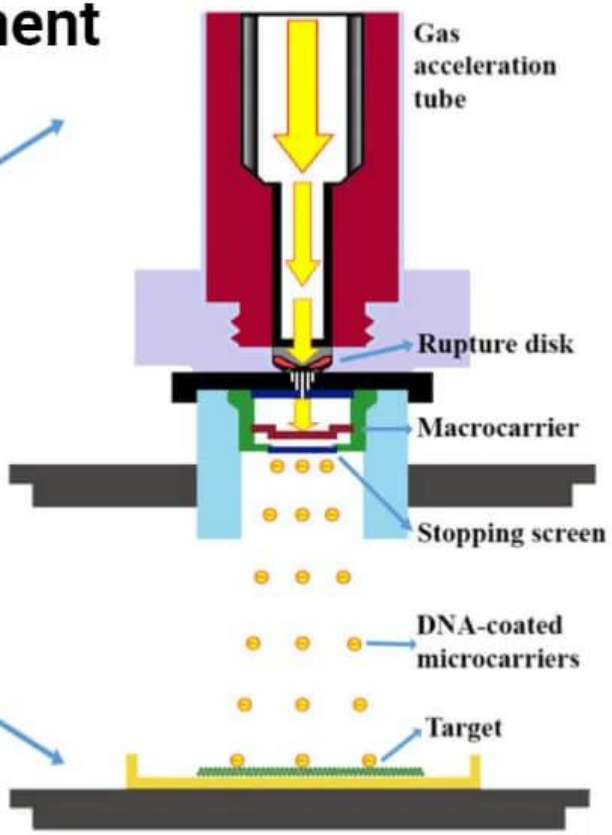
This technique results transfer of gene into many cells at a time.

Successfully used for transformation in many crops.

Universal in its application and are utilized extensively irrespective of species and genotype.

Microprojectile Bombardment





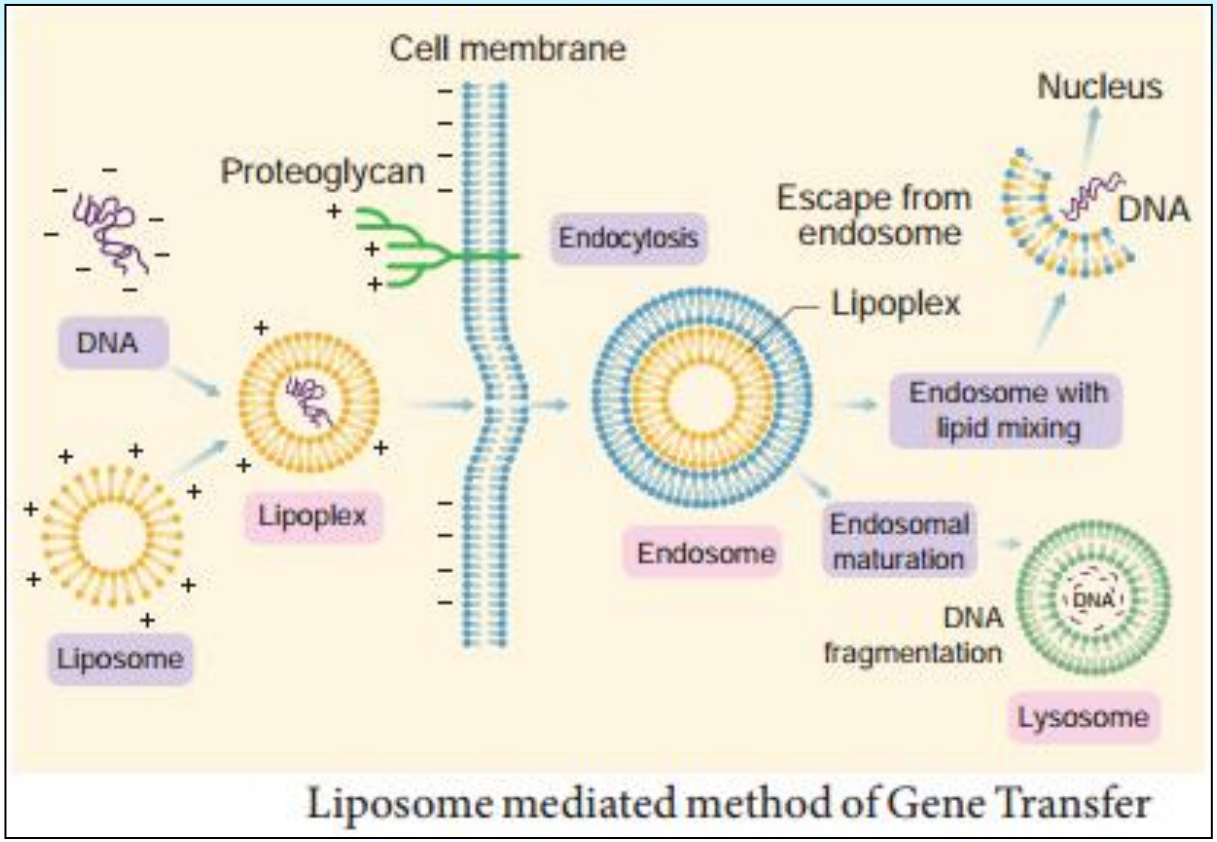
Electroporation

Short electrical pulses of high field strength are used, to increase the permeability of protoplast membrane and facilitates entry of DNA molecules into the cells.

Liposome mediated gene transfer

Liposomes - large number of plasmid enclosing small lipid bags.

Such bags are induced to fuse with protoplast by endocytosis, using PEG



Liposome mediated method of Gene Transfer

Liposome mediated gene transfer

Liposomes - large number of plasmid enclosing small lipid bags.

Such bags are induced to fuse with protoplast by endocytosis, using PEG

Advantages:

1. Protection of DNA/RNA from nuclease digestion
2. Low cell toxicity
3. Stability of nucleic acids due to encapsulation in liposomes
4. High degree of reproducibility and
5. Applicability to a wide range of cell types.

Calcium phosphate precipitation method

Foreign DNA can also be carried with the Ca^{++} ions, to be released inside the cell due to the precipitation of calcium in the form of calcium phosphate

Transformation using pollen or pollen tube, Incubation of dry seeds, embryos, tissues or cells in DNA, transformation by ultrasonication are also the possible methods applied for gene transfer, but none of these methods are absolutely fruitful.