STUDY MATERIAL

VIVEKANANDA COLLEGE THAKURPUKUR

NAAC Accredited Grade—A

BOTANY

(HONOURS)

Methods of Gene Transfer in Plants

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Gene Transfer in Plants

Transgenic plants, or **genetically modified (GM) plants**, are those that have been genetically transformed by artificially transferring suitable DNA molecules, *i.e.* **genes**, usually from other taxa, into their **nuclear** (or **organellar**) **genome** using the tools of **genetic engineering** to introduce new traits that do not occur naturally in them. Transgenic plants with improved or novel traits that are used in agriculture are called **genetically modified (GM) crops**. These are therefore a class of **genetically modified organisms (GMOs)**. The DNA molecules or genes that are artificially transferred from one organism to another are called **transgenes**. This artificial gene transfer is a form of **horizontal** (or **lateral**) **gene transfer**. The source of the transgenes are usually **other species or higher taxa** (even kingdoms: bacteria, fungi and animals), but occasionally from the **same species** but after appropriate modifications. Plant genetic engineering through gene transfer can overcome the limitations of **conventional plant breeding** for **crop improvement**. The use of multiple transgenes to introduce **metabolic pathways** for producing novel metabolites in crop plants is called **metabolic engineering**. The large-scale production of commercially-valuable substances (including pharmaceuticals) from transgenic crops is called **molecular farming** (or **molecular pharming**).

Methods of Gene Transfer

The physical, chemical and biological means of **gene transfer** in plants in the form of suitable **gene constructs** into target plant cells for their **integration** and **expression** in the **nuclear** (or **organellar**) **genome** are summarized below (Table 1). These methods may be direct or indirect. The **direct methods** do not involve any vector and **naked DNA** is directly transferred into the plant cell. The **indirect methods** involve a suitable **vector** for carrying the DNA that is finally transferred into the plant cell. Also, these procedures may be performed *in vitro* or *in planta*. The more common ***in vitro* methods** involve totipotent cells from excised plant organs or isolated plant tissues, cells or protoplasts as target cells and are maintained in the laboratory by *in vitro* culture. Transgenic plants are finally regenerated through *in vitro* techniques. Thus, **plant tissue culture** is essential for the conventional *in vit*ro methods. However, the recently developed ***in planta* methods** involve target cells in the form of **floral meristems** of field plants. Transgenic plants are directly obtained from their seeds. Thus, time-consuming plant tissue culture is not required for the innovative *in planta* methods.

Table 1: Methods of Gene Transfer in Plants

Physical Methods	Chemical Methods	Biological Methods
1. Electroporation 2. Microinjection 3. Macroinjection 4. Sonication 5. Silicon carbide whisker method 6. Laser microbeam method 7. Biolistic method	1. Polyethylene glycol method 2. Liposome-mediated method	1. *Agrobacterium*-mediated method

(Modified from Lindsey, 1998).

Electroporation

This physical method for direct *in vitro* gene transfer uses electric pulse. The target cells are in the form of plant protoplasts suspended in a buffer along with suitable divalent cations (Ca^{2+}), PEG and DNA in an electroporation cuvette. PEG, a high molecular weight polycation, helps in the adsorption of the DNA molecules to the surface of the plasma membrane. A **high-intensity electric pulse** (1-10 $kV.cm^{-1}$ for 5-200 µsec) is applied with the help of **glass capillary microelectrodes**. The electric pulse disrupts the membrane lipids and produces transient micropores to temporarily increase membrane permeability to DNA for its uptake. E.g. rice, wheat, maize, sorghum, tobacco and petunia.

Microinjection

This physical method for direct *in vitro* gene transfer uses glass micropipettes. The target cells are in the form of plant protoplasts suspended in a buffer and these are **immobilized** on a **solid support** (e.g. poly-L-lysin coated cover slip), embedded in a **substrate** (e.g. agarose, agar or sodium alginate) or held by a pipette under **suction**. Under a **micromanipulator**, the **micropipette** (0.5-10 µm diameter tip) can gently pierce the plasma membrane (and even the nuclear envelop) without injuring the protoplasts to deliver the DNA. E.g. rapeseed.

Macroinjection

This physical method for direct *in planta* gene transfer uses a standard injection syringe with needle diameter greater than the cell size. The target cells are the **floral meristems** of intact young plants. DNA suspension is directly, and rather crudely, injected into the soft tissues of field plants. The needle can pierce the outer hard tissue to reach the meristematic tissue to deliver the DNA into the germ line cells. E.g. rye.

Sonication

This physical method for direct *in vitro* gene transfer takes advantage of ultrasound. The target cells are in the form of plant protoplasts suspended in a buffer along with DNA. **Ultrasound** of 20 KHz is applied. This mild sonication produces fine temporary openings in the plasma membrane for the DNA uptake. E.g. sugar beet and tobacco.

Silicon Carbide Method

This physical method for direct *in vitro* gene transfer uses **silicon carbide whiskers** (SFWs) or fibres. The target cells are in the form of callus tissue suspended in a buffer with SFWs and DNA. This is thoroughly **vortexed**. The fibres (0.6 µm diameter and 10-80 µm length) during the vigorous shaking by vibration penetrate the cell wall and plasma membrane (and often even the nuclear envelop) to produce fine temporary pores for the DNA uptake. E.g. maize and tobacco.

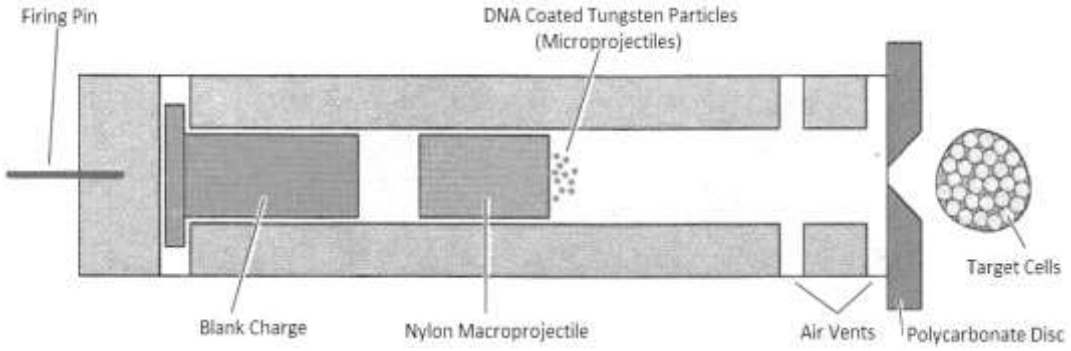
Laser Microbeam Method

This physical method for direct *in vitro* gene transfer uses laser light. The target cells are in the form of plant protoplasts (or even chloroplasts) suspended in buffer along with DNA. Under a **micromanipulator**, an **ultraviolet laser microbeam** (343 nm) is directed through an adjustable attenuator at the suspended plant protoplast. The laser beam can produce temporary micropunctures in the plasma membrane for the DNA uptake. E.g. rapeseed.

Biolistic Method

This physical method for direct *in vitro* gene transfer (also called the microprojectile, particle bombardment or popularly "**particle gun**" method) uses biological ballistics or **biolistics**. Although considerably costly and rather sophisticated, this is the most successful direct method of gene transfer in plants and can be applied to any taxa (especially the monocots). It is effective even with algae and fungi. The target cells are excised plant organs or callus tissue (and even zygotic embryos or pollen grains). DNA is coated on 1-3 µm particles (**microprojectiles**) of a heavy and inert metal such as **gold** or **tungsten**. Compressed air (130 $kg.cm^{-2}$) is usually used to accelerate **nylon** bullets (**macroprojectiles**) loaded with the particles at a **high velocity** (440 $m.sec^{-1}$). When a bullet is stopped by a **polycarbonate disc**, the particles are shot through its **central aperture** towards the target. The DNA-coated microprojectiles easily pass through the cell wall, plasma membrane (and even nuclear envelop) to deliver the DNA. E.g. rice, wheat, maize, cotton, tobacco.

Figure 1: Schematic diagram of a "particle gun" used for direct gene transfer in plants. The apparatus propels DNA-coated tungsten particles or microprojectiles through the rigid cell walls of plant cells.



(Source: Gardner *et al.*, 1991)

PEG Method

This chemical method for direct *in vitro* gene transfer uses polycations of which **polyethylene glycol** (PEG) is the most widely used agent. PEG is used at 15 to 25 %. The target cells are in the form of plant protoplasts suspended in a buffer along with divalent cations (Ca^{2+}), PEG and DNA, and incubated. PEG, a high molecular weight polycation, interacts with the slight negative charge present on the plant protoplast surface to increases the **adsorption** of DNA to the plasma membrane. It also stimulates **endocytosis** in the plasma membrane which results in the DNA uptake. E.g. petunia.

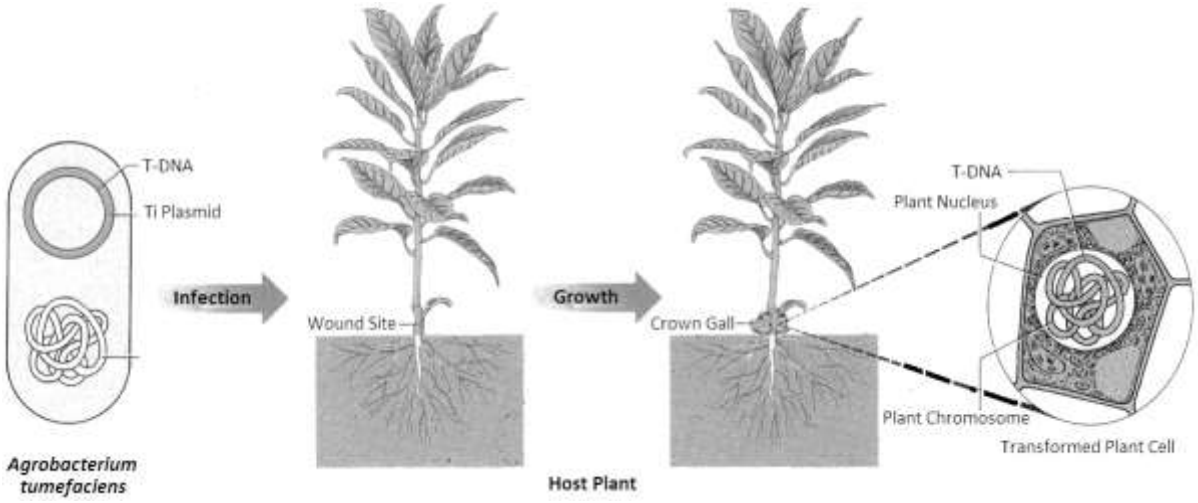
Liposome-mediated Method

This chemical method for indirect *in vitro* gene transfer (also called **lipofection**) uses artificially-prepared small lipid vesicles called **liposomes**. DNA is encapsulated in these liposomes (vectors). The target cells are in the form of isolated plant protoplasts suspended in buffer along with divalent cations (Ca^{2+}), PEG and liposome-encapsulated DNA, and incubated. PEG interacts with both the plant protoplasts and liposomes and stimulates the fusion of liposomes with the plasma membrane of plant protoplasts which results in the DNA uptake. E.g. tobacco and wheat.

Agrobacterium-mediated Method

This biological method for indirect *in vitro* (and *in planta*) gene transfer uses ***Agrobacterium***, a Gram negative, soil bacterium, that can naturally transfer its genes to dicots to produce disease in them. This pioneering method of gene transfer in plants is still the most important and widely-used (although largely in the dicots). DNA is integrated (covalently inserted) into the **transferred DNA** (**T-DNA**) region of a **megaplasmid** of the bacterium (vector). The target cells are excised plant organs or callus tissue that are simply co-cultured with the bacteria. Recent *in planta* gene transfers developed for *Arabidopsis thaliana* involve the direct application of the bacteria on intact plants by **floral-dip**, **floral-spray** or **vacuum infiltration** methods. *A. tumefaciens*, the most widely used species, causes the **crown gall disease** of **dicots** (Fig. 2). **Phenolic compounds** from **wound exudates** of the host attract the pathogen. Infection at the wound site causes cell proliferation, resulting in **crown galls** (tumours) that can synthesize novel arginine-derivatives called **opines** (*viz.* nopaline or octopine) as the bacteria's energy-source. The T-DNA (23 Kb) region of its megaplasmid (200-250 Kb), called **the tumour-inducing (Ti) plasmid**, contains tumour producing genes or **oncogenes** and opine (such as nopaline) synthesis genes.[1] The T-DNA region has two **borders** (imperfect repeats of 25 bp) required in cis-position for its excision and transfer. The transformed plant cells in the tumour contain the T-DNA segment of the Ti plasmid transferred from the bacteria, and these were randomly integrated into the DNA of the plant chromosomes. This process is controlled by the **virulence** (*vir*) region (35 Kb) of the Ti plasmid present outside the T-DNA region and harbours genes that are required in either cis- or trans-positions. The *vir* operon is induced by phenolic compounds (e.g. **acetosyringone**) present in the plant exudates. Therefore, foreign DNA present between the T-DNA borders of the different Ti vectors (with the *vir* genes present in the same plasmid or in another in the same protoplasm as described below) is transferred to the targeted plant genome. E.g. tobacco, tomato, potato, rice.

Figure 2: Transformation of the plant cells of a dicot plant by *Agrobacterium tumefaciens* harbouring wild type Ti plasmids. The bacterium infects the host at wound sites. The plant cells in the tumour contain the T-DNA segment from the bacteria's Ti plasmid that are randomly integrared into the DNA of the plant chromosomes.



(Source: Gardner *et al.*, 1991)

[1] Opine (such as nopaline) catabolism genes are present in the Ti plasmid outside the T-DNA region.

Disarmed Ti Vectors

The tumour producing **oncogenes** in the T-DNA region of the wild type Ti plasmid of *A. tumefaciens* are transferred along with the foreign gene into the plant genome. Their presence in the transformed plant cells result in the loss of normal control of cell division. The transgenic cells form tumours and the regeneration of transgenic plants becomes difficult. It is only the cis-acting borders of the T-DNA that are necessary for its transfer, and the rest can be deleted to produce **non-oncogenic** or **disarmed** Ti plasmids. The use of disarmed Ti vectors for plant gene transfer requires a **reporter gene**[2] (to replace the oncogenes) in the T-DNA region for the later identification of the non-oncogenic transformed plant cells.

Cointegrate Ti Vectors

The large (200 Kb) Ti plasmid of *A. tumefaciens* is difficult to manipulate. Thus the foreign DNA is initially cloned into a smaller (3-10 Kb) **cloning vector** called **intermediate donor plasmid**. The oncogenes in the T-DNA of the Ti plasmid are replaced with DNA from a plasmid of *E. coli* such as the common cloning vector pBR322. This **disarmed Ti plasmid** that has homology with the cloning vector is called **acceptor plasmid**. The cloning vector (with foreign DNA) present in *E. coli* is transferred to *A. tumefaciens* by **conjugative transfer**. The origin of replication site of this plasmid is specific for *E. coli* and therefore it cannot replicate in *A. tumefaciens*. It can only survive in this bacterium by **cointegrating** with the disarmed Ti plasmid to form a larger plasmid called **cointegrate**. The cloning vector and its foreign DNA is covalently inserted into the T-DNA region by **recombination** between the homologous regions of the two plasmids (unfortunately involving reversible **single crossover** events with probability of only 10^{-4}-10^{-5}). Thus, the use of cointegrate Ti vectors for plant gene transfer requires a **marker gene**[3] in the cloning vector to select bacterial cells harbouring it.

Binary Ti Vectors

The *vir* region of the Ti plasmid of *A. tumefaciens* can be trans-acting, thus allowing it to function from a different DNA molecule in the same protoplasm. In **bipartite vectors** the T-DNA region is in one plasmid (**binary Ti vector**) and the *vir* region in another (**disarmed Ti vector**). The binary vector is similar to the intermediate donor plasmid *i.e.* small sized (8-12 Kb allowing easy manipulation). To make it independent of recombination unlike the cointegrate Ti plasmid, it has a **T-DNA border** (functioning as a **direct tandem repeat** due to the small size of the plasmid which along with the foreign DNA is transferred like a T-DNA segment into the plant genome) and a **broad host range** (with multiple origins of replication sites) allowing it to **autonomously replicate** in both *E. coli* and *A. tumefaciens*.

[2] Reporter genes, also known as screenable or scoreable marker genes, have protein products that can be readily assayed subsequent to the gene transfer procedure to identify the transgenic cells. E.g. β-glucuronidase (GUS) gene which encodes this enzymatic protein that converts a colourless substrate (X-gal) into a blue product, and green fluorescent protein (GFP) gene which encodes this fluorescent protein that fluoresces green in the presence of ultraviolet light.

[3] Marker genes, also known as selectable marker genes, have protein products that can detoxify toxic selection agents present in the nutrient medium subsequent to the gene transfer procedure to preferentially select the transgenic cells. E.g. neomycin phosphotransferase II (NPTII) gene which encodes this enzymatic protein that phosphorylates and inactivates neomycin and other aminoglycoside antibiotics, and phosphinothricin acetyltransferase (PAT) gene which encodes this enzymatic protein that acetylates and inactivates the herbicide phosphinothricin.

Further Reading

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