Genetic Transformation Methods in Plants- "A Review"

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Abstract: Plant transformation is currently a crucial research tool in plant biology and a useful technique for the growth of transgenic plants. There are numerous tested techniques for introducing novel genes steadily into the nuclear genomes of various plant species. Thus, for many plant species, the development and use of practical transformation methods are no longer constrained by gene transfer and regeneration of transgenic plants. *Agrobacterium tumefaciens*, particle bombardment, and DNA uptake into protoplasts are some of the traditional methods used to modify plant genetics. These techniques produce transgenic events that contain transgenes that are randomly incorporated into the plant genome. Many issues with the current methods of gene transfer are avoided by novel techniques that facilitate the integration of foreign genes at certain pre-determined places. Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindrome repeats have all become popular tools for gene targeting in recent years (CRISPR). The current review focuses on the many strategies for plant gene targeting and how well they work, and it makes recommendations for future research in key areas of plant molecular biology.

Keywords: Plant transformation, *Agrobacterium tumefaciens*, ZFNs, TALENs, CRISPR.

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1 Introduction

Gene Transfer is the insertion of exogenous DNA into plant cells, organs or tissues employing direct or indirect means developed by molecular and cellular biology. This process is known as transformation. It is a sequential pattern process which involves the selection of plants first, those are capable of giving rise to whole transformed plants. The study of gene functions and connections, protein-protein interactions, developmental processes, applications for crop improvement, and the creation of plant bioreactors to create vaccines are just a few of the many basic research fields where plant transformation is a critical tool. Effective and repeatable transformation technologies are crucial for the creation of transgenic plants as well as for additional uses including gene editing and studies of transient gene expression (Hopp *et al*., 2022).

There are some other methods also which involves transformation without regeneration and vice versa, but these are of limited value such as *in vitro* regeneration done in meristem culture, pollen culture, organogenesis or embryogenesis using callus phase. The major limitation regarding *in vitro* regeneration in plants is the genome stress, being imposed by, especially when they undergoes regeneration via callus phase that would lead to somaclonal variation among them. Somaclonal variation refers to the genetic abnormalities or chromosomal rearrangement developed due to plant tissue culture methods. Also, genetic manipulation has been done by plant breeders for long years with great success. These types of variations can be genetic or epigenetic in origin (Bidabadi and Jain., 2020).The process of classical plant breeding is uncertain as well as slow where in order to transmit a gene of interest requires a sexual cross between two lines and then repeated back crossing between the hybrid offspring and one of the parents until a plant with the desired characteristic is obtained. Plant breeding is a lengthy process, taking ten to fifteen years to produce and to release a new variety. This process, however, is limited to those plants which can sexually hybridize, and genes in addition to the desired gene will be transferred. Recombinant DNA technologies circumvent these limitations by enabling plant geneticts to identify and clone specific genes for desirable traits. Plants have a number of unique biological features that can be explored with recombinant DNA technologies (Glick and Patten., 2022).

In this review, the basic techniques used to manipulate plants genetically are discussed i.e. from exploitation of the natural gene transfer system of *Agrobacterium* to the chemical treatment of isolated protoplasts by polyethylene glycol. It also includes physical procedures of DNA introduction, including electroporation of protoplasts and tissues, microinjection and silicon carbide fibre-mediated transformation. The methods are classified into the given categories.

2. Vectors for Genetic Transformation

A DNA molecule used as a vehicle to artificially carry the foreign genetic material into another cell is termed as a vector. It consists of a transgene and a large sequence serving as a backbone of the vector. The vectors used for plant transformation are plasmids, generally, designed specifically to generate transgenic plants. Binary vectors are most commonly used as it has the ability to replicate in both bacterium, *E.coli* (common lab bacteria) as well as *Agrobacterium tumefaciens* (a vector used to insert recombinant DNA into plants). The common features of a suitable transformation vector as given below (Low *et al*., 2018):

- Multiple unique restriction sites (synthetic polylinker)
- Bacterial origin of replication eg. *Col E1*
- Selectable marker gene
- Bacterial antibiotic resistance gene

Mostly, vectors carry these selectable marker genes that allows the recognition of transformed cells. Various types of selectable markers are now know but among all, the most common is nptII (Neomycin phosphotransferase), providing kanamycin resistance.

Agrobacterium Ti plasmids are preferred over all other types as it consists of wide host range of bacterial system and its capacity to transfer genes due to presence of T-DNA border sequences. In 1907, *Agrobacterium tumefaciens* was discovered to be the cause of crown gall disease. In the 1970s, Zaenen *et al*., 1974 identified the bacterial component that causes tumour induction as a DNA plasmid known as the Ti plasmid (Hopp *et al*., 2022).

Methods of Gene Transfer in Plants

The uptake of DNA into isolated protoplasts is mediated by chemical processes, electroporation, or the employment of high-velocity particles in a number of gene transformation approaches (particle bombardment). Both steady transformation and momentary gene expression benefit from direct DNA absorption (Narusaka *et al*., 2012). Finding and introducing novel genes into current elite cultivars is how genetic engineering has transformed the crop development process. The efficiency of the gene transfer into plant cells has been continually improved using a variety of techniques .

Methods of Gene Transfer:

- **Direct Method**

  Biolistic/Particle Gun
  PEG
  Liposome Silicon
  Carbide
  Macroinjection
  Microinjection
  Ultrasonication
  Electroporation

- **Indirect Method**

  Agrobacterium tumifaciens or Agrobacterium rhizogenes

Physical Methods of Gene Transfer

- Biolistic Gene Gun Method:

The concept of transfering DNA-coated particles directly into cells was first conceived by Sanford and co-workers in 1984. This was also known as gene gun technique or microprojectile bombardment, employs high velocity gold or tungsten particles (0.2-0.4 μm) to deliever DNA into plant cells. The coated particles are loaded into a particle gun and accelerated to high speed either by the electrostatic energy released from a droplet of water exposed to high voltage or using pressurized helium gas; the target could be plant cell suspensions, callus cultures, or tissues. The projectiles penetrate the plant cell walls and membranes. As the microprojectiles enter the cells, transgenes are released from the particle surface for subsequent incorporation into the plant's chromosomal DNA. The method was developed with the aim to transform monocoltyledons, that are recalcitrant to transformation with *Agrobacterium* (Narusaka *et al*., 2012). Compared to the agroinfiltration and protoplast transformation approaches, transient expression experiments in plant tissues by biolistics take a lot less time overall. 24 hours after the vector is prepared, transient expression following biolistic administration can be seen. In addition to the 2 days needed for the transformation of the *Agrobacterium* cells with the DNA construct, the inoculation of *Agrobacterium* requires the establishment of a bacterial culture 24 hours beforehand and the detection of transitory expression 2–4 days following inoculation (Lacroix and Citovsky., 2020).

There are few applications of this method such as its versitality, simplicity as well as efficiency. This method enables to enlighten the concept of gene expression and its regulation. Also, this method of microprojectile bombardment allows more efficient transformation via *Agrobacterium* as it can be used to wound plant tissues. This technique significantly reduces the time required for production of genetically modified plants. With that, it helps in the transformation of several major cereals, including barley, maize, wheat, rice, pearl millet, together with other monocotyledons such as tulip and orchids.

Due to its simplicity, adaptability, and great efficacy, biolistic DNA transport has been regarded as a universal technique for genetic modification to transfer exotic genes to cells or tissues. In the majority of monocot crops, it has been the method of choice for examining the function of plant genes. In 1993, biolistic DNA delivery led to the successful regeneration of the first transgenic sorghum plants, with a transformation efficiency of only 0.3%. Since then, enormous progress has been made, with the highest transformation efficiency reported in recent years being 46.6% (Liu *et al*., 2020).

- **Electroporation**

Transient pores are created in the plasma membranes of prokaryotic and eukaryotic cells through the electrical transformation process known as electroporation. The electropores or

microscopic pores that are produced by the applied field in the cell membrane enable the entry and/or exit of micro- or macromolecules. Electrical pulses are applied to a suspension of protoplasts with DNA placed between electrodes in an electroporation cuvette. Short high- voltage electrical pulses induce the formation of transient micropores in cell membranes allowing DNA to enter the cell and then the nucleus. The method is used to transport biochemical substrates like lipids, proteins, RNA and DNA to cell interior (Ozyigit *et al*., 2020). Some of the applications of the method includes the creation of kanamycin-resistant plants from transgenic rice (*Oryza sativa* L.) plants electroporated with plasmid harbouring the nptII gene revealed the viability of protoplast in genetic engineering investigations (Saunders *et al*., 1989). The genomes of transgenic rice plants (*O. sativa* L.) were complexly electrotransformed with multiple copies of inserted genes. The study led to the identification and expression of HPT in the developmental history of transformants (Tada *et al*., 1990).

- **Microinjection**

Dr Marshall A.Barber invented this method initially to clone bacteria and to confirm the germ theory of Koch and Pasteur. Transformation through a microprojectile is a direct and precise delievery of DNA into the plant cell through a glass microcapillary injection pipette. DNA or other material is injected into the cell for later integration and/or expression using very small bore glass needles (outer diameter typically less than 0.2 μm) (Dean., 2013).

Its importance for plant transformation is rather limited due to the characteristics of plant cell walls, which contain a thick layer of lignins and cellulose. The plant cell wall is a barrier for glass micro tools. The method allowed the incorporation not only of DNA plasmids but also of whole chromosomes into plant cells. The transformation efficiency is ten times lower when compared to the biolistic gene gun method. Also, the injection of inheritable material like immature embryos, meristems, immature pollens, germinating pollen etc using a hypodermic syringe is known as macroinjection. The crops in which microinjection and macroinjection has been done are maize, tobacco, rice, soybean, wheat, cotton, nut, grass, algae, Rye, watermelon and petunia.

- **Silicon-carbide whisker-mediated transformation**

Si C mediated transformation (SCMT) method was proposed in 1990 to transform maize and tobacco. Silicon carbide filters are added to a suspension of tissue (cell clusters, immature embryos, callus and plasmid DNA using a vortex, shaker or blender. The fibres are used which are single crystals of silica organic minerals like silicon carbide which possess an elongated shape, having a diameter of 0.6 mm and a length of 10–80 mm. DNA coated fibers

will penetrate the cell membrane through small holes created by collisions between the plant cells and fibres. The method is least complicated and allows the stable transformation of different plants but having the disadvantage of low transformation efficiency. Plant material, fiber size, parameters of vortexing, shape of the vessels used, and the characteristics of the plant cells, especially the thickness of the cell wall are the factors on which transformation efficiency depends. The method being done has been reported in case of transformation of cotton (*Gossypium hirsutum* L.) and regeneration of salt tolerant plants (Arshad *et al*., 2013). The transformation of monocot and dicot plant species embryo and cell suspension cultures has been carried out using silicon carbide and other whiskers from various sources (Asad andArshad., 2016).

- **Ultrasound mediated gene transfer**

Ultrasound-mediated membrane perforation known as sonoporation has the potential to pierce tough cell walls and plasma membranes on a presumably reversible basis, which is advantageous for gene transfection and plant biotechnology.
The method involves rupturing the cellular membranes by acoustic waves which leads to the possibility to non-invasive introduction of molecules like DNA into the interior of cell. The membrane permeability increases and thereby, the entrance of molecules into the cells is facilitated. This phenomena leads to generate the microscopic channels that favour the exposure of internal plant tissue increasing the transient expression level of transferred DNA even to double. DNA has been introduced to the protoplasts of beetroot and tobacco by 20kHz ultrasound at 0.5 to 1.5 W/cm^2 **(Zolghadrnasab *et al*., 2021)**.

Chemical Methods of Plant Transformation

- **Gene Transfer by Polyethyleneglycol**

The method leads to the physicochemical uptake of DNA via endocytosis. The protoplast are kept in polyethylene glycol (PEG) solution. The concentration of PEG used is 15% having 8000 Dalton molecular weight. After exposure of protoplasts to exogenous DNA in presence of PEG and other chemicals, PEG is removed and intact protoplast are then cultured to form cells with walls and colonies in turn. Due to the simplest transformation protocol, the method is most widely used among all.
Without removing their cell walls, *E. coli* and *Saccharomyces cerevisiae* are both susceptible to the genetic change caused by polyethylene glycol (PEG). The PEG-mediated transformation of E. coli is technically straightforward and produces transformants with an efficiency of 10^6–10^7 transformants/μg DNA **(Klebe *et al*., 1983)**.

- **Liposome mediated encapsidation**

Lipofection, a method developed to protect the foreign DNA using liposomes during the transfer process first described in 1965 as a model of cellular membranes. Liposomes are artificial phospholipid vesicles used for delievery of molecules into the cells, multi-lamellaror unilamellar vesicles with a size range of 0.1-10µm or 20-25 nm respectively. The method of transformation followed by transgenic plant regeneration has been reported in tobacco and wheat **(Zhu *et al*., 1993).**
In terms of vectors for delivering genes to plant cells, liposomes may have the following benefits: Nucleic acids are protected from nuclease activity, nucleic acids are targeted to specific cells, nucleic acids are delivered into a range of cell types other than protoplasts through entry through plasmodesmata, and nucleic acids are delivered with intact tiny organelles **(Gad *et al*., 1990).**

DEAE- Dextran (Diethylaminoethyl dextran)

DEAE-dextran is a soluble polycationic carbohydrate that promotes interactions between DNA and endocytotic machinery of the cell. Negatively charged DNA and positively charged DEAE-dextran form aggregates through electrostatic interaction and forms a polyplex. The osmotic shock using DMSO or glycerol improves the complex DNA delievery with DEAE-dextran. The method is simple and inexpensive but sometimes toxic when used at high concentrations. The method is initially reported by Vaheri and Pagano in 1965 for enhancing viral infectivity of cell but later adapted as a method for plasmid DNA transfer **(Kujawa *et al*., 2021)**.

Indirect or Biological Method of Gene Transfer

- ***Agrobacterium* mediated plant transformation**

The ability of the bacterial pathogen *Agrobacterium tumefaciens* to introduce foreign genes into a variety of host plants is a key component of *agrobacterium*-mediated transformation (AMT). It is the most commonly used method for plant genetic engineering. The pathogenic soil bacteria *Agrobacterium tumefaciens* that causes crown gall disease has the ability to introduce part of its plasmid DNA into the nuclear genome of infected plant cells. The genetic material that is introduced is known as T-DNA located on a Ti-plasmid. A Ti-plasmidis a circular piece of DNA found in almost all bacteria. Due to its high efficiency, it is the most efficient method of transformation. During transformation, several components of Ti-

plasmid enables the effective transfer of genes of interest into the plant cells such as T-DNA border sequences, vir genes; for transferring T-DNA region to the plant genome but are not transferred themselves. The advantage of using this method over other methods are reduction in transgene copy number; intact and stable integration of transgene into the plant genome **(Bartlett *et al*., 2008).**

Seven steps make up the protocol, which can be summed up as follows: Stage (I) involves thepreparation of sterilised seed or samples and inoculum; Stage (II) entails explant preparation, infection, and cocultivation with *A. tumefaciens*; Stages (III) and IV involve selection; Stage (V) involves acclimatisation and molecular identification of T_0; Stage (VI) involves cultivation and self-crossing of T_0; and Stage (VII) involves T1 plant analysis **(Pratiwi and Surya., 2020)**.

Also, to cause crown gall formation, T-DNA encodes genes for the production of **auxin or indole-3-acetic acid** via the IAM pathway. This biosynthetic pathway is not used in many plants for the production of auxin, so it means the plant has no molecular means of regulating it and auxin will be produced constitutively. Genes for the production of **cytokinin** are also expressed. This stimulates cell proliferation and gall formation.

Example of T-DNA genes in Ti plasmids

Gene	Product	Function
ocs	octopine synthase	opine synthesis
nos	nopaline synthase	opine synthesis
tms1	tryptophan-2-mono-oxygenase	auxin synthesis
tmr	isopentyl transferase	cytokinin synthesis
ags	agropine sunthase	opine synthesis

T-DNA transfer and integration into the genome is mediated by various vir genes such as vir A and vir G both expresses constitutively. Vir A codes for kinase spans the bacterial membrane and for phenolic molecules (acetosyringone) it acts as receptor, released by wounded plant cells. This binding of acetosyringone causes vir A to get autophosphorylated on histidine residue. This phosphate group transferred to aspartate residue in vir G. Both vir A and vir G genes acts as activator for other vir genes. T-DNA transfer occurs through a conjugative pilus formed as a result of vir gene expression, initiated by the products of vir D1 gene (helicase) and vir D2 gene (act as endonuclease). The representation of *Agrobacterium* T-DNA transfer is given below:

In planta transformation

Since the first time *Agrobacterium* was used to transform plant explants, there has been a lot of interest in creating transformation techniques that do not rely on tissue culture regeneration, can provide high throughput transformation, require little to no labour, money, or expertise, and lower the rate of unintended mutagenesis and somaclonal variations induced by in vitro culture. Feldman first created an *in planta* transformation procedure in the A. thaliana model plant **(Ziemienowicz., 2014)**.

Role of gene transfer methods in crop improvement

- Genetic engineered wheat has led to increase the grain yield and minimize the crop loss due to unfavourable weather conditions and has developed resistance in crop against various pathogen and pests. The first transgenic wheat plants were produced by microprojectile bombardment as a method of DNA delievery. Genetic transformation with a single target gene has been used for the production of transgenic wheat expressing tolerance to herbicide, resistance to fungal and viral diseases.
- A coat protein-mediated resistance to viruses, introduced into rice via protoplast transformation, was transferred then to maize and barley via particle gun bombardment. The resistance to sulfonylurea (herbicide) conferred by the *als* gene of *Arabidopsis thaliana* was also transferred to maize by particle gun technology. Maize has been reported to get transformed by Silicon carbide fiber-mediated DNA delivery system. Whiskers-mediated maize transformation has also been reported.
- Transformation in rice leads to double the food supply and improved the quality as well as quantity. Biolistic was successfully used for transformation of immature embryos of rice . Reports were also maderegarding the transformation of indica and javanica rice in addition to other japonica rice. Fujimoto et al. were the first to engineer japonica rice through electroporation with modified d-endotoxin gene (*cry*) from *Bacillus thuringiensis* (**Khan., 2009)**.

3. Conclusion

One of the most difficult elements of plant science is arguably the development of gene transfer systems in plants. *Agrobacterium*-mediated and biolistic-mediated DNA delivery technologies are now the two best options. Two decades ago, the primary goal of the development of transformation technology was the creation of transgenic crops with enhanced agronomic properties for increased crop productivity.

The challenge of applying transformation strategies to higher plants with a higher frequency to produce GT events has not yet been solved. The transgene-carrying *Agrobacterium* strains can easily transform *Arabidopsis* plants by dipping the plants in the solution. It takes a lot of work to alter and screen higher plants for GT events. By using gene knocking, the creation of the GT technique represents a significant advance in furthering our understanding of how a single gene functions in the context of its genome. It also has the potential to boost public acceptability of molecularly-based plant gene manipulation.

Conflicts of Interest

The authors declare no conflict of interest.

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