TRANSGENIC BREEDING

Varsha Kundu[1*]

[1]Ph.D. Research Scholar, Department of Genetics and Plant Breeding,

Institute of Agricultural Science, University of Calcutta

51/2 Hazra Road, Kolkata, W.B.,

PIN- 700019.

Corresponding author email: varshakundu1997@gmail.com

Contact Number: 8967061844

ABSTRACT

Plant modification enables the transfer of the desired gene, making it possible to create unique transgenic plants that would be challenging to grow using traditional methods. To transfer transgenes from genotypes that are adaptable but unfavourable from an agricultural standpoint, into superior breeding lines using the backcross breeding approach, plant breeding must be included. Large-scale production of transgenic plants is challenging, labour-intensive, and expensive. To serve economic objectives, it is worthwhile to make simultaneous attempts to incorporate the transgene into enhanced plant material. Plant transformation techniques let farmers to get greater yield from less land and produce more with less money spent on inputs. With the help of plant transformation technologies, many crops are becoming accustomed to our desired traits. This chapter describes the technology of plant transformation, the sequential phases of transgenic crop development, and the advantages of transgenic plants.

Key words

Back cross breeding, Gene of interest (GOI), Genetically modified crop (GM crop), Plant transformation, Restriction endonuclease, Transgenic, Transgenic breeding, Transformation methods.

I. Introduction

Through traditional breeding, humans have been genetically altering plant crops for a very long time. Through breeding and selection, the desirable phenotypic qualities are transmitted to the progeny. The reproductive barrier between species, phylogenetic isolation barriers between and within genetic groupings, a smaller gene pool, and linkage drag are some disadvantages of these traditional methods of genetic modification. In addition to the extended period of time typically needed for favourable qualities to transfer, all of these disadvantages exist. Additionally, this era has witnessed a rapid increase in urbanisation and population, which has resulted in a massive loss of agricultural land; as a result, food security, particularly for the impoverished people, is of utmost concern. This will demand a 70 % increase in food production by 2050, according to estimates. Transgenic breeding can be one of the greatest ways to solve these issues in light of the aforementioned facts. The commercialization of varieties and hybrids with novel transgenic features, like disease, insect-pest resistances, and herbicide tolerance[1],[2] has effectively illustrated the promise of transgene technology in crop improvement. Furthermore, with the encouragement and enforcement of complementary national biotechnology laws that protect human, national trade, and the environment; transgenic crop varieties can be advantageous to both large cropping systems in the advanced nations and small farms in the developing nations[3]. Transgene technology is thus evolving into a crucial component of plant breeding. Modern developments in this quickly evolving field of transgenic technology are covered in this chapter.

II. Basic concepts of transgenic crops and transgenic breeding

Gordon and Ruddle coined the term "transgenic" in 1981 to refer to genetically altered creatures (plants, animals, or microorganisms). The term "transgenic breeding" refers to the genetic enhancement by biotechnology of domesticated animals, crop plants, and beneficial microorganisms. In particular, transgenic plant breeding describes the use of transgenes to improve the genetics of crop plants. Crops that have undergone genetic modification, also reffered as GM crops, are those whose genetic makeup has been edited through genetic engineering in order to give the plant a new trait that does not normally occur in that species[4]. In 1983 the first transgenic plant was created by Fraley *et al*. in the tobacco in 1983. The technology of plant breeding has been extended via transgenic breeding. It gives breeders the chance to; (i) introduce novel genetic variation that is absent in the breeding gene pool at the moment, and (ii) produce desired phenotypes from known genes. Globally, a variety of GM types and hybrids are created and grown[5]. GM crops in close collaboration with plant breeding approaches can address resilient crop systems throughout wide agro-ecosystems under a myriad of problems[6].

Table 1: Comparison between conventional breeding and transgenic breeding

Sl. No.	Particulars	Conventional breeding	Transgenic breeding
1.	Definition	Traditional breeding typically uses selection while utilising sexual and asexual reproduction that occurs naturally.	Transgenic breeding bypasses the sexual process and uses a way of introducing genetic material through a gene gun or other direct gene introduction methods, or by a specially designed bacterial vehicle, which does not occur in nature.
2.	Methods involved	Hybridization between two genotypes	Tissue culture and genetic engineering (rDNA technology)
3.	Transfer of genes from microbes and animals	Not possible	Possible
4.	Chances of linkage drag	Present	Absent
5.	Manipulation of genes	Indirectly by selecting offspring with desired traits	Direct manipulation of one or more genes
6.	Frequency of desirable plants	Adequate	Low
7.	Technical skill required	Moderate	Very high
8.	Expenditure involved	Low	High
9.	Time required for releasing new variety	10 to 15 years	3 to 4 years
10.	Equipment and facilities required	Simple, mainly field facilities required	Sophisticated, well-equipped lab

III. Development of transgenic crops

Although, there present many different and intricate processes to create of transgenic crops, its fundamental ideas are straightforward. It is essential to comprehend how genes are regulated, how to use genes and gene products carefully as well as how biochemical and physiological pathway function. However, in order to create genetically modified plants, a series of five steps must be successfully completed, including isolation of nucleic acid *i.e.*, DNA or RNA from the organism containing the desired gene, cloning of the gene, packaging and designing of the desired gene, cell transformation, or the insertion of the desired gene into a specific plant cell, and backcross breeding.

1. Isolation of nucleic acid

This is the beginning of the transgenic plant development process. It is necessary to extract nucleic acid (DNA, RNA) or a gene from the target organism in order to work with it. A variety of protocols are used to remove the nucleic acid from a sample of an organism containing the desirable gene. To isolate the nucleic acid, three fundamental stages are required: cell lysis; removal of contaminating proteins, nucleic acids, and salts; deactivation of DNAases or RNAases; and, finally, the recovery of DNA or RNA. Despite the fact that these fundamental processes are the same for all crops, the actual protocol for isolating the nucleic acid varies.

2. Cloning of the gene

During this phase, the target gene is sorted using restriction enzymes from the donor organism's whole DNA before being cloned into an appropriate vector and make thousands of copies of it.

Restriction endonuclease

Restriction endonuclease (molecular scissors), a bacterial enzyme shreds dsDNA into bits after identifying particular nucleotide sequences called recognition or restriction site. Hamilton and his co-workers isolated a restriction enzyme, HindII, from the bacterium Haemophilus influenzae strain Rd in 1970 for the first time. These enzymes are considered as an important tool for genetic engineering. Restriction endonucleases are classified into of four groups viz., Type I, Type II, Type III and Type IV, based on their constituents, characteristics of the cleavage site and the co-factor requirements.

Table 2: Comparative characteristics of different types of restriction endonucleases

Characteristics	Type I	Type II	Type III	Type IV
Nature	Bi-functional, have both endonuclease and methylase activity	Uni-functional	Bi-functional, have both endonuclease and methylase activity	Target modified DNA such as methylated, hydroxymethylated and glucosyl-hydroxymethylated DNA
Protein structure	Three different subunits	Two identical subunits	Two different subunits	
Co-factors	ATP, Mg^{2+}, S-adenosylmethionine	Mg^{2+}	ATP, Mg^{2+}	Mg^{2+}
Cleavage site	Random, up to 1000 bp away from the restriction site	At or near restriction site	25-27 bp 3’ to restriction site	Close to or within the restriction site
Example	EcoB	EcoRI	EcoPI	McrBC

As they fragment DNA at locations outside of the recognition sites, Type I and III endonucleases are not so effective for gene coning because they produce a unpredictable cleavage pattern. On the other hand, Type II endonucleases are frequently used for cloning and mapping practices because they recognise particular sites and cleave only at these locations.

Any cloning experiment goes through five basic steps, which include;

a. Generation of donor DNA fragments or gene by using suitable restriction endonuclease enzymes or reverse transcriptase enzymes,

b. Selection of appropriate cloning vector that must be self-replicating inside the host cell, must possess restriction site for RE enzymes, must contain a selection marker gene for later identification of recombinant cell. The primary function of a cloning vector is to duplicate the introduced DNA fragment within the host cell. Ti plasmid, co-integrative and binary vectors and plant virus vectors like *Cauliflower mosaic virus* (CaMV), *Tobacco mosaic virus* (TMV), *Alfalfa mosaic virus* (AMV), *Potato virus X* (PVX), *Cowpea mosaic virus* (CPMV) etc. can be utilized as vectors in order to create transgenic plants.

c. Deployment of recombinant vector into an appropriate host.

d. Careful selection and separation of recombinant cell from non-recombinant cells through plasmid vector's flag (marker) gene.

e. Amplification of the inserted gene in the host in growth media.

3. Packaging and designing of the desired gene

After the interested gene has been cloned, it needs to be connected to other DNA segments that will regulate how the gene will function once it has been inserted into the plant genome. These bits of DNA will activate (promoter) and deactivate the inserted gene's expression. By substituting an existing promoter with a new one and including a selectable marker gene, one can construct or package genes. Promoters allow for varied gene expression, which means that while certain promoters cause the inserted gene to be expressed constantly, others only permit it to be expressed at specific phases of plant development, in specific plant tissues, or in response to environmental cues. Additionally, the promoter also regulates how much of the gene product will be expressed.

In order to make it easier to recognise the gene of interest once it is inside the plant tissues, selectable marker genes are frequently linked to it. This makes it possible to choose the cells that have successfully absorbed the desired gene, saving both money and time. Modern genetic engineers analyse plant tissues for the insert using an antibiotic resistance marker gene. The presence of an inserted gene can be detected in cells that survive the addition of antibiotics to the growth media. Due to certain worries that the use of antibiotic resistance marker genes will enhance antibiotic resistance towards antibiotics in both humans and animals, the genes coding for resistance to non-medicinally essential antibiotics are recommended in this situation. Meanwhile, alternative types of marker genes are also occurring.

It is then injected into a bacterium to enable the production of several copies of the gene package after the gene of interest, promoter, and the marker gene have been packaged together.

4. Gene insertion into a plant cell

Once the gene package is prepared, it must be inserted into the plant cells that are undergoing modification, a procedure known as cell transformation or gene insertion. Three important organelles, the nucleus, chloroplast, and mitochondria, each with their own genome, are present in every plant cell. Two of these three had their genomes routinely altered to add new features to cultivated plants. While chloroplast transformation is clearly more successful at modifying therapeutic qualities, nuclear transformation is more effective at modifying agricultural charascteristics in crops. Chloroplasts are preferred to express features linked to health rather than agriculture because they are polyploid at the organellar and genome levels and offer natural gene confinement. It is common practise to generate transgenic crops to express beneficial and useful features, however it is crucial to talk about the technology involved in their creation. Higher plants can receive exogenous DNA by a variety of direct and indirect mechanisms, including protoplast electroporation, particle bombardment (biolistic), and *Agrobacterium*-mediated gene transfer.

4.1 Indirect methods

4.1.A Genetic transformation using *Agrobacterium sp.*

This method involves combining the transgene with a vector, which is then introduced into the desired cells for integration. The term "plant gene vector" refers to promosing carriers of genetic material from bacteria, fungus, and other organisms to plants as well as between plants. The ability of the host plant to recreate is closely associated with the vector-mediated gene transfer. Plasmids or viral genomes are the bases for plant vectors.

In 1983, the *Agrobacterium* system made a significant advancement in plant genetic engineering by becoming the first and most commonly utilised transformation system in history. The two common species of gram-negative, naturally occurring *Agrobacterium* are *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. These are regarded as the world's natural genetic engineers because of their capacity to alter plants.

A. tumefaciens have Ti (tumor-inducing) plasmid and *A. rhizogenes* have Ri (root-inducing) plasmid. These are employed as vectors in plants. *A. rhizogenes* naturally develops hairy-root disease, which is brought on by Ri plasmids. On the other hand, by introducing a specific DNA fragment *i.e.*, T-DNA or transfer DNA from its Ti plasmid into the nuclear genome of plant cells, *A. tumefaciens* mostly affects dicot plants and causes crown gall disease.

(i) Ti plasmid-based vector

Ti plasmid of *A. tumefaciens* is a huge, 200 kb plasmid that contains T-DNA, a cluster of vir genes, and a replication origin. A particular DNA segment called T-DNA is introduced into plant cells. Depending on the strain, it ranges in size from 15 to 30 kb. It has border sequences, which are 25 bp flawed direct repeats that are not fully transferred to the plant genome but participate in the transfer process. The proper border sequence must be transported and merged into the plant genome (deletion of right border prevents T-DNA transfer). The specific junction of T-DNA that is integrated into a plant genome maintains one to two base pairs of the right repeat, whereas the left junction can fall up to 100 base pairs short of the left repeat. For effective T-DNA transfer, a cis-element (the "overdrive sequence") close to the right border is necessary outside the T-DNA area. Plant cells express the genes for three different forms of opines; nopaline, octopine, and agropine-as well as the phytohormones auxin and cytokinin.

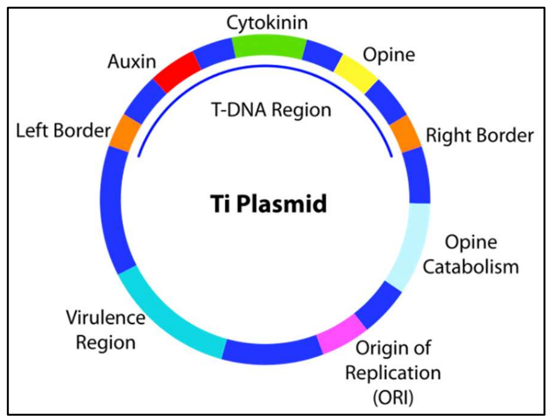

Fig 1: Schematic representation of Ti plasmid

The **virulence (*vir*) genes,** positioned into several operons *viz.,* ***vir*A**, ***vir*B**, ***vir*C**, ***vir*D**, ***vir*E**, ***vir*F**, ***vir*G** and ***vir*H** on the Ti plasmid, are responsible for transferring and incorporating the T-DNA into the plant genome.

Mechanism of transfer of T-DNA

- The two-component system of VirA and VirG reacts to the specific phenolic substances that are released by injured plant cells.
- VirA binds to acetosyringone and become auto-phosphorylated on histidine. After that the phosphate group switched to aspartate residue of VirG (the product of *vir*G gene).

- Now, in order to enhance the transcription of these operons, the activated VirG binds to the specific 12 bp DNA sequences known as ***vir* box enhancer elements** that are present in promoters of other *vir* genes (*vir*A, *vir*B, *vir*C, *vir*D, and *vir*E operons).

- After *vir* genes are expressed, some Vir proteins generates a single stranded T-DNA. Proteins "VirD1" and "VirD2" act as endonucleases. VirD2 cuts the phosphodiester bond at the 5' end and remain covalently attached to the nicked DNA via tyrosine residue. The nicked DNA is eventually moved from the plasmid by 5' to 3', giving **single-stranded T-DNA** (called **T-strand**).

- The interaction of VirD2 with the 5' end of T-strand inhibits the exonucleolytic damage and also distinguish the 5' end as leading end of the T-DNA complex during transfer. VirE2, a sequence nonspecific ssDNA binding protein wraps and shields the T-DNA against nucleases and targets T-DNA to the plant-cell nucleus.

- For the process of delivering T-DNA between bacterial and plant cells, the VirB and VirD4 proteins are involved in the generation of conjugal tubes.

- A signal sequence on VirD2 promotes it into the plant cell's nucleus while it is still attached to 5'end of T-DNA.

- VirC1 specifically binds to overdrive sequence and stimulates the transfer process. The transport of T-DNA is a polar process that begins at the right border and ends at the left border.

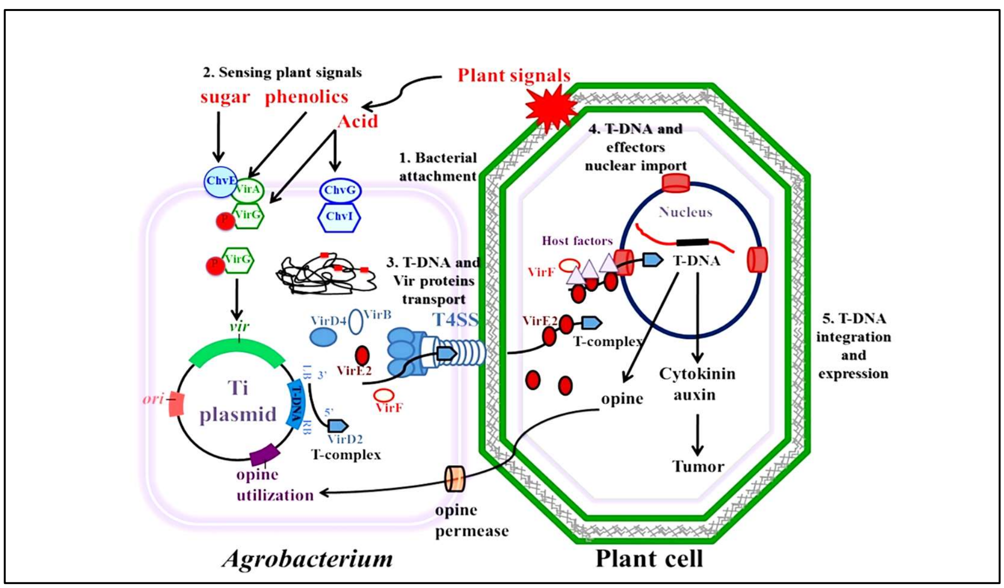

Fig 2: Major steps of the *A. tumefaciens* mediated plant transformation process

Source: *The Arabidopsis book, 2017; BioOne*

Integration of T-DNA into plant genome

With the help of VirE2, T-DNA initially enters plant cells as single stranded structure, but it quickly changes to a double stranded form and syncs at random locations across the host plant genome. At the target location, 23-79 base pairs are deleted for insertion. The genes for auxins, cytokinins and opines are activated and express themselves after T-DNA introduction into plant genome, leading to abnormal development in the form of tumor.

The Ri plasmid

The Ri plasmid of *Agrobacterium rhizogenes* has attracted interest in the past for use in plant cloning vector development. The key distinction between Ri and Ti plasmids is that when T-DNA from the Ri plasmid is transferred to a plant, it does not cause a crown gall but rather a hairy root disease, which is characterised by a tremendous proliferation of a highly branching root system. In order to potentially acquire significant quantities of protein from plant genes that have been cloned, biotechnologists have investigated the prospect of growing modified roots at high density in liquid culture.

4.1.B Virus mediated transfection

The technique of virus-mediated transfection is widely used[7]. This technique involves replacing, introducing, or complementing a desired gene into the plant virus genome. The desired gene is subsequently transferred into the recipient cell by virus infection using this virus as a vector.

4.2 Direct methods

When foreign DNA is introduced directly into an organism's genome, the phrase "direct transfer of gene" is employed. Making physical and chemical alterations to the plant's cell walls and membranes is necessary for the direct transfer of genes into the target cells' nuclei. The two main categories of direct DNA transfer techniques are physical gene transfer techniques like electroporation, microinjection, and particle bombardment (biolistics) and chemical gene transfer techniques like poly-ethylene glycol (PEG) mediated, calcium phosphate precipitation and diethyl amino ethyl (DEAE) dextran-mediated methods.

4.2.A Physical gene transfer methods

(i) Particle bombardment or biolistics or gene gun mediated transformation

John Standford proposed particle bombardment, originally known as biolistics (a combination of biological and ballistics), as an alternate method for plant transformation in 1987 to circumvent the host range limits of *Agrobacterium*. It is a typical way for incorporating genes into plant mitochondria, chloroplasts, and nuclei. A modified shotgun is used in this technique to propel tiny (1-4 m) metal (gold or tungsten) particles wrapped with DNA into plant cells at a speed high enough to breach the cell wall (around 250 m/s). Prior to coating, DNA is precipitated using spermidine, polyethylene glycol, and calcium chloride. Once within the cell, the DNA elutes from the particles and either becomes momentarily expressed or stably integrates into the host genome[8], which solely depends on cellular components[9].

Numerous significant crops have been altered using this technique, including wheat[10], rice, maize, oats, sugarcane, barley, cotton, and soybean. Interferon was recently effectively delivered into tobacco chloroplast DNA using a gene gun, and it was claimed that protein expression was higher than that of nuclear transformed plants[11].

(ii) Protoplast electroporation

DNA is inserted into plant cells using the electroporation technique, which creates tiny pores in the cell membrane. Although this procedure was initially created for protoplasts, it has produced results that are just as good for cells and tissues, and it makes it simple to recover plantlets that have been rejuvenated. Metaphasic cells are best suited for electroporation because of an exceptional permeability of the plasma membrane and the absence of the nuclear envelop. A vector carrying the desired exogenous DNA and a selection gene must be obtained, much as with the other plant transformation methods. This method uses a suspension of purified protoplasts, vectors, and carrier DNA in an electroporation cuvette. Extremely high voltage electrical pulses (4000–8000 V/cm) are then administered in brief bursts-a few milliseconds at a time-to the suspension. In order to achieve stable or transitory DNA expression, temporary micropores are generated in the plasma membrane that allow the vector carrying the desired exogenous gene to enter. The reversibility of these micropores enhances plant cell survival. Through electroporation, numerous plants, including rice, maize, sugar beet, sugarcane, barley, cowpea, tobacco, basil, ryegrass, etc., are altered to take on varied features.

(iii) Microinjection

Microinjection is an essential tool with significant plant transfection potential because of its wide applicability to a variety of plants. It is a physical technique that directly introduces foreign DNA into the cytoplasm or nucleus of recipient cells using a glass micropipette or microinjector (0.1–10 m). The recipient cell may be the one identified from intact cell, protoplasts, callus, embryos, meristems etc. The cells are kept immobilised in agarose embedding and held by a suction holding pipette after gene transfer. Transgenic plants are subsequently created by cultivating the injected cells in vitro. By using this technique, transgenic tobacco, rapeseed, and numerous other plants have been successfully created.

(iv) The pollen-tube pathway method

Zhou *et al*. first proposed the pollen-tube pathway method in 1983 by taking advantage of the germplasm cells of the plant's own reproductive system. The basic idea behind this technique is to change egg cells, zygotes or early embryonic cells, such, by introducing foreign DNA into the embryo sac through pollen tube channels. The crop enhancement programme currently uses this strategy as well.

4.2.B Chemical gene transfer methods

(i) DNA transfer by calcium phosphate

This approach involves making a suitable mixture of calcium phosphate, calcium chloride, and foreign DNA in order to precipitate the DNA with the calcium phosphate. The target cells are then exposed to the DNA precipitation[13].

(ii) Polyethylene glycol (PEG) mediated transfer

The chemical PEG is most frequently used to alter plant cells. For this method of transformation, protoplast, or plant cells devoid of a cell wall, is the initial substance needed. Protoplasts of the target plant cells are combined with molecules of linearized plasmid DNA encoding foreign gene and put in a transformation medium rich in Mg^{2+} ions rather than Ca^{2+} ions, after which 20% PEG is added. After the treatment, Ca^{2+} concentration is increased and PEG concentration is decreased to encourage the transformation frequency.

4.3 Nano-particle based plant transfection strategies

In the past, researchers considered encapsulating target genes in carriers or chemically attaching them to nanocarriers to facilitate gene delivery. Due to their small size, nanoparticles have a fast-increasing particular surface area and energy. This causes a huge number of unsaturated chemical bonds to form, which creates a large number of chemically active sites. Due to their simple interactions with genes, these active sites enable robust synthetic gene vectors and great loading efficiency[14],[15]. Since they do not cause physical harm to cells or protoplasts or pose a risk of infection, synthetic nano-vectors may serve as a universal platform for transferring genes to plant cells. Additionally, DNA can be shielded by nano-carriers from denaturation or enzymatic destruction[16]. To transfer genes into plants, several nano-carriers are present there. These include phospholipids (liposomes), various inorganic nanoparticles, and cationic polymers like polyethyleneimine (PEI) and chitosan.

Table 3: Applications of nanocarriers in gene delivery to plant components

Carriers	Foreign gene	Conjugation	Transfection target	Method	Transfection efficiency	Reference
PEI	GFP plasmid DNA (pDNA)	Electrostatic adsorption	*Arabidopsis* protoplasts	Co-incubation	65%	Li *et al.*, 2011
			Saffron cells		Increased by 2 times under ultrasound	Firoozi *et al.*, 2018
Chitosan	GFP pDNA	Electrostatic adsorption	*Arabidopsis* protoplasts	Co-incubation	1%	Song *et al.*, 2009
			Onion skin	Gene gun	8%	Feng-hua *et al.*, 2009
Cadmium selenide-chitosan nanoparticles	GFP pDNA	Electrostatic adsorption	Jatropha calluses	Co-incubation	Successfully express GFP	Wang *et al.*, 2011a
Single walled carbon nanotubes	ssDNA	π-bond stacking	Tobacco BY-2 cells	Co-incubation	80%	Liu *et al.*, 2009
PEI modified carbon nanotubes	Cy3-DNA	Electrostatic adsorption	*N. benthamiana* Mesophyll cells	Leaf injection penetration	62%	Demirer *et al.*, 2019
Chitosan-modified carbon nanotubes	YFP pDNA	Electrostatic adsorption	Arugula Mesophyll cell chloroplasts	Localized infiltration	47%	Kwak *et al.*, 2019
PEI modified triferric oxide nanoparticles	GUS pDNA	Electrostatic adsorption	Cotton pollens	Pollen magnetofection	Successfully express GFP	Zhao *et al.*, 2017

Triethylene glycol modified mesoporous silicon-gold particles	GFP pDNA	Electrostatic adsorption	Tobacco leaves and corn calluses	Gene gun	GFP transient expression	Tornry *et al*., 2007
Nano-silicon plating gold powder	GFP pDNA	Electrostatic adsorption	Onion skin, tobacco and corn leaves	Gene gun	76%	Martin-Ortigosa *et al*., 2012b
Calcium phosphate nanoparticles	GUS pDNA	Internal embedding	Mustard cotyledon hypocotyls	Co-incubation	80.7%	Naqvi *et al*., 2012
Layered double hydroxides	GUS dsRNA	Internal embedding	*N. tabacum* leaves	Spray on the leaves	Successfully express GUS dsRNS	Mitter *et al*., 2017

Green fluorescent protein (GEP), Yellow fluorescent protein (YEP), Double-stranded RNA (dsRNA), Single stranded RNA (ssRNA)

Source: Li-Hua *et al.,* 2021.

4.4 In planta transformation method

Despite being a viable approach for plant regeneration, tissue culture requires more steps to produce a plant with the necessary characteristics. To solve this problem, it is required to create a transformation that drastically reduces the current, time-consuming tissue culture steps. Transformation techniques in plants offer such a chance. Planta transformation methods are those that entail delivering transgenes directly to intact plant floral parts in the form of naked DNA[30]. These techniques create a large number of plants with the necessary characteristics while omitting the steps involved in tissue culture and using a quick, straightforward process that saves time. The first plant to undergo successful planta transformation was Arabidopsis thaliana. In contemporary technology, genetic material is transferred straight from the plant to pollen, ovule and newly forming cells during the flowering stage. There are several planta techniques, such as vacuum infiltration transformation of germination seeds and floral dipping, floral spray, pollen transformation, and embryo transformation[31]. Compared to the processes of vacuum infiltration and floral dip, planta transformation yields higher results.

4.5 Combination of Agrobacterium and in planta transformation method

The newest Agrobacterium-based transformation technique in Arabidopsis and some other important plants crops are "floral dip" method. The step of tissue culture stage is eradicated[32]. In this, In planta method of transformation. When the plant has the most unopened floral outgrowths or bud clusters, it is substituted by being submerged in an Agrobacterium suspension[33]. A cleansing agent, which lowers surface tension, is also added to the suspension, along with some sugar. A microbial adjustment takes place in the growing flowers, transferring T-DNA[34]. The production of transgenic seeds with a selectable marker can then be implanted on medium. The plants that thrive in the chosen medium are transplanted, where they can then be examined or screened[35].

Table 4: Advantages and disadvantages of different gene insertion methods

Methods	Advantages	Disadvantages
Agrobacterium mediated gene transformation	• Simple as well as comparatively less expensive • High transformation efficiency • Obtained Transgenic crops have higher fertility percentage • Protocols for both dicot and monocot are accessible • Transfer of rather long DNA segments is possible	• Time taking • Cannot be used to treat all types of cells by this approach • Sometimes results in false positive
Virus mediated transformation	• Efficient and high expression of transgenes • Shorter production time than agrobacterium mediated transformation • No species range limitation	• Low stability. • Size of the inserted foreign gene is limited • Can cause host plant pathogenesis as some modified plant viral vectors can diffused to other plant parts after forming in infected leaves
Particle bombardment or biolistics	• Wide range of target receptors • No host restriction (Altpeter *et al.*, 2005)	• Application cost is high • Exogenous gene inactivation is risky • Silencing during transfection is tough (Travella *et al.*, 2005)
Protoplast electroporation	• In comparison to gene gun method the Micropores produced by electroporation method are reversible which increases the plant cell survival rate	• The major difficulty with this technique is the regeneration of plants from the transformed protoplasts. • Regenerated plants may also have fertility problems
Microinjection	• Wide acceptability to a variety of plant species	
Pollen-tube pathway method	• Utilizes the natural reproductive process without requiring further tissue culture and regeneration afterward (Chen et al., 1998). • Higher transformation efficiency than Agrobacterium can be achieved (Xian *et al.*, 2017)	• Working time is restricted by flowering time • Difficulties in operating crops with small flowers.
DNA transfer by calcium phosphate	• No chimeras	• Tedious • Protoplast regeneration needed

Polyethylene glycol (PEG) mediated transfer	• No chimeras	• Confined to protoplasts. • Problems encountered when regenerating these protoplast cells into viable plants. • Low transformation efficiency.
Liposomes	• Minimally cytotoxic • Gives DNA an effective protection from nuclease degradation • High transfection rate for plant RNA (Nagata *et al*., 1981)	• Difficulty in transformation of intact cells with cell walls. • Only suitable for protoplasts
Polymeric nanoparticles (PEI-Pilyethyleneimine)	• Well organised plant transfection vector	• Difficulty in transformation of plant cells with cell walls
Chitosan nanoparticles	• Can improve the germination of plant seeds like cucumber (Jogaiah *et al*., 2020), maize (Saharan *et al*., 2016) and chilli (Vanti *et al*., 2020).	• Very low transfection efficiency (Sang *et al*., 2009)
Silicon fibre mediated	• Easy	• Toxic to cells
Pistil transformation	• Easy • No tissue culture	• Demonstrated only in few species
Floral dip method with *Agrobacterium*	• Easy • No need of tissue culture	• Demonstrated only in few species

4.6 Strategies for specific gene editing in plants

Genetic engineering can now insert target genes into plants via a variety of transgenic techniques thanks to improvements in gene delivery technologies. The majority of these transgenic technologies, however, are unable to modify specific plant genes. Randomness in the host genome spot where the transgene is placed could result in redundant mutation since it would disrupt active plant genes[36],[37]. A variety of site-directed nucleases, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindrome repeats (CRISPRs), have been used for targeted gene editing in plants to address the issues listed above.

(A) Homologous recombination (HR)

Because of the inserted gene's sequence is homologous to one at this locus, HR transfers foreign genes into recipient cells' chromosomes. To achieve the purpose of gene editing, a new gene fragment may be used in lieu of the original gene fragment by a single or double exchange. However, HR is often accompanied by non-homologous end joining (NHEJ).

(B) Zinc finger nucleases (ZFNs)

Artificial restriction enzymes, ZFNs, are created by combining the restriction enzyme FokI, which cleaves DNA or nuclease domain through peptide bonds, with the zinc-finger DNA binding domain, which identifies a particular DNA sequence[38]. In order to properly alter the endogenous gene loci in eukaryotic organisms, the zinc finger domain Cys2-His2 built at the N-terminus may detect novel DNA sequences in complicated genomes.

Most frequently, electroporation is utilised to introduce ZFNs into cells[39]. Currently, ZFNs have accomplished targeted plant mutagenesis for genes including ABA INSENSITIVE4 in *Arabidopsis*[40], stress response regulator, and heat-shock promoter[41]. Maize[42], tobacco43], bread wheat[44], soybeans[45] and other plants all use ZFNs to modify endogenous genes. The designed nuclease will, however, be off-target and inactivated if the Zinc Finger domain is not correctly specific for the target site or the target is not unique in the eukaryotic genome.

(C) Transcription activator-like effector nucleases (TALENs)

As an alternative to ZFNs[46], TALENs are developed by fusing transcription activator-like effectors (TALEs) to the catalytic domain of FokI endonuclease. TALENs are currently a popular choice of engineered nucleases in human cell lines and animal species, however their usage in plants is limited because to the lengthy production period of these enzymes. But in 2012, Li et al. produced specific ALS gene alterations in transformed tobacco protoplasts using TALENs. Gene targeting effectiveness of up to 14%[47]. Recently, the TALENs method enabled the synthesis of monoclonal antibodies and glycol-engineered tobacco[48], showing that TALENs may be a highly effective tool for precise gene editing in plants.

(D) Clustered regularly interspaced short palindrome repeats (CRISPRs)

The latest tool, CRISPR/Cas9, developed in 2013, is a revolutionary technology that allows for the addition, deletion, and insertion of desirable traits[49]. In a single plant, it is also used to change many genes simultaneously by very accurate and precise CRISPR gene editing at a specific target spot. With the aid of RNA guider genes, the enzyme Cas9 cuts the DNA at very precise locations. To create a genetic plant with the necessary characteristics, it is a very efficient, speedy, and reliable method of gene transfer in the plant[50].

In order to increase rice yield, this ground-breaking technique has been applied to plants to increase grain weight[51],[52] and blast resistance[54]. With the extensive use of nano-delivery platforms in plant research recently, the CRISPR and nanotechnology combo has arisen, which can address the issues brought on by conventional delivery techniques. It is crucial to properly apply CRISPR/Cas9 technology to agricultural applications given the rapid growth of delivery techniques, and significant advancements can be expected.

5. Re-generating plants from transformed cells

Using a variety of the aforementioned approaches, the new gene is injected into some of the cells. Each method's primary objective is to deliver the new gene(s) to the plant cell's nucleus or another organelle (such the plastid or mitochondria) without harming the cell.

It is not possible to put a copy of the transgene into every cell in plants due to the vast number of cells that exist. Because they are totipotent and can develop into any type of plant tissue, undifferentiated plant cells are propagated in large numbers via tissue culture. Calli (undifferentiated cells) arise when explants or protoplasts are exposed to a selected regeneration media. These are the cells that get the new transgene. Following this regeneration stage, the calli are placed in an elongation medium for around 30 days. The differentiated and extended shoots are divided into sections and placed in the rooting medium. Rooted plants with developed shoots are then transferred to a substrate and grown to maturity in the greenhouse. Once the transgene's expression has been verified, the hemizygous transgenic plant (also known as T_0) is self-fertilized to produce homozygous transgenic progeny.

The development of homozygous lines in crop species requires a few additional steps, including; (i) germination of T_1 transgenic progeny seeds on selective media, (ii) evaluation for transgenic trait expression (if there is differential expression between homozygotes and heterozygotes), (iii) Basta paint tests, and (iv) real-time PCR (RT-PCR). RT-PCR can be used to differentiate between heterozygotes and homozygotes, but it is expensive and needs specialist knowledge and tools. In a rare-instances, homozygous lines can be produced by seed germination on conditions containing selection agents like antibiotics (hygromycin or kanamycin) or herbicides like Basta (glufosinate ammonium)[55]. By regenerating plants through anther-culture, heterozygous lines can be instantly diploidized.

6. Backcross breeding

The fifth and last step in developing a genetically modified crop is backcross breeding. To integrate the desired features of elite parents and the transgene into a single line, transgenic plants are crossed with elite breeding lines using conventional plant breeding techniques. In order to create a high producing transgenic line, the progenies are repeatedly backcrossed to the elite line. The outcome will be a plant that expresses the characteristic encoded by the new transgene and has a yield potential comparable to present hybrids.

Numerous studies have demonstrated that, if molecular markers are utilised to expedite recovery of the recurrent parent germplasm in the backcross generations, two backcrosses with non-transformed parental line are adequate to create an isogenic line bearing transgene. If molecular markers are also utilised to identify plants homozygosity for the event of interest, virtual recovery of the recurrent parent can be accomplished by two generations, involving one generation of self-pollination to fix transgene expression[56].

Two backcrosses with non-transformed parents are preferred to evaluate the efficiency of the transgene at its best while minimising the undesirable impacts of the non-elite transformed donor. Instead of having to create ever more events in order to achieve the best possible transgene expression, this aids in realising the appropriate choice of event. By carefully selecting genetic backgrounds, plant breeding systems can be developed to accumulate transgenic loci. Based on the agronomic superiority at the target environment, the recurrent parent for the transgene deployment can be chosen.

For any crop species, the entire genetic transformation process is fundamentally similar. The target gene, crop species, present resource, and legal authorizations all affect how long it takes to accomplish all five processes from beginning to end. Perhaps it will take anywhere from 6 to 15 years or more before a unique transgenic hybrid is good enough for release to be cultivated.

IV. Achievements in transgenic breeding

During the previous three decades, there has been a significant advancement within the process of introducing foreign genes into plants. 1257 transgenic plants in various crops have been created and disseminated globally as of 1992. This figure would be rather high. Opportunities have arisen to alter crops to boost yields, confer resilience to biotic[57],[58],[59] and abiotic stresses[60],[61] and enhance nutritional quality[62],[63]. Oil seed rape has had the most transgenic plants released (290), followed by potato (133), tobacco, tomato, and maize (65), as well as flax seed (49), soybean (40), cotton (37), sugar beet (28), and lucerne (21). Despite this, there are still transgenic plants present in a variety of field crops, including wheat, barley, oats, peanuts, cotton, sunflowers, brinjal, etc.

Table 5: Character wise transgenic achievements in some crop species

Crop plants	Character modified	Source of transgene	Transgene product
Cotton	Resistance to *Helicoverpa*	*Bacillus thuringiensis*	Bt. Insecticidal protein
	Herbicide resistance to		
	a. Glyphosate	Microbial gene	Analogue to EPSP synthase
	b. Bromoxynil	*Klebsiella ozaenae*	Bromoxynil specific nitrilaze
	c. 2,4-D	*Acaligenes eutrophus*	2,4-D mono-oxygenase
Maize	Resistance to European corn borer	*Bacillus thuringiensis*	Bt. Insecticidal protein
	Herbicide resistance to sulphonyl urea	*Arabidopsis thaliana*	Acetolactate synthase
Wheat	Herbicide resistance to glufosinate	Streptomyces	Phosphinothricin acetyltransferase
Tobacco	Insect resistance	*Vigna unguiculata*	Trypsin inhibitor protein
	Disease resistance to		
	a. Brown spot resistance	*Serrati marcescens*	Chitinase
	b. Seedling blight resistance	*Phaseolus vulgaris*	Bean endochitinase
	Herbicide resistance to 2,4-D	*Arabidopsis thaliana*	2,4-D mono-oxygenase
	Cold resistance	*Arabidopsis thaliana*	Glycerol-3-phosphate acyltransferase
	Freezing resistance	Winter flounder fish	Antifreeze protein
	Increased mannitol	*Escherichia coli*	Mannitol dehydrogenase
	Protein quality	Wheat	Glutenin gene
Potato	Disease resistance to		
	a. Potato leaf roll virus	Potato leaf roll virus	Viral coat protein
	b. Potato virus X and Y	Potato virus Xand Y	Viral coat protein
	Herbicide resistance to glufosinate	*Streptomyces hygroscopicus*	Phosphinothricin acetyltransferase
	Increased starch content	*E. coli*	ADP glucose phosphorylase
	Serum albumin	*Homo sapiens*	Human serum albumin
Tomato	Insect resistance	*Bacillus thuringiensis*	Bt. Insecticidal protein
	Resistance to Tomato mosaic virus	Tomato mosaic virus	Viral coat protein
	Herbicide resistance to ghlufosinate	Streptomyces	Phosphinothricin acetyltransferase
	Freezing resistance	Winter flounder fish	Antifreeze protein

Rapeseed	Resistance to seedling blight	*Phaseolus vulgaris*	Bean endochitinase
	Herbicide resistance to		
	a. Sulfonyl urea	*Arabidopsis thaliana*	Acetolactate synthase
	b. Glufosinate	Streptomyces	Phosphinothricin acetyltransferase
	Increased stearic acid	*Brassica rapa*	Antisense stearoyl ACP desaturase
	Increased methionine	*Bertholletia exceisa*	Seed storage protein
	Increased lauric acid	*Umbellularia californica*	Lauroyl ACP thiosterase
	Keeping quality improvement	*Lycopersicon esculentum*	Antisense
	Male sterility system	*Bacillus amyloliquefaciens*	Ribonuclease inhibitor
Soybean	Herbicide resistance to glyphosate	Microbial genes	Analogue of EPSP synthase
	Increased methionine	*Bertholletia exceisa*	Seed storage protein
Flax seed	Herbicide resistance to		
	a. Glyphosate	Microbial genes	Analogue of EPSP synthase
	b. Sulfonyl urea	*Arabidopsis thaliana*	Acetolactate synthase
Alfalfa	Protein quality	Chicken	Chicken ovalbumin
Petunia	Orange flower colour	*Zea mays*	Dihydroflavonal-4-reductase (FR)
Arabidopsis	Biodegradable thermoplastic	*Acaligenes eutrophus*	Polyhydroxy butyrate (PHB)
Canola	Altered fatty acid composition (increased laurate levels)	California bay tree *Umbellularia californica*	Lauroyl ACP thiosterase
Plum	Resistance to Plum pox virus	Plum pox virus	Viral coat protein

V. Advantages

(i) Transgenic breeding is a quick way to improve crops. By using this technology, stable transgenic plants can be created in 3–4 years as opposed to the 12–15 years needed to create a new variety using traditional breeding techniques.

(ii) Transgenic plant development avoids the sexual phase, allowing it to get over cross-incompatibility obstacles.

(iii) It is helpful in finding solutions to issues that cannot be resolved by conventional means.

(iv) Because transgenic breeding allows for gene transfer between different plant species, it's possible that it will occasionally result in the emergence of whole new plant species. As a result, it will have an impact on natural evolution.

(v) It can be used to improve crop plants that are both autogamous and allogamous genetically. Transgenes can be used to enhance species that are vegetatively and seed propagated.

(vi) For the genetic enhancement of monogenic traits, it has been found to be particularly efficient.

(vii) Gene transfer from any source, including animals, is allowed.

(viii) It enhances output and increases farmers' income. The traditional sowing and cultivation methods that Indian farmers continue to use call for scientific intervention to increase their output. Consequently, it is one of the steps taken to increase farm productivity.

(ix) It lessens the use of pesticides and insecticides during farming, which may be excellent steps for improving the availability of food.

(x) It can feed a population that is growing quickly since it exhibits considerably higher yields.

(xi) It can grow more crops on a smaller plot of land, crop productivity can rise.

VI. Limitations

(i) Transgenic plants cannot be immediately introduced to cultivation after generation because, not all the superior or commonly grown lines of a crop species are compatible to genetic manipulation and prolonged operation of genetic transformation causes in genetic drag of the initial variety.

(ii) Transgenic plants can occasionally perform inconsistently depending on the character being considered. For instance, in Petunia, the hemizygous condition of the flower colour transgene was permanent in the greenhouse but not in the wild.

(iii) The site where the new gene is fully integrated into the host genome tends to affect the transgene's capability of expressing itself within the transformant (position effect). In the particle bombardment approach of gene transfer, neither the position of integration nor the number of foreign genes can be controlled.

(iv) Only monogenic characteristics have been modified thus far using genetic engineering approaches.

(v) In the cell culture medium, transgenic cells are recovered at a very low frequency.

(vi) Due to the potential for one organism to be favoured by the "superior" qualities created by modifying genes, the production carries significant dangers for the destruction of ecosystems and biodiversity. Consequently, it may eventually interfere with how genes move naturally.

(vii) It raises the expense of production and makes farming more market-oriented and focused on enduring profits.

(viii) In addition to endangering farmers, transgenic crops also threaten the environment and the trade.

(ix) Over time, the costly manufacture of genetically modified foods will become inefficient due to the possibility that the pests they were designed to fend against may evolve resistance to them.

VII. Conclusion

Transgene technology will unquestionably be essential to crop enhancement in the future. The promising transgenic breeding strongly relies on the ability to send those genes between and within biological species, their expression with preferred phenotypes and satisfactory genetic behavioural patterns and their stability with endogenous genes inside the host genomes. We now have a wide range of genes available for selection in plant breeding thanks to the rapid growth of genomics research. In order to maintain development as more and more transgenic features are being introduced into a variety of crops, supplementary breeding for biotechnology efforts and an equal amount of funding must be diverted. We can maximise the benefits of genetic transformation for crop development by carefully selecting the starting material and carefully integrating it into farmed genotypes.

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