

Genetic Engineering

by

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Genetic engineering is a method of modifying plants for the improvement of characters by introducing foreign genetic material or by enhancing the expression of endogenous genes. Genetic engineering involves the artificial manipulation of genes and the transfer of genes from one organism to another to improve its character as well as to understand the basic function of a gene. Genetic engineering methods complement plant breeding efforts by increasing the diversity of genes and germplasm available for incorporation into crops. It also shortens the time required for the production of new varieties and hybrids. Sources of variation in genetic engineering not only come from plants but also from bacteria, viruses, animals, and other unrelated plant species. Synthetic genes have also been used in order to increase the expression of a particular character. The method is utilized to lengthen the shelf life of fruits and upgrade quality of seeds – improving its amino acid and fatty acid composition, modifying its storage proteins, producing novel carbohydrates and reducing anti-nutritional factors. Genetic engineering of plants offers new opportunities for the agrochemical, food processing, specialty chemical, and pharmaceutical industries to develop new products and manufacturing processes.

Gene transfer to plants has developed into an important tool for studies in basic plant biology, allowing the analysis of gene structure and regulation to reach a high level of sophistication. Much knowledge has arisen from the development of methods for the introduction of foreign DNA into intact plant cells and from the study of transient and integrative transformation in both homologous and heterologous systems. The refinement in plant regeneration from cultured cells, efficient vector constructs, and availability of defined selectable marker genes have contributed significantly in the production of a series of transgenic plants that include rice, maize, rye, tomato, and several other crops. In addition, transgenic plants in model plant species such as tobacco, petunia and *Arabidopsis* are being routinely produced carrying both selectable markers and other cloned genes for herbicide tolerance, insect resistance, virus protection, storage proteins and others.

Gene transfer for the genetic engineering of crop plants imposes very specific requirements on the technology. There are four components for its success, including (1) the identification and isolation of suitable genes to transfer, (2) the delivery systems to introduce the desired genes into recipient cells, (3) expression of the new genetic information in the recipient cells, and (d) stability of the genes transferred. For genetic engineering to provide impact to crop improvement, genes for important characters not found in the gene pool of the

organism should first be identified and isolated. Genetic engineering can be resorted for the improvement of crops which takes a long time to be conducted through conventional breeding and if the character is hard to transfer due to the effects of the environment.

Success in genetic engineering also depends on the delivery system to the recipient cells used. It requires methods that work efficiently with virtually every variety of every plant species. It also requires production of normal fertile plants which express the newly introduced gene. Transformation is achieved either through physical methods or use of plasmid vector. Some of the methods that were useful but have been replaced by more efficient transformation methods include:

- a. **Polyethylene glycol-mediated transformation.** Treatment of plant protoplasts with poly-1-ornithine or polyethylene glycol containing Ca^{2+} facilitates the absorption of DNA by isolated plant protoplasts. Some of these foreign DNA demonstrate stable integration and expression. Maize and other cereals (Lorz et al. 1986, Potrykus et al, 1985, Golovkin et al. 1993) were successfully transformed by this method. The fundamental problems of transformation methods based on protoplast cultures are the loss of embryogenic capacity with time, genotype dependence, somaclonal variation and relatively high input of labor and energy and the low rate of transformation.
- b. **Electroporation.** The plant protoplasts and DNA are placed in a chamber and electrical charges are applied to the solution containing them. The electrical pulses produce transient openings in the cell membrane and leads to uptake of the DNA (Paoletta 1998). Efficiencies of transformation on the order of 0.1% to 1% have been achieved (Paoletta 1998). Rhodes et al. (1998) and Fennell and Hauptman (1992) delivered DNA into maize using this technique.
- c. **Microinjection.** In this method the cell walls are enzymatically digested and the resulting protoplasts directly injected with the foreign DNA using microscopically fine needles. This has been used in the transformation of *Brassica* and tobacco protoplasts. Compared to other transformation methods, microinjection has limited success.

Current transformation methods that are widely used in dicots and monocots are:

1. Particle Bombardment

The use of microprojectile bombardment has allowed the production of transgenic plants in a variety of species that were not previously accessible to transformation including the organelles. The biolistic process was developed by Sanford in 1987 at Cornell University. It is the introduction of substances into intact cells and tissues through the acceleration of microprojectiles through biolistic device (Sanford, 1990). It is primarily a mechanism for breaching cell walls and cell membranes, which are the principal barriers to DNA delivery. A microprojectile can be defined as any small coherent particle capable of being accelerated, such that it penetrates cells and tissues. A microprojectile should be small enough to enter a cell or tissue in a non-lethal manner, and should be capable of carrying DNA on its surface or in its interior. Typically,

microprojectiles are made of high-density metals such as tungsten or gold which are more or less spherical and approximately 1.5 – 3.0 μm in diameter. At present, DNA is carried on the surface of such particles, rather than within their interiors. Currently, biolistic devices primarily employ macroprojectiles as means for accelerating microprojectiles. Typically, cells or tissues are configured to provide a maximal amount of surface for bombardment, over an area of about 5 cm in diameter (Sanford, 1990).

Potrykus (1990) and Christou (1992) were unanimous in saying that the advantages of microprojectile bombardment are: (a) it is relatively easy to handle; (b) one shot yields many hits; (c) cells survive the intrusion of particles and the genes coated onto the particles assume biological activity in the cells; (d) target tissue can be organized cells and maybe as different as yeast, algae, pollen suspension cultures, callus cultures, scutellum, leaves and seedlings with the shoot apex exposed; (e) rapid recovery of transformed T1 seed: universal delivery system; and (f) transformation of recalcitrant species. Once the gun is fired, the pellets penetrate the cell walls and membranes so that the foreign DNA is introduced into the nucleus in the cell wall and plasma membrane. The pellets also remain in the cytoplasm but cause no harm.

The primary limitation of this approach is the large number of apical meristems that must be isolated and bombarded and the need to analyze the large number of plants for the presence of the introduced genes. Other tissues from which plants can be readily regenerated are being explored as targets for particle bombardment. For example, transgenic rice plants have been produced by bombarding the scutella of embryos (Christou et al, 1991).

Particle Acceleration. A number of methods can be used to accelerate particles into living cells. The basic system employs a macroprojectile (or macrocarrier), a mechanism for accelerating the macroprojectiles and means of stopping the macroprojectiles. The DNA-coated particles (generally gold or tungsten powders) are placed as a suspension in a small aqueous volume, on the front end of a bullet-like plastic macroprojectile. In the first systems described (Klein et al, 1987), the macroprojectile is accelerated to a velocity of about 2500 ft/sec by a gunpowder charge. Other systems for acceleration of microprojectiles include shock waves by electric discharge through a drop of water (Christou et al, 1988), or the sudden release of compressed air or nitrogen. These systems accelerate a macroprojectile into a stopping plate or screen. Sautter et al (1991) have designed a device that uses a gas impulse to directly accelerate the particle-DNA suspension through a capillary tube, without the use of a macroprojectile. This device targets very small areas of the tissue.

Another recent development involves the use of compressed helium for acceleration of microprojectiles. The PDS-1000/He (Bio-Rad) uses a shock wave generated by the sudden release of compressed helium to accelerate a thin plastic sheet into a metal screen. The microprojectiles are dried onto the surface of the carrier sheet from an ethanol suspension. This device has been applied to transfer genes to several cell and tissue types. In all cases, it was found to have higher rates of gene transfer when compared to device

using gun powder for acceleration. The enhanced rates of gene transfer were attributed to decreased damage to the target tissue due to a more even distribution of microprojectiles.

Factors Affecting Transformation by Particle Bombardment. A number of critical variables have been identified as considerations in using particle bombardment in genetic transformation. Physical parameters include the nature, chemical, and physical properties of the metal particles used to carry the foreign DNA into cells; nature, preparation and binding of DNA onto the particles; and target tissue. *Environmental variables* include such parameters as temperature, photoperiod, and humidity of donor plants, explants and bombarded tissues. Biological factors include choice and nature of explant, pre- and post-bombardment culture conditions, and interactions between the introduced DNA and cytoplasmic or nuclear components.

2. The *Agrobacterium* Ti-plasmid-mediated transformation

Transformation could also be achieved using *Agrobacterium tumefaciens*. DNA to be transferred is inserted in the plasmid of the bacterium, which naturally transfers it to the genome of a host plant. The method involves the co-cultivation of the explant with *Agrobacterium* for several days, subsequent transfer of the infected explant to a callus induction / selection medium, and then to a regeneration medium. Following this, the resulting putative transformants are transferred to soil. The method is routinely used in dicots. More recently, the method has been extended to important cereals like barley, maize, and rice.

Agrobacterium tumefaciens is a bacterium belonging to the family Rhizobiaceae. It is characterized by the presence of a Ti (tumor-inducing) plasmid, a large, double-stranded, circular, closed extrachromosomal DNA. In one region, the T-DNA (transferred DNA) is located, which is naturally exported and integrated into the recipient cell nucleus. The following accounts regarding the Ti plasmid and the T-DNA are based on the works of Taiz and Zeiger (1991), Salisbury and Ross (1992) and Westhoff et al, (1998).

The transfer DNA (T-DNA). The T-DNA region is bounded by the left (LB) and the right (RB) borders, each consisting of 25 base pair repeating sequences. These serve as markers for excision of the functional sequence between them. T-DNA transfer appears to be a polar process initiating at the right border, progressing leftwards and terminating at a left border. There are reports, however, of left border repeat-initiated T-DNA transfer.

Several genes are contained in the T-DNA. Genes *tmr* (tumor morphology root) and *tms* are involved in the synthesis of cytokinin and indole-3-acetic acid respectively. Expression of these genes causes tumor formation in plants infected with *Agrobacterium*. In addition, the T-DNA also contains a *nos* or *ocs* genes. Their gene products, nopaline synthetase and octopine synthetase, catalyze nopaline and octopine synthesis, respectively, which serve as source of nitrogen for the bacterium. Different plasmids are

contained in various strains of *Agrobacterium* and the types are distinguished based on the kind of opines that they make the host plant synthesize. None of the above T-DNA genes are expressed in the bacterium because opine genes have eukaryotic regulatory sequences for expression. Only transformed plant cells that incorporated the T-DNA in their genome do. Other genes located on the T-DNA include those that interfere with the plant's hormone metabolism, which are necessary for tumor induction as well as promoters to activate bacterial genes.

The virulence region. This region encodes proteins that are involved in the transfer of T-DNA, but this segment stays within the bacterium. Its gene products participate in recognizing the wounded plant through a variety of phenolic compounds like acetosyringone that plants excrete. These signal substances activate *vir* genes resulting in the excision of the T-DNA and its subsequent movement through the bacterial plasma membrane, the cell wall, the plant cell's plasma membrane, and finally the pores of the cell's nucleus. The *vir* region is 30 kilobases (kb) long and contains seven complementation groups (*virA* to *virH*) that can each be assembled by several genes (e.g. *virD1* and *virD2* to make VIRD, or *virB1* to *virB11* to make VIRB). Other plasmid genes are involved in determining the host specificity of the *Agrobacterium*. Chromosomal genes (*chva* and *chvb*) are necessary for the bacteria to attach to plant cells.

Use of T-DNA modification in plant improvement

Agrobacterium –mediated transformation is one way of improving crop plants. According to Westhoff et al (1998), when used for this purpose, the Ti plasmid is “disarmed”, that is, *tms* and *tmr* genes are removed in the T-DNA so that T-DNA is merely transferred into the host plant without producing tumor. The desired foreign gene is said to be introduced between the LB and RB of the T-DNA including the promoters and terminators that could be recognized by the host plant. In addition, a marker gene which serves to distinguish transformed cells from those that were not also exported with similar regulatory sequences. More recently, the binary system of *Agrobacterium*-mediated transformation had been developed. In this technique, the functions of the Ti plasmid are given up to two plasmids. The larger plasmid which remains in the bacterium has the *vir* region required for infection and mobilization of the T-DNA while the smaller one contains the T-DNA foreign genes, the borders and other sequences required for its integration into the hosts' genome. With smaller plasmid, manipulation of foreign gene is easier to manage. The smaller plasmid with foreign DNA is cloned in *Escherichia coli*. Subsequently, the plasmid is transferred to *Agrobacterium* by electroporation or freeze-thaw method.

Host of *Agrobacterium tumefaciens*

A. tumefaciens has been routinely used for dicot genetic engineering and monocots as a group were considered to be outside its host range. However, information reviewed by Smith and Hood (1995) on tumor formation and opine production in some monocots demonstrate

that they could be infected by the bacterium. Other evidences cited by Smith and Hood (1995) regarding competence of *A. tumefaciens* to infect monocots include: a) The study of agro-infection by Grimsley et al (1987), wherein the cDNA of maize streak virus was delivered by *Agrobacterium* and the plants became systemically infected and b) the works of Li et al (1992) and Shen et al (1993) where β -glucuronidase gene delivered to maize shoots by *A. tumefaciens* was expressed.

Several possibilities were put forward to account for the inability of *Agrobacterium* to infect other monocots. One possibility is the inability of monocots to produce phenolic compounds, or if they do, then it may be insufficient to serve as signal molecules to activate *vir* genes. A second possibility is the absence of attachment sites in monocots for *Agrobacterium*, since attachment to the plant cell wall is the initial step during infection. Thirdly, since monocots in general lose early the ability to dedifferentiate, the age and type of tissue may also be important consideration. This is because of three possible reasons: a) in the process of differentiation, wall changes may be involved which inhibit or reduce bacterial attachment, b) age and type of tissue determine their competence to dedifferentiate and form tumor, and c) age and type of tissue may differ in ability to produce / vary in the level of production of *vir* inducing compounds. A fourth possibility for absence of tumor in monocots is the lack of transcription of opine genes or because of a difference in endogenous hormone physiology between monocots and dicots. Lastly, absence of tumor could be attributed to incorrect strain of bacterium (virulent vs avirulent) or incompatible strain to targeted monocot plant species.

Chan et al, 1993 on successful T-DNA expression, added phenolic compounds from wound exudates of potato to the *A. tumefaciens* suspension to induce *vir* genes. Using embryonic tissue (i.e. immature rice embryos about 10-12 days after pollination), a monocot gene promoter (maize *Adh* promoter) and the virulent strain of bacteria (supervirulent strain A281), successful transformation was obtained. Transformation and integration of the T-DNA into genomic DNA was confirmed by Southern blot analysis of the progeny and sexual inheritance of the genes in a 3:1 ratio was confirmed. While the transformation frequency was low, their work made it seem probable that experimental parameters could be found which could permit more efficient *Agrobacterium*-mediated transformation of rice.

Improved transformation efficiency using *Agrobacterium tumefaciens* in rice

For a long time monocots have been thought to be outside the host range of *A. tumefaciens* so that other genetic engineering techniques mentioned above had been used to transform them. In the case of rice, transgenic plants have been formed by polyethylene glycol-mediated uptake of DNA into protoplasts, electroporation of protoplasts, and particle bombardment. However, as mentioned previously, a major problem with the above transformation mechanisms is the generally low efficiency of transformation and the difficulty of inducing plant regeneration, in the case of protoplast culture.

Besides higher transformation efficiency, the *Agrobacterium* system of plant transformation also offers several additional advantages over the other transformation

methods so that there is continuous effort to try applying this transformation mechanism to monocots. One of the advantages is the fact that it naturally inserts a portion of its DNA into one of the chromosome of the recipient cell without any requirement of sophisticated laboratory or expertise. Other advantages include defined transgene integration, transfer of relatively large segments of DNA with little rearrangement, potentially low copy number of genes, and preferential integration into transcriptionally active regions of the chromosome. According to Brettschneider et al. (1997) the reason for multiple copy integration in direct DNA uptake is the fusion of DNA molecules by homologous and non-homologous recombination before its integration into the plant genome. The *Agrobacterium*-mediated transformation results in transgenic plants, which are generally fertile, and foreign genes are transmitted in an expected Mendelian manner.

A recent research by Hiei et al (1995) on *japonica* rice that was later confirmed by Aldemita and Hodges (1996) and extended to *indica* rice, a recalcitrant variety, substantively demonstrate that rice can be efficiently transformed through *Agrobacterium* mediation. The key factors facilitating the transformation of rice by *A. tumefaciens* appeared to be the presence of extra copies (duplicate) of certain *vir* genes on the binary vector of pTOK233, the maintenance of high concentrations of acetosyringone for inducing the *vir* gene during co-cultivation of embryos with *Agrobacterium*, use of hygromycin as a selection agent (which does not interfere with rice regeneration) and the use of embryos as explant. Succeeding researches focused on ways to improve efficiency of *Agrobacterium*-mediated transformation in monocots. Aldemita (1996) tried different promoters and compared their activities in driving T-DNA expression in *indica* rice. Reconsideration of the factor of temperature is predicted to improve the efficiency of transformation protocols. Since plant regeneration is an important component of transformation protocols, the different regeneration potentials of *indica* rice varieties were also investigated.