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Genetic Transformation of Plants

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ABSTRACT

Currently, plant genetic transformation techniques are most widely used to obtain new improved plant variety. The introduction of exogenous genes into plant cells, tissues or organs employing direct or indirect means developed by molecular and cellular biology is called genetic transformation of plants.*Agrobacterium*-mediated transformation method is used for the indirect introduction of genes into plant cell, while Polyethylene glycol (PEG)-mediated Transformation method, Microinjection, Electroporation and Biolistics (Particle Bombardment) are direct transformation methods. *Agrobacterium*-mediated transformation method is most popular method for the development of transgenic plants because it is most efficient, stable and cost effective method. Biolistic gene gun method is second most popular transformation method.With the help of microinjection we can introduce whole chromosome in the plant cell, but generally microinjection used for the transformation of animal cells and has limited application for genetic modification of plants. Remarkable success has been achieved to transform the cereals like maize, rice and barley using electroporation. Desired gene isolated from the selected species and introduced in the plant cell by appropriate transformation methods. These cells are allowed to grow on selective medium containing chemical agents. Only transformed cells are selected and allowed for further growth using tissue culture techniques. The use of tissue culture in genetic transformation process is its main limitation as it requires highly efficient and regenerable cells for the growth, also requires high technical skill and expensive methods.

KEY WORDS: Genetic transformation, Electroporation, Microinjection, Selectable markers, Recombinant DNA.

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1. INTRODUCTION

For centuries, people from all over the world have developed different types of new crops by selecting plants from the existing collection. Plant breeding is the technique which involves the identification and selection of desirable parental traits and combining them to obtain a new improved plant variety[1]. Currently, there are lot of changes brought about in the genetic structure of a plant which forms the basis of the plant breeding. Each plant cell has approximately 30,000 genes and these genes codes the information for a plant's phenotype. Mendel's laws of genetics proved to be an important area of study in genetics since 1900, and his laws provided the scientific basis for plant breeding. As all traits of a plant are controlled by genes located on chromosome, conventional plant breeding can be considered as the manipulation of the combination of chromosome[2]. Conventional plant breeding is nothing but changing the genes of a plant to develop a variety that is better than the existing ones and new in its own individual way. The aim is to combine those traits that are favourable in both parent plant varieties. Conventional plant breeding may also make use of 'wider crosses' that involve crossing species or even genera that are quite unrelated. But these crosses cannot take place without help – and thus a number of sophisticated techniques like genetic transformation were taken in use[3].

Recently, scientists combined molecular biology, tissue culture, and gene transfer techniques to form a powerful tool for the introduction of new traits into a particular plant. This particular tool has enabled a scientist to introduce genes from animals, microorganism, or different plant species into the genome of a recipient plant in a controlled manner, independent of fertilization. This technique eliminated the sexual barriers between species, as well as the barriers between those of the organism of the eubacteria, archea, and eukarya, a phylogenetic domains[4].

Plant genetic transformation can be defined as "The introduction of exogenous genes into plant cells, tissues or organs employing direct or indirect means developed by molecular and cellular biology"[5].The inserted gene sequence is known as transgene, which may come from a distantly related plant or from a completely different species[6].

2. PLANT TRANSFORMATION METHODS

To introduce foreign genes into plants different methods have been developed over the course of time. A common thing in all these methods is that the transforming DNA first needs to enter the plant cell by penetrating both the plant cell wall and the plasma membrane. It must then reach the nucleus and integrate into the resident chromosomes. Gene transfer is carried out, for the majority of species, using explant competent of regeneration to obtain complete, fertile plants. Through this, the development of a tissue culture technology is frequently seen as becoming an art. Gene transfer

technology has become a routine in working with several plant species, yet the limiting step in others is not the transformation itself but rather the lack of efficient regeneration protocols[7].

Most widely used and successful method of plant transformation is the '*Agrobacterium*-mediated plant transformation'[8]. Other methods for plant transformation are direct transformation methods such as 'Microinjection'[9], 'Polyethylene glycol-mediated transfer'[10], 'Electroporation'[11] and 'Gene gun methodology'[12].

2.1 Agrobacterium Mediated Transformation of Plants

Agrobacterium tumefaciens is a soil living bacterium that causes a "crown gall" disease in the infected plants. Through the advancement and innovation of molecular biology technology during past few decades, different types of tumor inducing bacterial and plant genes were identified. With the help of complete knowledge of the interaction of *A. tumefaciens* with host cells, *A. tumefaciens*become the most widely used tool for the plant transformation[13]. *A. tumefaciens* naturally attracted to wound sites of plant cell by chemotaxis and transfer a DNA segment of the tumor inducing (Ti) plasmid called the transfer DNA (T-DNA) into the genome of host plant cell[14].

Agrobacterium rhizogenes is also a soil bacterium used for the plant transformation, which generates the "hairy root" syndrome in infected plants and is characterized by the neoplastic outgrowth of roots[15]. This phenomenon is based on the transfer and integration of the T-DNA, which is a specific part of the "root-inducing" (Ri) plasmid of *A. rhizogenes*, into the genome of host cells[16,17,18].

At the beginning of the last decade, when there was not much known molecular features, the first record on transgenic tobacco plant appeared which expresses the foreign genes[19]. *A. tumefaciens* naturally infects only dicotyledonous plants. The transformed plants obtained from *Agrobacterium*-mediated methods are generally fertile and the foreign genes are transmitted to the next generation in Mendelian manner[20]. Consistent and efficient methodologies developed for rice[21,22], banana[23], corn[24], wheat[25], and sugarcane[26,27,28], makes the *Agrobacterium*-mediated transformation successful in monocotyledonous plants.

T-DNA carries a set of oncogenes and opine-catabolism genes and its expression is responsible for the neoplastic growth of the transformed plant tissues and the production of opines is used as a nitrogen source by the bacteria[29]. Other than T-DNA region there is also present a virulence (vir) region on the Ti plasmid of the *A. tumefaciens.* The T-DNA flanked by conserved 25-base pair imperfect repeats at the both ends of the T-region called border sequences. Virulence (vir) region is composed of at least seven major loci (virA, virB, virC, virE and virG) which codes for the components of the bacterial protein machinery required for T-DNA processing and transfer. VirA

and VirG regulate and activate the expression of other vir genes on the Ti plasmid. The VirB, VirC, VirD and VirE are involved in the processing, transfer, and integration of the T-DNA from *A. tumefaciens* into host plant cell[13].VirC and VirF determine the range of plant species that could be transformed by the *Agrobacterium*[30].

Basic process of *A. tumefaciens* mediated genetic transformation involves the following steps: (1) Sensing of chemical signals of plants and inducing of virulence (vir) proteins. Wounded part of the plant release the chemical signals, which are perceived by a VirA/VirG two component system of *A. tumefaciens*, which leads to the transcription of vir gene promoters and so the vir proteins are expressed. (2) T-DNA processing. T-DNA is nicked by VirD2/VirD1 from the T-region of Ti plasmid and which forms the T-strand which is linear and single stranded, and VirD2 molecule attached at the 5' end of the T-strand by covalent bond. (3) Attaching of *A. tumefaciens* to plant transfer a T-complex to plant cell. With the help of VirD4/B T4SS transport system the *A, tumefaciens* attaches to the plant cell and transfer the T-complex into the host plant cell. (4) Targeting of T-complex to plant cell nucleus and integration of T-DNA into plant genome. T-complex integrate with the plant genomic DNA after reaching to the nucleoplasm with the help of some host proteins. (5) Expression of T-DNA in plant cell and inducing plant tumor formation. The T-DNA genes codes for the phytohormones synthases due to which it forms the uncontrolled plant cell growth and opine synthases which helps to provide nutritive compound to the *Agrobacterium*[31].

Agrobacterium-mediated transformation has the following advantages over other transformation methods: It has more stable and low copy number transgenic events[24,32]. Due to low copy number of transgene, it leads to a very few number of problems with transgene cosuppression and instability[33,34]. Because it is a single-cell transformation system, it does not form the mosaic plant which is commonly formed in the direct transformation methods. It is a highly efficient method. By using this method we can transfer the larger DNA segments into the recipient cells[24,26,27,32,35].

Agrobacterium-mediated transformation of plants has the following disadvantages: It has limited application for the transformation of monocot plants including some important crop plants[13]. *Agrobacteriumsp*. infects the limited range of host plants[36]. Expression level of transgene is variable[30].

2.2 Polyethylene glycol (PEG)-mediated Transformation of Plants

At present, the PEG technique is ranked as the third most common gene transfer technique for plant transformation. However, it was the first technique to report the successful integration of the foreign genes in to plant cell. This technique is mostly used to introduce the foreign genes or DNA into the protoplast of cell, because it was proven that call walls be a significant barrier in other transformation methods. Protoplast of plant cell receives the foreign DNA by the reversible permeabilization of the plasma membrane, which is induced by the PEG. Due to increase in its permeability, molecules of various range of size from small gene sequences or plasmids to large molecules like chromosomes or micronuclei can be easily pass through the plasma membrane in presence of the PEG. This ability to transfer the different molecules contributes to the PEG system to being one of the most simple and promising transformation system applicable to large scale, irrespective of the plant species. The main limitation of this system is that, it is used for only those plants, whose protoplasts are efficiently regenerated. PEG-mediated gene transfer is an efficient, reliable, cost effective and simple technique for the transformation of plant, only if is possible to regenerate plants from the protoplasts. Regeneration of transformed plant can be achieved under in vitro and only in optimized growth conditions. The most efficient DNA uptake occurs in the pro-metaphase in actively growing cells[37].

2.3 Microinjection

In this technique, the gene of interest is injected into the nucleus of the recipient cell[38]. Glass microcapillary-injection pipette is used to directly introduce the foreign gene into the cell or nucleus[39,40]. This method does not allow the introduction of only plasmid into the plant cell but whole chromosome also introduced in to the cell[41,42]. This method is generally used to transfer the foreign genes into the large animal cells. Application of this method for transformation of plant cell is limited because thick layer of plant cell wall act as a barrier for micropipettes. During the introduction of foreign DNA into the nucleus or cell, recipient cells are immobilized by the holding pipette and gentle suction and then DNA is injected into the cell by micropipette[43]. Each glass micropipettes having the 0.5-10 μm diameter tip are used to transfer macromolecules into the recipient cell[38]. Low melting point agarose also used to hold the protoplast during microinjection[44]. Regeneration of the transformed protoplast is not always efficient so to overcome this problem immature structures like embryos, meristems, immature pollen, germinating pollen, etc. used[45,46]. Microinjection method is used to transform the plants like, tobacco[47], petunia[41], oilseed rape[48], and barley[49].

The disadvantages of this technique include that, the likelihood for the production of chimeric plants with only a part of the plant transformed. However from this chimeric plant, transformed plants of single cell origin can be obtained. It is very slow, tedious and expensive method[50]. Frequencies of somaclonal variations are high in transformation by microinjection[51].

2.4 Electroporation

Electro oration is a method in which presence of electric field creates small pores in the plasma membrane of the cells, which makes it permeable for the substances like piece of DNA, molecular probes or any drug[52]. The pores are transient and after some time reseal automatically and comes to its natural state, this process is known as reversible electroporation[52,53]. For reversible electroporation it is needed to keep the membrane potential below the critical value, but if it exceeds its critical value, irreversible electroporation takes place which cause the damage to cell by permanently making pores in membrane and loss its viability[54]. It can be used to transfer DNA into a large number of cells in a very short period of time[52]. Study using DNA in suspension under an artificial bilipid layer suggests that, when we apply the electric field, DNA will interact with the membrane and promote the formation of pores in the membrane[55]. Larger electric field is required to permeabilize the cells having small radius[56]. Remarkable success has been achieved to transform the cereals like maize, rice and barley[57,58,59]. However, electroporation has a limited use in transformation of some species like wheat because of its lower efficiency[60,43]. Production of fertile transgenic wheat plants depends on the quality of the material used for the transformation[61].

2.5 Biolistics (Particle Bombardment)

Since 1980's, Biolistic Gene Gun method or particle bombardment is used to introduce the foreign gene in to the plant cells. This method is known as Biolistics gene gun method or Particle Bombardment. In this method piece of DNA is introduced into the recipient plant tissue plated on the selective medium, using the 0.5 mm Gold or Tungsten particles coated with DNA, which are shot with high velocity with the help of compressed Helium and driven to plant cell[62]. High density and chemically inert micro particles like Gold or Tungsten are used to carry the DNA[4]. Gene gun system is made up of high and low pressure chamber having membrane at the middle. Due to excess pressure this membrane is ruptured and created pressure difference shot the DNA coated micro projectiles with high velocity, which pass through barrel and hits the porous stop screen and allows only microparticles coated with DNA to pass further. After reaching to plant tissue it punch holes in the cell walls of plant tissue kept in petri dish and release transgene into the cell which co integrate with the chromosomal DNA[50]. This technique is also used to deliver the foreign gene in organelles like chloroplast and mitochondria[52]. The first commercially available transgenic maize and soybean

in markets were produced by biolostic method[62]. Many parameters influence the process of transformation by biolistic method such as physical parameters which include size of microparticles used for bombardment, properties of DNA attachment on particles, use of different types of instruments etc., biological parameters involves the selection of gene of interest which expresses itself at desired level, cells having different size, turgidity of cells, how old are the cell culture etc. and environmental parameters like temperature, humidity, quality and intensity of light, period of exposure to light etc.[63].

3. DEVELOPMENT OF GENETICALLY MODIFIED PLANTS

For the development of transgenic plants, there are many strategies available for the physical transfer of DNA into cells. Generally these strategies are applicable to transformation of all plants, but some of these are possible for the cells taken from specific sources. These approaches and strategies are called recombinant DNA technology[64]. The following are the laboratory steps for the development of genetically modified plants:

3.1 Isolation and Cloning of Gene of Interest

Desired gene have been isolated from the parent cell and inserted in the vector by treating both of them with same restriction endonuclease enzyme to produce site specific excision which generate the sticky ends. This sticky ends of DNA and vector are joined with each other by the enzyme DNA ligase and as a result Recombinant DNA molecule is formed. Recombinant DNA molecule is multiplied in a bacterial cell[65]. A transgene to deliver in plant cell contains two or more expression cassettes. Each cassette has a promoter region, target gene and terminator sequence. A transgene necessarily contain the cassette for the desired phenotypic character in transgenic plant and other for the selection of transgenic plant[66]. Promoter region located at the 5' upstream of gene direct the switch on/off of the gene expression[67]. The promoters usually are of three types: 1) Constitutive promoters, 2) Tissue-specific promoters, and 3) Inducible promoters[68].

3.2 DNA Transfer in Plant Tissues

Plant transformation is the fundamental tool of research in plant biology and is a practical tool in development of transgenic plants[69]. Several methods used to transfer the foreign DNA in plant cell as described above but among all other methods, Agro bacterium-mediated and Biolistics are most widely used for the plant transformation[66].

3.3 Integration of the Transgene

In the plant cell, either stable or transient transformation achieved. In the stable transformation, transgene introduced in the nucleus of plant cells, successfully integrate with the genome of plant cell and both are replicated together, which enables the inheritance and expression of transgene in the next generation. However, in transient transformation transgene expressed transiently because transgene does not replicate itself in host cell and gets degraded after some time. This both type of transformation depends up on the type of plasmid used as a vector or transgene construct[67].

3.4 Selection of Transformed Tissues

After completing the DNA transfer process, plant tissues are allowed to grow on particular medium which contain the chemical substance such as antibiotic or herbicide, choice of chemical agents depends upon the type of selectable marker used for Gene Construct. Only those plants survive which are transformed and express the antibiotic resistance gene[65].

3.4.1 Selectable Marker Genes

Selectable marker gene provides the resistance to some chemical agents like antibiotics and herbicides and hence used to identify the transformed plant cell. Marker gene codes for the protein which provides resistance by deactivating the selective chemical substance or by increasing tolerance[65]. Generally, marker genes used for the selection are antibiotic resistance gene such as neomycinphosphotransferase-II gene, hygromycine-phosphotransferase (hpt) and herbicide resistance genes such as BAR, EPSPS, PAP etc.[66].

3.4.2 Reporter Genes

Reporter genes are such genes which are attached to the foreign gene and used to find the expression of the transgene and also for the determination of the place of proteins which are expressed[70]. High sensitivity, stability and versatility are some characteristics of the best reporter genes[71].

3.5 Regeneration of Plant Tissue

After selection, explant and protoplast are allowed to grow on selective regeneration medium to form calli. After that, calli is transferred to elongation medium for about 30 days. Then, differentiated and elongated shoots are separated and allowed to grow on rooting medium. After roots gets developed, plantlets are transferred to greenhouse for grow in controlled environment. After successful establishment, they are tested for the transgene expression[4].

3.6 Plant Breeding and Testing

After producing the transgenic plant, it is tested for the activity and stable inheritance of the inserted gene and to know whether it has any adverse effects on other plant function, growth or yield. After initial evaluation, it will be repeatedly crossed with improved variety to recover most of the characters from the improved variety and transgene from the transformed variety. Later it will be grown at different location in different season to analyse the effects of transgene and overall performance[65].

4. DISCUSSION

Genetic transformation of plants leads to generation of large number of plants with improved agronomic traits[62]. Nowadays, many transformation methods are available for the insertion of novel genes. *Agrobacterium*-mediated methods are comparatively more desirable for transformation of many dicot and monocot species. However, many times biolistics is used for the transformation of some angiosperm and non-angiosperm plant species. Other methods are not generally used for the routine basis[72].

Currently, transformation of plants carried out by any biological or physical methods faces major challenges[50]. Hwang *et al.*, stated that *Agrobacterium sp.* infects only limited number of host species. Therefore, application of *Agrobacterium*-mediated methods is limited to transformation of few species as, it is not used for the species which does not get infected by the *Agrobacterium sp.* PEG-mediated gene transfer is an efficient, cost-effective and reliable but dependency on regeneration capacity of protoplast limits its application. Protoplasts with low regeneration capacity are not transformed by PEG-mediated gene transfer technique. Slow, tedious and expensive nature of microinjection and lower efficiency of electroporation reduce their popularity for development of genetically modified plants[43,50,60].

Development of efficient and reproducible protocols is required for the transformation of wide range of plant species and to increase the expression level of the transgene for novel characteristics. In-planta transformation methods overcome limitation generated due to low efficiency of protoplast regeneration. Plant breeders, researchers and biotechnologist can develop various transgenic lines with different expression level by collaborative contribution.

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