

Gene delivery methods in plants

For production of transgenic animals, DNA is usually microinjected into pronuclei of embryonic cells at a very early stage after fertilization, or alternatively gene targeting of embryo stem (ES) cells is employed. This is possible in animals due to the availability of specialized *in vitro* fertilization technology, which allows manipulation of ovule, zygote or early embryo.

Such techniques are not available in plants. In contrast to this in higher plants, cells or protoplasts can be cultured and used for regeneration of whole plants. Therefore, these protoplasts can be used for gene transfer followed by regeneration leading to the production of transgenic plants. Besides cultured cells and protoplasts, other meristem cells (immature embryos or organs), pollen or zygotes can also be used for gene transfer in plants. The enormous diversity of plant species and the availability of diverse genotypes in a species, made it necessary to develop a variety of techniques, suiting different situations. These different methods of gene transfer in plants are discussed.

Target cells for gene transformation

The first step in gene transfer technology is to select cells that are capable of giving rise to whole transformed plants. Transformation without regeneration and regeneration without transformation are of limited value. In many species, identification of these cell types is difficult. This is unlike the situation in animals, because the plant cells are totipotent and can be stimulated to regenerate into whole plants *in vitro* via organogenesis or embryogenesis. However, *in vitro* plant regeneration imposes a degree of 'genome stress', especially if plants are regenerated via a callus phase. This may lead to chromosomal or genetic abnormalities in regenerated plants a phenomenon referred to as soma clonal variation.

In contrast to this, gene transfer into pollen (or possibly egg cells) may give rise to genetically transformed gametes, which if used for fertilization (*in vivo*) may give rise to transformed whole plants. Similarly, insertion of DNA into zygote (*in vivo* or *in vitro*) followed by embryo rescue, may also be used to produce transgenic plants. Another alternative approach is the use of individual cells in embryos or meristems, which may be grown *in vitro* or may be allowed to develop normally for the production of transgenic plants.

Vectors for gene transfer

Most vectors carry marker genes, which allow recognition of transformed cells (other cells die due to the action of an antibiotic or herbicide) and are described as selectable markers. Among these marker genes, the most common selectable marker is npt II, providing kanamycin resistance. Other common features of suitable transformation vector include the following: (i) multiple unique restriction sites (a synthetic polylinker); (ii) bacterial origins of replication (e.g. ColE1).

The vectors having these properties may not necessarily have features, which facilitate their transfer to plant cells or integration into the plant nuclear genome. Therefore, *Agrobacterium* Ti plasmid is preferred over all other vectors, because of wide host range of this bacterial system and the capacity to transfer genes due to the presence of T - DNA border sequences.

Gene delivery methods

To achieve genetic transformation in plants, we need the construction of a vector (genetic vehicle) which transports the genes of interest, flanked by the necessary controlling sequences *i.e.* promoter and terminator, and deliver the genes into the host plant. The two kinds of gene transfer methods in plants are:

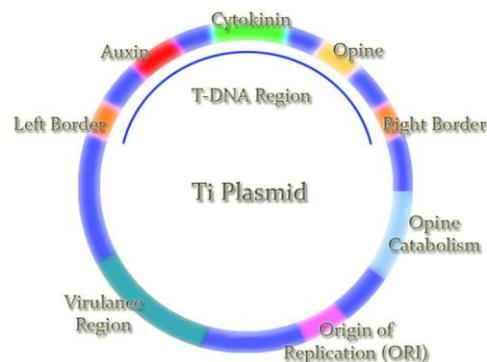
Vector-mediated or indirect gene transfer

Among the various vectors used in plant transformation, the Ti plasmid of ***Agrobacterium tumefaciens*** has been widely used. This bacterium is known as “natural genetic engineer” of plants because these bacteria have natural ability to transfer T-DNA of their plasmids into plant genome upon infection of cells at the wound site and cause an unorganized growth of a cell mass known as crown gall. Ti plasmids are used as gene vectors for delivering useful foreign genes into target plant cells and tissues. The foreign gene is cloned in the T-DNA region of Ti-plasmid in place of unwanted sequences. To transform plants, leaf discs (in case of dicots) or embryogenic callus (in case of monocots) are collected and infected with ***Agrobacterium*** carrying recombinant disarmed Ti-plasmid vector. The infected tissue is then cultured (co-cultivation) on shoot regeneration medium for 2-3 days during which time the transfer of T-DNA along with foreign genes takes place. After this, the transformed tissues (leaf discs/calli) are transferred onto selection cum plant regeneration medium supplemented with usually lethal concentration of an antibiotic to selectively eliminate non-transformed tissues. After 3-5 weeks, the regenerated shoots (from leaf discs) are transferred to root-inducing medium, and after another 3-

4 weeks, complete plants are transferred to soil following the hardening (acclimatization) of regenerated plants. The molecular techniques like PCR and southern hybridization are used to detect the presence of foreign genes in the transgenic plants.

Structure and functions of Ti and Ri Plasmids

The most commonly used vectors for gene transfer in higher plants are based on tumour inducing mechanism of the soil bacterium *Agrobacterium tumefaciens*, which is the causal organism for crown gall disease, A closely related species *A. rhizogenes* causes hairy root disease. An understanding of the molecular basis of these diseases led to the utilization of these bacteria for developing gene transfer systems. It has been shown that the disease is caused due to the transfer of a DNA segment from the bacterium to the plant nuclear genome. The DNA segment, which is transferred is called T - DNA and is part of a large Ti (tumour inducing) plasmid found in virulent strains of *Agrobacterium tumefaciens*. Similarly Ri (root inducing) megaplasmids are found in the virulent strains of *A. rhizogenes*.



Most Ti plasmids have four regions in common, (i) Region A, comprising T-DNA is responsible for tumour induction, so that mutations in this region lead to the production of tumours with altered morphology (shooty or rooty mutant galls). Sequences homologous to this region are always transferred to plant nuclear genome, so that the region is described as T-DNA (transferred DNA). (ii) Region B is responsible for replication. (iii) Region C is responsible for conjugation. (iv) Region D is responsible for virulence, so that mutation in this region abolishes virulence. This region is therefore called virulence (v) region and plays a crucial role in the transfer of T-DNA into the plant nuclear genome. The components of this Ti plasmid have been used for developing efficient plant transformation vectors.

The T-DNA consists of the following regions: (i) An one region consisting of three genes (two genes *tms* and *tms2* representing 'shooty locus' and one gene *tmr* representing 'rooty locus') responsible for the biosynthesis of two phytohormones, namely indole acetic acid (an auxin) and isopentyladenosine 5'-monophosphate (a cytokinin). These genes encode the enzymes responsible for the synthesis of these phytohormones, so that the incorporation of these genes in plant nuclear genome leads to the synthesis of these phytohormones in the host plant. The phytohormones in their turn alter the developmental programme, leading to the formation of crown gall (ii) An *os* region responsible for the synthesis of unusual amino acid or sugar derivatives, which are collectively given the name opines. Opines are derived from a variety of compounds (e.g. arginine + pyruvate), that are found in plant cells. Two most common opines are octopine and nopaline. For the synthesis of octopine and nopaline, the corresponding enzymes octopine synthase and nopaline synthase are coded by T- DNA.

Depending upon whether the Ti plasmid encodes octopine or nopaline, it is described as octopine-type Ti plasmid or nopaline-type Ti plasmid. Many organisms including higher plants are incapable of utilizing opines, which can be effectively utilized by *Agrobacterium*. Outside the T-DNA region, Ti plasmid carries genes that, catabolize the opines, which are utilized as a source of carbon and nitrogen. The T-DNA regions on all Ti and Ri plasmids are flanked by almost perfect 25bp direct repeat sequences, which are essential for T-DNA transfer, acting only in cis orientation. It has also been shown that any DNA sequence, flanked by these 25bp repeat sequences in the correct orientation, can be transferred to plant cells, an attribute that has been successfully utilized for *Agrobacterium* mediated gene transfer in higher plants leading to the production of transgenic plants.

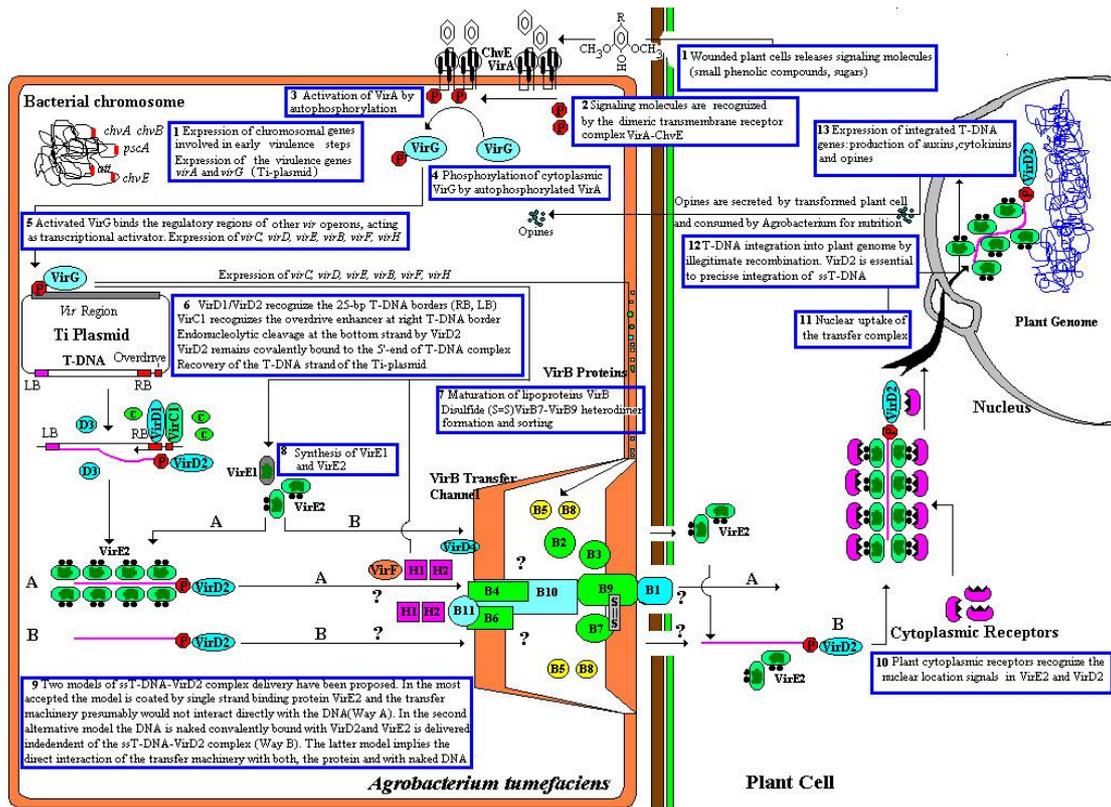
Besides 25bp flanking border sequences (with T DNA), *vir* region is also essential for T-DNA transfer. While border sequences function in cis orientation with respect to T - DNA, *vir* region is capable of functioning even in trans orientation. Consequently physical separation of T-DNA and *vir* region onto two different plasmids does not affect T-DNA transfer, provided both the plasmids are present in the same *Agrobacterium* cell. This property played an important role in designing the vectors for gene transfer in higher plants, as will be discussed later. The *vir* region (approx 35 kbp) is organized into six operons, namely *vir A*, *vir B*, *vir C*, *vir D*, *vir E*, and *vir G*, of which four operons (except *vir A* and *vir G*) are polycistronic. Genes *vir A*, *B*, *D*,

and G are absolutely required for virulence; the remaining two genes vir C and E are required for tumour formation. The vir A locus is expressed constitutively under all conditions.

The vir G locus is expressed at low levels in vegetative cells, but is rapidly induced to higher expression levels by exudates from wounded plant tissue. The vir A and vir G gene products regulate the expression of other vir loci. The vir A product (Vir A) is located on the inner membrane of *Agrobacterium* cells and is probably a chemoreceptor, which senses the presence of phenolic compounds (found in exudates of wounded plant tissue), such as acetosyringone and β -hydroxyacetosyringone. Signal transduction proceeds via activation (possibly phosphorylation) of Vir G (product of gene vir G), which in its turn induces expression of other vir genes.

Transformation techniques using *Agrobacterium*

Agrobacterium infection (utilizing its plasmids as vectors) has been extensively utilized for transfer of foreign DNA into a number of dicotyledonous species. The only important species that have not responded well, are major seed legumes, even though transgenic soybean (*Glycine max*) plants have been obtained. The success in this approach for gene transfer has resulted from improvement in tissue culture technology. However, monocotyledons could not be successfully utilized for *Agrobacterium* mediated gene transfer except a solitary example of *Asparagus*. The reasons for this are not fully understood, because T-DNA transfer does occur at the cellular level. It is possible that the failure in monocots lies in the lack of wound response of monocotyledonous cells.



Vectorless or direct gene transfer

In the direct gene transfer methods, the foreign gene of interest is delivered into the host plant cell without the help of a vector. The gene transfer system using genetically engineered vectors do not work out well particularly in monocot species. Considering the problem, direct gene transfer methods have been tried and the methods used for direct gene transfer in plants are:

Chemical mediated gene transfer

Direct DNA uptake by protoplasts can be stimulated by chemicals like polyethylene glycol (PEG). This method was reported by **Krens** and his colleagues in 1982. The technique is so efficient that virtually every protoplast system has proven transformable. PEG is also used to stimulate the uptake of liposomes and to improve the efficiency of electroporation. PEG at high concentration (15-25%) will precipitate ionic macromolecules such as DNA and stimulate their uptake by endocytosis without any gross damage to protoplasts. This is followed by cell wall formation and initiation of cell division. These cells can now be plated at low density on selection medium. Initial studies using the above method were restricted to *Petunia* and *Nicotiana*.

However, other plant systems (rice, maize, etc.) were also successfully used later. In these methods, PEG was used in combination with pure Ti plasmid, or calcium phosphate precipitated Ti plasmid mixed with a carrier DNA. Transformation frequencies upto 1 in 100 have been achieved by this method. Nevertheless, there are serious problems in using this method for getting transgenic plants and all these problems relate to plant regeneration from protoplasts.

Microinjection and Macroinjection

Plant regeneration from transformed protoplasts, still remains a problem. Therefore cultured tissues, that encourage the continued development of immature structures, provide alternate cellular targets for transformation. These immature structures may include immature embryos, meristems, immature pollen, germinating pollen, isolated ovules, embryogenic suspension cultured cells, etc. The main disadvantage of this technique is 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 subsequently obtained. Utilizing this approach, transgenic chimeras have actually been obtained in oilseed rape (*Brassica napus*).

When cells or protoplasts are used as targets in the technique of microinjection, glass micropipettes with 0.5-10 μ m diameter tip are used for transfer of macromolecules into the cytoplasm or the nucleus of a recipient cell or protoplast. The recipient cells are immobilized on a solid support (cover slip or slide, etc.) or artificially bound to a substrate or held by a pipette under suction (as done in animal systems). Often a specially designed micromanipulator is employed for microinjecting the DNA. Although, this technique gives high rate of success, the process is slow, expensive and requires highly skilled and experienced personnel.

The microinjection method was introduced by two groups of scientist led by **Crossway** and **Reich** in 1986. Recently a method known as "holding pipette method" was introduced. In this, the protoplasts are isolated from cell suspension culture are placed on a depression slide, by its side with a microdroplet of DNA solution. Using the holding pipette, the protoplast has to be held and the DNA to be injected into the nucleus using the injection pipette. After the micro injection the injected cells are cultured by hanging droplet culture method.

DNA macroinjection employing needles with diameters greater than cell diameter has also been tried. In rye (*Secale cereale*), a marker gene was macroinjected into the stem below the immature floral meristem, so as to reach the sporogenous tissue (De la Pena *et al.*, 1987) leading to successful production of transgenic plants. Unfortunately, this technique could not be successfully repeated with any other cereal, when tried in several laboratories. Therefore, doubt is expressed about the validity of earlier experiments conducted with rye (Potrykus, 1991).

Electroporation method

Electroporation is another efficient method for the incorporation of foreign DNA into protoplasts, and thus for direct gene transfer into plants. This method was introduced by **Fromm** and his coworkers in 1986.

This method is based on the use of short electrical impulses of high field strength. These impulses increase the permeability of protoplast membrane and facilitate entry of DNA molecules into the cells, if the DNA is in direct contact with the membrane. In view of this, for delivery of DNA to protoplasts, electroporation is one of the several routine techniques for efficient transformation. However, since regeneration from protoplasts is not always possible, cultured cells or tissue explants are often used. Consequently, it is important to test whether electroporation could transfer genes into

walled cells. In most of these cases no proof of transformation was available.

The electroporation pulse is generated by discharging a capacitor across the electrodes in a specially designed electroporation chamber. Either a high voltage (1.5 kV) rectangular wave pulse of short duration or a low voltage (350V) pulse of long duration is used. The latter can be generated by a home made machine. Protoplasts in an ionic solution containing the vector DNA are suspended between the electrodes, electroporated and then plated as usual. Transformed colonies are selected as described earlier. Using electroporation method, successful transfer of genes was achieved with the protoplasts of tobacco, petunia, maize, rice, wheat and sorghum. In most of these cases cat gene associated with a suitable promoter sequence was transferred. Transformation frequencies can be further improved by (i) using field strength of 1.25kV/cm, (ii) adding PEG after adding DNA, (iii) heat shocking protoplasts at 45°C for 5 minutes before adding DNA and (iv) by using linear instead of circular DNA.

Microprojectiles or biolistics or particle gun for gene transfer

In 1987, **Klein** and his colleagues evolved a method by which the delivery of DNA into cells of intact plant organs or cultured cells is done by a process called Projectile Bombardment. The micro-projectiles (small high density particles) are accelerated to high velocity by a particle gun apparatus. These particles with high kinetic energy penetrate the cells and membranes and carry foreign DNA inside of the bombarded cells. This method is otherwise called as "Biolistics Method". In recent years, it has been shown that DNA delivery to plant cells is also possible, when heavy microparticles (tungsten or gold) coated with the DNA of interest are accelerated to a very high initial velocity (1,400 ft per, sec). These microprojectiles, normally 1-3µm in diameter, are carried by a 'macroprojectile' or the 'bullet' and are accelerated into living plant cells (target cells can be pollen, cultured cells, cells in differentiated tissues and meristems) so that they can penetrate cell walls of intact tissue. The acceleration is achieved either by an explosive charge (cordite explosion) or by using shock waves initiated by a high voltage electric discharge. The design of two particle guns used for acceleration of microprojectiles.



Gene gun

Transformed plants using the above technique have been obtained in many cases including soybean, tobacco, maize, rice, wheat, etc.. Transient expression of genes transferred in cells by this method has also been observed in onion, maize, rice and wheat. There is no other gene transfer approach, which has met with so much of enthusiasm. Consequently considerable investment has been made in experimentation and manpower for development of this technique.

Sonication Method: This is a simple technique recently (1990) formulated by **Xu** and his coworkers. In this method the explants (especially leaves) are excised and cut into segments, immersed in sonication buffer containing plasmid DNA and Carrier DNA in a sterile glass petridish. Then the samples were sonicated with an ultrasonic pulse generator at 0.5 c/cm² acoustic intensity for 30 minutes. After 30 minutes, the explants were rinsed in buffer solution without DMSO and transferred to the culture medium.

Transformation

This method is used for introducing foreign DNA into bacterial cells e.g. *E. Coli*. The transformation frequency (the fraction of cell population that can be transferred) is very good in this method. E.g. the uptake of plasmid DNA by *E. coli* is carried out in ice cold CaCl₂ (0-50C) followed by heat shock treatment at 37-450C for about 90 sec. The transformation efficiency refers to the number of transformants per microgram of added DNA. The CaCl₂ breaks the cell wall at certain regions and binds the DNA to the cell surface.

Conjunction

It is a natural microbial recombination process and is used as a method for gene

transfer. In conjunction, two live bacteria come together and the single stranded DNA is transferred via cytoplasmic bridges from the donor bacteria to the recipient bacteria.

Liposome mediated gene transfer or Lipofection

Liposomes are small lipid bags, in which large number of plasmids are enclosed. They can be induced to fuse with protoplasts using devices like PEG, and therefore have been used for gene transfer. The technique, offers following advantages: (i) protection of DNA/RNA from nuclease digestion, (ii) low cell toxicity, (iii) stability and storage of nucleic acids due to encapsulation in liposomes, (iv) high degree of reproducibility and (v) applicability to a wide range of cell types.

In this technique, DNA enters the protoplasts due to endocytosis of liposomes, involving the following steps: (i) adhesion of the liposomes to the protoplast surface, (ii) fusion of liposomes at the site of adhesion and (iii) release of plasmids inside the cell. The technique has been successfully used to deliver DNA into the protoplasts of a number of plant species (e.g. tobacco, petunia, carrot, etc.).

Gene transformation using pollen or pollen tube

There has been a hope that DNA can be taken up by the germinating pollen and can either integrate into sperm nuclei or reach the zygote through the pollen tube pathway. Both these approaches have been tried and interesting phenotypic alterations suggesting gene transfer have been obtained. In no case, however, unequivocal proof of gene transfer has been available. In a number of experiments, when marker genes were used for transfer, only negative results were obtained. Several problems exist in this method and these include the presence of cell wall, nucleases, heterochromatic state of acceptor DNA, callose plugs in pollen tube, etc. Transgenic plants have never been recovered using this approach and this method, though very attractive, seems to have little potential for gene transfer.

Calcium phosphate precipitation method for gene transfer

Foreign DNA can also be carried with the Ca^{++} ions, to be released inside the cell due to the precipitation of calcium in the form of calcium phosphate. In the past, this method was considered to be very important for gene transfer in plants.

Incubation of dry seeds, embryos, tissues or cells in DNA

Incubation of dry seeds, embryos, tissues or cells in known DNA (viral or non viral having defined marker genes) has been tried in many cases and expression of

defined genes has been witnessed. However, in no case proof of integrative transformation could be available. In all these cases, plant cell walls not only work as efficient barriers, but are also efficient traps for DNA molecules. It would be very surprising if DNA can cross cell walls efficiently without permeabilizing them either by PEG, or by electroporation or by any other device.

Selection of transformed cells from untransformed cells

The selection of transformed plant cells from untransformed cells is an important step in the plant genetic engineering. For this, a marker gene (e.g. for antibiotic resistance) is introduced into the plant along with the transgene followed by the selection of an appropriate selection medium (containing the antibiotic). The segregation and stability of the transgene integration and expression in the subsequent generations can be studied by genetic and molecular analyses (Northern, Southern, Western blot, PCR).

Though several methods have been described for gene transfer using naked DNA, the recovery of genetic recombinants, otherwise called as "transgenic plants" appears to be a rare phenomenon. A concerted effort

- To accurately identify genes which can be shown to influence agronomically important characters
- To apply the technique to clone the isolated genes
- To anneal them to appropriate vectors and
- To evaluate their expression in agronomically important crop varieties will solve the deficiencies in the conventional breeding procedures.

The last five years have seen successful outcomes and transgenic plants have been produced in some crop species by using both vector mediated and direct gene transfer techniques. However, all the programmes were not successful because of lack of proofs for the integrative gene transfer. Considering the needs to have integrative gene transfer, **Potrykus** points out that all the successful gene transfers should have the following proofs.

1. Serious controls for treatments and analysis.
2. A tight correlation between treatment and predicted results.
3. A tight correlation between physical (Southern blot, *in situ* hybridization) and phenotypic data.

4. Complete Southern Analysis to show the hybrid fragments of host DNA and foreign DNA, and the absence or presence of contaminating fragments.
5. Data that allow discrimination between false positives and correct transformants in the evaluation of the phenotypic evidence.
6. Correlation of the physical and phenotypic evidence with transmission to sexual offspring, as well as genetic and molecular analysis of offspring populations.

Questions

1. The vectors used in genetic engineering should possess
 - a). Multiple unique restriction sites
 - b). Bacterial origins of replication
 - c). Marker genes, which allow recognition of transformed cells
 - d). All the above

2. Agrobacterium Ti plasmid is preferred over all other vectors because
 - a). Wide host range
 - b). Capacity to transfer genes due to the presence of T - DNA border sequences
 - c). Both a and b**
 - d). None of the above

3. The natural genetic engineer is
 - a). *Agrobacterium tumefaciens***
 - b). *Bacillus subtilis*
 - c). *E. coli*
 - d). None of the above

4. *Agrobacterium tumefaciens* causes
 - a). Crown gall**
 - b). Hairy root
 - c). *Root rot*
 - d). None of the above

5. *Agrobacterium rhizogenes* causes
 - a). Crown gall
 - b). Hairy root**
 - c). *Root rot*
 - d). None of the above

6. Ti plasmids have regions in common.
 - a). 4**
 - b). 5
 - c). 3
 - d). None of the above

6. Region which is responsible for tumour induction in Ti plasmids is
 - a). Region A**
 - b). Region B
 - c). Region C
 - d). Region D

7. T-DNA region of Ti plasmids is
 - a). Region A**
 - b). Region B
 - c). Region C
 - d). Region D

8. Region B is responsible for
 - a). Tumour induction
 - b). Replication**
 - c). Conjugation
 - d). Virulence

9. Region A is responsible for

- a). **Tumour induction**
c). Conjugation
- b). Replication
d). Virulence
10. Region C is responsible for
- a). Tumour induction
c). Conjugation
- b). Replication
d). Virulence
11. Region D is responsible for
- a). Tumour induction
c). Conjugation
- b). Replication
d). Virulence
12. The gene responsible for shooty locus in T-DNA region is
- a). tms
c). tmr
- b). tms2
d). tms and tms2
13. The gene responsible for rooty locus in T-DNA region is
- a). tms
c). tmr
- b). tms2
d). None of the above
14. The chemical mediated (PEG) transfer is proposed by.....
- a). Krens**
c).
- b).
d). None of the above
15. The optimum concentration of PEG for DNA transfer is.....
- a). 15-25%**
c). 10%
- b). 30-40%
d). None of the above
16. The microinjection method was introduced by.....
- a). Crossway and Reich**
c). Klein
- b). Fromm
d). Xu
17. The electroporation method was introduced by.....
- a). Crossway and Reich
c). Klein
- b). Fromm**
d). Xu
18. The particle gun for gene transfer was introduced by.....
- a). Crossway and Reich
c). Klein
- b). Fromm
d). Xu
19. The sonication method was introduced by.....
- a). Crossway and Reich
c). Klein
- b). Fromm
d). Xu

Additional reading...

http://www.biotechnology4u.com/plant_biotechnology_gene_transfermethods_plants.html

<http://depts.washington.edu/agro/>

<http://www.ejbiotechnology.info/content/vol1/issue3/full/1/bip/>

<http://arabidopsis.info/students/agrobacterium/>