Agrobacterium-Mediated Gene Transfer

The gene transfer techniques in plant genetic transformation are broadly grouped into two categories:

I. Vector-mediated gene transfer

II. Direct or vector less DNA transfer

The salient features of the commonly used gene (DNA) transfer methods are given in Table 49.1.

Table 49.1 Gene transfer (DNA delivery) methods in plants

Method	*Salient features*
I. Vector–mediated gene transfer	
Agrobacterium (Ti plasmid)-mediated gene transfer	Very efficient, but limited to a selected group of plants
Plant viral vectors	Ineffective method, hence not widely used
II. Direct or vectorless DNA transfer	
(A) Physical methods	
Electroporation	Mostly confined to protoplasts that can be regenerated to viable plants. Many cereal crops developed.
Microprojectile (particle bombardment)	Very successful method used for a wide range of plants/ tissues. Risk of gene rearrangement high.
Microinjection	Limited use since only one cell can be microinjected at a time. Technical personnel should be highly skilled.
Liposome fusion	Confined to protoplasts that can be regenerated into viable whole plants.
Silicon carbide fibres	Requires regenerable cell suspensions. The fibres, however, require careful handling.
(B) Chemical methods	
Polyethylene glycol (PEG)-mediated	Confined to protoplasts. Regeneration of fertile plants is frequently problematical.
Diethylaminoethyl (DEAE) dextran-mediated	Does not result in stable transformants.

Method # I. Vector-Mediated Gene Transfer:

Vector-mediated gene transfer is carried out either by Agrobacterium-mediated transformation or by use of plant viruses as vectors.

Agrobacterium-Mediated Gene Transfer:

Agrobacterium tumefaciens is a soil-borne, Gram-negative bacterium. It is rod shaped and motile, and belongs to the bacterial family of Rhizobiaceae. A. tumefaciens is a phytopathogen, and is treated as the nature's most effective plant genetic engineer.

Some workers consider this bacterium as the natural expert of inter-kingdom gene transfer. In fact, the major credit for the development of plant transformation techniques goes to the natural unique capability of A. tumefaciens. Thus, this bacterium is the most beloved by plant biotechnologists.

There are mainly two species of Agrobacterium:

i. A. tumefaciens that induces crown gall disease.

ii. A. rhizogenes that induces hairy root disease.

Crown Gall Disease and Ti Plasmid:

Almost 100 years ago (1907), Smith and Townsend postulated that a bacterium was the causative agent of crown gall tumors, although its importance was recognized much later. As A. tumefaciens infects wounded or damaged plant tissues, in induces the formation of a plant tumor called crown gall (Fig. 49.1). The entry of the bacterium into the plant tissues is facilitated by the release of certain phenolic compounds (acetosyringone, hydroxyacetosyringone) by the wounded sites.

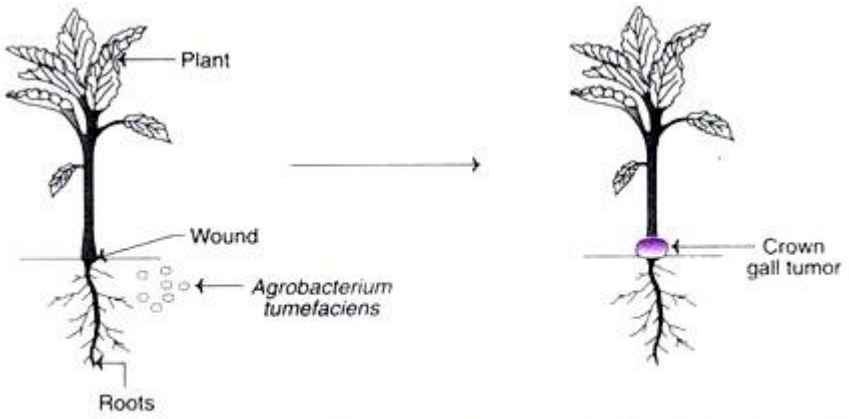

Fig. 49.1 : Formation of a crown gall tumor in a plant infected with Agrobacterium tumefaciens.

Crown gall formation occurs when the bacterium releases its Ti plasmid (tumor- inducing plasmid) into the plant cell cytoplasm. A fragment (segment) of Ti plasmid, referred to as T-DNA, is actually transferred from the bacterium into the host where it gets integrated into the plant cell chromosome (i.e. host genome). Thus, crown gall disease is a naturally evolved genetic engineering process.

The T-DNA carries genes that code for proteins involved in the biosynthesis of growth hormones (auxin and cytokinin) and novel plant metabolites namely opines — amino acid derivatives and agropines — sugar derivatives (Fig. 49.2).

The growth hormones cause plant cells to proliferate and form the gall while opines and agropines are utilized by A. tumefaciens as sources of carbon and energy. As such, opines and agropines are not normally part of the plant metabolism (neither produced nor metabolised). Thus, A. tumefaciens genetically transforms plant cells and creates a biosynthetic machinery to produce nutrients for its own use.

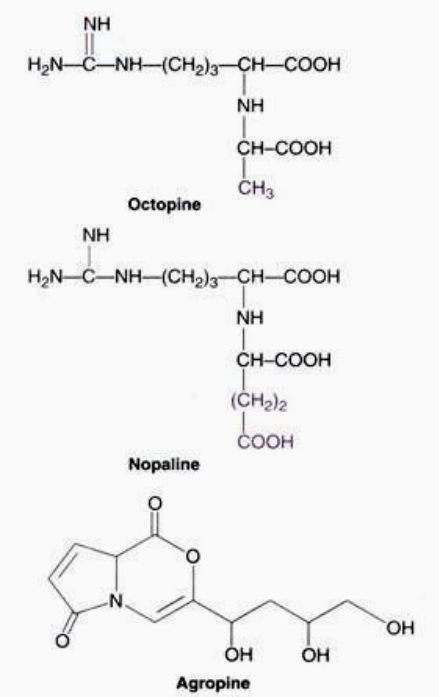

Fig. 49.2 : Structures of three opines–octopine, nopaline, and agropine.

As the bacteria multiply and continue infection, grown gall develops which is a visible mass of the accumulated bacteria and plant material. Crown gall formation is the consequence of the transfer, integration and expression of genes of T-DNA (or Ti plasmid) of A. tumefaciens in the infected plant.

The genetic transformation leads to the formation of crown gall tumors, which interfere with the normal growth of the plant. Several dicotyledonous plants (dicots) are affected by crown gall disease e.g. grapes, roses, stone-fruit trees.

Organization of Ti plasmid:

The Ti plasmids (approximate size 200 kb each) exist as independent replicating circular DNA molecules within the Agrobacterium cells. The T-DNA (transferred DNA) is variable in length in the range of 12 to 24 kb, which depends on the bacterial strain from which Ti plasmids come. Nopaline strains of Ti plasmid have one T-DNA with length of 20 kb while octopine strains have two T-DNA regions referred to as T_L and T_R that are respectively 14 kb and 7 kb in length.
A diagrammatic representation of a Ti plasmid is depicted in Fig. 49.3. The Ti plasmid has three important regions.

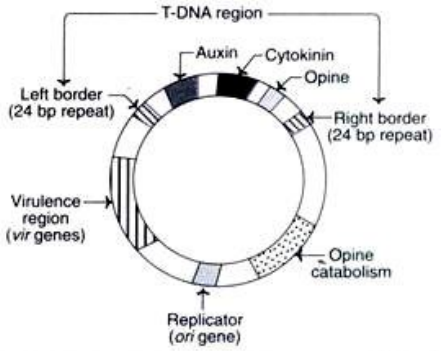

Fig. 49.3 : A dagrammatic representation of a Ti plasmid.

1. T-DNA region:

This region has the genes for the biosynthesis of auxin (aux), cytokinin (cyt) and opine (ocs), and is flanked by left and right borders. These three genes-aux, cyto and ocs are referred to as oncogenes, as they are the determinants of the tumor phenotype.

T-DNA borders — A set of 24 kb sequences present on either side (right and left) of T-DNA are also transferred to the plant cells. It is now clearly established that the right border is more critical for T-DNA transfer and tumori-genesis.

2. Virulence region:

The genes responsible for the transfer of T-DNA into the host plant are located outside T-DNA and the region is referred to as vir or virulence region. Vir region codes for proteins involved in T-DNA transfer. At least nine vir-gene operons have been identified. These include vir A, vir G, vir B_1, vir C_1, vir D_1, D_2 and D_4, and vir E_1, and E_2.

3. Opine catabolism region:

This region codes for proteins involved in the uptake and metabolisms of opines. Besides the above three, there is ori region that is responsible for the origin of DNA replication which permits the Ti plasmid to be stably maintained in A. tumefaciens.

T-DNA transfer and integration:

The process of T-DNA transfer and it integration into the host plant genome is depicted in Fig. 49.4, and is briefly described.

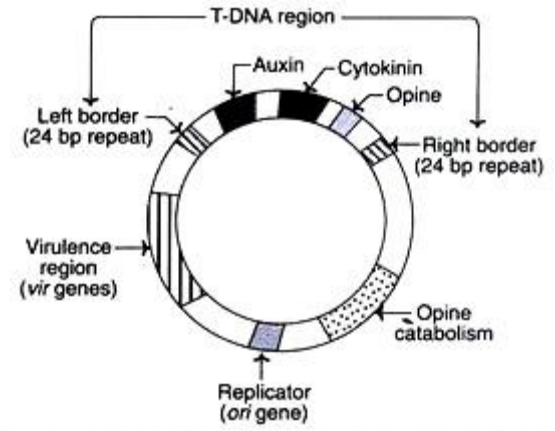

Fig. 49.3 : A dagrammatic representation of a Ti plasmid.

1. Signal induction to Agrobacterium:

The wounded plant cells release certain chemicals- phenolic compounds and sugars which are recognized as signals by Agrobacterium. The signals induced result in a sequence of biochemical events in Agrobacterium that ultimately helps in the transfer of T-DNA of T-plasmid.

2. Attachment of Agrobacterium to plant cells:

The Agrobacterium attaches to plant cells through polysaccharides, particularly cellulose fibres produced by the bacterium. Several chromosomal virulence (chv) genes responsible for the attachment of bacterial cells to plant cells have been identified.

3. Production of virulence proteins:

As the signal induction occurs in the Agrobacterium cells attached to plant cells, a series of events take place that result in the production of virulence proteins. To start with, signal induction by phenolics stimulates vir A which in turn activates (by phosphorylation) vir C. This induces expression of virulence genes of Ti plasmid to produce the corresponding virulence proteins (D1, D2, E_2, B etc.). Certain sugars (e.g. glucose, galactose, xylose) that induce virulence genes have been identified.

4. Production of T-DNA strand:

The right and left borders of T-DNA are recognized by vir D_1/vir D_2 proteins. These proteins are involved in the production single-stranded T-DNA (ss DNA), its protection and export to plant cells. The ss T-DNA gets attached to vir D_2.

5. Transfer of T-DNA out of Agrobacterium:

The ss T-DNA — vir D_2 complex in association with vir G is exported from the bacterial cell. Vir B products form the transport apparatus.

6. Transfer of T-DNA into plant cells and integration:

The T-DNA-vir D_2 complex crosses the plant plasma membrane. In the plant cells, T-DNA gets covered with vir E_2. This covering protects the T-DNA from degradation by nucleases; vir D_2 and vir E_2 interact with a variety of plant proteins which influences T-DNA transport and integration.

The T-DNA-vir D_2-vir E_2 — plant protein complex enters the nucleus through nuclear pore complex. Within the nucleus, the T-DNA gets integrated into the plant chromosome through a process referred to illegitimate recombination. This is different from the homologous recombination, as it does not depend on the sequence similarity.

Hairy Root Disease of A. Rhizogenes — R_i Plasmids:

Agrobacterium rhizogenes can also infect plants. But this results in hairy roots and not crown galls as is the case with A. tumefaciens. The plasmids, of A. rhizogenes have been isolated and characterized. These plasmids, referred to as Ri plasmids, (Ri stands for Root inducing) are of different types. Some of the Ri plasmid strains possess genes that are homologous to Ti plasmid e.g. auxin biosynthetic genes.

Instead of virulence genes, Ri plasmids contain a series of open reading frames on the T-DNA. The products of these genes are involved in the metabolism of plant growth regulators which gets sensitized to auxin and leads to root formation.

Vectors of A. rhizogenes:

As it is done with A tumefaciens, vectors can be constructed by using A. rhizogenes. These vectors are alternate strategies for gene transfer. However, employment of A. rhizogene-based vectors for plant transformation is not common since more efficient systems of A. tumefaciens have been developed.

Importance of hairy roots:

Hairy roots can be cultured in vitro, and thus are important in plant biotechnology. Hairy root systems are useful for the production of secondary metabolites, particularly pharmaceutical proteins.

Ti Plasmid-Derived Vector Systems:

The ability of Ti plasmid of Agrobacterium to genetically transform plants has been described. It is possible to insert a desired DNA sequence (gene) into the T-DNA region (of Ti plasmid), and then use A. tumefaciens to deliver this gene(s) into the genome of plant cell.

In this process, Ti plasmids serve as natural vectors. However, there are several limitations to use Ti plasmids directly as cloning vectors:

i. Ti plasmids are large in size (200-800 kb). Smaller vectors are preferred for recombinant experiments. For this reason, large segments of DNA of Ti plasmid, not essential for cloning, must be removed.

ii. Absence of unique restriction enzyme sites on Ti plasmids.

iii. The phytohormones (auxin, cytokinin) produced prevent the plant cells being regenerated into plants. Therefore auxin and cytokinin genes must be removed.

iv. Opine production in transformed plant cells lowers the plant yield. Therefore opine synthesizing genes which are of no use to plants should be removed.

v. Ti plasmids cannot replicate in E. coli. This limits their utility as E. coli is widely used in recombinant experiments. An alternate arrangement is to add an origin of replication to Ti plasmid that allows the plasmid to replicate in E. coli.

Considering the above limitations, Ti plasmid- based vectors with suitable modifications have been constructed.

These vectors are mainly composed of the following components:

1. The right border sequence of T-DNA which is absolutely required for T-DNA integration into plant cell DNA.

2. A multiple cloning site (poly-linker DNA) that promotes the insertion of cloned gene into the region between T-DNA borders.

3. An origin of DNA replication that allows the plasmids to multiply in E. coli.

4. A selectable marker gene (e.g. neomycin phosphotransferase) for appropriate selection of the transformed cells.

Two types of Ti plasmid-derived vectors are used for genetic transformation of plants— co-integrate vectors and binary vectors.

Co-integrate vector:

In the co-integrate vector system, the disarmed and modified Ti plasmid combines with an intermediate cloning vector to produce a recombinant Ti plasmid (Fig. 49.5).

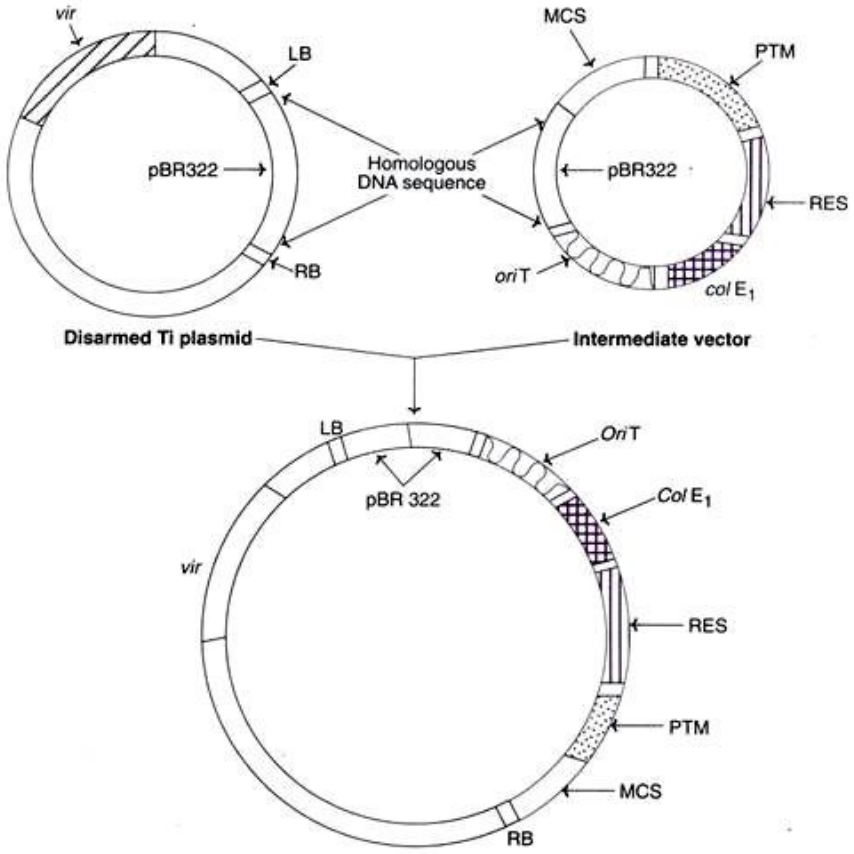

Fig. 49.5 : Cointegrate vector system (vir-Ti plasmid virulence region; pBR322-Bacterial plasmid 322; LB-Left border; RB-Right border; MCS-Multiple cloning site; PTM-Plant transformation marker; RES-Bacterial resistance marker; col E_1-Origin of a replication from col E_1 plasmid; ori T-Origin of transfer site for conjugative plasmid mobilization).

Production of disarmed Ti plasmid:

The T-DNA genes for hormone biosynthesis are removed (disarmed). In place of the deleted DNA, a bacterial plasmid (pBR322) DNA sequence is incorporated. This disarmed plasmid, also referred to as receptor plasmid, has the basic structure of T-DNA (right and left borders, virulence genes etc.) necessary to transfer the plant cells.

Construction of intermediate vector:

The intermediate vector is constructed with the following components:

i. A pBR322 sequence DNA homologous to that found in the receptor Ti plasmid.

ii. A plant transformation marker (PTM) e.g. a gene coding for neomycin phosphotransferase II (npt II). This gene confers resistance to kanamycin in the plant cells and thus permits their isolation.

iii. A bacterial resistance marker e.g. a gene coding for spectinomycin resistance. This gene confers spectinomycin resistance to recipient bacterial cells and thus permits their selective isolation.

iv. A multiple cloning site (MCS) where foreign genes can be inserted.

v. A Co/E$_1$ origin of replication which allows the replication of plasmid in E. coli but not in Agrobacterium.

vi. An oriT sequence with basis of mobilization (bom) site for the transfer of intermediate vector from E. coli to Agrobacterium.

Production and use of co-integrate vectors:

The desired foreign gene (target-gene) is first cloned in the multiple cloning site of the intermediate vector. The cloning process is carried out in E. coli, the bacterium where the cloning is most efficient. The intermediate vector is mated with Agrobacterium so that the foreign gene is mobilised into the latter.

The transformed Agrobacterium cells with receptor Ti plasmid and intermediate vector are selectively isolated when grown on a minimal medium containing spectinomycin. The selection process becomes easy since E. coli does not grow on a minimal medium in which Agrobacterium grows.

Within the Agrobacterium cells, intermediate plasmid gets integrated into the receptor Ti plasmid to produce co-integrate plasmid. This plasmid containing plant transformation marker (e.g. npt II) gene and cloned target gene between T-DNA borders is transferred to plant cells. The transformed plant cells can be selected on a medium containing kanamycin when the plant and Agrobacterium cells are incubated together.

Advantages of co-integrate vector:

i. Target genes can be easily cloned

ii. The plasmid is relatively small with a number of restriction sites.

iii. Intermediate plasmid is conveniently cloned in E. coli and transferred to Agrobacterium.

Binary vector:

The binary vector system consists of an Agrobacterium strain along with a disarmed Ti plasmid called vir helper plasmid (the entire T-DNA region including borders deleted while vir gene is retained). It may be noted that both of them are not physically linked (or integrated). A binary vector with T-DNA can replicate in E. coli and Agrobacterium.

A diagrammatic representation of a typical binary vector system is depicted in Fig. 49.6. The binary vector has the following components.

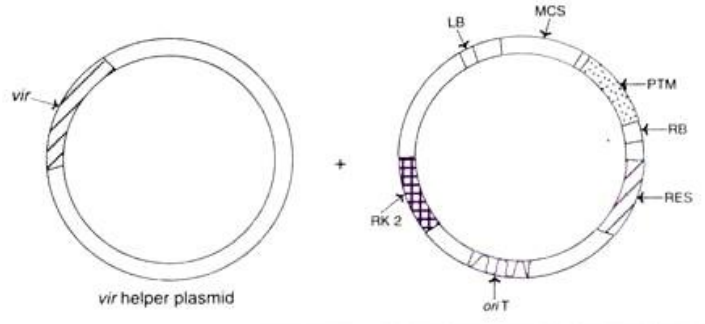

Fig. 49.6 : Binary vector system (vir-Ti plasmid virulence region; LB-Left border; RB-Right border; MCS-Multiple cloning site; PTM-Plant transformation marker; RES-Bacterial resistance marker; oriT-Origin of transfer site for conjugative plasmid mobilization; RK_2-Origin of replication from plasmid).

1. Left and right borders that delimit the T-DNA region.

2. A plant transformation marker (PTM) e.g. npt II that confers kanamycin resistance in plant transformed cells.

3. A multiple cloning site (MCS) for introducing target/foreign genes.

4. A bacterial resistance marker e.g. tetracycline resistance gene for selecting binary vector colonies in E. coli and Agrobacterium.

5. oriT sequence for conjugal mobilization of the binary vector from E. coli to Agrobacterium.

6. A broad host-range origin of replication such as RK_2 that allows the replication of binary vector in Agrobacterium.

Production and use of binary vector:

The target (foreign) gene of interest is inserted into the multiple cloning site of the binary vector. In this way, the- target gene is placed between the right and left border repeats and cloned in E. coli. By a mating process, the binary vector is mobilised from E. coli to Agrobacterium. Now, the virulence gene proteins of T-DNA facilitate the transfer of T-DNA of the vector into plant cells.

Advantages of binary vectors:

i. The binary vector system involves only the transfer of a binary plasmid to Agrobacterium without any integration. This is in contrast to co-integrate vector system wherein the intermediate vector is transferred and integrated with disarmed Ti plasmid.

ii. Due to convenience, binary vectors are more frequently used than co-integrate vectors.

Plant Transformation Technique Using Agrobacterium:

Agrobacterium-mediated technique is the most widely used for the transformation of plants and generation of transgenic plants. The important requirements for gene transfer in higher plants through Agrobacterium mediation are listed.

i. The explants of the plant must produce phenolic compounds (e.g. autosyringone) for activation of virulence genes.

ii. Transformed cells/tissues should be capable to regenerate into whole plants.

In general, most of the Agrobacterium-mediated plant transformations have the following basic protocol (Fig. 49.7)

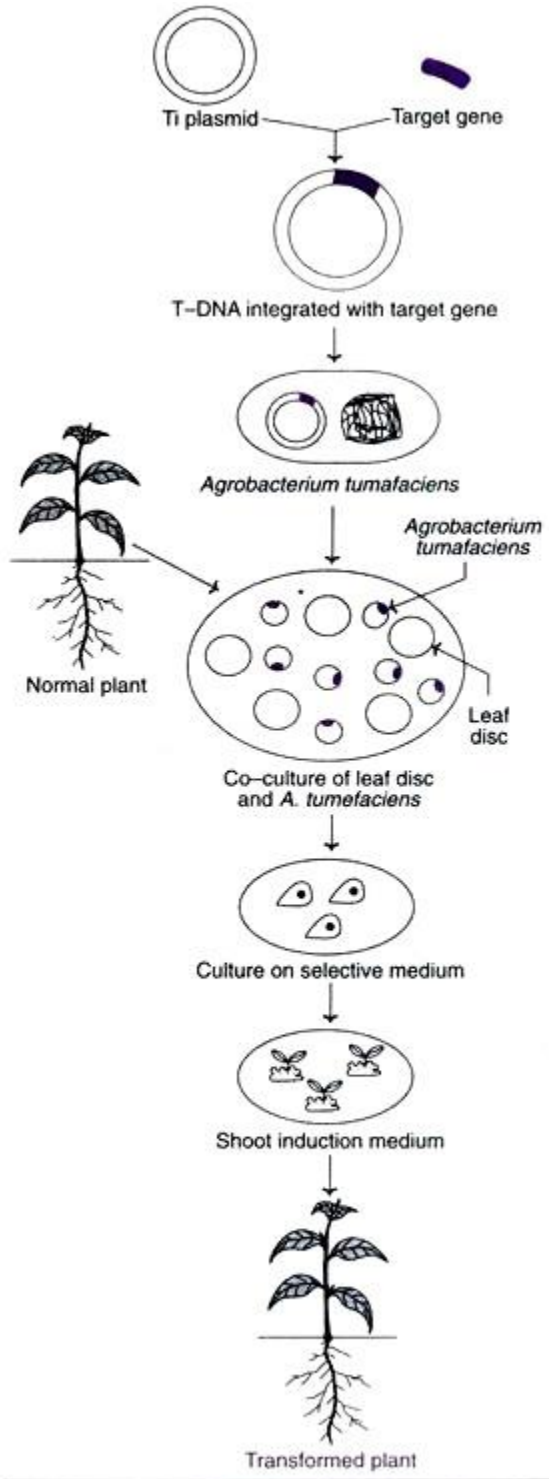

Fig. 49.7 : Transformation technique using Agrobacterium-mediated gene transfer.

1. Development of Agrobacterium carrying the co-integrate or binary vector with the desired gene.

2. Identification of a suitable explant e.g. cells, protoplasts, tissues, calluses, organs.

3. Co-culture of explants with Agrobacterium.

4. Killing of Agrobacterium with a suitable antibiotic without harming the plant tissue.

5. Selection of transformed plant cells.

6. Regeneration of whole plants.

Advantages of Agrobacterium- mediated transformation:

i. This is a natural method of gene transfer.

ii. Agrobacterium can conveniently infect any explant (cells/tissues/organs).

iii. Even large fragments of DNA can be efficiently transferred.

iv. Stability of transferred DNA is reasonably good.

v. Transformed plants can be regenerated effectively.

Limitations of Agrobacterium- mediated transformation:

i. There is a limitation of host plants for Agrobacterium, since many crop plants (monocotyledons e.g. cereals) are not infected by it. In recent years, virulent strains of Agrobacterium that can infect a wide range of plants have been developed.

ii. The cells that regenerate more efficiently are often difficult to transform, e.g. embryonic cells lie in deep layers which are not easy targets for Agrobacterium.

Virus-Mediated Gene Transfer (Plant Viruses as Vectors):

Plant viruses are considered as efficient gene transfer agents as they can infect the intact plants and amplify the transferred genes through viral genome replication. Viruses are natural vectors for genetic engineering. They can introduce the desirable gene(s) into almost all the plant cells since the viral infections are mostly systemic.

Plant viruses are non-integrative vectors:

The plant viruses do not integrate into the host genome in contrast to the vectors based on T-DNA of A. tumefaciens which are integrative. The viral genomes are suitably modified by introducing desired foreign genes. These recombinant viruses are transferred, multiplied and expressed in plant cells. They spread systemically within the host plant where the new genetic material is expressed.

Criteria for a plant virus vector:

An ideal plant virus for its effective use in gene transfer is expected to posses the following characteristics:

i. The virus must be capable of spreading from cell to cell through plasmodesmata.

ii. The viral genome should be able to replicate in the absence of viral coat protein and spread from cell to cell. This is desirable since the insertion of foreign DNA will make the viral genome too big to be packed.

iii. The recombinant viral genome must elicit little or no disease symptoms in the infected plants.

iv. The virus should have a broad host range.

v. The virus with DNA genome is preferred since the genetic manipulations involve plant DNA.

The three groups of viruses — caulimoviruses, Gemini viruses and RNA viruses that are used as vectors for gene transfer in plants are briefly described.

Caulimoviruses as Vectors:

The caulimoviruses contain circular double- stranded DNA, and are spherical in shape. Caulimoviruses are widely distributed and are responsible for a number of economically important diseases in various crops. The caulimovirus group has around 15 viruses and among these cauliflower mosaic virus (CaMV) is the most important for gene transfer. The other caulimoviruses include carnation etched virus, dahlia mosaic virus, mirabilis mosaic virus and strawberry vein banding virus.

Cauliflower mosaic virus (CaMV):

CaMV infects many plants (e.g. members of Cruciferae, Datura) and can be easily transmitted, even mechanically. Another attractive feature of CaMV is that the infection is systemic, and large quantities of viruses are found in infected cells.

A diagrammatic view of the CaMV genetic map is depicted in Fig. 49.8. The genome of CaMV consists of a 8 kb (8024 bp) relaxed but tightly packed circular DNA with six major and two minor coding regions. The genes II and VII are not essential for viral infection.

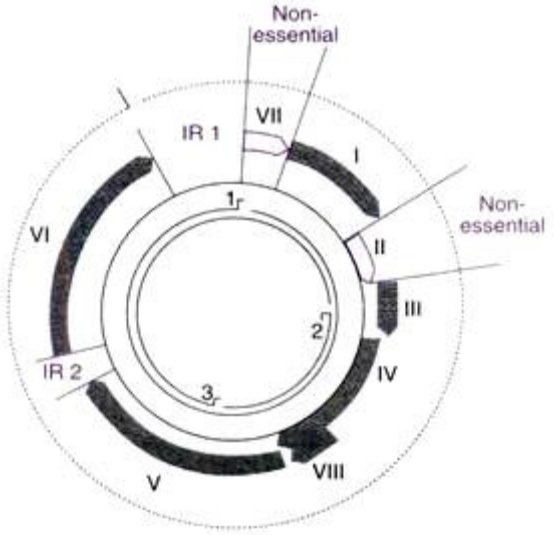

Fig. 49.8 : *A diagrammatic representation of the genetic map of cauliflower mosaic virus genome (I...VIII represent coding regions; IR 1and IR 2 are intergeneric regions; The outside dotted circle represents 30S transcript; The two circular lines at the centre indicate viral DNA strands).*

Use of CaMV in gene transfer:

For appropriate transmission of CaMV, the foreign DNA must be encapsulated in viral protein. Further, the newly inserted foreign DNA must not interfere with the native assembly of the virus. CaMV genome does not contain any non-coding regions wherein foreign DNA can be inserted. It is fortunate that two genes namely gene II and gene VII have no essential functions for the virus. It is therefore possible to replace one of them and insert the desired foreign gene.

Gene II of CaMV has been successfully replaced with a bacterial gene encoding dihydrofolate reductase that provides resistance to methotrexate. When the chimeric CaMV was transmitted to turnip plants, they were systemically infected and the plants developed resistance to methotrexate.

Limitations of CaMV as a vector:

i. CaMV vector has a limited capacity for insertion of foreign genes.

ii. Infective capacity of CaMV is lost if more than a few hundred nucleotides are introduced.

iii. Helper viruses cannot be used since the foreign DNA gets expelled and wild-type viruses are produced.

Gemini Viruses as Vectors:

The Gemini viruses are so named because they have geminate (Gemini literally means heavenly twins) morphological particles i.e. twin and paired capsid structures. These viruses are characterized by possessing one or two single-stranded circular DNAs (ss DNA). On replications, ss DNA forms an intermediate double-stranded DNA.

The Gemini viruses can infect a wide range of crop plants (monocotyledons and dicotyledons) which attract plant biotechnologists to employ these viruses for gene transfer. Curly top virus (CTV) and maize streak virus (MSV) and bean golden mosaic virus (BGMV) are among the important Gemini viruses.

It has been observed that a large number of replicative forms of a Gemini virus genome accumulate inside the nuclei of infected cells. The single-stranded genomic DNA replicates in the nucleus to form a double-stranded intermediate.

Gemini virus vectors can be used to deliver, amplify and express foreign genes in several plants/ explants (protoplasts, cultured cells). However, the serious drawback in employing Gemini viruses as vectors is that it is very difficult to introduce purified viral DNA into the plants. An alternate arrangement is to take the help of Agrobacterium and carry out gene transfer.

RNA Plant Viruses as Vectors:

There are mainly two type's single-stranded RNA viruses:

1. Mono-partite viruses:

These viruses are usually large and contain undivided genomes for all the genetic information e.g. tobacco mosaic virus (TMV).

2. Multipartite viruses:

The genome in these viruses is divided into small RNAs which may be in the same particle or different particles, e.g. brome mosaic virus (BMV). HMV contains four RNAs divided between three particles. Plant RNA viruses, in general, are characterized by high level of gene expression, good efficiency to infect cells and spread to different tissues. But the major limitation to use them as vectors is the difficulty of joining RNA molecules in vitro.

Use of cDNA for gene transfer:

Complementary DNA (cDNA) copies of RNA viruses are prepared in vitro. The cDNA so generated can be used as a vector for gene transfer in plants. This approach is tedious and cumbersome. However, some success has been reported. A gene sequence encoding chloramphenicol resistance (enzyme- chloramphenicol acetyltransferase) has been inserted into brome mosaic virus genome. This gene expression, however, has been confined to protoplasts.

Limitations of Viral Vectors in Gene Transfer:

The ultimate objective of gene transfer is to transmit the desired genes to subsequent generations. With virus vectors, this is not possible unless the virus is seed-transmitted. However, in case of vegetatively propagated plants, transmission of desired traits can be done e.g. potatoes. Even in these plants, there is always a risk for the transferred gene to be lost anytime. For the reasons referred above, plant biotechnologists prefer to insert the desired genes of interest into a plant chromosome.