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RESEARCH ARTICLE

GENE TRANSFER IN HIGHER PLANTS FOR THE DEVELOPMENT OF GENETICALLY MODIFIED CROPS (GM CROPS)

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ABSTRACT

Plant transformation using Ti-plasmid of Agrobacterium tumefaciens has become the most powerful method of foreign gene introduction into plant cells and subsequent regeneration of transgenic plants. The genetic engineering of plants actually started with the discovery of a pathogenic bacterium, Agrobacterium tumefaciens, causing a disease known as crown gall (tumor). A. tumefaciens, a rod shaped spore-forming Gram negative pathogenic soil bacterium. The bacterium is considered as natural genetic engineer in higher plants. The property of natural tendency of gene transfer to plants comes from the genes carried by Ti-plasmid (> 200 kbp) of Agrobacterium, which catalyzes the transfer of T-DNA to plant genome naturally. This feature is exploited in plant transformation, where a foreign gene inserted into T-DNA region (~23 kbp) is naturally transferred to plant. Thus bacterial Ti-plasmid is designated as key component in the gene transfer mechanisms. Natural Ti-plasmid is not suitable for gene transfer because of its large size and virulence genes content, which may create disease not desirable in transgenic system. Disarmed Ti-plasmid is used without any virulence genes on itself. Virulence genes are necessary for gene transfer processes but supplied by other helper plasmid. Only the T-DNA region flanking (25 bp direct repeat) with left and right border is transferred into the plant nucleus where it integrate with the genome for expression. Natural T-DNA mainly carries plant hormone genes (auxin and cytokinin) to develop tumoregenic growth (crown gall) by misbalancing the hormonal balance of the plant. T-DNA region also carries some special kinds of metabolizing gene for the synthesis of opines (amino acid derivatives- nopaline/octopine). These compounds serve as sources of carbon and/or nitrogen and energy and also stimulate plasmid conjugative processes as well as virulence genes (vir genes) expression. The genes encoding enzymes for opine catabolism is located outside the T-DNA. Natural Ti-plasmids are used in vector construction are as follows -pTiAch5 (octopine type) and pTiC58 (nopaline type). It was the findings that Agrobacterium can infect only dicots but nowadays routinely used in gene transfer in monocots including cereals crops. A number of plants have been transformed using this Agrobacterium-mediated gene transfer technique. Most prominent vectors such as co-integrate vectors (pGV2260) and binary vectors (pBIN19 and helper vector pAL4404) are used in foreign gene introduction into plant. The recombinant vectors with foreign gene are then used for transformation of plant explants (protoplast, tissues, leaf disks). Co-cultivation is widely used for transformation of dicot as well as monocot species. Ri-plasmid is another type plasmid present in Agrobacterium rhizogenes, which is responsible for root induction in plants and has been used in gene transfer system.

INTRODUCTION

The first evidences indicating that this bacterium as the causative agent of the crown gall goes back to more than ninety years (Smith and Townsend, 1907). The bacterium transfers a set of genes from its plasmid into infected plant cells, where the transferred genes are expressed and produced gall at the junction of root and stem (*i.e*., crown region of the plant). This discovery revolutionized the plant transformation system through which any gene can be introduced into plant genome to change their genetic make-up for commercial benefits, spanning from disease resistance to the production of pharmaceutically important proteins. Plant transformation depends on the stable insertion of transgene(s) into the plant genome. Various methods of gene transfer to plants have been adopted to transform them and many plant species have been successfully transformed. The gene transfer methods in higher plant are mainly grouped into the following categories-

Vector-mediated gene transfer methods

i. *Agrobacterium*-mediated gene transfer
ii. Virus-vector mediated gene transfer
    a) CaMV based vector
    b) Gemini virus based vector

Vectorless or Direct gene transfer methods

i) Physical based technique
   a) Microprojectile Bombardment (Biolistic)
   b) Silicon carbide fibers
   c) Electroporation and Electrophoresis
   d) Microinjection
   e) Sonication
   f) Macroinjection

ii) Chemical based technique
   a) Liposome fusion and lipofection
   b) PEG-mediated DNA delivery

Vector-mediated gene transfer methods

Vector-mediated gene transfer method is achieved either by *Agrobacterium*-mediated gene transfer or by use of plant viruses as transformation vectors. Main focus has been given to the *Agrobacterium*-mediated gene transfer method in the present context.

Natural genetic engineer of the plant

Agrobacterium tumefaciens will transfer genes (T-DNA region of Ti-Plasmid) into the plant that produces its food or nutrient called opines (opines are not needed for plants). Apart from that, it also integrates genes for cell division and proliferation leading to crown gall disease. So that more number of cells results in more production of opines (for bacterial carbon source).

Agrobacterium is valuable in many ways. It is the only prokaryote known that is capable of transfering DNA to eukaryotic cells (Bundock *et al*., 1995). In 1983, the era of plant transformation was initiated when *Agrobacterium*-mediated gene delivery system was used to develop transgenic tobacco (*Nicotiana tabacum*), a dicotyledonous plant (Fraley *et al*,1983).This system has an obvious limitation that its inability to transform monocotyledonous plants. It has been observed in case of corn (*Zea mays* L.) that the secondary metabolite *dimboa* has an inhibitory effect on *Agrobacterium* growth and consequently not allowing to infect corn, a monocots (Sahi *et al*, 1990). More recently, so many monocotyledonous cereal crops such as rice, wheat have been transformed using *Agrobacterium*-mediated gene delivery system. Now, it is possible to transform a wide range of plants for desire agronomic importance. Among the dicots, soybean, cotton have been transformed with genes coding for herbicide resistance, insect resistance, viral protection, improved seed storage proteins and increased starch content.
Agrobacterium tumefaciens is a soil-borne, Gram negative, sporulating, phytopathogenic bacterium. It is rod shaped, motile and belongs to the bacterial family Rhizobiaceae, present in the rhizosphere around the roots. There are three species of *Agrobacterium*-

Agrobacterium tumefaciens

Causes crown gall disease in dicotyledonous plants and gymnosperms. Strains are as such - Ach5, A6, B6, C58, T37, Bo542 and B023955.

Agrobacterium rhizogenes

*C*auses hairy root disease; strains are as such- NCPPB1855, ATCC23834 and A4.

Agrobacterium radiobacter

It is an avirulent strain, can be used as biocontrol agent to control crown gall disease.

Reason for Crown gall and hairy root disease

Agrobacterium tumefaciens is a causative agent of *"Crown-gall"* disease, infecting a wide range of broad leaved dicotyledonous plants that was identified by Erwin F. Smith and Townsend of the U.S. Department of Agriculture (USDA) in the year 1907. It causes considerable damage to perennial crops notably in grapes, peach, walnuts, apples, roses, ornamental plants, other flowers and fruit trees. Crown gall was the first tumor in any organism, for which the causative agent was identified before the discovery of 'ROUS' sarcoma virus by several years. The ability to cause *'crown-gall'* (tumor growth) depends on the transfer ability of tumor inducing genes from bacterium into the plant genome. It has been demonstrated that the infective process is a natural forms of genetic engineering to develop disease on host plant by transferring bacterial genes into the plant genome. This natural process of gene transfer of *Agrobacterium* has been used widely as a major tool to manipulate the plant genome by which a new gene can be put into plants for expressing thereon to give rise a new variety of the plant with desired traits.

In the 1980s, Jeff Schell and Mare Van Montagu and their groups at the State University of Ghent, had shown that a large size of plasmid present in all virulent but not in avirulent strains of *A. tumefaciens*, which has clearly shown that the plasmid is responsible for tumor formation. This plasmid can be transferred to avirulent strain through conjugation. Virulence is lost when the bacteria are cured of plasmid. Both the virulent strains of the bacteria, *A. tumefaciens* and *A. rhizogenes* carry the characteristics large megaplasmids. The plasmid found in *A. tumefaciens* is named as Ti-plasmid (***T***umor ***i***nducing plasmid), which is responsible for the development of *'crown-gall'* tumors on appropriate host plants. *A. rhizogenes* strain carrying Ri-plasmid (***R***oot ***i***nducing-plasmid) is the causative agent of hairy root disease. Ri-plasmid can also be used as a gene transfer vectors to develop healthy genetically modified plants. *Agrobacterium rhizogenes* is a Gram negative soil bacterium that incites hairy root disease in dicots. A plasmid called Ri- plasmid is responsible for this disease. Ri-plasmids are analogous to *A. tumefaciens* Ti-plasmid. Ti-plasmid is considered one of the most successful gene delivery vector systems in higher plants.
Ri-plasmid organization: It is large in size approximately 250 kbp, contains two T-DNA fragments Tr-DNA and Tl-DNA (left and right) separated by 15 kb segment. The Tr-DNA is similar to T-DNA. It has genes for auxin and agropine synthesis. The Tl-DNA has four genes- rolA (for hairy root formation), rolB (induces root initiation and callus formation), rolC (promotes root growth) and rolD (suppresses callus growth) (rol means = root loci). The Ri-plasmid has a

virulence genes region that mediate T-DNA transfer and an ori same as Ti-plasmid. It is used in gene transfer vector construction in higher plants. They can be used to induce rooting in clones where rooting from callus is difficult.

Crown-gall disease

Agrobacterium tumefaciens is the causative agent of crown gall disease in dicotyledonous plants and few gymnosperm. The crown gall is characterized by unlimited plant cell proliferation (forming gall or tumor) near the junction of root and stem (crown part). *A. tumefaciens* generally present in the rhizosphere, the region around the root/stem junction of plants. They normally survive on nutrients generally released from the roots of the nearby host plants, and can not infect the plants. They only can infect the plants if there is any kind of wound or damage occurs near the rhizospheric region of the plant and cause disease symptoms. The damaged or wounded part of the plant releases some kind of chemicals (sugar and phenolic compounds) which acts as chemotaxis compound by which the bacteria are attracted towards the wound region and attached with the wounded cells. *A. tumefaciens* has the potentiality to transfer a particular DNA segment of the Ti-plasmid (which is termed as Transfer DNA or T-DNA) into the nucleus of the recipient host cells where the T-DNA is stably integrated into the plant genome and expressed efficiently, causing the crown gall disease (Chilton *et al*, 1977; Nester *et al.,* 1984). The expression of T-DNA genes in side the host cell leads to the transformed phenotype. They are expressing the tumor inducing genes which encodes the enzymes responsible for the synthesis of phytohormones (*i.e.* auxin and cytokinin) and the enzymes for the biosynthesis of novel plant metabolites. The novel metabolites are the opines and agropines. The opines are amino acid derivatives and produced by transformed cells only. In 1960s, Georges M. Morel and his groups, at the Institute National de la Recherche Agronomique in Versailles, observed that crown gall cells synthesize a class of novel chemical compounds which are normally not produced by the normal cells of the same plant, are named as opines. Opines are two types, octopines and nopaline both are derivatives of amino acids. Octopines are derived from the amino acid arginine and pyruvic acid, nopaline is a combining product of arginine and α-ketoglutaric acid. Bacteria produce either octopine or nopaline not both by a single bacterium. A third category of opines also synthesized by some strain of *Agrobacterium species*, referred to as agropines, which are sugar derivatives. Agropines and mannopine metabolizing genes are also carried by *Ri*-plasmid. Both the opines (octopine and nopalines) and agropines are synthesized and excreted by the crown gall cells and consumed by *Agrobacterium* as the sole source of carbon and energy. The plant hormones (auxin and cytokinin) produced by the genes carried by the T-DNA causes the plant cells to proliferate and form characteristics gall (tumor). The novel compounds nopaline, octopine or agropines are transported into the bacterial cells and catabolized by the enzymes produced by the genes present on the **Ti**-plasmid (or ***Ri***-plasmid) which has not been transferred to the plant cells. So, crown gall formation is the consequence of the transfer, integration and expression of genes of T-DNA (of Ti-plasmid) of *A. tumefaciens* in the infected plant. This gall formation is interfere with the normal growth of the plants and thus referred to as plant disease and which cause considerable damage to the host plants.

Opines and Genetic Colonization

The Ti-plasmid of *Agrobacterium sp*. has been classified into three classes on the basis of opines they are synthesized and catabolized. These are as follows i) octopine ii) nopaline and iii) agropine. The most common opine are being octopine, nopaline, (amino acid derivatives); or mannopine, agropine, succinamopine and leucinopine (sugar derivatives). The octopine is a carboxy-ethyl derivatives of arginine where as nopaline is a dicarboxypropyl derivatives of arginine. The enzymes octopine synthase (ocs) and nopaline synthase (nos) are encoded by the genes which are present in the T-DNA and transferrd into the plant genone for the synthesis of octopine and nopaline respectively. The opines are accummulated in the transformed plant cells but can not be metobolized by them. Instead, they are secreted out from the transformed plant cells and accumulated in the soil at the transition zone from root to stem (crown) in the rhizosphere. These opines are useful to the bacterium since they serve as a sole source of carbon and nitrogen. Only the rhizospheric *Agrobacterium* can take them up and use them as nitrogen, carbon and energy source. The enzymes required for catabolism of these opines, are synthesized by the Ti-plasmid of the respective bacterium. In addition of the energy source, opines can induce the conjugal transfer of Ti-plasmid between the bacteria. The presence of such genes responsible for opine synthesis and opine catabolism selectively favors the growth of *Agrobacteria* to utilize these opines and thus providing a competitive advantage near the crown region of the rhizosphere over other soil borne microorganisms and they established a favored ecological niche. The establishment of such favored ecological niche only by the *Agrobacterium*, is referred to as genetic colonization, because it starts with the transfer of the genes of the T-DNA segment of the Ti-plasmid into the recipient plant genome. It is maintained stably untill and unless spontaneous reversion occurred. It has been found through the molecular analysis that methylation of the cytosine residues of the T-DNA genes or T-DNA deletion can be responsible for the reverted phenotype. The methylation mechanism playing an important role in transgene silencing in host plant, which is important for the application of plant genetic engineering specifically to the plant breeding.

Structural features of Ti-plasmid

The Ti-plasmid of *Agrobacterium* is a autoreplicating, large double stranded circular DNA molecule of 200 kb in size, which is about 3-8% of the *Agrobacterium* chromosome. Different strains of *Agrobacterium* have different types of Ti-plasmid (octopine, nopaline and agropine types) but containing some common structural features which are as follows

- It has own origin of replication *i.e.* autoreplicating genetic element
- A genetic element responsible for conjugative transfer (*tra*-region)
- Opine synthesizing gene
- Opine catabolizing gene

- Contains T-DNA (Transfer-DNA) region, one or more than one to transfer into the plant genome which are flanked by left and right border
- A virulence region (*vir*-genes)

Among the above genetic components, two are more important, which are required for gene transfer in plants, the virulence (*vir*) region, and T-DNA region. Beside these a set of gene products encoded by bacterial chromosomal genes (chvA, chvB, chvE, pscA, attR genes) are also required for T-DNA transfer into plant cells.

Main structural features of T-DNA

Nopaline T-DNA

The T-DNA of nopaline type Ti-plasmid (pTiC58) is a single continuous segment of DNA of about 22 kb long. The T-DNA has a specific boundary known as border, at both the ends, referred to as left border (LB) and right border (RB), which are 25bp imperfect direct repeats (Zambryski *et al*, 1982). The border sequences of nopaline T-DNA is as follows- left border 5′ TGGCAGGATATATTGTGGTGTAAAC 3′, right border 5′ TGACAGGATATATGGCGGGTTAAAC3′. The border sequences act as a *cis*-element signal for the transfer apparatus. Any foreign DNA is placed between the borders are transferred into the plant cell. Through the investigation it has been demonstrated that this portion of the Ti-plasmid is integrated into the nuclear genome of the plant cell, and subsequently termed as 'transferred DNA' (T-DNA), which is responsible for tumour induction in plant.

Octopine T-DNA

The T-DNA of octopine type Ti-plasmid is divided into two different segments and designated as transferred left T-DNA segment (T_L-transfer left) and transferred right T-DNA (T_R-transfer right). The T_L is of 14 kb in size containing eight ORFs and T_R segment is of 7-kb in size. Each of the T-DNA is flanked by separate 25bp border sequences as found in the nopaline T-DNA. The T_R and T_L can independently be transferred and integrated into the plant nuclear genome due to the presence of these boundary sequences. Left border of T_L-T-DNA is as follows 5′ CGGCAGGATATATTCAATTGTAAAT 3′ and right border 5′ TGGCAGGATATATACCGTTGTAATT 3′. The T_L DNA carries the tumor inducing function (oncogenes) but T_R-DNA contains opine synthesizing genes. T-DNA of octopine Ti-plasmid contains an overdrive or enhancer sequence outside of the T-DNA but close to right border required for optimal T-DNA transfer. (*e.g*. octopine plasmid; pTiAch5). The T_L T-DNA is similar to that of T-DNA of nopaline Ti-plasmid. It is a uniqueness of the T-DNA genes that although carried by the bacterium but only express in plant cells.

Eukaryotic features of T-DNA

T-DNA genes are as like as the eukaryotic genes. Some molecular evidence support this features. The α-amanitin inhibits the transcription of T-DNA genes, suggesting that T-DNA genes are transcribed by RNA polymerase II, which is available in the eukaryotic nucleus. Eukaryotic structure of the T-DNA genes later have been confirmed by S1 mapping to know the mRNA initiation and termination sites. T-DNA genes show structurally common features with the eukaryotic genes. As like as the eukaryotic genes, a TATA box is present at –30 promoter region in the initiation site, which is a eukaryotic characteristics, whereas in prokaryotic gene this consensus sequences are located in the -10 and -35 regions. There is also another conserved sequence present in the T-DNA genes at –80 region, which is known as CAAT box, an important feature of eukaryotic promoters, which is involved in correct initiation of mRNA transcription. Besides these TATA and CAAT boxes, there are one specific region present on the *nos* promoter at -100 to -170 spanning some direct repeat as well as some inverted repeat sequences. Like a eukaryotic gene, the 3′ end of the mRNA of *nos* gene contains an untranslated region containing one or more consensus polyadenylation signal sequence AATAAA, which is also a feature of eukaryotic genes. The T-DNA genes do not contain introns just like a many other eukaryotic genes.

Tumor inducing oncogenes

T-DNA contains two categories of genes, the oncogenic genes and the opine synthesizing genes. Oncogenic gene consisting of auxin and cytokinin synthesizing genes along with the tumor size determining gene.

Table 1

Genes in T-DNA	Function of the genes
iaaM (aux1, tms1)	Auxin biosynthesis- codes for enzyme tryptophan-2-monooxygenase that converts tryptophan to indole-3-acetamide (IAM).
iaaH (aux2, tms2)	Synthesize indole-3-acetamide hydrolase, that converts IAM to indole-3-acetic acid (auxin biosynthesis).
Ipt	Cytokinin biosynthesis- synthesize enzyme isopentenyl transferase that catalyzes formation of isopentenyl adenine.
Nos	Produce enzyme nopaline synthase that form nopaline from pyruvic acid and arginine.
25 bp left and right border of direct repeats	It is site of endonuclease recognition during T-DNA transfer, and it is mandatory for gene transfer to plant cell.

The oncogenic region of transferred DNA includes the two auxin synthesizing genes namely ***tms1***: tryptophan mono-oxygenase, or iaaM; ***tms2***: indole-3-acetamide hydrolase or iaaH and one cytokinin synthesizing gene, ***tmr***: isopentenyl transferase (*ipt* or *cyt* gene); and one ***tm1*** gene to determine the tumor size. Two genes *tms1* and *tms2* are responsible for the synthesis of the phytohormone indole-3-acetic acid (auxin) from amino acid, tryptophan. The *tmr* gene encodes an enzyme isopentyl transferase (*ipt*), which catalyzes the formation of cytokinin, isopentyl adenosine-5′-monophosphate from isopentenyl pyrophosphate. The *tms1* (auxA, also known as iaaM) encodees tryptophan mono-oxygenase and *tms2* (auxB or iaa H) encodes indole acetamide-hydrolase, responsible for the biosynthesis of plant hormone auxin (IAA). The enzyme tryptophan mono-oxygenase converts tryptophan to indole-3-acetamide, which is again converted to indole-3- acetic acid (auxin) by indole-3-acetamide hydrolase encoded by *tms2* gene. Apart from the genes for auxin synthesis, T-DNA also contains gene necessary for cytokinin synthesis. The *tmr* (also known as *cyt* or *ipt*) isopentenyl transferase that catalyzes the most essential step in cytokinin production. Both the phytohormones auxin

and cytokinin induces the tumor formation *i.e.* crown gall in plants and hence designated as 'oncogenes'.

Opine synthesizing genes

The T-DNA contains either nopaline synthase (*nos*) for the synthesis of nopaline or octopine synthase (*ocs*) for the synthesis of octopine. The *ocs* gene generally carried by T_R-T-DNA of the octopine Ti-plasmid. The above two genes are responsible for the production of opines which are secreted outside of the plant cells and used as sole source of energy (carbon and nitrogen source) by the *Agrobacterium*. Surprisingly no other soil bacteria have the property to utilize this opines as energy source. They synthesize either octopine or nopaline not both at a time by an individual Ti-plasmid containing bacterium. The T_R T-DNA of octopine Ti-plasmid, encodes the enzymes responsible for the production of other type of opines such as mannopine, agropine and fructopine. The Ri-plasmid can also carried the genes for the synthesis of agropine and mannopines.

Opine catabolyzing and tra genes

The opine catabolising genes *noc* (***no***paline ***c***atabolising gene) and *occ* (***oc***topine ***c***atabolising gene) are located in the Ti-plasmid but outside of the T-DNA and these are responsible for the catabolism of nopaline and octopine respectively. The *tra* gene responsible for conjugative transfer of the Ti-plasmid to avirulent *Agrobacterium* strains present near theopine catabolizing gene. Ti-plasmid also contains the genes responsible for its own integrity and stability.

The virulence genes and their induction

T-DNA itself has no ability to transfer them from bacterial cell to plant cell but a set of genes responsible for this T-DNA transfer present on the outside of the T-DNA region, generally termed as virulence genes (*vir*-genes). The *vir*-genes located in a 40 kb size of the Ti/Ri-plasmid. The vir-region consists of about 25 genes organized in nine operons, namely virA, virB, virC, virD, virE, virG, virF, virJ and virH. They are co-regulated and thus form a regulon. The vir-genes are coding for functions responsible for plant cell recognition, bacterial attachment, excision, transfer and possibly the integration of T-DNA into the targeted plant genome (see table 1). Acetosyringone (AS) and α-hydroxysyringone (OH-AS) induces the *virA gene*. Sinapinic acid is a lignin precursor can also induce *vir-genes* (lignin is a cell wall components of the vascular plants). But the analogues of these compounds lacking the methoxy or hydroxyl groups cannot induce the vir-genes. Normal leaf tissues produce both the components AS and OH-AS in a lesser quantity but in the wounded leaf tissues they produce at least ten-fold higher amounts than the intact tissues. They exudates proportionately in more amounts in the outside than in the leaf tissue itself.

It has been reported by Ashby *et al* (1987) that *Agrobacteria* are positively attracted and showing chemotactic response to AS at an optimum concentration of 10^{-7} M. The most monocots are not producing AS at a concentration sufficient to induce *vir*-gene expressions otherwise they would have been also infected with the *Agrobacterium sp*. The host range of *Agrobacterium* may be artificially extended to the monocotyledonous plant also by adding AS to the *Agrobacterium*-monocot cell *co-cultivation* inoculation method and it has been achieved. The natural physiological role of these compounds (AS, OH-AS, sinapinic acid, flavonoids *etc*) may be associated with wound healing and also inhibit the growth of invading pathogens as a phytoalexins. Some of the flavonoids inhibit the growth of the some pathogens and act as a phytoalexins (Darvill and Albershein, 1984). Some signaling compounds are related to lignin, required for repairing the wounded cells. Both the *vir-genes*, virA and virG are expressed constitutively but the virG gene can be induced by AS to higher levels of expression. The reason is that un-induced virG gene transcribes a shorter mRNA in compare to induced mRNA transcript because the constitutively transcribed mRNA is without Shine-Dalgarno

Table 2 The virulence genes (*vir*-genes) of *Agrobacterium sp* and their role in T-DNA transfer.

Vir genes	Functional role	Nature of the gene
Vir A (2.5 kb)	Transmembrane phenolic chemosensor kinase detect the small phenolic compounds released by wounded plant cells, after sensing, the vir-A protein autophosphorylate itself and consequently phosphorylates virG-protein.	Monocistronic, expressed constitutively
VirG (1.2 kb)	VirG is a transcription factor, activates all other vir-genes	Monocistronic, inducible (also low level constitutive)
VirJ	T-DNA export	-
VirD (4.5kb)	Regulate the virD2 activity and essential for T-DNA processing	Polycistronic (4-cistron), inducible
VirD1	Recognize the 25bp border sequences with the help of virD1 and make a single stranded nick in the bottom strand of each border and remains tightly associated with the 5′end of the ssT-DNA	
Vir D2	to protect them from nucleases. Nuclear localization signal is present on the virD2 protein for targeting the T-DNA into the nucleus. Interact with plant protein such as importin and cyclophilins during the ssT-DNA transfer.	
VirF(23 kDa)	Helps in T-DNA integration into the plant genome, by regulating the cell cycle, prolonged S-phase	Monocistronic, inducible.
VirE	VirE1, acts as a chaperone of virE2 protein, required to maintain its structure and function.	Polycistronic (2-cistrons), inducible
VirE1 (2kb)	Single stranded DNA binding protein, protect ssT-DNA from nuclease degradation, it also carries NLS, for nuclear targeting of the ssT-DNA and involve in passage through nuclear pore complex (NPC). Interacts with plant protein VIP1 and VIP2, to facilitate the T-DNA to move	
VirE2	near the active chromatin region for integration.	
VirC1 (2kb)	Recognize the overdrive sequence of the Ti-plasmid and enhances the efficiency of ssT-DNA transfer.	Polycistronic (2-cistrons), inducible.
VirB1-B11(9.5 kb)	Make a transfer complex apparatus at the junction of bacterial and plant cell surface for transfer of T-DNA.	Polycistronic (11-cistrons), inducible.
VirH(virH1 & H2)	Detoxification of certain plant compounds	Polycistronic, (2-cistrons), inducible

sequences, which is essential for efficient translation. The feature can be compared with the CI gene regulation by pRE and pRM in the bacteriophage lambda (λ-Phage), and considered as a reminiscent of the same. So, *virA* is expressed constitutively and probably functions as a chemoreceptor which senses the availability of the phenolic signaling molecules *i.e.* AS, OH-AS and transmits this signal to the bacterium by phosphorylating the virG protein, which is a transcription factor, activates the transcription by binding to the operator sequences within the promoter region of the inducible *vir-operons* (all *vir-genes*). Inducible *vir-genes* do not have –35 consensus sequences in the promoter but they carry a hexanucleotide sequences common to the entire region at –35 and act as *cis*-acting regulatory sequences for the *vir*-gene induction.

The mechanism of T-DNA mobilization and crown gall tumourigenesis

Three genetic components are essential to transfer the T-DNA from virulent *Agrobacterium* strain to the plant cells.

Vir region

At least 25 genes are co-ordinately regulated in this vir-region, so it is so complex. In the absence of any induction, virA and virG genes are expressed constitutively at a low level. Induction of the vir-genes are prerequisite to transfer the T-DNA into the plant genome. Vir-genes can be induced by a variety of plant phenolic compounds, plant monosaccharide and can be enhanced by some other factor such as opines, flavonoid compounds. Induction also depends on the low temperature (below 28°C) and acidic pH.

The T-DNA itself

The *Ti* or *Ri* plasmid possesses a DNA segment, which will be transferred to the plant cell during the formation of crown gall, and is termed as T-DNA (Transferred DNA). T-DNA only contains the genes responsible for oncogenicity and octopine synthesis (*oc*), no genes for T-DNA excision and transfer. Only the *cis*-acting two conserved elements are required for T-DNA transfer, these are the 25bp direct repeat border sequences at both ends of the T-DNA. An overdrive sequence (enhancer sequence) is only located outside the T-DNA close to the right border of the octopine Ti-plasmid not in nopaline Ti-plasmid, which acts as a transformation enhancer.

Chromosomal virulence genes (chv)

The third genetic component is required for T-DNA transfer consists of a set of genes located on the bacterial chromosome. They are necessary for the bacterial colonization and in the attachment of *A. tumefaciens* to the plant cell surface. The bacteria move towards the wound sites of the plant through chemotactic movements. The co-induction of the *vir-genes* can also be obtained by monosaccharide. The genes *chv*A, *chv*B, *chv*E, pscA and attR, are present on the bacterial chromosome which are necessary for T-DNA transfer, and tumourigenesis, and subsequently referred to as ***ch***romosomal ***v***irulence genes (*chv gene*). Genes *chv*A and *chv*B are involved in the synthesis of cyclic β-1,2 glycans and excreted out from the bacterial cells, which is required for attachment of the bacterium to the plant cells. The gene *chv*E, is involved in the synthesis of glucose/galactose transporter for the enhancement of the sugar concentration, which subsequently induces the *vir* genes. The *psc*A (*exo*C) gene is responsible for the synthesis of polysaccharide but only induces in the presence of succinoglycan which is synthesized by the gene *att*R, and ultimately produces polysaccharide that facilitates the initial attachment between bacteria and plant cells. Subsequently the *Cel-gene* produces cellulose fibril, which forms a mesh like structure at the attachment site. It has been demonstrated that the mutation in the *chv*A or *chv*B genes convert the *Agrobacterium* into avirulent strain. The mechanism of transfer seems to be essentially identical for both *Ti* and *Ri* plasmid derived T-DNAs. The process of T-DNA transfer and integration into the plant genome is illustrated diagramatically in and which has been described in the following stages:

Contacts between bacteria and plant cells

Attachment to plant cells

Wounded plant cells release some kinds of chemical compounds to heal the wound region and also release some other types of compounds to resist pathogenic attack as a defense strategy, these are the derivatives of phenolic compounds such as acetosyringone, vanillin, ethylferulate, sinapinic acid and some sugar compounds (D-mannose, D-galactose). They act as a chemo-attractants to attract the *Agrobacterium* cells near the wounded region (*i.e.,* rhizosphere). *Agrobacteria* are attracted to the wound sites of the plant by sensoring these signal molecules, such as phenolics, sugars that are released by the wounded plant cells. These molecules are actually released as a defense strategies for the synthesis of phytoallexins and lignins, to protect the plant from being pathogenic attack. And also acts as a signaling molecule to attract the *Agrobacterium*. Attracted bacteria multiply in the wound sap and attach to the walls of plant cells. Monosaccharides are also effective chemoattractants for *Agrobacteria* and responsible for the long distance attraction of virulent and non-virulent strains of *Agrobacterium* to the rhizosphere of wounded plants but phenolic compounds such as acetosyringone acts as specific chemoattractants for virulent *Agrobacterium* strain only. The product of virA and virG genes are also involved in this chemotaxis mechanism, these two proteins regulate the signal transduction mechanism to make a close contact between plant cells and bacterial cells. The product of virA gene is a transmembrane sensor protein kinase recognizes the signal of phenolic molecules which in turn autophosphorylates itself. Signal molecule (*e.g.,* acetosyringone) after binding with the vir-A protein, stimulates autophosphorylation of its own at a histidine residue in the cytoplasmic C-terminal domain. Phosphorylated virA protein then can able to phosphorylate the virG gene product and activates it, which is a transcriptional factor, recognizes the *vir-box* region in all the *vir-genes* and induces all the inducible *vir*-genes.

Agrobacterium tumefaciens in the soil swims towards wounded plant cells in a positive chemotactic response to acetosyringone (AS), a signaling molecule released by wounded plant cells. Bacterial cells attach to the plant cells at

this region by producing polysaccharide by the chromosomal *att*R gene initially and then made cellulosic fibrillar mesh by the product of bacterial chromosomal gene *Cel*, by which bacteria can clump together and firmly anchor to the plant cells. Subsequently, it has been observed by genetic analysis that two constitutively expressing neighboring genes *chv*A (1.5 kb) and *chv*B (5 kb) are responsible for bacterial attachment to the plant cells, which are present on bacterial chromosome. Mutation in these two chromosomal virulence genes (*chv*A and *chv*B) can only make defective attachment. The *chv*A and *chv*B genes are responsible for the production and secretion of β-1, 2 glucan, mutant in the two genes, fail to attach the bacterial cell to the plant cells because bacteria do not synthesize and secrete this glucan. It is observed that monocots are not generally susceptible to infection by *Agrobacterium tumefaciens* because of failure of attachment to the plant cells. So, constitutively expressed products of several chromosomal genes (*chv*A, *chv*B, *chv*E, *psc*A and *att*R) in *Agrobacterium sp* are responsible for their attachment to the plant cells and the specific attachment of *Agrobacterium* to plant cells is a prerequisite for subsequent transfer of T-DNA of *Ti/Ri* plasmid. Chromosomal virulence genes are concerned with the synthesis and secretion of glucans, cellulose fibrils and cell surface proteins and plays a vital role in bacterial attachment to the plant cell surface.

Induction of bacterial virulence genes

Genes responsible for the transfer of T-DNA are clustered in a region referred to as virulence region (*vir*-region) of about 30-40 kb in size outside the T-DNA in the *Ti*-plasmid. Vir-region is consisting of at least six essential operons (*vir*A, *vir*B, *vir*C, *vir*D, *vir*E, *vir*G) and three non-essential operons (*vir*F, *vir*H, *vir*J). The *vir*A and *vir*G genes are expressed constitutively and they are monocistronic, all other *vir*-genes are inducible and polycistronics. The virA, virG have only one gene, virE, virC, virH have two genes and virD and virB have four and eleven genes respectively. The virA gene is expressed constitutively under all conditions. The virG gene is expressed at low levels in vegetative cells but is rapidly induced to higher expression levels by exudates from wounded plant cells. The vir-genes are exclusively plant inducible and can be induced by the small phenolic compounds like acetosyringone, released by wounded plant tissue, and induces the vir-genes. It has been demonstrated that the virA and virG gene products regulate the expression of the other vir-genes. The virA is transmembrane dimeric sensor protein kinase that detects signal molecules, mainly small phenolic compounds, such as acetosyringone, released from wounded plant cells. The signal molecules may be other phenolic compounds, or acidic pH condition, and certain class of monosaccharide which acts synergistically with phenolic compounds. After receiving signal, *vir*A protein autophosphorylates and subsequently phosphorylate virG protein by transferring its phosphate to a conserved aspartate amino residue of the cytoplasmic DNA binding protein *vir*G and activate them. Phosphorylated active *vir*G acts as a transcriptional factor and induces the expression of all *vir*-genes (including virA, virG). This transcriptional factor finds to the operator region of the *vir*-genes and induces their expression. The C-terminal part is responsible for the DNA binding activity while the N-terminal part acts as a phosphorylating domain. Certain monosaccharide particularly, glucose, galactose, xylose enhances *vir*-genes induction. The induction by this system is only possible if the periplasmic sugar (glucose/galactose) transporter protein *chv*E is expressed to import the sugar inside the bacterial cells and which in turn interacts with virA to induce the other vir-genes. Temperature and pH condition may play important role in vir-gene induction. At temperature greater than 32°C, the vir-genes are not expressed because of a conformational change occurred in the folding of virA protein which inactivate its functional properties. The expression of these vir-genes are tightly regulated and only expressed when the *Agrobacterium* infect a plant. Wound sap induces the vir-genes and most effective inducers are monocyclic phenolic compounds such as acetosyringone. The virA and virG form a two component regulatory system activating the transcription of the other vir-genes. Virulence inducing signals are first recognized by virA protein, which then activates virG by phosphorylation. The virG is a transcriptional factor which up regulates the expression of all the virulence genes virB, virC, virD, virE, virF, virH, virJ including virA and virG gene.

Production of ssT-DNA transfer complex and its transportation

As soon as the *vir*-genes are expressed and produced the respective *vir*-proteins they start to generate a single stranded transfer DNA (ssT-DNA) molecule to transfer it to the plant cell. This ssT-DNA molecule represents the copy of the bottom strand of the T-DNA. The proteins virD1 and virD2 encoded by the *vir*D locus starts the T-DNA excision by recognizing the T-DNA border sequences and nicking (endonuclease activity) the bottom strand at each border. The virD2 protein has the specificity to cut within the conserved 25 bp T-DNA borders, the origin of transfer (oriT) in association with virD1 protein(Wang *et al*,1984; 1990). VirD2 protein recognizes these border repeats (LB and RB) and makes single stranded nicks between the third and fourth bases in the bottom strand of the T-DNA from the 3′ end, with the help of virD1, virC, and also virD3 proteins. The VirD1 protein has topoisomerase activity and assisting the endonuclease cleavage in this T-DNA excision mechanism, that helps the virD2 to cleave the supercoiled double stranded T-DNA. Then the both ended nicked bottom strand is displaced by synthesizing a new DNA strand in the 5′ to 3′ direction using upper T-DNA strand as a template through rolling circle mechanism of DNA replication. DNA repair mechanism is also involved in this ssT-DNA formation. The strand displacement is controlled by the helicase activity, which is a inherent property of virD2 protein. The newly synthesized DNA strand is remained as a bottom strand with the Ti-plasmid. The displaced single-stranded T-DNA (ss-T-DNA or T-strand) is remains covalently attached with virD2 protein at the 5′ end only and the T-strand is coated with virE2 protein, which protect the T-strand from being attack of exo-endonucleases and also facilitates export to the plant cells. VirB loci (11 cistrons) produce various proteins, which are involved in the formation of transfer membrane channel in association with other proteins like virD4. The single–stranded T-DNA (ssT-DNA) associated with virD2 and virE2 form a transfer complex and is exported to the plant cell through this transfer channel like a conjugal transfer. During this transfer virF proteins also transferred to the plant cells. The ssT-DNA molecule is also designated as T-strands

(transfer strand of T-DNA), which is an intermediate molecule in the transfer mechanism of the T-DNA. In addition to that the 5′ end acts as a leading end of the T-DNA transfer complex. It has been observed, when any mutation or deletion occurs at the right border (RB) of the T-DNA, there was completely loss of T-DNA transfer capacity. Mutation or deletion in the left border results in lowering the transfer efficiency but can not abolished the transfer process. It is proving that T-strand synthesis is started at the right border, which proceeds in the 5′ to 3′ direction, reason may be the presence of specific ***enhancer sequence*** sometimes called as ***'overdrive sequence***'. It is consisting of 24bp long DNA sequences (5′TAAGTCGCTGTGTATGTTTGTTTG 3′) and is located next to the right border outside of the T-DNA region. The *overdrive sequence* is recognized specifically by virC1 protein and directs the movement in a 5′ to 3′ direction initiating from the nicked end in the right border, not transferring itself into the plant cell. The 25 bp border repeat acts as a *cis*-acting element in the T-DNA transfer process and its polarity is maintained due to the presence of this overdrive sequence outside of the T-DNA but close to the right border. VirE2 proteins then coat the ssT-strand to make the transfer complex (T-complex). Virtually any DNA sequence flanked by such border repeats can be transferred to plant cell by the virulence system. The virE2 protein is a large single stranded DNA-binding protein and may stabilize the T-strand during transport to the plant nucleus.

The role of T-DNA borders in T-DNA transfer

The T-DNA of *Ti/Ri* plasmids is flanked by imperfect direct repeats of 25 bp and designated as the left border (LB) and right border (RB). T-DNA transfer process is prevented if the RB is deleted but not the left border (LB). It is also observed that the 25 bp right border alone can promote the T-DNA transfer in one direction only (5′ to 3′ direction of the T-strand), without any size restriction (upto 50 kb of DNA). These findings allow the researchers to modify *Agrobacterium* Ti-plasmid to be used as transfer vehicle to transform the plant cells. The right border nick provides the initiation of DNA replication whereas the left border nick gives the signal for termination of the replication and also transfer the same. If the left border is deleted, termination process will occur but at a distal site. In principle, as both the LB and RB contain the similar 25 bp repeat sequences and can function in similar way and can initiate transfer of *T*-DNA to the left of its, as a result there will be no T-DNA transfer (with foreign gene) into the plant genome, consequently there was no tumourigenesis. Instead of the left border, the right border is preferred site for initiation of T-DNA transfer as because of the presence of overdrive/enhancer sequence adjacent to the RB of the Ti-plasmid outside of the T-DNA and enhances the transfer efficiency. The 'overdrive sequence' is recognized by the virC1 protein and enhances its ability and polarity of the T-DNA transfer. Some genetic experiments have proved that these 25 bp repeat sequences, particularly those on the right border of the T-DNA, are absolutely required for T-DNA transfer and functions in *cis*-condition and directs the polarity of the T-DNA transfer. It is also evident that any DNA sequence can be transferred to plant cells if it is flanked by the 25 bp left and right borders, transported in a correct orientation (Rubin, 1986). The transferred DNA into the plant genome is identified by the presence of these 25 bp repeat sequences (partially or fully).

The T-DNA transfer i.e., ssT-DNA complex targeted to the nucleus

There is a controversy about the intermediate molecular structure of the transferring ssT-DNA, in which intermediate forms it will be transferred into the plant cells. That intermediate forms may be any one of the following structures-

i. Single-stranded T-DNA molecules (*i.e.* T-strand)
ii. Double-stranded T-DNA intermediate or
iii. Covalently closed circular T-DNA molecule

The most accepted one is the single-stranded T-DNA (ssT-DNA), which makes a complex with proteins. VirD2 proteins are remained covalently attached to the 5′ end of the ssT-DNA and protects it from exonucleolytic degradation. In addition, the virD2 acts as a capping protein to determine the polarity and also guides the T-strand mobilization towards the plant nucleus, during plant cell transformation. This ssT-DNA-virD2-complex is coated by the 69kDa virE2 proteins (600 molecules per 20kb T-DNA). The virE2 is the single stranded DNA-binding proteins, which protects the ssT-DNA from being nuclease attack and reduces the complex diameter of the ssT-DNA-virD2-complex at least 2nm, maintaining the structure somewhat in a linear fashion and make it easier to pass through membrane channels. The virE1 protein is essential for the export of virE2 to the plant cell, and it acts as a chaperon of virE2 proteins, to give proper conformation to the virE2 protein. From the various genetic analysis, it has been suggested that the transfer complex is a single strand DNA covalently bound at its 5′ end with virD2 and not coated by virE2. virE2 exported independently to the plant cell and there it is interacted with the transported T-DNA-virD2 complex.

The virB and virD4 proteins are responsible for the formation of a membrane channel (*i.e.* T-pilus, or conjugal pore) these are the type IV secretion system between the bacterium and the plant cell(Zupan *et al*., 2000). The virE2, virD2 and virF are transported out from the bacterial cells along with other proteins involved in this mechanism (Zupan *et al*, 1995;1996). The ssT-DNA protein complex travels through the T-pilus, and reached into the cytoplasm of plant cells with the help of proteins encoded by virB and virD4 operon. Some of the proteins of the membrane channel acts as a ATPase activity, probably the B11 protein, which provides the essential energy required for the passage of the T-strand by its autophosphorylating property. Although virD4 is a transmembrane protein but predominantly present at the cytoplasmic side of the cell membrane. Some of the virB proteins are assembled as a membrane spanning channel covering both the membranes, but other virB proteins may be extra cellular. The virB1 protein is always present extracellularly on surface and virB2 is with the extracellular functions. VirB4 and virB11 are with the ATpase activity, they provide necessary energy for active T-DNA transfer. VirB3 has the likely functional activity with that of virB4, whereas virB7 provides the correct conformation to the T-

DNA complex during transfer. VirB9 is unstable, but stable when makes heterodimer with virB7 through disulfide bridges. The virB7-virB9 heterodimer is assumed to stabilize other *vir* proteins during the formation of functional transmembrane channels. VirB1 is a transglycosidase and may be involved in the formation of transmembrane channel. Both the associated proteins (virD2 and virE2) of the ssT-DNA complex interact with the different plant proteins and facilitate the entry of T-DNA-complex into the nucleus. T-DNA-virD2 complex is correctly localized into the nucleus because virD2 contains a nuclear localisation signal (NLS) sequence in its amino acids composition. The NLS interacts with the plant proteins and ultimately NLS has been recognized by a group of proteins termed as importins and make the T-DNA-virD2-complex feasible to transport through the nuclear pore complex (NPC). Nuclear localisation signal also carried by virE2 proteins but two in numbers, these NLS can only be recognized by importin protein of the plant cells *via* another plant protein, named as VIP1. If VIP1 is associated with the T-DNA-virD2-virE2 complex then only the NLS present on the virE2 is recognized by importins and facilitates its passage through NPC. The NLS present on virE2 protein mainly helps to transport the large DNA molecules. VirE2 proteins in the T-DNA-virD2 complex also provide correct conformation to the T-DNA-virD2 complex. The virH operon encodes some proteins which are involved in the detoxification of the certain toxic molecules produced by the plant cells that might otherwise adversely affect the growth of *Agrobacterium.* Tumour formation efficiency is increased by bacteria having the virH gene as compared to those lacking the same. VirD2 protein has a special role for integration of the T-strand into the plant nuclear genome.

Integration of T-DNA into the plant nuclear genome and their expression

T-DNA Integration

After entering into the plant cytoplasm, the ssT-DNA-virD2-virE2 transfer complex is interacted with the various plant proteins specifically; the nuclear localization signal (NLS) present on the virD2 is recognized by cytoplasmic importins which facilitates the import of ssT-DNA transfer complex to the nucleus through the nuclear pore complex (NPC). NLS present on the virE2 proteins is also recognized by plant importin protein *via* the other type of cytoplasmic protein, named, as VIP1 and VIP2. Then the T-DNA integrates into the plant nuclear genome *via* illegitimate recombination although the mechanism is not fully known but possibly required plant encoded recombinases and DNA polymerases in association with other plant protein such as cyclophilin, importin, histoneH2A *etc*. Whatever the principles have been considered to describe the phenomenon of T-DNA integration into the nuclear genome, but it is not involving only the bacterial encoded proteins and enzymes but maximum components are used in this process coming from the plant sources. Because *Agrobacterium* can infect the wounded plant cells only and wounded cells undergo cell division for the wound healing purposes. The replication of the DNA is necessary to proceed towards the cell division and the necessary enzymes for DNA replication and repair are available during the cell division and the some of the proteins and enzymes of this mechanism can also be utilized during the bacterial T-DNA transfer to the wounded cells for integration into the plant genome. virE2 proteins coat the T-DNA virD2 complex and protects it from nuclease degradation, which also facilitates transportation into the nucleus through nuclear pore complex (NPC) by providing correct conformational structure.Then the T-DNA-virD2 complex interact with the another virE2 interacting proteins, VIP2, which direct this complex towards the chromatin and plays vital role in integration to the nuclear genome. Another plant protein, cyclophilin interacts with the virD2 protein of the T-DNA-virD2-virE2-complex and might be facilitate the integration process. The T-DNA integrate into the host plant nuclear genome by a process, which is referred to as *illegitimate recombination*. The process is not like the homologous recombination, and does not depend on any kind of sequence similarity during recombination. Once transferred to the plant nucleus, the T-DNA derived from *Ti/Ri* plasmid is covalently integrated into the nuclear genome either as a single copy or several copies (on an average 2-3 copies) randomly through out the genome. The random integration process may insert multiple copies of the T-DNA including direct or inverted tandemerization or truncation of T-DNA or make aberrant integration. After its integration, T-DNA adopts eukaryotic characteristics of chromatin organization in order to make nucleosome structure and sensitive to DNase I. Recent investigations suggested that integration is not occurred randomly instead integration occurs preferentially at the region of chromatin, which is transcriptionally active. In addition, homologous recombination is occurred with the short sequence similarity present on the nuclear genome. It is being observed through the transgenic production that the highly expressed open chromatin region is being the targeted region for preferential recombination. The virF protein functions as a cell cycle regulators, and prolong the S-phase for the T-DNA integration into the chromatin of the plant nuclear genome. Transfer of the T-strand from the cytoplasm to the plant nucleus is mediated by nuclear localisation signals (NLS) that are present in the amino acid residues of virD2 and virE2 proteins. Once within the nucleus, the endonuclease activity of virD2 might be help to integrate the T-DNA intermediate into the plant genome. VirF can discriminate the host and non-host plant, by interacting with the T-DNA transfer complex and determine the host range.

T-DNA of octopine type Ti-plasmid contains two accessory vir operons, virF and virH. virF encodes a protein of 23 kDa and involved in the integration of the T-DNA to the nuclear genome and control the cell cycle by prolonging the S-phase. On the other side, virH has two genes encoding virH1 and virH2 proteins, which are involved in the host range specificity by the bacterial strains. Different plant species are recognized by different strain of bacteria because the enzymes are encoded by these two genes (virH1 and virH2) can detoxify certain plant compounds that can inhibit the bacterial growth (Kanemoto *et al*., 1989). During the integration of the T-strand-virD2 protein is released from the 5′ end and provide energy by cleaving the phosphodiester bond between Tyr29 residue and the first nuleotide of T-strand, and allow the 5′ end to integrate into the plant DNA through illegitimate recombination (Gheysen *et al*, 1989). In this recombination, pairing of few bases between the T-strand and plant DNA is necessary, and termed as micro-homologies. (Tinland *et al*.,

1995). The integrated T-DNA is transcribed and translated into proteins in the plant cells because they possess plant promoters and polyadenylation signal sequences. The genes are expressed to produce phytohormones, auxin and cytokinin for tumourigenesis and opines for the bacterial growth and development.

Phytohormones production-regulation and tumorigenesis

Successful transformation leads to the transcription of the transferred oncogenic genes. In case of crown gall, the tms1, tms2, and tmr genes are expressed in a coordinated manner to produce the respective enzymes for the biosynthesis of phytohormones-auxin and cytokinin at a high level, which triggers the high mitotic activity and leads to the formation of neoplastic growth (*i.e.* crown-gall). Auxin synthesis is autoregulated by a product of T-DNA gene indole-3-lactate from tryptophan which is a antagonist of auxin and control the over production of the auxin and thus prevent toxic effects of the auxin overdose.

Hairy root formation by the T-DNA of Ri-plasmid

T-DNA of ***Ri***-plasmid (*Agrobacterium rhizogenes*) is transferred to plant cell and integrated into the nuclear genome almost in a similar way as the T-DNA of ***Ti***-plasmid The vir-genes of Ti and Ri plasmids share extensive homology and have the potentiality to complement each other. The phenotypes conferred by the transformed plant cells is different in ***Ti*** and ***Ri*** transformation because physiological basis of transformation is different. The hairy root phenotype is caused by the over production of auxin of some agropine type Ri T-DNAs. They contain similar auxin producing genes, tms1 and tms2 but not present in all hairy root forming *Ri*-plasmid. It has been found that the tms1 and tms2 are not responsible for hairy root phenotypes. The major genes responsible for hairy root transformation is exerted by another set of different genes located in all Ri-T-DNAs and referred to as ***rol*** genes (***ro***ot ***l***oci) such as rolA, rolB, rolC and rolD. These genes are expressed in the transformed plant cells and are involved in the metabolism of plant growth regulators which make the plant cell more sensitive to endogenous auxin. Transformed cells are more competence to give a response against increased level of auxin. So that, the root inducing potentiality is enhanced. It has been found that transformed cells are releasing the active auxin from inactive glucosylated storage forms by an enzyme indole-β-glucosidase, which is encoded by the rolB locus. Cytokinin is also released in the same way from the Ri-T-DNA transformed cells by the enzyme cytokinin-β-glucosidase, which is encoded by rolC gene. The two oncogenic region (rolB and rolC) of Ri-T-DNA is interfere with normal plant development by releasing phytohormones from their inactive glucoside conjugates rather than by catalyzing their synthesis. Both the genes rolB and rolC are inducible in nature and induced by auxin. So, there will be a phytohormones imbalance in the Ri-T-DNA transformed plant cells and produce hairy root phenotypes. Hairy roots can be cultured in the laboratory *in vitro* and may be utilized as a source of many secondary metabolites of the plant. At the advent of recombinant DNA technology, recently they have been used as a source of hairy root transformation and the transformed tissues can be transferred to regeneration medium to give a plant. In spite of this, the *A. tumefaciens*-mediated gene transfer in plants has been preferred, which is more efficient than the Ri-plasmid transformation.

Basic features of Ti-plasmid required to be used as a plant transformation vectors

Natural *Ti-plasmid* as such cannot be used as a plant transformation vectors because of the following reasons

- Large size (200 kb) Ti-plasmid can not be used as a suitable vector for direct DNA cloning and transfer; high yield circular plasmid cannot be prepared, more over it is without multiple cloning sites (MCS), where the foreign gene(s) are to be inserted to transfer to plant genome.
- Suitable selectable dominant markers are totally absent.
- Tumor inducing ***onc*** genes (auxin and cytokinin synthesizing genes) present on the T-DNA of ***Ti***-plasmid creates problem to regenerate normal plantlets and then whole plants because the auxin and cytokinin balance is disturbed due to the synthesis of phytohormones by the *oncogenes* (***onc***). But these phenotypes can also be used as dominant selectable markers in some cases. This property must preclude to be used as a plant vectors.
- As such cannot be replicated in *E. coli* cell and consequently not feasible for manipulation *in vitro*. So, it is clear that the goal of obtaining genetically transformed plants (transgenic) with desirable traits would require engineered Ti-plasmid to transfer foreign gene(s) into plant cell.

The above problems have been circumvented by the following ways:-

- The ***onc*** genes carried by the T-DNA is responsible for tumor formation but by any means not required for gene transfer mechanism. The ***onc*** genes containing region can easily be deleted from the engineered vector, which will be used to transfer the foreign gene into plant cells. The Ti-plasmid with out ***oncogenic*** region is referred to as ***disarmed Ti-plasmid*** and can be used as plant transformation vectors. The tumour inducing genes were replaced by the dominant selectable markers, these are generally bacterial genes conferring resistance to antibiotics *i.e.* kanamycin (Hoekema *et al*., 1983), strain is LBA4404.
- The T-DNA border sequences are the only components of the T-DNA of ***Ti-plasmids*** required for gene transfer processes, and accordingly imperfect direct repeat sequences of LB and RB are needed to be incorporated into the engineered Ti-plasmid vectors to transfer desired gene into plant cell for integration into the plant nuclear genome.
- The *E. coli* origin of replication is added to replicate in *E. coli* for routine manipulation.
- Dominant selectable markers such as drug resistance genes are included into the vector structure to select the transformed plant cells easily.
- Transformation vector size has been reduced to manipulate *in vitro* easily as because they are less liable to damage by shearing forces. A multiple cloning site

Table 3 Selectable marker and scorable reporter genes for plant gene transfer

Gene	Origin	Enzyme synthesized	Resistance against	Useful as Selectable marker	Useful as Scorable marker
npt II	Tn5	Neomycin phosphotransferase	G-418 Neomycin Kanamycin	++	+
npt I	Tn601	Neomycin phosphotransferase	G-418 Neomycin Kanamycin	+	+
cat	Tn9	Chloramphenicol acetyltransferase	Chloramphenicol	++	(+)
dhrf	Plasmid R67	Dihydrofolate reductase	Methotrexate	+	-
hpt	E. coli	Hygromycin phosphotransferase	Hygromycin	+	-
bar	*Streptomyces hygroscopicus*	Phosphinothricin acetyltransferase (Pat)	Phosphinothricin Bialaphos	++	-
aroA	*Salmonella typhimurium*	EPSP synthase	Glyphosate (Roundup)	++	-
gus	E. coli	Glucoronidase	NA	-	++
LuxA/B	*Vibrio harveyi*	Bacterial luciferase	NA	-	++
Luc	Photinus pyralis	Luciferase	NA	-	++
LacZ	*E. coli*	β-galactosidase	NA	-	+
ocs	T-DNA	Octopine synthase	Aminoethyl-cysteine	(+)	+
nos	T-DNA	Nopaline synthase	NA	-	+
ble	Tn5	Bleomycin resistance gene	Bleomycin Phleomycin	+	-
spt	Tn5	Streptomycin phosphotransferase	Streptomycin	+	-
AAC(3)	E. coli	Aminoglycoside-3-N-acetyltransferase	Gentamycin	+	-
aadA	E. coli	Aminoglycoside 3-0-adenylyltransferase	Spectinomycin	+	-

(MCS) has been included into the vector for easy insertion of the foreign gene (s).

- T-DNA promoters have been used to express the foreign gene in transformed plant cells because T-DNA promoters only expressed in plant cells not in bacteria. (A generalized diagrammatic representation of T-DNA transfer and integration into the plant nuclear genome).

Principle behind Agrobacterium mediated gene transfer

Basic principle of Agrobacterium -mediated gene transfer is that if any DNA fragment can replace T-DNA of a Ti-plasmid it will be transferred and integrated into the plant genome under certain conditions.

Prerequisites for t-DNA transfer

It is necessary to replace the T-DNA with the gene of interest to transfer to plant cells. The LB and RB repeats should be maintained in intact configuration, Vir regions are needed to mediate transfer and chromosomal genes (chv genes) are important which facilitates intimate contact between bacterial cell and plant cell.

Types of transformation vectors based on Ti-plasmid

There are two types of plant transformation vectors have been designed on the basis of Ti-plasmid to reduce the size of the natural Ti-plasmid as well as make easy to transfer the foreign genes.

i) Co-integrative vectors and
ii) Binary vectors.

Cointegrative vectors

This vector based on disarmed Ti-plasmid containing the border sequences (LB and RB) only without hormone synthesizing genes. Two plasmids are required to create a cointegrative vector (Zambryski *et al* 1983; Bevan *et al*. 1983; Horsch *et al*. 1984). The transgene (foreign gene) and vir-genes reside on the same plasmid molecule (*cis* -acting *vir*-genes). Two plasmids are necessary to create co-integrate vector which are as follows- a) One acts as an acceptor vector generated by disarming the Ti-plasmid, and b) other one acts as intermediate vector, which will ultimately be incorporated into the acceptor vector. In this vector system, both the T-DNA with the gene of interest and *vir* region are present in the same vector used for transformation.

An intermediate vector is made in the first step using E. coli plasmid, Vir region, T-DNA borders and origin of replication including pBR322 sequences. Second type vector is a disarmed pTi vector which is like this- gene of interest, some marker genes, and pBR322 sequences. Thus homologous recombination is occurred between similar sequences and co-integration of the plasmids will take place within Agrobacterium. Therefore, a co-integrate vector with both T-DNA with foreign gene within the T-DNA border and vir region. The complete vector is used for transformation viz. pGV2260.

To prepare a cointegrative vector, a Ti-plasmid pGV 3850 used as an acceptor vector, which is, derived from the nopoline Ti-plasmid pTiC58 (Zambryski *et al*., 1983). The *onc* gene of the T-DNA of pGV3850 has been removed and replaced by an intermediate vector pGV1103. The intermediate vector pGV3850 contains a common small *E. coli* cloning vector pBR322. This construct will be acted as an acceptor vector (which is small and easy to manipulate *in vitro*). The acceptor vector pGV3850 has been designed in such a way that it has a homologous region with that of intermediate vector containing the pBR322 plasmid. The intermediate vector pGV1103, is constructed by Hain *et al*., (1985), based on pBR322, a small cloning vector of *E. coli*.

The gene intended to transfer to plants is cloned into the multiple cloning site (MCS) of the pBR322 region of the intermediate vector pGV1103. A colE1 origin of replication is present in this intermediate vector. This allows the replication of the plasmid only in the *E. coli* host cell, not in *Agrobacterium sp*. A plant selectable marker (PSM) gene *i.e*. neomycin phosphotransferase (*npt* II) has been added to the intermediate vector to isolate the plant cells those are transformed with the desired vectors. A dominant bacterial selectable marker gene (BSM) *i.e.* spectinomycin resistance gene is present on the intermediate vector, which allows selecting the bacterial cells that has been transformed with the desired plasmid vectors. Beside these, an *ori*T (with *bom* site) is added to the intermediate vector to mobilize the intermediate vector from *E. coli* to *Agrobacterium* cell. If the pBR322 region of intermediate vector is with the foreign DNA, which can easily be transferred between the T-DNA borders by homologous recombination and as a result a cointegrative vector will be formed. The pBR322 is used as a part of the intermediate vector in which the gene of interest has been cloned into the multiple cloning sites. Then the transgene will be inserted into an acceptor vector pGV3850 in a specific strain of *A. tumefaciens* via triparental mating. Triparental mating facilitates the integration of foreign gene cloned into the intermediate vector through reciprocal recombination between the intermediate vector and the T-DNA region of the acceptor vector. Now, the transgene is a part of the T-DNA region, which can be transferred to the plant cell for integration into nuclear genome. In this disarmed cointegrative vectors, the oncogenic part of the T-DNA has been replaced with antibiotic selectable markers, which was interfering with normal plant development. Since the border sequences are the only *cis* requirements for T-DNA transfer, T-DNA genes can be totally removed and replaced by passenger foreign DNA, the foreign DNA then transfer to plant cell for integration into the nuclear genome to create transgenic plant with desired traits. Selectable bacterial marker gene will not express in the plant system if they are with their own promoter. To express in side the plant cells, a promoter of a plant origin or plant virus based or T-DNA derived must be added to the marker gene and moreover a polyadenylation terminating signal is to be attached at the 3′ end. Then the bacterial antibiotic resistance gene will be expressed and which will allow selection of the transformed plant cells on media containing appropriate antibiotic (Bevan *et al*., 1983).

Strategy to create cointegrative vectors

The desired foreign gene (transgene) is to be transferred to the plant cells first cloned in the multiple cloning site of the intermediate vector. The intermediate vector is efficiently cloned into the *E. coli* host due to the presence of colE1 origin of replication. Then the intermediate plasmid pGV1103 is mobilized into the *Agrobacterium* through conjugal transfer system when they are mated together. Inside the *Agrobacterium*, the intermediate vector does not replicate itself instead it is integrated into the acceptor vector. As soon as the intermediate vector is integrated with the acceptor vector it gives a new type of vector and which is referred to as ***cointegrative*** vector (pGV3850:: 1103). Whole the process can be achieved through a triparental mating. After co-integration, the pGV3850::1103 T-DNA contains the whole of the intermediate vector (pGV1103) along with the foreign gene. The unwanted pBR322-DNA sequences are introduced into the transformed plants if it is used as such. This can be minimized by using a so called *Split End Vector* (SEV) designed by Fraley *et al.* (1985). As for example, pTiB6S3-SE is a SEV, combining disarmed Ti-plasmid pTiB6S3-SE with the intermediate vector pMon200. The pMon200 carries the RB, *nos* gene, MCS, BSM (Spt^R), PSM but pTiB6S3-SE contains LB, vir-genes, BSM (Kan^R) gene as a result produces SEV-pTiB6S3-SE**::** pMon200. This vector minimizes the inclusion of bacterial DNA into the plant genome.

Binary vectors

Binary vector consists of a pair of plasmids. (a). A disarmed Ti plasmid containing T-DNA with foreign gene of interest, ori for both *E. coli* and *Agrobacterium*. It is named as mini-Ti or micro Ti-plasmid such as pBin 19. (b). Helper Ti-plasmid has virulence region that mediates transfer of T-DNA in micro Ti-plasmid to the plant.

Most advanced type of vector, the binary vector has been developed to overcome the problems associated with the cointegrative and Split-End-Vector (SEV). Binary vector system is consisting of a pair of plasmid both of which replicate in *Agrobacterium i.e*., two plasmids are used at a time to transfer the foreign gene into the plant genome. One plasmid provides the virulence functions in *trans*, and considered as helper Ti-plasmid that is devoid of T-DNA (*e.g*. pLA4404). While the second plasmid acts as a vector itself, carrying a gene of interest within the T-DNA border. Beside these a mobilization function (bom), replication function, and a bacterial selectable marker reside on Ti-plasmid outside of the T-DNA borders of the second plasmid, which facilitates the plasmid maintenance, mobilization, replication between *E. coli* and *Agrobacterium*. The idea of the construction of binary vector came from the observation that vir genes products function in *trans*, not *cis*-essential for T-DNA transfer. This could be located on a different plasmid or even on the bacterial chromosome. But the T-DNA is placed on a separate second plasmid with wide host range replication origin to replicate in both *E. coli* and *Agrobacterium* and also conjugal transfer gene to facilitate the conjugal transfer between *E. coli* and *Agrobacterium*. The second plasmid is referred to as binary vector. The necessary genetic elements for replication, conjugal transfer are derived from plasmid, pRK_{252}. The virulence genes containing helper Ti-plasmid supply the vir genes in *trans* arrangement and termed as plasmid for virulence (pVIR). This helper plasmid has no T-DNA region and is not capable to transfer any foreign DNA into the plant nuclear genome. In contrast to that, the other plasmid is considered as a binary vector, which is smaller in size, at least 20 times less than the helper Ti-plasmid because they don't have to carry any *vir* genes, which is supplied in *trans* conditions by the helper Ti-plasmid (pVIR). There is no need of any kind of genetic manipulation to the helper Ti-plasmid so it can be stably maintained in a suitable *Agrobacterium* strain. The binary plasmid vector (pBV) contains T-DNA region along with the left and right border sequences, which is needed to transfer the transgene into the plant cells. The bacterial selectable marker genes are located on the binary vector outside of the T-DNA region for selection in *E. coli* and *Agrobacterium sp*. A plant selectable

marker gene is located on T-DNA region to select the transformed plant cells. This binary vector plasmid (pBV) is normally maintained in *E coli* host cell for *in vitro* manipulation (to clone the foreign gene *i.e.* transgene into this plasmid) (Klee *et al*, 2000). The binary vectors have the following features (binary vector pGA482; 13.2kb).

a) A broad host range replication origin (oriV) derived from the R-factor, RK_2. This oriV is compatible with the oriV of Ti-plasmid and allow replication of both the plasmids in the same cell.
b) oriV from colE1 plasmid for high copy number of the plasmid in *E. coli*, not in *Agrobacterium*, where it will not work.
c) Transposable elements that allow gene tagging in the host genome.
d) A selectable marker, tetracycline to select bacterial transformation.
e) Reporter genes are also included in this construct.
f) Multiple cloning sites (MCS) to insert the desired foreign gene construct into the binary vector.
g) A plant cell transformation marker, kanamycin resistance gene (*npt*II flanked by 5′ promoter and 3′ polyadenylation signal sequence of *nos* gene, sometimes, this construct, the *nos-npt*II fusion, is also expressed in bacteria at very low levels although they are devoid of bacterial promoter. The *nos-npt*II fusion is flanked by T-DNA border sequences (LB/RB) for DNA transfer to plant cells. The T-DNA also caries a multiple cloning site (MCS) to clone the desired gene.
h) Elements, which facilitate the rescue of transferred DNA from plants to phage or bacterial plasmid by involving lambda *cos* site or a plasmid *replicon* with the T-DNA border.

Production of binary vector:

The desired gene, X, first has to be cloned into the MCS of binary vector (*e.g.*, pGA482). Then transform the competent *E. coli* host cell for cloning this recombinant plasmid pGA482::X. Then the recombinant plasmid is transferred to a special strain of *A. tumefaciens* strain, which already harbour a helper plasmid such as pLA4404 (pVIR). The introduction of these two different kind of plasmids (pVIR and recombinant binary vector) into the *Agrobacterium* strain can be obtained by triparental mating: *E. coli* (pRK2013) X *E.coli* (pGA482::X) X *A. tumefaciens* (pLA4404). The newly created strain of *A. tumefaciens* will carry a new type of plasmid constructs which is as such pLA4404, and pGA482::X. The plasmid pRK2013 enters into the *Agrobacterium* strain along with the recombinant plasmid construction pGA482:X and then the plasmid pGA482::X is mobilized to the recipient strain of *Agrobacterium* which already harboured helper plasmid pLA4404. The plasmid pRK2013 has a colE1 oriV and is not able to replicate inside the *Agrobacterium*.

The pBIN19 was the first binary vector used to transform the plant cells in the early 1980s, which was one of the most widely used general purpose plant vectors (Bevan., 1984). The pBIN19 is 11.77kb in size, and an antibiotic resistant gene kanamycin (*npt*-II) is located close to the right border (RB). The multiple cloning site derived from pUC19, is housed within the LacZα region of β-galactosidase on the T-DNA, which facilitates the blue/white screening on IPTG/X-gal plates to distinguish recombinant from non-recombinant colonies. The MCS contains seven restriction enzyme cutting sites for the insertion of passenger DNA (foreign/transgene). The pBIN19 contains a prokaryotic kanamycin resistance gene (APH-I) for the selection of bacterial transformation outside of the T-DNA. The T-DNA contains a plant transformation selectable marker, kanamycin resistance gene (*npt*-II) derived from Tn_5, which is placed under the control of the *nos* promoter (nopaline synthase) and *nos* 3′ polyadenylation signal close to the right border. The origin of replication is derived from a wide host range plasmid pTiT37. The helper Ti-plasmid is derived from a octopine type plasmid pTiAch5. The helper Ti-plasmid can also be obtained from nopaline (T37-SE), octopine (pAL4404), and succinamopine strains (pEHA101).

The standard strains of *Agrobacterium*, such as LBA4404 are generally used to transform dicotyledonous plants. There are many cereal crops which are not normally infected by the standard vector containing *Agrobacterium* strain but can be infected with the more virulent so called *super-virulent* strain of *Agrobacterium* such as EHA101 or EHA105. They are derived from Ti-plasmid, pTiBo542, and contains disarmed version of pTiBo542. Alternatively*' super-binary'* vectors can also be used to transform the cereal crops (monocots), which contains an extra copy of the virulence region that includes virB, virE and virG genes. The vir-region was derived from the plasmid, pTiBo542 and which was carried by a small T-DNA containing plasmid (Komari, 1998) in a binary vector system in addition to the full set of its virulence genes. Such type of vectors and strains of *Agrobacterium* are very efficient to transform the monocotyledons. Generally, the antibiotics such as kanamycin are used to select the transformed plant cells with *Agrobacterium* but so many other selectable markers are also used to select the transformed plant cells (see table 3).

Limitations of pBin19

- Due to its large size difficult to manipulate *in vitro*.
- Sometime gives false positive response, because the kanamycin gene reside close to the right border and it is assumed that kanamycin resistant plant do not express the transferred gene due to truncation during transfer or not at all transferred but giving kan^R response due to its location near to RB, due to the polarity of the T-DNA transfer. Transfer originates from the right border so there is a chance to enter the selectable marker gene (kan^R gene) before the entry of desired gene (transgene) it is reside close to the right border.
- So, it may give kanamycin resistance transformed plant but without gene of interest or truncated transgene.
- Multiple cloning sites only contain eight restriction-cutting sites, which limits the gene manipulation system.

All the above limitations can be improved in a more advanced type of binary vector, which is known as pGreen, that is 4.632 kb in size, kanamycin selectable resistance gene is located close to the LB, MCS present in between *Sac*I and *Kpn*I

cutting sites with other over 15 restriction enzyme sites, that is situated in LacZα segment of the plasmid, facilitates the blue/white screening procedures. The binary vector is with pSa origin of replication but the enzymes (replicase) needed for replication supplied in *trans* conditions (*i.e.*, on another plasmid).

Advantages of Binary vectors:

- *Agrobacterium*-mediated gene transfer to plants mainly are obtained using the binary vectors than co-integrative vectors, due to small size, they are convenient to manipulate *in vitro.*
- In case of binary vector system, only the foreign DNA along with plant selectable markers are introduced into the plant genome but in co-integrative vector, unwanted bacterial plasmid DNA also introduced into the plant genome.

***Agrobacterium*-mediated plant Transformation Techniques**

The purpose of most plant transformation experiments in plant biotechnology is to produce whole, transgenic plants. *Agrobacterium*-mediated technique is the most widely used for the transformation of plants and regeneration of transgenic plants. The explants used in transformation experiments must therefore be capable of producing whole plants by regeneration and must be competent for transformation. So, tissue culture techniques have been playing important roles in most of the transformation methods available to date (only exception *in planta*-transformation). The tissue culture based conventional transformation techniques are referred to as *in vitro* transformation. Generally, *Agrobacterium*-mediated *in vitro* plant transformation techniques have been adopted to inoculate the plant explants to transfer the desired gene into the plant cells (Table 3). A variety of inoculation methods are available to transform plant cells for transferring foreign gene(s) into the higher plants through *Agrobacterium*-mediated gene delivery methods, these are as follows

i. Co-cultivation method.
ii. Leaf- disk transformation method
iii. *Agroinfection* method and
iv. *In planta* transformation method

Co-cultivation method

Co-cultivation is a method of infection of the plant explant specifically protoplast with *Agrobacterium* strain containing binary vector system with desired foreign gene to transfer desired gene to the plant cells. Other explants can also be used in co-cultivation for inoculation of *Agrobacterium,* such as stem segment, suspension cultured cells, micro-calli,

Table 4 Plant species currently transformed by *Agrobacterium tumefaciens*

Plant	Gene transferred	References (not included)
Gymnosperm		
Pinus strobes	-	Leve *et al*, 1999
Ginkgo biloba	-	Dupre *el al*, 2000
Dicotyledons		
Populus	Glutamine synthetase	Gallardo *et al*, 1999
	Herbicide resistance	Confalonieri *et al*, 2000
Eucalyptus camaldulensis		
	Insect and herbicide resistance	Harcourt *et al*, 2000
	Disease resistance	
Pyrus communis	Rooting in apple	Reynoird *et al*, 1999
Malus domestics	Disease resistance	Zhu *et al*, 2001
Actinidia deliciosa (Kiwi fruit)		Kobayashi *et al*, 2000
Glymax max	-	
		Donaldsonand Simmonds, 2000
Brassica oleracea	-	Pinus and Achar, 2000
Gossypium hirsutum	Disease, insect and herbicide	
Hevea brasiliensis	resistance	Wilkins *et al*, 2000
Coffea canephora	-	Montoro *et al*, 2000
Papaver somniferum	-	Hatanaka *et al*, 1999
Digitalis purpurea	-	Park and Facchini 200b
Mesembryanthemum crystallinum (ice	-	Koga *et al*, 200b
plant)	-	Ishimaru *et al*, 1999
Lactuca sativa		
	Ferritin gene	Goto *et al*, 2000
Monocotyledons		
Rice		
	MADS-box genes	Jeon *et al*, 2000b
	A BT gene	Shu *et al*, 2000
	β-carotein (golden rice)	Ye *et al*, 2000
	Chitinase gene	
		Nishi Zawa *et al*, 1999
Maize		Datta *et al*, 2000
	-	Takakura *et al*, 2000
Sorghum bicolor		Nomura et al, 200a,b
Sugarcane		Negrotto *et al*, 2000
Gene transfer by *A. rhizogenes*	-	Zhao *et al*, 2000
Ginseng (*Panax giseng*)	-	Arencibia *et al*, 1998
Crotolaria juncea		
	-	Yang and Choi, 2000
	-	Ohara *et al*, 2000

epidermal segment, cotyledon, hypocotyls and germinating seeds. Pollen of *Petunia* can also be transformed by *Agrobacterium* binary vector system in co-cultivation inoculation approach. Protoplasts are prepared by digesting the plant cell wall with a mixture of enzymes (cellulases and pectinases) and 2-3 days old protoplast cells (10^4-10^5 cells/ml) are incubated with 10^7-10^{-8} cells/ml of *Agrobacterium* strain and are incubated for 36-48 h in a liquid medium in a test tube or petri dish. *Agrobacterium* normally grows and maintains on YEP agar medium at 28^0C for 3 days before inoculation of the plant explant. During the co-cultivation, plant cells divide and dedifferentiate, bacteria also divides and T-DNA is beings transferred from *Agrobacterium* to plant cells. Transformation efficiency depends on the composition of the co-cultivation medium and the medium that supports the active division of plant cells. The signaling molecule acetosyringone is added to the medium to increase the transformation efficiency at a concentration of 100μM specifically in cereal transformation (rice and maize, Hiei *et al*, 1994; 1997). The temperature of co-cultivation plays an important role, some plant species transform successfully at temperature 22^0C (*Phaseolus*) but other are at 28^0C (rice). Other factors, such as acidic pH, high osmotic pressure are also played important role for the expression of *vir* genes, consequently control the transfer efficiency. Opines also induce the *vir* genes and some sugar, D-glucose; 2-deoxy-D-glucan can enhance the *vir*-gene expression. After co-cultivation, *Agrobacterium* cells are killed by adding antibiotics to the tissue culture media, such as carbenicillin, cefotaxime, which are not toxic to plant cells but to kill the *Agrobacterium.* Then the plant cell transfer to media containing amino-glycoside antibiotics such as kanamycin and G418 to select the transformants (transformed plant cells) and also add cefotaxime to kill the *Agrobacterium* if present any one. The gene for neomycin phosphotransferase (*npt* II, also called aphII or *neo*) is used to select the transformants on selective medium. Hygromycin phosphotransferase (*hpt*, *aph*-iv or *hph*) also confers resistance to the amino-glycoside antibiotic hygromycin. Other antibiotics spectinomycin, or chloramphenicol, gentimycin, herbicides such as bromoxynil, 2,4-dichlorophenoxyacetic acid, phosphinothricin, glyphosate, and imidazolinones. The bacterial *bar* gene for phosphinothricin acetyltransferase (PAT) is used to select the transformants specifically the cereals. The *bar* gene confers resistance to L-phosphinothricin (PPT), gluphosinate (an ammonium salt of PPT) and bialaphos (a derivative of PPT). Due to the public concern over the use of antibiotic resistance genes as selectable markers has been replaced by non-antibiotic resistance markers, which are safer and more readily acceptable. Mannose-6phosphate isomerase, xylose isomerase can be used as a plant selectable markers which enable plant cells to metabolise mannose and xylose respectively but non-transformed plant cells can not metabolise them. *Agrobacterium* gene, isopentenyl transferase (*ipt*) can also be used as selectable marker, which increase the production of cytokinin and which may induce shoot induction and subsequently used as a plant selectable markers. The transformed plant explants then transferred to regeneration medium containing auxin (NAA) and cytokinin (6-BAP) for organogenesis and ultimately whole plant regeneration.

Leaf disc transformation method

The co-cultivation method has some disadvantages such as preparation of protoplast by using a cocktail of cellulases and pectinases and all plant species are not responding well to regenerate whole plants *via* callus formation. The co-cultivation method only is applicable for plant species where the plants are regenerated from protoplast. To overcome the above problems, an alternative method of plant inoculation by *Agrobacterium* has been discovered by Horsch *et al* (1984), the technique is referred to as Leaf-disc transformation method. In this method, leaves are surface sterilized then cut into small pieces. The sterile leaf explants were inoculated with recombinant *Agrobacterium* and cultured on an appropriate medium. Subsequently transferred to medium containing antibiotic (cefotaxime/carbenicillin) to kill the *Agrobacterium* and consequently the transformants are selected containing second antibiotic, kanamycin. The survived transformants are allowed to form callus. The transgenic plantlets are regenerated from the selected calli. In the leaf-disc transformation, callus formation generally occurs from the cut edge of the leaf-disc and they are susceptible to *Agrobacterium* infection with high transformation efficiency. This method is much easier and faster for selection and regeneration of transformants than the co-cultivation method. Thus, this method has been widely adopted to transfer the foreign gene into plant cells by many laboratories of the world. Adding acetosyringone in leaf-disc culture in case of *Arabidopsis* can enhance transformation efficiency.

Agrobacterium-mediated virus infection (Agroinfection or Agroinoculation)

Virus vectors derived from RNA and DNA viruses are also used to transfer foreign genes into plant cells. Gemini virus, and Cauliflower Mosaic Virus (CaMV) can be used to create such type of virus vector. More recently, a combination of strategy has been developed (Grimsley *et al*, 1986 and 1987) by combining a virus vector with the Ti-plasmid to infect plant cells. In this strategy, two copies of the viral vector genome were inserted in a tandem fashion in-between the borders of the T-DNA and *Agrobacterium* carrying these constructs are used to infect plant cells *via* artificial wound. The T-DNA will be transferred to the plant cells along with the viral genome. Somehow viral DNA is released (escaped) from the T-DNA construct and start to produce many copies of the viral genome through replication and spreads systematically. Each of the viral genome can produce the disease symptoms *via* infection. This type of viral infection *via* the T-DNA of *Agrobacterium* is known as ***"Agroinfection"*** (Grimsley *et al*, 1986), or more recently ***"agroinoculation".*** The technique was used to infect the wheat plant (a monocot) by wheat dwarf virus, and rice with rice tungro bacilliform virus and turnip (*Brassica rapa*) by CaMV. A vector was constructed containing a tandemly repeated double stranded dimer of the DNA genome of maize streak virus (MSV-a gemini virus) to infect maize cells (Grimsley *et al*, 1987). The genome of the Gemini virus is a single-stranded circular DNA, can replicate to high copy number in side the plant nucleus via the replicative form (RF) autonomously. The cloned naked viral DNA cannot infect the plant cells because they are not infectious. They are infectious if transmitted through insect vectors (leafhopper and cause

stunting and formation of yellow-streaked leaves). The isolated MSV-DNA alone is not infectious and cannot infect the maize plants. To cause infection, MSV-DNA dimer is to be inserted in-between the T-DNA border of mini-Ti-vector. The *Agrobacterium* harboring this recombinant Ti-plasmid are used to infect the maize leaves *via* artificial wounding. The inoculated maize plants showed symptoms of viral infection after two weeks of inoculation. There was no symptom of viral infection if the virA gene is mutated in *Agrobacterium* or constructed vector lacking the T-DNA borders. That means viral infection is only possible in presence of functional vir-genes (virA, B, C, D and G) and T-DNA borders. These observations provide clear evidence that viral DNA is transferred from *Agrobacterium* to monocotyledonous cells and cause infection if they provided with *vir*-function in *trans* and T-DNA borders in *cis* arrangement. It also support that they can replicate inside the plant cell without integration with the plant genome and can cause disease symptoms. Therefore, ***agroinfection*** may be utilized to express foreign genes into host plants without its integration. In few cases, viral DNA can be integrated with the plant nuclear genome and can create transgenic plant if the viral genome carried the desired gene in-between the TDNA borders. So, ***agroinfection*** represents an alternative route to transfer foreign genes into higher plants specifically into monocotyledons where the *Agrobacterium*-mediated genes transfer is not so high.

In planta Transformation

This is a tissue culture free transformation method. The time for production of transgenic plants would be shortened dramatically, and problems associated with tissue culture such as somaclonal variation can be eliminated in this *in planta* transformation method. This technique is very efficient in transformation of *Arabidopsis* (Bent, AF, 2000). In this *in planta* transformation method, inflorescence shoots are cut at their bases and wound site are used for inoculation with *Agrobacterium* in association with vacuum infiltration. The vacuum infiltration step has been removed in " floral spray" or "floral dip" methods, where the *Arabidopsis* flower buds (just initiated the flower formation) were simply sprayed with or dipped in *Agrobacterium* suspension culture. After incubation they are allowed to grow until the seeds are shaded. Transformants are selected by germinating the seeds on a medium containing antibiotics. Recovery of transformants in this method was very high in comparison to other transformation methods in *Arabidopsis.* Transformants were all hemizygous, which provide the evidence that transformation occurs after divergence of male and female germline cells and before a very early stage of embryogenesis. There were some reports showing that ovules are the primary target for ***in planta*** transformation. Homozygous transformants were developed from inoculated *Medicago truncatula*, suggesting that the T-DNA integration steps took place at earlier stages than in *Arabidopsis*. This experimental success providing the clue that the other plant species can also be transformed *in planta* to produce transgenic plant without involving tissue culture procedures.

Advantages of *Agrobacterium*-mediated gene transfer method

i. *Agrobacterium* is capable of infecting any plant explants (plant cells, tissues or organs).
ii. It is as like as natural gene transfer in higher plants
iii. Large DNA segment (up to 50kb) can be efficiently transferred into the plant cells
iv. Without substantial rearrangements of the genomic DNA.
v. Integration of the T-DNA is not so random but it is precise.
vi. The stability of the transferred gene is reasonably good.
vii. Transformed plant explants can be regenerated into whole plant effectively.

Disadvantages

i) Some important cereal crops cannot be infected with *Agrobacterium* because they are belonging to monocotyledons. But recently, this limitation has been overcomed by using the ***super virulent*** strain of *Agrobacterium*, which can infect wide host range of plant species.
ii) More easily regenerable plant explants, the embryogenic cells are difficult to transform with the *Agrobacterium* because they are laid in deep layers of the tissue system and are not easily available for *Agrobacterium* infection or simply is not ideal target for T-DNA transfer.

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