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Production of Courseware e-Content for Postgraduate Subjects

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1. Details of Module and its Structure

Module Detail	
Subject Name	<Botany >
Paper Name	<Plant Genetic Engineering >
Module Name/Title	< Selectable and Screenable Markers>
Module Id	<>
Pre-requisites	A basic idea about plant genetic engineering
Objectives	To make the students aware of Selectable and Screenable markers to know whether the transgene has been transferred, where it is located, and when it is expressed.

Structure of Module / Syllabus of a module (Define Topic / Sub-topic of module)	
< Selectable and Screenable Markers >	<Sub-topic Name1>, <Sub-topic Name2>
Keywords	selectable, screenable markers,

2. Development Team

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TABLE OF CONTENTS (for textual content)

1. INTRODUCTION

Marker systems are tools for studying the transfer of genes into an experimental organism. In gene transfer studies, a foreign gene, called a transgene, is introduced into an organism, in a process called transformation. A common problem for researchers is to determine quickly and easily if the target cells of the organism have actually taken up the transgene. A marker allows the researcher to determine whether the transgene has been transferred, where it is located, and when it is expressed (used to make protein). Marker systems exist in two broad categories: selectable markers and screenable markers.

- Selectable markers are typically genes for antibiotic resistance, which give the transformed organism (usually a single cell) the ability to live in the presence of an antibiotic.
- Screenable markers, also called reporter genes or scorable genes, typically cause a color change or other visible change in the tissue of the transformed organism. This allows the

investigator to quickly screen a large group of cells for the ones that have been transformed.

Selectable and screenable markers are essential for genetic engineering in both prokaryotes and eukaryotes, and are often built into engineered DNA plasmids used for genetic transformation.

2. SELECTABLE MARKERS

A selectable marker gene encodes a product that allows the transformed cell to survive and grow under conditions that kill or restrict the growth of non transformed cells. Most such genes used in plants are dominant selectable markers that confer resistance to antibiotics or herbicides (Table 1).

Important aspects of the marker gene that has to be considered are:

1. Its structure (nucleic acid sequence), which will determine factors such as regulation of transcription (constitutive, environmental or developmental expression), rate of transcription, transcript stability and efficiency of translation.
2. The gene product itself, which is obviously responsible for the dominant expression of a suitable selective phenotype.

The selectable functions on most general transformation vectors are prokaryotic antibiotic-resistance enzymes which have been engineered to be expressed constitutively in plant cells. In some experiments, enzymes affording protection against specific herbicides have also been used successfully as dominant marker genes. The enzyme coding sequence is normally fused to promoters isolated from the T-DNA region of *Agrobacterium tumefaciens* or Cauliflower Mosaic Virus (CaMV) genome at the 5' end, and a polyadenylation signal, often again from a T-DNA gene, at the 3' end.

Table 1 : Selectable markers and selective agents used in plant transformations

Selectable marker	Source	Principle of selection
npt-II (neomycin phosphotransferase)	*Escherichia coli*	Confers resistance to the aminoglycoside antibiotics neomycin, kanamycin and G148 (geneticin)
hpt (hygromycin phosphotransferase)	*Klebsiella* spp.	Confers resistance to hygromycin B
bar and pat (*(phosphinothricin acetyltransferase)*	*Streptomyces hygroscopicus*	Confers resistance to phosphinothricin (glufosinate) and the herbicides bialaphos and Basta
epsps (5-enolpyruvylshikimate-3-phosphate synthase)	*Agrobacterium tumefaciens strain CP4*	Confers resistance to the herbicide glyphosate *N*-(phosphonomethyl)glycine - Round-up
Ble (glycopeptide- binding protein)	*Streptalloteichus hindustantus*	Confers resistance to the glycopeptide antibiotics bleomycin and pheomycin (and the derivative Zeocin)
dhfr (dihydrofolate)	Mouse	Confers resistance to methotrexate
dhfr (dihydrofolate reductase)	Mouse	Confers resistance to methotrexate
csr1–1 (acetolactate synthase)	*Arabidopsis thaliana*	Confers resistance to chlorsulphuron
tms2 (indoleacetic acid hydrolase)	*Agrobacterium tumefaciens*	Confers sensitivity to naphthalene acetamide (NAM)

Some of the common selectable markers are discussed in detail.

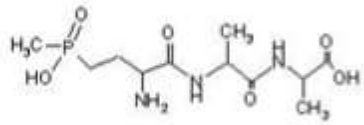
2.1. *npt*-II

- Neomycin phosphotransferase is a bacterial enzyme that confers resistance to the antibiotic **Kanamycin.**
- Kanamycin works by inhibiting protein synthesis in prokaryotes and eukaryotes.
- *npt*-II provides resistance by phosphorylating kanamycin, making it inactive.
- Very popular selection system for many plant species because most plant species are extremely sensitive to kanamycin.

2.2. *bar* and *pat*

- It consists of a glutamic acid analogue moiety, called phosphinothricin [PTC or glufosinate =2-amino-4-(hydroxymethylphosphinyl) butanoate] and two alanine residues.
- Bialaphos is an inhibitor of the key enzyme in the nitrogen assimilation pathway, glutamine synthetase (GS).
- A *bar* (bialaphos resistance) gene encodes a phosphinothricin acetyl transferase (PAT) enzyme
- It becomes active after removal of the alanine residues by intracellullar peptidases.
- The remaining glufosinate compound inhibits GS and as a result, leads to accumulation of toxic levels of ammonia in both bacteria and plant cells.
- The biochemical and toxicological characteristics of glufosinate have made it a popular, nonselective herbicide, which has been commercialized under the names Basta®, Buster® and Liberty® by Bayer Crop Science (formerly Aventis).
- Treatment of genetically modified plants carrying a *bar* gene with glufosinate or bialaphos provides a very efficient means of selection in genetic transformation protocols.

Bialaphos



phosphinothricin-alanyl-alanine

2.3. *hpt*

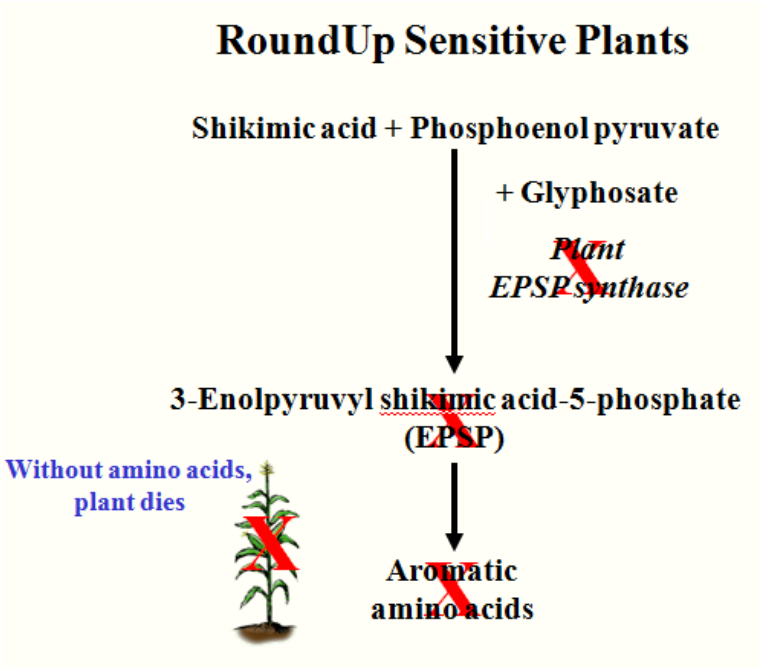
- The gene codes for hygromycin phosphotransferase (HPT), which detoxifies the aminocyclitol antibiotic hygromycin B.
- A large number of plants have been transformed with the *hpt* gene and hygromycin B has proved very effective in the selection of a wide range of plants, especially monocotyledons.
- Most plants exhibit higher sensitivity to hygromycin B than to kanamycin, for instance cereals.

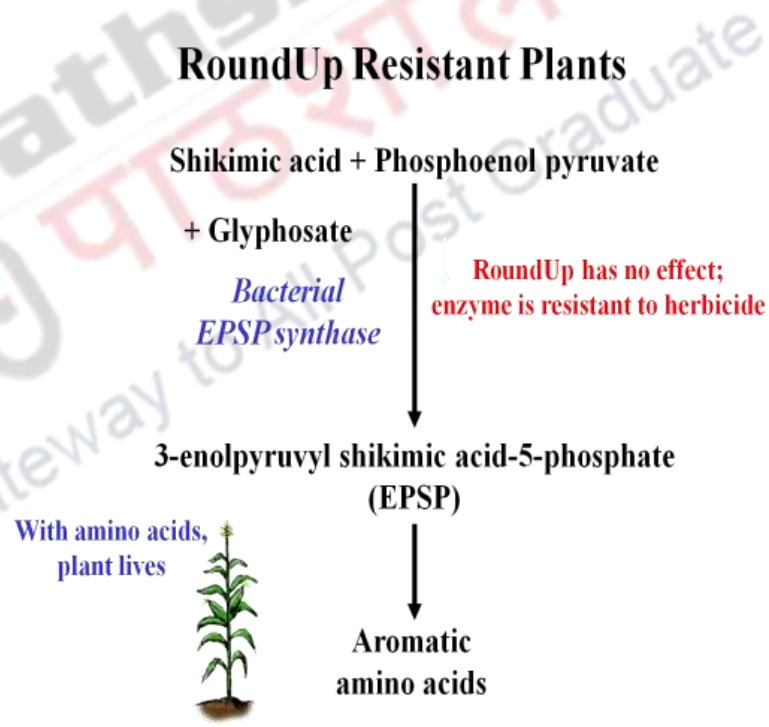
- The sequence of the *hpt* gene has been modified for its use in plant transformation. Deletions and substitutions of amino acid residues close to the carboxy (C)-terminus of the enzyme have increased the level of resistance in certain plants, such as tobacco.
- At the same time, the hydrophilic C-terminus of the enzyme has been maintained and may be essential for the strong activity of HPT.
- HPT activity can be checked using an enzymatic assay. A non-destructive callus induction test can be used to verify hygromycin resistance.

Hygromicin resistant rice callus

2.4. *epsps*

- Glyphosate (*N*-[phosphonomethyl]glycine) is a broad-spectrum herbicide that is the active ingredient of the commercial Roundup® formulations.
- It acts as an inhibitor of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) which is essential in the shikimate pathway for the biosynthesis of the aromatic amino acids.
- The chemical binds to the active site of the EPSPS enzyme. This enzyme is a key to the development of a group of amino acids called the aromatic amino acids.
- When this enzyme is bound by glyphosate, it can not synthesize those amino acids, and the plants die because protein synthesis is severely disrupted.
- Glyphosate will not bind to a particular genetically-engineered version of EPSPS. Therefore RoundUp Ready crops with this altered enzyme will survive when sprayed with the herbicide.





EPSPS synthesizes 3-enolpyruvly shikimic acid-5-phosphate is the essential precursor to aromatic amino acids. When plants are sprayed with a glyphosate-containing herbicide, such as RoundUp, this important precursor is not synthesized, and consequently the plant is starved of aromatic amino acids. The result is plant death.

An engineered version of EPSPS one that was discovered in a bacterium, is introduced into the plant. This enzyme cannot be bound by glyphosate. Therefore, if a field is sprayed with the herbicide, the introduced version of the gene produces a functional enzyme. The EPSP precursor is synthesized normally, and the plant produces enough aromatic amino acids to survive.

2.5 Other Dominant Selectable Markers

A wide range of dominant selectable markers are available for plants, including genes providing resistance to other antibiotics e.g. bleomycin, pheleomycin etc.,

Phleomycin and Bleomycin are novel antibiotics that belong to the bleomycin family of glycopeptides that act by site-specific, single- and double-stranded DNA cleavage.

3. SCREENABLE MARKERS

Screenable marker systems employ a gene whose protein product is easily detectable in the cell, either because it produces a visible pigment or because it fluoresces under appropriate conditions. Visible markers rarely affect the studied trait of interest, but they provide a powerful tool for identifying transformed cells before the gene of interest can be identified in the culture. They can also identify the tissues that have (and have not) been transformed in a multicellular organism such as a plant.

Table 2 : Screenable markers and selective agents used

Screenable marker	Source	Principle of selection
gusA (β-glucuronidase GUS)	*Escherichia coli*	Catalyses hydrolysis of β-glucuronides; variety of colorimetric, fluorometric and chemiluminescent assay formats; can be used for *in vitro* and *in vivo* assays
lux (Firefly luciferase)	The firefly *Photinus pyralis*	Light produced in the presence of luciferase, its substrate luciferin, Mg^{2+}, oxygen and ATP
gfp (green fluorescent protein)	The jellyfish *Aqueorea victoria*	Spontaneous fluorescence under UV or blue light.

Screenable marker genes are included on many transformation vectors for two reasons.

1. To allow independent verification of the transformed status of tissues growing on media containing selective antibiotics or herbicides.
2. As a principal means of identifying transformants in conditions where transformation frequencies are high.

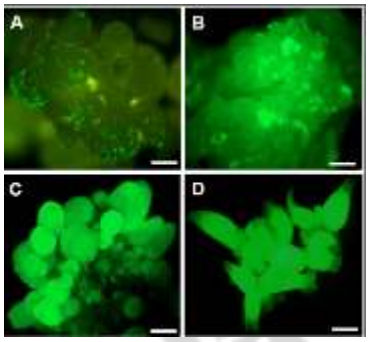
A specific use of a screenable marker is as a reporter gene; both in the development of transformation systems using transient expression assays to monitor success or to test out DNA sequences which may be able to regulate gene expression in stably transformed tissues.

The most commonly used screenable markers are:

3.1 Green fluorescent protein (GFP)

- GFP is a fluorescent protein from the **Jellyfish** - ***Aequorea victoria***.
- In the Jellyfish, it works with ***Aqueorin***, a blue fluorescent protein that converts ATP into light in the presence of calcium.
- When aqueorin gives off the blue light, it is absorbed by GFP which then fluoresces green light.
- Best use of GFP is for examinations of protein levels and protein distribution in intact cells.

- Because of its smaller size as compared with luciferase and GUS, the GFP protein is better for making protein fusions.
- Assay can be done on ***LIVE*** tissue and is ***not destructive*** to the sample.

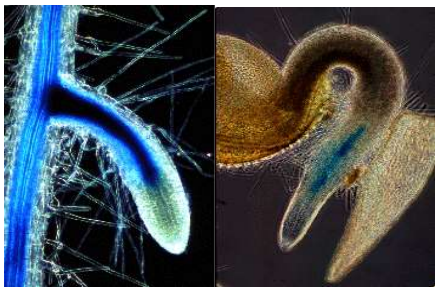


GFP fluorescence in embryogenic callus cultures transformed with *A. tumefaciens*

3.2 GUS assay

3.3 GUS encodes the β-glucuronidase enzyme from *E. coli* and it has been extensively used as a reporter gene in transgenic plants.

- The main advantage of the GUS reporter gene system is the stable expression of GUS enzyme in plant cells. GUS activity can readily be detected by chromogenic or fluorogenic enzyme substrates.
- Various β-glucuronic acid substrates are available for detection of GUS expression.
- The most widely used fluorogenic substrate for detection of β-glucuronidase activity *in vitro* is 4-methylumbelliferyl β-D-glucuronide (MUG, 4-methylumbelliferyl-beta D-glucuronic acid dihydrate, M-5700).



Transgenic plants that harbor the AGL12::GUS fusions show root-specific expression

3.3 Chloramphenicol Acetyltransferase or **CAT**

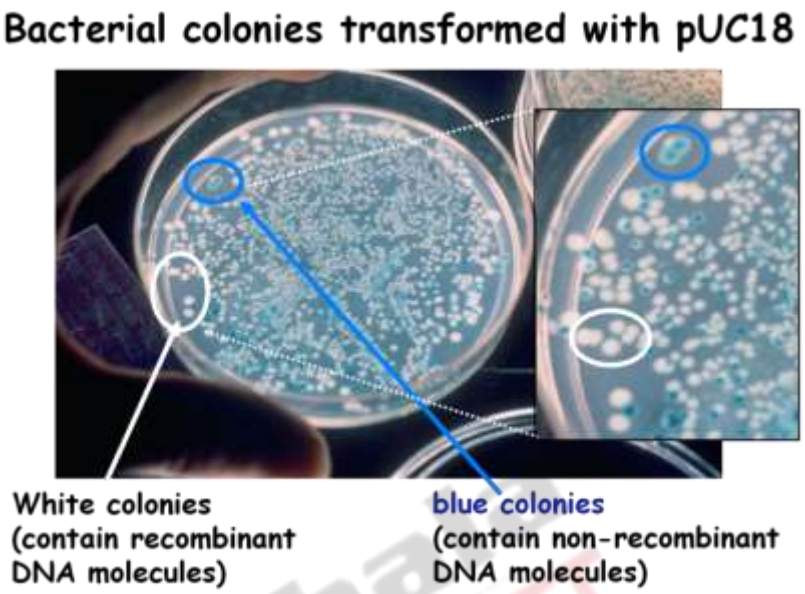
- Enzyme detoxifies Chloramphenicol by acetylation.
- Chloramphenicol is an antibiotic that inhibits protein synthesis on 70S ribosomes (prokaryotic).
- Main problem with CAT assays is that they work only in extracts and not in intact cells. Assay involves addition of 14C-chloramphenicol to extract and allowing acetylation by CAT.
- Chloramphenicol is acetylated in two positions, 1 or 3, producing three products, the 1 and 3 acetylation and the 1,3 double acetylation.
- Reaction products are separated from substrate by TLC and detected by autoradiography.

3.4 Luciferase

- *Luciferase* - class of enzymes that convert chemical energy into light.
- Most commonly used is from the **firefly** - ***Photinus pyralis.***
- Firefly luciferase encoded by single gene called the ***lux*** gene and requires the cofactor **Luciferin**.
- Luciferase/luciferin complex produces light by the hydrolysis of ATP.
- During Transformation, the expression of luciferase is detected by addition of luciferin to watering solutions of plants.
- *In vivo* detection of luciferase activity is accomplished by placing the plant or tissue on photographic film.
- For *in vitro* assays of plant extracts, luciferin and ATP are added to the extract the reaction is carried out in a scintillation counter which then measures light emission

3.5 Blue/white screening

Blue/white screening is used in bacteria. The **lacZ** gene makes cells turn blue in special media (containing X-gal). A colony of transformed cells can be seen with the naked eye.



4. APPLICATION OF REPORTER GENE/SCREENABLE MARKERS

4.1 Transformation and transfection assays

- Many methods of transfection and transformation - two ways of introducing a foreign or modified gene into an organism - are effective in only a small percentage of a population subjected to the techniques.
- Reporter genes used in this way are normally expressed under their own promoter independent from that of the introduced gene of interest.
- In the case of selectable-marker reporters such as CAT, the transfected population of bacteria can be grown on a substrate that contains chloramphenicol.
- Only those cells that have successfully taken up the construct containing the CAT gene will survive and multiply under these conditions.

4.2 Gene expression assays

- Reporter genes can also be used to assay for the expression of the gene of interest, which may produce a protein that has little obvious or immediate effect on the cell culture or organism.
- In these cases the reporter is directly attached to the gene of interest to create a gene fusion.
- The two genes are under the same promoter and are transcribed into a single polypeptide chain.
- In these cases it is important that both proteins be able to properly fold into their active conformations and interact with their substrates despite being fused.
- In building the DNA construct, a segment of DNA coding for a flexible polypeptide linker region is usually included so that the reporter and the gene product of will only minimally interfere with one another

4.3 Promoter assays

- Reporter genes can be used to assay for the activity of a particular promoter in a cell or organism.
- In this case there is no separate "gene of interest"; the reporter gene is simply placed under the control of the target promoter and the reporter gene product's activity is quantitatively measured.
- The results are normally reported relative to the activity under a "consensus" promoter known to induce strong gene expression.

Summary

Selectable and screenable markers are important tools in genetic engineering. Selectable markers enable selection of transformed bacterial cells or plant cells and tissues from the non-transformed ones. They are usually genes that confer antibiotic resistance to the transformed cells or tissues. Screenable markers are used to determine whether the gene of interest has been transferred, where it is located, and when it is expressed. These are

usually color markers that can be visualised in transformed cells. These include GUS markers, LUX markers or GFP markers. Screenable markers not only report on successful transformation, but also tell us whether the transformation is stable, whether the gene of interest is expressed and also about specificity of expression of a promoter.

These markers have greatly improved our understanding of the temporal and spatial expression of genes.

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1. Details of Module and its Structure

Module Detail	
Subject Name	**Botany**
Paper Name	**Plant Genetic Engineering**
Module Name/Title	**Agrobacterium mediated transformation**
Module Id	
Pre-requisites	**Basic knowledge about plant biology and biotechnology**
Objectives	**To create awareness in students on basic principles and applications of plant transformation mediate by Agrobacterium based vectors.**
Keywords	**Agrobacterium tumefaciens, binary vectors, cointegrate vectors, plant transformation**
Structure of Module/Syllabus of a module (Define Topic / Sub-topic of module)	
Strategies forAbiotic Stress Tolerance in Plants	**<Sub-topic Name1>**

2. Development Team

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TABLE OF CONTENTS

1. Introduction

Transformation is an important and powerful technique for functional analysis of a gene and producing transgenics which contribute to increase crop productivity. It involves introduction and integration of foreign DNA in the genome of the host cell such that it can be passed to subsequent generations.Although a variety of transformation methods are available,*Agrobacterium*mediated transformation is the most widely used method to transform economically important agricultural and horticultural crops ranging from dicots, monocots, fungi and even gymnosperms.

2. Plant transformation methods

Plant transformation was initially described in 1984 in tobacco and since then several developments have taken place in the methods for plant transformation. There are two main methods of transformation:

Directtransformation: It includes physical and chemical methods like particle bombardment or biolistics, electroporation, polymer based transfection, liposome mediated transfection, and protoplast transformation. These methods do not involve the use of vectors as transgene delivery systems in plants.

Indirect transformation: It includes biological methods like *Agrobacterium*mediated plant transformation and virus mediated transformation. These methods involve the use of vectors like *Agrobacterium*and virus for delivery of foreign DNA into plant cells.

Since the discovery of *A. tumefaciens* and its ability to integrate a part of its genome in host, extensive research has undergone regarding its biology, physiology, molecular machinery and applications in biotechnology. This strategy has become a routine for producing transgenic plants and deciphering the functions of a gene. Several transformation protocols have been developed and standardized to introduce *Agrobacterium*in plants and produce stable transgenic plants.

In this particular review, we will be discussing the *Agrobacterium*biology,history and developments in *Agrobacterium* transformation research, physiological and molecular basis of *Agrobacterium* mediated transformation, progress till date, limitations, advantages and applications of this strategy.

3. *Agrobacterium* as a pathogen

*Agrobacterium*is a soil borne bacteria belonging to family Rhizobiacaea. The genus contains both pathogenic or virulent strains like *A.tumefaciens,A. rhizogenes* and *A.vitis*and non pathogenic strains or non virulent like *A. radiobacter*. The pathogenic strains cause neoplastic tumor like growths,which includes crown gall disease caused by *A. tumefaciens* and hairy root disease caused by *A. rhizogenes*. The first report of crown gall disease was reported in 1853 and *A.tumefaciens* was identified as a causal agent of crown gall in 1907 in Paris daisy by Smith and Townsend. *A. tumefaciens* causes crown gall disease in more than 90 families of mainly dicotyledonous plants thus having the broadest host range of any plant pathogenic bacteria. The symptoms of crown gall include swellings on the roots and stem near the soil and resemble an unorganized callus containing aberrant stem and leaf like structures (Fig. 1). The tumor originates from dividing cells of cambiawhich are unable to differentiate into proper xylem and phloem vessels. Increasing tumor size results in crushing and distortion of nearby normal tissues. It affects transport of water and nutrients near gall region and results into wilting, stunted growth and ultimate death of the infected plant. *A.rhizogenes* was first identified in 1942 whichcauses hairy rootsphenotype in many dicotyledonous plants and it was termed as hairy root disease (Fig. 2). The hairy root disease is characterized by high degree of lateral branching and enhanced growth rate causing profusion of root hairs at the site of infection. Release of secondary metabolites by roots of infected plants is marked as the characteristic feature of this disease.

Figure 1 – Crown gall tumor caused by *A. tumefaciens*

Figure 2 – Hairy roots in tobacco plant caused by *A. rhizogenes*(Adapted from Agrios, 5th edition)

4. Major discoveries in *Agrobacterium* mediated transformation

After the discovery of *A.tumefaciens* as causal agent of crown gall disease by Smith and Towsend, research progressed in several related areas to know the cause of tumorformation by crown gall disease.

Table 1 -A short summary of important discoveries related to biology of *Agrobacterium* (Modified from *Escobar* and Dandekar, 2003)

Year ; Reference	**Discovery**
1941; Braun	- continuous presence of bacteria is not required to induce tumors - bacteria can produce an active principle which was called Tumor Inducing Principle (TIP)
1956; Lioret	- identified existence of opines which are amino acid derivatives in crown galls - specificity of opines (octapine or nopaline) in a particular strain

1974; Ivo Zaenen	Large plasmid present in bacteria could be involved in inducing tumors named as Tumor Inducing principle or Ti plasmid
1977; Chilton *et al*	Only a part of Ti plasmid; which is a T-DNA is transferred to the plant
1980; Lemmers *et al*	Neighbouring segments of T-DNA called TL segments were also transferred along with T-DNA. These segments were found to have auxin and cytokinin like functions and opine synthesising genes
1982; Yadav NS.*et al*	TL segments are short direct repeats of 21-25 bp that form left and right border. They are essential for tumor induction
1986; Satchel and Nester	Another plasmid was present along with Ti plasmid. The other plasmid harboured critical set of genes necessary for virulence and was called *virulence* genes or *vir* genes.
1987; Engstrom *et al*	The virulence factors are induced by plant phenolic acetosyringone. Other virulence proteins VIR A, VIR G, VIR B, VIR E were characterized

Similar to Ti plasmid in *A. tumefaciens*, Moore *et al* in 1979 isolated a high molecular weight plasmid from *A. rhizogenes* and was found to be correlated with induction of hairy roots. This plasmid was called Root Inducing or Ri plasmid. Ri plasmid share high similarity with Ti plasmid. White et al in 1985 identified four rol genes *ROLA, ROLB, ROLC, ROLD*involved in induction of hairy roots and genetic transformation of any foreign gene in plant.

5. Development of *Agrobacterium* as a tool for plant transformation

After the discovery that T-DNA and some neighbouring genes in the Ti plasmid are transferred to the host cell, several developments were made to use *Agrobacterium*as a tool for plant transformation. It was reported that any foreign DNA when inserted between the T-DNA border regions can be transferred into plant cells. Using this report, *Agrobacterium*was used as a vector for introduction of foreign DNA in the host cell by Zambryski *et al* in 1983. A modified Ti plasmid in

which all the oncogenic genes of T-DNA had been deleted or replaced with other DNA was able to efficiently integrate into the tobacco plant genome. The integration did not affect plant cell differentiation and transformed cells can regenerate into complete plants. This was the first time *A. tumefaciens* and its Ti plasmid was used as a vector system to produce transgenic plants. Many agricultural and horticultural crops like corn, soybean, tomato, cotton, flax, blueberry, apple etc are transformed till date. The first attempt to transform monocot plant maize which was beyond the normal host range was done by Chilton in 1983. Other monocots including cereals wheat, barley, rice and sorghum have been also efficiently transformed by *Agrobacterium*. The host range was extended to include *Saccharomyces cerevisiae*(yeast) which is a model fungus, filamentous fungi like *Aspergillus* widely used in food processing industries, and*Fusarium* to characterize genes important for pathogenicity of the fungal pathogen. AMT has also been efficiently established in mycorrhizal fungus as a tool for insertional mutagenesis. The host range is further extended to bryophytes like immature thalli of *Marcantia* which is also an emerging model for plant biology, gymnosperms like various conifers and living fossil *Gingko biloba*, pteridophytes like spores of ferns *Pteris* and *Ceratopteris*. Ri plasmid was also first time used for transformation in 1973 by Ackermann in tobacco, since then several dicotyledons and gymnosperms have been transformed using Ri plasmid.

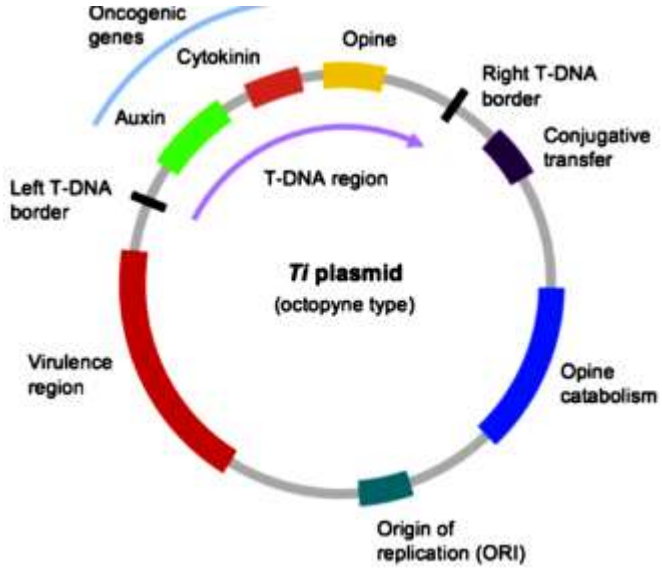
1. Molecular basis of *Agrobacterium*mediated transformation

A. Ti plasmid and various genes in the plasmid

Ti plasmid is a 200Kb large single copy plasmid containing several genes important for virulence, survival and infection of *Agrobacterium*(Fig. 3). Ti plasmid consists of:

- T-DNA region containing oncogenes and opine synthesis genes
- Vir region containing various virulence genes
- Genes producing opine catabolising enzymes
- *Tra* region which enables conjugative transfer of the plasmid
- Origin of replication

Figure 3 -A structure of a typical Ti plasmid (Adapted fromPacurar *et al*., 2011)



These genes are described as follows:

T-DNA or transfer DNA- This part of Ti plasmid gets transferred and integrated into the genome of the host. The process by which the DNA is integrated into the genome will be discussed in the next part. T-DNA region of the Ti plasmid is defined by homologous left and right border sequences (LB and RB) having 25bp of direct repeat needed for the transfer of T-DNA in the host genome. RB has shown to be more important for virulence than left border. The transmission of T-DNA is dependent on the orientation of the border repeat with respect to T-DNA. Any DNA present between the border segments gets transferred in the host.

T-DNA is organized into two distinct regions T-DNA left (TL) and T- DNA Right (TR). TL segment carries oncogenes while TR region carry genes for biosynthesis of opines. An overdrive sequence of 24bp is present adjacent to the Right border of T-DNA.

The oncogenes - The genes present in these region are*AUXA*or *TMS1* or*IAAM*which encodes tryptophan monooxygenase and*AUXB*or *TMS2*or *IAAH*which encodes indole acetamidehydrolase. These genes are involved in synthesis of auxin. Another gene *IPT*or *TMR* or *CYT*is present which encodes isopentyl transferase enzyme involved in cytokinin production. The production of auxin and cytokinin help in tumor formation and are principle determinants of tumor phenotype.

Opine synthesizing genes- Opines are amino acid sugar conjugates which are synthesized by T-DNA which acts as a nutrient source for the bacteria for survival. It also promotes growth of other *Agrobacterium*in the rhizosphere. There are opine catabolising genes on Ti-plasmid but none of the catabolising genes are present in plants.

*Agrobacterium*strains are classified on the basis of opines encoded by T-DNA in the Ti plasmid. On the basis of opines induced in the tumors they can be divided into three subgroups. Two strains belong to the *A. tumefaciens* octopine/agropine subgroup, two strains induces only agropine and the remaining 7 produces nopaline. They all differ in the organization of T-DNA. In Ti plasmids of nopaline strain, TL and TR segments are transferred as a single 22Kb fragment while in octapine strain TL and TR are transferred independently as 14Kb and 7Kb fragment. Octopine strain contains'*OCS*' gene encoding octapine synthase enzyme while '*NOS*' gene encodes nopaline synthase gene.

***TML* gene** – They are involved in determining the tumor size. Deletions of this gene results in very large tumors although not much is known about the molecular mechanism.

Virulence genes (*vir* genes)

Virulence genes are a set of genes present in the Ti plasmid which are responsible for the transport and integration of T-DNA in the host genome. The vir region consists of approximately 35 genes coding for 9 operons which are described in the table 2.

Table 2 -Various virulence genes characterized in vir region of Ti plasmid and their functions (modified fromSlater *et al.*, 2008, Oxford Publications)

Virulence operon (genes)	**Function in *Agrobacterium***	**Function in plant**
Vir A (1)	Functions as an environmental sensor; Part of a two component signalling system with Vir G	
Vir G (1)	Phosphorylated by virA, controls transcription of other vir operons	

Vir B (11)	Membrane proteins; Forms a transfer apparatus for efficient transfer of T-DNA	
Vir C (2)	Helicase activity; Binds and enhances T-DNA transfer	
Vir D (4)	Directs the transfer of T-DNA through the transfer apparatus formed by Vir B	Vir D2 contains NLS and interacts with karyopherin- α family
Vir E (2)	Forms the part of T-DNA –VirD2 complex	It binds to VIP1 protein in plant and prevents T-DNA degradation
Vir F		Interacts with plant Skp1 protein ; Proteolysis of some host proteins
Vir H		Detoxification of phenolics produced in defence by the plant
Vir J	Movement of substrates lacking signal peptide from the periplasm into the host cell	

B. Mechanism of T-DNA transfer from *Agrobacterium* into host cell

The basic process of T-DNA transfer includes:

- Chemotaxis, signal recognition and bacterial attachment to the host cell
- Induction of virulence system
- Generation of T-DNA transfer complex
- Translocation of T-DNA out of bacterial cell to the host cell
- Integration of T-DNA into plant genome

Chemotaxis, signal recognition and bacterial attachment to the host cell:*Agrobacterium*possesses a highly sensitive chemotaxis system and respond to wide range of amino acids, phenolics and sugars which are also a part of plant defence system and produced by

wounded tissues.The initial attachment of bacterial cell wall is mediated by acidic polysaccharides produced by *Agrobacterium*. The genes which encode these polysaccharides are located in the att region of the bacterial chromosome. The tight attachment of the bacteria such that no shear forces can remove them are mediated by genes producing and transporting β-1,2-glycan which help in binding of bacteria to plant cells. These genes are identified to be *CHVB*which synthesizes cyclic β-1,2-glycan, chvA , homologous to ATPase are involved in export of glycans from the cytoplasm.

Induction of virulence system: VirA and VirG are involved in induction of virulence system. VirA and VirG form a part of two component signalling.VirA consists of two structural domains; TM1 which senses the plant signal mainly acetosyringone and transmits it to the receiver TM2. TM2 is a kinase domain in coordination with membrane transporter CheV,autophosphorylates at His-294 (Histidine- 294). The activated Vir A then transfers the phosphate group to the aspartate residue of the VirG present in the N- terminal region while C-terminal region acts as an activator and induces transcription of other Vir genes required for transport and integration of T-DNA in the host cell.

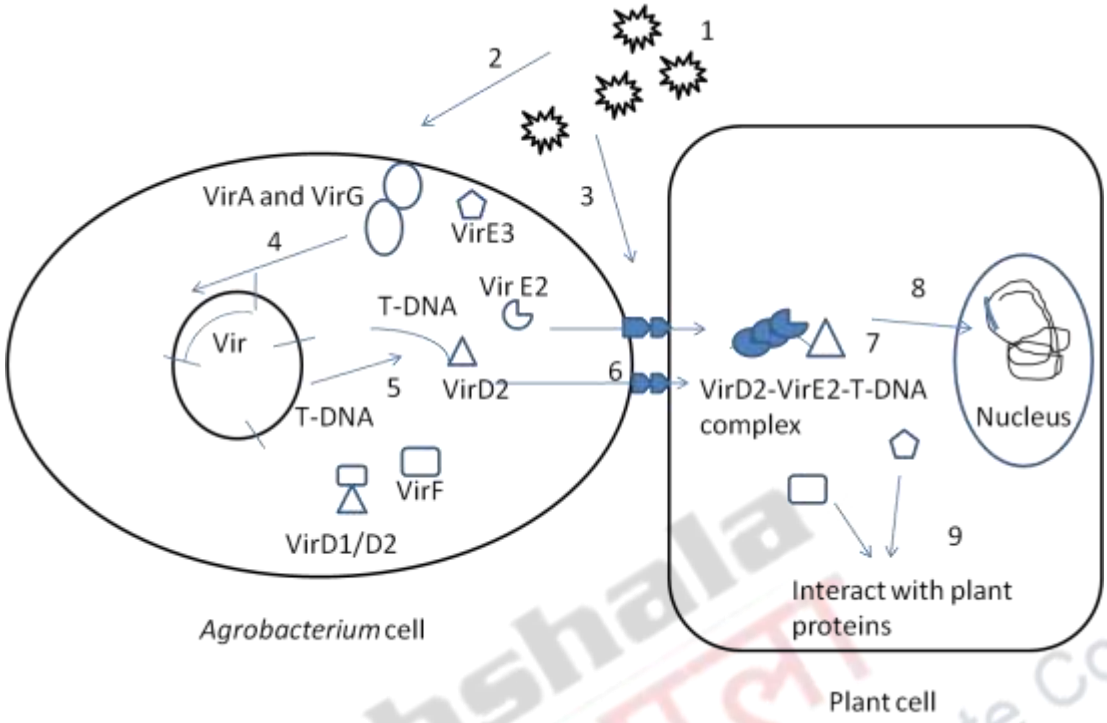
Generation of T-DNA transfer complex: The T-DNA is processed before being transported to the host cell. VirD and VirE proteins along with T-DNA form a T-DNA complex. VirE2 binds strongly to ssDNA and forms coating along the whole T-DNA and VirD2 is associated towards the 5' end of the DNA and both are transferred in the host cell along with T-DNA. Both VirD2 and VirE2 have nucleus localization signals (NLS) and interact with proteins in the host cell.

Translocation of T-DNA out of bacterial cell to the host cell: All the eleven VirB proteins along with VirD4 a membrane protein forms a Type IV secretion apparatus (T4SS) to transport the T-DNA into the host cell. T4SS is a cell envelope spanning complex and forms a channel or pore through which the DNA is delivered to the recipient cell. VirB1 and VirB2 has extracellular functions while VirB3 to VirB11 are hydrophilic requiring ATP for active transfer of T-DNA. VriB2, VirB5 and VirB7 make up the pilus which helps in bringing bacteria to close proximity to the host. Vir B4 allows conformational changes in the formation of channel. VirB7 – VirB11 heterodimer stabilize other Vir proteins for formation of a transmembrane channel. VirE2 helps the T-DNA to pass through the nuclear pores of the host and VirE1 acts as a chaperon for VirE2 and doesnot directly bind to T-DNA. VirE2 also protects T-DNA from nuclease present in the cytoplasm of the host. VirD2 interacts with Karyopherin-α family involved in recognition and

transport of protein through the Nuclear Pore Complex while VirE2 interacts with the VirE2 interacting protein (VIP1) which is a functional homologue of VirE3 present in bacteria. VIP1 interacts with the chromatin protein histone H2A important for targeting T-DNA into its integration site. These interactions facilitate the movement and integration of T-DNA into the nucleus of the host nucleus

Integration of T-DNA in the host genome: T-DNA does not have any targeted sites rather is randomly integrated into any part of host DNA. The process of integration of T-DNA is known as illegitimate recombination. The terminal ends of T-DNA have been shown to have homology with short segments of host target sequences. The truncated T-DNA ends are joined to the breakpoints of the target DNA by annealing to the target DNA through partial homology. VirD2 is involved directly in the integration process by recognizing nicks in the plant DNA and by assisting in the T-strand ligation to the target DNA.

Figure 4 -Representation of molecular mechanisms in T-DNA transfer and integration.
1.Release of acetosyringone by plant cells;**2.**Activation of VirA-VirG system;**3.**Attachment of bacterial cell wall to the host cell; **4.**Activation of other Vir genes by VirG; **5.**Binding of VirD2 with T-DNA;**6.** Transport of T-DNA and other Vir genes in the host cell; **7.** Formation of T-DNA complex (VirD2-VirE2-T-DNA) in host cell;**8.** Interaction of other Vir genes with plant proteins to protect T-DNA complex;**9.** Integration of T-DNA randomly in the genome (modified from Tzfira T, Citovsky V, 2006).

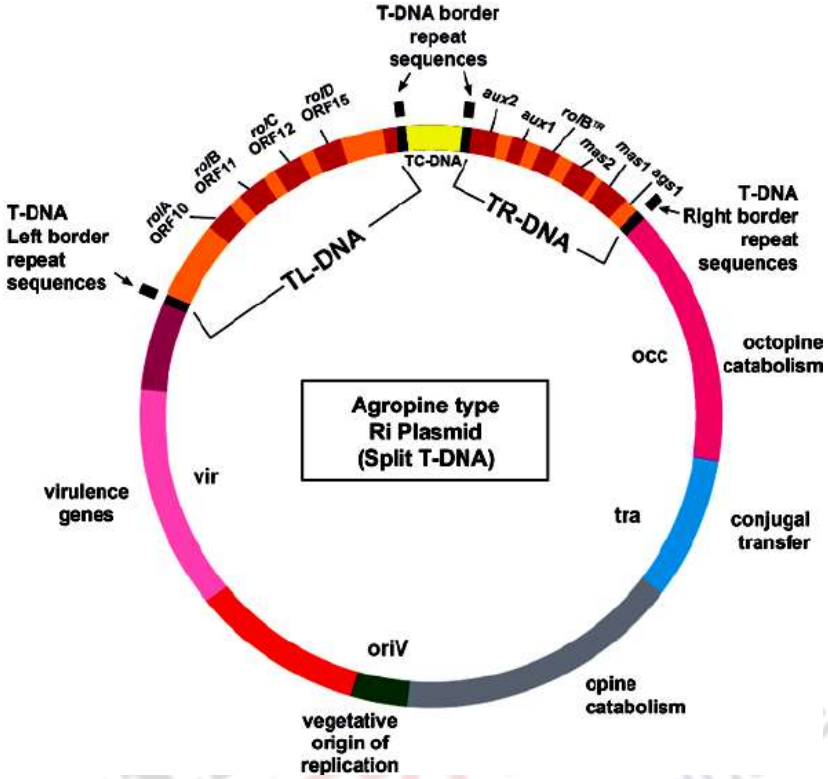


C. Mechanism of T-DNA transfer from *Agrobacterium* into host cell

Ri plasmid show structural and functional similarity to Ti plasmid. T-DNA, virulence genes, opine catabolising genes, auxin and cytokinin producing genes are present in the Ri plasmid having similar functions. The mechanism of T-DNA processing, transfer and integration are common in both the plasmids. However there are some differences in the composition in T-DNA of both the plasmids which are highlighted below:

Opine catabolising genes and classification of A. *rhizogenes* strains– The strains produce two type of opines. The strain which produce a few agropine type opines like agropine, manopine,agroponic acid, and mannopinic acids are known as agropine-type strains while other strain which produces all agropine type opines except agropine are known as mannopine type strain.

Figure 5 -Structure of Ri plasmid (Adapted from Oziyigit *et al.*, 2013 , K. R. Hakeem *et al.* (eds.), Crop Improvement)



T-DNA of Ri plasmid consists of two segments T_L DNA and T_R DNA. T_R DNA consists of the same genes as in T-DNA of *A. tumefaciens* while TL DNA consists of rol genes *ROLA, ROLB, ROLC, ROLD*essential for hairy root induction. The specific function of each rol genes have been identified and discussed below:

ROL Aconsists of 10 ORFs and is involved in causing hormonal imbalance in the host.

ROLBconsists of 11 ORFs and is involved in regulating the activity of auxin. It codes for a β-glucosidase which releases active auxins by hydrolyzing β-glucosides. It has been shown to have tyrosine phosphatise activity which plays a major role in auxin transduction.

ROL Cconsists of 12 ORFs and involved in regulating activity of cytokinin. It encodes a cytokinin β-glucosidase which releases active cytokinins by hydrolyzing cytokinin β-glucosides.It also regulates sugar transport and metabolism by influencing source-sink relationship.

ROLDis present in agropine-type strains only and codes for ornithine cyclodeaminase enzyme which catalyzes the conversion of ornithine to proline.

Ri plasmid does not contain virE2 and VirE1 genes to protect T-DNA from nucleases and assist them in nuclear import. Another protein called *galls* substitute for the functions of VirE genes. GALLS contain ATP-binding and helical motifs which help in conjugation but the mechanism is not much known.

2. Molecular basis of *Agrobacterium*mediated transformation

Agrobacterium based vectors should have basic requirements present in all commercial vectors required for efficient transformation. Thus wild Ti plasmid has to undergo some modifications before using it as a vector.

A. Development of Ti plasmid as a vector: Co-integrate and binary vectors

Wild Ti plasmid cannot be used as a vector and some modifications are necessary to construct an efficient gene vector system . The limitations and modifications are:

- Removal of growth regulator (auxin and cytokinin) genes as they are tumurous and interfere with regeneration of normal and fertile plants
- The Ti plasmid is very large in size (200Kb) which makes manipulation of this plasmid difficult
- Introduction of selectable markers to select transformed*Agrobacterium* and plants transformed by *Agrobacterium*. They are generally genes conferring resistance to antibiotics or herbicides
- Introduction of origin of replication for autonomous replication in *E.coli as* Ti plasmid cannot replicate in *E.coli* where genetic manipulation is easy
- Addition of a Multiple Cloning Site (MCS) to clone for insertion of gene of interest

The Ti plasmid derived plant vectors developed for introduction of new genes in the plasmid are:

Co-integrating vectors -This method involves the use of *E.coli* vector plasmid and Ti plasmid. The co-integrating vectors are constructed by recombination of a bacterial plasmid with the endogenous

T-DNA of the Ti plasmid. The recombination is based on the presence of homology present between them. The *E.coli* plasmid is called an intermediate vector and are generally Pbr322 based plasmids containing some part of T-DNA, gene of interest in the MCS region along with plant transformation marker or selection marker conferring Kanamycin resistance, bacterial resistance marker conferring Spectinomycin resistance, oriT sequence for transfer of intermediate vector to the *Agrobacterium*vector. The *Agrobacterium*vector is modified and a gene of pBR322 is inserted in place of T-DNA, right border of T-DNA, virulence genes and opines synthesizing genes. The PBR322 sequence of *E.coli* integrates to PBR322 in Ti plasmid by single crossover homologous recombination creating a disarmed Ti plasmid with all the genes necessary for genetic transformation.

SEV (Split and end vector)systems are examples of co-integrate vectors which includes pTiB6S3 which is a disarmed Ti plasmid and pMON plasmids of *E.coli*. The two vectors co-integrate in the region of LIH (homology regions) by homologous recombination and form a complete Ti plasmid with gene of interest.

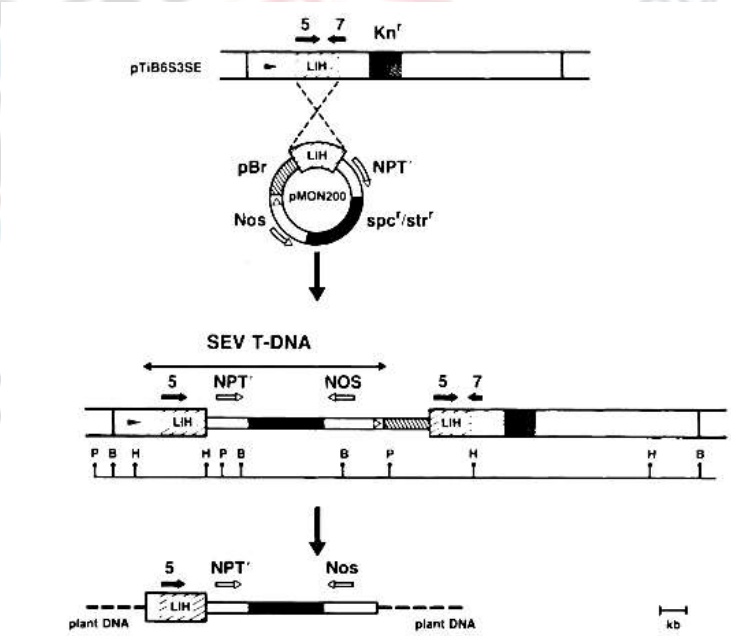


Figure 6 -Diagrammatic representation of Co-integrate vectors: pMON is an *E. coli* plasmid and TiB653 is a Ti plasmid. The integration happens between the homology regions (LIH) making an entire hybrid T-DNA (Adapted from Fraley *et al*., 1985)

Binary vector – The concept of binary vector was first introduced by Hoekema *et al* 1983 who determined that the vir region and the T-DNA could be split into two different replicons. The transfer and processing of T-DNA is possible until they are in the same *Agrobacterium*cell. This strategy has been used widely since its discovery. In binary vectors the vir genes are in separate plasmid called helper plasmid while T-DNA region and other genes related to selection isin another plasmid called disarmed Ti plasmid. The disarmed Ti plasmid consists of T-DNA along with its left and right borders, plant transformation marker conferring Kanamycin resistance, Multiple cloning site region to introduce our gene of interest, bacterial resistance marker, oriT sequence for transfer of binary vector from *E.coli* to *Agrobacterium*, a broad host range vector RK2 which allows replication in *Agrobacterium*. The binary vector is most frequently used in transformation as it involves only transfer of plasmid and does not involve integration as in co-integrate vectors. The transformation rate of binary vectors is much higher than co-integrate vectors.

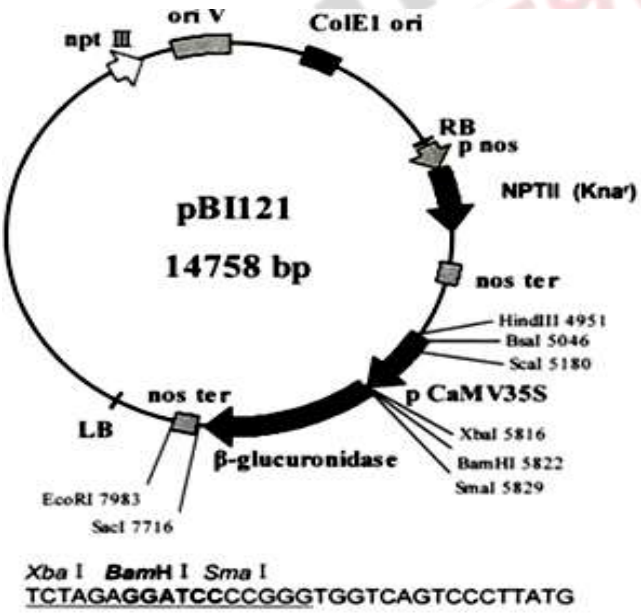


Figure 7 -Binary vector and their components (Adapted from snapgene.org) **P35S**–Promoter 35S transcript; **Pnos**–Promoter for nopaline synthase; **nptII**–plant selectable marker; neomycin phosphatase transferase; **OriV**–origin of replication in *Agrobacterium;* **ColE1** – origin of replication in *E.coli;* **β-gluc**- Reporter gene; **LB -** Left border; **RB -**Right border; and **NptIII –** bacterial selection marker; Kanamycin resistance.

B. Components of binary vector T-DNA system

Promoters and terminators – Promoters are vital and should be present with any gene that needs to be expressed while terminator sequences are present to ensure that the transcription stops at the correct position. In *Agrobacterium*vectors there are many genes which need to be expressed like gene for selectable marker, reporter gene and the gene of interest. For selectable marker gene expression in dicots 35S promoter derived from 35SrRNA of *Cauliflower mosaic virus* is generally used and in monocots maize ubiquitin promoter is used. However promoters derived from Ti plasmid are widely used in *Agrobacterium*vectors like nos (nopaline synthase) and ocs (octopine synthase).

Selectable marker genes – Selectable marker genes allows identifying the transformed tissues as it is always expressed and co-transformed with the gene of interest. For co-transformation it is situated between the left and the right border along with the gene of interest. Most of the selectable markers are those which confer antibiotic resistance like Kanamycin, Hygromycin, Gentamycin, Spectinomycin etc. Most frequently used selectable marker in *Agrobacterium*is nptII conferring Kanamycin resistance and hptII conferring Hygromycin resistance. Though there are many selectable markers the efficiency of their resistance depends on the plant and the strain to be transformed. Herbicide resistance genes have also been used as selectable markers.

Reporter gene –Reporter genes are used as indicators of plant transformation and study the functionality and expression in a tissue in plants. Beta-galacturonidase (GUS), Green Fluorescent protein (GFP) and luciferase gene (luc) are widely used as reporter genes. They are easy to assay and have no endogenous activity in plant. GUS reporter gene has been widely used in *Agrobacterium*based vector and also has an intronic region such that it expresses only in eukaryotic system and does not interfere with GUS expression in *Agrobacterium*.

Origin of replication - Vectors require origin of replication for both*E.coli* and *Agrobacterium*. Earlier a broad host range origins like RK2 origin has been used for replication in both bacteria but this resulted in lower copy number in *E.coli*. So a new system was derived where different origin of replication was provided for both *E.coli* mainly ColE1 ori frompBR322 and a different one in *Agrobacterium*like PVS1 and pRI.

Left Border and Right border – LB and RB border sequencesare retained withinthe modified vector as it contain elements important for the transfer of the gene of interest. These are natural sequences and all the sequences to be transferred in the plant are placed between them.

Other elements include OriT for plasmids mobilization, trf region to enable proper replication of plasmid,MCS region which is mainly present in the lacZ gene to clone the gene of interest.

C. Methods of transformation

The modified vector needs to be introduced in the host to express the gene of interest. Several methods have been developed to transfer disarmed Ti plasmid. The earliest technique was developed by Horsch *et al*., 1985 known as leaf disc transformation which was done in tobacco. The leaves of tobacco were injured and were co-cultivated with bacteria to mediate transformation. Then this method was modified for using other explants tissue other than leaves. Some of the methods widely used for transformation are described below:

Co-cultivation - A suitable plant tissue is selected, injured and placed in a medium containing *Agrobacterium*disarmed Ti plasmid for about 30 minutes. The bacterium is allowed to attach to the plant cells which are known as co-cultivation. The time of co-cultivation depends on the type of crop species being used. The explants are then placed in MS media with suitable conditions and incubated for 2 to 3 days to mediate the transfer of T-DNA into the host cell. The explants are removed from incubation medium and washed in antibiotic solution like Cefatotaxime that kill the excess *Agrobacterium*cells. The explants are then transferred to fresh solid media with a selective agent (according to the plant selection marker) and plant growth regulators to promote regeneration of transformed plants.

Floral dip transformation –This method was first developed for *Agrobacterium*mediated transformation in *Arabidopsis* which is a model plant. The bacteria are transferred to the floral tissues which are female gametophytes of immature flowers of *Arabidopsis*. Plants in the flowering stage are dipped for 3-5 minutes in a infiltration medium containing*Agrobacterium*cells, sucrose and Silwet-77 which is a surfactant. The plants are then allowed maintained under high humidity for 16-20 hours and then transferred to normal growing conditions to set seeds. The harvested seeds are then allowed to grow on a selection medium containing the selection marker such as antibioticsand

screened for the gene of interest. The method is widely used as it directly produces transgenic seeds, less effort is required to perform, has a high transformation rate than co-cultivation and vacuum infiltration and does not involve tissue culture dependent methods.

Vacuum infiltration – This method has been mostly employed in transformation in monocots. Vacuum infiltration is almost same as floral dip but differs in application of vacuum in co-cultivation medium to drive the bacteria to enter the host cell.

Sonication Assisted *Agrobacterium*mediated transfer (SAAT) - This is a modified method to transform highly recalcitrant tissues. The plant tissue is subjected to brief periods of ultrasound by sonication which produces large number of small and uniform wounds for easy access of *Agrobacterium*in plant tissue.

The transformation protocols vary depending on the plant species. Extensive research has been done and is ongoing to standardize the explants type, time of incubation, modifications of components in media, finding the most suitable method in each plant species for high efficiency of transformation.

D. Commercial vectors for *Agrobacterium* mediated transformation

Since the discovery of binary vectors, many commercial vectors have been developed based upon the Ti plasmid like pZP, pCAMBIA, pGREEN, pMON etc. Pbin19 was the first modified plasmid commercialized for plant transformation. Novel binary system which has the ability to deliver multiple gene of interest simultaneously has also been designed by combining to other techniques like gateway cloning and Cre/loxP mediated recombination. Large binary vectors have been constructed based on Bacterial Artificial Chromosome (BAC) and Transformation-competent artificial chromosome (TAC)to transfer genes greater than 150Kb.Large, multi-cassette plant transformation vectors like pSAT system was developed having greater than seven expression cassettes. Super-binary vectors have been developed to transform recalcitrant crop species and are difficult to transform. Gateway compatible vectors like pEarlyGate, pGWTAC, pSITE have also been developed and used routinely.

8. Factors affecting *Agrobacterium*mediated transformation

The efficiency of transformation depends on the right combination of factors influencing the process of transformation. A lot of research has been done in optimization and manipulation of conditionsfor improving efficiency in each plant species.

A. Plant related factors

Selection of explants - A tissue capable of regeneration and which allows recovery of whole plant should be used as a target material for transformation. The type of explants to be used depends on the plant species to be transformed. Embryonic callus, mature embryos, meristems, root and shoot apices, stem segments, and leaf discs are few routinely used explants. For dicotyledonous host the explants used are germinated seedling while for monocots immature embryos are generally used.

Manipulation of explants- Osmotic treatment of explants with sugars like mannitol, sucrose and glucose increases the efficiency of T-DNA transfer and regeneration of whole plants. Desiccation of explants prior to transformation has also been shown to increase transformation rate in many plants. Tissue necrosis of explants is a limiting factor after infection due to production of reactive oxygen species by the plants as a defence strategy. So anti-necrotic treatments are given to explants which helps to increase the transfer efficiency. Silver nitate, L-cysteine, ascoebic acid have been used as anti-necrotic chemicals.

Plant species and genotype – Though most of the dicotyledonous are susceptible to *Agrobacterium*, the degree of infection varies among them. The degree of infection may vary within a species, variety and even an ecotype. A suitable transformation protocols have to be developed foreach plant species. The difference in susceptibility in plant genotype has been observed in legumes and only a few model genotypes have been used for transformation.

Wounding of explants – Different methods of physical wounding the explants have been employed ranging from simple wounds to particle gun mediated wounding. Wounding is an important factor

as it acts as a stimulus allows bacterium to infect the target tissue. Sonication has been used to enhance transformation in many plant species.

B. Inoculation and culturing medium

Surfactants – Addition of surfactants like Silwet L77 and pluronic acid in inoculation medium has been shown to enhance T-DNA delivery in the host.

Acetosyringone – The co-culturing medium for transformation in monocots generally contain acetosyringone as monocots cannot produce them. Addition of this compound has led to increase in transformation efficiency.

pH –*Agrobacterium*requires an acidic pH (i.e. <6) for efficient transformation.

Antibiotics – Antibiotics are applied to the co-culturing medium to kill excess *Agrobacterium*which has not been transformed but does not affect plant tissue. The major ones used are cefotaxime and cefoxitin. However the concentration of antibiotics have proved to be crucial for efficient transformation like antibiotic concentration above 100mg/L was found to have effects on plant regeneration. Other antibiotics used are timentin which has less inhibitory effects on plant regeneration. Type and concentration of antibiotics may have different effects on transformation efficiency in different *Agrobacterium*strains. Meropenem or moxolactum have been more successful than cefotaxime for controlling *Agrobacterium*strains EHA101 and LBA4404.

C. *Agrobacterium*related factors

***Agrobacterium*strain –** The strains of *Agrobacterium* are classified on the basis of the opines secreted by them. Different strains have different virulence system which influences the transformation efficiency. Development of super binary vectors like A281 strain with additional virulence genes has been proved to be more efficient. Different strains have varying virulence on different plant species. The hypervirulent strain EHA105 has been found suitable for transformation in cereals.

***Agrobacterium*cell density-** The cell density is also an important factor for influencing efficiency of transformation. Excessively high cells may cause tissue death after infection. The optimal density ranges from $0D_{600}$ 0.3 – 0.6 according to the strain to be used.

D. Physical conditions

Temperature – Temperature is an important factor for determining the efficiency of T-DNA transfer as it was found that the strains lose their plasmid above 30°C. The optimal temperature needs to be evaluated with specific explants and the strain involved. The optimal temperature is found to be 25° – 28°C. However in monocots the transformation has proved to be highly efficient in 20° to 23°C.

Light –Light conditions during co-culture vary with different transformation procedures. It was seen that co-cultures inoculated in darkness inhibited T-DNA transfer. Light also induces the phenolics for vir gene induction. Many transformation protocols use dark conditions to enable proper regeneration of callus by preserving light sensitive plant hormones.

9. Advantages and Limitations of *Agrobacterium* mediated transformation

Advantages:

- *Agrobacterium* is a natural genetic engineer and transfer T-DNA into the genome of plant and so is the most acceptable of all techniques
- The gene transferred is extremely stable and gives appropriate segregation ratios over generations
- It is capable of transferring large fragmentsefficiently without rearrangements
- *Agrobacterium* can infect intact tissues and organs and transformed tissues regenerated rapidly
- Integration of T-DNA is a precise process and one or more copies of gene is introduced in one or more loci in the intact genome
- Several commercial vectors are now available containing appropriate selectable markers and reporter genes, which allows the researcher to choose the best vector among all to insert the gene

- In planta transformation using floral dip and vacuum infiltration donot involve tissue culture techniques

Limitations:

- Limitation of host range; Many important food crops are recalcitrant to *Agrobacterium*and transformation protocols need to be highly optimized for these plants
- Regeneration of plants can be difficult from some tissues and plants

10. Applications of *A. tumefaciens* mediated transformation

Production of transgenic plants:*Agrobacterium* mediated transformation has revolutionized the generation of genetically engineered crops also known as transgenic plants. Transgenic plants express a foreign geneof interest delivered through *Agrobacterium*which can be transferred to further progenies. The transgenic plants are further used in crop improvement, improving nutritional quality, providing resistance to pests and pathogens, tolerance to various herbicides, production of edible vaccines etc.*Agrobacterium* is preferred for introducing genes as it results in high transgenic events with a single copy of transgene integrations and stable integration which are important considerations in plant transformation.

Fig. 8: Production of transgenic NERICA (rice cultivar in Africa) mediated by *Agrobacterium*. A) T-DNA region of pBIG-ubi::GUS; B) histochemical assays of GUS activity in immature embryos; C) regeneration of putatively transformed plants on regeneration medium with 20 mg/l hygromycin; D) growth of putative transformants on medium with 30 mg/l hygromycin. E) PCR analysis for the presence of the genes for HPT and GUS for putative transformants; F) GUS activity detected in the leaf of a transformed plant (lower) but no activity in a non-transformed plant (upper); G) a transformed plant grown in a greenhouse. (Adapted from T. Ishizaki and T. Kumashiro, Plant Cell Reporter, 2008)**.**

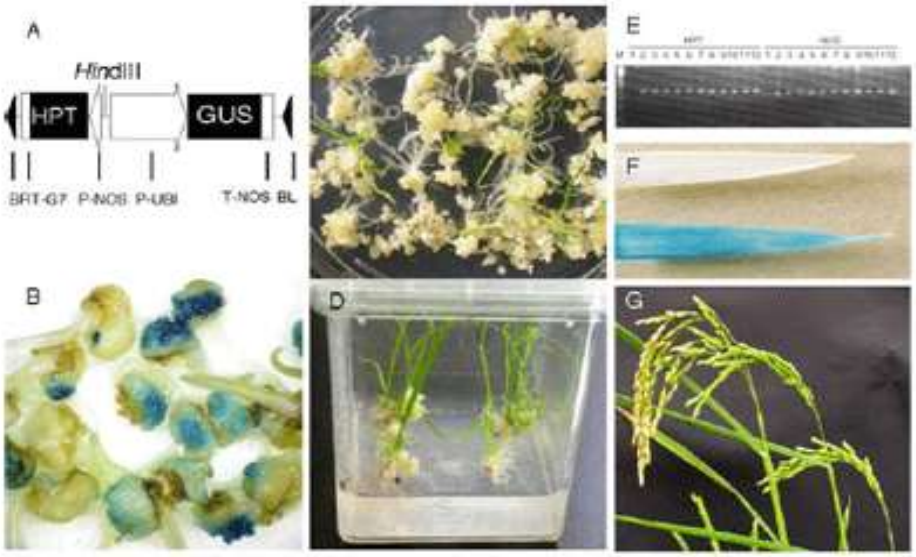


Table 3 -Some of the commercially released transgenics by *Agrobacterium* mediated transformation (modified from Birch, 1997)

Transgene commercial name	Crop improved	Trait	Gene introduced
Flavr Savr	Tomato	Incerased shelf life	Polygalacturonase gene in antisense direction
Roundup Ready	Soyabean	Herbicide (glycophosate) tolerance	5-enolschikimate-3-phosphate synthase (EPSPS) gene
Bollgard	Cotton	Insect resistance	Cry genes
Laurical/Calgene	Oilseed rape	Altered oil composition mainly lauric acid	Gene encoding enzyme ACP thioesterase
Amflora	Potato	Producing pure amylopectin	Antisense gene of Granule bound starch synthase protein (GBSS)
Freedom II	Squash	Virus resistance	Viral coat protein gene
BXN	Cotton	Herbicide(bromoxynil) resistance	bxn gene for oxynil tolerance
Maximizer	Corn	Insect resistance	Cry genes

Generation of marker free transgenic plants: Selectable marker genes are co-transformed with the gene of interest. The maintenance of these genes are unnecessary as they are required only to identify the transgene and also raises environmental and consumer concerns over generations. *Agrobacterium*strain containingselectable marker gene and gene of interest in two different T-DNA have been used to obtain marker free transgenics. If the two T-DNA are present in different positions, progenies containing only the gene of interest can be selected in the next generation after segregation analysis. Site specific recombinases are used near the selectable marker gene which excises the marker gene from the gene of interest. Marker free transgenic rice plants containing Rice ragged stunt virus derived resistance gene have been obtained by using twin T-DNA vectors. Similarly marker free soybean plants producing high levels of γ- linolenic acid and stearidonic acid using two T-DNA vectors introduced through *Agrobacterium* mediated transfer.

Insertional mutagenesis:*Agrobacterium*mediated transfer is a powerful tool for large scale random mutagenesis, targeted gene disruption and T-DNA tagging based on the transfer and integration of T-DNA at specific sites of the genome. It is further used for gene discovery and analysis of functions of various genes through loss of function mutations by T-DNA integration. T-DNA tagged mutations have been advantageous as they are stable through several generations, can act as a marker to identify mutations, they have high chances of finding genes with T-DNA insertions and is randomly integrated into the genome. This approach has been used to find regulatory elements and genes in model plant *Arabidopsis*. Identification of various pathogenecity genes and their functions in fungus like *Colletotrichum annum*, *Magnaporthe grisea*and *Fusarium* has been studied through T-DNA integration. T-DNA tagging has been used to label the gene mutations for the molecular characterization of mutants.

Identification of promoters: Isolation of new constitutive promoters suitable to drive expression in transgenics hasbeen possible through T-DNA tagging strategy. Studying of gene promoters is important for global regulation of gene expression in plants. Novel bidirectional promoters have been characterized in rice through *Agrobacterium* mediated transformation. T-DNA also provides as an excellent tool to study the interaction of promoters with transcription factors which bind on the promoters.

Gene traps: Several traps like promoter traps and enhancer traps have been developed which exploits transfer of T-DNA through*Agrobacterium* mediated transformation. The promoter-less reporter gene is inserted adjacent to the right border of T-DNA and allowed to integrate in the genome of the host. The reporter genes when placed in between the exons results in non-functional protein and altered expression or phenotype. The gene disrupted can be studied through expression of the reporter gene which will be transcribed along with the exons.

11.*A. rhizogenes*mediated transformation:Applications

A. rhizogenes have also been developed as binary vectors to transform T-DNA in host to induce hairy roots. The gene constructs of Ri plasmid is same as that of Ti plasmid. The reporter genes in modified Ri plasmid plays an important role in detection of expression of the gene of interest in roots. Several *A. rhizogenes* strains like K47, HR1and K599 serve as excellent candidates as vectors for transformation. Roots induced by *A. rhizogenes* have unique ability to grow in absence of exogenous plant growth regulators. The major disadvantage of this system is inability of regenerating whole plants from hairy roots.

Production of composite plants: These plants have transformed roots on non transformed shoots. These have been widely used to study the genes involved in nitrogen fixation and nodule formation in various legumes, pathogen interaction at the root level and enables in root testing .Composite plants are easy to regenerate under non-annexic conditions also and provides an advantage of cost, time and space over the traditional transgenic production by *A. tumefaciens*. Transgenic soyabean roots of composite plants were developed to study the function of various genes and their effect on development of nematodes which infect plants through roots.

Transformation in woody plants: The major limitations of genetic manipulation in trees are their long generation cycle. However, *A.rhizogenes*has been used to introduce foreign genes in the woody plant roots and successfully regenerate it. It has also been successful in inducing high intensity of rooting in various tree species like *Pinus*, apple and *Larix*.

RNA silencing: RNA silencing has been shown to be functional in hairy roots and has been used silence genes involved in symbiotic interactions and causing disease. RNA interference coupled

with *Agrobacterium rhizogenes* mediated transformation was done in roots of *Medicago truncalata* which is used as a model system for symbiosis and interaction with pathogens.

Production of secondary metabolites: Large scale productions of secondary metabolites are done from *A.rhizogenes* transformed hairy roots in bioreactors. The hairy roots have greater capacity to produce general or species specific metabolitecompared to normal roots. Several plants have been transformed to increase the production of metabolites they produce like metabolites secreted from medicinal plants which are used as medicines to cure many health problems- Atropine and scopolamine from *Atropa belladonna*, Nicotine from *Nicotiana tabacum*, Digitalin from *Digitalis* etc. The secondary metabolites have also been used for conservation purposes like production of barberin in high amount in *Barberis aristata* which is an endangered species reduce the risk of its overharvesting from its natural habitat.

Phytoremediation and environment interactions: Hairy roots secrete a large number of enzymes for conversion of toxic to non-toxic metabolites and metal chelating compounds which can be used to detoxify harmful chemicals.

12. *Agrobacterium* mediated transformation in non plant organisms and applications

Several fungi, gram positive bacteria like *Streptomyces*, algae like *Chlamydomonas* and human cells have been transformed through *Agrobacterium* mediated transfer. The ability of *Agrobacterium* to transform *Saccharomyces cerevisae* (yeast), which is a model organism has led to advances in discoveries of transformation procedure for other beneficial and harmful fungus. Transformation protocols has been standardized like addition of acetosyringone in induction medium as they donot secrete them, selection of selectable marker genes and promoters which can mediate transcription in that organism like URA3 and TRP1, which are auxotrophic markers and herbicide resistant markers like bar are used in yeast and other filamentous fungi. T-DNA integration at random positions has been useful in random mutagenesis studies and random gene tagging. The transfer of virulence proteins in the host has also been exploited to transfer several enzymes and heterologous proteins in yeast. Apart from random mutagenesis, T-DNA integration has also been done at predetermined position to increase the level of expression of the introduced gene of interest.

13. Conclusion

A.tumefaciens and *A.rhizogenes* are the two most successful plant genetic engineer. Several decades of intensive research has been applied to understand the molecular mechanisms and applying it in transformation studies. Modern agricultural biotechnology is heavily dependent on *Agrobacterium* mediated transformation to create transgenics for crop improvement and disease resistance. Extensive research is being done to improve and standardize protocols for transformation in each species, also in recalcitrant species. The host range of *Agrobacterium*mediated transformation has further extended to woody species and non plant organisms like mushrooms, yeast, fungus and even blue green algae. Over the years the unique ability of transforming gene of interest in host through *Agrobacterium* has provided us with means to understand plant-pathogen interaction and control of gene expression involved in differentiation and development in the host.

14. Suggested readings

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