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Agrobacterium - Mediated Plant Transformation

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Introduction

Agrobacterium is a gram-negative soil bacterium that uses horizontal gene transfer and naturally transformed plant cells. This system was historically the first successful plant transformation system. The breakthrough in gene manipulation in plants came by characterizing and exploiting plasmids carried by the bacterial plant pathogens *Agrobacterium tumefaciens* and *A. rhizogenes*. These provide natural gene transfer, gene expression, and selection systems. In recent times, *A. tumefaciens* has been treated as nature's most effective plant genetic engineer (Mehrotra *et al.*, 2012).

Agrobacterium tumefaciens infects plants through breaks or wounds and causes crown gall disease. Characteristics of crown gall disease are –

- Hormone independent growth of infected plant part
- Presence of opines in plant

Tumor formation is the result of the integration of T-DNA (Transfer DNA) from plasmid into the plant genome, which is incorporated at a semi semi-random location in the plant genome.

History

- Smith and Townsend (1907): said bacteria caused crown gall disease
- Brown and stonier (1958): proposed that not whole bacteria but some part of it causes disease
- Zaenen I *et al.* (1974) - noted virulent strain- *Agrobacterium tumefaciens*
- Chilton *et al.* (1977): reported Ti & Ri plasmid transfer to plant causing disease.

Ti plasmid

Tumor inducing plasmid (Ti Plasmid) found in *Agrobacterium tumefaciens.* The presence of Ti – Plasmid is essential to cause crown gall disease in plants. It exists as an independent extrachromosomal

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entity of size 200 kb.

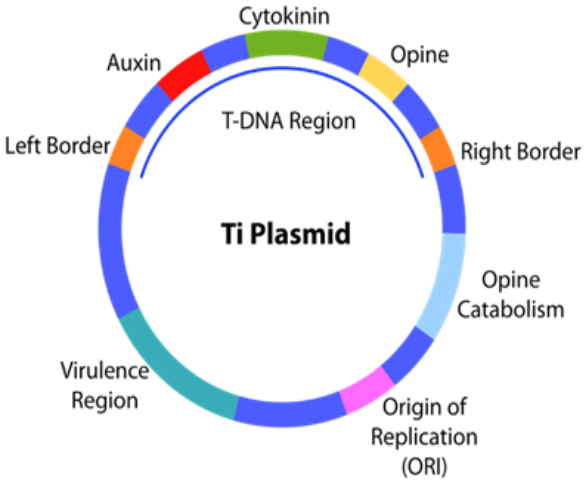

Figure: structure of Ti plasmid

This plasmid contains-

a. T- DNA region: 17-24kb long region has many subunits enclosed in the Right and Left border.
b. ORI region: this sequence is required for the origin of autonomous replication of plasmid
c. Opine catabolism: this helps to utilize opine synthesized in galls during crown gall disease in dicot plants.
d. Virulence region: this region contains a gene that helps in the transfer of T-DNA from bacteria to plant and it is 35 kb.

Ti plasmids can be classified according to the opines (Amino acid derivatives) produced:

a. Nopaline plasmids - carry genes for synthesizing nopaline in the plant and for utilization (catabolism) in the bacteria.
b. Octopine plasmids - carry genes to synthesize octopine in the plant and catabolism in the bacteria.
c. Agropine plasmids - carry genes for agropine synthesis and catabolism.

Transfer DNA (T- DNA)

Agrobacterium contains unique T-DNA in their plasmid, the nature of genes and T-DNA no. vary considerably. The bacterial T-DNA is about 24000 bp long and contains genes that code for opines and phytohormones (auxin and cytokinin).

T-DNA is bordered by both ends (Left and Right border). The transfer is initiated at the Right border and terminated at the Left border and requires vir-genes of Ti-Plasmid.

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Functions of T-DNA genes-

Genes	Products	Functions
ocs	Octopine synthase	Opine synthesis
nos	Nopaline synthase	Opine synthesis
trns1 (iaaH, auxA)	Tryptophan-2-mono-oxygenase	Auxin synthesis
trns2 (iaaM, auxB)	Indole acetamide hydrolase	Auxin synthesis
trnr (ipt, cyt)	Isopentyl-transferase	Cytokinin synthesis
trnL	unknown	Unknown, mutation affect tumor size
frs	Fructopine synthase	Opine synthesis
mas	Mannopine synthase	Opine synthesis
ags	Agropine synthase	Opine synthesis

Virulence gene (vir gene)

Virulence genes are unique regions and important components of both Ti & Ri plasmids. Most of the vir operons are involved in T-DNA transfer but their physical attachment is not essential (trans-acting). It consists of at least 10 organized operons of 35 kb having at least 21 genes -

1. Vir A – chemoreceptor of phenolic compound produced by plants and also activates vir G leading to constitutive expression of all alleles, autokinase activity
2. Vir G – transcriptional activation of vir box
3. Vir D2 – endonuclease and has a role in the integration of T-DNA in the host cell. Cuts T-DNA at the right border to initiate T-DNA synthesis
4. Vir D1 – topoisomerase activity, helps vir D2 to recognize and cleave within the 25bp border sequence
5. Vir C – promotes high-efficiency T-strand synthesis
6. Vir C1, C2 – helicase activity

7. Vir E1 – SSB protein, acts as chaperons and stabilize the Vir E2 in Agrobacterium
8. Vir E2 – binds to T-strand, protecting it from nuclease attack, and intercalates with lipids to form channels in the plant membranes through which the T-complexes passes
9. Vir B & Vir D4 – encodes membrane proteins and helps in the transfer of T-DNA. It assembles a secretion system (T4SS) which spans the inner and outer bacterial membranes. Required for export of the T-complex and Vir E2 into the plant cell
10. Vir B11 – ATPase activity
11. Vir H – detoxify the phenolic compounds
12. Vir F – degradation of the host cell during infection and it also helps in integration of T-DNA into the plant genome

Mode of action

The crown of the plant is usually located at the soil surface, this is where a plant is most likely to be wounded. During infection of the wound site by *A.tumefaciens*, two key events occur;

- The plant begins to divide and form tumors
- They begin to synthesize an arginine derivative called opine. Those opines are used as an energy source by infecting bacteria.

The entry of the bacterium is facilitated by the release of phenolic compounds (from wounded plant parts) like Acetosyringone and Hydroxysyringone. During the transformation process, T- DNA is excised from Ti plasmid, transferred to plant cells, and integrated into the DNA of plant cells.

Steps of T-DNA transfer

1. In a plant wound is created by any means
2. Phenolic compounds (acetosyringone and hydroxyacetosyringone) secreted by plants
3. Phenolic compound is sensed by Vir A gene of bacterium and it activates Vir G gene by adding phosphate (phosphorylation) on Vir G
4. Vir G activates all others Vir protein synthesis
5. Vir D2 (endonuclease) cut at Right border and attach to 5'end ss T-DNA and also helps in NLS (nucleus localization signal); Vir D1 (topoisomerase activity) helps D2
6. Vir B and Vir D4 forms T4SS

7. SS T-DNA moves to plant cell and Vir C promotes the high-efficiency transfer of T-DNA into plant cell; Vir E2 – binds to T-strand, protecting it from nuclease attack and intercalates with lipids to form channels in the plant membranes through which the T-complexes passes; Vir E1 – SSB protein, acts as chaperons and stabilize the Vir E2 in Agrobacterium
8. T-DNA moves to plant nucleus and integrates into the plant genome, Vir F helps in integration
9. Expression in a plant cell, plant cell divides rapidly due to high amount of auxin and cytokinin synthesis; form a tumor
10. Opine synthesis takes place in plant cells and it diffuses to bacteria; bacteria use opines as an energy source.

Introduction of a new gene into the plant by using Ti plasmid

It was realized very quickly that the Ti plasmid could be used to transport new genes into plant cells. It would be necessary to insert the new genes into the T-DNA and then the bacterium could do the hard work of integrating them into the plant genome. But Ti plasmid is of large size (200kb) and makes manipulation of the molecules very difficult. The main problem is the unique restriction site (impossibility with a plasmid 200 kb in size). So, novel strategies have to be developed for inserting new DNA into the plasmid-

a. The binary vector strategy
b. The Co-integrate vector strategy

The Binary Vector Strategy

The binary vector strategy is based on the observation that the T-DNA does not need to be physically attached to the rest of the T-DNA. The 2-plasmid system with the T-DNA relatively small molecule, and the rest of the plasmid in normal form, is just as effective at transforming plant cells. In fact, some strains of *A. tumefaciens* and related agrobacterium, have natural binary plasmid systems. The T-DNA plasmid is small enough to have a restriction site and to be manipulated using standard techniques.

The Co-Integrate Vector Strategy

The Co-integrate vector strategy uses an entirely new plasmid, based on an *E. coli* vector, but carrying a small portion of the T-DNA. The homology between the new molecule and the Ti-plasmid means if both are present in the same *A. tumefaciens* cell, recombination can integrate the *E. coli* plasmid into

the T-DNA region. The gene to be cloned is therefore into a unique restriction site on a small E. coli plasmid, introduced into an *A. tumefaciens* cell carrying a Ti- plasmid, and the natural recombination process is left to integrate the new gene into T-DNA. Infection of the plant leads to the new gene, along with the rest of the T-DNA, into the plant chromosome.

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