...tumefaciens.

Rhizobiaceae. ***A. tumefaciens*** is a phytopathogen, and is treated as the ***nature's most effective plant genetic engineer***. Some workers consider this bacterium as the ***natural expert of interkingdom gene transfer***. In fact, the major credit for the development of plant transformation techniques goes to the natural unique capability of *A. tumefaciens*. Thus, this bacterium is the most beloved by plant biotechnologists.

There are mainly two species of *Agrobacterium* :

- *A. tumefaciens* that induces crown gall disease.
- *A. rhizogenes* that induces hairy root disease.

CROWN GALL DISEASE AND Ti PLASMID

Almost 100 years ago (1907), Smith and Townsend postulated that a bacterium was the causative agent of crown gall tumors, although its importance was recognized much later.

As ***A. tumefaciens*** infects wounded or damaged plant tissues, in induces the formation of a ***plant tumor called crown gall*** (***Fig. 49.1***). The entry of the bacterium into the plant tissues is facilitated by the release of certain phenolic compounds (acetosyringone, hydroxyacetosyringone) by the wounded sites. Crown gall formation occurs when the bacterium releases its ***Ti plasmid*** (tumor-inducing plasmid) into the plant cell cytoplasm. A ***fragment*** (segment) ***of Ti plasmid***, referred to as ***T-DNA***, is actually transferred from the bacterium into the host where it gets integrated into the plant cell chromosome (i.e. host genome). Thus, ***crown gall disease is a naturally evolved genetic engineering process.***

The T-DNA carries genes that code for proteins involved in the biosynthesis of growth hormones (auxin and cytokinin) and novel plant metabolites namely ***opines*** — amino acid derivatives and ***agropines*** — sugar derivatives (***Fig. 49.2***). The growth hormones cause plant cells to proliferate and form the gall while opines and agropines are utilized by *A. tumefaciens* as sources of carbon and energy. As such, opines and agropines are not normally part of the plant metabolism (neither produced nor metabolised). Thus, *A. tumefaciens* genetically transforms plant cells and creates a biosynthetic machinery to produce nutrients for its own use.

As the bacteria multiply and continue infection, grown gall develops which is a visible mass of the accumulated bacteria and plant material. Crown gall formation is the consequence of the transfer, integration and expression of genes of T-DNA (or Ti plasmid) of *A. tumefaciens* in the infected plant. The genetic transformation leads to the formation of crown gall tumors, which interfere with the normal growth of the plant. Several ***dicotyledonous plants (dicots)*** are affected by crown gall disease e.g. grapes, roses, stone-fruit trees.

Organization of Ti plasmid

The Ti plasmids (approximate size 200 kb each) exist as independent replicating circular DNA molecules within the *Agrobacterium* cells. The T-DNA (transferred DNA) is variable in length in

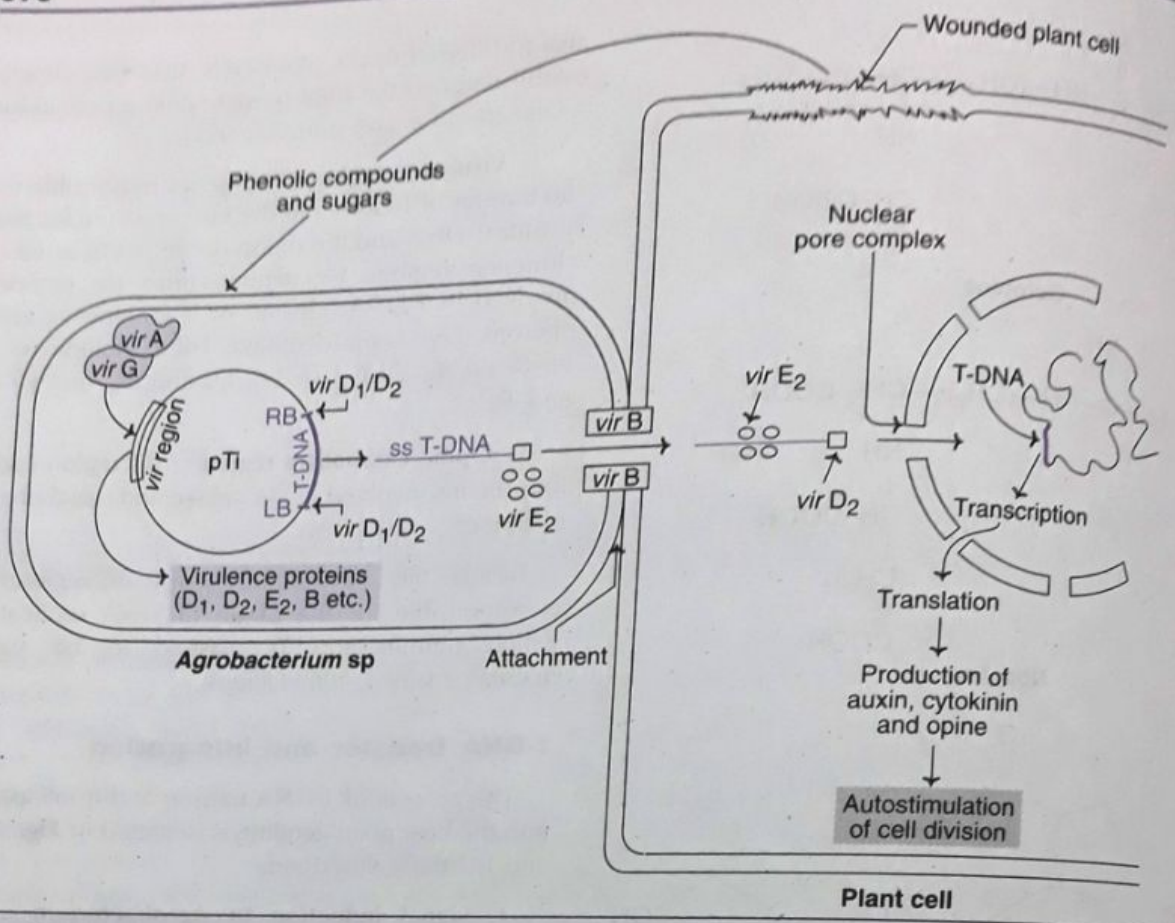

***Fig. 49.4** : A diagrammatic representation of T-DNA transfer and its integration into host plant cell genome (pTi-Ti plasmid; RB-Right border; LB-Left border; ss-Single-stranded).*

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

3. **Production of virulence proteins :** As the signal induction occurs in the *Agrobacterium* cells attached to plant cells, a series of events take place that result in the production of virulence proteins. To start with, signal induction by phenolics stimulates *vir A* which in turn activates (by phosphorylation) *vir G*. This induces expression of virulence genes of Ti plasmid to produce the corresponding virulence proteins (D_1, D_2, E_2, B etc.). Certain sugars (e.g. glucose, galactose, xylose) that induce virulence genes have been indentified.

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

5. **Transfer of T-DNA out of *Agrobacterium* :** The ss T-DNA — vir D_2 complex in association with vir G is exported from the bacterial cell. *Vir B* products form the transport apparatus.

6. **Transfer of T-DNA into plant cells and integration :** The T-DNA-vir D_2 complex crosses the plant plasma membrane. In the plant cells, T-DNA gets covered with vir E_2. This covering protects the T-DNA from degradation by nucleases. vir D_2 and vir E_2 interact with a variety of plant proteins which influences T-DNA transport and integration. The T-DNA-vir D_2-vir E_2 — plant protein complex enters the nucleus through nuclear pore complex. Within the nucleus, the T-DNA gets integrated into the plant chromosome through a process referred to ***illegitimate recombination***. This is different from the homologous recombination, as it does not depend on the sequence similarity.

HAIRY ROOT DISEASE OF A. RHIZOGENES — R_i PLASMIDS

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

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

Vectors of A. *rhizogenes*

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

Importance of hairy roots

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

Ti PLASMID-DERIVED VECTOR SYSTEMS

The ability of Ti plasmid of *Agrobacterium* to genetically transform plants has been described. It is possible to insert a desired DNA sequence (gene) into the T-DNA region (of Ti plasmid), and then use *A. tumefaciens* to deliver this gene(s) into the genome of plant cell. In this process, Ti plasmids serve as natural vectors. However, there are several ***limitations to use Ti plasmids directly as cloning vectors***.

- Ti plasmids are large in size (200–800 kb). Smaller vectors are preferred for recombinant experiments. For this reason, large segments of DNA of Ti plasmid, not essential for cloning, must be removed.
- Absence of unique restriction enzyme sites on Ti plasmids.
- The phytohormones (auxin, cytokinin) produced prevent the plant cells being regenerated into plants. Therefore auxin and cytokinin genes must be removed.
- Opine production in transformed plant cells lowers the plant yield. Therefore opine synthesizing genes which are of no use to plants should be removed.
- Ti plasmids cannot replicate in *E. coli*. This limits their utility as *E. coli* is widely used in recombinant experiments. An alternate arrangement is to add an origin of replication to Ti plasmid that allows the plasmid to replicate in *E. coli*.

Considering the above limitations, ***Ti plasmid-based vectors with suitable modifications have been constructed***. These vectors are mainly composed of the following components.

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

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

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

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

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

Cointegrate vector

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

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