SEM IV

MICROBIOLOGY CORE

Paper- MBIOCC408
Microbial Genetics

Topic- Ti Plasmid vector

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Why genetically engineer plants?

- To improve the agricultural or horticultural value of plants
- To serve as living bioreactors for the production of economically important proteins or metabolites
- To provide a powerful means for studying the biological action of genes and gene products

Smith and Townsend postulated that bacterium is the causative agent of Crown gall tumors.

Agrobacterium tumifaciens infects wounded plants and causes the formation of crown gall.

Crown gall occurs when the bacterium releases its Ti plasmid into the plant cell cytoplasm.

Agrobacterium tumefaciens is a gram negative, soil bacterium and a plant pathogen that induces tumour-like growths on plants called crown gall tumours.

Gene transfer from the bacterium to the plant occurs naturally, resulting in tumours.

Tumours can also be induced in gymnosperms and dicotyledonous angiosperms by inoculation of wound sites with *A. tumefaciens*.

Ti plasmid-: The large-sized tumor inducing plasmid found in *Agrobacterium tumefaciens*. It directs crown gall formation in certain plant species.

- A fragment 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.

- Thus, crown gall disease is a naturally evolved genetic engineering process.

- Crown gall formation is the consequence of the transfer integration and expression of genes of T-DNA of A.tumefaciens in the infected plant.

- The Ti plasmid (approx.size 200 kb each) exist as independent replicating circular DNA molecules within the ***Agrobacterium*** cells.
- The T-DNA is variable in length in the range of 12 to 24 kb.
- The Ti plasmid has three important region:-

1. **T-DNA region:** This region has the genes for the biosynthesis of *auxin* (*aux*), *cytokinin* (*cyt*) and *opine* (*ocs*), and is flanked by left and right borders.

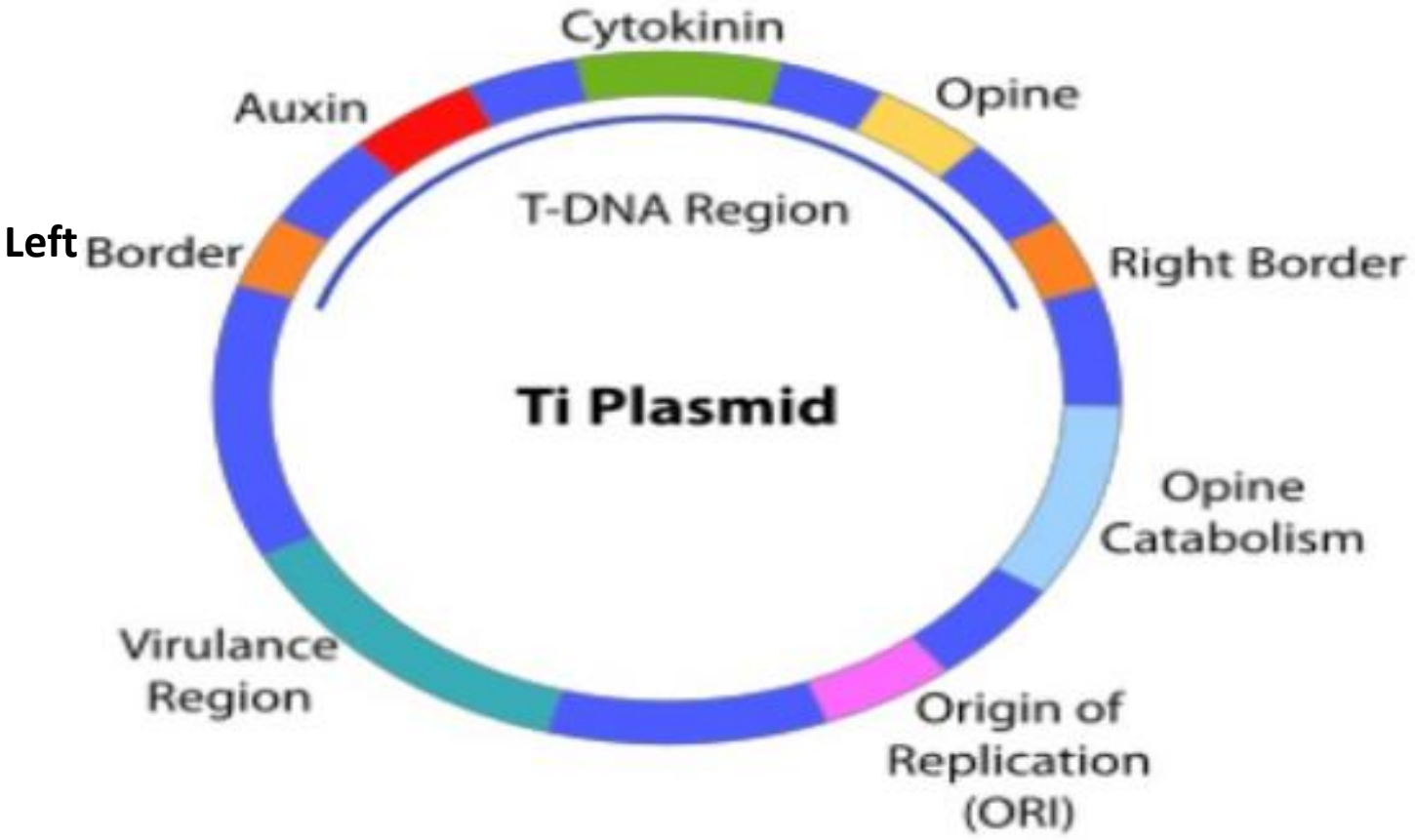
- T-DNA borders- A set of 24 kb sequences present on either side (right & left) of T-DNA are also transferred to the plant cells.
- It is clearly established that the right border is more critical for T-DNA transfer.

2. **Virulence region:** The genes responsible for the transfer of T-DNA into host plant are located outside T-DNA and the region is reffered to as *vir* or virulence region .

- At least nine vir-gene operons have been identified. These include *vir A*, *vir G*, *vir B1*, *vir C1*, *vir D1*, *D2*, *vir D4* and *vir E1,E2*.

3. **Opine catabolism region:** This region codes for proteins involved in the uptake and metabolisms of opines.

- Besides the above three there is *ori region* that responsible for origin of DNA replication which permit the Ti plasmid to be stably maintain in A. tumefaciens.



Structure of Ti plasmid

Virulence Region

- Genes in the virulence region are grouped into the operons *virABCDEG*, which code for the enzymes responsible for mediating transduction of T-DNA to plant cells.
- ***virA*** codes for a receptor which reacts to the presence of phenolic compounds such as acetosyringone, which leak out of damaged plant tissues.
- ***virB*** encodes proteins which produce a pore/pilus-like structure.
- ***virC*** binds the overdrive sequence.
- ***virD1*** and ***virD2*** produce endonucleases which target the direct repeat borders of the T-DNA segment,
- ***vir E*** Binds to T-strand protecting it from nuclease attack, and intercalates with lipids to form channels in the plant membranes through which the T-complex passes, beginning with the right border.
- ***virG*** (TRANSCRIPTIONAL FACTOR) activates vir-gene expression after binding to a consensus sequence, once it has been phosphorylated by *virA*.

Opines

- Derivatives of **amino acids** synthesized by T-DNA
- Ti plasmids can be classified according to the opines produced :
  1. Nopaline plasmids
  2. Octopine plasmids
  3. Agropine plasmids

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

Modification in Ti plasmid

- The right border sequence of T-DNA is absolutely required for T-DNA integration in to plant cell DNA.
- A multiple cloning sites that promotes the insertion of cloned gene in to the region between T-DNA borders.
- Reduction of size of Ti plasmid by removal of non essential genes.
- An origin of DNA replication that allows the plasmids to multiply in *E.coli*.
- A selectable marker gene (e.g. neomycin phosphotransferase) for appropriate selection of the transformed cells.

The Transformation strategy:

- The process of T-DNA transfer and it integration into the host plant genome are as follows:-

1. **Signal induction to Agro bacterium:-**

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

2. **Attachment of Agro bacterium to plant cells:-**

The Agro bacterium 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 Agro bacterium cells attach to plant cell, 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 gene of Ti-plasmid to produce the corresponding virulence proteins (D1,D2,E2,B etc.).
- Certain sugars (eg. Glucose, galactose, xylose) that induce virulence genes have been identified.

The Transformation strategy:

4. **Production of T-DNA strand:-**

The right and left borders of T-DNA are recognized by vir D1/vir D2 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 D2.

5. **Transfer of T-DNA out of Agro bacterium:-**

The ss T-DNA –vir D2 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 D2 complex crosses the plant plasma membrane.In the plant cells, T-DNA gets covered with *vir E2*. This covering protects the T-DNA from degradation by nucleases. *Vir D2* and *vir E2* interact with a variety of plant proteins which influences T-DNA transport and integration.

- The T-DNA – *vir D2, vir E2*- 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.*

There are two types of Ti plasmid vectors are used for genetic transformation of plant they are cointegrate vector and binary vector.

1. **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.

Production of disarmed Ti plasmid:-

The T-DNA genes for hormone biosynthesis are removed. 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 &left borders,virulence genes etc.) necessary to transfer the plant cell.

Construction of intermediate vector:-

The intermediate vector is constructed with the following components.

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

- ***A plant transformation marker(PTM):-***
- For e.g. a gene coding for neomycine phosphotransferase 2 *(npt2)*.These gene confers resistance to kanamycin in the plant cells and thus permits their isolation.

Co-integrated Vectors

- **co-integrated vectors** or **hybrid Ti plasmids**, were among the first types of modified and engineered Ti plasmids devised for *Agrobacterium* -mediated transformation, but are not widely used today.
- These vectors are constructed by homologous recombination of a bacterial plasmid with the T-DNA region of an endogenous Ti plasmid in *Agrobacterium*. Integration of the two plasmids requires a region of homology present in both.

ADVANTAGES OF COINTEGRATE VECTOR:-

Target genes can be easily cloned.

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

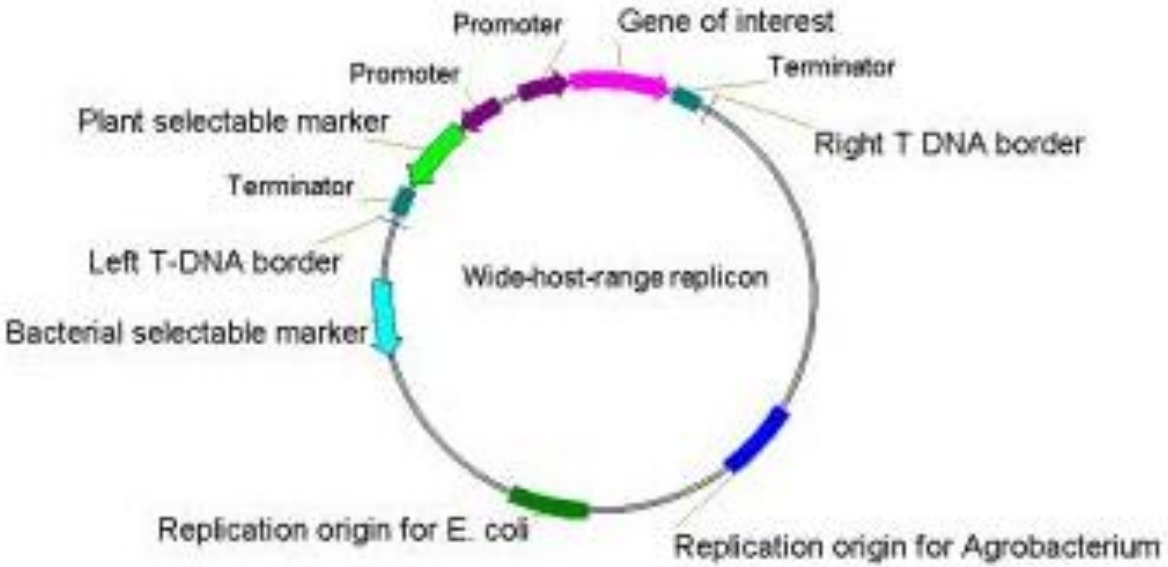
Intermediate plasmid is convenientiy cloned in *E.coli* and transferred to Agrobacterium.

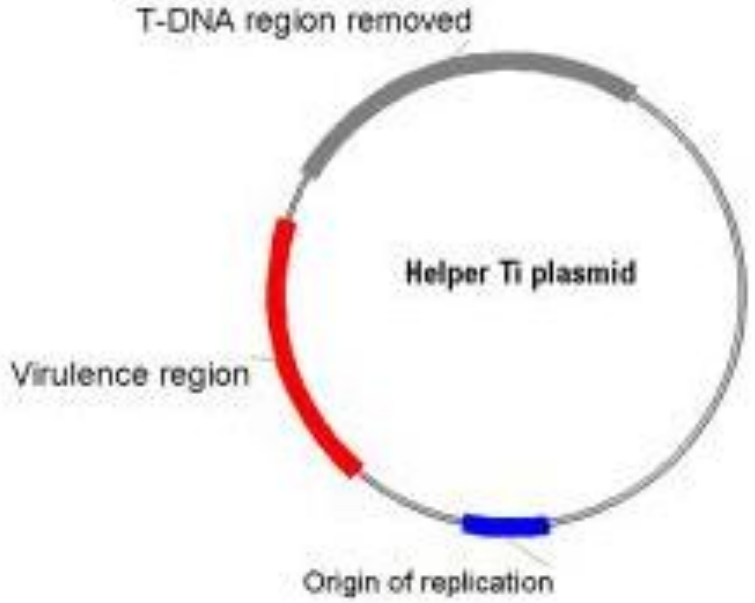
Binary Vector strategy

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

The binary vector has following components-:

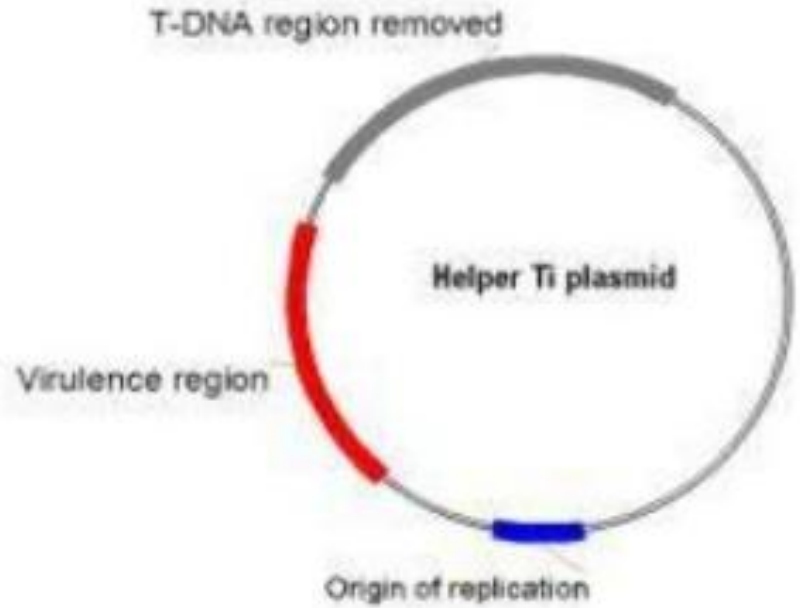
1. Left and right borders that delimit the T-DNA region.
2. A plant transformation marker (PTM) e.g. *npt2* that confers kanamycin resistance in plant transformed cells.
3. A multiple cloning site (MCS) for introducing target/foreign genes.
4. A bacterial resistance marker e.g. tetracycline resistance gene for selecting binary vector colonies in E.coli and Agrobacterium.
5. oriT sequence for conjugal mobilization of the binary vector from E.coli to Agrobacterium.
6. A broad host- range origin of replication such as *RK2* that allows the replication of binary vector in Agrobacterium.


Promoter
Gene of interest
Promoter
Terminator
Plant selectable marker
Right T DNA border
Terminator
Left T-DNA border
Wide-host-range replicon
Bacterial selectable marker
Replication origin for E. coli
Replication origin for Agrobacterium


T-DNA region removed
Helper Ti plasmid
Virulence region
Origin of replication

a **helper Ti plasmid**, harbored in *A. tumefaciens*, which lacks the entire T-DNA region but contains an intact *vir* region.

- In general, the transformation procedure is as follows:
- the recombinant small replicon is transferred via bacterial conjugation or direct transfer to *A. tumefaciens* harboring a helper Ti plasmid,
- the plant cells are co-cultivated with the *Agrobacterium*, to allow transfer of recombinant T-DNA into the plant genome, and
- transf(te conditions.



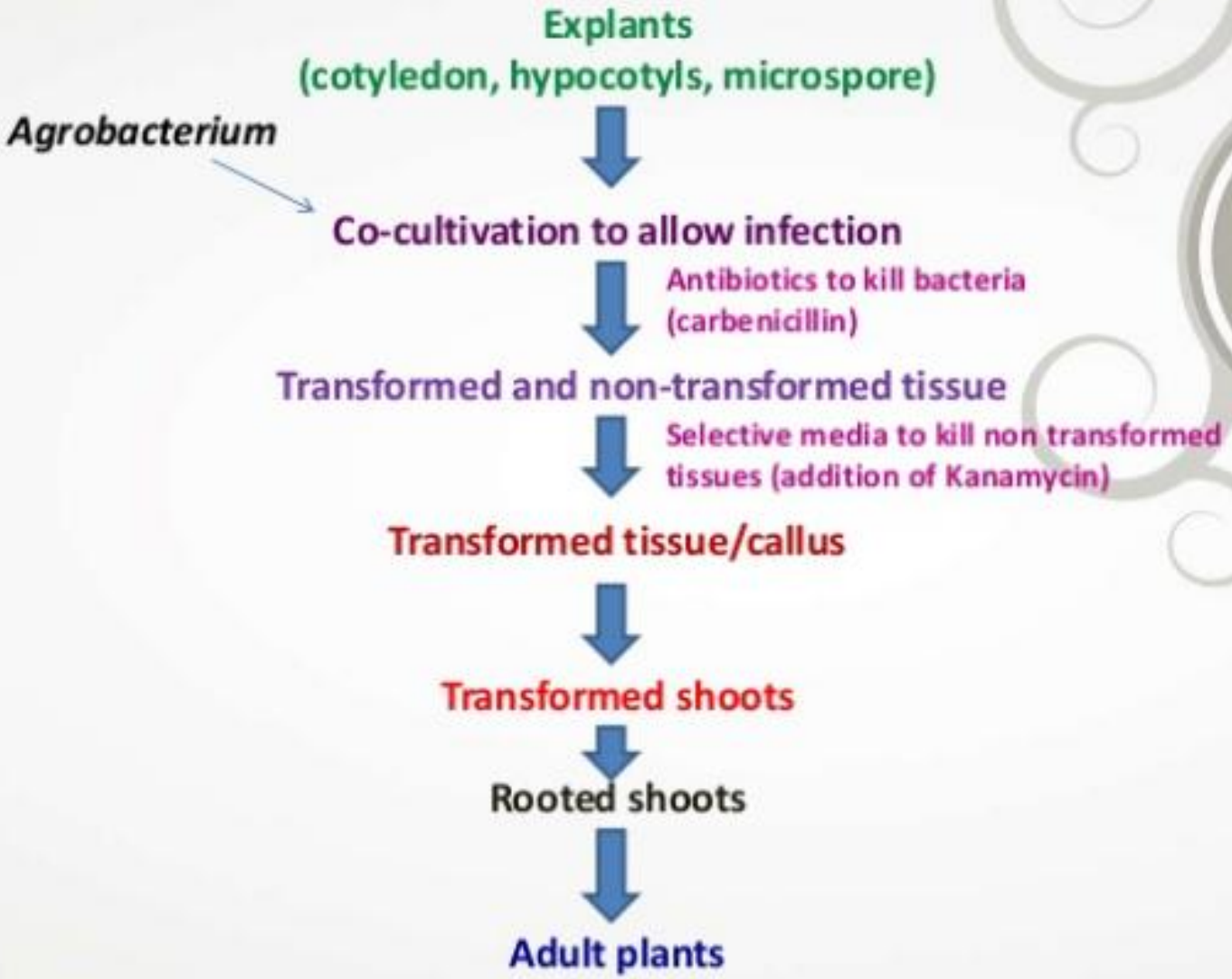
The Binary Vector has the following components:

- Left and right borders that delimit the T-DNA region.
- A multiple cloning site (MCS) for introducing target/foreign genes.
- Ori for *E.coli* and *Agrobacterium.*

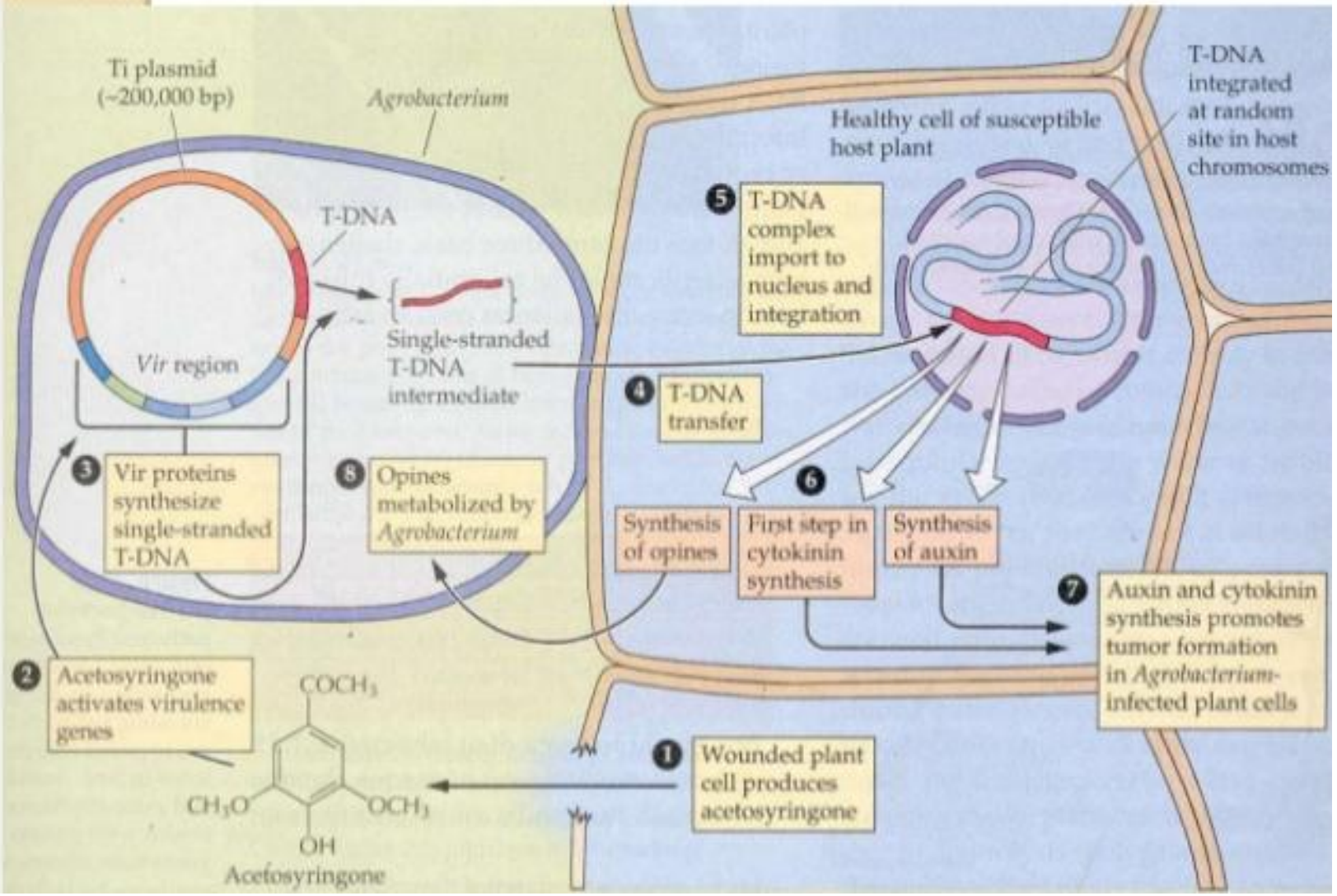
Advantages

- Compared with co-integrated vectors, binary vectors present some advantages:
- No recombination process takes place between the molecules involved.
- Instead of a very large, recombinant, disarmed Ti plasmid, small vectors are used, which increases transfer efficiency from *E. coli* to *Agrobacterium*

Process of Transformation



Overview of the Infection Process



Reference books

1. TA Brown RDT
2. Slater Plant Biotechnology
3. B.D. Singh Biotechnology