Manipulation of *Agrobacterium* for genetic engineering purposes

For more than two decades, scientists have used *Agrobacterium*-mediated genetic transformation to generate transgenic plants through replacing wild-type T-DNA coding region (mentioned in previous lecture) by any DNA sequence without any effect on its transfer from *A. tumefaciens* to the plant inspired the promise that *A. tumefaciens* might be used as gene vector to deliver genetic material into plants. Initial technologies to introduce any genes of interest into the transfer DNA (T-DNA) region of large tumor-inducing plasmids (Ti-plasmids) of *Agrobacterium* involved complex microbial genetic methodologies that inserted those genes. Basic frameworks of the current vectors for transformation of higher plants were by the removal of wild-type T-DNA, which causes tumors (tumor-inducing genes = oncogenic genes) and inhibits plant regeneration, from Ti plasmids to generate "disarmed strains" such as LBA4404.

Earlier attempts at the introduction of engineered T-DNA into *A. tumefaciens* involved the placement of genes in *E. coli* vectors that could be integrated into a disarmed Ti plasmid (*E. coli* is easier to isolate and manipulate *in vitro*, faster in growing and has higher copy number for the plasmid compared with *Agrobacterium*). This was a reasonably efficient system, but a limitation was that the final product is a plasmid larger than 150 kb in *A. tumefaciens*.

Then the binary vector system was invented, exploiting the fact that the process for transfer of T-DNA is active even if the virulence genes and the T-DNA are located on separate replicons in an *A. tumefaciens* cell. An artificial T-DNA is constructed within a plasmid that can be replicated in both *A. tumefaciens* and *E. coli*. Although the term ***binary vector*** literally refers to the entire system that consists of two replicons, one for the T-DNA and the other for the virulence genes, the plasmid that carries the T-DNA is frequently called a binary vector while the other is called **helper plasmid**.

We follow this popular and convenient terminology in the following sections. An improved binary vector called a ***'super-binary vector'*** was latterly developed. It carries additional virulence genes, such as *virB*, *virE*, and *virG*, which exhibit certain gene dosage

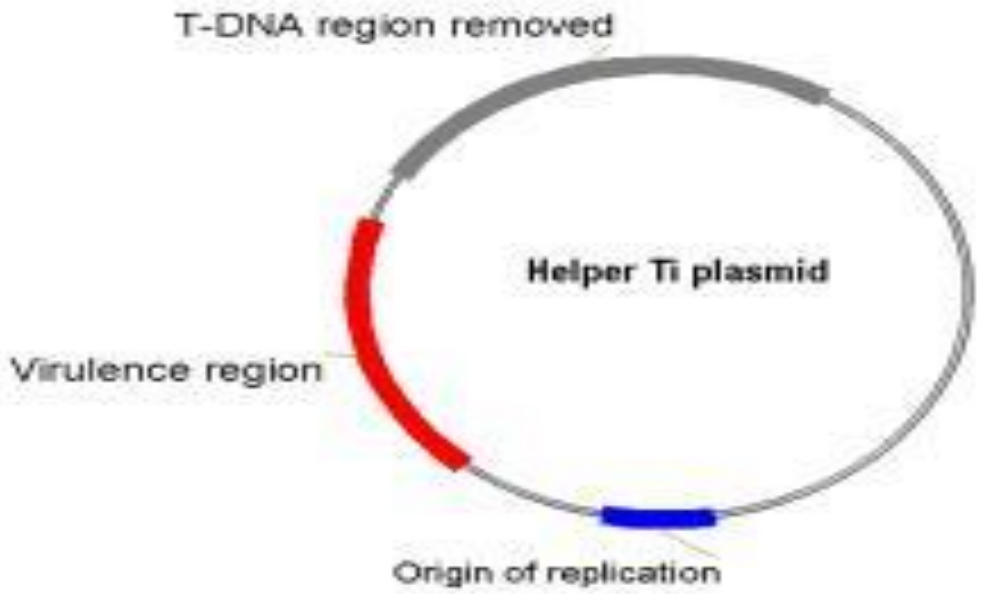
effects. Super-binary vectors exhibit very high frequency of transformation, so played an important role in the development of transformation technologies for monocotyledons.

Binary Vector system

Binary vectors systems are constructed and introduced into competent *E. coli* cells for replication then transferred to Agrobacterial cell that becomes ready to be used in transformation.

In the binary vector system, the two different plasmids employed are:

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



2) a **wide-host-range small replicon (Binary vector)**, which has a T-DNA and a vector backbone.

* The Vector backbone carries:

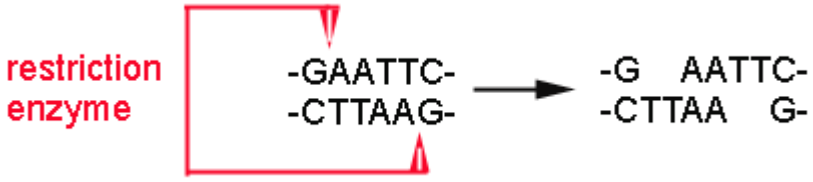
1. **bacterial selection markers:** for selection and maintenance of both manipulated *E. coli* and *A. tumefaciens* cultures. Encodes for resistance to antibiotic to **screen for bacterial** cells received the vector.
2. **an origin of replication (ori)** that permits the replication and maintenance of the plasmid in a wide range of bacteria including *E. coli* and *Agrobacterium*.

3. In addition, it carries **plasmid mobilization function** to transfer the plasmid from *E. coli* to *A. tumefaciens.* Transfer is aided by a conjugal helper plasmid.

The incompatibility group of the plasmid, with function related to the specific origin of replication, can be important if several plasmids need to co-exist in the bacterium. As such, these plasmids must belong to different incompatibility groups. In some instances, origins of replication may function in both *Agrobacterium* and in *E. coli*. These broad host range replication origins include those from RK2 (incPa; e.g. pBIN19 and derivatives), pSa (incW; e.g. pUCD plasmid derivatives), and pVS1 (e.g. pPZP derivatives). Other origins of replication that function in *Agrobacterium*, such as those from Ri-plasmids (e.g. pCGN vectors), do not function in *E. coli*; thus, a ColE1 origin (such as the one used in pUC and pBluescript plasmids) is added to the vector. Different origins of replication replicate to different extents in *Agrobacterium*. The pSa origin replicates to two to four copies per cell, the RK2 and pVS1 origins replicate to seven to 10 copies per cell, and the pRi origin replicates to 15 to 20 copies per cell.

* The T-DNA of the plasmid typically contains:

1. **introduced foreign DNA** in place of T-DNA, It may be safe to retain a few hundred bases of natural sequences adjacent to the T-DNA. A multiple cloning site (MCS) must be present to enable insertion of genes to be transferred into plant. It contains sites for restriction endonuclease produces sticky ends following treatment with any of restriction enzymes, which able the gene of interest to be inserted in for example in binary vectors, such as pBIN19 and pCAMBIA, contained a few restriction endonuclease cloning sites in a lacZ alfa fragment.



2. **the borders:** right border (RB) and left border (LB) (or at least the right T-border) that determine the T-DNA.

3. **selectable markers and screenable markers for plants:** T-DNA may contains plant selectable marker cassette (to kill non transformed cells under selection pressure) and/or plant reporter gene cassette (to demonstrate success of transformation process). The most commonly used selection systems employ are for antibiotic or herbicide resistance. Aminoglycoside antibiotics such as kanamycin or hygromycin, herbicides such as phosphinothricin/gluphosinate, or herbicide formulations such as Basta or Bialophos. Early binary vectors had these markers placed near the T-DNA right border. However, because of the polarity of T-DNA transfer (RB to LB), recent vectors contain the selectable marker near the LB to assure transfer of the gene of interest. When reporter genes being transferred, they produce certain visible changes or activities that can be easily detected through enzyme assay or histochemical reaction.
4. **Promoter and terminator:** Genes must be preceded with promoters to start transcription and followed with terminators to terminate the process. Each gene requires a promoter to decode it into a protein.
   - Terminator is a DNA fragments of a few hundred bases derived from the 3' ends of the CaMV 35S transcript, *Agrobacterium* Nos (nopaline synthase) and other T-DNA genes are carried by many of the binary vectors. It is used to stop transcription.
   - The stronger the promoter the faster the gene can be decoded and as a consequence more protein can be produced. Promoters used in biotechnology are of different types according to the intended type of control of gene expression. They can be generally divided into 4 types:
     a) **Constitutive promoters.** These promoters direct expression in virtually all tissues and are largely, if not entirely, independent of environmental and developmental factors as Cauliflower mosaic virus (CaMV) 35S, plant ubiquitin (Ubi) and Actin 1 (Act1). **CaMV 35S** is the most commonly used constitutive promoter responsible for the transcription of the whole CaMV genome and have high levels of gene expression in

dicot plants. Maize Ubi and rice Act1 are the most commonly used constitutive promoters for monocots. **Ubiquitin** is a small protein that is found in almost all cellular tissues, which helps to regulate the processes of other proteins in the body. **Actin 1** highly conserved proteins that are involved in cell cytoskeleton; plays an important role in cytoplasmic streaming, cell shape determination, cell division, organelle movement and extension growth.

b) Tissue-specific or development-stage-specific promoters. These direct the expression of a gene in specific tissue(s) or at certain stages of development. The transgenes driven by these type of promoters will only be expressed in tissues where the transgene product is desired, leaving the rest of the tissues in the plant unmodified by transgene expression.

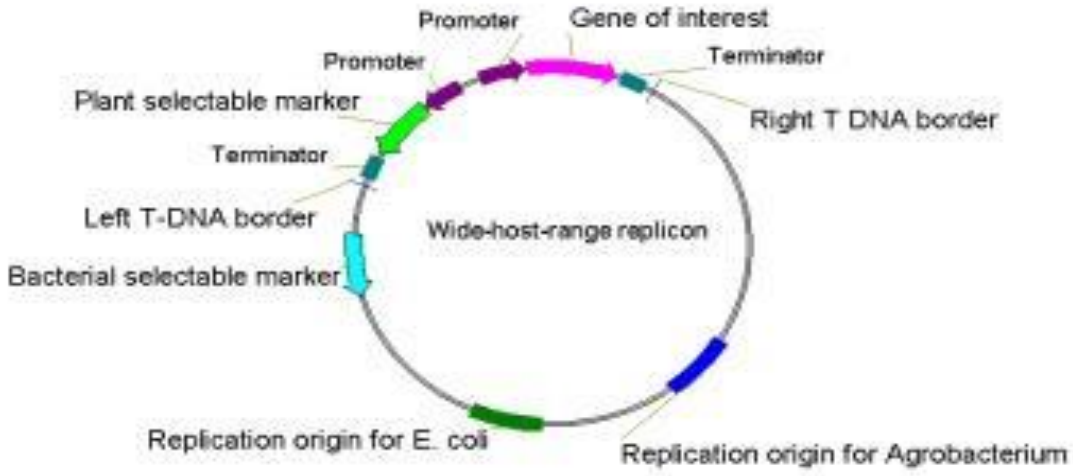
c) Inducible promoters. Their performance is not conditioned to endogenous factors but to environmental conditions and external stimuli that can be artificially controlled (turned on/off). Within this group, there are promoters modulated by chemical or physical factors:

ii. Chemically-regulated promoters, including promoters whose transcriptional activity is regulated by chemicals eg: alcohol, tetracycline, steroids, metal and other compounds.

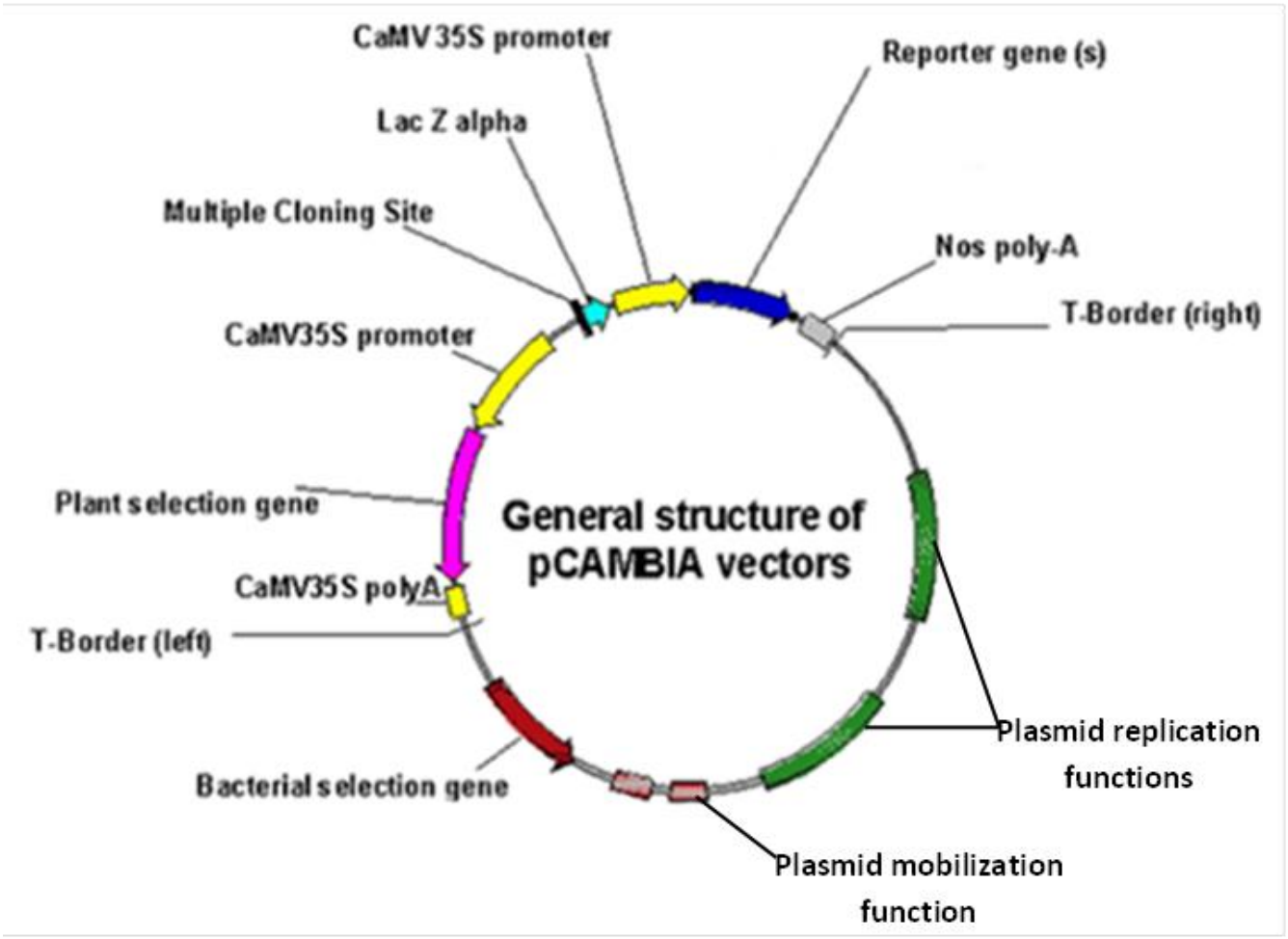
iii. Physically-regulated promoters, including promoters whose transcriptional activity is regulated by physical factors eg: light, temperatures, salinity, drought…etc.

d) Synthetic promoters. Their DNA sequences do not exist in nature and which are designed to regulate the activity of genes, controlling a gene's ability to produce its own uniquely encoded protein. Promoters made by bringing together the primary elements of a promoter region from diverse origins as the TATA element, which is the site where the

TATA-binding protein (TBP) binds. This protein is part of a complex of polypeptides that recruit the RNA polymerase II to begin transcription.



Example of a binary vector



The basic protocol used for any *Agrobacteruim* mediated transformation experiments

1. **Identification a suitable explant**: It includes selection of the explant from which a complete plant can be regenerated through a well-defined regeneration protocol.
2. **Cultivation of *Agrobacterium* in liquid medium:** *Agrobacterim* is inoculated into liquid medium (LB or YEB medium) containing the antibiotic (the resistance for which is encoded by the bacterial selectable marker gene on T-plasmid backbone) to get rid of bacterial cells that lost the vector. Cultures are maintained on shaker till its $O.D_{600}$ reach 1-1.5. **YEB medium for Agrobacterium** (1 Liter) Beef extract 5 g, Yeast extract 1 g, Peptone 5 g, Sucrose 5 g, MgSO4 300 mg. **LB medium**: 10 g/L tryptone, 10 g/L NaCl and 5 g/L yeast extract.
3. **Co-cultivation of explant with the Agrobacterium**: The excised explant is placed into a liquid culture of *Agrobacterium* supplemented with phenolic compound (eg: acetosyringone) that increases virulence of *Agrobacterium*. Different combinations between $O.D_{600}$ of bacterial culture (obtained by diluting the culture having $O.D_{600}$ value of 1-1.5 with liquid MS medium) and time of incubation are tested to determine the best combination. Too low OD_{600} or time of incubation not allow transformation while sever treatments cause necrosis of explants.

   The explants are subsequently removed from the bacterial culture and placed onto the MS medium that contain no selective agent (the antibiotic whose resistance is encoded by the plant selectable marker gene on T-DNA). The incubation time of explants with *Agrobacterium* also needed to be optimized to allow maximum transformation efficiency and avoid necrosis of explant.
4. **Killing the Agrobacterial cell with a suitable antibiotic**: The explants are removed from the medium and washed in antibiotic (cefotaxime) solution that kills Agrobacterial cells.
5. **Selection of transformed explants and regeneration of transformed plants**: The explants are transferred to fresh solid medium (the medium used for regeneration, either directly or indirectly) supplemented with a selective agent (the antibiotic whose resistance is encoded by the plant selectable marker gene on T-DNA). The medium should be also supplemented with cefotaxime to ensure killing of any remaining

Agrobacterial cells. Only transformed explants will survive and regenerate plant while explants that did not received T-DNA will die.

Regeneration on media containing the plant selectable marker is not an evidence for transformation. Plant cells may acquire resistance for such marker due to genetic variations occurring *in vitro* (somaclonal variation). Consequently, the regenerated plants must be subjected to several tests to confirm their transformation. These tests include test for presence of the reporter gene, histochemical test, PCR-based test for the gene(s) of interest and southern blot. Western blot can be also used to confirm the presence of protein encoded by transgene(s). Transgenes must be followed in next generations to confirm their presence.