The demonstration that wild-type T-DNA coding region can be replaced 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.

Basic frameworks of the current vectors for transformation of higher plants were developed in the early and mid-1980s, soon after it had been elucidated that crown gall tumorigenesis represented the genetic transformation of plant cells. The first achievement was the removal of wild-type T-DNA, which causes tumors 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 (*E. coli* is easier and faster in growing and has higher copy number for the plasmid compared with *Agrobacterium*) that could be integrated into a disarmed Ti plasmid. 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 played an important role in the development of transformation technologies for monocotyledons.

Components of a Binary Vector

Binary vectors are constructed and introduced into competent E. coli cells for replication then transferred to Agrobacterial cell that becomes ready to be used in transformation. Generally, binary vector components are divided into components on T-DNA and others on the vector backbone.

On T-DNA T-DNA borders (right border (RB) and left border (LB)) that determine the 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 enzymes produces sticky ends following treatment with any of restriction enzymes whose restriction sites present on it.

In addition to MCS, 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). A list for the most widely used selectable marker and reporter genes are listed in the next table. Genes must be preceded with promoters to start transcription and followed with terminators to terminate the process. Promoters used in biotechnology are of different types according to the intended type of control of gene expression. They can be generally divided into:

1- Constitutive promoters. These promoters direct expression in virtually all tissues and are largely, if not entirely, independent of environmental and developmental factors. Cauliflower mosaic virus (CaMV) 35S and plant ubiquitin (Ubi are examples of this type. CaMV 35S is the most commonly used constitutive promoter for high levels of gene expression in dicot plants. Maize Ubi and rice Act-1 are the most commonly used constitutive promoters for monocots.

2- 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

Selectable Markers (S) and Reporter Genes (R) Employed in Binary and Super-binary Vectors

Common abbreviations	Protein	S or R	Used for		Selection pressure
			Plants	Bacteria	
NptII, Aph 3' II, Kan	Neomycin phosphotransferase II Aminoglycoside 3' phosphotransferase II	S	Yes	Yes	Kanamycin, G418, paromomycin
Hpt, Hph, AphIV, Hyg	Hygromycin phosphotransferase Aminoglycoside phosphotransferase IV	S	Yes	Yes	Hygromycin
NptI, Kan	Aminoglycoside phosphotransferase I	S		Yes	Kanamycin
NptIII, Kan	Aminoglycoside phosphotransferase III	S		Yes	Kanamycin
Bar, Pat	Phosphinothricin acetyl transferase	S	Yes		Phosphinothricin (BASTA), bialaphos
PMI	Phosphomannose isomerase	S	Yes		Mannose as sole carbon source
Ble	Bleomycin binding protein	S	Yes		Bleomycin, phleomycin
Sul	Mutant dihydropteroate synthase	S	Yes		Sulfonamide
BSD	Blasticidin deaminase	S	Yes		Blasticidin S
Als	Mutant acetolactate synthase	S	Yes		Sulfonylurea, imidazolinone, bispyribac-sodium
AHAS	Mutant acetohydroxy acid synthase	S	Yes		Chlorsulfuron
DHFR	Dihydroforate reductase	S	Yes		Methotrexate
Gen	Gentamycin acetyltransferase	S	Yes	Yes	Gentamycin
EPSP	5-Enolpyruvylshikimate-3-phosphate synthase	S	Yes		Glyphosate (Round-up)
Ipt	Isopentenyl transferase	S	Yes		Cytokinin free
AadA, SPT, Spec	Aminoglycoside-3"-adenyltransferase Aminoglycoside nucleotidyl transferase	S	Yes	Yes	Spectinomycin, streptomycin
CAT, Cm	Chloramphenicol acetyltransferase	S, R	Yes	Yes	Chloramphenicol
Bla, Amp, Carb	β-Lactamase	S		Yes	Ampicillin, carbenicillin
Tet, TetA, TC	Tetracycline efflux protein	S		Yes	Tetracycline
Cah	Cyanamide hydratase	S	Yes		Cyanamide

Tdc	Tryptophan decarboxylase	S	Yes		4-Methyl tryptophan
XylA	Xylose isomerase	S	Yes		D-Xylose as sole carbon source
hemL, GSA-AT	Mutant glutamete-1-semialdehyde aminotransferase	S	Yes		Gabaculine
TfdA, DPAM	2,4-Dichlorophenoxyacetate monooxygenase	S	Yes		2,4-D
Bxn	3,5-Dibromo-4-hydroxybenzoic acid nitrilase	S	Yes		Bromoxynil
Pflp	Ferredoxin-like-protein	S	Yes		*Erwinia carotovora*
PPO	Mutant protoporphyrinogen oxidase	S	Yes		Butafenacil (herbicide)
DOGR	2-Deoxyglucose-6-phosphate phosphatase	S	Yes		2-Deoxyglucose
Gus, UidA	β-Glucuronidase	R	Yes		
Luc	Luciferase	R	Yes		
GFP	Green fluorescent protein[a]	R	Yes		
LacZ	β-Galactosidase	R	Yes	Yes	
Nos	Nopaline synthase	R	Yes		
R-nj	Anthocyanin	R	Yes		
OxO	Oxalate oxidase	R	Yes		

[a]A number of useful, distinctive derivatives of GFP and fluorescent proteins with different characteristics are available and reviewed in the literature

where the transgene product is desired, leaving the rest of the tissues in the plant unmodified by transgene expression.

3- Inducible promoters. Their performance is not conditioned to endogenous factors but to environmental conditions and external stimuli that can be artificially controlled. Within this group, there are promoters modulated by chemical or physical factors.

   Chemically-regulated promoters, Including promoters whose transcriptional activity is regulated by chemicals eg: alcohol, tetracycline, steroids, metal and other compounds. As prerequisites, the chemicals influencing promoter activity typically not be naturally present in the organism where expression of the transgene is sought; not be toxic; affect only the expression of the gene of interest and easy application or removal.

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

4- Synthetic promoters. Promoters made by bringing together the primary elements of a promoter region from diverse origins:

   - 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;
   - The transcription start site;
   - The CCAAT consensus sequence.

Terminator is a DNA fragments of a few hundred bases derived from the 3' ends of the CaMV 35S transcript and *Agrobacterium* Nos (nopaline synthase) and other T-DNA genes are carried by many of the binary vectors. It is used to stop transcription.

The vector backbone carries bacterial selectable marker gene and plasmid replication functions enable replication in *Agrobacterium* and *E. coli.* In addition to these components, vector backbone carries plasmid mobilization function to transfer the plasmid from *E. coli* to *A. tumefaciens.* Transfer is aided by a conjugal helper plasmid.

Binary vector may carry accessory components including:

1- Device to suppress transfer of non-T-DNA segments.

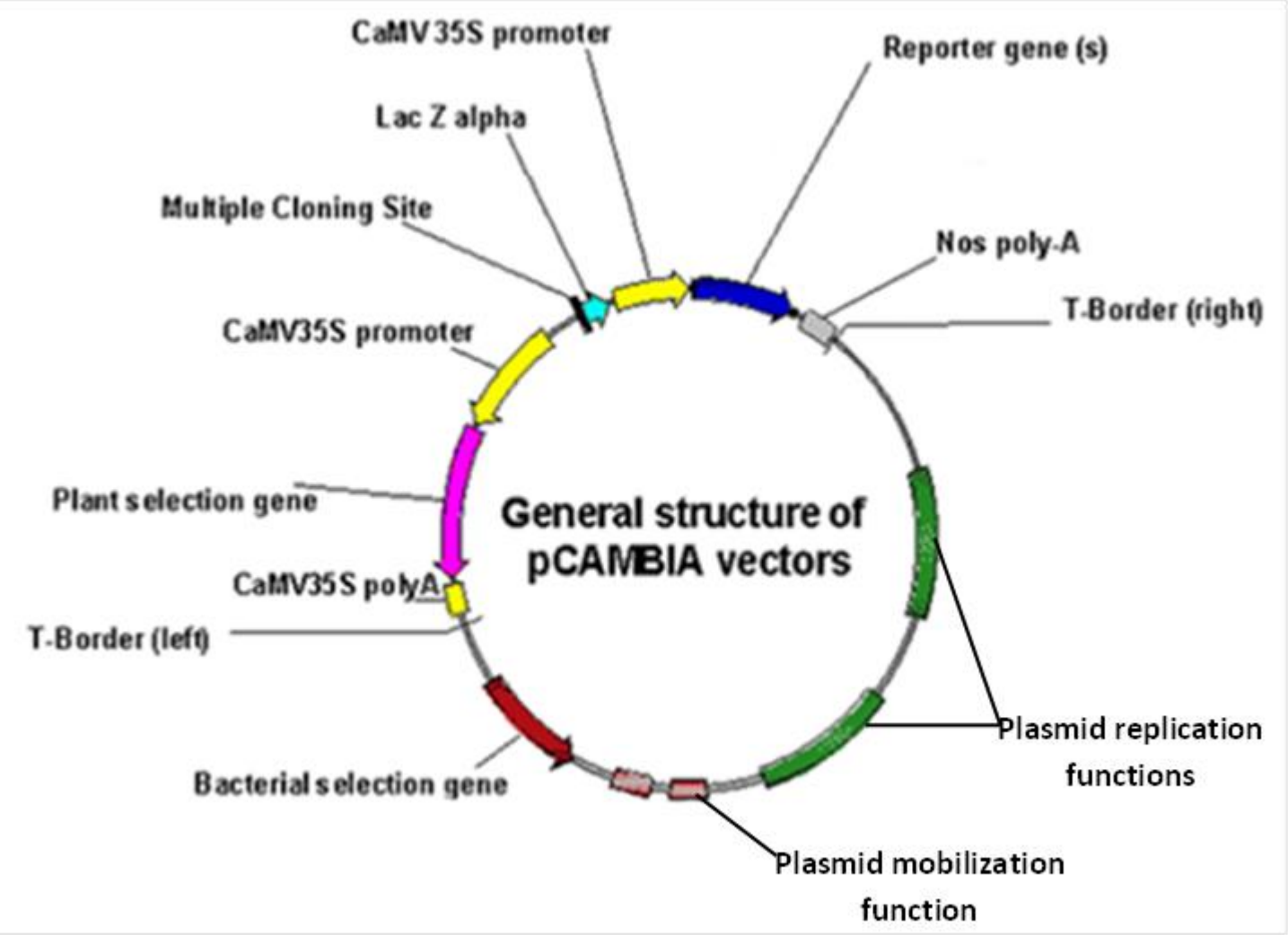
a- Multiple left border repeats. Transfer of so-called "backbone sequences" from binary vectors to higher plants is not uncommon and has raised considerable concerns over genetically modified plants. A simple method is to place additional LB sequences close to the original LB; transfer of the backbone sequences is then suppressed in a nearly perfect fashion.

b- Killer gene. Another method is to place a gene, whose gene product is lethal to cells, outside the T-DNA to eliminate transformed cells that acquired the backbone.

2- Device for removal of selectable marker genes.

Site-specific recombination systems. Many authors have reported vectors exploiting site-specific recombination systems derived from phages or fungi, such. In such a vector, a marker gene is flanked by the short target DNA sequences for a specific recombinase. After the integration of the T-DNA to plant cells, the recombinase is provided to the cells by various sophisticated means so that the marker gene is excised out. Cotransformation is another strategy to eliminate selectable marker genes. Two separate T-DNAs are used. One T-DNA carries a selectable marker gene, and the other does genes of interest. There is a good chance that these T-DNAs, segregate independently, and marker-free progeny plants are identified.

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 LEB 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.

3. **Co-cultivation of explant with the Agrobacterium**: The 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 *Agrobacterium* with a suitable antibiotic**: The explants are removed from the medium and washed in antibiotic (cefotaxime) solution that kill *Agrobacterium* 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, 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