Ti plasmid derived plant vector systems: binary and co-integrative vectors
transformation process; regeneration of the transformed lines

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Constraints of Wild type Ti/Ri-plasmid

- Very large

- **Low copy number** in *Agrobacterium*

- Difficult to isolate and manipulate in vitro

- Do not replicate in *Escherichia coli*, the favored host for genetic manipulation.

- **T-DNA regions** from wild-type Ti-plasmids are generally large and do not contain unique restriction endonuclease sites suitable for cloning a gene of interest.

- In addition, scientists wanted to eliminate *oncogenes* from T-DNA to regenerate normal plants. The phytohormone produced by transformed cells growing in culture prevents their regeneration into mature plants. Hence **auxins and cytokinin** genes must be removed from the Ti –plasmid derived cloning vector.

- **Opine synthase genes** were also generally deemed superfluous in constructions designed to deliver goi to plants.
Features of efficient vector for plant transformation

- A selectable marker gene that confers resistance to transformed plant cells. As these marker genes are prokaryotic origin, it is necessary to put them under the eukaryotic control (plant) of post transcriptional regulation signals, including promoter and a termination-polyadenylation sequence, to ensure that it is efficiently expressed in transformed plant cells.

- An origin of replication that allows the plasmid to replicate in *E. coli*.

- The right border sequence of the T-DNA which is necessary for T-DNA integration into plant cell DNA.

- A polylinker (MCS) to facilitate the insertion of cloned gene into the region between T-DNA border sequences.
Co-integrate vectors

• Co-integrated vectors or **Hybrid Ti** plasmids

• 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.
Co-integrate vectors

Three vectors are necessary in this system:

A. Disarmed Agrobacterium Ti plasmids

In these Ti plasmids, the oncogenes located in the T-DNA region have been replaced by exogenous DNA.

Examples of these vectors include:

- **SEV series**: the right border of the T-DNA together with the phytohormone genes coding for cytokinin and auxin are removed and replaced by a bacterial kanamycin resistance gene while the left border and a small part of the left segment (TL) of the original T-DNA (referred to as Left Inside Homology (LIH)) are left intact.

- **pGV series**: the phytohormone genes are excised and substituted by part of pBR322 vector sequence. The left and right border sequences as well as the nopaline synthase gene of the Ti plasmid are conserved.
Ti-plasmid derived Plant vectors: Disarmed Ti-plasmid vectors
Prototype disarmed Ti vectors

- Wild-type Ti plasmids not suitable as vector because T-DNA contains oncogenes that cause disorganized growth of the recipient plant cells.
- Substitute pBR322 sequences for almost all of the T-DNA, leaving only the left and right border regions and the nos gene to construct pGV3850.
- *Agrobacterium* carrying pGV3580 transferred to plant cells, no tumor cells were produced, but transformed cells can produce nopaline.
- besides *ocs* and *nos*, drug resistance genes and herbicide resistance genes are widely used as selectable markers
Co-integrate vectors

Three vectors are necessary in this system:

B. Intermediate vectors
- Small pBR322-based plasmids (E. coli vectors) containing a T-DNA region.
- Used to overcome the problems derived from the large size of disarmed Ti plasmids and their lack of unique restriction sites.
- Intermediate vectors are replicated in *E. coli* and are transferred into Agrobacterium by conjugation. They cannot replicate in *A. tumefaciens* and therefore, carry DNA segments homologous to the disarmed T-DNA to permit recombination to form a co-integrated T-DNA structure.

C. Helper vectors
These are small plasmids maintained in E. coli that contain transfer (tra) and mobilization (mob) genes, which allow the transfer of the conjugation-deficient intermediate vectors into Agrobacterium.
• Conjugation between the two *E. coli* strains transferred the helper plasmid to the carrier of the intermediate vector, and then transferred to *A. tumefaciens*.

• Homologous recombination between the T-DNA in intermediate vector and Ti plasmid to form a large co-integrate plasmid which is facilitated by native *Agrobacterium* rec functions.

• The recombinant T-DNA is transferred to plant genome.
Intermediate vector transferred into *Agrobacterium* by conjugation. Unable to replicate autonomously in *Agrobacterium*.

Disarmed Ti-vector pGV3850 resident in *Agrobacterium*.

Homologous recombination between DNA regions derived from pBR322.

Cointegrate formation. Select for maintenance of cointegrate by kanamycin.

Cointegrate disarmed Ti-plasmid.
A resulting co-integrated plasmid assembled by in vitro manipulation normally contains:

- the **vir genes**,
- the left and right **T-DNA borders**, 
- an **exogenous DNA sequence** between the two T-DNA borders,
- plant and bacterial (**E. coli and A. tumifaciens**) **selectable markers**,
- **E. coli** functional **origin of replication that doesn’t operate in Agrobacterium**

**Disadvantages:**

- Long region of homologies required between the Ti plasmid and the E. coli plasmids (pBR322 based intermediate vectors) making them difficult to engineer and use
- Relatively inefficient gene transfer compared to the binary vectors
**Binary vector strategy: two vector strategy**

- Systems in which T-DNA and vir genes are located on separate replicons were eventually termed T-DNA binary systems.
- Consists of a pair of autonomously replicating plasmid vectors.
- Based on the knowledge that vir region need not be in the same plasmid along with T-DNA for transfer.
Binary vector strategy: two vector strategy
Schematic diagram of co-integration/exchange systems and T-DNA binary vector systems to introduce genes into plants using Agrobacterium-mediated genetic transformation.
• Binary vector/shuttle vector:
  – disarmed Ti-plasmid with gene of interest between T-DNA borders + ori for both *E. coli* and *Agrobacterium*
  – also called as mini-Ti or micro Ti plasmid

• Helper Ti-plasmid:
  – with virulence region that mediates transfer of T-DNA in micro Ti-plasmid to the plant
  – Constructed by removing the T-DNA
• Two different approaches have been mediated:
  – Binary vector with two origin of replication: one for *E.coli* and the other for *A. tumifaciens*
  – Binary vector with single broad host range origin of replication

• In either case no *vir* genes are present on binary cloning vector

• The *vir* genes present in a disarmed Ti plasmid from which the T-DNA has been removed synthesize vir proteins that eventually mobilize the T-DNA region of the binary cloning plasmid vector into the target plant cells
Advantages of Binary vector

- Binary vectors don’t demand in-vivo recombination as in co-integrate vectors
- Binary vectors are more efficient and easier to obtain
- In binary system the binary plasmids exist as separate replicons and thus their copy number remains flexible
- The size of the binary vectors are relatively small and thus easier to manipulate
Genetically engineered Ti-plasmid vectors

**Binary systems**

- Needs 2 vectors:
  - Disarmed Ti plasmid with gene of interest (no vir genes)
  - Helper vector for infection (with vir genes)

**Co-integrated vectors**

- Needs 3 vectors
- Disarmed Ti plasmid capable for infection
- Intermediate vector with T-region and gene of interest (transferred by conjugation)
- Helper vector for transfer of intermediate plasmid into *A. tumifaciens*

Form co-integrated plasmid after homologous recombination on T-DNA
Super Binary Vectors

- One of the approaches toward enhancing the frequency of transformation by binary vectors is to employ additional virulence genes, such as virB, virE, and virG, which exhibit certain gene dosage effects.

- In the super-binary vector system, a DNA fragment that contains virB, virC, and virG from pTiBo542 is introduced into a small T-DNA-carrying plasmid.

- *A. tumefaciens* strains that carry pTiBo542 are wider in host range and higher in transformation efficiency than strains that carry other Ti plasmids, such as pTiA6 and pTiT37.

- Super-binary vectors are highly efficient in the transformation of various plants.
ALTERNATIVE T-DNA BINARY SYSTEMS

- Although T-DNA binary vector systems almost always consist of T-DNA and vir regions localized on plasmids, it is not essential that they function this way.
- Replicons containing T-DNA or vir genes do not need to be plasmids. Indeed, several laboratories have shown that T-DNA can be integrated into an Agrobacterium chromosome and launched from this replicon, and specialized vectors have been generated to facilitate integration of DNA into a specific neutral region of the chromosome of A. tumefaciens C58.
- Although launching T-DNA from the Agrobacterium chromosome can result in lower transformation frequencies, this process has the beneficial consequences of reducing integrated transgene copy number and almost completely eliminating integration of vector backbone sequences into the plant genome.
• Regeneration of transformed lines

- Small discs punched from leaf
- Surface-sterilize
- Inoculation: Culture overnight in liquid with *Agrobacterium* to infect cut edges of disc
- Blot disc dry
- Shoot-inducing solid medium + kanamycin + carbenicillin
  - Culture for about 20 days
- Leaf disc
  - Filter paper
  - A layer of feeder cells, previously grown in suspension
- Shoot-inducing solid medium (high in cytokinin)
  - Culture for 2 days
- Root-inducing solid medium (high in auxin) + kanamycin + carbenicillin
- Excise shootsed callus, transfer to root-inducing medium
- Transfer plantlets to soil as soon as roots appear 4–7 weeks after inoculation
Assignment

• Explain the constraints of using wild type Ri plasmid. [3]
• Elaborate about the binary vector system and their variations. [7.5]
• Differentiate between Binary and Co – integrate vectors. [3]
• Differentiate between Super Binary and Co – integrate vectors. [1]
• Explain about various types of vectors used for preparation of Co – integrate and Binary Vectors. [3]