Transgenic plants

All stable transformation methods consist of three steps:

- Delivery of DNA into a single plant cell.
- Integration of the DNA into the plant cell genome.
- Conversion of the transformed cell into a whole plant.

PRODUCTION OF TRANSGENIC PLANTS

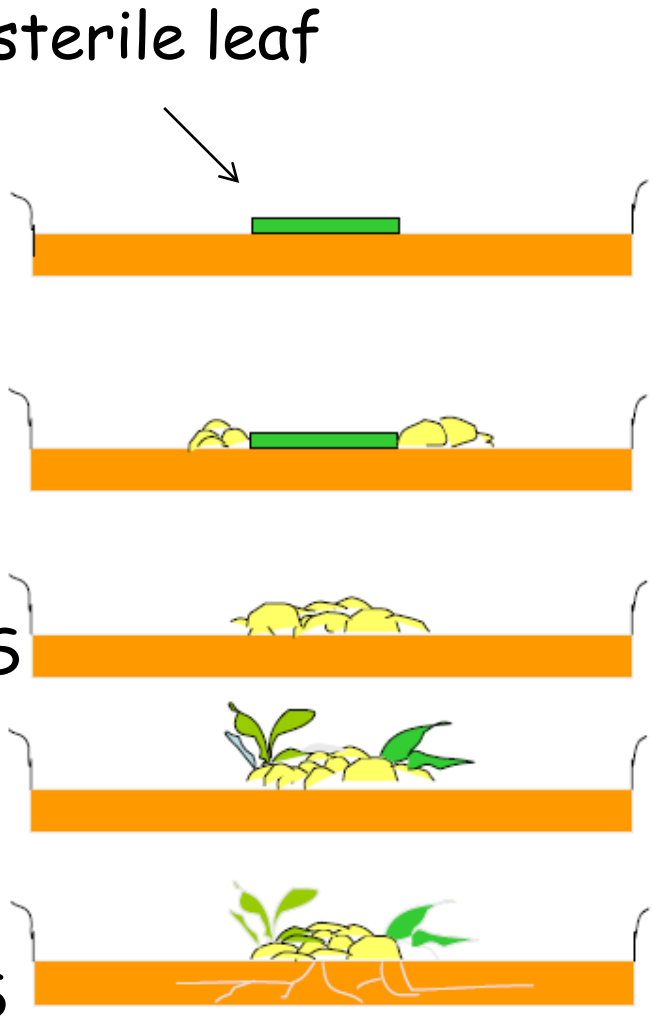
Plant cells are TOTIPOTENT → base to create transgenic plants

Possibility to create a entire plants from a single differentiated cell

in vitro it is possible to generate a plant by using different auxin and cytochin ratio (both are plant hormon that regulate proliferation and differentiation)

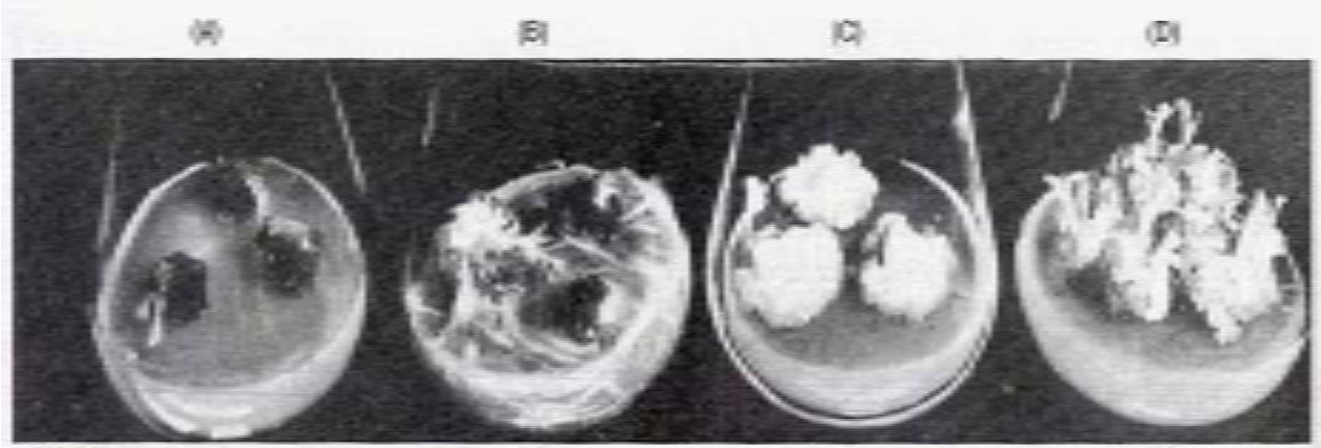
- CALLUS : cellular mass not committed.
- BUD
- ROOT

IN VITRO PLANT RIGENERATION



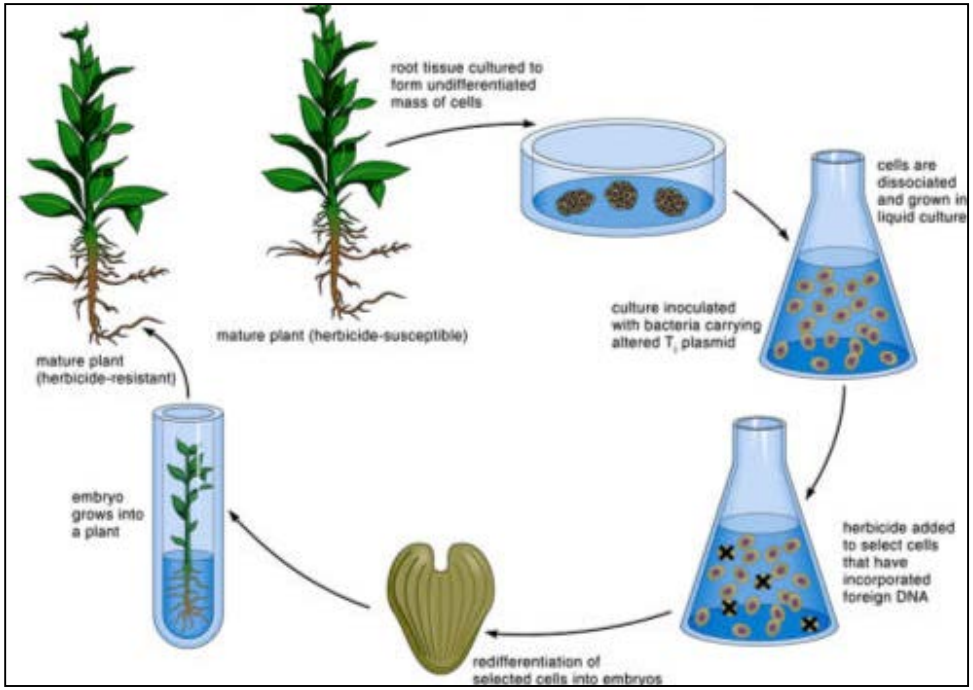
IN VITRO PLANT RIGENERATION

roots buds



Auxina/Citochina Auxina/**Citochina**

PRODUCTION OF TRANSGENIC PLANTS



1) Agrobacterium tumefaciens
2)Gen gun

Genetic Engineering of Plants

- Must get DNA:
  1. into the cells
  2. integrated into the genome (unless using transient expression assays)
  3. expressed (everywhere or controlled)

- For (1) and (2), two main approaches for plants:
  1. *Agrobacterium* - mediated gene transfer
  2. Direct gene transfer

- For (3), use promoter that will direct expression when and where wanted – may also require other modifications such as removing or replacing introns.

Agrobacterium - mediated Gene Transfer

- Most common method of engineering dicots, but also used for monocots
- Pioneered by J. Schell (Max-Planck Inst., Cologne)

- *Agrobacteria*
  - soil bacteria, gram-negative, related to *Rhizobia*
  - species:

    tumefaciens- causes crown galls on many dicots

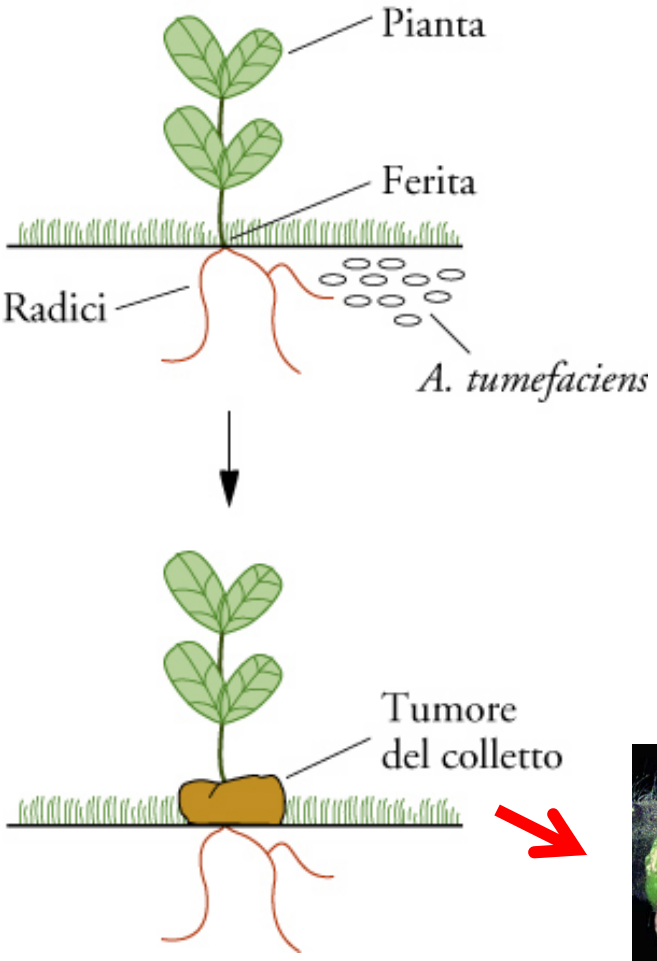
    rubi- causes small galls on a few dicots

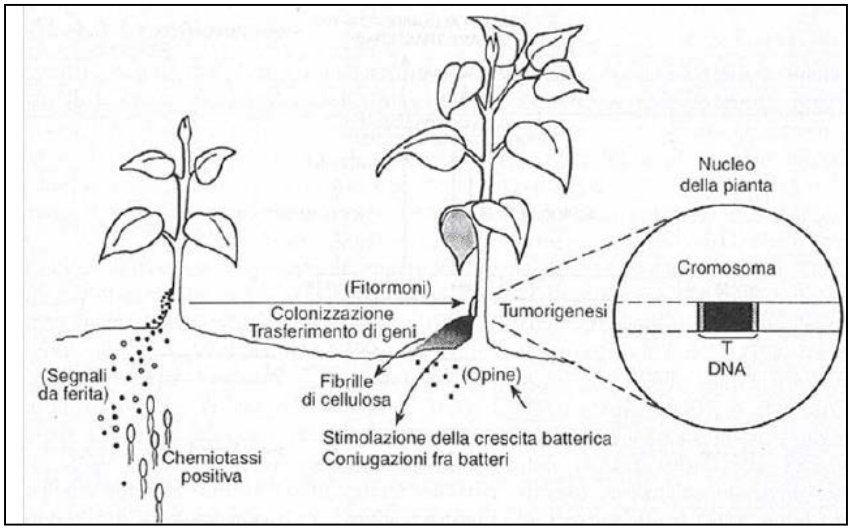
    rhizogenes- hairy root disease

    radiobacter- avirulent

Agrobacterium tumefaciens

Batterio del suolo Gram-
Fitopatogeno che trasforma geneticamente le piante colpite
COLPISCE LE DICOTILEDONI (vite, rose, piante frutto carnoso e seme legnoso)





Infection and tumorigenesis

- Infection occurs at wound sites
- Involves recognition and chemotaxis of the bacterium toward wounded cells
- galls are “real tumors”, can be removed and will grow indefinitely without hormones
- genetic information must be transferred to plant cells

Tumor characteristics

1. Synthesize a unique amino acid, called “opine”
   - octopine and nopaline - derived from arginine
   - agropine - derived from glutamate
2. Opine depends on the strain of *A. tumefaciens*
3. Opines are catabolized by the bacteria, which can use only the specific opine that it causes the plant to produce.
4. Has obvious advantages for the bacteria, what about the plant?

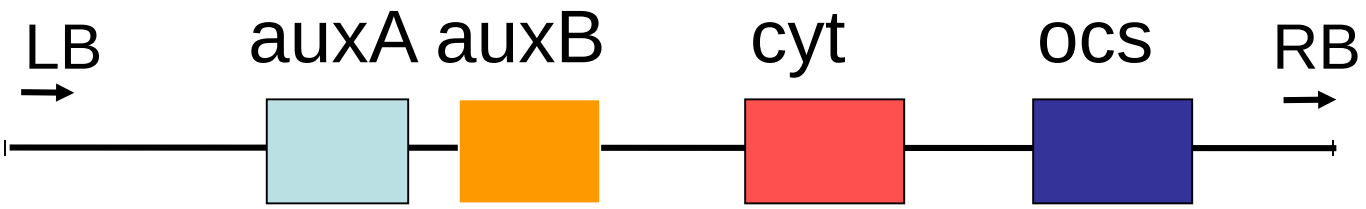
Elucidation of the TIP (tumor-inducing principle)

- It was recognized early that virulent strains could be cured of virulence, and that cured strains could regain virulence when exposed to virulent strains; suggested an extra-chromosomal element.
- Large plasmids were found in *A. tumefaciens* and their presence correlated with virulence: called tumor-inducing or Ti plasmids.

Ti Plasmid

1. Large (□200-kb)
2. Conjugative
3. ~10% of plasmid transferred to plant cell after infection
4. Transferred DNA (called **T-DNA**) integrates semi-randomly into nuclear DNA
5. Ti plasmid also encodes:
   - enzymes involved in opine metabolism
   - proteins involved in mobilizing T-DNA (*Vir* genes)

T-DNA



LB, RB – left and right borders (direct repeat)
auxA + *auxB* – enzymes that produce auxin
cyt – enzyme that produces cytokinin
Ocs – octopine synthase, produces octopine

These genes have typical eukaryotic expression signals!

auxA *auxB*

Tryptophan → indoleacetamide → indoleacetic acid (auxin)

cyt

AMP + isopentenylpyrophosphate → isopentyl-AMP (a cytokinin)

- Increased levels of these hormones stimulate cell division.

- Explains uncontrolled growth of tumor.

Vir (virulent) genes

1. On the Ti plasmid
2. Transfer the T-DNA to plant cell
3. Acetosyringone (AS) (a flavonoid) released by wounded plant cells activates *vir* genes.
4. *virA,B,C,D,E,F,G* (7 complementation groups, but some have multiple ORFs), span about 30 kb of Ti plasmid.

Ti plasmids and the bacterial chromosome act in concert to transform the plant

1. *Agrobacterium tumefaciens* chromosomal genes: chvA, chvB, pscA required for initial binding of the bacterium to the plant cell and code for polysaccharide on bacterial cell surface.

2. Virulence region (*vir*) carried on pTi, but not in the transferred region (T-DNA). Genes code for proteins that prepare the T-DNA and the bacterium for transfer.

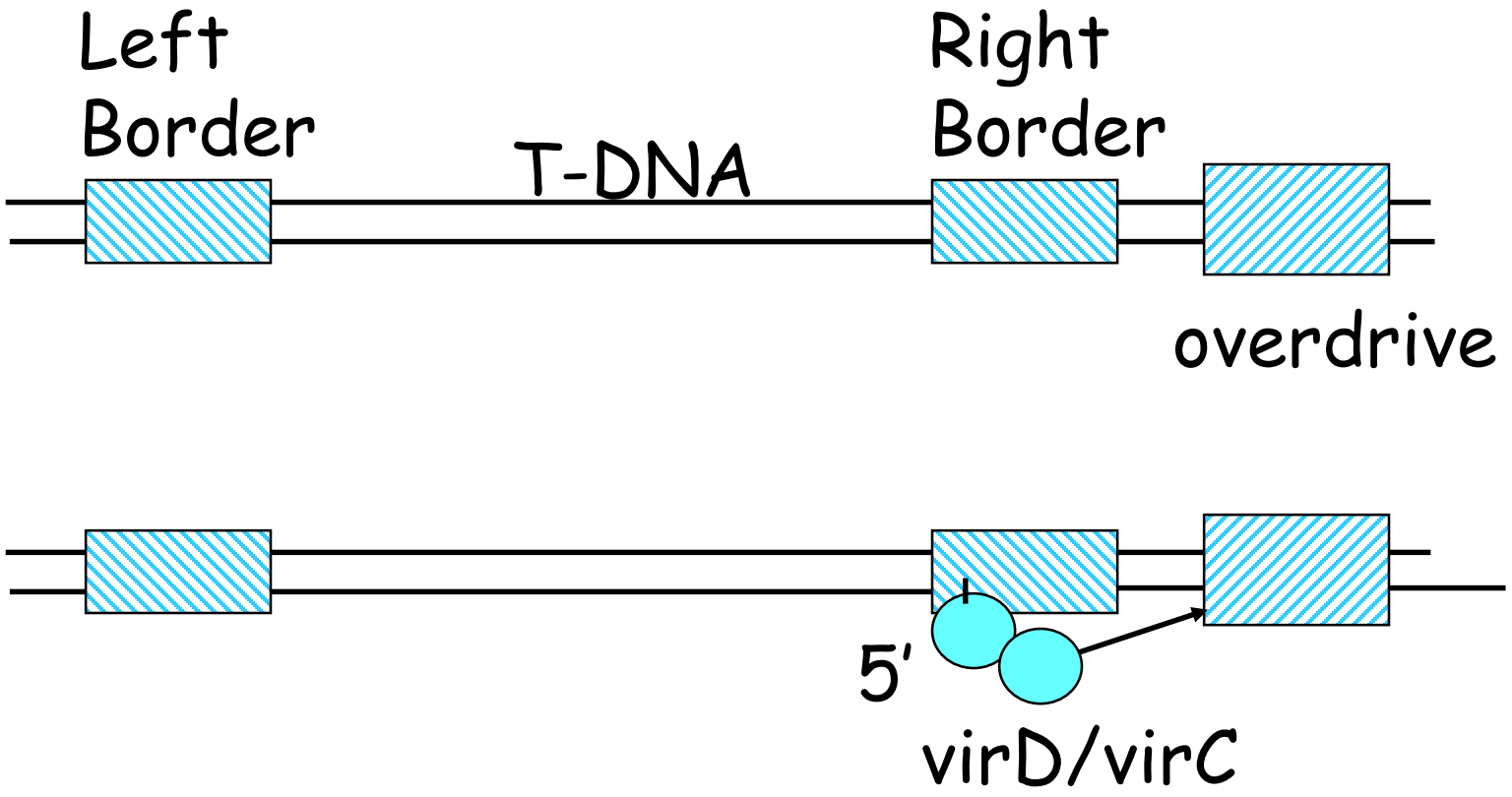
3. T-DNA encodes genes for opine synthesis and for tumor production.

4. *occ* (opine catabolism) genes carried on the pTi allow the bacterium to utilize opines as nutrient.

Vir gene functions (cont.)

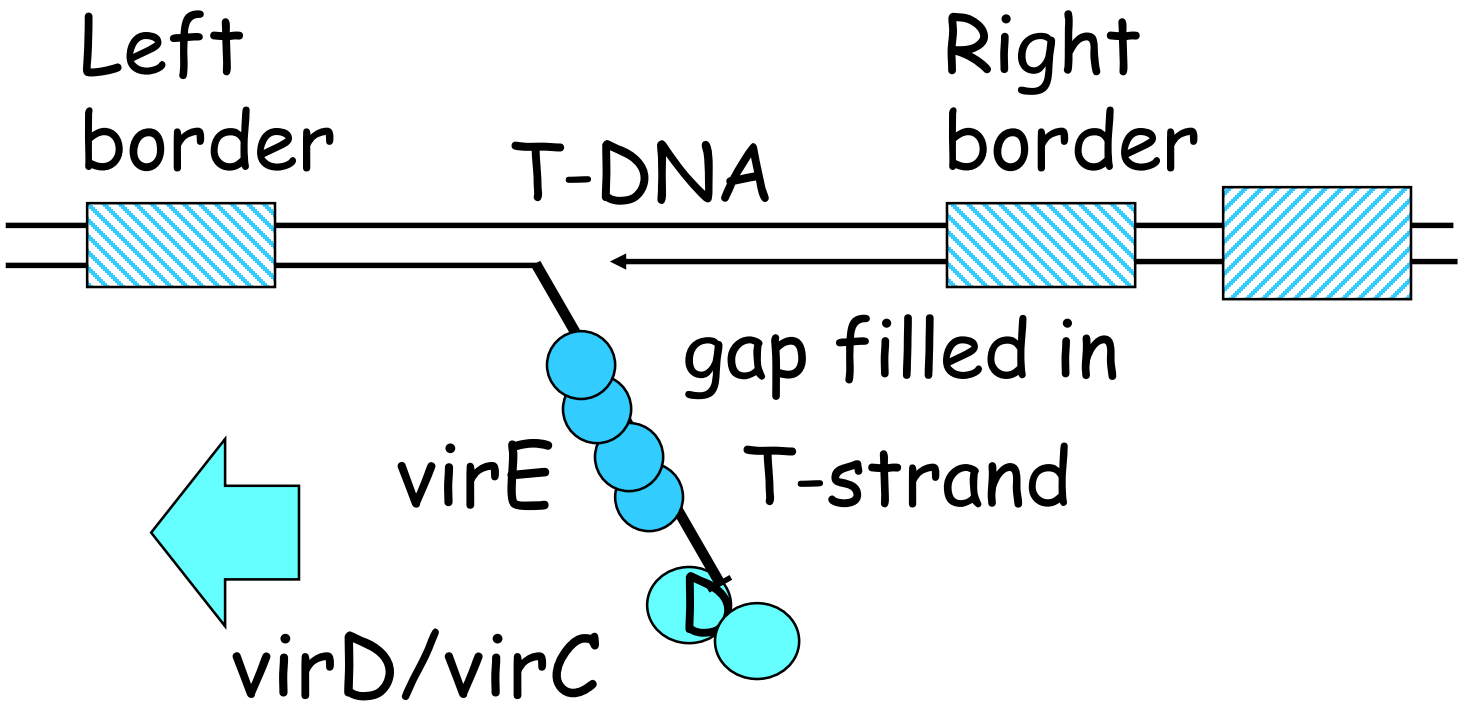
- *virA* - transports AS into bacterium, activates *virG* post-translationally (by phosphoryl.)
- *virG* - promotes transcription of other *vir* genes
- *virD2* - endonuclease/integrase that cuts T-DNA at the borders but only on one strand; attaches to the 5' end of the SS
- *virE2* - binds SS of T-DNA & can form channels in artificial membranes
- *virE1* - chaperone for *virE2*
- *virD2* & *virE2* also have NLSs, gets T-DNA to the nucleus of plant cell
- *virB* - operon of 11 proteins, gets T-DNA through bacterial membranes

Generation of the T-strand



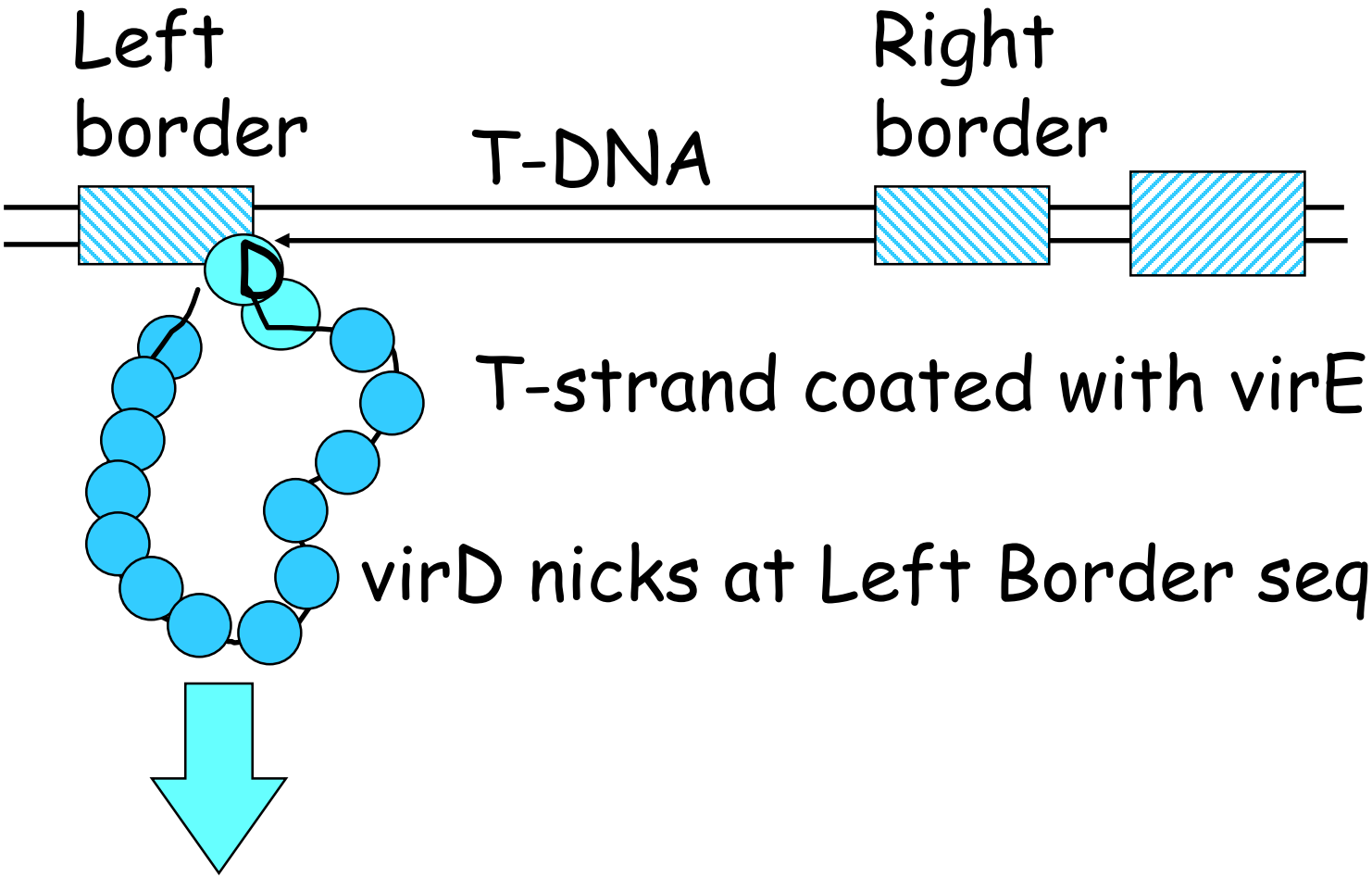
VirD nicks the lower strand (T-strand) at the right border sequence and binds to the 5′ end.

Generation of the T-strand



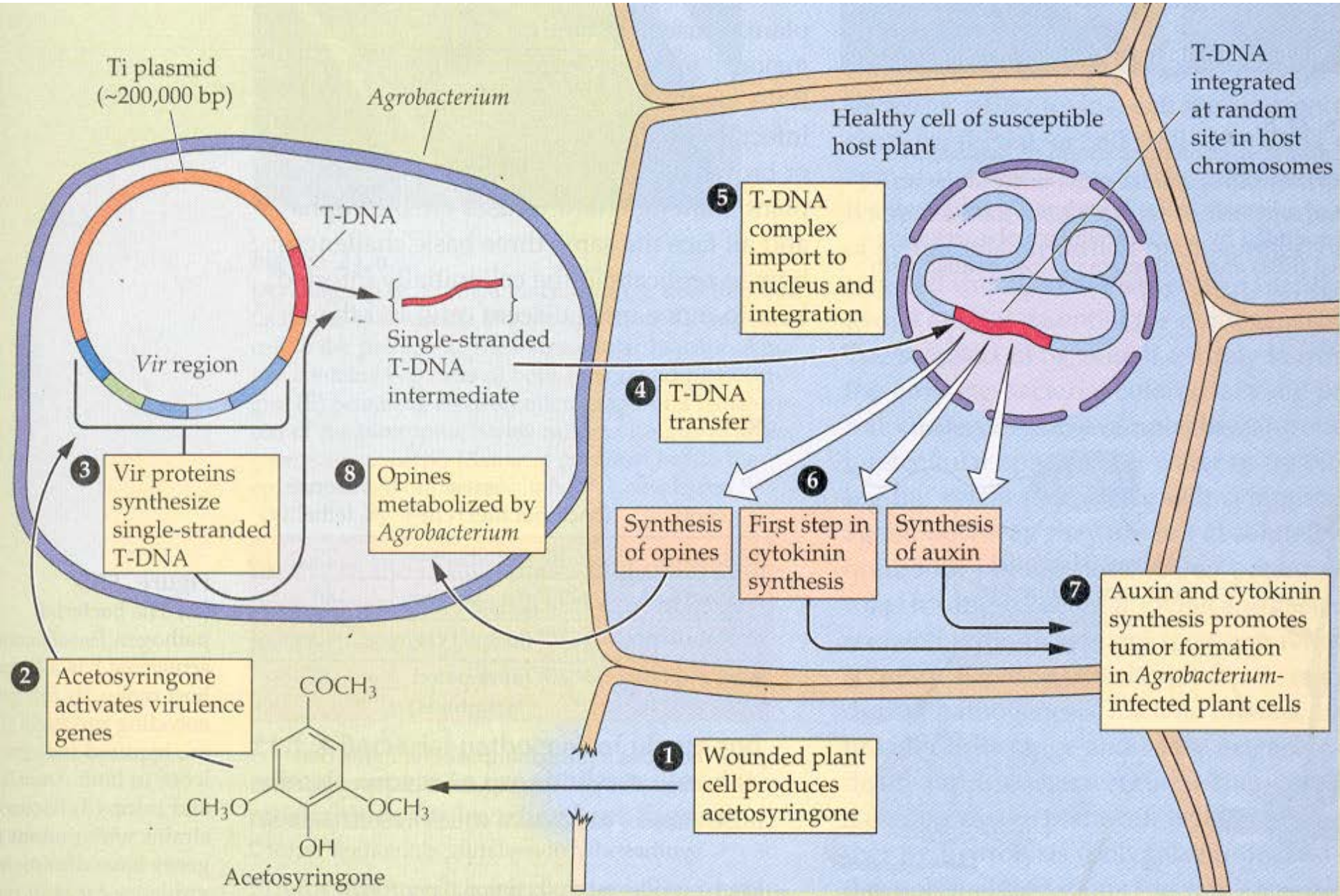
1. Helicases unwind the T-strand which is then coated by the virE protein.

2. ~one T-strand produced per cell.

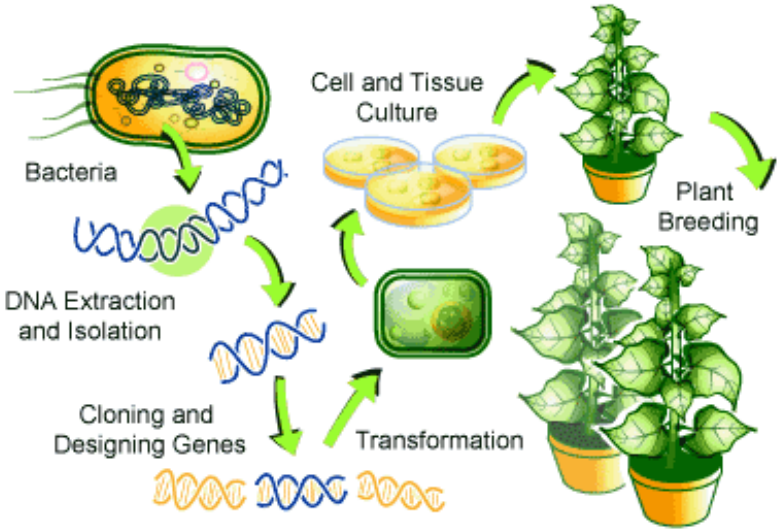


1. Transfer to plant cell.
2. Second strand synthesis
3. Integration into plant chromosome

Overview of the Infection Process



Agrobacterium tumefaciens for TRANGENIC PLANTS



Drawbacks:

1) Auxine/Cytochine made by T-DNA do not allow proper plant regeneration
2) Opine is not usefull for plant
3) Ti plasmids are big (200-800Kb)
4) Monocots don't produce AS in response to wounding.
5) couldn't regenerate plants from tumors

Important: Putting any DNA between the LB and RB of T-DNA it will be transferred to plant cell!

Agrobacterium tumefaciens for TRANGENIC PLANTS

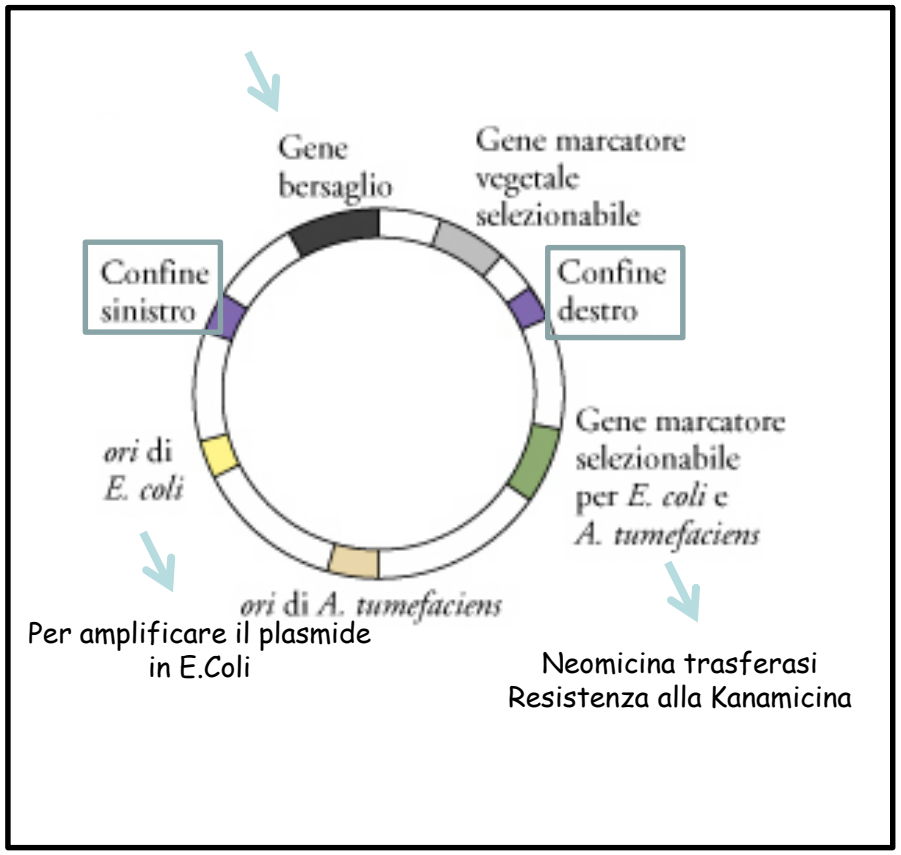
2 ways

1) Binary vector system

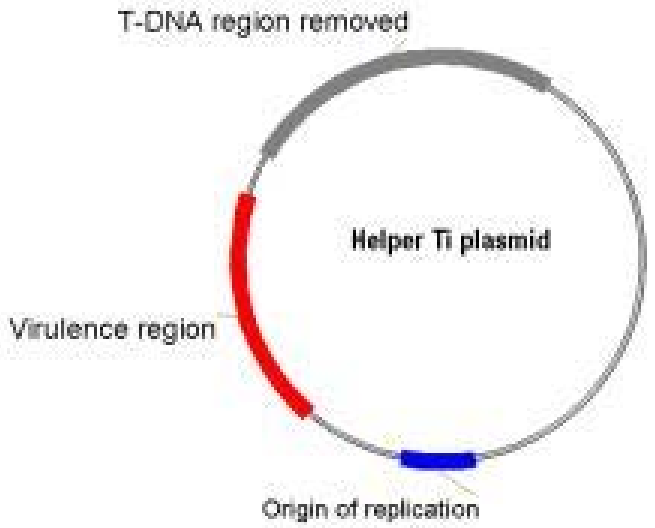
Strategy:

1. Move T-DNA onto a separate, small plasmid.
2. Remove *aux* and *cyt* genes.
3. Insert selectable marker (kanamycin resistance) gene in T-DNA.
4. *Vir* genes are retained on a separate plasmid.
5. Put foreign gene between T-DNA borders.
6. Co-transform *Agrobacterium* with both plasmids.
7. Infect plant with the transformed bacteria.

Binary vector system



No geni VIR

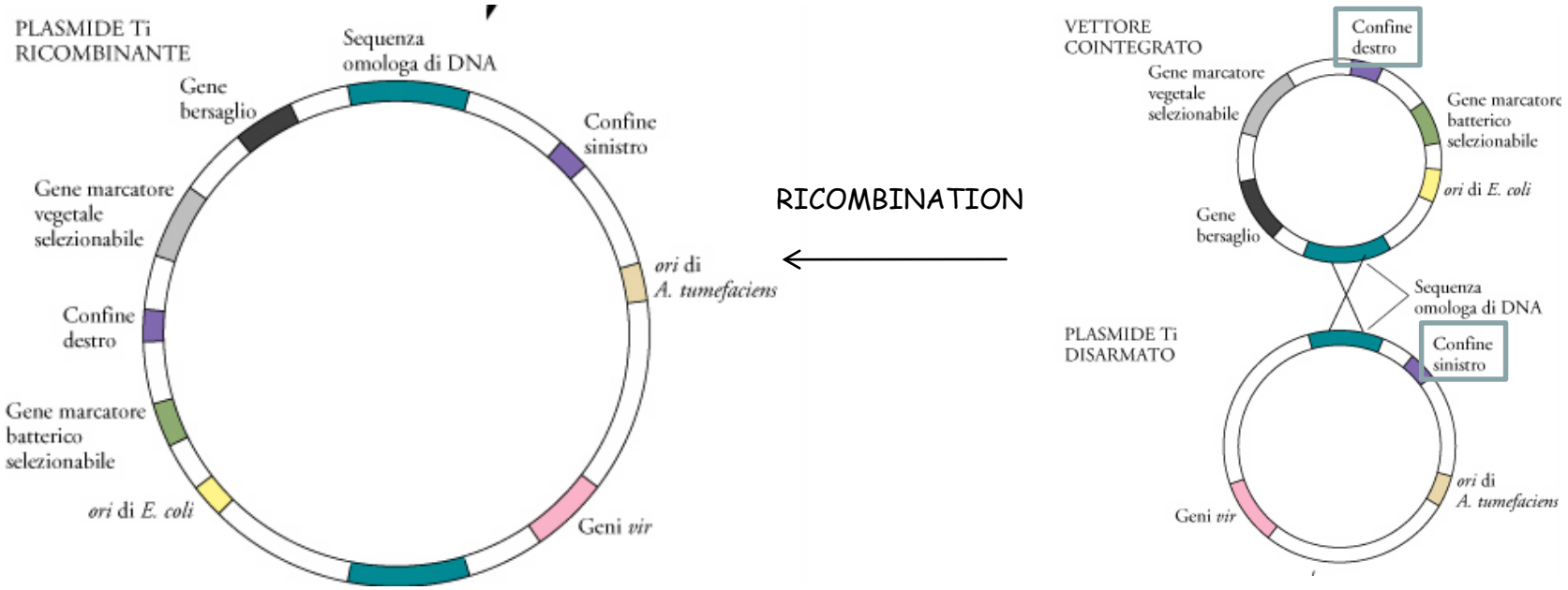


geni VIR

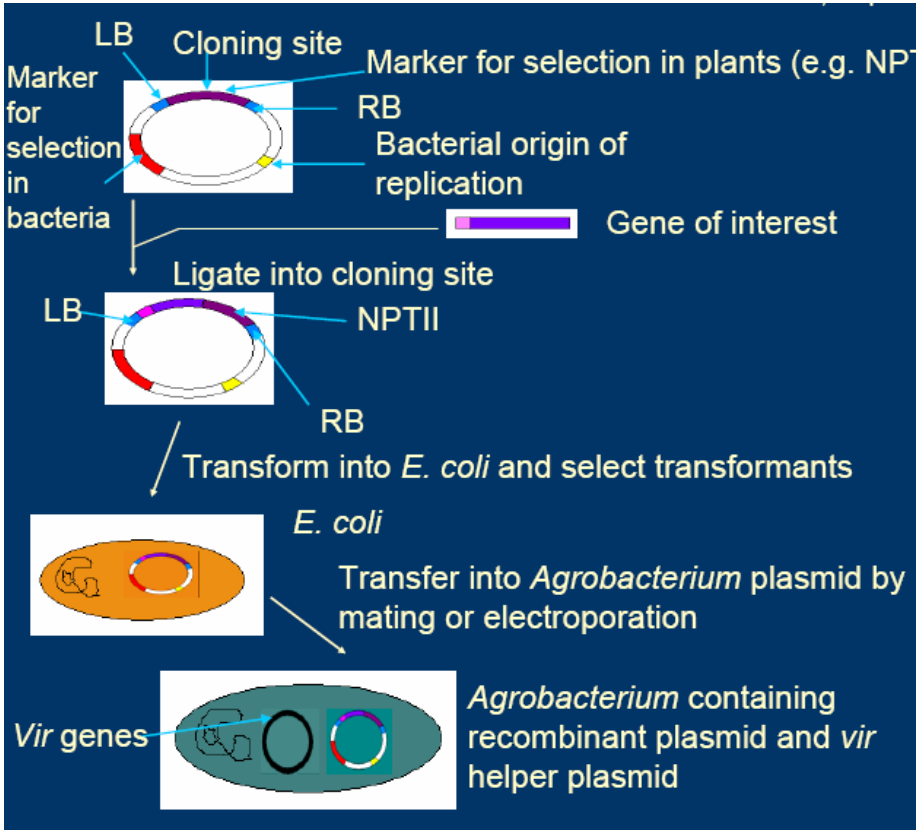
Help transfer of T-DNA

Agrobacterium tumefaciens for TRANGENIC PLANTS

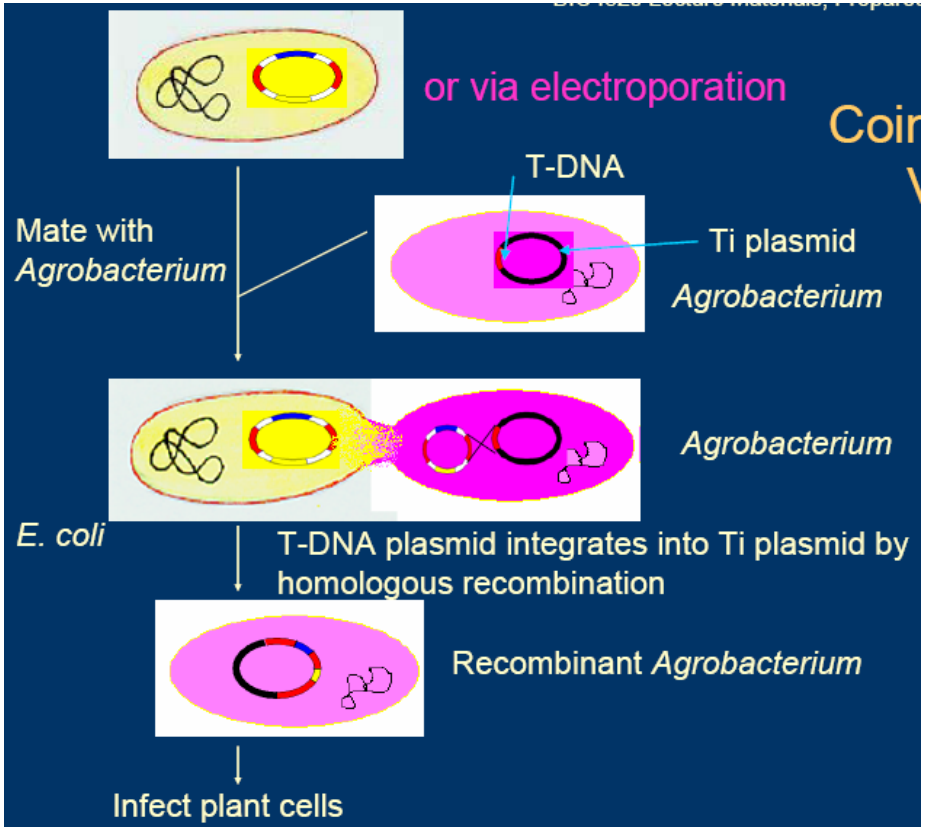
2) COINTEGRATED VECTOR

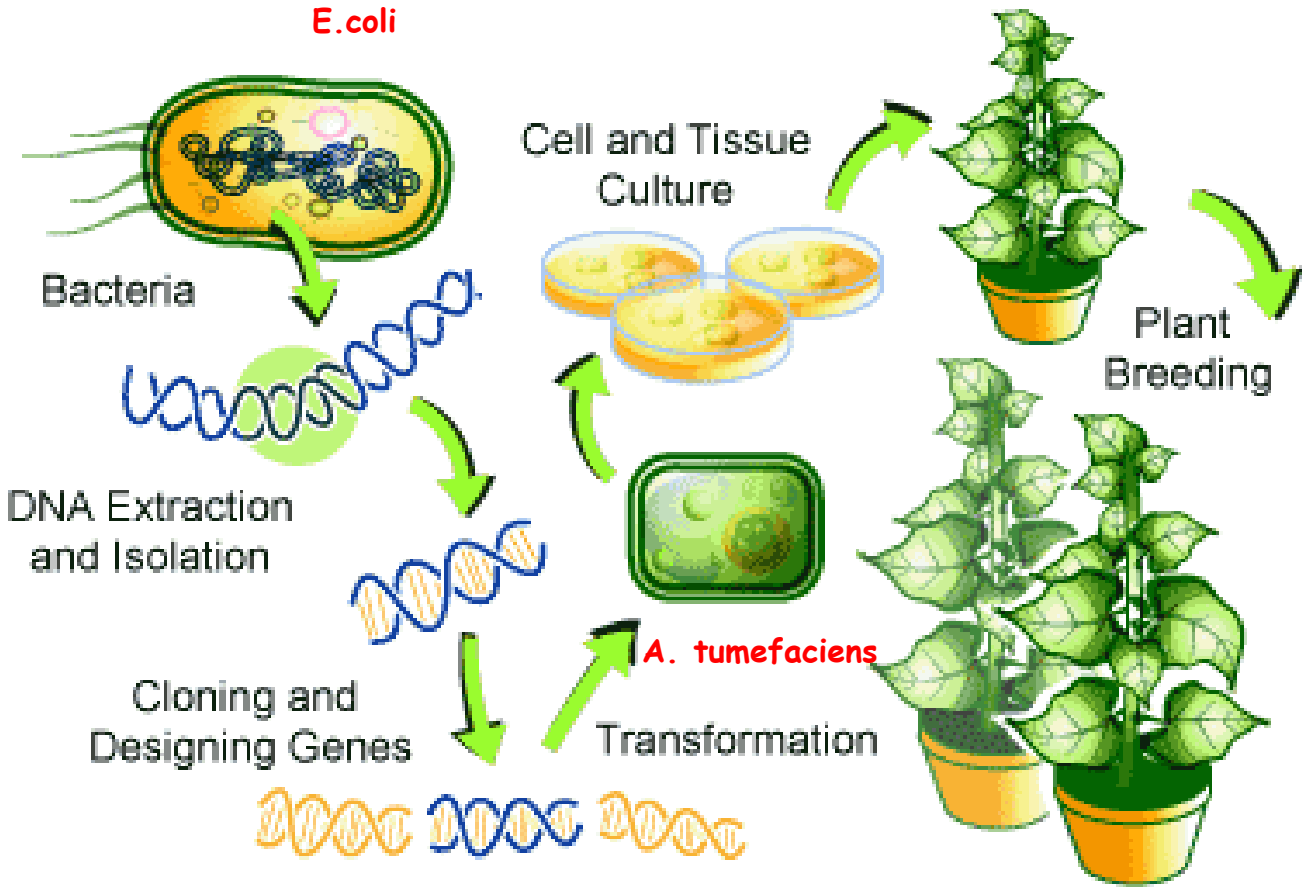


1) BINAR VECTOR



2) COINTEGRATED VECTOR




E.coli
Bacteria
Cell and Tissue Culture
Plant Breeding
DNA Extraction and Isolation
A. tumefaciens
Cloning and Designing Genes
Transformation

TOBACCO TRANSFORMATION

Preparazione degli espianti, formazione della ferita

Co-coltivazione con *A. tumefaciens*

Rigenerazione e selezione (mezzo selettivo)

Trasferimento su terreno

GEN GUN

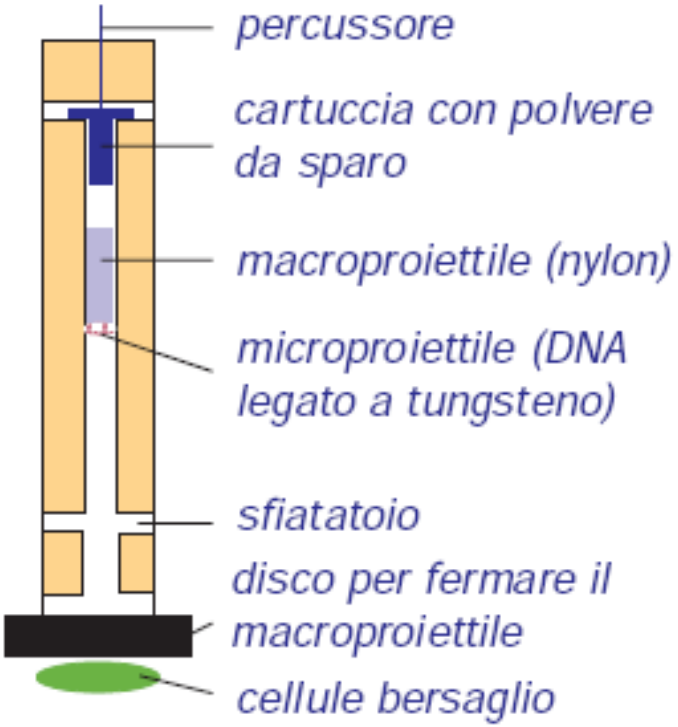
Used for most of Monocotyledons

- Low frequency of stable integration
- Multiple insertion allowed

Usefull to introduce DNA into:

- Monocotyledons
- Plant cell suspensioni
- Callus colture
- Pollen
- Mitochondria and chloroplast

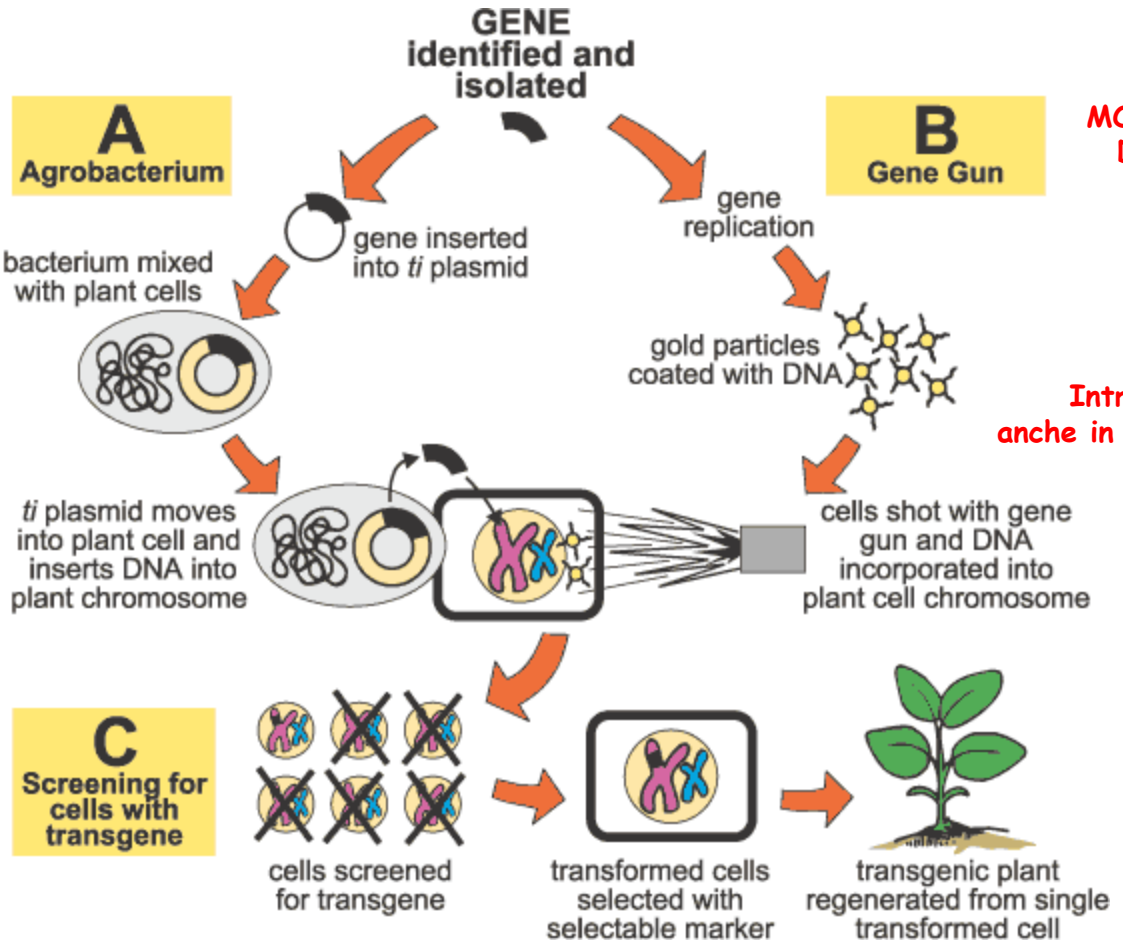
Gene Gun


percussore
cartuccia con polvere da sparo
macroproiettile (nylon)
microproiettile (DNA legato a tungsteno)
sfiatatoio
disco per fermare il macroproiettile
cellule bersaglio

Agrobacterium and Gene gun

Infetta solo DICOTILEDONI

Introduzione del DNA solo nel NUCLEO



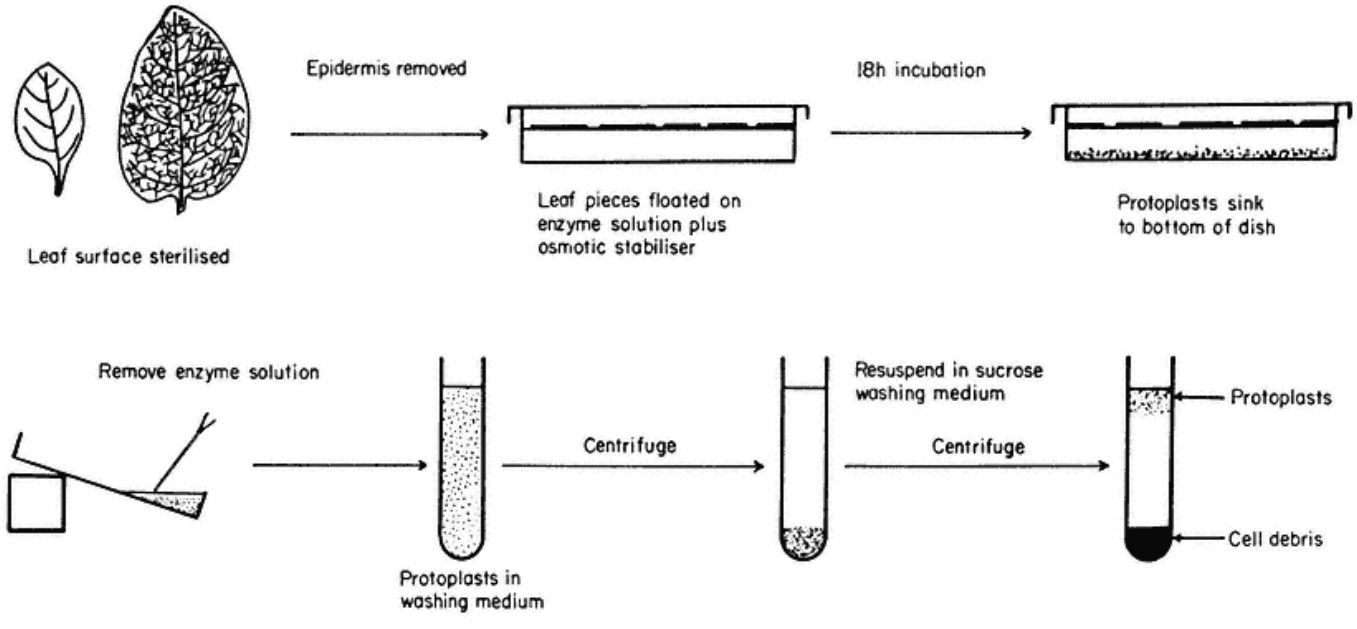
Infetta MONOCOTILEDONI DICOTILEDONI EMBRIONI etc

Introduzione del DNA anche in cloroplasto/mitocondrio

Other methods…

Trasferimento genico mediato dal plasmide Ti	Sistema eccellente e molto efficiente, limitato a pochi tipi di piante
Bombardamento con microproiettili	Applicato ad un elevato numero di piante e tessuti, facile e poco costoso
Vettori virali	Metodo poco efficace
Trasferimento del gene nei protoplasti della pianta	Si può applicare solo ai protoplasti delle cellule che si prestano ad essere rigenerati in piante vitali
Microiniezione	Limitata utilità, si può iniettare una cellula per volta, richiede elevata manualità
Elettroporazione	Limitata ai protoplasti delle cellule che si prestano ad essere rigenerati in piante vitali
Fusione dei liposomi	Si può applicare solo ai protoplasti delle cellule che si prestano ad essere rigenerati in piante vitali

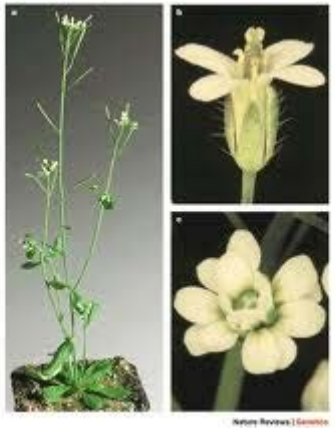
PROTOPLAST preparation



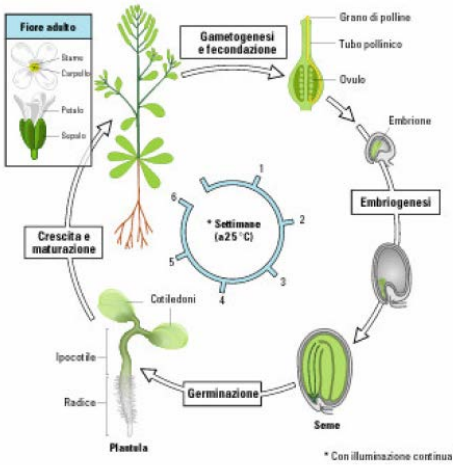
Arabidopsis Thaliana

The *Arabidopsis thaliana* is a good MODEL:

Small size



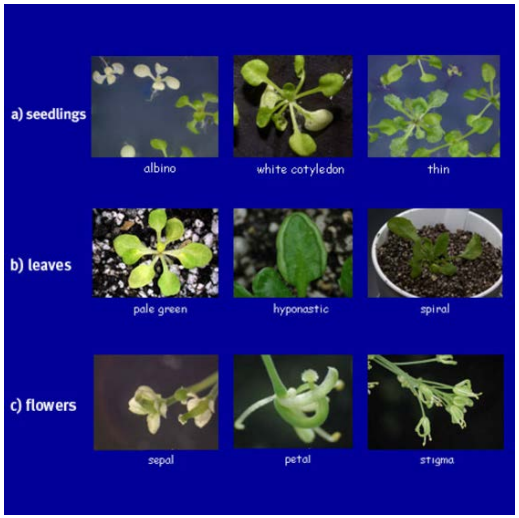
Fast growth



Small genome (only 125 Mb)

The *Arabidopsis thaliana* is a good MODEL:

Easy to manipulate



Big choise of mutants

Elevated number of seeds
(1 plant: 10.000 seeds)

d:0,5mm

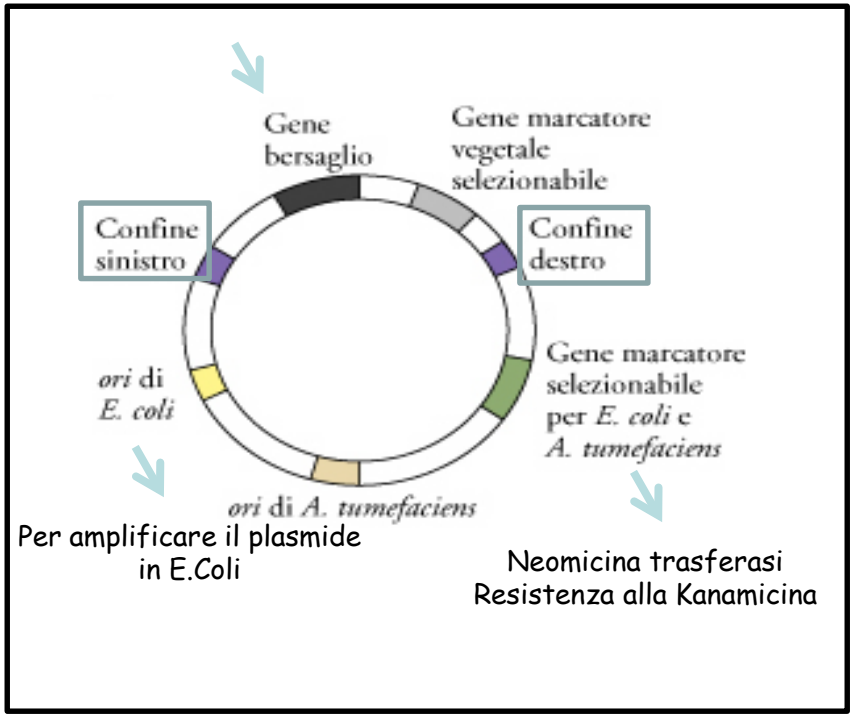
GENI REPORTER for plants

GFP: Green Fluorescent protein

LUCIFERASI

GUS: β-D-glucuronidasi

Production of transgenic plants without MARKER GENES



MARKER GENES COULD BE TOXIC AND/OR ALLERGENIC upon ingestion.
Moreover gene that confer antibiotic resistence could be transferred to bacteria of intestinal flora

Production of transgenic plants without MARKER GENES

1) Using Marker Genes at the beghinning and then its removal

2) Using two genes approach (GENE x + GENE RESISTANCE)
They segregate as two distint alleles

3) Screening by PCR