

# Mutants and transgenic material in science and biotechnology

# Isolation, generation and characterisation of mutants

- **natural mutants**
- **chemical-mediated mutants (ethylmethane sulfonate, EMS)**
  - ethylation of G
  - G > A
- **x-ray**
- **insertions**
- **statistical insertion of foreign DNA**
  - resistance genes
  - flanking DNA sequences
  - transposons (DNA, RNA)
- **CRISPR/Cas-Method (site-directed insertion)**

**loss-of-function mutants**  
**gain-of-function mutants**

**Mutagenesis**

- seeds
- pollen

**EMS mutagenesis**

**Mutagenesis of seeds**

“

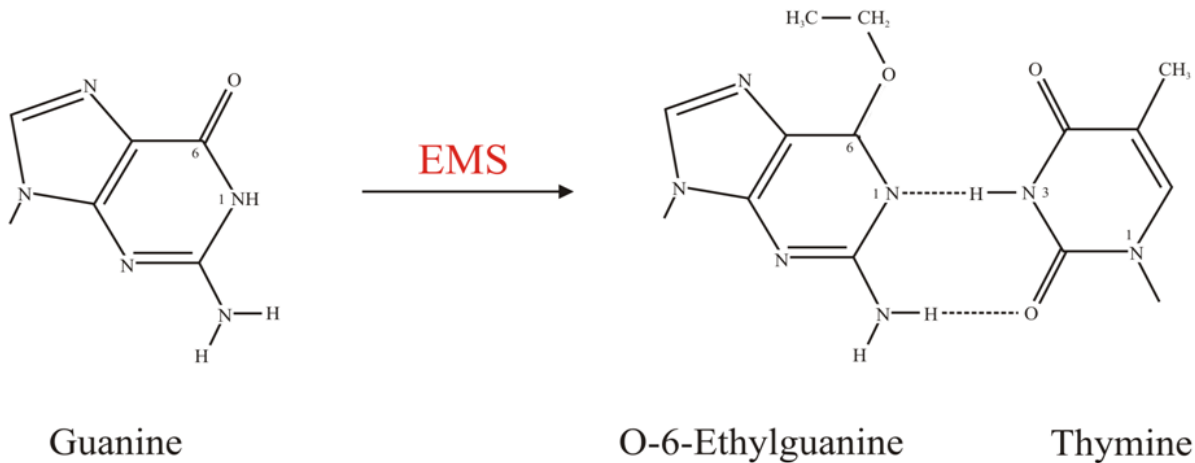
**Self pollination**

“

**F<sub>1</sub> – heterozygote**

“

**F<sub>2</sub> – screening for phenotype**



T pairs with A instead of G  
' point mutation

low dosis ' few mutants ' screening of many individuals  
 high dosis ' many mutants ' screening of less individuals

however ....

identified mutant has many (high dosis) or few (low dosis) EMS mutations in the genome

## X-ray mutagenesis

- deletion
- insertion
- rearrangement

## Insertion mutagenesis

- insertion of known DNA which can be identified in the genome
  - resistance gene under eukaryotic promoter
  - reporter gene
  - gene of interest
  - transposable element (DNA-, RNA-based)
- 
- Integration in intergenic region, regulatory elements (promoters), coding sequences



**GUS**



**Luciferase**



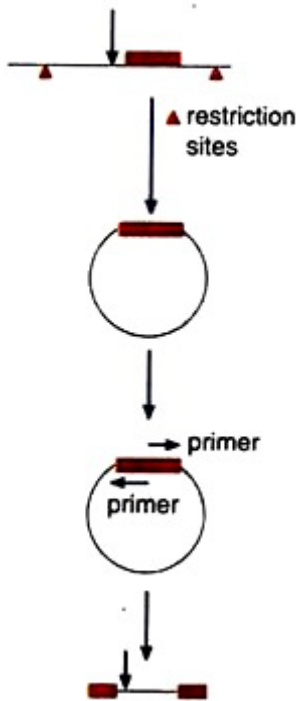
**GFP**



**AC-DS element in maize**

## Insertion mutagenesis

- PCR amplification of flanking sequences of known insertion
  - confirmation by insertion lines



or

- cut genomic DNA
- ligate adapter
- PCR with insertion and adapter primers

- Point (EMS) mutation ” insertion / x-ray  
Mutation
- Nucleotide (amino acid?) exchange allows epitope analyses
  - Insertion destroys gene



## **EMS mutagenesis: identification of mutated gene by**

- 1. Gene mapping**
- 2. Chromosome walking**
- 3. Genome sequencing**

## EMS mutagenesis: identification of mutated gene by

### 1. Gene mapping

### 2. Chromosome walking

### 3. Genome sequencing

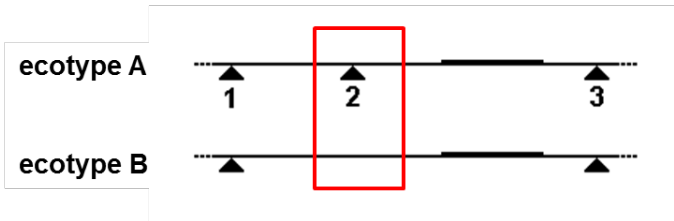
- cross mutant with wild-type of another ecotype
- **ALWAYS:** select offspring with mutant phenotype in  $F_2$ , discard all other offspring
- $F_2$ : identify chromosome origin in individual  $F_2$  offspring with ecotype-specific molecular markers
- Identification of chromosome with mutated gene
- $F_3$ : due to cross-over: identify mutant ecotype DNA on chromosome by chromosome walking

screen for phenotype in  
offspring populations

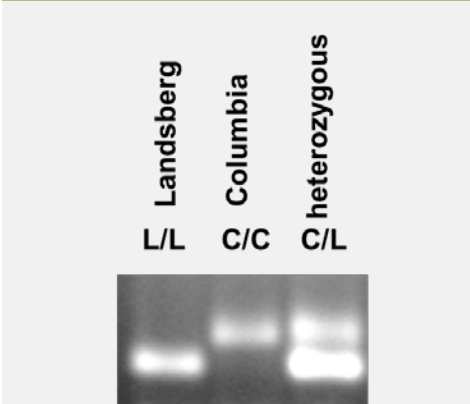


**Molecular marker**: any DNA sequence that differs between the two ecotypes

**Restriction fragment length polymorphism (RFLP) marker**



**Simple sequence length polymorphism (SSLP) marker**



**Cleaved amplified length polymorphism (CAPS) marker**

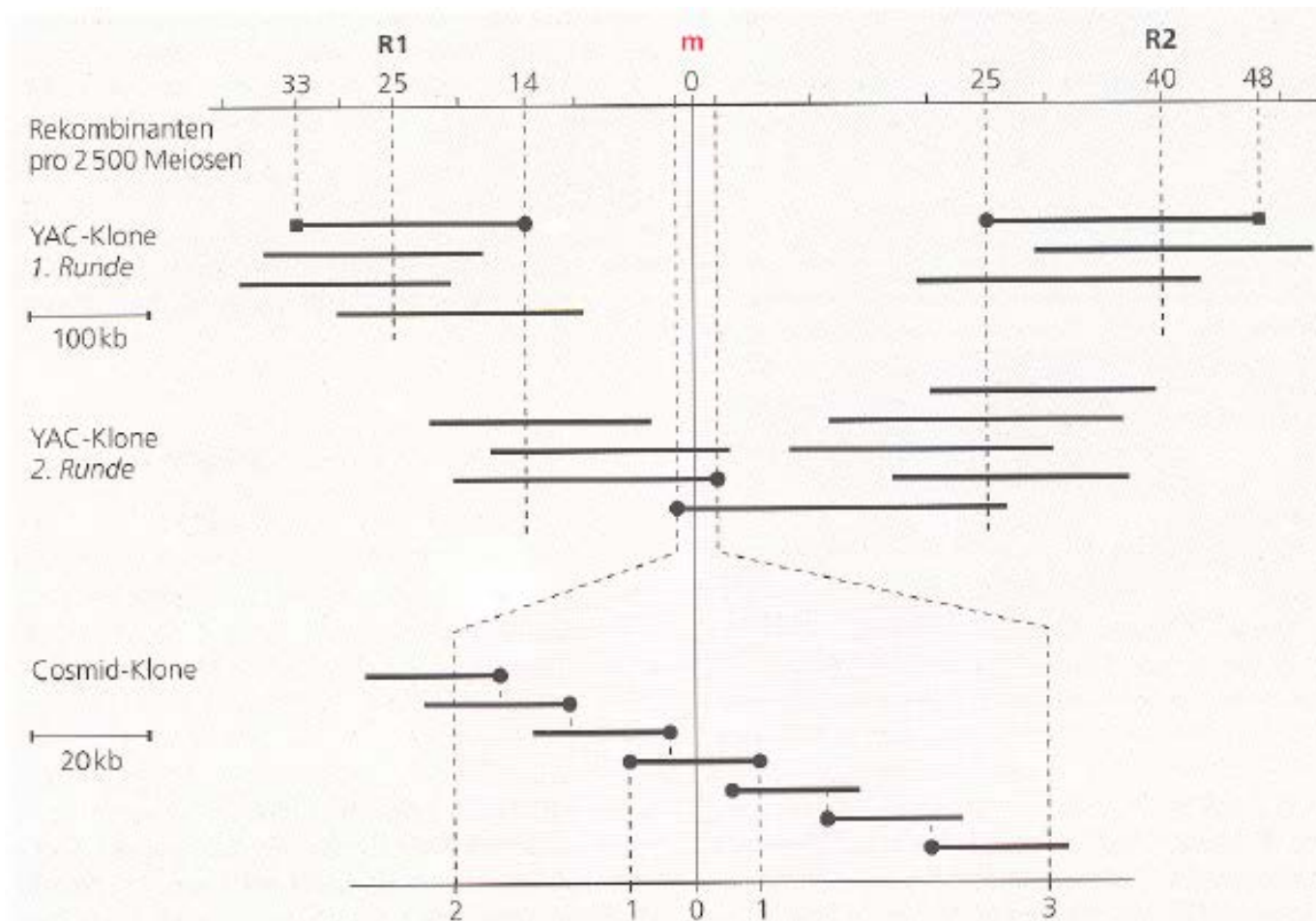


# EMS mutagenesis: identification of mutated gene by

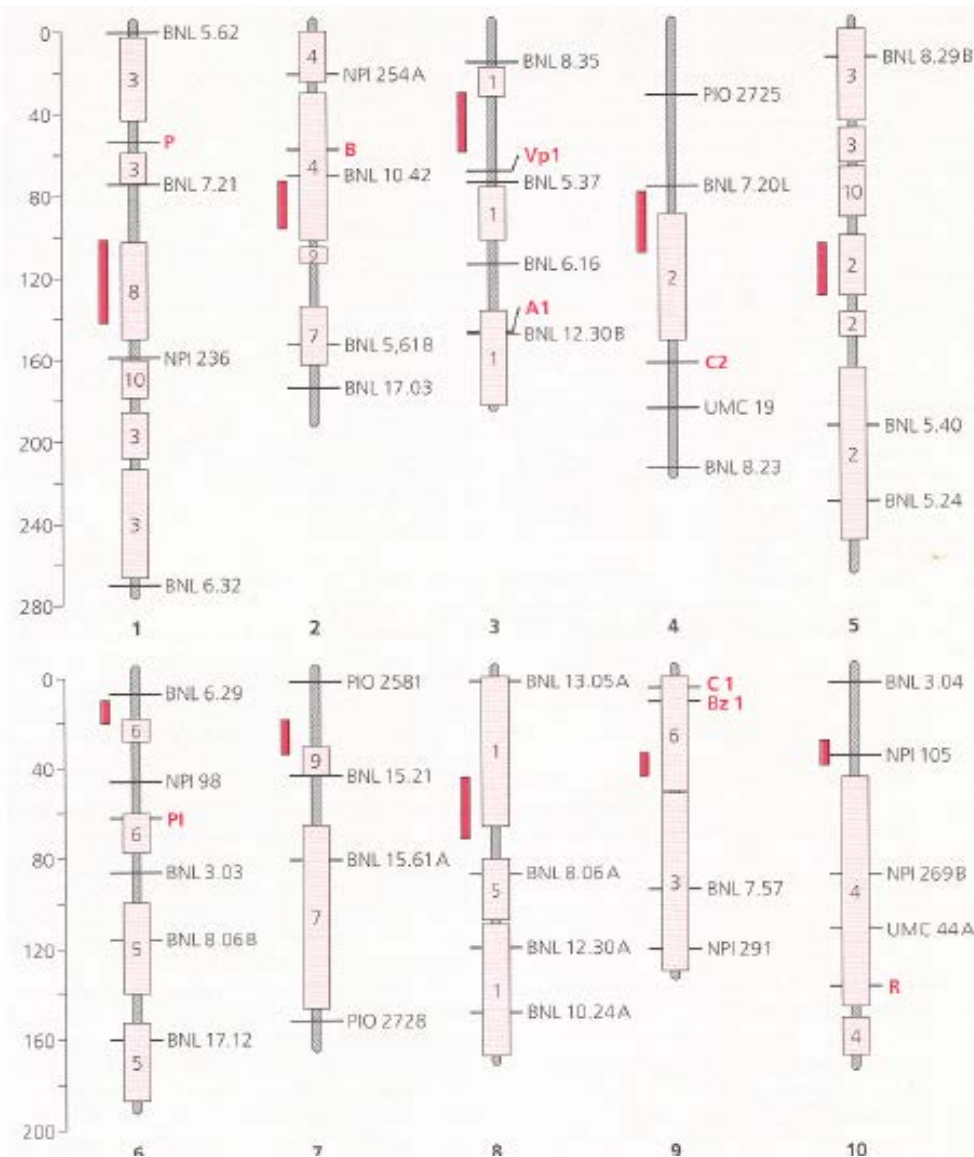
## 1. Gene mapping

## 2. Chromosome walking

## 3. Genome sequencing



# Colinearity of genomes



Maize (10 chromosomes in black) and rice (red)

## EMS mutagenesis: identification of mutated gene by

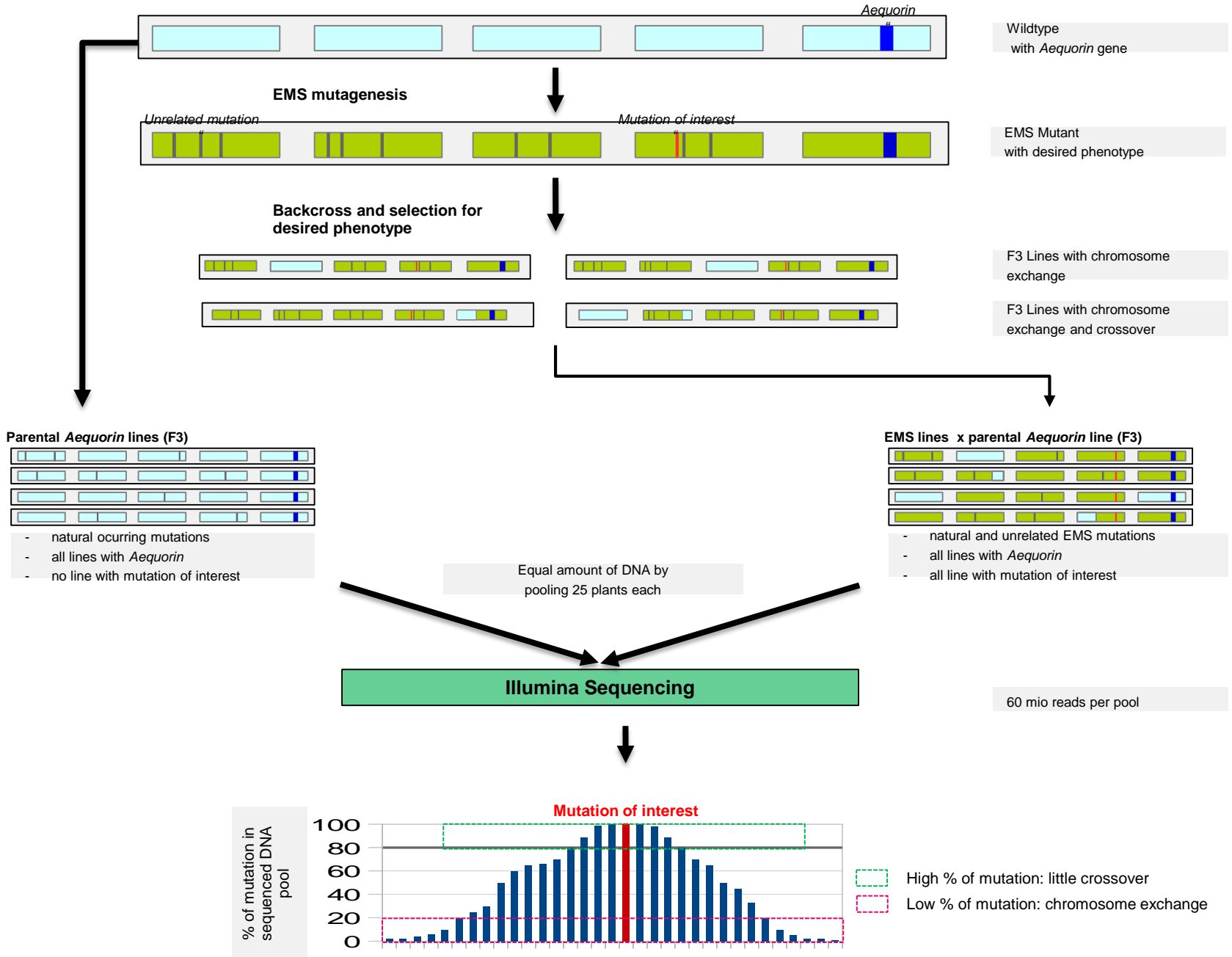
1. Gene mapping

2. Chromosome walking

3. Genome sequencing

- cross mutant with wild-type of another ecotype
- **ALWAYS:** select offspring with mutant phenotype in  $F_2$ , discard all other offspring
- mix >25 individual mutant  $F_2$  offspring DNA and compare to DNA from the other ecotype

# Gene mapping by Illumina sequencing





# Integration of foreign genetic information into plants

## 1. Totipotence, regeneration

## 2. Techniques

- Agrobacterium
- particle gun
- electroporation
- microinjection

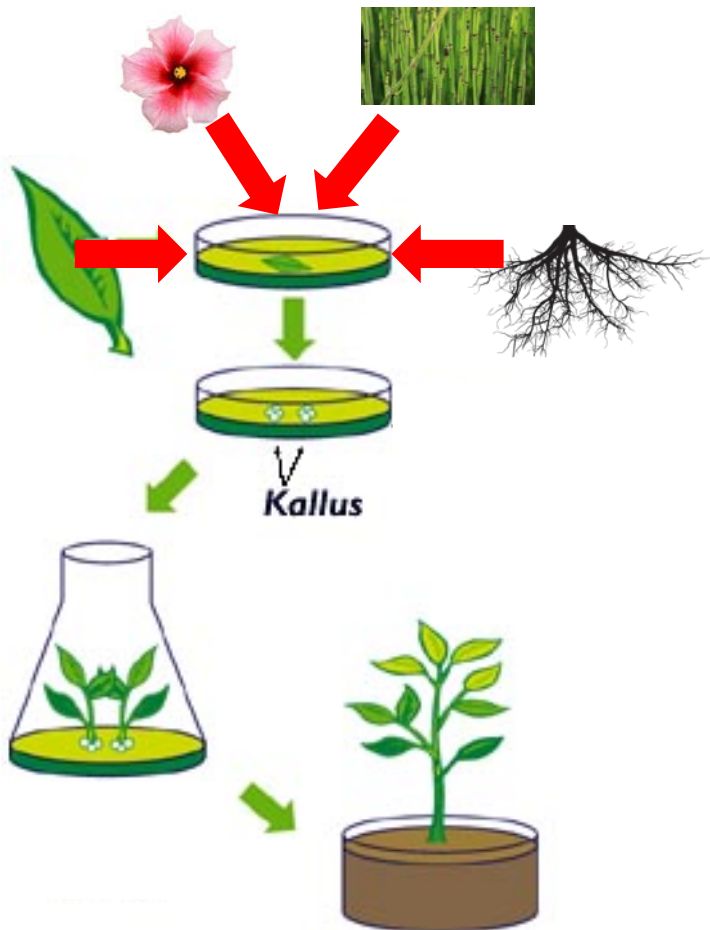
## 3. Nucleus

- Random integration
- **CRISPR/Cas-Method**

## 4. Plastids

- Chloroplast transformation

# 1. Totipotence, regeneration



All living plant cells are totipotent and can be used for generation of a genetically identical new plant,

however ....

.... the regeneration capacity differs substantially

**Good regeneration:**

- tobacco
- *Petunia*
- (*Arabidopsis*)

**Bad regeneration:**

- crops

# Foreign DNA into

germ line (egg cell)  
(animals)

differentiated cell  
(plants)

↓  
loss of specification

↓  
totipotent cell

↓  
callus

**auxin / cytokinin >**

↓  
organogenesis

↓  
new organism  
(genetically identical to  
origin)

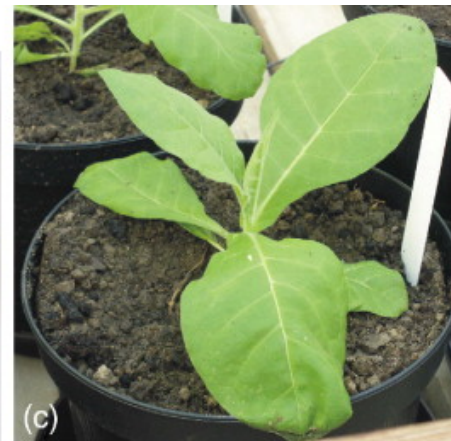
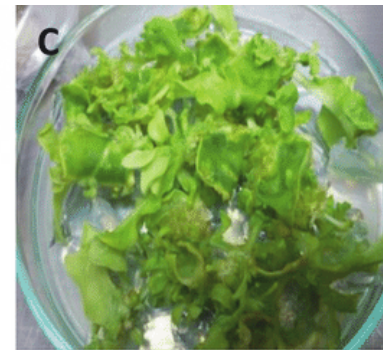
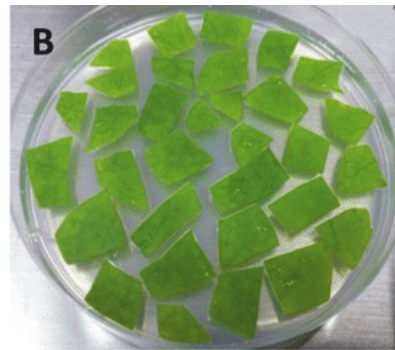
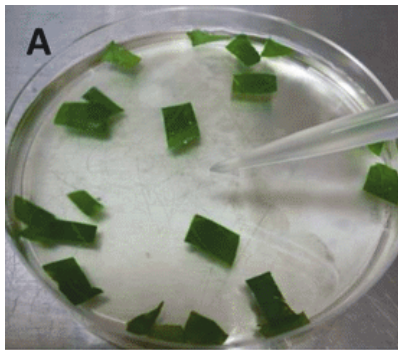


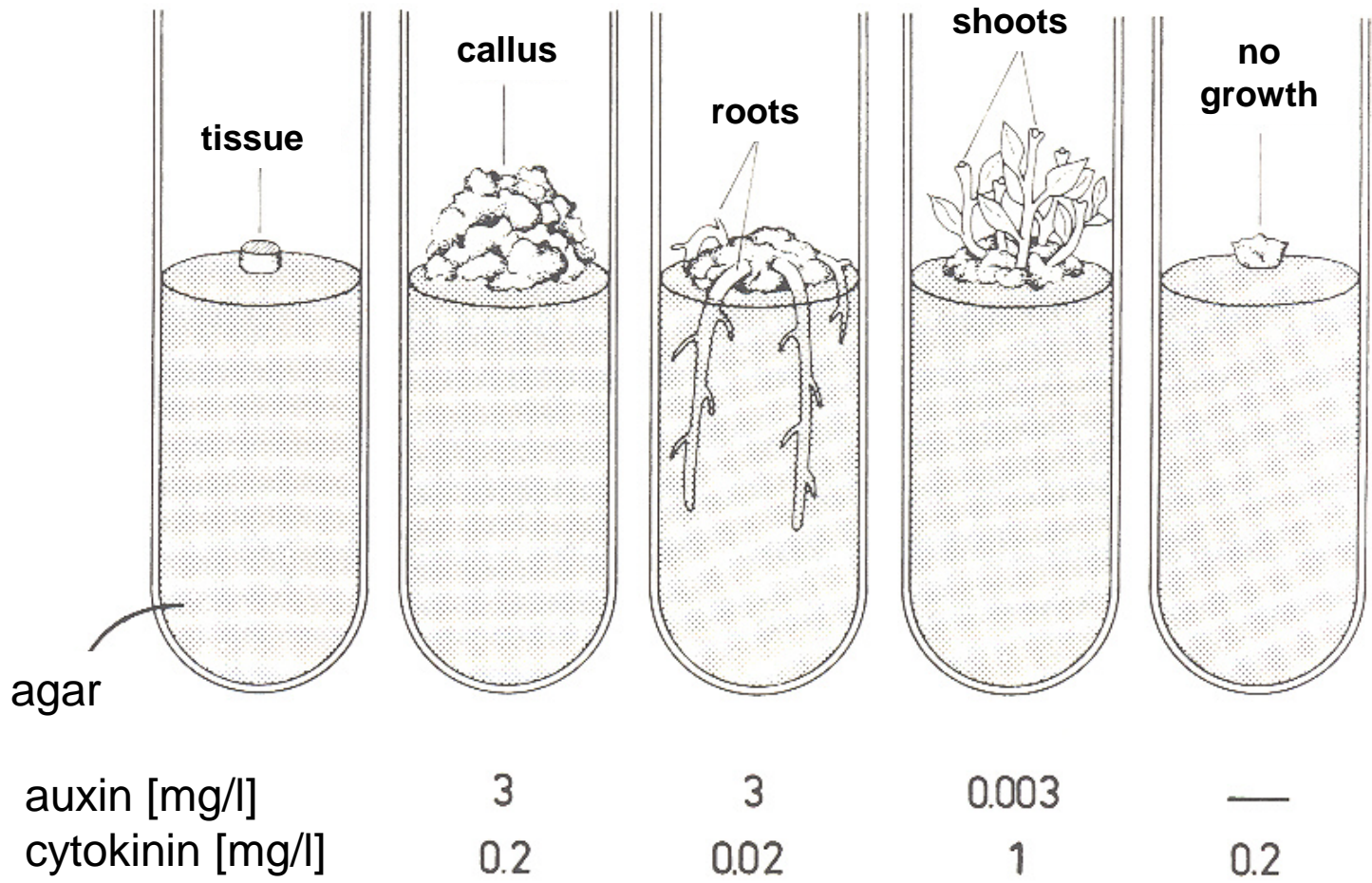
tissue culture  
↓  
pots

Only a few cells obtain foreign DNA

**selection marker** in the medium

Kan<sup>R</sup>, Amp<sup>R</sup>, Rif<sup>R</sup>, Hyg<sup>R</sup>, BASTA<sup>R</sup>, *etc.*

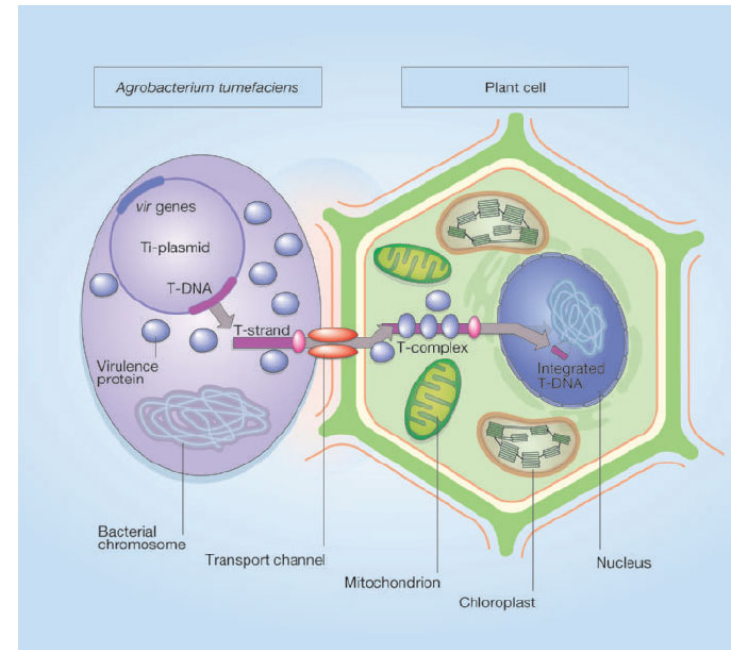
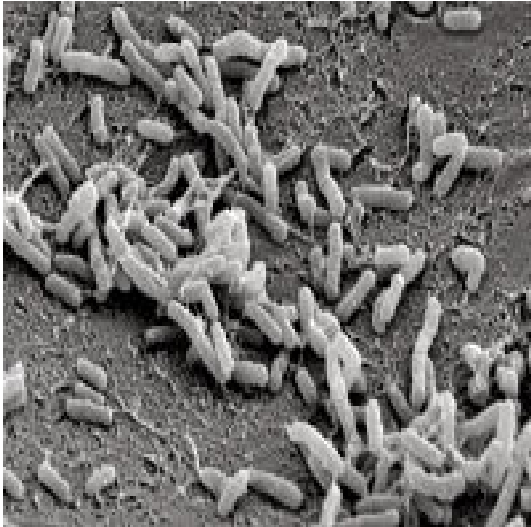




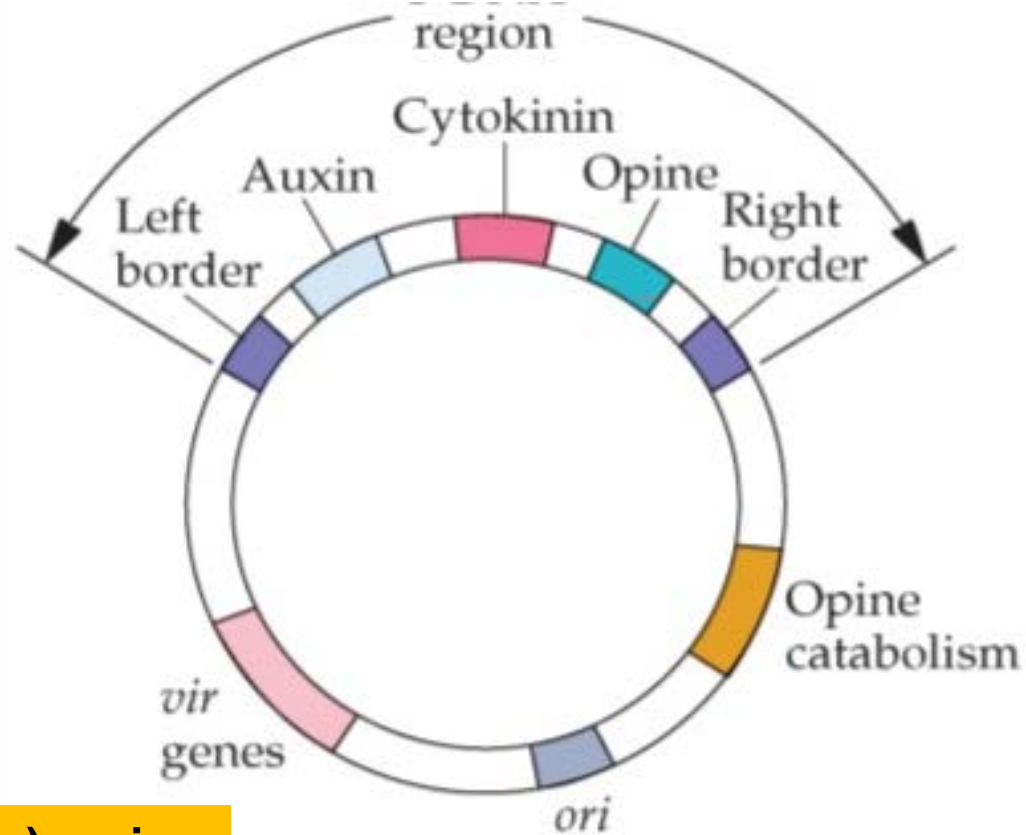
## 2. Techniques

- *Agrobacterium*
- particle gun
- electroporation
- microinjection

# *Agrobacterium tumefaciens* – gene transfer in nature



## T-DNA (transfer DNA)



### ***vir* (virulence) region**

1. Wounded plant cell releases flavonoids (replaced in lab by acetosyringon)
2. Flavonoids activate genes in *vir* region.
3. Gene products of *vir* region recognize Left and Right Borders of T-DNA and nick the DNA.



# *vir* region

## Flavonoids activate genes A-F on - *vir* region

### Gene products:

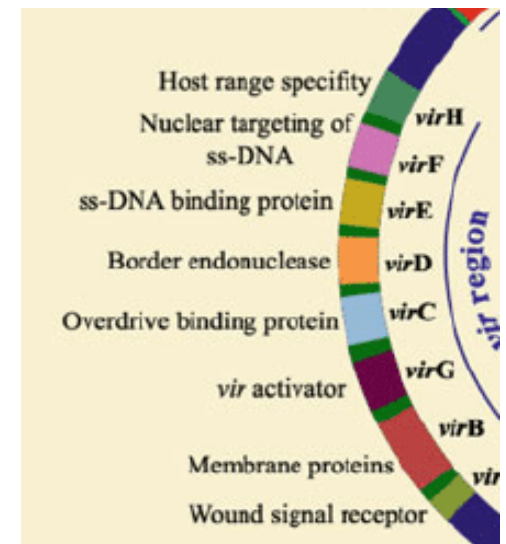
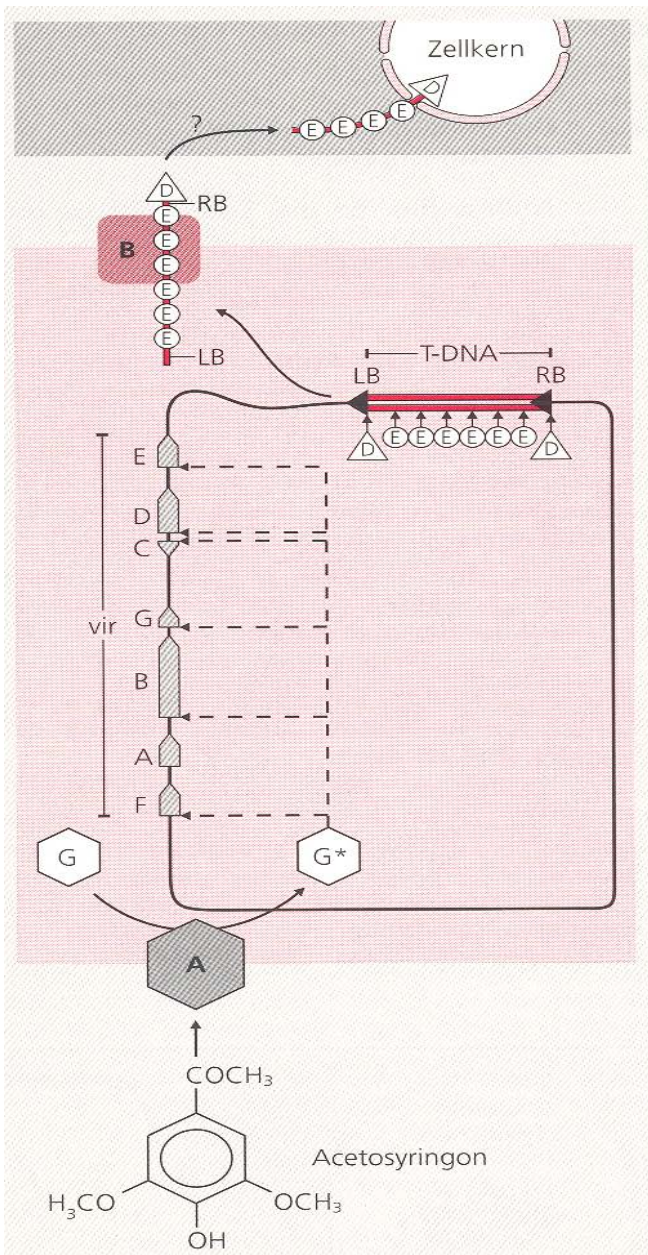
**F:** transcription factor

**D:** single-stranded nuclease

**E:** single stranded DNA-binding protein with  
NLS

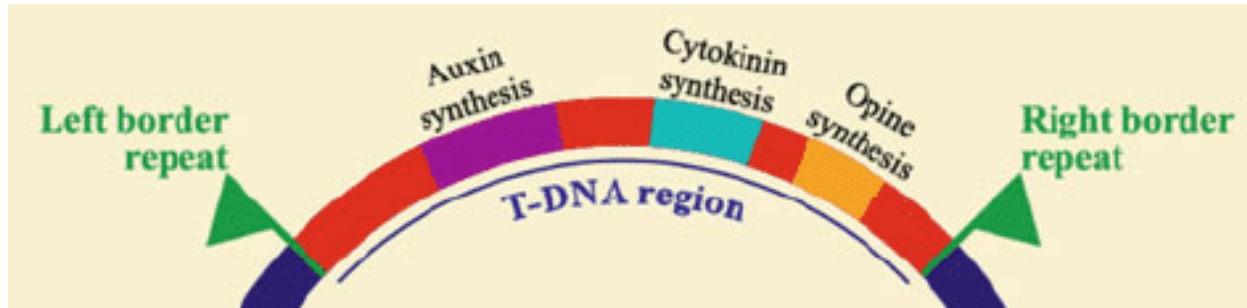
**B:** bacterial exporter

**A:** flavonoid-recognizing membrane protein





# gene transfer in nature

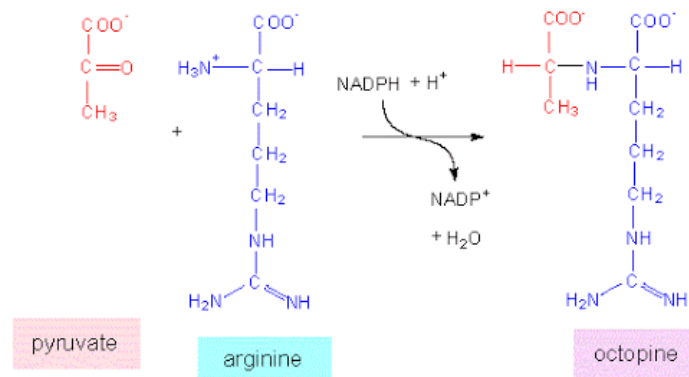


## Auxin/cytokinin biosynthesis genes:

- > high levels of auxin and cytokinin
- > uncontrolled cell division
- > tumor development (in tissue culture: callus development)

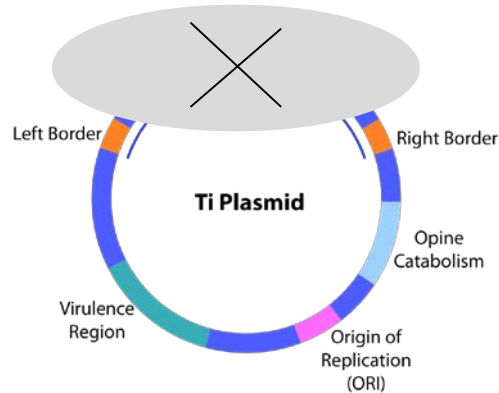
## Octopine (nopaline) biosynthesis genes:

- > generation of N source for bacterium

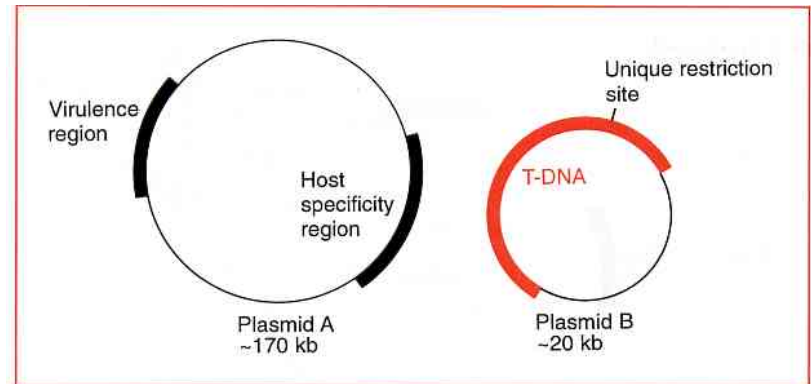


# *Agrobacterium* used as transformation system

Remove genes between LB and  
RB:  
No tumor induction



Separation of *vir* region and T-DNA  
region on 2 vectors:  
Binary vector



Insert between LB and RB:

Gene(s) of interest with eukaryotic elements  
(promoter, mRNA signals)  
Selection marker gene (with eukaryotic  
elements)

# *Agrobacterium* used as transformation system

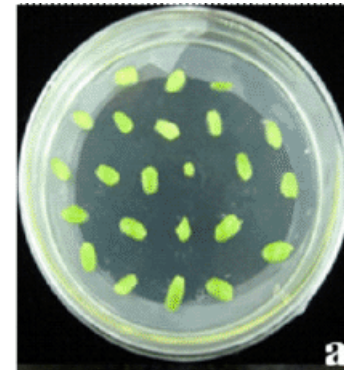
## Triparental mating

Mix on plates:

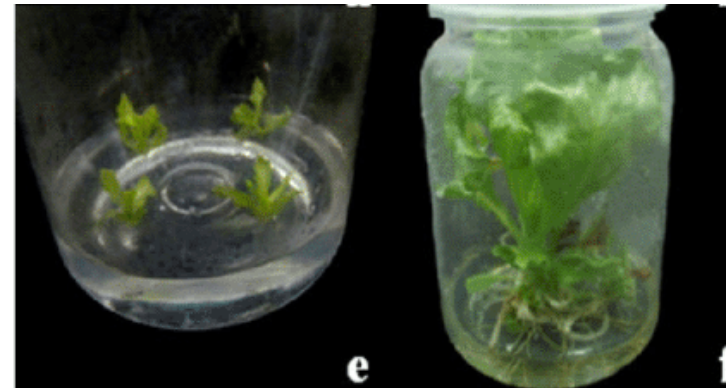
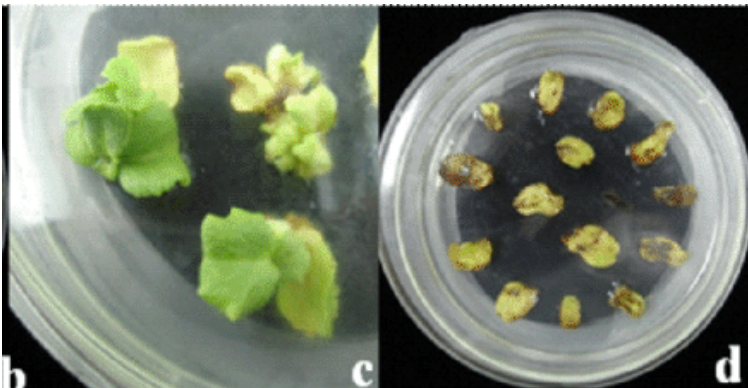
- *Agrobacterium* (no plasmid)
- *E. coli* with *vir* plasmid
- *E. coli* with T-DNA construct

Select: *Agrobacterium* with both plasmids

*Agrobacterium* infection of wounded plant pieces (leaves)



Selection on plates



## You have to know:

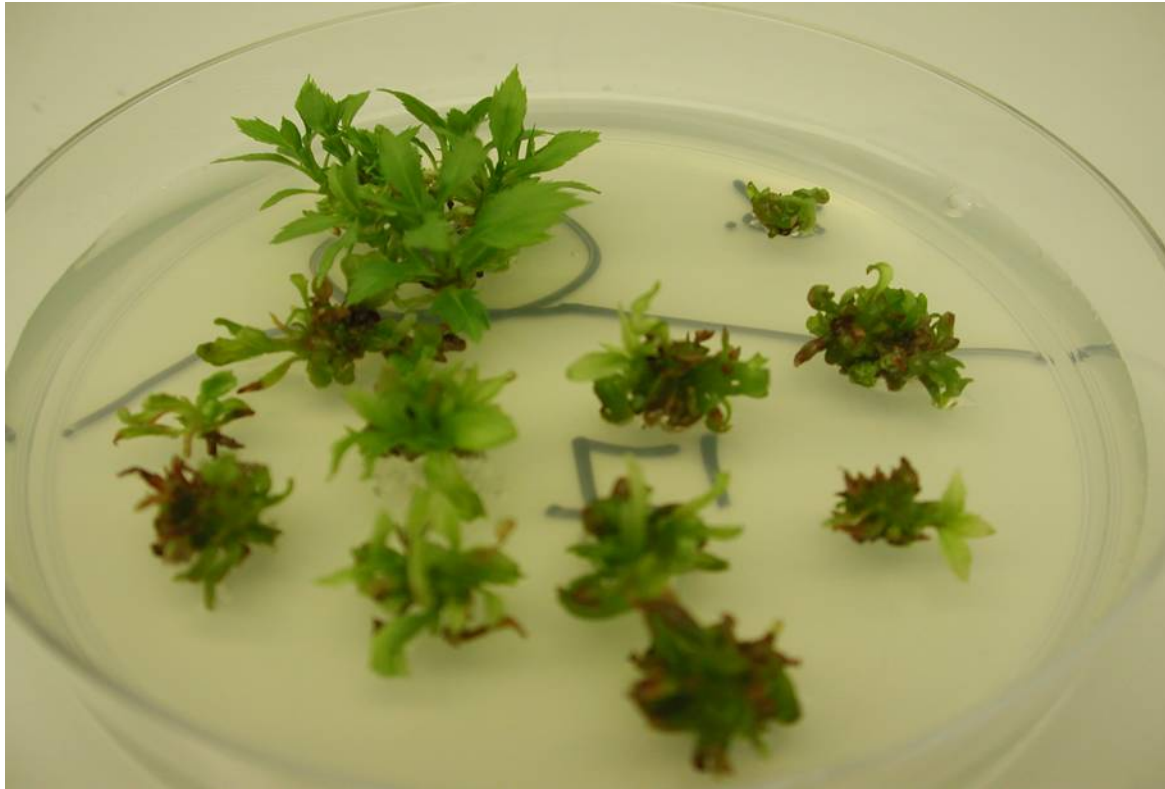
- Random insertion into genome
- One or multiple copies inserted
- Transgenic lines differ: number and loci of insertions



### Independent transformation events

Plantlets derive from different transformed cells. Genetically different

***Agrobacterium tumefaciens* – plant regeneration on resistance media**

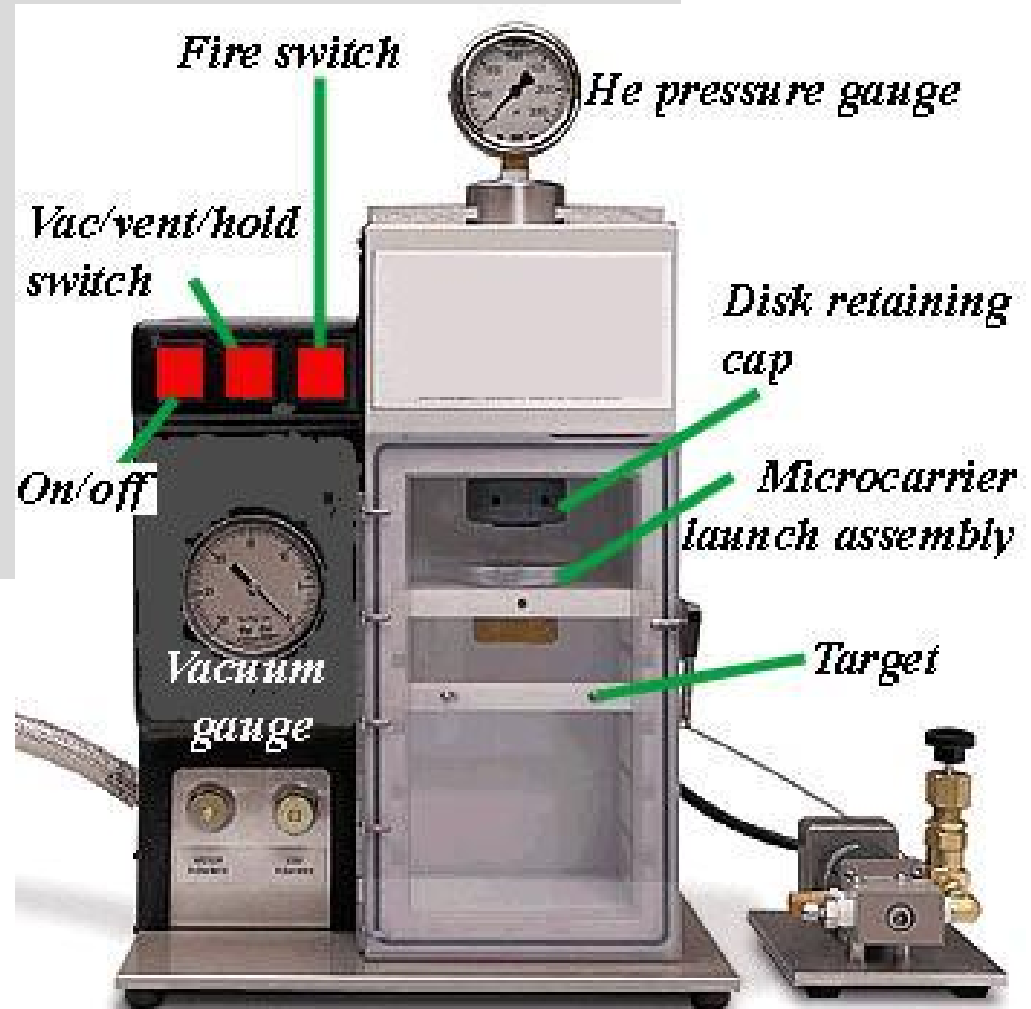


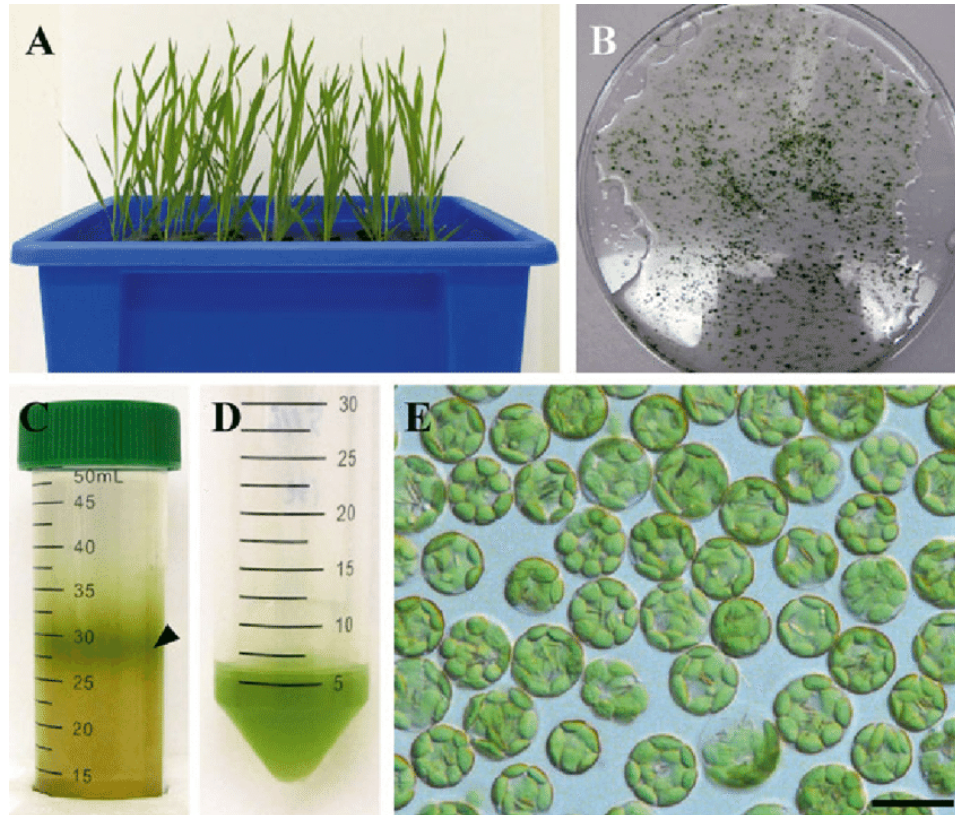
## 2. Techniques

- Agrobacterium
- particle gun
- electroporation
- microinjection



- Mix plasmid with gold or tungsten particles
- Plasmid binds to metal surface
- Particle bombardment
- Particles are
- integrated into
- a single cell.
- Targets:
  - (crop) protoplasts
  - leaf discs
  - microorganisms





## Isolation of protoplasts

(a) hydroponically grown plants

(b) chopped leaves in solution

(c) enzymatic digestion of the cell wall

(d) fractionation by sucrose density gradient yielded protoplasts at the interface

(e) visualization under microscopy using bright-field filter

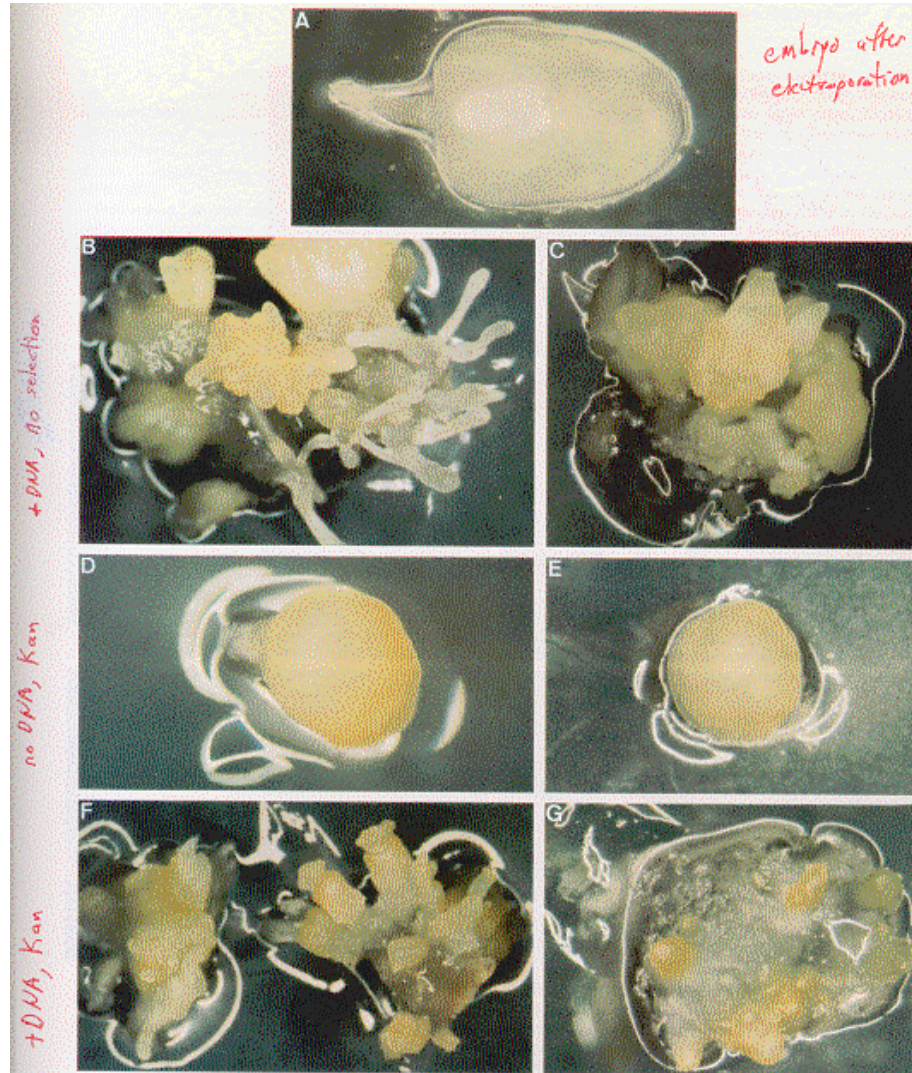
## 2. Techniques

- Agrobacterium
- particle gun
- **electroporation**
- microinjection



- protoplasts, *etc.*
- DNA (plasmids)
- PEG
- electrical pulse

# Transformation of maize embryonic tissues by electroporation

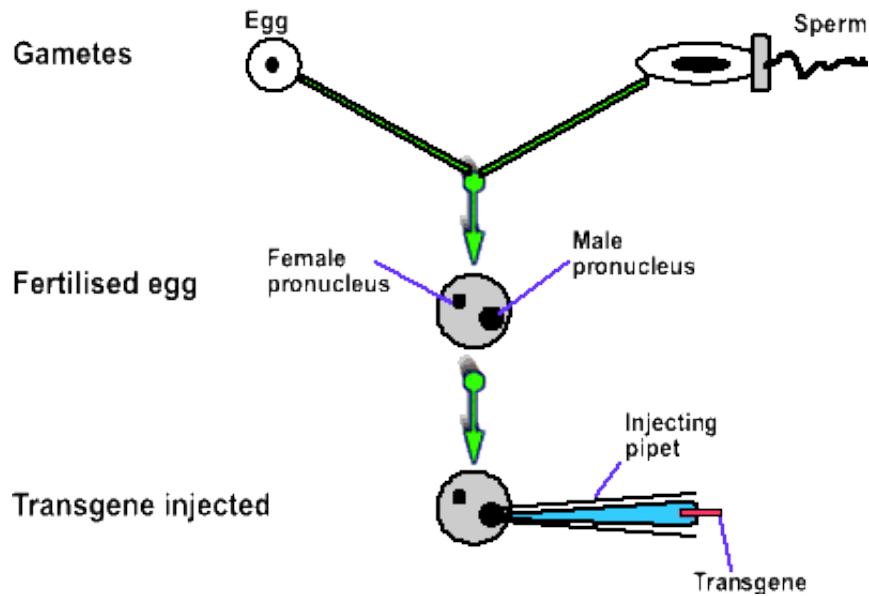


## 2. Techniques

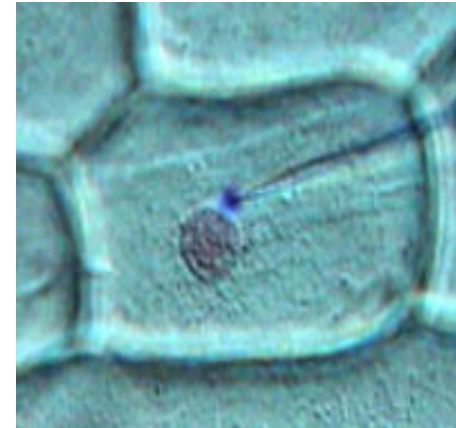
- Agrobacterium
- particle gun
- electroporation
- **microinjection**



# Germ line for animal transformation



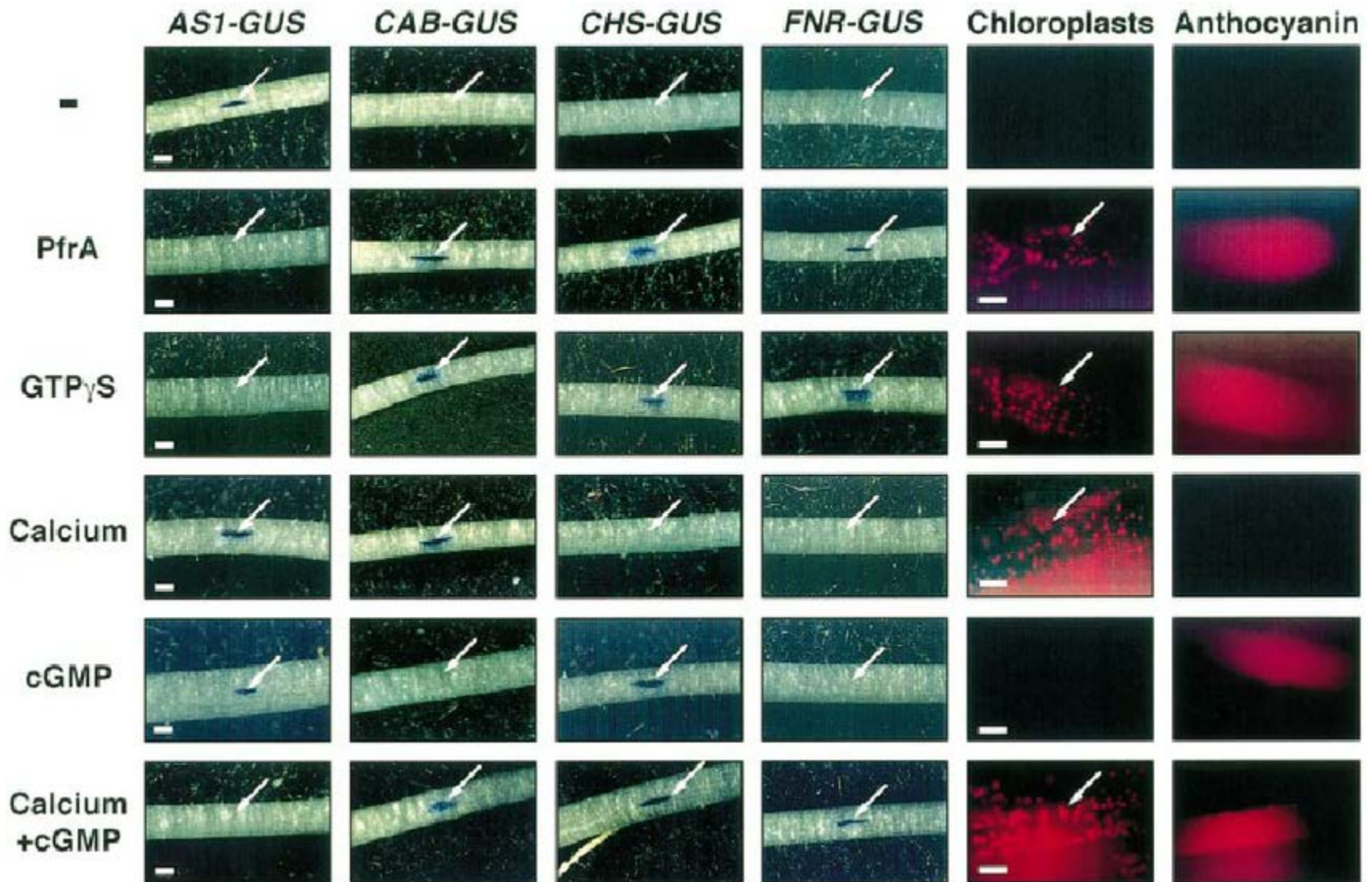
# Plants any cell



regeneration

Reporter gene  
expression

# Co-injection of $\text{Ca}^{2+}$ , cGMP and reporter gene constructs



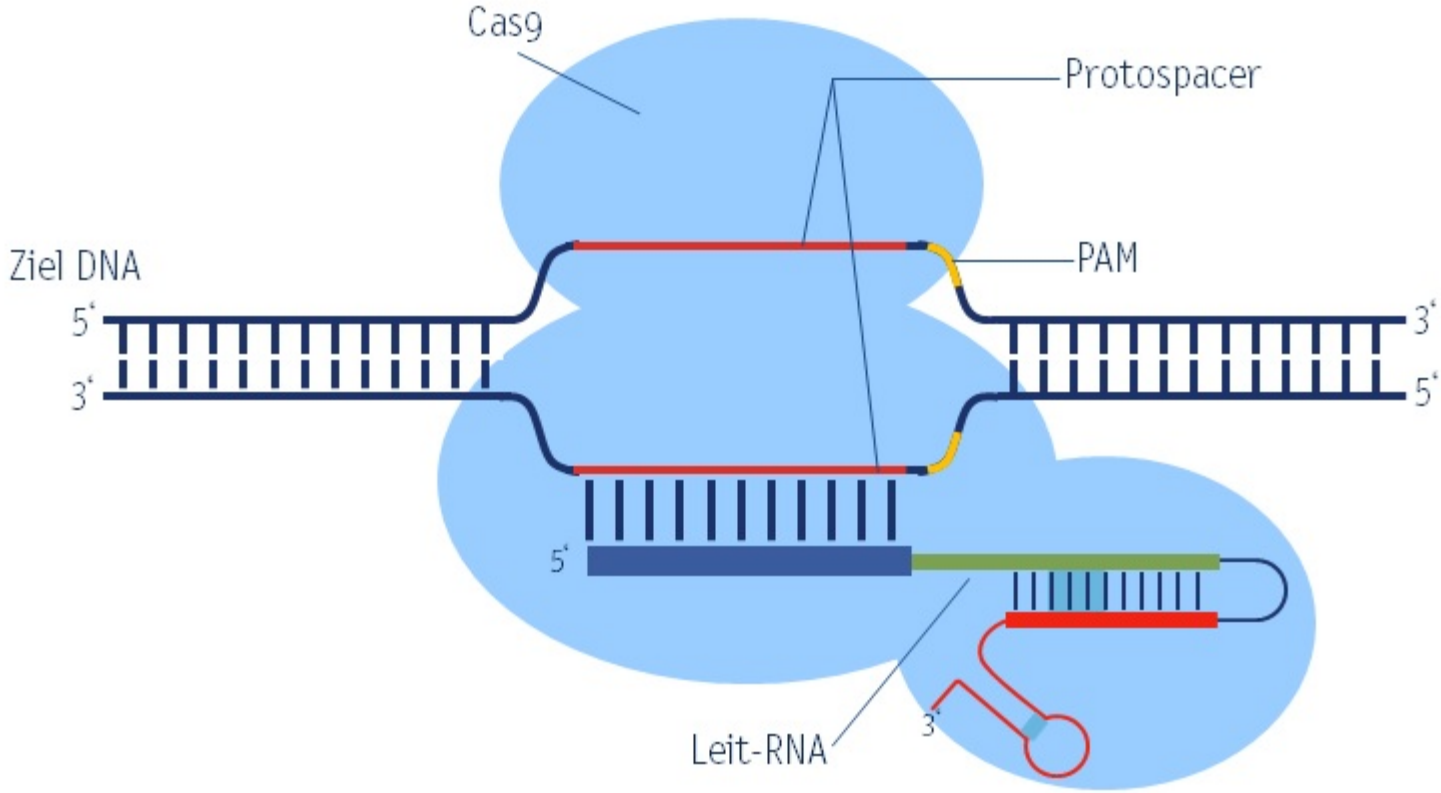


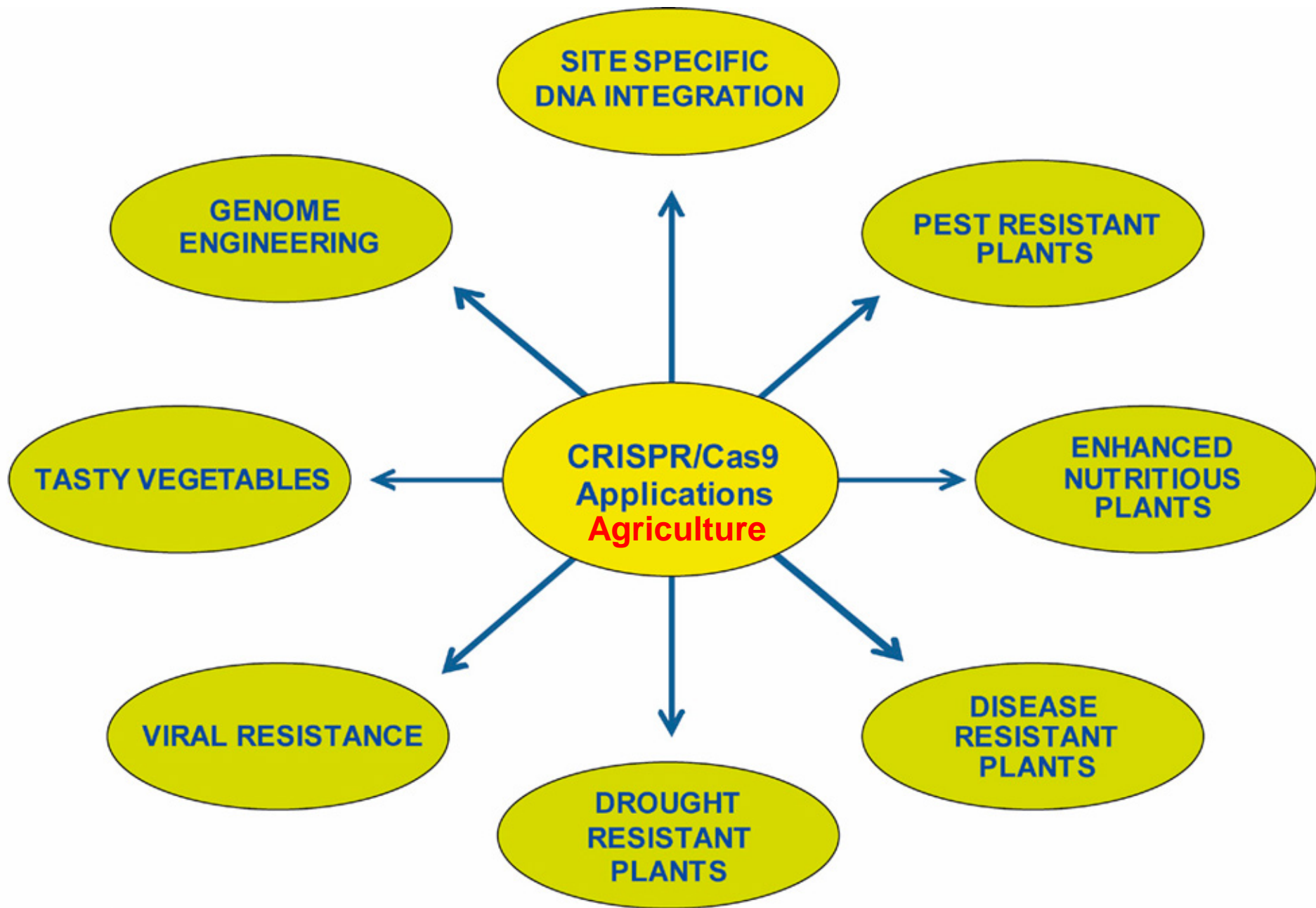
### 3. Nucleus

- Random integration
- **CRISPR/Cas-Method**

### 3. Nucleus

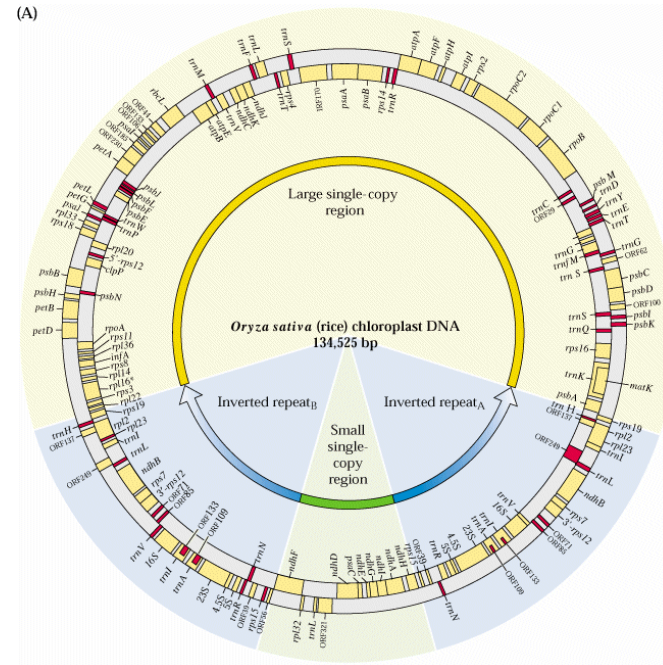
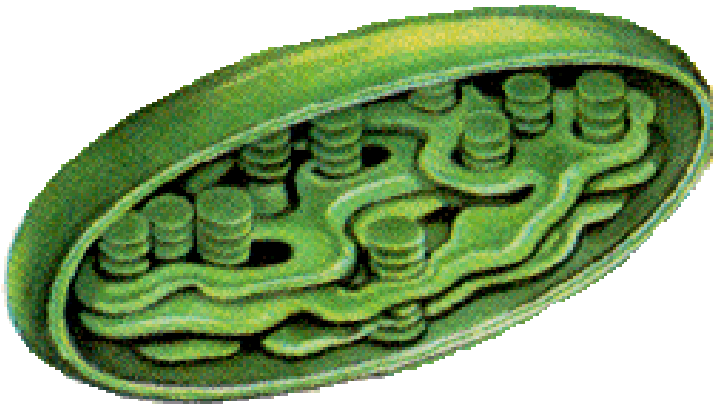
- Random integration
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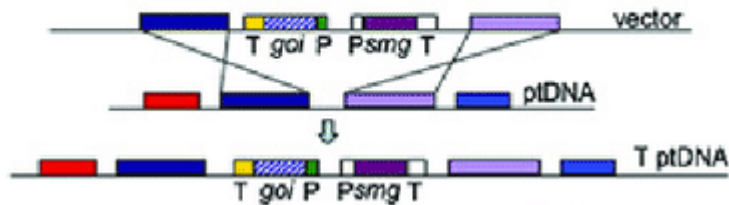


## 4. Plastids

- Chloroplast transformation



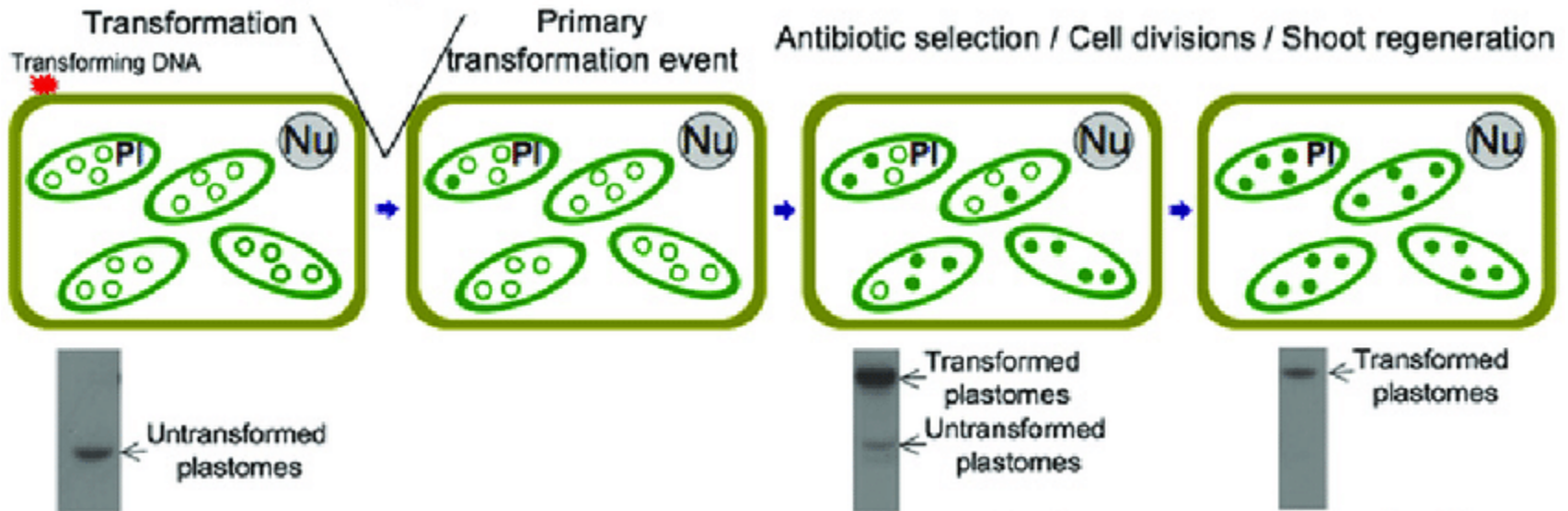
# Homologous recombination



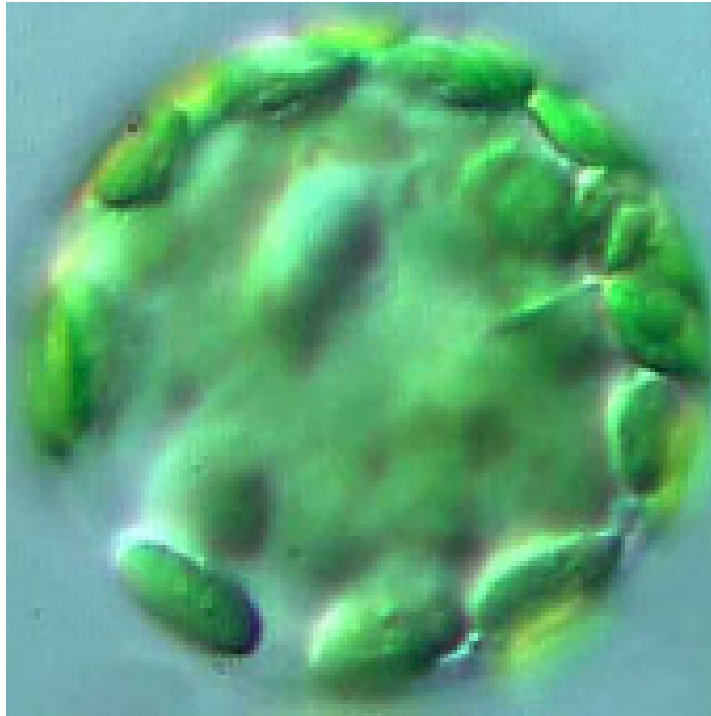
- untransformed plastid DNA
- transformed plastid DNA

# Growth on selection medium

wild-type , , , , , , , , , heteroplasmic , , , homoplasmic



## Transformation: particle gun and protoplasts of leaf cells

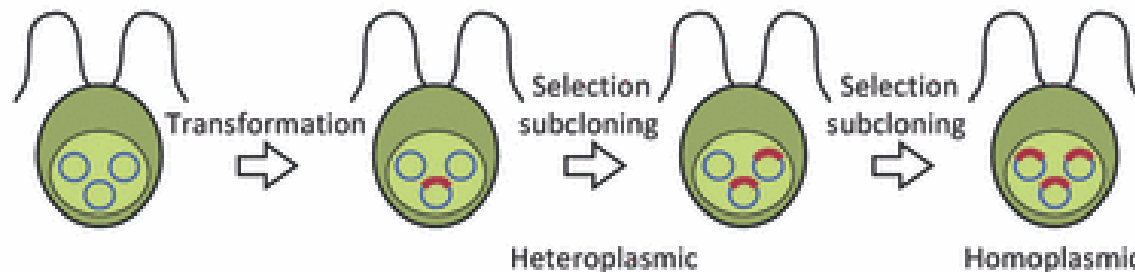
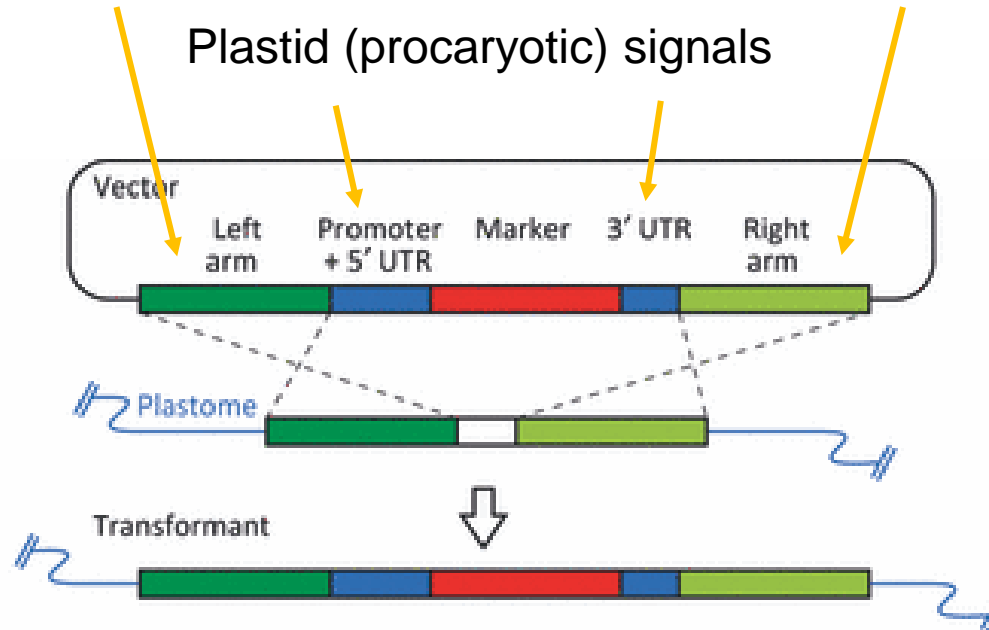


# *aad* as selection marker

aminoglycoside adenylyltransferase (*AAD*) ' spectinomycin- or streptomycin-resistance

Sequences for homologous recombination

Plastid (procaryotic) signals

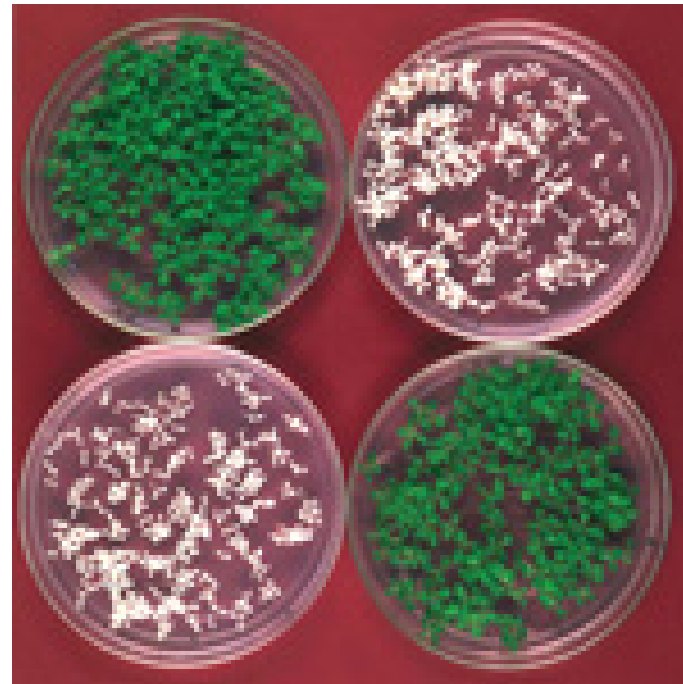




” polymerase subunit



Reciprocal crosses (WT + aad line)  
show maternal inheritance



# Advantage / disadvantage of pt transformation

## disadvantage

- low transformation efficiency

## advantage

- huge amounts of proteins with very little number of plants
- vitamins
- hormones
- industrial precursors
- enzymes
- any protein or peptide
  
- Techniques available for biotechnological removal of selection marker gene, once transformation has occurred.
  
- maternal inheritance (agriculture)
  
- Application to eatable tissue: potato and tomato

# ..... advantage

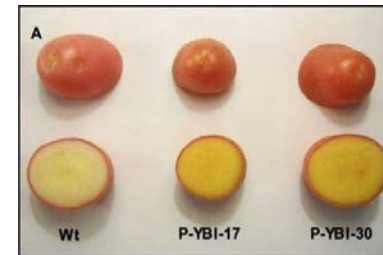
**improved  
resistance**



**Even after boiling:  
vaccines active**



**Improved vitamin A  
and E**



**Sweet potato  
features**



**Cellulose  
improvement for  
paper industry**

**Improved resistance**



**Freshness**



**No boiling:**

**e.g.**

- human growth hormone
- insulin
- Ca<sup>2+</sup>- or Fe<sup>2+</sup>- binding proteins
- Covid 19 vaccine



**No boiling:**

**calmodulin**

**Taiwan: 16.500 babies die yearly because  
of a  $\text{Ca}^{2+}$ -uptake disease**