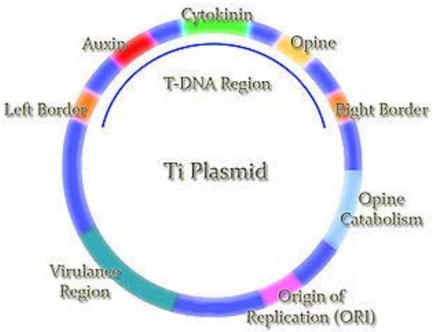
Genetic Engineering of Plants



Roman Sobotka

Terminology matters...

Plant breeding (even conventional like selectively mating plants) is a kind of **genetic engineering** and has existed for a very long time.

Terminology matters...

Plant (green) biotechnology .. usually covers ‘modern’ plant breeding methods like mutation breeding (chemical mutagenesis, X-ray mutagenesis), breeding of polyploids, generation of haploids, tissue cultures, protoplast fusion ... (**conventional plant breeding**)

~ 1960 - **Green Revolution‘** – breeding, plant management (fertization, pest control..)

1983 - first transgenetic (GM) plants

Transgenic, Genetic modified (GM) plants – juridical definition, no clear scientific definition exists

EU - *genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination* (1991)

Many ‘classical’ breeding methods in EU = GMO, a *long list of exceptions ....*

USA, Canada - an organism containing a **transgene** introduced by technological (not breeding) methods, a plant that expresses trait(s) that result from the introduction of foreign DNA

Terminology matters...

New breeding techniques (GM?).. based on research progress in last two decades. A similar to conventional plant breeding —but faster and more precise (genome editing).

CRISPR/CAS9

Mutagenesis using **Zinc-finger a TALEN nucleazes.** No clear in EU, not GM in US but it is GMO in New Zealand (2014)

Cisgenosis and **intragenesis** (cisgenosis = GMO in EU, 2012)

Agro-infiltration

RNA-dependent DNA methylation

Oligonucleotide-directed mutagenesis (ODM) – a canola already marketed in Canada

Grafting onto GM rootstock (apple, wine ...)

CRISPR/CAS9 – a system for precise mutagenesis

Not GMO in US, China, Japan, Brazil and Australia ... white button mushroom (*Agaricus bisporus*) bred by CRISPR/CAS9 entered US marked (2016)

July, 2018 - the decision of the Court of Justice of the European Union:

Crops and food created using gene-editing techniques (CRISPR/CAS9) will be subject to the same regulations as those governing GMO (labelling etc)

...random mutagenesis techniques (chemical, X-ray mutagenesis) that have conventionally been used in a number of applications and have a long safety record are exempt from those obligations...

Future of plant biotechnology in EU?

Position of UK after Brexit?

How to control what is a CRISPR/CAS9 product?

A perspective of CRISPR/CAS9 patents holt by EU universities? ... CRISPR patents for agricultural use (2020): **18 in Europe, 61 in the United States and 259 in China**

- Lecture #1

Methodology – how to create a ‘classical’ GM plant

The first generation of GM crops

Lecture #2

- The next generation of GM crops
- Regulation of GM crops/food, safety, public issues

Why people are producing GM plants?

A pure science ... to study gene functions and regulations:

- gene silencing, inactivation
- mutagenesis,
- analysis of gene expression...

A new way for crop breeding:

- herbicide and pesticide resistance
- better nutritional/industrial value
- virus, pests resistance
- tolerance to drought, salinity...

Other biotech applications:

- new sources of bioengineered drugs
- cleaning of contaminated lands
- rapidly growing biomass
- ....

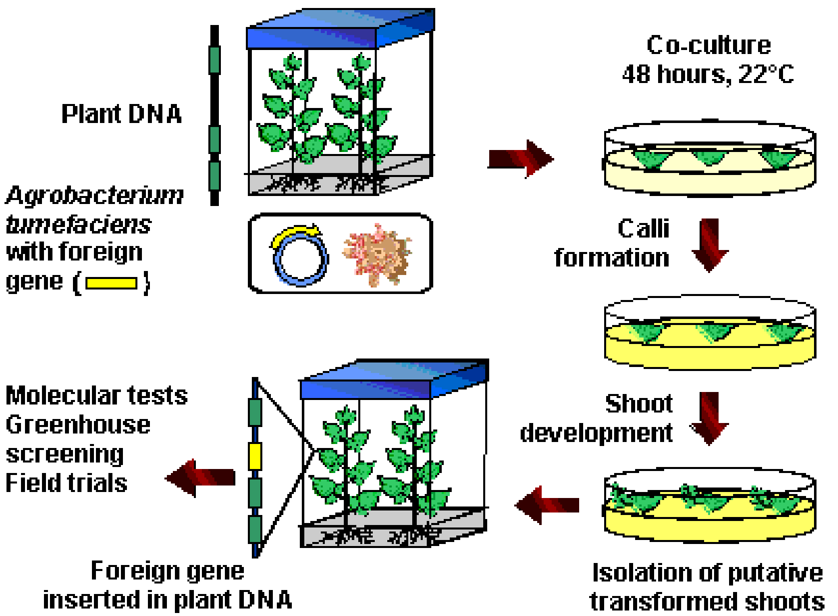
Construction of GM plants in four steps

1.Create recombinant bacteria with desired gene(s)

2. Allow the bacteria to “infect" the plant cells (direct DNA transfer as an alternative..)

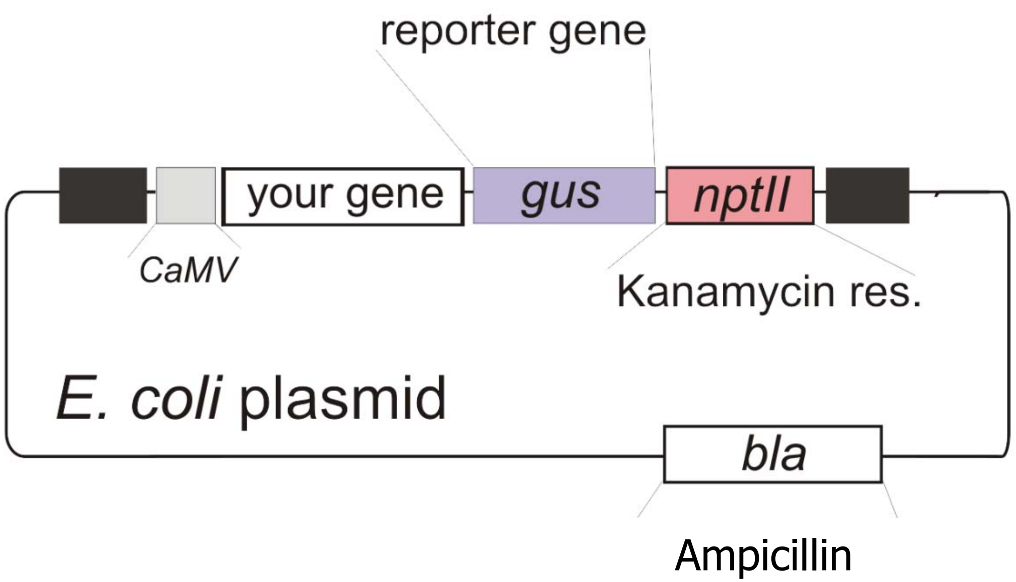
3. Select plant cells with desired gene inserted into chromosomes

4. Regenerate whole plants from plant cells (callus, hairy roots)



#1. A few pieces of DNA have to be assembled together

- promoter + gene(s) you want to express in a plant
- a gene providing resistance against a compound to select transformed cells
- a reporter gene to see that your genes are expressed in the plant cell (optional)



Suitable gene promoters

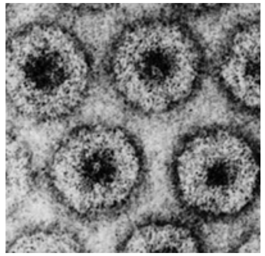
- Constitutive promoters

Cauliflower mosaic virus (CaMV) promoter

Opine promoters – originated from *Agrobacterium tumefaciens*

Plant ubiquitin (Ubi)

Rice actin 1 (Act-1) ...



CaMV

- Tissue-specific promoters:

Phosphoenolpyruvate carboxylase (PEP) promoter - expression only in cells that are actively involved in photosynthesis (green parts)

Glutelin – express specifically in endosperm (seeds)

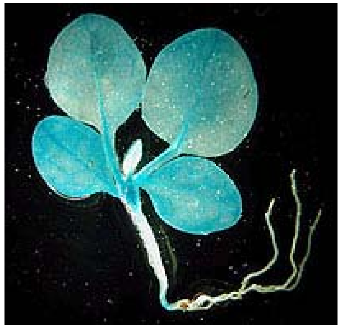
Resistance and reporter genes

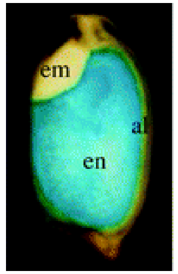
- Resistance

nptII – Neomycin phosphotranferase - detoxifies **kanamycin**

other resistance genes -> to **hygromycin, streptinomycin, gentamycin..**

Basta – PAT (**Glufosinate** resistance - herbicide)

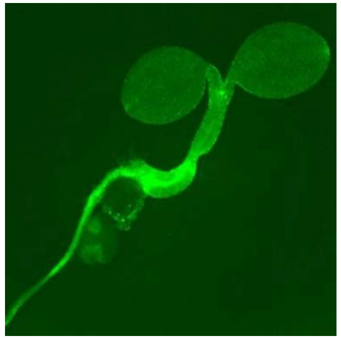




- Reporter genes

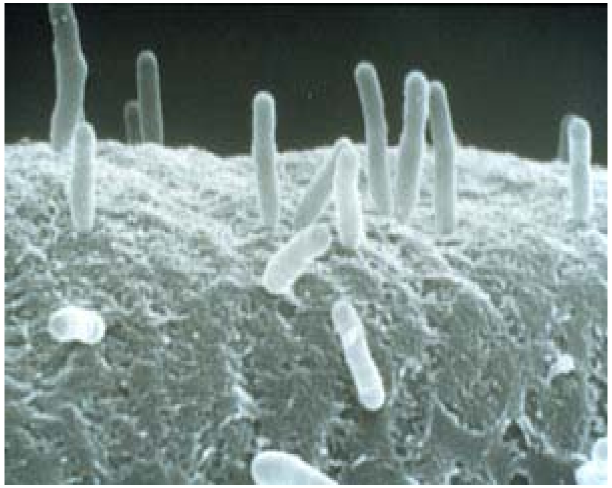
gus - β-glucuronidase - with substrate turns blue

gfp - Green fluorescent protein - fluoresces under UV light, needs no substrate



Agrobacterium sp. – natural tool for plant transformation

- Soil bacteria, gram-negative, related to *Rhizobia* species
- Causes crown gall disease by first transferring part of its DNA into an opening in the plant
- The **DNA then integrates itself** into the plant's genome and causes the formation of the gall



Agrobacterium sp.

Agrobacterium *tumefaciens*- causes crown galls on many dicots
rhizogenes- hairy root disease
rubi- causes small galls on a few dicots
radiobacter- avirulent

A. tumefaciens - specie of choice for engineering dicot plants; monocots are generally resistant (but you can get around this .. see later)

Genome has been sequenced; 4 chromosomes; ~ 5500 genes

Some dicots more resistant than others

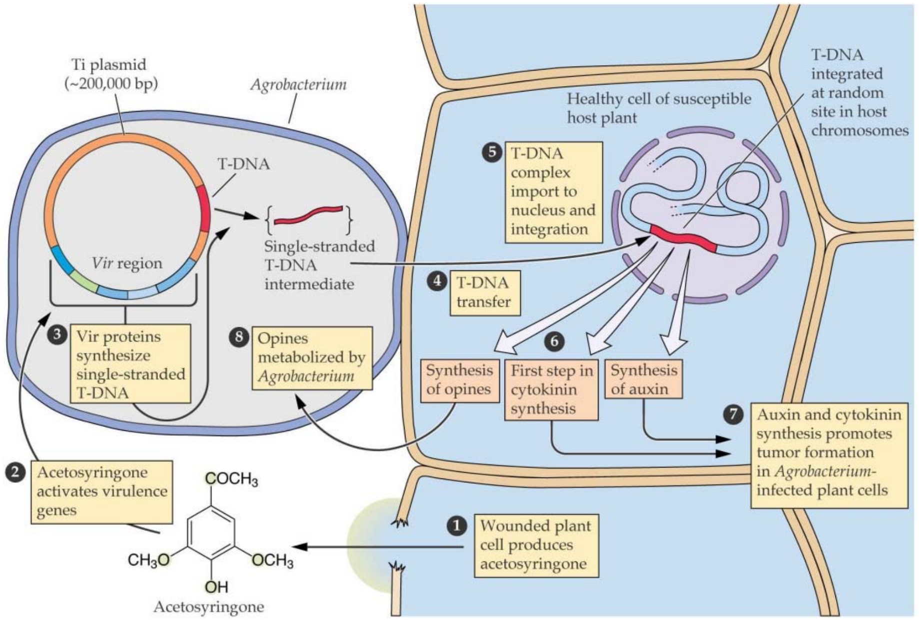
A. rhizogenes – better for some applications (hairy roots culture)

Agrobacterium - 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

Synthesize unique (nitrogen-rich) amino acids called **opines**

- octopine and nopaline - derived from arginine
- agropine - derived from glutamate

Opines are catabolized by the bacteria, which can use only the specific opine that it causes the plant to produce.

Ti plasmid

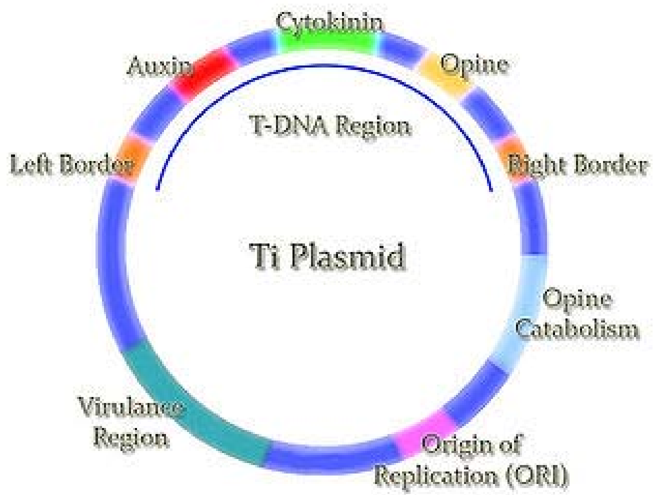
• *A. tumefaciens* contains large plasmids, their presence correlated with virulence. Called tumor-inducing (Ti) plasmids.

• Large (~ 200 Kb) conjugative, ~10% of plasmid transferred to plant cell after infection

• Transferred DNA (called T-DNA) integrates semi-randomly into nuclear DNA

• Ti plasmid also encodes enzymes involved in opine metabolism proteins involved in mobilizing T-DNA (*vir* genes)

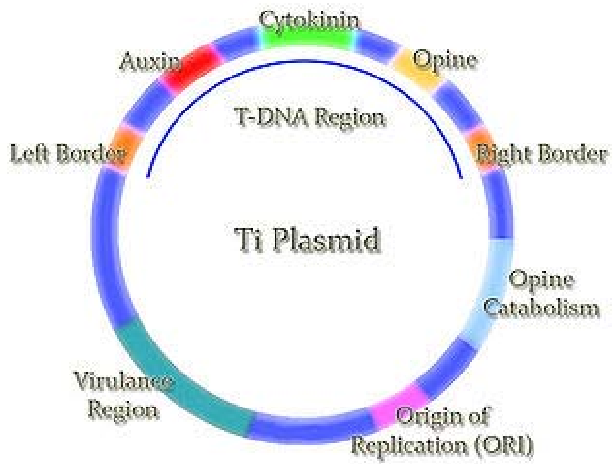
***A. rhizogenes* –> Ri plasmid (root inducing)**



Ti plasmid

T-DNA – integrated into plant chromosome

auxA + *auxB* – enzymes that produce auxin
cyt – enzyme that produces cytokinin
Ocs – octopine synthase, produces octopine



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

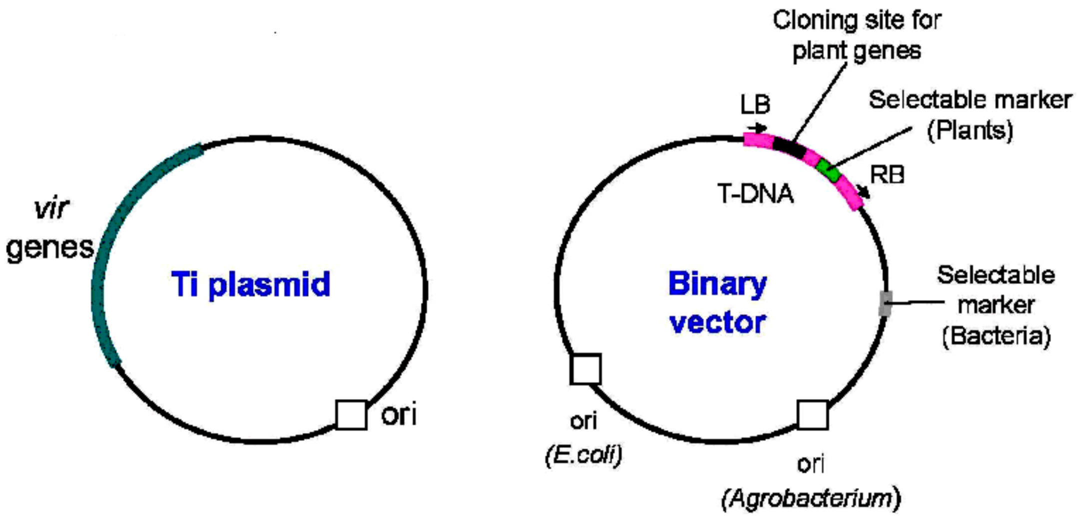
Engineering plants with *Agrobacterium* - two problems had to be overcome:

- Ti plasmids large, difficult to manipulate
- how to regenerate plant from the tumor

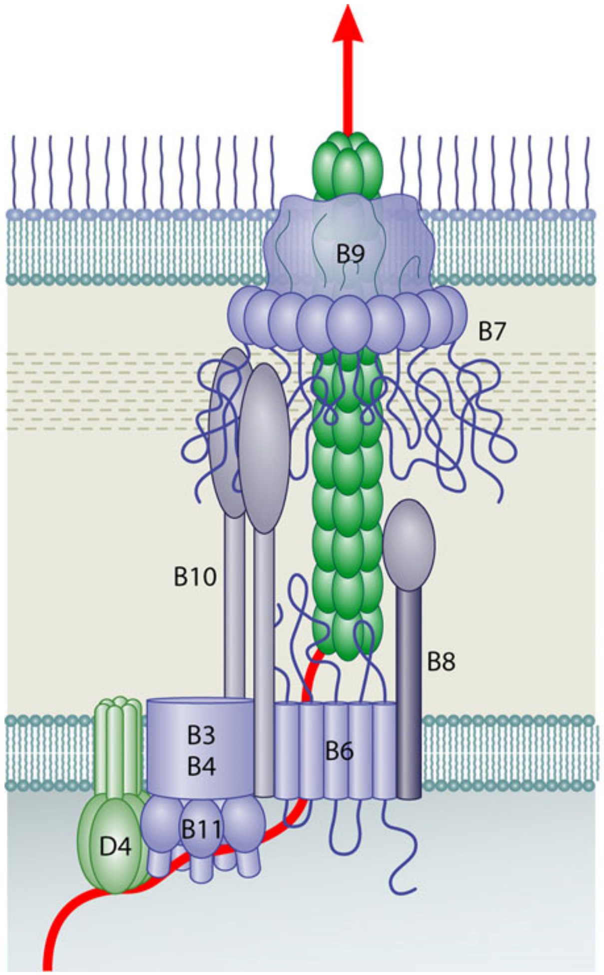
Binary vector system

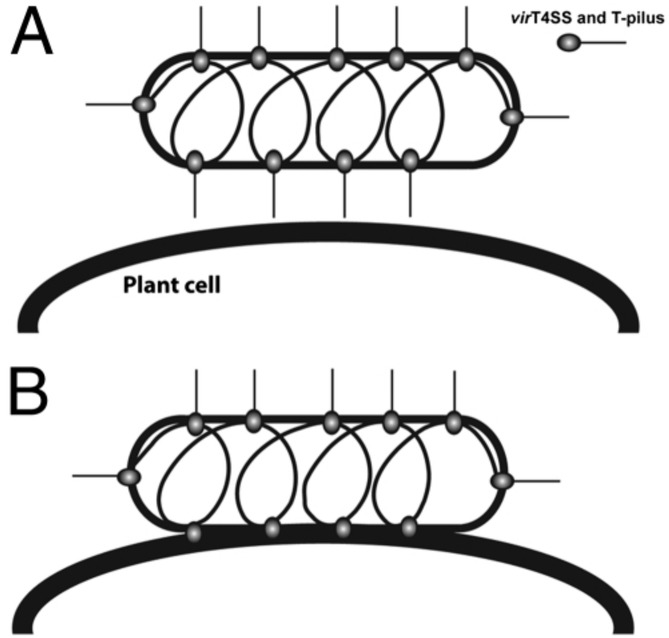
Strategy:

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.



Vir apparatus – inject DNA into plant cell



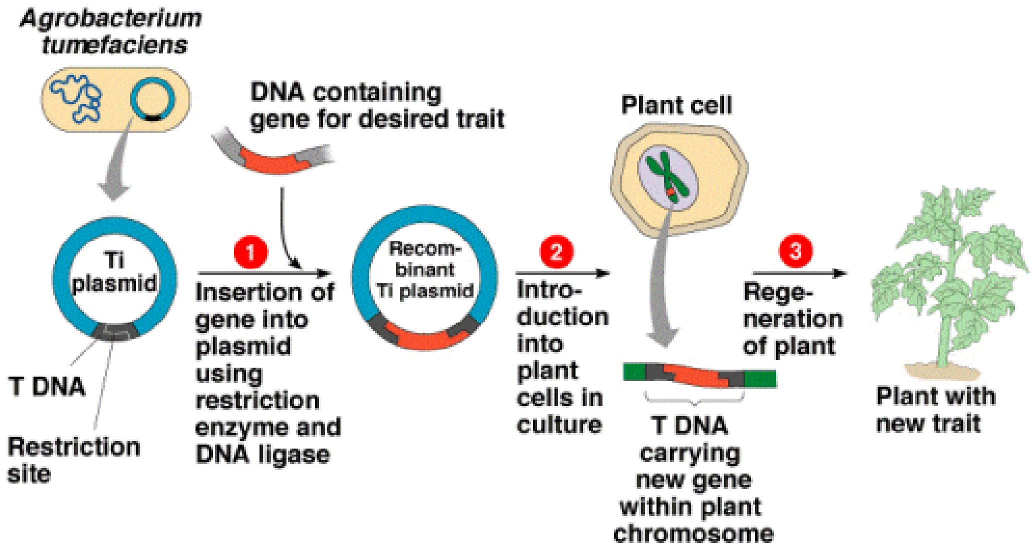


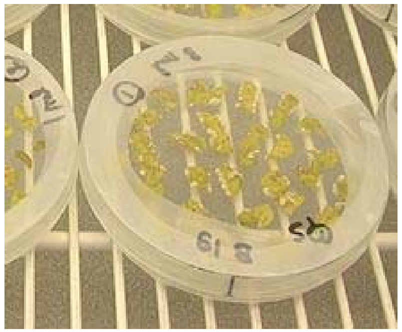
Agrobacterium - Transformation Protocols

Tissue must be capable of developing into normal plants

Leaf-disc transformation - after selection and regeneration with tissue culture, get plants with the introduced gene in every cell

Floral dips, germinating seed, immature embryos – does not require tissue culture. Reproductive tissue is transformed and the resulting seeds are screened for drug-resistant growth

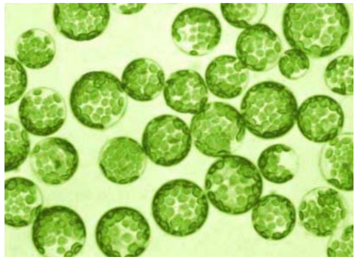


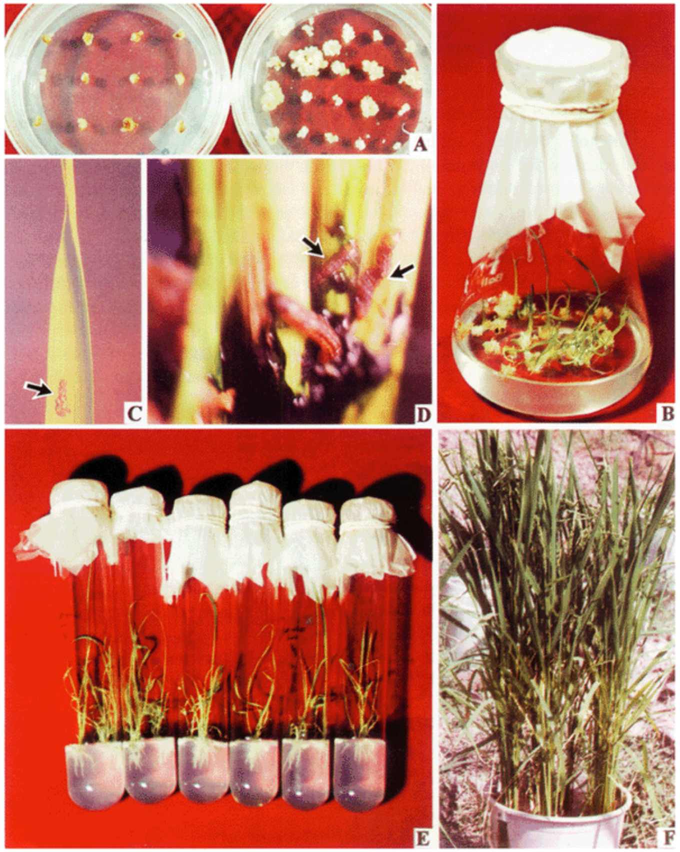


Agrobacterium - Transformation of monocots

An enormous research effort has allowed the engineering of monocots with *Agrobacterium* – most important crops (maize, rice, barley, wheat ...)

- transformation of protoplasts, media with acetosyringone ...





Alternatives to *Agrobacterium* -> direct DNA transfer

- Introduce naked DNA into cells; low effectiveness, not easy to regenerate plants from single protoplasts

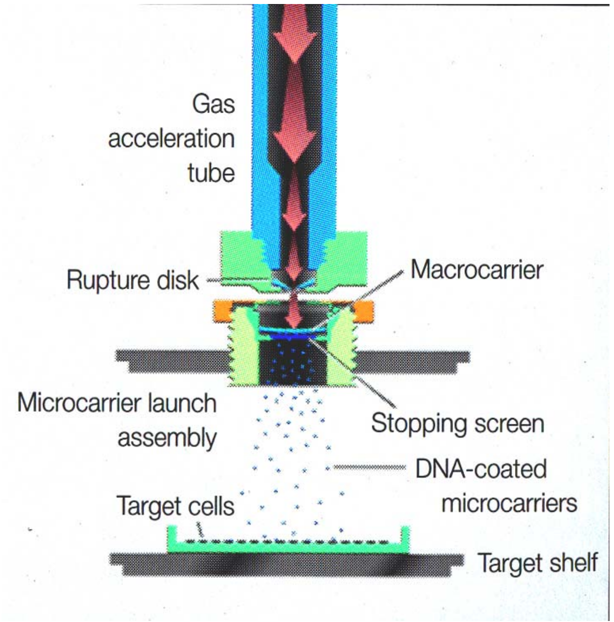
- Chemically-induced transformation (protoplasts)
- Electroporation (protoplasts)
  .... high-voltage pulses cause pores
- Particle bombardment - Bioballistics

Bioballistics

Very small slivers of metal are coated in genetic material.

The coated slivers are propelled into the cell using a shot gun.

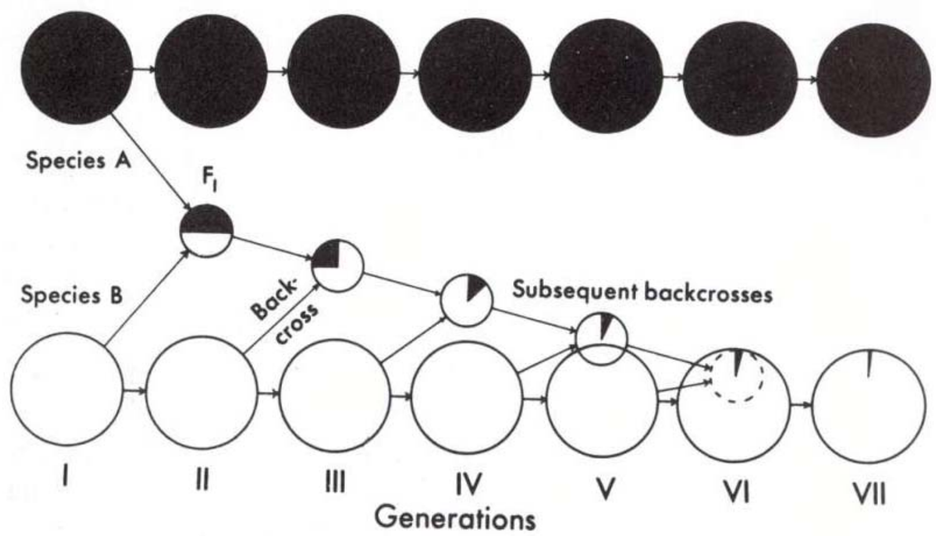
Once inside the cell, the genetic material is taken to the nucleus and incorporated into the recipient's DNA



Next step – > conventional plant breeding

• Lines that survive transformation and tissue culture well are typically lower yielding than current elite lines

• To make these transgenic lines marketable, plant breeders must use breeding techniques to transfer the transgene into a high yielding elite line –> **backcrossing**



Next step – > field and clinical trials

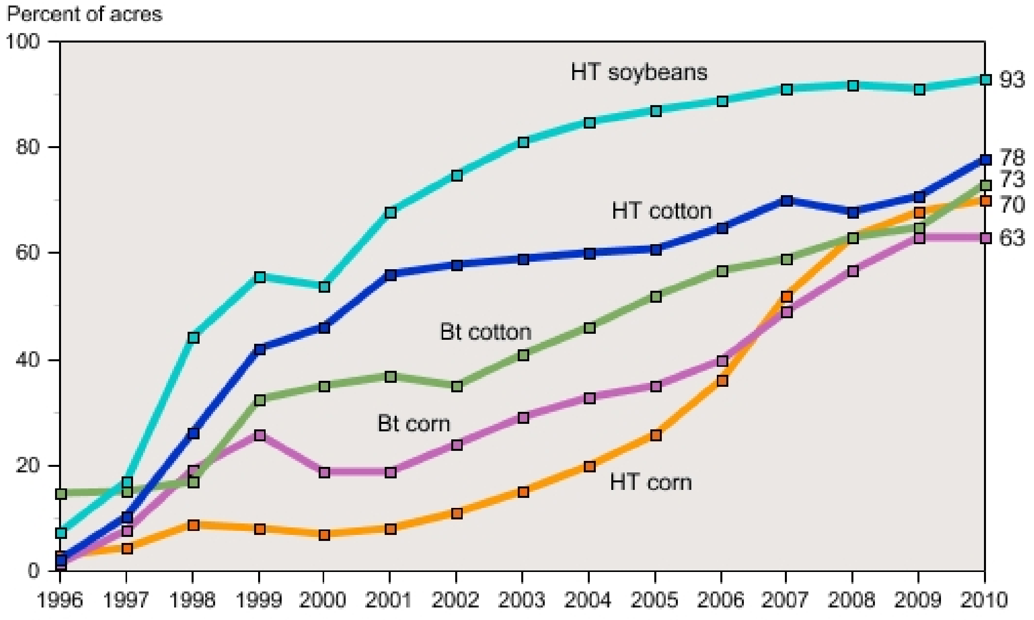
For approval field trials generally required separately in every country

Stringent safety (clinical) testing for GM food, takes long time, expensive

The first generation of GM crops

- produce δ-endotoxin (Bt insecticide), resistant to insect pests
- posses resistance to herbicides

Rapid growth in adoption of genetically engineered crops continues in the U.S.



Data for each crop category include varieties with both HT and Bt (stacked) traits.
Sources: 1996-1999 data are from Fernandez-Cornejo and McBride (2002). Data for 2000-10 are available in the ERS data product, Adoption of Genetically Engineered Crops in the U.S., tables 1-3.

FlavrSavr tomato

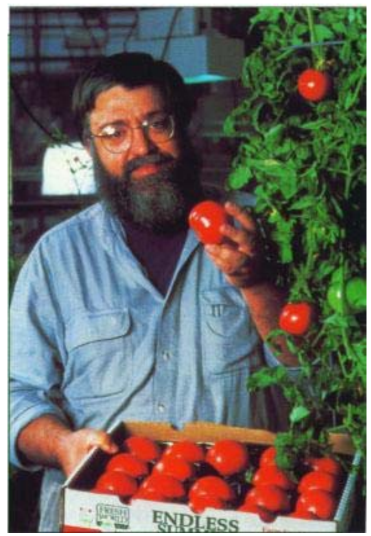
The first GM plant approved for market (1994), small company Calgene (US)

Genetically engineered to have reduced polygalacturonidase - enzyme that breaks down pectin in ripening fruit walls -> delayed ripening

Antisense technology was used to turn off (silence) the polygalacturonase (PG) gene

Gene encoding antisense RNA was inserted into tomato cells

The antisense RNA finds the normal RNA and hybridizes, the cell then degrades this complex, preventing the normal RNA from being translated



FlavrSavr tomato

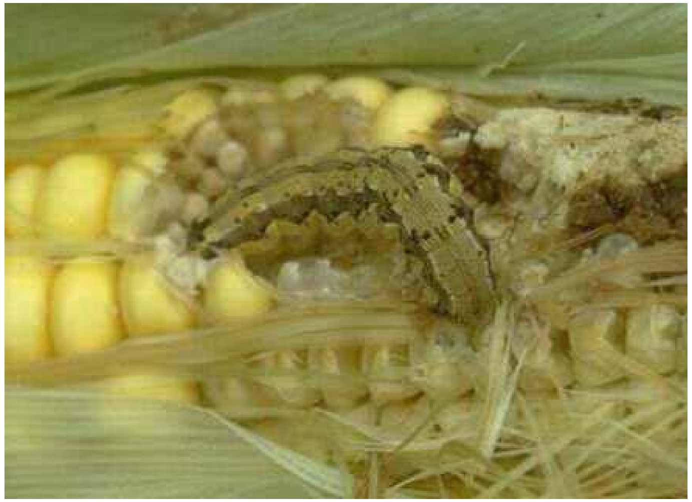
Picked later, prolonged shelf life, reduced shipping loss, improved quality → 20% cheaper

Adopted by Zeneca for UK market, canned tomato puree (1.8 mil can sold),

Withdrawn from market 1998 after ‘Pusztai affair’ → negative consumer response

Resistance to insect pests - Bt crops

Corn earworm, European corn borer, Corn rootworm **...** paintfull losses on maize and cotton, the insects are already resistant to many pesticides

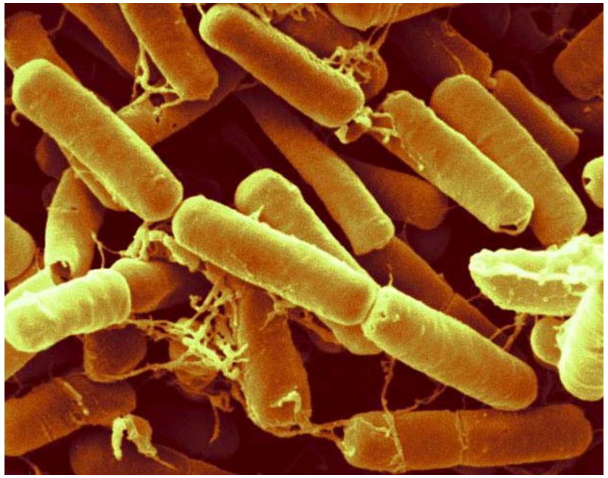


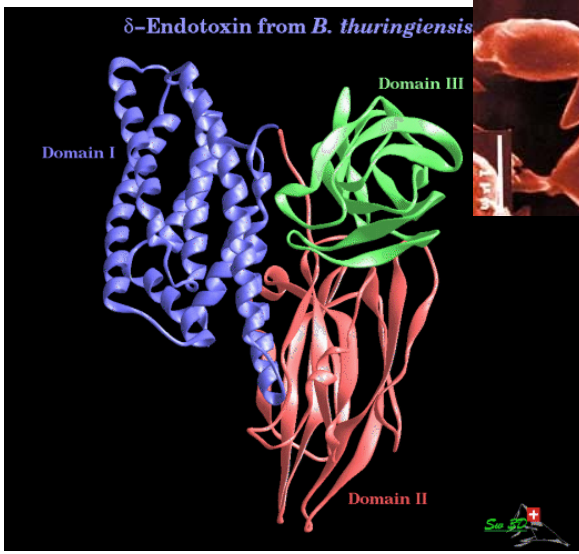
Resistance to insect pests - Bt crops

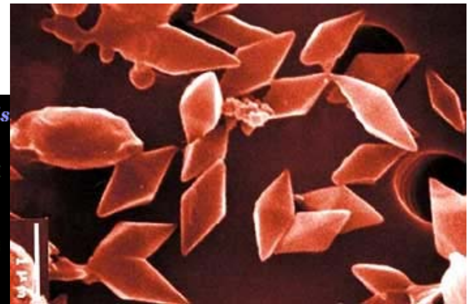
Bacillus thuringiensis is an insecticidal bacterium, marketed worldwide for control of many important plant pests

The protein is very selective - highly effective at controlling *Lepidoptera* larvae, generally not harming insects in other orders (such as beetles, flies, bees and wasps), not toxic for vertebrates

Pest must ingest the toxin crystal to be effective, required high pH and a specific receptor







Bt crops

Bt products represent about 1% of the total ‘agrochemical’ market (fungicides, herbicides and insecticides)

Bt toxin coded by B. *thuringiensis cry* genes (>200 genes isolated in different strains), different effect on insect species

Combining PEP Carboxylase with *cry* gene will produce a plant that poisons pests only if they eat the green parts of the plant

Introduced into maize, cotton, soya, canola, tabaco ...

Sunflower with white mold resistance

transgene = oxalate oxidase from wheat

Oxalate oxidase enhances white mold resistance in cultivated sunflower by degrading oxalic acid, which contributes to white mold pathogenicity

Herbicide tolerance

- Methods used to promote crop growth also promote weeds
- Weeds often outgrow crops and reduce farm output
- Even though there are about 100 chemical herbicides, weeds still reduce crop productivity by ~12%
- Problem is that many herbicides kill both crops & weeds
- This has led to the creation of herbicide tolerant crops

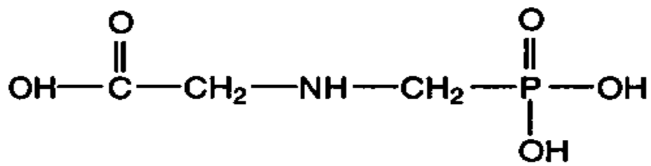
Roundup Ready crops

Glyphosate - inhibits 5-enol pyruvylshikimate
3-phosphate synthase = EPSPS. Involved in synthesis of aromatic amino acids

Roundup = Monsanto herbicide, glyphosate brand, patent expired in 2014

Breaks down quickly in the soil, eliminating residual carry-over problems

GLYPHOSATE (Roundup®)



N-(phosphonomethyl)glycine
(isopropylamine salt

Roundup action

Shikimic acid + Phosphoenol pyruvate

Plant EPSP synthase

Glyphosate

3-Enolpyruvyl shikimic acid-5-phosphate (EPSP)

Aromatic amino acids

Without amino acids, plant dies

Roundup tolerant crops

One strategy is to incorporate a soil bacterium gene that produces a glyphosate-tolerant **form of EPSPS**.

Another way is to incorporate a different soil bacterium gene that produces a **glyphosate degrading enzyme**.

Before **After**

Roundup Ready crops (Monsanto/Bayer)

Corn
Alfalfa
Soybeans
Canola
Sorghum
Cotton
Tomato
Potato
Wheat
...

All patents for the Roundup Ready technology expired ...
but glyphosate going to be banned in EU

Other herbicide systems

BASTA L phosphinothricin (PPT) (**Glufosinate**) -> inhibits **glutamine synthetase**

Bromoxynil –> inhibit **photosystem II**, iolseed rape with a bacterial enzyme degrading Bromoxynil

Sulfonylurea -> inhibits **Acetohydroxyacid synthase (AHAS)** – synthesis of amino acids **,** cotton with mutation in AHAS

Stacked cultivars – both herbicide tolerant and *Bt* resistance, ~ 26% of the total area

Stacked cultivars

Figure 12. Global Area of Biotech Crops, 1996 to 2016: by Trait (Million Hectares)