MOLECULAR BIOTECHNOLOGY

SQG3213

TRANSGENIC PLANTS

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What are transgenic plants?

- **Transgenic** indicates gene transfer using recombinant DNA technology. The transferred gene is usually, but not necessarily, from outside the normal range of sexual compatibility.

- **Synonyms:**

  Genetically modified organism (GMO)

  Genetically engineered organism (GEO)

Plant breeding includes two basic steps

I. Generation (or identification) of variation.

- Collection from wild or farmers
- Hybridization (crossing 2 or more plants)
- Induced mutation, induced polyploidy

II. Selection for desired characteristics. The earliest grain farmers most likely selected for large seed size, seed dormancy, and non-shattering seed heads.

Hybridization can draw upon a range of germplasm resources

Primary gene pool (same species)

- **Elite cultivars**
- **Landraces (primitive cultivars)**
- **Wild plants of the same species**

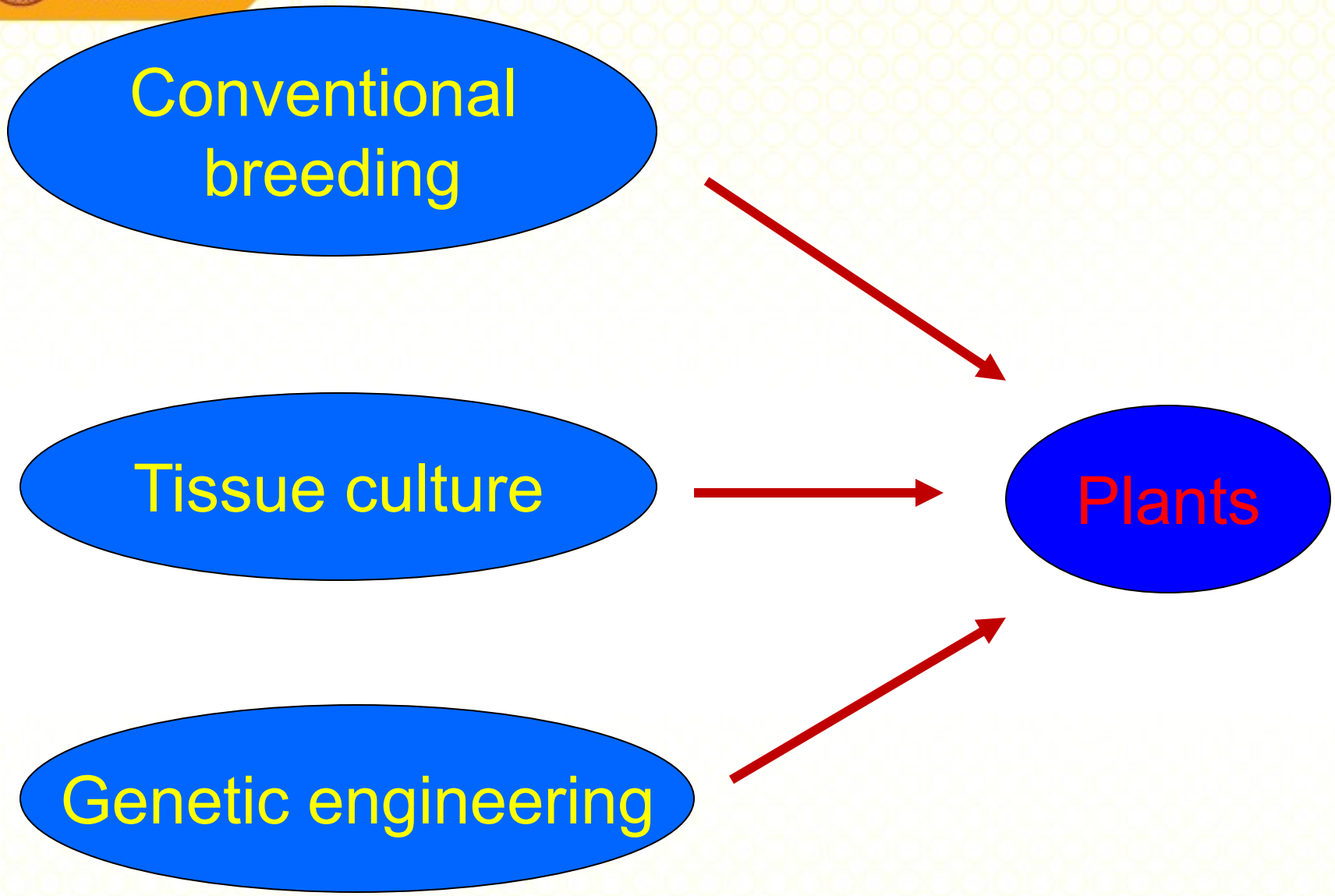
Secondary gene pool

- **Cultivars, landraces, or wild plants of different species or genera. “Wide crosses”**

Wide crosses and induced mutations are not uncommon

- The grain crop triticale is an artificial cross between wheat (*Triticum*) and rye (*Secale*).
- TAM107, a wheat cultivar that contains a rye chromosome arm, is a popular stress-tolerant variety in Colorado.
- Clearfield wheat is herbicide tolerant due to a chemically induced mutation.

Manipulation of Plants


Conventional breeding
Tissue culture
Genetic engineering
Plants

Conventional breeding

- Selection

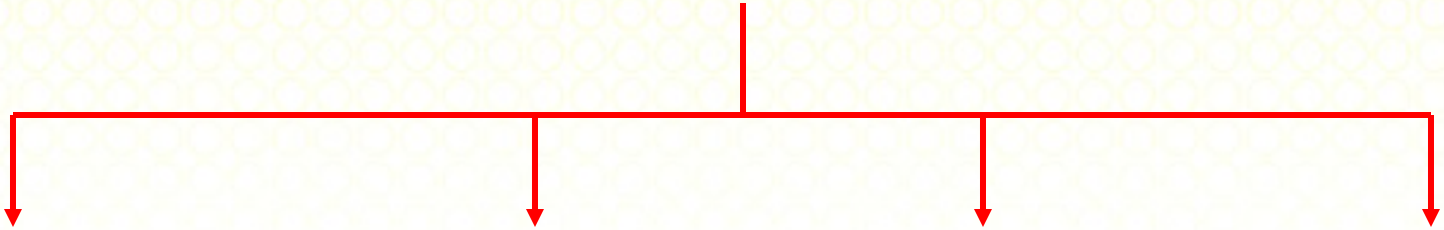
Tissue culture

- Without *in vitro* selection
- With *in vitro* selection

Plant Transformation

- Plants are the easiest of higher organisms to transform
- Both physical and biological methods exist for transformation
- Until recently, only transgenic organisms in wide public release were plants

Plant Transformation Methods



Physical

Microinjection
Pressure
Biolistics - gene gun/ particle bombardment
Electroporation
Microinjection
Silica/carbon fibers
Lazer mediated

Chemical

PEG
DEAE-dextran
Calcium phosphate
Artificial lipids
Proteins
Dendrimers

Biological

A. Tumefaciens
A. Rhizogenes

Virus-mediated

In planta

Is transgenic technology an extension of traditional plant breeding, or is it a revolutionary new development?

- Draws upon genetic variation across kingdoms, rather than within a species or genus.
- Gene transfer is more precise than previous methods.
- But the two basic steps of plant breeding are still followed: generate variation, then select.

Transgenic Plants

- **Why?**

1. Study gene function and regulation
2. Making new organismic tools for other fields of research
3. Curing genetic diseases in people
4. Improving agriculture and related raw materials
5. New sources of bioengineered drugs (use plants instead of animals of bacteria)

Transgenic Plants In Use or About to be on a Large Scale

- Herbicide-resistant plants
- Pest-resistant plants
- Vaccine plants (just starting to be used)

Herbicide-resistant plants

- Resistant to herbicide “Round-up” (Glyphosate)
- Contain bacterial EPSP synthase
- Advantages: better weed control, less tillage
- soybeans, corn, rice, wheat

Herbicide-resistant plants

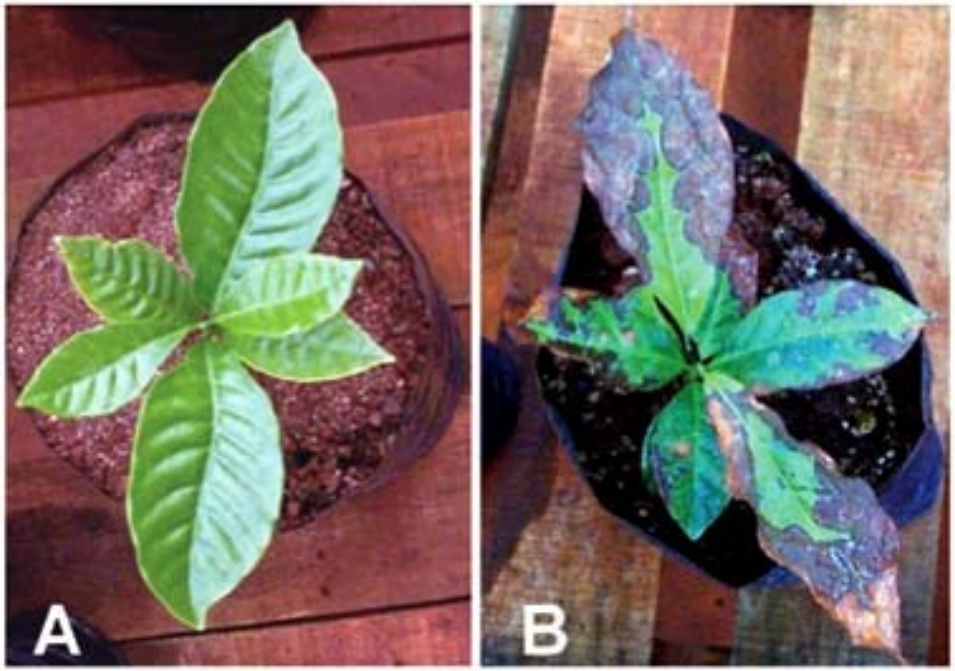


Figure 1. Herbicide tolerant coffee plant (A) and non-transformed plant (B), one week after spraying with ammonium glufosinate at 200 mg.L^{-1} (Ribas et al., 2006).

Pest-resistant plants

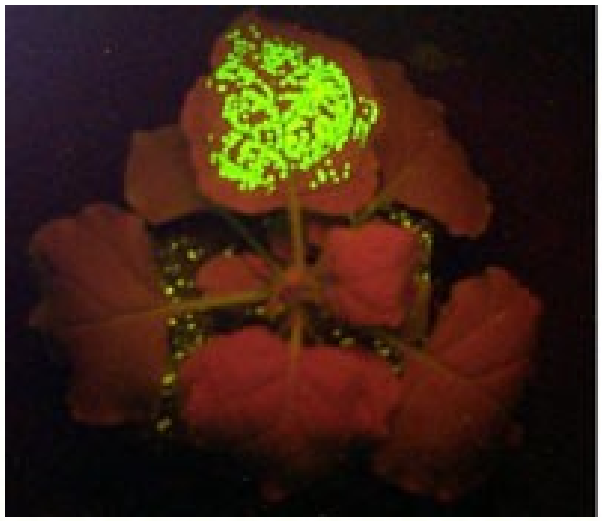
- Resistant to certain insects
- Plants carry gene(s) for *Bacillus thuringiensis* (*Bt*) toxin
- Advantage: less insecticide required, better yield
- corn, cotton, potatoes

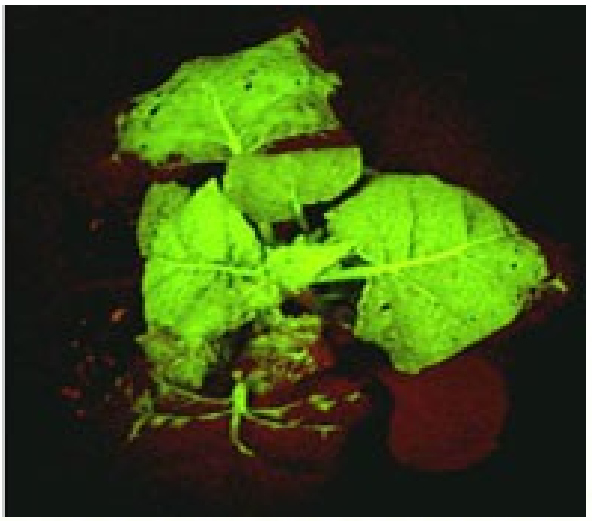
Pest-resistant plants

Vaccine plants

- Pioneered by Charlie Arntzen
- cheap vaccine-delivery system
- use plants producing pathogen protein to induce immunity
- being developed for a number of human and animal diseases, including measles, cholera, foot and mouth disease, and hepatitis B and C.
- potatoes, bananas

Vaccine plants

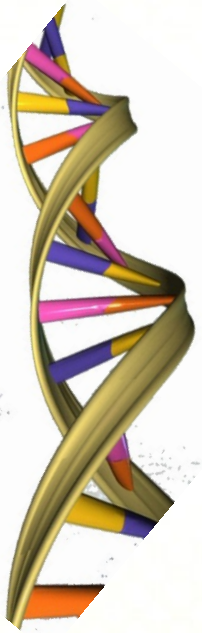




Source: C.J. Arntzen et al. (2005) Plant-derived Vaccines and Antibodies: Potential and Limitations. *Vaccine* 23, 1753-1756

HOW?

A gene is a DNA segment that encodes a specific protein that contributes to expression of a trait.



mRNA → protein ⇢ trait

translation

transcription

expression

Producing transgenic plants

- Isolate and clone gene of interest
- Add DNA segments to initiate or enhance gene expression
- Add selectable markers
- Introduce gene construct into plant cells (transformation)
- Select transformed cells or tissues
- Regenerate whole plants

Identify and clone the gene of interest

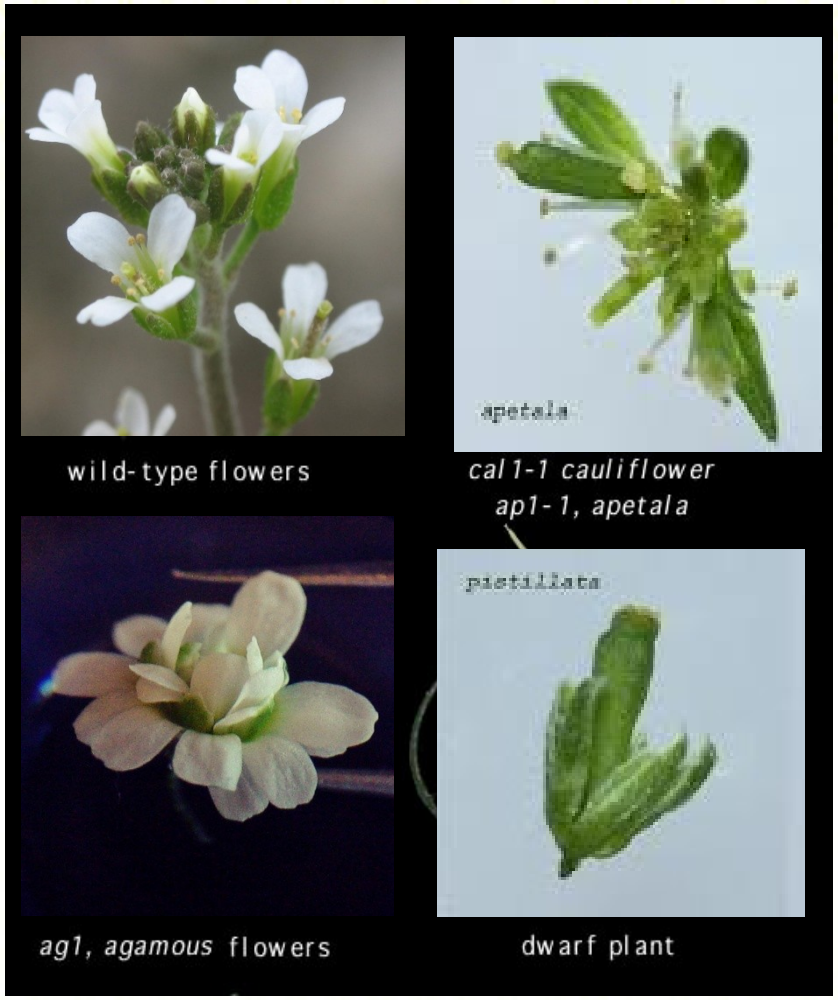
- The most limiting step in the transgenic process.
- Public and private labs are directing huge efforts to locate, identify, characterize, and clone genes of agricultural importance.

Arabidopsis thaliana

- Genome sequence completed in Dec., 2000. Contains ~120 Mb of DNA, and 25,000 genes.
- Tentative functions assigned to 70% of genes.
- Duplicated regions make up 58% of the genome, likely due to a whole-genome duplication event 100 million years ago.

Lessons from *Arabidopsis* genome

- Many more protein-kinase genes than expected, indicating the importance of cell signaling mechanisms in plants.
- Genes for basic cell function are well conserved between humans and *Arabidopsis*, but genes for cell communication are very different, implying
  - Genes for basic cell function existed in a common ancestor of all organisms,
  - but multicellularity evolved separately in plants and animals.



Arabidopsis **mutants generated through transgenic "knock-out" technology, provide clues about gene function.**

Future plant genome objectives

- Determine function of all *Arabidopsis* genes by 2010.
- Sequence the rice genome (smallest genome of grain crops), both public and private sectors.
- Sequence *Medicago truncatula* as a model system for legume biology.
- Sequence selected gene-rich regions of crops with large genomes, e.g., corn, wheat.

Bt genes

- Spores of the soil bacterium *Bacillus thuringiensis* (Bt) contain a crystalline (Cry) protein. In the insect gut, the crystal breaks down and releases a toxin that binds to and creates pores in the intestinal lining.

- A truncated Cry gene is used in Bt crops.

Not required for active toxin

Toxin-encoding sequence

Add DNA segments to control gene expression

Promoter

Bt coding sequence

Termination sequence

- **Promoter** initiates transcription; affects when, where, and how much gene product is produced.
- **Termination sequence** marks end of gene.

Transgene promoters:

- Most commonly used is the CaMV 35S promoter of cauliflower mosaic virus. It is a constitutive promoter (turned on all the time in all tissues), and gives high levels of expression in plants.
- More specific promoters are under development: tissue-, time-, and condition-specific.

Termination sequence:

- Most commonly used is the nopaline synthase (*nos*) transcription terminator sequence from *Agrobacterium tumefaciens*.

Types of promoters:

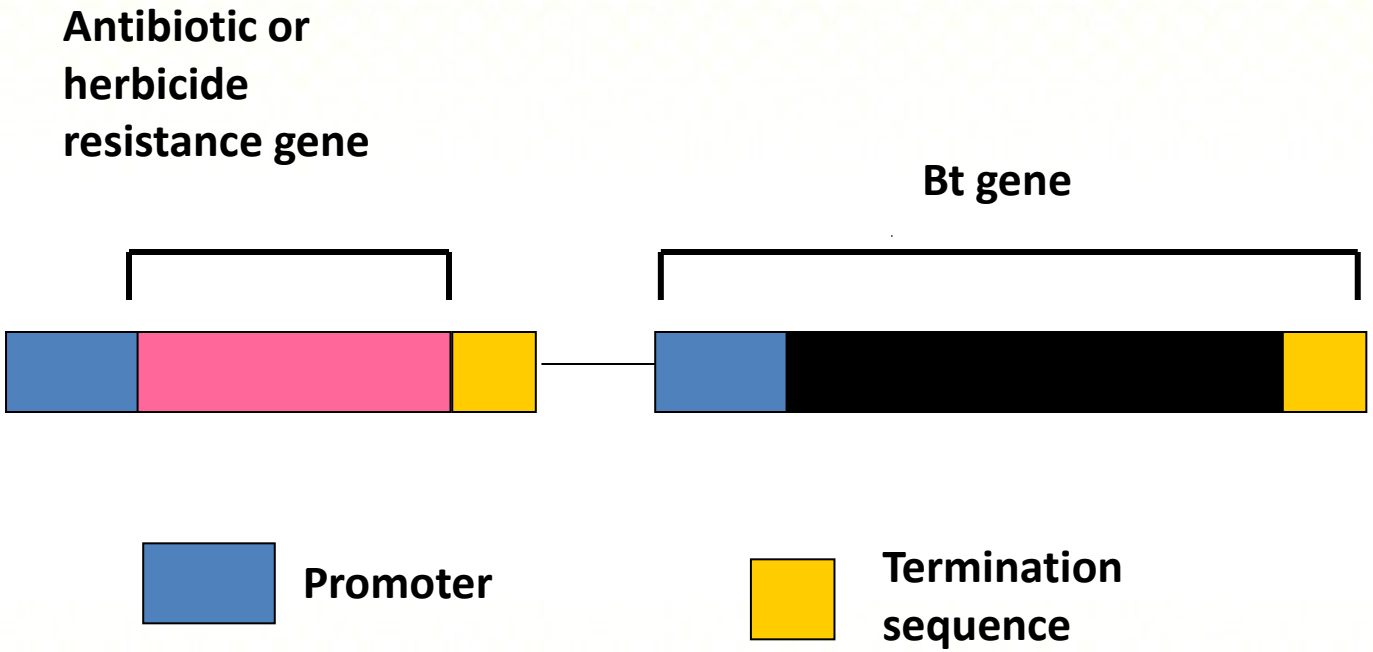
- Constitutive – direct expression in most tissues
  - independent of environment & development cues
- Tissue-specific – direct expression in specific tissue or certain stages of development
- Inducible – expression may be stimulated by environmental condition & external stimuli
- Synthetic – may be made by combining primary element of promoter region from various origins.

Add selectable markers

- Because gene transfer is an inefficient process (1 to 5% success rate), a system is needed to identify cells with the new genes.

- Typically, antibiotic or herbicide resistance genes are used as markers.

Bt gene construct



Introduce gene construct into plant cells (transformation)

- **Direct gene transfer via:**
  - **“Gene gun” (synonyms: biolistics, microprojectile bombardment)**
  - **Chemical**
  - **Electroporation**
- ***Agrobacterium* infection**

Plant tissues used for transformation

The choice of tissue depends on the species, but some common ones are immature embryos, leaf disks, and apical meristems.

Latest craze - the plastids.

The tissue must be capable of generating callus (undifferentiated tissue), from which the complete plant can be produced.

***Arabidopsis* buds can simply be sprayed with a solution of the transgene and vector.**

THE METHODS

Two main ways of introducing DNA into plant chromosomes:

1. Direct gene transfer

2. Biological transformation by *Agrobacterium*- mediated gene transfer

Direct DNA Transfer

- Introduce naked DNA into cells
- Enable immediate expression assay of the gene, or selection of cells that are permanently transformed
- DNA introduction methods:

1. Chemical
2. Electroporation
3. Particle bombardment (Biolistics)

Chemically-induced transformation

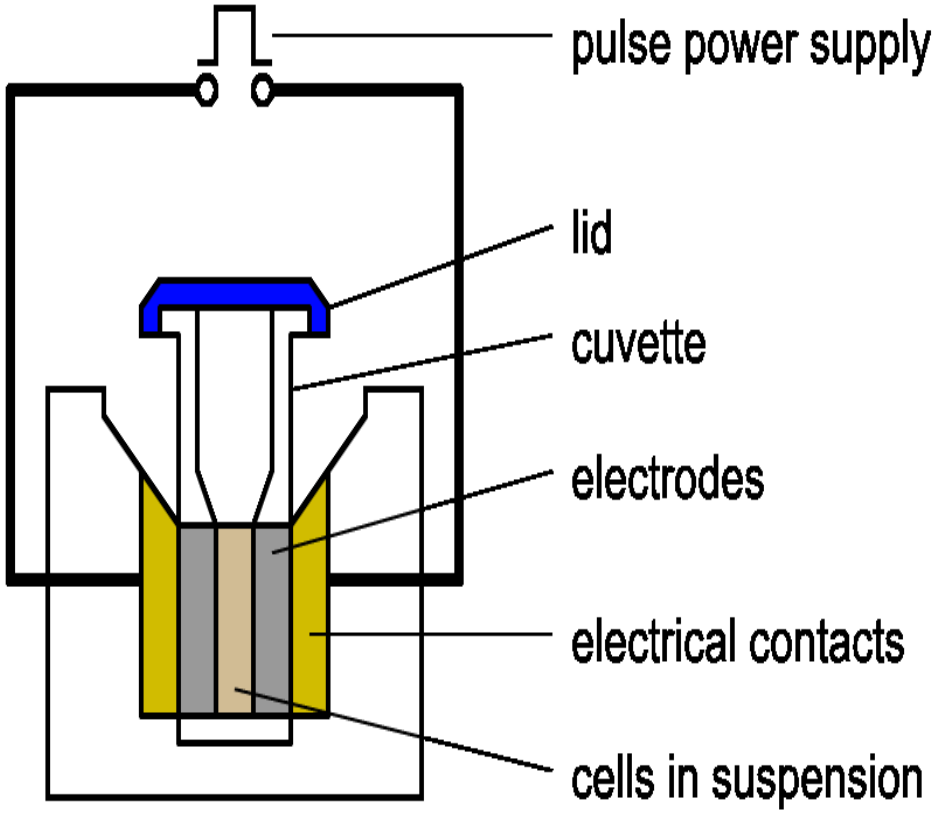
- Usually use on cells without walls

- Multiple protocols:

1. put DNA inside artificial membranes (liposomes), they will fuse with plasma membrane
2. Bind DNA with polycations to neutralize charge, some cells endocytose the complex
3. Combine (2) and (1)

Electroporation

- Use on cells without walls (plant protoplasts or animal cells)
- Used on monocots (maize, rice, etc.)
- High-voltage pulses cause pores to form transiently in cell membrane, DNA slips in
- Drawback - its more cumbersome to regenerate plants from single protoplasts than from the tissue transformations with *Agrobacterium*

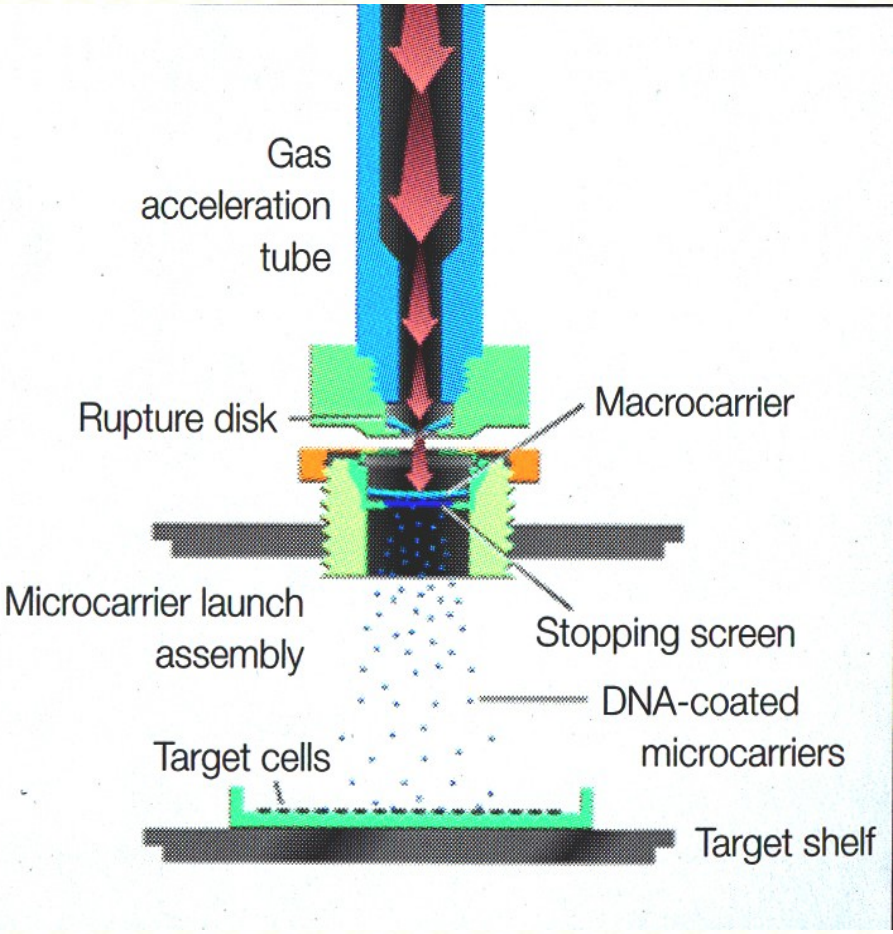


Particle Bombardment (Biolistics)

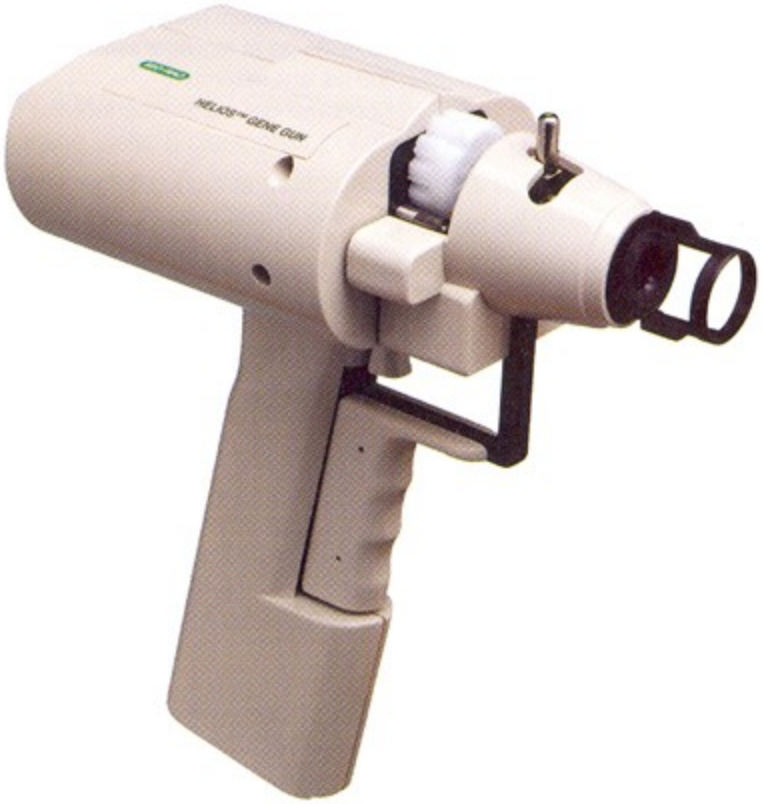
- Less limitations than electroporation
- Can use on cells with walls, or essentially any tissue
- Can transform organelles
- Method:

1. Precipitate DNA onto small tungsten or gold particles.
2. Accelerate particles to high speeds to penetrate cells and tissues.
3. Perform selective growth and regeneration of transgenic plants as described for Agro-mediated transformation.

The Helium Gas Gun – Biolistic



The Hand-Held Gas Gun (Gene Gun)

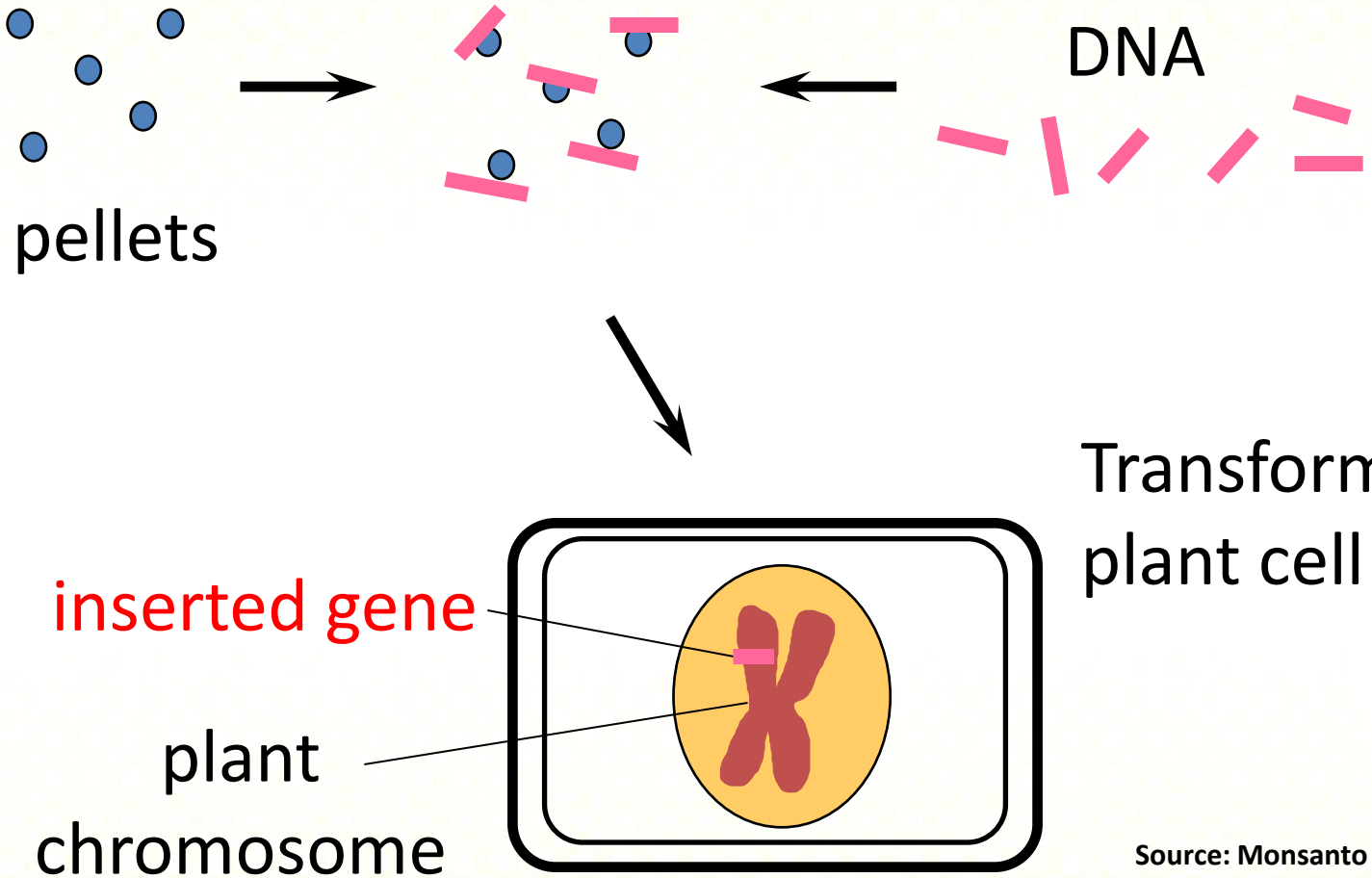


Source: Bio-Rad

Purpose:
Introduce DNA into cells that are below the top surface layer of tissues (penetrate into lower layers of a tissue)

One interesting use:
Making DNA Vaccines in whole animals.

"Gene gun" method



Source: Monsanto

Agrobacterium -mediated Gene Transfer

- Used in dicots and monocots (monocots are more resistant towards *A.tumefaciens* so does some dicots)
- 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 (cane gall disease)
    rhizogenes- hairy root disease
    radiobacter- avirulent species, causes crown gall
    vitis- galls on grapes and a few other plant species

Agrobacterium tumefaciens, a natural plant genetic engineer

- Soil bacterium, related to *Rhizobium*
- causes crown galls (tumors) on many dicots
- Infection occurs at wound sites
- complex bacterium – genome has been sequenced; 4 chromosomes with ~ 5500 genes

Infected Tobacco w/teratoma

Source: Brief recitation in Weaver, pp. 85-89

Agrobacterium tumefaciens inserts part of its DNA into cells of many ornamental and fruit species, causing tumors or galls.

Source: Ohio State Univ.

Agrobacterium infection and tumorigenesis

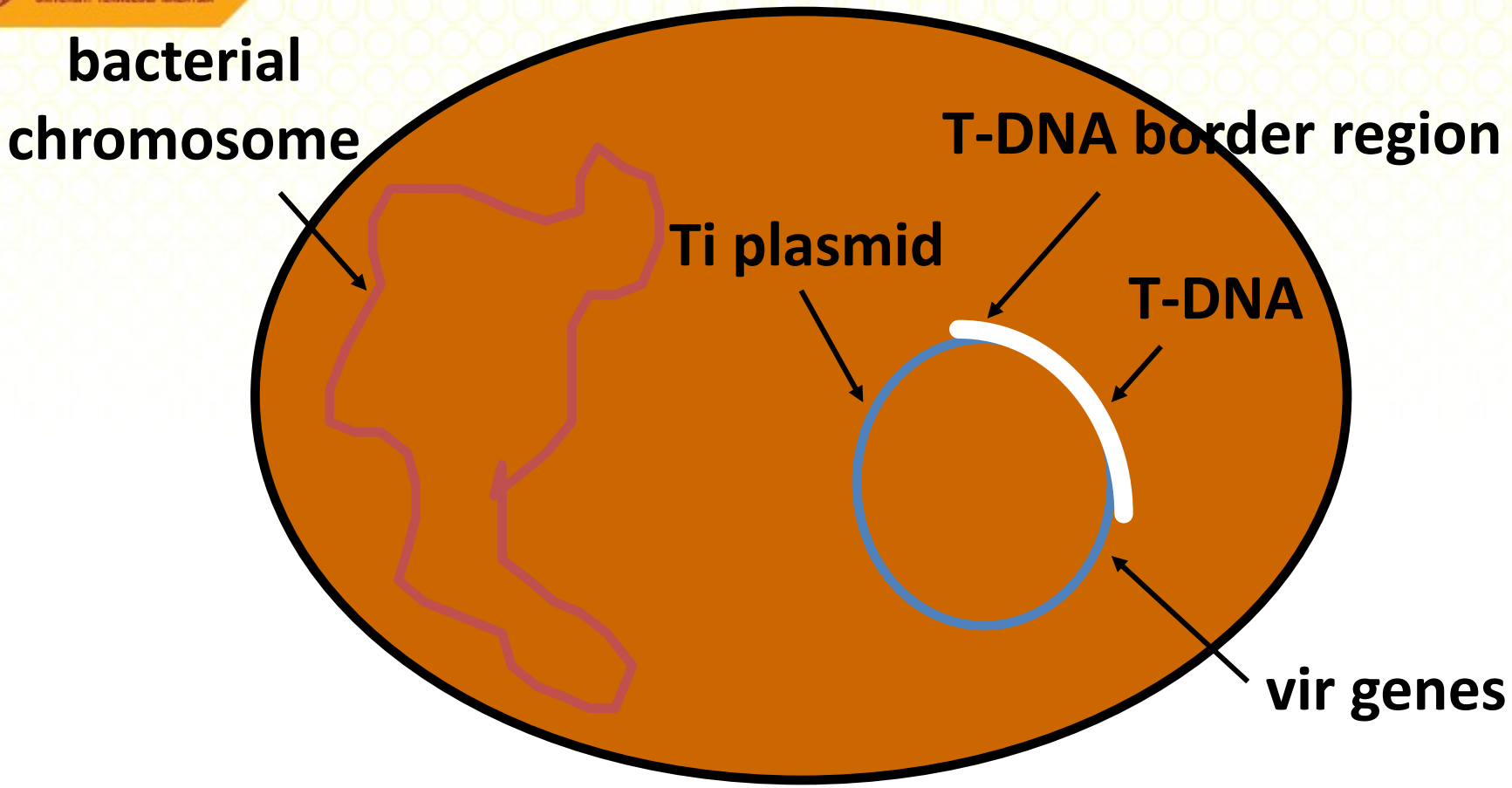
- Infection occurs only at wound sites
- Involves recognition and chemotaxis of the bacterium toward wounded cells
- galls are “real tumors”, can be removed and grow indefinitely without hormones
- genetic information must be transferred to plant cells
- Possible plant compounds, that initiate *Agrobacterium* to infect plant cells:
    - Acetosyringone,
    - ferulic acid,
    - gallic acid,
    - Hydroxybenzoic acid,
    - pyrogallic acid,
    - vanillin etc.

Tumor characteristics

- Altered hormone (auxin & cytokinin) levels causing abnormal growth
- synthesize a unique amino acid, called “opine”
  - octopine and nopaline (derived from arginine)
  - agropine (derived from glutamate)
- specific opine depends on the strain of *A.tumefaciens*
- opines are catabolized by the bacterium, the bacterium cause the plant to produce specific opines for usage

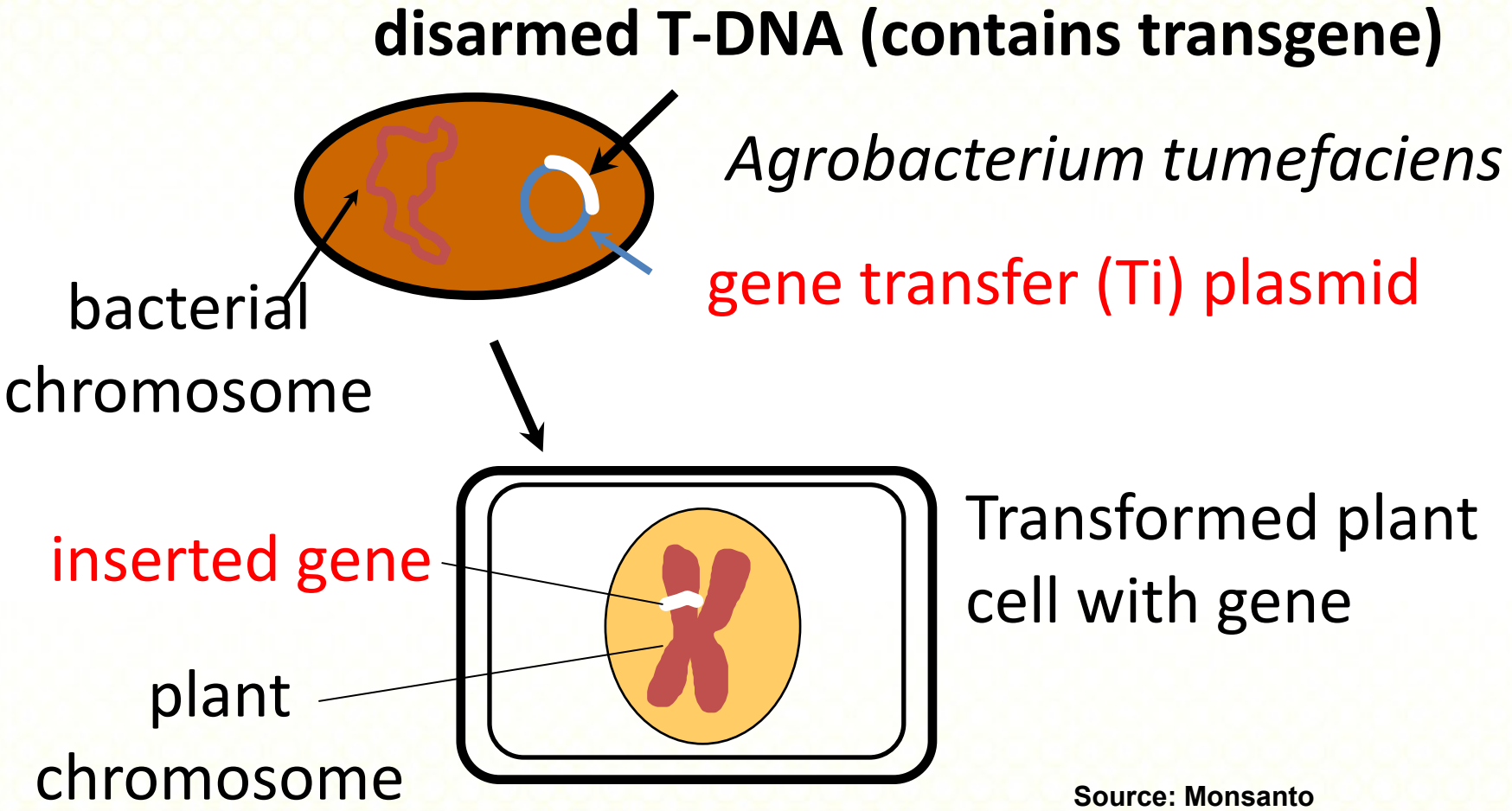
Elucidation of the TIP (tumor-inducing principle)

- Virulence within virulent strains could be cured
- Cured strains could regain virulence upon exposure to virulent strains
- These reversible effect suggested an extra-chromosomal element
- Large plasmids were found in *A. tumefaciens* & were associated with virulence referred to as **tumor-inducing or Ti plasmids.**



In response to chemical signals, the vir genes become activated and direct a series of events to transfer the T-DNA to the plant cell.

Agrobacterium method



Source: Monsanto

Agrobacterium infection

Different *vir* genes

- **Copy the T-DNA.**
- **Attach a product to the copied T-DNA strand to act as a leader.**
- **Add proteins along the length of the T-DNA, possibly as a protective mechanism.**
- **Open a channel in the bacterial cell membrane, through which the T-DNA passes.**

Agrobacterium infection

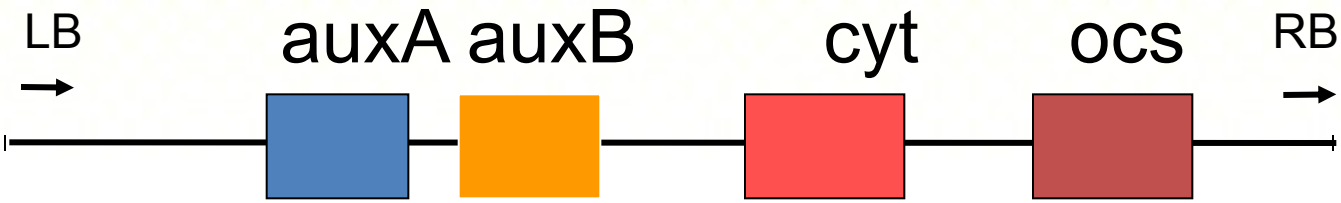
The T-DNA enters the plant cell through a wound, then somehow moves to the nucleus and becomes integrated into the plant chromosome.

One speculation is that the T-DNA waits until the plant DNA is being replicated or transcribed, then inserts itself into the exposed plant DNA.

Ti Plasmid

- 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:
  1. enzymes involved in opine metabolism
  2. 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

- Increased levels of hormones stimulate cell division
- Explains uncontrolled growth of tumor

Vir (virulent) genes

- Found on the Ti plasmids
- Transfer the T-DNA to plant cell
- acetosyringone (AS) (a flavonoid) released by wounded plant cells activates *vir* genes
- *virA,B,C,D,E,F,G* (A-E are operons with multiple ORFs), span about 30 kb of Ti plasmid

Vir genes functions (cont.)

- *virA* - transports AS into bacterium, activates *virG* post-translationally
- *virG* - promotes transcription of other *vir* genes
- *virA* & *virG* – sense phenolic compound from wounded cells & induce expression of virulence genes
- *virD1* - Topiosomerase; Helps Vir D2 to recognise and cleave within the 25bp border sequence
- *virD2*- endonuclease that cuts T-DNA at the borders but only on one strand to initiate synthesis; attaches to the 5' end of the SS
- *virC* - Binds to the 'overdrive' region to promote high efficiency T-strand Synthesis
- *virE2*- DNA-binding protein, binds SS of T-DNA
- *virE1* - chaperone for *virE2*
- *virD2* & *virE2* also help T-DNA get to nucleus in plant cell, they have NLSs
- *virB* - 11 ORFs, helps DNA-protein complex get through cell membranes.
- *virB* & *virD4* - Assemble into a secretion system which spans the inner and outer bacterial membranes. Required for Export of the T-complex and Vir E2 into the plant cell

- Monocots don't produce AS in response to wounding.

- **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:

(1) Ti plasmids large, difficult to manipulate

(2) couldn't regenerate plants from tumors

Binary vector system

Strategy:

1. Move T-DNA onto a separate, small plasmid
2. Remove *aux* and *cyt* genes
3. Insert selectable marker (drug 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

Plant Transformation Methods

Virus-mediated gene transfer
(Plant viruses as vectors)

Caulimoviruses – ds DNA – CaMV

Geminiviruses - 2ss DNA – maize streak virus

RNA plant viruses - TMV

2 Common Transformation Protocols

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

2. Floral Dip – does not require tissue culture. Reproductive tissue is transformed and the resulting seeds are screened for drug-resistant growth

(Clough and Bent (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant Journal 16, 735–743)

Selectable Markers

- A gene encoding an enzyme
- Antibiotic resistance
- Herbicide resistance
- Positive selection genes
  - genes that allow use of some necessary media component.
  - *nptII* - kanamycin (antibiotic)
  - *hpt* - hygromycin
  - PMI (Phosphomannose isomerase)- changes mannose to useable carbohydrate

Novel Selection Genes

- Luciferase - gene from fireflies – substrate
- Green Fluorescent Protein - from jellyfish - under lights and filter the transgenic plants - GFP
- GUS - glucuronidase gene will convert added substrate to blue color.

Production of transgenic plants

Isolate and clone gene of interest

Add DNA segments to initiate or enhance gene expression

Add selectable markers

Introduce gene construct into plant cells (transformation)

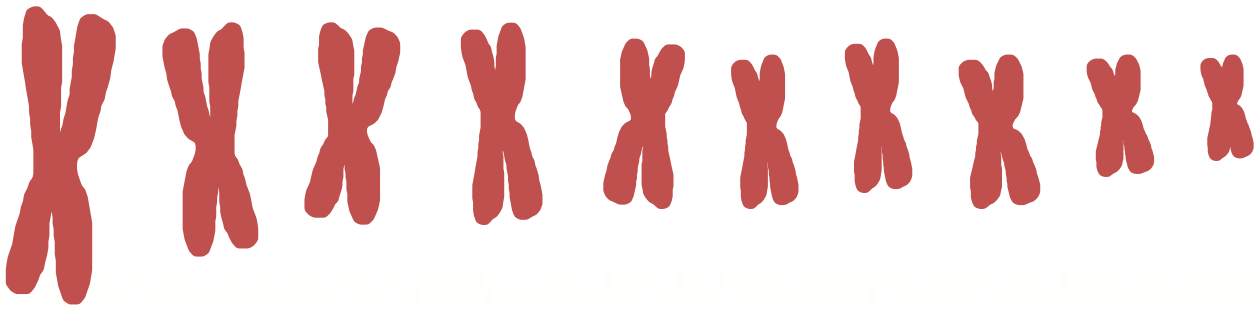
Select transformed cells or tissues

Regenerate whole plants

Transgenic "event"

Event = Successful transformation

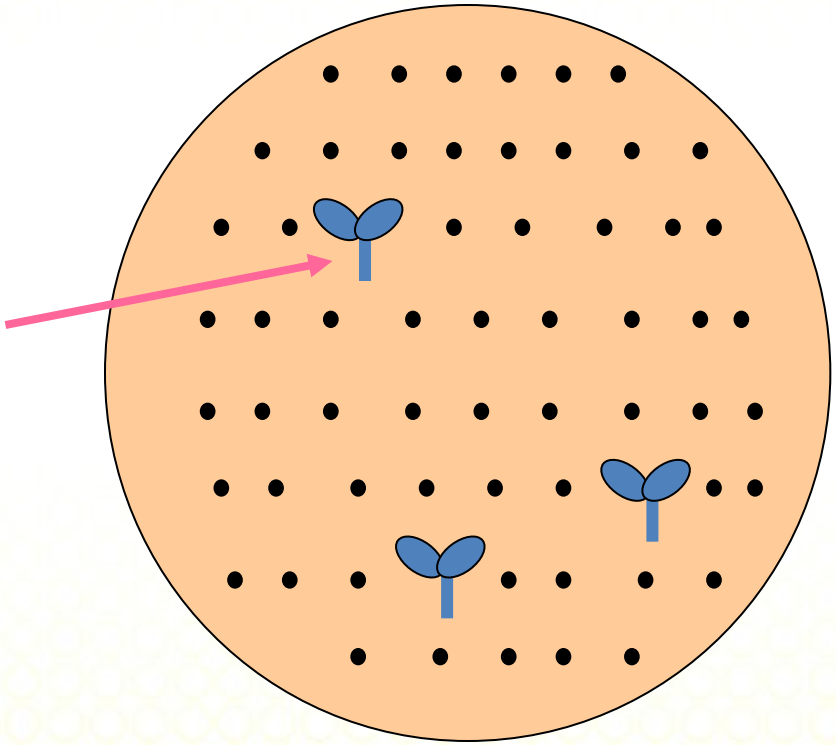
Events differ in the specific genetic components, and in the place of insertion of the foreign DNA into the host chromosome.

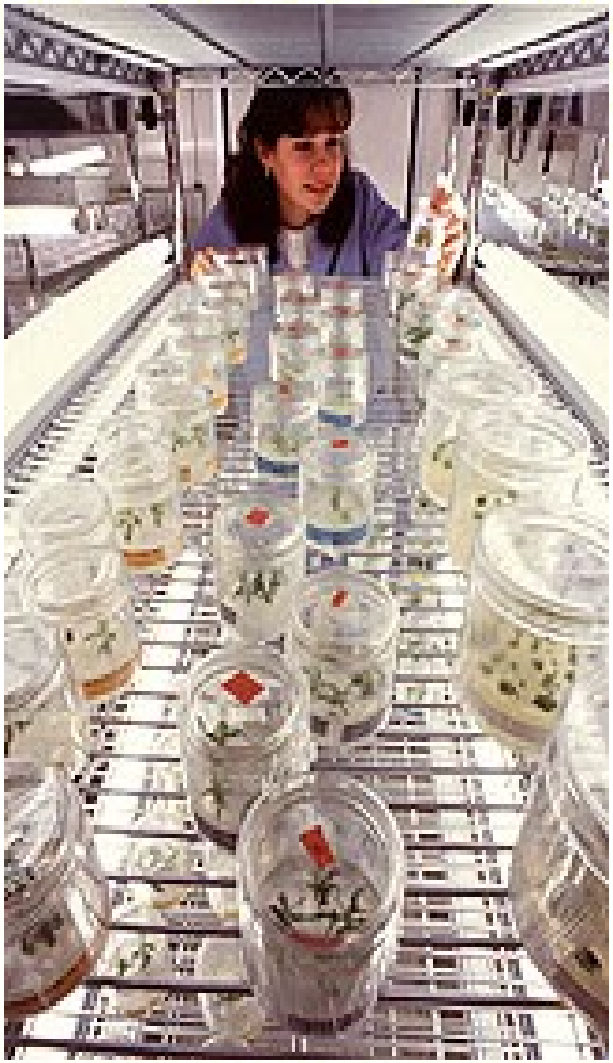


Corn has 10 chromosomes, any of which might incorporate the transgene.

To identify cells/tissues in which new genes are incorporated into plant's DNA, grow in media containing antibiotics or herbicides.

Successful transformant





Whole plants with inserted genes are regenerated through tissue culture.

Source: USDA

Selection & Regeneration

- Cells which contain the selectable marker gene can grow
- All plants that develop are transgenic
- Plant transformation using physical or biological methods requires a tissue culture stage

Analysis of T_1 plants

Morphology

Physiology

Yield characters

GUS expression

Gene expression

Confirmation with selectable marker, Screenable marker, Negative & Positive control

Evaluate transformed plants

- Presence and activity of introduced gene
- Other effects on plant growth
- Environmental effects
- Food or feed safety

Presence and activity of introduced gene

- Southern blot -- is the introduced DNA present in the plant's genome?
- Northern blot -- is mRNA produced?
- Western blot -- is the protein produced?
- Is the expected phenotypic trait observed?

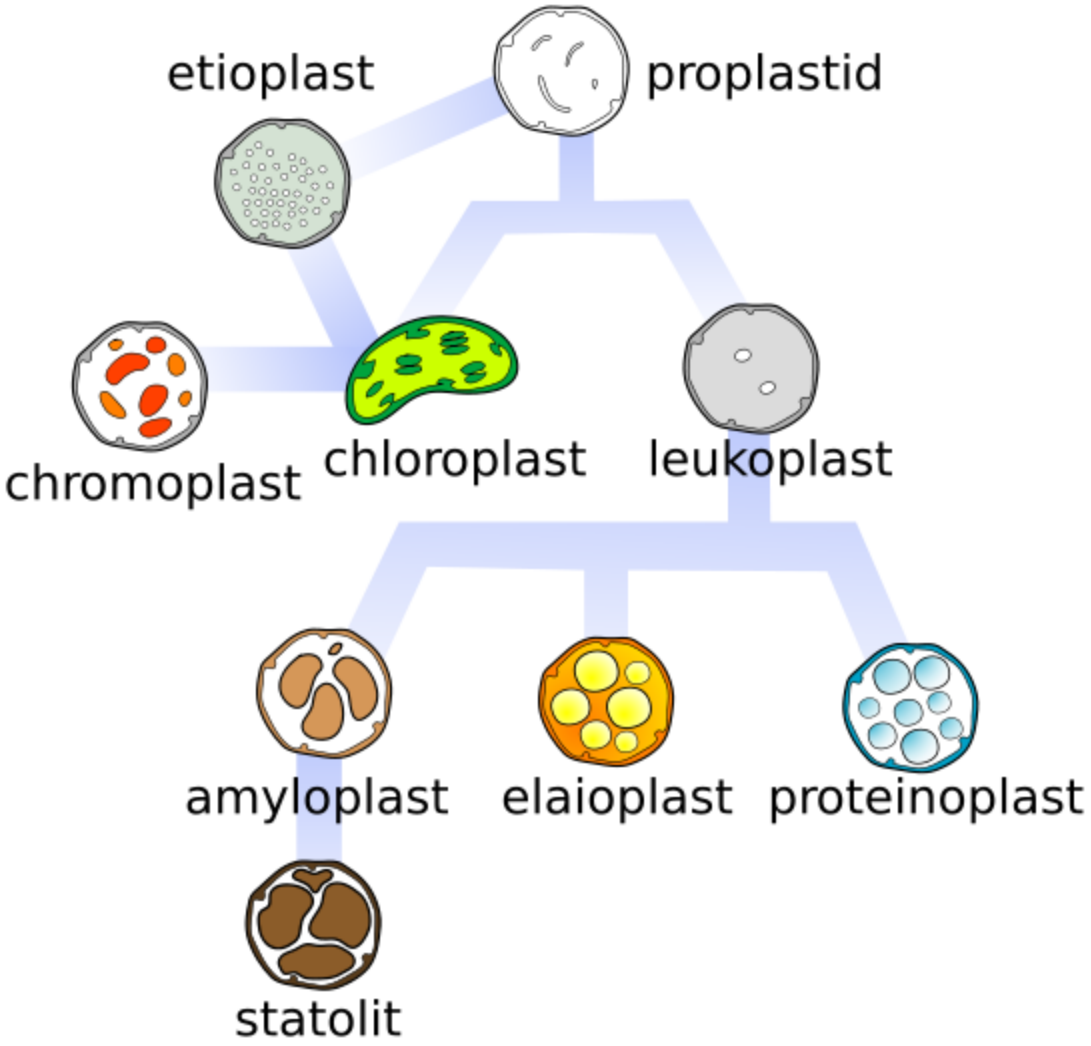
Backcross transformed plant into an improved variety

- For most plant species, only a few lines or varieties will give high rates of transformation. Often they are lines with poor agronomic or quality characteristics.
- Therefore, an improved variety must be backcrossed for several generations to the transformed plant.

Plastid Transformation

- Cytoplasmic organelles of photosynthetic cells found in plants and algae

- Various kinds of plastids



Basic Principle of Plastid Transformation

1. Coating of the gene or genes to be introduced into the plastid genome with microscopic gold particles (0.6-1 pm in diameter).

2. Bombardment of DNA-coated gold-particles into plant cells using a helium-driven biolistic gun.

3. Selection of transformed plant cells (plant cells that contain a plastid or plastids with the gene of interest) are selected

4. Regeneration of a new transplastomic plant from the plant cells.

With antibiotics-based selection method, selection & regeneration are prone to errors but there are ways beyond it.

Future of transgenic technology

New techniques will improve efficiency and may resolve some health or environmental concerns.

- **Insertion at specific points in the genome**
- **New marker genes to replace antibiotic resistance markers**
- **Better control of gene expression (only when and where needed)**
- **Transformation of chloroplasts rather than nuclei**

References:

- Bernard R. Glick. (2008) Molecular Biotechnology: Principles & Applications of Recombinant DNA - John Wiley & Sons, Inc., USA.
- Acquaah, G. (2004) Understanding Biotechnology: An Integrated and Cyber-Based Approach. Pearson, Prentice Hall, New Jersey.
- C.J. Arntzen et al. (2005) Plant-derived Vaccines and Antibodies: Potential and Limitations. *Vaccine* 23, 1753-1756
- Clough and Bent (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant Journal* 16, 735–743