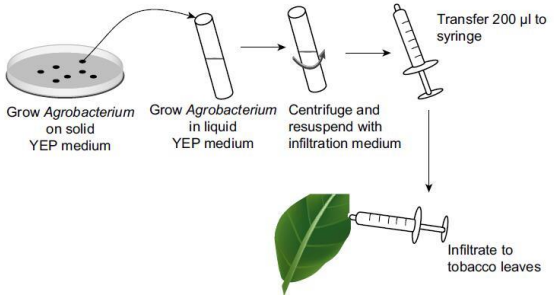
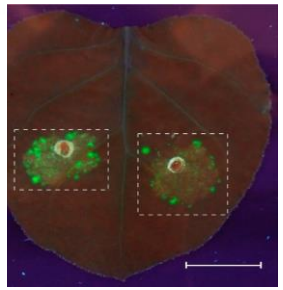
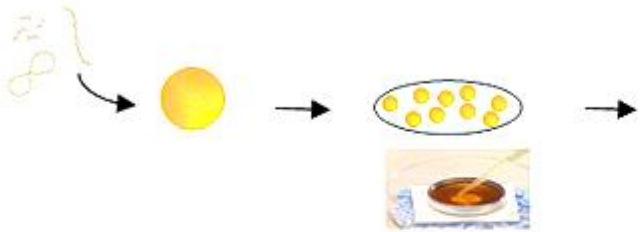


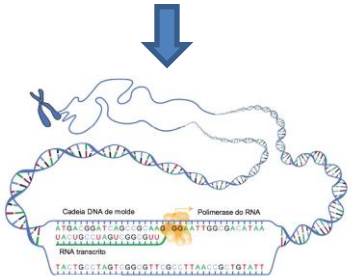


Transient Genetic Transformation Methods for Protein Localization

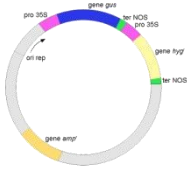
Theoretical-practical
October 2024
smserrazina@fc.ul.pt



Organism



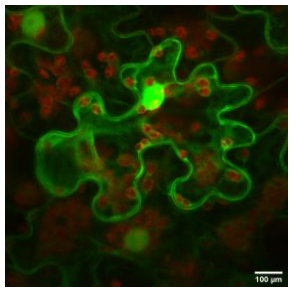
Gene



Plasmid

Methods of genetic transformation (model organism)

Expression and localization of the reporter protein



Serrazina S., Malhó R. et al. (2021), Front. Plant Sci. 12:628697

Leaf epidermis of *Nicotiana benthamiana*: GFP

Transient transformation: short-lived expression, with no purpose to be passed on to offspring.

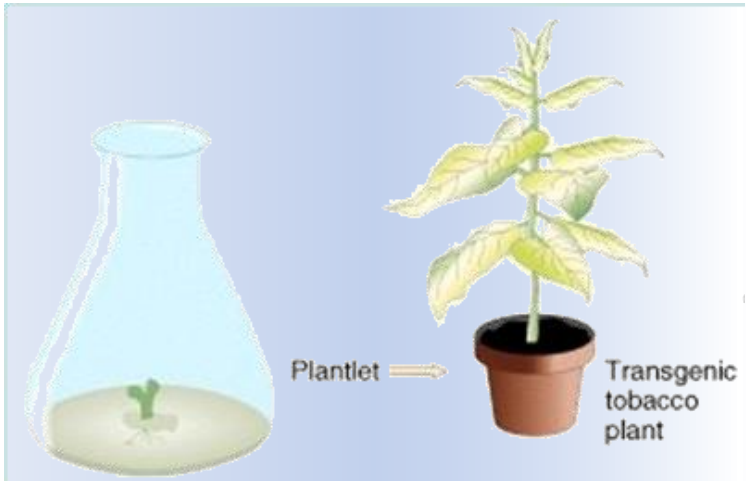
A reporter gene must be associated with the gene of interest for follow-up.

Stable transformation: viable integration of introduced genetic material into genomic DNA. Selection gene(s) (e.g. antibiotic resistance) and molecular analyses must be applied to isolate the transformed tissue. Long time span.

In vitro culture of *Nicotiana tabacum* in selective conditions

Serrazina S.(2004)

Molecular analyses

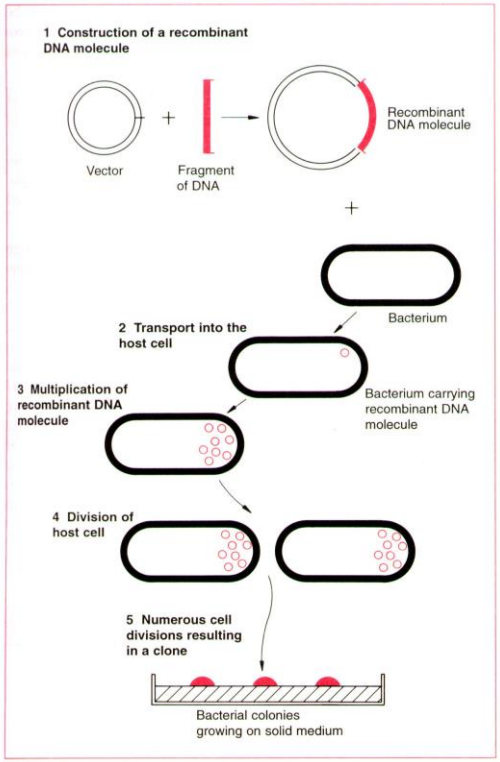


Remembering the basics of Molecular Biology…

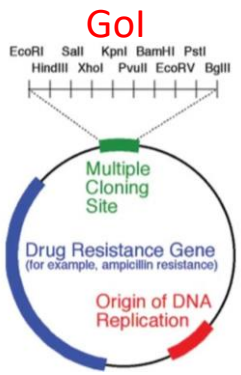
Gene – Protein of Interest: GoI, PoI

1. Isolation from DNA, RNA (e.g. PCR) – many copies of GoI

2. Initial cloning:
→ insertion of GoI into a cloning plasmid (e.g. restriction enzymes + ligases)
→ insertion in *E. coli*, solid medium with selection
→ **many copies of the plasmid with GoI and bacterium storage at -80ºC (stable)**
→ liquid medium and isolation of the recombinant plasmid by mini-prep



3. Cloning for insertion of the GoI into different expression vectors, larger and low-copy plasmids



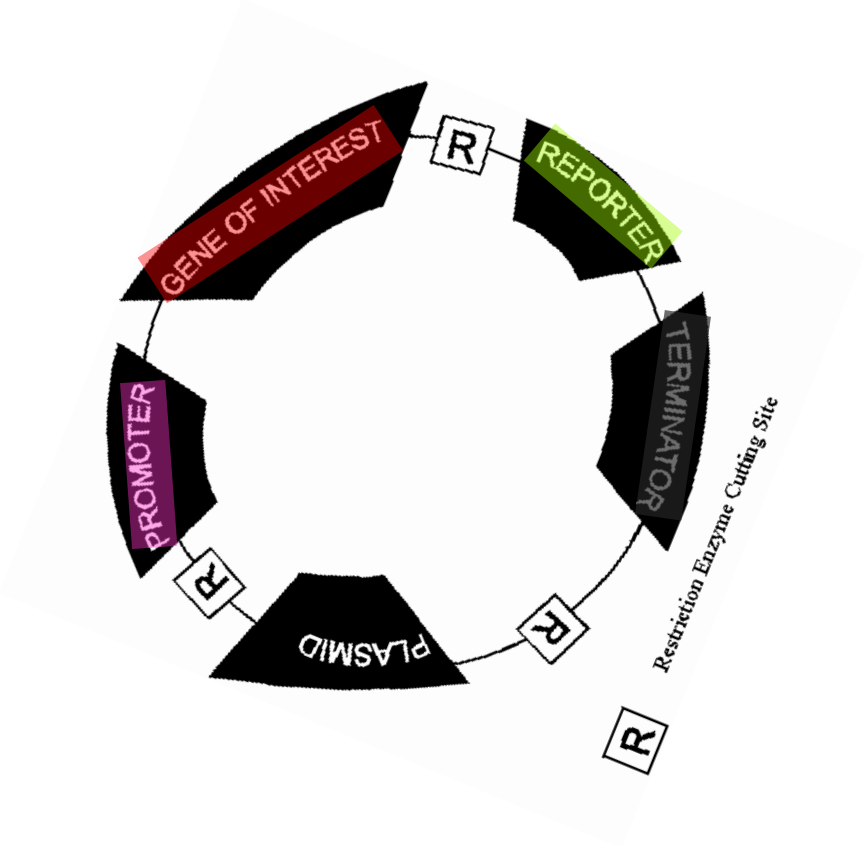
cloning plasmid

GoI → expression vector with a reporter gene

GoI → expression vector to isolate PoI

3. Cloning of GoI in a expression vector

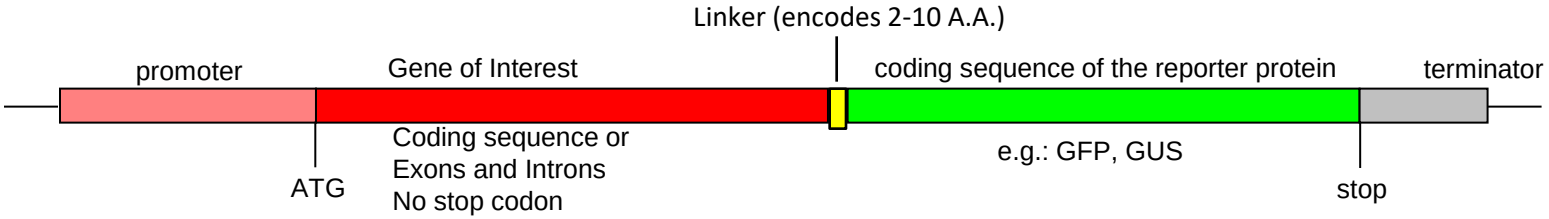
insertion of GoI in an expression cassette, associated to a reporter gene

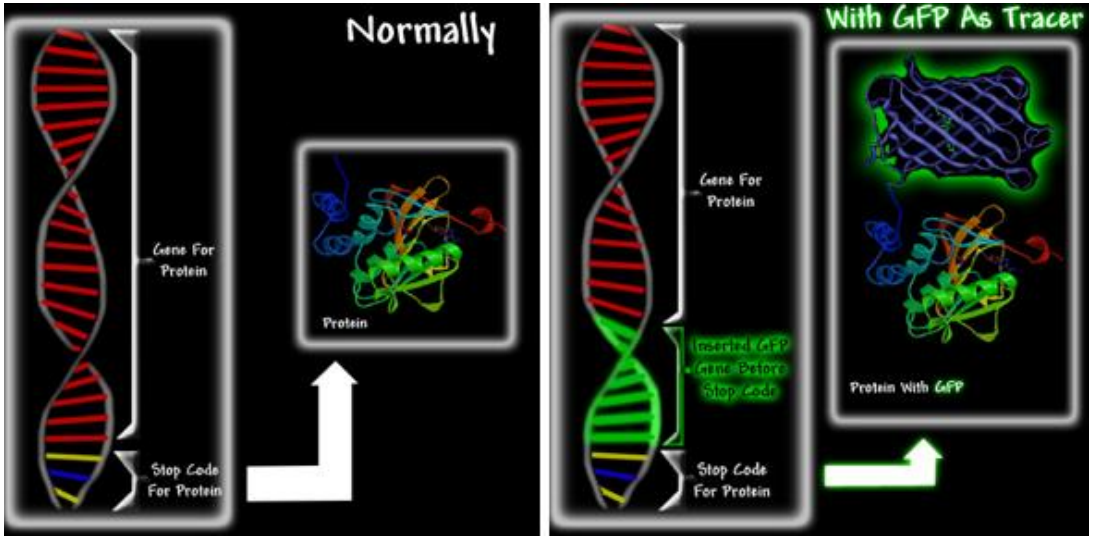


3. Construction of the expression cassette

GoI must be *in frame* with the reporter protein gene – Fusion protein

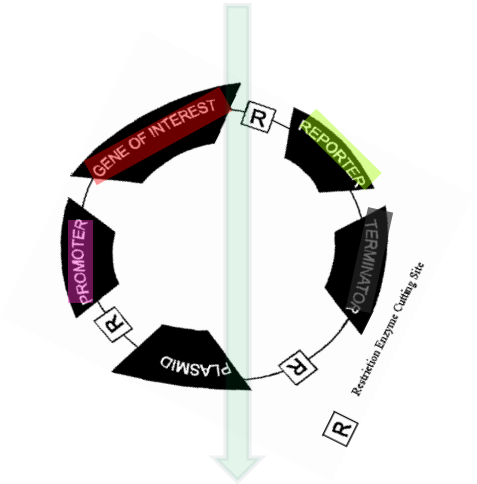
⇨ subcellular localization of proteins through co-localization with the Reporter Protein; protein trafficking; protein-protein interaction; **characterization, function**.





3. Expression cassette in the transformation vector → *E. coli* → vector replication

→ plasmid isolation (mini/midi-prep)

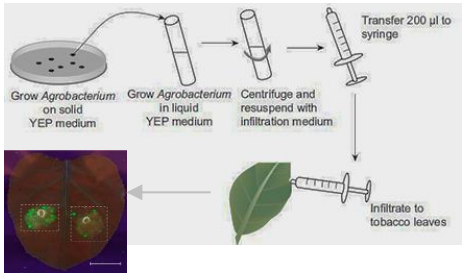


Transient genetic transformation methods

4. Agroinfiltration

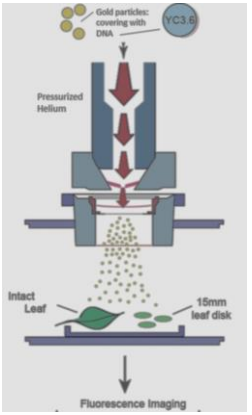
Insertion of the vector in the transformation agent **Agrobacterium**

→ Infiltration of *Nicotiana benthamiana* leaves



5. Biolistics

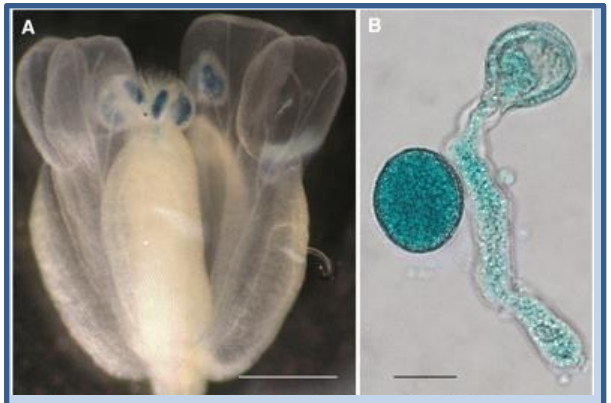
Direct insertion of the vector into tissue/cells



Reporter protein

strong expression (easy detection)

not endogenous of the organism to be transformed



Flower (A) e pollen (B) of Arabidopsis.
Sousa E., Malhó R. (2008). The Plant Cell, 20: 3050–3064.

• GUS (β-glucuronidase)

• gene isolated from *E. coli*

• *in situ* localization after an histochemical test → destructive detection

• easy cloning and precise detection.

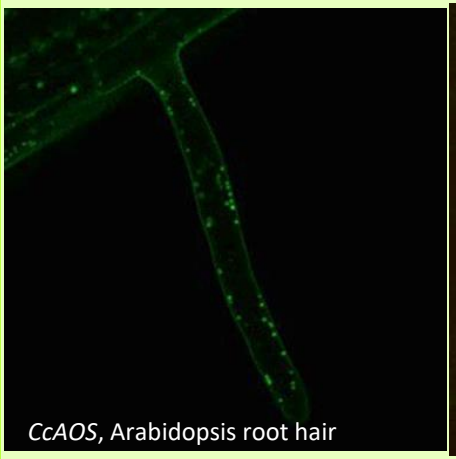
•GFP (green fluoresecent protein)

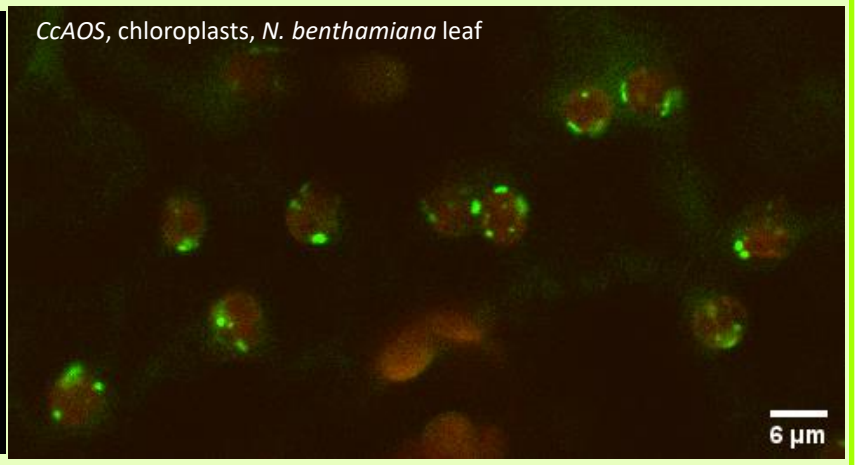
• Gene isolated from the cnidarian *Aequorea victoria* and modified

• *in vivo* localization by fluorescence (exitation: blue; emission: green)

→ enables real-time and progressive detection

• widely used in subcellular localization.

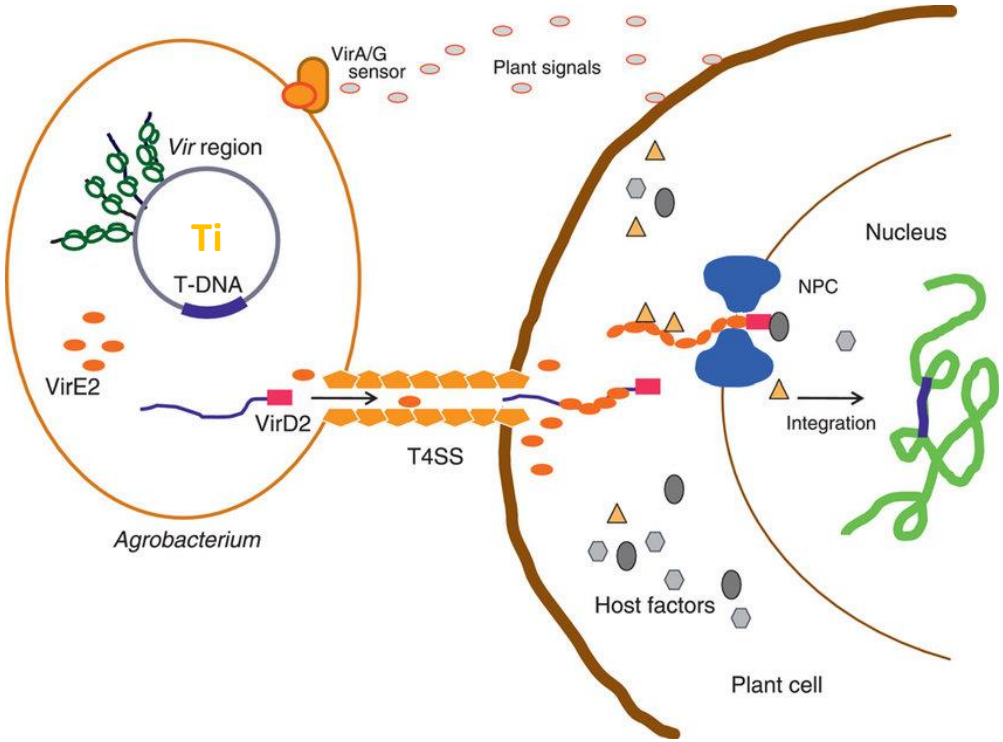

CcAOS, Arabidopsis root hair



Serrazina S., Malhó R. et al. (2021), Front. Plant Sci. 12:628697

Agrobacterium: Gram-negative bacteria

A. tumefaciens can transfer part of its DNA (T-DNA) of the plasmid Ti (tumor-inducing) into plant cells



T-DNA in **wild-type**:

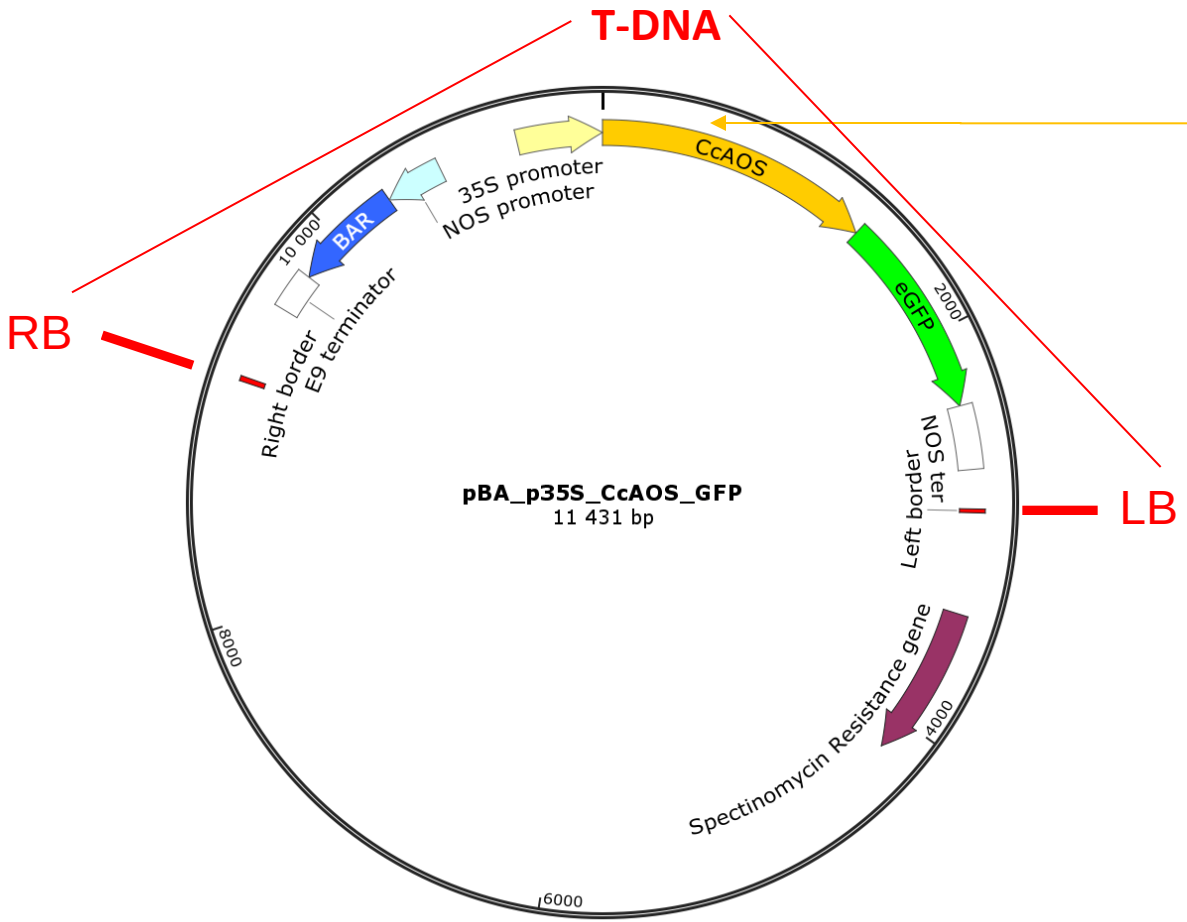
Enzymes for the production of A.A. octopin e nopalin, sources of C e N to the bacteria

Plant hormones:
auxins e cytokinins →
high celular division→ tumor

Agrobacterium genetically modified- Genetic Engineering tool

Ti plasmid:
- deletion of *vir* genes of the T-DNA in wild-type;
- maintenance of the LB e a RB ends;
- cloning of the expression cassettes inside the T-DNA → transformation vector

Example:

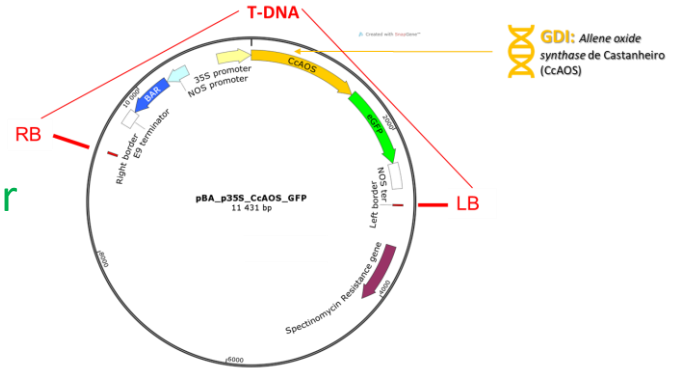


GoI: *Allene oxide synthase* of Chestnut (*CcAOS*)

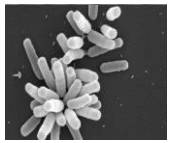
Constitutive promoters→
strong expression:
-35S (viral)
-NOS (bacteria)

BAR gene : resistance to the herbicide Bialaphos, a glufosinate precursor.

Transformation Vector



4. Introduction into the Agrobacterium strain GV3101



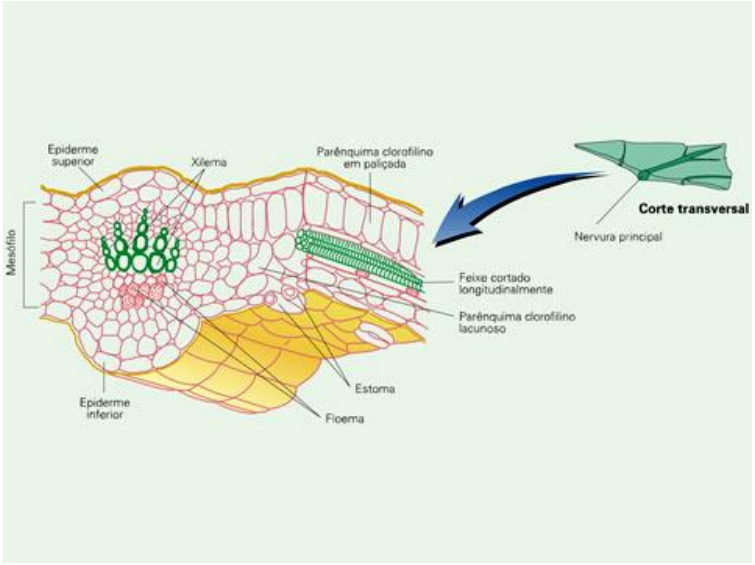
Agroinfiltration in *Nicotiana benthamiana* leaves

4. Agroinfiltration

An Agrobacterium suspension with acetosyringone* is injected into the leaf tissue, on the abaxial side, passes through the stomata into the lacunous parenchyma.

The bacteria stays in the intercellular spaces and transfers the expression cassette in the T-DNA to plant cells.

PoI expression verification: 2 a 4 days after infection.



*Acetosyringone: increases the virulence of Agrobacterium→ promotes the transfer of T-DNA to the plant. Phenolic compound secreted by dicotyledon plants at wound sites, involved in plant recognition by Agrobacterium. Increases transient transformation rate.

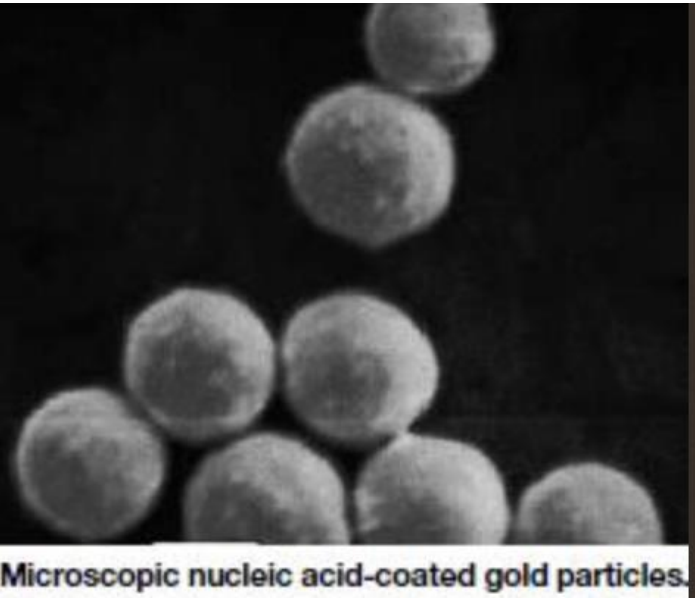
5. Biolistics

Method developed in the 1980s

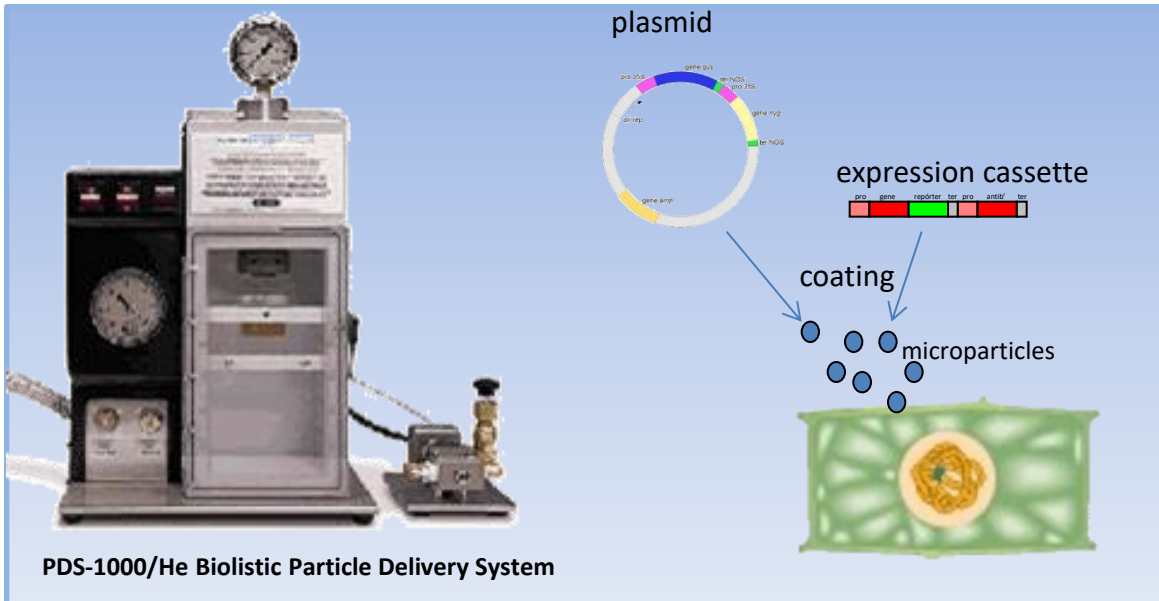
Objective: rice improvement (monocotyledon) for insect resistance, salinity and drought

Direct insertion of DNA into cells:
- coating of microparticles (gold, tungsten) with DNA
- targeted cell/tissue bombardment.

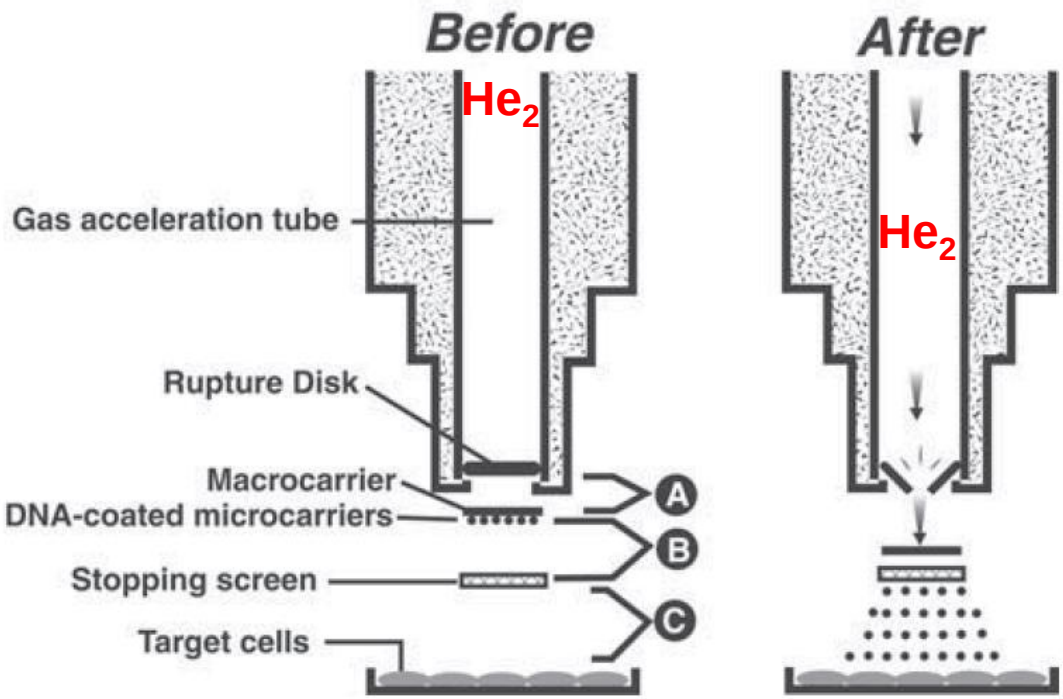
DNA: transformation vector or expression cassette (e.g. promoter-GoI-Reporter-terminator)



Microscopic nucleic acid-coated gold particles.



PDS-1000/He Biolistic Particle Delivery System



Pressurized helium gas

Cells/tissues in vacuum

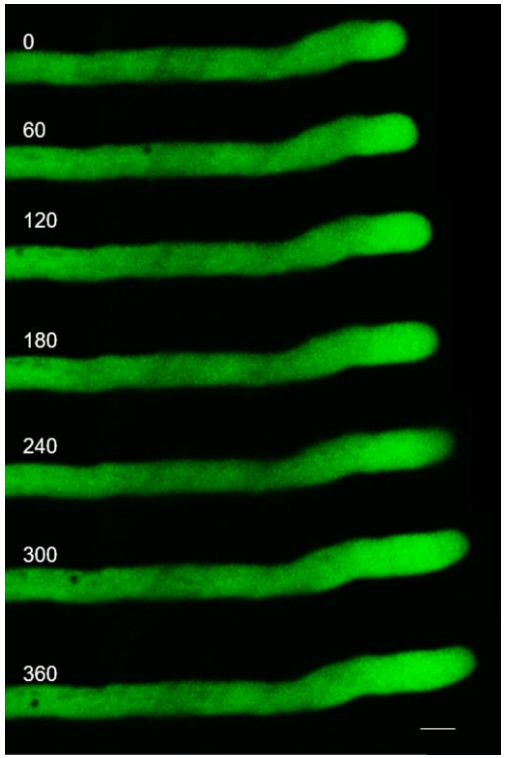
Coated particles entering and leaving the cell leave the DNA→ resuspension inside the cell (nucleoplasm, cytosol…)

Random integration in gDNA

Expression of the PoI 1 day after the bombardment.

Biolistics – bombardment of pollen grains of *Nicotiana tabacum*

Pollen germination after transformation and observation under confocal microscopy

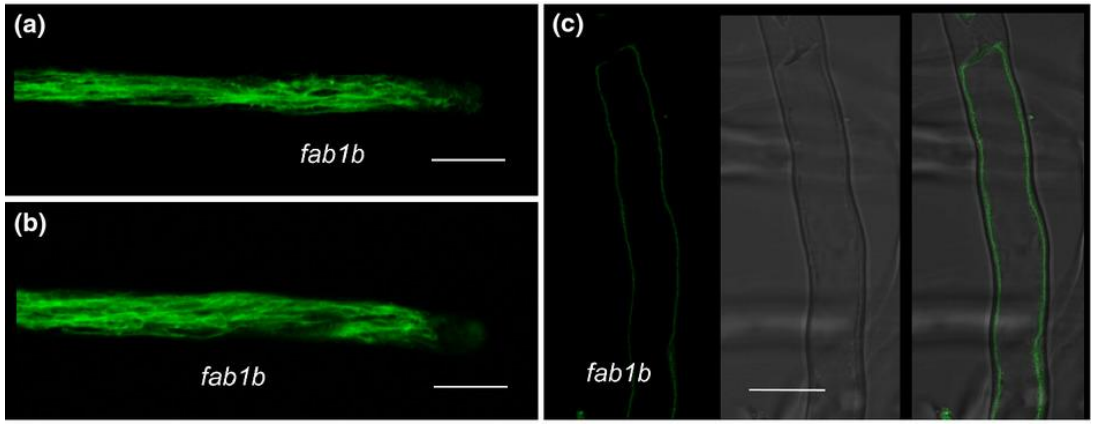


Coating of microparticles with
pLAT52:DGK4:mGFP4

Location: cytosol.

Function: signaling of secretion events related with pollen tube growth.

F. Vaz Dias, S. Serrazina, R. Malhó et al. 2019, New Phytologist 222 (3)



Coating of microparticles with pLAT52:FAB1B:mGFP4

Location: endosome near the apex; tonoplast in distal zones.

Function: membrane reorganization and endocytosis related with pollen tube growth.

S. Serrazina, F. Vaz Dias, R. Malhó 2014, New Phytologist 203 (3)

Arsenault and O'Brien *BMC Research Notes* 2013, **6**:544
http://www.biomedcentral.com/1756-0500/6/544

BMC Research Notes

RESEARCH ARTICLE Open Access

Optimized heterologous transfection of viable adult organotypic brain slices using an enhanced gene gun

Jason Arsenault and John A O'Brien*

Abstract

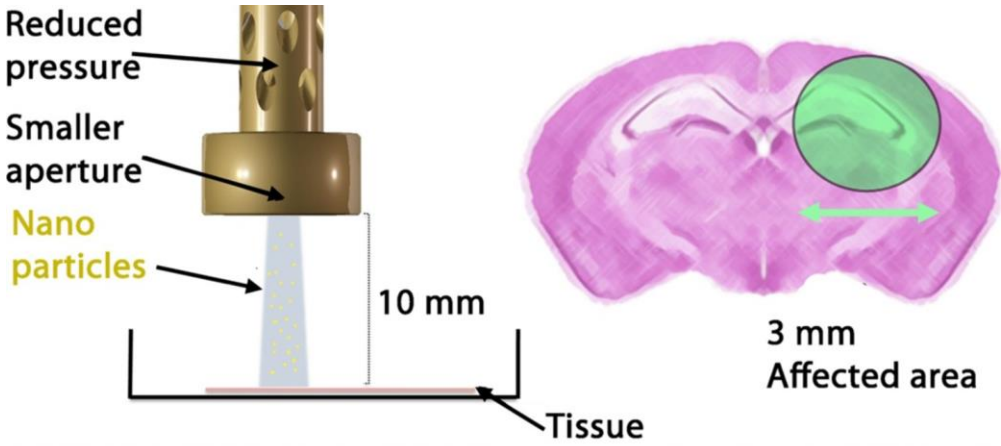
Background: Organotypic brain slices (OTBS) are an excellent experimental compromise between the facility of working with cell cultures and the biological relevance of using animal models where anatomical, morphological, and cellular function of specific brain regions can be maintained. The biological characteristics of OTBS can subsequently be examined under well-defined conditions. They do, however, have a number of limitations; most brain slices are derived from neonatal animals, as it is difficult to properly prepare and maintain adult OTBS. There are ample problems with tissue integrity as OTBS are delicate and frequently become damaged during the preparative stages. Notwithstanding these obstacles, the introduced exogenous proteins into both neuronal cells, and cells imbedded within tissues, have been consistently difficult to achieve.

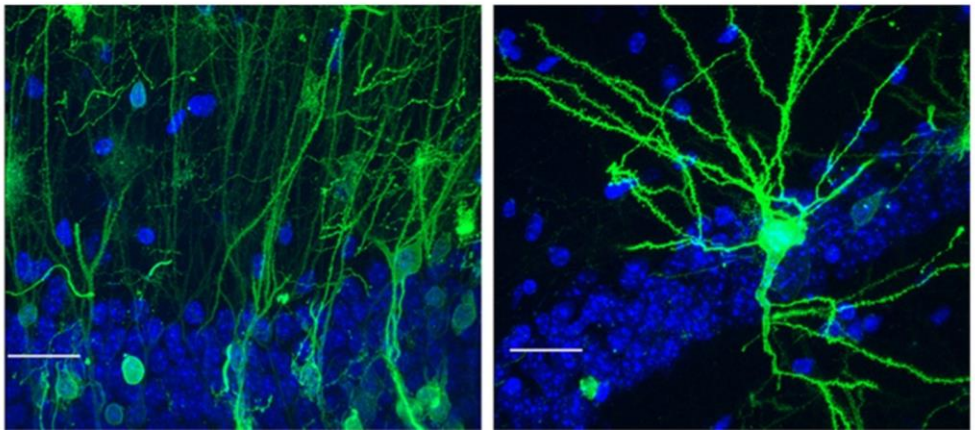
Results: Following the *ex vivo* extraction of adult mouse brains, mounted inside a medium-agarose matrix, we have exploited a precise slicing procedure using a custom built vibroslicer. To transfect these slices we used an improved biolistic transfection method using a custom made low-pressure barrel and novel DNA-coated nanoparticles (40 nm), which are drastically smaller than traditional microparticles. These nanoparticles also minimize tissue damage as seen by a significant reduction in lactate dehydrogenase activity as well as propidium iodide (PI) and dUTP labelling compared to larger traditional gold particles used on these OTBS. Furthermore, following EYFP exogene delivery by gene gun, the 40 nm treated OTBS displayed a significantly larger number of viable NeuN and EYFP positive cells. These OTBS expressed the exogenous proteins for many weeks.

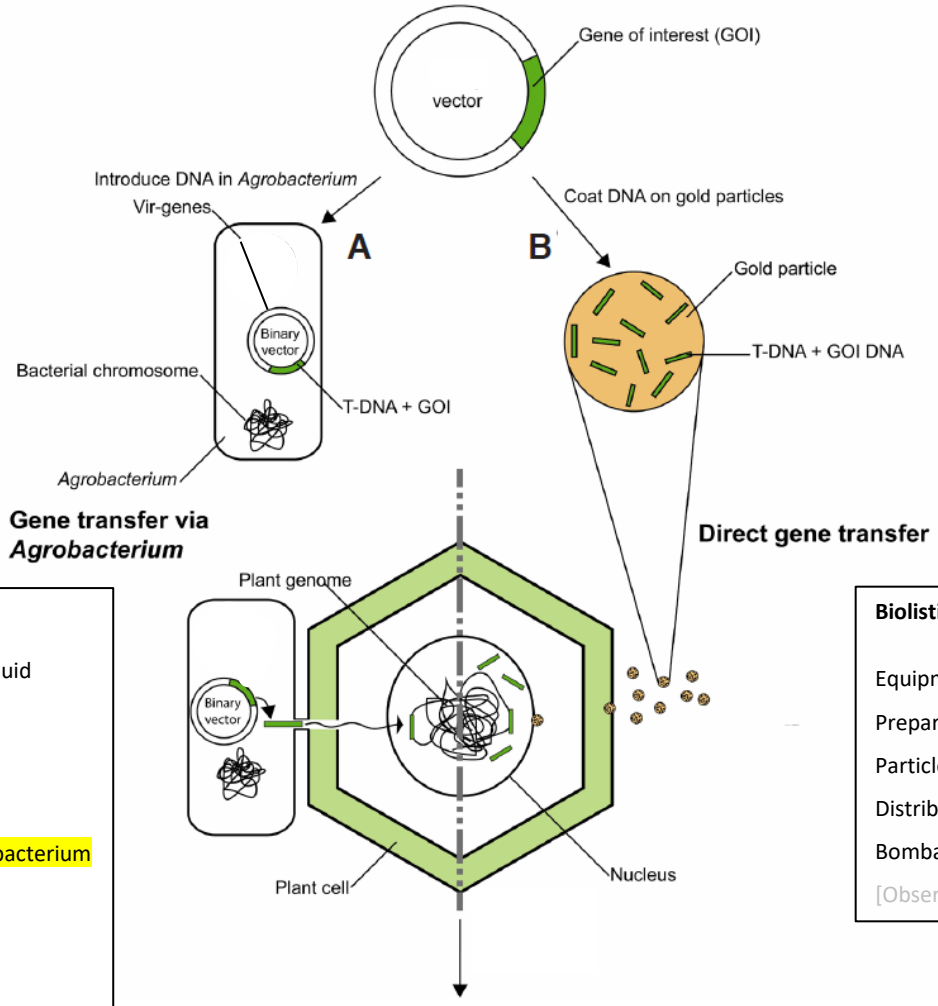
Conclusions: Our described methodology of producing OTBS, which results in better reproducibility with less tissue damage, permits the exploitation of mature fully formed adult brains for advanced neurobiological studies. The novel 40 nm particles are ideal for the viable biolistic transfection of OTBS by reducing tissue stress while maintaining long term exogene expression.

Keywords: Organotypic brain slices, Vibroslicer, Gene gun, Biolistic transfection, Nanoparticles, Tissue slicer

E.g.: Mouse neural cells







Agroinfiltration:

1st day (yesterday) - Growth of Agrobacterium in liquid medium with selection agents.

Day 2 (today!)

- Preparation of the infiltration medium
- Addition of the infiltration medium to Agrobacterium
- Measurement of the optical density (OD)
- Adjustment of the OD
- Leaf infiltration

Observation of the PoI expression next week (CcAOS-GFP)

Biolistics (demonstration in video):

Equipment and sample

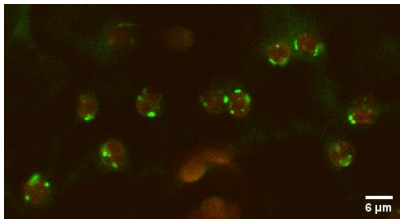
Preparation of gold particles

Particle coating with plasmid DNA

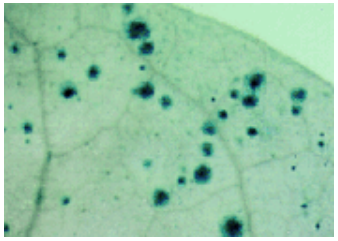
Distribution by carriers

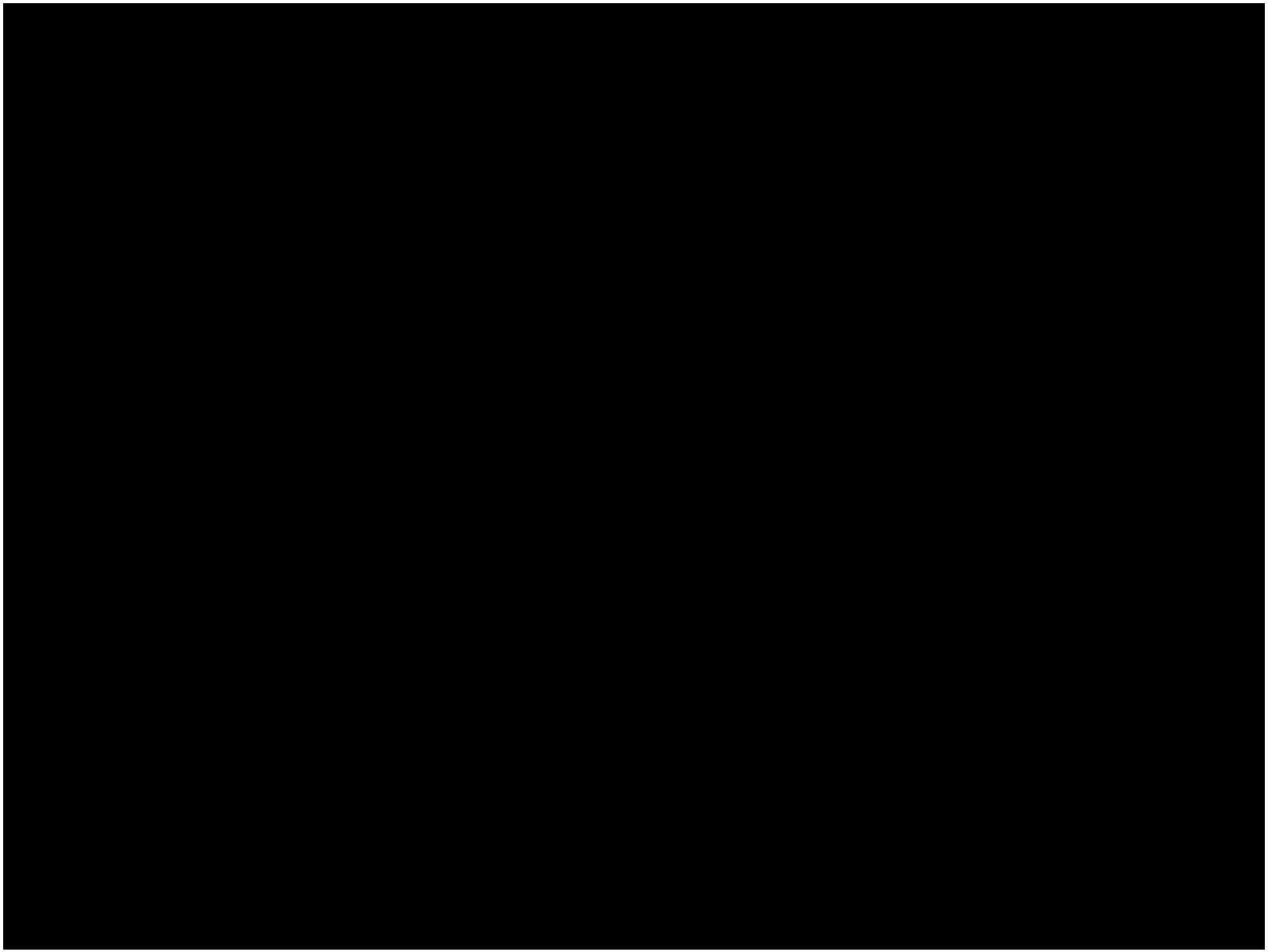
Bombardment preparation and firing

[Observation after histochemical test (GUS)]



Expression after Transient Transformation
(confocal microscopy, fluorescence microscopy, histochemistry…)





https://www.youtube.com/watch?v=GHc7PU_jG2M

Agroinfiltration Protocol

1. Preparation of the infiltration medium

- 0,050 g D-Glucose
- 1 mL MES [2-(N-morpholino)ethanesulfonic acid, 10x stock]
- 1 mL $Na_3PO_4.12H_2O$ (10x stock)
- 25 uL acetosyringone (200 mM stock)
- Add dH_2O to the final volume of 10 mL.

2. Agrobacterium preparation

- Centrifuge the Agrobacterium culture 5-10 min at 4000 rpm.
- Remove the supernatant inverting the tube.
- Add 1 mL of infiltration medium and resuspend the pellet (agitate or use the vortex).
- Make a 1:10 dilution for a plastic cuvette: 100 uL of Agrobacterium to 900 uL of infiltration medium. Measure optical density at 600 nm.
- Dilute the Agrobacterium suspension in the infiltration medium to OD=0.1, in a final volume of 1 mL (1000 uL). Use the obtained absorbance value in the formula

$$Abs_i \times V_i = Abs_f \times V_f$$

$$(Abs_i \times 10) \times V_i = 0.1 \times 1000$$

Prepare the dilution in 2 mL tubes.

3. Infiltration (GLOVES)

- Choose the youngest fully expanded leaves.
- Fill the syringe (without the needle) with circa 0.5 mL of Agrobacterium.
- Press gently on the abaxial page (bottom), outside the midrib area and slowly press the plunger until a dark-green infiltrated area appears, or the entire leaf area. Repeat on another part of the same leaf or change to other leaf.
- Mark the infiltrated area or leaf.