Agroinfiltration basics

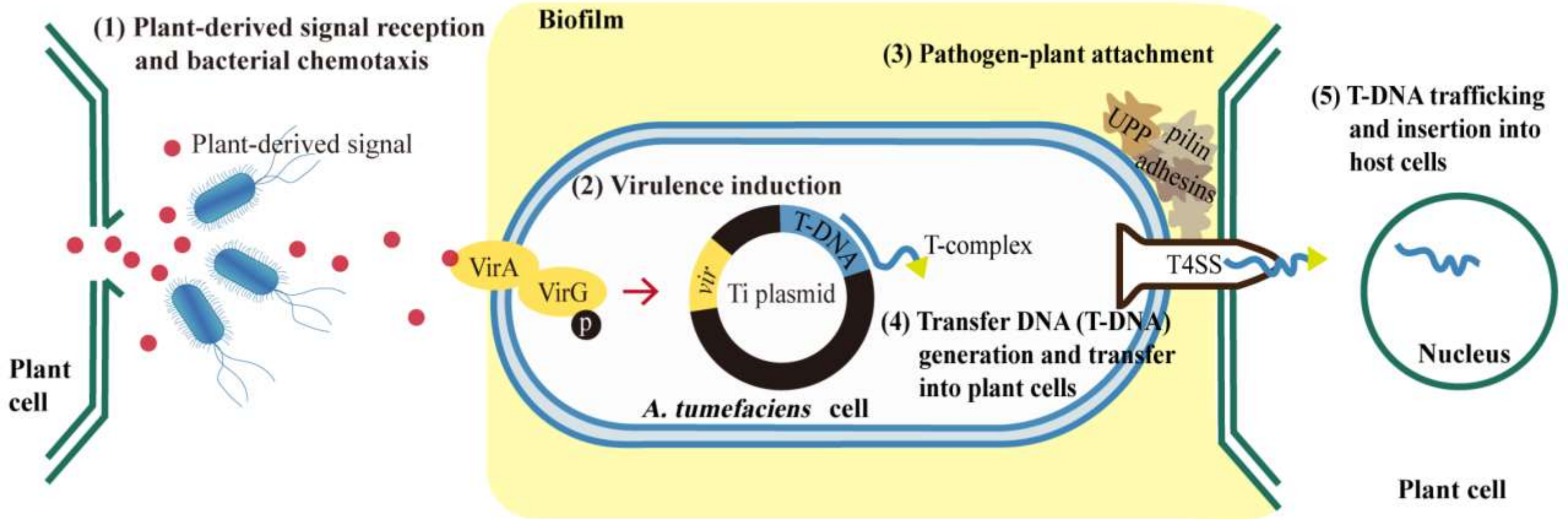
Animal and plant cell culture

Practicum: Plant tissue culture

2024/2025

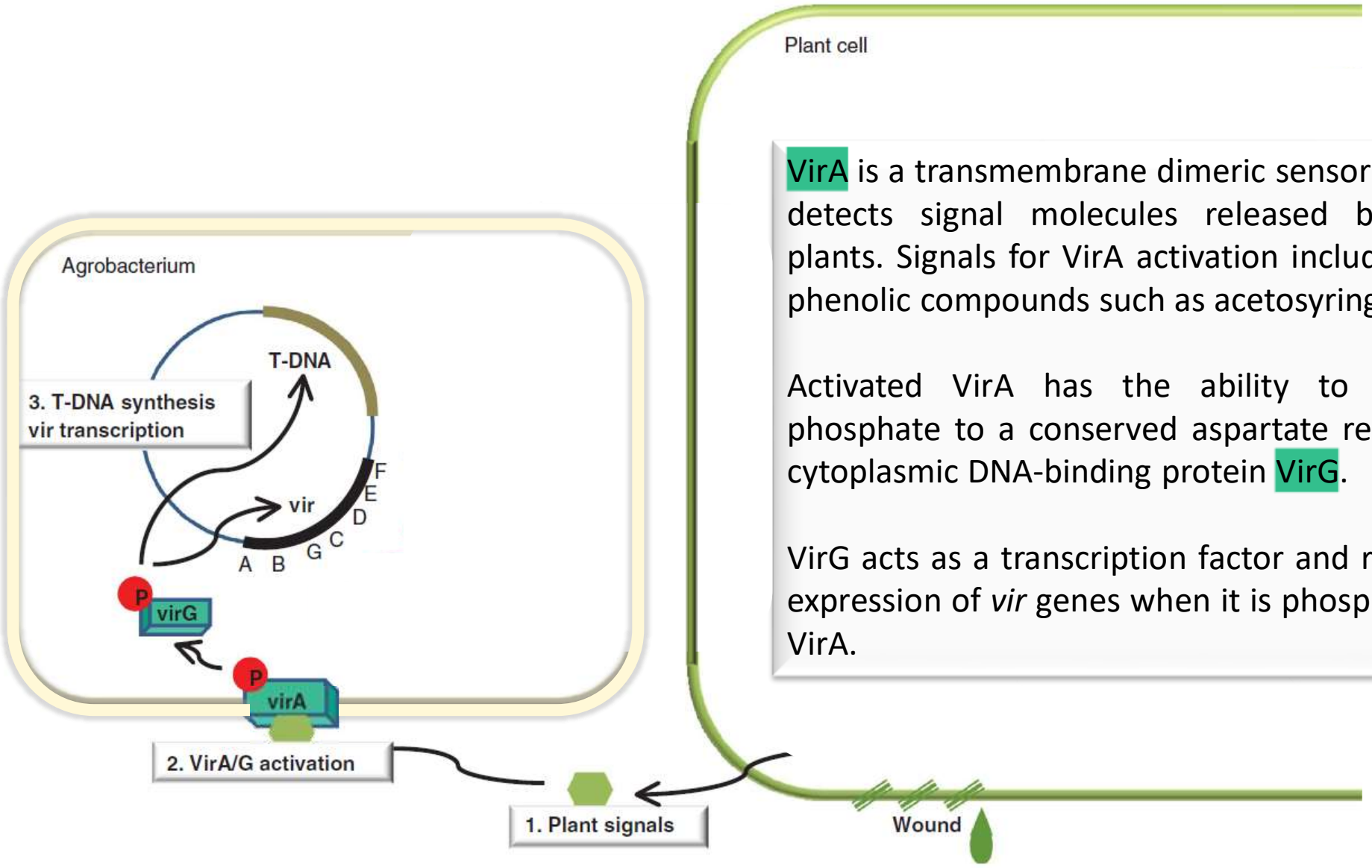
Virulent strains of *Agrobacterum tumefaciens*

T-DNA transfer is mediated by products encoded by the 30-40 kb *vir* region of the Ti plasmid (a large plasmid, more than 200 kb, with a key role in tumor induction). This region consists of at least six essential operons (*vir A, vir B, vir C, vir D, vir E, virG*) and two non-essential ones (*virF, virH*). The only constitutively expressed operons are *virA* and *virG*, which encode a two-component system (VirA-VirG) that activates transcription of the other vir genes.



The T-DNA contains two types of genes: the oncogenic genes coding for enzymes involved in the synthesis of auxins and cytokinins that are responsible for tumor formation, and the genes coding for the synthesis of opines that are produced and secreted by the crown gall cells and consumed by *A. tumefaciens* as a source of carbon and nitrogen.

Induction of the bacterial virulence system

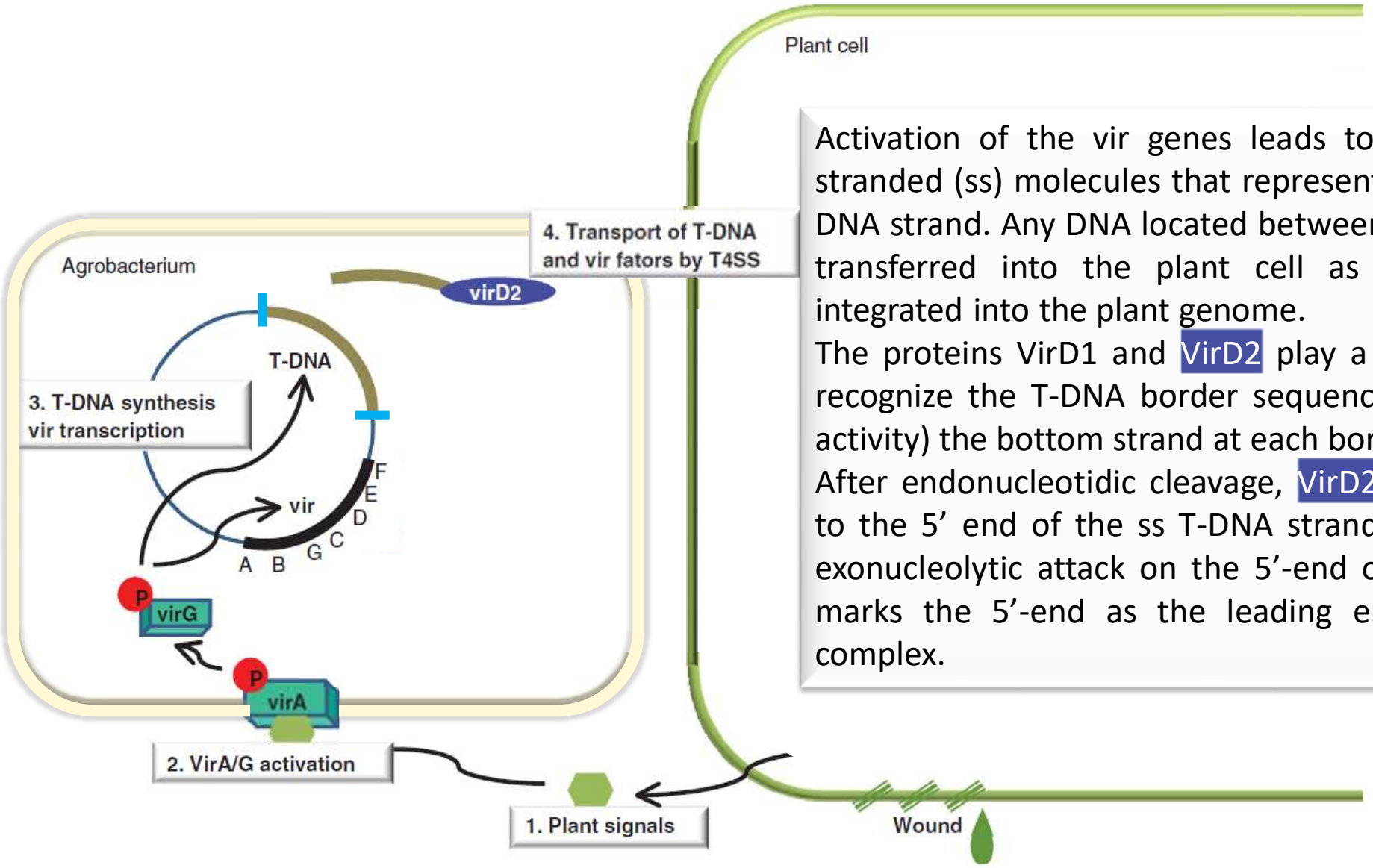


VirA is a transmembrane dimeric sensor protein that detects signal molecules released by wounded plants. Signals for VirA activation include acidic pH, phenolic compounds such as acetosyringone.

Activated VirA has the ability to transfer its phosphate to a conserved aspartate residue of the cytoplasmic DNA-binding protein VirG.

VirG acts as a transcription factor and regulates the expression of *vir* genes when it is phosphorylated by VirA.

Generation of the T-DNA transfer complex

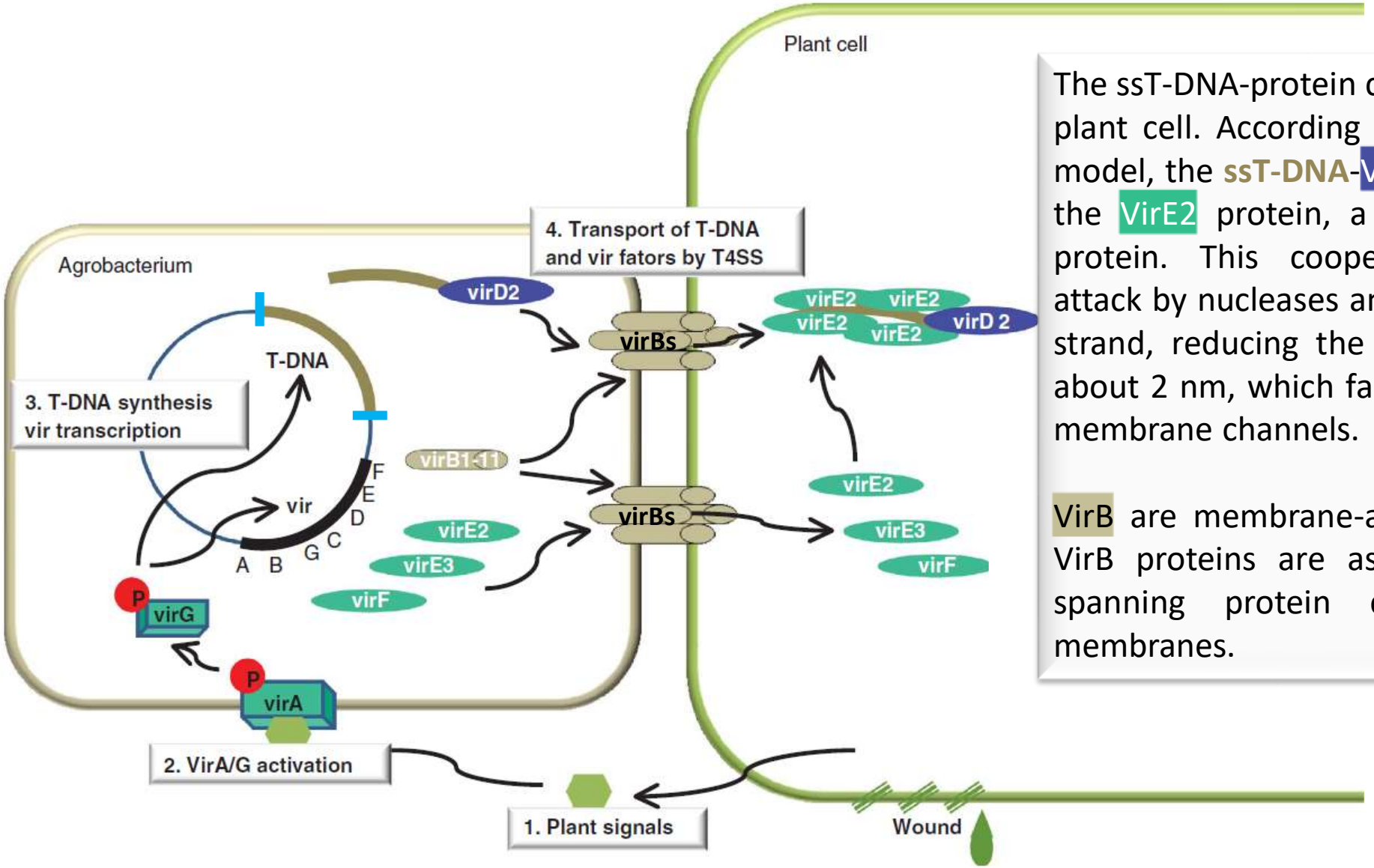


Activation of the vir genes leads to the generation of single-stranded (ss) molecules that represent the copy of the bottom T-DNA strand. Any DNA located between the T-DNA borders will be transferred into the plant cell as single-stranded DNA and integrated into the plant genome.

The proteins VirD1 and VirD2 play a key role in this step. They recognize the T-DNA border sequences and nick (endonuclease activity) the bottom strand at each border.

After endonucleotidic cleavage, VirD2 remains covalently bound to the 5’ end of the ss T-DNA strand. This association prevents exonucleolytic attack on the 5’-end of the ss-T-DNA strand and marks the 5’-end as the leading end of the T-DNA transfer complex.

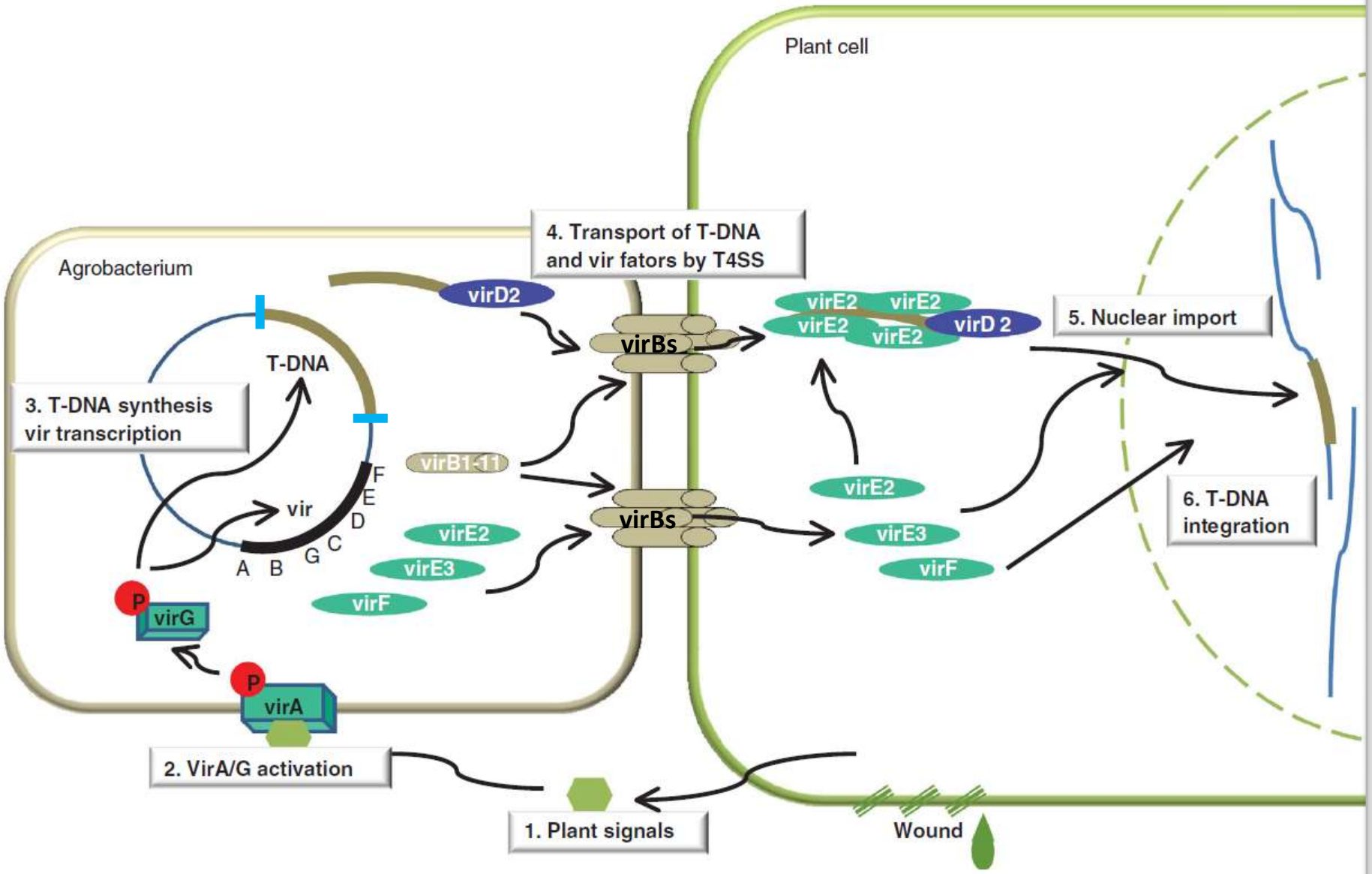
Translocation of the T-DNA complex



The ssT-DNA-protein complex is transferred into the plant cell. According to the most widely accepted model, the ssT-DNA-VirD2 complex is enveloped by the VirE2 protein, a single-stranded DNA-binding protein. This cooperative association prevents attack by nucleases and also elongates the ssT-DNA strand, reducing the diameter of the complex to about 2 nm, which facilitates translocation through membrane channels.

VirB are membrane-associated proteins and most VirB proteins are assembled into a membrane-spanning protein channel that spans both membranes.

Integration of the T-DNA into the plant genome

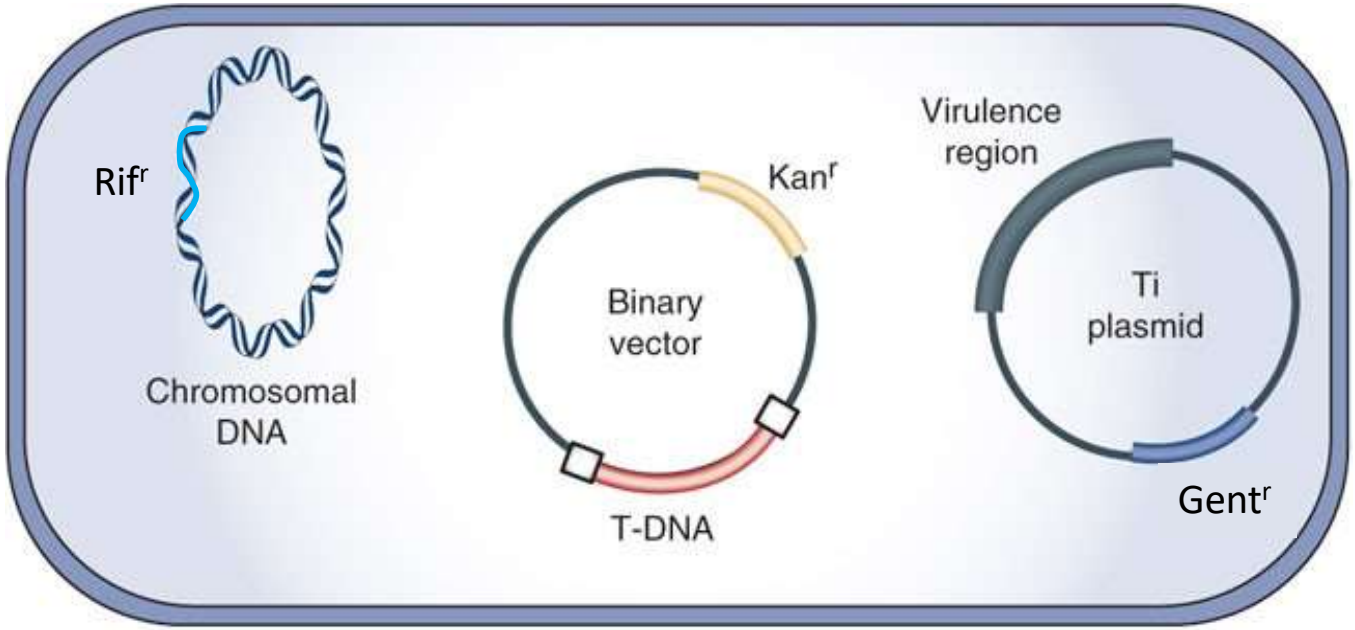


Within the plant cell, the ssT-DNA complex is transported through the nuclear membrane into the nucleus. It was found that two Vir proteins play an important role in this step: VirD2 and VirE2. It is hypothesized that the two **NLS**s of VirE2 are important for the continuous nuclear import of the ss-T-DNA complex.

The final step of T-DNA transfer is its integration into the plant genome. It is assumed that integration occurs by illegitimate recombination. The cleavage of some bases confers only minimal specificity to the recombination process by positioning VirD2 for ligation.

Pitzschke and Hirt. The EMBO Journal (2010) 29, 1021–1032

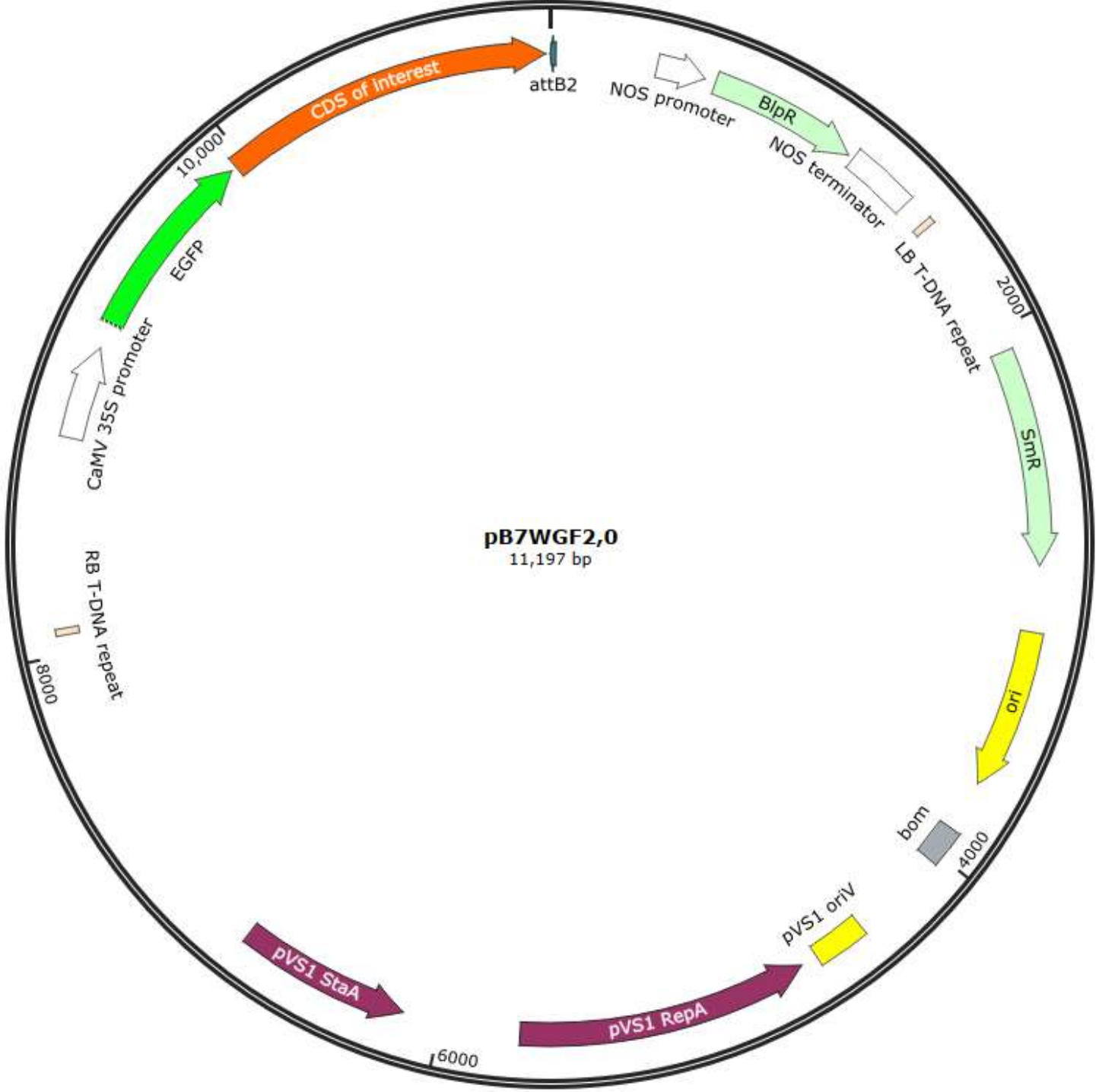
Agrobacterium tumefaciens strain GV3101



The *Agrobacterium tumefaciens* strain GV3101 used for transformation harbors the C58 chromosomal backbone containing **rifampicin** resistance and carries two plasmids. A non-oncogenic disarmed tumor-inducing plasmid (**Ti plasmid**) containing the virulence (*vir*) genes and resistance to **gentamicin**, but lacking the T-DNA region. The T-DNA region is present on a second plasmid, the **binary vector**. The selection marker in the binary vector is the **kanamycin** resistance gene. DNA sequences to be transformed are cloned between the left and right border sequences of the T-DNA region and transferred to the host.

Binary vectors

Binary vectors are a standard tool in the transformation of higher plants mediated by *Agrobacterium tumefaciens*. It is composed of the borders of T-DNA, multiple cloning sites, replication functions for *Escherichia coli* and *A. tumefaciens*, selectable marker genes, reporter genes, and other accessory elements that can improve the efficiency of and/or give further capability to the system.



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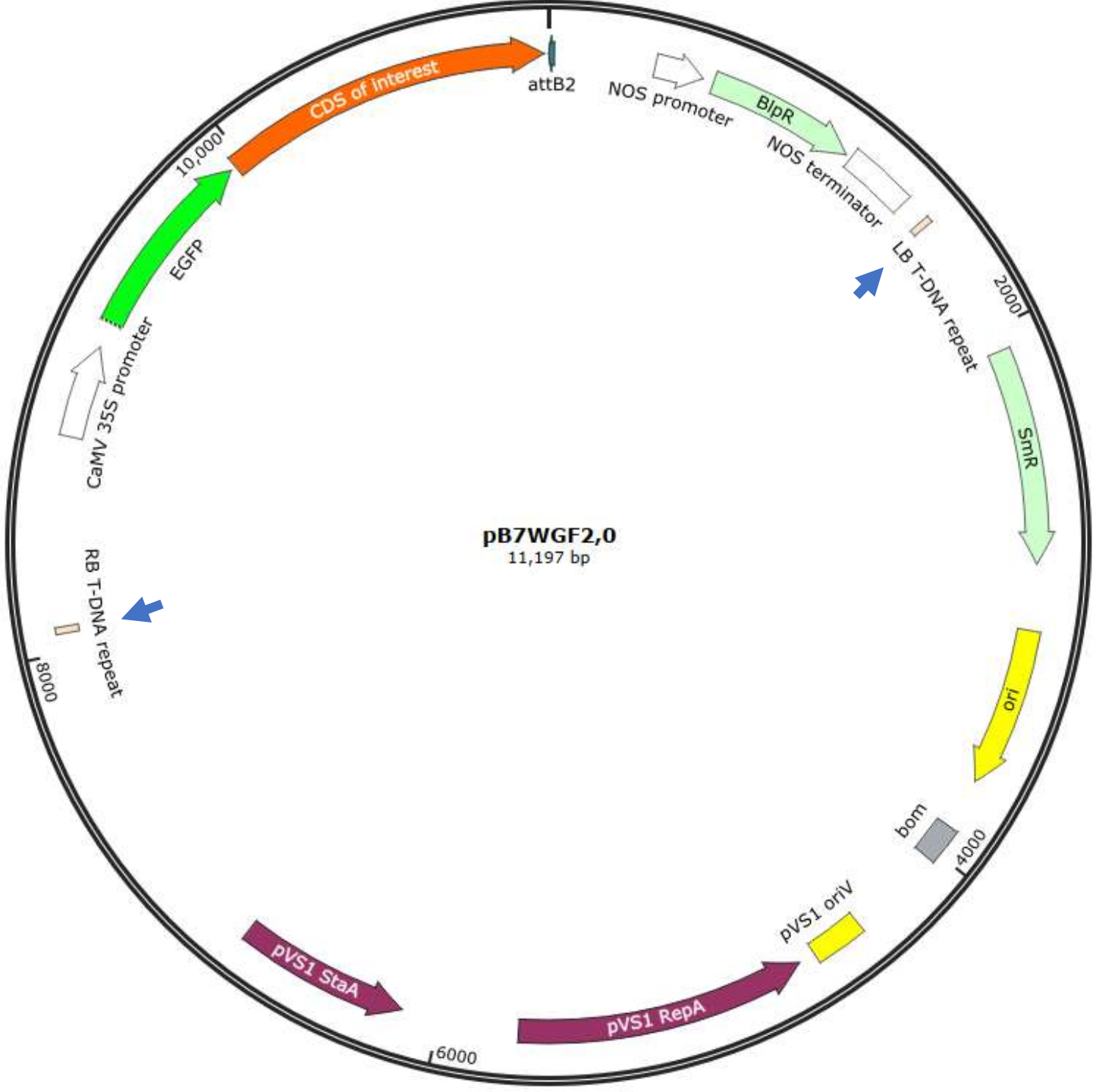

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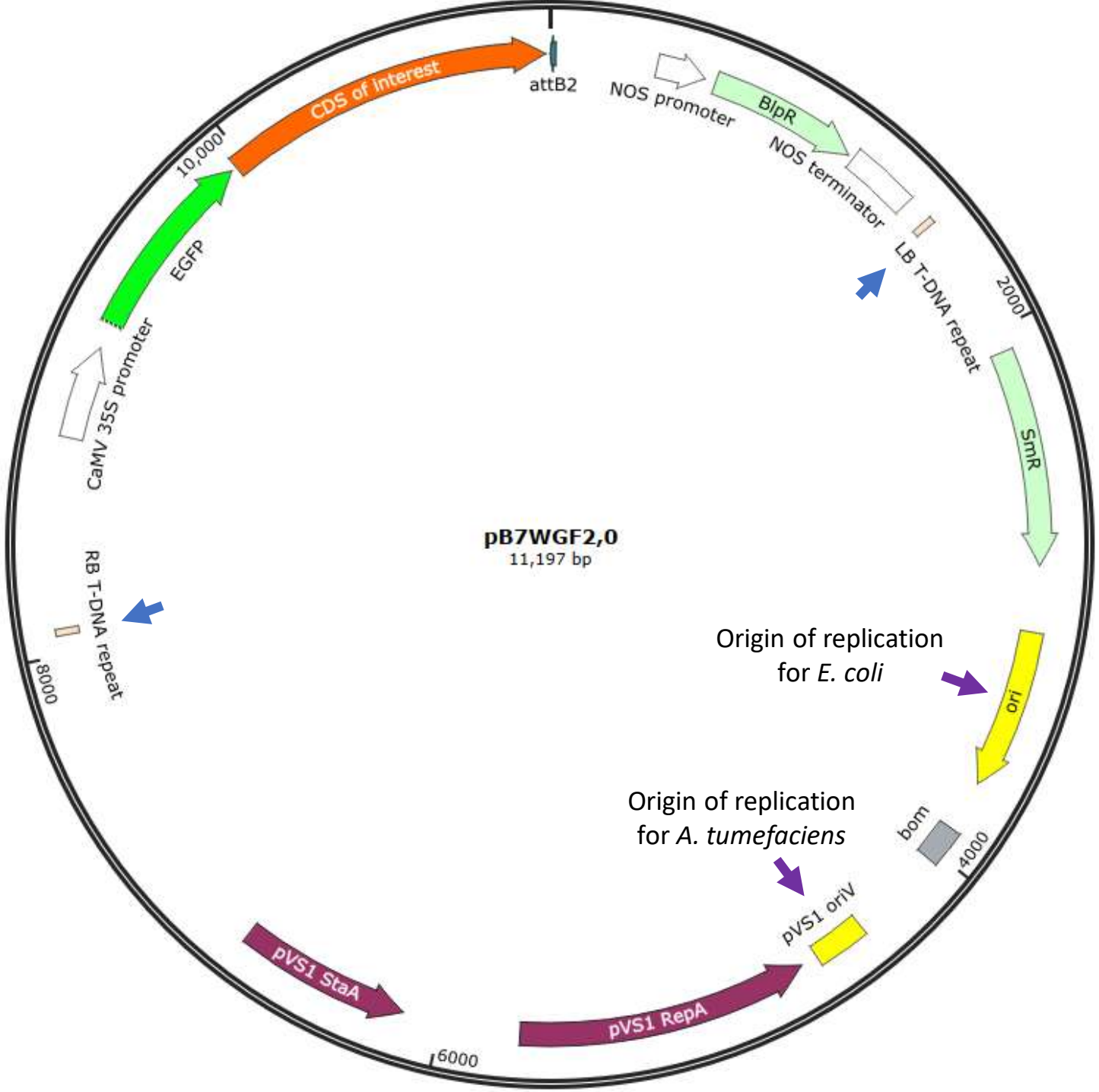

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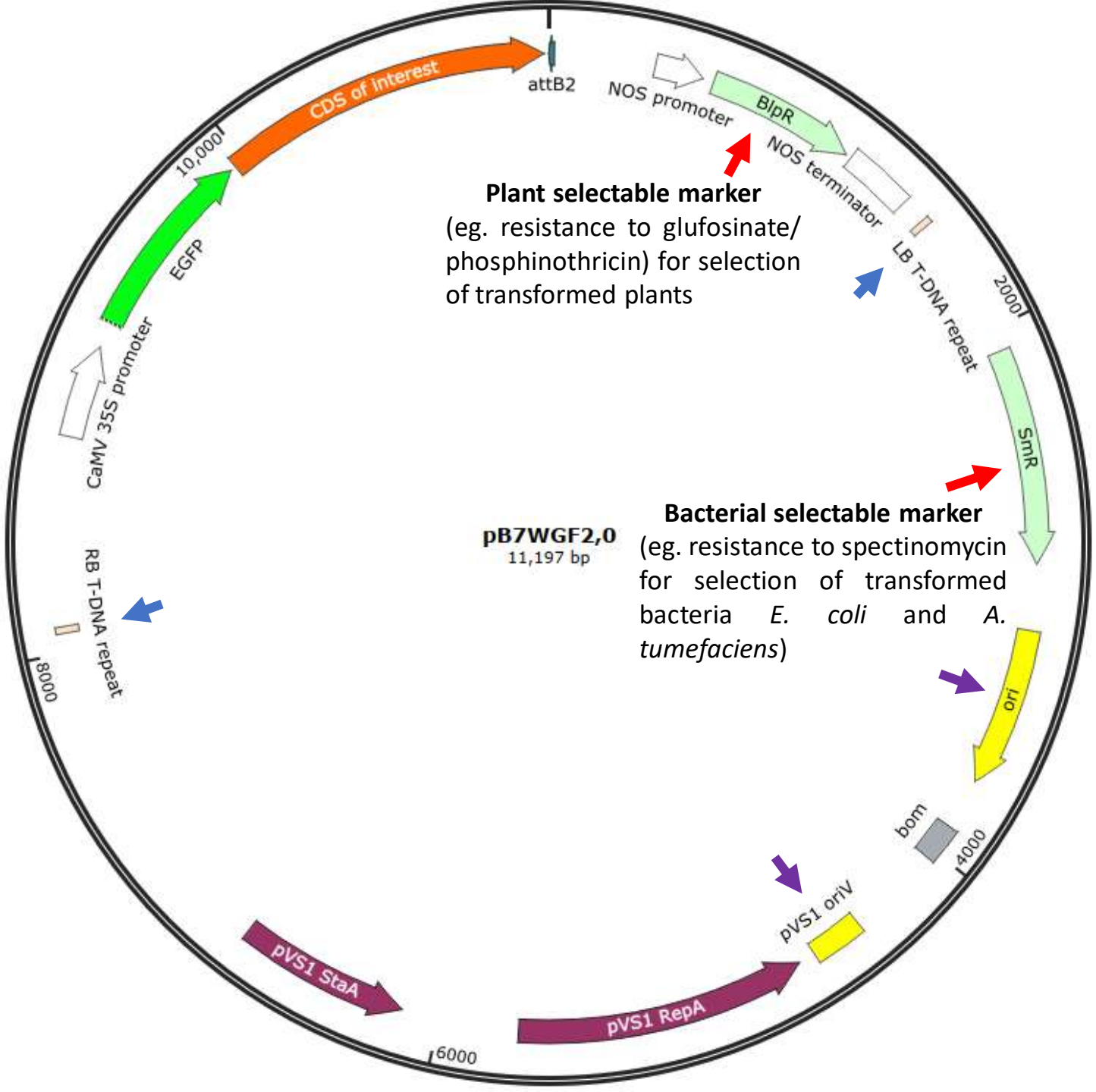

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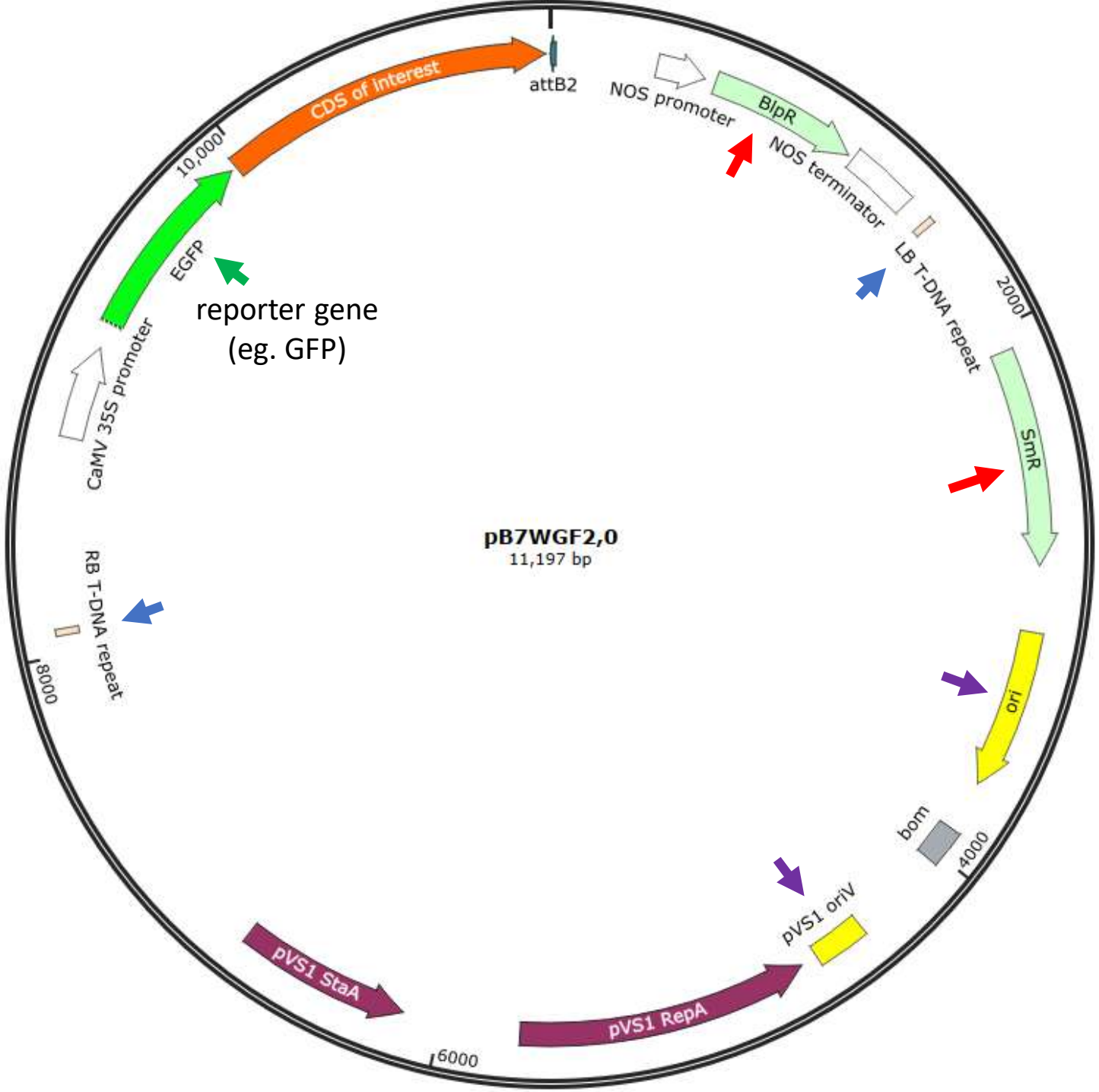
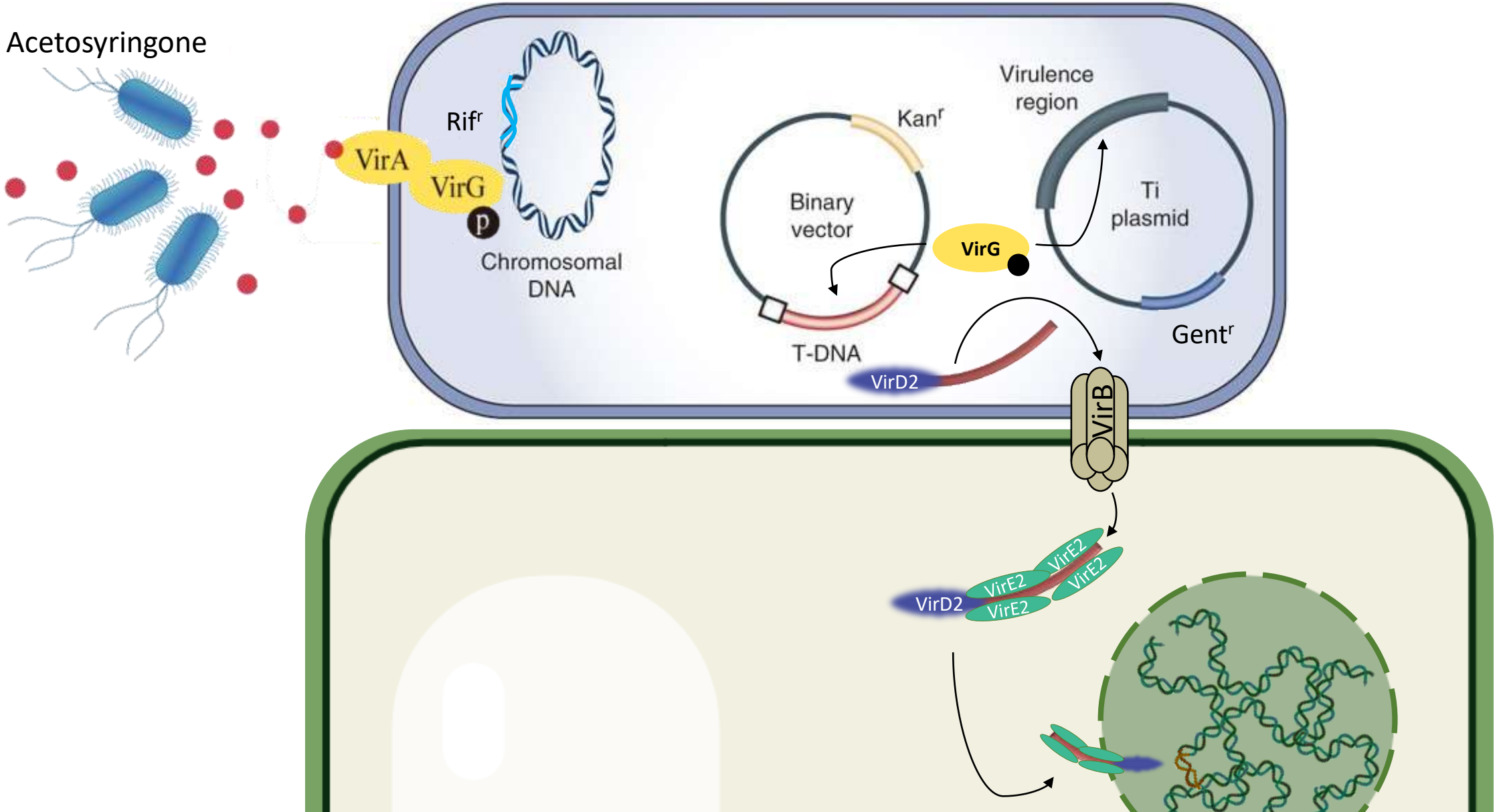
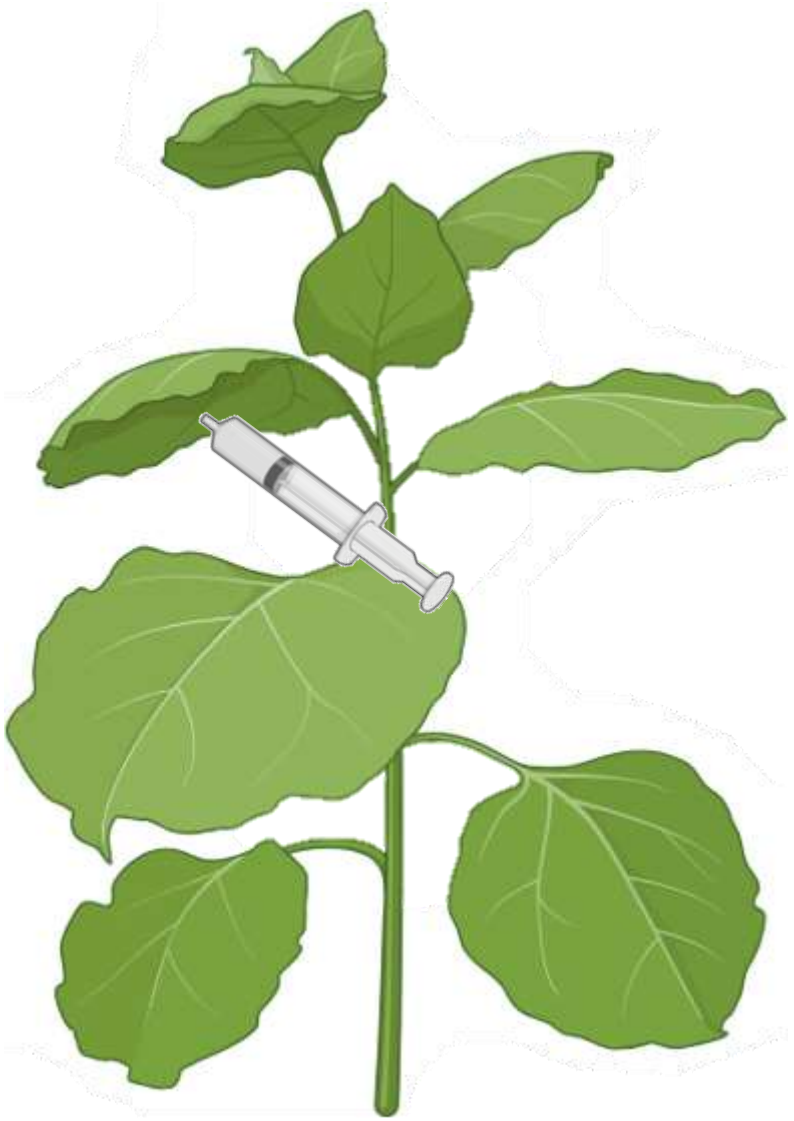


Agrobacterium tumefaciens strain GV3101

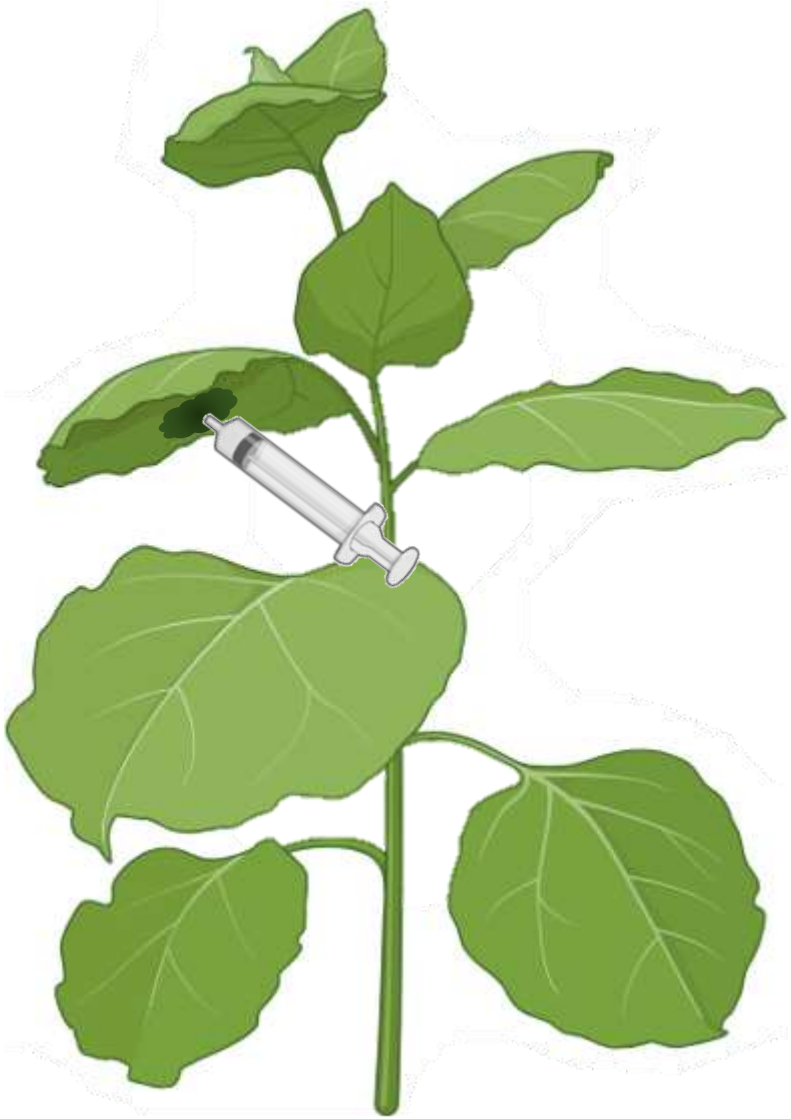


Agroinfiltration



Nicotiana benthamiana

Agroinfiltration



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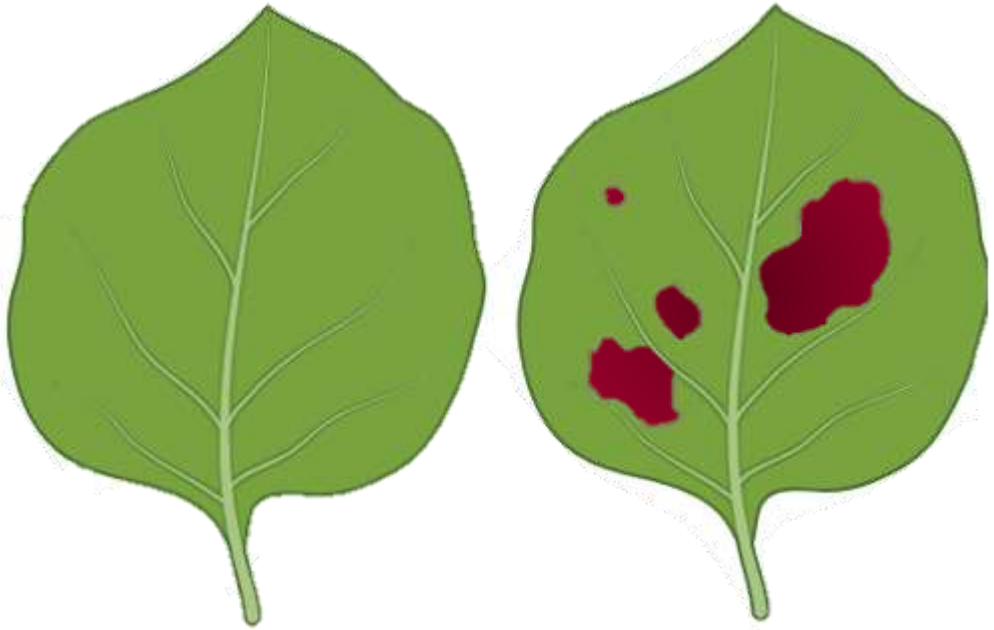
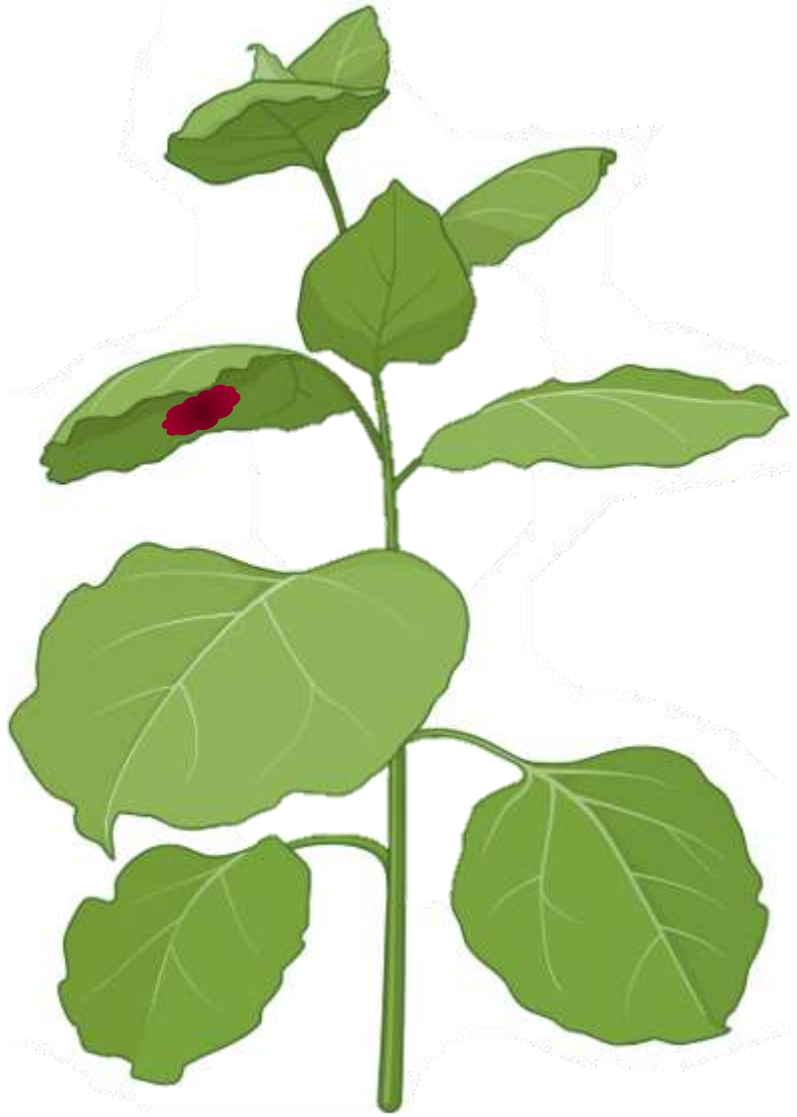
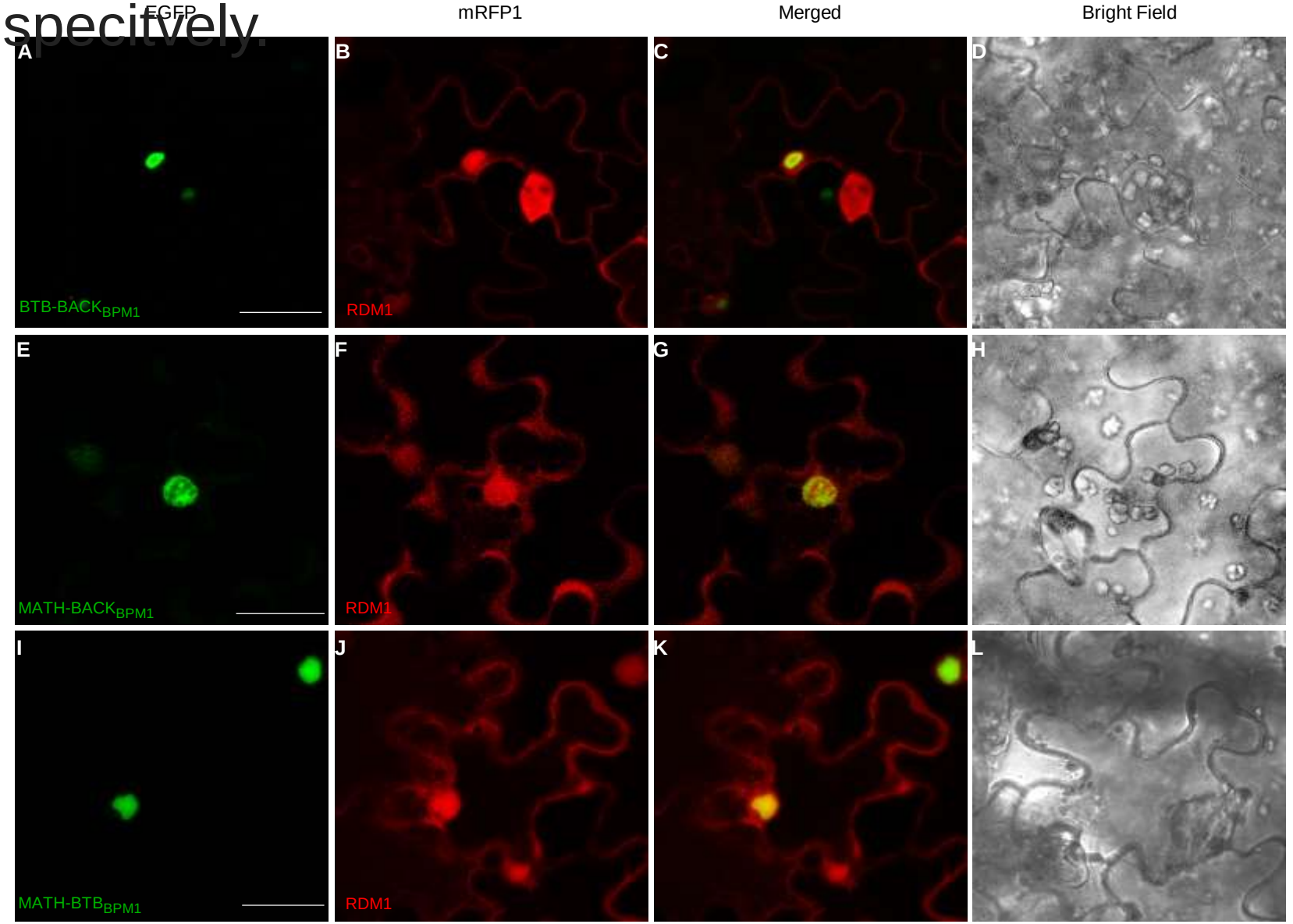




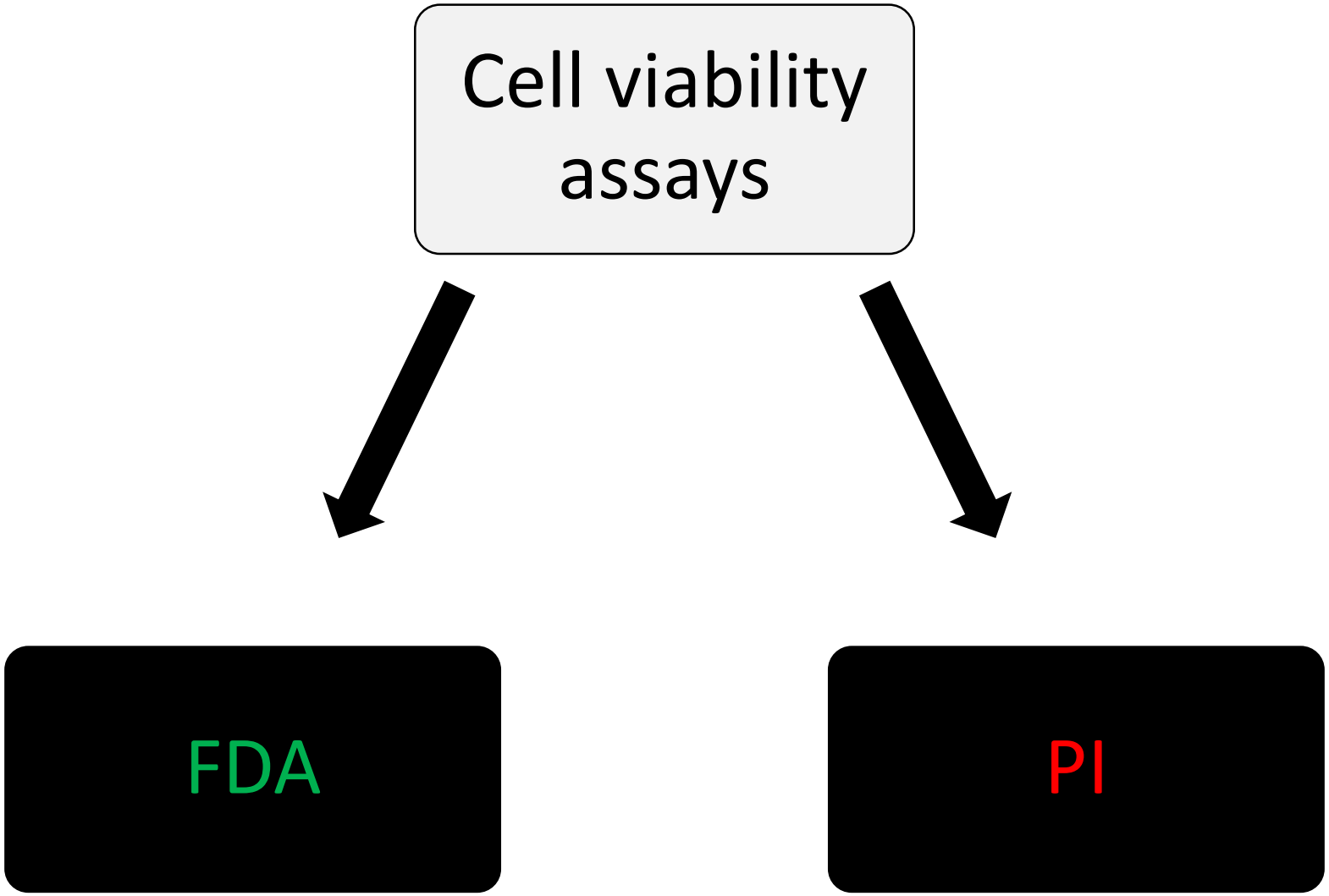
Nicotiana benthamiana

Confocal microscopy after argoinfiltration with plasmids encoding GFP and RFP-tagged proteins, respecitvely.

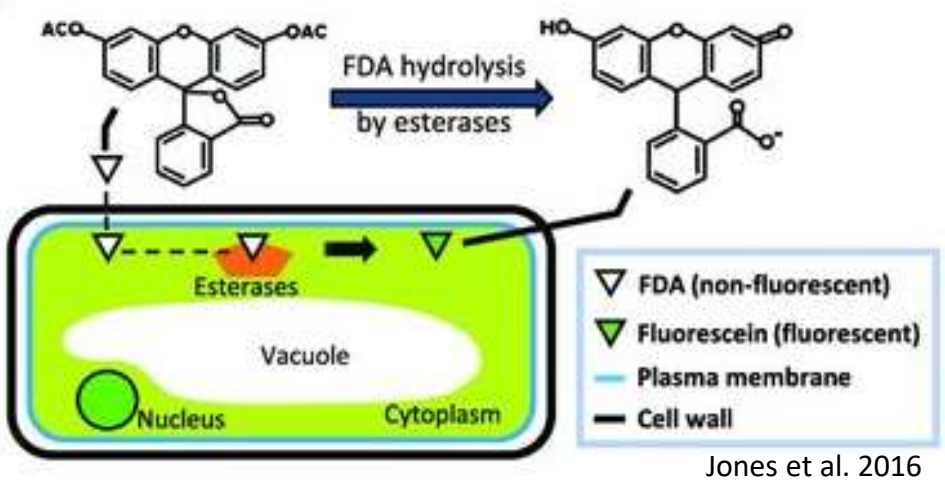


Nicotiana benthamiana leaf epidermal cells.

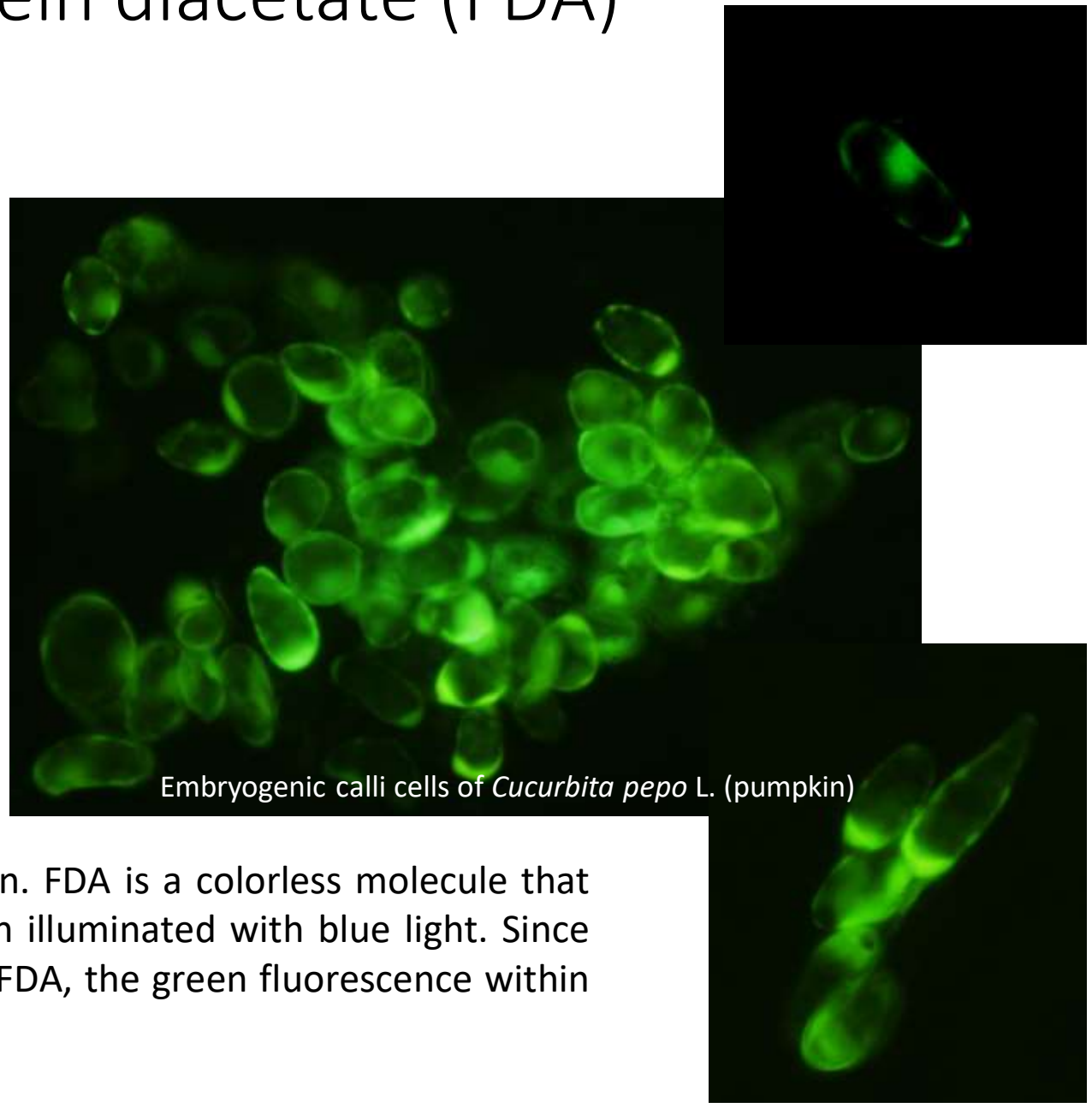
Jagić. PhD (2023)


Cell viability assays
FDA
PI

Cell viability assay - fluorescein diacetate (FDA)



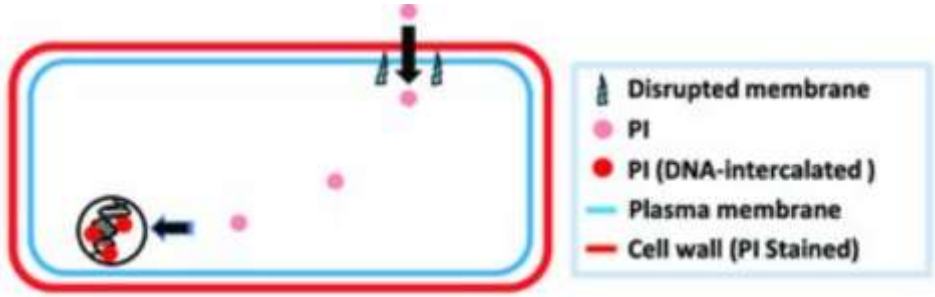
Jones et al. 2016



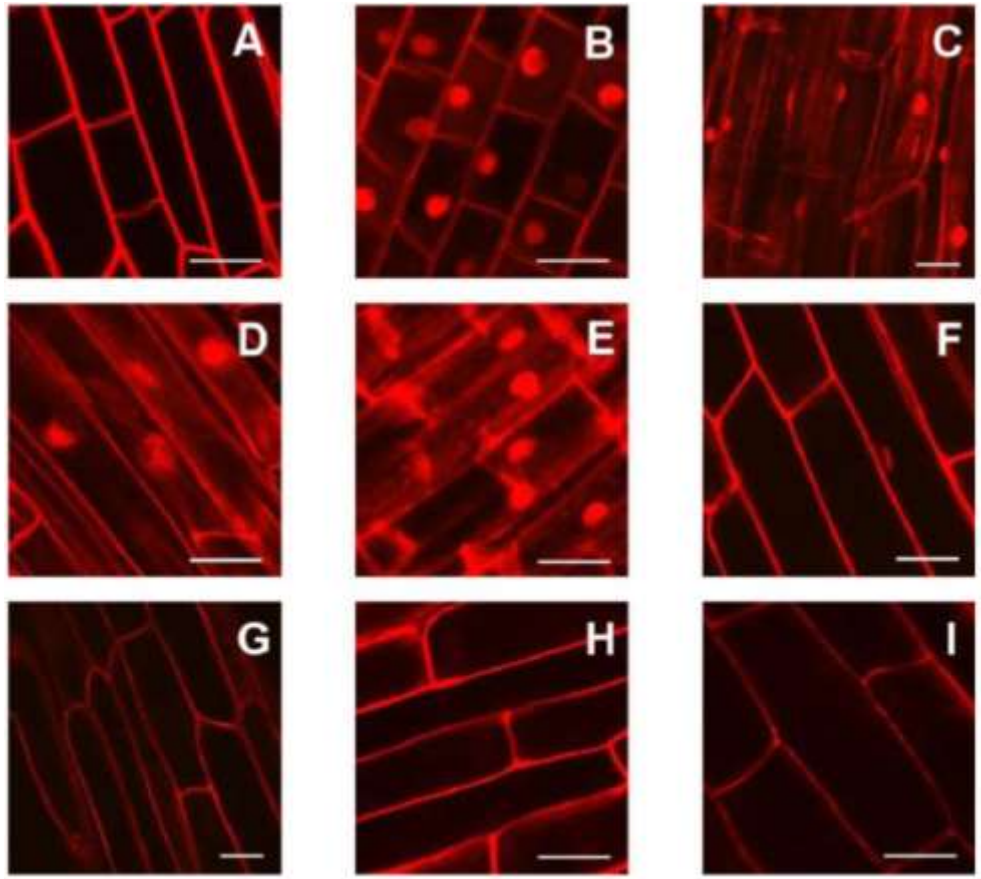
Embryogenic calli cells of *Cucurbita pepo* L. (pumpkin)

FDA enters the cells, which then convert it into fluorescein. FDA is a colorless molecule that does not fluoresce, and fluorescein fluoresces green when illuminated with blue light. Since only living cells have active esterases that can metabolize FDA, the green fluorescence within the cell is used as an **indicator of viable cells**.

Cell viability assay - propidium iodide (PI)

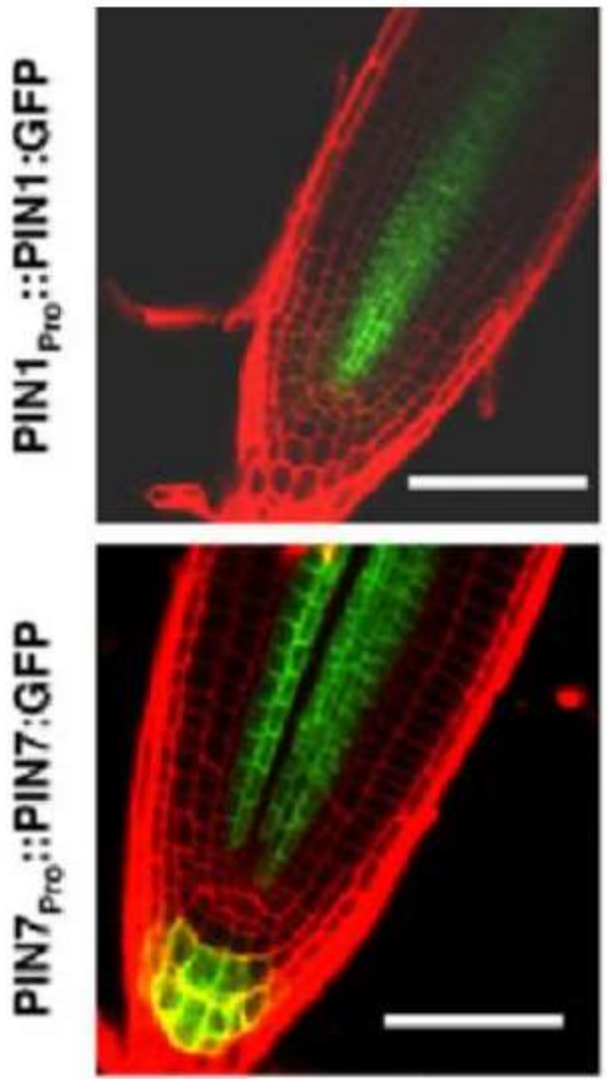


PI is a dye that **intercalates into the DNA of dead cells**, as it cannot pass through the cell membranes of living cells, and fluoresces red in green light. PI is also used in plant biology to make cell walls visible.



Coskun et al. Journal of experimental botany (2011) 63(1), 151–162

Confocal micrographs showing propidium iodide staining of the cell wall and nuclei of damaged cells from lateral root tips of intact barley (*Hordeum vulgare* L.) seedlings treated with silver ions.



Application of propidium iodide for the labeling of cell walls in the analysis of GFP-tagged PIN1 and PIN7 protein localization.