

# Métodos de Transformação Genética em Microrganismos

Profa. Dra. Chirlei Glienke

- Fungos
- Bactérias
- Trypanossomas



# Pré-requisitos para a transformação genética

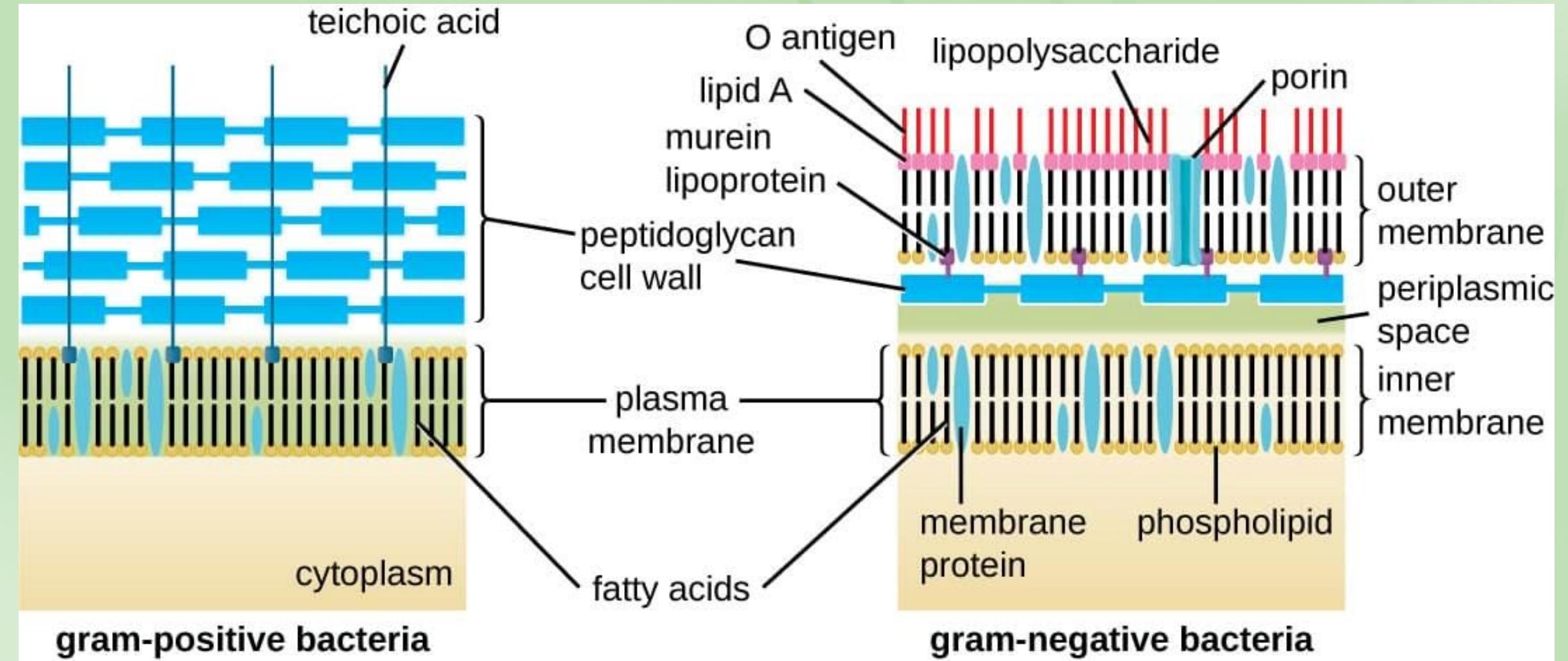
- (1) grandes quantidades de DNA de transformação puro
- (2) células altamente competentes de uma linhagem receptora que permite a passagem de DNA exógeno através da matriz da parede celular
- (3) um mecanismo que entrega o DNA em transformação através da membrana plasmática para dentro do citoplasma

# Pré-requisitos para a transformação genética de fungos

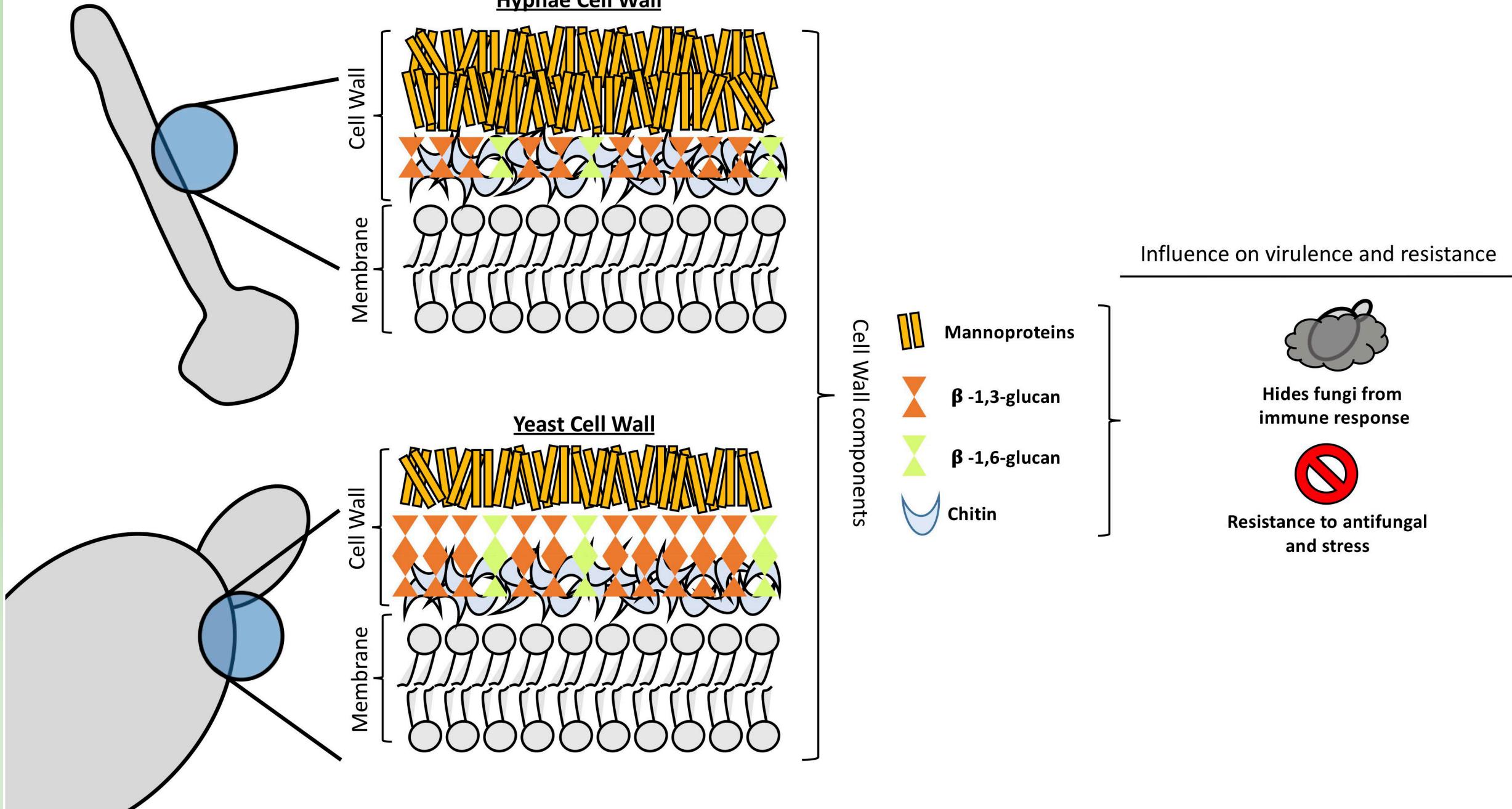
- (4) um mecanismo que permite que a entrega do DNA através da membrana nuclear
- (5) um mecanismo que expressa o DNA transformante (tanto integrado ao genoma do fungo/trypanossoma ou mantido em um plasmídeo de replicação autônoma)
- (6) um procedimento de recuperação e seleção que permita o crescimento e isolamento de transformantes positivos e estáveis

**TABLE 1***Traditional and current transformation techniques used in filamentous fungi*

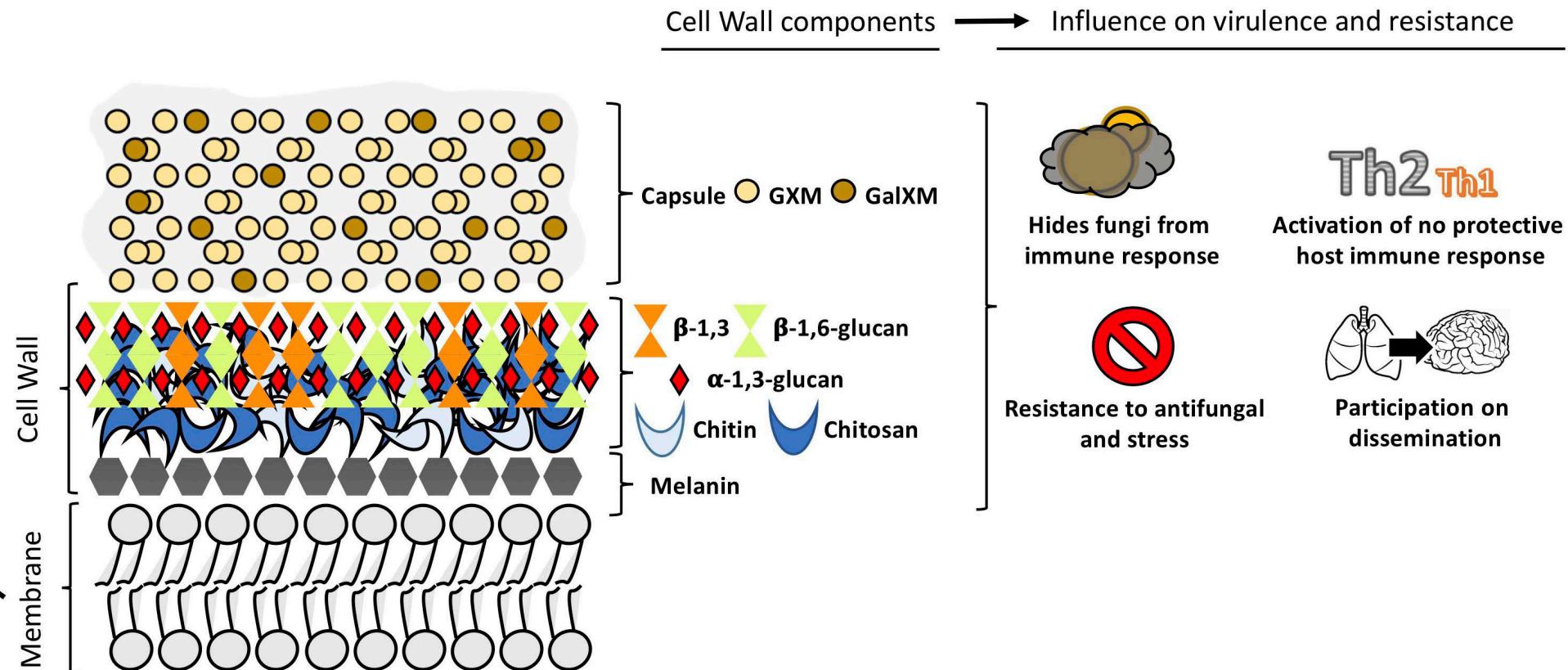
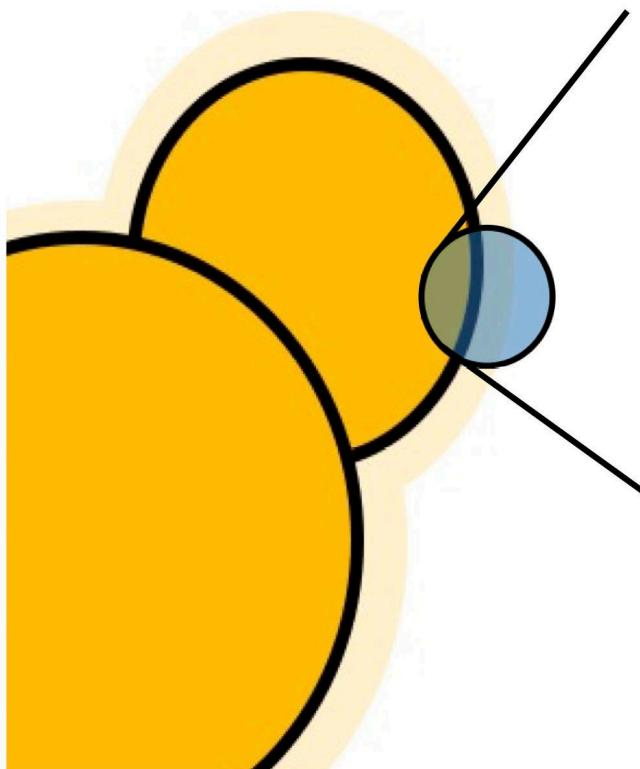
<i>Transformation technique</i>	<i>Working Principle</i>	<i>Advantages</i>	<i>Limitations</i>
Protoplast-mediated transformation (PMT)	Uses cell wall hydrolyzing enzymes with a combination of chemicals like PEG or calcium ions for DNA uptake [20, 38].	Effective in a wide number of filamentous fungi, can be used for hyphal tissues, spores, as well as germlings [29, 37].	The transformation rate is highly dependent on the lytic enzymes. Moreover, PEG/Ca sometime causes a toxic effect on the cell [22, 29].
Electroporation (EP)	An electric current creates permeability in the membrane, making it enable for the uptake of foreign genetic materials (DNA) [19, 41].	Easy to operate and less time-consuming. Highly effective for young mycelia, germinating, and conidia [48].	High voltage may be lethal to some fungal cell, while the precise mechanism for proper optimization is still unknown [49, 50].
<i>Agrobacterium</i> -mediated transformation (AMT)	Ti plasmid from the <i>A. tumefaciens</i> , act as a binary vector. Transformation is attained by cocultivation with the fungus [38, 64].	Minimum possibility of cell death with no need of protoplast preparation. Applicable for both direct and indirect mutagenesis [23, 61].	The preparation of the binary vector relies on complicated and laborious operation process. Its efficiency is highly dependent on various parameters. [60, 68].
Biostatic transformation/particle bombardment (BT)	Genetic material is absorbed on to a gold or tungsten surface and injected inside the host cell by applying high pressure [52, 53].	Useful for those strains which are difficult to culture, also in which the mechanism of PMT is not fully understood [56, 57].	Robust technology is required. Moreover, it does not apply to targeted and specified mutagenesis [29, 45].
Shock waves-mediated transformation (SWMT)	High-intensity underwater shock waves, induces collapse of a cell with microbubbles containing genetic material [70, 71].	A novel approach claimed to transform any type of conidia, intact spores, and mycelia [68, 70].	Transformation ration is dependent on high cell density [71]. Noneconomical and require adequate amount of plasmid <i>in vitro</i> [38, 50, 72].

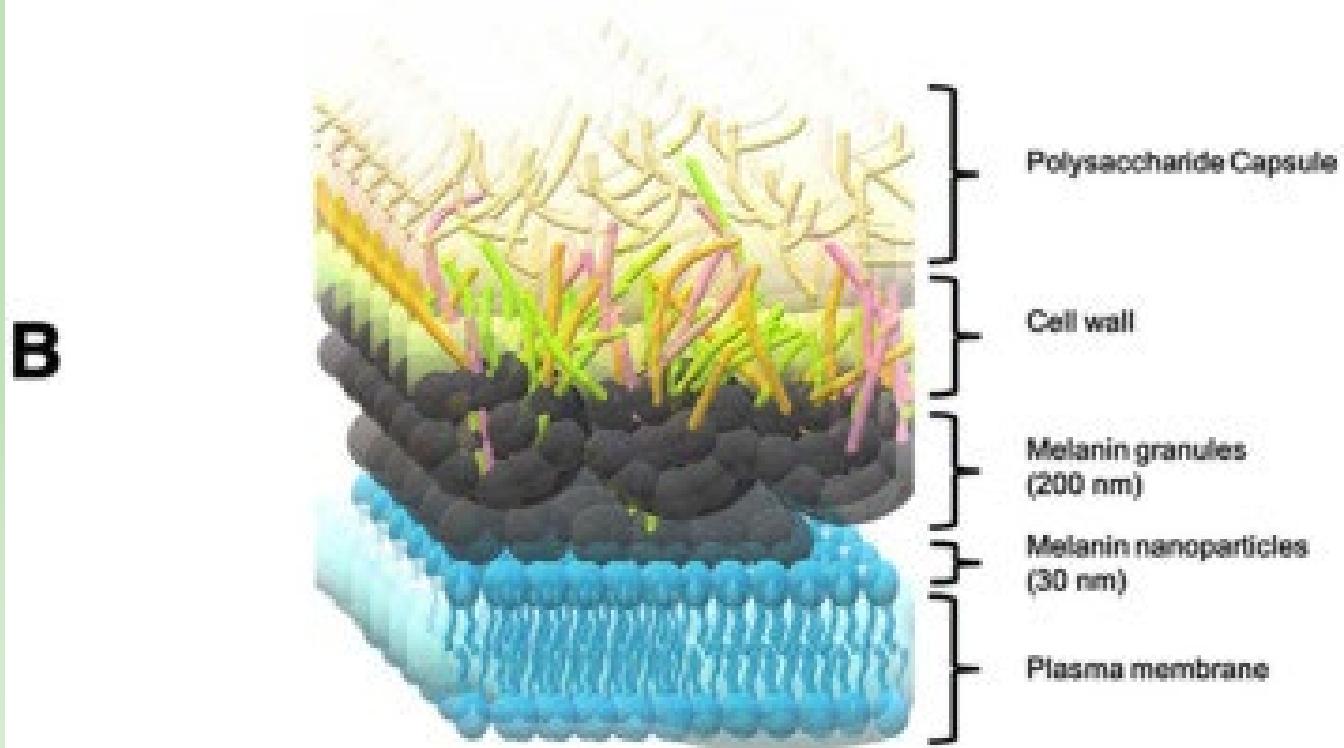
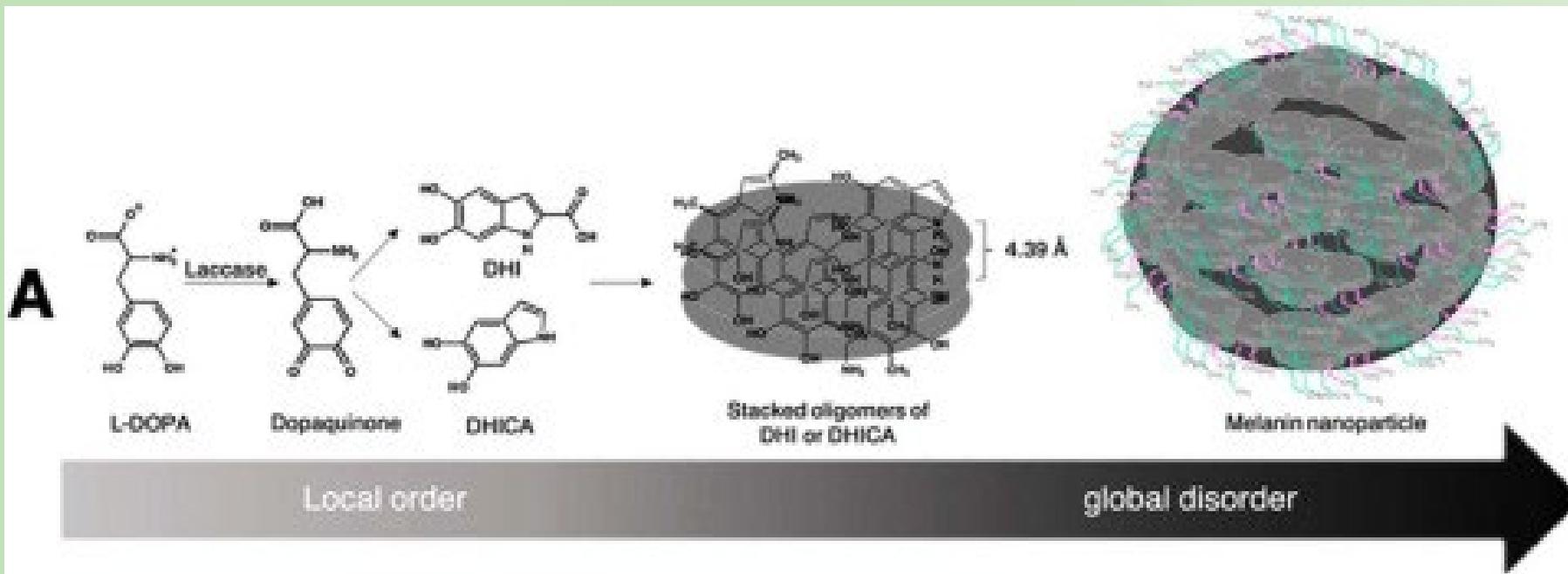


# The *Candida* cell wall

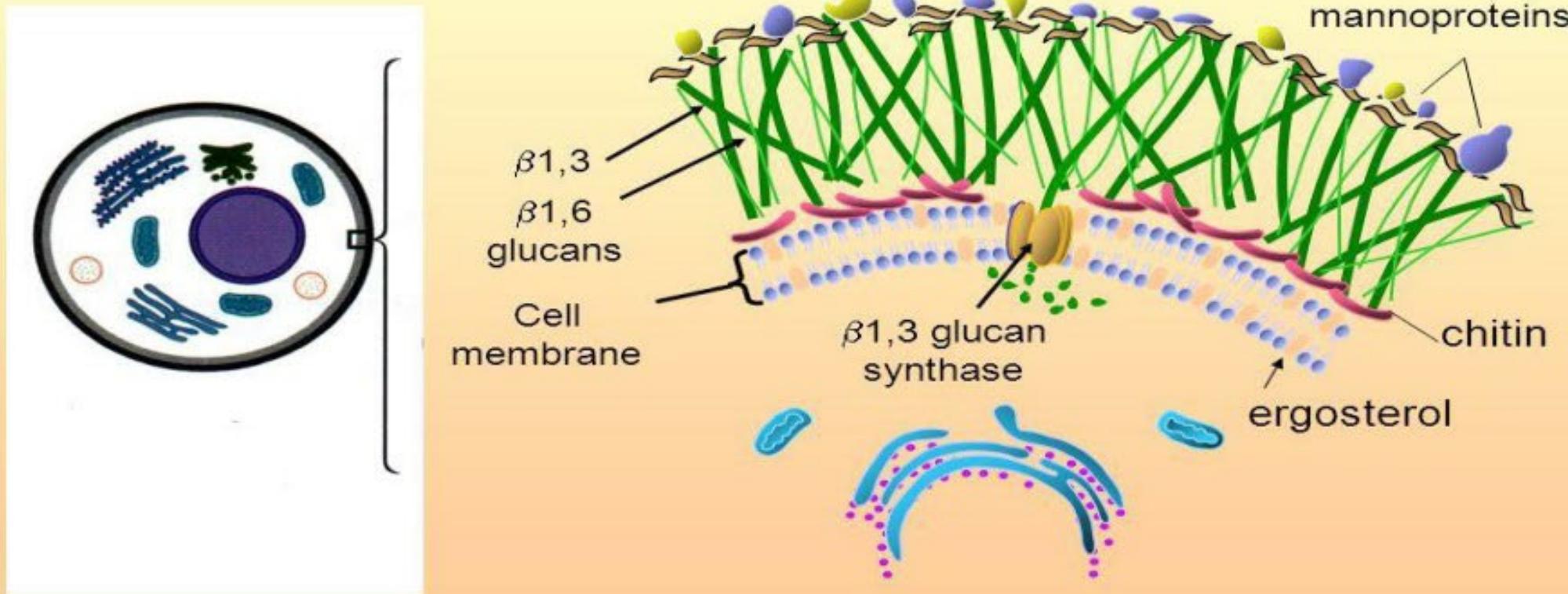


## The *Cryptococcus* cell wall





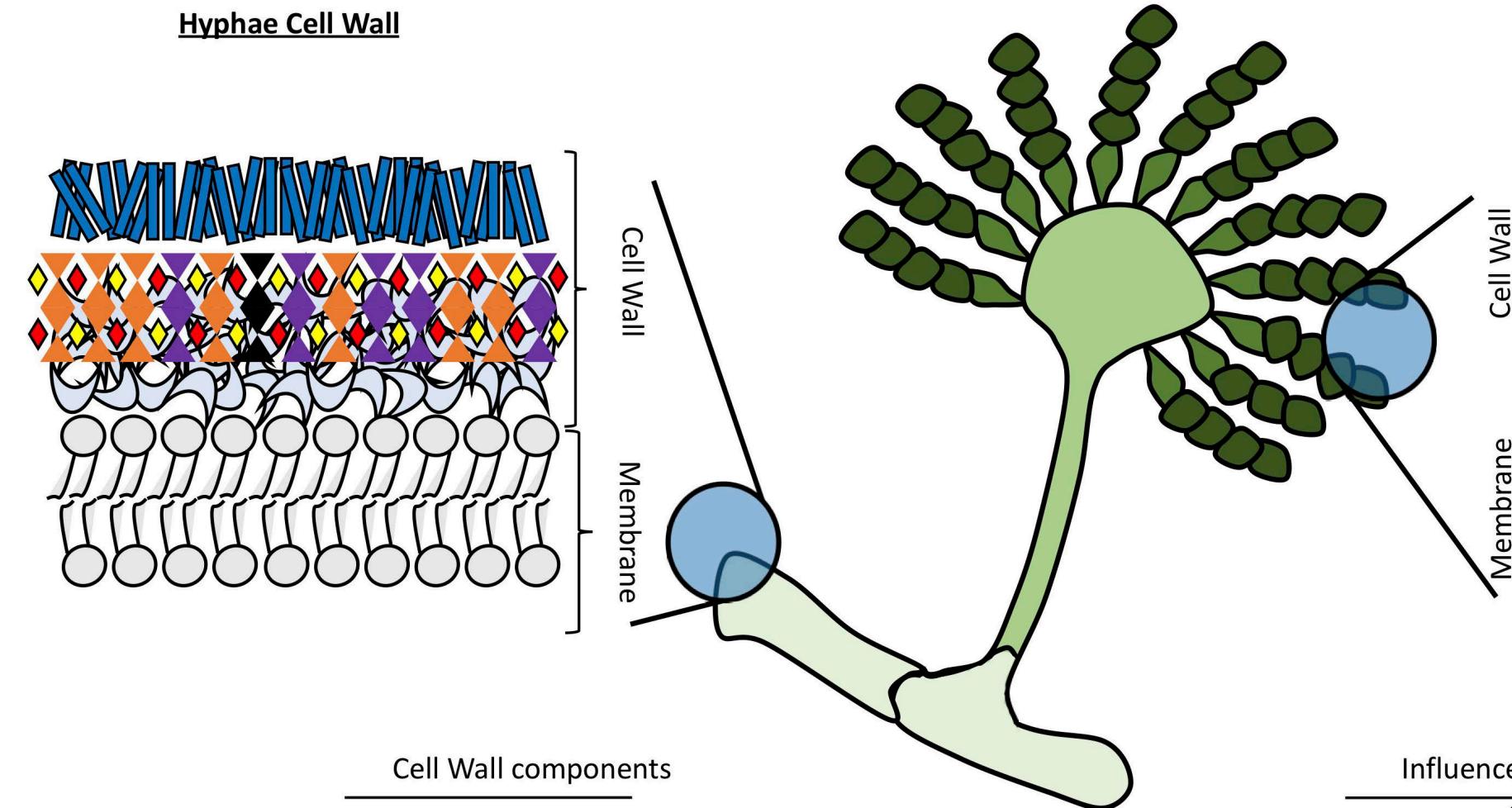
# Parede celular de fungos filamentosos



Atlas of fungal Infections, Richard Diamond Ed. 1999  
Introduction to Medical Mycology, Merck and Co. 2001

# The *Aspergillus fumigatus* cell wall

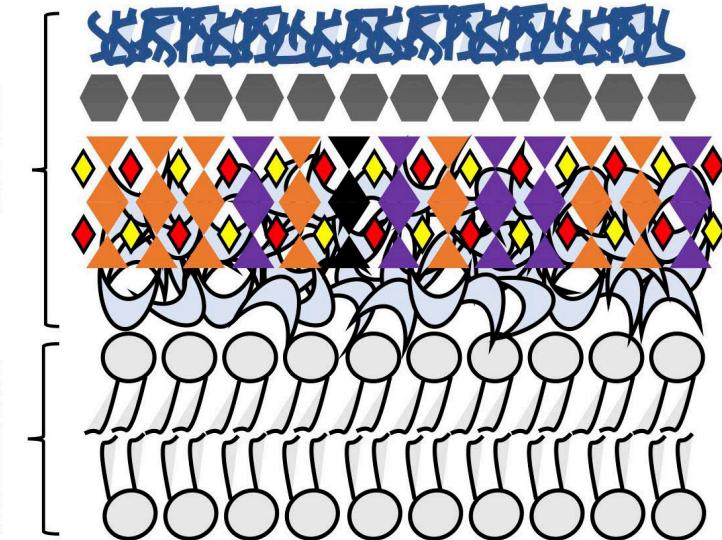
## Hyphae Cell Wall



### Cell Wall components



## Conidium Cell Wall



### Influence on virulence and resistance



Hides fungi from immune response

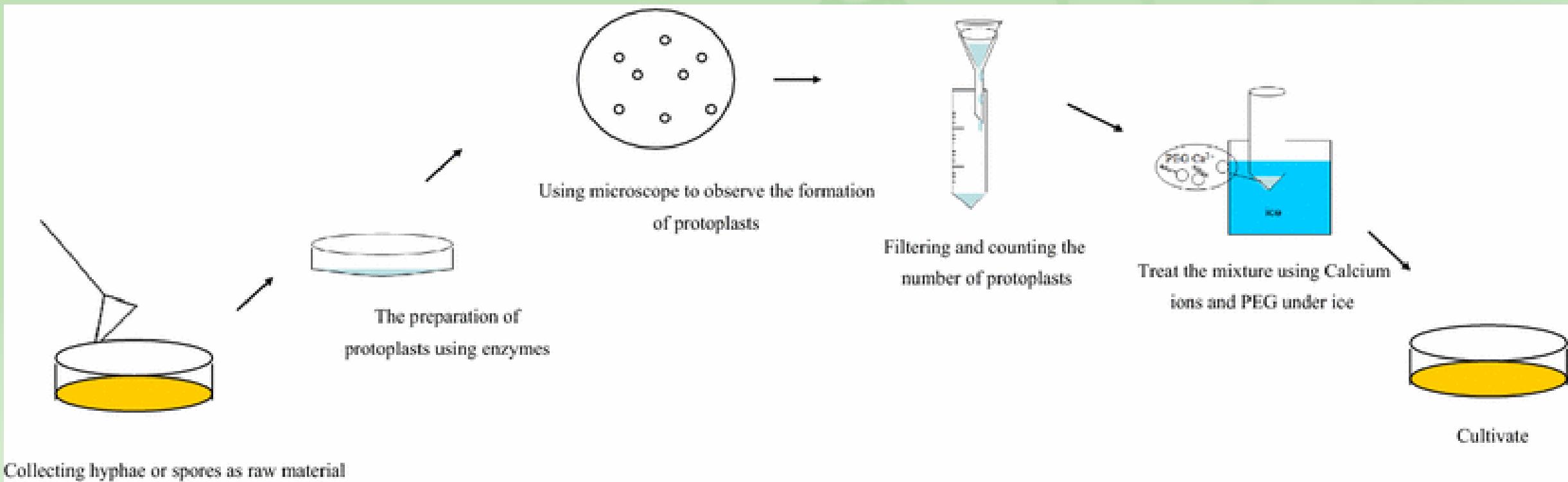
Th<sub>2</sub> Th<sub>1</sub>

Activation of no protective host immune response

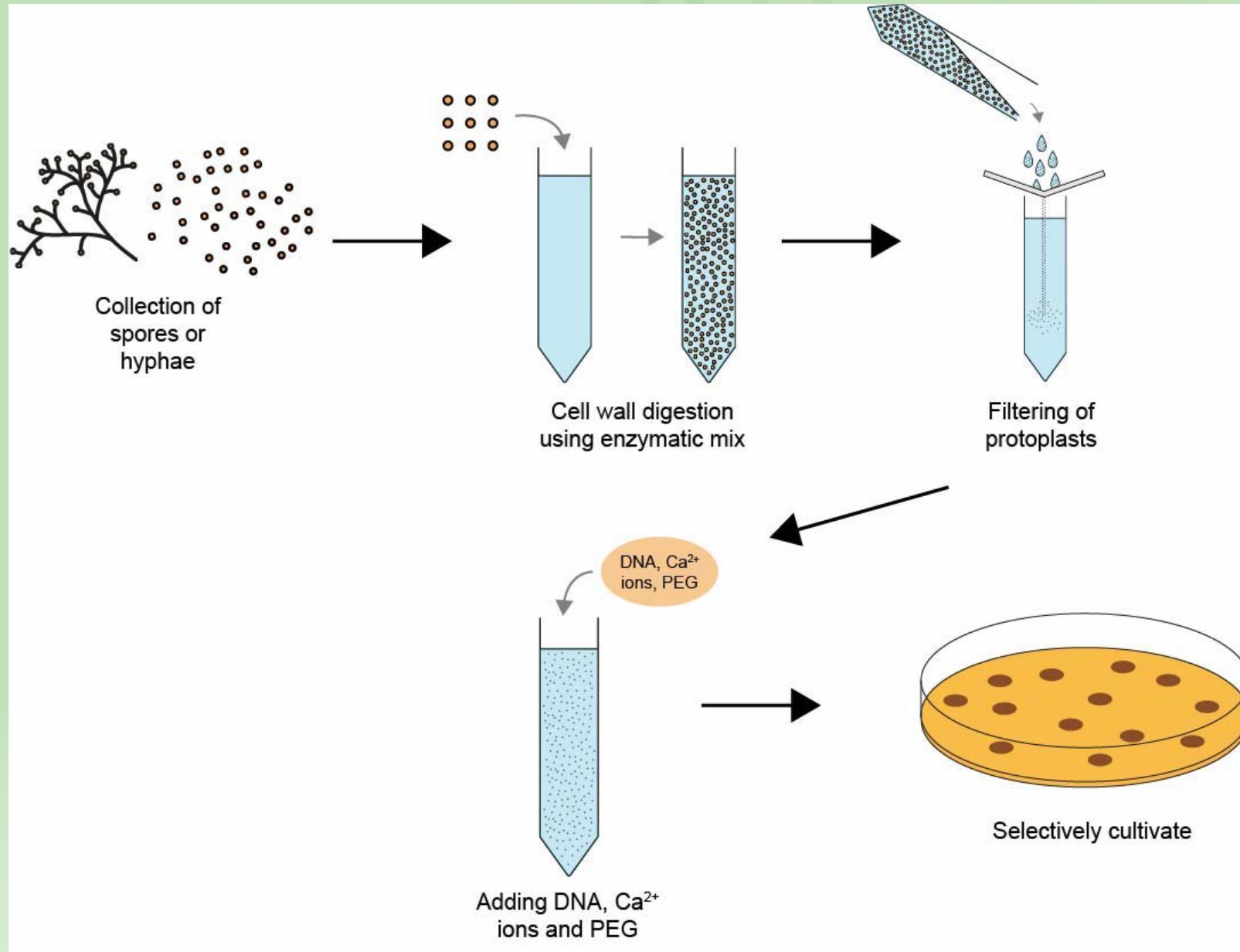
# PMT (protoplastos)

- Foi aplicado pela primeira vez em *Saccharomyces cerevisiae* em 1978
- Usa enzimas de hidrólise da parede celular disponíveis comercialmente com uma combinação de produtos químicos
  - polietileno glicol
  - íons de cálcio

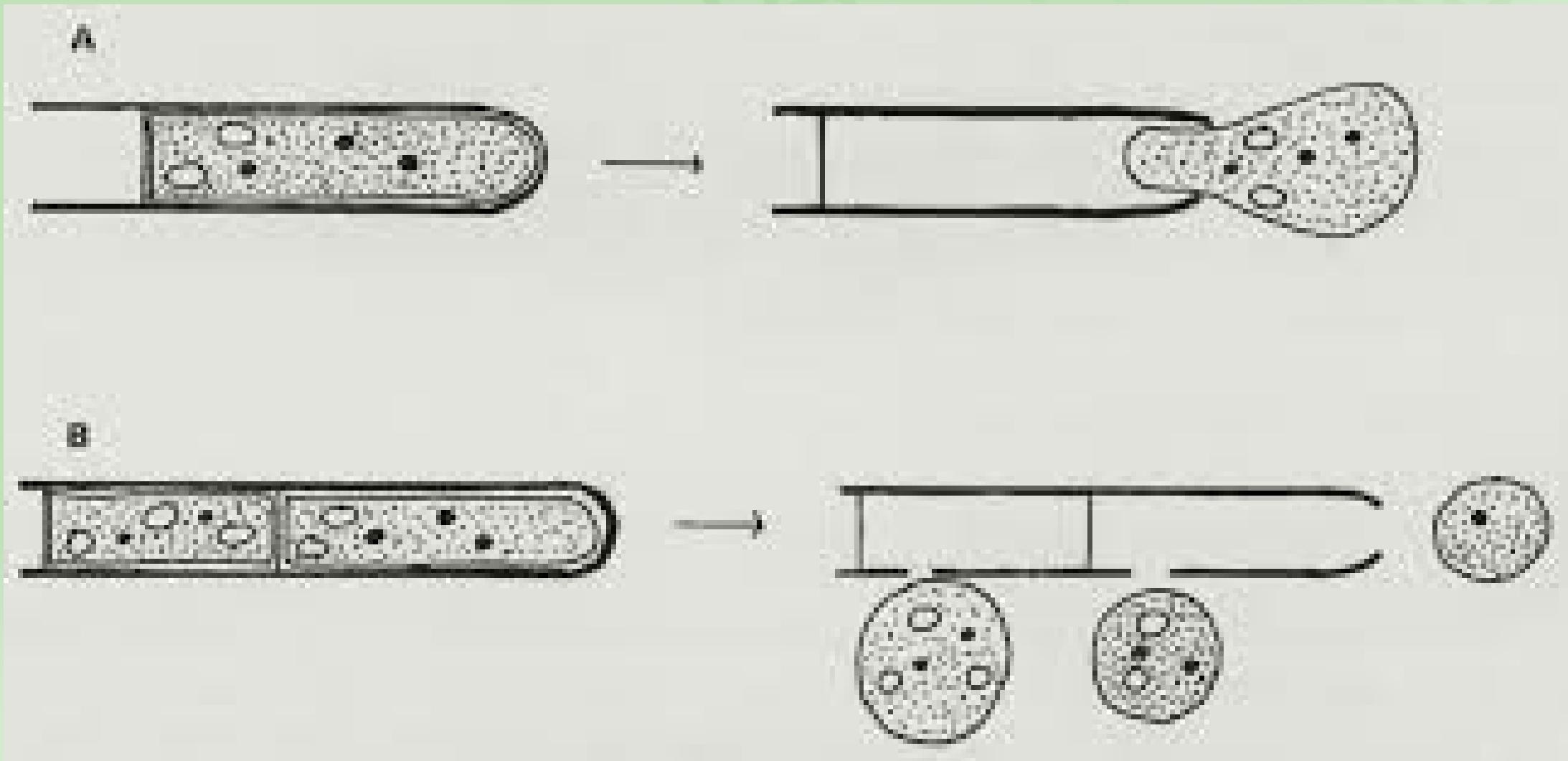
# PMT (protoplastos)



# Transformação de protoplastos



# Produção de protoplastos



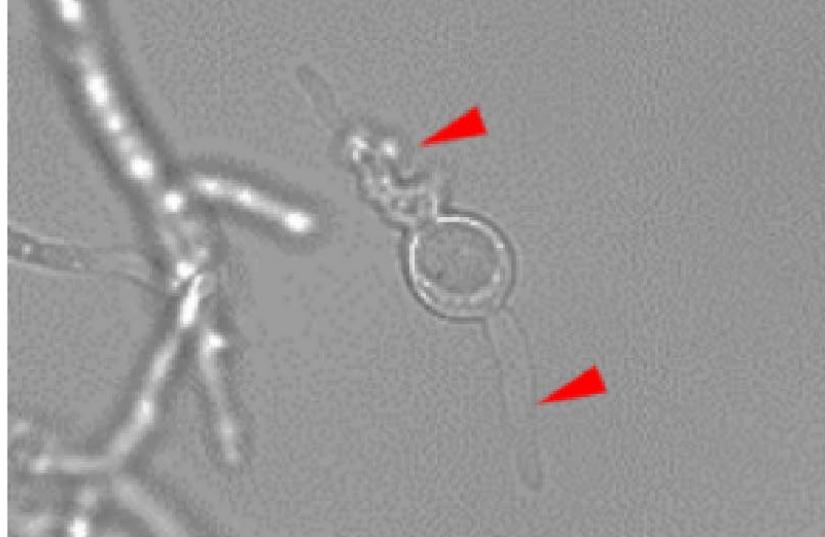


# Regeneração de protoplastos

0h



24h



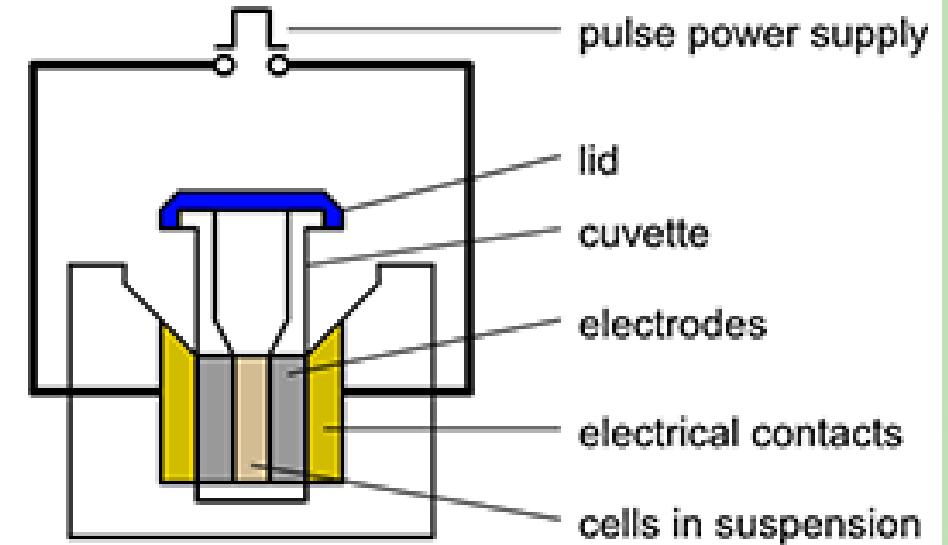
48h

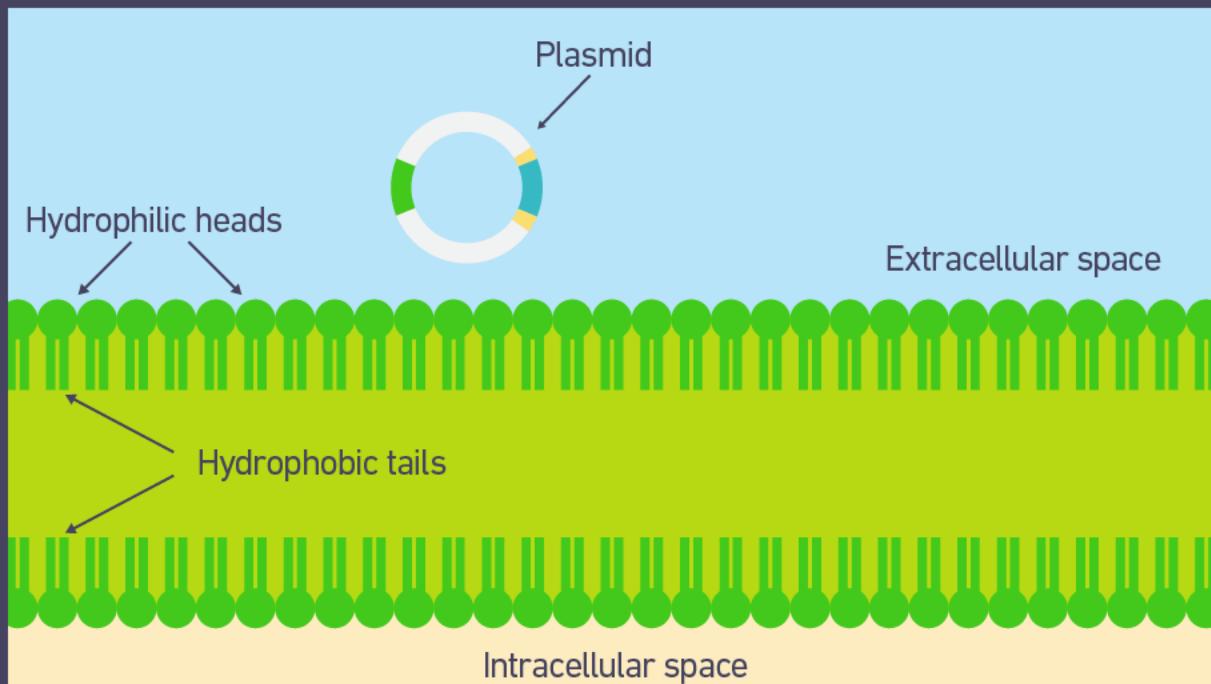


**Table 2 Summary of protoplast preparation parameters for some common fungal species**

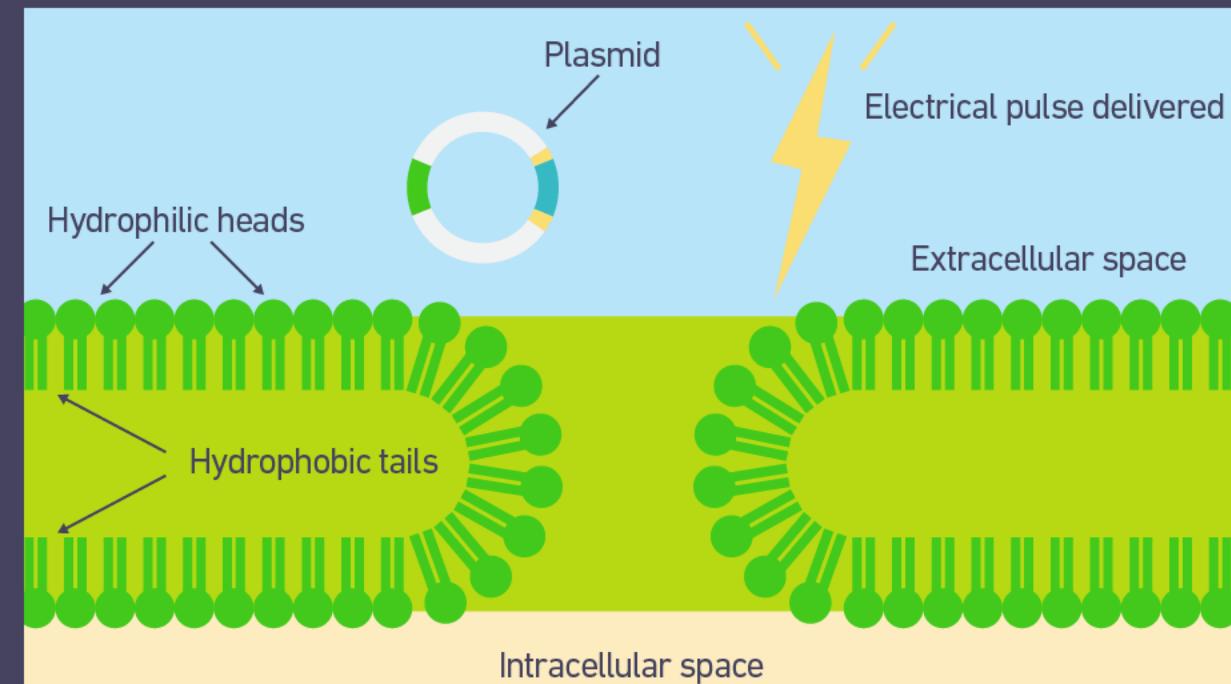
Strain	Starting material	Enzyme	Enzymolysis conditions	Osmotic stabilizer	Protoplast yield (protoplasts/mL)	References
<i>A. niger</i> K10	NA	Lyophilized snail	Incubate at 31 °C for 3 h on a laboratory reciprocal shaker (78 strokes/min, amplitude 3 cm)	0.7 M NaCl	$8.9 \times 10^6$	[31]
<i>A. niger</i> N583	Mycelium	Lysing enzymes, chitinase griseus and $\beta$ -glucuronidase	Incubate in a 100 mL glass bottle for 2 h at 37 °C and 130 rpm	0.8 M sorbitol	NA	[32]
<i>Aspergillus nidulans</i> A89	Mycelium	Yatalase, Kitalase	Incubate 6 h at 30 °C with constant shaking (60–80 rpm)	1 M sorbitol	NA	[33]
<i>Aspergillus nidulans</i> G191	Mycelium	Novozym 234	Incubate 1.5 h at 30 °C	0.6 M KCl	NA	[34]
<i>Trichoderma atroviride</i> T11	Mycelium	Lysing enzymes from <i>Trichoderma. harzianum</i>	Incubate at 30 °C for 2 h with shaking at 80 rpm	1 M sorbitol	$1\text{--}5 \times 10^6$	[26]
<i>Penicillium simplicissimum</i>	Mycelium	Driselase, caylase, cellulase, enzyme cocktail II from Merck company	Incubate at 30 °C for 20 or 180 min	1.2 M sorbitol	$2 \times 10^9$	[26]
<i>Trichoderma reesei</i> QM 9414	Mycelium	Lysing enzymes from <i>T. harzianum</i>	Incubate at 28–30 °C for 2 h	1 M sorbitol	NA	[17]
<i>Trichoderma reesei</i> QM 9414 and VTT-D-79125	Mycelium	Novozym 234	Incubate at 28 °C for approx. 1.5 h	1 M and 1.2 M sorbitol	$5 \times 10^7$ to $5 \times 10^8$	[16]
<i>N. crassa</i> M246 (qa-2 mutation) and M6-11 (arom-9 mutation)	Mycelium	Proteinase K	Incubate at 1 h at room temperature	1 M sorbitol	$1 \times 10^7$	[19]
<i>Aspergillus fumigatus</i> 2085	Mycelium	$\beta$ -glucuronidase	Incubate at 37 °C for 1 h with occasional shaking	0.7 M KCl	$1 \times 10^6$	[35]
<i>A. oryzae</i> NRRL 492	Mycelium	Novozyme 234 and $\beta$ -glucuronidase	Incubate at 30 °C for 1.5 h with shaking at 80 rpm	0.6 M sorbitol and 1 M sucrose	Each 400 mL culture yielded $5\text{--}10 \times 10^7$ protoplasts	[20, 21]
<i>Rhizopus oryzae</i> AS 3.819	Mycelium	Helicase, cellulase, lyticase	Incubate at 35 °C for 140 min with gentle shaking	0.6 M sorbitol	$1 \times 10^7$	[36]
<i>Monascus purpureus</i>	Mycelium	Glucanex, lysing enzyme from <i>T. harzianum</i> $\beta$ -glucuronidase and Caylase	Incubate at 28 °C for 4 h with orbital shaking (100 rpm)	0.5 M KCl and 0.1 M MgSO <sub>4</sub>	$1 \times 10^9$	[22]
<i>Penicillium expansum</i>	Mycelium	0.6% cellulase and 0.6% snailase	Incubate at 25–35 °C for 3–3.5 h	0.6 M NaCl	$2.36 \times 10^8$	[37]

# Eletroporação - células, esporos, protoplastos

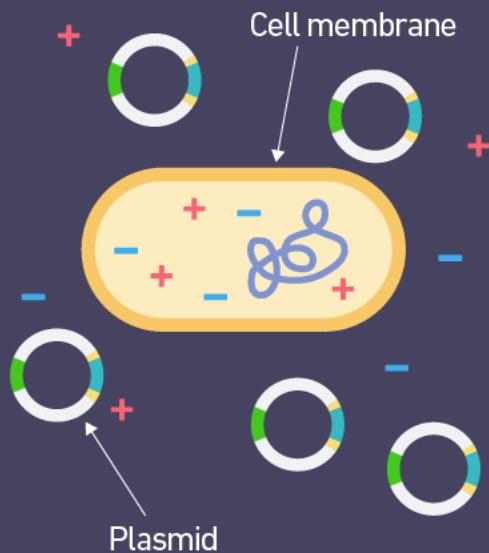




Before electroporation



After electroporation

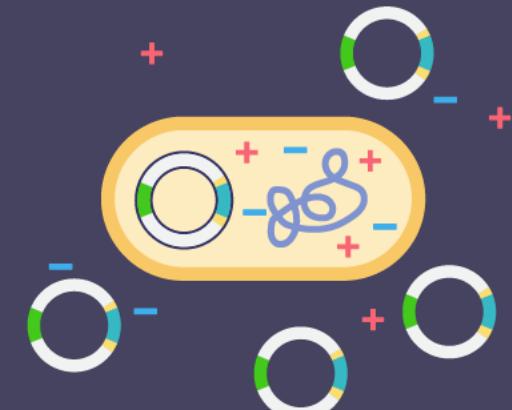
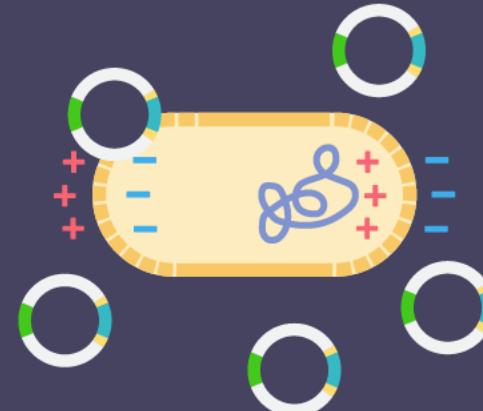
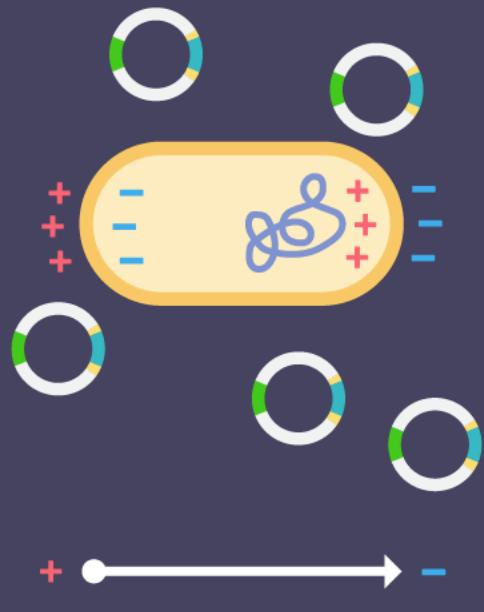


Cells and  
plasmids are  
mixed together

An electrical pulse  
is applied, causing  
pores to form

Plasmids enter  
the cells through  
the pores

Pores seal with  
the plasmid inside  
transformed cells

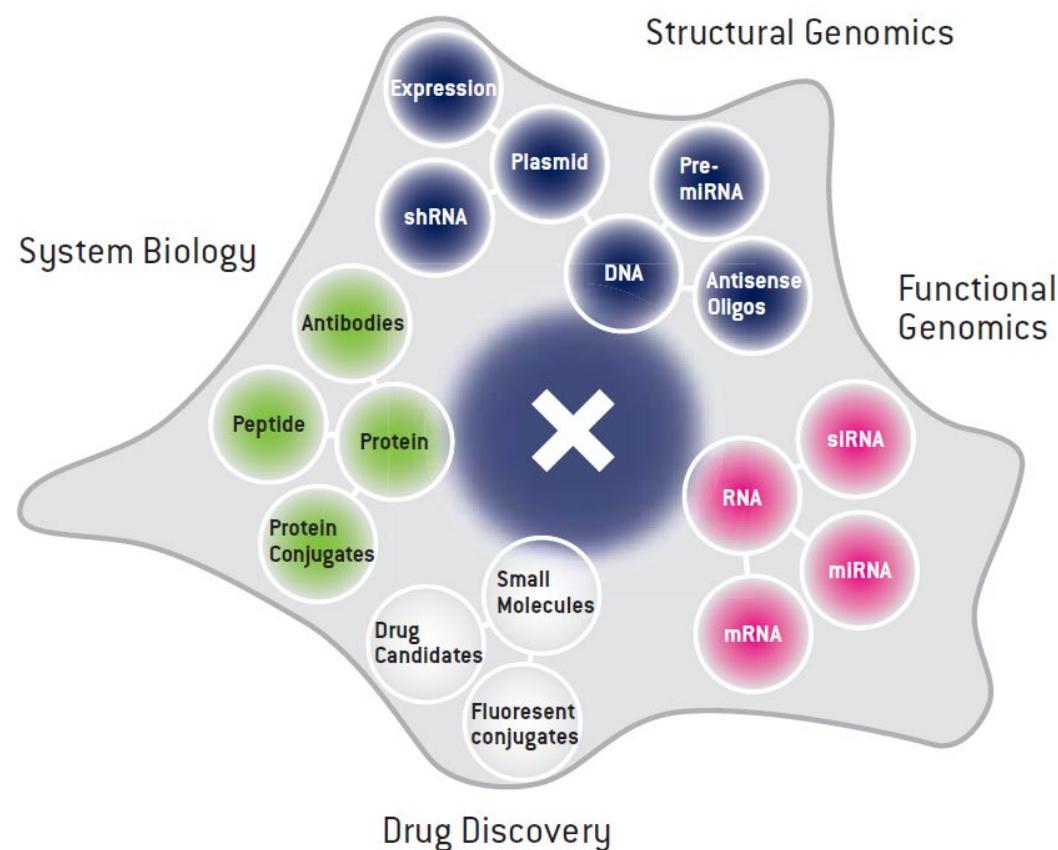


**Table 5 Summary of electroporation-mediated transformation protocols for different fungal species**

Strain	Waveform	Instrument	Electroporation parameters	Raw material	Number of cell	Name and size of vectors	Amount of DNA	Selective markers	Transformation efficiency (per µg DNA)	References
<i>Rhizopus oryzae</i> AS 3.819	NA	Multiporator (Eppendorf)	12 kV/cm	Protoplasts	$1 \times 10^7$	pBS-hgro- <i>ldhA</i> (8533 bp)	5 µg	<i>hygB</i>	10.2 transformants	[36]
<i>R. oryzae</i> AS 3.819	NA	Multiporator (Eppendorf)	15 kV/cm	Germinated spores	$1 \times 10^7$	pBS-hgro- <i>ldhA</i> (8533 bp)	5 µg	<i>hygB</i>	8.8 transformants	[36]
<i>A. niger</i> ATCC 20739	Exponential decay waveform	Electro Gene Transfer Unit	6 kV/cm and 3 ms time constant	Germinated spores	$1 \times 10^7/0.4$ mL	pXbaI92 (5.7 kb) and pBXba2 (6.0 kb)	1–10 µg	<i>argB</i> <sup>–</sup>	1.2 transformants for integrative vector and 100 colonies for plasmid DNA.	[52]
<i>Penicillium urticae</i> NRRL 2159A	Exponential decay waveform	Gene Pulser (Bio-Rad) Multiporatr (Eppendorf)	12.5 kV/cm, 8.6 ms time constant and 25 µF	Germinated conidia	$8 \times 10^6$	pCSN44(NA)	1–5 µg	<i>hygB</i>	$1.8 \times 10^3$ transformants	[64]
<i>A. oryzae</i> ATCC 14895	Exponential decay waveform	Gene Pulser (Bio-Rad) Multiporatr (Eppendorf)	11–12.5 kV/cm, 4.6–4.8 ms time constant and 25 µF	Germinated conidia	$2.5 \times 10^6$	pBEN(NA)	1–5 µg	Benomyl <sup>R</sup>	$2.6 \times 10^3$ transformants	[64]
<i>Leptosphaeria maculans</i> "Virulent"	Exponential decay waveform	Gene Pulser (Bio-Rad) Multiporatr (Eppendorf)	12.5 kV/cm, 4.8 ms time constant and 25 µF	Germinated conidia	$1.2 \times 10^6$	pCSN44(NA)	1–5 µg	<i>hygB</i>	$1–6 \times 10^2$ transformants	[64]
<i>N. Crassa</i> R-206A	Exponential decay waveform	Gene Pulser (Bio-Rad) Multiporatr (Eppendorf)	12.5 kV/cm, 5 ms time constant and 25 µF	Germinated conidia	$3–6 \times 10^6$	Bsqa(NA)	1–5 µg	<i>hygB</i>	$5.7 \times 10^3$ transformants	[64]

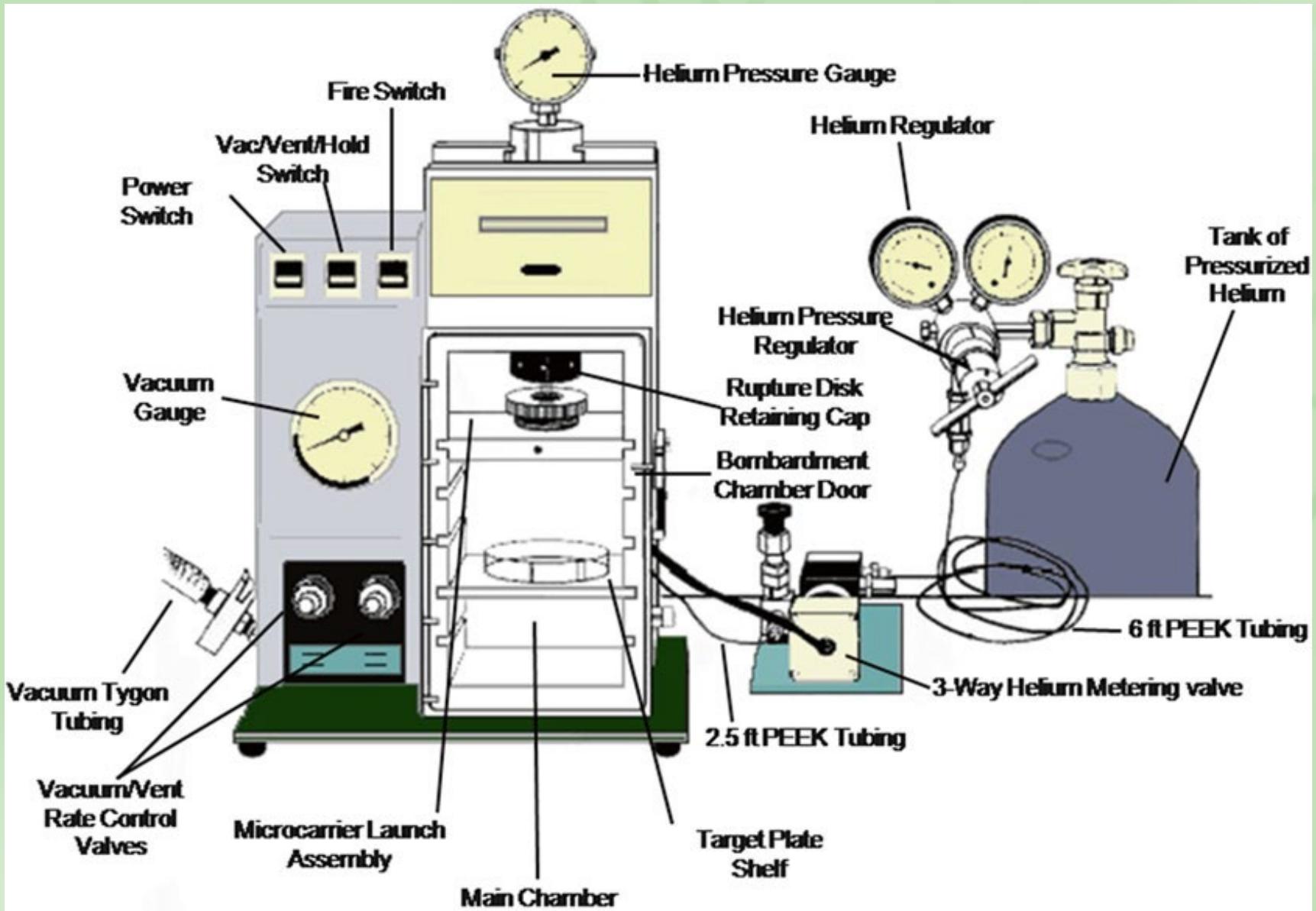


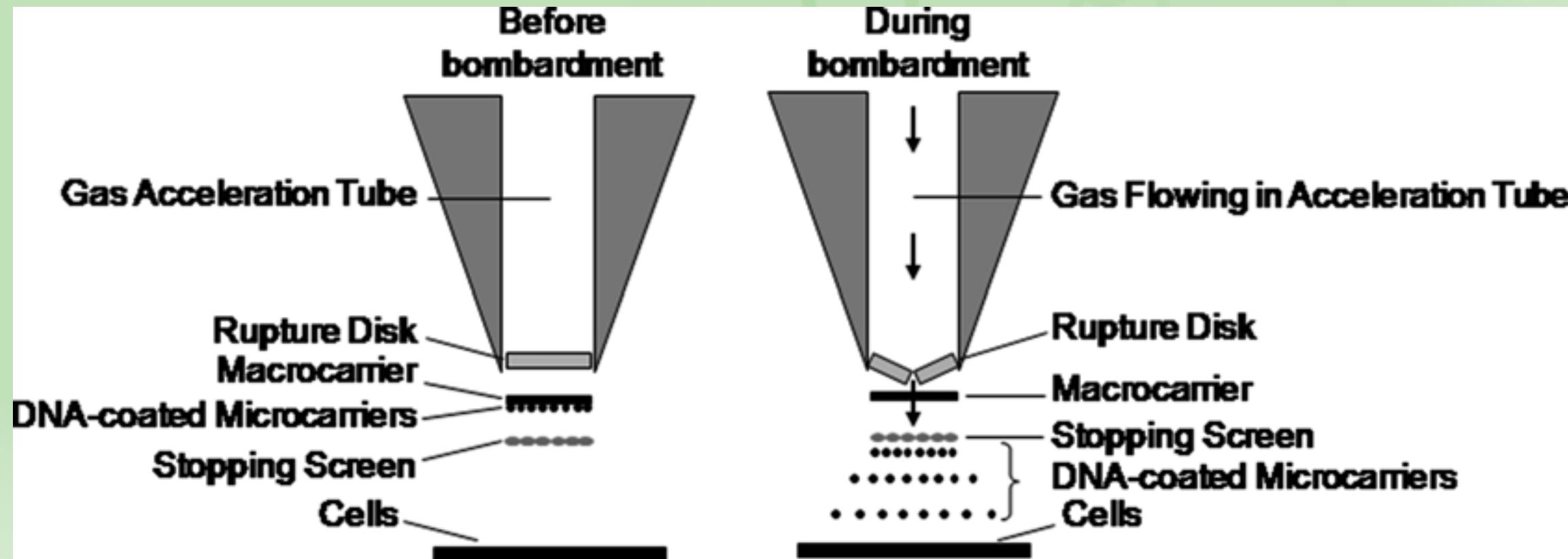
## Nucleofector® Technology – delivers the widest range of substrates



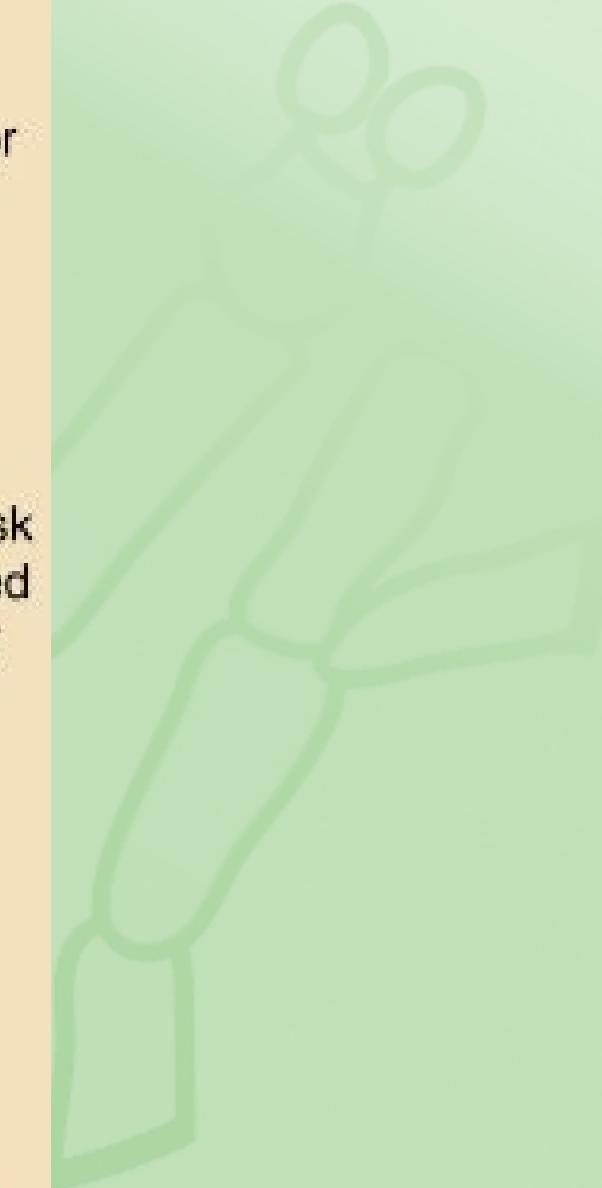
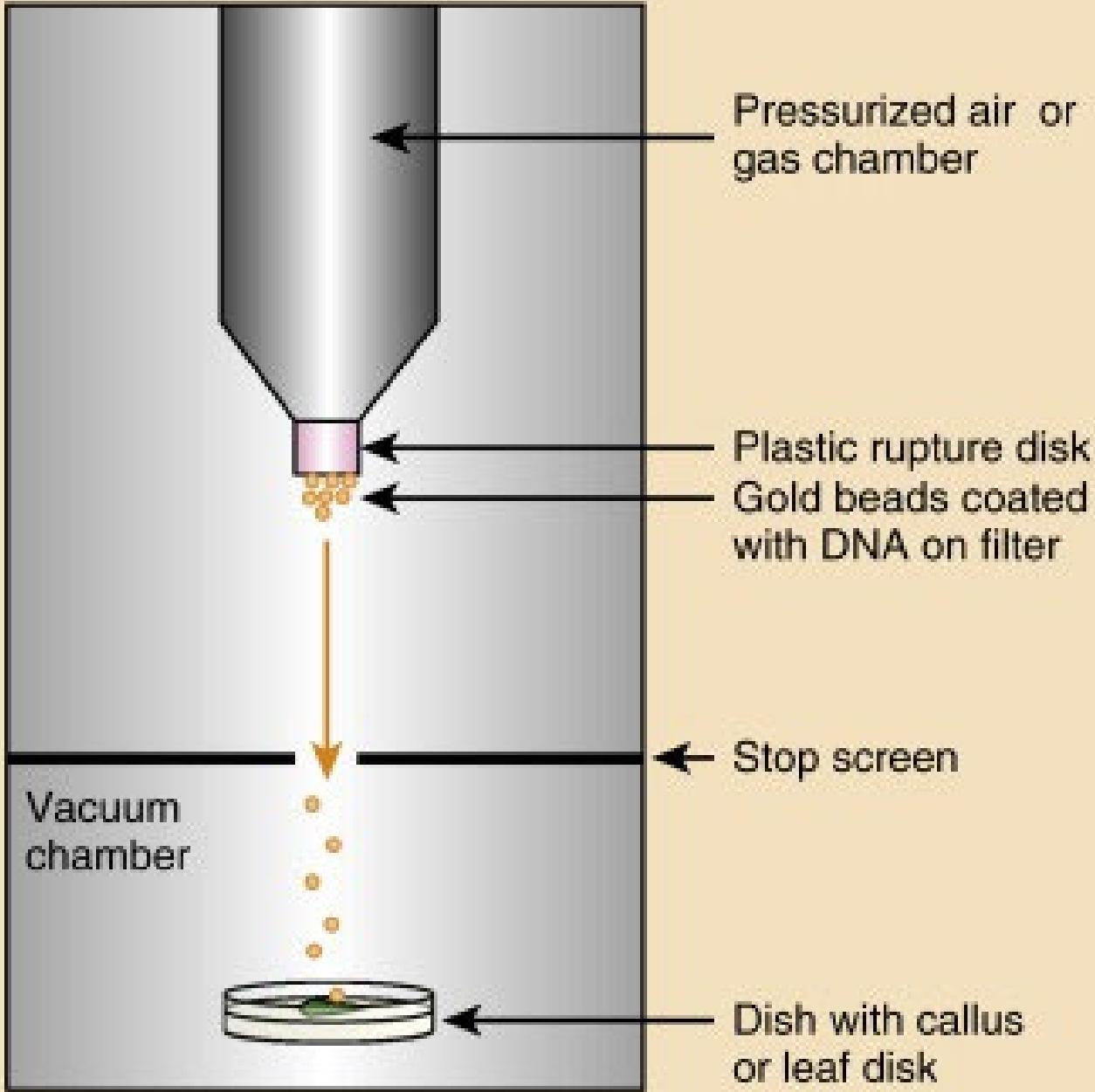
**Figure 5.** Overview of substrates that can be transfected into primary cells and cell lines using Nucleofection®.

# Bombardeamento de micropartículas

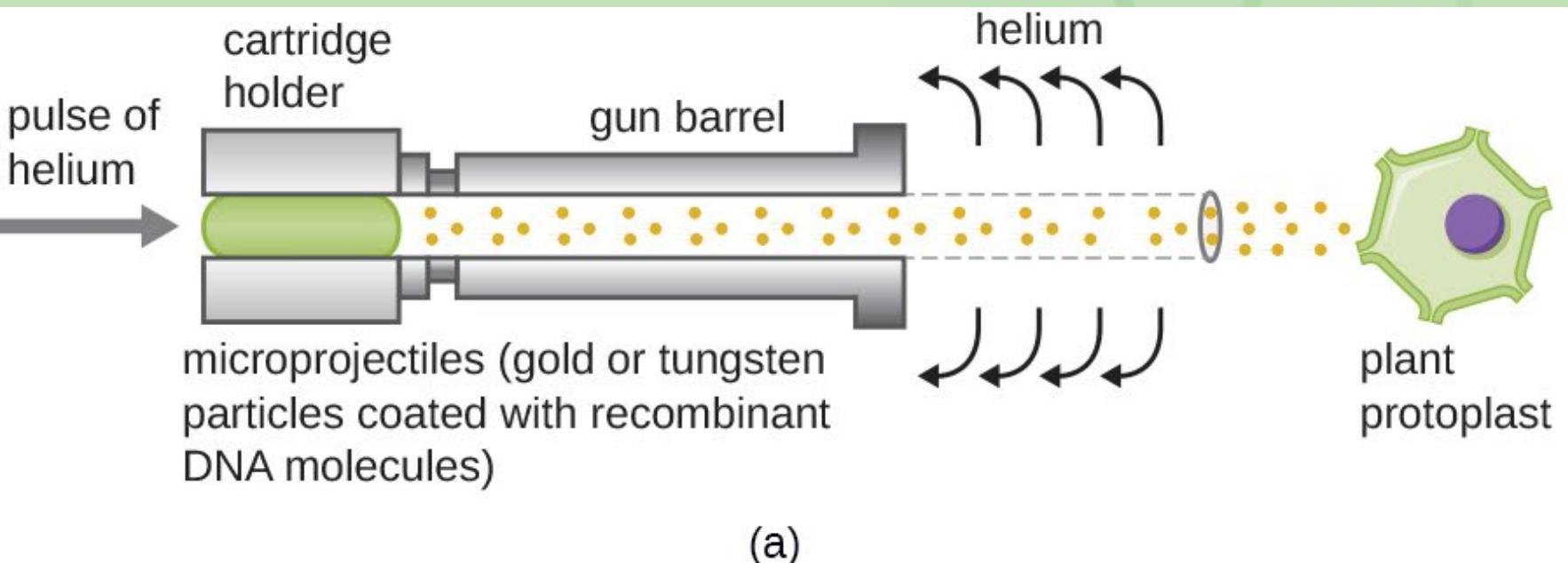




A



# Bombardeamento



# Agrotransformação

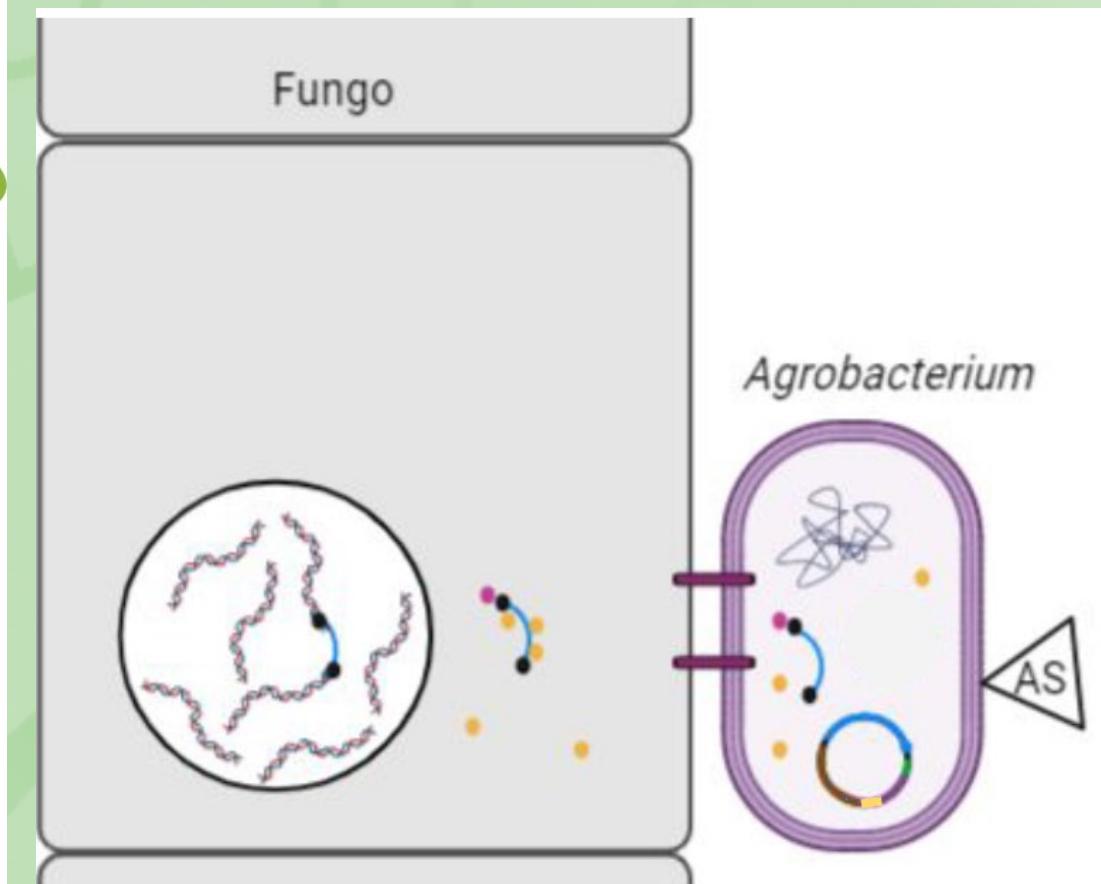
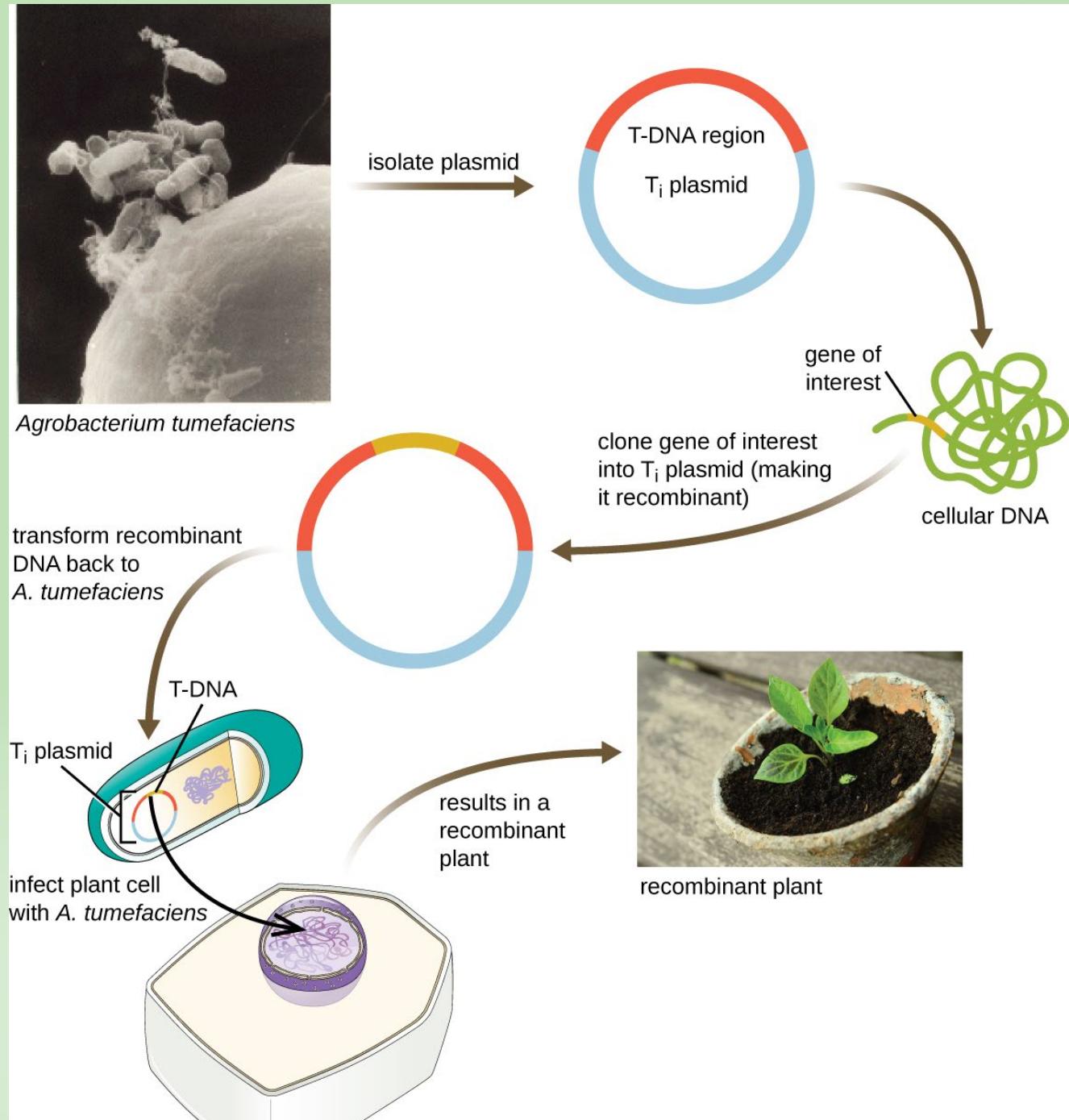
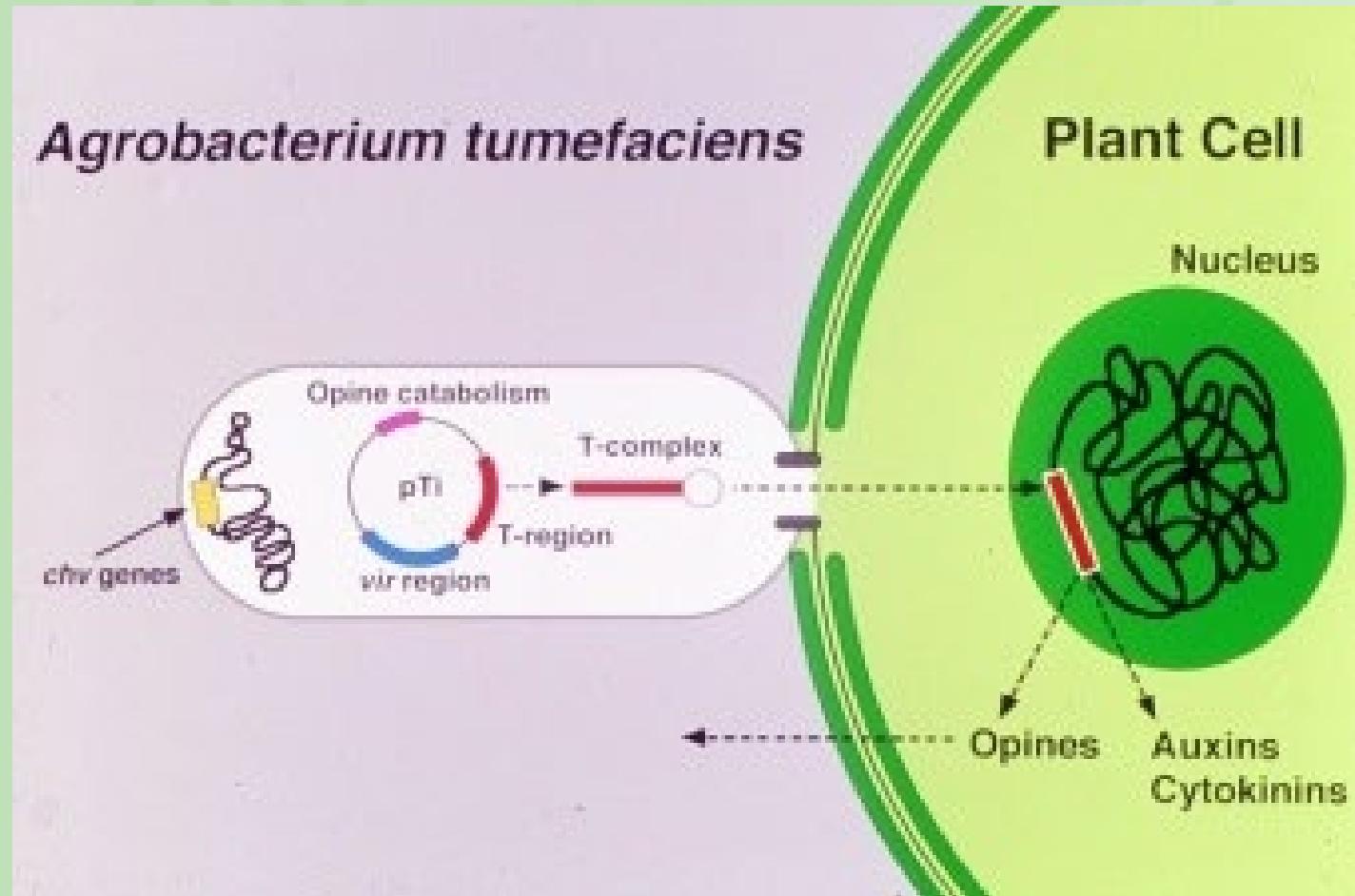
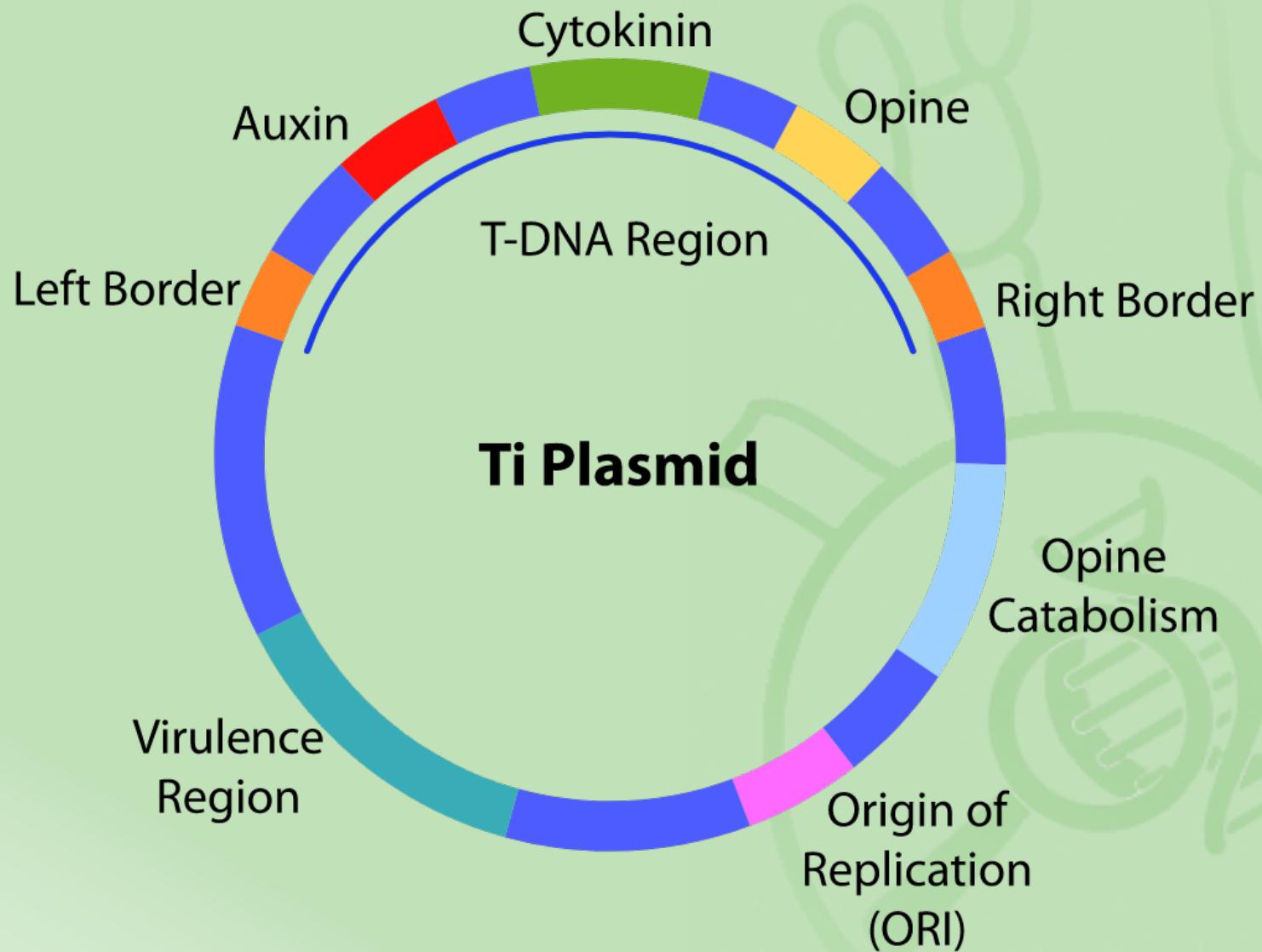


Fig. 2 Esquema da agrotransformação adaptado de Idnurm et al., 2017





<https://www.youtube.com/watch?v=wTO-KmpZQgQ>



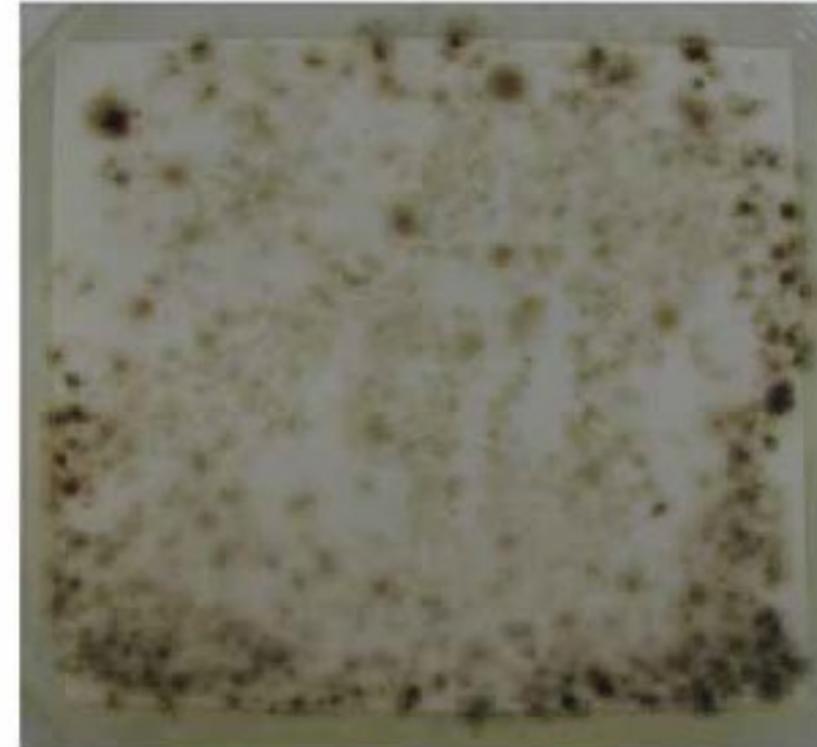
## T DNA do plasmídio Ti

- Indutor de Tumor

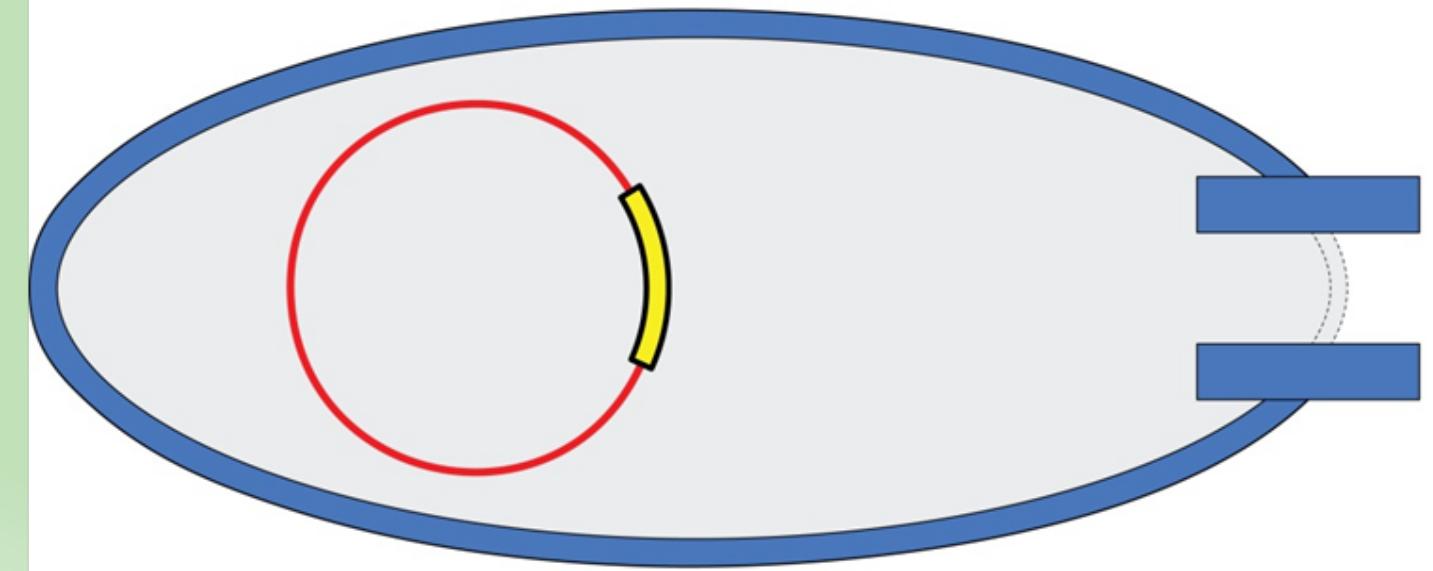
## Efeito da acetoseringona



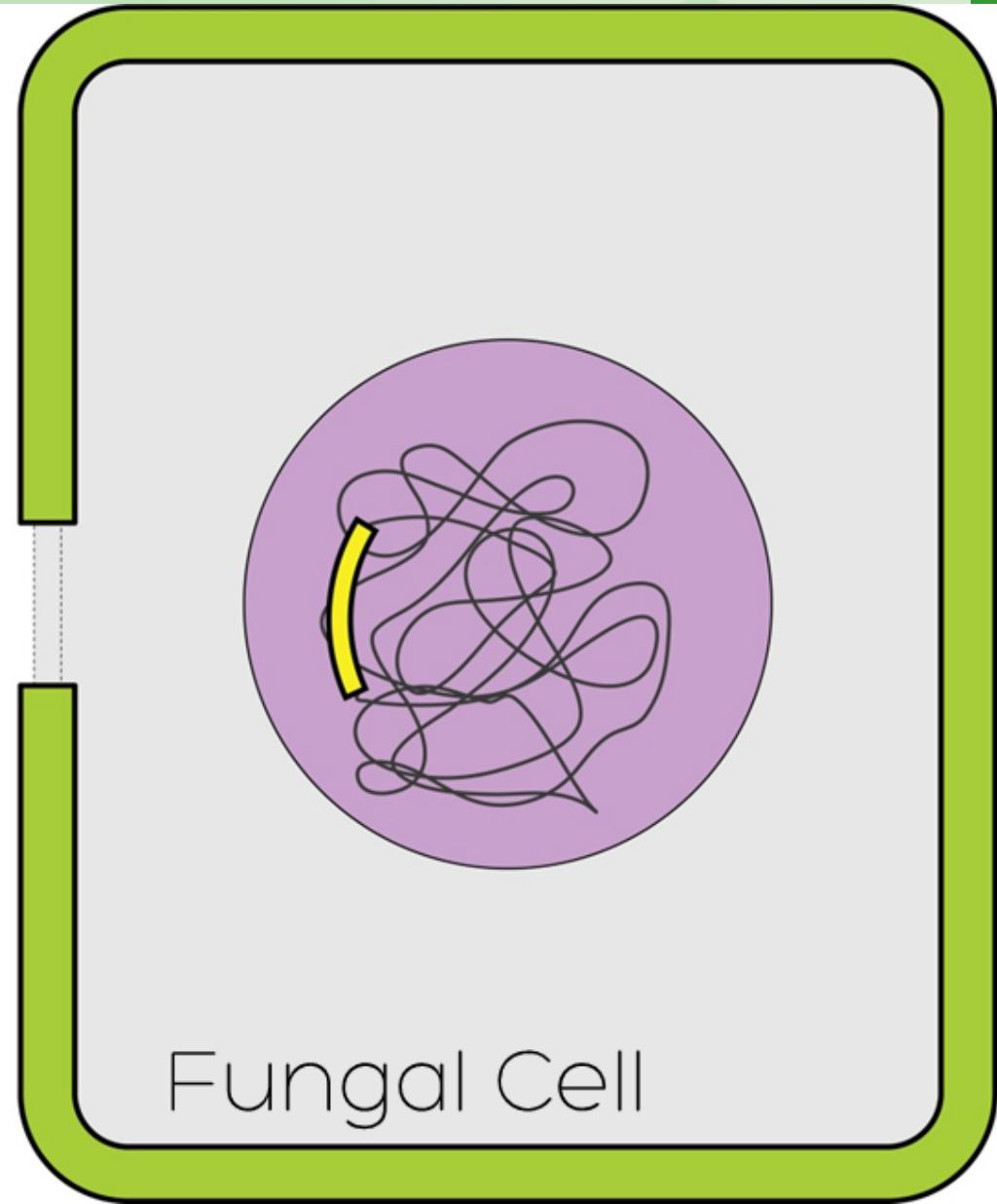
– Acetosyringone



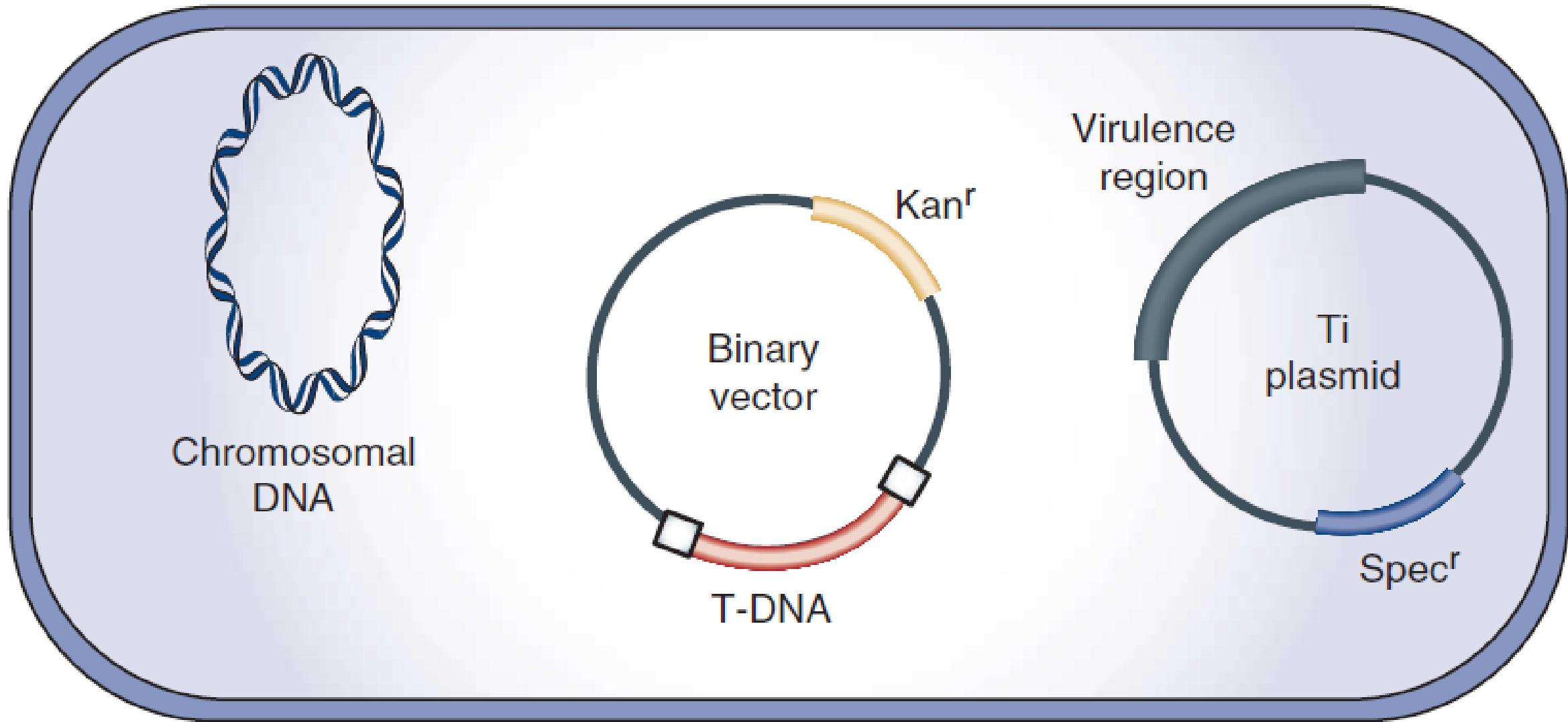
+ Acetosyringone



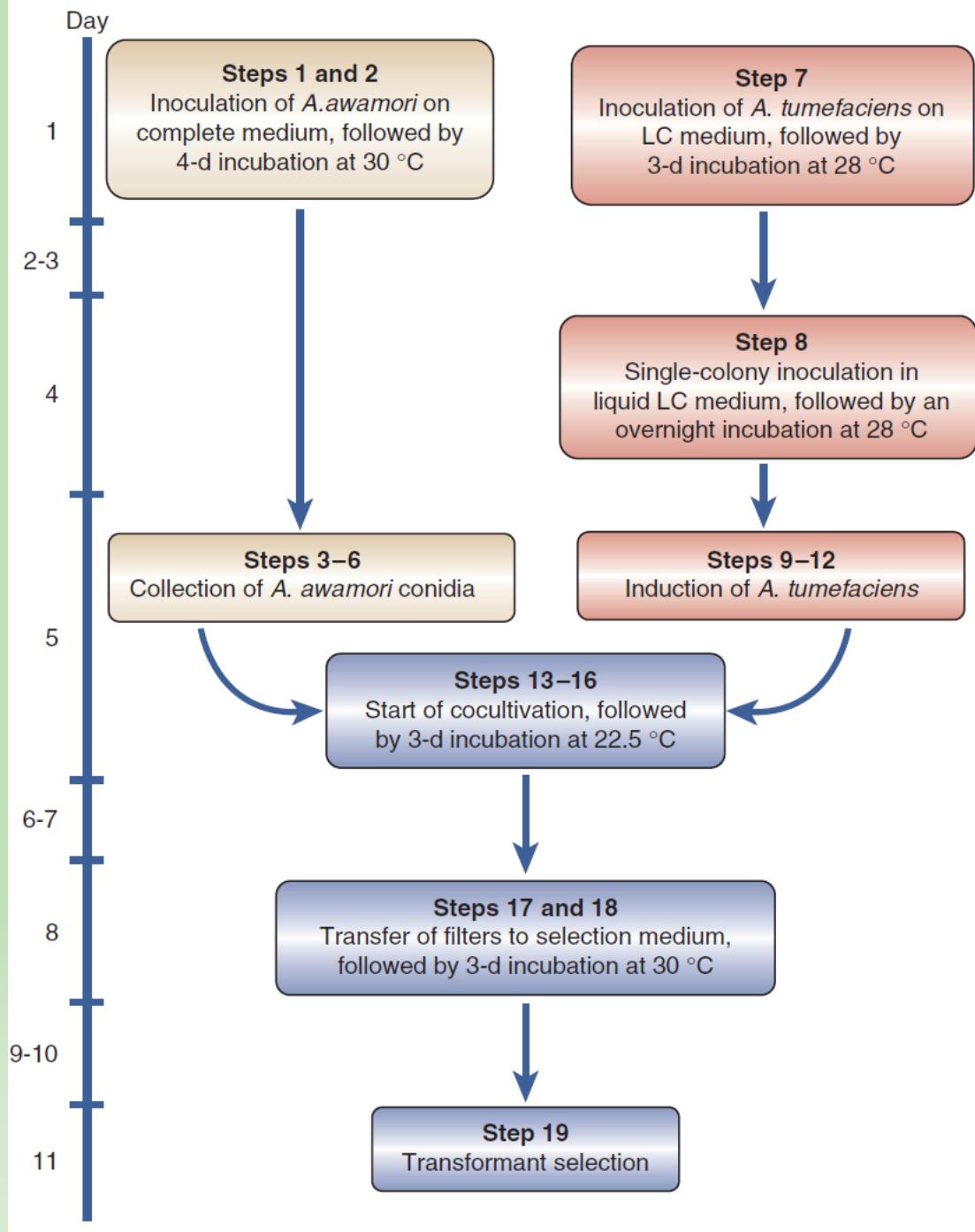
*Agrobacterium tumefaciens*



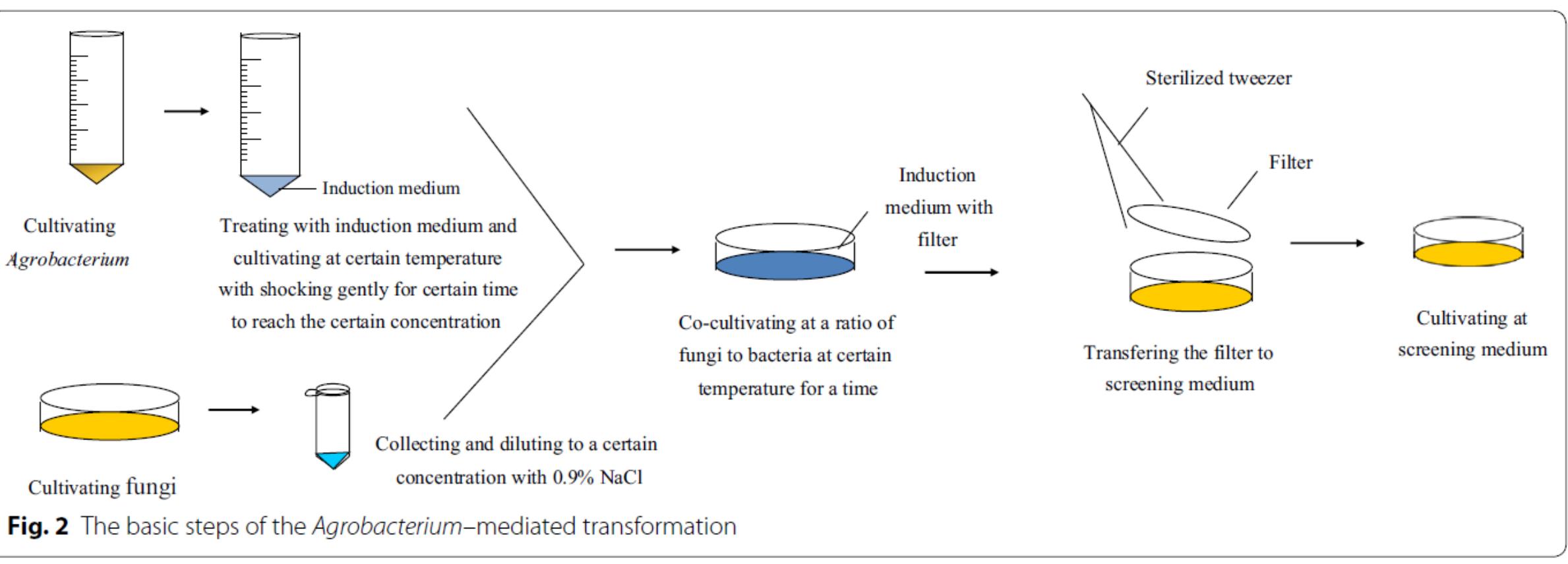
Fungal Cell



*A. tumefaciens*



# ESQUEMA DE AGROTRANSFORMAÇÃO EM FUNGOS



**Fig. 2** The basic steps of the *Agrobacterium*-mediated transformation

**Table 3 Summary of Agrobacterium-mediated transformation protocols for different fungal species**

Fungus	Agrobacterium	Starting material	The number of fungal cells	Pre-culturing of Agrobacterium	The conditions of co-culturing	Name of vector (size)	Selective marker	Transformation efficiency	References
<i>Aspergillus awamori</i>	<i>A. tumefaciens</i> LBA1100	Conidiospores	$1 \times 10^6$	Incubate at 28 °C 4–5 h with 0.2 M AS and 100 rpm to OD600 nm of 0.8	Incubate the plates for 3 days at 22.5 °C	NA	<i>hygB</i>	200–250 transformants per $10^6$ conidiospores	[45]
<i>Aspergillus fumigatus</i> B-5233	<i>A. tumefaciens</i> EHA105	Conidia	$1 \times 10^7$	Further incubation at 28 °C for 24 h to a ratio of 1:10 (conidia to bacteria)	Incubated at 37 °C for 3 days	pDHt/hph (NA)	<i>hygB</i>	100 transformants per $10^7$ conidia	[46]
<i>Trichoderma reesei</i> QM 9414	<i>A. tumefaciens</i> AGL-1	Protoplasts and conidia	$1 \times 10^7$	Further incubation for 6 h at 28 °C to OD600 nm of 0.15	Incubation at 25 °C for 48 h	pBI-hph (15846 bp)	<i>hygB</i>	Start from protoplasts: 2000 to 9000 transformants per $10^7$ protoplasts. Start from conidia: 200–500 transformants per $10^7$ conidia	[47]
<i>Mucor circinelloides</i> ATCC1216b	<i>A. tumefaciens</i> AGL-1	Spores	$1 \times 10^5$ spores per mL and	Incubate for 4 h at 20 °C to OD600 nm of 0.6	Incubation at 15 °C for 6 days	pTiBo542 (NA)	<i>hygB</i>	2000–9000 transformants per $10^7$ sporangiospores	[48]
<i>Aspergillus giganteus</i> IfGB15/0903	<i>A. tumefaciens</i> LBA1100	Germinated conidia	$5 \times 10^7$	Incubate for 6 h at 30 °C with 200 µM AS to OD 600 nm of 2.5	Incubation at 24 °C for 3 days	pUR5750 (NA)	<i>hygB</i>	500 to 7900 transformants per $10^8$ conidia	[23]



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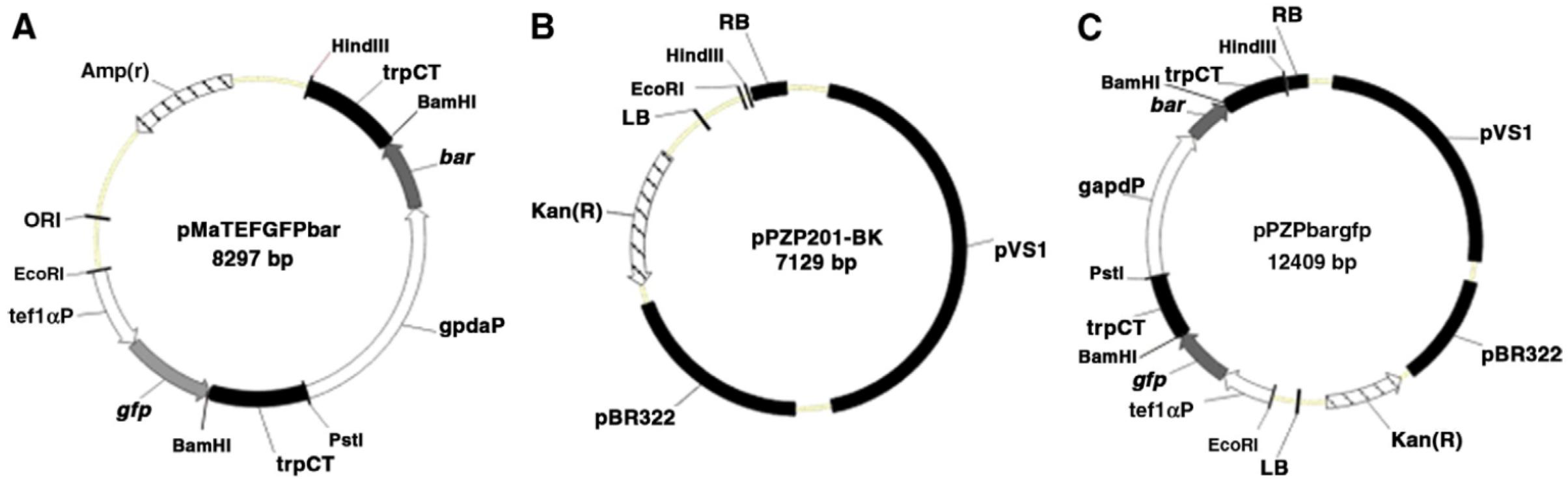


## *Agrobacterium tumefaciens*-mediated transformation of *Guignardia citricarpa*

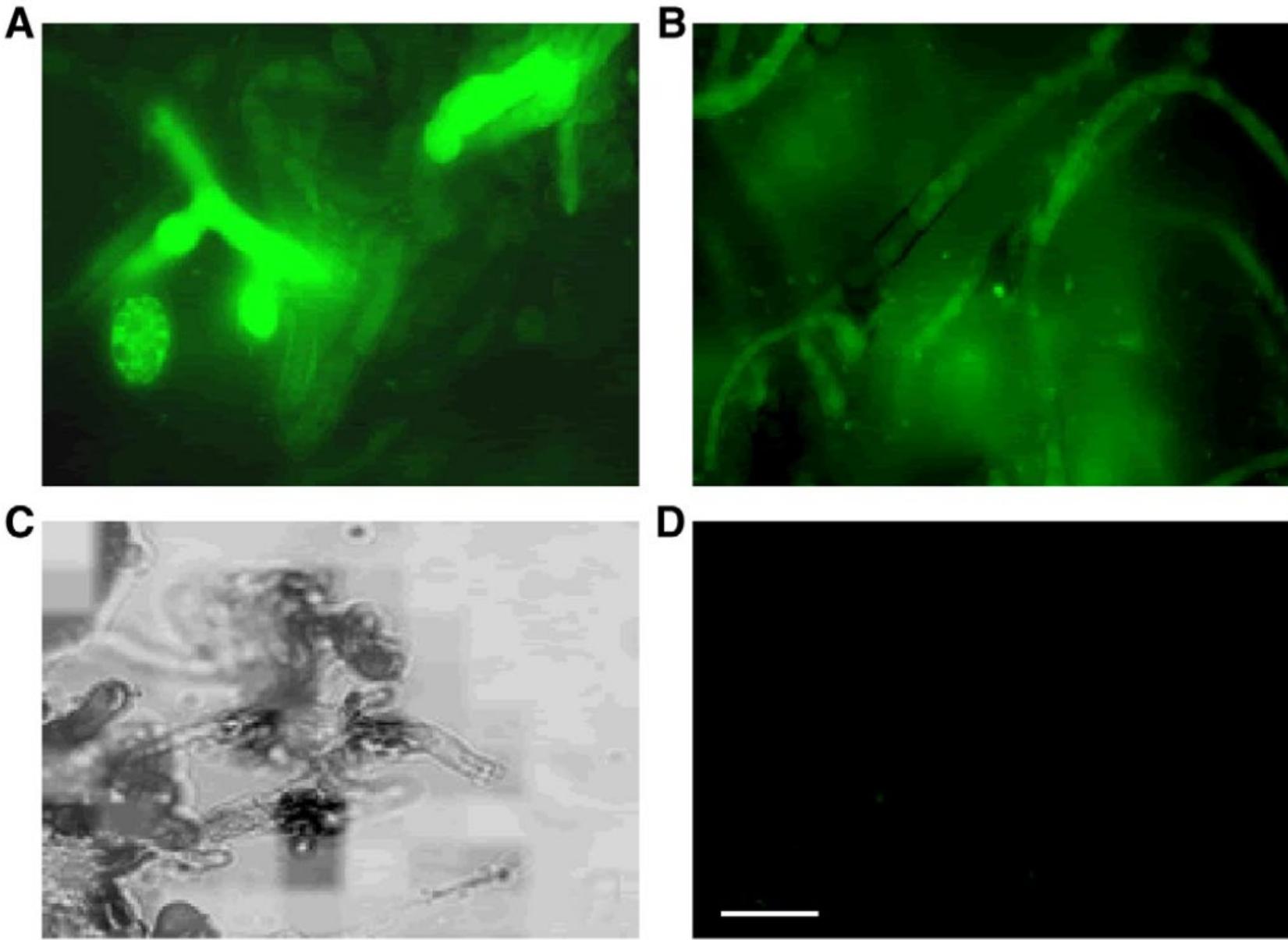
J.G. Figueiredo <sup>a,1</sup>, E.H. Goulin <sup>a,2</sup>, F. Tanaka <sup>a,2</sup>, D. Stringari <sup>a,3</sup>, V. Kava-Cordeiro <sup>a,3</sup>, L.V. Galli-Terasawa <sup>a,4</sup>, C.C. Staats <sup>b,5</sup>, A. Schrank <sup>b,6</sup>, C. Glienke <sup>a,\*</sup>

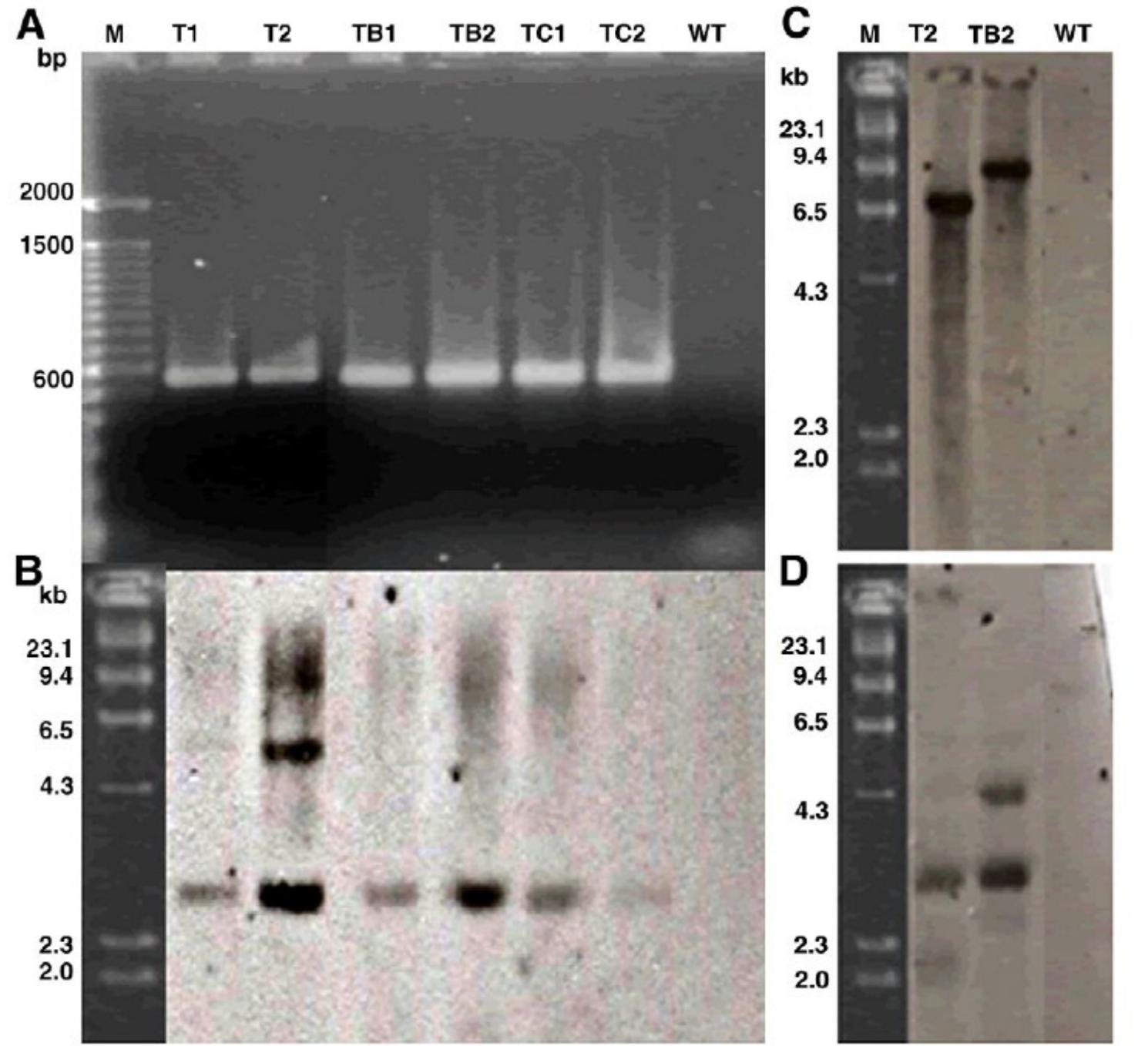
<sup>a</sup> UFPR, Department of Genetics, Curitiba, PR, Brazil

<sup>b</sup> UFRGS, Biotechnology Center, Porto Alegre, RS, Brazil



**Fig. 1.** Construction of pPZPbargfp. The *gfp* and *bar* expression cassette was obtained by subcloning an EcoRI–HindIII restriction fragment from pMaTEFGFPbar (A – Nakazato et al., 2006) into the binary vector pPZP201-BK (B – Covert et al., 2001). The pPZPbargfp (C) plasmid T-DNA contains an ammonium gluconate resistance gene (*bar*) expression cassette (*Aspergillus nidulans* *gpdA* promoter and *trpC* terminator) and a *gfp* expression cassette (*Metarrhizium anisopliae* *tef* promoter and *A. nidulans* *trpC* terminator between its left and right borders).





## Bibliotecas de Mutantes

- Importantes para estudos de genética funcional
- Muitos mutantes produzidos

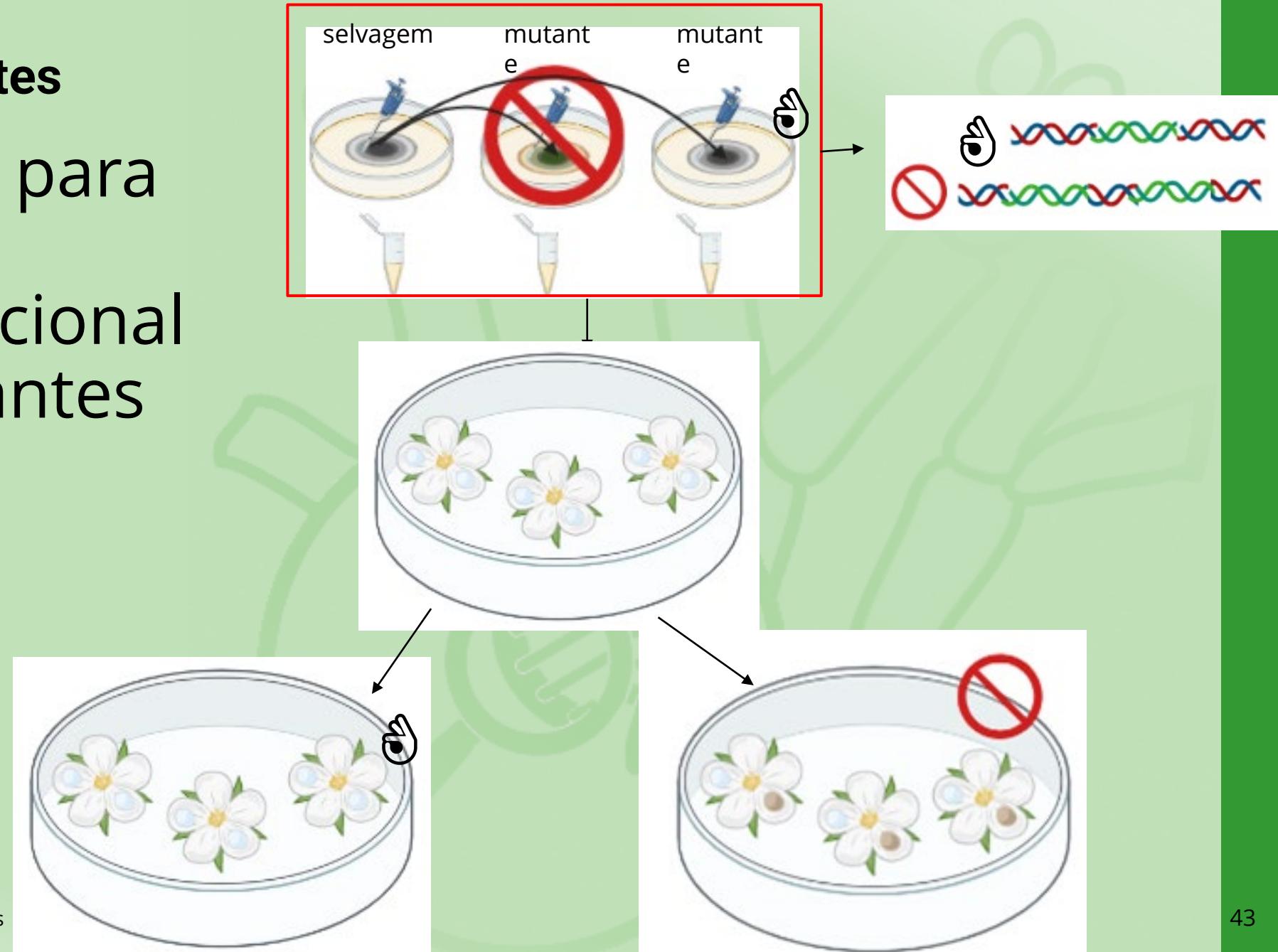
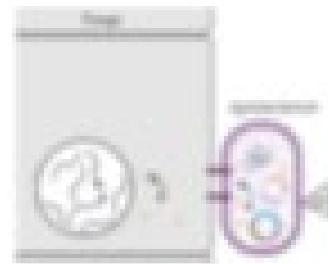


Fig. 3 Esquema representando mutantes pesquisados  
Fonte: o autor (2021)

Agrotransformação



Microscopia



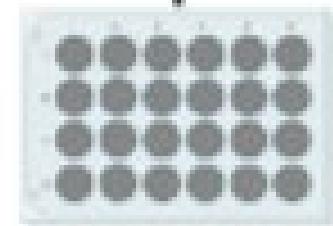
Extração DNA



Southern blot



Biblioteca de mutantes



Electroporação

Teste de  
patogenicidade



PCR



Achar locus de  
inserção



# Eletroporação da *A. tumefaciens*

- *A. tumefaciens* linhagem EHA105



**Fig. 4** Placa com EHA105 após eletroporação com pPZPbargfp  
Fonte: o autor (2018)

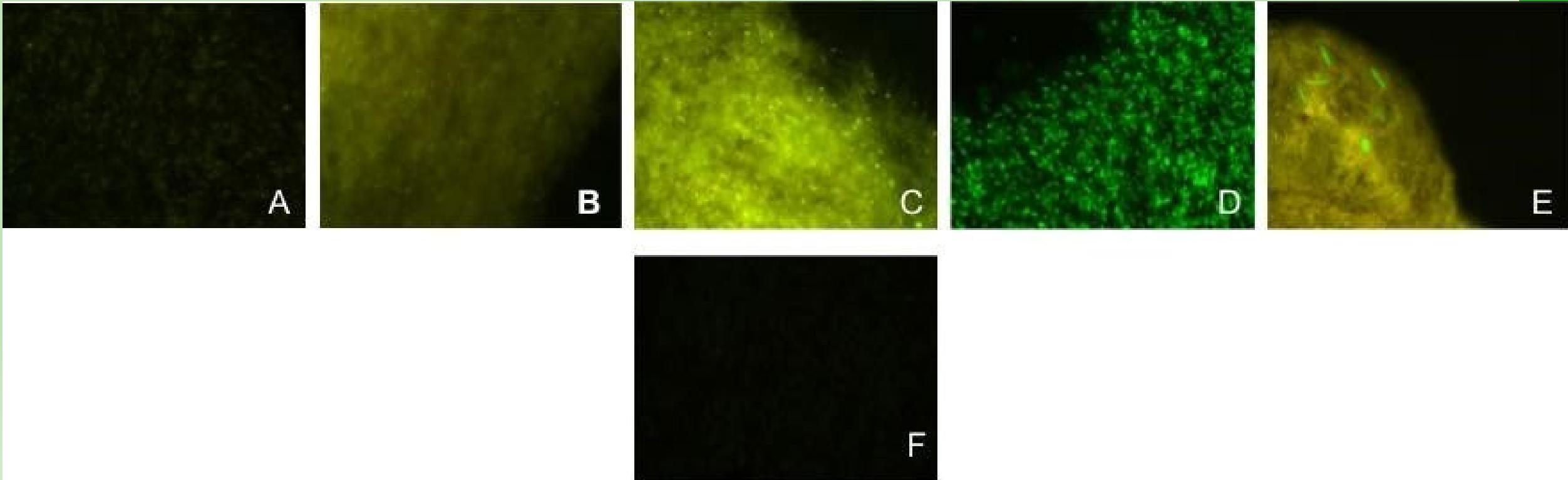
# Agrotransformação

- 230 mutantes com resistência ao glufosinato e semelhantes a linhagem IAC142

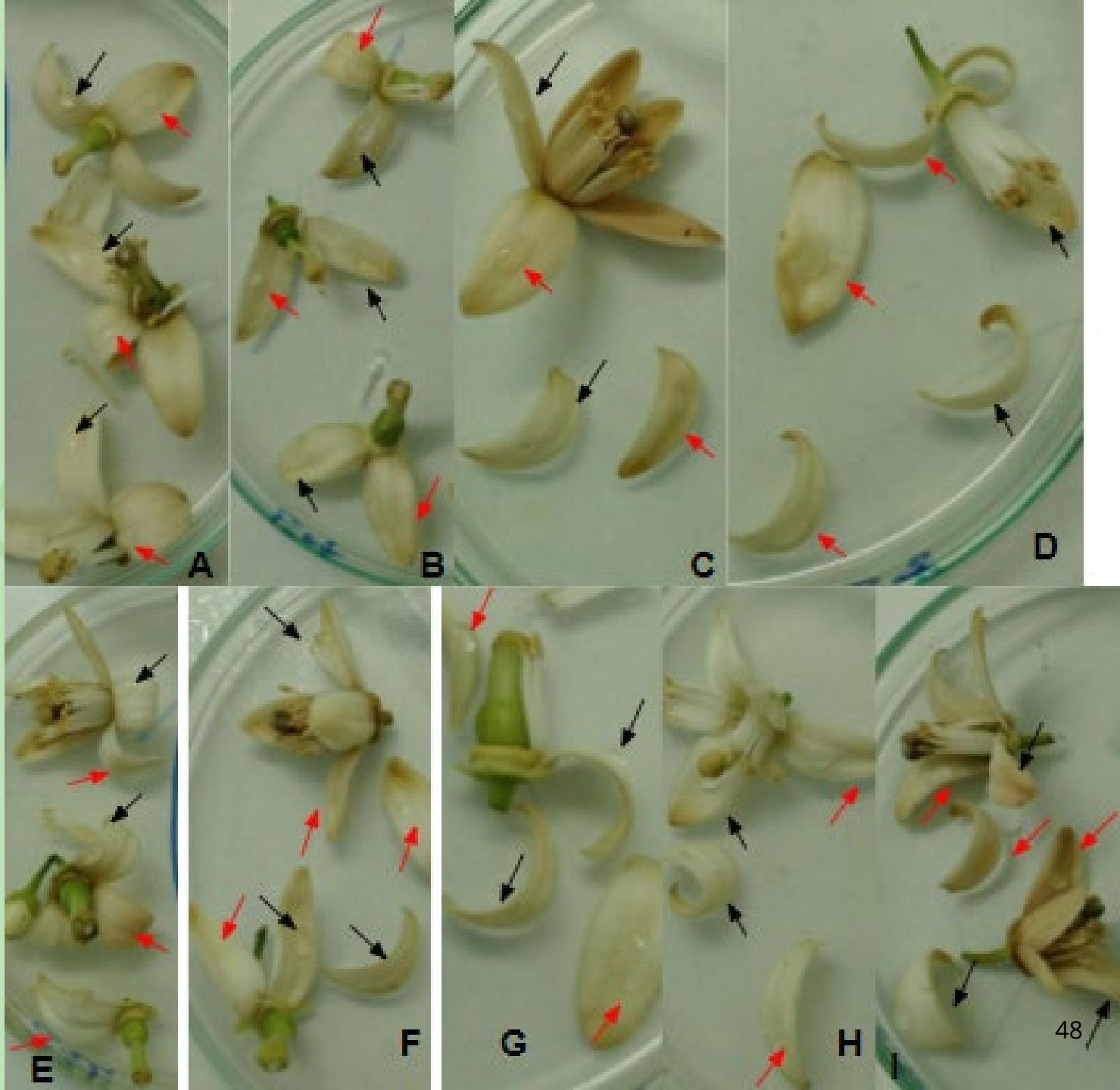


# Análise da Expressão do Gene Repórter *gfp*

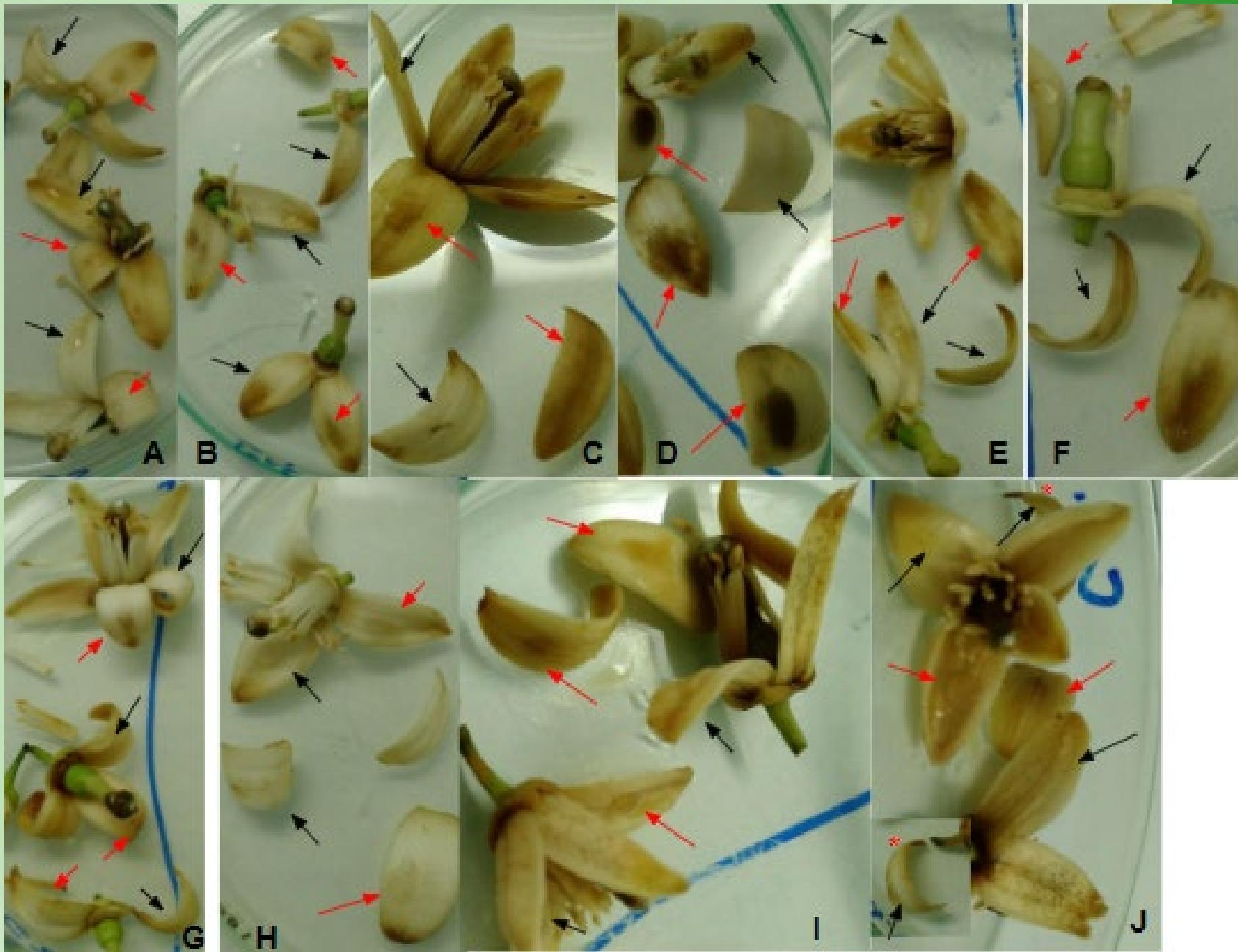
- 220 transformantes apresentaram expressão do gene *gfp*



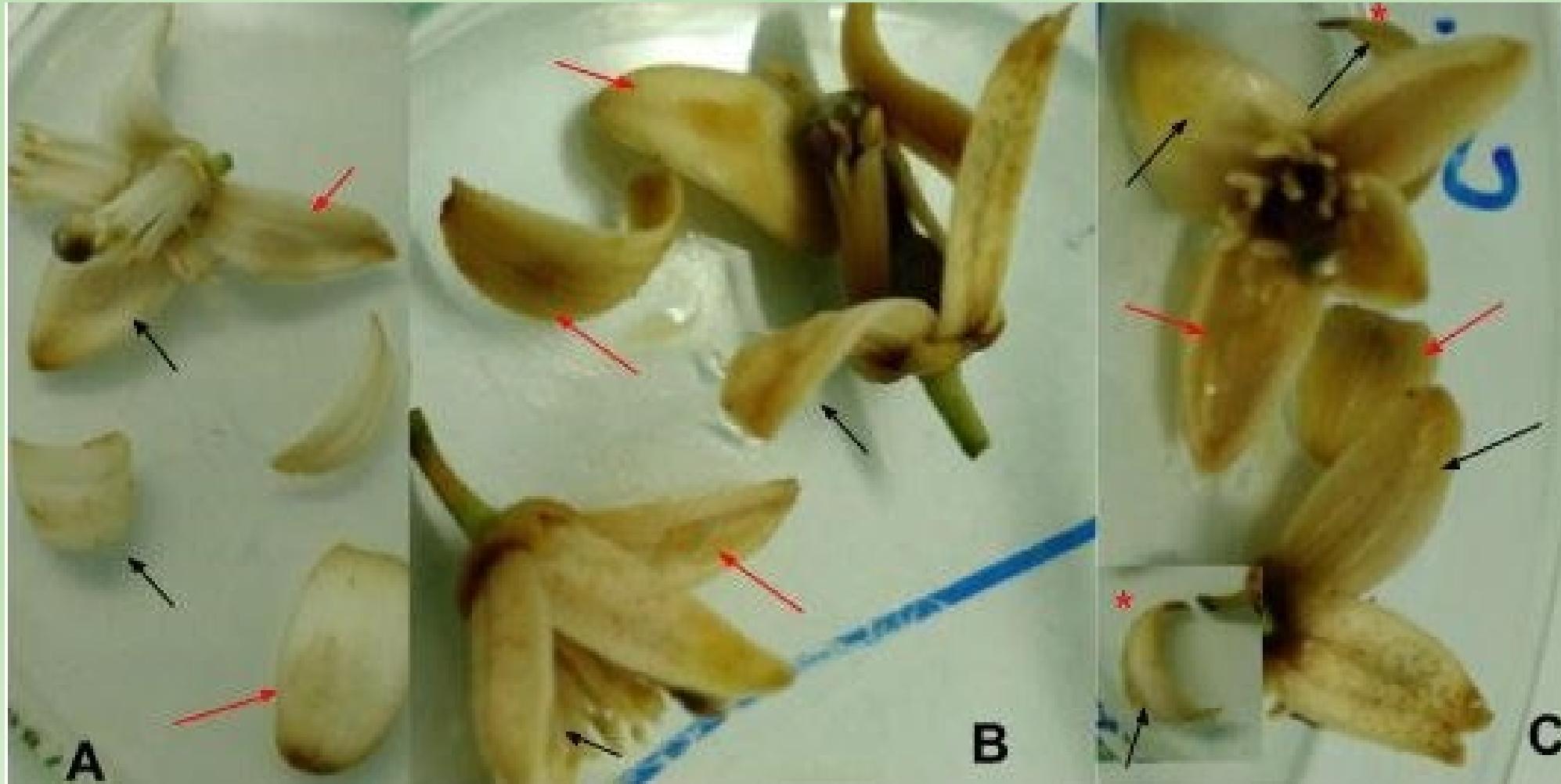
# Teste de patogenicidade em flor de citros



# Teste de patogenicidade em flor de citros



# Teste de patogenicidade em flor de citros



# Genome walking

