

Metodologias para o estudo funcional de genes

Profa. Dra. Chirlei Glienke

Era Genômica

Se temos genomas disponíveis, qual a necessidade de ainda trabalharmos com metodologias de manipulação genética, como genética funcional?

Metodologias que podem ajudar a responder perguntas

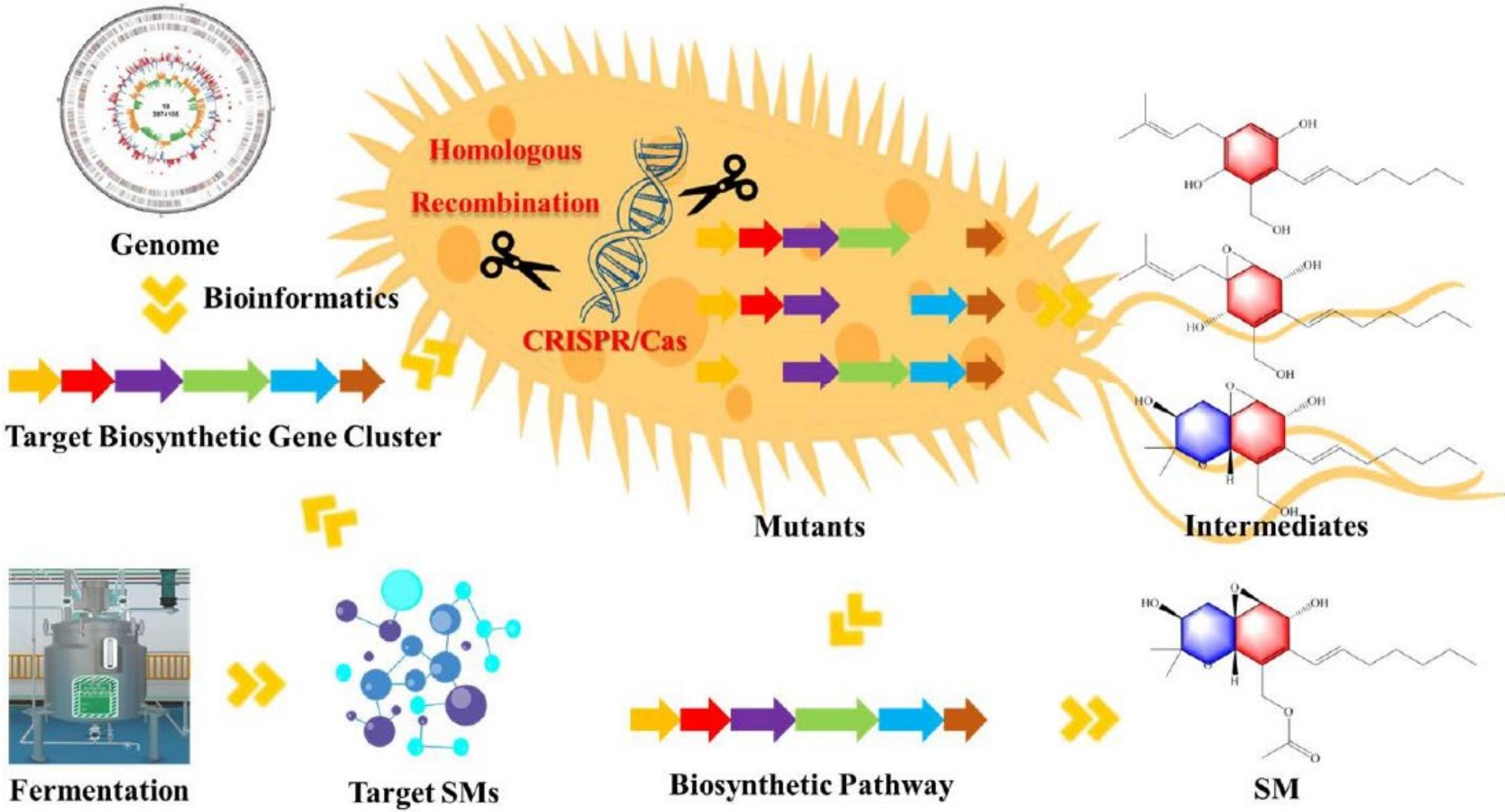
- Métodos disponíveis para a manipulação genética:
 - integração aleatória (AMT)
 - restriction enzyme mediated integration (REMI)
 - transposon-arrayed gene knockout (TAGKO)
 - mutação/modificação dirigida de genes (Gene targeting) - baseada em recombinação homóloga
 - edição de genes com nucleases - TALEN
 - tecnologias de RNA
 - edição genética - CRISPR

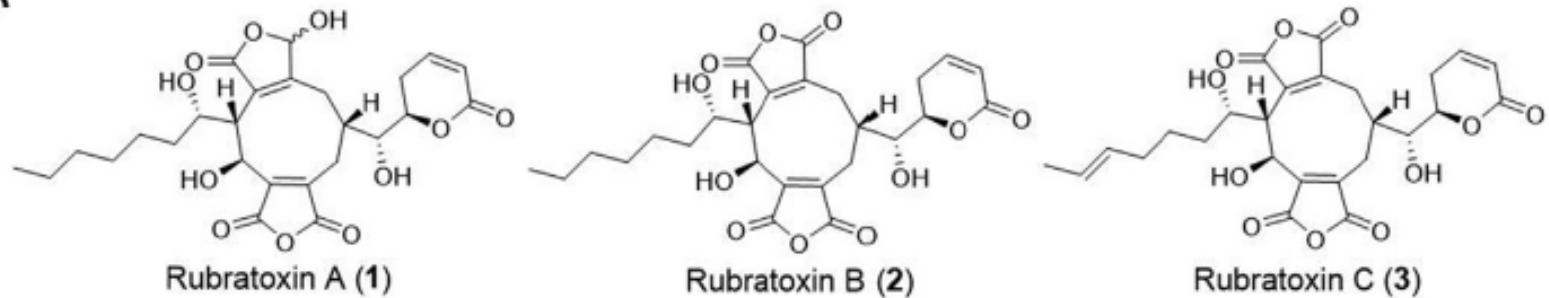
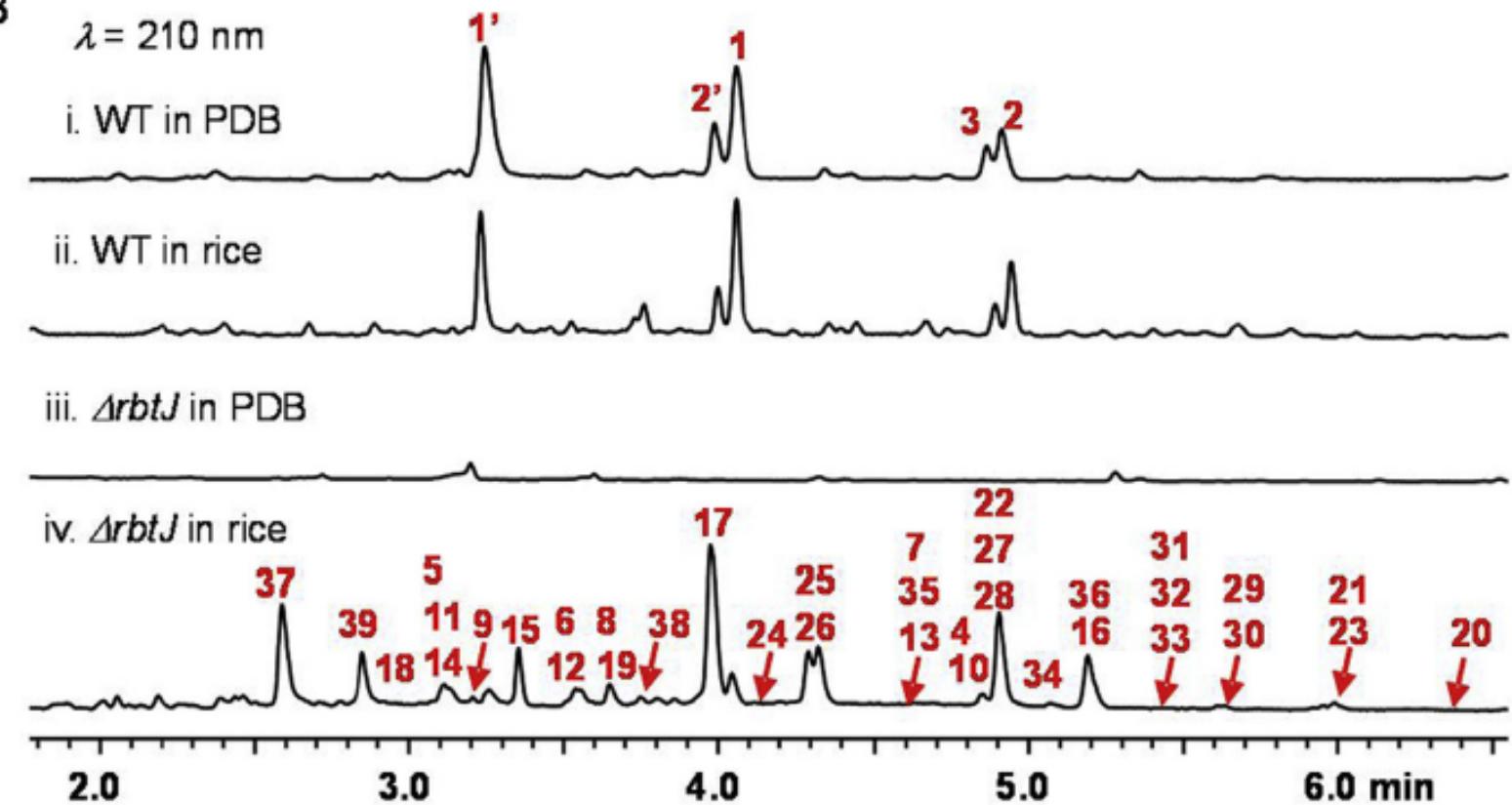
Metodologias que podem ajudar a responder perguntas

- Deleção de genes (knockout)
 - Entender um patossistema: genes associados à patogenicidade
 - Encontrar alvos para controle (químico ou via biotecnologia)
 - Validação de genes – análise comparativa de genomas
 - Validação de gene clusters de metabólitos secundários

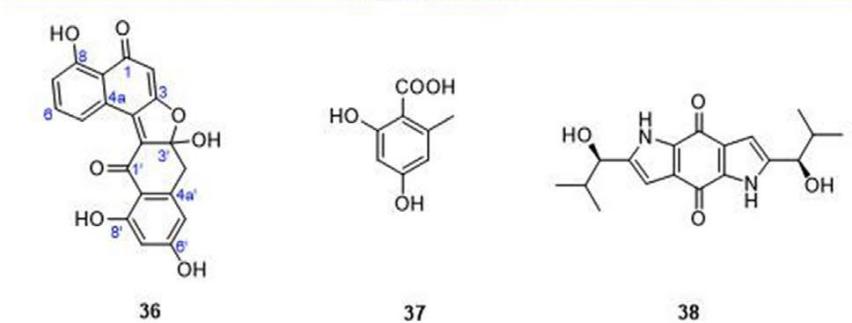
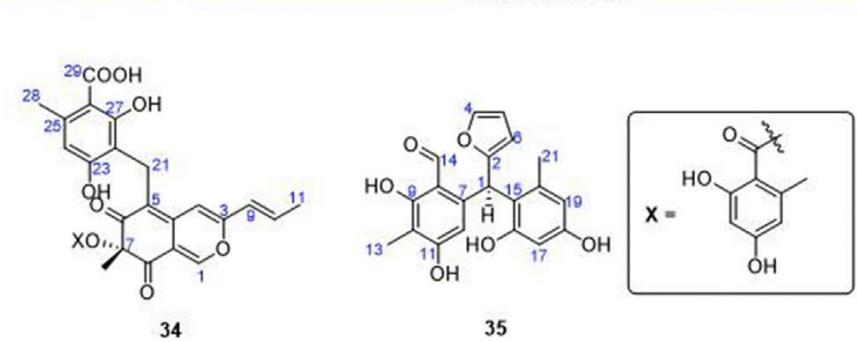
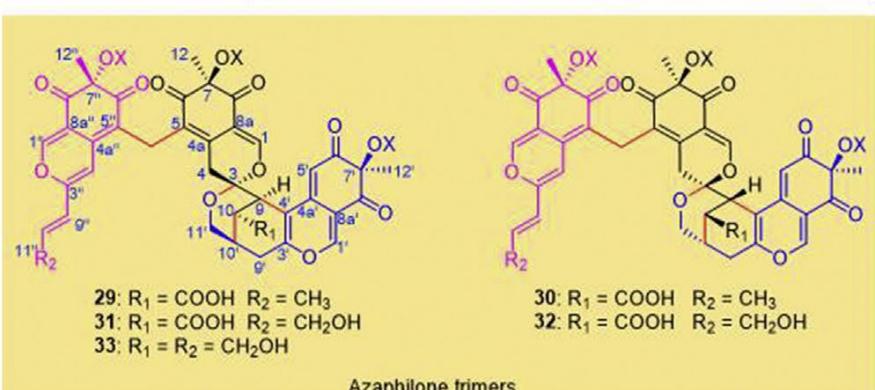
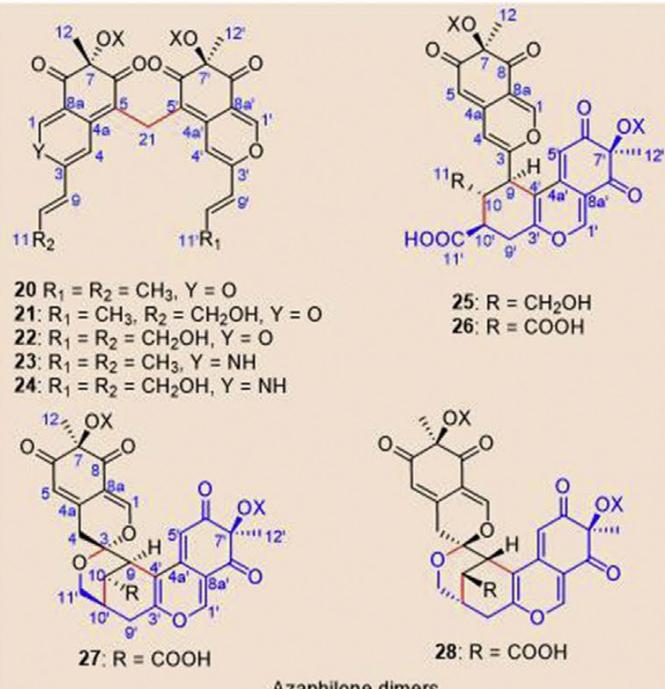
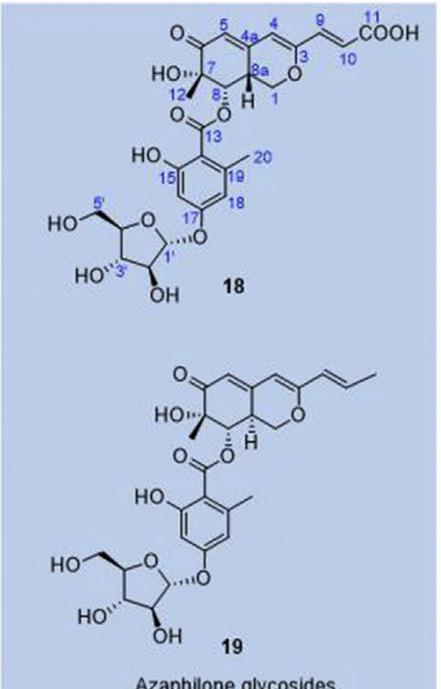
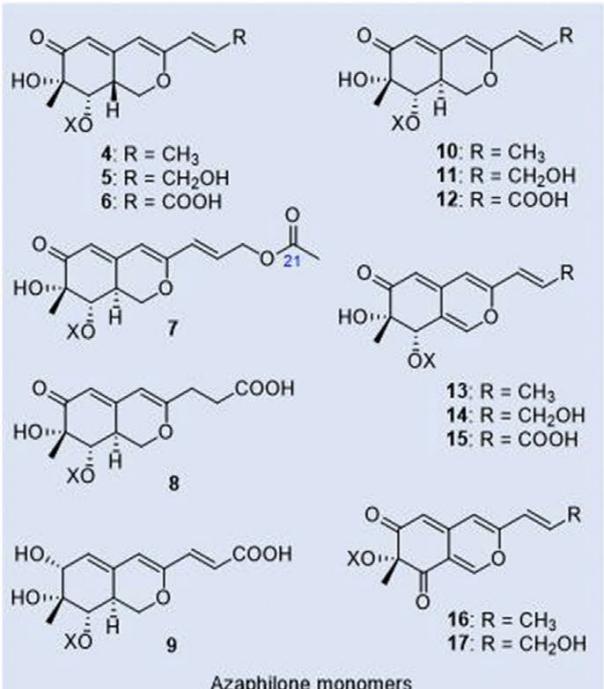
Metodologias que podem ajudar a responder perguntas

- Deleção de genes (knockout)
 - Validação de gene clusters de metabólitos secundários – mineração de genes
 - knockout gênico no estudo de metabólitos secundários: determinação de BGCs alvo, construção de mutantes e identificação de produtos intermediários, que verifica a via biossintética
 - SMs (policetídeos, terpenóides, saponinas e peptídeos não ribossômicos)
 - possuem atividade biológica de interesse (antibacterianas, antifúngicas, antitumorais e imunorreguladoras)



A**B**

- Mutantes deletados produzidos por recombinação homóloga
 - Substituição do gene de PKS pelo gene de resistência à higromicina
 - Clonagem com levedura como intermediário



Compounds purified from *P. dangereardii* DrbtJ mutant strain

Metodologias que podem ajudar a responder perguntas

- Deleção de genes (knockout)

Phytopathology • 2020 • 110:1530-1540 • <https://doi.org/10.1094/PHYTO-04-20-0114-R>

Mycology

e-Xtra*

Molecular Characterization of the Purine Degradation Pathway Genes *ALA1* and *URE1* of the Maize Anthracnose Fungus *Colletotrichum graminicola* Identified Urease as a Novel Target for Plant Disease Control

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Accepted for publication 27 April 2020.

Metodologias que podem ajudar a responder perguntas

- Silenciamento de genes (knockdown) - RNAi
 - Estudo de genes essenciais
 - Estudo de genes próximo a sequencias repetitivas
 - Estudo de genes em fungos de difícil transformação

The Plant Cell, Vol. 25: 2356–2378, June 2013, www.plantcell.org © 2013 American Society of Plant Biologists. All rights reserved.

- Exemplo:

Infection Structure-Specific Expression of β -1,3-Glucan Synthase Is Essential for Pathogenicity of *Colletotrichum graminicola* and Evasion of β -Glucan-Triggered Immunity in Maize^W

Ely Oliveira-Garcia^a and Holger B. Deising^{a,b,1}

^a Faculty of Natural Sciences III, Institute for Agricultural and Nutritional Sciences, Phytopathology and Plant Protection, Martin-Luther-University Halle-Wittenberg, D-06120 Halle (Saale), Germany

^b Interdisciplinary Center for Crop Plant Research, Martin-Luther-University Halle-Wittenberg, D-06120 Halle (Saale), Germany

ORCID IDs: 0000-0001-5789-4269 (H.B.D); 0000-0003-0322-8716 (E.O-G).

Metodologias que podem ajudar a responder perguntas

- Superexpressão de genes
 - Expressão constitutiva de genes para estudo de função
 - Mudança de promotor
 - Inserção de cópia extra do gene com um promotor constitutivo

The Plant Cell, Vol. 25: 2356–2378, June 2013, www.plantcell.org © 2013 American Society of Plant Biologists. All rights reserved.

- Exemplo:

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Metodologias que podem ajudar a responder perguntas

- Fusão de proteínas de interesse (ou promotores) com proteínas fluorescentes (gfp, DsRed e etc)
 - Saber quando um gene é expressão (fase, indução)
 - Saber a localização da proteína



- Exemplo:

The Plant Journal (2016) 87, 355–375

doi: 10.1111/tpj.13205

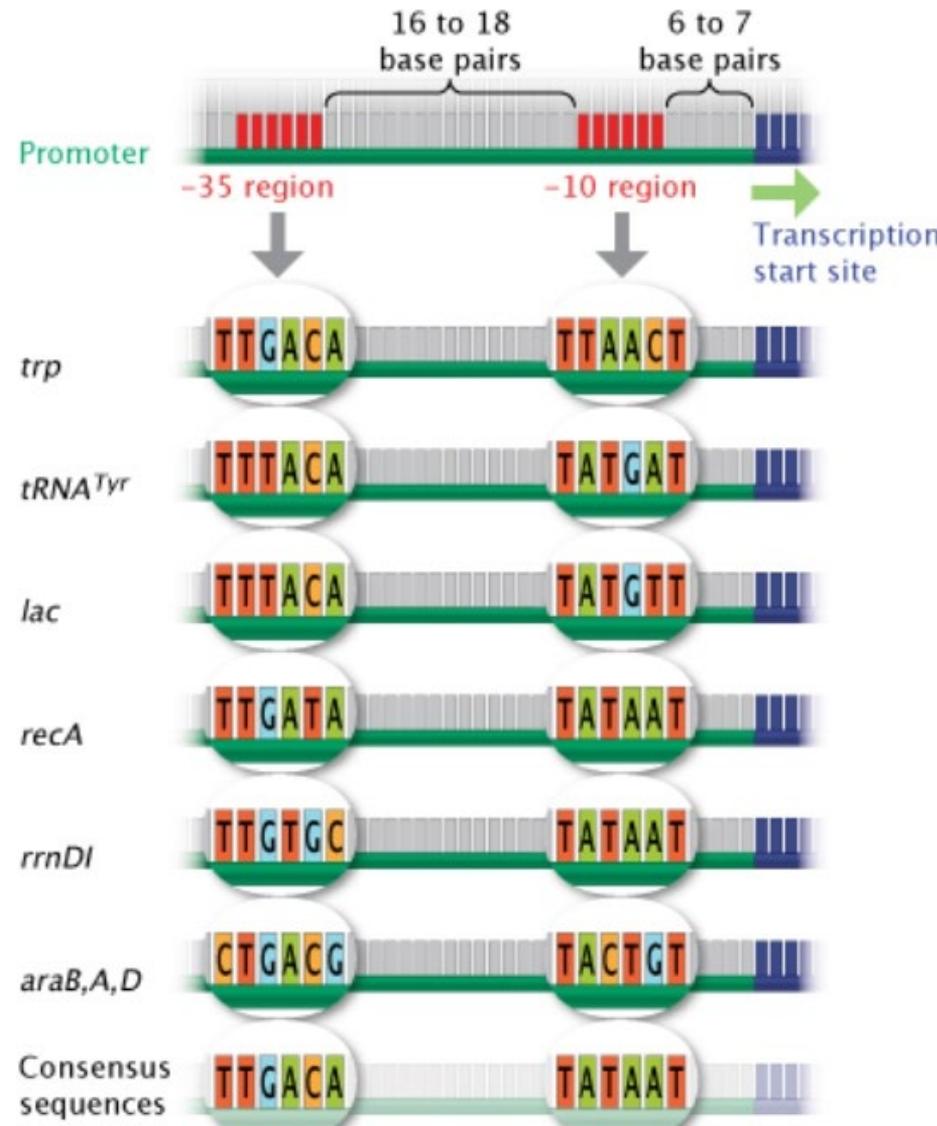
Attenuation of PAMP-triggered immunity in maize requires down-regulation of the key β -1,6-glucan synthesis genes *KRE5* and *KRE6* in biotrophic hyphae of *Colletotrichum graminicola*

Ely Oliveira-Garcia^{1,†} and Holger B. Deising^{1,2,*}

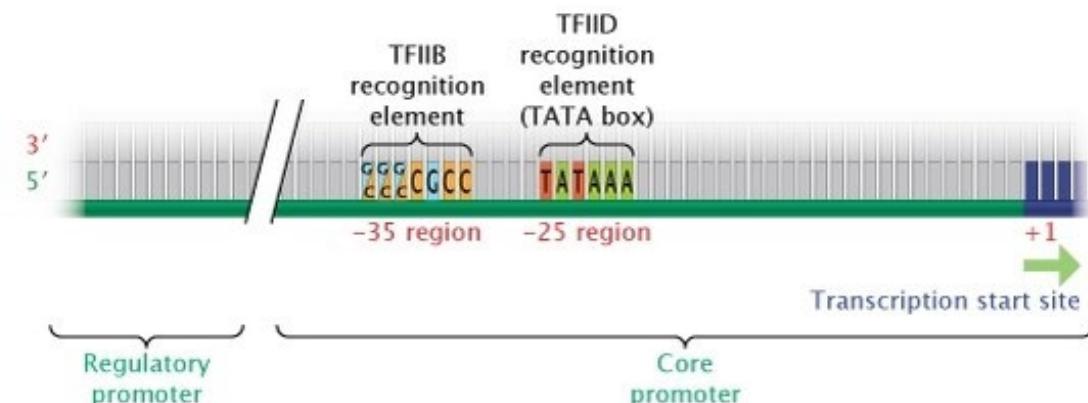
¹Martin-Luther-Universität Halle-Wittenberg, Naturwissenschaftliche Fakultät III, Institut für Agrar- und Ernährungswissenschaften, Phytopathologie und Pflanzenschutz, Betty-Heimann-Str. 3., D-06120 Halle/Saale, Germany, and

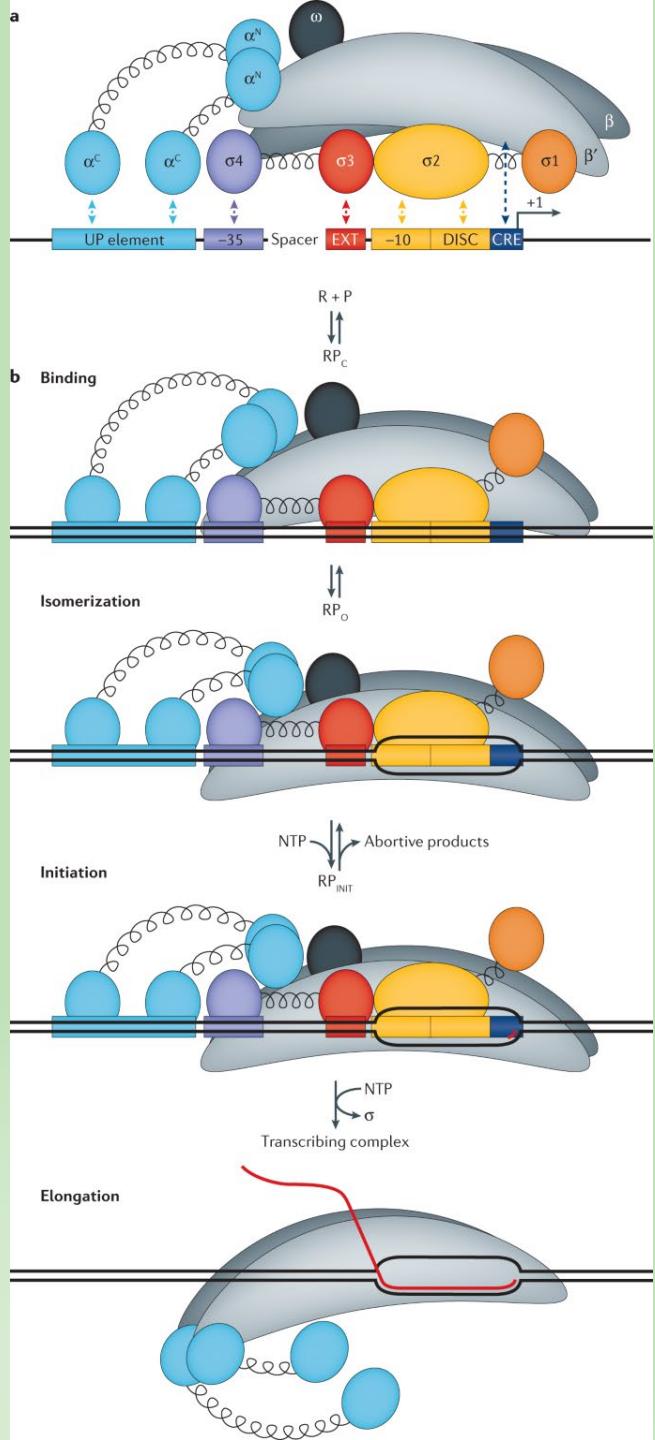
²Martin-Luther-Universität Halle-Wittenberg, Interdisziplinäres Zentrum für Nutzpflanzenforschung, Betty-Heimann-Str. 3., D-06120, Halle/Saale, Germany

Promotor procariótico



Promotor eucariótico

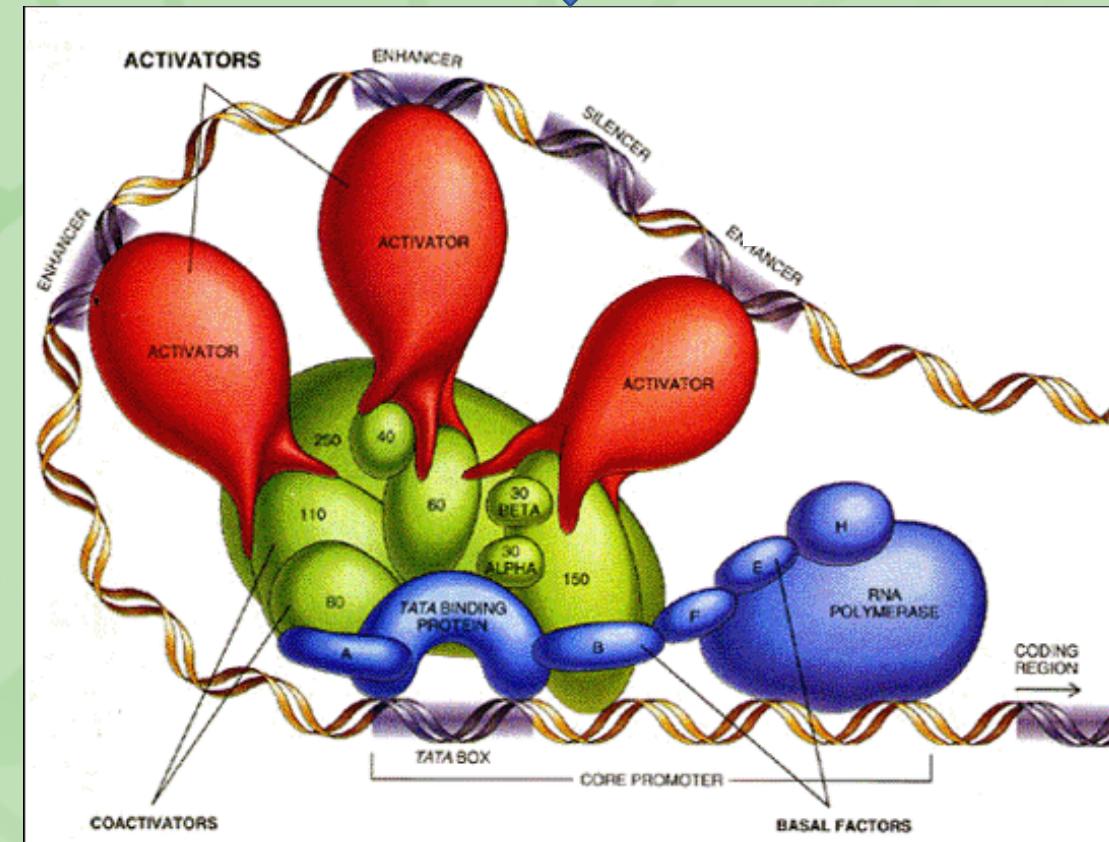




- Complexos de transcrição em procariotos e

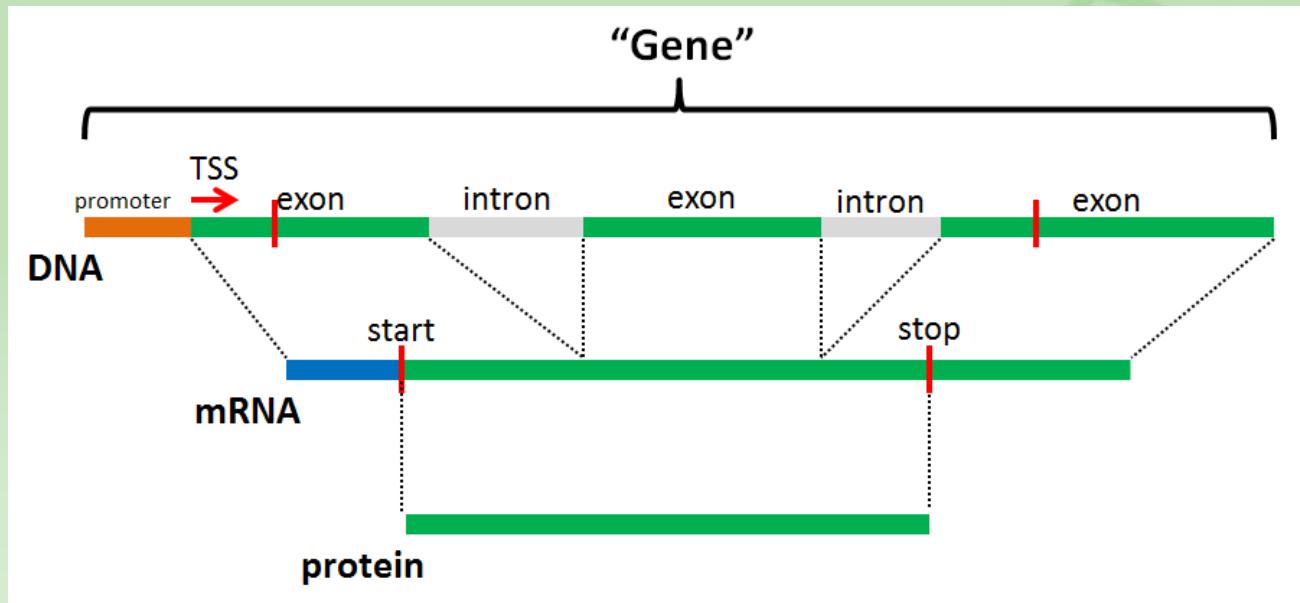


- em eucariotos

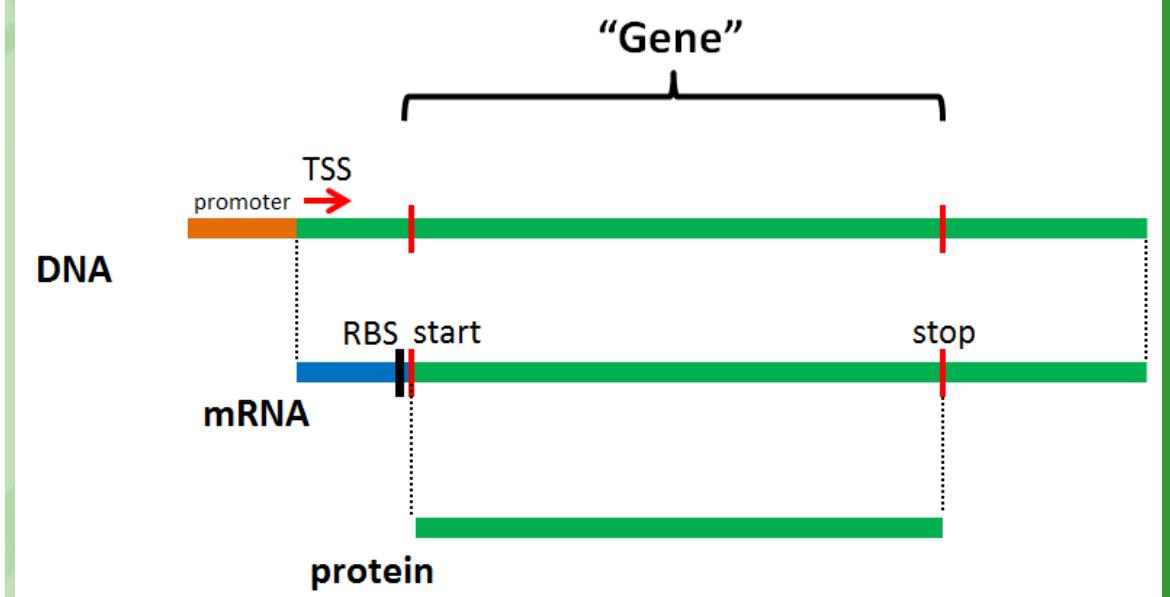


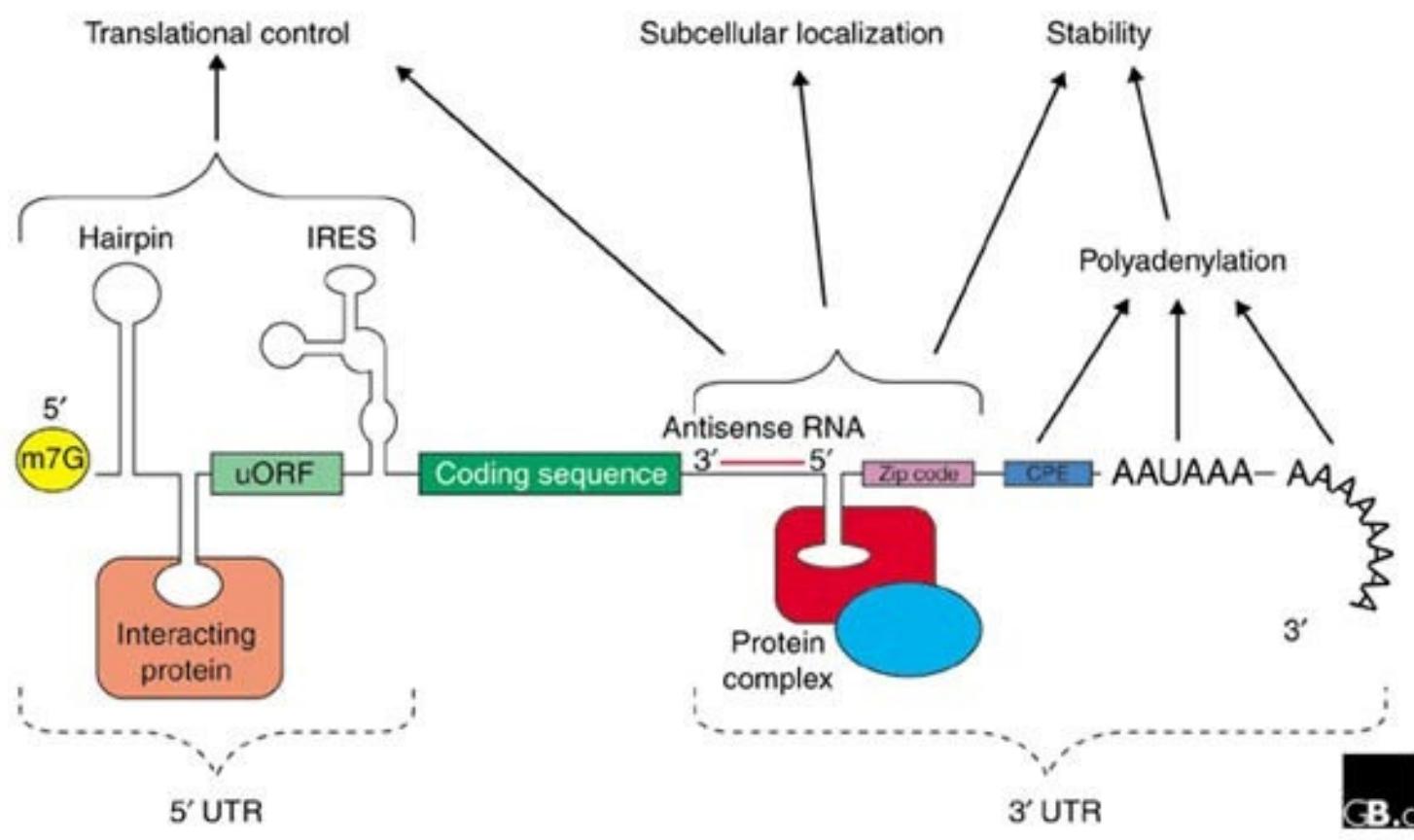
Estrutura de genes

Eucariotos



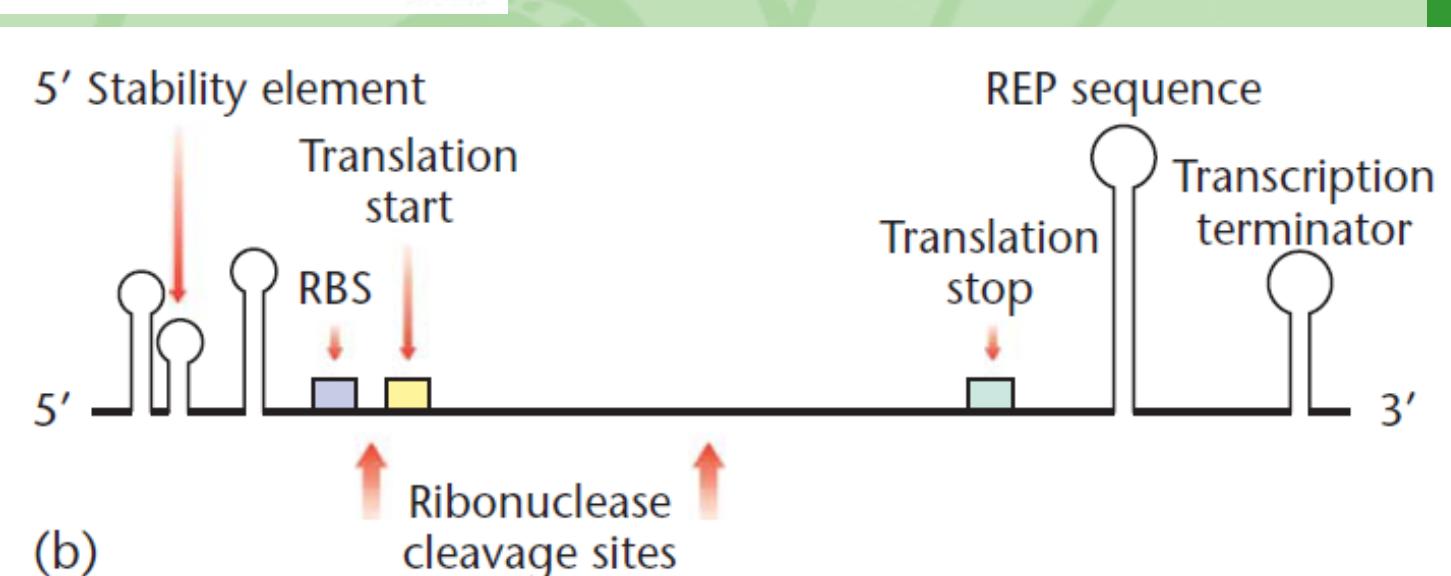
Procaríotos



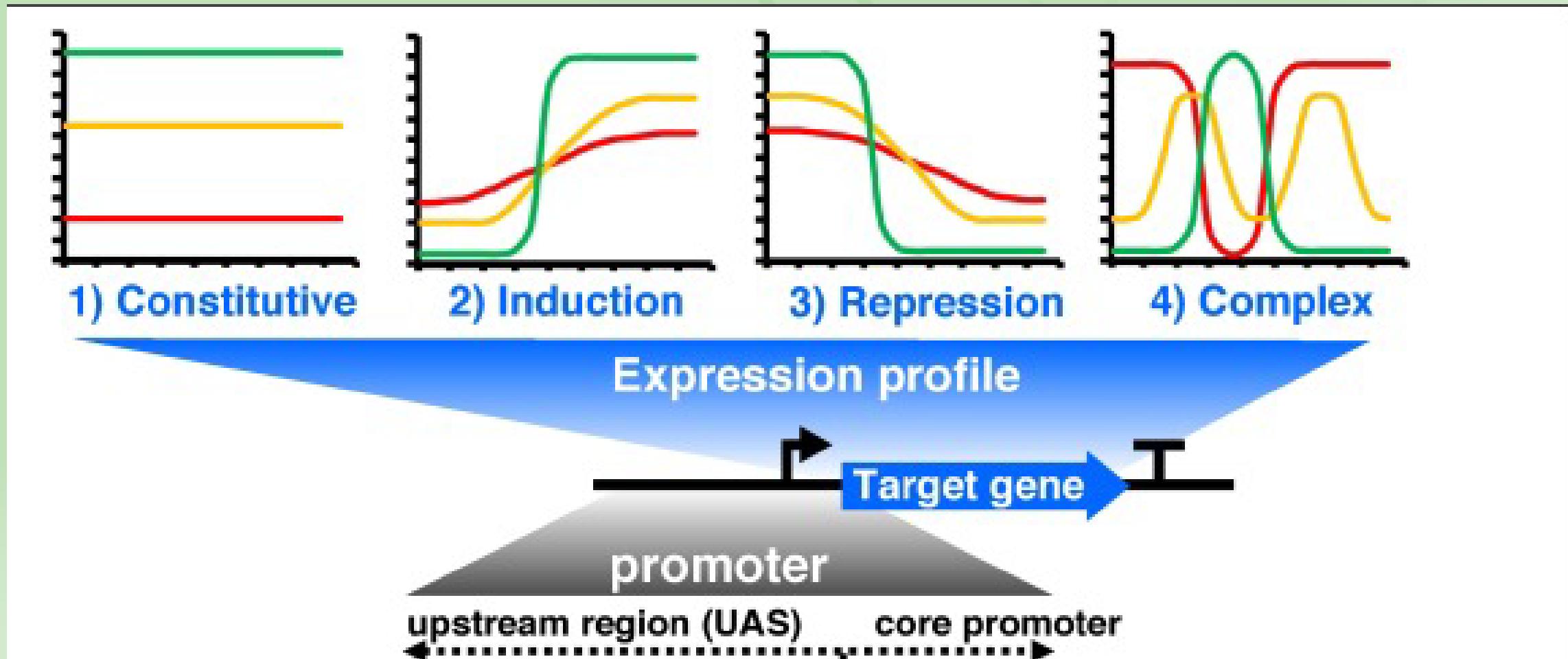


UTR, untranslated region; m7G, 7-methyl-guanosine cap; hairpin, hairpin-like secondary structures; uORF, upstream open reading frame; IRES, internal ribosome entry site; CPE, cytoplasmic polyadenylation element; AAUAAA, polyadenylation signal.

- REP elements (repetitive extragenic palindromes) Ribosome-Binding Site (RBS)

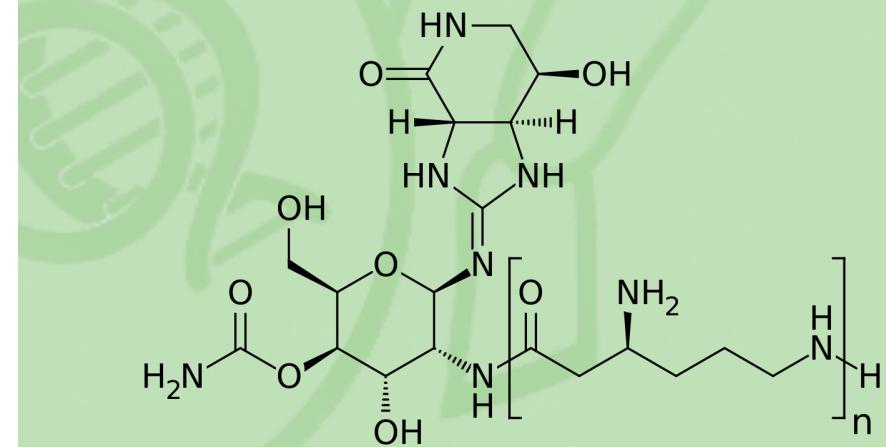
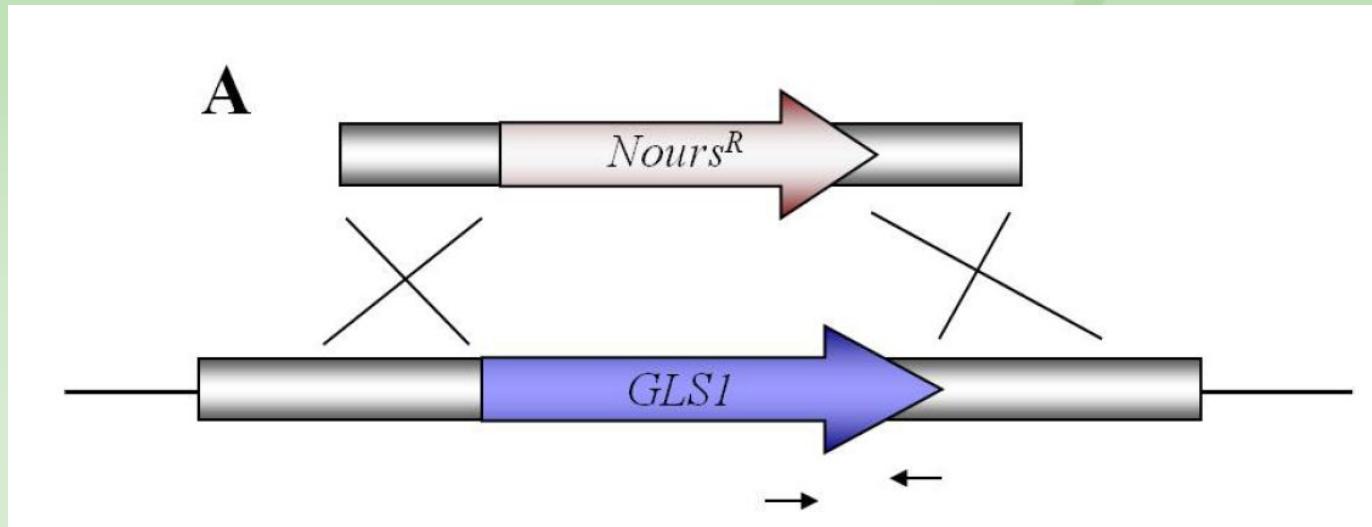


Tipos de expressão gênica



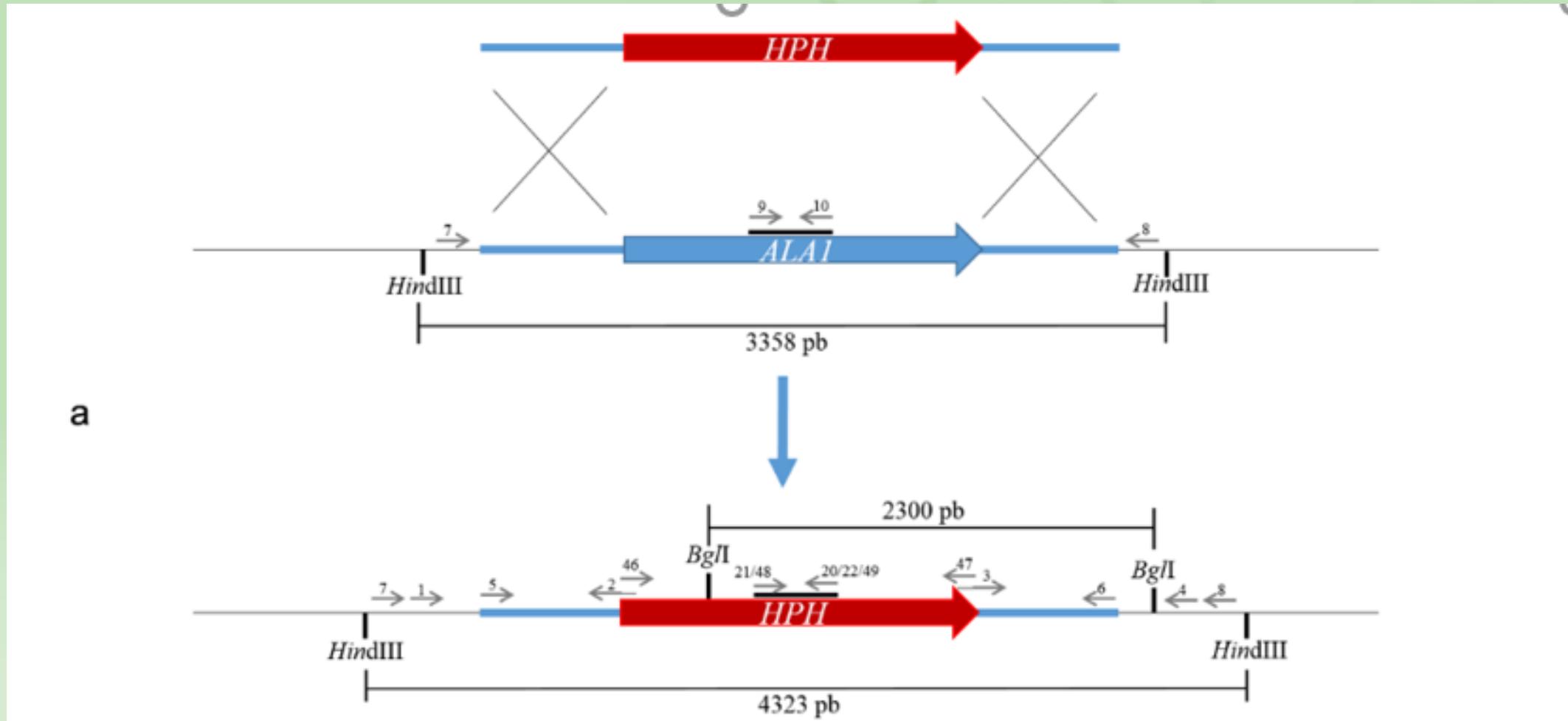
Deleção de genes

- Substituir um gene de interesse (alvo) por um gene de resistência à drogas (para seleção)
- Exemplo:
 - Deleção do gene do fungo *GLS1* (B,1-3 glucan sintetase)
 - Substituição pelo gene *Nours^R* (resistência à Nourseothricin - nourseothricin N-acetyl transferase)

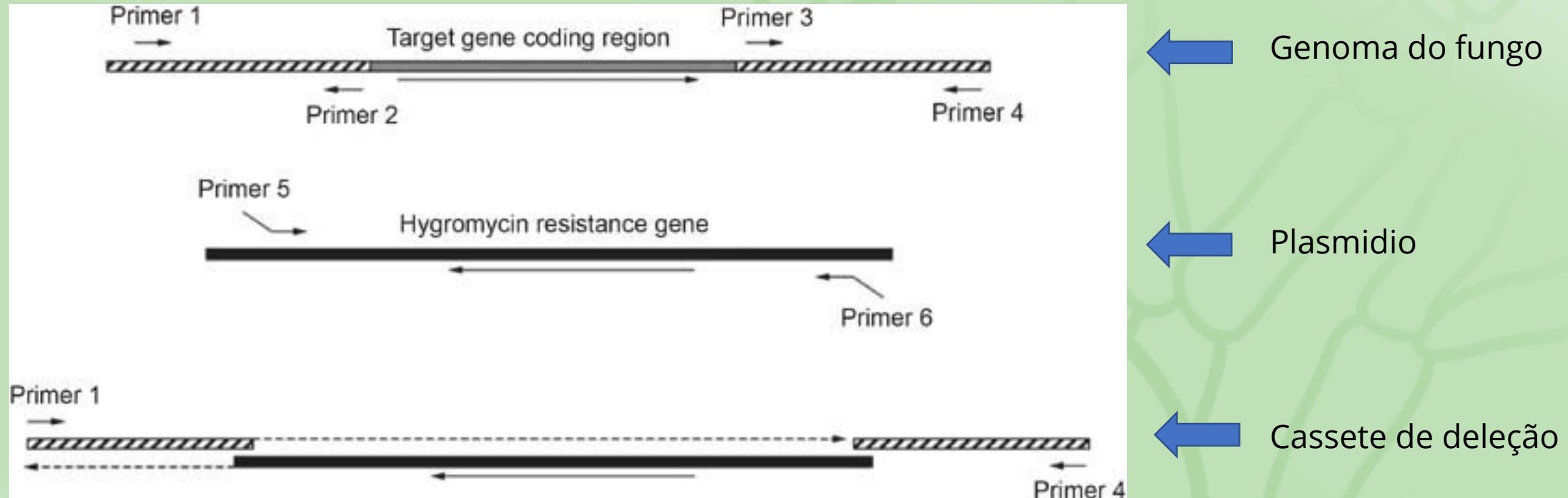


Dependente de recombinação homóloga

- Em torno de 500 pares bases em fungos filamentosos
- Quais regiões estão incluídas no cassete?

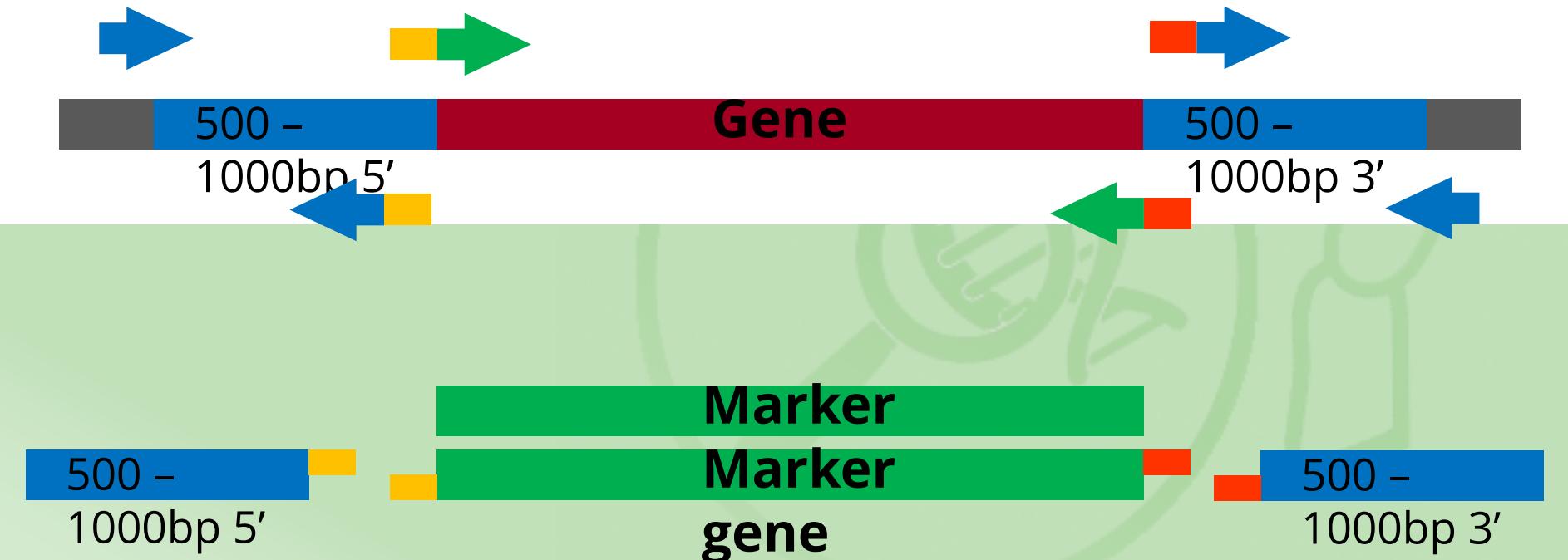


Dependente de recombinação homóloga



Gene Deletion

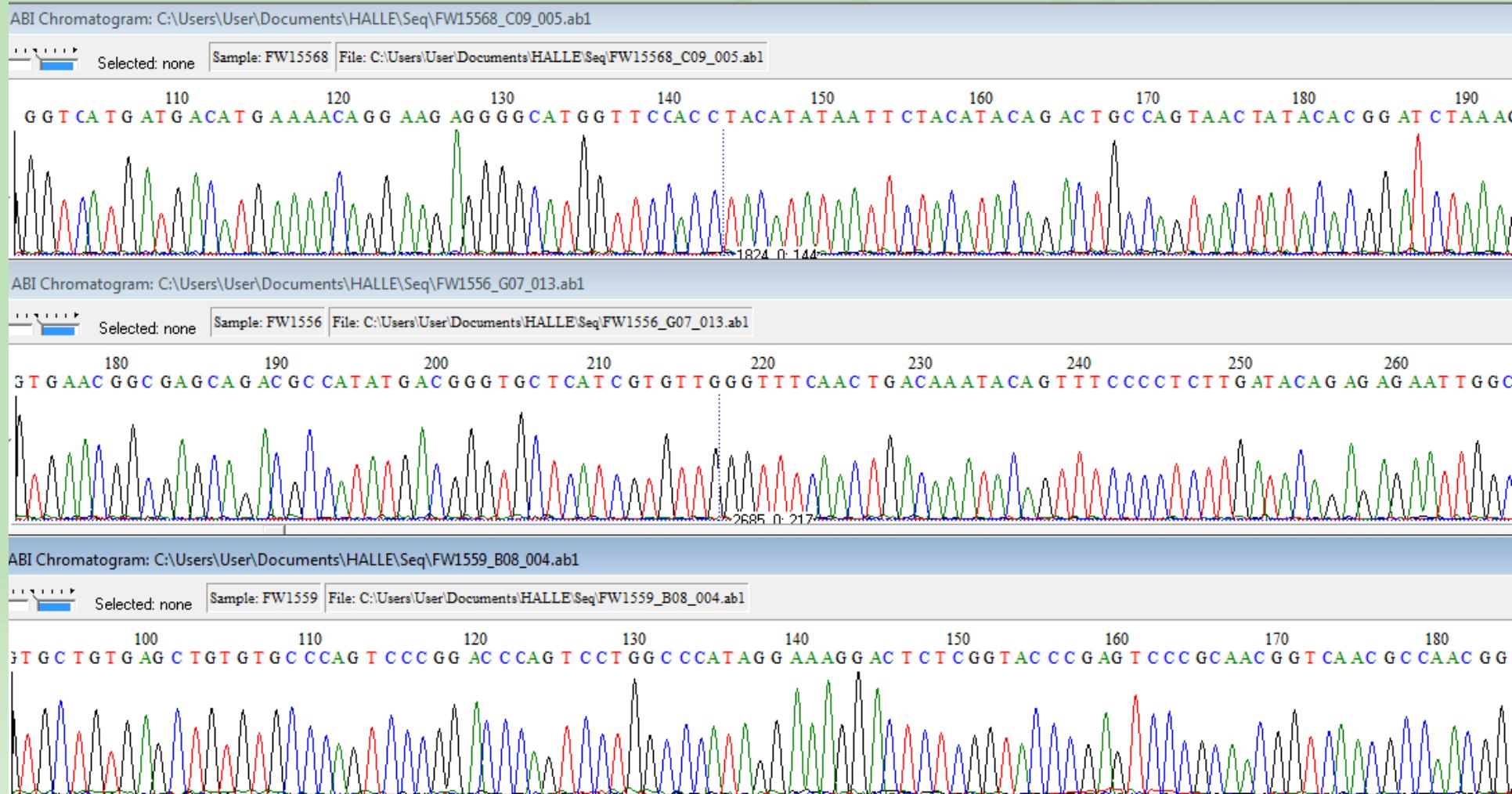
- SEARCH FOR THE GENOME (NCBI:
<https://www.ncbi.nlm.nih.gov/genome/>)
- Primers design for flank regions (3' and 5') amplification:



Deleção de genes

- Problemas encontrados
 - heterocromatina, regulação epigenética associado à fase do desenvolvimento, presença do hospedeiro, etc
- Critérios para publicação
 - 3 mutantes independentes e de cópia única (southern blot)
 - Complementação com o próximo gene do selvagem
 - Complementação de mutantes de *Saccharomyces cerevisiae*

Deleção de genes



Deleção de genes – complementação de mutantes em levedura

- Demonstrar a função

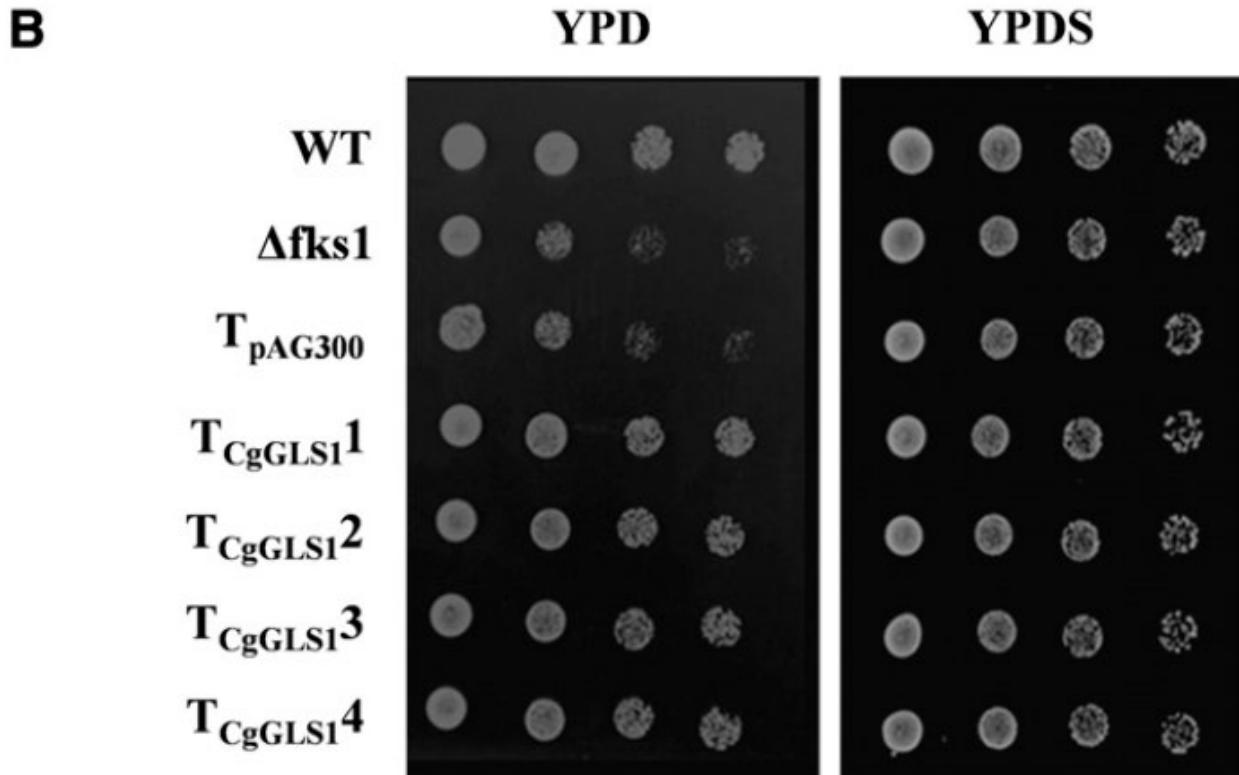
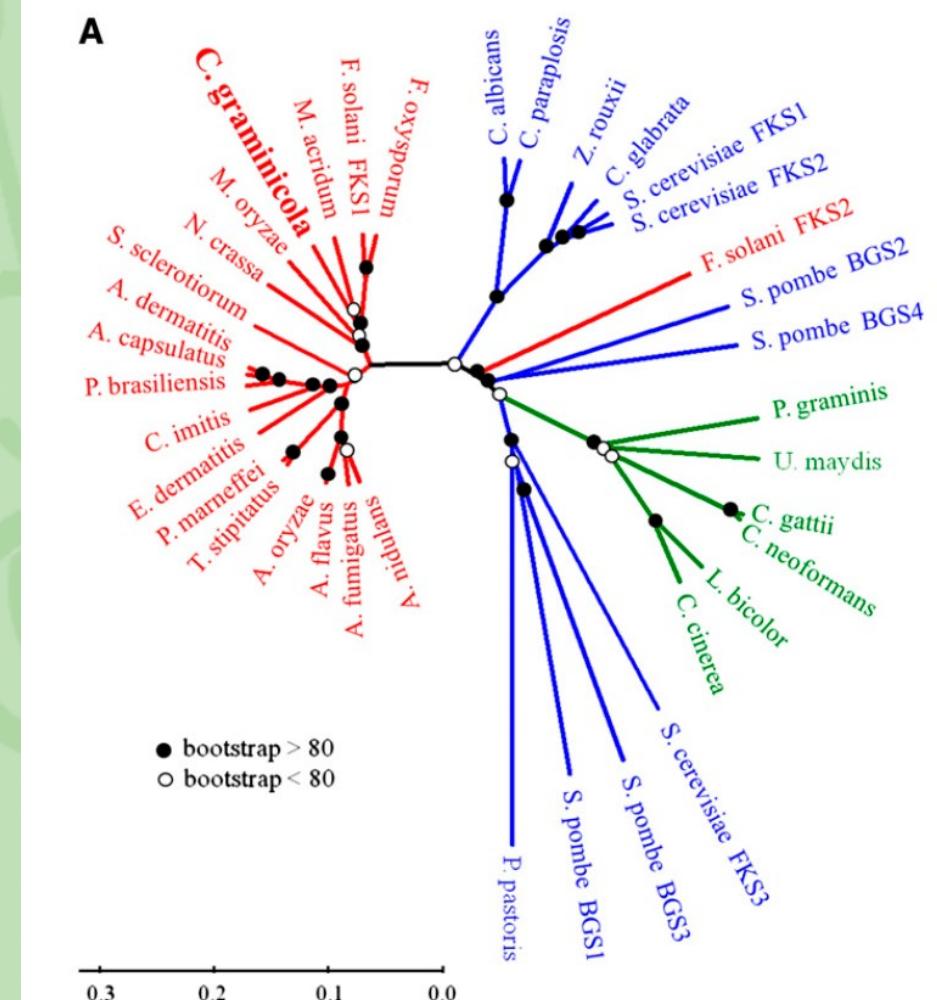


Figure 1. *GLS1* of *C. graminicola* Encodes a Functional GLS.

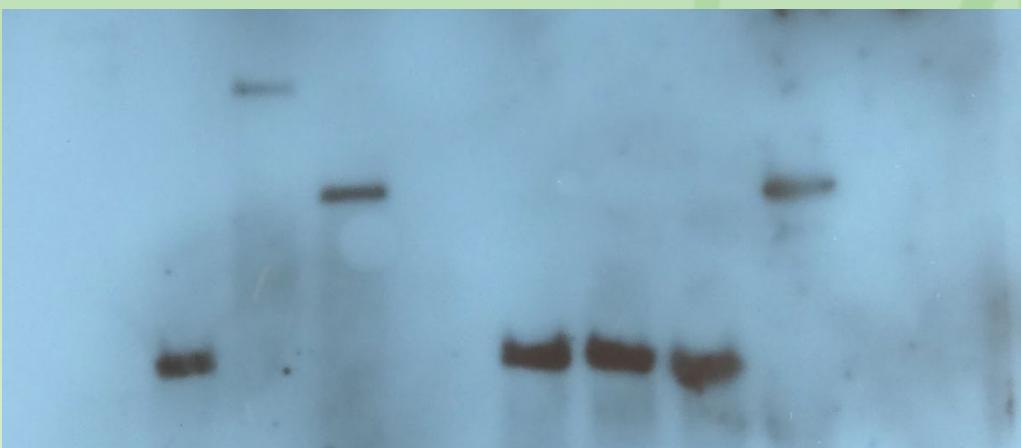


Deleção de genes

$\Delta 1$ $\Delta 2$ $\Delta 4$ $\Delta 103$ $\Delta 6$ $\Delta 12$ $\Delta 13$ $\Delta 14$ $\Delta 15$ $\Delta 18$ $\Delta 19$ $\Delta 24$ $\Delta 28$ $\Delta 29$ $\Delta 30$ WT M



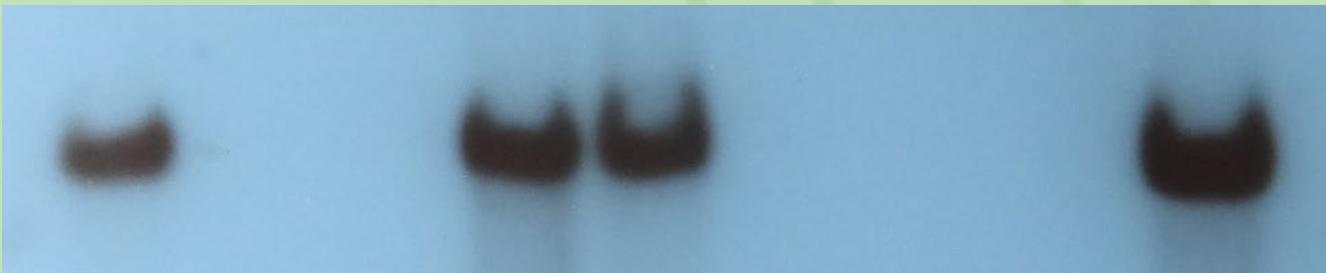
$\Delta 1$ $\Delta 2$ $\Delta 4$ $\Delta 5$ $\Delta 7$ $\Delta 13$ $\Delta 14$ $\Delta 15$ $\Delta 19$ WT



Allantoicase Mutants +
Bg/I and *HindIII*
Probe: Hygromycin

Deleção de genes

Δ102 Δ15 Δ18 Δ19 Δ24 Δ28 Δ29 Δ30 WT

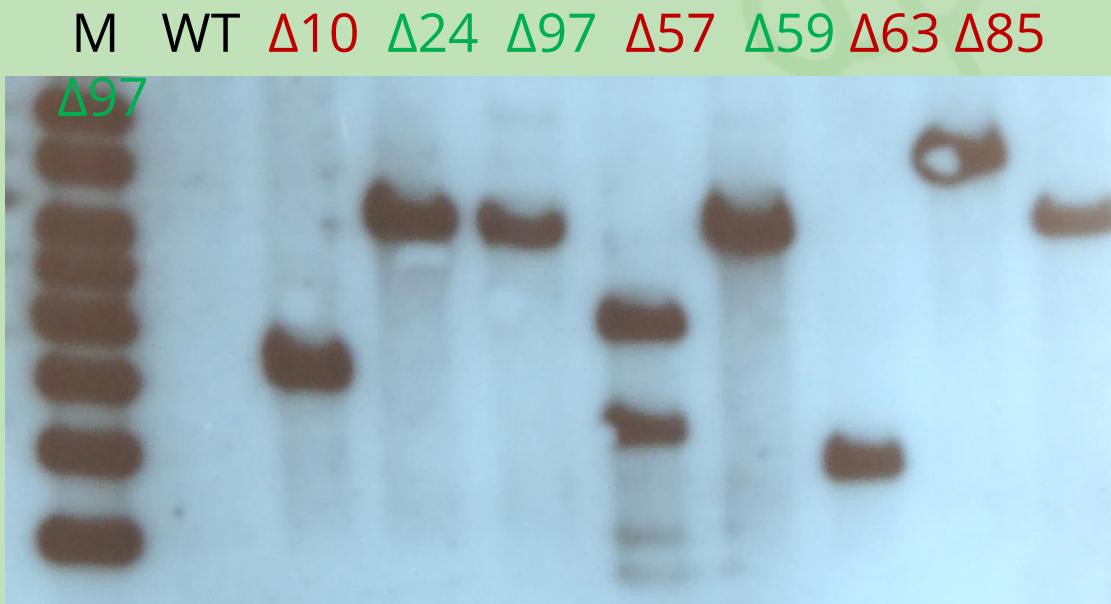


WT Δ2 Δ103 Δ4 Δ5 Δ6 Δ12 Δ13 Δ14

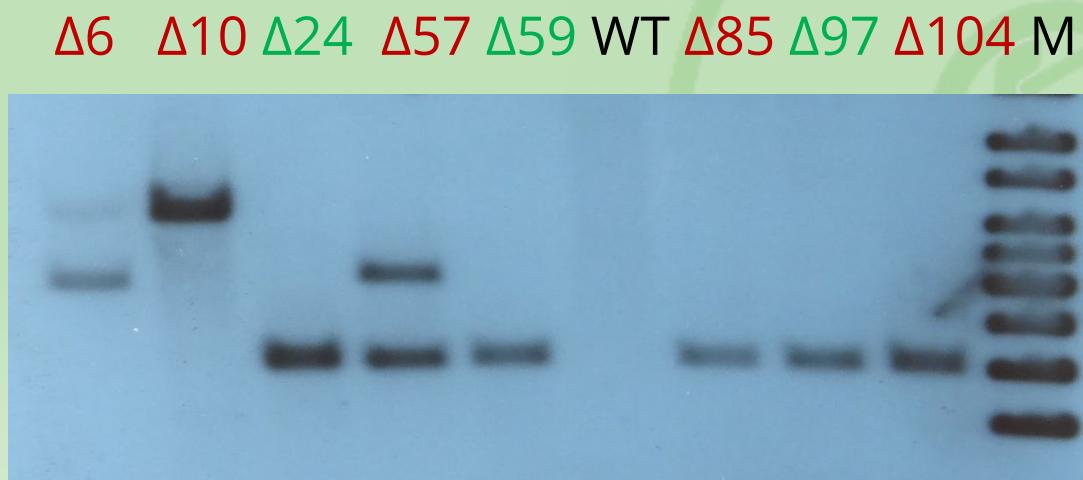


**Allantoicase Mutants + *Hind*III
Probe: Allantoicase**

Deleção de genes



Urease Mutants +
Scal
Probe: Hygromycin

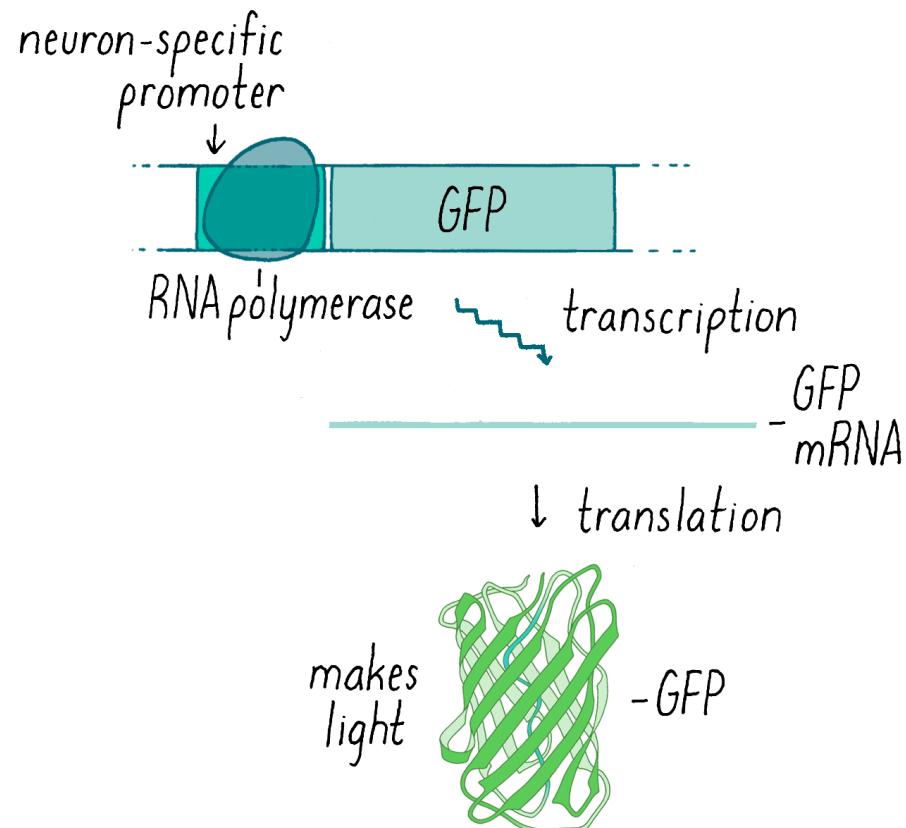


Urease Mutants + *Ncol*
Probe: Hygromycin

Fusão promotor - GFP

Verificar quando um gene é expresso

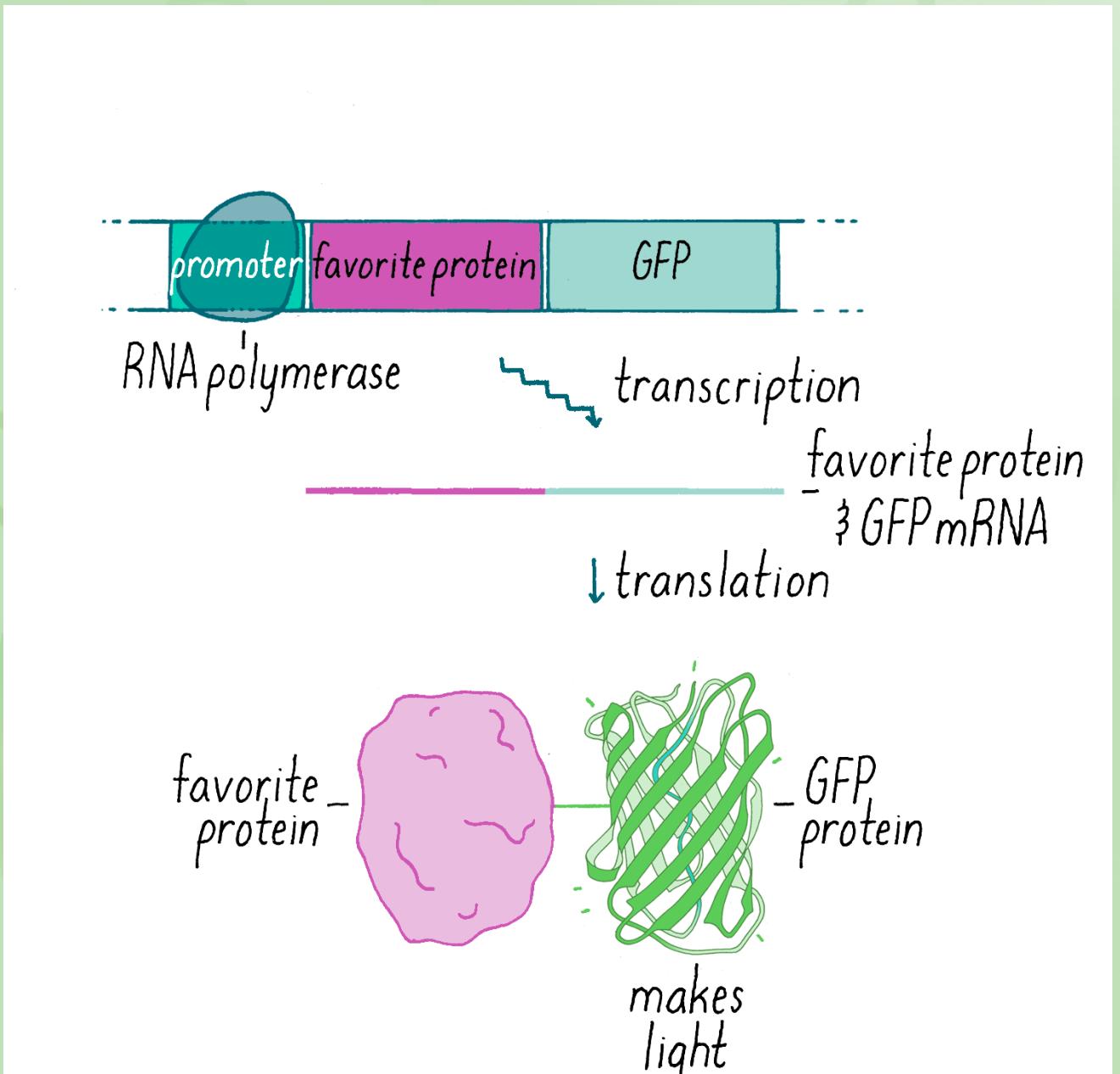
GFP TRANSCRIPTION AND TRANSLATION IN NEURONS



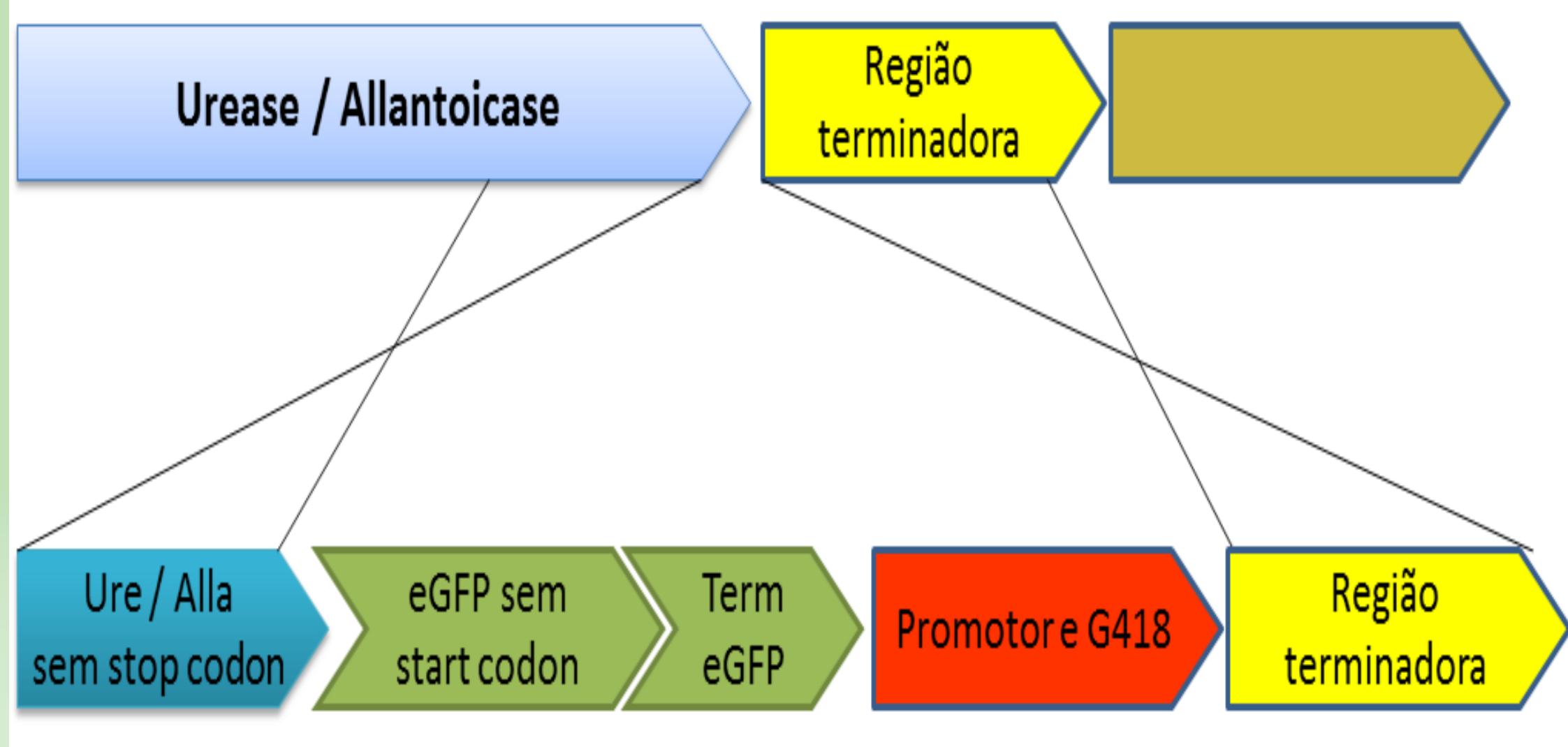
Fusão gene - GFP

1. Verificar quando um gene é expresso

2. Localização da proteína



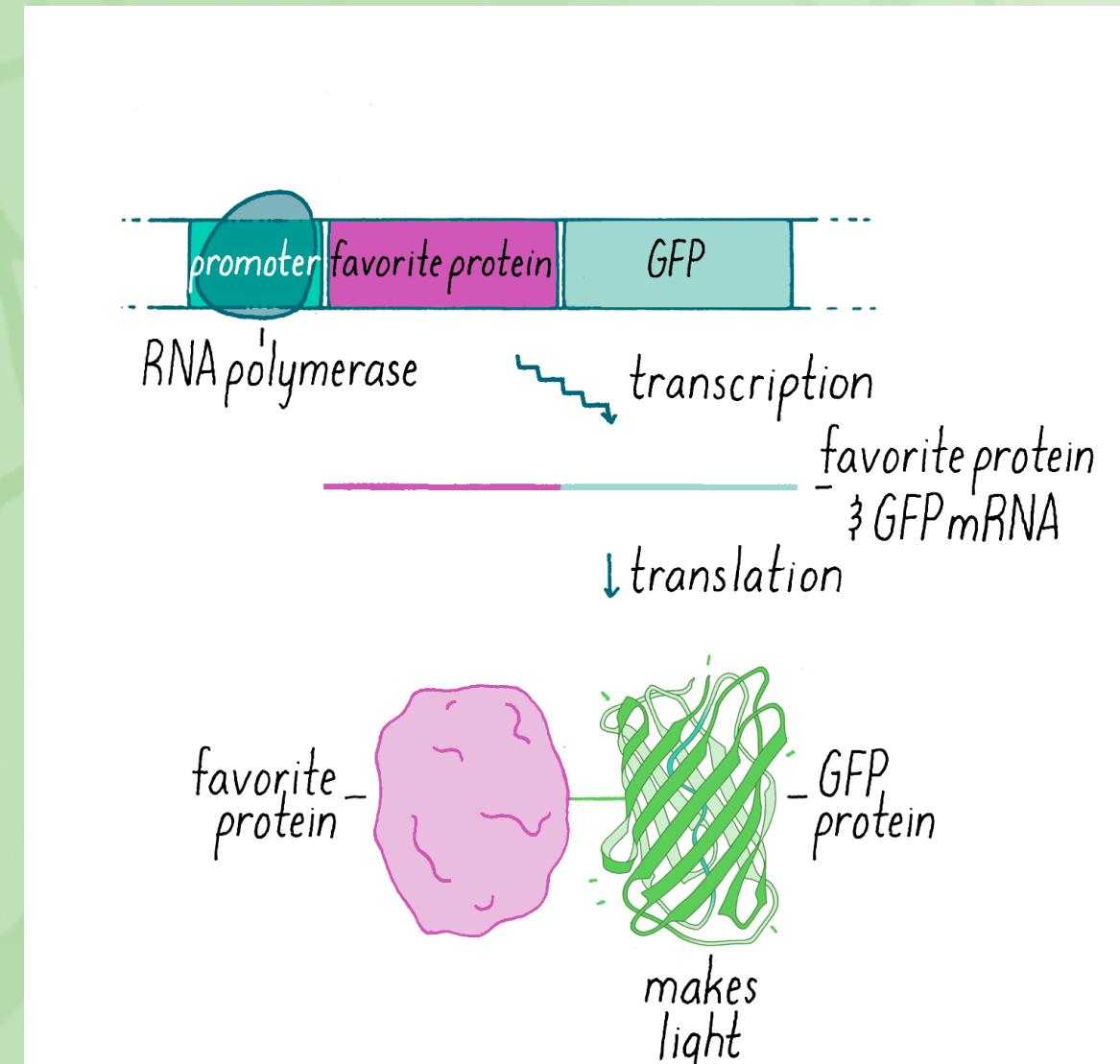
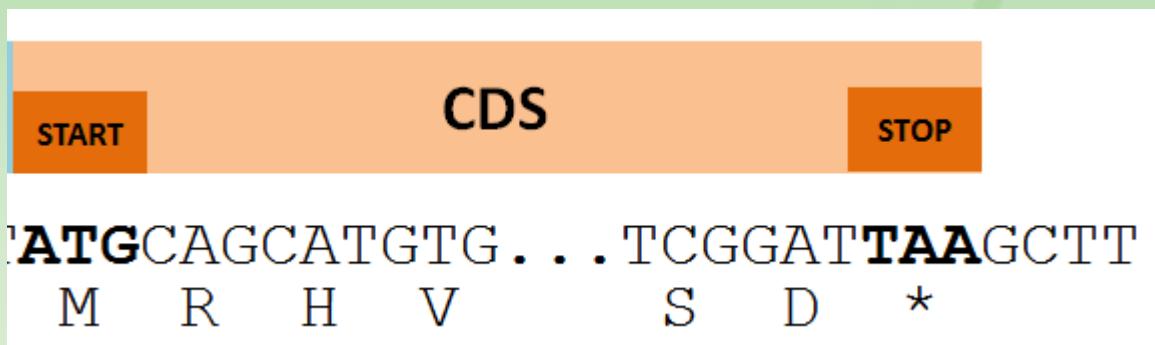
Estratégia para montagem de cassete de fusão



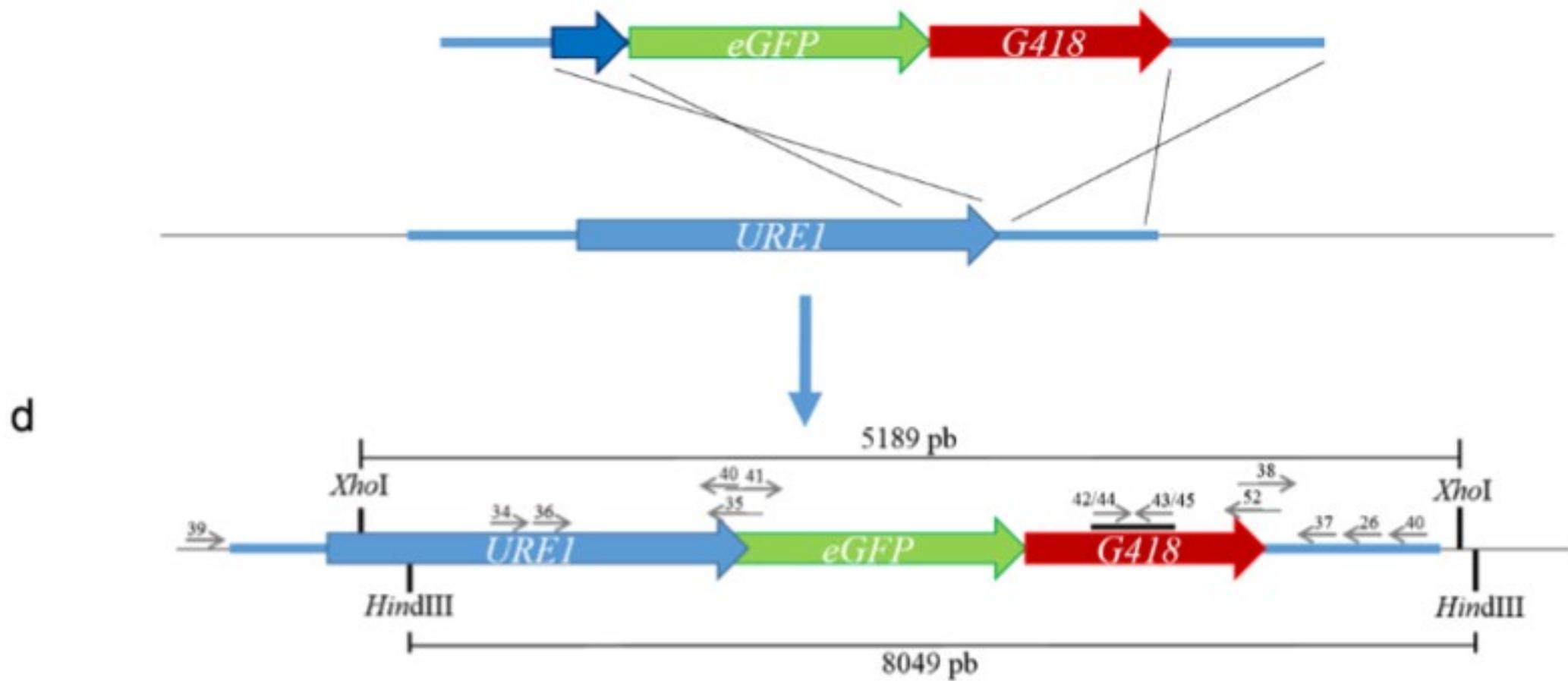
Fusão gene - GFP

Gene de interesse sem o
STOP codon

Gene GFP sem o **START**
codon

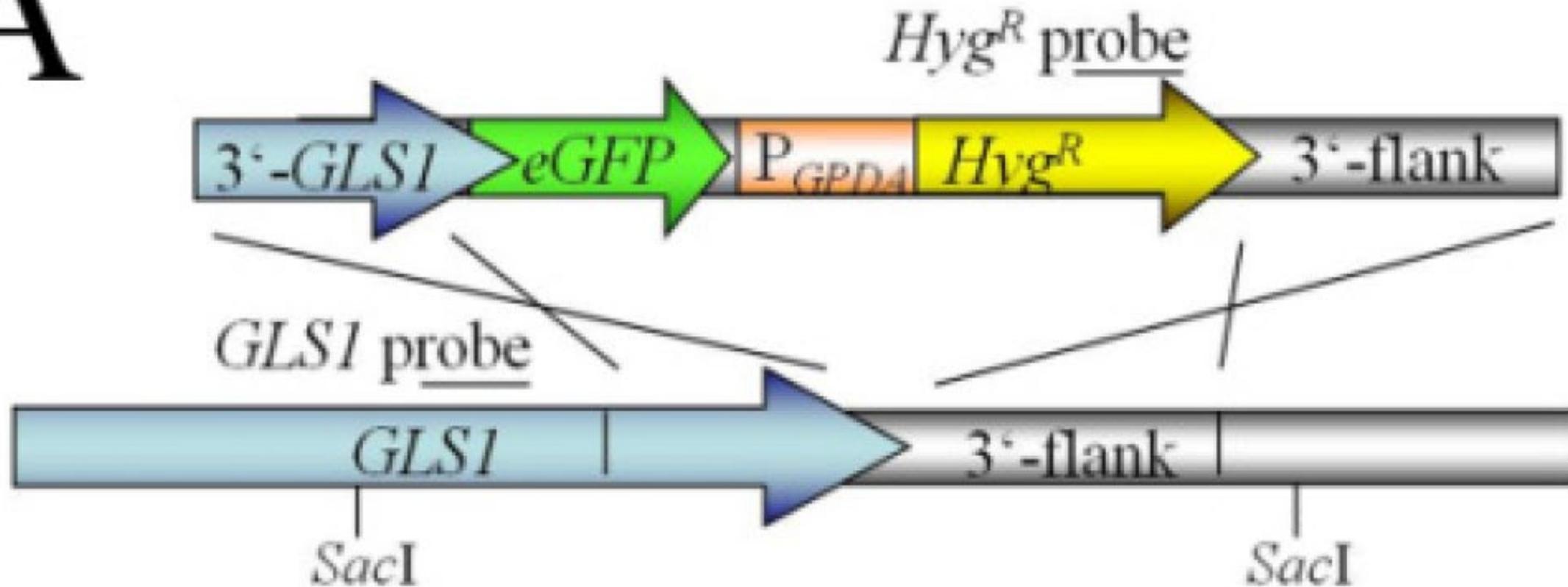


Fusão com gene para proteína fluorescente (eGFP)

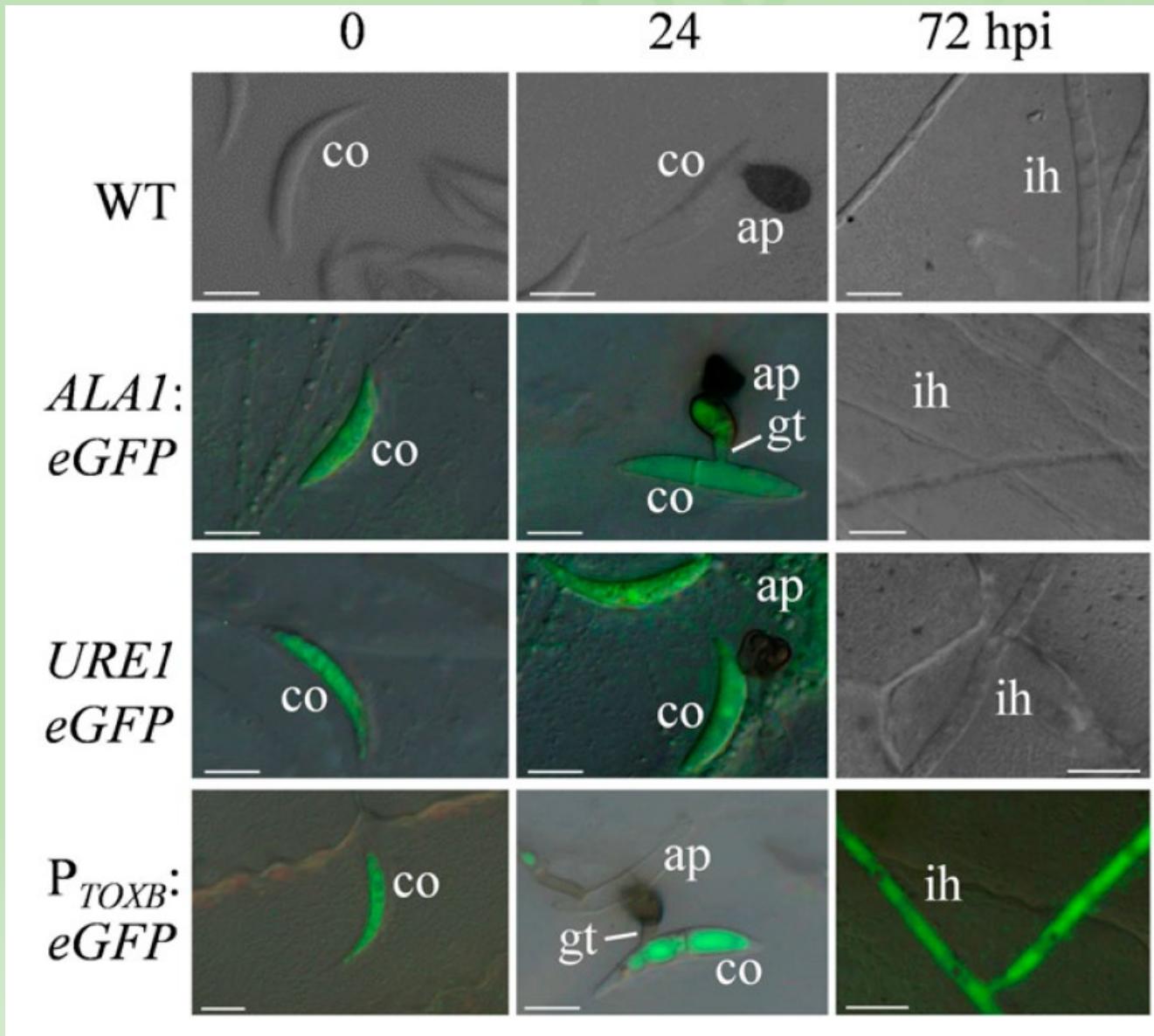


Fusão com gene para proteína fluorescente (GFP)

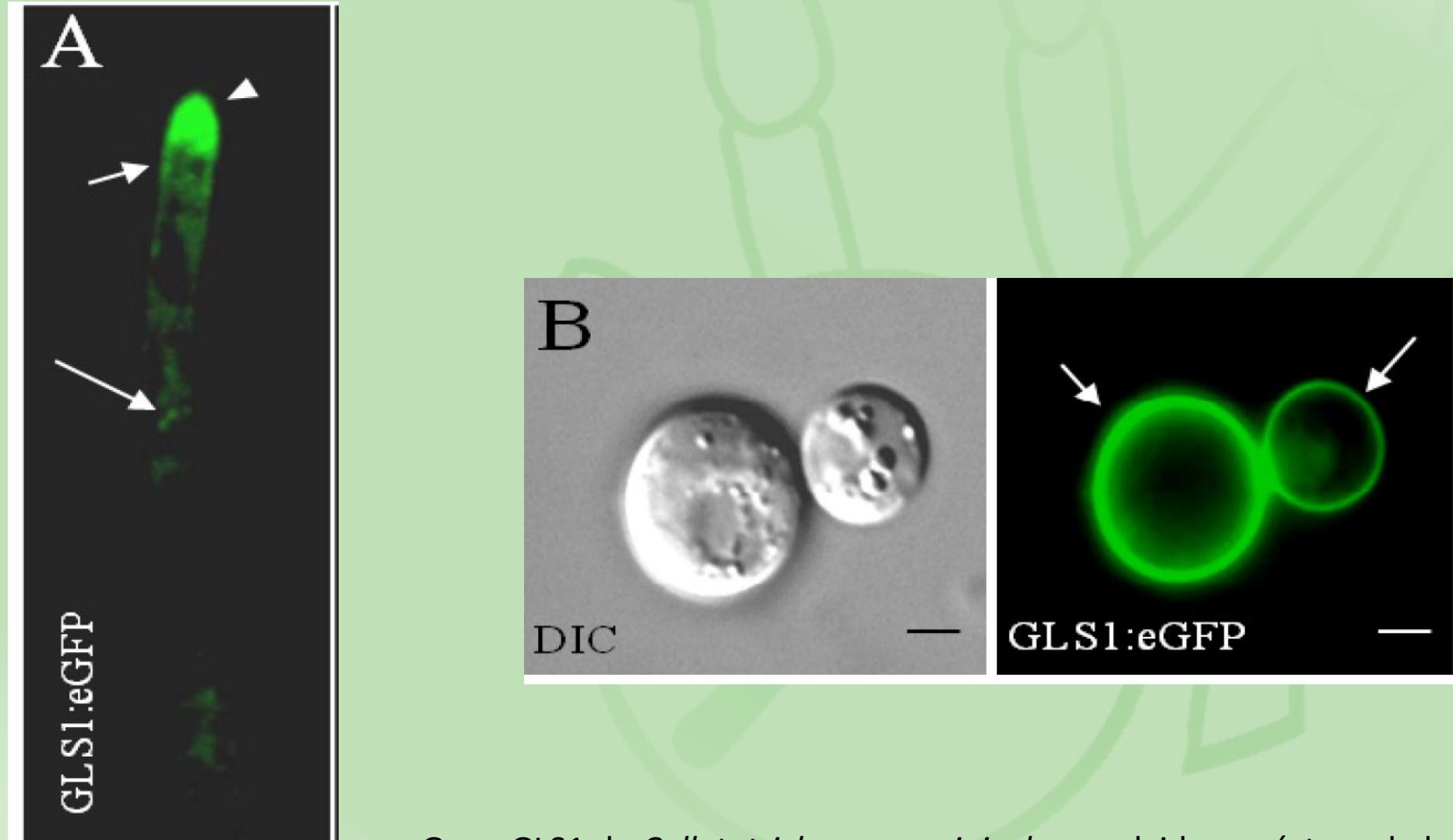
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Análise temporal da expressão do gene

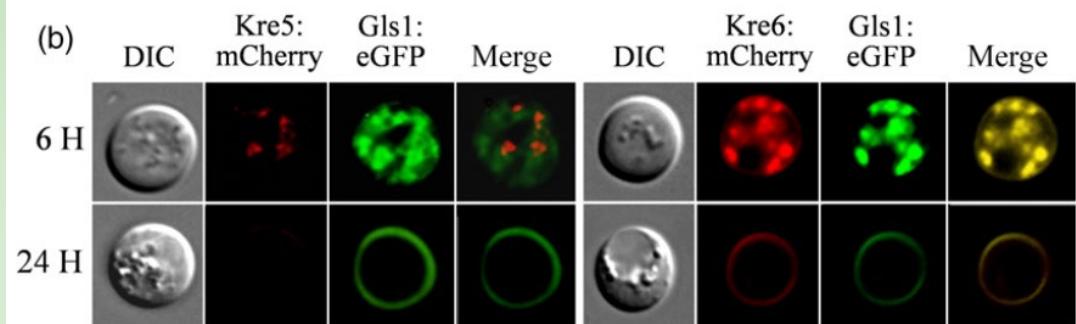
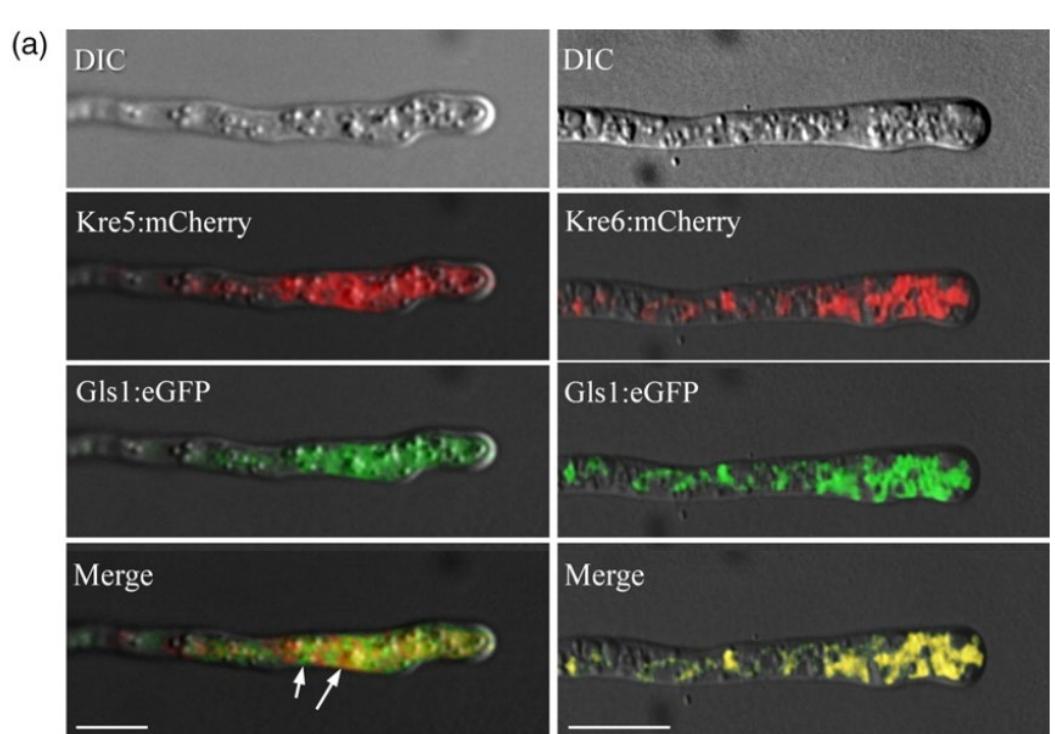


Análise temporal da expressão do gene e localização da proteína



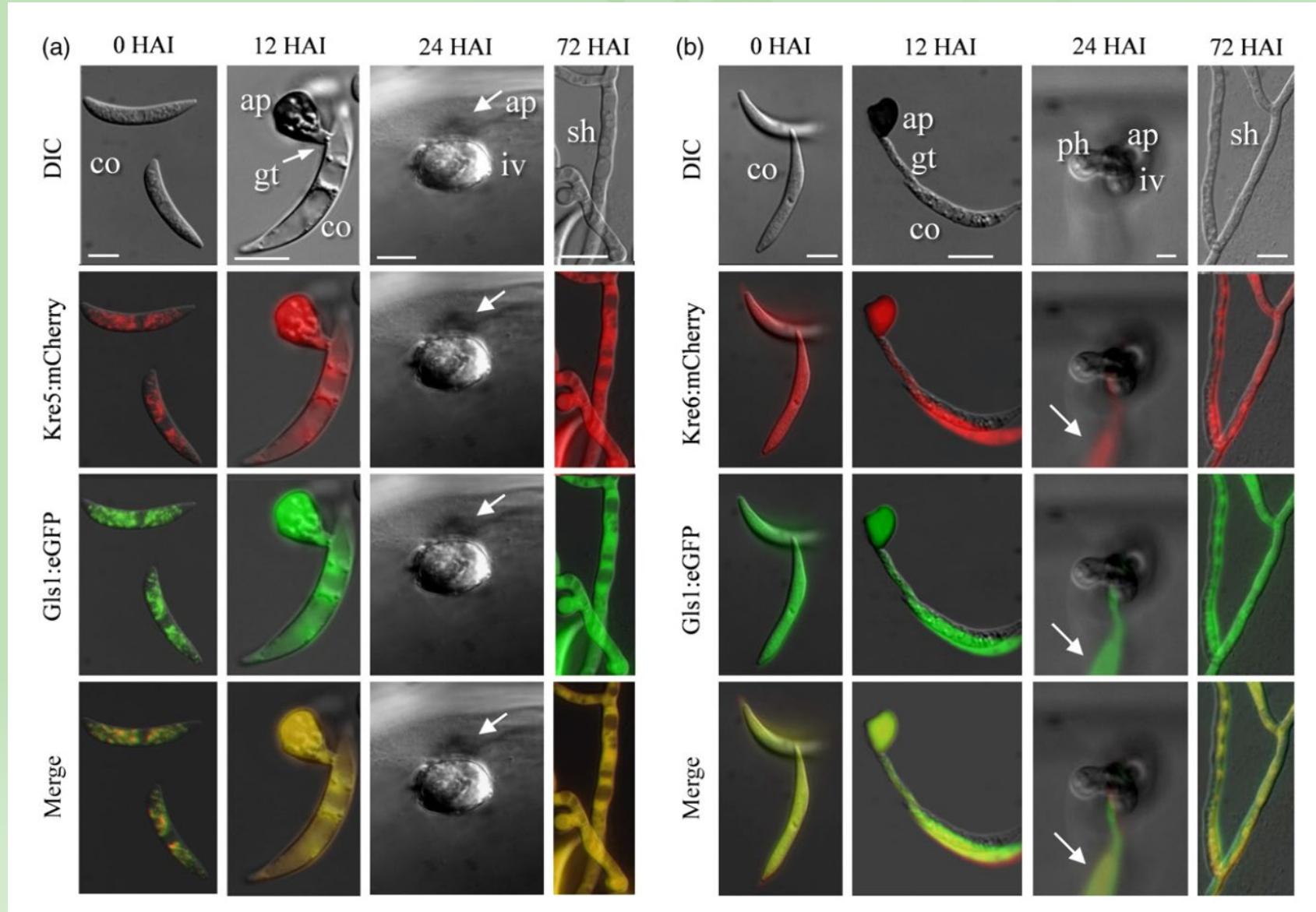
Gene *GLS1* de *Colletotrichum graminicola* envolvido na síntese de b-1,3-glucan

Uso de mCherry e eGFP para estudo da expressão de dois genes ao mesmo tempo

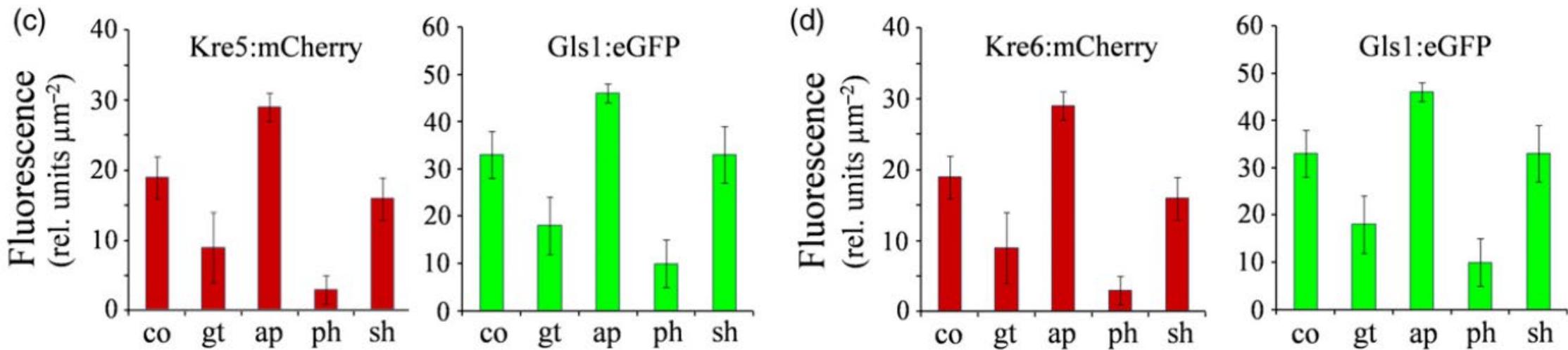


Genes KRE5 e KRE6 de *Colletotrichum graminicola* envolvidos na síntese de b-1,6-glucan
Gene GLS1 de *Colletotrichum graminicola* envolvido na síntese de b-1,3-glucan

Uso de mCherry e eGFP para estudo da expressão de dois genes ao mesmo tempo



Uso de mCherry e eGFP para estudo da expressão de dois genes ao mesmo tempo



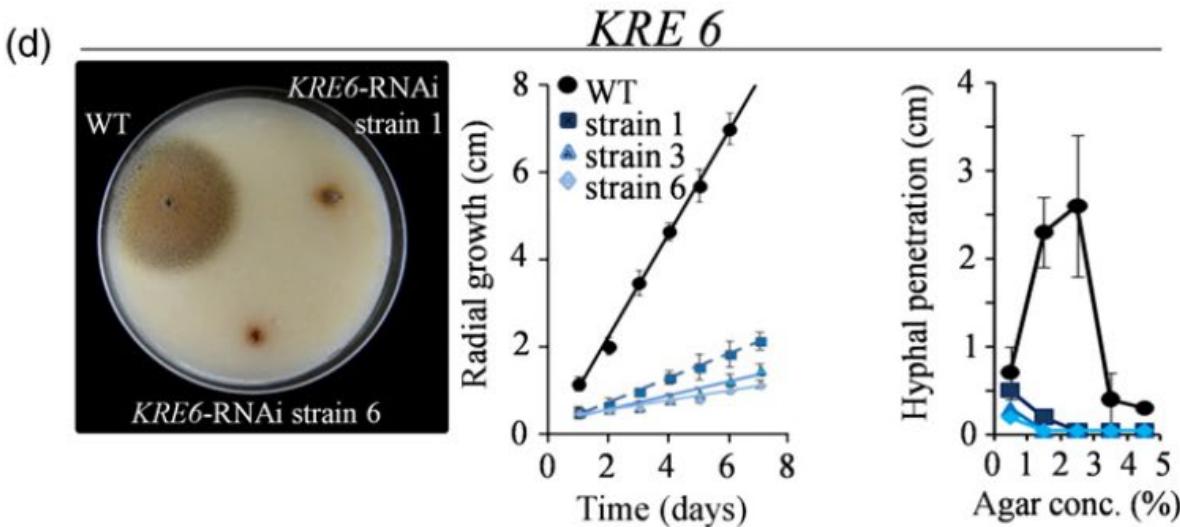
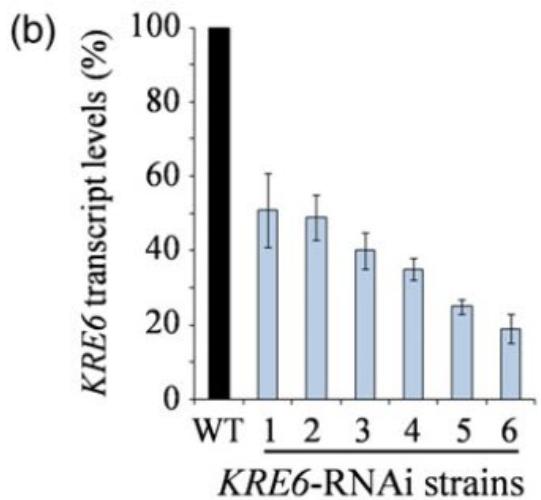
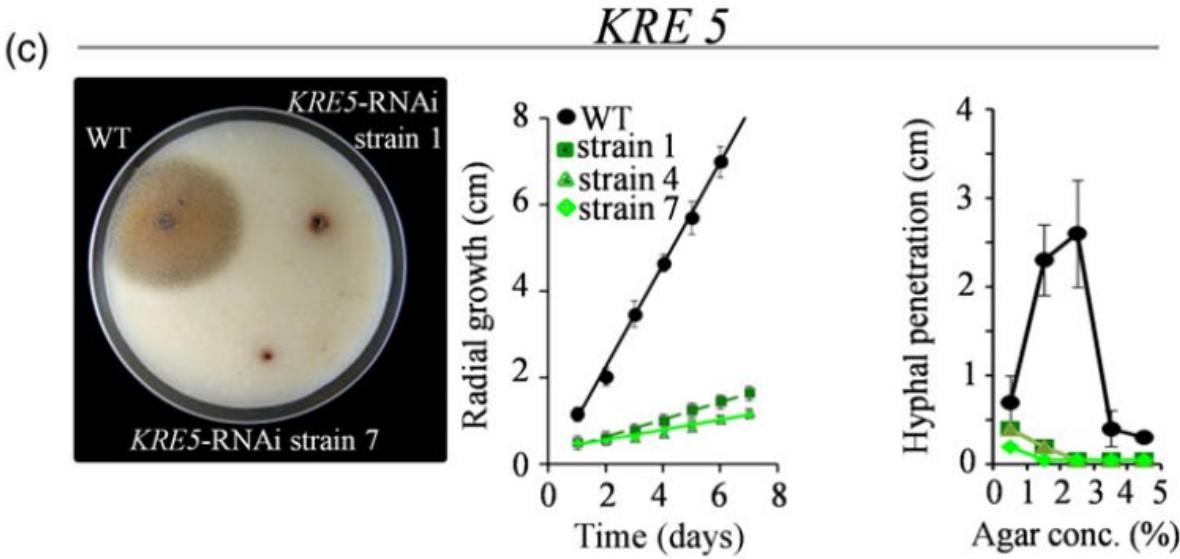
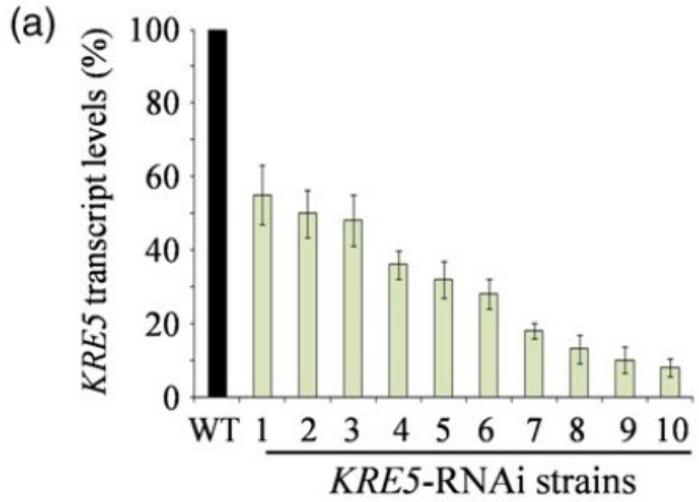
Genes KRE5 e KRE6 de *Colletotrichum graminicola* envolvidos na síntese de b-1,6-glucan

Gene GLS1 de *Colletotrichum graminicola* envolvido na síntese de b-1,3-glucan

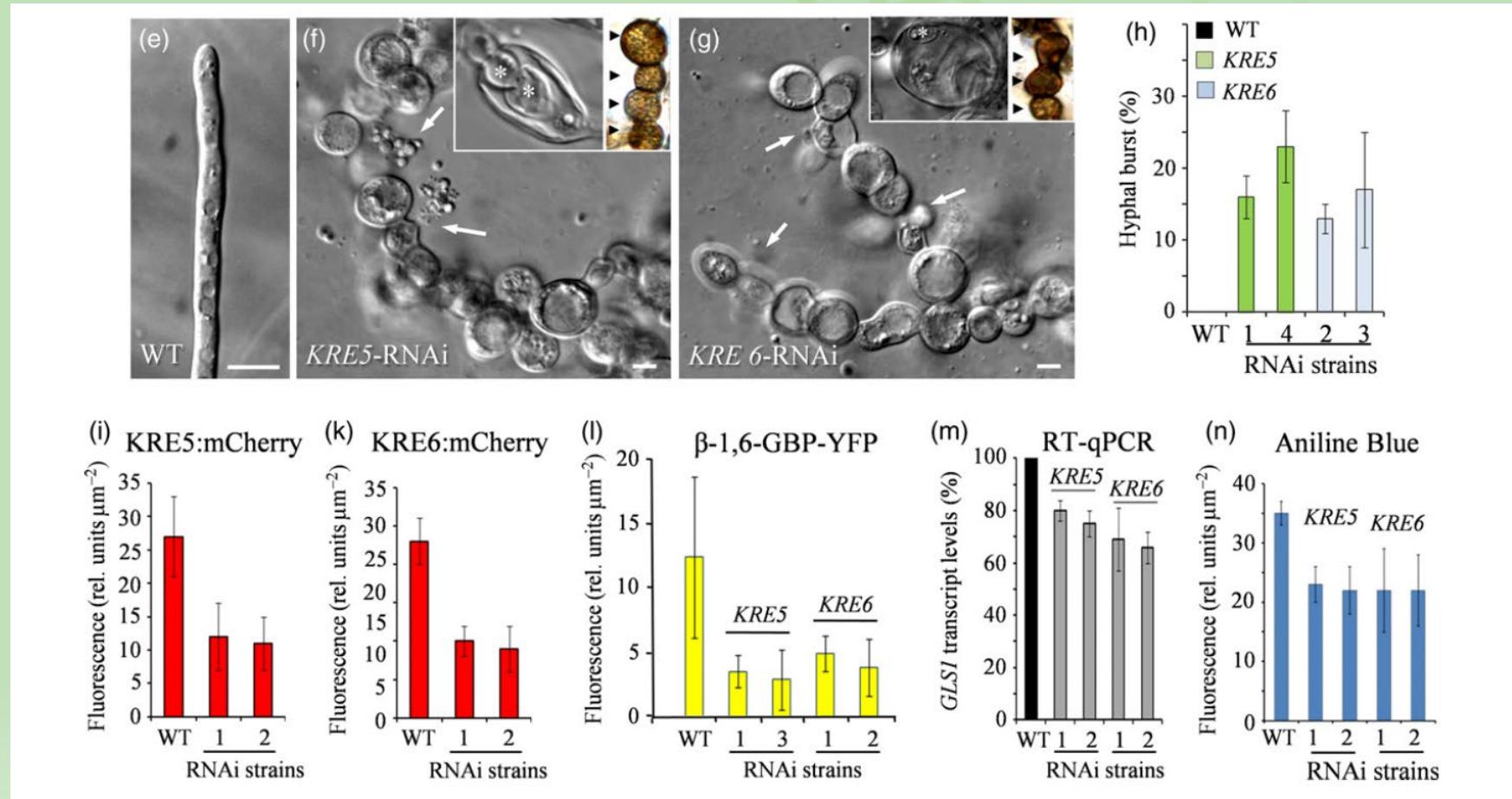
Microscopia de fluorescência quantitativa

Quantitative fluorescence levels of KRE5:mCherry and KRE6:mCherry expressing transformants of *C. graminicola* were evaluated at 0, 12, 24, and 72 HAI, using a Zeiss Observer Z1 inverted microscope equipped with a Plan Apochromat 963/1.40 oil immersion objective and an AxioCam MRm camera. Epi-illumination analyses employed filter set 49 for aniline blue fluorochrome and filter set 38HE for mCherry. Image acquisition and analysis were performed by using Zeiss AxioVision 4.8.2 (06-2010) software with the Physiology module (all from Carl Zeiss, Oberkochen, Germany).

RT-qPCR: Avaliar o nível de transcrição (mRNA)



Linhagens KRE:mCherry + iRNA KRE



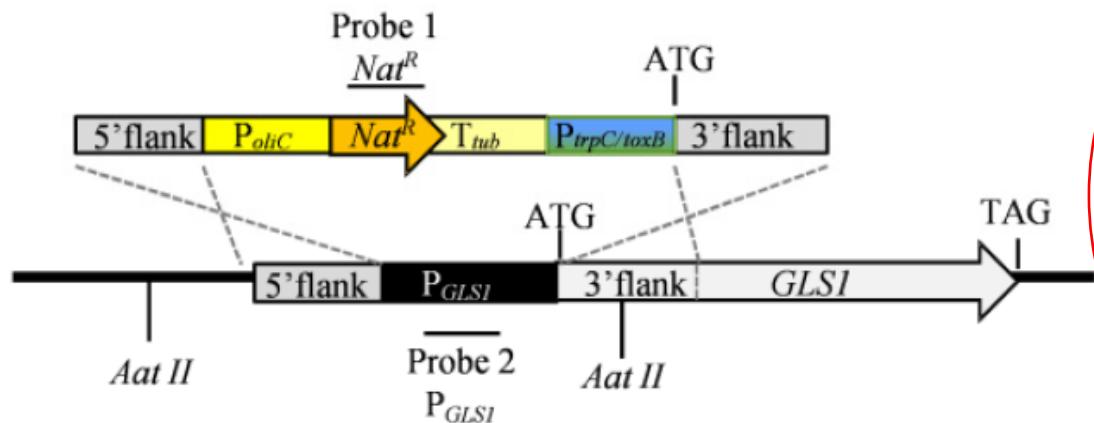
Verificar redução das proteínas além da redução dos mRNAs

Superexpressão de genes

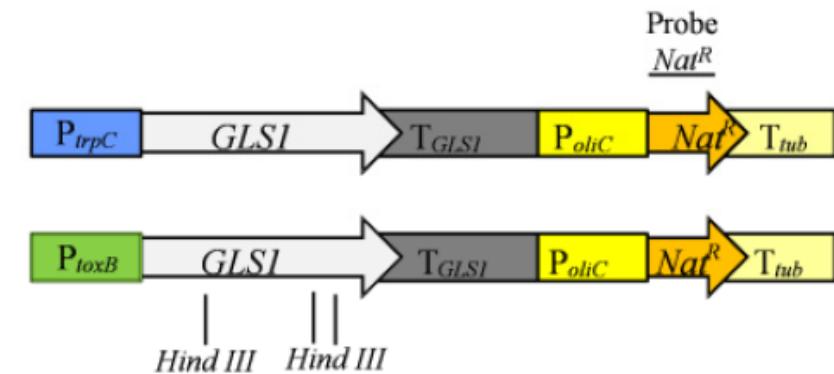
Expressão constitutiva ou aumento da expressão

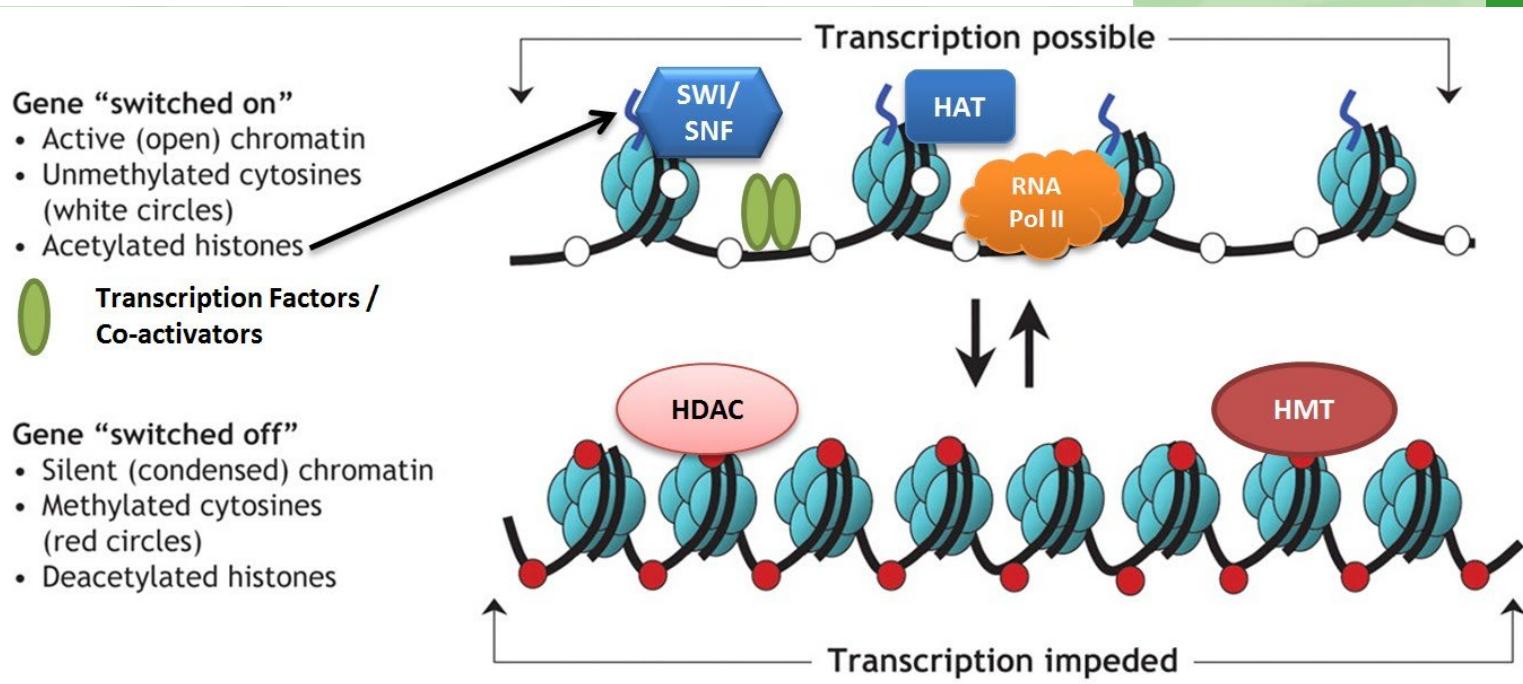
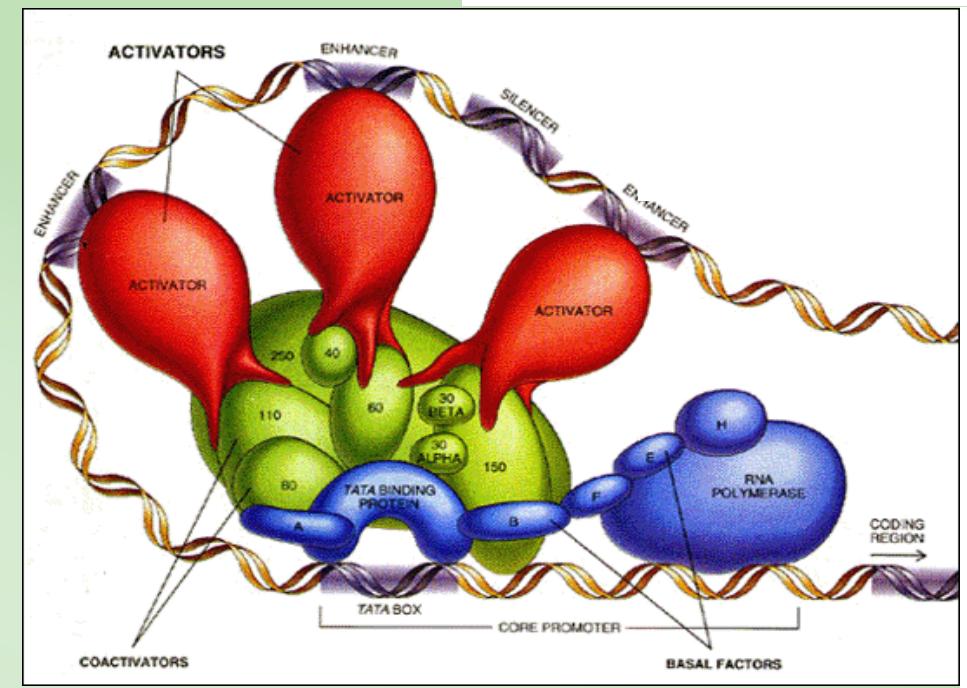
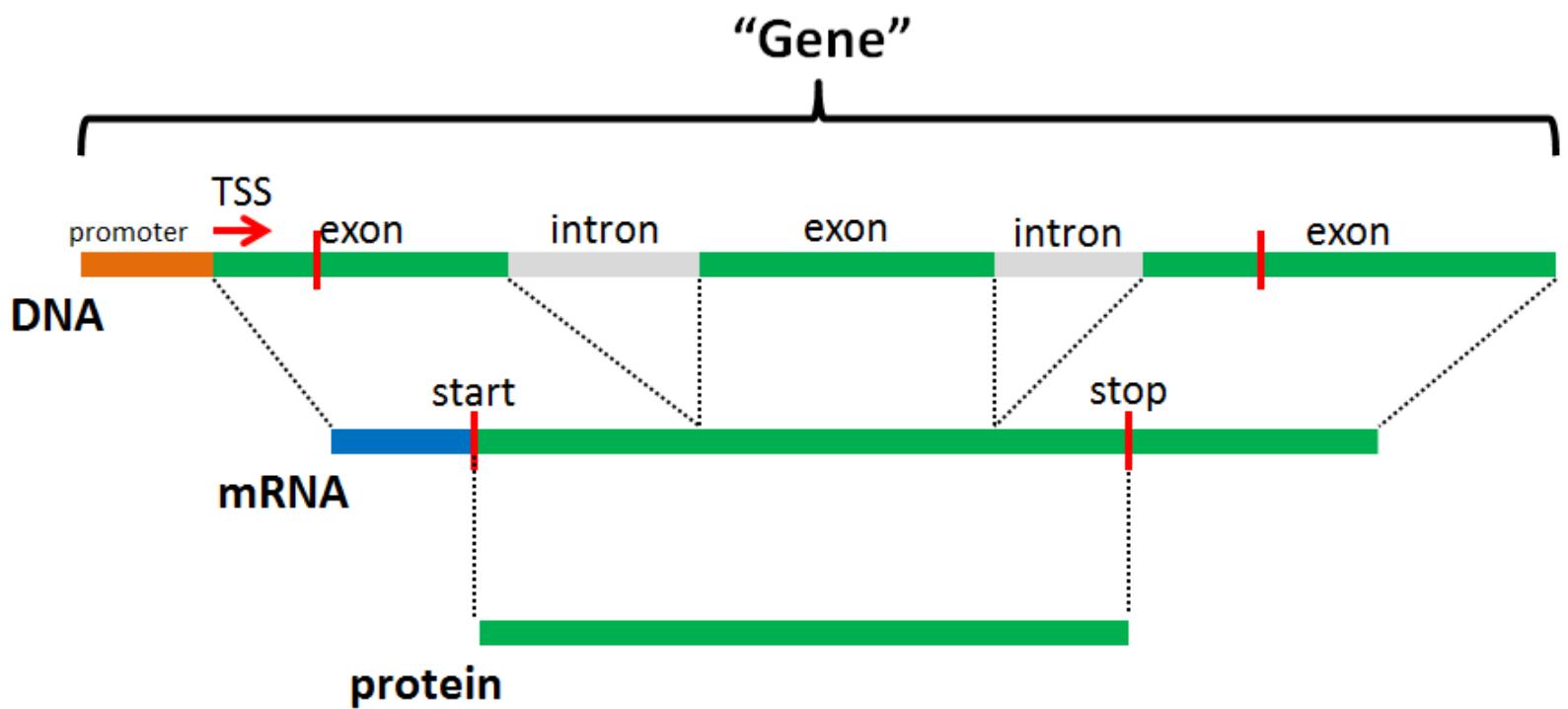
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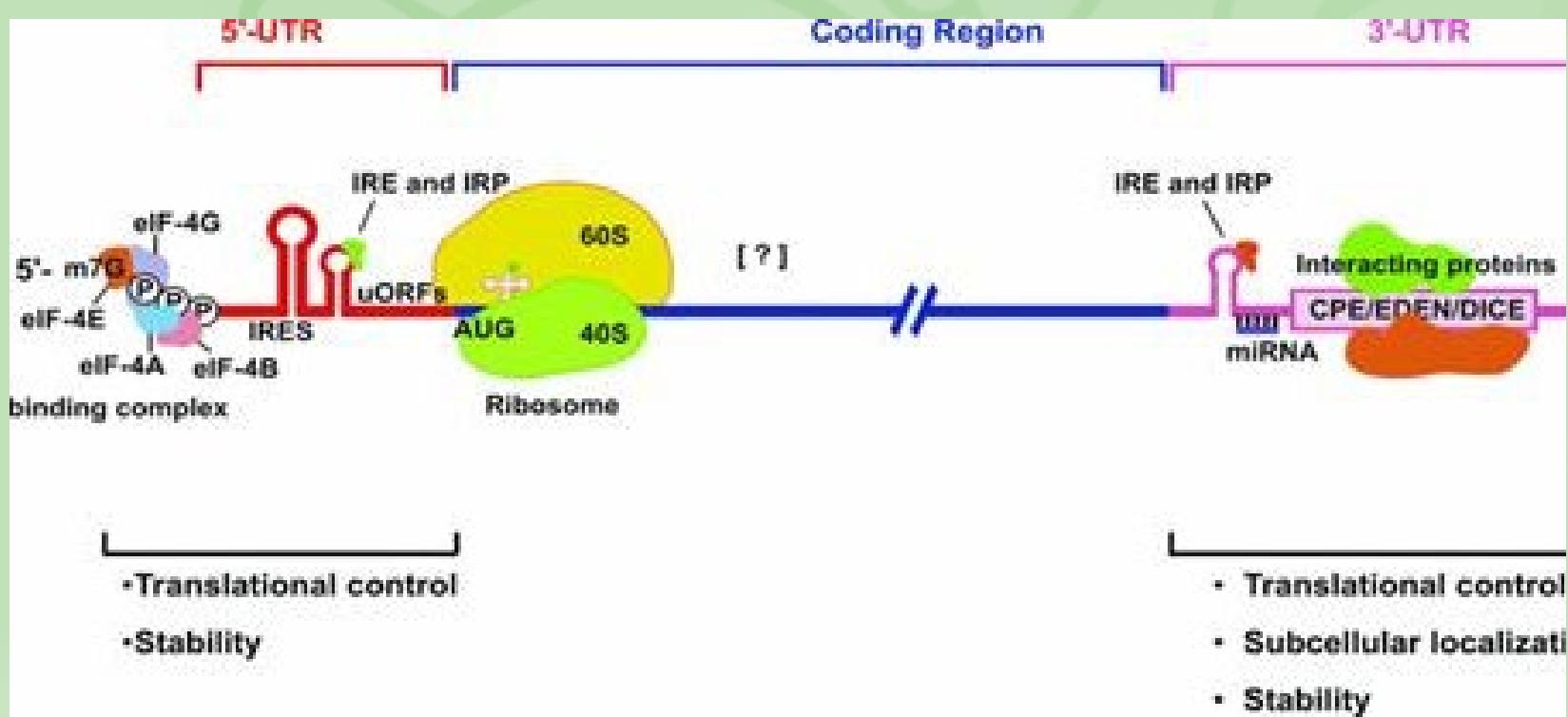
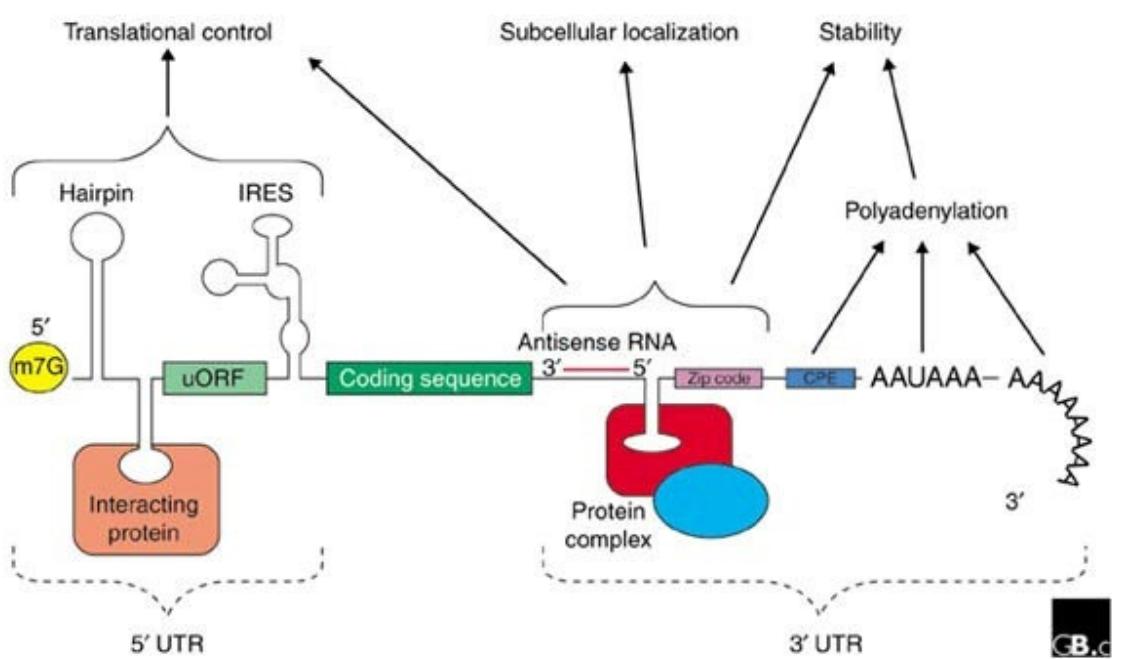
promoter exchange



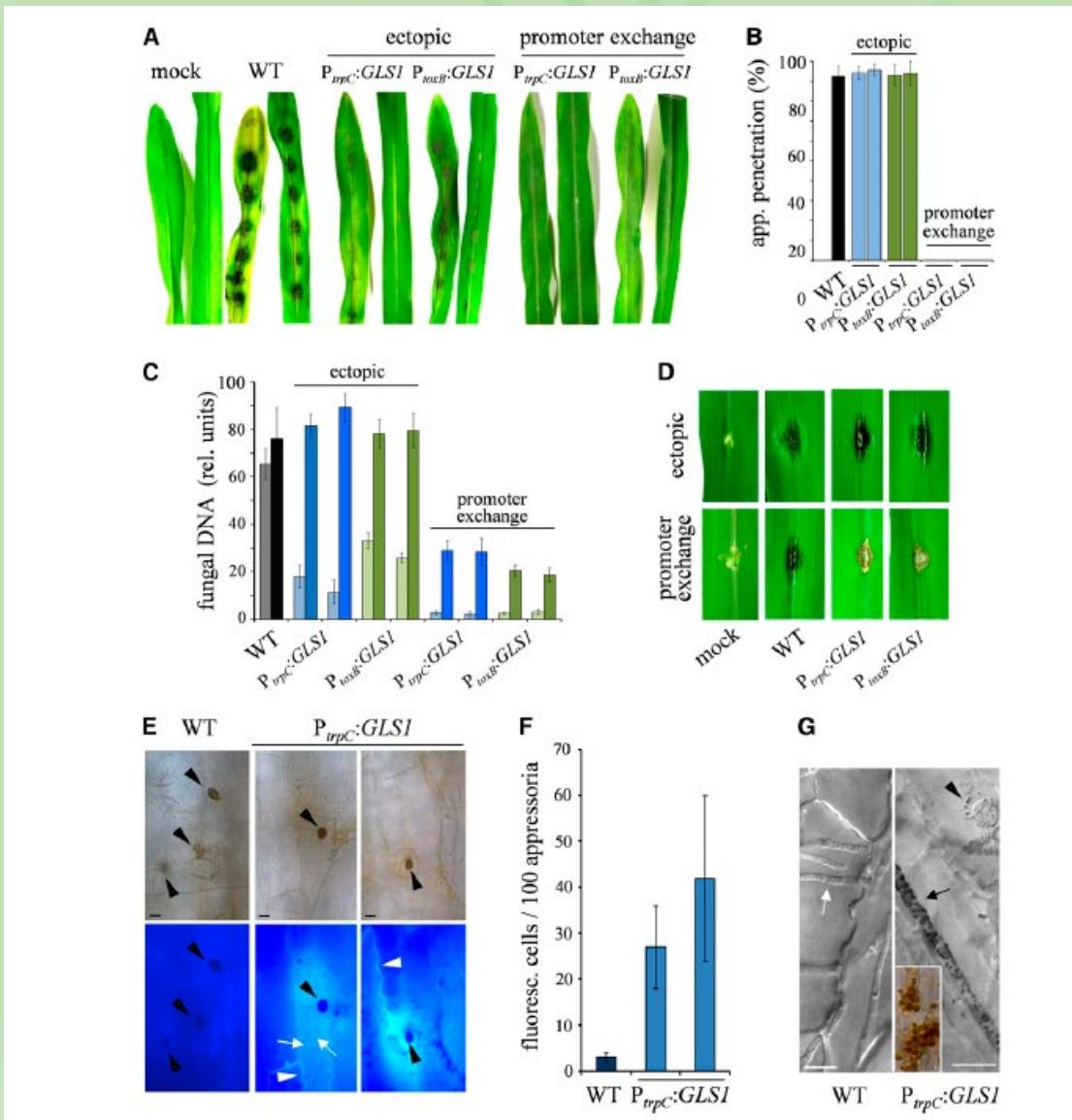
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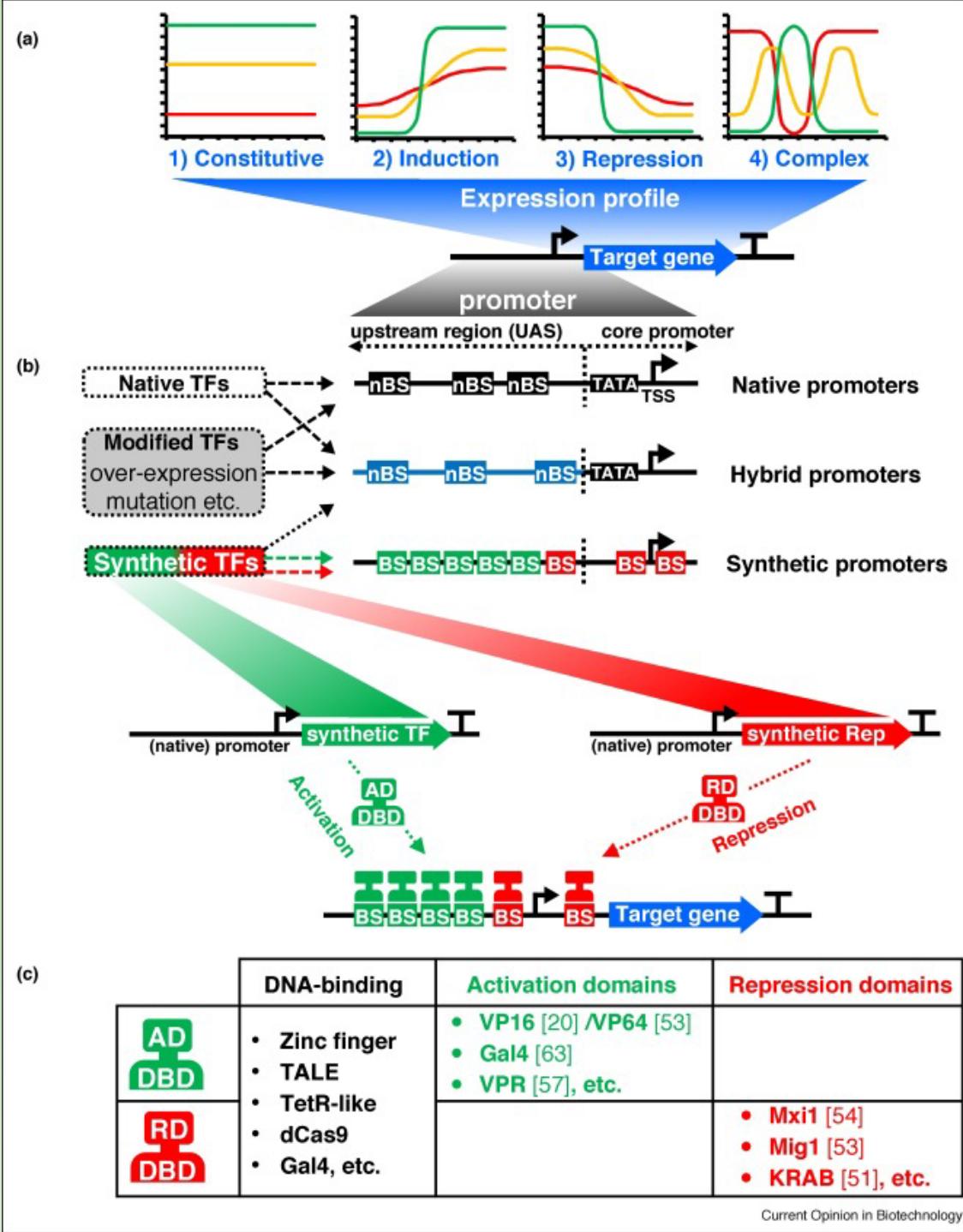


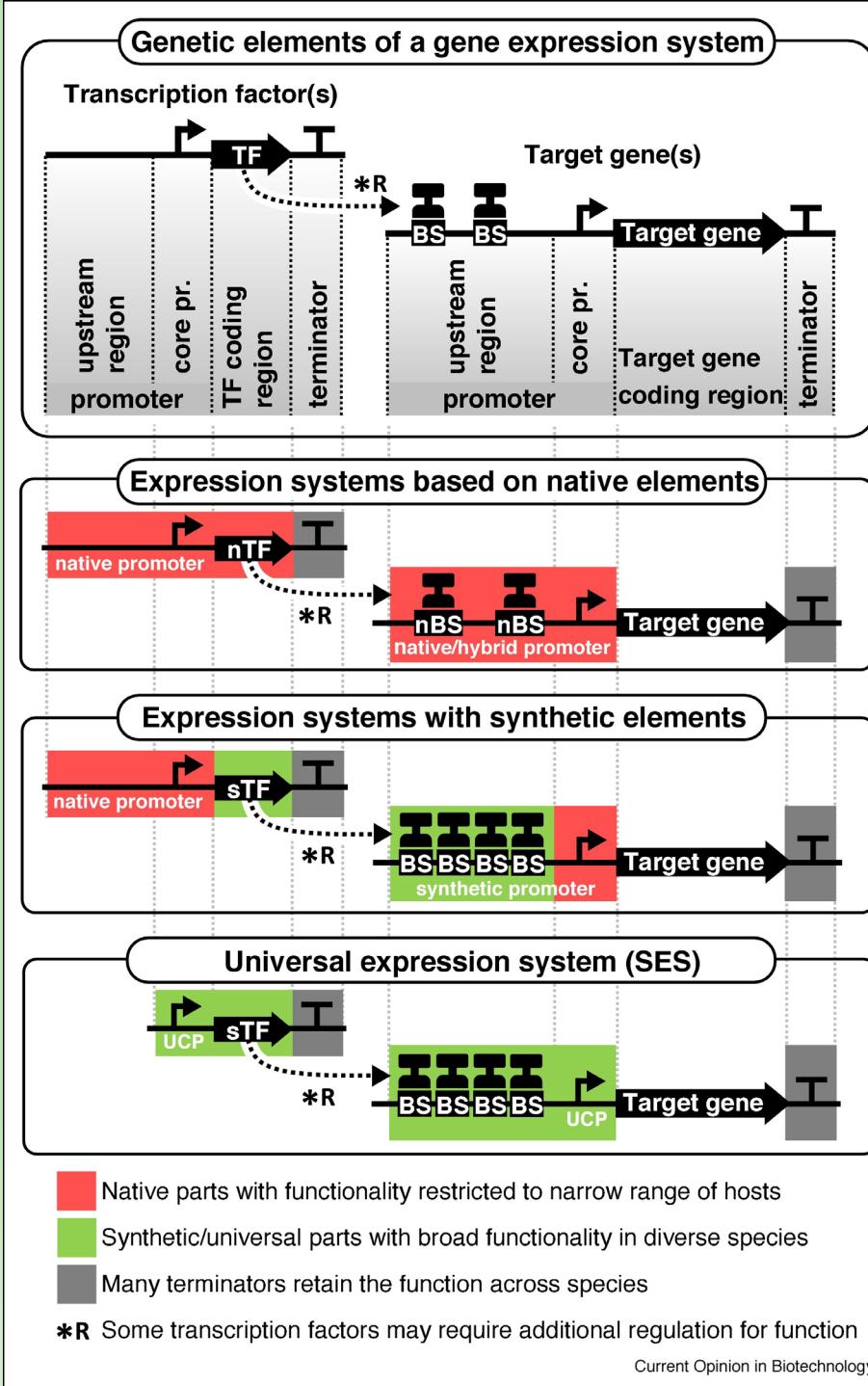




Expressão forçada do gene *GLS1* em hifas biotróficas







Current Opinion in Biotechnology 2019, 59:141–149

This review comes from a themed issue on **Tissue, cell and pathway engineering**

Edited by Eveline Peeters and Marjan De Mey

For a complete overview see the [Issue](#) and the [Editorial](#)

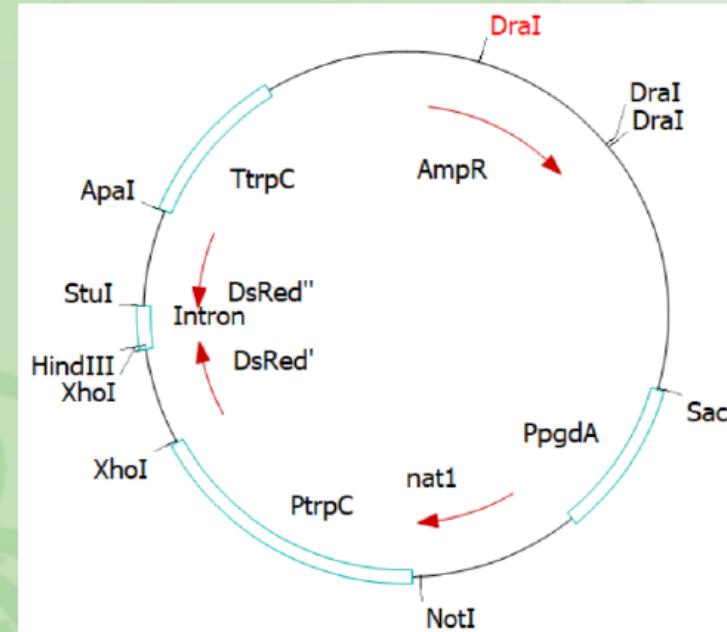
Available online 30th May 2019

<https://doi.org/10.1016/j.copbio.2019.04.007>

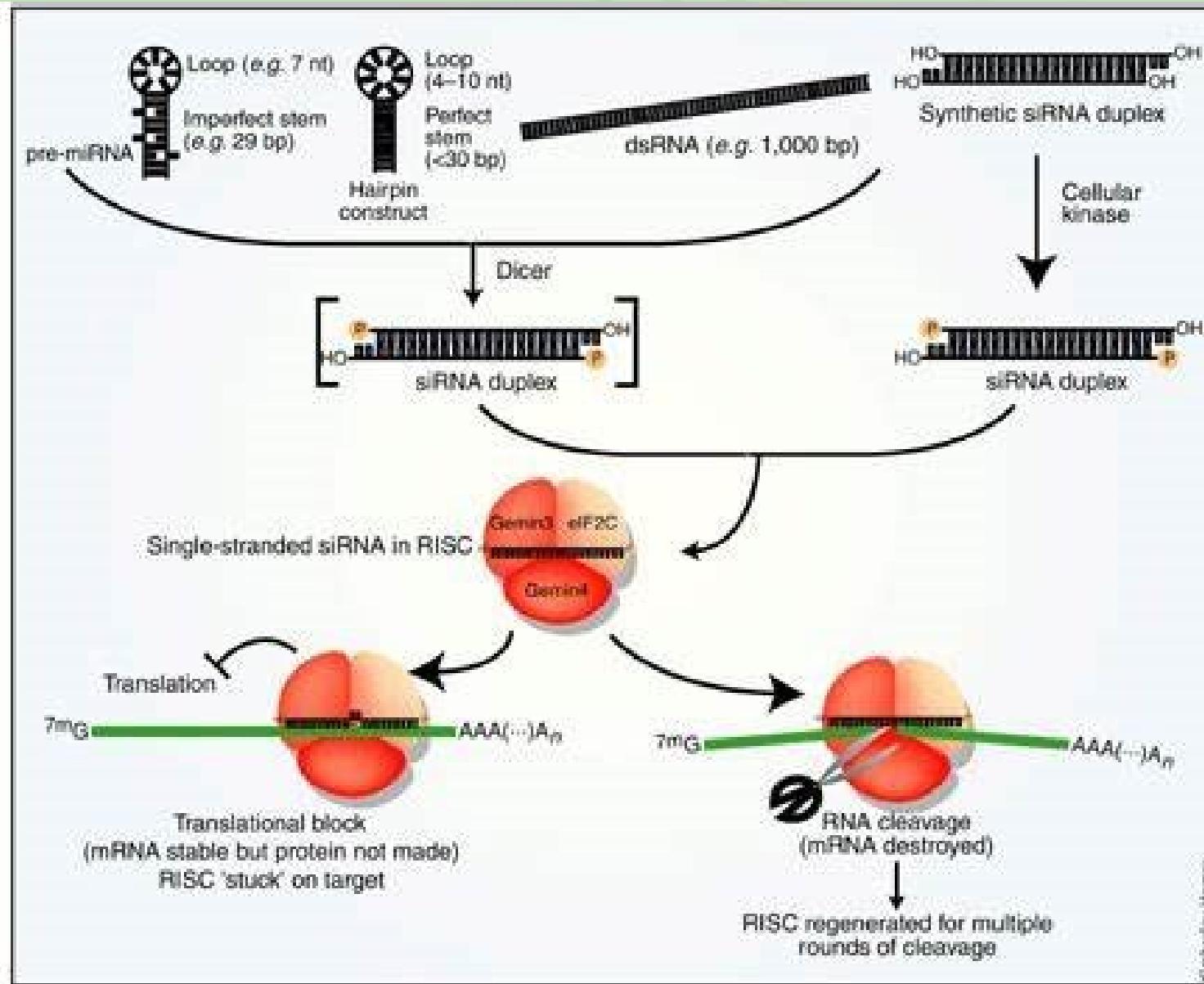
Silenciamento via iRNA

Estudo de caso: Montagem de Cassete de RNAi

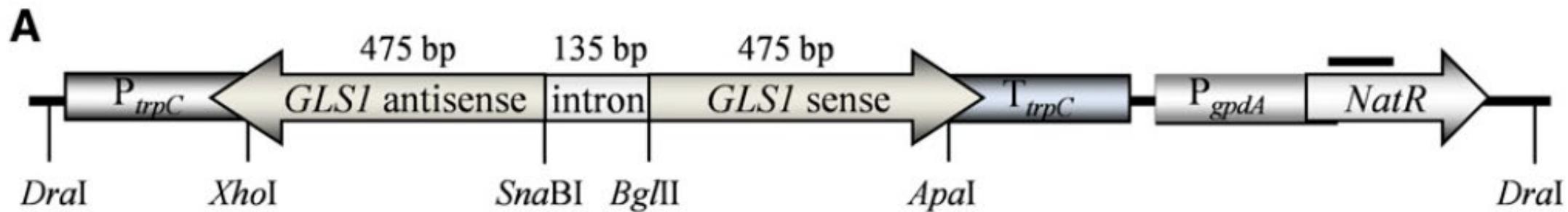
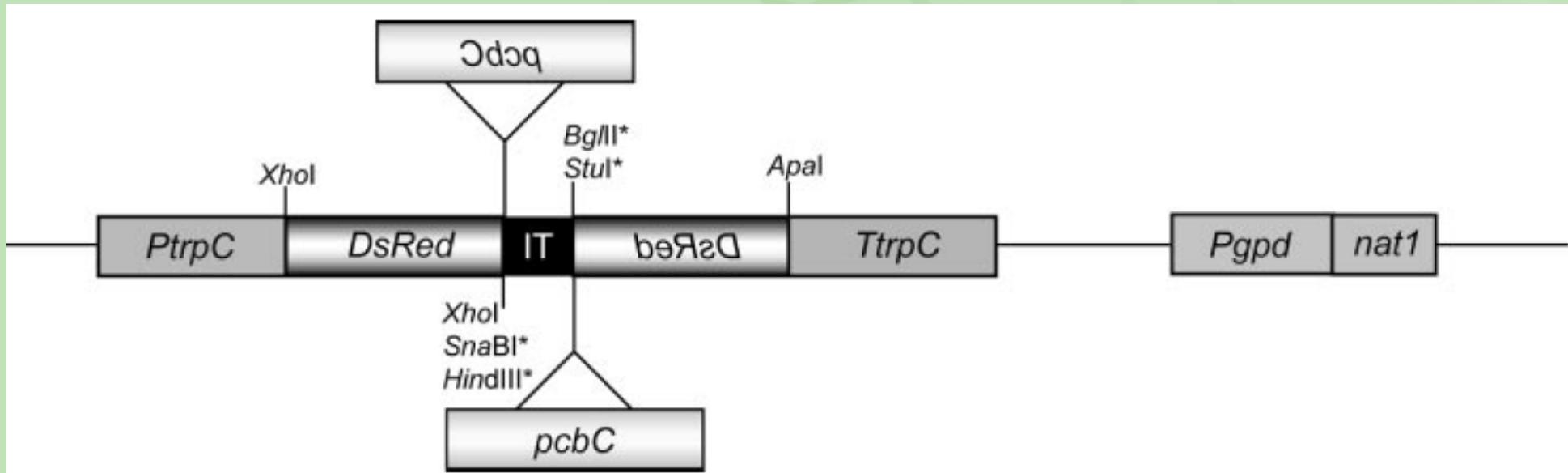
- Plasmídeo base: pRedi
 - Votor já contendo cassete de RNAi para o gene DsRed
 - Verificar a possibilidade de reutilizar esse votor para o gene alvo
 - Estudar as digestões e junções necessárias
- Gene Alvo
 - Predição de siRNA no cDNA do gene
 - Ferramenta DSIR: <http://biodev.cea.fr/DSIR/DSIR.html>
 - Conferir sítios de restrição compatíveis
 - *ApaI/BgII* e *HindIII/Xhol*
 - Desenhar primers para fragmento de 400-500 pb com caudas



Silenciamento via iRNA



Silenciamento via iRNA



Silenciamento via iRNA

Montagem do primeiro fragmento no Cassete

- Digestão do plasmídeo
 - Retirar o fragmento atual para poder inserir o novo
 - Fragmento 1:
 - Digerir com *Hind* III e *Xho* I (1 µg de plasmídeo)
 - Separar em gel de agarose, purificar e quantificar a banda do plasmídeo
- Amplificação do fragmento
 - Amplificar por PCR e digerir diretamente ou purificar antes
 - Digerir produto de PCR com *Hind* III e *Xho* I (500-1000 ng)
- Ligação
 - Fechar o plasmídeo com o fragmento novo
 - Proporção plasmídeo x produto: 1:3 ou 1:1 (tamanhos semelhantes)