

Metodologias para o estudo funcional de genes

Profa. Dra. Chirlei Glienke
BIOGEMM-UFPR

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

– Exemplo:

Phytopathology • XXXX • XXX:X-X • <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}

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^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

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- 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*

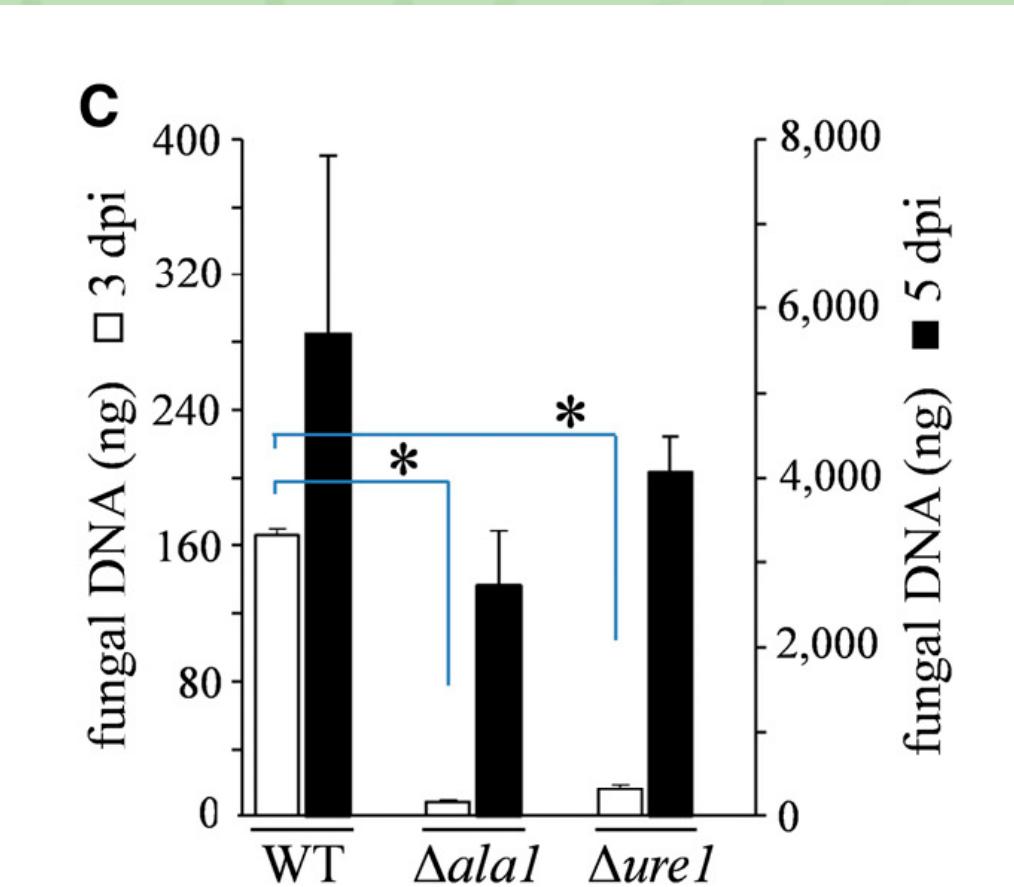
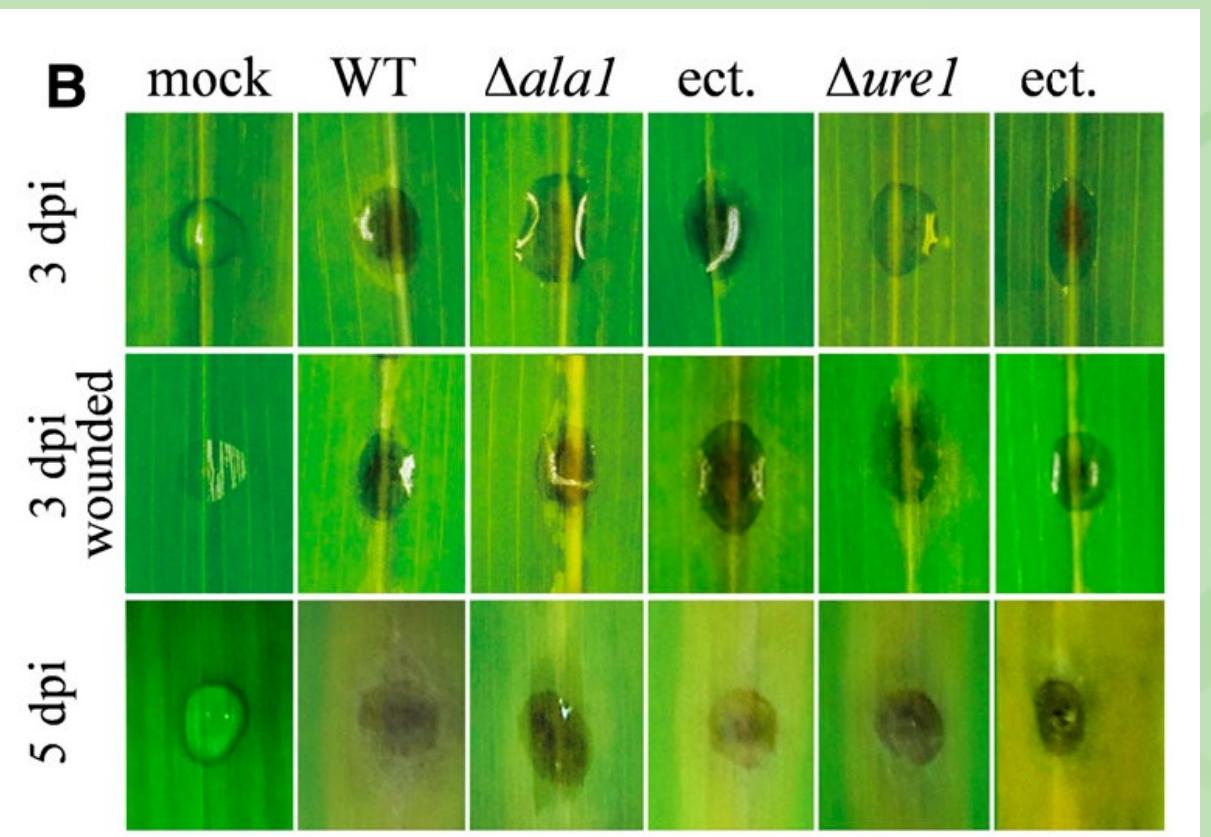
Ely Oliveira-Garcia^{1,†} and Holger B. Deising^{1,2,*}

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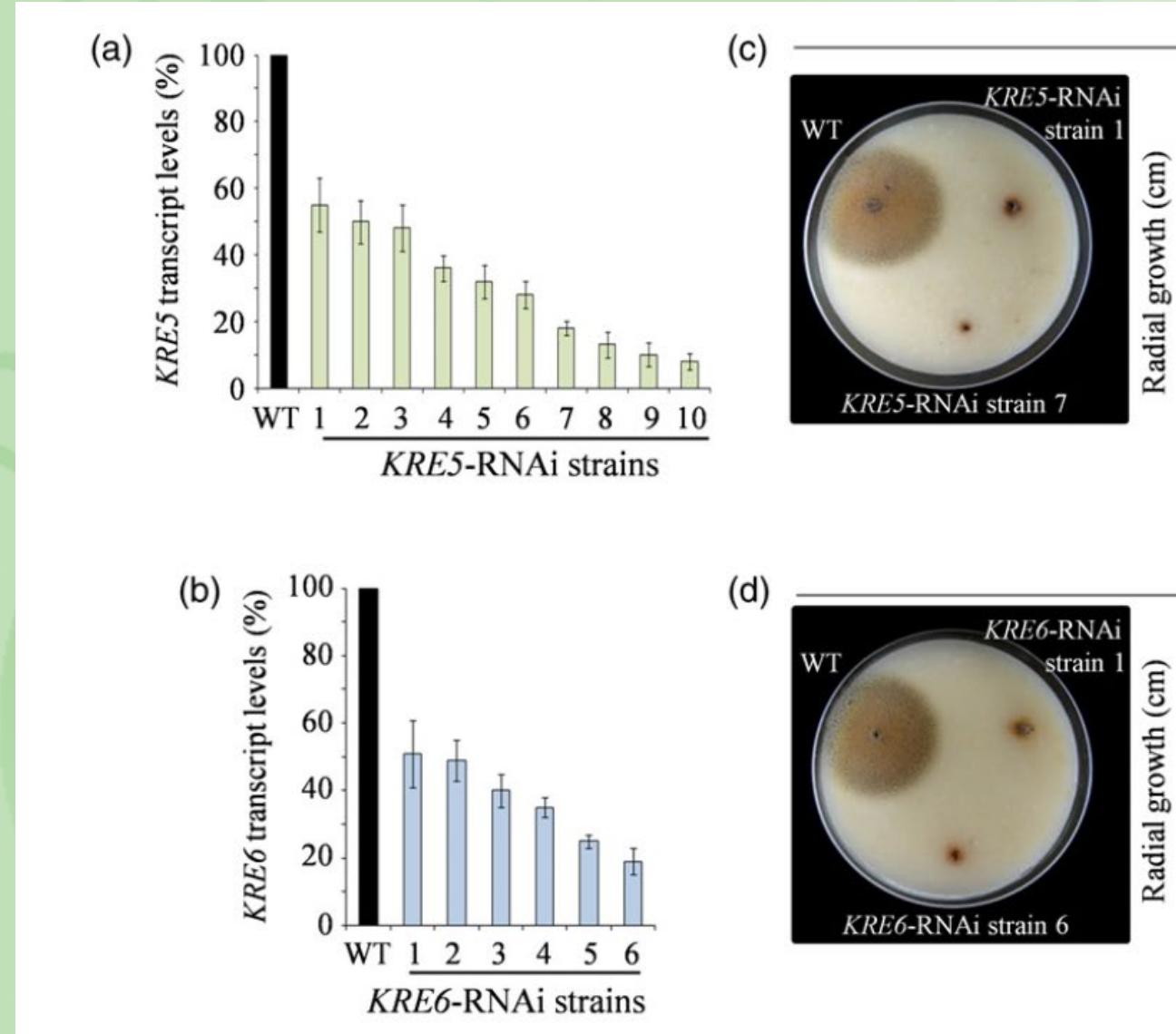
Metodologias que podem ajudar a responder perguntas

- qPCR: Análise comparativa de massa fúngica em lesões
 - Uso de DNA da lesão (avaliar quantidade de DNA do fungo na lesão)

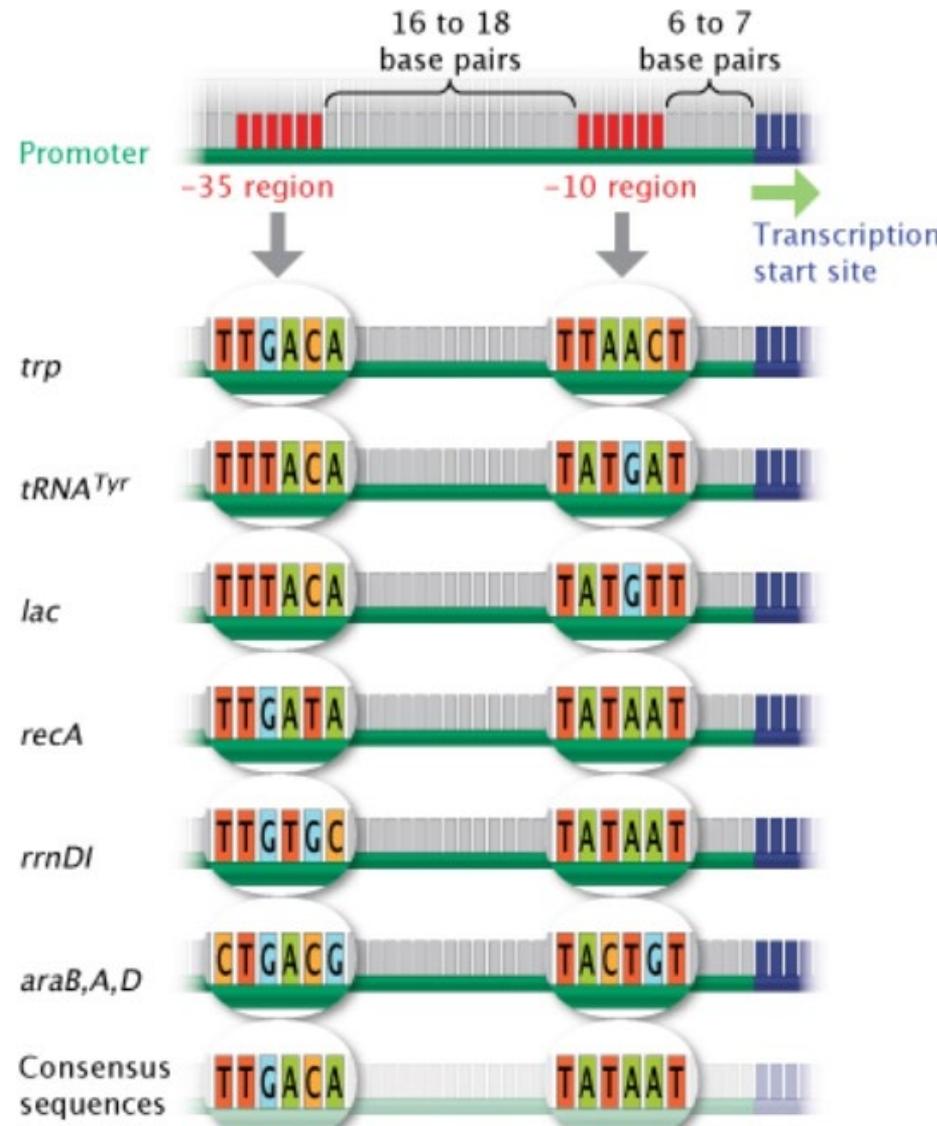


Metodologias que podem ajudar a responder perguntas

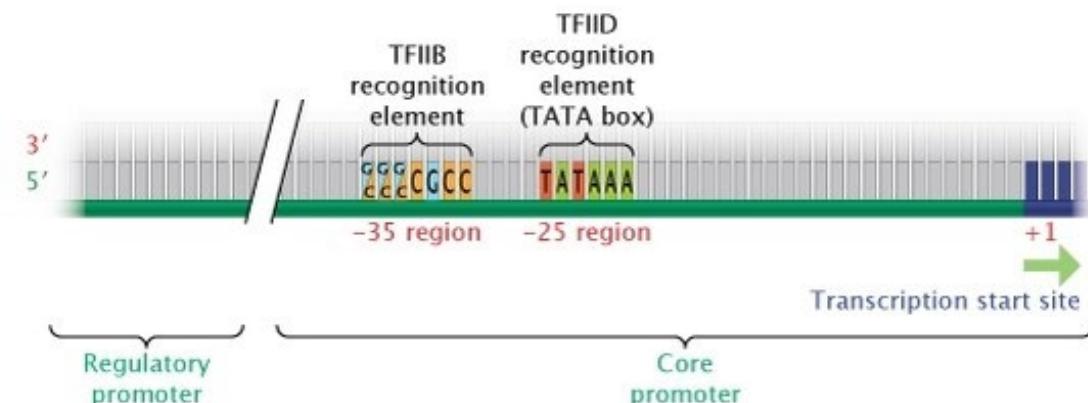
- RT-PCR: Análise de expressão de genes (nível de mRNA)
 - Se, quando e quanto um gene é expresso
 - Uso de mRNA do gene específico (avaliar o quanto um gene específico foi expresso em cultivo ou na lesão)

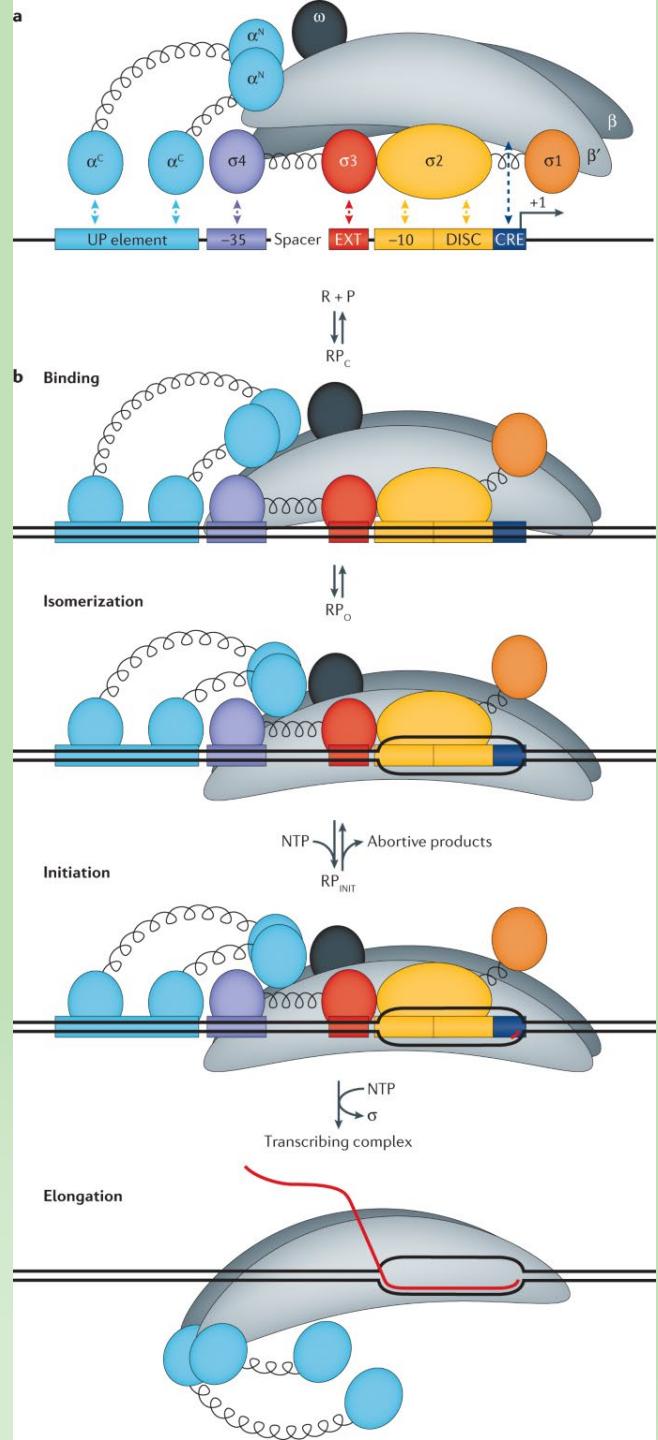


Promotor procariótico



Promotor eucariótico

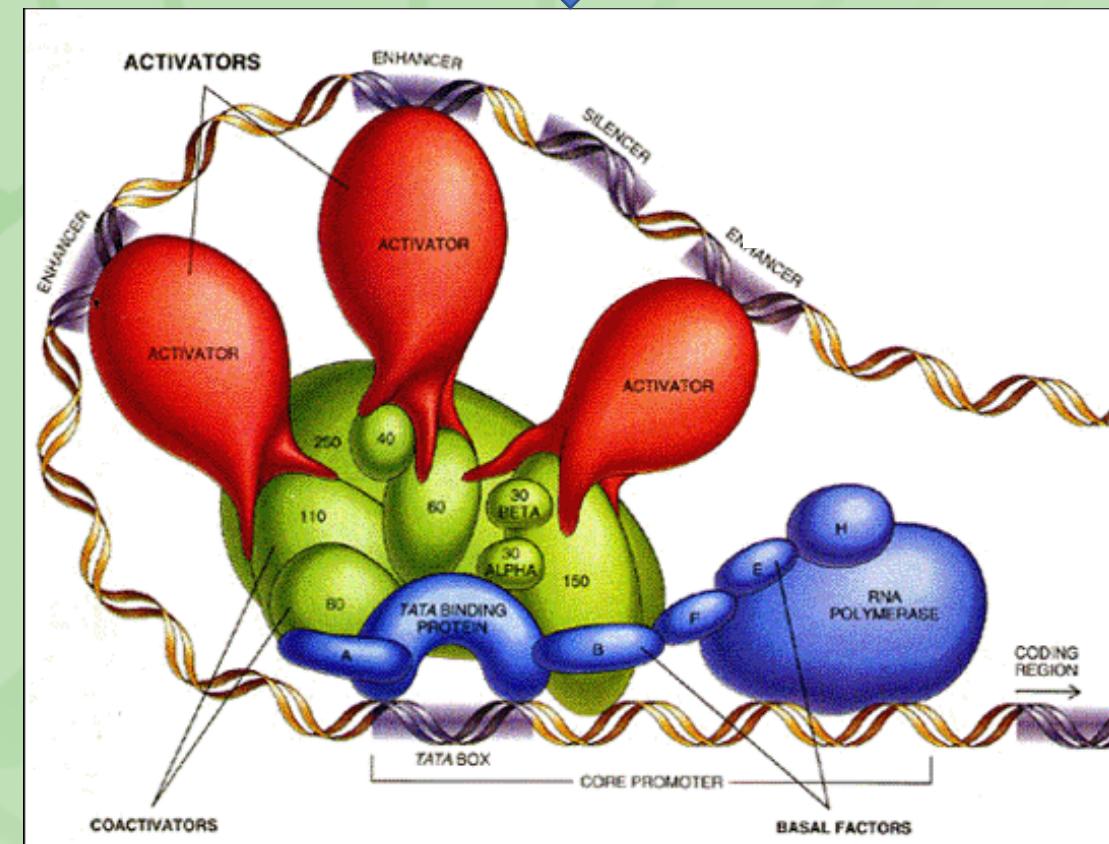




- Complexos de transcrição em procariotos e

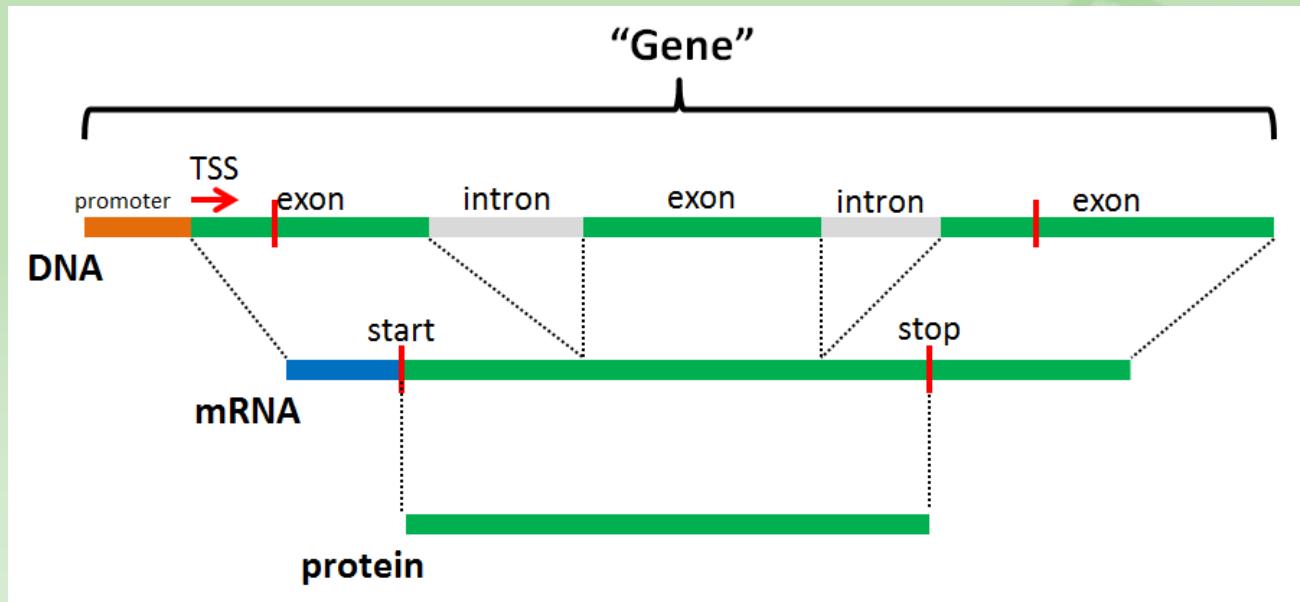


- em eucariotos

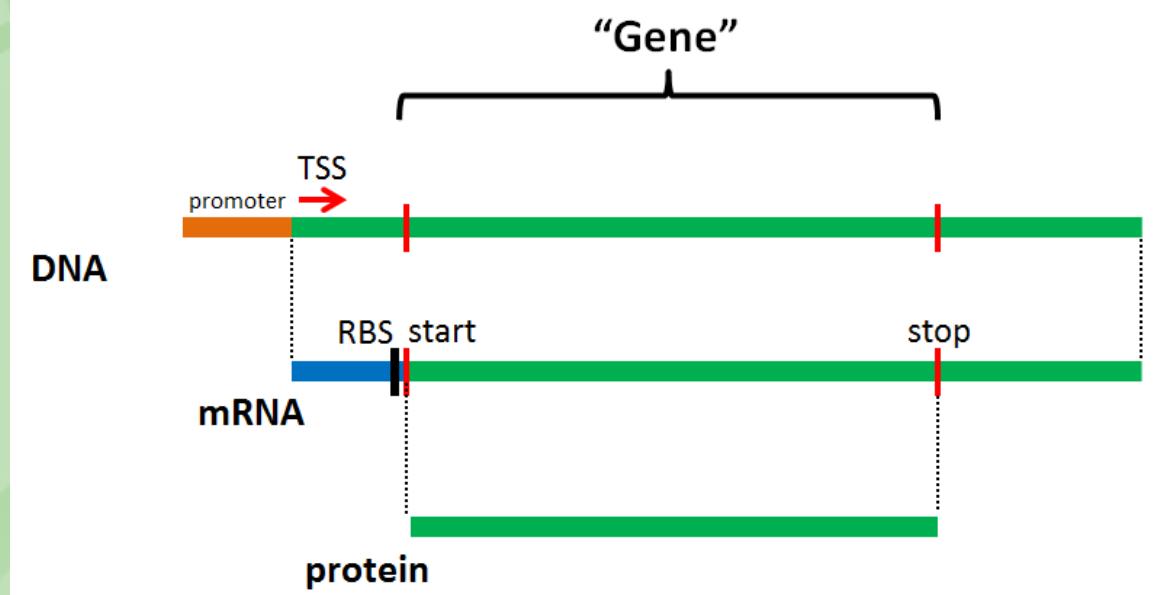


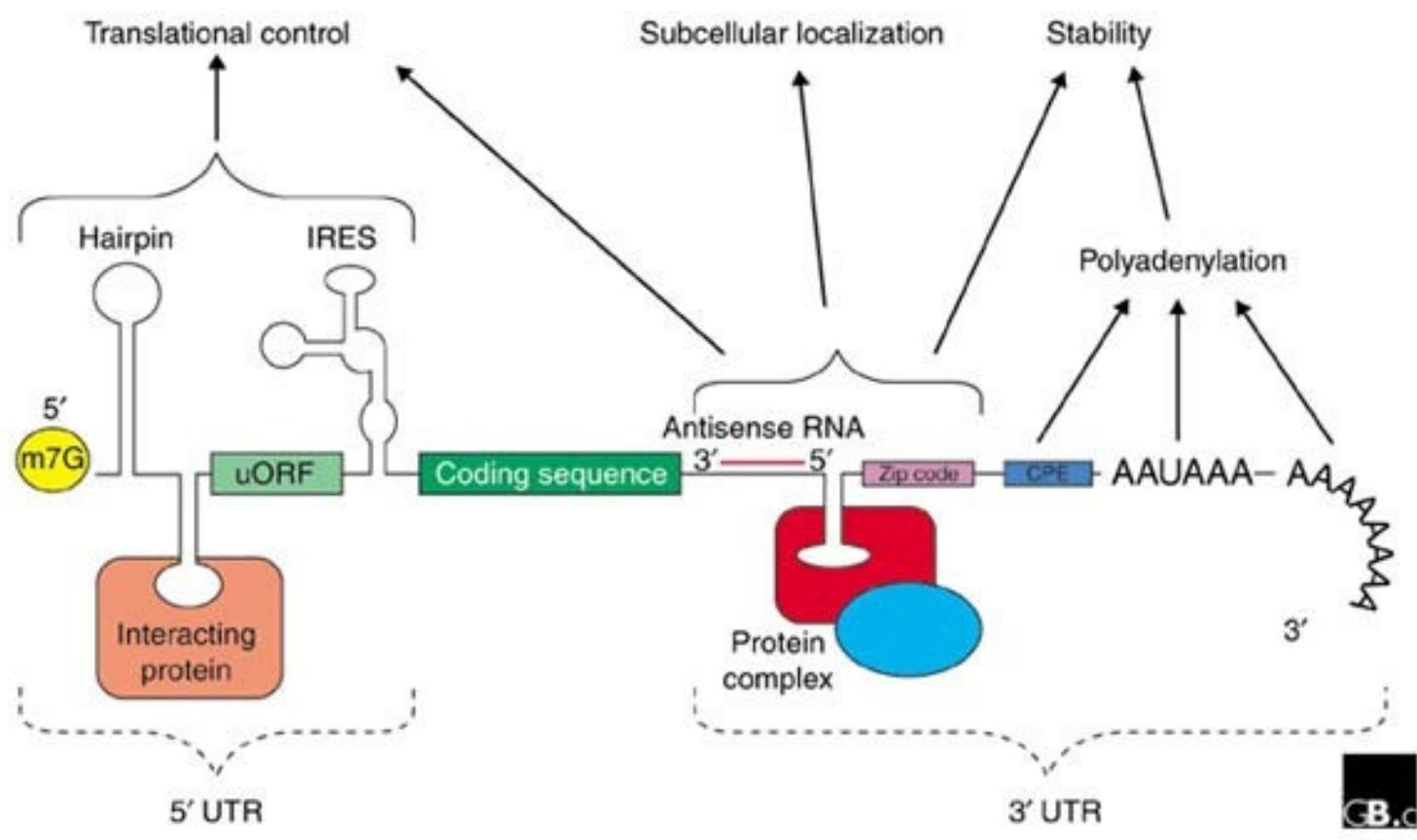
Estrutura de genes

Eucariotos



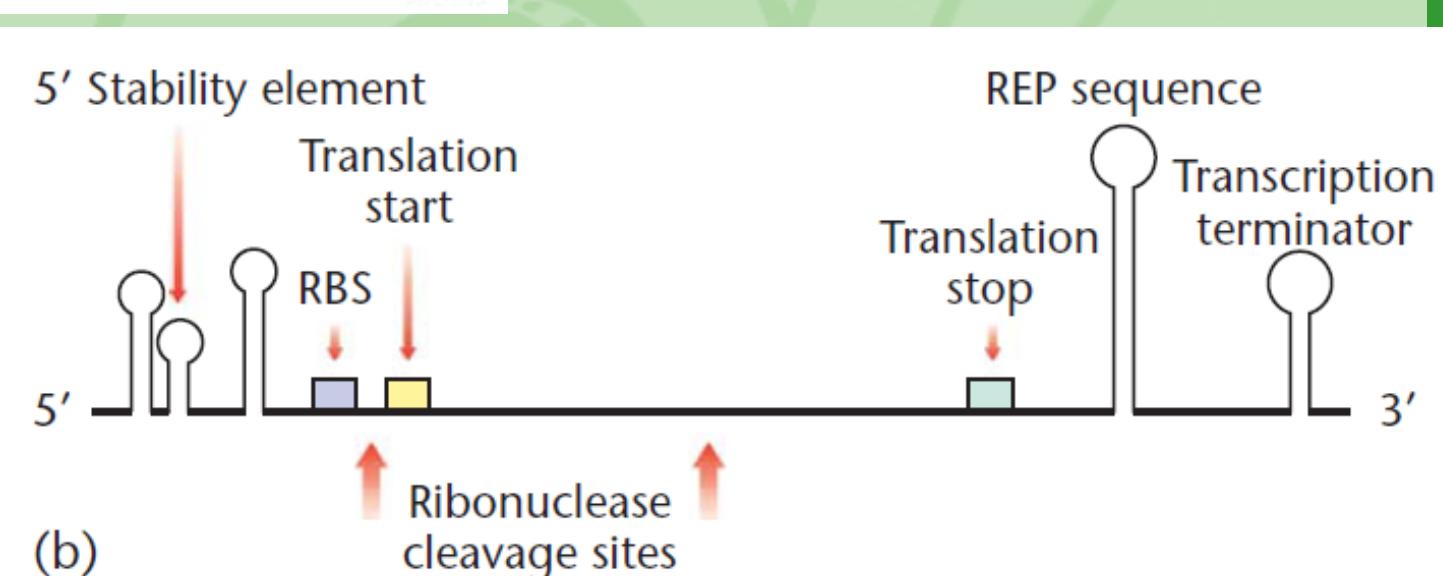
Procaríotos



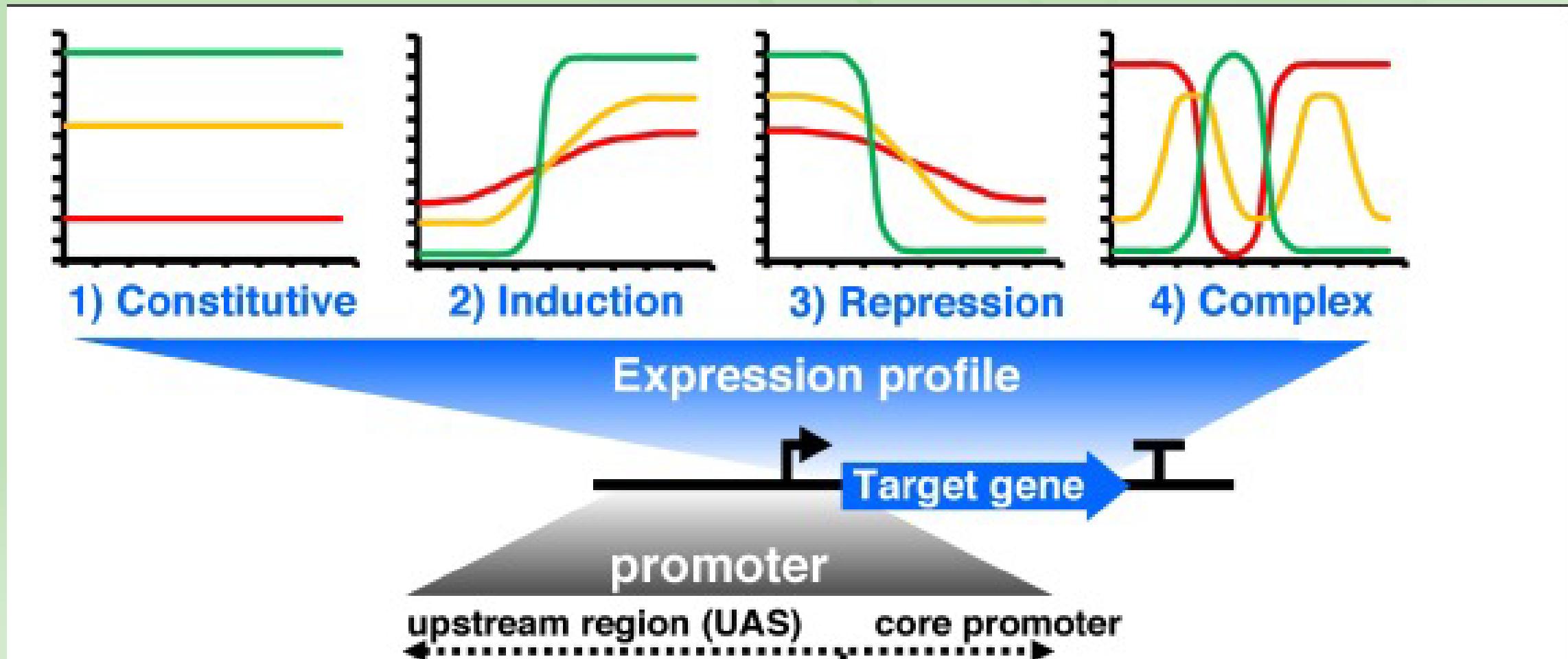


UTR, untranslated region; m7G, 7-methyl-guanosine cap; hairpin, hairpin-like secondary structures; uORF, upstream open reading frame; IRES, internal ribosome entry site; CPE, cytoplasmic polyadenylation element; AAUAAA, polyadenylation signal.

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

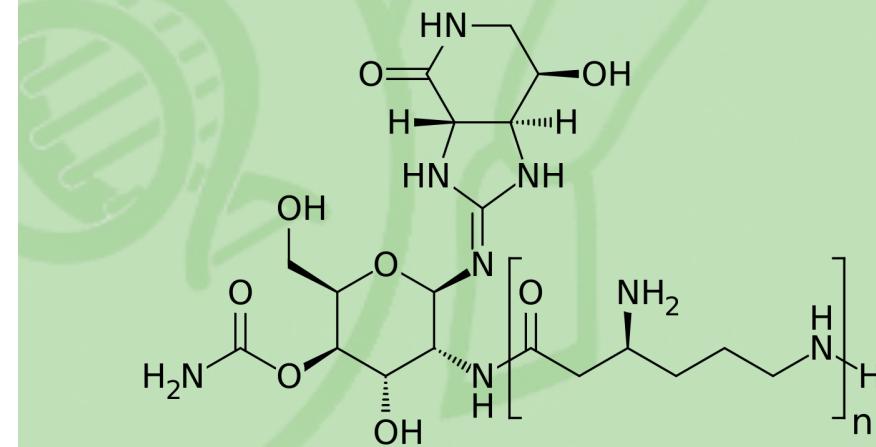
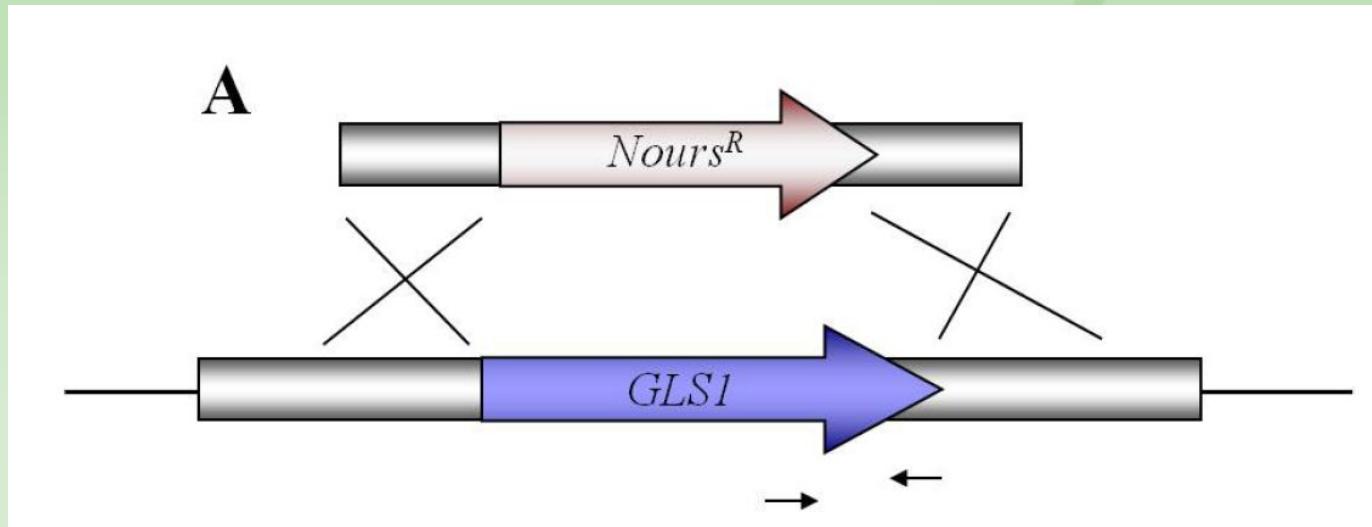


Tipos de expressão gênica



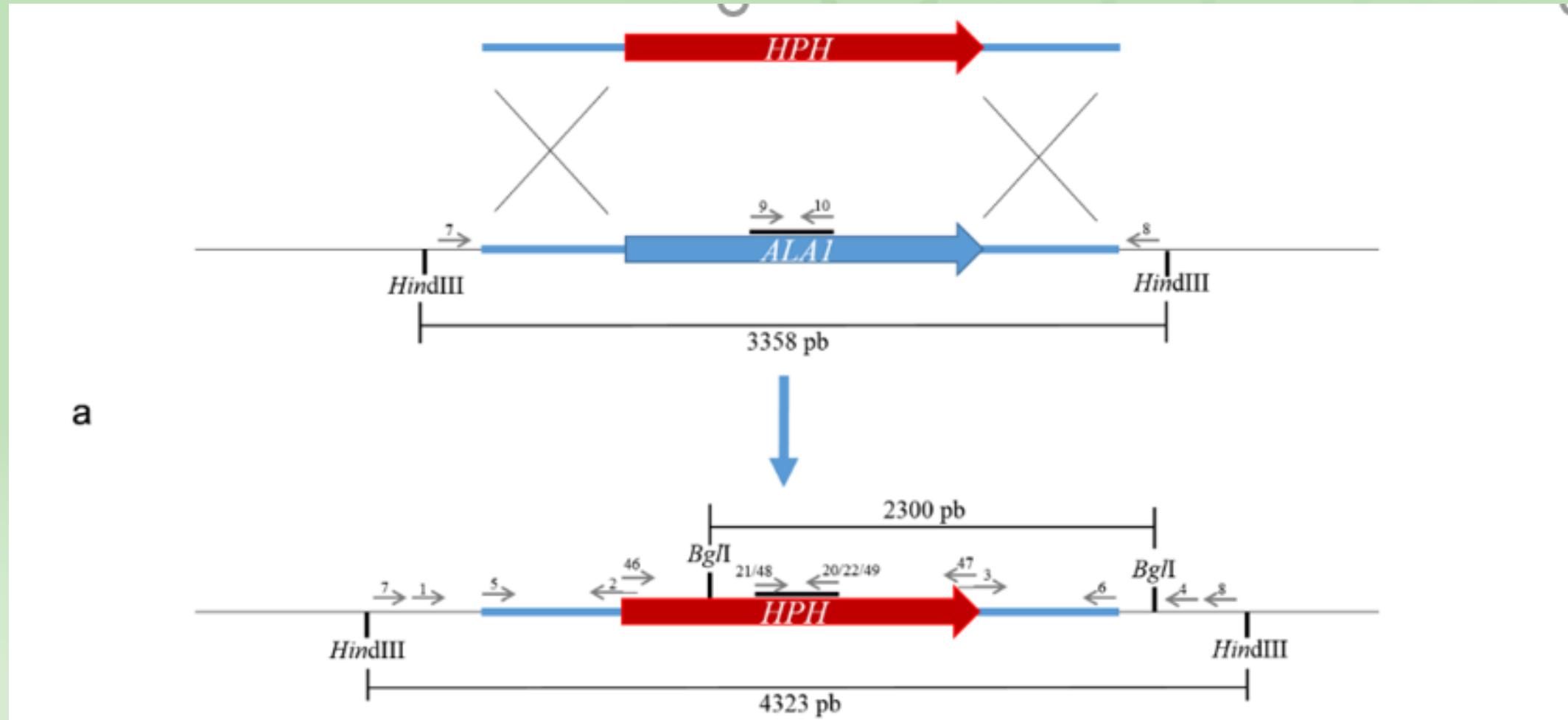
Deleção de genes

- 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)

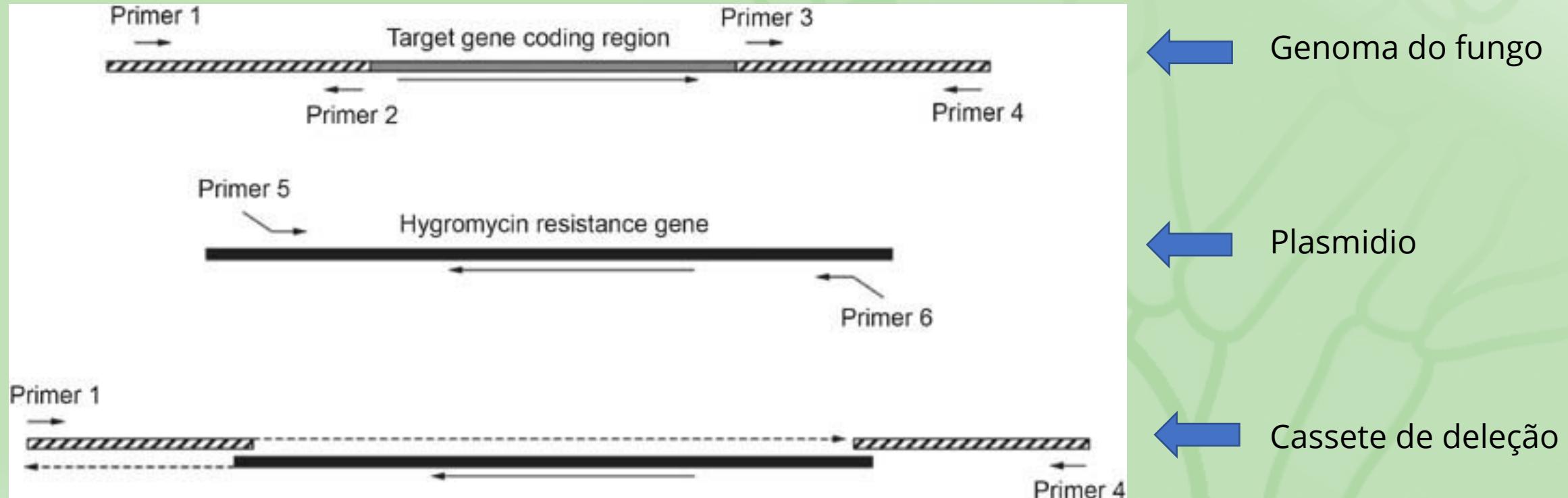


Dependente de recombinação homóloga

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



Dependente de recombinação homóloga

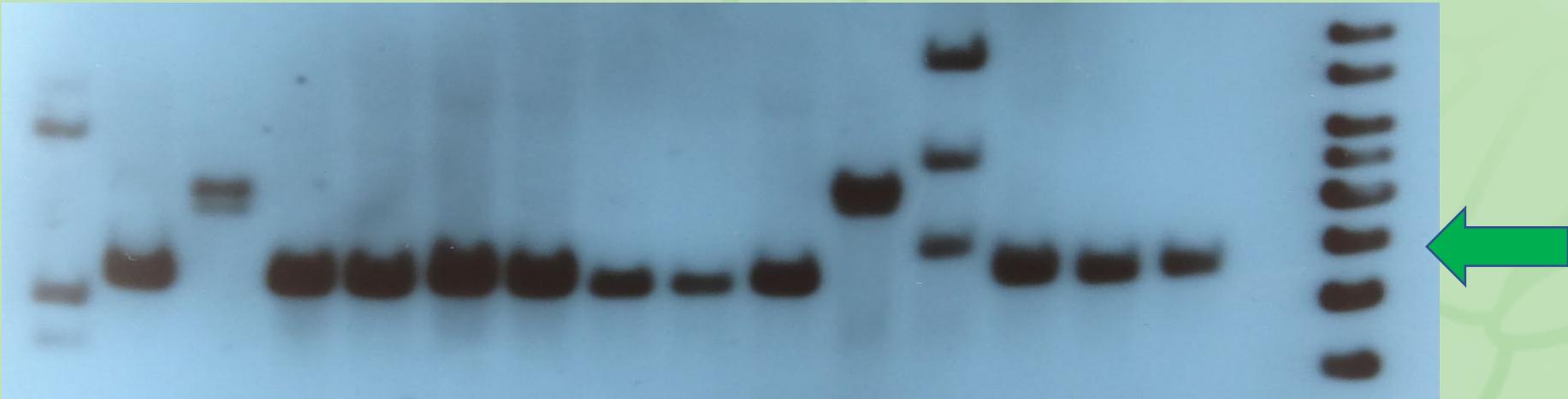


Deleção de genes

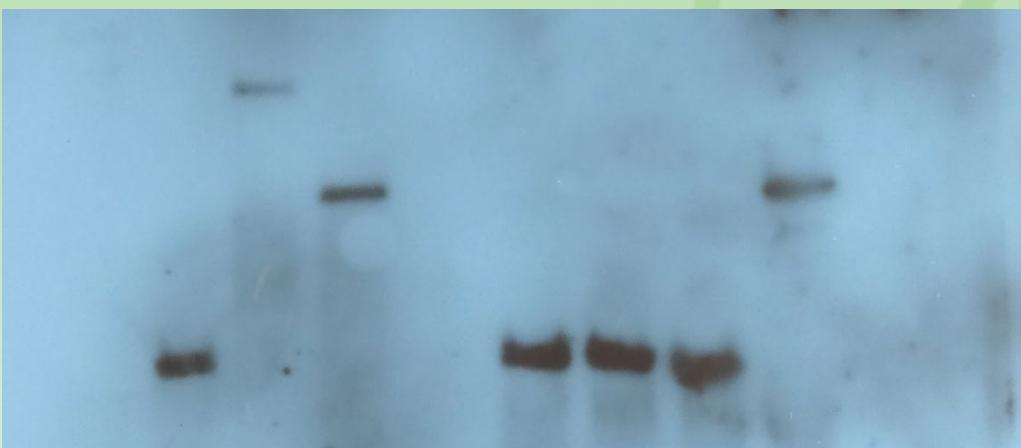
- Problemas encontrados
 - heterocromatina, regulação epigenética associado à fase do desenvolvimento, presença do hospedeiro, etc
- Critérios para publicação
 - 3 mutantes independentes e de cópia única (southern blot)
 - Complementação com o próximo gene do selvagem
 - Complementação de leveduras

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

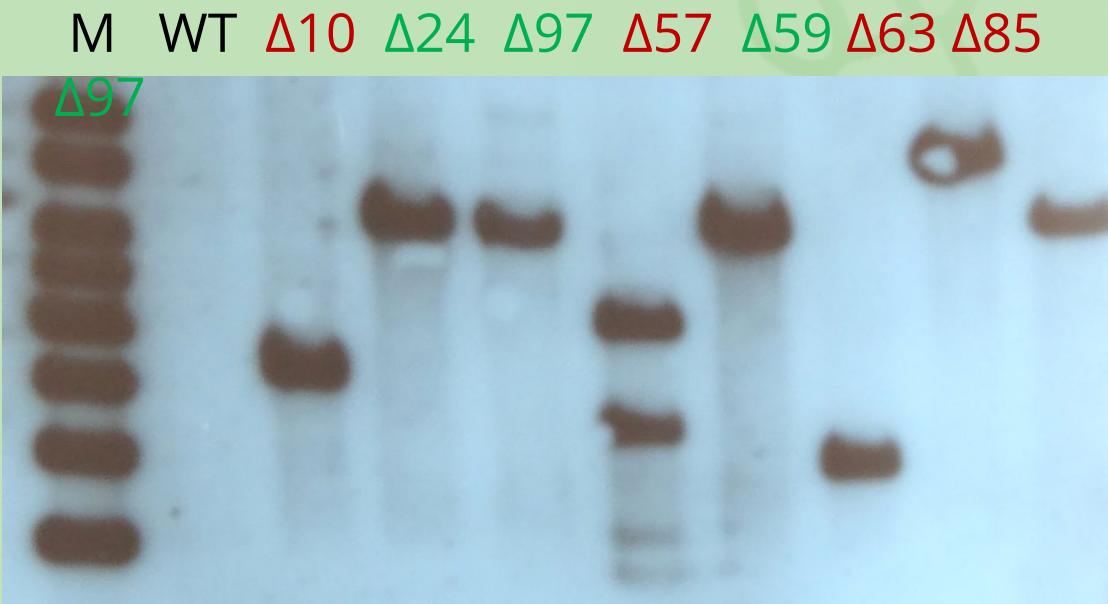


WT Δ2 Δ103 Δ4 Δ5 Δ6 Δ12 Δ13 Δ14

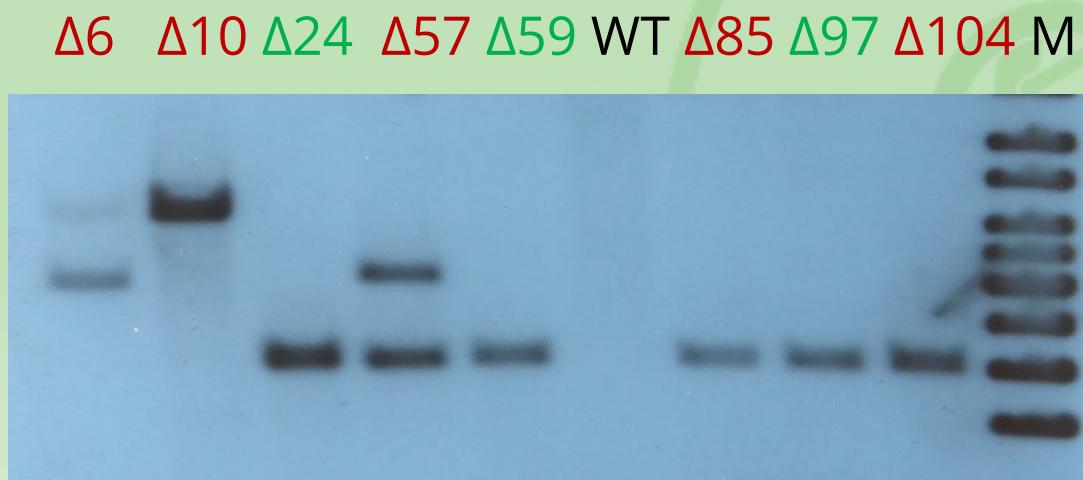


**Allantoicase Mutants + *Hind*III
Probe: Allantoicase**

Deleção de genes



Urease Mutants +
Scal
Probe: Hygromycin

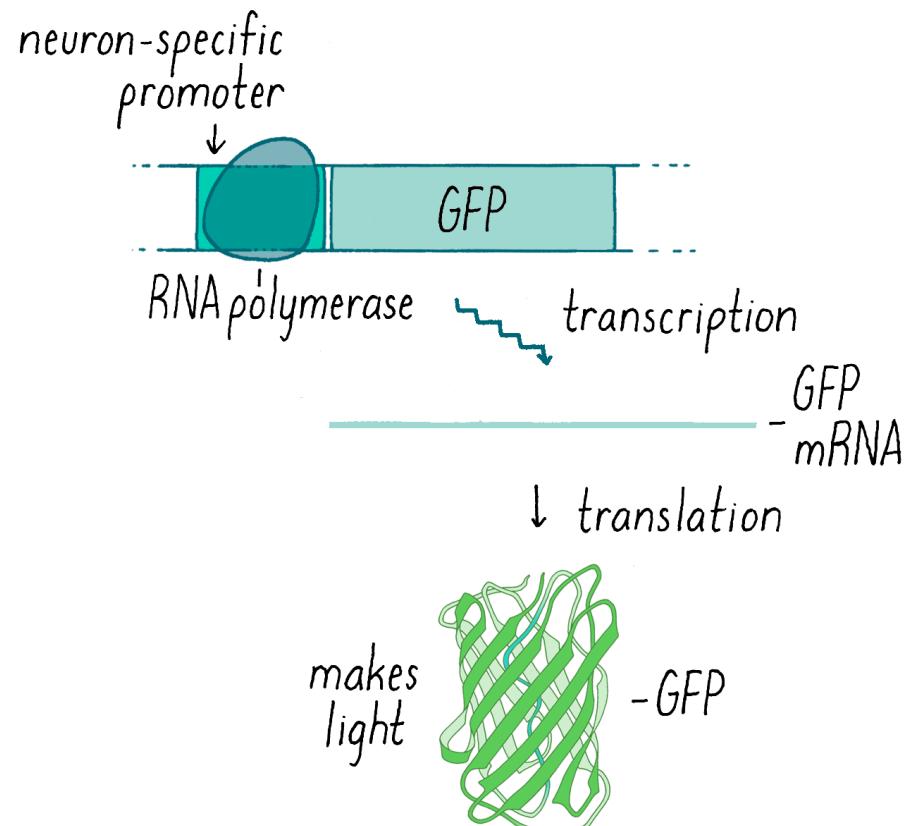


Urease Mutants + *Ncol*
Probe: Hygromycin

Fusão promotor - GFP

Verificar quando um gene é expresso

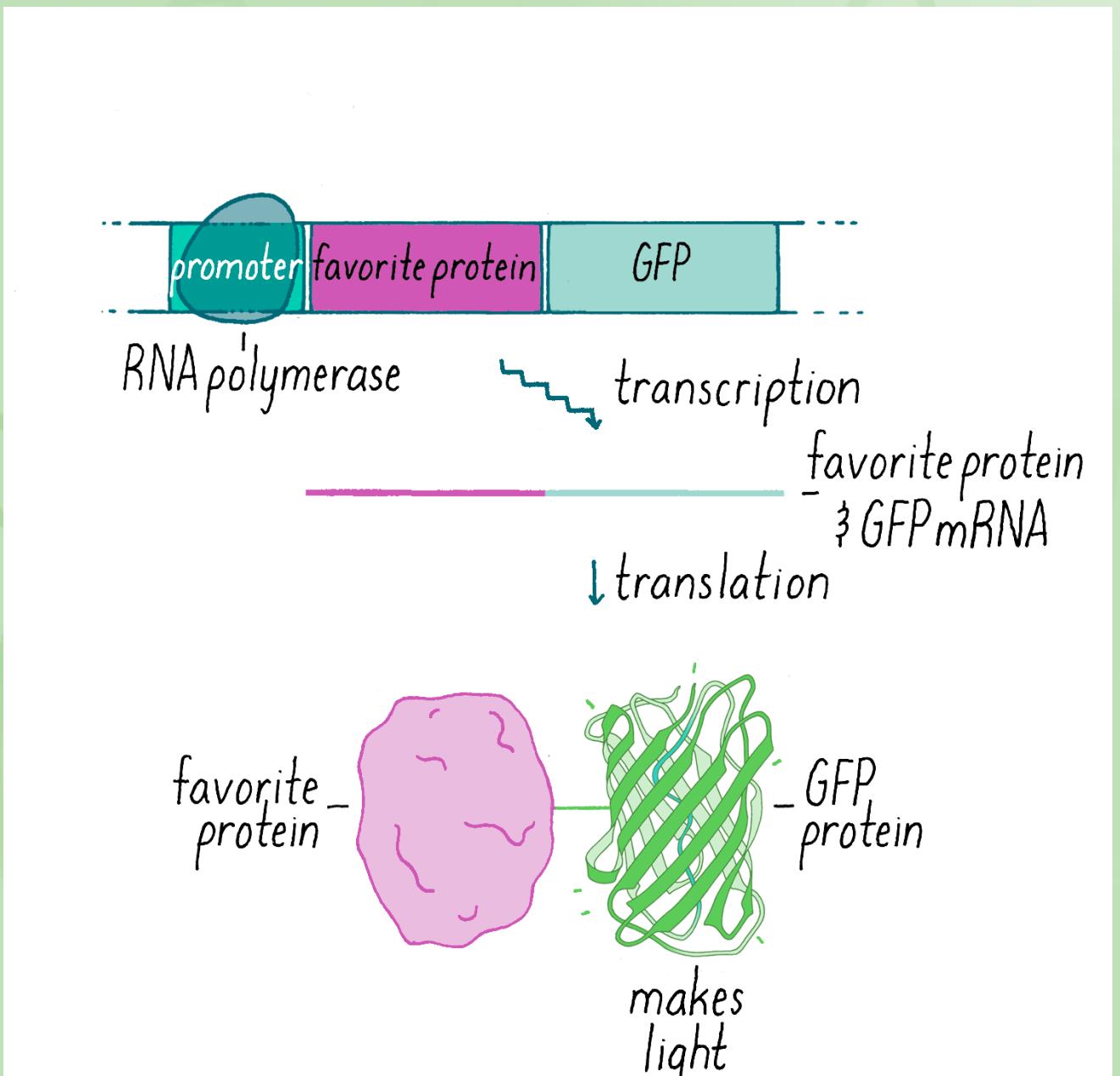
GFP TRANSCRIPTION AND TRANSLATION IN NEURONS



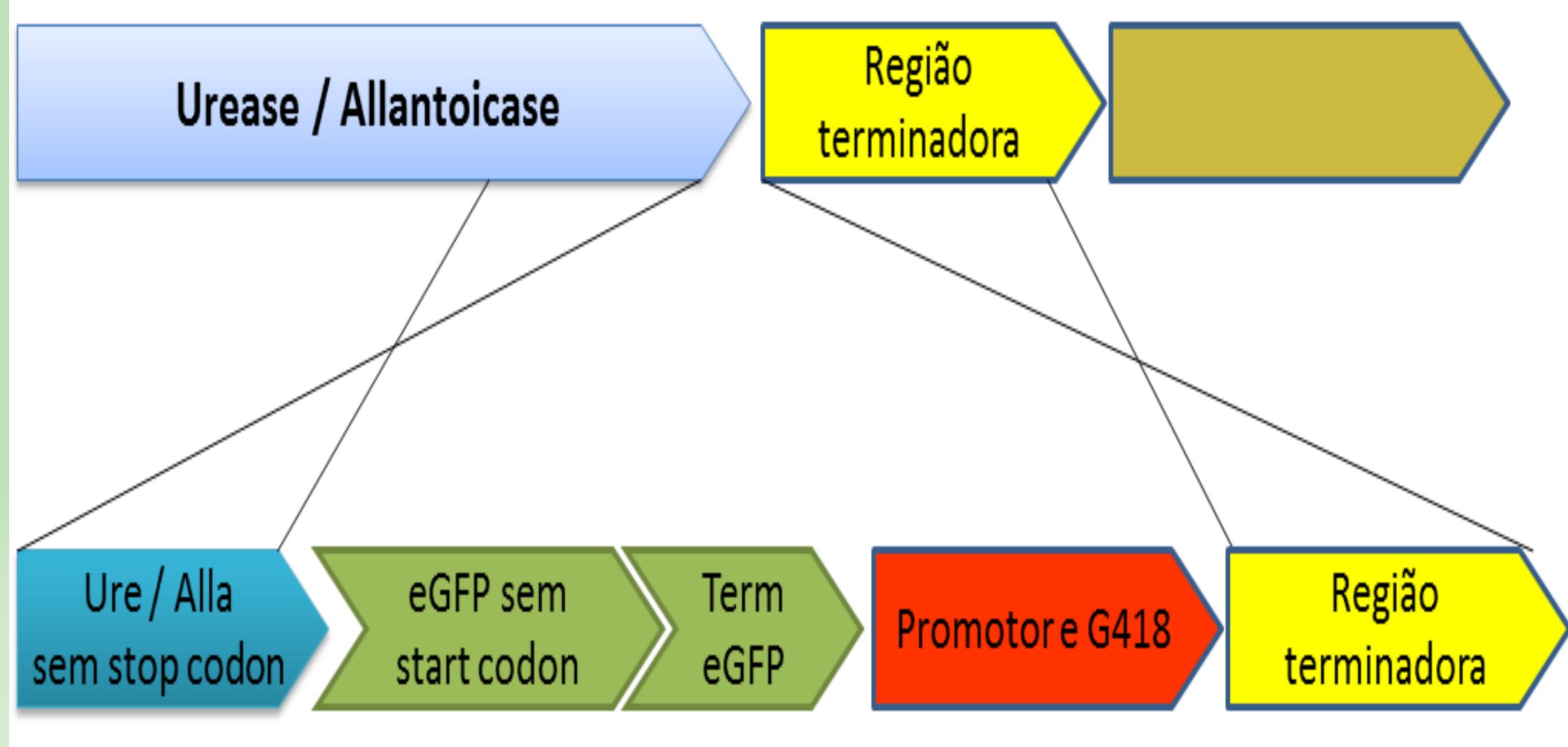
Fusão gene - GFP

1. Verificar quando um gene é expresso

2. Localização da proteína



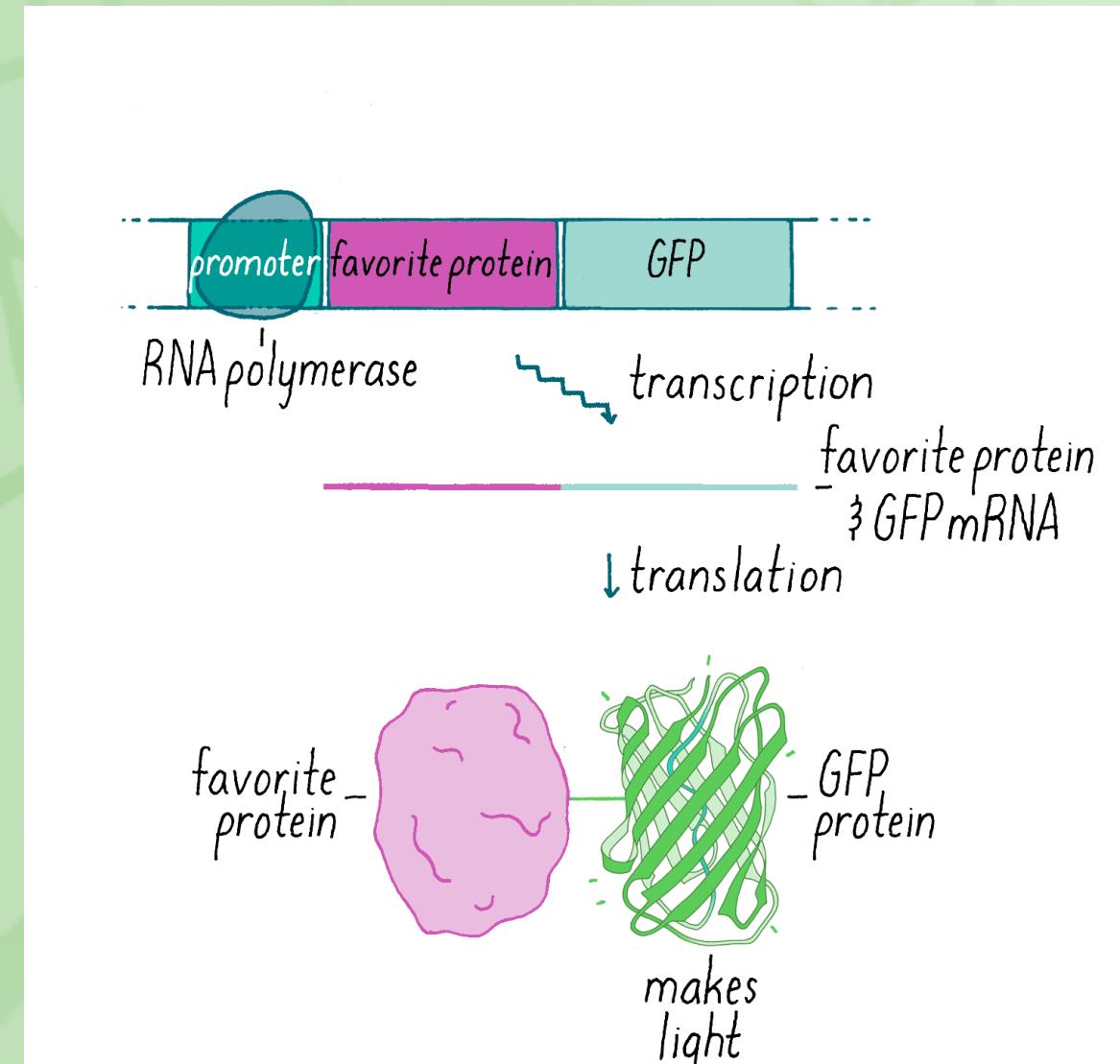
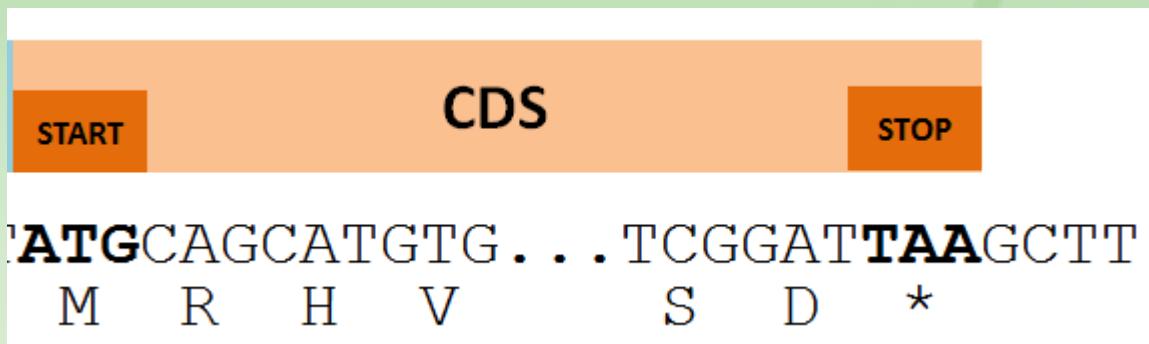
Estratégia para montagem de cassete de fusão



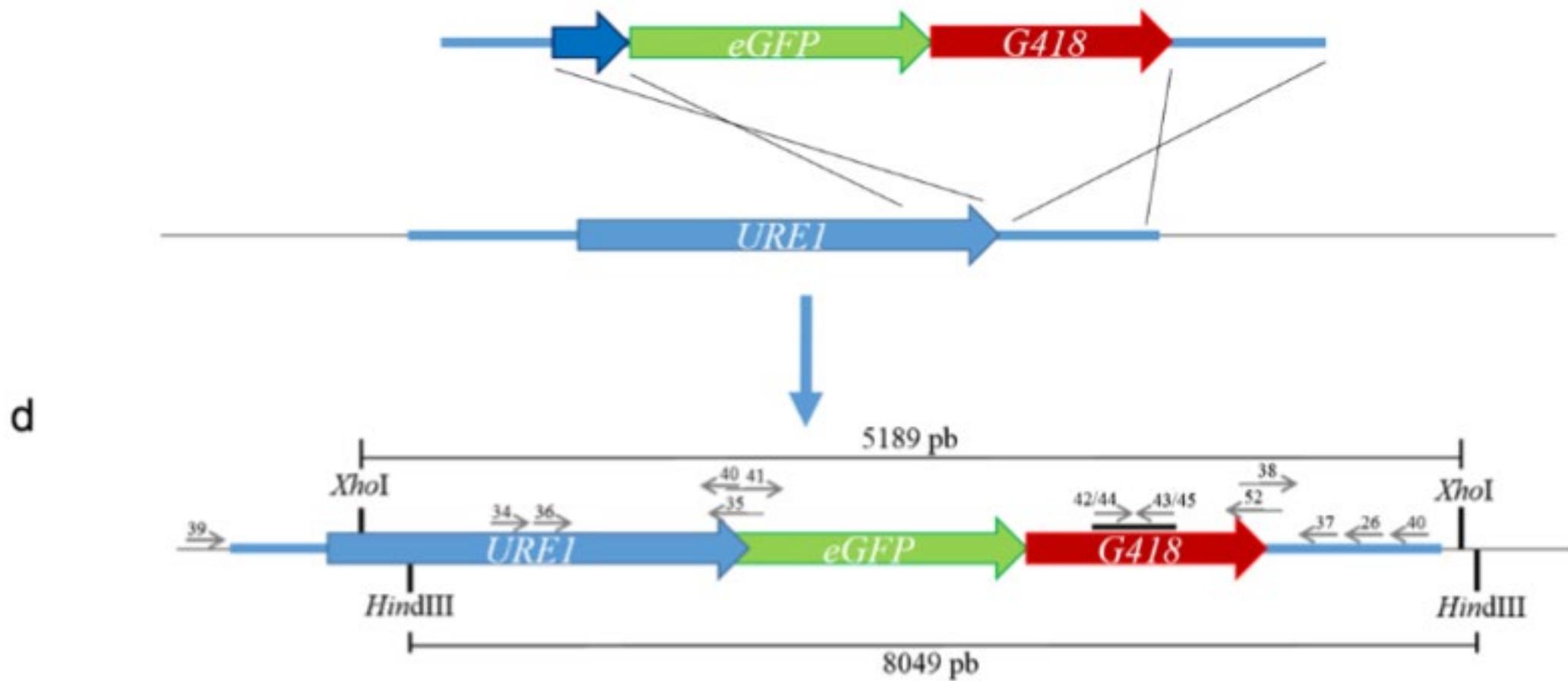
Fusão gene - GFP

Gene de interesse sem o
STOP codon

Gene GFP sem o **START**
codon

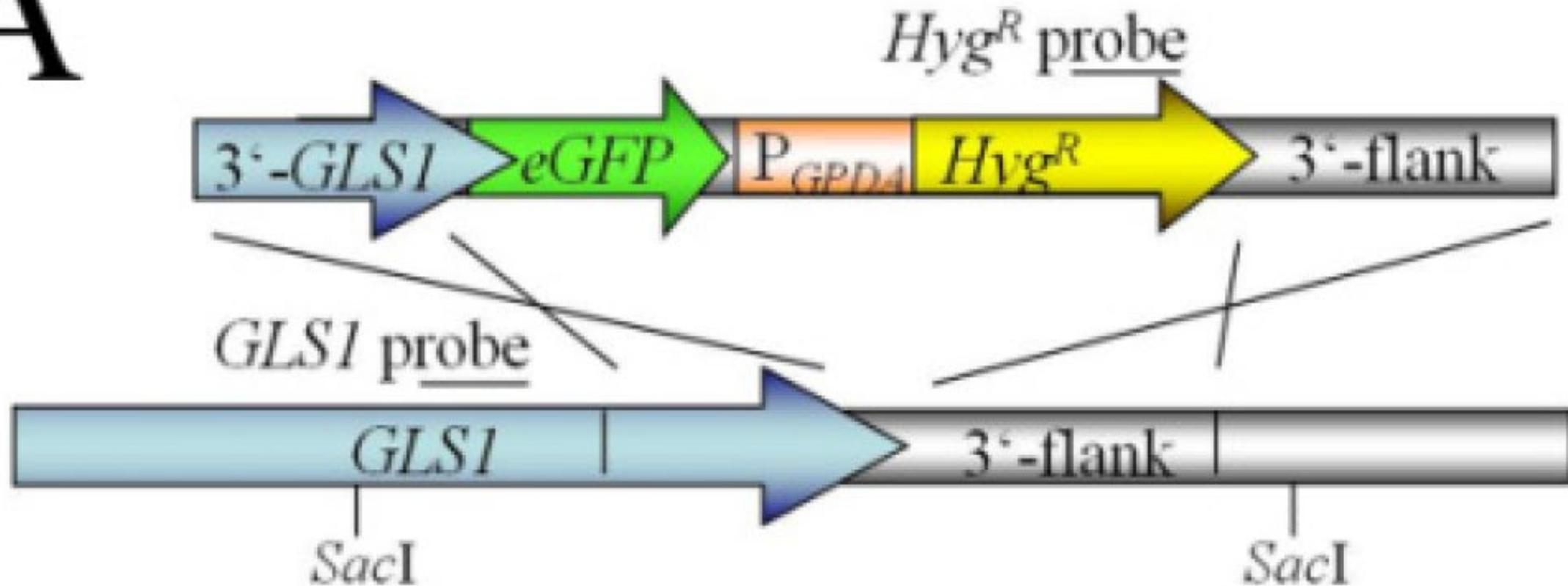


Fusão com gene para proteína fluorescente (eGFP)

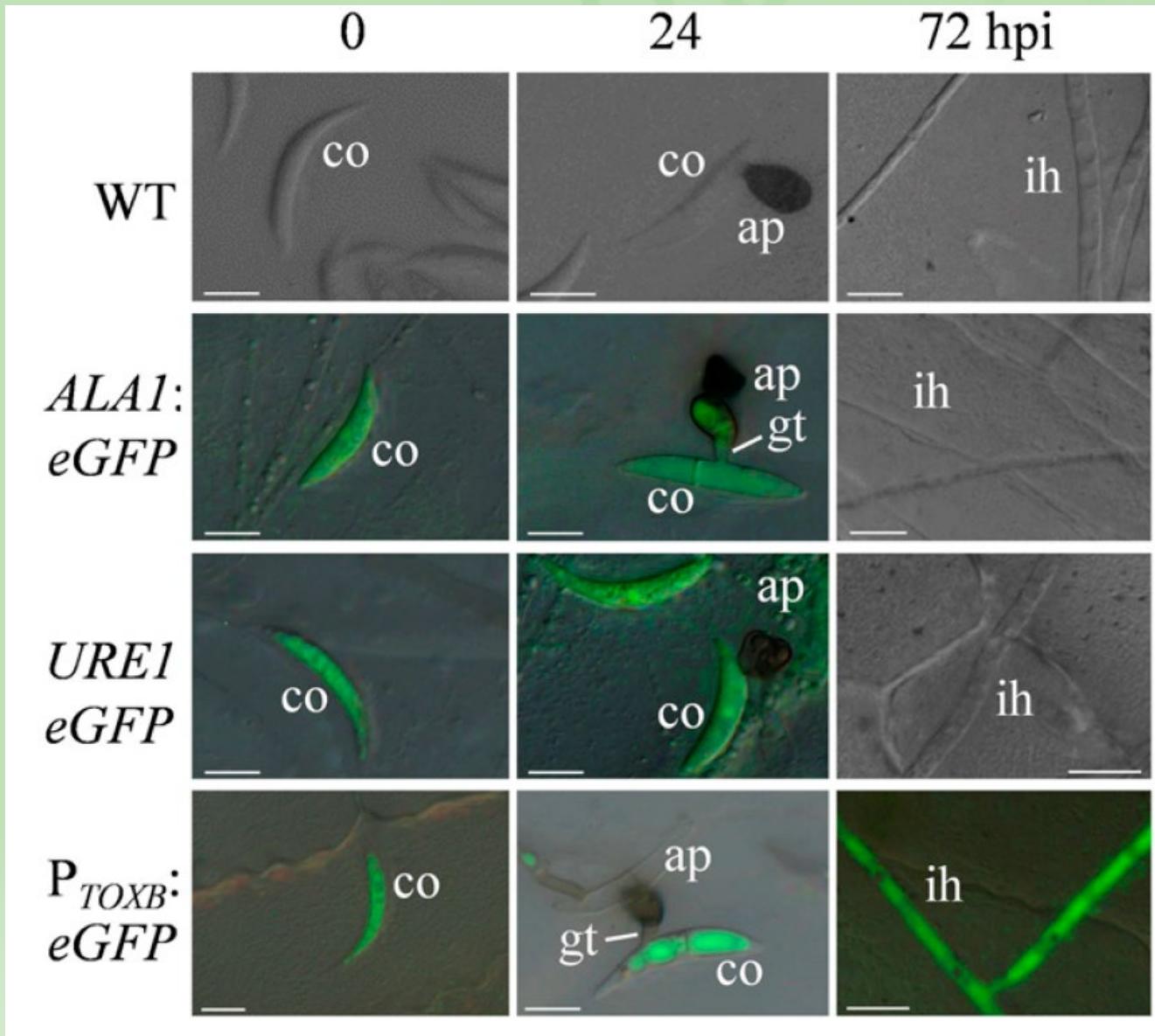


Fusão com gene para proteína fluorescente (GFP)

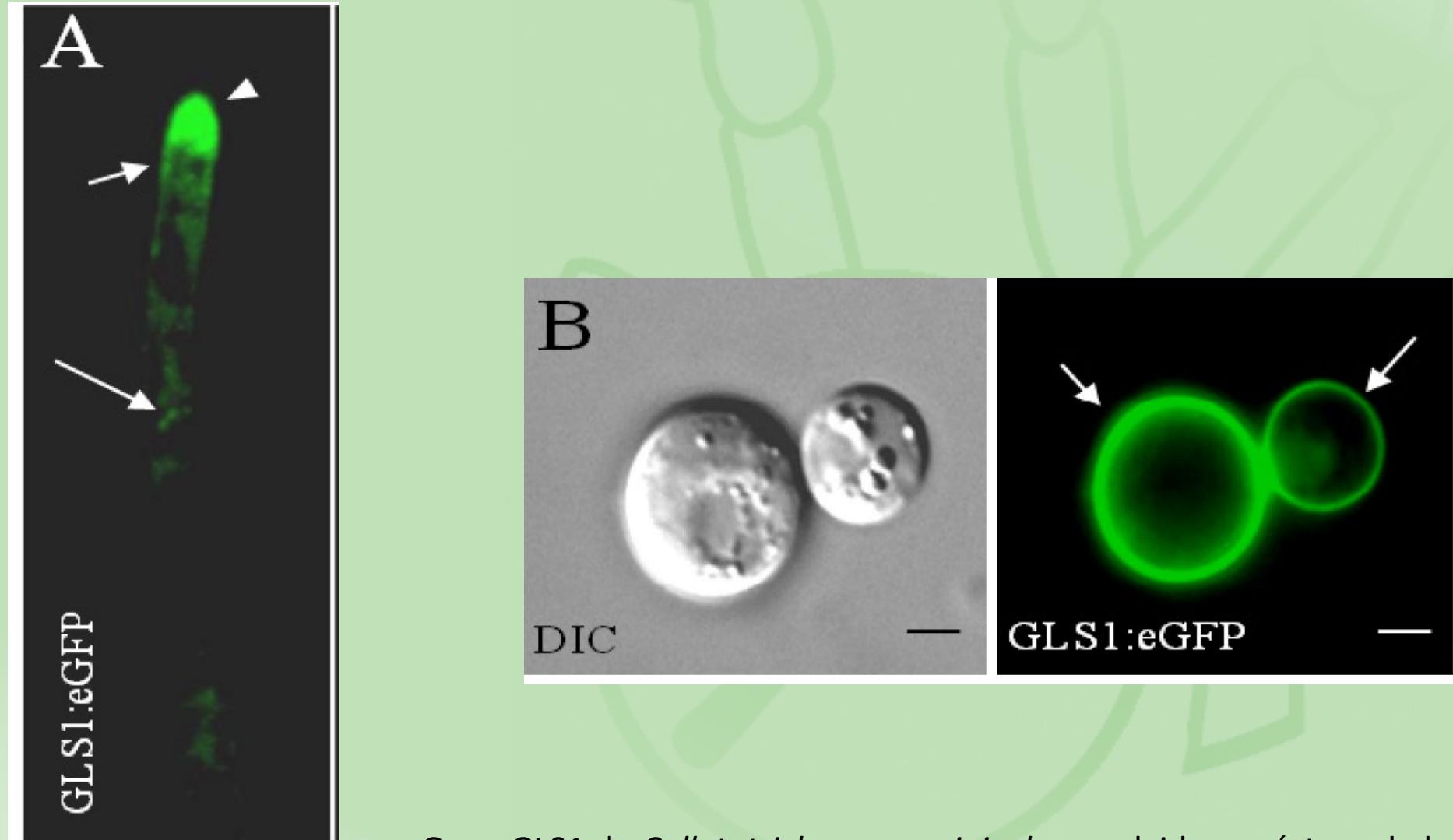
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Análise temporal da expressão do gene

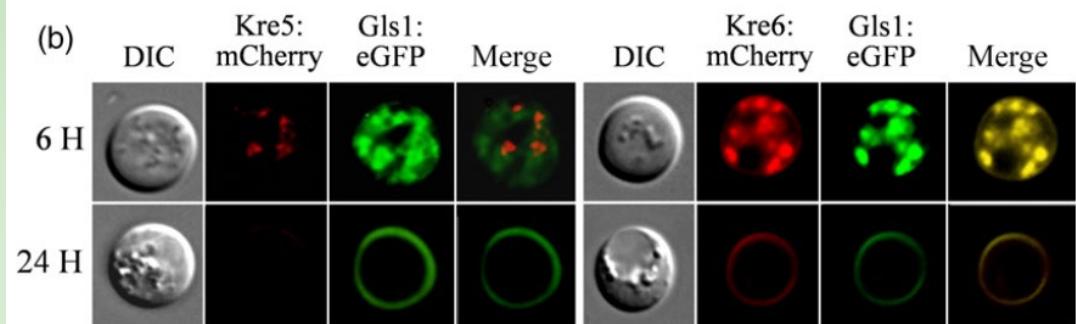
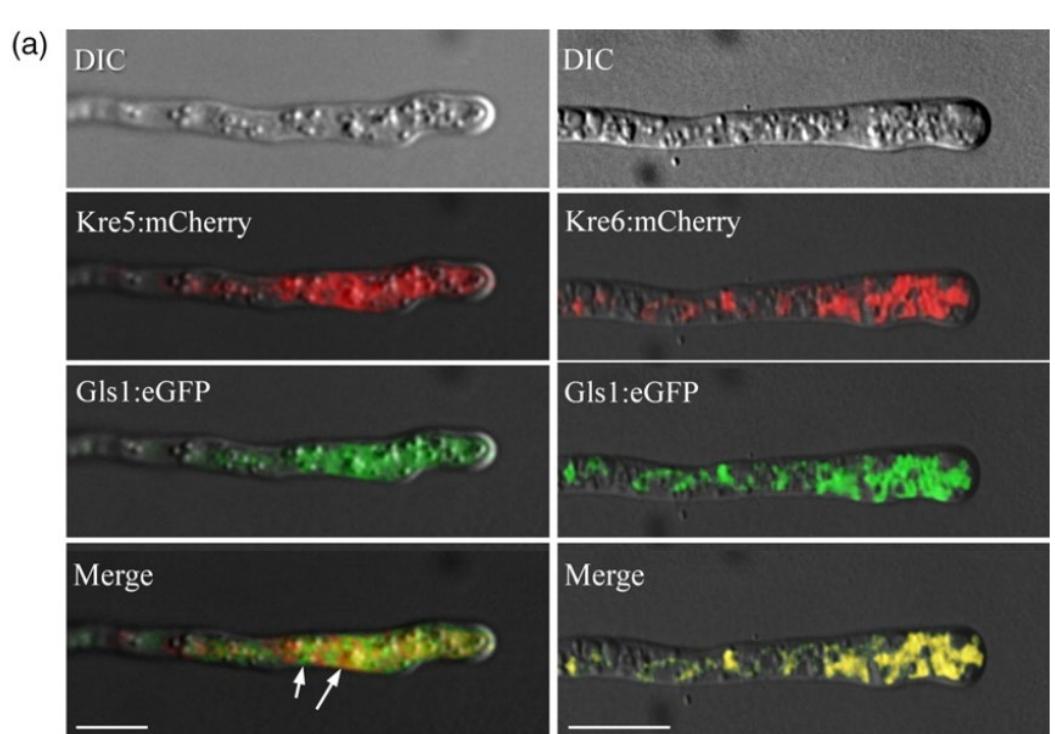


Análise temporal da expressão do gene e localização da proteína



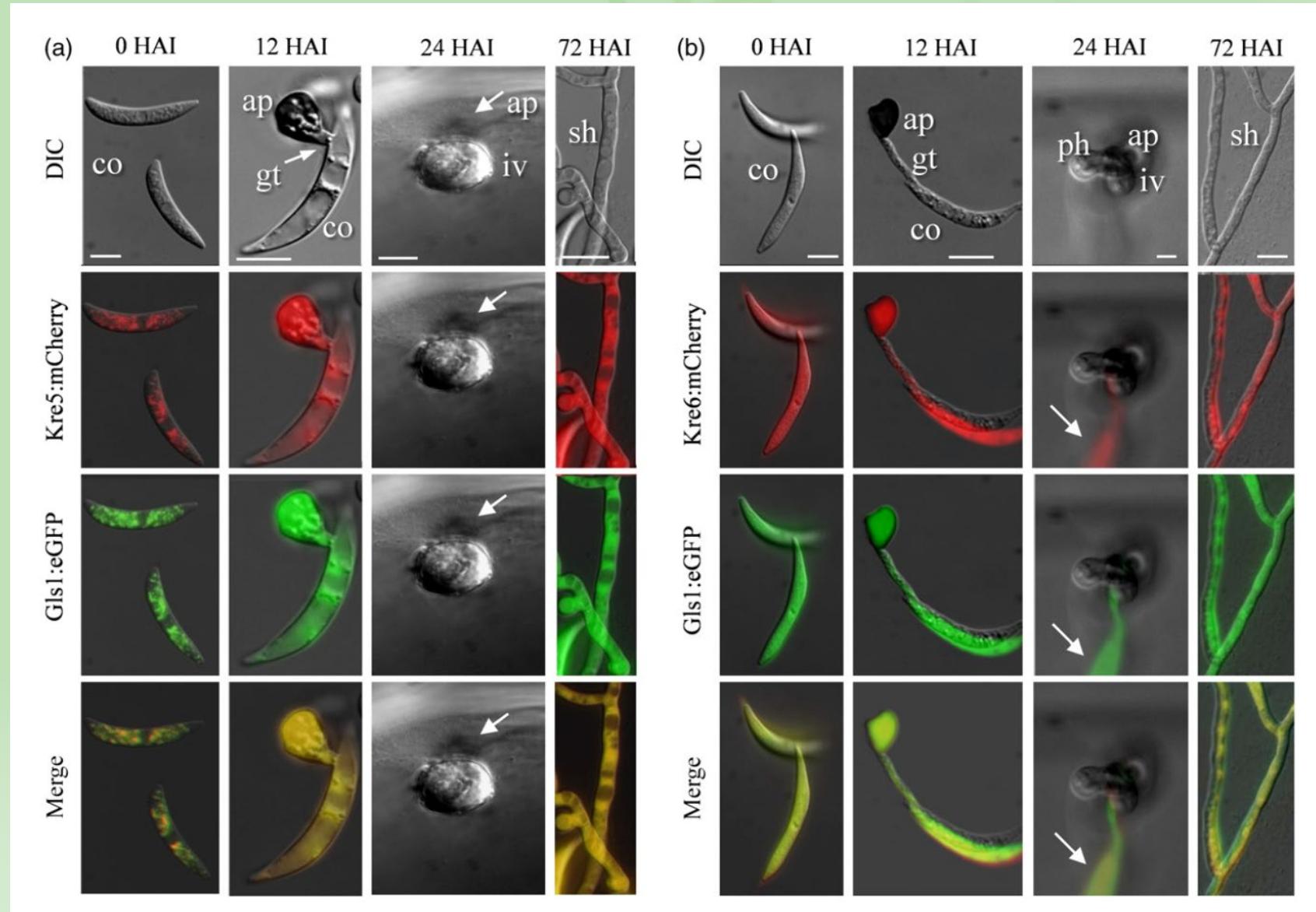
Gene *GLS1* de *Colletotrichum graminicola* envolvido na síntese de b-1,3-glucan

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

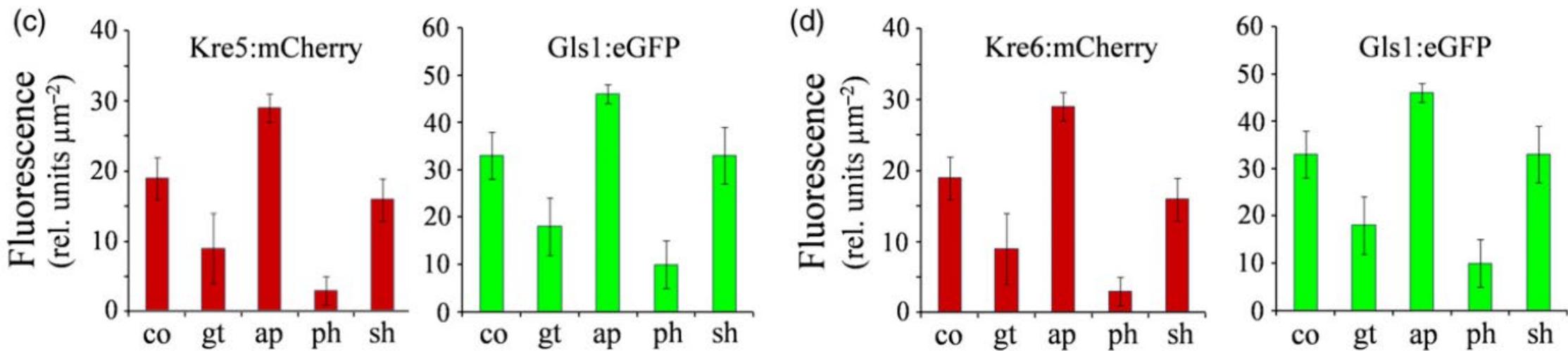


Genes KRE5 e KRE6 de *Colletotrichum graminicola* envolvidos na síntese de b-1,6-glucan
Gene GLS1 de *Colletotrichum graminicola* envolvido na síntese de b-1,3-glucan

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



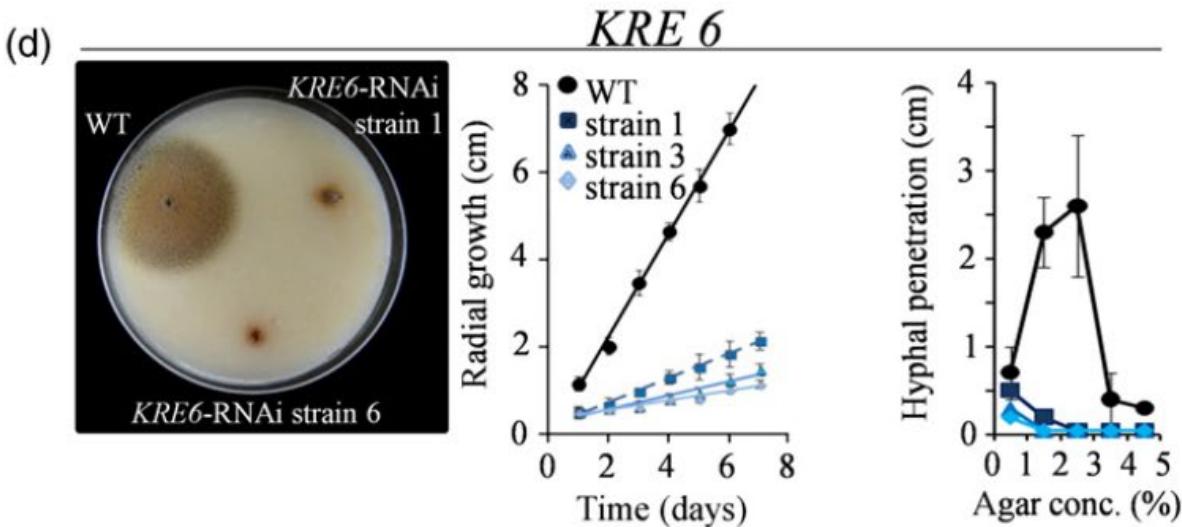
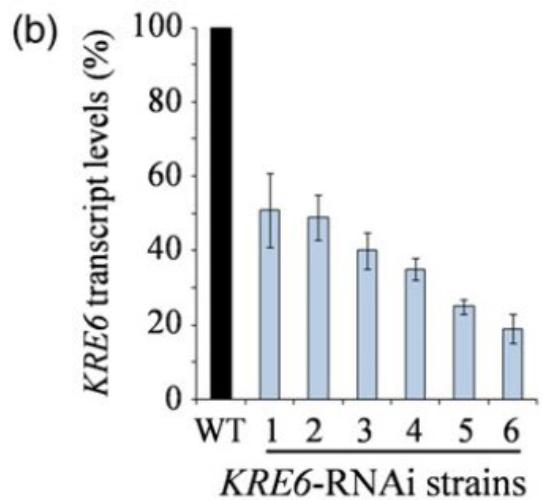
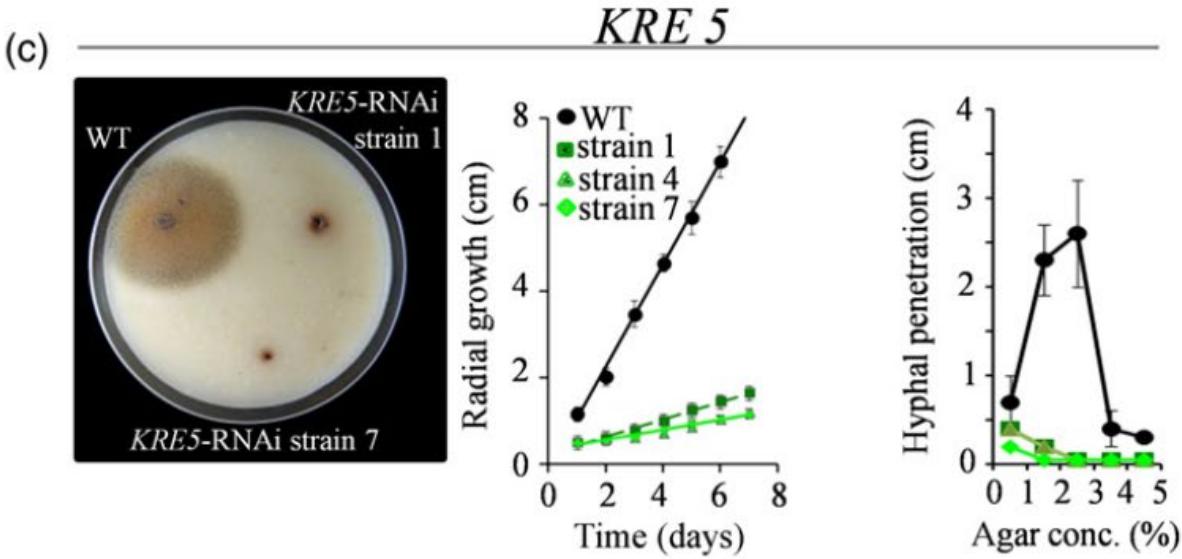
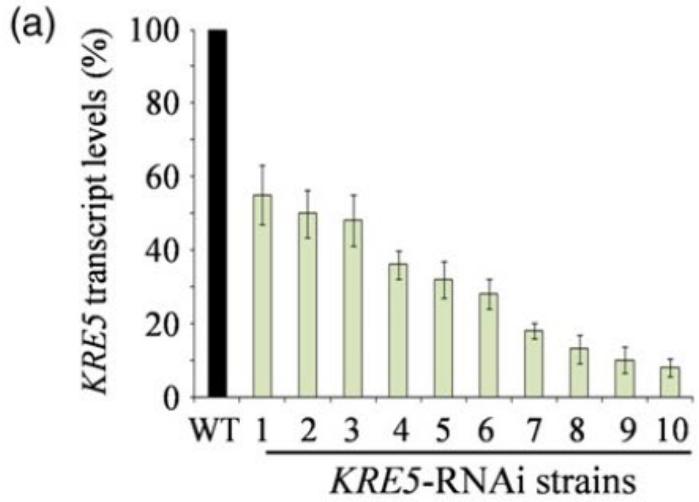
Genes KRE5 e KRE6 de *Colletotrichum graminicola* envolvidos na síntese de b-1,6-glucan

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

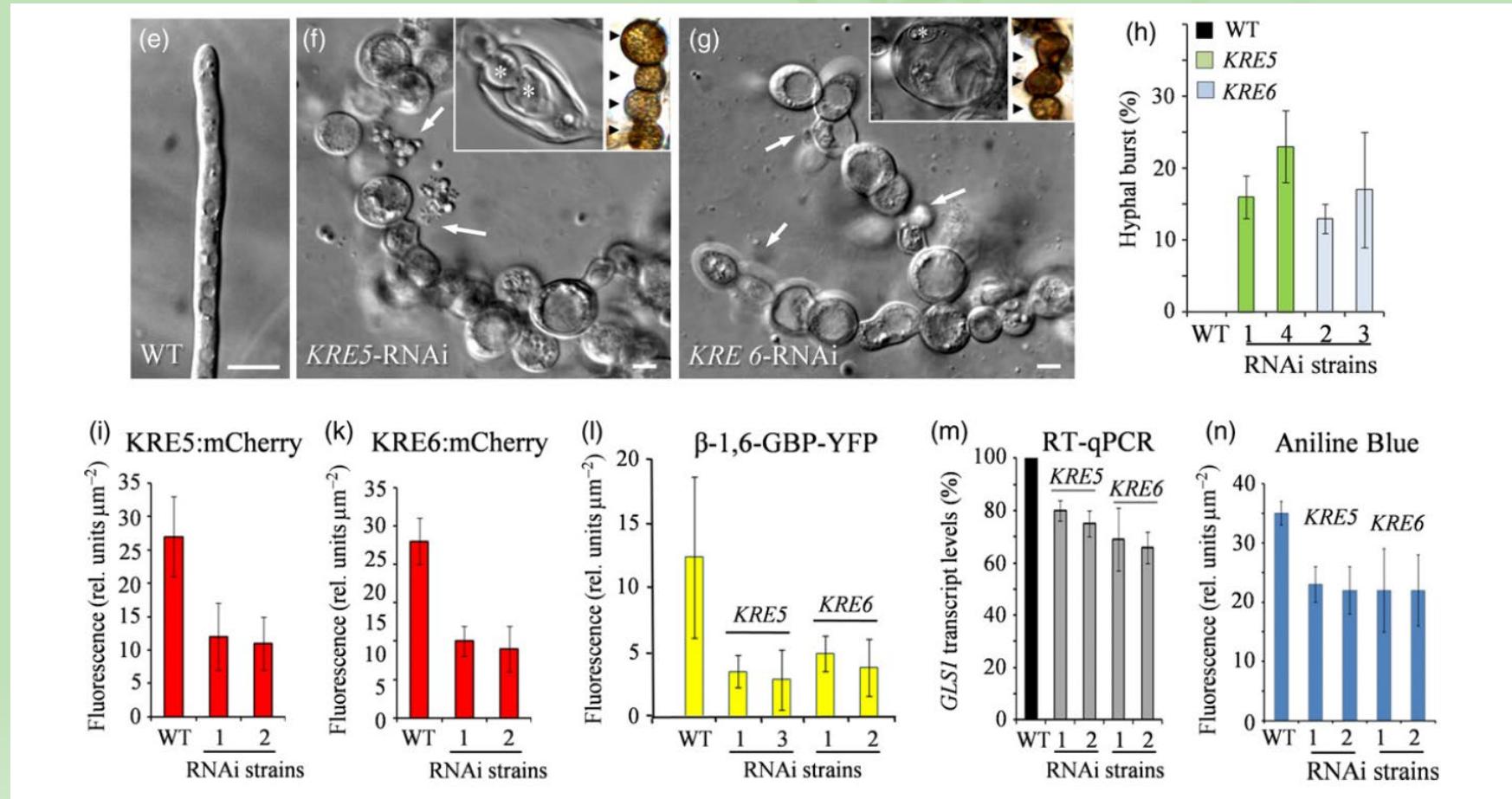
Microscopia de fluorescência quantitativa

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



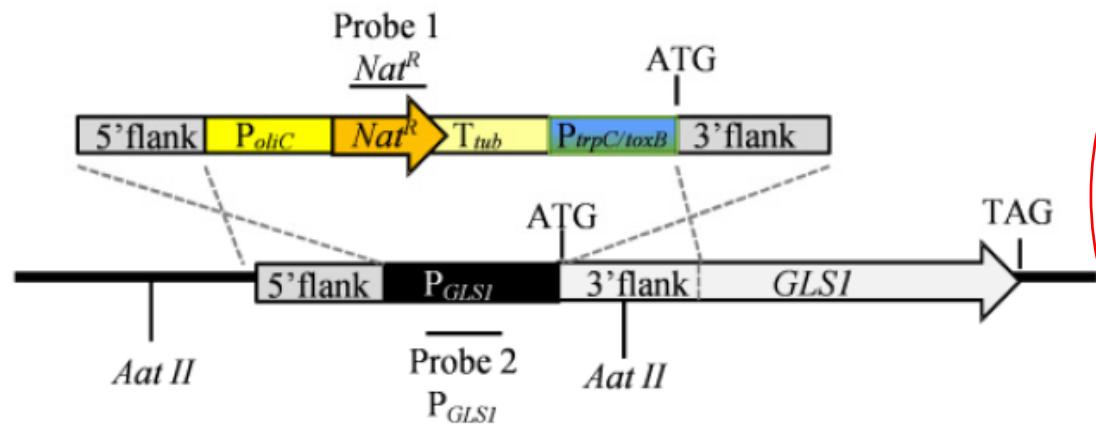
Verificar redução das proteínas além da redução dos mRNAs

Superexpressão de genes

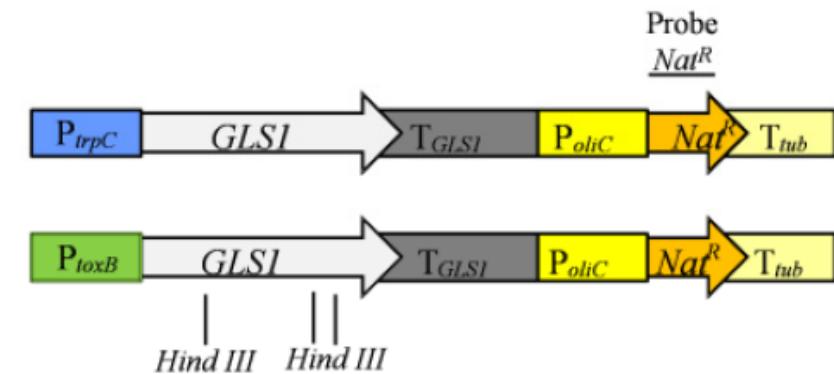
Expressão constitutiva ou aumento da expressão

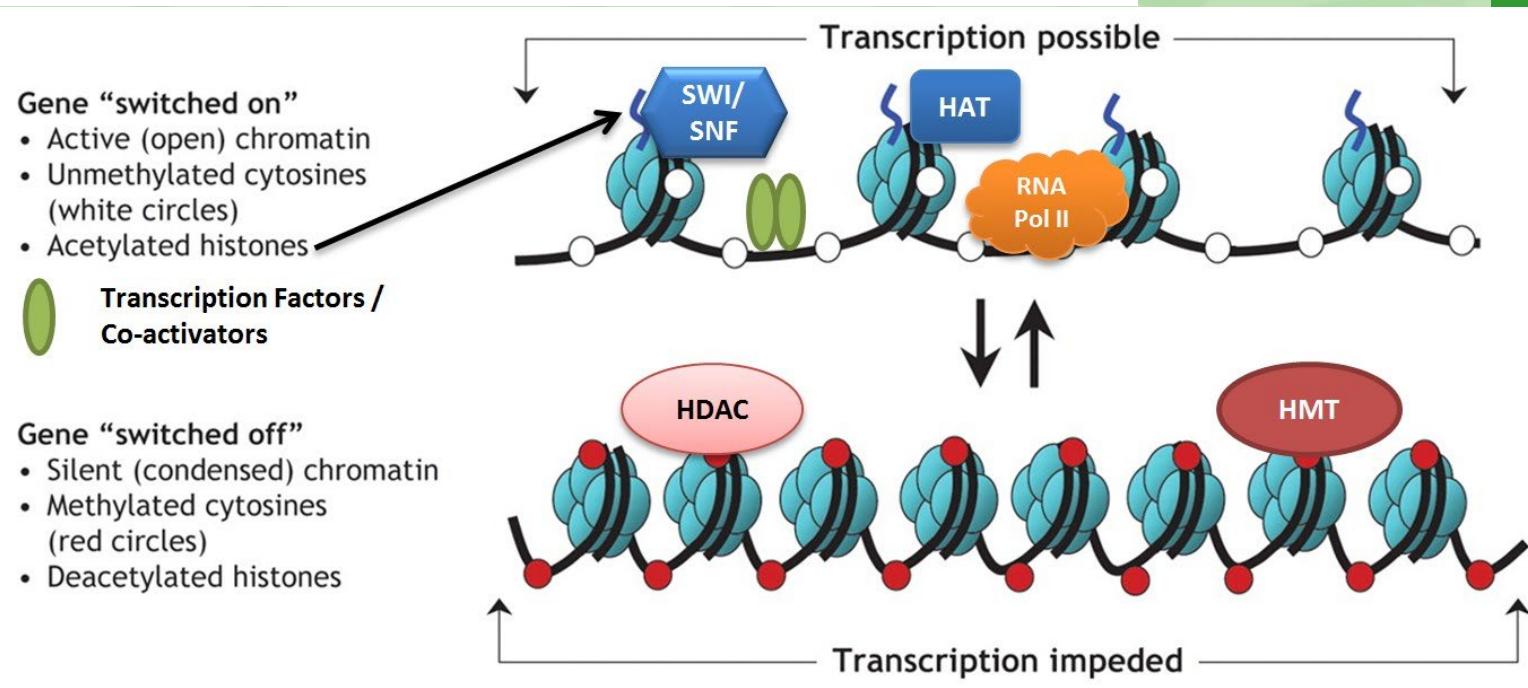
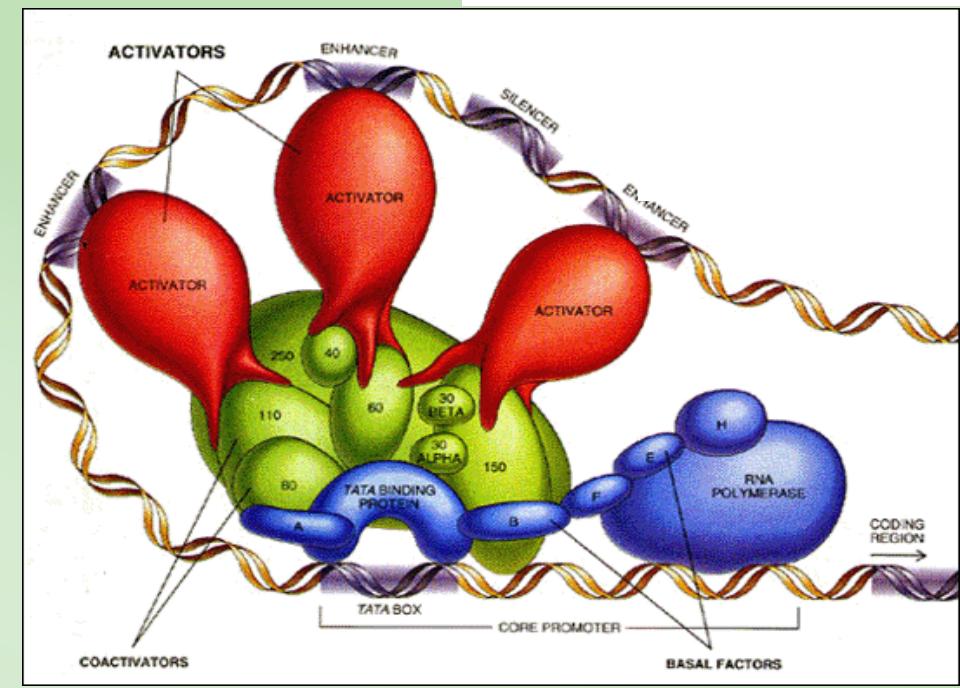
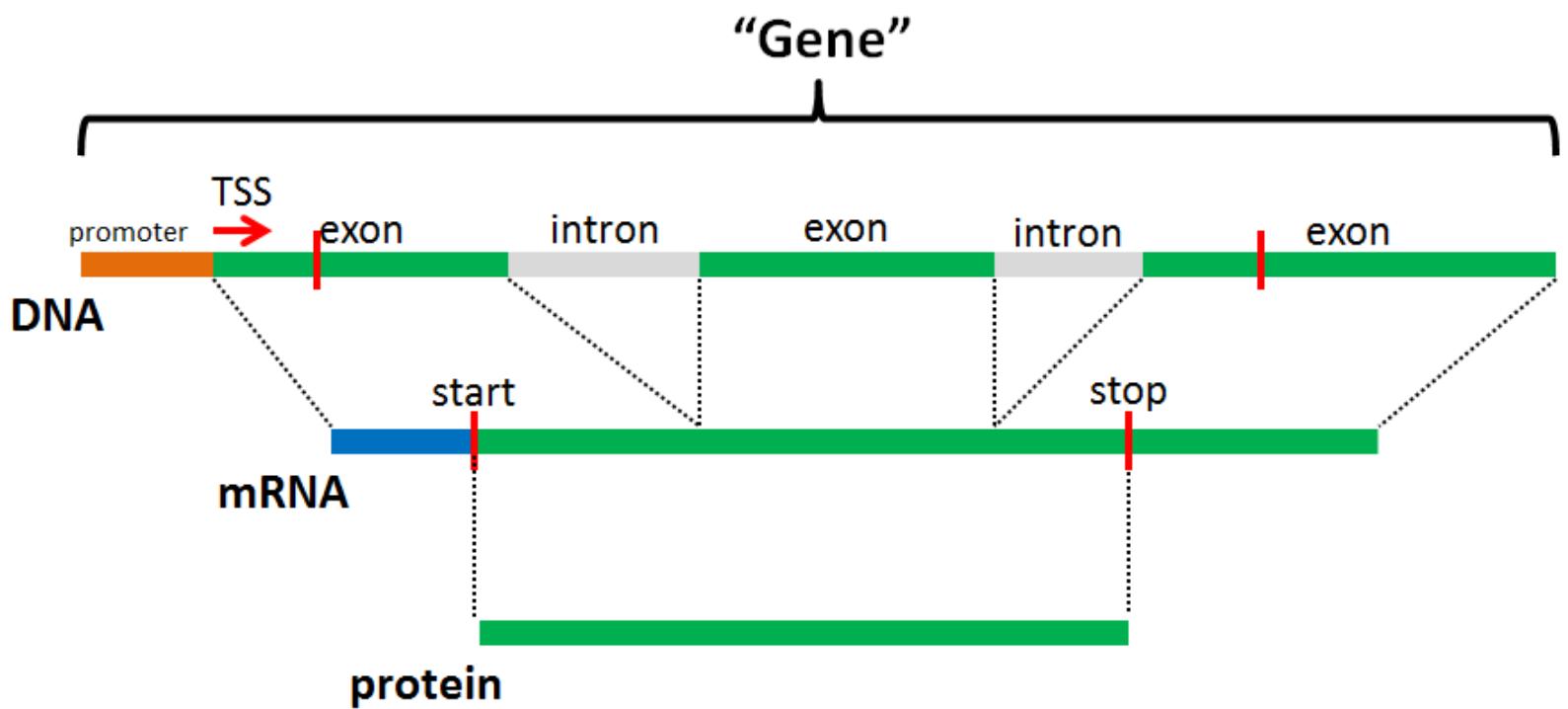
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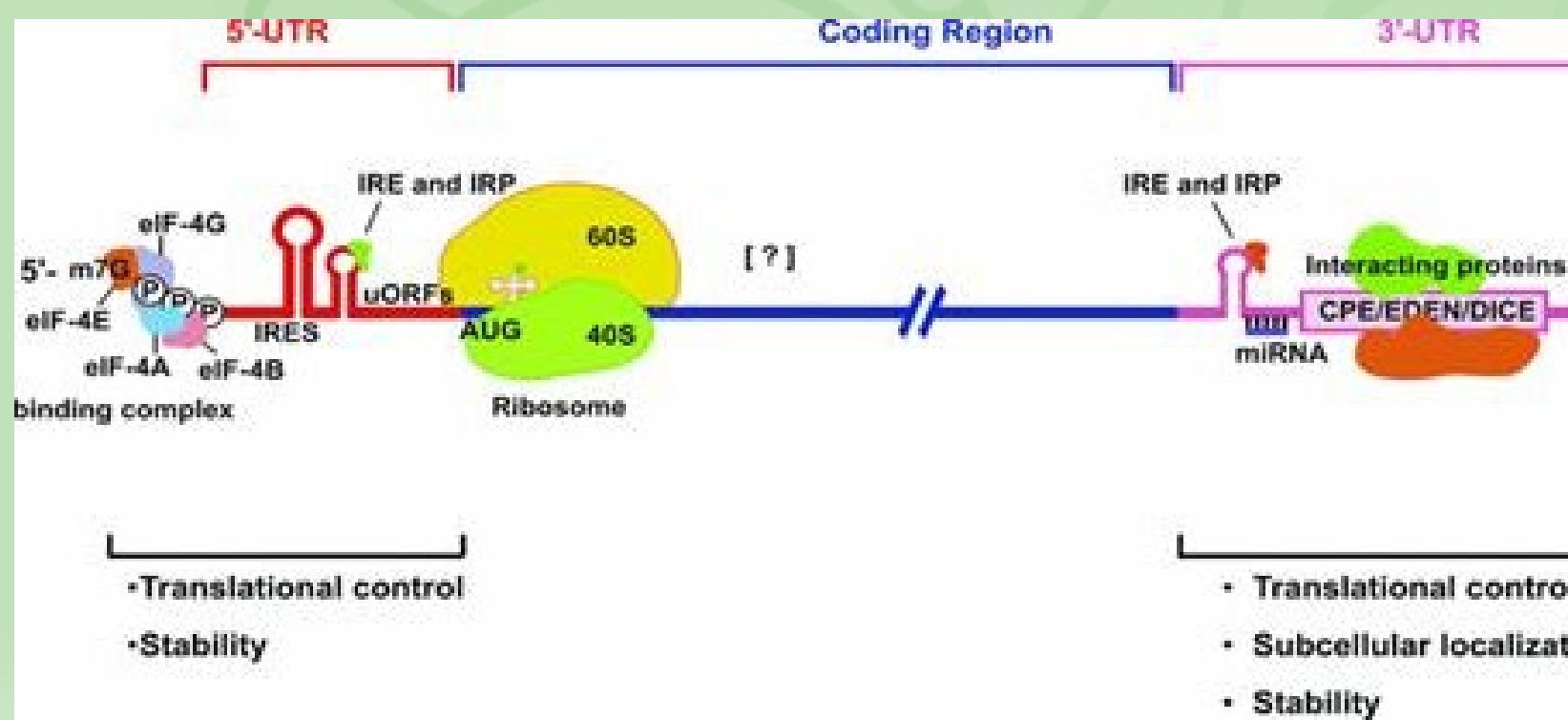
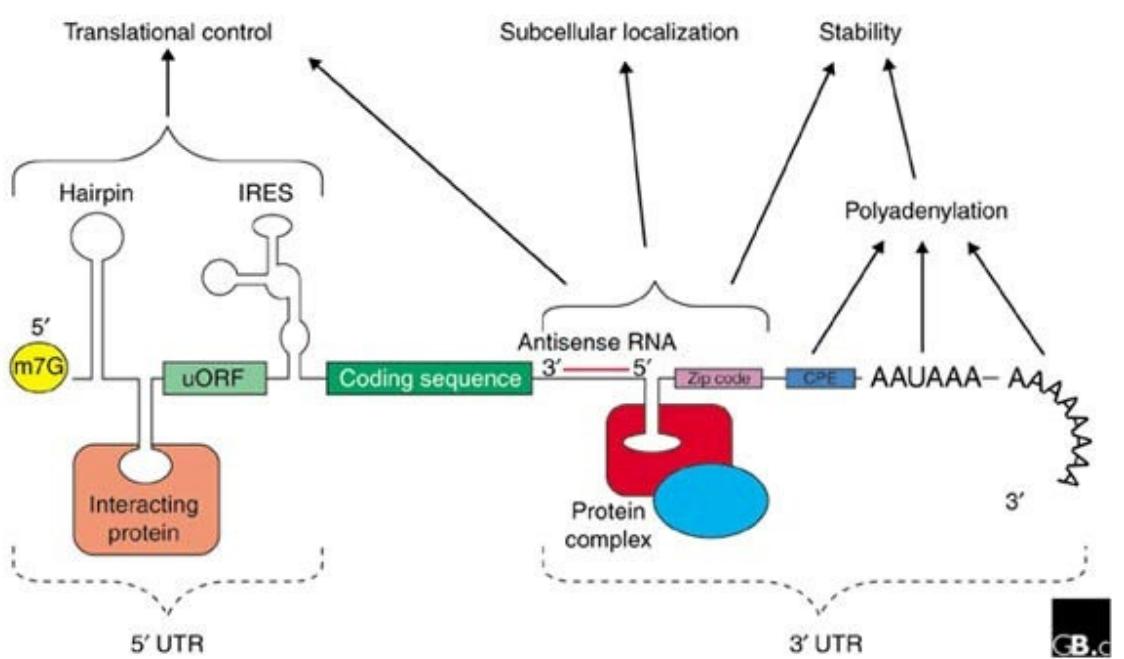
promoter exchange



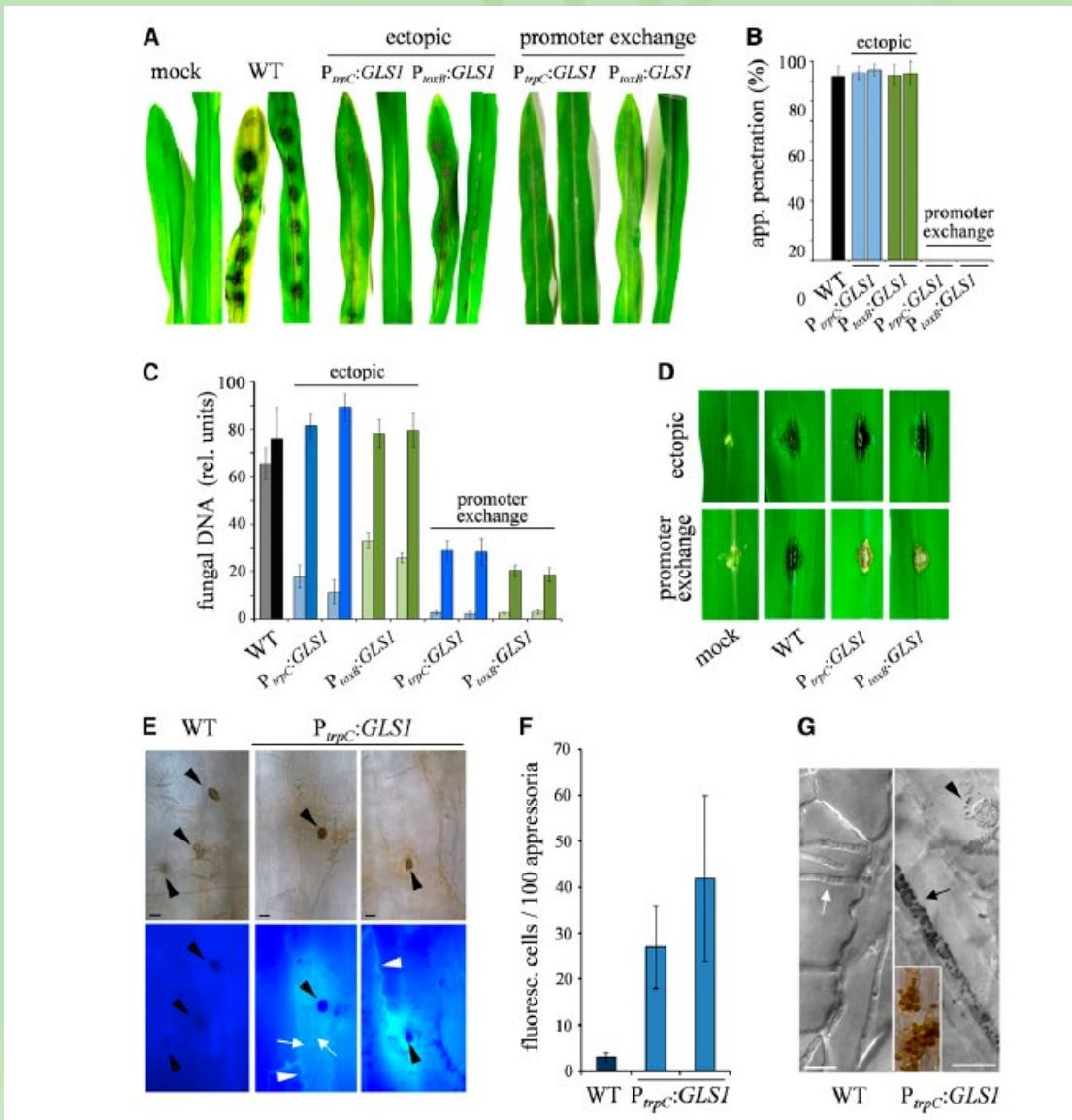
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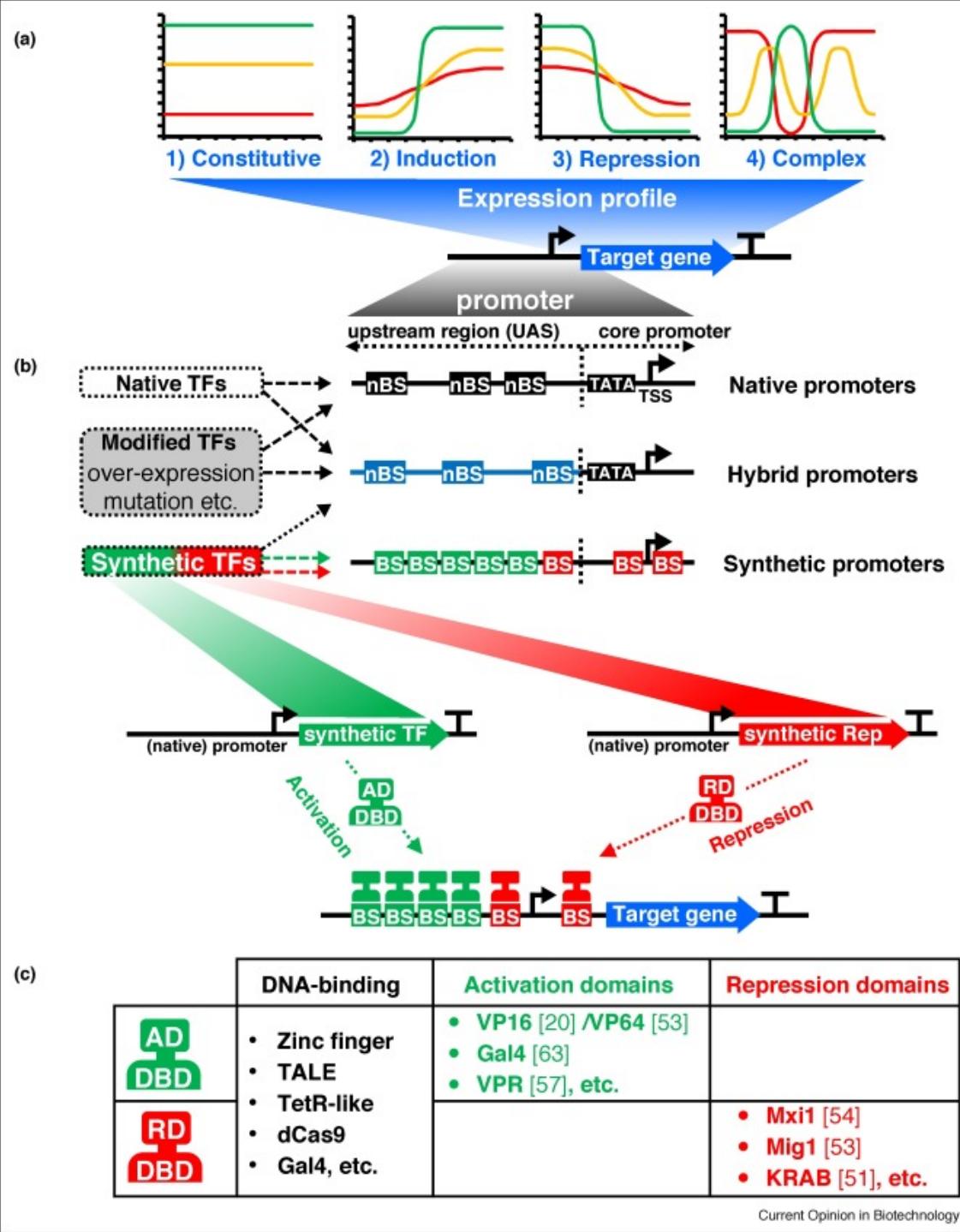






Expressão forçada do gene *GLS1* em hifas biotróficas





Current Opinion in Biotechnology 2019, 59:141–149

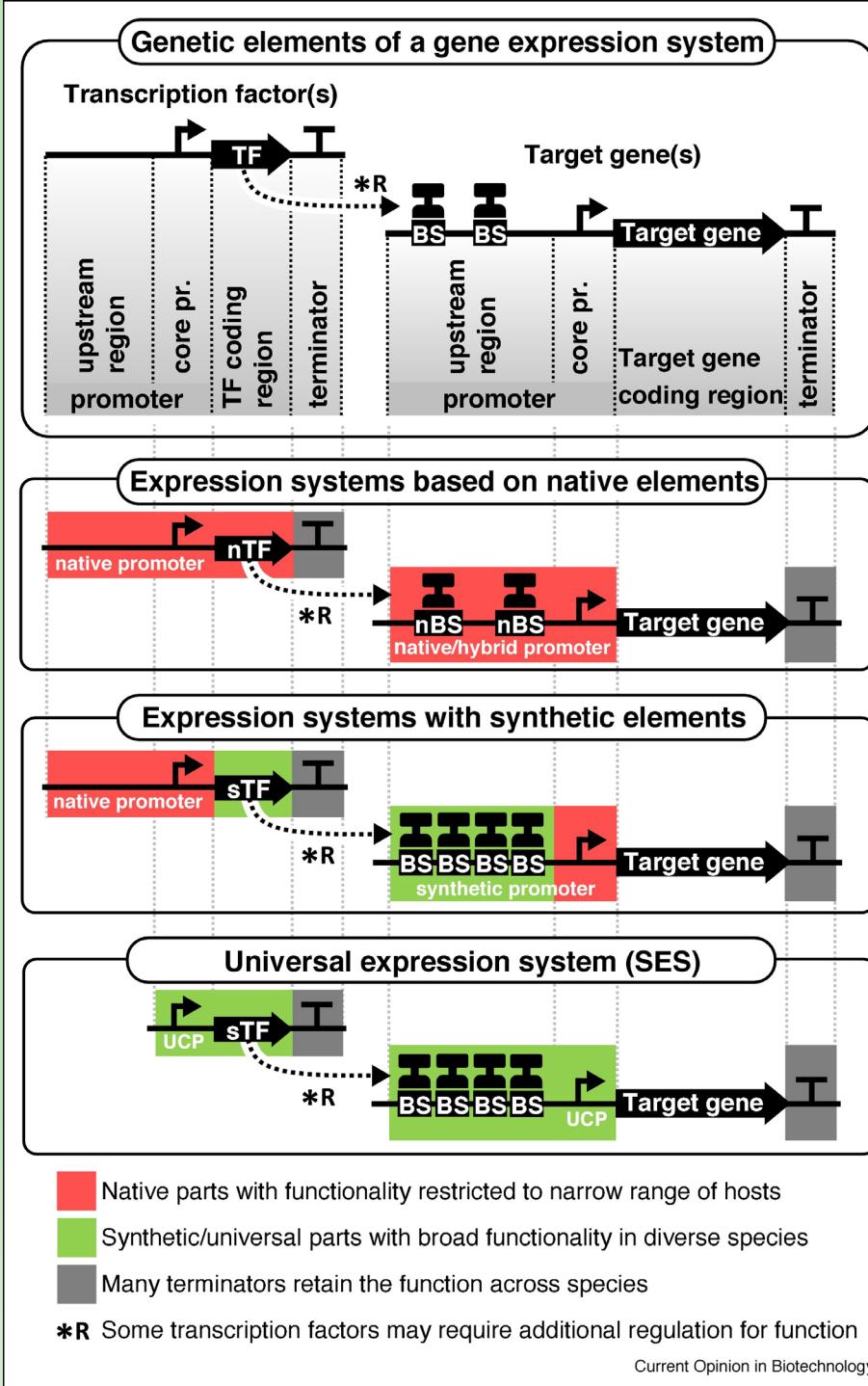
This review comes from a themed issue on **Tissue, cell and pathway engineering**

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>



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