

# Metodologias para o estudo funcional de genes

Profa. Dra. Chirlei Glienke

# 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**

Elvio Henrique Benatto Perino,<sup>1</sup> Chirlei Glienke,<sup>1,2,†</sup> Alan de Oliveira Silva,<sup>1,2</sup> and Holger B. Deising<sup>2,†</sup>

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# 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 $\beta$ -1,3-Glucan Synthase Is Essential for Pathogenicity of *Colletotrichum graminicola* and Evasion of $\beta$ -Glucan-Triggered Immunity in Maize<sup>W</sup>

Ely Oliveira-Garcia<sup>a</sup> and Holger B. Deising<sup>a,b,1</sup>

<sup>a</sup> 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

<sup>b</sup> 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:

## Infection Structure-Specific Expression of $\beta$ -1,3-Glucan Synthase Is Essential for Pathogenicity of *Colletotrichum graminicola* and Evasion of $\beta$ -Glucan-Triggered Immunity in Maize<sup>W</sup>

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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 $\beta$ -1,6-glucan synthesis genes *KRE5* and *KRE6* in biotrophic hyphae of *Colletotrichum graminicola*

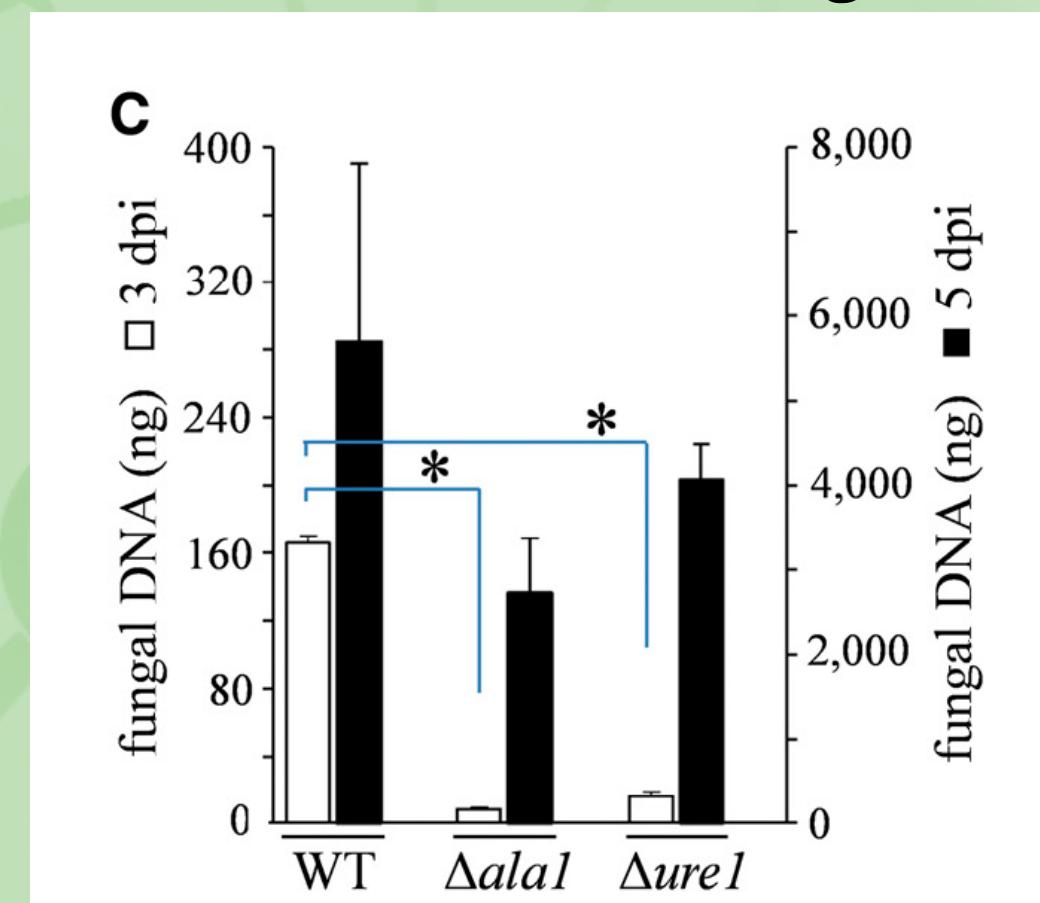
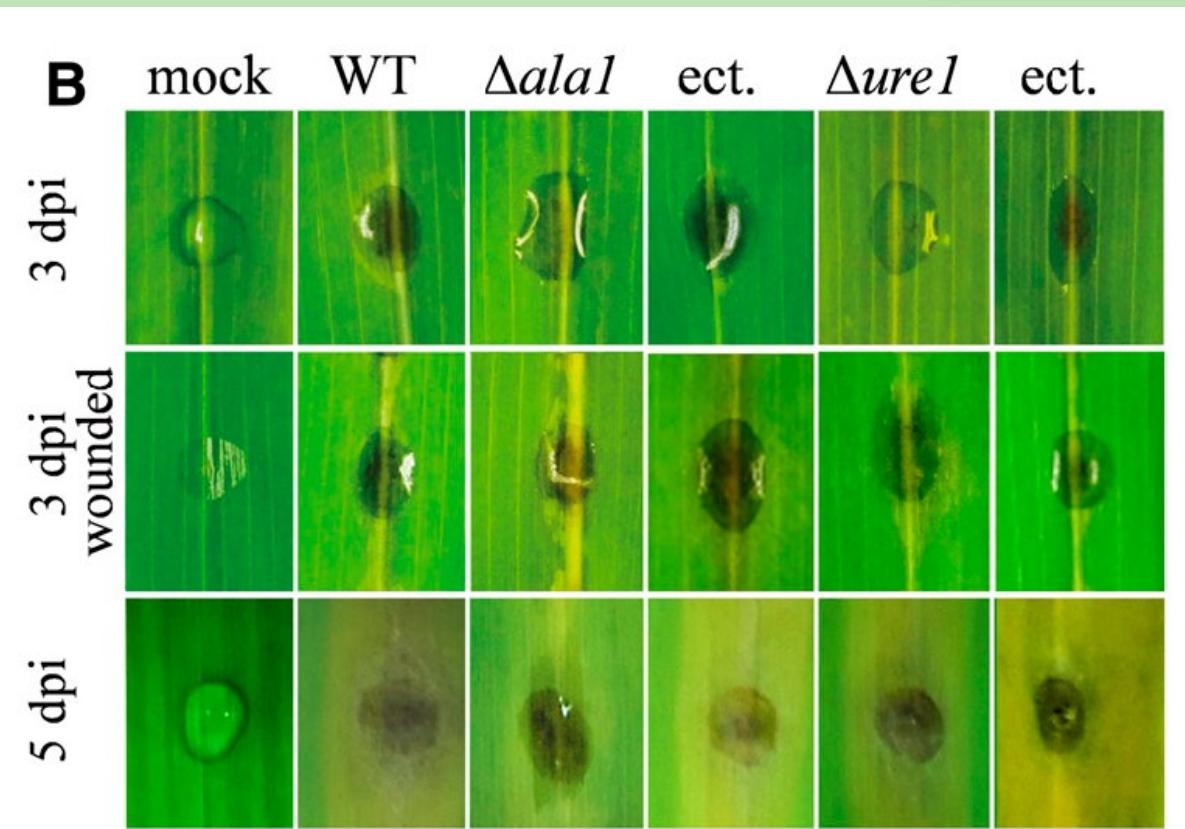
Ely Oliveira-Garcia<sup>1,†</sup> and Holger B. Deising<sup>1,2,\*</sup>

<sup>1</sup>Martin-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

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

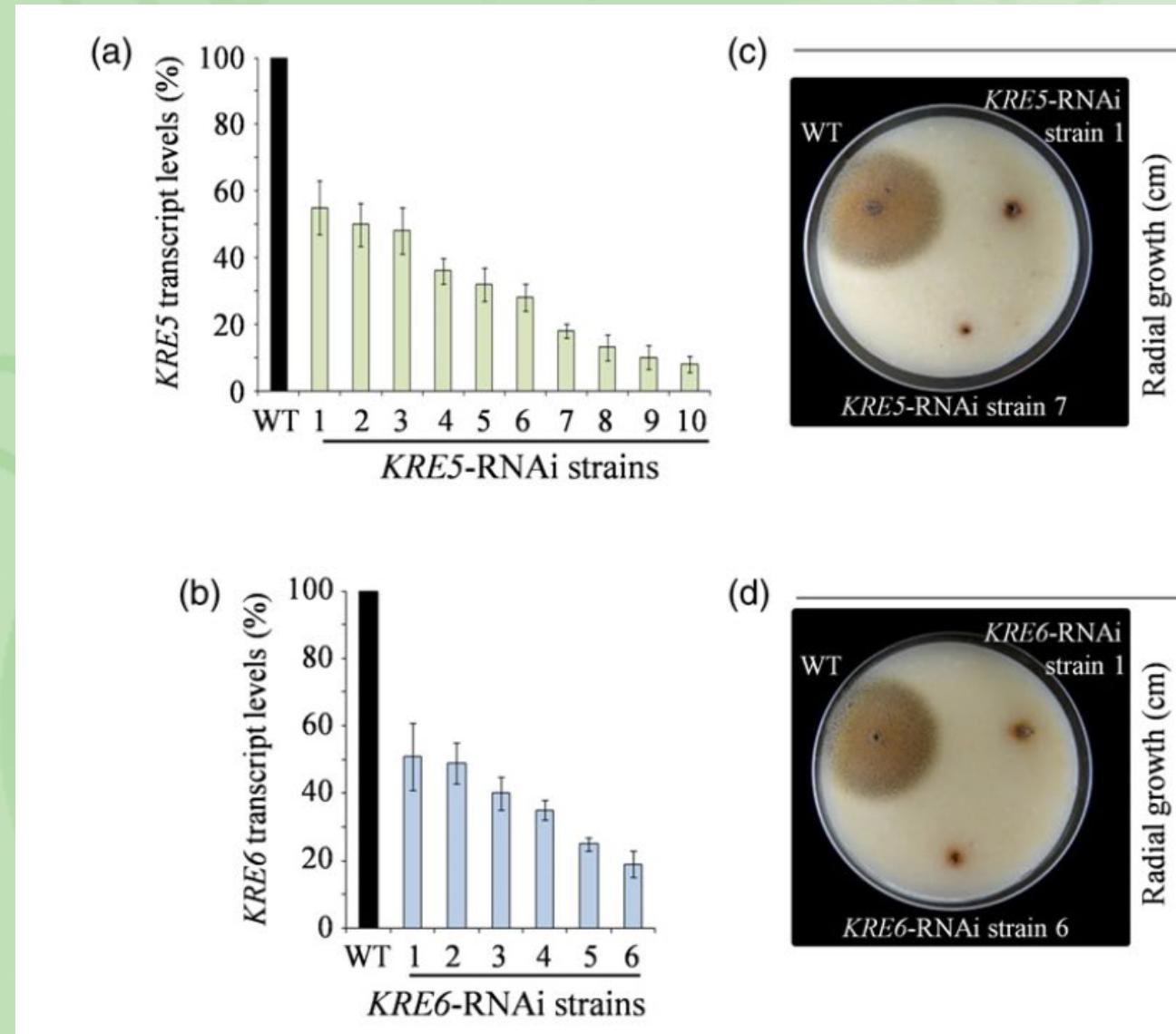
# Metodologias que podem ajudar a responder perguntas

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

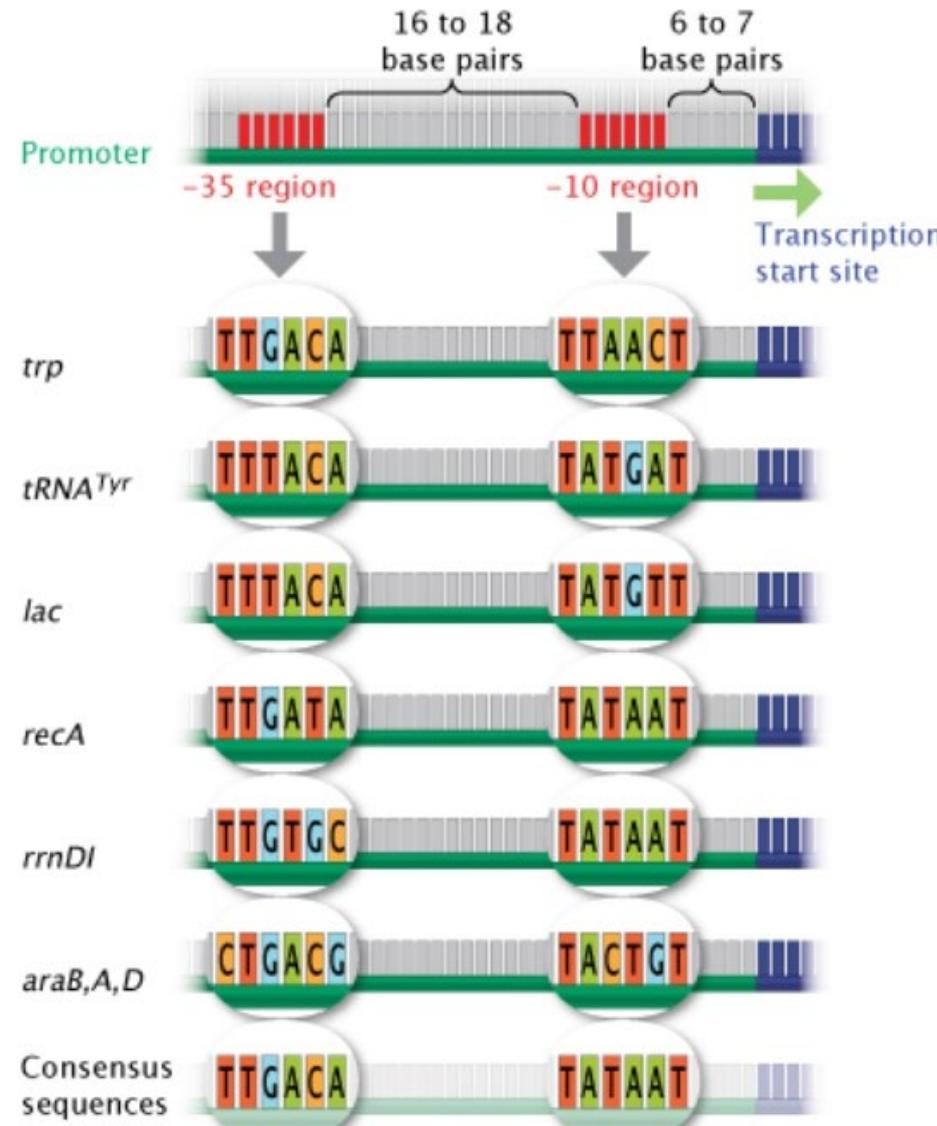


# Metodologias que podem ajudar a responder perguntas

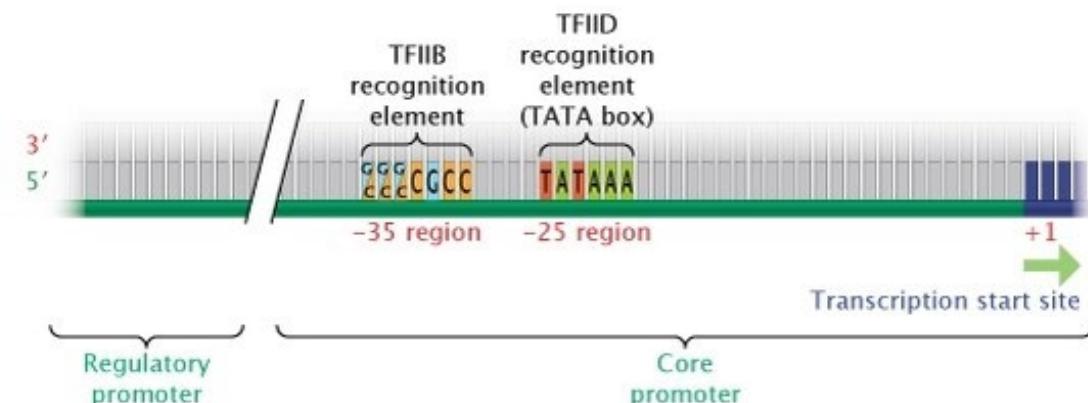
- RT-PCR: Análise de expressão de genes (nível de mRNA)
  - Se, quando e quanto um gene é expresso
  - Uso de mRNA do gene específico (avaliar o quanto um gene específico foi expresso em cultivo ou na lesão)

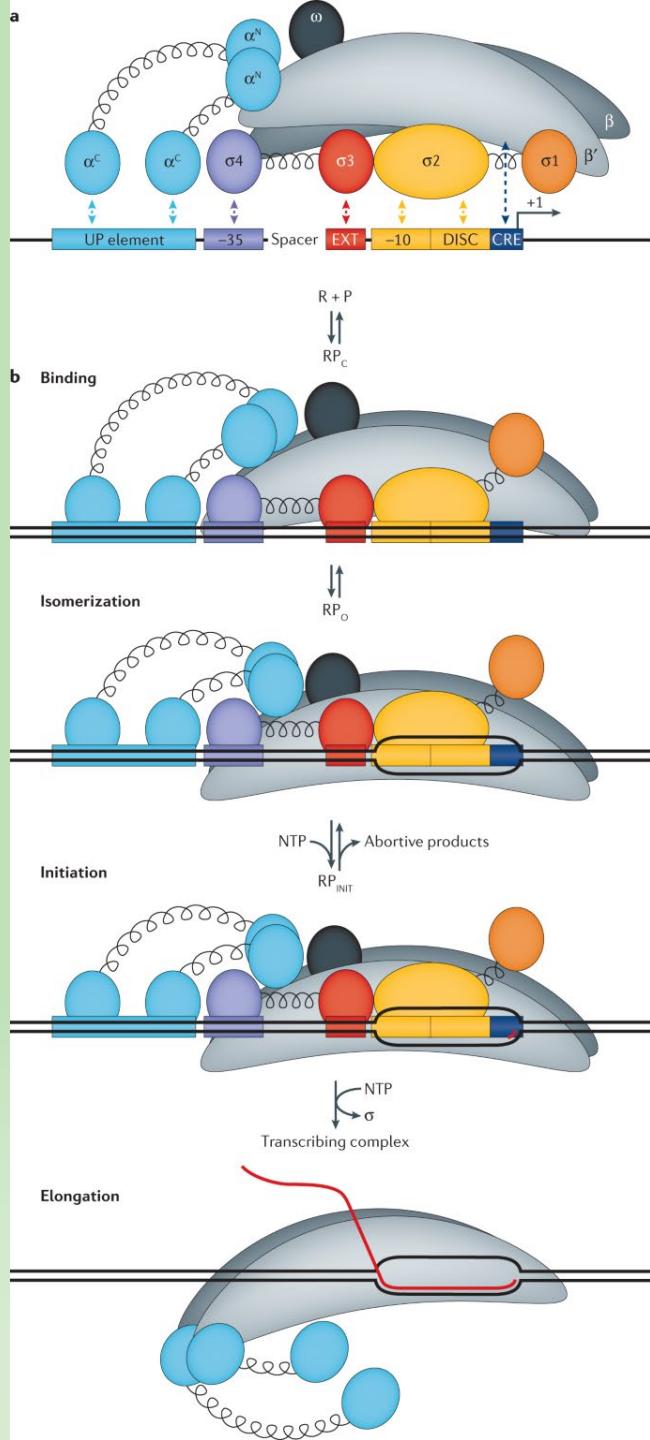


# Promotor procariótico



# Promotor eucariótico

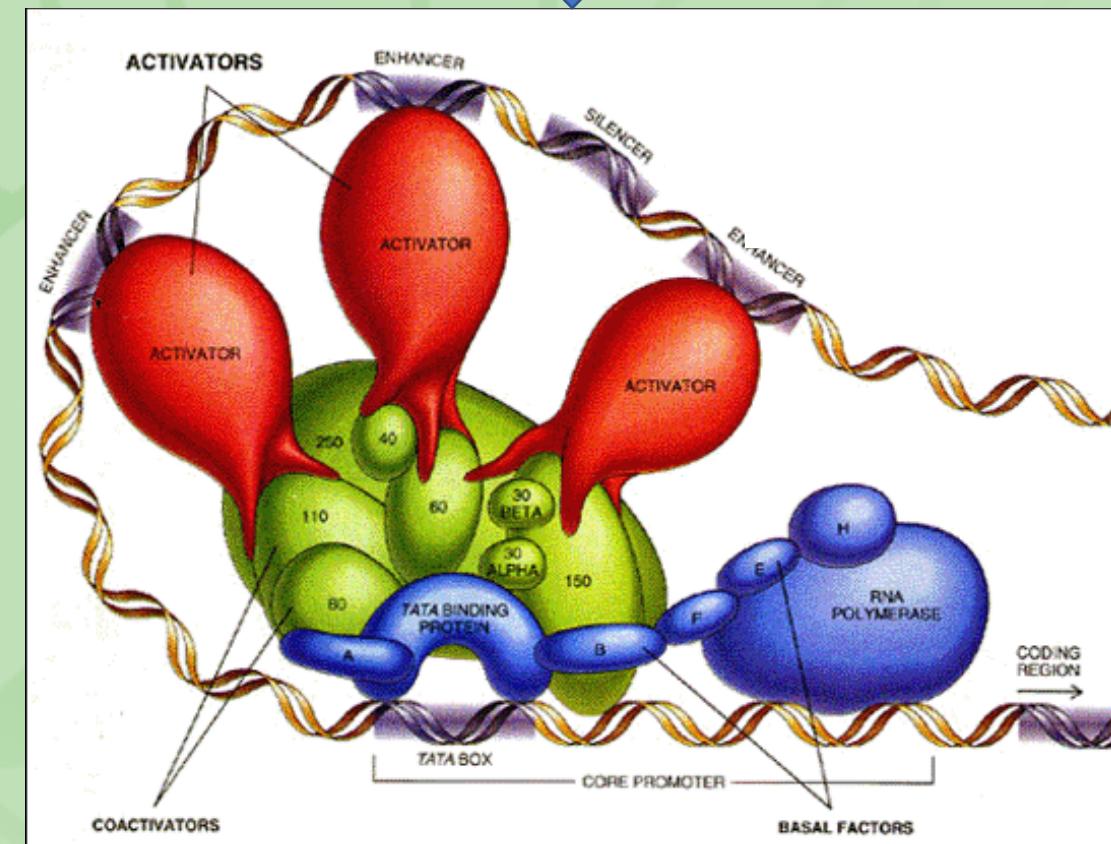




- Complexos de transcrição em procariotos e

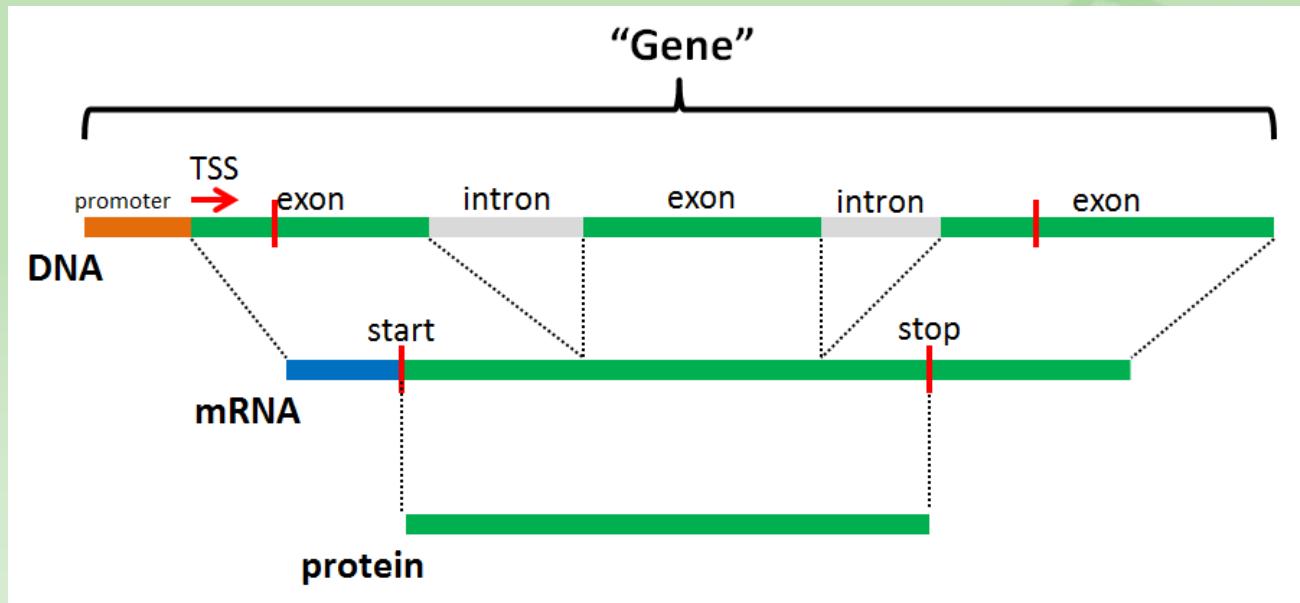


- em eucariotos

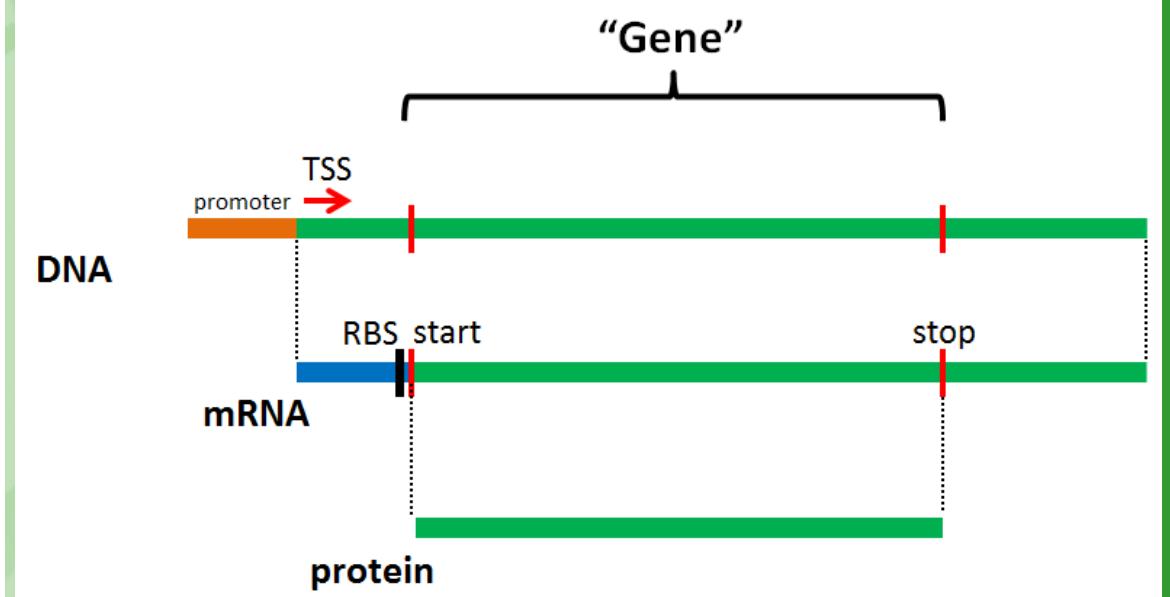


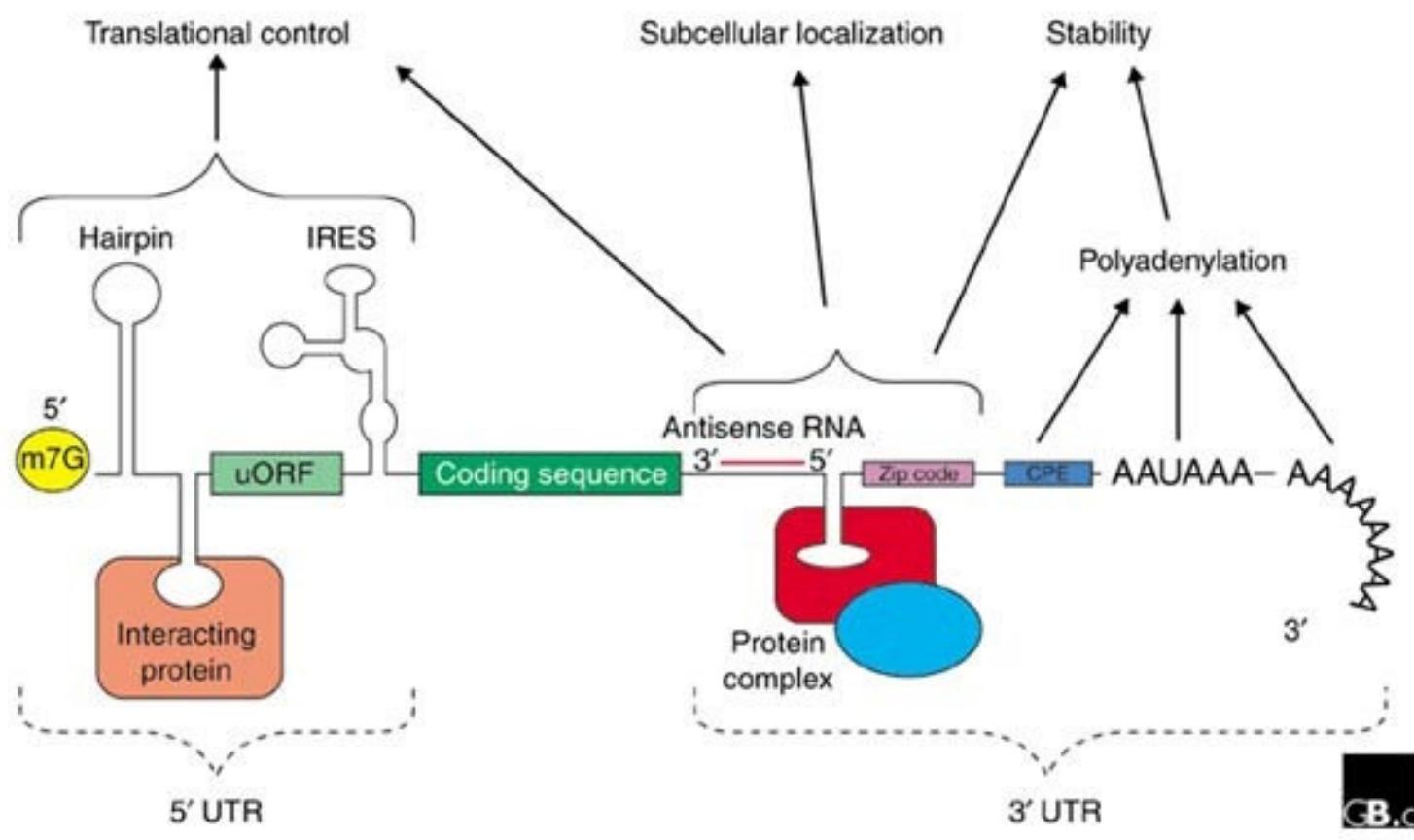
# Estrutura de genes

## Eucariotos



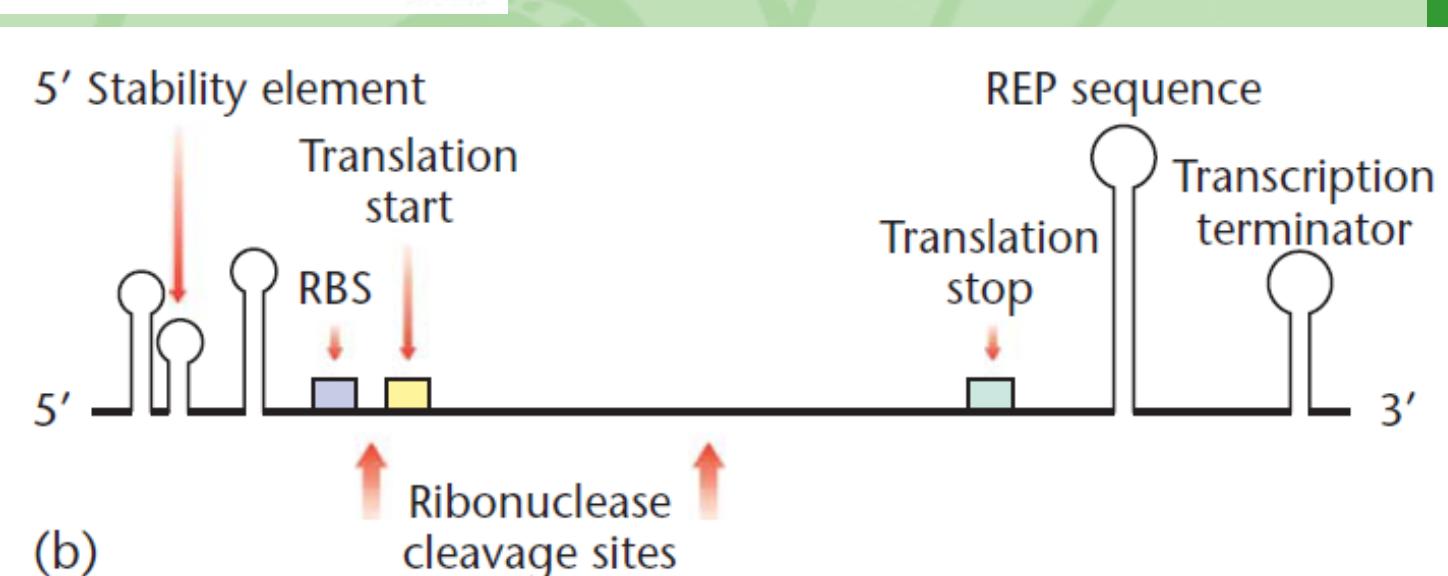
## Procaríotos



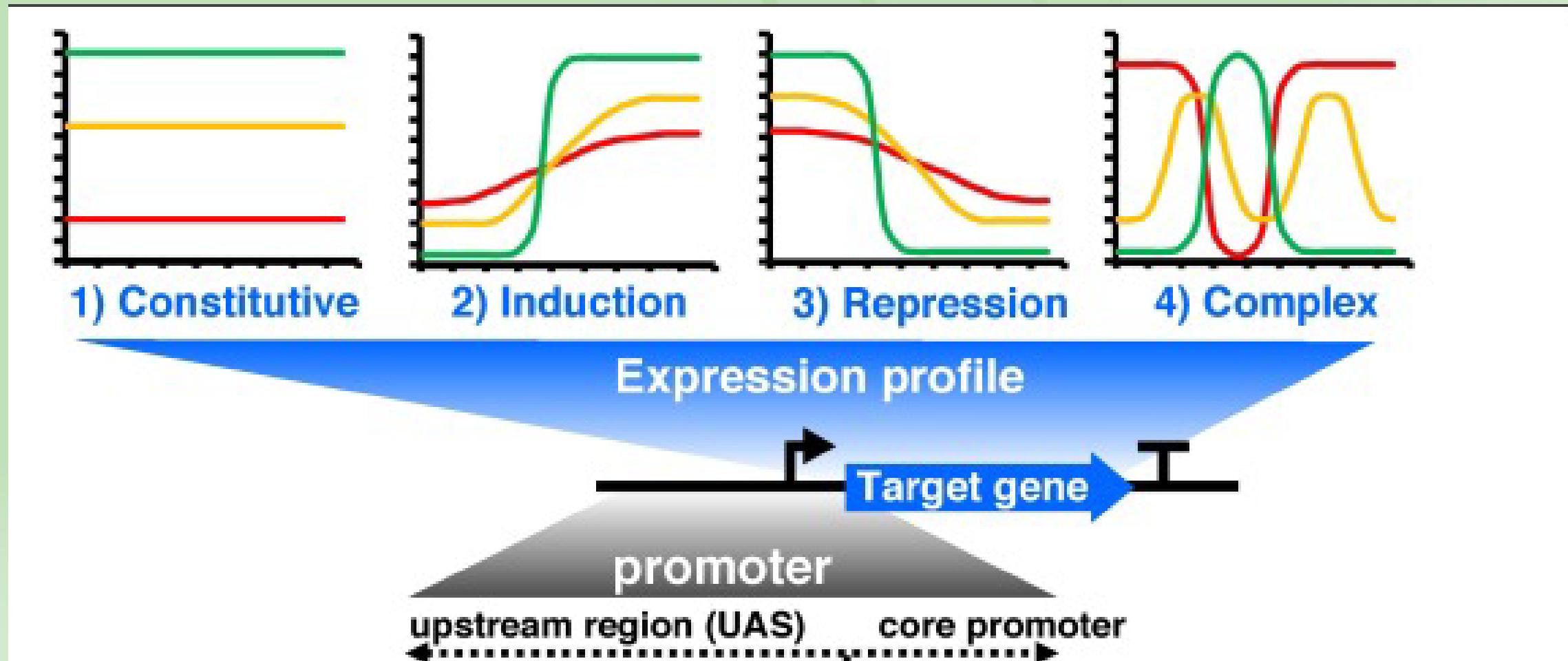


UTR, untranslated region; m7G, 7-methyl-guanosine cap; hairpin, hairpin-like secondary structures; uORF, upstream open reading frame; IRES, internal ribosome entry site; CPE, cytoplasmic polyadenylation element; AAUAAA, polyadenylation signal.

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

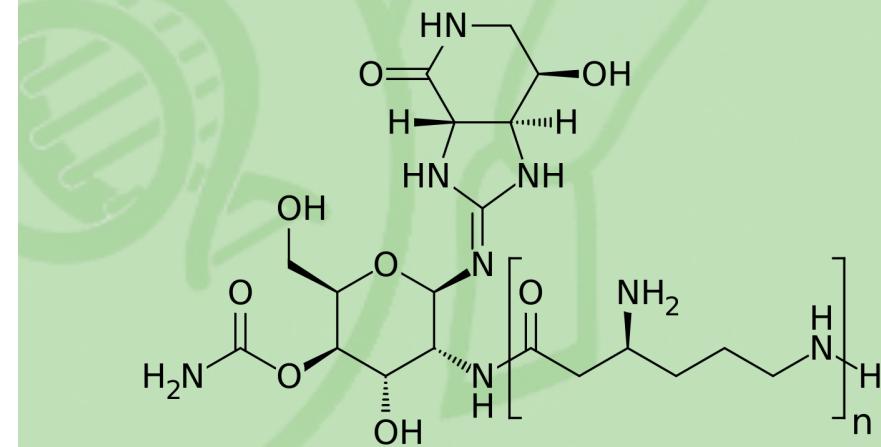
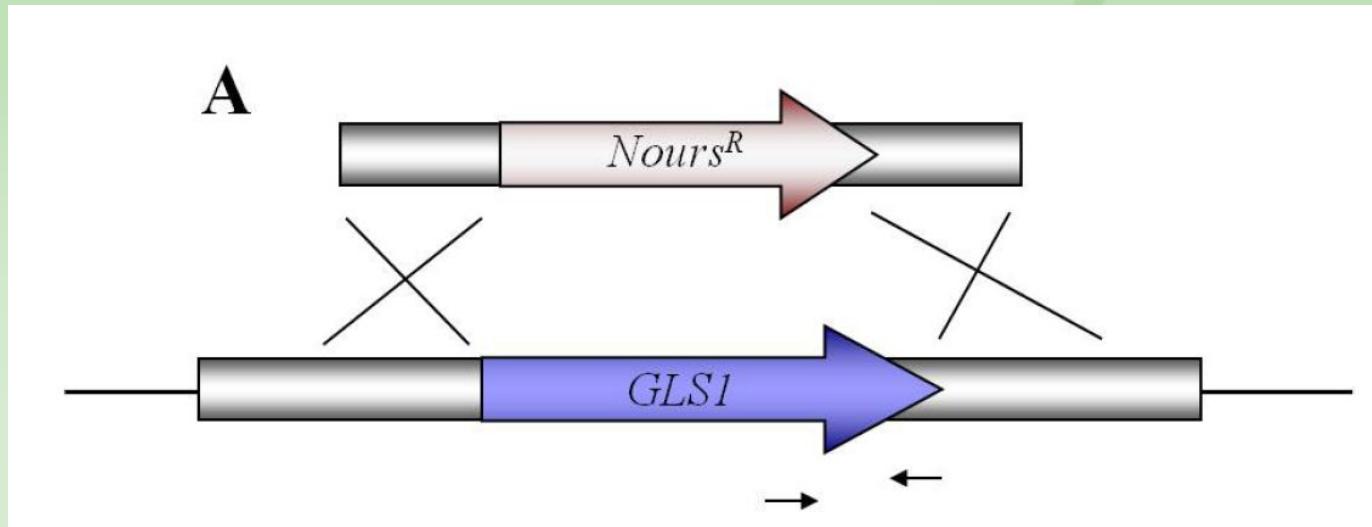


# Tipos de expressão gênica



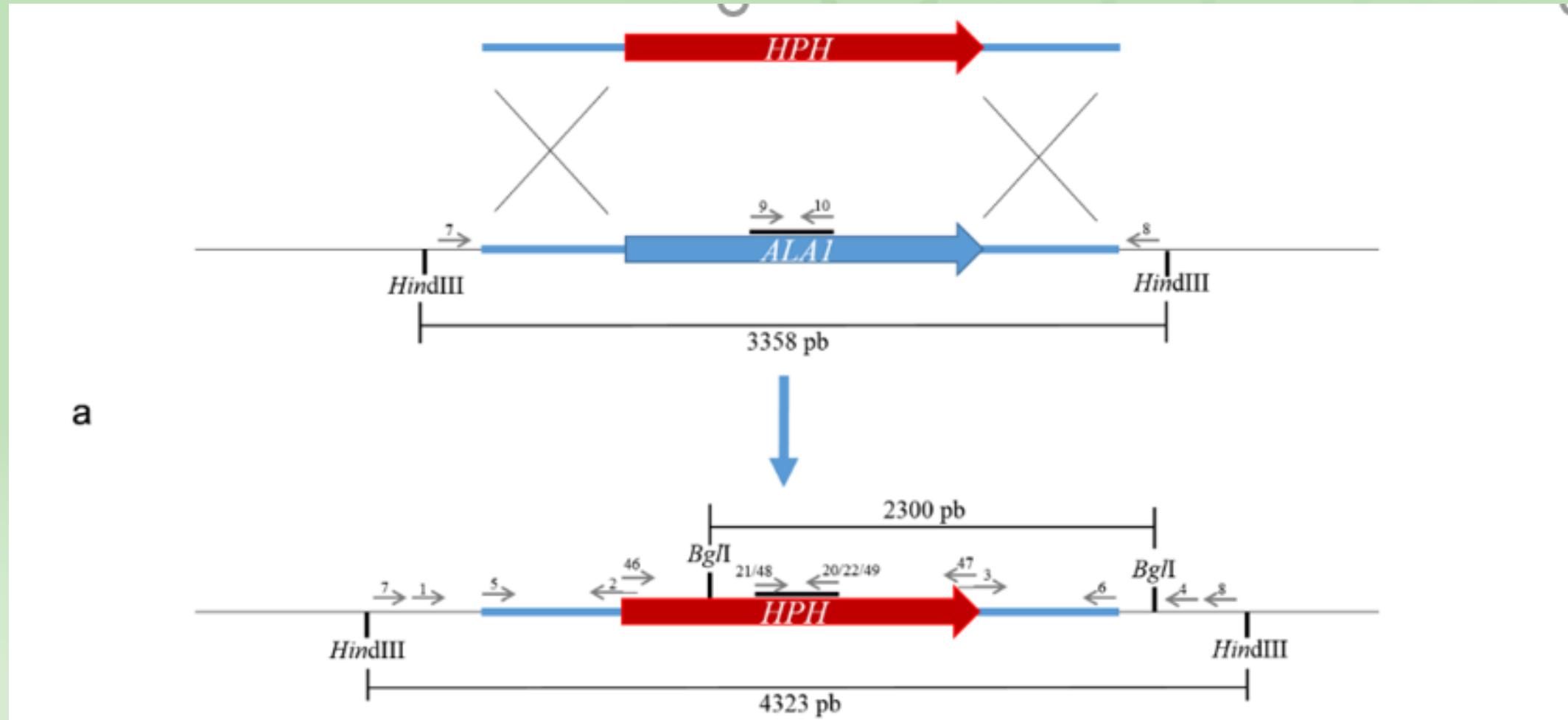
# Deleção de genes

- 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<sup>R</sup>* (resistência à Nourseothricin - nourseothricin N-acetyl transferase)

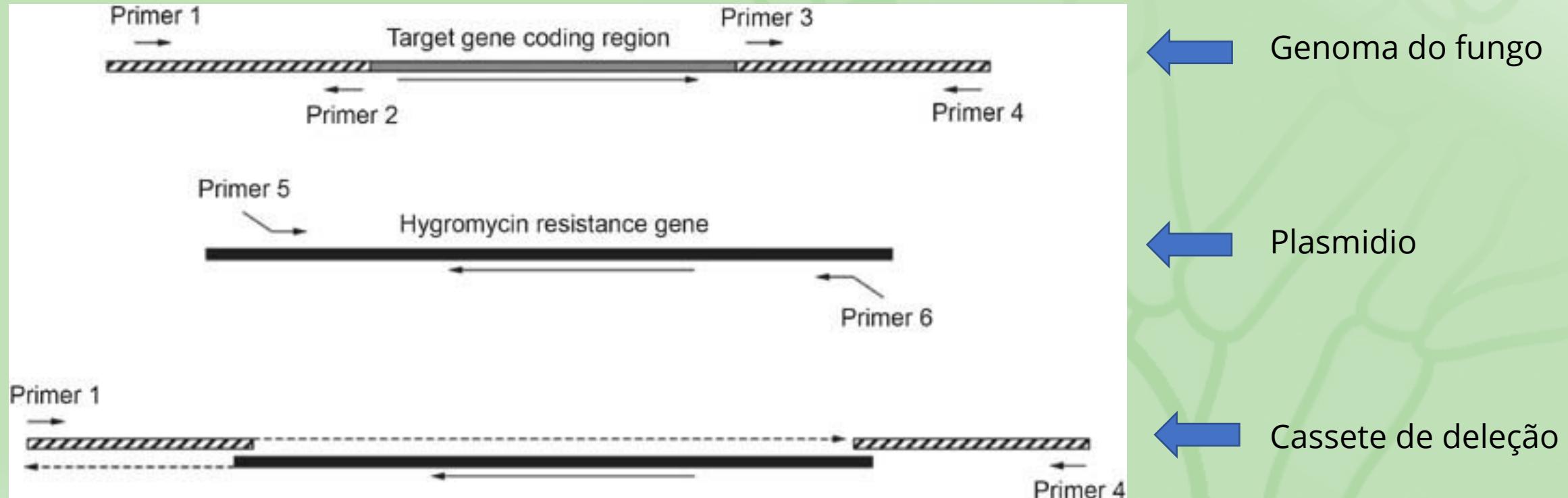


# Dependente de recombinação homóloga

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

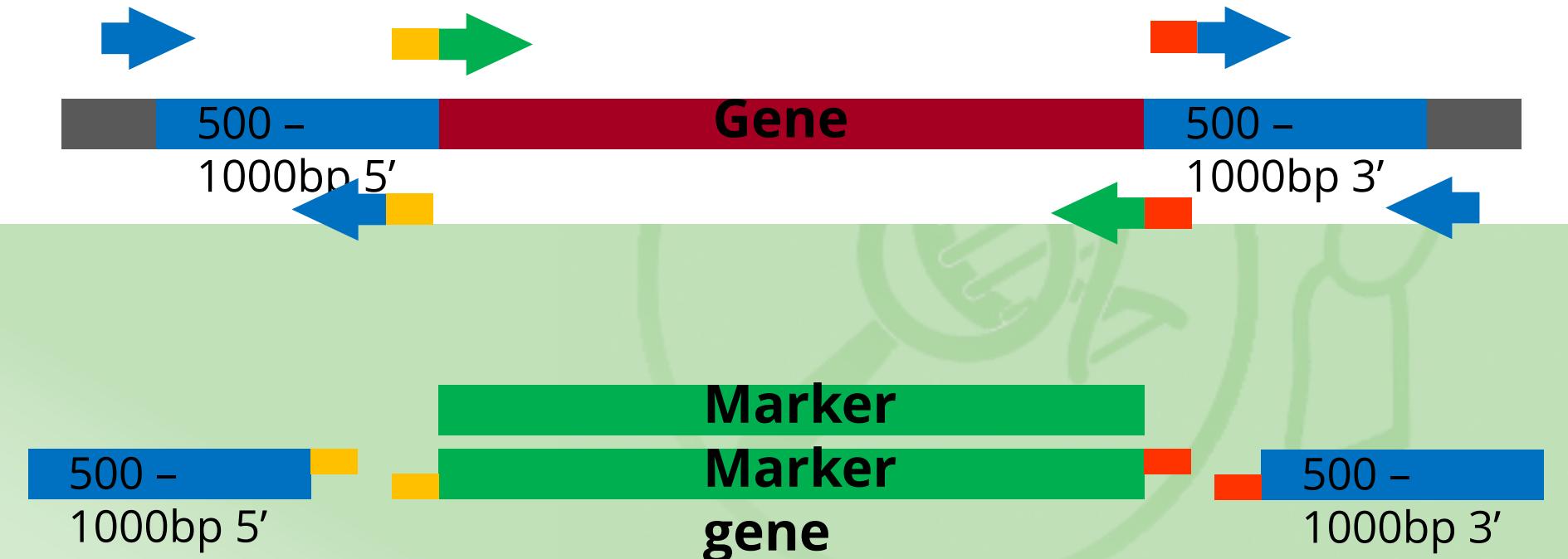


# Dependente de recombinação homóloga



# Gene Deletion

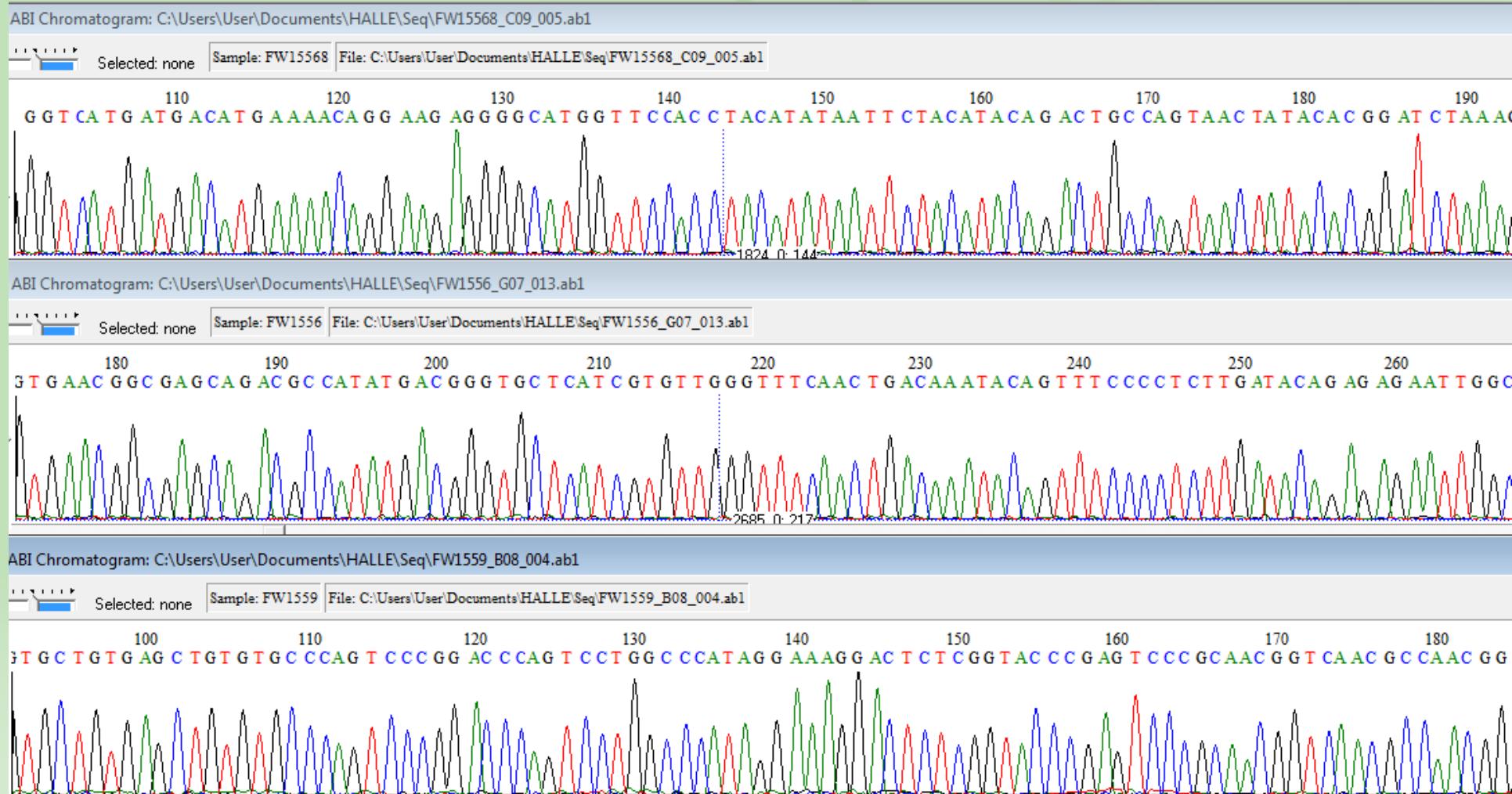
- SEARCH FOR THE GENOME (NCBI:  
<https://www.ncbi.nlm.nih.gov/genome/>)
- Primers design for flank regions (3' and 5') amplification:



# 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

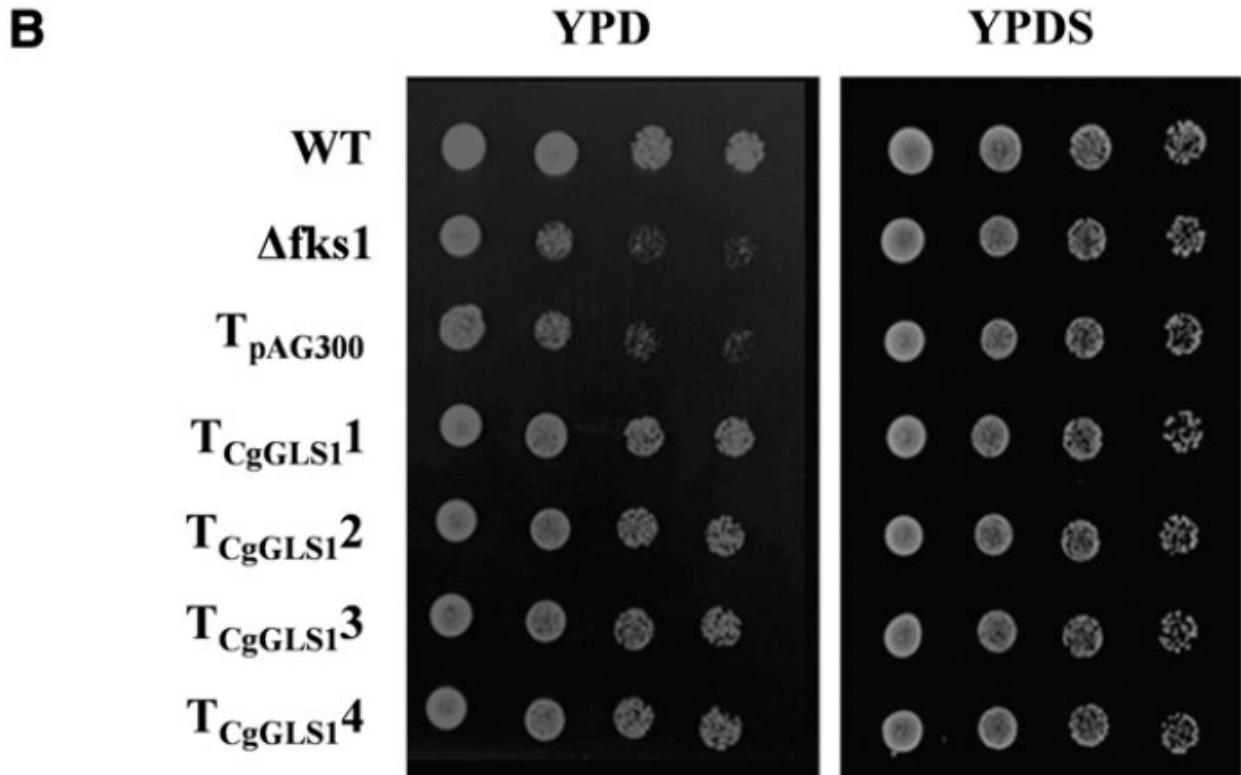
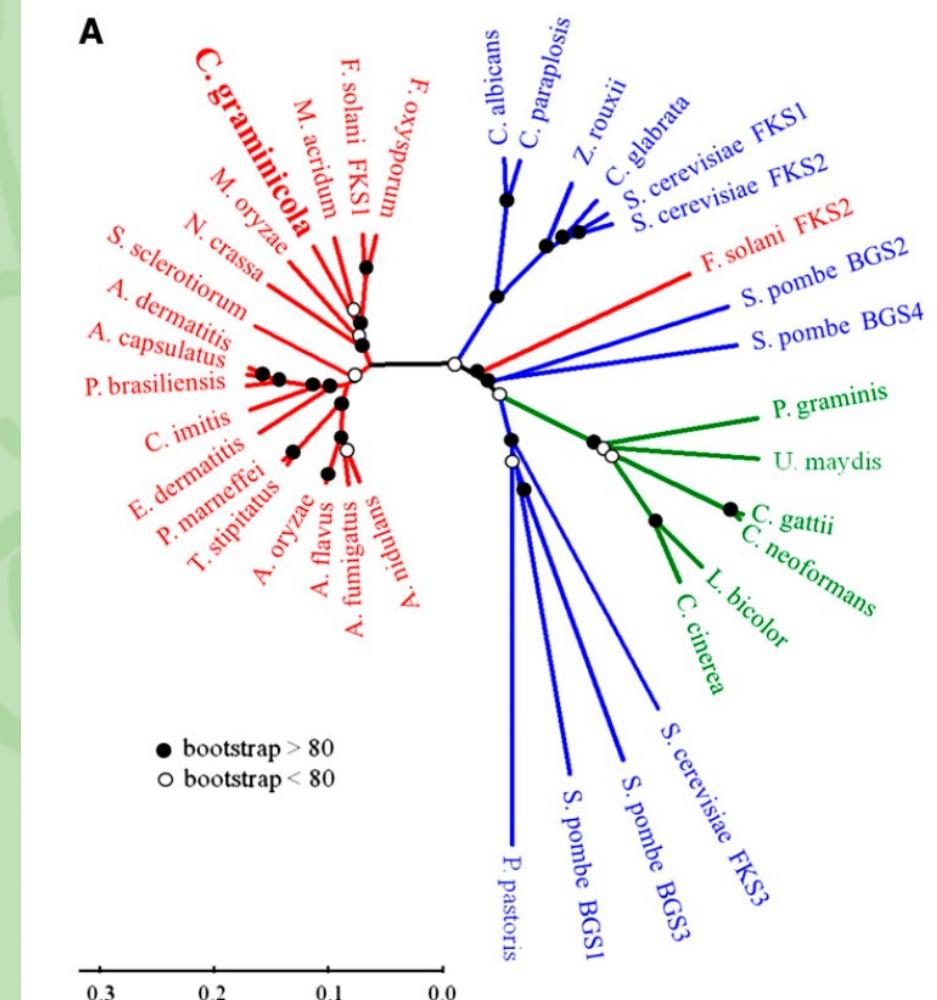
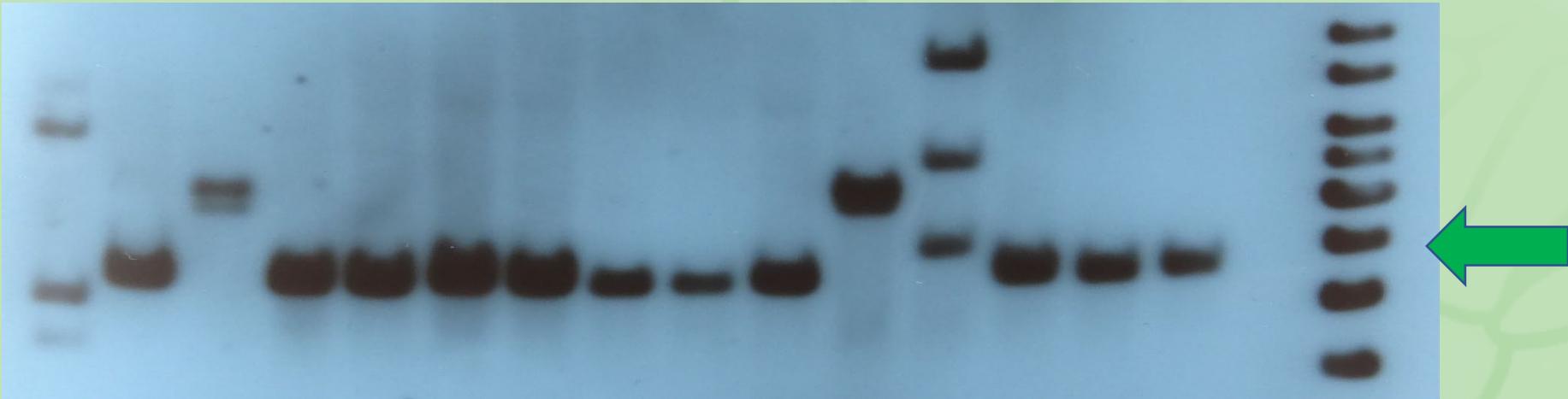


Figure 1. *GLS1* of *C. graminicola* Encodes a Functional GLS.

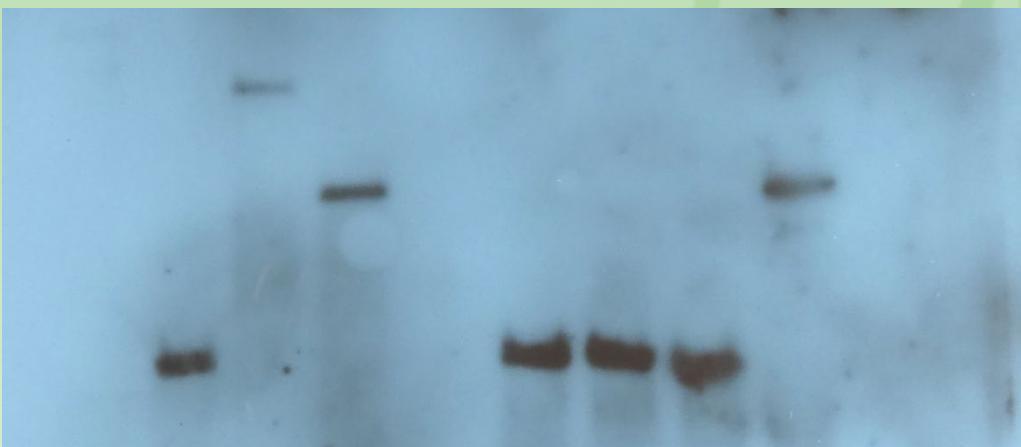


# Deleção de genes

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

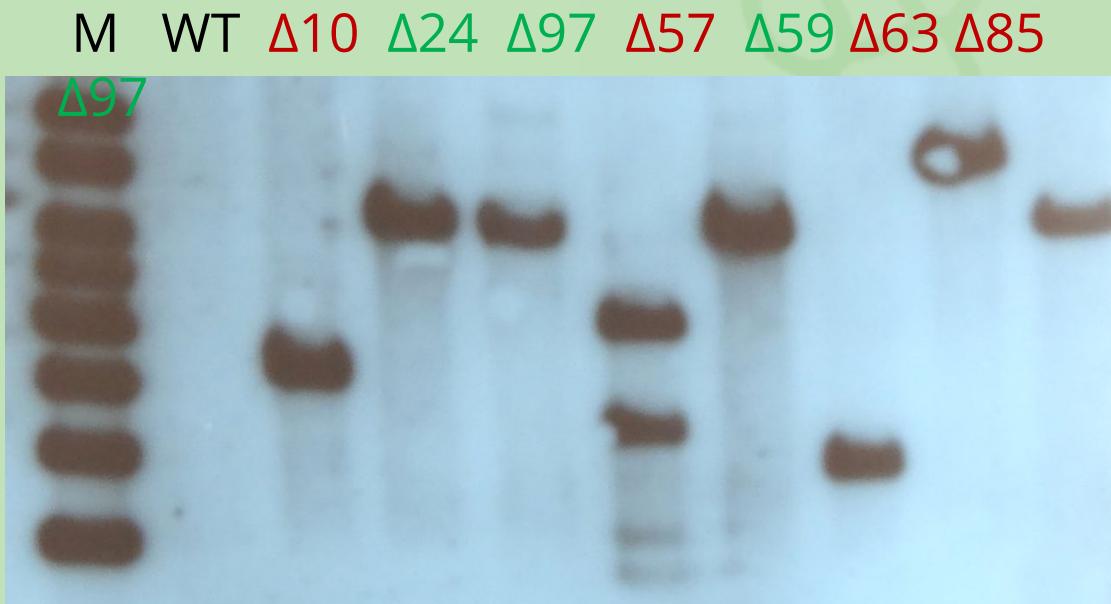


**WT Δ2 Δ103 Δ4 Δ5 Δ6 Δ12 Δ13 Δ14**

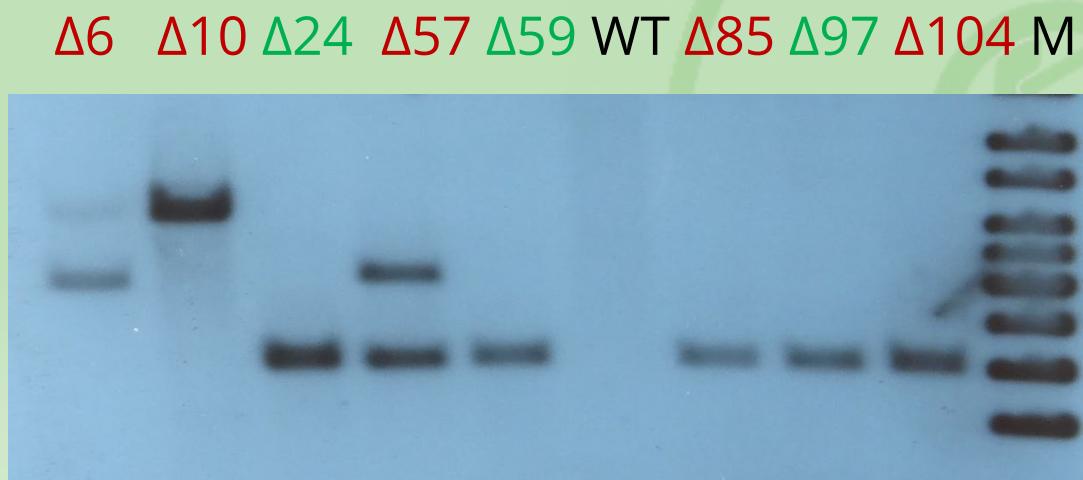


**Allantoicase Mutants + *Hind*III  
Probe: Allantoicase**

# Deleção de genes



Urease Mutants +  
*Scal*  
Probe: Hygromycin

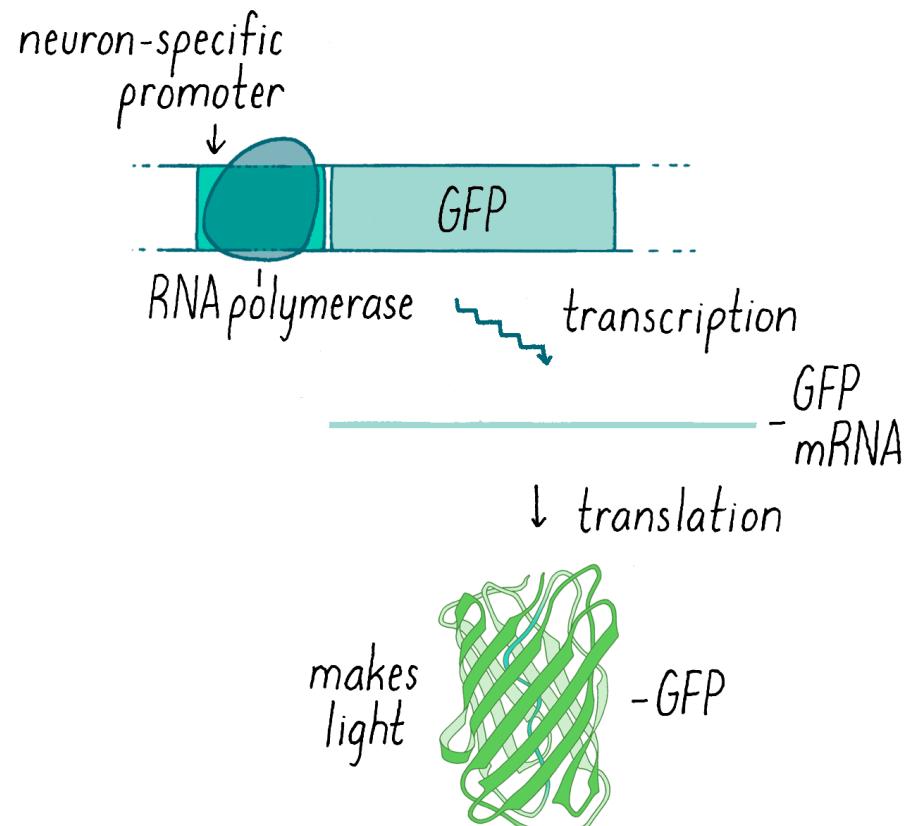


Urease Mutants + *Ncol*  
Probe: Hygromycin

# Fusão promotor - GFP

Verificar quando um gene é expresso

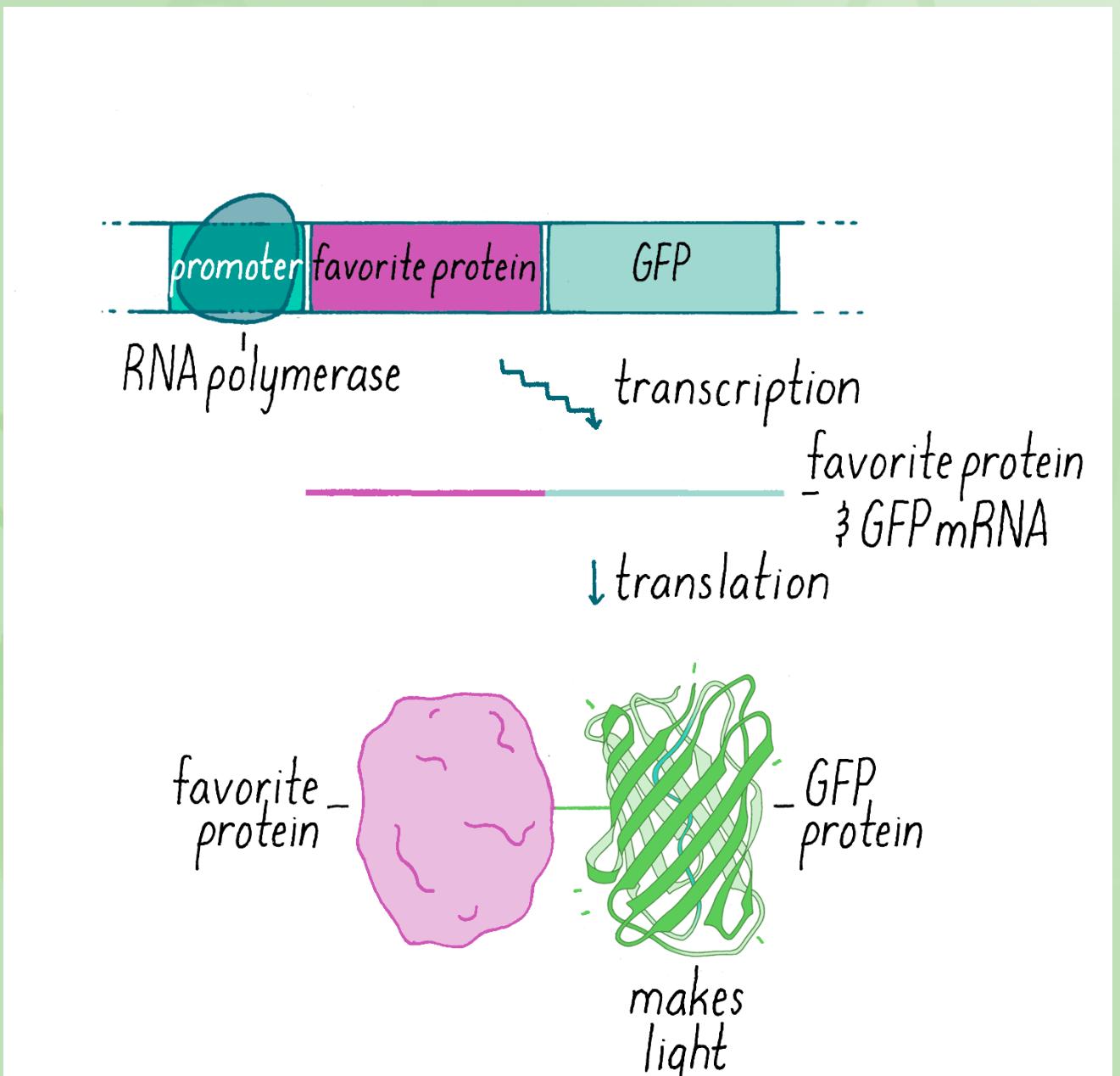
GFP TRANSCRIPTION AND TRANSLATION IN NEURONS



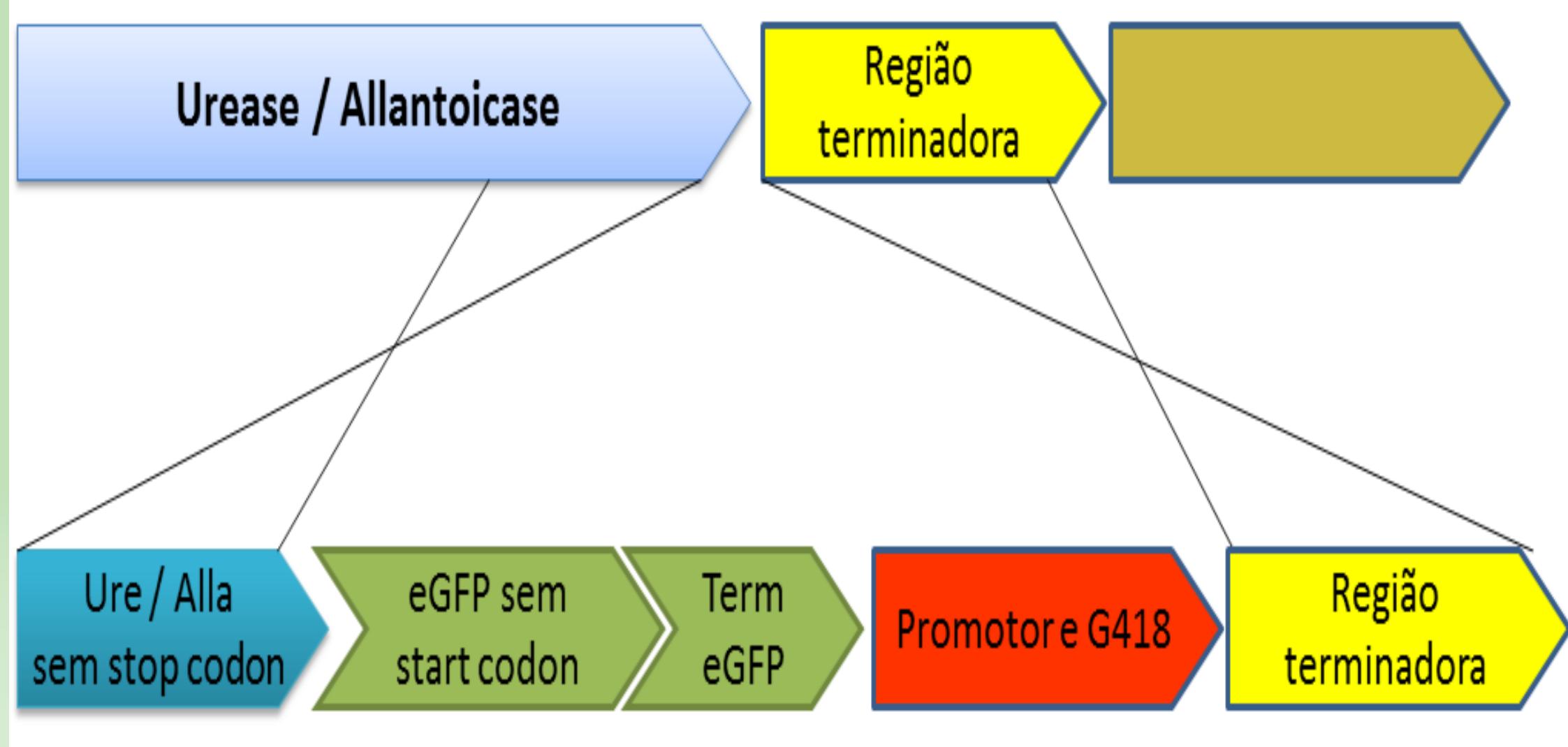
# Fusão gene - GFP

1. Verificar quando um gene é expresso

2. Localização da proteína



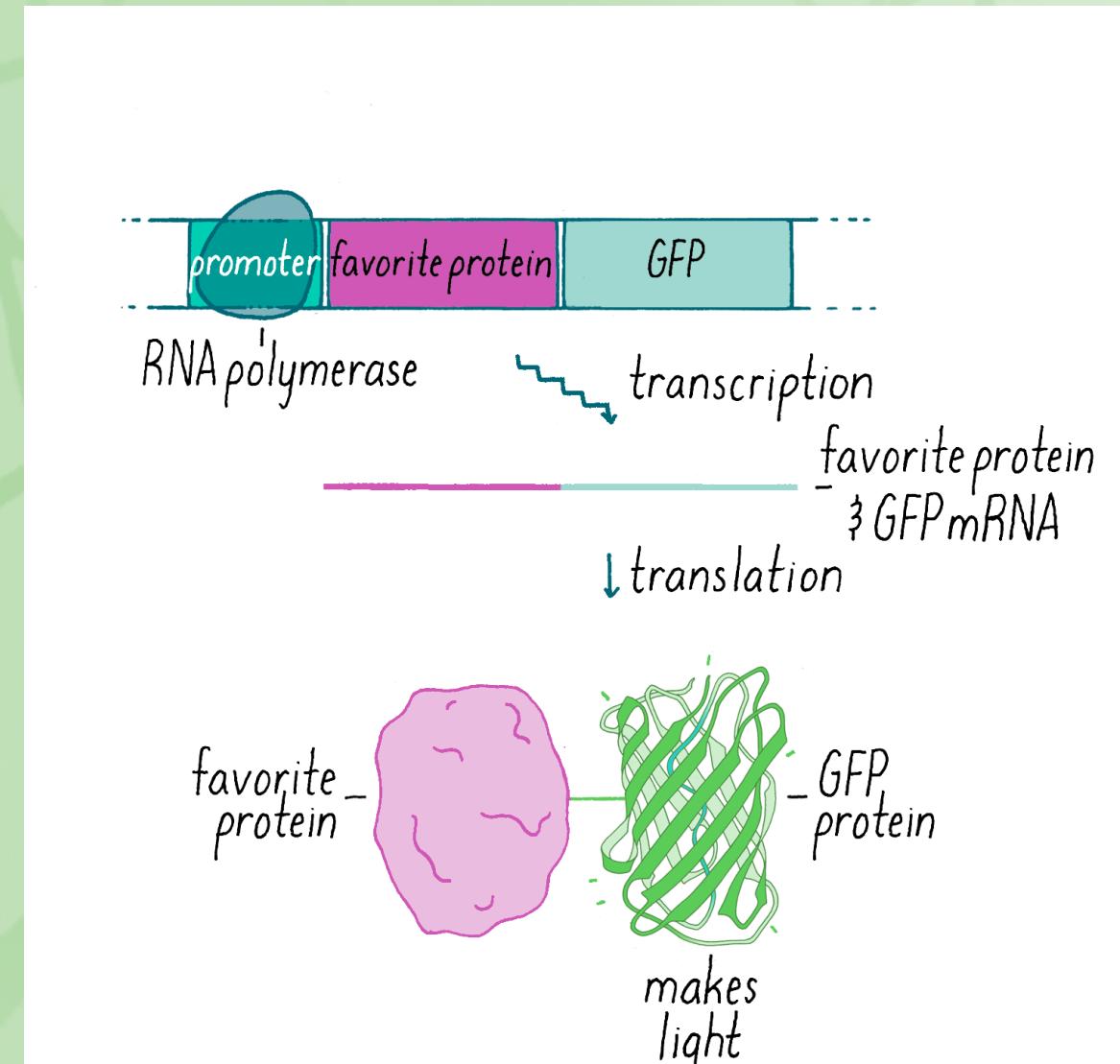
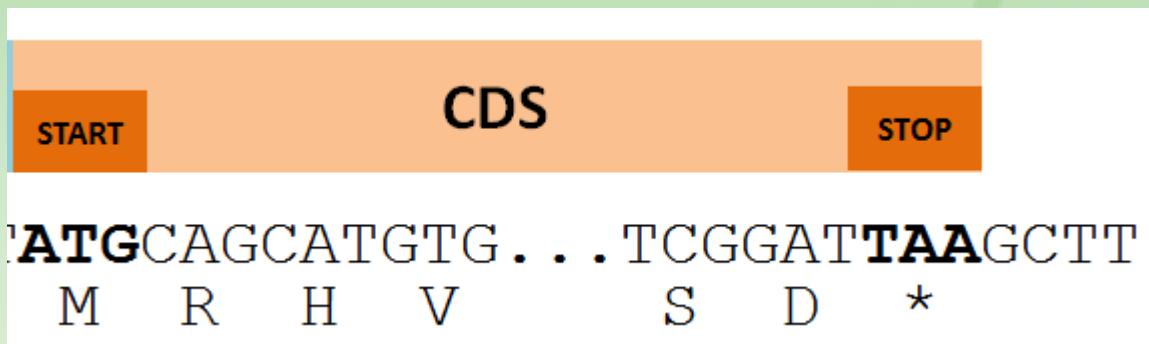
# Estratégia para montagem de cassete de fusão



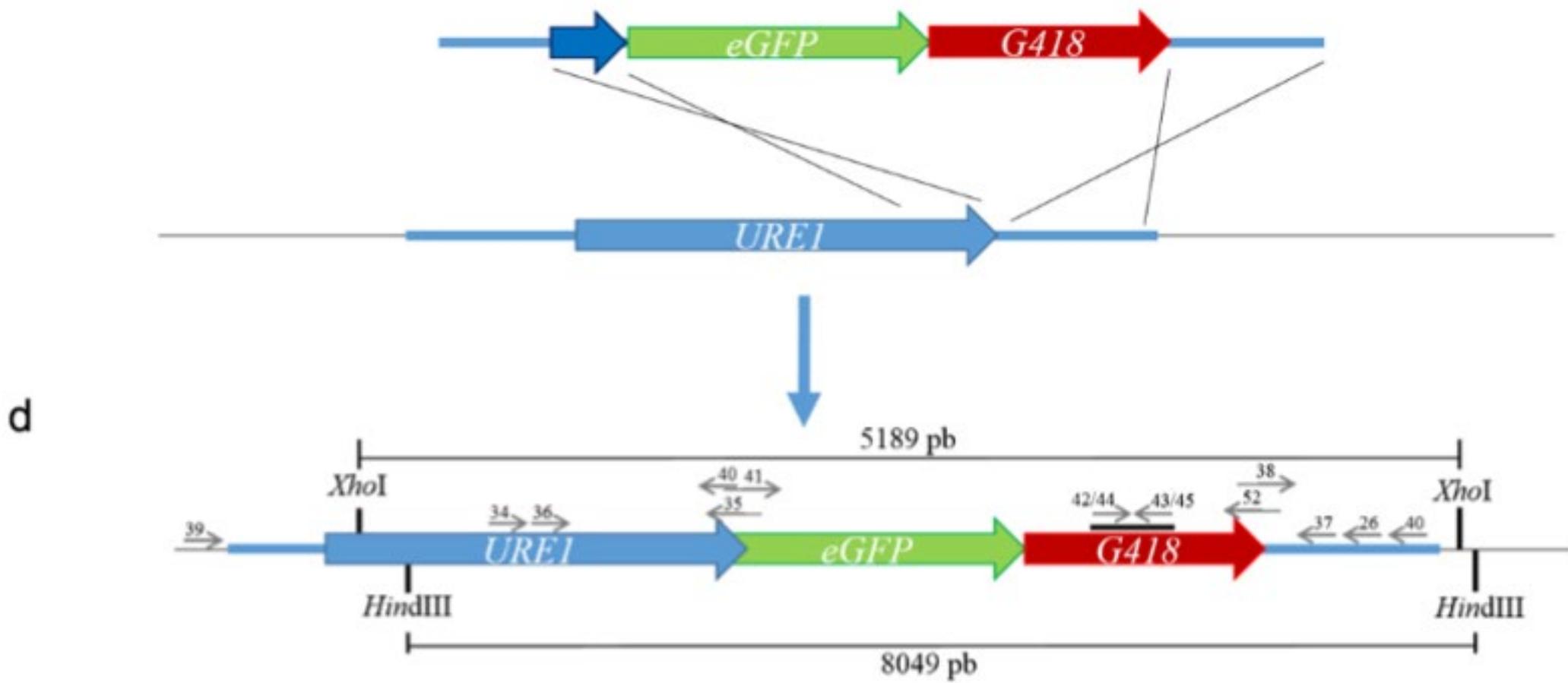
# Fusão gene - GFP

Gene de interesse sem o  
**STOP** codon

Gene GFP sem o **START**  
codon

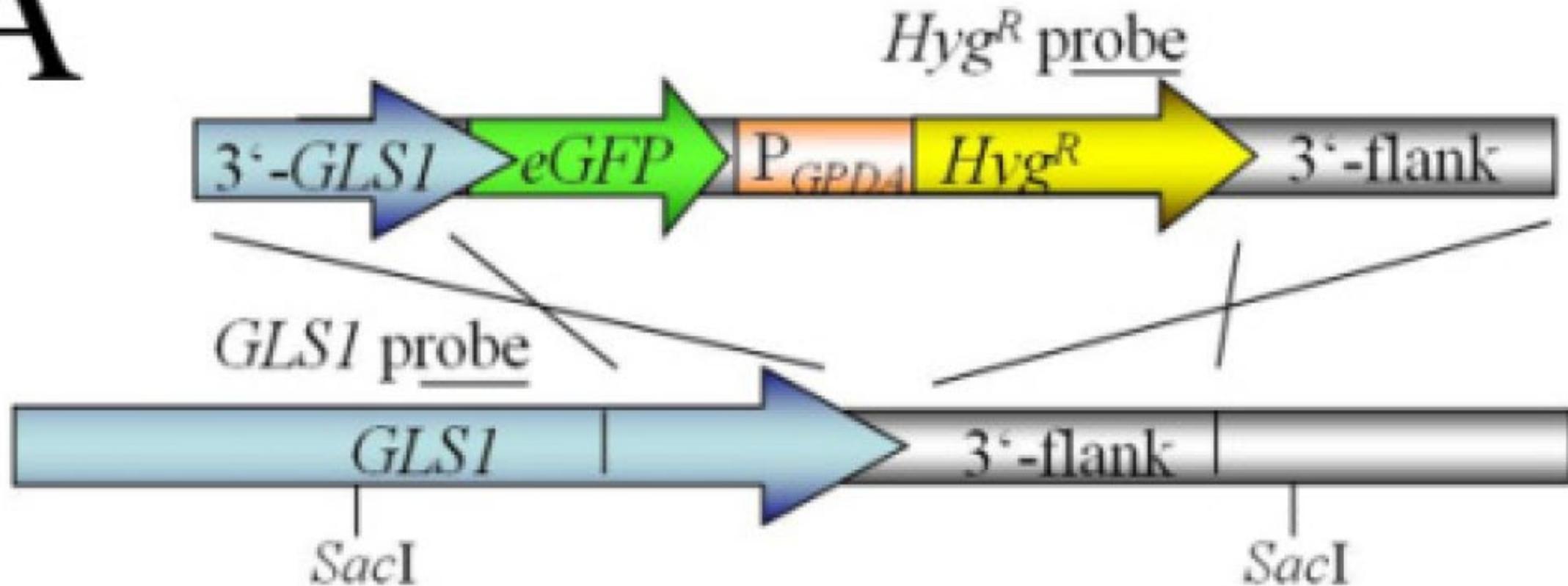


# Fusão com gene para proteína fluorescente (eGFP)

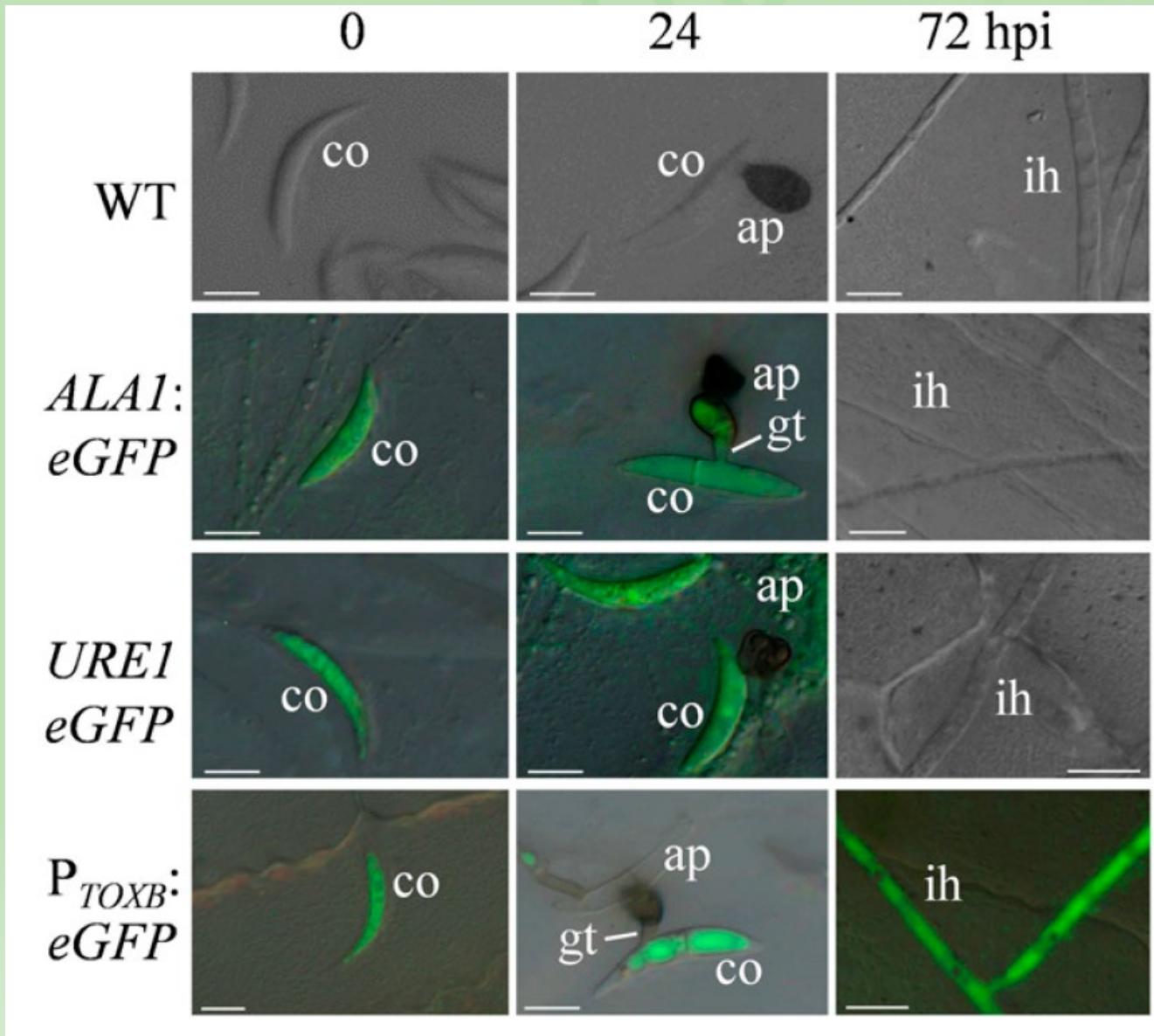


# Fusão com gene para proteína fluorescente (GFP)

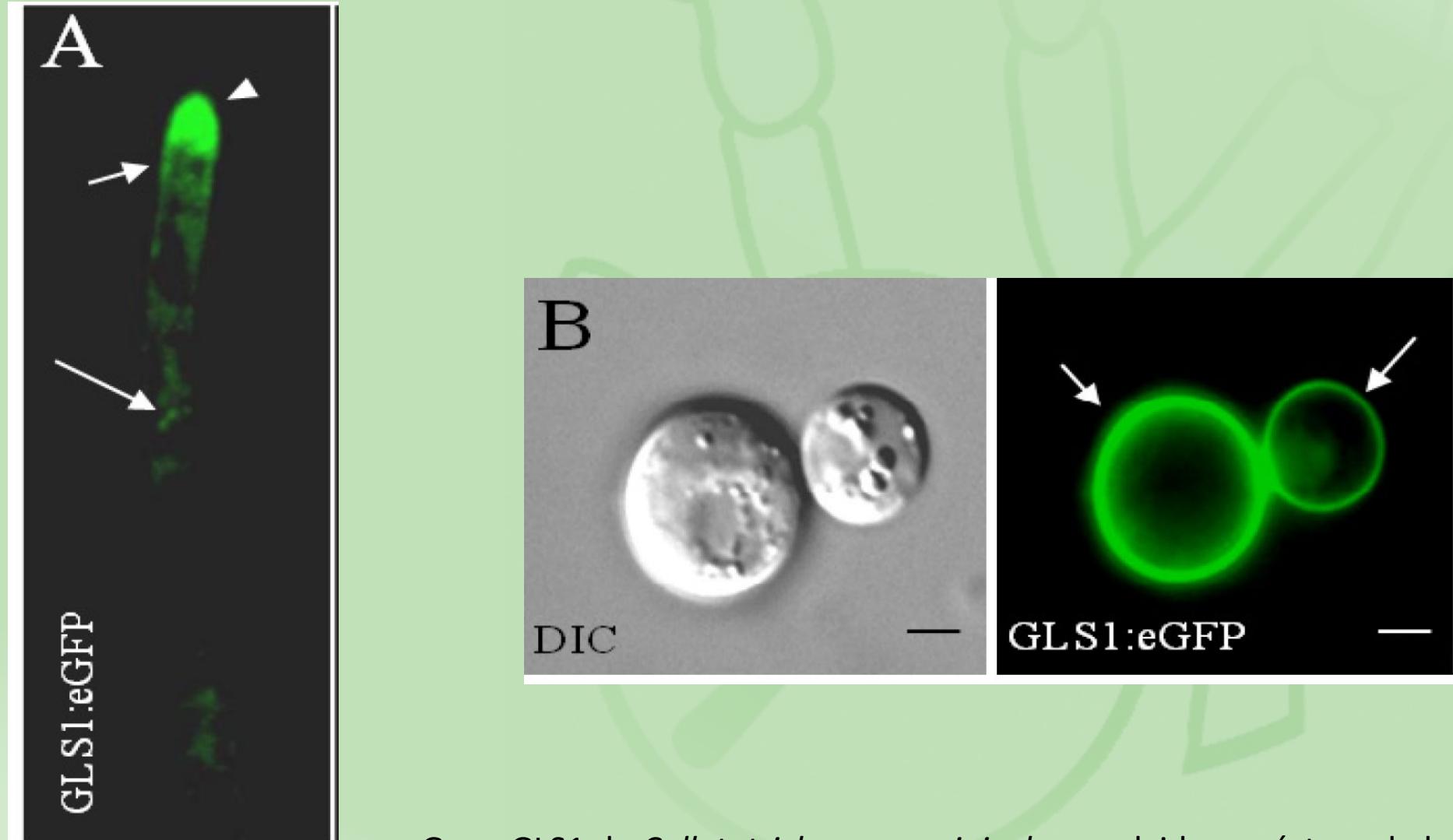
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# Análise temporal da expressão do gene

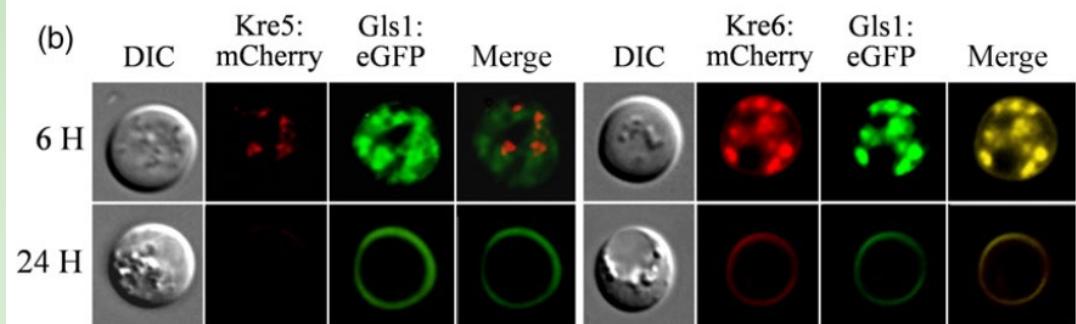
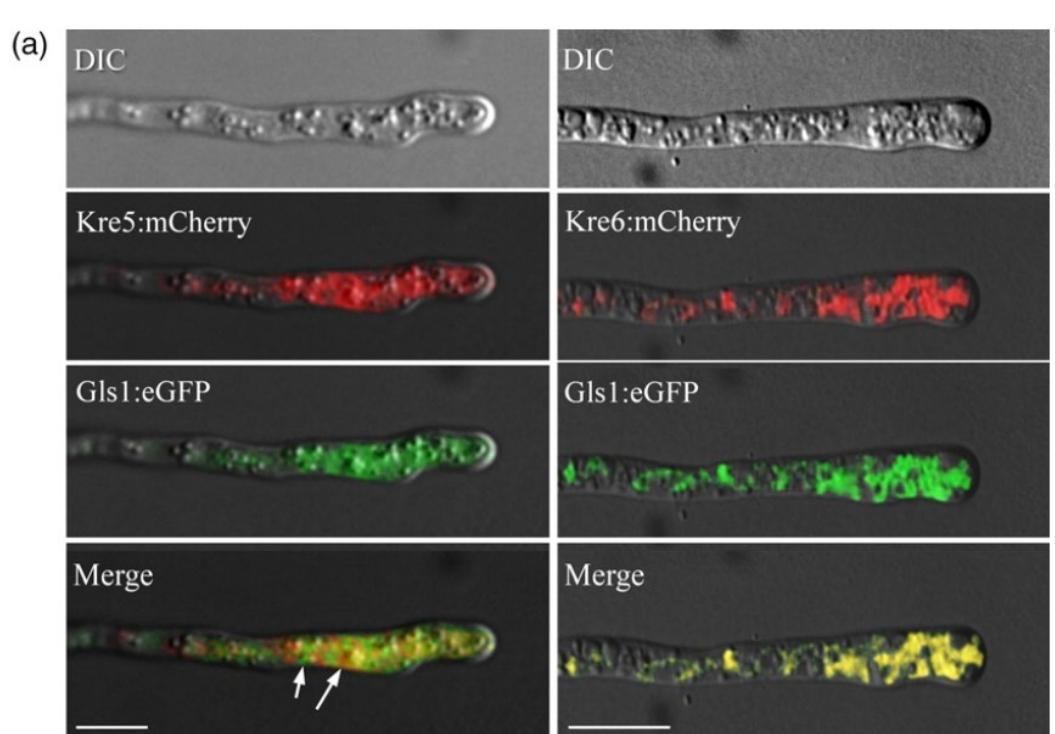


# Análise temporal da expressão do gene e localização da proteína



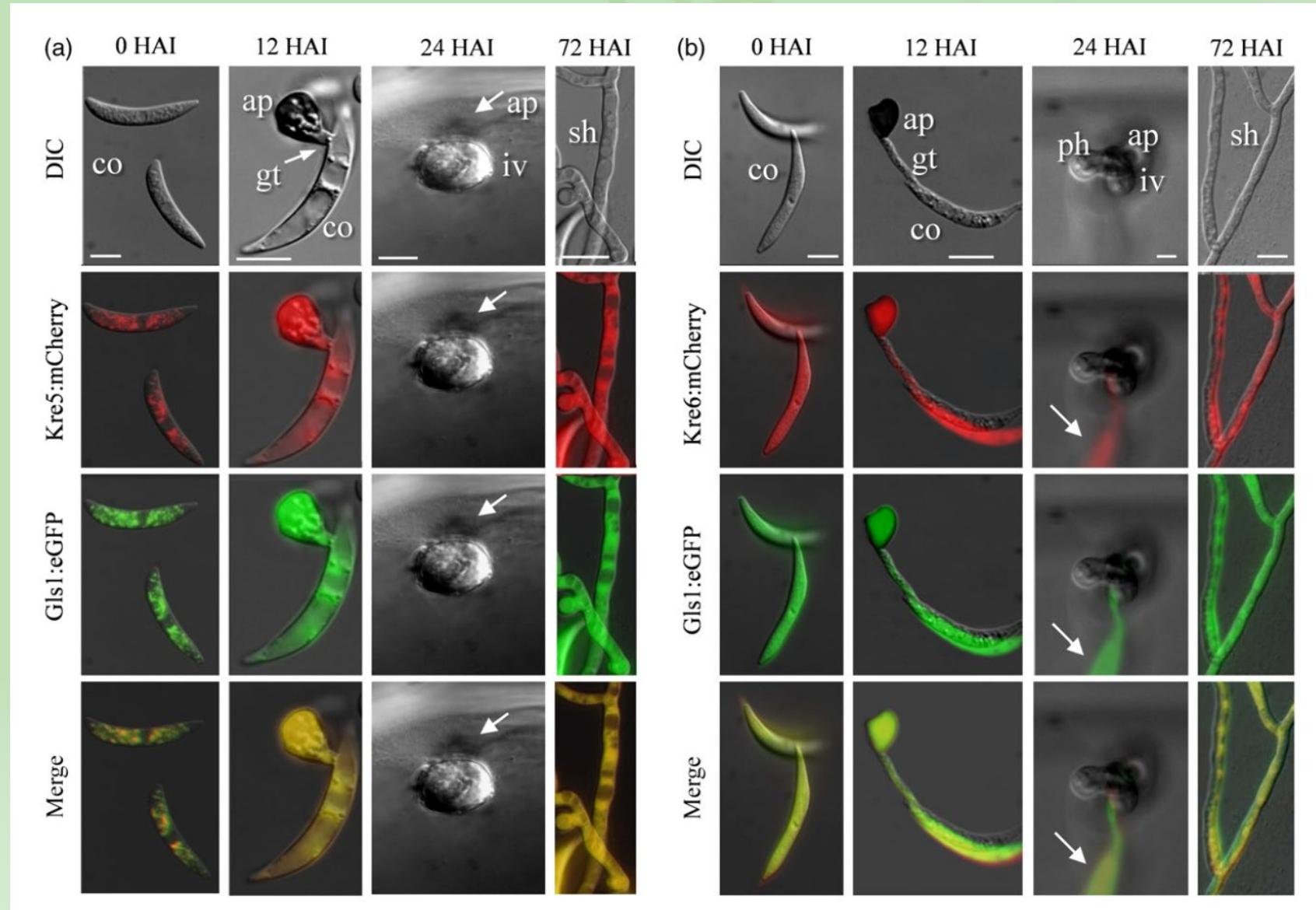
Gene *GLS1* de *Colletotrichum graminicola* envolvido na síntese de b-1,3-glucan

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

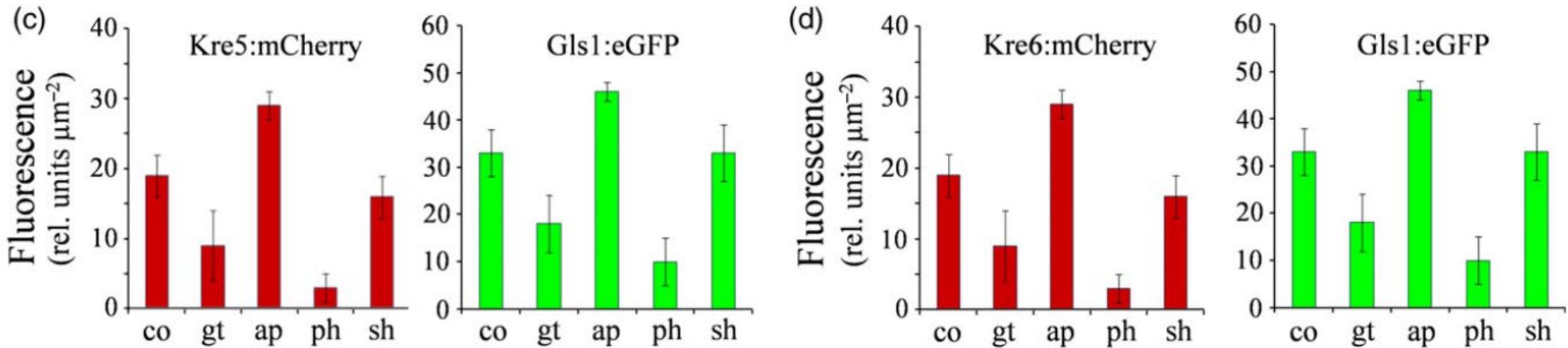


Genes KRE5 e KRE6 de *Colletotrichum graminicola* envolvidos na síntese de b-1,6-glucan  
Gene GLS1 de *Colletotrichum graminicola* envolvido na síntese de b-1,3-glucan

# 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



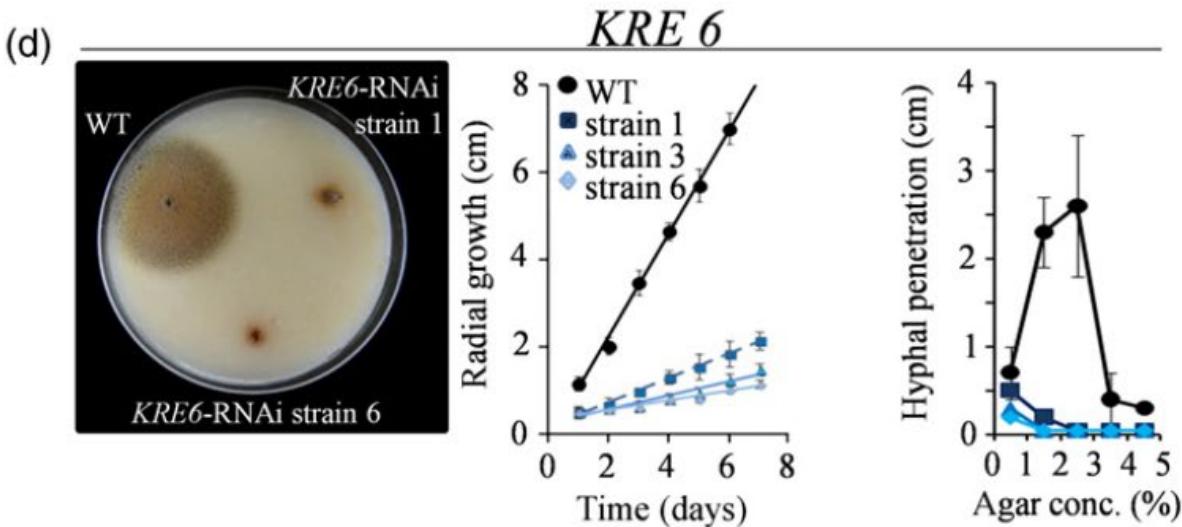
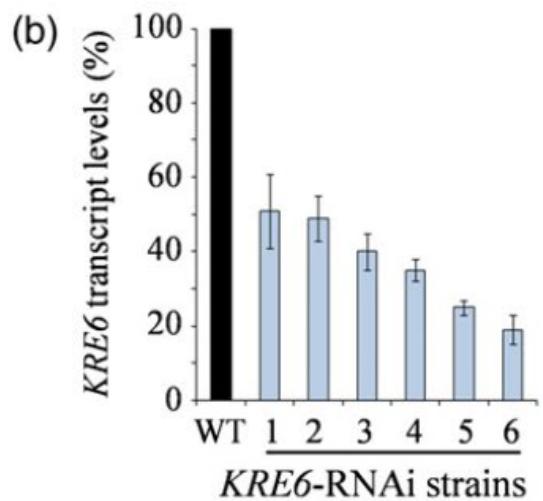
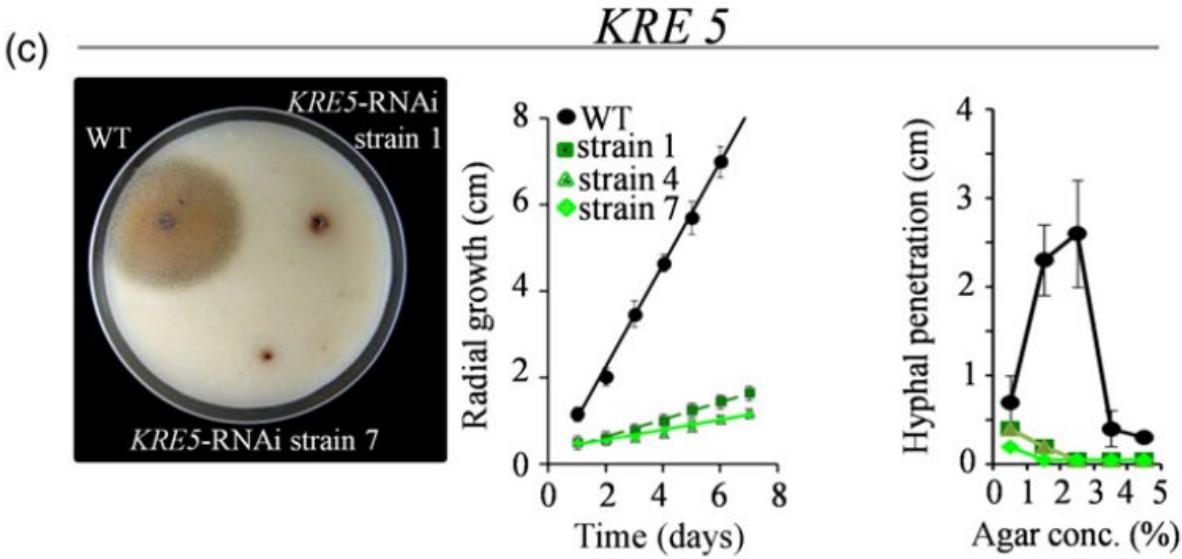
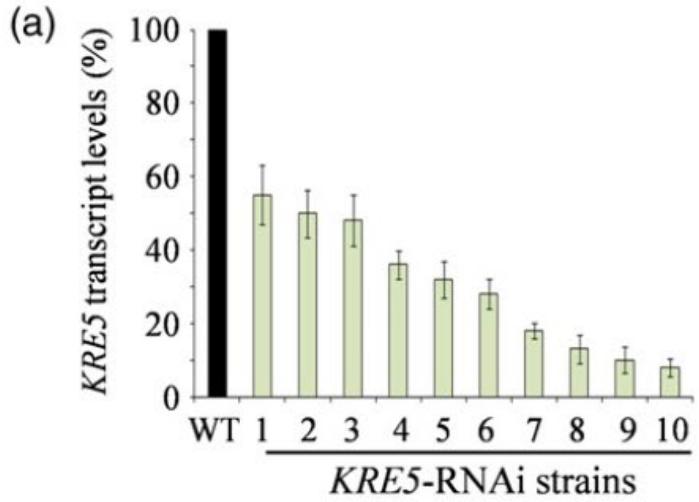
Genes KRE5 e KRE6 de *Colletotrichum graminicola* envolvidos na síntese de b-1,6-glucan

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

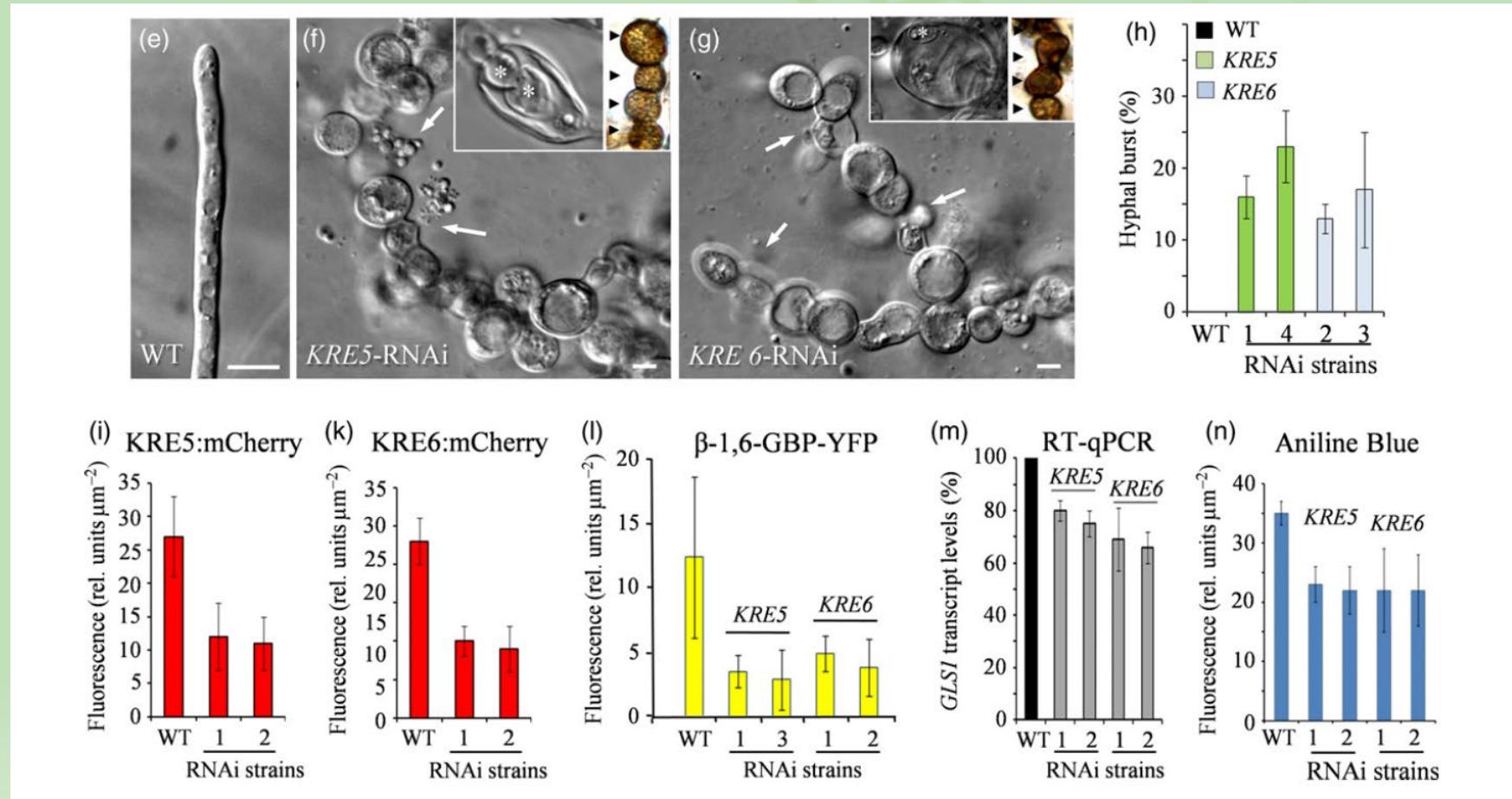
## Microscopia de fluorescência quantitativa

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



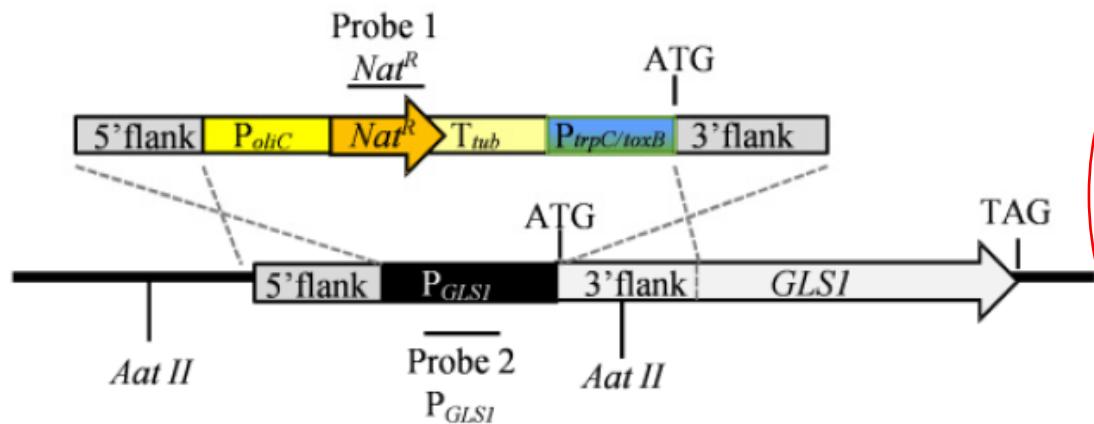
Verificar redução das proteínas além da redução dos mRNAs

# Superexpressão de genes

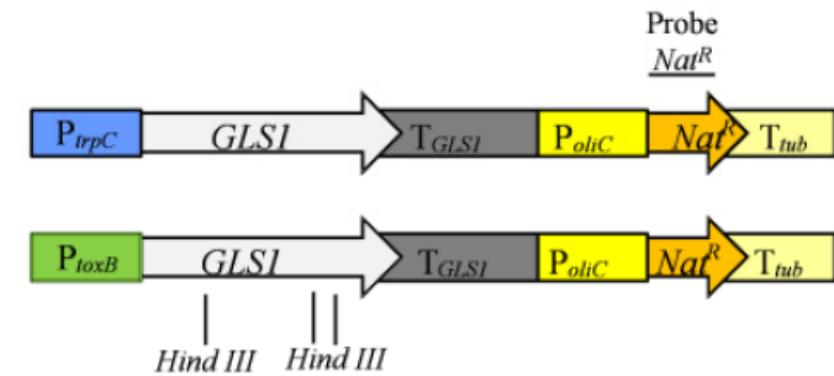
Expressão constitutiva ou aumento da expressão

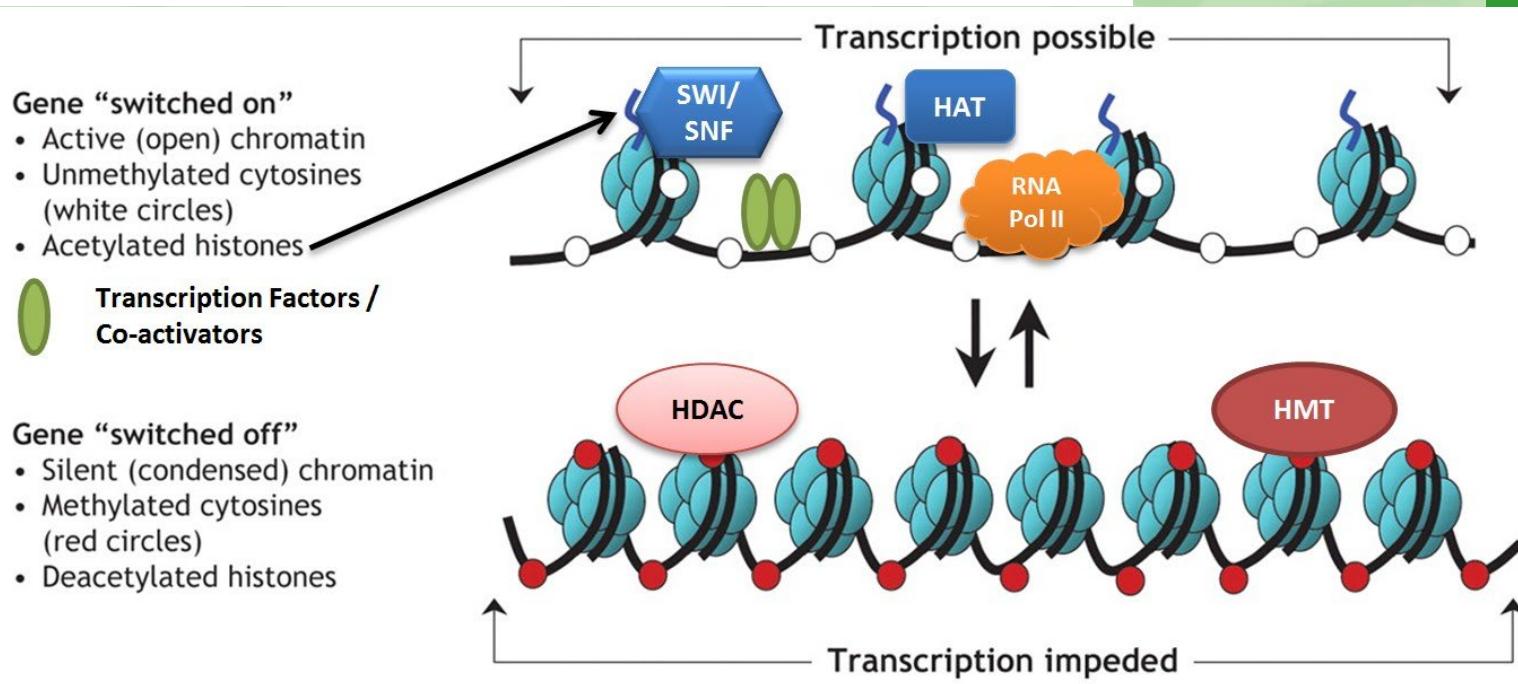
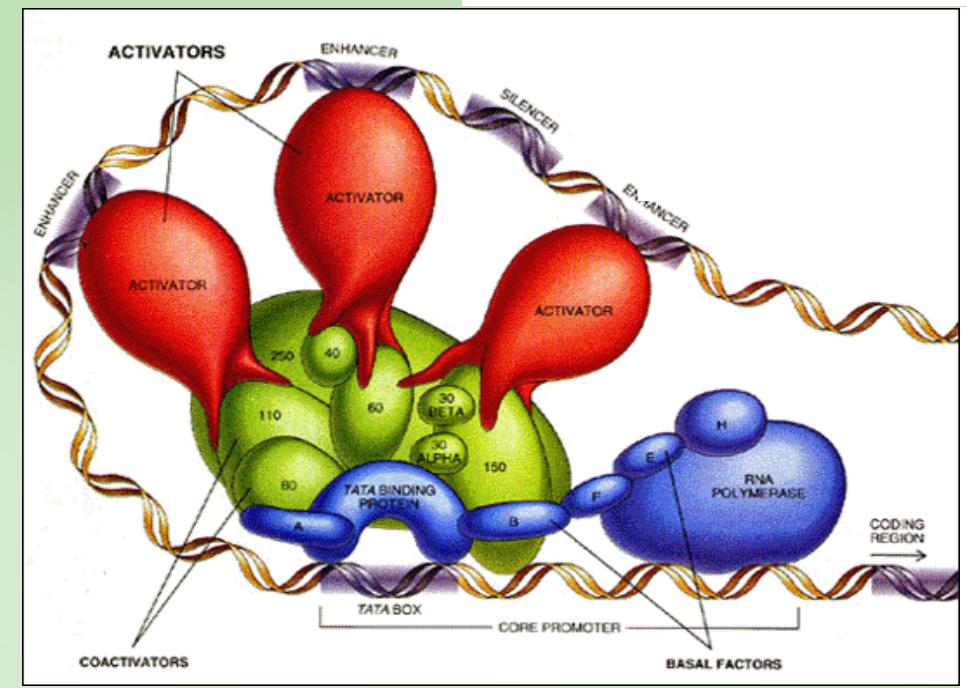
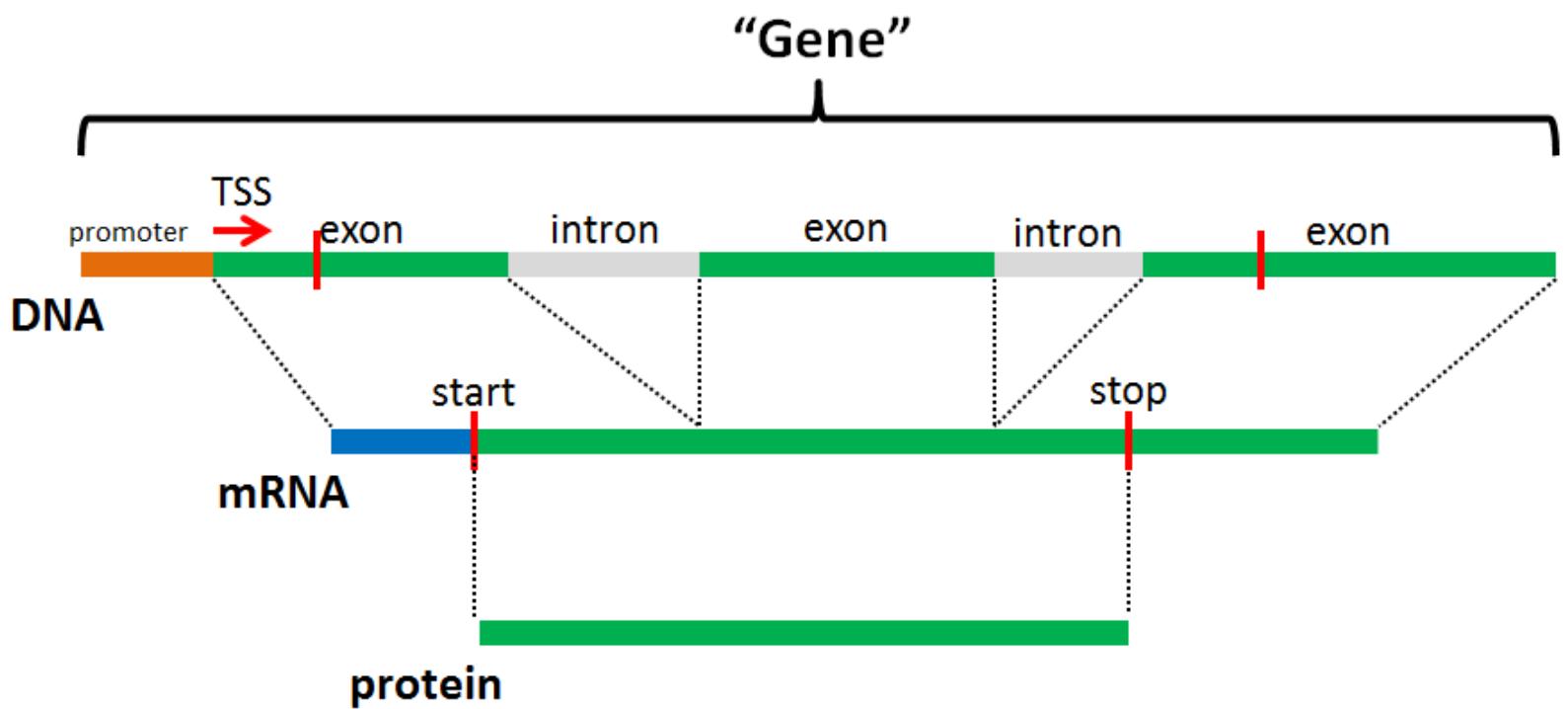
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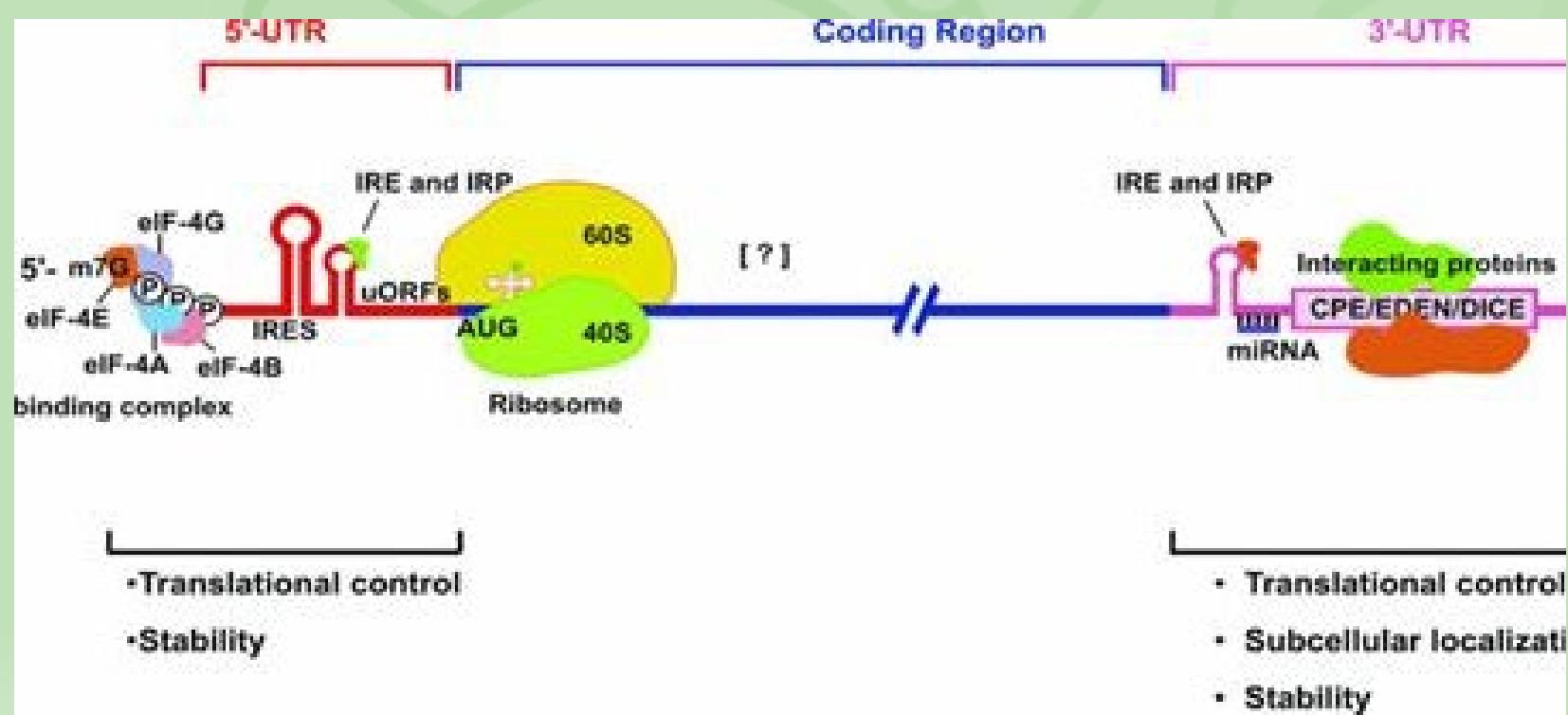
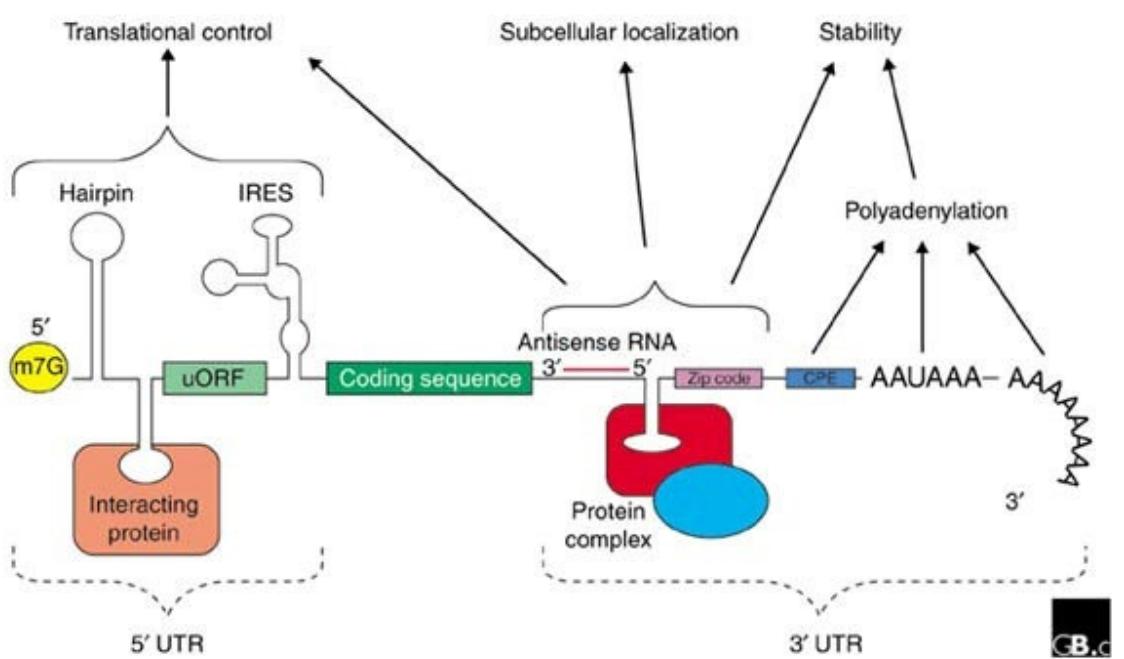
promoter exchange



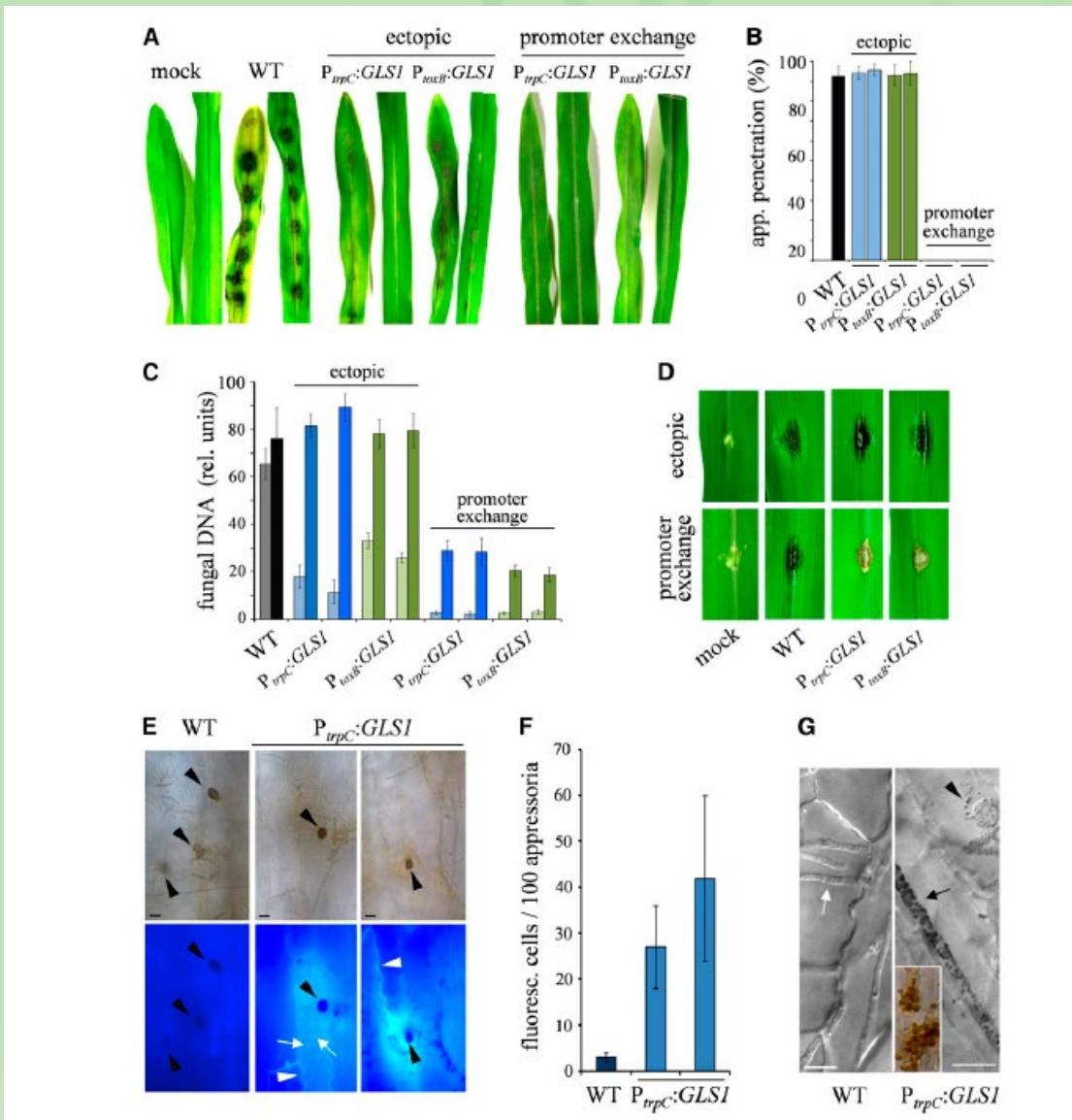
ectopic

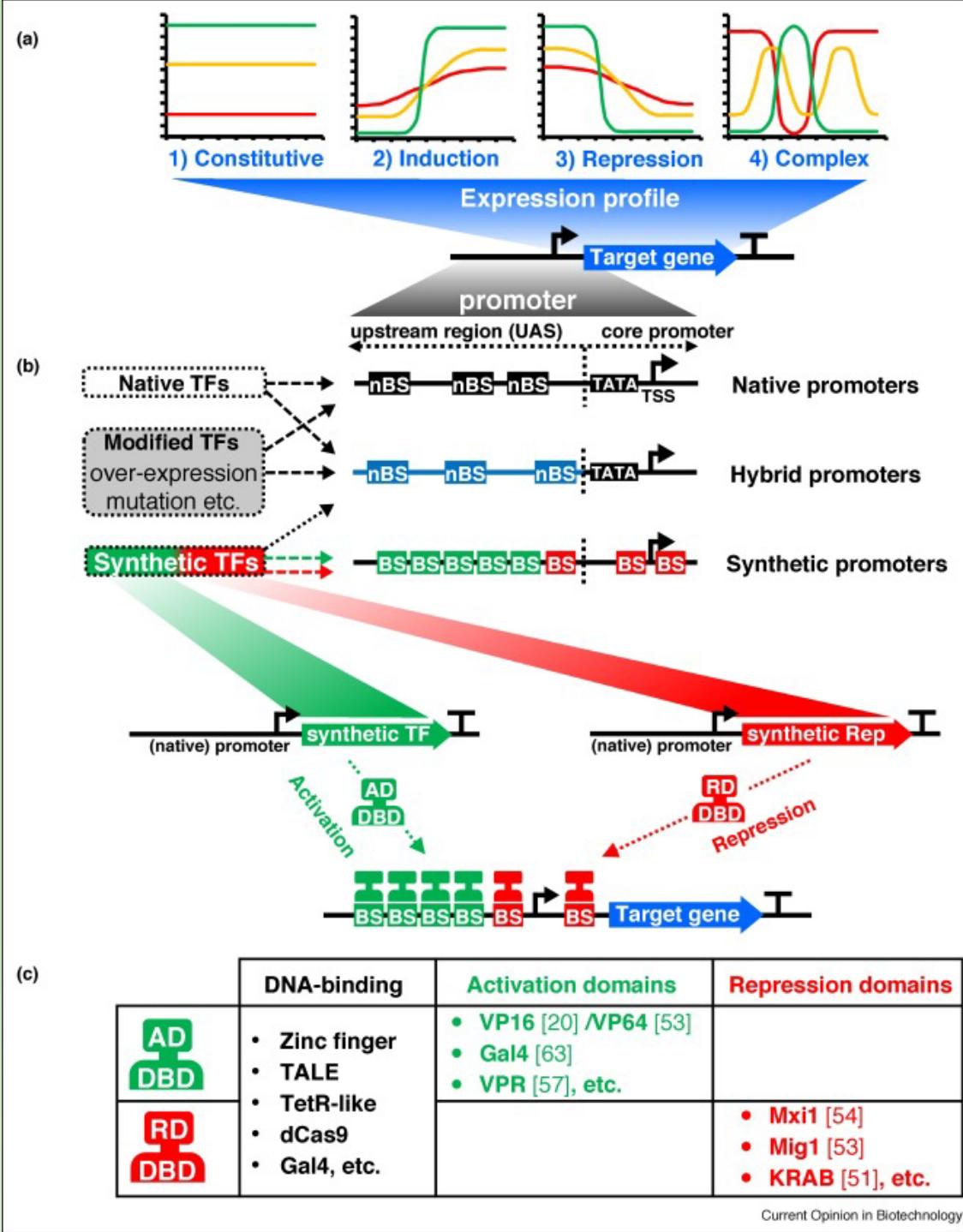


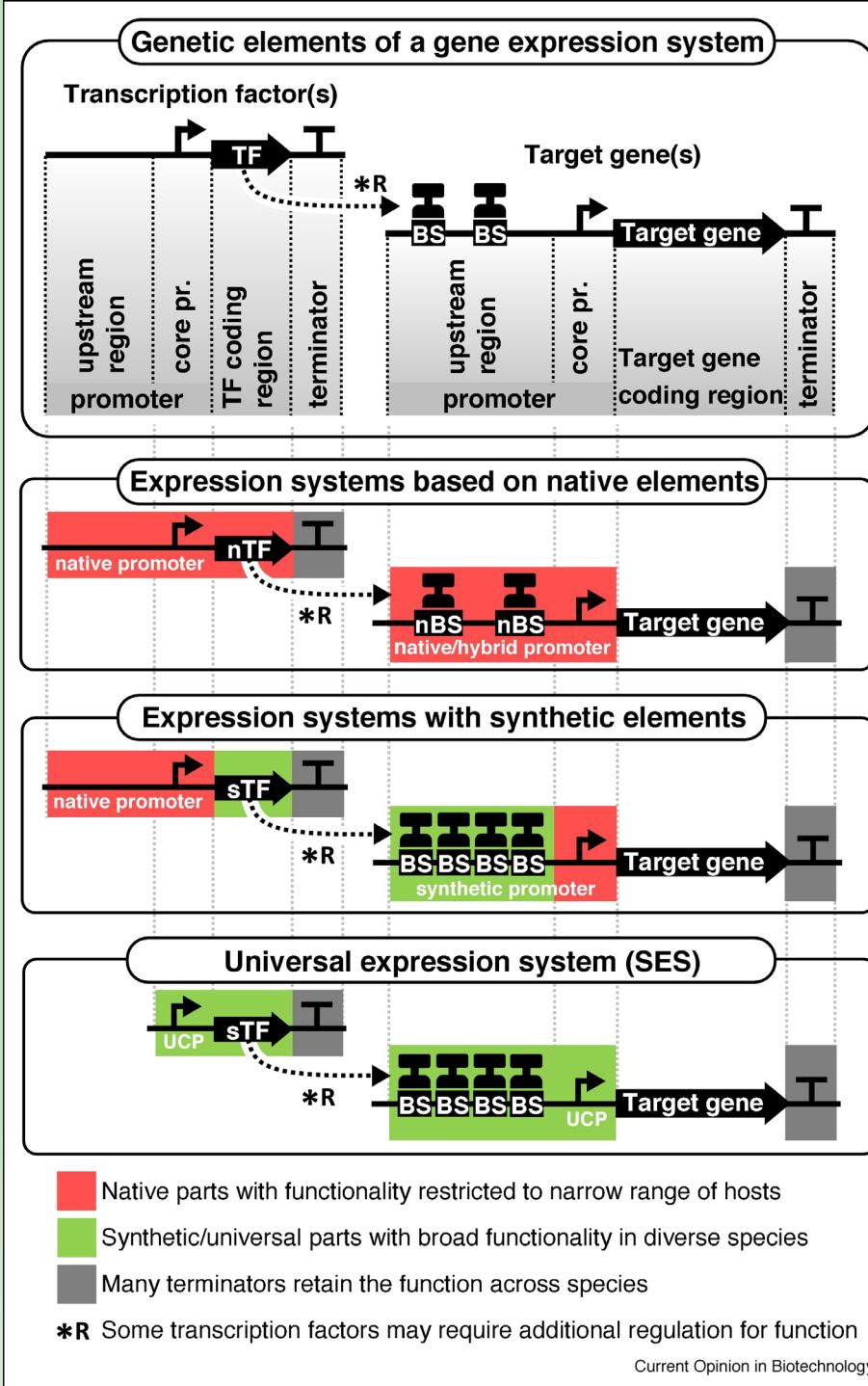




# Expressão forçada do gene *GLS1* em hifas biotróficas







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](#)

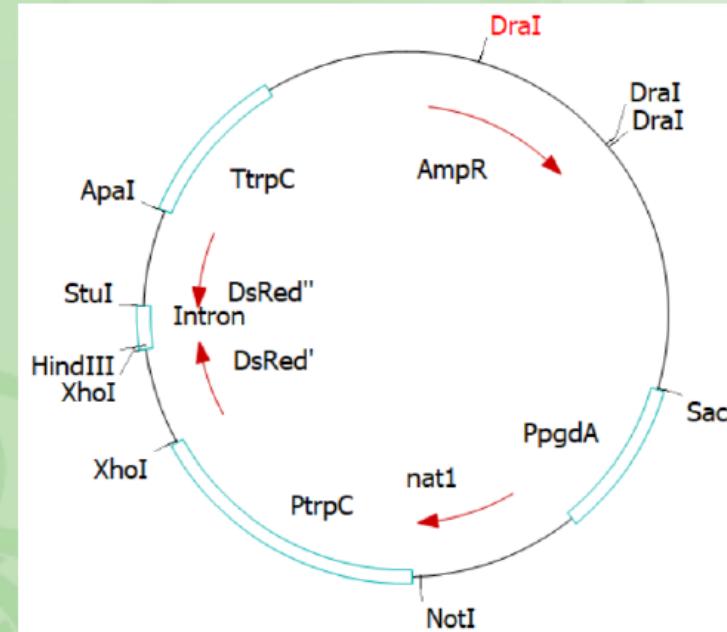
Available online 30th May 2019

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

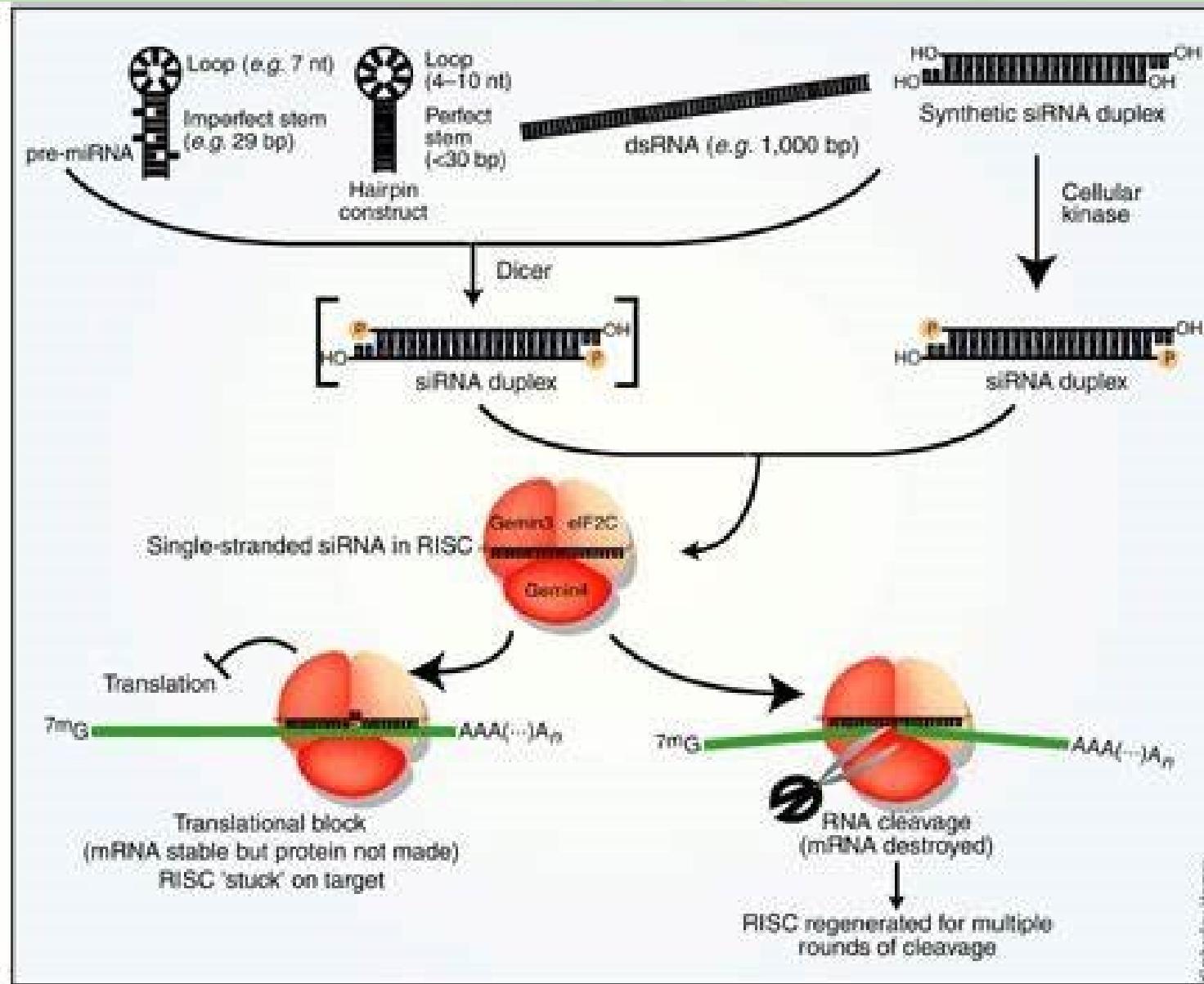
# Silenciamento via iRNA

## Estudo de caso: Montagem de Cassete de RNAi

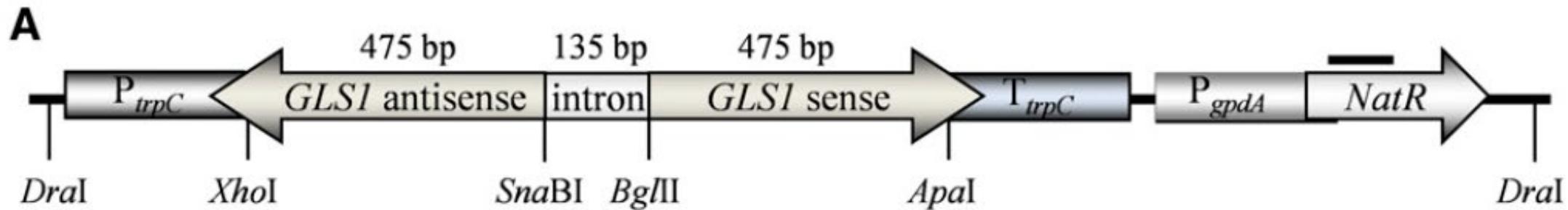
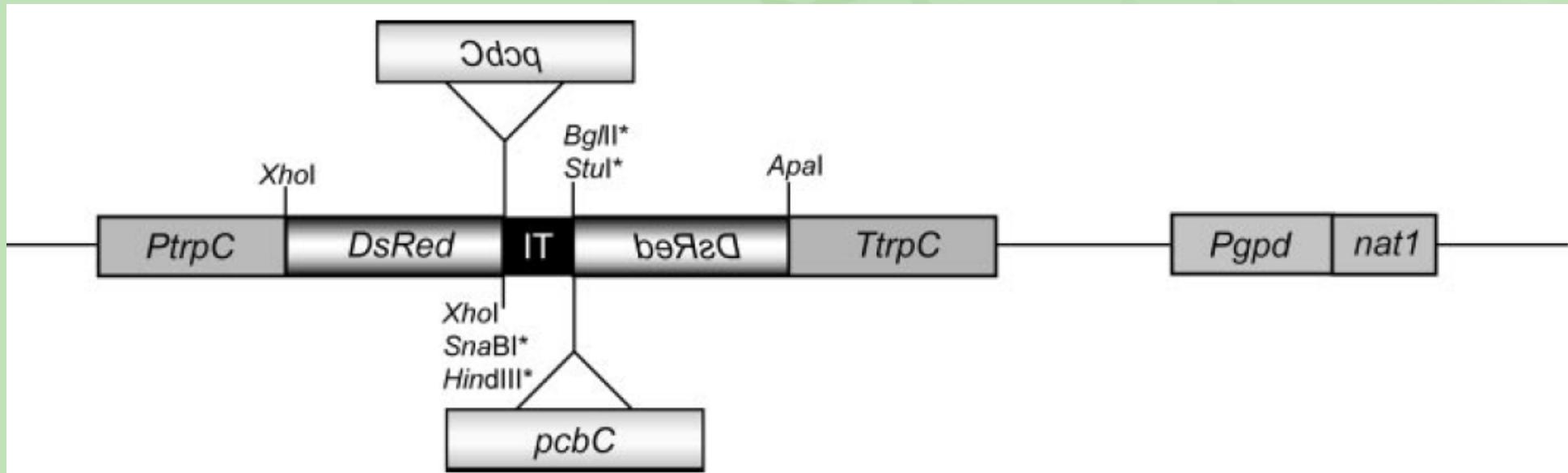
- Plasmídeo base: pRedi
  - Votor já contendo cassete de RNAi para o gene DsRed
    - Verificar a possibilidade de reutilizar esse votor para o gene alvo
    - Estudar as digestões e junções necessárias
- Gene Alvo
  - Predição de siRNA no cDNA do gene
    - Ferramenta DSIR: <http://biodev.cea.fr/DSIR/DSIR.html>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



# Silenciamento via iRNA



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## 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)