UNIVERSIDADE DE BRASÍLIA
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA
E BIODIVERSIDADE

ISOLAMENTO E CARACTERIZAÇÃO DE PROMOTORES DE GENES INDUZÍVEIS EM RESPOSTA A ESTRESSES BIÓTICO E ABIÓTICO

ELINEA DE OLIVEIRA FREITAS

Orientadora: Maria Fátima Grossi-de-Sá

Brasília-DF/ Brazil
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Tese apresentada ao Departamento de Biotecnologia e Biodiversidade do Instituto de Biologia da Universidade de Brasília, como parte dos requisitos para a obtenção do título de Doutor em Biotecnologia e Biodiversidade.

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RESUMO

Freitas, Elinea de Oliveira, Universidade de Brasília. Julho de 2019. **Isolamento e caracterização de promotores de genes induzíveis em resposta a estresses biótico e abiótico.** Orientadora: Maria Fátima Grossi de Sá.

A biotecnologia vegetal têm alcançado progressos através do uso da engenharia genética como uma aliada na geração de organismos geneticamente modificados. Contudo, o sucesso da tecnologia de transformação genética de plantas, desde a pesquisa básica até o desenvolvimento de novas cultivares está diretamente associado à obtenção de um nível de expressão apropriado do DNA exógeno, que depende da correta seleção do promotor a ser utilizado. Assim, a busca por promotores induzíveis têm sido uma poderosa estratégia biotecnológica para controlar a expressão de genes envolvidos em respostas a estresses bióticos e abióticos. No primeiro capítulo deste estudo foi descrito o isolamento e análise funcional de três promotores de genes de algodão (*Gossypium hirsutum*) induzidos pelo estresse biótico causado pelo desenvolvimento da larva do bicudo-do-algodoeiro em botões florais de plantas de algodão. Inicialmente, foram selecionados 20 genes com padrão de expressão induzível identificados no transcriptoma de botões florais de algodão infestados com larvas do bicudo. A expressão desses genes foi analisada por RT-qPCR após diferentes tempos de alimentação da larva do bicudo. Botões florais de algodão inoculados com um ovo do bicudo-do-algodoeiro, contendo um embrião ativo, foram analisados após 2h, 6h, 12h, 24h e 96h de inoculação. Entre os genes analisados, *GhERF17-like, GhERF105-like* e *GhNc-HARBI1* apresentaram um perfil de expressão aumentado, principalmente em respostas tardias (12h, 24h e 96h). Essas análises confirmaram que estes genes são induzíveis pelo estresse biótico causado pelo desenvolvimento da larva do bicudo-do-algodoeiro em botões florais de plantas de algodão. As sequências dos promotores desses três genes foram isoladas e analisadas quanto à presença de elementos *cis* regulatórios e foram identificados, nos três promotores, elementos *cis* do tipo ERF, MYB e muitos fatores de transcrição W-box (conhecidos como local de ligação do fator de transcrição do tipo WRKY): WRKY71OS, WBOXNTERF3, WBBOXPCWRKY1, WBOXATNPR1. Além disso, também foram identifidas sequências *cis*-regulatórias envolvidas na indução de ácido salicílico (SA). Posteriormente, esses três promotores foram clonados no vetor de expressão estável que contém a fusão GUS-GFP. A atividade do gene repórter GFP, controlada pelos promotores p*GhERF17-like*, p*GhERF105-like* e p*GhNc-HARBI1-like*, foi monitorada e comparados com o promotor *CaMV35S* em folhas de plantas transgênicas de *A. thaliana* sob estímulo com ácido salicílico (AS). As análises indicaram que os três promotores foram induzíveis pelo AS, com destaque para o promotor p*GhNc-HARBI1-like* cuja fluorescência de GFP foi mais intensa, quando submetida ao tratamento com AS, enquanto nenhuma fluorescência foi observada no tratamento controle. Já em plantas com o promotor *CaMV35S* observou-se intensa fluorescência com e sem o tratamento com AS. Estudos adicionais estão sendo realizados para proporcionar uma cacterização completa desses três promotores que, possivelmente, poderão ser indicados como potenciais ferramentas biotecnológicas para impulsionar a expressão gênica induzível em plantas. No segundo capítulo o objetivo foi identificar e caracterizar o promotor *GmRD26* (p*GmRD26*), que está envolvido na regulação das respostas das plantas ao estresse hídrico. O perfil de expressão do gene *GmRD26* obtido por qRT-PCR sob condições de estresse e sem

estresse confirmou que o *GmRD26* é induzido sob condições de déficit hídrico. A caracterização da região promotora *GmRD26* foi realizada sob condições de estresse com ácido abscísico (ABA), polietilenoglicol (PEG) e seca em plantas de *A. thaliana* contendo a construção completa de p*GmRD26*::GUS (2.054 pb) e dois módulos, p*GmRD26A*::GUS (909 pb) e p*GmRD26B*::GUS (435 pb), controlando a express do gene β-glucuronidase (*uidA*). Os módulos p*GmRD2*6 e p*GmRD26A* conferiram expressão forte e induzida de transgenes. Os dados demonstraram que, de acordo com análises da atividade de GUS, o p*GmRD26A* induziu maior expressão de transgenes do que o promotor usado como controle positivo, *AtRD29*, e que os outros módulos, dependendo do tipo de tratamento analisado. O módulo p*GmRD26A* forneceu maior capacidade de transcrição do gene *uidA* do que outros módulos, especialmente em resposta a tratamentos com polietilenoglicol e seca. Em resumo, este estudo indica que o p*GmRD26A* pode se tornar uma ferramenta biotecnológica promissora para aplicação no desenvolvimento de plantas modificadas tolerantes à seca ou outras plantas projetadas para resposta ao estresse.

Palavras chave: Promotor induzível, elementos *cis* regulatórios, *Gossypium hirsutum,* estresse biótico, *Glycine max*, tolerância à seca.

ABSTRACT

Freitas, Elinea de Oliveira, **University of Brasilia. July 2019. Isolation and characterization of inducible gene promoters in response to biotic and abiotic stresses.** Doctoral advisor: Maria Fátima Grossi de Sá.

Plant biotechnology has made progress through the use of genetic engineering as an ally in the generation of genetically modified organisms. However, the success of plant genetic transformation technology, from basic research to the development of new cultivars, is directly associated with obtaining an appropriate level of expression of exogenous DNA, which depends on the correct selection of the promoter to be used. Thus, the search for inducible promoters has been a powerful biotechnological strategy to control the expression of genes involved in responses to biotic and abiotic stresses. In and analyzed for the presence of regulatory cis elements and the cis-elements of the ERF, MYB and many W-box transcription factors (known as the binding factor transcription of type WRKY): WRKY71OS, WBOXNTERF3, WBBOXPCWRKY1, WBOXATNPR1. In this study, we also identified cis-regulatory sequences involved in the induction of salicylic acid (SA). Subsequently, these three promoters were cloned into the GUS-GFP fusion-stable expression vector. The activity of the GFP reporter gene controlled by the p*GhERF17-like,* p*GhERF105-like* and p*GhNc-HARBI1-like* promoters was monitored and compared to the *CaMV35S* promoter on leaves of *A. thaliana* transgenic plants under stimulation with salicylic acid (SA). The analyzes indicated that the p*GhERF17-like,* p*GhERF105-like* and p*GhNc-HARBI1-like* promoters were inducible by SA. In plant leaves containing p*GhNc-HARBI1-like*, more intense GFP fluorescence was observed when subjected to SA treatment and no fluorescence was observed in the control treatment. In plants with the *CaMV35S* promoter, intense fluorescence was observed with and without SA treatment. Further studies are being conducted to provide a complete characterization of these three promoters that may possibly be indicated as potential biotechnological tools to boost inducible gene expression in plants. In the second chapter our objective was to identify and characterize the *GmRD26* promoter (p*GmRD26*), which is involved in the regulation of plant responses to water stress. The expression profile of the *GmRD26* gene was investigated by qRT-PCR under stress conditions and without stress. Our data confirm that *GmRD26* is induced under water deficit conditions. Characterization of the *GmRD26* promoter region was performed under stress conditions with ABA, polyethylene glycol (PEG) and dry in *A. thaliana* plants containing the complete construct of p*GmRD26*::GUS (2.054 bp) and two null promoters, p*GmRD26A*::GUS (909 bp) and p*GmRD26B*::GUS (435 bp), controlling the expression of the *β*-glucuronidase gene (*uidA*). The p*GmRD26* and p*GmRD26A* modules conferred strong and induced expression of transgenes. The data demonstrated that, according to analyzes of GUS activity, p*GmRD26A* induced more transgene expression than the promoter used as a positive control, *AtRD29*, and that the other modules, depending on the type of treatment analyzed. The p*GmRD26A* module provides increased *uidA* transcription capacity than other modules, especially in response to treatments with polyethylene glycol and dry. In summary, this study indicates that p*GmRD26A* may become a promising biotechnological tool for application in the development of modified drought tolerant plants or other plants designed for stress response.

Key words: Inducible promoter, regulatory cis elements, *Gossypium hirsutum*, biotic stress, *Glycine max*, drought tolerance.

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Identification and characterization of the *GmRD26* soybean promoter in response to abiotic stresses: potential tool for biotechnological application

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Lista de Abreviaturas ou Siglas

ABA: ácido abscísico

BLAST: *basic local alignment search tools*

Bt: *Bacillus thuringensis*

CaM: calmodulinas

CaMV: *Cauliflower mosaic virus*

DAMPs: Padrões moleculares associados ao dano

ET: etileno

GFP: *green fluorescent protein*

GM: geneticamente modificadas

GUS: enzima β-glucuronidase

H2O2: peróxido de hidrogênio

JA: ácido jasmônico

MAMPs ou PAMPs ou HAMPs: do inglês, *microbe-or pathogen-or herbivore associated molecular patterns*

MAPK: proteínas quinases ativadas por mitógenos

OGM: organismos geneticamente modificados

pb: pares de base

PCR: reação em cadeia da polimerase

PRRs: receptores de reconhecimento padrão

PTI: imunidade desencadeada por PAMP

qPCR: PCR quantitativa em tempo real

ROS: espécies reativas de oxigênio

RT-PCR: transcrição reversa seguida de reação em cadeia da polimerase

SA: ácido salicílico

SAR: resistência sistêmica adquirida

TF: fatores de transcrição

TSS: do inglês, *transcription start site*

uidA: gene codificante da enzima β-glucuronidase

(Ca^{2+}): cálcio

(K+): potássio

(O2-): superóxido

REVISÃO BIBLIOGRÁFICA

1. Biotecnologia e engenharia genética de plantas

Nos últimos anos a biotecnologia agrícola alcançou progressos através do uso da engenharia genética como uma aliada na geração de organismos geneticamente modificados (OGM). O desenvolvimento de pesquisas na área genômica e os recentes avanços com o sequenciamento de genomas tornarão mais rápida e direcionada a criação de novas variedades (Carpenter 2010, Abdallah et al., 2015; Georges 2017). Com isso, a transformação genética de plantas vem sendo utilizada em diversos países com a finalidade de controlar insetos, tolerância a seca e, consequentemente, aumentar a produtividade, preservar o meio-ambiente e a saúde (Grover et al., 2003; Baulcombe et al., 2009; Gregory et al., 2009; Smyth e Phillips 2015; Nakashima and Suenaga 2017). Atualmente, o Brasil ocupa a segunda posição no ranking mundial de países que adotam a biotecnologia em suas lavouras, sendo que, em 2017 cultivou 50,2 milhões de hectares com culturas transgênicas, dos quais 26% representam o cultivo mundial de soja, milho e algodão obtido a partir de ferramentas biotecnológicas (Serviço Internacional para a Aquisição de Aplicações em Agrobiotecnologia, ISAAA, 2017).

A transformação genética de plantas permite a introdução de genes específicos em genomas alvo capazes de conferir ao organismo uma característica desejável e vem sendo amplamente empregada no desenvolvimento de novas cultivares de interesse comercial. Esta tecnologia é uma ferramenta importante que tem auxiliado nos programas de melhoramento, uma vez que possibilita a transferência de genes entre plantas de espécies filogeneticamente distantes, fato que não ocorre por meio de cruzamentos sexuais ou fusão de gametas. (Singh e Hymowitz, 1999, Abdallah et al., 2015; Bradshaw, 2017).

Para que a transferência de genes ocorra com sucesso, três etapas fundamentais são necessárias: a identificação, o isolamento e introdução do gene de interesse no DNA da planta, a identificação e seleção de plantas transformadas (Brasileiro et al., 1999). Os dois métodos mais utilizados para inserir genes em plantas são a biobalística, no qual a planta é bombardeada por partículas de ouro ou de tungstênio cobertas pelo DNA (Rech e Aragão 1998) e via *Agrobacterium sp*., uma bactéria de solo capaz de transferir um segmento de seu DNA para plantas por meio do plasmídio *Ti* (tumor inducing) (Chilton et al., 1977, Chilton 1983, Binns e Campbel 2001). Entre as principais metodologias de transformação genética via *Agrobacterium*, o método de imersão floral (*floral dip*) de *Agrobacterium tumefaciens*, amplamente utilizado em

Arabidopsis e já demonstrado também em *Camelina* (Clough e Bent, 1998, Liu et al 2012), é uma metodologia fácil, rápida e eficiente para dicotiledôneas, capaz de produzir centenas de progênies transgênicas prontas para análise do fenótipo de interesse (Brasileiro et al., 1999; Andrade et al., 2003; Gelvin 2000).

Plantas modelo, como Arabidopsis, que possui genoma de tamanho reduzido, bem anotado e entrega de genes de baixo custo, constitui uma excelente ferramenta para estudos de expressão gênica e caracterização de promotores para posterior aplicação biotecnológica (Delatorre e Silva, 2008). Dessa forma, *A. thaliana* vem sendo usada como modelo tanto em estudos de crescimento e desenvolvimento, assim como nos processos de resposta a estresses bióticos e abióticos de várias espécies de plantas dicotiledôneas (Mc Conn et al., 1996; Menke et al., 2004; Ikegami 2009; Bihmidine et al., 2013; Nobres et al., 2016).

Para selecionar plantas transformadas, além dos genes de interesse, genes marcadores de seleção também são utilizados. Estes genes conferem às células transformadas resistência a determinados agentes seletivos, como antibióticos ou herbicidas. Existem diferentes tipos de genes de seleção, incluindo o gene *bar* (que codifica a enzima phosphinothricin-N-acetyltransferase - PAT) de Streptomyces, conferindo resistência ao herbicida glufosinato de amônio (Thompson et al., 1987). Esta enzima é usada como um marcador de seleção e como fonte de resistência aos herbicidas do grupo fosfinotricina (PPT) (Almeida e Ulbrich, 1999).

Além dos genes de seleção, as células e os tecidos transformados podem ser identificados pela expressão de genes repórteres ou marcadores, que codificam proteínas, geralmente de atividade enzimática, cujo produto é facilmente detectável. Exemplos de genes repórteres são: GFP, que codifica a proteína (*Green fluorescent protein*), visível quando submetida à luz ultravioleta (Chalfie et al., 1994), e o gene *uidA* - β-glucuronidase (GUS), visualizado por análise histoquímica com o X-GLUC (5-bromo-4cloro-3-indolil glucuronida) (Jefferson et al., 1987).

A regulação da expressão do transgene será feita, em maior parte, pelo promotor, uma vez que a transcrição é o primeiro processo de regulação gênica. A expressão do transgene, porém, não é uniforme em todas as plantas geradas sob as mesmas condições, pois ele está sujeito a outros mecanismos de regulação endógenos da planta. A escolha de um promotor adequado para regular o transgene pode diminuir essa variabilidade de expressão e aumentar a eficiência da técnica (Potenza et al., 2004).

2. Promotores utilizados em biotecnologia vegetal

O gene é constituído por uma região promotora, codificadora e terminadora. Promotores são regiões do genoma responsáveis pela regulação da expressão de um gene, uma vez que contém os sítios de ligação para os fatores de transcrição e para a RNA polimerase (responsável pela transcrição gênica) e as informações sobre o controle local e temporal, além de respostas a estímulos ou sinalizações (Potenza et al., 2004; Lewin, 2004).

Compreende por definição a região 5' da sequência a ser transcrita, podendo se estender por algumas centenas de pares de base (pb). A região promotora de um gene eucarioto contém uma sequência conservada (T/A)A(A/T), denominada TATA Box, localizada a aproximadamente 30 pb do sítio de início da transcrição (TSS – "*transcription start site*") da RNA polimerase II. Além disso, possui elementos promotores proximais localizados a cerca de 100 pb (CCAAT Box) e 200 pb (GC Box) acima do ponto de início da transcrição (Stephen et al., 2003; Vernimmen et al., 2015; Chatterjee et al., 2017). Os elementos contidos em tais sequências determinam o ponto correto do início e a taxa da transcrição, bem como o padrão espacial e o momento em que este processo biológico deverá ocorrer (Vernimmen et al., 2015). A região mínima de sequência contínua de DNA necessária para dirigir corretamente o início da transcrição de um gene pela maquinaria da RNA polimerase II, que inclui o próprio sítio de início de transcrição (+1), bem como cerca de 35 nucleotídeos acima e abaixo do mesmo, é denominada promotor principal (*core promoter*) (Kadonaga 2012; Kwak et al., 2013).

Além do promotor principal, a regulação da expressão gênica pode ainda ser modulada pela interação do promotor a elementos *cis* regulatórios, que são sequências reconhecidas que regulam a transcrição em resposta a diversas situações, como ao estímulo exógeno (luz, pressão, umidade, temperatura, etc.) ou endógeno (auxinas, giberelinas, ácido salicílico, acido jasmônico, etc.), através de interação específica com proteínas reguladoras, desencadeando mudança na atividade do promotor (Kadonaga 2012; Schor et al., 2017). De acordo com a localização, existem três classes de elementos *cis*-regulatórios em eucariotos: 1. O **Promotor principal**: região de ligação da RNA polimerase II e fatores gerais de transcrição próximos ao TSS; 2. **Elementos *cis* potenciadores**: sequências próximas ao promotor que auxiliam a ligação da RNA polimerase II e modulam sua atividade; 3. **Elementos intensificadores e silenciadores:** influenciam a taxa de transcrição, podendo agir para ativar ou reprimir a transcrição

independentemente de sua distância relativa do TSS e orientação (Maston et al., 2006; Shlyueva et al., 2014).

A depender do tipo de regulação, os promotores usados na biotecnologia vegetal são divididos em diferentes categorias e podem ser classificados como: (i) promotores constitutivos são aqueles continuamente ativados na maioria dos tecidos; (ii) promotores espaço-temporal são aqueles ativados em diferentes estádios de desenvolvimento ou tecido específico e (iii) promotores induzíveis são aqueles regulados por estímulos hormonal, físicos ou químicos internos ou externos (Potenza et al., 2004). Em síntese, a identificação de um amplo espectro de promotores, que diferem na sua capacidade de regular o padrão de expressão temporal, espacial e na magnitude adequada de um transgene pode aumentar o sucesso da aplicação da tecnologia de transgênicos.

Na maioria dos casos, a expressão de um gene de interesse em plantas geneticamente modificadas é controlada por promotores constitutivos, que determinam um padrão de expressão elevado e em todos os tecidos da planta e durante todo o desenvolvimento dos vegetais, como o promotor *CaMV35S*, derivado do vírus do mosaico da couve-flor (CaMV - *Cauliflower mosaic virus*) (Odell et al., 1985), o promotor BSV, derivado do vírus da banana (Schenk et al., 2001), o promotor CsVMV, derivado do vírus do mosaico de nervuras de mandioca (Li et al., 2001) e o promotor do vírus Multan de enrolamento da folha de algodão (CLCuMV - *Cotton leaf curl Multan virus* (CLCuMV) (Xie et al., 2003). Entre estes, o promotor majoritariamente empregado para direcionar a expressão de transgenes comerciais ainda tem sido o *CaMV35S*, considerado constitutivo/ectópico (Amarasinghe et al., 2006). Contudo, a expressão, em todos os tecidos vegetais, de genes expressos somente em situações de estresse, apesar de aumentar a resistência da planta, pode causar retardamento no crescimento ou ocasionar problemas no desenvolvimento (Chen et al., 2009; Cominelli e Tonelli, 2010). Além disso, existem algumas limitações na utilização de promotores de origem viral na engenharia genética de plantas, pois possui limitações no que diz respeito ao silenciamento transcricional do gene sob sua regulação, pois as células vegetais podem reconhecer essa sequência como estranha e inativá-las (Potenza *et al.,* 2004; Elmayan e Vaucheret, 1996), utilizando mecanismos de silenciamento gênico transcricional que incluem mecanismos como metilação, remodelação da cromatina e excisão de DNA estrangeiro (Kumpatla et al., 1998).

O sucesso da tecnologia de transformação genética de plantas, desde a pesquisa básica até o desenvolvimento de novas cultivares independentemente da sua finalidade, está

diretamente associado à obtenção de um nível de expressão apropriado do DNA exógeno, que depende da correta seleção do promotor a ser utilizado e dos propósitos do projeto (Potenza et al., 2004; Porto et al., 2014). Para que isso ocorra, faz-se necessário a identificação e caracterização de novas regiões promotoras de genes, preferencialmente endógenos, que possuam padrões de expressão potencialmente interessantes para esta aplicação, como por exemplo, gene expresso especificamente em condições de estresse biótico, como ataque de patógenos, ou estresse abióticos, como déficit hídrico (Yoshida e Shinmyo, 2000). Deste modo, o emprego de promotores induzíveis é importante para diminuir os efeitos pleiotrópicos da expressão constitutiva.

2.1. Promotores induzíveis

Promotores induzíveis utilizam componentes endógenos e/ou exógenos para o controle da regulação precisa da expressão do transgene após a percepção de vários tipos de estresses, tanto bióticos quanto abióticos, que levam a indução de um grande número de genes (Zuo e Chua, 2000 Maruyama et al., 2012*).* Alguns aspectos importantes devem ser considerados para garantir um sistema de expressão induzível confiável, como por exemplo: o sistema deve ser altamente induzível, rápido e responder apenas na presença de indutores específicos, após a indução. Além disto, deve ativar especificamente os genes-alvo e a ativação do próprio sistema não deve ser tóxica nem causar outros efeitos indesejáveis não específicos na planta (Zuo e Chua, 2000).

Em plantas, vários tipos de estresses bióticos e abióticos induzem a expressão de grande número de genes de defesa ou tolerância. Dentre os fatores abióticos, podemos citar as mudanças ambientais como calor, frio, luz UV, alta concentração de sal ou de minerais/metais pesados no solo, déficit hídric e, entre os bióticos, o ataque de patógenos e herbívoros que causam dano em tecidos da planta (Taiz e Zeiger, 2009). A resposta da planta em condições de estresse é ativar genes que são mobilizados para defesa e reparo e induzem a mudanças de desenvolvimento, bioquímicas e fisiológicas, que ajudarão a superar os efeitos deletérios das circunstâncias prejudiciais (Potenza et al., 2004). A identificação de genes induzidos por estresse e de defesa têm produzido uma série de promotores com alto valor biotecnológico, pois constituem ferramentas de grande importância que direcionam a expressão do transgene diretamente no local do dano ou infecção (Logemann et al., 1989; Yamaguchi-Shinozaki and Shinozaki, 1994; Aoyama e Chua 1997; Kang et al., 1999; Van et al., 2006; Charng et al., 2007; Gulbitti-Onarici

et al., 2009; Dubey et al. 2013; Mafra et al. 2013 Kong et al., 2018). Assim, a identificação e caracterização de promotores induzíveis podem oferecer vários benefícios como, a diminuição de gastos energético desnecessários da planta para suprir uma falsa demanda, diminuição na toxicidade para organismos não alvos, no caso de plantas que abrigam proteínas inseticidas pode reduzir o silenciamento transgênico pela planta hospedeira, além de permitir respostas de defesa mais eficiente.

Numerosos promotores induzivéis de planta foram descritos, caracterizados e efetivamente utilizados em plantas, como por exemplo, o promotor induzido por estresse abiótico em *Arabidopsis rd29A* (Yamaguchi- Shinozaki and Shinozaki, 1994), que foi usado para mediar a expressão específica do gene DREB1A em amendoin transgênico, sob déficit hídrico, resultando em plantas mais tolerantes sem apresentar nenhum efeito deletério desenvolvimento das mesmas (Bhatnagar-Mathur 2014). O promotor do gene de arroz *Wsi18*, envolvido na síntese e sinalização de ABA, foi altamente induzível em arroz transgênico após tratamentos de ABA, seca e alta salinidade (Yi et al., 2014). Os elementos *cis* reguladores que comumente estão presentes em promotores que respondem à desidratação incluem o elemento responsivo à desidratação DRE (A/GCCGAC) implicado na regulação das respostas ao frio e à desidratação em *Arabidopsis* e o elemento ABRE (ACGTGG/ TC, elemento de resposta a ácido abscísico, ABA) que regula as respostas à desidratação, alta salinidade e baixa temperatura em *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki, 1994).

Diversos promotores de resposta a defesa (dano e patógenos) também já foram identificados, como por exemplo, o promotor de defensina (*PRPI*) do arroz e do trigo induzidos por ferimentos (Kovalchuk et al., 2010), os promotores *wun1* (Keil et al., 1990) e proteinase inibidor (*pin2*) (Xu et al., 1993) de batata, induzíveis por ferimento e/ou patógeno e o promotor de cevada *Germin-Like GER4*, altamente induzível após infecção por biotróficos e patógenos necrotróficos (Himmelbach et al., 2010). O promotor induzido por ferida (*AoPR1*), isolado de *Asparagus officinalis*, foi utilizado em tabaco para conduzir a expressão de um gene *Bt* (*Cry1Ac*) e conferiu controle contra as lagartas *Heliothis virescens* e *Manduca* (Gulbitti-Onarici et al., 2009). Esses promotores apresentam pouca ou nenhuma expressão constitutiva sem a presença do estímulo. Esta característica deve ser idealmente encontrada em um promotor induzível, no qual nenhum nível basal de expressão do transgene deve ser observada na ausência do agente indutor, e a expressão deve ser dose-dependente e reversível (Gatz e Lenk, 1998).

Elementos *cis* induzidos por dano ou patógenos geralmente contém múltiplos motivos W-boxes com um motivo típico do núcleo TGAC, que são vitais para a alta afinidade de ligação

dos fatores de transcrição WRKY, um motivo necessário para induzir atividade de defesa contra o patógeno (Berri et al., 2009; Phukan et al., 2016). Diversas famílias de FTs como WRKY, bZIP e MYB foram identificados na regulação ao estresse abiótico, enquanto que os fatores de transcrição MYC, WRKY bZIP e EREB/AP2 estão envolvidos na regulação da defesa da planta infectada por patógenos, sob ataque de pragas e de outros processos biológicos e podem ser bons candidatos para identificar promotores induzíveis por respostas de defesa (Phukan et al., 2016; Li et al., 2015; Alves et al, 2013; Chen et al., 2010). A utilização de promotores induzível por ferimento pode ser uma das melhores alternativas para controlar genes inseticidas, pois fornecem proteção durante a colonização de patógenos, ou ataque de insetos, e pode facilitar uma regulamentação mais abrangente de genes de resposta de defesa (Potenza et al., 2004).

3. Mecanismos de defesas dos vegetais contra estresse biótico

As plantas, por serem organismos sésseis, desenvolveram e aperfeiçoaram no decorrer de milhões de anos uma diversidade de mecanismos de defesa contra os estresses bióticos (ex. herbívoros, patógeneos) e abióticos (ex. seca, salinidade, frio), a fim de garantir a sobrevivência (Jones e Dangl, 2006; Rosales et al., 2012; Mitchell 2016). As estratégias de desfesa contra estresses bióticos podem ser respostas de defesa constitutivas, que incluem mecanismos de defesa, que estão sempre presentes. A maioria dos metabólitos secundários atua como compostos de defesa constitutiva, assim com tricomas, espinhos, cutícula e espessamento da parede celular (Lucas 2000; War 2012; Bixenmann et al., 2016). As defesas das plantas também podem ser induzidas em resposta aos stresses, como por exemplo, à herbivoria, e, nesse caso, as respostas iniciam somente após a ocorrência do dano e podem ser divididas em **defesas indiretas**: produção de substância com odor volátil para atrair o predador do inseto herbívoro; e **defesas diretas**: afetam diretamente os herbívoros ao impedir que se alimentem, incluindo produção de inibidores de proteases ou de α-amilase (que dificultam a digestão e diminuem a disponibilidade de nutrientes), compostos secundários tóxicos e enzimas que podem afetar o crescimento e desenvolvimento do inseto (Taiz e Zeiger, 2009; War, 2012; Scholz et al., 2016; Bixenmann et al., 2016). Estas defesas induzidas requerem menor investimento de recursos vegetais do que os mecanismos constitutivos, uma vez que reduzem o investimento em mecanismos de defesa, retardam a adaptação e o desenvolvimento de resistência dos herbívoros (Agrawal et al., 1999; Scholz et al., 2016).

O sucesso das plantas para uma resposta de defesa apropriada perante o estresse biótico depende da sua capacidade de reconhecer rapidamente, decifrar o sinal de entrada, e responder

adequadamente aos ataques do patógeno ou herbívoro (Maffei et al., 2007; Fürstenberg et al., 2013). Este reconhecimento ocorre a partir da percepção de padrões moleculares associados micróbios ou patógeno ou herbívoros (MAMPs ou PAMPs ou HAMPs do inglês, *microbe-or pathogen-or herbivore associated molecular patterns*), os quais são detectados por receptores de reconhecimento padrão (PRRs) e também pelas moléculas elicitoras endógenas que as plantas produzem após infecção. Estas moléculas são denominadas Padrões moleculares associados ao dano (DAMPs) que são liberadas após a infecção e/ou injúria e reconhecidas pelos PRR, desencadeando as reações de defesa (Boller 2009; Tang et al., 2012; Macho et al., 2014).

As rotas metabólicas induzidas na planta após o reconhecimento de um patógeno ou inseto abrangem diferentes cascatas de sinalização capazes de reprogramar a expressão gênica e responder ao ataque. Estes incluem uma rede interligada de diferentes vias de transdução de sinal que abrange genes que codificam diferentes fatores de transcrição, principalmente associados a sinais moleculares envolvidos no aumento da emissão de três hormônios: ácido jasmônico (AJ), etileno (ET) e ácido salicílico (AS), induzidos após o ataque de herbívoros (Dicke e Baldwin, 2010; Delia et al., 2013; Higenaga et al., 2016). Após a herbivoria, ocorre uma despolarização do potencial da membrana e um influxo de cálcio (Ca^{2+}) (Chin et al., 2000; Fürstenberg et al., 2013). A lagarta *Spodopter littoralis* ao se alimentar de *Phaseolus lunatus* causa um aumento transiente nos níveis de Ca^{+2} citosólico nas células adjacentes ao local de alimentação (Howe et al., 2008). Esse aumento no nível de Ca^{+2} citosólico ativa calmodulinas (CaM) e outras proteínas sensoras de Ca^{+2} que, subsequentemente, promovem eventos de sinalização, incluindo fosforilação de proteínas e ativação de respostas transcricionais (Finn et al., 1995; Chin et al., 2000; Scholz 2016).

O íon Ca^{+2} também está associado a resposta induzida pelo dano mecânico (degradação e morte celular), que produz acumulação de espécies reativas de oxigênio (ERO ou ROS) como superóxido (O2-) e peróxido de hidrogênio (H_2O_2), além do óxido nítrico, que possuem funções regulatórias de defesa (Chin et al., 2000; Arimura et al., 2005; Wu e Baldwin, 2009). A geração de ROS inicia a explosão respiratória ou oxidativa, que é uma resposta de defesa da planta após o reconhecimento do patógeno conduzindo a resposta hipersensitiva (HR) ou reação de hipersensibilidade. O H_2O_2 pode ser diretamente tóxico ao patógeno atuando diretamente como um agente antimicrobiano, formando uma barreira mecânica efetiva (Wu e Baldwin, 2009). O peróxido de hidrogênio atua também como mensageiro secundário, sendo responsável pela ativação da hidrolase do ácido benzóico, enzima responsável pela conversão do ácido benzóico

em ácido salicílico (AS) (Vernooij et al., 1994). A explosão oxidativa poderá ocorrer nos tecidos distantes conduzindo à resistência sistêmica adquirida (SAR), a qual é mediada pelo AS como um sinal que imuniza toda planta (Gaffney et al., 1993; Ryals et al., 1996; Pieterse et al., 2012). O padrão espaço-temporal do acúmulo sugere que o AS é essencial para a resposta de morte celular hipersensível, geralmente associada com resistência (Alvarez 2000; Zheng et al., 2015).

O sinal de resposta ao dano mecânico contínuo estimula a fosforilação de proteínas quinases, que levam a ativação de enzimas envolvidas na biossíntese do ácido jasmônico (JA) (Seo et al., 1999; Rocha et al., 2007; Pieterse et al., 2012; Hettenhausen et al., 2014). As quinases são proteínas responsáveis pelo controle pós-traducional de proteínas alvo, agindo como reguladores críticos de muitas cascatas de sinalização. Em plantas, as MAPKs (*Mitogen-activated protein kinase*) participam de vários processos como resistência a doenças, crescimento, desenvolvimento, percepção de hormônios e respostas a estresses (Xu e Zhang 2015; Pitzschke 2015).

O ácido jasmônico (JA) é um importante regulador de respostas de defesa, atua como estímulo principal na resposta da planta à herbivoria, ativando os genes que respondem local e sistematicamente ao stress. Estes genes envolvidos na síntese de compostos de defesa incluem os terpenos sintases para a produção de voláteis que atraem os inimigos naturais (Hettenhausen et al., 2014). A demonstração direta da ação do ácido jasmônico na resistência a insetos tem sido resultado de pesquisas em linhagens mutantes de *Arabidospsis* com níveis baixos de AJ, os quais apresentam uma alta susceptibilidade a insetos praga (McConn et al., 1997).

Embora os jasmonatos sejam moléculas-chave na ativação de respostas de defesa, o Etileno (ET) também atua em sinergia com AJ para ativar um conjunto específico de genes de defesa. Experimentos de ganho de função confirmaram que o fator de transcrição *At*MYC2 regula a ativação da sinalização do AJ e atua em combinação na via de fatores responsivos a etileno (ERF) e induz ERF1 e ORA59, ambos fatores de transcrição responsivos a AJ/ET que ativam genes responsivos, como o *Plant Defensin1.2* (*PDF1.2*) (Lorenzo et al., 2004; Pré et al., 2008). Ademais, as respostas de defesa ao estresse biótico não são feitas exclusivamente por um regulador, ou seja, é uma defesa induzida pela interconexão das vias de transdução do sinal de JA, SA e ET (Durrant et al., 2004; Sánchez e Alina 2017).

4. Sinalização de déficit hídrico por ácido abscísico

A regulação da resposta ao déficit hídrico em plantas tem início a partir de uma via de transdução de sinais que se manifesta logo após a percepção do estresse, o qual desencadeia uma cascata de eventos moleculares, sendo finalizada em vários níveis de respostas fisiológicas, metabólicas e de desenvolvimento. Essas respostas podem resultar em tensões como seca, alta salinidade e baixas temperatura. Essas tensões envolvem mudança no potencial osmótico, através da membrana plasmática, e pode ser a maior causa de respostas ao estresse hídrico em nível molecular (Bray, 1993).

Após a percepção da perda de água as plantas transmitem os sinais de déficit hídrico para órgãos distantes através das raizes (Christmann et al., 2013). O turgor celular reduzido em resposta às condições de estresse de desidratação é um importante mediador de potencial hídrico das raízes para as células-guarda. Estas mudanças hidráulicas podem transmitir sinais das raízes às folhas, induzindo fechamento estomático, que ocorre principalmente em resposta à perda de turgescência, mantida pelo ABA (Brodribb et al., 2011; Wilkinson et al., 2012). Os níveis elevados de ABA durante estresse hídrico induzem abertura dos canais de cálcio (Ca^{2+}) da membrana plasmática e inibição da bomba de prótons H^{+}ATPase e, com isso, ocorre bloqueio dos canais de entrada de potássio (K+). O ABA também ativa o canal de saída de K^{+} através da alcalinização do citosol (o pH passa de 7,6 para 7,9). Dessa forma, os íons de cálcio Ca^{2+} e o pH afetam os canais da membrana das células-guarda por meio da inibição do canal de entrada de K^{+} e pelo fechamento estomático (Taiz e Zeiger, 2004; Yoshida e Fernie 2018).

Outras vias metabólicas de resposta ao estresse hídrico são geradas em plantas, porém o ABA por desempenhar papel crítico no desenvolvimento da manutenção de respostas ao estresse abiótico e biótico, tem sido um dos hormônios mais estudadado, tendo em vista sua participação na indução da expressão de vários genes com diferentes funções envolvidas na resistência ao estresse da desidratação em tecidos e órgãos (Wilkinson e Davies, 2002; Li et al., 2010; Kuromori et al., 2018).

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OBJETIVO GERAL

Este estudo teve o objetivo de isolar e caracterizar promotores de genes responsivos ao estresse biótico causado pelo desenvolvimento da larva do bicudo-do-algodoeiro em botão floral de algodão. Além disso, objetivou-se identificar e caracterizar o promotor de soja *GmRD26* induzido pelo estresse hídrico.

Objetivos específicos

- Avaliar e confirmar, pela técnia de qRT-PCR, a expressão de genes induzidos pelo ataque da larva no botão floral e selecionar os genes promissores para isolar o promotor;

- Clonar dos promotores de algodão p*GhERF17-like,* p*GhERF105-like e* p*GhNc-HARBI1-like* em vetores binários de transformação contendo a fusão dos genes repórteres GUS-GFP e transformação de *A. thaliana;*

- Analisar funcionalmente os promotores p*GhERF17-like,* p*GhERF105-like e* p*GhNc-HARBI1-like* em comparação com o promotor constitutivo *CaMV35S* em plantas transgênicas de *Arabidopsis* através de indução com elicitor químico ácido salícico (AS);

- Analisar o perfil de expressão gênica do gene *GmRD26* em soja (*Glycine max*), sob estresse hídrico, por meio da técnica de qRT-PCR;

- Clonar o promotor *GmRD26* e seus módulos em vetores binários de transformação contendo a fusão dos genes repórteres GUS-GFP e transformar plantas de *A. thaliana;*

- Avaliar a atividade do promotor *GmRD26* e seus diferentes módulos e em comparação com o promotor *AtRD29* em plantas transgênicas de *Arabidopsis*, por meio de ensaios histoquímicos, fluorimétricos e de qRT-PCR.

APRESENTAÇÃO DOS RESULTADOS DA TESE

Os resultados desta tese estão apresentados em dois capítulos:

O capítulo I se refere ao artigo intitulado "Isolation and characterization of inducible gene promoters in cotton buds (*Gossypium hirsutum* L.) for the control of insect pests".

O capítulo II se refere ao artigo intitulado "Identification and characterization of the *GmRD26* soybean promoter in response to abiotic stresses: potential tool for biotechnological application" (Aceito para publicação na revista *BMC Biotechnology*).

CAPÍTULO I

Isolation and characterization of inducible cotton (*Gossypium hirsutum* L.) promoters for insect pests control

Isolation and characterization of inducible cotton (*Gossypium hirsutum* L.) promoters for insect pests control

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ABSTRACT

Cotton (*Gossypium hirsutum*) is the main source of fiber for the textile industry and a major source of seed oil. However, one of the greatest challenges to crop productivity is the constant attack of pests, among them the cotton boll weevil, *Anthonomus grandis* (Coleoptera: Curculionidae), considered to have the greatest impact. This insect-pest attacks the floral buds for feeding and oviposition, compromising the production of the fiber. In addition, its endophytic habit makes control difficult, since the larvae are protected from the action of insecticides. Considering the importance of this pest, the objective of the present study was to evaluate by qPCR the expression of genes induced by the attack of the cotton boll weevil larva (CBWL) in the floral bud and to select target genes for the isolation and characterization of promoters. For this, 20 genes with inducible expression pattern identified in a transcriptome of cotton buds infested with bollworm larvae were selected. The expression of these genes was analyzed by RT-qPCR after different feeding times of the CBWL. Floral cotton buds were inoculated with a cotton boll weevil egg containing an active embryo and then the flower buds were analyzed after 2h, 6h, 12h, 24h and 96h after inoculation. Among the analyzed genes, *GhERF17-like, GhERF105-like* and *GhNc-HARBI1* showed an increased expression profile, mainly in late responses (12h, 24h and 96h). These analyzes confirmed that these genes are induced by the biotic stress caused by the development of CBWL in cotton flower buds. The promoter sequences of these three genes were isolated and analyzed for the presence of regulatory *cis* elements. All three promoters have *cis* elements of ERF, MYB and many W-box transcription factors (known as WRKY type transcription factor binding site): WRKY71OS, WBOXNTERF3, WBBOXPCWRKY1, WBOXATNPR1. In this study, we also identified *cis*-regulatory sequences involved in the induction of salicylic acid (SA) as GT1CONSENSUS, MYBCOREATCYCB1 and ASF1MOTIFCAMV. Subsequently, the promoters were cloned into the stable expression vector containing the GUS-GFP fusion. The activity of the GFP reporter gene controlled by the p*GhERF17-like*, p*GhERF105-like* and p*GhNc-HARBI1-like* promoters was monitored and compared with *CaMV35S* promoter on *A. thaliana* transgenic leaves under stimulation with salicylic acid (SA). The analyzes indicated that p*GhERF17-like*, p*GhERF105-like* and p*GhNc-HARBI1-like* promoters were inducible by SA. On the other hand, plants transformed with *CaMV35S* showed fluorescence with or without SA treatment. Comparing the different cotton promoters, GPF fluorescence was more intense in p*GhNc-HARBI1-like* plant leaves when undergoing treatment with SA. Additional studies are in progress. However, these promoters indicate that they are potential biotechnological tools to boost inducible gene expression in plants.

Key words: Cotton, *Anthonomus grandis*, Inducible promoter, Salicylic acid, Green fluorescent protein (GFP).

Introduction

Cotton (*Gossypium hirsutum* L.) is the most widely cultivated cotton species in the world and the main source of natural fiber as one of the main oil crops. (John 1997, Khan et al, 2007, Khan et

al, 2010). Grown in 70 countries, it provides subsistence for more than 180 million people (Rahman et al., 2012).

World cotton production is based on arboreal (*Gossypium barbadense* L.) and herbaceous (*Gossypium hirsutum* L.) types. The herbaceous species, an annual shrub, is widely cultivated in Brazil and accounts for more than 95% of world cotton production (Beltrão et al., 2004). In addition to fiber, Brazil is also the fourth largest producer of oil extracted from cottonseed. According to the US Department of Agriculture, fiber is the most important natural resource used in the textile industry. World cotton consumption in 2018/19 should reach 123.6 million bales, increase 0.9 percent over the previous year (http://www.fas.usda.gov, em Julho/2019).

Despite the great importance of cotton, pests are still a limiting factor of production. About 1326 species of insects have been reported for attacking cotton. Among these species, the aphid (*Aphis gossypii*), carpipe (*Spodoptera frugiperda*), apple caterpillar (*Heliothis virescens*), *Helicoverpa armigera* and cotton bollfoot (*Anthonomus grandis*) are the major plagues for cotton farming (Dubey et al., 2013). However, the most important pest in the Brazilian cotton crop is the boll weevil. It is a pest of great economic importance, due to its rapid reproductive capacity and destruction. Infestation levels increase rapidly and losses can reach up to 100% of production if control measures are not suitable (Degrande, 1998; Greenberg et al., 2004; Martins et al., 2007; Grossi-De-Sá et al., 2007).

The cotton boll weevil (CBW), *Anthonomus grandis* Boheman, is a phytophagous insect. The adult female oviposites and feeds on flower buds and/or cotton fruits. The larvae have an endophytic habit, that is, they remain inside the cotton reproductive organs and, after hatching, they feed inside the flower buds and the apples, destroying fibers and seeds in formation. This behavior also contributes to the difficulty in chemical control of this pest, since the larvae are protected from the action of insecticides throughout the initial development (Haynes and Smith, 1992; Busoli et al., 1994; Papa and Celoto, 2015). Around 50% of insecticide costs used in the cotton crop is directed to the control of the CBW, with sprays aimed at the control of adults (Specht et al., 2013). Another strategy in the control of beet populations is integrated pest management (IPM) (Luttrell et al., 1994). However, in South America, insect populations are still causing great damage to cotton plantations, destroying flower buds and cotton capsules (Freire 2011). Thus, in view of the need to minimize the damage caused by the cotton boll weevil and to preserve human health and the environment, biotechnology tools through the use of genetic engineering has the potential to address some of the major agriculture challenges including the development of pest resistance GMOs.

Among the approaches adopted to control insect pests is the production of resistant transgenic plants. The widely used and commercially used strategy to date is the use of genes for *Bt* toxins, derived from the bacterium *Bacillus thuringiensis* (*Bt*), a soil bacterium that forms spores during its stationary phase of growth. These spores contain crystals, predominantly composed of one or more types of Cry proteins (and/or Cyt, also known as δ-endotoxins) (Schnepf et al 1998; Abulreesh et al., 2012). These toxins accumulate in the bacteria in the form of crystals, which, upon ingestion by the insect, are degraded under conditions of alkaline pH and in the presence of specific proteases of their digestive tract releasing the active toxin that binds to specific receptors and affects the membrane permeability of the mesenteric epithelial cells. In this way the affected cells rupture and the larva dies. The need for alkaline conditions, proteases and specific receptors explain why *Bt* toxins are harmless to mammals, birds, amphibians and reptiles and can target a restricted group of insects (preserving e.g. bees) (Glare and O'Callaghan, 2000). *Bt* toxins have been deeply studied and modified to be increasingly specific for their hosts and have gained worldwide importance as an alternative to chemical insecticides (Palma et al., 2014).

Some Cry proteins, such as Cry1Ba6, Cry8Ka, Cry1Ia12 and Cry10Aa, have been described as entomotoxic against *A. grandis* (Grossi-de-Sá et al., 2007; Martins et al ., 2010; Aguiar et al ., 2012; Oliveira et al., 2016; Ribeiro et al., 2017). Identification of different toxic Cry proteins has a beneficial effect on the generation of transgenic plants resistant to insect pests. But, when using multiple traits in the generation of genetically modified plants, different promoter regions should be employed for targeting specifically each of these traits, in order to minimize sequence dependent gene silencing (Furtado et al., 2008).

Despite significant efforts for the isolation and characterization of plant genes, only a small number of promoters have been isolated and functionally characterized (Lescot et al., 2002; Peremarti et al., 2010). The promoter mostly employed to direct protein expression in commercial transgenic plants has been the *CaMV35S* cauliflower mosaic virus derivative (Amarasinghe et al., 2006), used in more than 80% of genetically modified plants (Hull et al., 2002). However, in recent years the use of the *CaMV35S* promoter in genetically modified plants, including genes expressed in cotton (Bakhsh et al., 2009; Dong and Li, 2007), has been questioned (Dong and Li, 2007; Wessel et al., 2001). One of the problems observed is the variation of *Bt* protein expression in different tissues and throughout the development of the plant and the fact that ectopic expression requires a high energy expenditure by the plant (Rawat et al., 2011). The variation of Cry protein expression has been a major concern in the adoption of

Bt cotton strains. This not only increases the costs for pest control, but also favors the appearance of insect resistant to transgenic varieties (Dong and Li, 2007).

In plants, several types of biotic and abiotic stresses induce the expression of a large number of defense genes. When a plant suffers damage, genes are mobilized for defense and repair, and proteins and signaling molecules are synthesized to signal the plant that an attack occurred. The study of stress and defense inducible genes has allowed identifying a series of promoters that can be used to drive the transgene expression directly into the site of the damage or infection (Potenza et al., 2004; Logemann et al., 1989; Keil et al., 1990; Xu et al., 1993).

The search for inducible promoters is often based on the identification of genes that are expressed in a physiological condition through gene expression studies. A large number of stress responsive genes during *A. grandis* feeding, oviposition and larval development were significantly altered upon larvae infestation in cotton floral buds, mainly such related to chitin and signaling, kinase cascades, transcriptions factors (WRKY and ERF), Ca2+ influx, ROS, as well as phytohormone signalling pathways (Artico et al., 2014).

The objective of this study was to isolate and characterize inducible cotton promoters. For this, we initially confirmed the inducible expression of genes previously identified *G. hirsutum*-CBW larvae interaction (Artico et al., 2014). The promoter sequences from three genes were analyzed for the presence of regulatory *cis* motifs and were subsequently cloned into the stable expression vector pCK1407, which contains the GUS-GFP fusion. In addition, the activity of the GFP reporter gene controlled with p*GhERF17-like*, p*GhERF105-like* and p*GhNc-HARBI1-like* promoters was monitored and compared to the *CaMV35S* promoter on leaves of transgenic *A. thaliana* plants under SA stimulation.

Material and methods

A. grandis infestation assay

Three-month-old cotton (BRS Cedro) plants grown under controlled temperature (27 ± 2 °C) and natural photoperiod conditions were used for the infestation assay. Populations of *A. grandis* (Coleoptera: Curculionidae) were obtained at Embrapa-Cenargen insect rearing platform, maintained at 27 ± 2 ° C, 70 ± 10% relative humidity and photoperiod of 14h. The insects were kept on the usual rearing diet (Monnerat et al., 2000). An *A. grandis* egg containing an active

embryo was inoculated into the 6mm floral buds, previously drilled with an approximately 0.5 mm-diameter neddle (Figure 1). The orifice resulted from the perforation was sealed with Vaseline to avoid egg dehydration. The larvae were removed with a magnifying glass and clamp after 2h, 6h, 12h (initial response), 24h and 96h (late response) of inoculation and were immediately frozen in liquid nitrogen in order to isolate total RNA to perform expression analysis of the previously selected genes. The insect infection experiments were carried out in three biological replicates (nine flower buds of different plants for each time analyzed). The control sample consisted of only perforated cotton buds with no egg inoculated.

Real-time qPCR analysis to confirm the expression of inducible genes by the response to infection with cotton boll weevil larvae

To determine which genes induce early and late defense responses in cotton, we selected 20 inducible genes (**Table 1**), identified in the transcriptome of cotton buds infested with CBW larvae for 48h (Artico et al., 2014). Total RNA was extracted from 100 mg of cotton floral buds challenged with *A. grandis*, in parallel to the control, using the Invistab Spin Plant Mini RNA kit (Invitek) according to the manufacturer protocol.The quality and quantity of RNA were determined using Nanodrop 2000 (Thermo Scientific). Equal amounts of RNA (2µg) were used for the synthesis of cDNA with transcriptase reverse M-MLV (Invitrogen) according to the manufacturer's recommended protocol and the first 10 mM nvDT30. The cDNA was stored at -20 °C.

The gene expression profile was determined by quantitative real-time PCR (RT-qPCR). The analysis was performed using an ABI 7500 Fast instrument, SYBR Green reagent (Invitrogen, USA), specific primers (**Table 2**) and three cDNA-independent biological samples. All analyzes were performed in biological and experimental triplicate conditions. The reference genes *GhEF1A* and *GhUBQ* were used to normalise the qPCR data (Artico et al., 2010). The Miner software (http://www.miner.ewindup.info/Version2) was used to o calculate the efficiency and Ct (cycle threshold) for qRT-PCR from individual PCR reactions (Zhao and Fernald, 2005). Relative fold changes in expression was calculated using the Relative Expression Software Tool (REST©, http://www.gene-quantification.de/rest-2009-index.html) (Pfaffl, 2002), comparing inoculated and control samples. The relative quantification was measured by using *2-ΔΔCt* method (Bustin, 2000).

Identification and isolation of promoter sequences

We examined the sequences within 1.500 base pairs (bp) upstream of the start codon (ATG) of each promoter (p*GhERF17-like*, p*GhERF105-like* and p*GhNc-HARBI1-like*) sequence by using the BLASTn tool in the Cotton Genome Database (Cotton Research Institute of CAAS, Anyang, Henan, China. Cotton Genome Project-CGP (http://cgp.genomics.org). The promoter's sequences were obtained by using the Integrative Genomics Viewer (IGV) 2.3 software (Robinson et al., 2011).

The promoter regions were analyzed using the software "Signal Scan Search" of the "Plant Cis-acting Regulatory DNA Elements" (PLACE) (Higo *et al.,* 1999) and Plant-PAN (Chang *et al.,* 2008) softwares in order to identify the *cis*-acting elements.

Construction of expression vectors used for *Arabidopsis* transformation

The p*GhERF17-like*, p*GhERF105-like* and p*GhNcHARBI1* (1.500 bp) promoter regions were synthesized individually by the company Epoch Biolabs (Sugar Land, TX, EUA) in a binary expression vector pCK1407, containing the GFP reporter gene and generating the recombinant target clones p*GhERF17*::GFP, p*GhERF105*::GFP and p*GhNc-HARBI1-like*::GFP. Plasmids have the GUS-GFP fusion and the plant selection marker gene *bar*. The sequence of the constitutive promoter *CaMV35S* (Odell et al., 1985), used as a control, was cloned into the same plasmid. The recombinant plasmids were introduced into *Agrobacterium tumefaciens* (GV3101) by the thermal shock method (Brasileiro and Carneiro 1998) and were used to transform the *A. thaliana* Columbia (Col-0) ecotype by the floral immersion method (Clough and Bent 1998).

Biotic stress with salicylic acid in transgenic plants of Arabidopsis

Salicylic acid (SA) was identified as a signaling molecule crucial for the induction of plant defense responses (Qi et al., 2018). Thus, we decided to boost expression of the GFP reporter gene under the control of the p*GhERF17-like*, p*GhERF105-like*, p*GhNc-HARBI1-like* and p*CaMV35S* promoters using the chemical SA elicitor. For this, 30-day-old *A. thaliana* plants (T2) were maintained for 24h in half-stregth Murashige and Skoog (MS) liquid medium containing SA (0.5Mm) (Sarkar et al., 2018). Control samples consisted of plants maintained only in MS medium. To analyse the GFP expression, *Arabidopsis* leaves subjected to SA stress and control were observed on a Zeiss Axiophot epifluorescence microscope (Zeiss, Mannheim, Germany) equipped with an excitation filter of 450 nm/ 500 to 550 emissions.

Results

Identification and selection of genes with inducible expression by *A. grandis*

To validate the transcriptomic data and indicate the inducible cotton genes with greater potential to isolate the promoter, we selected genes expressed under the biotic stress caused by feeding the larva of the boll weevil in cotton (**Table 1**), esses genes estão envolvidos na resposta ao estresse biótico e foram anotados em processos biológicos como resposta à quitina e/ou morte celular (Artico et al., 2014).

For this study we considered as potencies genes to isolate promoters those that showed preferentially a constant or increased expression pattern between the initial and/or late responses (**Figure 2**). At all times analyzed the amounts of transcript accumulation in infested floral buds were compared to the control (floral buds without larva). In general, the expression patterns obtained indicate that all genes had some late expression, but few genes had initial responses after feeding the larvae of the bulrush, corroborating with data previously obtained by Artico et al., (2014).

Among the analyzed genes that showed the greatest expression in response to bollworm larvae attack, we selected contig24042 (initial and late response) and Contig13134 and Contig931 (late response) (**Figure 3**). According to the NCBI database and the transcriptome of buds infested with boll weevil larvae, these genes are involved in biological processes associated with insect defense. Contig13134 and Contig24042 are responsive to ethylene in Gossypium hissutum, thus they were named as *GhERF105-like*, *GhERF17-like* respectively. The Contig931 is a *HARBI1-like* Nuclease, involved in the regulation of programmed cell death, then was named *GhNc-HARBI1-like*. These genes were significantly expressed in the different cotton organs (root, stem, branch, leaf, flower and fruit), with expression being stronger in roots (**Figure 4**).

Analysis of the promoter sequences of p*Gh*ERF17, p*Gh*ERF105 and p*Gh*Nc-HARBI1-like

As these genes are predominantly expression in the presence of the injury (herbivory) stimulus, we decided to isolate their regions and evaluate them as potential candidates to direct biotic stress induced expression. The 1.500bp regions of *GhERF17-like, GhERF105-like* and *GhNc-HARBI1-like* were isolated from sequences from the Genome database of cotton. The activity of

the promoter region was evaluated based on the presence of several regulatory motifs induced by biotic stress in cotton flower buds. The p*GhERF17-like*, p*GhERF105-like* and p*GhNc-HARBI1-like* promoter regions were analyzed for the presence of putative regulatory motifs using the PLACE and Plant-PAN databases. Several cis-regulatory elements were predicted, such as TATA-box and CAAT-box (**Tables 3, 4 e 5**). In addition, all the promoters isolated in this study have cis-elements related to the early signaling mechanisms of defense responses, such as mitogen-activated protein kinases (MAPK).

The regions of these three promoters present ERF, MYB and many W-box transcription factors (known as WRKY type transcription factor binding site): WRKY71OS, WBOXNTERF3, WBBOXPCWRKY1, WBOXATNPR1. In this study we also identified cis-regulatory sequences involved in salicylic acid (SA) induction as GT1CONSENSUS (in the three promoters), MYBCOREATCYCB1 and ASF1MOTIFCAMV (p*GhERF17-like*, p*GhERF105-like*). The detailed lists of cis elements for each promoter are available from the **Tables 3, 4 e 5**.

Cotton promoters activate the GFP reporter gene by treatment with SA

To investigate whether the cotton promoters under study are capable of inducing expression of the GFP-reporter gene, we used transgenic *Arabidopsis* plants with T-DNA insertion identified by Glufosinate-ammonium selection and confirmed by PCR (**Figure 5**), in treatments of stress with SA, a chemical elicitor involved in the activation of plant defense pathways.

After treatment with SA a fluorescent green signal, GFP activity was observed between the cells of the *Arabidopsis* leaves. The green signal mixed with intense red signals, which occupy most of the cells, corresponds to the chlorophyll fluorescence. When we analyzed plants with the p*GhNc-HARBI1*::GFP promoter it was possible to observe many regions with intense green signal, already in the p*GhERF17*::GFP, p*GhERF105*::GFP promoters the green signal was also observed, but to a lesser extent (**Figure 6**). When cotton promoters were analyzed without SA treatment, no fluorescence was visible. In plants with the p*CaMV35S*::GFP control promoter intense green fluorescence was observed with and without SA treatment, in WT plants no fluorescence was visible, as expected (**Figure 6**).

Discussion

Plants in natural habitat are exposed to biotic stresses such as insect or pathogen attack and respond with the activation of multiple defenses involving the expression of a large number of genes whose products may be involved in various adaptive functions stress conditions (Rosales et al., 2012; Mitchell 2016.). The key to understanding plant defense responses lies in the elucidation of the signaling pathways involved in their regulation. For this, transcriptomic studies of plants that are exposed to herbivory have recently been carried out, and other investigations involving the analysis of signaling and resistance to disease, allow the identification of several genes and mechanisms involved in important defense signaling pathways regulated by signaling molecules, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Sánchez e Alina 2017; Pré et al., 2008; Artico et al, 2014; Hettenhausen et al., 2014; Dubey et al. 2013; Mafra et al. 2013; Xu et al. 2011; Little et al. 2007; Berrocal-Lobo e Molina, 2004). These studies are important because they provide useful data for research on the mechanisms, genes and promoters that may be applied in the future for the improvement of pest-resistant plants.

Of the genes that have been analyzed, two are ERFs (Factors Responsive to Ethylene). Plants under attack by pathogens produce high levels of ethylene, which play important roles in plant immunity (Li et al., 2012). Studies have shown that the overexpression of ERF family genes in *Arabidopsis* or tobacco has conferred greater resistance to biotic and abiotic stresses (Sharma et al., 2010; Pan et al., 2010; Xu et al., 2007). The members of the ERF subfamily bind to the cis-regulatory element, called GCC-box in the promoter regions of genes related to pathogenesis regulating its expression in response to ethylene (Hao et al., 1998). Chromatin immunoprecipitation assays in *Arabidopsis* showed the direct binding of WRKY33 to W-boxes motifs in the promoters of the ACS2 and ACS6 genes in vivo, suggesting that WRKY33 is directly involved in the activation of ACS2 and ACS6 expression downstream of the MPK3 / MPK6 cascade in response to pathogen invasion (Li et al., 2012).

It has been demonstrated that mechanisms that activate WRKY transcription factors (TF) may be involved in MAP kinase cascades and/or calcium signaling. In the analysis of transcriptome of BF cotton infested with larvae of *A. grandis* 21 WRKYs were regulated. Among these genes, cotton WRKY genes have been identified that have putative *Arabidopsis* homologues, such as *AtWRKY64* and *AtWRKY70* (positively modulate SAR), *AtWRKY40* (involved in regulating resistance to plant diseases), *AtWRKY33* (required to activate the synthesis of the antimicrobial

substance) and the *GhWRKY22-like* gene serve as the first activated WRKY proteins in response to MAPK signaling triggered by PAMP (Ülker et al; 2007; Pandey e Somssich, 2009; Rushton et al. 2010; Artico et al., 2014).

Du and Chen (2000), in search of promoter regions of genes that contained the W-box motif, identified four genes encoding protein kinases whose expression is induced by AS in the presence of a pathogen in Arabidopsis. These authors have demonstrated that the *AtWRKY18* protein is able to bind to these cis-regulatory sequences and, above all, that the induction by SA depends on the WRKY type transcription factors. In *Vitis vinifera* the WRKY1 protein binds specifically to the W-box elements present in the promoter regions of two pathogen defense genes, PR1 in parsley and NPR1 in *Arabidopsis* (Chloe et al 2007).

Another gene isolated and characterized was the *GhNc-HARBI1-like* promoter, involved in the regulation of programmed cell death (PCD). PCD is an active, genetically controlled process in which cells are selectively eliminated in a highly coordinated, multi-step manner through the involvement of specific proteases and nucleases. Thus, only the cells destined to die are destroyed and no damage to neighboring cells is inflicted (Gadjev et al., 2008). In arabidopsis, the *AtWRKY6* promoter may be a PDC regulator, and has been found to be induced by injury, infection by bacterial pathogens and pathogen elicitors, and after treatment with SA, JA, and ethylene (Robatzek e Somssich 2002).

The role of salicylic acid has been widely studied by being involved in the induction of resistance genes to pathogens (PR), pathways of disease resistance, including PCD and local and acquired systemic resistance (SAR) (Alvarez, 2000; Lu et al., 2016; Hartmann e Jürgen 2019). The treatment of exogenous SA induces many defense genes, phytoalexins and promotes ROS and PCD generation (Vásquez et al., 2015; Wang et al., 2019). SA-mediated gene expression and resistance to diseases in *Arabidopsis* require the involvement of the NPR1 gene (non-expressing PR1 gene) (Radojičić et al., 2018; Withers et al., 2016).

Salicylic acid has also been widely applied in studies of gene promoters induced by pathogens, pests or involved in plant defense pathways. In arabidosis the interaction of TGA3 and WRKY53 transcription factors in the Caulimoviral CmYLCV (*Cestrum yellow leaf curling virus*) promoter resulted in an increase in the promoter activity via salicylic acid dependent on NPR1 ("Non-expressor of PR1") signaling (SARKAR et al. ., 2018). In this study, the promoters p*GhERF17-like*, p*GhERF105-like* and p*GhNc-HARBI1-like* also showed activity when induced with SA.

Conclusions and perspectives

In our experiment p*GhNc-HARBI1*::GFP showed more intense GFP blossom, indicating to be stronger than p*GhERF17*::GFP, p*Gh**ERF105*::GFP when submitted to SA treatment. These data are not conclusive to date, however, further analyzes, with other chemical and pathogenic elicitors are being performed. The complete study of the activity of these promoters inducible by biotic stresses may provide the possibility of obtaining potential biotechnological tools capable of controlling the activity of resistance genes to pathogens or pests in crops of commercial interest and mainly in cotton.

Figures

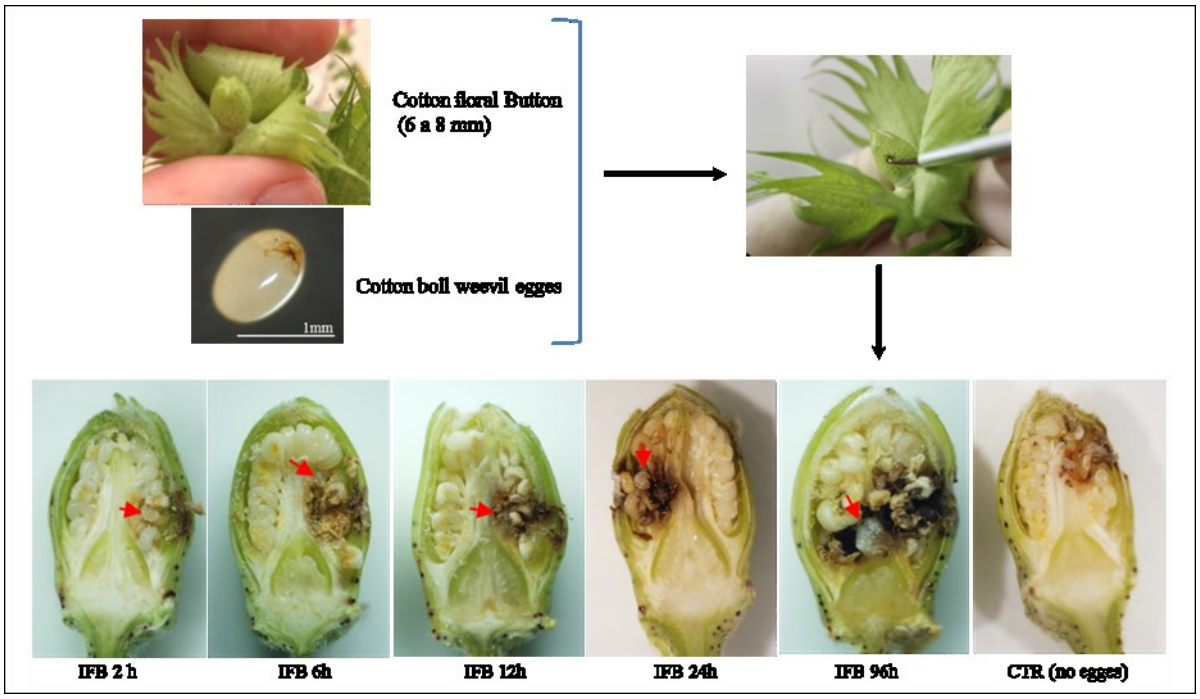

Figure 1: Infestation test of cotton buds with eggs of the cotton beet. A hole was made for inoculation of the *A. grandis* egg, the flower buds were sealed with petroleum jelly to avoid dehydration of the egg. The flower buds were collected after 2h, 6h, 12h, 24h and 96h of inoculation.

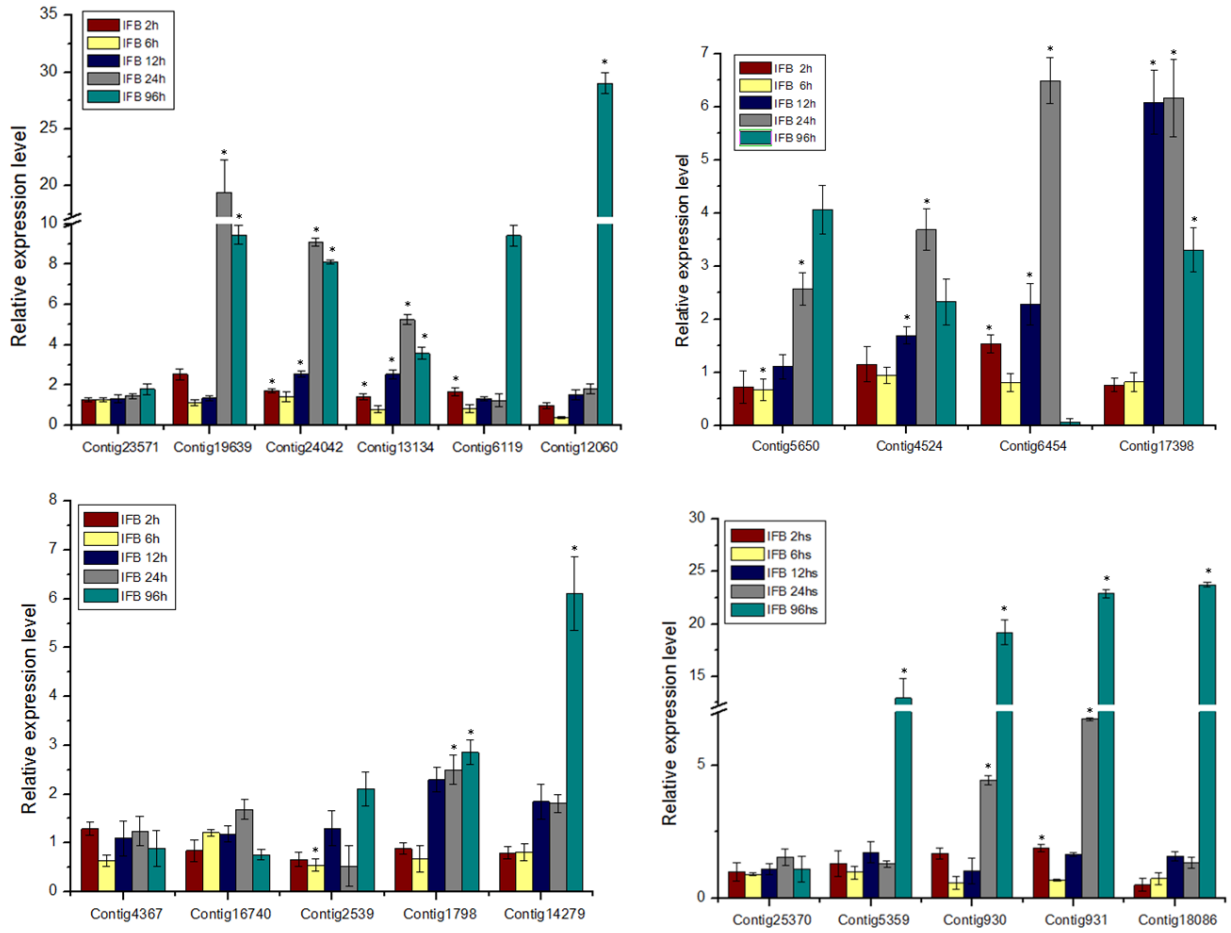

Figure 2: Relative expression pattern of the genes analyzed by qRT-PCR, in response to feeding of larvae of the cotton boll weevil (After 2h, 6h, 12h, 24h and 96h of infestation). The expression patterns obtained indicated that all genes exhibited late expression, but few exhibited early responses.

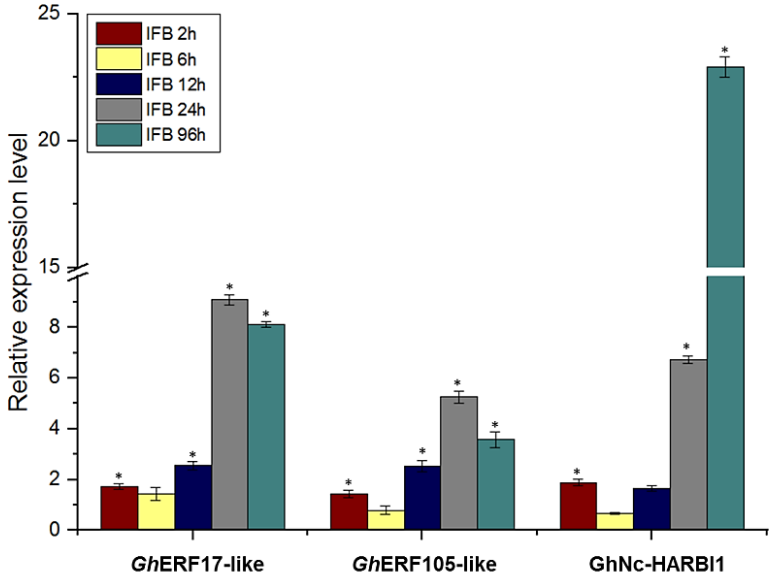

Figure 3: Relative expression of the three target genes (*GhERF17-like, GhERF105-like*, *GhNc-HARBI1-like*) in cotton plants in response to feeding of cotton boll weevil larvae after different times of infestation.

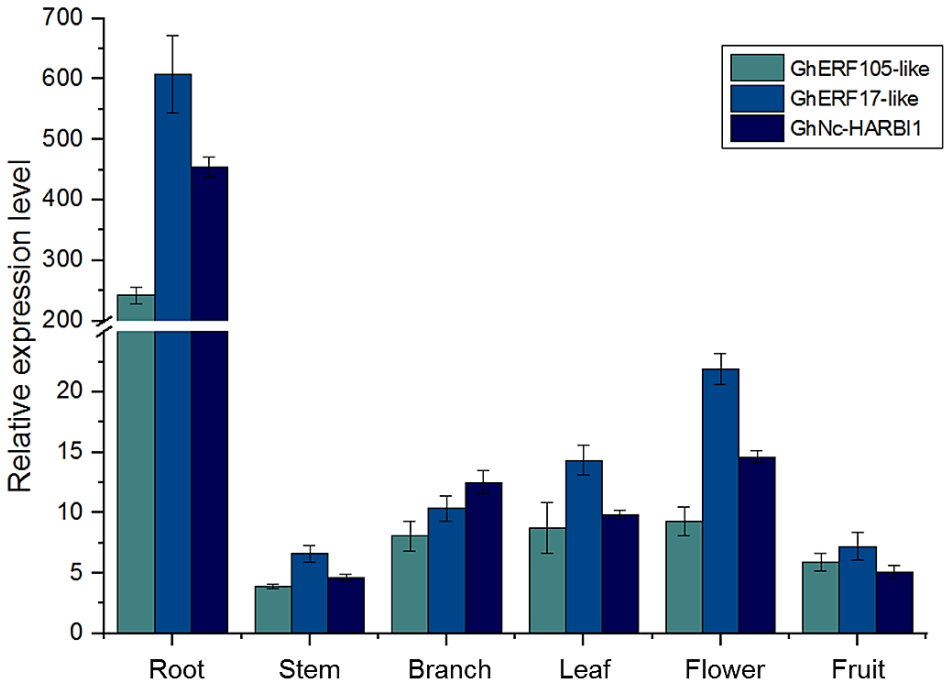

Figure 4: Relative expression of the *GhERF17-like, GhERF105-like, GhNc-HARBI1-like* genes in different organs of the plant.

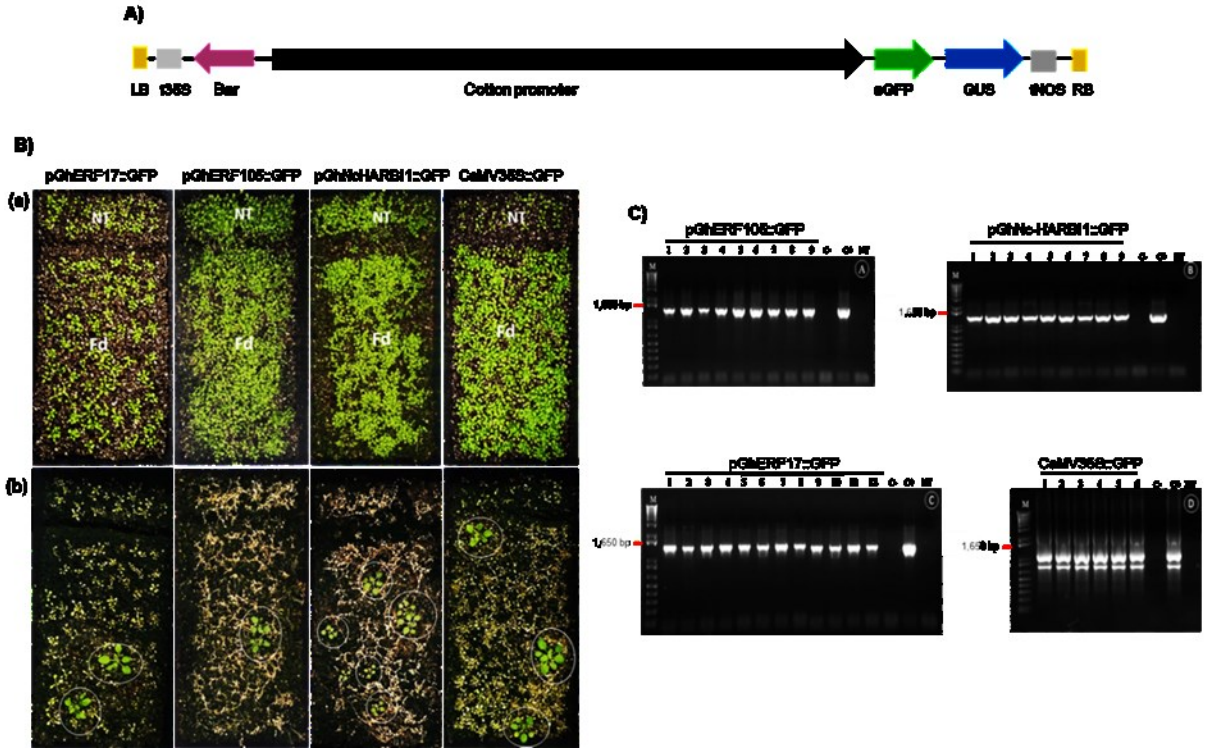

Figure 5: Obtaining transgenic lineages with constructs of cotton promoters. **A**: Scheme of the expression cassette of the cotton promoters in the expression vector pCK1407. **B**: Selection of transformed plants: (**a**) Seedlings with 10 days of age before spraying with ammonium Glufosinate. (**b**) Plants after 15 days of spraying. **C**: Ethidium bromide stained agarose gel containing PCR amplification products to confirm T-DNA insertion in *Arabidopsis* (T_1) plants. NT: Col-O (non-transgenic) seedlings, Fd: *Arabidopsis* seedlings submitted to floral dip.

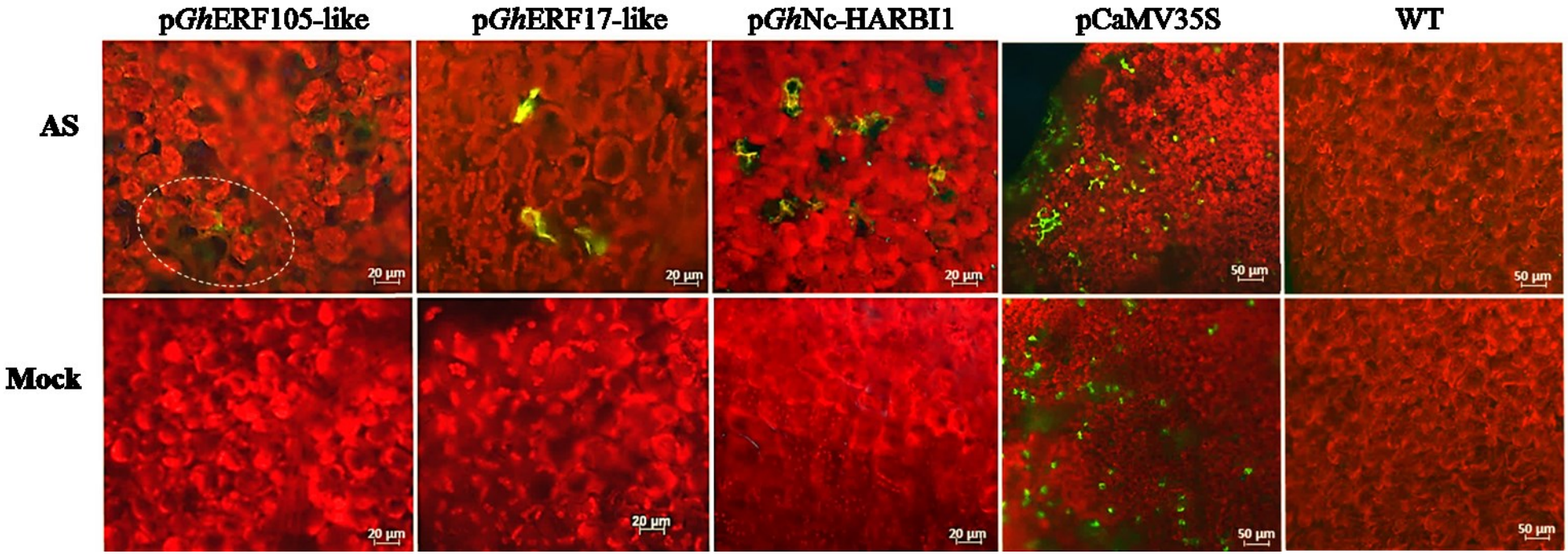

Figure 6: Expression of the GFP reporter gene in *Arabidopsis thaliana* (T2) leaf cells under the control of cotton promoters induced by the chemical elicitor 0.5mM salicylic acid (SA) for 24 h. Merged image of chlorophyll fluorescence is shown in red and activity of the p*GhERF105-like*::GFP, p*GhERF17-like*::GFP, p*GhNc-HARBI1*::GFP, p*CaMV35S*::GFP is seen in green, GFP fluorescence. Mock: MS medium, without SA. Leaves were observed under a Zeiss Axiophot epifluorescence microscope. WT = Leaves of wild-type plants

Tables

Table 1. List of differentially expressed genes (DEGs) in cotton buds in response to infestation of cotton boll weevil larvae that were analyzed in this study

Unigene identifier	Description	Log Fold Change
contig23571	Encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family	7,288793115
contig14729	NAC domain containing protein 90 (NAC090)	6,795997792
contig24042	Encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family	6,348039826
contig17398	Alpha/beta-Hydrolases superfamily protein	6,268344508
contig1798	Encodes one of the mitochondrial dicarboxylate carriers (DIC)	6,130070301
contig1674	Encodes a cell wall-modifying enzyme, rapidly upregulated in response to environmental stimuli	5,838022547
contig931	Putative nuclease HARBI1	5,687358898
contig18086	Ankyrin repeat family protein	5,572637191
contig2539	Encodes a cell wall-modifying enzyme, rapidly upregulated in response to environmental stimuli	5,570674053
contig4524	ATPase E1-E2 type family protein / haloacid dehalogenase-like hydrolase family protein	5,529668657
contig565	Related to Cys2/His2-type zinc-finger proteins found in higher plants	5,401573164
contig5359	Member of WRKY Transcription Factor	5,369745959
contig13134	Encodes a member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family.	5,25328749
contig2537	Xyloglucan endotransglycosylase-related protein (XTR6)	5,229409248
contig6454	Related to Cys2/His2-type zinc-finger proteins found in higher plants	5,221254352
contig4367	Ubiquitin-like modifier, polypeptide covalently attached to various intracellular protein targets.	5,137664447
contig930	unknown protein	5,072655207
contig6119	Encodes a member of the DREB subfamily A-1 of ERF/AP2 transcription factor family (CBF4)	5,028486841
contig12060	Encodes a member of the DREB subfamily A-4 of ERF/AP2 transcription factor family	5,023658474
contig19639	Encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family	5,018342262

Table 2. Sequence of the primers used for analysis of the genes under study, with the respective amplicon size and annealing temperature

Contig cotton	Primer foward (5'-3')	Primer reverse (5'-3')	Amplicon (pb)	Temperatura
contig23571	GGATTGGTCCTCGTTTTTGA	TAGAATTGATCGCCGGGTAA	122	60°C
contig14729	CAAATGGGAGGAAAACCAAA	TATCGAATGCTCGAGAGCTG	150	60°C
contig24042	CTATGGACGGGTCGTTTCTG	ACGATGATCCGAGAAAAGCA	120	60°C
contig17398	GCACTTTGGTACCTGCGTTT	TTCTTCGCATGTCAATGGAG	132	60°C
contig1798	GCTTTGGATTGTGCACTGAA	GCTCCAATGTCACAAACAGC	138	60°C
contig1674	AGCTGCCCATCTCCATATTC	TATTCGACATGCACCTCAGC	148	60°C
contig931	AATAAAAATTGCCGCCGTCT	TCATGGAAATTGGGTCTTCC	125	60°C
contig18086	GTGCAGCTCTTCAAATGCAA	AGGCGTTCTGCCATTACTGT	152	60°C
contig2539	AGCAAAGCAGCAAAGGCTAC	AAATCTCTTGGCGTCAGTGC	112	60°C
contig4524	AATCCAAGGCTTCAACTCCA	CGGGTCTCCATTGACAGAAT	122	60°C
contig565	TCCTTGTATGGCAGGGAAAA	AGTAGCTGACGGCGAAGAAA	105	60°C
contig5359	ATGGGCAAAAAGACATCCTG	TCAAAGATGGTGGGATCTTCA	127	60°C
contig13134	CAATGGCGGTATCAAAGGTT	ACGAGCAGAGGCATTACAGG	120	60°C
contig2537	TAAACGGGAACGAGGAGACA	TAGCTTGGTTCACCGAATCC	142	60°C
contig6454	GGGGCTTAGACCTCAACCTT	ATCACGAGACGTGGCTTTTT	118	60°C
contig4367	TTTCCAACTCATCCGGAGTC	CAGGACGGGAATGAAGTGTT	160	60°C
contig930	TGCTCGTGCTTCATTTCATC	ACGGCGACAAGTTTTATTGG	125	60°C
contig561	ATTGAACACTCGTGGGACCT	GCCACCGTAAACTTTCAGGA	111	60°C
contig6119	AACACGGAGATGCAGAAAGG	TCATACCGGCAGCCATATTT	118	60°C
contig12060	GGATCAAGGCAACCTTCAAA	CCACCATCAAATTGGGAAAG	123	60°C
contig19639	ATGGGTTCGGGTAATGGTGT	CGGAATCAAAATCCCATCAA	132	60°C

Table 3. Relevant putative *cis* elements in the p*GhERF105-like* inducible promoter

Cis elemento	Sequence	Description	Quantity
CAATBOX1	CAAT	"CAAT promoter consensus sequence" found in legA gene of pea;	6
GT1CONSENSUS	GRWAAW	Involved in plant responses to environmental factors for light and salicylic acid	10
POLLEN1LELAT52	AGAAA	One of two co-dependent regulatory elements responsible for pollen specific activation of tomato; AGAAA are required for pollen specific expression;	8
WBOXATNPR1	TTGAC	(pathogen- and SA-responsive)"W-box" found in promoter of *Arabidopsis thaliana* (A.t.) NPR1 gene;They were recognized specifically by salicylic acid (SA)-induced WRKY DNA binding proteins	4
WRKY71OS	TGAC	(GA-responsive) - Binding site of rice WRKY71, a transcriptional repressor of the gibberellin signaling pathway	4
WBOXNTERF3	TGACY	(wounding-responsive)-WBOXNTERF3 (TGACY) é um W-box promoter motive functioning in response to signal wound	3
GT1GMSCAM4	GAAAAA	"GT-1 motif" found in the promoter of soybean (Glycine max) CaM isoform, SCaM-4; Plays a role in pathogen- and salt-induced SCaM-4 gene expression	3
WBBOXPCWRKY1	TTTGACY	Biotic stress-related	2
ASF1MOTIFCAMV	TGACG	SA-responsive cis-regulatory elements in the promoter sequences Tomato	1
MYBCOREATCYCB1	AACGG	SA-responsive cis-regulatory elements in the promoter sequences Tomato	1
TATABOX5	TTATTT	"TATA box"; TATA box found in the 5'upstream region of pea (Pisum sativum) glutamine synthetase gene; a functional TATA element by in vivo analysis; TATA; glutamine; synthetase.	2

Table 4. Relevant putative *cis* elements in the p*GhERF17-like* inducible promoter

Cis elemento	Sequence	Description	Quantity
CAATBOX1	CAAT	"CAAT promoter consensus sequence" found in legA gene of pea;	6
GT1CONSENSUS	GRWAAW	involved in plant responses to environmental factorsfor light and salicylic acid	5
POLLEN1LELAT52	AGAAA	One of two co-dependent regulatory elements responsible for pollen specific activation of tomato; AGAAA are required for pollen specific expression;	2
WBOXATNPR1	TTGAC	(pathogen- and SA-responsive)"W-box" found in promoter of *Arabidopsis thaliana* (A.t.) NPR1 gene;They were recognized specifically by salicylic acid (SA)-induced WRKY DNA binding proteins	2
WRKY71OS	TGAC	(GA-responsive) - Binding site of rice WRKY71, a transcriptional repressor of the gibberellin signaling pathway	3
WBOXNTERF3	TGACY	(wounding-responsive)-WBOXNTERF3 (TGACY) é um W-box promoter motive functioning in response to signal wound	3
GT1GMSCAM4	GAAAAA	"GT-1 motif" found in the promoter of soybean (Glycine max) CaM isoform, SCaM-4; Plays a role in pathogen- and salt-induced SCaM-4 gene expression; See also S000198 (GT-1 consensus); GT-1 box; Glycine max (soybean)	1
WBBOXPCWRKY1	TTTGACY	Biotic stress-related	1
MYBCOREATCYCB1	AACGG	SA-responsive cis-regulatory elements in the promoter sequences Tomato	1
TATABOX5	TTATTT	"TATA box"; TATA box found in the 5'upstream region of pea (Pisum sativum) glutamine synthetase gene; a functional TATA element by in vivo analysis; TATA; glutamine; synthetase;	4

Table 5. Relevant putative cis elements in the p*GhNc-HARBI1-like* inducible promoter

Cis element	Sequence	Description	Quantity
CAATBOX1	CAAT	"CAAT promoter consensus sequence" found in legA gene of pea;	5
GT1CONSENSUS	GRWAAW	involved in plant responses to environmental factorsfor light and salicylic acid	6
POLLEN1LELAT52	AGAAA	One of two co-dependent regulatory elements responsible for pollen specific activation of tomato; AGAAA are required for pollen specific expression;	3
WRKY71OS	TGAC	(GA-responsive) - Binding site of rice WRKY71, a transcriptional repressor of the gibberellin signaling pathway	4
WBOXNTERF3	TGACY	(wounding-responsive)-WBOXNTERF3 (TGACY) é um W-box promoter motive functioning in response to signal wound	3
GT1GMSCAM4	GAAAAA	"GT-1 motif" found in the promoter of soybean (Glycine max) CaM isoform, SCaM-4; Plays a role in pathogen- and salt-induced SCaM-4 gene expression.	3
OSE2ROOTNODULE	CTCTT	One of the consensus sequence motifs of organ-specific elements (OSE) characteristic of the promoters activated in infected cellsof root nodules	1
TATABOX5	TTATTT	"TATA box"; TATA box found in the 5'upstream region of pea (Pisum sativum) glutamine synthetase gene; a functional TATA element by in vivo analysis; TATA; glutamine; synthetase;	4

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CAPÍTULO II

Identification and characterization of the *GmRD26* soybean promoter in response to abiotic stresses: potential tool for biotechnological application

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Identification and characterization of the *GmRD26* soybean promoter in response to abiotic stresses: potential tool for biotechnological application

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Abstract

Background

Drought is one of the most harmful abiotic stresses for plants, leading to reduced productivity of several economically important crops and, consequently, considerable losses in the agricultural sector. When plants are exposed to stressful conditions, such as drought and high salinity, they modulate the expression of genes that lead to developmental, biochemical, and physiological changes, which help to overcome the deleterious effects of adverse circumstances. Thus, the search for new specific gene promoter sequences has proved to be a powerful biotechnological strategy to control the expression of key genes involved in water deprivation or multiple stress responses.

Results

This study aimed to identify and characterize the *GmRD26* promoter (p*GmRD26*), which is involved in the regulation of plant responses to drought stress. The expression profile of the *GmRD26* gene was investigated by qRT-PCR under normal and stress conditions in Williams 82, BR16 and Embrapa48 soybean-cultivars. Our data confirm that *GmRD26* is induced under water deficit with different induction folds between analyzed cultivars, which display different genetic background and physiological behaviour under drought. The characterization of the *GmRD26* promoter was performed under simulated stress conditions with abscisic acid (ABA), polyethylene glycol (PEG) and drought (air dry) on *A. thaliana* plants containing the complete construct of p*GmRD26*::GUS (2.054 bp) and two promoter modules, p*GmRD26A*::GUS (909 pb) and p*GmRD26B*::GUS (435 bp), controlling the expression of the β-glucuronidase (*uidA*) gene. Analysis of GUS activity has demonstrated that *pGmRD26* and *pGmRD26A* induce strong reporter gene expression, as the *pAtRD29* positive control promoter under ABA and PEG treatment.

Conclusions

The full-length promoter p*GmRD26* and the p*GmRD26A* module provides an improved *uidA* transcription capacity when compared with the other promoter module, especially in response to polyethylene glycol and drought treatments. These data indicate that

p*GmRD26A* may become a promising biotechnological asset with potential use in the development of modified drought-tolerant plants or other plants designed for stress responses.

Key words: Stress-responsive promoter**,** Drought tolerance, Abscisic acid, Promoter modules analysis, gene-promoter characterization

Background

Drought is one of the most limiting and severe abiotic stresses for field crops because it causes significant losses in plants production on a global scale [1, 2]. Under drought conditions, plants trigger many physiological, biochemical and molecular responses. The sign of abiotic stress is perceived by cellular receptors and secondary messengers, culminating in the gene expression reprogramming to improve plant tolerance, adaptation, and survival. In signal transduction cascade, transcription factors (TFs) emerge as one of the most important messengers in plant adaptation, because they are capable of modifying specific gene expression, encompassing different physiological changes [3, 4]. For example, abiotic factors such as drought, salinity, and heat (high evaporation) alter the osmotic balance in plants' cell, inducing the biosynthesis of abscisic acid (ABA), a vital phytohormone involved in the expression of drought-related genes [5, 6].

Several TFs are involved in water stress tolerance, including ABA-responsive element (ABRE), nitrogen assimilation control (NAC), dehydration-responsive element binding (DREB), basic leucine zipper (bZIP), myeloblastosis (MYB) and myelocytomatosis (MYC) proteins. All of these TFs are mediators of the classic ABA-dependent or ABA-independent signaling pathways [7, 8, 9, 10, 11]. These transcription factors bind preferentially to the dehydration-responsive element (DRE) core sequence (A/GCCGAC) of gene-responsive promoters and regulate several stress-induced genes [12]. The DRE sequence is present in the *A. thaliana RD29A* (*AtRD29*) promoter region and is used extensively to drive expression in a stress-inducible manner in different plants, such as tobacco [13], potato [14], and soybean [15].

The expression of DREB1A under the control of the *AtRD29* promoter in *A. thaliana* increased the survival rate of plants stressed with freezing, drought, high salinity, and high temperature [16]. Similar results were also observed in tobacco plants [13]. In both cases, the use of inducible promoter *AtRD29* displays a higher gene expression than the Cauliflower mosaic virus 35S (CaMV35S) constitutive promoter, as it reduces the pleiotropic effects on growth due to the overexpression of DREB1A [13, 16]. The expression of genes of interest under the control of the *AtRD29* promoter has been widely used to regulate drought tolerance-associated genes in different plant species [16, 17, 18, 19]. However, compared with *AtRD29*, some specific inducible promoters can achieve higher levels of expression. An example is the promoter of the *Coffea arabica CaHB1*2 gene [20]. For this reason, the identification, isolation, and

characterization of new specific promoters inducible by abiotic stress have been crucial to ensure the successful application of gene modification and, consequently, the development of new cultivars resistant to water deprivation stress.

In this study, we have investigated the gene expression profile of *GmRD26* in soybean (*Glycine max*), homologous to the *A. thaliana AtRD26* gene (ANAC072). *GmRD26* is highly induced by ABA, PEG, and drought according to our gene expression analysis it displays similar expression profile in comparison with *AtRD26* and *GmNAC085*, a soybean *GmRD26* gene-paralogue already characterized [11, 21]. These genes belong to SNAC-A subfamily (ATAF), as well as *GmRD29*, the *AtRD29* orthologous, extensively used as a model of drought-inducible gene. Previous studies of the GENOSOJA project have demonstrated that *GmRD29* was not differentially expressed during severe water deprivation as *GmRD26* [22], selected as the focus for this study.

Many SNAC-A genes are involved with different abiotic stress responses and senesce progression. In soybean, 44% of NAC genes are differentially expressed (DE) during age triggered senescence, being 90% of genes from SNAC-A subfamily [21]. In *A. thaliana*, all SNAC-A gene members - *ANAC055* (AT3G15500), *ANAC019* (AT1G52890), *ANAC072/RD26* (AT4G27410), *ANAC002/ATAF1* (AT1G01720), *ANAC081/ATAF2* (AT5G08790), *ANAC102* (AT5G63790), and *ANAC032* (AT1G77450) - are induced by age triggered leaf senescence [21, 23]. *At*RD26 acts as a transcriptional activator in ABA-mediated dehydration response, positively regulating NYE1, which triggers chlorophyll degradation [24]. The *Gm*RD26 paralogue in soybean (*Gm*NAC085) is also a positive regulator of leaf senescence, displaying high expression during age triggered senescence and classic senescence symptoms when transiently expressed in *Nicotiana benthamiana* [21].

We subsequently isolated and characterized the *GmRD26* promoter (p*GmRD26*). The transcriptional activity of p*GmRD26* and its modules were evaluated in transgenic *A. thaliana* plants under the stress conditions with abscisic acid (ABA), polyethylene glycol (PEG) and drought (air dry) to evaluate the activities of the different regions of the p*GmRD26*.

Results

Soybean RD26 gene expression profile in distinct soybean lines under different stress conditions

To identify and characterize the orthologous gene of *A. thaliana AtRD26* (ANAC072) in soybean, an in-silico approach was applied. The *AtRD26* (AT4G27410) sequence was accessed and compared against the Williams 82 soybean reference genome. A putative *RD26* orthologous (*GmNAC043* – Glyma.06G248900) was identified by neighbour-joining analysis, which revealed that at least four genes (Glyma.13G279900, Glyma.12G221500, Glyma.06G248900, and Glyma.12G149100), closely related to

AtRD26, are present in the soybean genome **(Fig. 1)**. A comparative amino acid deduced sequence analysis of candidate genes was performed, and *GmNAC043*, called *GmRD26,* (Glyma.06G248900) displayed a relatively high amino acid similarity with *AtRD26*.

To evaluate whether the *GmRD26* soybean transcription factor is induced during water stress, its expression pattern was analyzed in cDNA subtractive libraries related to dissection experiments available in the GENOSOJA LGE (Genomics and Expression Laboratory: GENOSOJA Project) database and from these analyses, the presence of *GmRD26* was confirmed. To evaluate the *GmRD26* expression profile and its relation to multiple stress responses in soybean, the transcript levels were analyzed in the leaves and roots of Williams 82 soybean seedlings by qRT-PCR. The expression pattern was also evaluated for *GmNAC085*, a paralogue of *GmRD26* gene whose stress induction profile is reported previously [21]. As expected, *GmRD26* is highly expressed under the use of PEG (10% m/v) in leaves but is also induced by ABA (150 mM) and drought in leaves and roots **(Fig. 2)**. This gene expression profile is similar to the *GmNAC085* expression **(Additional file 1: Figure S1)**. In addition, both related genes are repressed by tunicamycin (Tun) in leaves and roots, showing an expressive induction by salicylic acid (SA) (5 mM) treatment in roots **(Fig. 2; Additional file 1: Figure S1)**.

The expression profile of *GmRD26* was also determined in two contrasting soybean genotypes in response to drought tolerance under simulated drought stress **(Fig. 3)** and ABA exogenous stimuli **(Fig. 3-A)**. It is expected that positive regulators of drought perception, signal transduction, and drought avoidance-associated genes are expressed higher in tolerant lineages than in susceptible lineages, as shown in the gene expression analysis results. In addition, the gene expression-folding is extensively high in BR16 and Embrapa48 cultivars when compared with the expression in Williams 82.

GmRD26 was differentially expressed in both leaves and roots of contrasting cultivars, and the tissues display a similar induction pattern as observed in Williams 82 under PEG (10% m/v) stress **(Fig. 2 and 3)**. In the roots, the gene expression was considerably lower than in the leaves. The difference between the cultivars is the gene expression levels: the susceptible cultivar BR16 had a significantly lower *GmRD26* transcript accumulation in comparison with the tolerant cultivar Embrapa48 at all times of stress progression **(Fig. 3)**. The gene expression significantly increased beginning at 125 min, showing that the *GmRD26* gene is strongly induced under severe stress conditions. The ABA response was also analyzed. As observed in *Arabidopsis*, the results revealed that *GmRD26* is also up-regulated by ABA in both soybean cultivars and the mRNA levels are significantly higher in tolerant cultivar Embrapa48, as observed in drought treatment **(Fig. 3A and B)**.

Analysis of water deficit-responsive *cis*-elements frequency

To investigate the transcriptional activity of the *Gm*RD26 soybean promoter under different stress conditions, the full-length promoter sequence (2.054 bp) was analyzed using PLACE and Genomatix for *cis*-regulatory element mapping. The promoter sequence analysis revealed some conserved TATA- and CAAT-box regions that are essential for transcription initiation complex assembly and gene transcription in eukaryotes. Potential *cis*-regulatory element families such as the ABRE, DREB, G-box, MYC and MYB families, which can respond to many environmental signals, abiotic stresses and phytohormones were also found in the p*GmRD26* sequence **(Fig. 4 and Table 1)**. The families' distribution in each promoter module used for *A. thaliana* genetic transformation is represented in **Fig. 4B**. Our analysis also revealed some specific drought-responsive *cis*-elements, MYB2AT and ACGTATERD1, as well as ABA-responsive ones, ABRERATCAL, ABREATCONSENSUS, DPBFCOREDCDC3, and EBOXBNNAPA Moreover, in the p*GmRD26* sequences, some doubly responsive elements, MYB2CONSENSUSAT, ABREZMRAB28, MYBCORE, and G-box, have been identified that respond to both drought and ABA. **(Fig. 5 and Table 1)**. The most frequent *cis*-elements identified in the modular p*GmRD26A* (909 bp) and p*GmRD26B* (435 bp) were DPBFCOREDCDC3, ABRERATCAL, and ABREATCONSENSUS, required in ABA-signaling and MYCCONSENSUSAT, ACGTATERD1 and MYBCORE, involved in dehydration-responses (**Table 1**). High-salinity responsive *cis*-elements are also present. These stress-associated *cis*-elements were also found in *AtRD29* promoter, and it was observed that *GmRD26* promoter has nine of thirteen dehydration and ABA responsive *cis*-elements, as found in p*AtRD29* promoter (**Additional file 2: Table S1**).

GUS activity and expression in transgenic *A. thaliana* lineages under p*GmRD26* control during different stress treatments

Homozygous T_2 *A. thaliana* lineages carrying the full-length p*GmRD26*::GUS and the promoter modules p*GmRD26A*::GUS and p*GmRD26B*::GUS were used to analyse promoter induction under drought stress through GUS activity. The GUS histochemical assay was performed after 12h of treatment with ABA and PEG in transgenic lineages and the controls p*AtRD29*::GUS (positive control) and non-treated plants (negative control). Plants carrying the p*GmRD26*::GUS and p*GmRD26A*::GUS displayed intense GUS activity in their foliar vascular tissue after ABA treatment, as well the positive control p*AtRD29* **(Fig. 6A- a, e, m).** In contrast, the p*GmRD26B*::GUS is not strongly inducible by ABA, according to its GUS activity **(Fig. 6A - i),** although ABRE elements are abundantly distributed in this promoter module. Under PEG treatment, the GUS activity pattern was the same **(Fig. 6A - b, f, j, n)**, with a discrete decline in activity in the p*GmRD26*::GUS plants when compared with ABA and drought (air dry) treatment **(Fig. 6A - b)**. In the p*GmRD26A*::GUS construct, a strong GUS-derived staining was observed in almost all the leaves surfaces in PEG treatment **(Fig. 6A - f)**. Under the

drought treatment, GUS activity was strongly detected in all analyzed leaves, mainly in the modular constructs p*GmRD26A*::GUS, p*GmRD26B*::GUS **(Fig. 6A - g and k)**. The basal expression in the control plants (without stress conditions) was low but detectable **(Fig. 6A - d, h, l, and p).** In our study, *pGmRD26A* displays activity in all treatments, but this activity is higher under desiccation conditions **(Fig. 6A - g)**, reinforcing the role of RD26 in desiccation-triggered protective mechanisms in plants.

To confirm the induction profile of p*GmRD26* revealed by histochemical assays, GUS activity was also monitored in transgenic lineage plants. Under ABA treatment, full-length p*GmRD26* encompassed the same results when compared to the p*At*RD29 positive control and the module p*GmRD26A* displays the higher GUS activity **(Fig. 6B)**. These results contrast with the PEG treatment, in which the full-length promoter and the p*GmRD26A* module exhibit higher activity when compared with the positive control, p*AtRD29*, and the p*GmRD26B* **(Figure 6B)**. When GUS activity was analyzed under drought treatment, p*GmRD26A* shows the same activity of the full-length promoter, higher than the smaller module p*GmRD26B*, but lower than the positive control. The activity of the p*GmRD26A* module was higher than the other fragments and the p*AtRD29* control under ABA and PEG treatments **(Fig. 6B)**. In addition, p*GmRD26A* transgenic lines display high levels of *uidA* mRNA after PEG treatment, while p*GmRD26* lines display high levels of *uidA* transcripts under ABA treatment. In the drought treatment, p*AtRD29* control lines presented higher expression level than the p*GmRD26* promoter and its modules. However, when we analyzed the differences between the three fragments after drought treatment, p*GmRD26A* showed higher expression levels compared to p*GmRD26* and p*GmRD26B* **(Fig. 7)**.

The results of qPCR demonstrate that transcriptional GUS activity, driven by the promoters p*GmRD26* and p*GmRD26A* was similar but not the same during ABA treatment. According to our data, p*GmRD26* display a similar expression when compared with p*AtRD29* (positive control) and higher mRNA accumulation when compared with p*GmRD26A* module **(Fig. 7A)**. This data is compatible with the histochemical assay. Under PEG treatment, the module *pGmRD26A* displayed a higher transcriptional activity, followed by the full-length *pGmRD26* and the positive control p*AtRD29* **(Fig. 7B)**. The module p*GmRD26B* continued to displaying lower GUS transcriptional activity. As expected, under drought condition, p*AtRD29* displayed higher GUS expression, followed by considerable GUS expression driven by modules p*GmRD26A* and p*GmRD26B* and the full-length promoter p*GmRD26* **(Fig. 7C)**.

Discussion

In this study, we confirmed that the *GmRD26* gene is induced under different simulated drought conditions. In view of the potential of this gene as a target for the development of strategies for the genetic engineering of resistant plants, we decided to isolate and

characterize the *GmRD26* promoter region. Our results show that p*GmRD26* and its modules activated the reporter gene *uidA* under different water deprivation stress conditions. These results are consistent with the characteristics of the *cis*-regulatory elements identified by *in silico* analyses of p*GmRD26* sequence. In Arabidopsis, *At*RD26 is an important member of ABA-dependent drought tolerance, and its overexpression is associated with a drought-tolerant phenotype [25].

The *GmRD26* gene, as well as its *A. thaliana* orthologous (*AtRD26*/ANAC072), belongs to the subfamily SNAC-A, whose members have some correlation of functional conservation with the *ATAF1* gene (AT1G01720), which has been shown to be a regulator of ABA biosynthesis and responsive to water stress [26, 27, 28, 29, 30]. Analysis of *GmRD26* expression in two contrasting drought-tolerant soybean cultivars, BR16 and Embrapa48, and in the Williams 82 (soybean reference genome) demonstrated that the expression profile of the *GmRD26* gene is compatible with phylogenetic and molecular characteristics already described for orthologous genes, reinforced by *GmNAC085* expression profile, a phylogenetically close-related gene in soybean [26, 31]. Our gene expression analysis reveals that the induction fold of G*mRD26* is not the same along the three analyzed cultivars, displaying an unexpectedly high expression level in BR16 and Embrapa48. The different genetic background of these cultivars should proportion this difference, once the BR16 and Embrapa48 are commercial cultivars, obtained by genetic breeding programs. In our study, *GmRD26* was responsive to osmotic stress during PEG treatment, desiccation and exogenous ABA stimulation in both leaves and roots, while the tolerant soybean variety displays higher gene expression level than susceptible variety. The leaves exhibited a more significant folding variation, suggesting that the physiological mechanism triggered by *Gm*RD26, mainly related to ABA-dependent responses, is more effective in the leaves than in the roots. In general, genes involved in ABA-mediated stress responses are involved in leaf morphophysiological changes, including stomatal closure, leaf area adjustment, photosynthesis, transpiration index and osmolyte accumulation [32, 33]. In Arabidopsis, it was already demonstrated that *Gm*NAC085 overexpression confers drought tolerance, improving the plant physiological performance during water deprivation stress. The transgenic lineages display a more robust antioxidative response under stress and many readouts genes, involved in ABA-dependent signalling, are up-regulated [11]. These results, associated with the determined *GmRD26* and *GmNAC085* gene expression profile, may justify the drought inducibility of SNAC-A genes during abiotic stress and confirm their potential to drive expression of genes involved with plant adaptability.

Compared with p*AtRD29*, a previously characterized drought-associated promoter, the promoter p*GmRD26* was also enriched in stress-related *cis*-elements, responsive to salinity, dehydration, ABA and temperature. These results can be directly related to the excellent performance of the soybean promoter under ABA and PEG treatments. During drought treatment, the promoter displays some reasonable activity but is not capable of being compared with p*AtRD29*. This broad responsive promoter activity can be applied

in soybean molecular breeding programs. In ABA-dependent pathways, ABREs (ABA-responsive elements) are the main phytohormone-responsive *cis*-element [27]. The occurrence of three ABRE motifs from five total ABA-responsive elements indicates a strong promoter induction under drought conditions, which can trigger increased drought-responsive gene expression by p*GmRD26* during stress. This effect is reinforced by the presence and frequency of the ACGT motif, a characteristic and important *cis*-element in drought-responsive promoters [34]. *Cis*-acting elements of the G-box family, found in several plant genes' promoters are known to interact with bZIP transcription factors, mediating responses to different stimuli. Studies comparing the patterns and evolution of the G-box family core (ACGT) in *O. sativa*, *S. bicolor*, *A. thaliana* and *G. max* suggest that this is the family with the most conserved elements between species and leads to responses to exogenous stresses, especially water and salt stress [35]. Other stress-responsive elements are also present in p*GmRD26*, such as MYCs/MYBs, which exhibit rapid induction in response to ABA treatment and water stress. These elements are targets of a large TF family in soya. MYC and MYB transcription factors are necessary for the early response to osmotic stress [36, 27].

In this study, we also the activity of the soybean promoter p*Gm*RD26 and two-promoter modules, p*Gm*RD26A and p*Gm*RD26B, in transgenic *A. thaliana* plants that were submitted to simulated (ABA and PEG) and real drought stress. The p*GmRD26*, p*GmRD26A*, and p*GmRD26B* promoters were induced by all stress treatment assays, showing greater or similar GUS activity than p*AtRD29* (positive control) under ABA, PEG, and drought treatment. Differences in induction intensity between p*GmRD26* and modules under different types of abiotic stress are probably related to the distribution of specific *cis*-elements in their sequences involved in the control of water stress response [37, 38]. The transcriptional activation of some genes depends not only on the promoters' *cis*-acting elements and their sequences but also on their position and the presence of enhancers, regulatory sequences and other synergistic *cis*-elements [39, 40]. It is important to highlight that some differences between transcriptional and translational activity are common on promoter's genes analysis. Our data demonstrate that the induction profile of p*GmRD26* is similar in the tested conditions, demonstrating that the full-length promoter and its modules respond to the same conditions. Also, promoters' modules enriched in *cis*-acting elements drive more consistent gene expression, reinforcing the idea of a synergistic effect of *cis*-elements in gene promoter sequences.

Similar results were obtained in the characterization of the α-galactosidase soybean promoter (GlymaGAL) responsive to water stress; the smallest fragment, pGAL-1kb, showed no significant difference in GUS activity compared to the control and treated samples (PEG and dry). The full-length fragment promoter, pGAL-2kb, however, led to a significant increase in GUS expression. This increase in GUS expression of pGAL-2kb was associated with a high number of ABRE, MYCATERD1, G-box, and DRE *cis*-elements [41]. Other studies have also reported the importance of distal promoter regions in responses associated with water stress in other species [42, 43].

Conclusions

In this study, we analyzed the expression profile of *GmRD26* gene, which is expressed under simulated osmotic and drought conditions in soybean. The stressed soybean seedlings display a high *GmRD26* expression under ABA exogenous-stimuli in leaves and roots, as well under PEG and air dry treatment. This gene expression pattern raised the hypothesis of drought-inducible *cis*-elements enriched promoter of *GmRD26*. Our analysis showed that the *GmRD26* promoter region is enriched with essential *cis*-elements associated with drought stress, such as ABRE, DREB, MYB, MYC, and G-BOX. Molecular characterization of p*GmRD26* in *A. thaliana* has demonstrated that the full promoter (p*GmRD26*) and two different promoter-modules (p*GmRD26A* and p*GmRD26B*) are inducible under simulated osmotic and drought stress conditions, confirming the soybean gene expression profile. In addition, our data also revealed that the full-length promoter and the p*GmRD26A* module, with higher *cis*-acting elements incidence compared to the other module, displayed a slightly higher level of expression than p*GmRD26B* and the p*AtRD29*, an *A. thaliana* promoter used as a model to drought inducible gene studies, during ABA and PEG treatment. The complete characterization of p*GmRD26* and its modules suggests that the promoter or the fragment p*GmRD26A* may become a potential biotechnological tool capable of inducing expression of genes of interest under specific conditions, such as drought or other abiotic stress related with osmotic imbalance to improve the tolerance associated to physiological performance in genetically modified plants.

Methods

Identification of the drought marker gene *GmRD26* in soybean

The *A. thaliana* RD26 (AT4G27410) deduced amino acid sequence (available in TAIR database - https://www.arabidopsis.org/) was used to identify the closely related orthologous gene (*GmRD26*/*Gm*NAC043 - Glyma.06G248900) in the soybean genome (Williams.82 v2.2-available in Phytozome: https://phytozome.jgi.doe.gov) [44]. For sequence comparison, BLASTP (https://blast.ncbi.nlm.nih.gov) was used and the alignment was confirmed using the online tool ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2). To determine the phylogenetic relationship between the *Arabidopsis* and soybean genes, the neighbour-joining clustering method derived from a distance matrix from a Poisson model was used, and the tree was reconstructed using MEGA software [45].

To evaluate whether the selected putative soybean gene *GmRD26* is induced during drought stress, its expression pattern was analyzed from cDNA subtractive libraries related to dissection experiments available in the GENOSOJA database LGE (Genomics and Expression Laboratory: GENOSOJA Project) [22].

Soybean plant growth conditions and stress treatments

For the *GmRD26* gene expression profile analysis, soybean (Williams 82) seeds were germinated in the soil and grown under greenhouse conditions (12h of light, 25-35°C, 70% relative humidity) until the V2-V3 development stage. To simulate multiple stress conditions, the seedlings were first transferred to Hoagland hydroponic solution for 24h. After acclimation, the soybean roots were immersed in the same solution supplemented with 10% (w/v) PEG 8000 to induce osmotic stress, 5 μg/mL tunicamycin (Tun) to induce endoplasmic reticulum stress, 150 mM ABA and 5 mM salicylic acid (SA) to simulate drought and biotic stress conditions, respectively. For the drought treatment, the plants were removed from the hydroponic solution and placed on plates with cotton. Leaf discs and roots of treated and control (0h - untreated plants were collected one time) seedlings were collected after 0.5h, 2h, 4h and 12h of stress treatment and immediately frozen in liquid nitrogen. All treatments were performed at a three-plants pool, and samples were collected in triplicate.

Embrapa48 and BR16 soybean cultivars were used to determine the expression profile during the ABA and drought treatments in drought responses contrasting soybean lineages. The BR16 variety is considered as a model of drought sensitivity, while Embrapa48 is considered as drought tolerant cultivar. The seeds were germinated in watered germination test paper and then transferred to a hydroponic box system filled with Hoagland solution. The seedlings in stages V3-V4 were grown under the same conditions as the Williams 82 seedlings. The drought stress was generated by removing the plants from the hydroponic solution and placing them in empty boxes for different water deprivation periods: 0 min (T0 - control), 25 min (T25), 50 min (T50), 75 min (T75), 100 min (T100), 125 min (T125) and 150 min (T150). Roots and leaf disc samples from three plants of each cultivar were collected during the exposure to water and were immediately frozen in liquid nitrogen for RNA extraction and gene expression analysis.

The contrasting soybean cultivars were also submitted to exogenous ABA treatment. Plants germinated and grown under the same conditions were sprayed with water (control) or ABA solution (300 ppm). Three biological replicates were used, consisting of three plants per treatment. After 6h, leaf discs were collected and immediately frozen in liquid nitrogen for RNA extraction.

RNA extraction, cDNA synthesis and *GmRD26* gene expression analysis

The total RNA of soybean leaf and roots was extracted according to the TRIzol® manual (Invitrogen, USA). RNA quantification was performed using a NanoDrop™ Spectrophotometer ND-1000 (Thermo Scientific, USA) and the RNA integrity was assessed by 1% agarose gel electrophoresis. A total of 2 μg of RNA was used for cDNA synthesis with MMLV reverse transcriptase protocol (Invitrogen, USA).

The gene expression profile was determined by qPCR. The analysis was performed using an ABI 7500 Fast instrument, SYBR Green (Invitrogen, USA) reagent, specific primers (**Additional file 3: Table S2**) and three independent cDNA pools. All the analyses were performed using three biological and two technical replicates, originated from a five soybean plants pool. The reaction was performed as follow: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 94 °C for 15 sec and 60 °C for 1 min. The *CYP* and *ELF* soybean genes [46] were used as endogenous controls for expression normalization and relative gene expression calculated by the $2^{-\Delta\Delta Ct}$ method. The endogenous gene stability was determined by G-norm algorithm (https://genorm.cmgg.be/), from Q-base package, and the M-value is 0.89 and 0.91 for *CYP* and *ELF*, respectively. The *GmRD26* orthologue gene, *GmNAC085*, was used as a comparative control in Williams 82 for gene profile determination.

Analysis of p*GmRD26* soybean *cis*-acting elements

The p*GmRD26* promoter sequence (2.054 bp) was obtained from the soybean genome available in the Phytozome database (https://phytozome.jgi.doe.gov) [44]. The *cis*-acting elements responsive to drought-, salinity-, osmotic- and ABA-induced stress were identified, analyzed, and mapped using the Genomatix (https://www.genomatix.de/online_help/matinspector/matinspector). For this study, we considered only the *cis*-elements statistically significant, with a p-value ≤ 0.05 [47, 48].

Construction of p*GmRD26* plasmids

The full-length *GmRD26* soybean promoter region was considered as the 2.054 bp gene-promoter and A and B promoter-modules contain 909 bp and 435 bp, respectively, considering the distribution of the drought-responsive *cis*-acting elements. The sequences were transcriptionally fused in frame to the GUS gene in a binary expression pC1407 vector backbone, synthesized by Epoch Biolabs (Sugar Land, TX, USA). The generated recombinant plasmids were called p*Gm*RD26::GUS (2.054 bp), p*GmRD26A*::GUS (909 bp) and p*GmRD26B*::GUS (435 bp). The plasmids carry out the translational GUS-GFP fusion and Bar plant selection marker gene. The *AtRD29A* (*AtRD29*) promoter gene sequence [49] was cloned into the same plasmid as a positive control of drought-inducible promoters.

Transgenic *A. thaliana* plants

The recombinant plasmids were introduced into *Agrobacterium tumefaciens* GV3101 strain, which was then transferred to *A. thaliana* ecotype Columbia (Col-0) by floral dip method [50]. Transgenic plants with a T-DNA insertion were identified by glufosinate-ammonium selection and confirmed by PCR. Three homozygous independent lines

were obtained for each construction and T_2 plants expressing GUS-GFP used in abiotic stress treatments and promoter characterization.

Drought, PEG and ABA treatment of *A. thaliana* transgenic lineages

A. thaliana seeds were germinated in the soil and grown under growth chamber-controlled conditions (12h photoperiod, 21°C temperature and 70% relative humidity). After 10 days, the seedlings were sprayed three times at intervals of five days with glufosinate-ammonium (100 mg/L) for positive transgenic plant selection. Four weeks old transgenic plants were carefully removed of soil moisture, and their roots were immersed in Hoagland hydroponic solution supplemented with 5% (w/v), PEG (MW 8.000) and 50 μM ABA solution to simulate drought conditions. For the drought treatment, the plants were removed from the hydroponic solution and placed on open plates. The non-stressed controls consisted of plants that were kept in Hoagland hydroponic solution. Two leaves of three plants for each full-length or modular promoter were collected after 12h of treatment and immediately frozen in liquid nitrogen and stored at -80°C for further extraction of RNA.

Histochemical GUS assays

To detect GUS activity in transgenic *A. thaliana* lineages, fresh leaves were incubated for 12-16h at 37°C in 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) solution [51]. After X-Gluc incubation, the leaves were washed with water, and the chlorophyll was removed with ethanol (70% v/v) for approximately 10h. The leaves were washed and then observed under Leica Wild Heerbrugg M3Z Stereozoom Microscope (Leica, Wetzlar, Germany). For each construct, leaves were collected from at least three different transgenic plants lineages.

Fluorimetric GUS assay

The *A. thaliana* transgenic plants were grouped into three-plant pools and subjected to stress treatments (PEG, ABA, and drought) as previously described. For plants pool protein extraction, extraction buffer with 100 mM NaH_2PO_4, 0.01% SDS, 10 mM EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100 and 1 mM DTT was used. Protein extraction was performed with frozen tissue powder (~100 mg) and samples were manipulated on ice. The total soluble proteins were quantified by the Bradford method [52] and used for a fluorimetric assay. The fluorimetric GUS assay was performed in a 500 μL reaction consisting of 400 μL of protein extract and 100 μL of 10 mM 4-methylumbelliferyl β-D-glucuronide (MUG; Sigma, USA). The reaction was incubated for 1 h at 37 °C. At the start point, a 50 μL reaction aliquot was removed and added to 450 μL of 0.2 M Na_2CO_3 stop buffer. The fluorescence of 4-methylumbelliferone (4-MU) was monitored using a Versa Fluor Fluorometer (BioRad) with excitation at 365 nm and emission at 455 nm. Each sample was analyzed in triplicate, and values were calculated according to a reference range of MU. GUS

activity was expressed in nanomoles of MU produced per minute per microgram of soluble protein.

All GUS fluorimetric assays were repeated at least three times. The results were expressed as the mean of independent experiments with the respective standard error. Different lowercase letters above the bars indicate significant differences at $P < 0.05$.

GUS gene expression analysis

GUS gene expression was analyzed in transgenic *Arabidopsis* plants expressing p*GmRD26*::GUS, p*GmRD26A*::GUS, p*GmRD26B*::GUS and p*AtRD29*::GUS. The gene expression level was monitored by qRT-PCR using three biological and two technical replicates, as previously described for soybean genes; the expression levels were normalized using *ACT2* (AT3G18780 [53]) and *GAPDH* (AT1G13340 [54]) as endogenous controls. The endogenous gene stability was determined by G-norm algorithm, from Q-base package, and the M-value is 0.86 and 0.79 for *ACT2* and *GAPDH*, respectively. The primers used are described in **Additional file 3: Table S2**.

Table 1. *Cis*-regulatory elements related to drought in the p*GmRD26* soybean promoter

***Cis*-regulatory element**	**Core sequence**	**Description**	**References**
ACGTATERD1	ACGT	Dehydration	[55]
MYCCONSENSUSAT	CANNTG	Dehydration, ABA and Cold	[56, 57]
ACGTABREMOTIFA2OSEM	ACGTGKC	Dehydration and ABA	[58]
DRE2COREZMRAB17	ACCGAC	Dehydration and ABA	[59]
MYB2CONSENSUSAT	YAACKG	Dehydration and ABA	[60]
ABREZMRAB28	CCACGTGG	ABA-responsive	[61]
ABREATCONSENSUS	YACGTGGC	ABA-responsive	[62, 63]
MYBCORE	CNGTTR	Dehydration and ABA	[64, 65]
MYB1AT	WAACCA	Dehydration and ABA	[60]
MYB2AT	TAACTG	Dehydration	[64]
G-box	CACGTG	Dehydration, high salinity, ABA	[66]
EBOXBNNAPA	CANNTG	ABA-responsive	[67, 56]
DPBFCOREDCDC3	ACACNNG	ABA-responsive	[68, 69]
ABRERATCAL	MACGYGB	ABA-responsive	[70]

Note: K=G/T; R=G/A; W=A/T; N=A/C/G/T; Y =*T C*

Figures

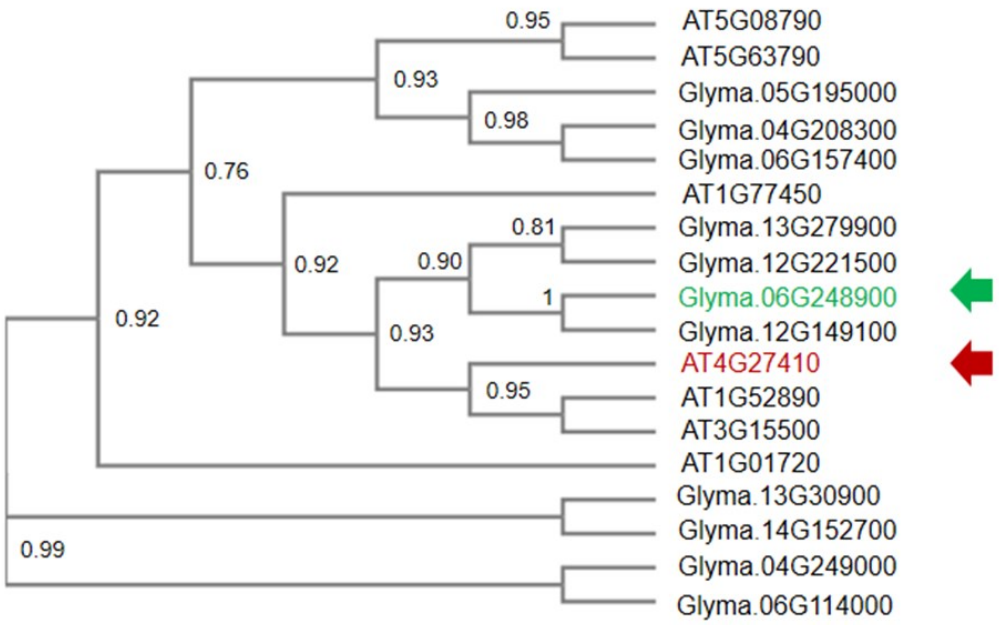


Fig. 1. Phylogenetic reconstruction of ATAF soybean genes, members of the NAC transcription factor subfamily. The deduced amino acid sequences of soybean and Arabidopsis were used to perform a multiple alignment using BLASTP and ClustalW2. The phylogenetic tree was constructed using MEGA4.0 software via the neighbour-joining method with a consensus of 10.000 bootstraps. The red arrow indicates the orthologous *A. thaliana* reference gene (AT4G27410), and the green arrow indicates *GmRD26* (Glyma.06G248900).

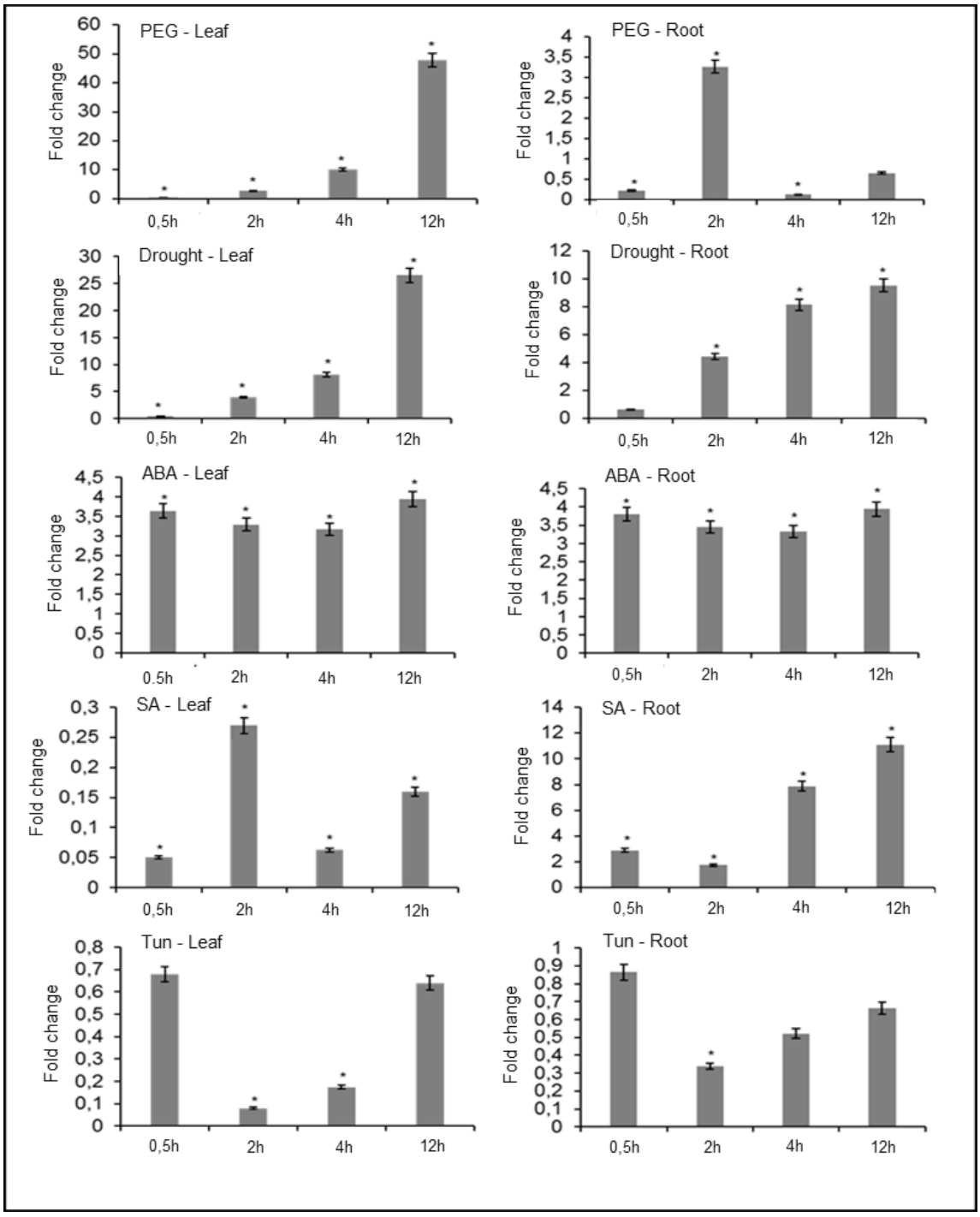


Fig. 2. *GmRD26* expression profile in soybean (Williams 82) under multiple stresses. To determine the gene expression profile of the *GmRD26* gene, the soybean seedlings were submitted to different stress conditions (ABA, PEG, SA, Tun and drought), and the gene expression in leaves and roots was analyzed by qRT-PCR. The fold change values were calculated in relation to untreated plants (0h), considering the relative expression in these plants as 1. *CYP2* and *ELF1A* were used as endogenous controls for normalization. The relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method in biological triplicates (n = 3). The bars represent standard errors and the asterisks (*) indicate statistical significance determined by the Student's t-test ($P \leq 0.05$).

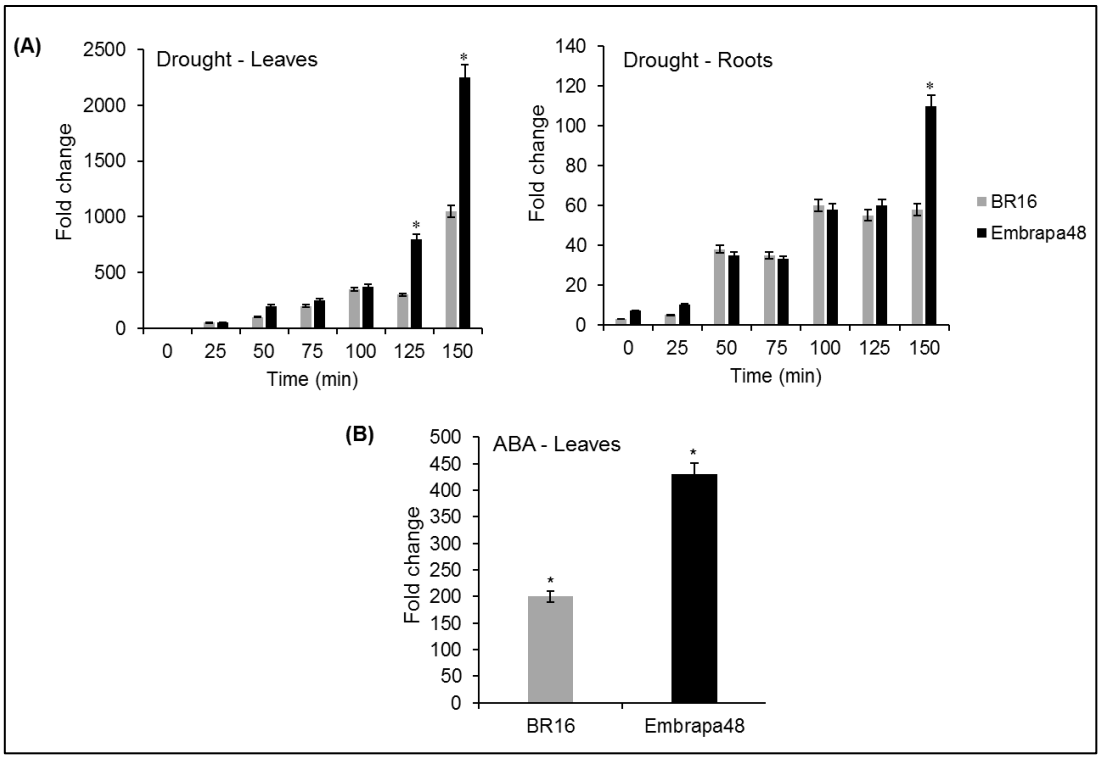


Fig. 3. *GmRD26* expression profile in two soybean cultivars, the drought-susceptible BR16, and the drought-tolerant EMBRAPA48. **(A)** Expression profile of the *GmRD26* gene under drought conditions, the gene expression pattern was determined at 25, 50, 75, 100, 125, and 150 min after water deprivation. **(B)** Expression profile of the *GmRD26* soybean gene in the leaves of contrasting genotypes BR16 and EMBRAPA48 after 6h of exogenous ABA stimuli. *CYP2* and *ELF1A* were used as endogenous controls for normalization. The relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method in biological triplicates (n = 3). The bars indicate the standard errors and the asterisks (*) indicate statistical significance determined by Student's t-test ($P \leq 0.05$).

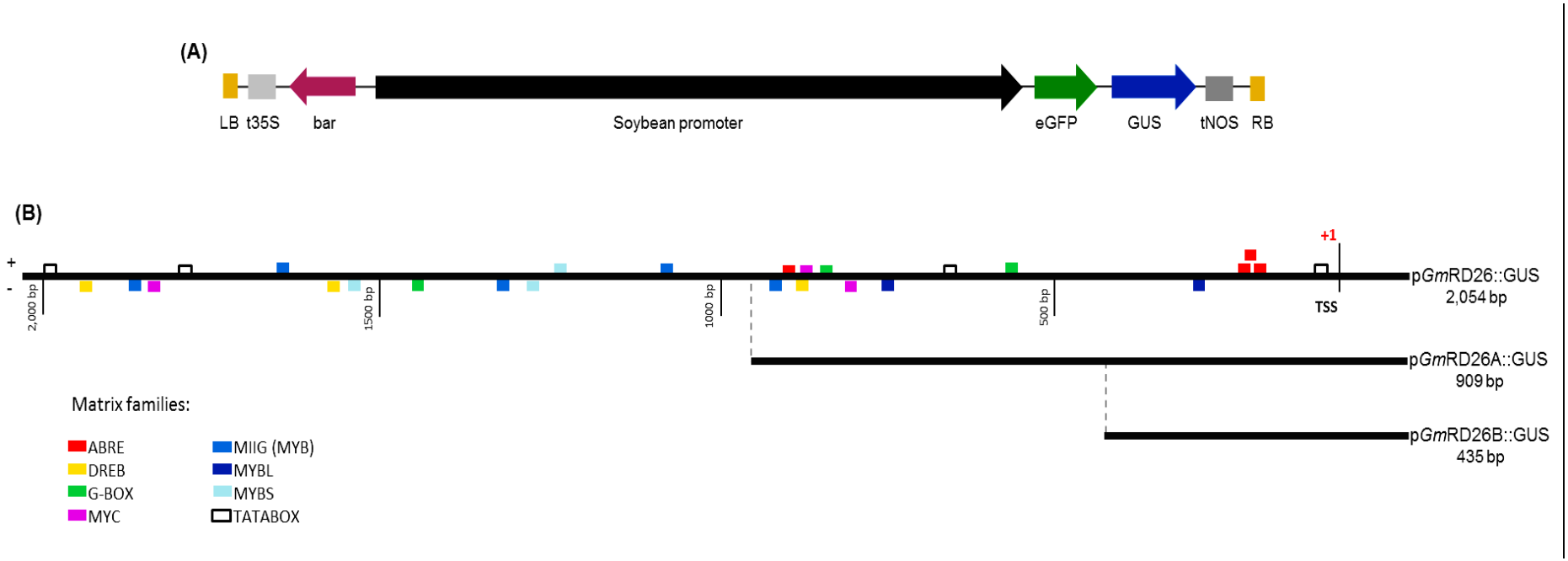


Fig. 4. Schematic representation of the *GmRD26* promoter regions controlling the expression of the GUS reporter gene. (**A)** Schematic drawing of the soybean promoter expression cassette in the pC1149::GUS expression vector. (**B)** Diagram of the main *cis*-acting elements in the full-length p*GmRD26* (2.054 bp) promoter and the modular promoters p*GmRD26A* (909 bp) and p*GmRD26B* (435 bp). The families of *cis*-elements were identified using the Genomatix data bases (p-value ≤ 0.05) and are represented by coloured boxes.

Fig. 5. Physical map of the *GmRD26* promoter. The transcription start site is highlighted in red and is designated as +1. The TATA-box is highlighted in bold. The numbers on the left side indicate the distance from the transcription start site. The sequences were analyzed by Genomatix databases (p-value ≤ 0.05). The putative *cis*-elements provided in p*GmRD26* are indicated by a bar and their names. Sense acting motifs (5′– 3′) are indicated by a superior bar, while antisense acting motifs (3′– 5′) are indicated by an inferior bar. All the stress-responsive motives are represented by different colours.

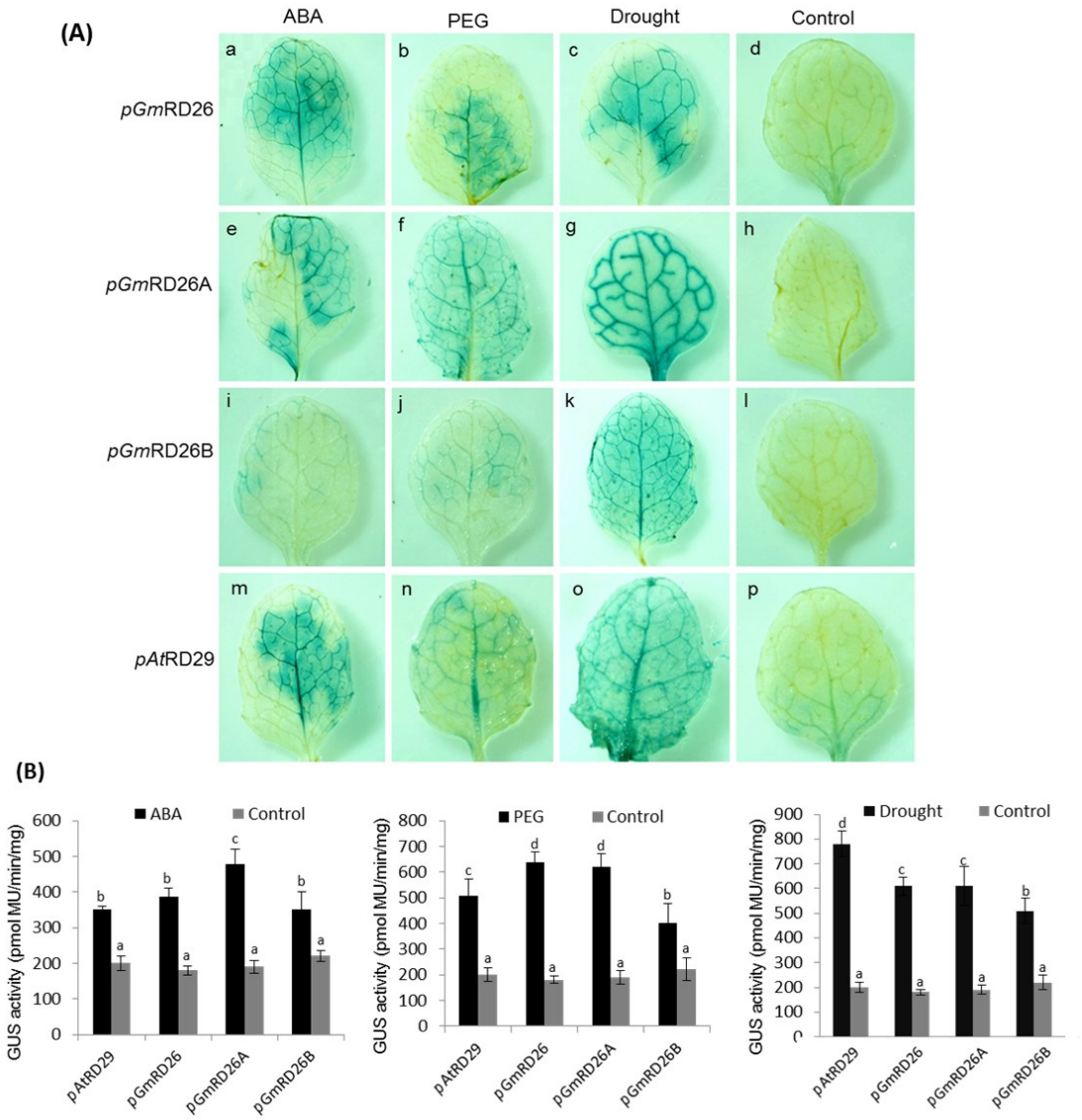


Fig. 6. Histochemical and quantitative fluorimetric analysis of different *GmRD26* promoter deletion constructs in transgenic *A. thaliana* plants. The stress treatments for GUS activity analysis was performed on 4 weeks *A. thaliana* plants under 12h treatments with ABA, PEG, or drought. **(A)** Histochemical localization of GUS activity in transgenic *A. thaliana* plants harboring promoter-GUS constructs. **(B)** The quantitative fluorimetric assay for GUS activity was carried out in three replicates. The soybean promoter and its modules were compared with the p*AtRD29* and wild type plants. Control samples consisted of untreated plants. Bars indicate standard error and different lowercase letters indicate significant differences at $P < 0.05$ on Tukey's Test. The data shown are representative of three independent lines (n = 3).

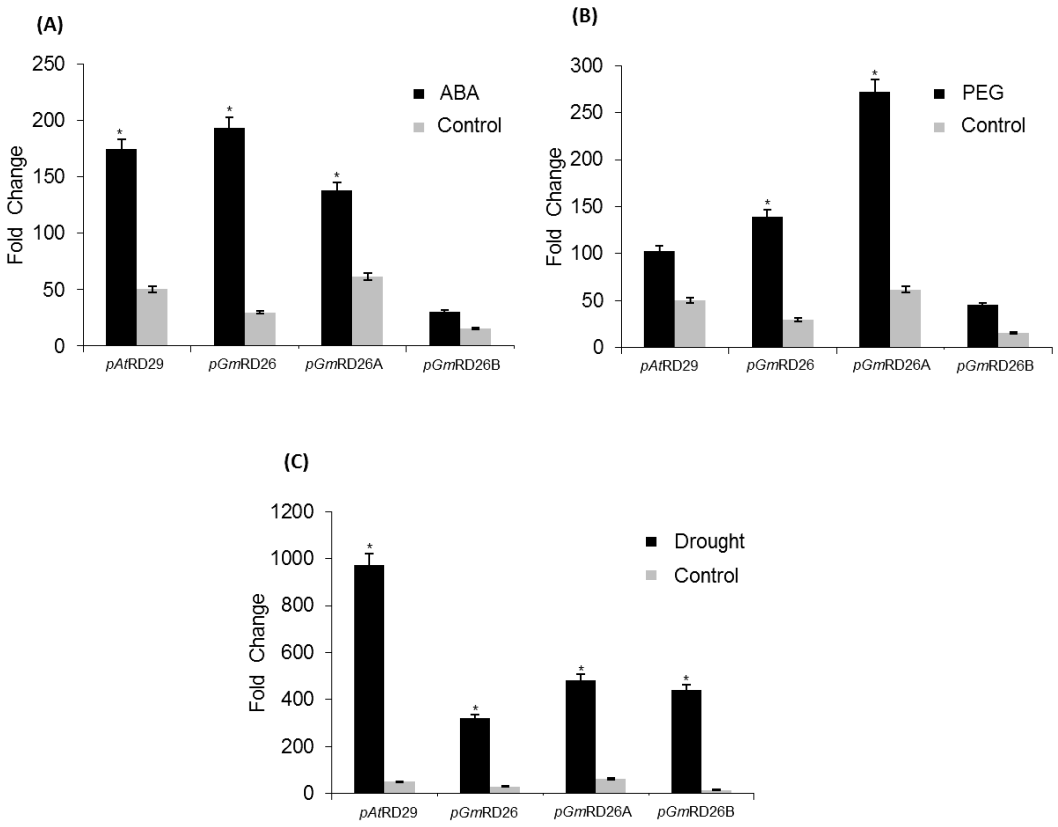
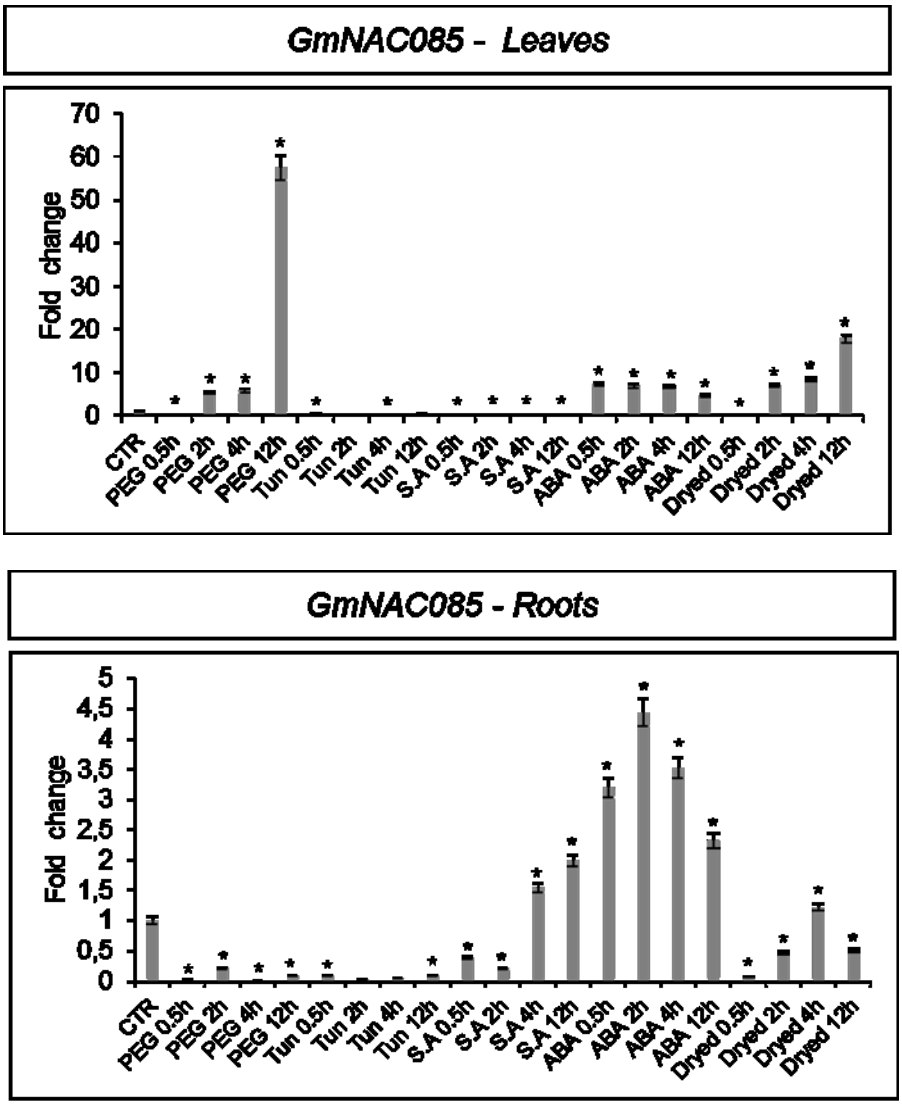


Fig. 7. Transcriptional GUS activity in transgenic *A. thaliana* under 12h of ABA, PEG or drought treatments. Levels of *uidA* mRNA of non-treated (control) and treated transgenic plants were measured using real-time PCR. The expression levels were normalized using *ACT2* and *GAPDH* as endogenous controls. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The bars represent standard error and the asterisks (*) indicate statistical significance determined by Student's t-test ($P \leq 0.05$). The data shown are representative of three independent lines (n = 3).



Additional file 1: **Figure S1**. *GmNAC085* expression profile in soybean (Williams 82) under multiple stresses. To determine the gene expression profile of the *GmNAC085* gene, the soybean seedlings were submitted to different stress conditions (ABA, PEG, AS, Tun, and drought), and the gene expression were analyzed in leaves and roots by qRT-PCR. The fold change values were calculated in comparison of plants treated with untreated plants (0h). *CYP2* and *ELF1A* were used as endogenous controls for normalization. The relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method in biological triplicates (n = 3). The bars represent standard errors and the asterisks (*) indicate statistical significance determined by the Student's t-test ($P \leq 0.05$).

Additional file 1: Table S1. *Cis*-regulatory elements related to drought revealed in the p*Gm*RD26 soybean promoter and the *A. thaliana* promoter RD29

***Cis*-regulatory element**	**Core sequence**	**Number of *Cis*-regulatory elements**		**Description**
		***Gm*RD26**	***At*RD29A**	
ACGTATERD1	ACGT	5	5	Dehydration
DREDR1ATRD29AB	TACCGACAT	0	2	Dehydration; high salt
DRECRTCOREAT	RCCGAC	0	4	Drought
DRE2COREZMRAB17	ACCGAC	3	3	Dehydration and ABA
MYCCONSENSUSAT	CANNTG	4	2	Dehydration, ABA and Cold
ACGTABREMOTIFA2OSEM	ACGTGKC	3	1	Dehydration and ABA
MYB2CONSENSUSAT	YAACKG	1	0	Dehydration and ABA
ABREZMRAB28	CCACGTGG	1	0	ABA responsive
ABREATCONSENSUS	YACGTGGC	2	0	ABA responsive
MYBCORE	CNGTTR	4	0	Dehydration and ABA
MYB1AT	WAACCA	1	1	Dehydration and ABA
MYB2AT	TAACTG	1	0	Dehydration
MYCATERD1	CATGTG	0	1	Dehydration
MYCATRD22	CACATG	0	1	Dehydration; ABA
LTRECOREATCOR15	CCGAC	0	4	Cold; drought, ABA
G-box	CACGTG	4	0	Dehydration, high salinity, ABA
EBOXBNNAPA	CANNTG	4	2	ABA responsive
DPBFCOREDCDC3	ACACNNG	3	2	ABA responsive
ABRERATCAL	MACGYGB	4	1	ABA responsive

Additional file 2: Table S2 - Primer sequences used in the qRT-PCR analysis

Gene name	**Forward primer sequence [5′-3′]**	**Reverse primer sequence [5′-3′]**
Glyma.06G248900	ATTCTTCCCGCAAACACAAC	CATTTATCTCCGGCAACGAT
CYP2	CGGGACCAGTGTGCTTCTTCA	CCCCTCCACTACAAAGGCTCG
ELF1A	GACCTTCTTCGTTTCTCGCA	CGAACCTCTCAATCACACGC
GUS	TTGGGCAGGCCAGCGTATCGT	ATCACGCAGTTCAACGCTGAC
ACT2	TTTCACTATATGCCAGTGGTCG	CTTCGTAGATCGGGACAGTGTG
GAPDH	GGTCATGGGAGATGACATGGTC	CAGGGTTTGTCTCGCAAAAATC

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