UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE BIOCIÊNCIAS

DEPARTAMENTO DE GENÉTICA

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

O papel dos fatores de transcrição bHLH162 e bHLH63 na adaptação das plantas ao estresse hídrico e ao ataque do fungo *Phakopsora pachyrhizi*

Graciela Castilhos

Porto Alegre

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Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para a obtenção do grau de Doutor em Ciências.

Orientadora: Marcia Pinheiro Margis

Coorientador: Maria Helena Zanettini

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SUMÁRIO

Resumo

A soja [*Glycine max* (L.) Merrill] é uma espécie pertencente a família Fabacea (leguminosas), de grande importância econômica no mundo. Por isso, foi recomendada como planta modelo para estudos genéticos e moleculares (Gepts et al., 2005). O sucesso na cultura dessa espécie pode ser limitado por diversos fatores bióticos e abióticos. Dentre os fatores bióticos, destacam-se as doenças, entre elas, a Ferrugem Asiática causada pelo fungo *Phakopsora pachyrhizi*, enquanto a desidratação causada pela seca é um dos fatores abióticos de maior relevância. A introdução de cultivares resistentes tem se mostrado uma alternativa eficiente para a redução de perdas na produtividade causada por esses estresses por patógeno e pela seca. Vários genes que codificam fatores de transcrição têm sido utilizados como tentativa para aumentar a tolerância de plantas a condições adversas. Entre as famílias de fatores de transcrição, a família bHLH (basic helix-loop-helix) destaca-se como a maior família de fator de transcrição de plantas e está envolvida em diferentes rotas regulatórias conferindo maior tolerância a diferentes estresses ambientais. Neste contexto, o objetivo deste trabalho foi a caracterização funcional de genes que codificam fatores de transcrição bHLH em resposta aos estresses gerados por *Phakopsora pachyrhizi* e seca. Na tentativa de esclarecer o papel de genes bHLH no desenvolvimento de planta de soja, foram produzidas plantas transgênicas de *Arabidopsis thaliana* superexpressando o gene bHLH (Gm06g01430) de soja, o qual mostrou-se diferencialmente expressos em condições de estresses. Estas plantas transgênicas apresentaram a emissão e a abertura dos cotilédones, assim como a emissão das folhas precocemente, em relação aos mesmos parâmetros observados na planta selvagem. Ainda, a fim de elucidar prováveis rotas sinalizadoras em que os fatores de transcrição bHLH estão envolvidos, identificou-se, através de um *screening* de duplo híbrido em leveduras, uma interação da proteína bHLH (Gm05g02110) com a proteína polyamina oxidase 2 (PAO2). Essa interação foi confirmada em ensaios de BiFC em protoplastos de arabidopsis que demonstrou que esta interação ocorre no citoplasma e no núcleo das células. Os resultados sugerem que Gm05g02110 está relacionado à resposta de suscetibilidade ao patógeno causador da ferrugem asiática. A proteína por ele codificada, provavelmente liga-se ao motivo de DNA G-box (CACGTG) e sua interação com a proteína PAO2 pode estar relacionada ao mecanismo de regulação da resposta a estresses bióticos e abióticos.

Abstract

Soybean [Glycine max (L.) Merrill] is a species belonging to Fabacea family (legumes), of great economic importance in the world. Therefore, it was recommended as a model plant for genetic and molecular studies (Gepts et al., 2005). The successful culture of this species may be limited by various biotic and abiotic factors. Among the biotic factors, there are the diseases, among them, the soybean rust caused by *Phakopsora pachyrhizi*, while the dehydration caused by drought is one of the abiotic factors most relevant. The introduction of resistant cultivars has proven to be an effective alternative for reducing yield losses caused by these stresses by pathogen and drought. Several genes encoding transcription factors have been used in an attempt to increase the tolerance of plants to adverse conditions. Among the families of the bHLH (basic helix- loop - helix) transcription factor,family stands out as the largest family of transcription factores plants and is involved in different regulatory routes conferring tolerance to various environmental stresses. In this context, the aim of this work was the functional characterization of genes encoding bHLH transcription factor in response to stresses generated by *Phakopsora pachyrhizi* and dry. In an attempt to clarify the role of the bHLH genes in the development of soybean plants, transgenic *Arabidopsis thaliana* plants overexpressing the bHLH gene (Gm06g01430) of soybean, which proved to be differentially expressed under conditions of stress were produced. These transgenic plants showed the emission and the opening of the cotyledons, and the emission of advance sheets to the same parameters observed in wild plant. Further, in order to elucidate signaling likely routes that are involved bHLH transcription factor, it was identified through a two-hybrid *screening* in yeast, the interaction of the bHLH protein (Gm05g02110) with polyamina oxidase 2 (PAO2) proteins. This interaction was confirmed in assays BiFC in *Arabidopsis thaliana* protoplasts which demonstrated that this interaction occurs in the cytoplasm and nucleus of cells. The results suggest that Gm05g02110 response is related to susceptibility to the causative pathogen of soybean rust, probably binds to the motif of DNA G -box (CACGTG) and its interaction with the protein PAO2 can be related to the mechanism of regulation of response to stress biotic and abiotic.

INTRODUÇÃO GERAL

1. Soja

A soja [*Glycine max* (L.) Merrill] pertence a família Fabacea (leguminosas), originária da Ásia (Embrapa, 2013), sendo destaque entre as oleaginosas produzidas no mundo (Silva et al., 2009). Em meados dos anos 60, a área cultivada para a produção de grãos começou a crescer de forma exponencial, não apenas nos EUA, como também no Brasil e Argentina. Atualmente, são produzidos, por ano, cerca de 200 milhões de toneladas deste grão. O Brasil aparece como o segundo maior produtor e exportador mundial de soja, atrás apenas dos EUA (Embrapa, 2013). Segundo levantamentos da safra de 2012/2013 no Brasil, a área de soja plantada foi de 27 milhões de hectares e a produção foi estimada em 81,5 milhões de toneladas (http://www.cnpso.embrapa.br/index.).

Devido a sua grande importância, a comunidade científica internacional que trabalha com leguminosas, recomendou a soja como planta modelo para estudos genéticos e moleculares (Gepts et al., 2005).

2. Estresses ambientais

Entre os diversos fatores bióticos que limitam a obtenção de altos rendimentos da soja, as doenças estão em destaque entre os mais importantes e difíceis de controlar. A expansão das culturas para novas áreas a monocultura e a utilização de práticas ineficazes de manejo têm aumentado o número de doenças causadas por fungos, bactérias, vírus e nematóides, além de outras anormalidades desconhecidas (Suzuki & Yuyama, 2005). Apesar do uso de fungicidas e outras técnicas que visam o controle ou a eliminação dos patógenos, as moléstias fúngicas são responsáveis por grandes prejuízos nas colheitas todos os anos.

Diversos fungos atacam as culturas da soja. Os patógenos mais comumente envolvidos com podridão de sementes e morte de plântulas são: *Pythium* sp., *Phytophtora sojae*, *Rhizoctonia solani* e *Fusarium* sp. Esses dois últimos, juntamente com *Macrophomina phaseoli*, *Sclerotium rolfsii* e *S. sclerotiorum*, são também responsáveis por podridões radiculares, do colo e da haste (Reis & Casa, 2002). Os autores salientam que

em soja não há fonte de resistência para a maioria desses fungos, sendo este um grande desafio para os programas de melhoramento.

A Ferrugem Asiática, causada pelo fungo *Phakopsora pachyrhizi* Sydow, foi detectada no Brasil há mais de uma década e, de acordo com as informações da safra de soja de 2001/2002, perdas de até 90% foram constatadas no estado da Bahia, onde a doença ocorreu com alta severidade (Yorinori et al., 2002). O desenvolvimento da infecção pode ser visto a partir da presença de pústulas em ambas as faces da folha, formando lesões de coloração amarelo-avermelhadas facilmente visíveis. O avanço da doença resulta no amarelecimento e queda prematura das folhas, evitando a plena formação dos grãos, comprometendo a qualidade e rendimento de grãos da cultura (Embrapa 2002).

Nas lavouras do Rio Grande do Sul, os prejuízos causados pela Ferrugem Asiática têm sido uma constante, tendo sido registrados 246 focos da doença na safra de 2008/2009, seguidos por 405 casos em 2009/2010 (Embrapa, 2010). Na safra de 2010/2011 foi evidenciado o primeiro foco de Ferrugem Asiática em propriedades do município de Júlio de Castilhos**,** com estimativas de perdas de até 80% das lavouras, gerando uma redução drástica no rendimento de grãos (Canal Rural, 2011). Além de propriedades no RS, o Consórcio Antiferrugem registrou a ocorrência da Ferrugem Asiática da soja em outras áreas comerciais na safra 2013/2014, como foi registrado no Paraná, Goiás e Mato Grosso (Embrapa 2014).

Assim como ocorre para outros fungos, não havia registros de cultivares resistentes à Ferrugem Asiática até a safra de 2009. Porém, uma nova cultivar com resistência parcial a Ferrugem Asiática, foi desenvolvida pela Embrapa em parceria com a Secretaria de Agricultura, Pecuária e Abastecimento do Estado de Goiás (SEAGRO). A cultivar de soja BRSGO 7560 é portadora de um gene maior recessivo, que confere resistência vertical à ferrugem. A nova cultivar pode evitar ou minimizar as perdas de produtividade (Embrapa 2009). Entretanto, até o momento, o controle da Ferrugem da soja não se mostrou eficiente. A cultivar recentemente lançada apresenta resistência vertical à Ferrugem asiática, e está sujeita à quebra dessa resistência devido à variabilidade genética do fungo *Phakopsora pachyrhizi*, causador da doença. Ainda, o gene *Rpp4* de resistência à Ferrugem Asiática, foi identificado como primeiro gene de resistência a esta doença, porém a perda deste gene durante o processo de seleção tem mostrado a dificuldade de se obter plantas resistentes à Ferrugem Asiática em soja (Meyer et al., 2009).

A tentativa de controle através de fungicidas se mostra cara e ineficiente, devido à agressividade do fungo e à dificuldade de detecção nos estágios iniciais da infecção. Além disso, o controle químico causa forte impacto ambiental. (Utiamada, 2003; Suzuki & Yuyama, 2005; Calvo et al., 2008).

Além de doenças, outro fator, como o estresse hídrico, limita a obtenção de altos rendimentos na cultura da Soja. Entre todos os desafios do melhoramento genético de plantas, a tolerância à seca tem se mostrado urgente, principalmente no Rio Grande do Sul, devido à destruição das lavouras pela falta de chuva. Vários projetos de pesquisa visando à obtenção de cultivares de soja transgênica tolerante à seca vêm sendo atualmente realizados no Brasil. Um dos estudos mais avançado está sendo desenvolvido pela Embrapa Soja de Londrina em parceria com o JIRCAS (Japan International Research Center for Agricultural Sciences), que visa o aumento da expressão dos genes DREB (Dehydration Responsive Element Binding protein) (http://www.cnpso.embrapa.br/noticia/ver_noticia.php?cod_noticia=670). O fator de transcrição DREB1 é descrito como ativador da expressão de vários genes responsivos a estresses em *Arabidopsis thaliana*. A superexpressão do gene *ZmDREB1* em plantas transgênicas de milho resultou em um aumento na tolerância a seca, salinidade e frio, sugerindo que este fator de transcrição é um alvo potencial para ser utilizado como ferramenta na engenharia genética (Qin et al., 2004).

Diversas famílias de fatores de transcrição estão presentes no genoma da soja, sendo que de um total de aproximadamente 43.000 lócus que codificam proteínas, 5.671 são prováveis genes que codificam fatores de transcrição, ou seja, 12,2% dos genes. Em termos de comparação, em *Arabidopsis thaliana* este número é de 7,1% (Schmutz et al., 2010). Entre os genes que codificam fatores de transcrição em soja, diferentes famílias gênicas foram identificadas como: BZIP, C2H2 (Zn), NAC, WRKY, MADS, bHLH, SNF2, MYB, ABI3/VPI, AP2/EREBP, AUX-IAA-ARF, entre outros (Schmutz et al., 2010).

3. Fatores de transcrição basic helix-loop-helix (bHLH)

Os fatores de transcrição são definidos pela capacidade de se ligarem a um ou mais domínios de ligação ao DNA presentes na região promotora de genes alvos e

regularem a transcrição. São identificados como reguladores chave do metabolismo e desenvolvimento e são classificados de acordo com o tipo de domínio de ligação ao DNA (Hughes 2011). Alterações na estrutura da cromatina facilitam a ligação de fatores de transcrição em seus respectivos sítios de ligação ao DNA, ativando a transcrição de genes (Latchman, 2008). A capacidade intrínseca de ligação ao DNA é apenas um dos vários parâmetros que podem determinar seu local de ligação no genoma. O local pode ser influenciado pela acessibilidade dentro da cromatina, pela cooperação ou competição com outras proteínas a sequências específicas de ligação ao DNA, por interações com proteínas da cromatina e por modificações da cromatina (Hughes 2011).

As proteínas basic helix-loop-helix (bHLH) correspondem a maior família de fatores de transcrição presentes em todos os organismos eucariotos, e atuam em diferentes processos regulatórios (Jin et al., 2014: http://planttfdb.cbi.pku.edu.cn/). Grande parte da diversidade das proteínas bHLH vegetais teve origem em plantas terrestres ancestrais há mais de 440 milhões de anos. As proteínas bHLH são monofiléticas e constituem 26 subfamílias (Pires & Dolan, 2010).

As subfamílias são caracterizadas pela presença do domínio bHLH altamente conservado. O domínio bHLH consiste de 50-60 aminoácidos com a formação de dois segmentos distintos: um trecho de 10-15 contendo predominantemente aminoácidos básicos (região básica) e uma seção de cerca de 40 aminoácidos prevista para formar duas α-hélices anfipáticas constituída de resíduos hidrofóbicos, separadas por um loop de comprimento variável (região helix-loop-helix) (Pires & Dolan, 2010). O domínio HLH promove a interação proteína-proteína, permitindo a formação de complexos homodimérico ou heterodimérico (Massari & Murre, 2000) e, os aminoácidos conservados na região básica servem para determinar o reconhecimento de sequências consensu E-Box 5'-CANNTG-3', enquanto outros resíduos proporcionariam especificidade para um determinado tipo de E-box (por exemplo, o G-box [5'-CACGTG-3']) (Carretero-Paulet et al., 2010). A especificidade ao motivo E-box requer o reconhecimento de dois resíduos específicos de aminoácidos na região básica (Glu-13 e Arg-16), o qual está presente em pelo menos 359 proteínas bHLH de plantas em uma análise feita com proteínas bHLH de *Arabidopsis thaliana* , arroz, musgos e algas. Para a especificidade de ligação ao DNA, são necessários ao motivo G-box resíduos como His/Lys-9, Glu-13 e Arg-17, os quais têm sido identificados em 280 proteínas bHLH (Carretero-Paulet et al., 2010).

Em plantas vasculares foram encontradas seis subfamílias bHLH, as quais são classificadas em seis grupos maiores (A-F), baseados na sua capacidade de ligação ao DNA. Diferentes estudos filogenéticos propõem a classificação das proteínas bHLH em 15-25 subgrupos (Li et al. 2006b). Em plantas, 53% das proteínas bHLH possui o grupo B (H_5-E_9-R_{13}) característico de proteínas bHLH presentes em animais e apenas 8% tem o grupo A (R_8-E_9) também típico de animais. Apenas 11% das proteínas vegetais têm um motivo conservado Q_5-A_9-R_{13} que, por sua vez, diferentemente dos demais motivos, não está presente em animais (Pires & Dolan, 2010). O alto grau de similaridade de sequência entre os domínio bHLH de proteínas vegetais e animais, em especial na região compreendendo a região de aminoácidos básicos que interage com o DNA e os aminoácidos hidrofóbicos que estabilizam a hélice, mostra que a estrutura molecular e a atividade das proteínas bHLH são altamente conservadas entre animais e plantas (Pires & Dolan, 2010).

Dentre os genes que codificam fatores de transcrição que apresentam o domínio bHLH, destacam-se: (i) o gene LC, envolvido na rota de biossíntese de antocianina; (ii) os genes *PIFs*, responsáveis pela sinalização do fitocromo; (iii) o gene *ICE1*, que atua como regulador positivo na tolerância ao frio; (iv) o gene *SPCH*, envolvido no desenvolvimento de estômatos; (v) o gene *GL3*, responsável pelo desenvolvimentos de tricomas; (vi) o gene *BIGPETAL*p, envolvido na morfogênese floral; (vii) genes de resposta ao ABA e (viii) genes de percepção da deficiência ao ferro (Ko et al., 2009).

A relação de genes *bHLH* e estresses abióticos tem sido relatada (Zhou et al. 2009; Liu et al., 2013). Segundo Zhou et al. (2009), plantas de *Oryza rufipogon* (arroz silvestre) superexpressando o gene *OrbHLH2* mostraram aumento na resistência ao estresse salino, porém, não exibiram tolerância ao congelamento. Como consequência da superexpressão de *OrbHLH*, os níveis da expressão de genes responsivos ao estresse salino foram induzidos. Além disso, *OrbHLH* possui um papel importante na transdução de sinal no estresse de seca por induzir *DREB1A/CBF3*, cruciais na resposta à seca. *OrbHLH2* pode funcionar *upstream* de *DREB1/CBF*, que, por sua vez, liga-se aos elementos *CRE/DRE* nos genes *RD29A* e *KIN1*, que conferem melhor tolerância das plantas ao estresse salino. Em *Arabidopsis thaliana* , o gene *bHLH122* está relacionado à resposta ao estresse por seca, sal e estresse osmótico, uma vez que plantas superexpressando *bHLH122* apresentaram maior resistência a tais estresses. Este aumento na tolerância destas plantas pode estar

relacionado com a capacidade do gene *bHLH122* regular a expressão de genes relacionados a estresses (Liu et al., 2013).

Diferentes fatores de transcrição bHLH têm sido relacionados a estresses bióticos via rotas dependentes de fito-hormônios como jasmonato (Boter et al., 2004; Todd et al. 2010). O fator de transcrição ORCA3 que participa da rota de biossíntese de alcalóides pode ser induzido pela ação de bHLHs, que por sua vez, modulam as funções dependentes de jasmonato em *Arabidopsis thaliana* (Boter et al., 2004). Todd et al. (2010) mostraram que os fatores de transcrição bHLH1 e bHLH2 atuam como ativadores na biossíntese do jasmonato em *Nicotiana benthamiana* (Todd et al., 2010). A superexpressão e o silenciamento dos genes *NbbHLH1* e *NbbHLH2* alteraram pouco a expressão de outros fatores de transcrição. Entretanto, em plantas RNAi, a expressão de genes envolvidos na biossíntese de alcalóides aumentou mais de dez vezes (Todd et al., 2010).

A subfamília 25 de proteínas bHLH está envolvida com a sinalização de hormônios tais como os Brassinosteróides (BR), os quais regulam o desenvolvimento vegetal (Carretero-Paulet et al., 2010). Wang e colaboradores (2009), destacam a importância das proteínas bHLH na regulação nuclear na rota de sinalização de BR, evidenciando duas famílias de proteínas bHLH capazes de modular a sinalização de BR, tais como as proteínas BEE1 e BEE3 em *Arabidopsis thaliana* . Os elementos E-box, reconhecidos por proteínas bHLH, estão presentes nos promotores de muitos genes responsivos aos BR. As proteínas BEE1, BEE2 e BEE3, contendo domínios bHLH podem também regular outros hormônios como o ácido abscísico (ABA), antagonista de BR. A superexpressão destas proteínas reduziram a resposta ao ABA em plantas, sugerindo que as proteínas BEE podem funcionar como sinalizadores intermediários em múltiplas rotas (Friedrichsen et al., 2002).

Em soja, foram identificados 480 bHLH (Jin et al.2014). Embora se conheça a importância de bHLH em diferentes estresses, a possível relação de proteínas bHLH de soja com estresses tem sido pouco relatada. Osório et al. (2012) identificaram pelo menos 14 genes *bHLH* de soja envolvidos em respostas a estresses bióticos e abióticos. O gene RD29 é um exemplo bem consolidado do papel de bHLH na resposta da planta a estresses, como por exemplo estresse de seca. Plantas transgênicas têm sido geradas usando o promotor do gene bHLH *RD29* para dirigir a expressão de genes alvos e assim aumentar a tolerância ao deficit hídrico em soja (Bihmidine et al., 2012).

Os resultados realizados neste trabalho serão apresentados em quatro capítulos:

O capítulo 1 apresenta uma revisão de fatores de transcrição bHLH envolvidos na resposta das plantas ao estresse ocasionado pela seca. Este estudo resultou em um artigo intitulado: “Possible roles of basic helix-loop-helix transcription factors in adaptation to drought”, publicado na revista *Plant Science*.

O capítulo 2 apresenta dados de expressão do gene bHLH codificado por *Glyma05g02110* em plantas de soja submetidas a estresse por *Phakopsora pachyrhizi* e ao estresse por seca, assim como a identificação da interação do produto deste gene com a proteína Poliamina oxidase 2 de soja, a partir de um *screening* de duplo híbrido em levedura, utilizando uma biblioteca de cDNA de folhas de soja submetida ao estresse por tunicamicina.

O capítulo 3 descreve os resultados obtidos a partir da superexpressão do gene bHLH *Glyma06g01430* em plantas transgências de Arabidospsis. Além disso, foi também realizado um estudo do padrão de expressão deste gene em resposta a estresses biótico e abiótico.

Por fim, o capítulo 4 refere-se aos dados obtidos durante o doutorado sanduíche na Universidade Nova de Lisboa sob a orientação do Dr. Nelson Saibo. O estudo apresenta-se em fase preliminar e objetivou elucidar prováveis rotas regulatórias em que o gene bHLH estão envolvidos. Nesse trabalho, o modelo de estudo foi o arroz, *Oryza sativa,* devido à disponibilidade, em nosso grupo, de plantas contendo níveis endógenos alterados de peróxido de hidrogênio que superexpressam um gene que codifica um fator de transcrição bHLH, mostrando que essa espécie reativa de oxigênio faz parte das vias que regulam a expressão desses genes. Assim, para tal estudo, foi utilizado o gene bHLH *Os01g06640* como isca e foram identificados dois fatores de transcrição que se ligam a região promotora deste gene, a partir de um *screening* de mono-híbrido em levedura, utilizando uma biblioteca de cDNA de plantas de arroz submetidas ao estresse por frio.

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OBJETIVO

O objetivo geral deste trabalho foi caracterizar os genes que codificam proteínas bHLH em soja, em especial aqueles relacionados com os processos de resposta ao ataque do fungo *Phakopsora pachyrhizi* e ao estresse hídrico.

Objetivos específicos

a) Identificar, no genoma da soja, genes que codificam proteínas bHLH;
b) Analisar *in silico* o padrão de expressão dos genes bHLH162 e bHLH63;
c) Determinar, através de PCR quantitativo após reação de transcriptase reversa (RT-qPCR), o perfil de expressão dos genes identificados no item "b" como diferencialmente expressos em resposta ao fungo *Phakopsora pachyrhizi*;
d) Determinar, através de RT-qPCR, o perfil de expressão dos genes identificados no item "b" como diferencialmente expressos em resposta ao déficit hídrico;
e) Caracterizar funcionalmente o gene bHLH63 potencialmente responsivos à infecção com o fungo e ao déficit hídrico em soja, através da superexpressão dos mesmos em plantas transgênicas de *Arabidopsis thaliana* ;
g) Identificar e caracterizar genes que codificam proteínas que interagem com os fatores de transcrição bHLH162.
h) Determinar o potencial de ativação da transcrição de sequências-alvo dos fatores de transcrição bHLH162.
i) Identificar prováveis fatores de transcrição que se ligam a região promotora do gene *Os01g06640* de arroz, a fim de esclarecer rotas metabólicas em que os fatores de transcrição bHLH podem estar envolvidos.

Artigo Publicado

Título do trabalho: Possible roles of basic helix-loop-helix transcription factors in adaptation to drought

Autores: Graciela Castilhos, Fernanda Lazzarotto, Leila Spagnolo-Fonini, Maria Helena Bodanese-Zanettini, Márcia Margis-Pinheiro.

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Capítulo II –

Caracterização do gene *GmbHLH162* relacionado às respostas a estresses em soja.

GmbHLH162 interacts with polyamine oxidase and participates in drought and Rust infection responses

Graciela Castilhos, Fernanda Lazzarotto, Leila Spagnolo-Fonini, André Cordeiro, Maria Helena Bodanese-Zanettini, Nelson Saibo, Luciano Fietto, Marcia Margis-Pinheiro.

Artigo em preparação

Introduction

Soybean [*Glycine max* (L.) Merrill] is one the major oilseeds produced in the world (Silva et al., 2009). Environmental stresses represent serious threats to agriculture. Several researches have been done in plants to permit the development of strategies to allow plants to better respond to the stress conditions and then to cope with environmental challenges (Castilhos et al., 2014).

Transcription factors are involved in the regulation of transcription of responsive gene to stresses (Shinozaki et al., 2007). bHLH transcription factor are consider the major family of transcription factors in plants being represented by 225 members in *Arabidopsis thaliana* and 480 members in soybean (Jin et al., 2014: http://planttfdb.cbi.pku.edu.cn/). The bHLH transcription factor are characterized by the presence of a highly conserved domain consisting of 50-60 amino acids with two distinct segments: a basic region responsible to DNA-binding and two amphipathic α-helices separated by a loop of variable length (helix-loop-helix region or HLH), which facilitates protein-protein interactions, enabling the formation of homodimeric or heterodimeric complexes. The basic region of bHLH domain allow the recognition of the E-Box (5'-CANNTG-3') and/or G-Box (5'-CACGTG-3') in the promoter sequences of target genes (Carretero-Paulet, et al 2010).

Glyma05g02110 is described as a MYC-type bHLH transcription factor in the Plant Transcription Factor Database v3.0 (Jin et al., 2014: http://planttfdb.cbi.pku.edu.cn/). We nominated *Gm05g02110* gene as *GmbHLH162*, which is homologous to the *AtbHLH162* gene (accession number: AT4G20970.1) (Carretero-Paulet et al., 2010). The MYC proteins are related to several stress responses activating or repressing gene transcription (Chen et al., 2011; Song et al., 2013). MYCs protein are involved in transcriptional networks related to defense response (Woldemariam et al. 2013) and confer tolerance to multiple abiotic stresses such as cold, osmotic and salt (Feng et al., 2013). MYC2 is the best characterized among the MYC bHLH transcription factores, presenting multifunctional role in the Jasmonate (JA) signaling pathway (Kazan and Manners, 2013). MYC2 regulates the expression of downstream and upstream genes of the jasmonate pathway such as the JASMONATE-ZIM-DOMAIN (JAZ) repressors, which acts upstream from MYC2. Kazan and Manners (2008) showed that MYC2 negatively regulates the expression of pathogen defense response genes, such as PDF1.2 (Dombrecht et al., 2007). The MYC2 repression mechanism includes the dimerization of MYC2 with repressors interfering in the bind to the G-box motif. Therefore, the activation of the repressor mechanism requires the binding of MYC2 to the G-box and the recruitment of co-repressor complexes such as histone deacetylases (HDAC) and DNA methyl transferases to the target promoters (Kazan and Manners, 2013).

In this study, we identified a MYC-type *GmbHLH162* transcription factor from soybean. To elucidate the potential role of *GmbHLH162* in stress response, we analyzed the transcript level of *GmbHLH162* in plant tissues as well as in response to *Phakopsora pachyrhizi* and drought conditions. In addition, we detected by two hybrid *screening* an interaction between GmbHLH162 and a polyamine oxidase 2 (GmPAO2) in the cytosol and nucleus of the cell.

Results

The *GmbHLH162* gene expression profiles in different organs and in response to stresses.

The *GmbHLH162* gene expression profile was analyzed in different plant organs by RT–qPCR. The lowest transcript level detected was used to normalize the transcript levels in other organs and thereby to quantify transcript accumulation. The *GmbHLH162* gene expression was detected in all organs analyzed without statistical significant differences among them. Therefore, this gene presents ubiquitous expression in the plant (Figure 1).

To verify the *GmbHLH162* gene expression patterns occurring in response to biotic stress, soybean cultivars with contrasting susceptibility to *Phakopsora pachyrhizi* were challenge by the pathogen (Figure 2). *GmbHLH162* transcript increased 1h and 48h post-inoculation (hpi) with a peak of expression in 12 hpi in the susceptible plant (EMBRAPA 48). The resistent cultivar (PI561356) did not presented alteration significantly after the expression level of *GmbHLH162* after different hours of infection with *Phakopsora pachyrhizi* fungus.

Changes in the *GmbHLH162* gene expression pattern in the leaves and root of plants submitted to drought stress were detected (Fig. 3). In the tolerant cultivar Embrapa 48 a pick of expression in 50 minutes after stress exposition was observed in leaves, whereas in roots this pick of expression, with about 6 folds, occurred after 100 minutes after exposition (Fig. 3A). In the sensitive cultivar BR16, there was no difference in the level of transcripts in roots at different times of exposure to dehydration stress, whilst in leaves the transcript level was reduced after 50 minutes of exposure to drought stress (Fig. 3B).

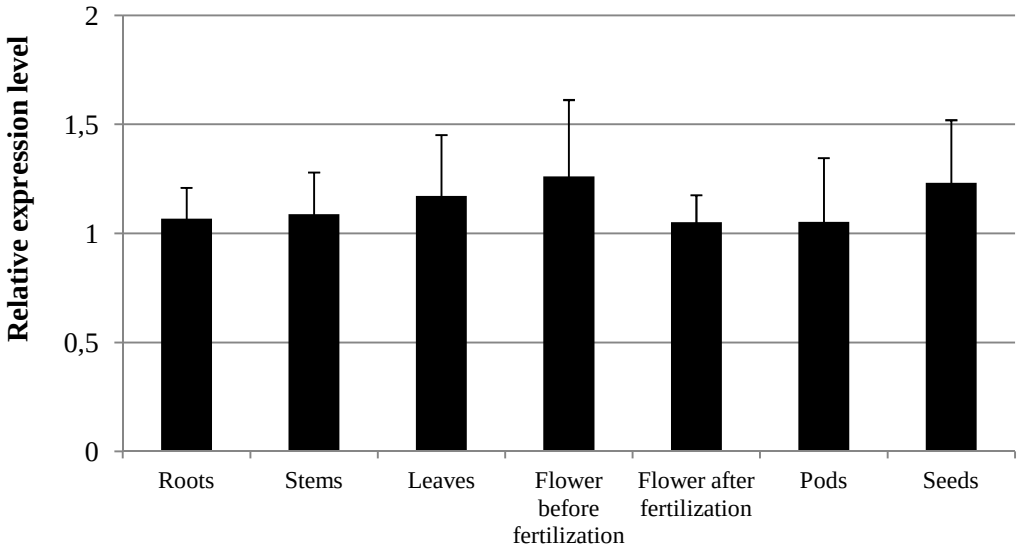


Figure1. Relative expression levels of the *GmbHLH162* gene in different organs of the Conquista Brazilian soybean cultivar. The ACTII, CYP2, and metalloprotease reference genes were used as internal controls to normalize for the amount of mRNA present in each sample. The lowest transcript level was used to normalize the transcript levels in other organs.

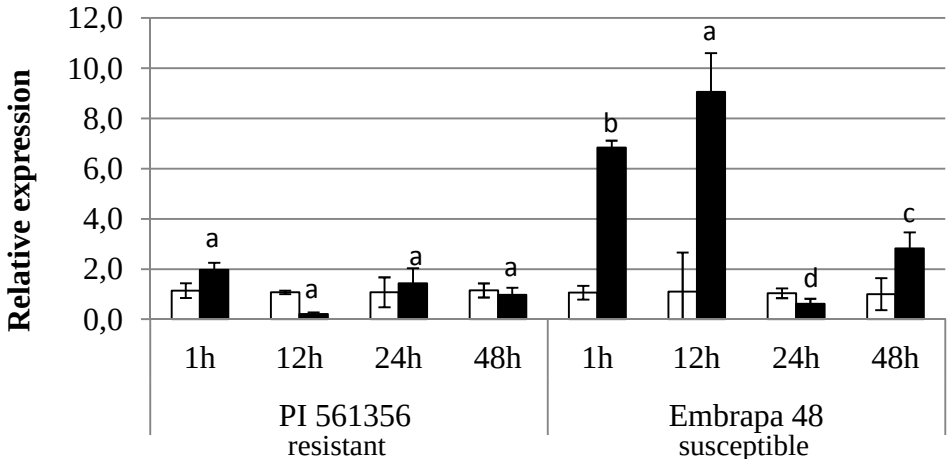


Figure 2. *GmbHLH162* gene expression profiles in response to *Phakopsora pachyrhizi* infection in the leaves of PI561356 (resistant) and EMBRAPA48 (susceptible) soybean genotypes. The relative expression levels of *Gm05g02110* gene was measured by RT-qPCR at 1, 12, 24, 48 hpi (hours post-inoculation). The white bars represent the mock plants (non-infected), and the black bars represent the infected plants. The values are the means of three biological replicates with four technical replicates each. The means that are labeled identically (with a letter) in the same cultivar do not differ significantly (Bonferroni multiple comparison test ($p<0.05$). The *f-box* and *metalloprotease* reference genes were used as internal controls to normalize for the amount of mRNA present in each sample. The transcript levels from the mock-inoculated plants were used to normalize the transcript levels from the inoculated plants.

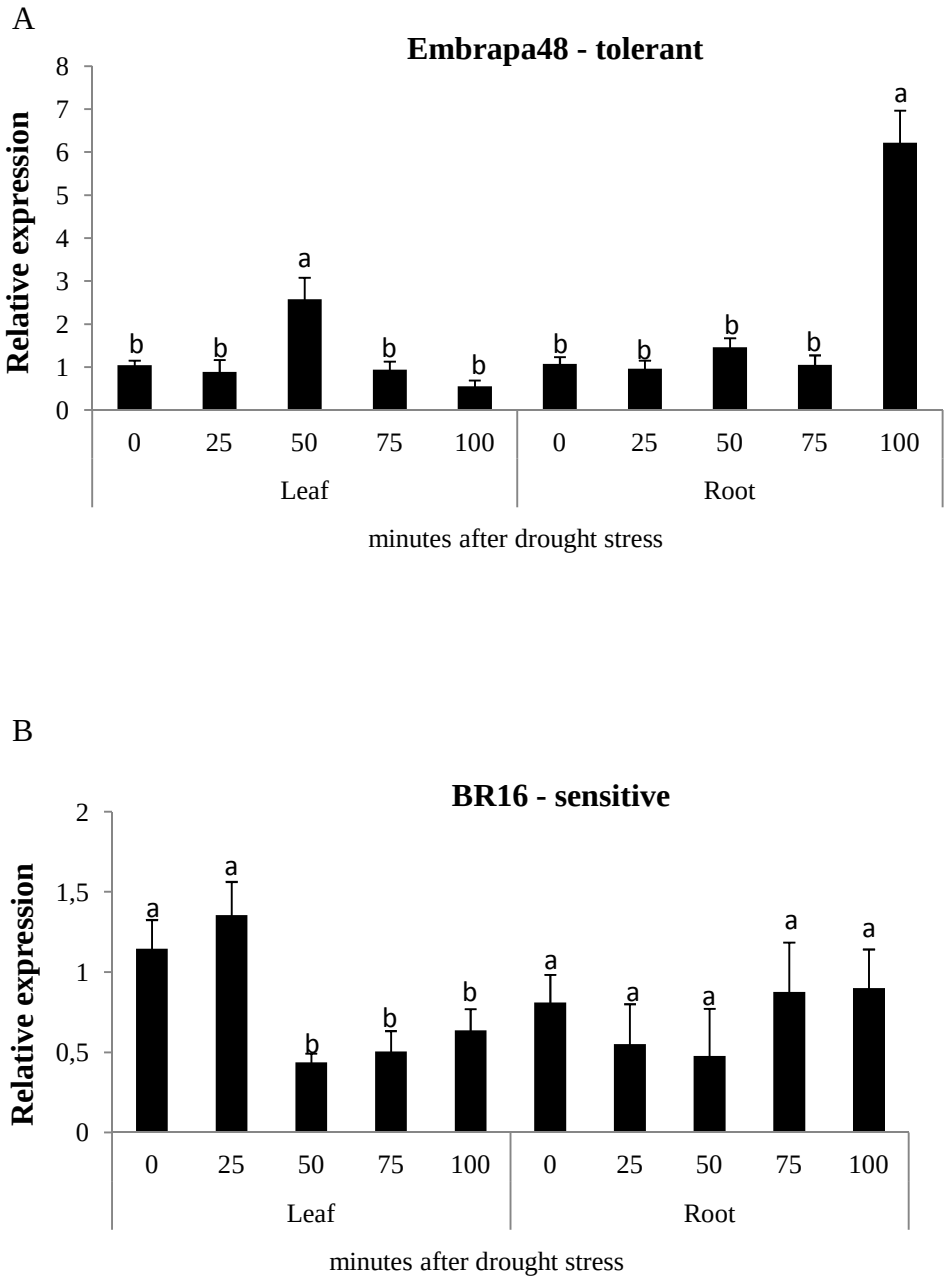


Figure 3. *GmbHLH162* gene expression in the leaves and roots of (A) EMBRAPA48 (tolerant) and (B) BR16 (sensitive) cultivars in response to dehydration stress. The relative expression levels were measured by RT- qPCR at 0 (control), 25, 50, 75 and 100 minutes of dehydration stress. The values are the means of three biological replicates with four technical replicates each. The means that are labeled identically (with a letter) in the same cultivar and the same organ (leaf or root) do not differ significantly (Bonferroni multiple comparison test, $p<0.05$). The transcript levels from the plants at 0 minutes (control) were

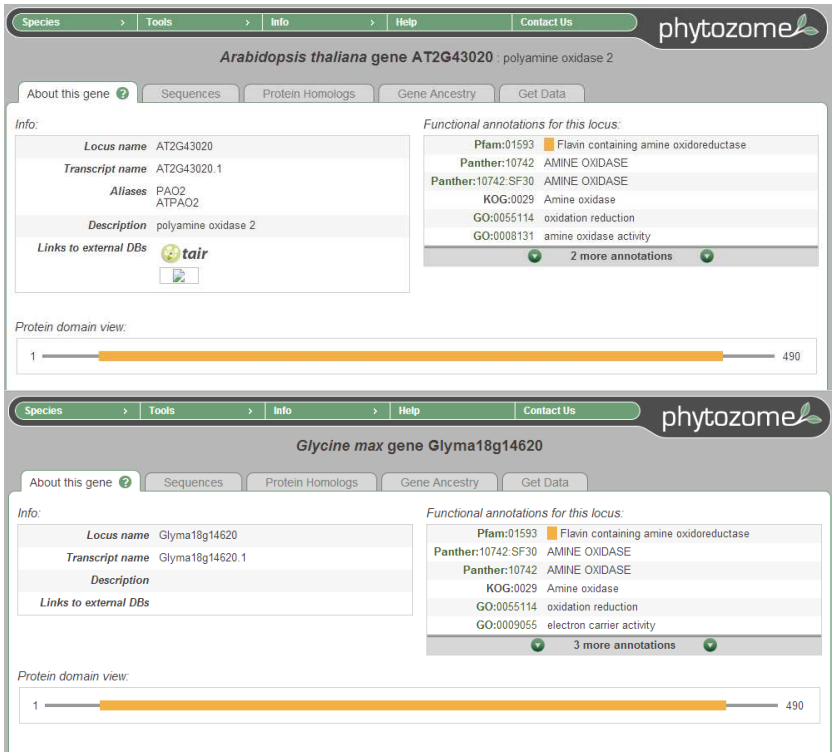
used to normalize the transcript levels from the plants subjected to dehydration stress. The *f-box, ACTII* and *ELF* (Jian et al. 2008) genes were used for normalization.

GmbHLH162 protein interacts with a Polyamine oxidase 2 (GmPAO2)

The coding sequence of the *GmbHLH162* soybean gene was isolated and used as bait to isolate probable targets proteins that interacts through the yeast two-hybrid (Y2H) system. For this, a cDNA library derived from soybean leaves treated with tunicamycin and PEG was used. We have screened $3x10^6$ (ufc) and one candidate that displayed growth in media with auxotrophic selection (SD-LTH) was selected for further testing. The cDNA clone isolated in this *screening* harbor about half-length (770pb) sequence encoding a soybean amine oxidase (Phytozome accession Glyma18g14620) that is similar to polyamine oxidase 2 (PAO2) proteins (Phytozome accession AT2G43020) of *Arabidopsis thaliana* with oxireductase activity (Figure 4). Due this sequence similarity between *Arabidopsis thaliana* and soybean protein, we have named this protein as GmPAO. After, the full-length coding sequence of the gene (1481pb) encoding the GmPAO was amplified and used to transform yeasts. First, the ability to self-activate transcription of the Glyma05g02110 and GmPAO proteins was tested by increasing concentrations of 3-AT and these proteins showed ability to self-activation in medium lacking histidine but the self-activation can be controlled when the medium was supplemented with 5mM 3-AT (Fig. 5A and 5B). The protein interaction was confirmed by growing yeast in selective medium without leucine, tryptophan and histidine supplemented with 0mM, 5mM, 10mM and 20mM 3-AT. The interaction was higher with full-length protein than the interaction observed with partial protein length, which was isolated in the Y2H *screening*, at all 3-AT doses tested. The interaction remained stable until 20mM 3-AT (Fig. 5C).

In order to confirm if the interaction between Gm05g02110 and GmPAO is effective, a BiFC assay was performed in *Arabidopsis thaliana* leaves protoplasts (Fig. 6). The fluorescence signal was clearly localized in the cytosol and nucleus of the cell, indicating that GmbHLH162 and GmPAO proteins interact forming a complex localized in both cytosol and nucleus. This system confirmed the interaction GmbHLH162 and GmPAO proteins previously observed in the Y2H assay (Fig. 6).

A



B

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                 *         20        *         40        *         60        *         80        *        100        *
AtPAO2      : MESRKNSDRQMRRANCRSAGRRMRTRSPSVIVIGGGFCGISAARTLQDASFQVMVLESRDRIGGRVHTDYSFGFPVDLGASWLHGVCRENPLAPVIGRLGLPLYRTSGDNSVLYDHD : 117
Gm18g14620  : MESRTKSNRQLTRALCYGNDCKQQCRSPSVIVIGGGMCGIAAARALFNASFQVVLLESRDRIGGRIHTDYSFGFPVDLGASWLHGVSNENPLASVIGRLGLPLYRTSGDNSVLYDHD : 117
              MESR  S1 Q6 RA C5    4   RSPSVIVIGGG  GI AAR L 1ASFQV66LESRDRIGGR6HTDYSFGFPVDLGASWLHGV  ENPLA VIGRLGLPLYRTSGDNSVLYDHD

                 120       *       140       *       160       *       180       *       200       *       220       *
AtPAO2      : LESYALFDMDGNQVPCELVTCICVTFFRILEEINKVRTPQDADISISQAFSIVFSRKPELRLEGLAHNVLQWYVCRMEGWFAADAPTISAKCWDQELLPGGHGLMVRGYRPVINTL : 234
Gm18g14620  : LESYALFDMDGKQVPEELVTKVCEIFETILQETDKIRCESSEDMSVLRGLSIVFERKPELRLEGLAHKVLQWYLCRMEGWFAADSLTISLKCWDQEVLLPGGHGLMVRGYEPVINTL : 234
              LESYALFDMDG QVP ELVT 6G  FE IL2E 1K6R E   D6S6    SIVF RKPELRLEGLAH VLQWY6CRMEGWFAAD  TIS K WDQE LLPGGHGLMVRGY PVINTL

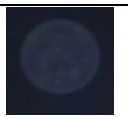
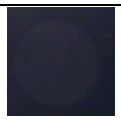
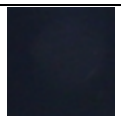
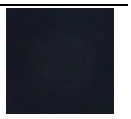
                 240       *       260       *       280       *       300       *       320       *       340       *
AtPAO2      : AKGLDIRVGHRVTKVRRYNGVKVTVENGQTFVADAAVIAVPLGVLKSGTIKFEPKLPEWKQPAINDLGVGIENKIILHFEFVFWEKVEFLGVVAETSYGCSYFLNLHKATGHPVLV : 351
Gm18g14620  : AKGLDIRLGHRVTKVVRRYNGVKVTVESGETFSADAAVIAVPLGVLKAKKIIFKPKLPDWKEPAIRDLGIGLENKIILHFENVFWENVEFLGVVADTSYGCSYFLNLHKAAGHAVLV : 351
              AKGLDI 6GHRVTK6VRRYNGVKVT E G TF ADAAVIAVPLGVLK   I F PKLP WK2 AI DLG6G6ENKIILHFE VFWP VEFLGVVA TSYGCSYFLNLHKA GH VLV

                 360       *       380       *       400       *       420       *       440       *       460
AtPAQ2      : YMPAGQLAKDIEKMSDEAANFAYLQICRILPDALPPVQYLVSRWGSDVNSMGSYSYDIVGKPHDLYERLRVPVDNLFFAGEATSSSFPGSVHGAYSTGLMAAEDCRMRVLERYGEI : 468
Gm18g14620  : YMPSGQLAKDVEKMSDEAAVNFAFMQIKKILPDASSPIQYLVSRWGSDINSLGSYSYDAVGKPHPLYERLRVPVDNLFFAGEATSKSYPGSVHGAFSTGMMAAEDCRMRVLERYGEV : 468
              YMP GQLAKD6EKMSDEAA NFA 6QL 4ILPDA  P6QYLVSRWGSD6NS6GSYSYD VGKPH LYERLRVPVDNLFFAGEATS S5PGSVHGA5STG6MAAEDCRMRVLERYGE6

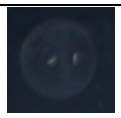
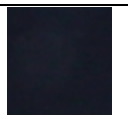
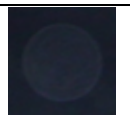
                 *       480       *
AtPAO2      : DLFQPVMGEEGPASVPLLISRL* : 490
Gm18g14620  : DLFQPVMGEEASISIPLQISRL* : 490
              DLFQPVMGEE   S6PL ISRL
```

Figure 4. Similarity of *Polyamine Oxidase 2* (*PAO2*) of the *Arabidopsis thaliana* and *Glyma18g14620* genes of the soybean (A) Search for soybean gene, found in yeast two-hybrid *screening* as probably target gene of the *GmbHLH162*, was carried out by Blast tool available on Phytozome (http://www.phytozome.net/). (B) Sequence similarity between Gm18g14620 soybean protein and AtPAO2 was verified through protein sequences alignments using ClustalW tool (Thompson et al., 1994) from MEGA v4.0 (Tamura et al., 2007).

A

AD/BD	**PAO**		
Empty			
-TH (3AT)	0 mM	+5 mM	+10 mM
AD/BD	**GmbHLH162**		
Empty			
-TH(3AT)	0 mM	+5 mM	+10 mM

B

AD/BD	**Empty**		
PAO			
GmbHLH162			
-LH (3AT)	0 mM	+ 5 mM	+10 mM

C

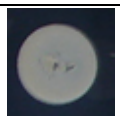
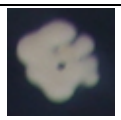
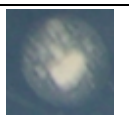
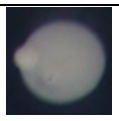
AD/BD	**GmbHLH162**			
PAO 770 pb				
PAO 1482 pb				
-LTH (3AT)	0 mM	+5 mM	+10 mM	+20 mM

Figure 5. Interaction between GmbHLH162 and GmPAO2 identified by yeast two-hybrid *screening* assay using a cDNA library derived from soybean leaves treated with tunicamycin and PEG, using GmbHLH162 as bait. (A) and (B): Ability to self-activation

in selective medium. In (A) GmbHLH162 and GmPAO2 were cloned into pDEST32 yeast expression vector, which contain the GAL4 binding domain. The self-activation was verified by yeast growth in medium lacking Leucine and Histidine (-LH) amino acids. (B) GmbHLH162 and GmPAO2 were cloned into pDEST22 yeast expression vector, which contains GAL4 activation domain. The self-activation was verified by yeast growth in medium lacking Tryptophan and Histidine (-TH) amino acids. The basal growing was eliminated supplementing the medium with 5mM 3-Amino-1, 2, 4-triazol (3-AT). (C) Interaction between GmbHLH162 and GmPAO2 proteins. Yeast colonies grown until concentration of the 20 mM 3-AT in the medium lacking Leucine, Tryptophan and Histidine (-LTH). The yeast growth was verified either with half (770pb) or full (1481pb) - length of the protein GmPAO2. The 3-AT doses used in this experiment were: 0mM, 5mM, 10mM and 20mM of the 3-AT.

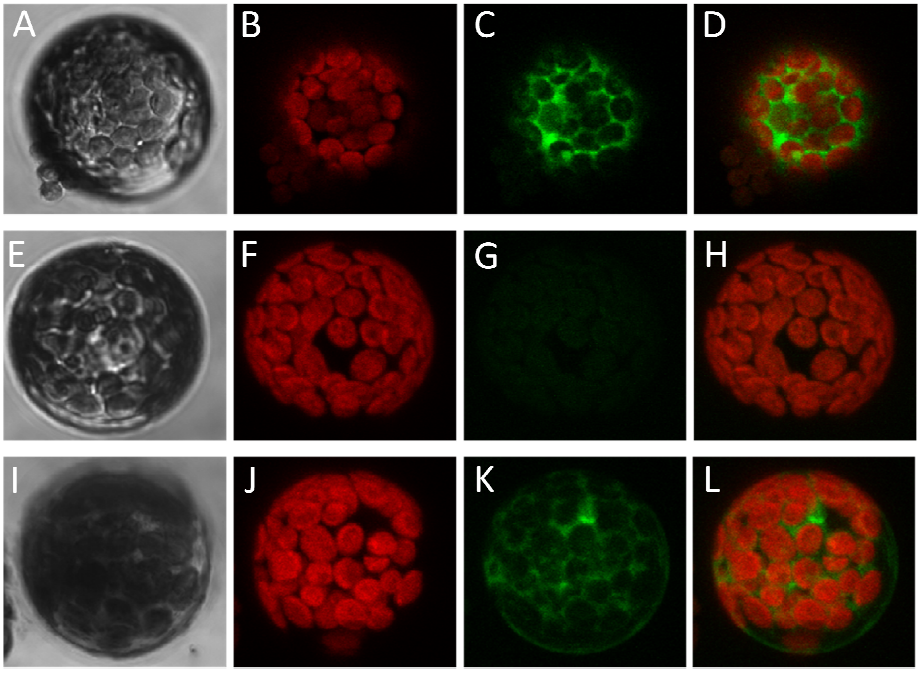


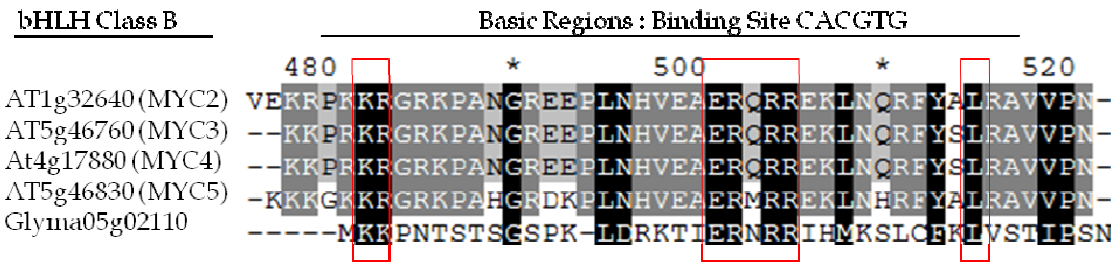
Figure 6. GmbHLH162 interacts with GmPAO in *Arabidopsis thaliana* leaf protoplasts by BiFC. Green signals indicate YFP fluorescence (C-K) and red signals indicate chlorophyll autofluorescence (B-F-J). BiFC system showing protein–protein interaction through YFP fluorescence restoration. (D-L) Positive control of the interaction using 35S-GUS::YFPN and 35S-GUS::YFP C cassettes. (A-B-C-D) Negative control without Restoration of YFP

fluorescence. (E-F-G-H) Restoration of YFP fluorescence through interaction between Gm05g02110 and GmPAO (I-J-K-L).

Analysis of the capacity of *GmbHLH162* to bind the G-box cis-element

To determine whether *GmbHLH162* interacts with G-box motifs *in vivo*, a transactivation assay was performed. According the domain similarity, *GmbHLH162* is a MYC-like bHLH showing conserved amino acids in the basic region of bHLH domain (Fig. 7). We aligned the sequence of four different MYC *Arabidopsis thaliana* protein (MYC2, MYC3, MYC4 and MYC5) with *GmbHLH162* soybean protein sequence (Fig 7A). The presence of conserved amino acids residues in the basic region of the bHLH domain suggests that GmbHLH162 is a MYC-like bHLH protein. According the domain classification proposed by Dang et al. (1992), this MYC-like protein belongs to bHLH class B that contains the CACGTG binding site, which is a g-box motif (Fig. 7A). Based on this information we constructed a synthetic promoter (pCAMBIA::G-box).

A



B

Forward promoter sequence

GAACGTTTTCTC**CACGTG**ACAAGAACCAAATGTTTCTC**CACGTG**ATGATTATCTCATTAAATTA AAA**CACGTG**TTTTAAATGTG

Reverse promoter sequence

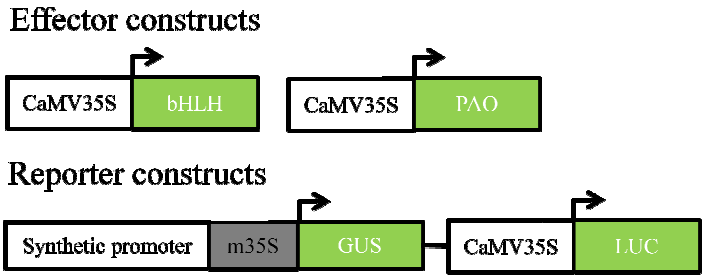
TCGACACATTTAAAACACGTGTTTTAATTTAATGAGATAATCATCACGTGGAGAAACATTTGGT TCTTGTCACGTGGAGAAAACGTTCTGCA

Figure 7. Design of the synthetic promoter containing the g-box motif. (A). Alignment of the GmbHLH162 with different MYC *Arabidopsis thaliana* proteins. Sequence similarly among GmbHLH162 and MYC proteins of the bHLH Class B, core DNA binding site and amino acid residues of the bHLH protein basic regions (red box) were established

according to Dang et al., 1992; (B) synthetic promoter sequence: bold is a g-box cis-element; sequence in box is a BCAT4 promoter which contain two g-box cis-elements; underlined sequence is a JAZ2 promoter; red sequence is a restriction site of the Pst I and Sal I enzymes.

The result of transactivation assay demonstrated that when the reporter was infiltrated in *Arabidopsis thaliana* protoplast, a substantial amount of GUS/LUC activity was detected (Figures 8B), indicating that endogenous factors of *Arabidopsis thaliana* may activate the expression of the G-box reporter. The expression of *GmbHLH162* did not change the GUS/LUC intensity (Figure 8B). It was also observed that when *GmbHLH162* co-expressed with GmPAO (pCAMB G-box: bHLH x pCAMB G-box: PAO) the GUS/LUC expression was not significantly different of the GUS expression level in comparison with control (pCAMB:G-box) (Fig. 8B). Thus, these results suggest that there are endogenous expressions of others transcription factors and when the *GmbHLH162* is co-expressed with *GmPAO*, the effect of this interaction cannot be measured. This methodology was not sufficient to determinate if *Gm05g02110* is a repressor or an activator of the transcription of the target genes.

A



B

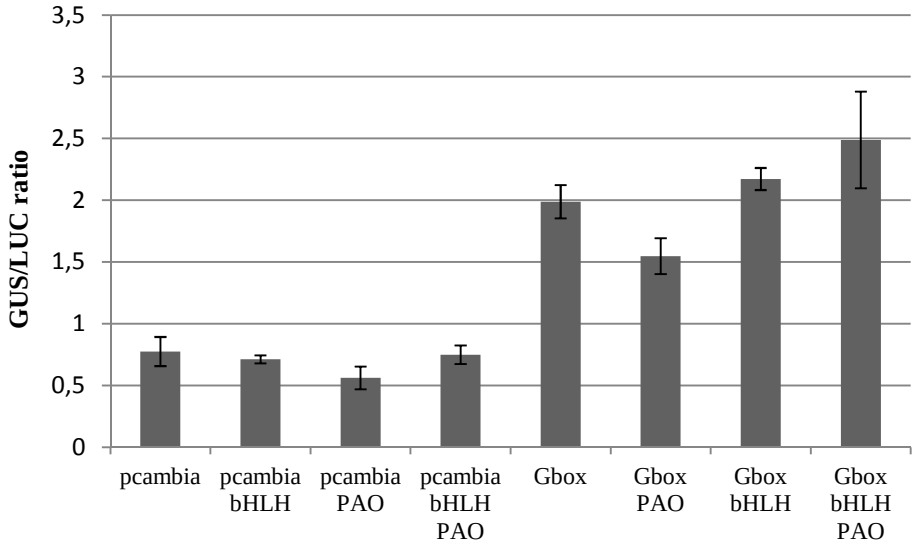


Figure 8. (A) Constructs used for *Arabidopsis thaliana* protoplast transformation. Effector constructs used correspond to the transcription factor coding region under the control of the full CaMV35S promoter. Reporter constructs contain the GUS gene driven by the minimal CaMV35S promoter (m35S) plus the fragment of the synthetic promoter containing g-box (CACGTC) motif. The LUC gene under the control of the full 35S promoter was used to normalize GUS expression levels. (B) Transactivation analysis as a GUS/LUC activity ratio. The reporter vector used was the one carrying the synthetic promoter fragment. Values shown are multiples of the GUS/LUC ratio obtained with the reporter vector without effector. Differences to the GUS/LUC values of the reporter vector alone are not statistically significant (t-test, $P < 0.1$). bHLH: GmbHLH162; PAO: GmPAO.

Discussion

Analyzing the expression pattern in different plant tissues in normal growth condition, *GmbHLH162* gene exhibited ubiquitous expression with same intensity in soybean organs either in vegetative or reproductive stage of the development (Fig. 1). The *ERF1* (ETHYLENE-RESPONSE-FACTOR1) gene also presents constitutive expression,

howevwe, its overexpression conferred resistance to necrotrophic fungi (Berrocal-Lobo et al., 2002).

GmbHLH162 gene was recently identified as an MYC-Type bHLH transcription factor in the Plant Transcription Factor Database v3.0 (Jin et al., 2014: http://planttfdb.cbi.pku.edu.cn/) in soybean. We performed an alignment and verified a sequence similarity of the *GmbHLH162* with others AtMYC proteins (Fig. 7A). MYC proteins are involved in the regulation of plant defense and developmental processes, and are described either positive (Song et al., 2013) or negative (Chen et al., 2011) regulators of the jasmonate-mediated defense response.

Regarding the expression of *GmbHLH162*, when EMBRAPA48 susceptible cultivar was challenged with *Phakopsora pachyrhizi*, occurred a faster increasing in the transcript level at early time of exposure, whilst in resistant cultivar (PI561356) no differences were observed in the gene expression levels in different exposure times (Fig 2). This result suggests that *GmbHLH162* may be modulating some stress-related target genes leading to susceptible response to *Phakopsora pachyrhizi* fungus. Anderson et al. (2004) identified a reduced susceptibility to pathogen was observed in *myc2* mutants plants, which exhibited increased resistance to the fungal pathogen *Fusarium oxysporum* (Anderson et al., 2004). This increased pathogen response in *myc2* mutants was related with reduced JA sensibility leading to the attenuation of lesion development induced by *Fusarium oxysporum* (Anderson et al., 2004).

bHLH genes have been described as transcriptional repressor in pathogen defense responses. The HOMOLOG OF BEE2 INTERACTING WITH IBH1 (HBI1) is a bHLH transcription factor characterized as a negative regulator in defense response. Transgenic *Arabidopsis thaliana* lines over-expressing *HBI1* showed an enhanced disease susceptibility to phytopathogenic bacteria leading negative impacts in innate immunity (Malinovsky et al., 2014). Expression studies with bHLH subgroup IIId, using mutant plants demonstrated an increased in jasmonate-mediated resistance against fungus (*B. cinerea*) whereas its over-expression reduced the jasmonate response. These results suggest that these *bHLH* genes act as transcription repressor of the jasmonate-mediated plant defense in *Arabidopsis thaliana* (Song et al., 2013).

Besides the participation of the bHLH transcription factors in the pathogen defense response, its evolvements in abiotic stress have been investigated. In transgenic tobacco

plants, the INDUCER OF CBF EXPRESSION (ICE1) bHLH over-expression conferred enhanced chilling, osmotic and salt tolerance by up-regulation of the expression of stress-tolerant genes as CBF/DREB and its expected targets genes (Feng et al., 2013).

Concerning drought stress, *GmbHLH162* showed a contrasting pattern of gene expression in tolerant and sensitive cultivars. Whereas in leaves of the tolerant cultivar the gene expression level increased at 50 minutes after stress exposure, in the sensitive plants the transcript level decrease at 50 minutes after dehydration. In roots, the gene expression level increased five folds at 1,5 h after drought stress and this expression pattern was observed only in the tolerant (Embrapa48) cultivar (Fig. 3A and 3B). Therefore, these results suggest that *GmbHLH162* may be a positive regulator involved in the responses to drought stresses and the increased level of *GmbHLH162* expression observed in roots of the tolerant cultivar, could assist the plant to mitigate the damages caused by drought stress. In *Arabidopsis thaliana*, the MYC2 bHLH was described as a JA-mediated repressor of the auxin pathway, down-regulating auxin-related *PLETHORA* genes expression, which is evolved in root development. The *PLETHORA* repression via MYC2 leads to inhibition of root growth, retaining the growth plasticity during root meristem development when exposure to adverse environmental condition and then increasing the adaptation of the plant to the stresses (Chen et al., 2011).

Moreover, an increased in *GmbHLH162* transcription level in leaves of tolerant cultivar, strengthens the hypothesis that *GmbHLH162* may be a positive regulator in drought stress responses. It is known that MYC2 is a positive regulator in ABA response alleviating drought damages in plants (Abe et al., 2003). Therefore, more detailed studies, evaluating the root growth as well as hormones responses in transgenic plants, are necessary to clarify the potential role of *GmbHLH162* in the drought stress responses.

In order to elucidate in which signaling pathway *GmbHLH162* operates, we carried out an Y2H *screening*. Ours results revealed an interaction between *GmbHLH162* and a GmPAO in yeast assay (Fig. 5) in the cytosol and the nucleus of the cell (Fig. 6). In *Arabidopsis thaliana* genome there are five *AtPAO* genes (*AtPAO1* to *AtPAO5*) encoding five isoforms of the polyamine oxidase related to polyamine catabolism (Takahashi et al., 2010). The GmPAO found in Y2H *screening* is very similar to AtPAO2 protein sequence (Fig. 4B), which was recently identified in soybean (Guerrero-Gonzalez et al., 2014) and has been described as a peroxisomal protein (Takahashi et al., 2010) with regulatory

function (Guerrero-Gonzalez et al., 2014). The physiological role and regulation mechanisms of PAOs are still not well understood.

Polyamines are low-molecular weight aliphatic amines expressed ubiquitously in plant cells. They have ROS-scavenging and membrane-protecting properties, increasing plant tolerance to the stressful condition (Kasukabe et al., 2004). Increasing of PA levels has been observed in stress tolerant plants under multiple environmental stresses conferring freezing, salt, drought and osmotic tolerance (Kasukabe et al., 2004). Transcriptomic analyzes showed alteration in different stress related genes in plants overexpressing polyamine biosynthesis enzymes and suggested that PAs are molecular signals involved in the stress response (Wimalasekera et al., 2011). The increase in the PA level in response to the osmotic stress in plants is consistent with the results in which PAO and a bHLH interacted in the Y2H *screening*, once the cDNA library was prepared with plants submitted to osmotic stress (Fig. 5).

The PAO is a polyamine catabolism enzyme that participates in several biological processes through its reaction products from the PA catabolism. Among these products, the H_2O_2 has a key role in the plant development and defense responses, mediating cell death and promoting a hypersensibility response to the pathogen (Cona et al., 2006). Also, H_2O_2 produced by PAO are required in ABA-induced stomatal closure improving water loss stress tolerance (Wimalasekera et al., 2011). Other secondary metabolic produced by PAO via PA catabolism is the 1-3 diaminopropane, which is also involved in stress tolerance (Cona et al., 2006).

It is known the PAO can promote gene expression and increase the DNA-binding activity of transcription factors (Kasukabe et al., 2004). Many genes exhibit responsive elements to polyamines and their transcription appear to be regulated by the redox status of the cell (Pegg, 2009). We are speculating that this interaction with the PAO may modulate the activity of the *GmbHLH162* transcription factor.

In *Arabidopsis thaliana*, the over-expression of the *SPERMIDINE SINTASE* gene, a product of PA catabolism by PAO, resulted in an up-regulation of genes encoding transcription factors as DREB, WRKY and MYB, which are able to regulate the expression of several stress-responsive genes (Kasukabe et al., 2004). Furthermore, there are evidences that in luminosity stress, the increase of PAO in response to high light

intensity could inhibit the extension growth in the mesocotyl apex, and this is correlated with decrease in the auxin levels in the mesocotyl epidermis (Cona et al., 2006).

Studies reveal that AtPAO1 participates in a repressor complex of the gene expression by hyperacetylation of histones, acting in the flowering pathway inhibiting the FLOWERING LOCUS C gene, which is a MADS-box transcription factor that blocks the transition from vegetative to reproductive development (He et al., 2003). There are evidences that PAO can participate of the co-repressor complex of transcriptional gene silencing in animals and plants. This co-repressor complex is composed by two components: SWIRM domain PAO-like protein (AtSWP1), and a plant-specific C2H2 zinc finger-SET domain histone methyltransferase (AtCZS). These two factors interact and promote the repression of targets genes through histone hypoacetilation in *Arabidopsis thaliana*. Also, this transcriptional complex co-repressor involving a PAO may acts as a gene expression global regulator in plants (Krichevsky et al., 2007). Moreover, is possible that the PAO-like protein acts as histone demethylase, kinases or ubiquitin ligases. Consequently, this AtSWP1/ AtCZS co-repressor complex is a multifunctional chromatin modifier (Krichevsky et al., 2007).

In summary, *GmbHLH162* may affect the expression state of stress related genes. The transactivation assay using GmbHLH162 as effector in *Arabidopsis thaliana* protoplasts was not efficient to determinate if this gene acts as a repressor or activator of the gene expression. Therefore, we need a more detailed study to conclude if the interaction of the bHLH and PAO2 promotes increasing or decreasing in its target genes. In addition, it is necessary to identify the potential target gene of the *GmbHLH162* transcription factor for then proceed a new transactivation assay to elucidate the role of *GmbHLH162* and PAO interaction in the gene regulation.

Together, our results support the hypothesis that *GmbHLH162* could be a negative regulator of genes related to the biotic stress signaling pathway, suggesting that *GmbHLH162* is associates with susceptibility response to *Phakopsora pachyrhizi*. Conversely, under abiotic stress, *GmbHLH162* is a positive regulator in leaves and roots of plants submitted to drought stress which can mitigate the damages occurred by dehydration in plants. Further studies are necessary to elucidate the effect of the interaction between GmbHLH162 and GmPAO proteins.

Materials and Methods

In silico analyses of *GmbHLH162*

Expression profiles of the identified bHLH sequences in both biotic and abiotic situations were obtained from subtractive libraries experiments available in the LGE Soybean Genome database (http://bioinfo03.ibi.unicamp.br/soja/). Soybean plants access PI 561356 (resistant genotype) were inoculated with the pathogen *Phakopsora pachyrhizi* for the construction of the subtractive libraries. Making use of this database generated by the GENOSOJA project, we selected (expressed sequence tags) differentially expressed ESTs. Specific primers were designed to confirm the expression pattern by RT-qPCR.

Phakopsora pachyrhizi infection

Soybean reaction to Asiatic Rust infection was assessed by an experiment carried out in Embrapa Soja, Londrina, PR, Brazil. Soybean plants were grown in a pot-based system and maintained in a greenhouse at 28±1ºC with 16 h light/dark at a light intensity of 22.5 $\mu Em^{-2}s^{-1}$. The Embrapa-48 genotype was used as a susceptible standard, which develops a Tan lesion (Van de Mortel et al., 2007), and the PI561356 genotype was used as the resistant standard, which carries the *Rpp1* resistance gene to soybean rust (Kim et al., 2012). The soybean leaves used in these assay were sprayed with a fungal spore suspension according Wiebke-Strohm et al. (2012). One trifoliolate leaf from each plant in V2 stage was collected at 1, 12, 24 and 48 hours after inoculation, frozen in liquid nitrogen, and stored at -80 °C. Three biological replicates from each genotype were analyzed for both treatments.

Drought stress

A highly sensitive BR16 cultivar and a tolerant EMBRAPA48 cultivar (Oya et al. 2004) were grown in a greenhouse according to the method described by Kulcheski et al. (2011) and were submitted to dehydration stress as described by Martins et al. (2008). Briefly, seedlings in the V2 stage were removed from a hydroponic solution and kept in a tray in the dark without nutrients. Leaves and roots were collected at 0 (control), 25, 50, 75 and 100 min after the initiation of dehydration stress and were frozen in liquid nitrogen at

−80°C until RNA extraction. Three biological replicates (three plants/replicate) were sampled for each treatment point.

Expression pattern in different organs

Leaves, roots, and stems of plants in the vegetative (V) phase and seeds, pods and flowers before and after fertilization were harvested from the MGBR-46 Conquista Brazilian soybean cultivar grown under greenhouse conditions. Tissues were harvested and immediately frozen in liquid nitrogen before being stored at -80º C until RNA extraction. Three biological replicates, with three plants per replicate, were sampled for each plant organ.

Quantitative real-time PCR (RT-qPCR)

To *Phakopsora pachyrhizi* infection assay, primer pairs designed to amplify an *f-Box* (Libault et al., 2008) and *Metalloprotease* (Libault et al., 2008) coding sequences were used as internal controls to normalize the amount of mRNA present in each sample. The *f-box*, *ACTII* and *ELF* (Jian et al. 2008) genes were used for normalization in the drought stress, for the analysis of the expression in different organs the ACTII, CYP2 (Jian et al. 2008), and *metalloprotease* genes were used as normalizer genes. Reactions were performed with 2.5 ng/µL cDNA, 0.2 µM forward and reverse primers, 0.1 mM of dNTPs, 1x PCR Buffer, 1.5 mM $MgCl_2$, 0.1x Sybr Green and 0.25 U Platinum Taq DNA polymerase (Invitrogen). The PCR conditions included an initial step of denaturation of 5 minutes at 94ºC, followed by 40 cycles of 15 seconds at 94ºC, 10 seconds at 60ºC and 15 seconds at 72 ºC. Melting curve was performed by 15 seconds at 95ºC for denaturation, 1 minute at 60ºC for annealing, and measurement each 0.4ºC up to 95ºC. The method for evaluating the relative expression of Glyma05g02110 genes were $2^{-\Delta\Delta Ct}$ (Livak et al., 2001).

Statistical analysis

To *Phakopsora pachyrhizi* infection and drought stress experiments, the relative expression level of *Gm05g02110* gene in each treatment was statistically compared by variance analysis considering stress exposure time and cultivar. Data were transformed

using the weighted least squares method. Means were compared using the Bonferroni multiple comparison test with significance of the 5%.

To expression pattern in different organs, the expression level was measured by variance analysis, the data were not transformed and the means were compared using the Tukey multiple comparison test. For all expression analyses it was used the Statistical Analysis System (SAS) 9.2 and the Statistical Package for the Social Sciences (SPSS/PASWSTAT) 18 programs.

Yeast two-hybrid *Screening*

cDNA Library Construction

To determinate possible target proteins that interact with the *GmbHLH162* protein a *screening* of a Yeast two-hybrid library, kindly provided by Professor Dr. Luciano Fietto of the Universidade Federal de Viçosa (UFV), was performed. This library was prepared with cDNA of soybean plants (*Glycine max*, variety Conquista) submitted to two stress conditions: tunicamycin- and PEG-treated according Alves et al., 2011. cDNAs were synthesized and cloned into pEXP-AD502 yeast expression plasmids, transformed into *Escherichia coli* DH5α, allowing the expression of the fusion library with the activation domain of the GAL4 transcription factor.

GmbHLH162 Cloning and yeast transformation

A cDNA of 650bp corresponding to the GmbHLH162 coding sequence was amplified by PCR and inserted into the entry vector pENTR/D-TOPO (Invitrogen) and then was recombinated by LR Clonase II (Invitrogen) into the yeast expression vector pDEST32, which contain the sequence of the GAL4 DNA binding domain. The construction was confirmed by PCR, sequencing and cleavage with restriction enzymes. The bacterial transformations were carried out by heat shock with termcompetent DH5α bacteria (Appendix). The yeast strain used was AH109 (Clontech). Yeasts were transformed by the LiAc-mediated, ssDNA and PEG (Gietz and Woods, 2006) method. The auxotrophic markers used were tryptophan for pEXP-AD502, leucine for pDEST32, and HIS3 as reporter gene. The transformed cells were plated on synthetic dropout (SD) medium lacking leucine, tryptophan and histidine (SD-LTH). The ability to self-activation transcription of the GmbHLH162 protein was tested by increasing concentrations of de 3-

Amino-1, 2, 4-triazol (3-AT). This protein showed low self-activation transcription allowing conducting the secreening without 3-AT addition. The colonies that grew in SD-LTH were plated in SD-LTH media with 5mM of the 3-AT to impair the basal yeast growth and eliminate false positives. To confirm the protein interactions, colonies were plated in SD-LTH media containing 10mM or 20mM of the 3-AT. Positive interactions were characterized by growth on SD-LTH after seven days. Negative interactions were characterized by the absence of growth in SD medium without auxotrophic markers and reporter (SD-LTH).

Interaction assay using bipartite fluorescence complementation (BiFC) system

A BiFC assay was used for detection of GmbHLH162 and GmPAO interactions in plant living cells (Walter et al., 2004). The split- YFP constructs pX-NYFP, pY-CYFP (where X and Y correspond to the GmbHLH162 and GmPAO, respectively), pGUS-NYFP and pGUS-CYFP, were produced using the plasmids pE3136, pE3130. pGUS-NYFP and pGUS-CYFP contain in-frame fusions of GUS with N-terminal YFP and C-terminal YFP, respectively. The amplified cDNAs were introduced into the appropriate plasmids using the Gateway technology. The resulting vectors were used for protoplast transformation. Protoplast isolation was performed through Tape- *Arabidopsis thaliana* Sandwich as described by Wu et al. (2009). Transformed protoplasts were incubated in the dark for 24–48 h at 27ºC before imaging. Fluorescence microscopy was performed under an Olympus FluoView 1000 confocal laser scanning microscope equipped with a set of filters capable of distinguishing between green and yellow fluorescent protein (EGFP and EYFP, respectively) and plastid autofluorescence. Images were captured with a high-sensitivity photomultiplier tube detector.

Transient transactivation assay in *Arabidopsis thaliana* protoplasts

Firstly, a synthetic promoter containing in tandem g-box motifs was designed to be used as reporter plasmid for transactivation assay. Primers were designed from the union of two different promoter sequences: *Branched-chain aminotransferase* (BCAT4) gene (Schweizer et al., 2013), which has two g-box cis-elements near in its promoter sequence and the gene JAZ2 (Figueroa and Browse, 2012), which has promoter sequence rich in AAAA (up-stream) and TTTT (dowstream) flanking the g-box (CACGTG) motif (Fig 7b).

It is known that these nucleotides around the *cis*-element may increase the binding affinity of the transcription factor to DNA binding site. These two promoter sequences used for the purpose of construct the synthetic promoter have been described as a target of the MYC gene sequences. The forward and reverse primers were built with restriction sites to *Pst* I and *Sal* I enzymes (Fig. 7b). After, an annealing reaction using the forward and reverse sequences of the manufactured primers to amplify about 90pb was performed. For that, 1µl tris-HCl pH 8.0 1M; 1,7µl NaCl 3M; 0,2µl EDTA 0,5M; 1ul forward primer 100 picomol/µl; 1ul reverse primer 100 picomol/µl and 95,1µl H_2O were used. The annealing reaction conditions included 95ºC/5 min; 95ºC/1min decreasing 1ºC/min until reaching melting temperature (66ºC); 66ºC/30 min and decreasing 1ºC/min until reaching room temperature. The amplified product was introduced by T4 ligase reaction into pCAMBIA1391z previously cleaved with the same enzymes.

The reporter plasmid containing the g-box motif was built using the pCAMBIA1391z promoter-cloning vector backbone, which contains the kanamycin plant resistance gene, downstream of the full CaMV35S promoter according Figueiredo et al. (2012). Effector plasmids were constructed by cloning the coding region of the *GmbHLH162* into the pH7WG2-D plasmid (pH7WG2-D: bHLH) and *Gm18g14620* into the pH7W2F plasmid (pH7W2F: PAO), to be under the control of the full CaMV35S promoter. *Arabidopsis thaliana* protoplasts were prepared as described by Figueiredo et al. (2012). For each independent transformation, 5µg of reporter plasmid and 10µ g of effector plasmid were used. Each transformation was performed in triplicates. The cells were incubated for 24 h at 22ºC in the dark and then collected at 450 g for 1 min in a swing-out rotor. Cell lysis was performed by resuspension in 150 µl 100 mM K_2PO_4 (from a 1M pH 7.8 stock solution), 1 mM EDTA (from a 0.5 M pH 8 stock solution), 7 mM 2-mercaptoethanol, 1% Triton X-100, and 10% glycerol, followed by two freeze–thaw cycles. The lysate was then cleared by centrifugation for 2 min at 17,000 g. For the GUS quantification assay, 0.5 µl of 50 mM 4-methylumbel-liferyl-b- D -glucuronide were added to 20 µl of the lysate (in triplicate). Reactions were carried at 37ºC in the dark for 1 h and stopped with 180 µl of 200 mM Na_2CO_3. Fluorescence was detected using a spectrofluorimeter (Fluoromax-4 with Micromax plate reader, Horiba) with excitation at 365 nm, emission at 455 nm, and a slit of 1.5 nm. Readings were performed in triplicates. Luciferase levels were determined by adding 150 µl LUC reagent (20 mM Tricine pH 7.8, 5 mM $MgCl_2$, 0.1 mM EDTA, 3.3

mM DTT, and 2 mM ATP) to 20 µl of the cell lysate. An aliquot (75 µl) of 1.5 mM luciferin was added to each sample and light intensity was read for 10 seconds in a luminometer (Modulus Microplate, Turner Biosystems). Readings were performed in triplicates. Activation of gene expression was calculated as a GUS/LUC ratio.

Table 1. Primers used in the experiments

GmbHLH162	**Forward sequence**	**Reverse sequence**
Quantitative real-time PCR	CCACGTACATAAAGCGTCTGAAG	CCCGAACCCAAGTCCTTTATC
Yeast two-hybrid Screening	CACCATGAAAAAACCAAACACTAGTACT	ACTTAAGACTCACCCAAATCCAC
BiFC system	CACCATGAAAAAACCAAACACTAGTACT	ACTTAAGACTCACCCAAATCCAC
Transient transactivation assay	CACCATGAAAAAACCAAACACTAGTACT	ACTTAAGACTCACCCAAATCCAC
G-box synthetic promoter	GAACGTTTTCTCCACGTGACAAGAACCAAATGTTTCTCCACGTGATGATTATCTCATTAAATTAAAACACGTGTTTTAAATGTG	TCGACACATTTAAAACACGTGTTTTAATTTAATGAGATAATCATCACGTGGAGAAACATTTGGTTCTTGTCACGTGGAGAAAACGTTCTGCA

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Capítulo III – O gene GmbHLH63 antecipa o desenvolvimento inicial da planta e pode estar relacionado ao aumento da tolerância a estresse biótico em soja

Functional analysis of the *GmbHLH63,* a soybean bHLH transcription factor involved with plant development and biotic and abiotic stress responses.

Graciela Castilhos, Ciliana Rechenmacher, Douglas Jardim Messender, Fernanda Lazzarotto, Marcia Margis-Pinheiro, Maria Helena Bodanese-Zanettini.

Artigo em preparação

Introduction

The basic helix-loop-helix (bHLH) is a major transcription factor family in eukaryote organisms; there are about 225 members in *Arabidopsis thaliana* and 480 members in soybean (Jin et al., 2014: http://planttfdb.cbi.pku.edu.cn/). These transcription factores present a highly conserved domain composed of the basic region responsible to DNA-binding and two amphipathic α-helices separated by a loop of variable length (helix-loop-helix region or HLH) liable for protein-protein interactions (Carretero-Paulet et al., 2010). The bHLH transcription factors are often mentioned as stress responsive (Bihmidine et al., 2012; Liu et al., 2014). In soybean, the expression pattern of bHLH encoding-genes was analyzed through SuperSage data indicating that bHLH transcription factors are involved in responses to abiotic and biotic stresses (Osório et al., 2012).

Environmental stresses as drought can reduce crop production yields the drought stress in plants can be mitigated by the manipulation of genes that act directly in the plant protection such as chaperones proteins, or by genes that regulate the expression of other stress-related genes such as WRKY, E2F and bZIP transcription factors (Minh-Thu et al., 2013). RD29 bHLH transcription factor is an example of gene highly responsive to dehydration able to mitigate abiotic stress in soybean (Bihmidine et al., 2012).

Regarding biotic stress, *Phakopsora pachyrhizi* is a fungus responsible by Asian Soybean Rust (ASR) disease, which can defoliate soybean fields within a few days, representing an economical threat in the soybean production (Goellner et al., 2010). Due to

the facility of fungus to develop new races, the resistance mediated by Rpp resistance genes has been overcome (Bromfield and Melching 1982). More studies are needed to identify promising candidate genes to promote resistance into soybean against *Phakopsora pachyrhizi*, which could be used to genetic engineer plant in a sustainable way (Goellner et al., 2010). The participation of bHLH transcription factors in *Phakopsora pachyrhizi* defense response is not documented. Some bHLH transcription factors are related to pathogen responses, such as MYC transcription factor, which is described as transcriptional repressor and activator in jasmonate mediated defense responses, allowing the plant to survive in a changing environment (Song et al., 2013).

Gm06g01430 is a bHLH transcription factor called as cryptochrome-interacting basic-helix-loop-helix (CIB1), which is homologous to the bHLH63 in *Arabidopsis thaliana* (accession number: AT4G34530) in the Plant Transcription Factor Database v3.0 (Jin et al., 2014: http://planttfdb.cbi.pku.edu.cn/). We named *Gm06g01430* gene as GmbHLH63 due to the homology with *AtbHLH63*. The CIB1 genes, which are involved in blue-light signaling, interacts with cryptochrome 2 (CRY2) and promotes the activation of the FLOWERING LOCUS T (*FT*) gene (Liu et al., 2008). The *FT* gene encodes a mobile transcriptional regulator that migrates from leaves to apical meristem activating floral meristem identity in *Arabidopsis thaliana* (Liu et al., 2011). To data, 5-25% of the genes in *Arabidopsis thaliana* has its expression modulated by blue-light response mediated by CRY2 and in most of these genes the changes in expression is due to CRY2-CIBs pathway (Liu et al., 2011).

The involvement of the CIB1 in the modulation of blue-light responsive genes is well documented. However, it remains unclear how CIB1 is related to stress conditions in plants. Elucidate stress tolerance mechanisms as well as plant adaptation mechanisms in soybean represent an advantageous alternative in comparison to studies using model plants, once this knowledge could be useful in soybean cultivar breeding aiming to increase the tolerance to stress (Ge et al., 2011).

Finally, the transcription factor could be a potential target gene to genetic manipulation aiming to increase tolerance. Once transcription factor can participate in a range of regulatory pathways, they may increase tolerance to one or more stresses. In the present work, we have evaluated the expression profile of *GmbHLH63* in two different stress situations in soybean: drought stress and infection by *Phakopsora pachyrhizi*; as well

as evaluated the effect of the expression of *Gm06g01430* in *Arabidopsis thaliana* transgenic plants.

Results

Expression pattern of the *GmbHLH63* gene

The *GmbHLH63*gene expression pattern in soybean in different organs of the plant was performed. The lowest transcript level detected was used to normalize the transcript levels in other organs and thereby to quantify the relative transcript accumulation. The *GmbHLH63* gene was expressed ubiquitously in all organs analyzed (Fig. 1).

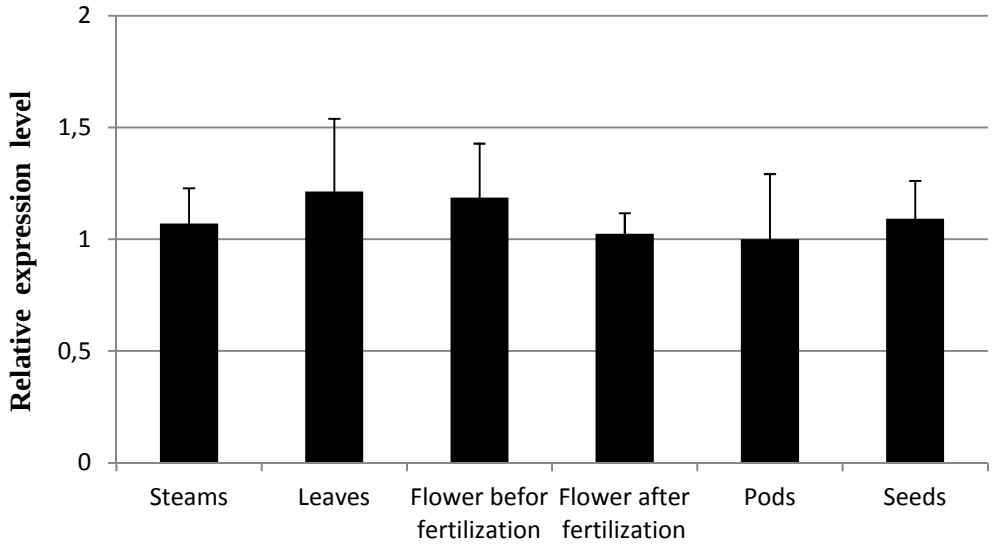

Figure 1. Relative expression levels of the *GmbHLH63* gene in different organs of the Conquista Brazilian soybean cultivar. The lowest transcript level was used to normalize the transcript levels in other organs.

The gene expression levels at successive time points after infection by *Phakopsora pachyrhizi* in leaves of the resistant and susceptible cultivars were compared (Fig. 2). The transcript accumulation of *GmbHLH63* was relatively enhanced 1,5 fold showing a peak of expression after about 12 h, followed by a decrease in 24 h in resistance cultivar. In contrast, no marked increase in the *GmbHLH63* transcription levels was observed in a susceptible plant under biotic stress (Fig. 2).

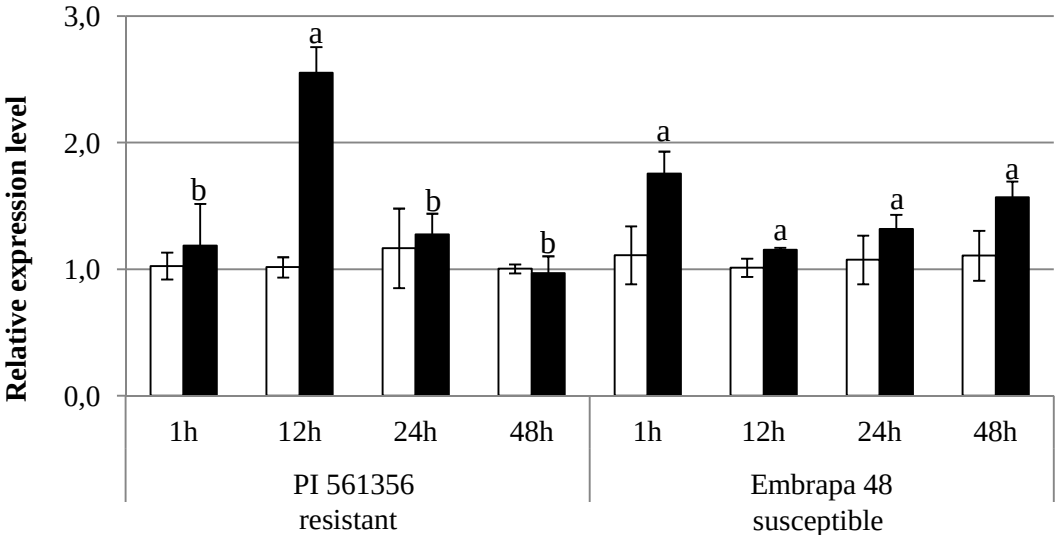

Figure 2. *GmbHLH63* gene expression profiles in response to *Phakopsora pachyrhizi* infection in the leaves of EMBRAPA48 (susceptible) and PI561356 (resistant) soybean genotypes at 1, 12, 24, 48 hpi (hours post-inoculation). The white bars represent the mock plants (non-infected), and the black bars represent the infected plants. The values are the means of three biological replicates with four technical replicates each. The means that are labeled identically (with a letter) in the same cultivar do not differ significantly (Bonferroni multiple comparison test ($p<0.05$).

Regarding abiotic stress, the expression levels of *GmbHLH63* in roots was not significantly altered in plants subjected to different time of drought exposure either in tolerant or sensitive cultivars tested (data not shown). However, in leaves of the two different cultivars the expression level decreased with increasing exposure to drought stress (Fig. 3). *GmbHLH63* exhibited relatively half of expression level after 50 minutes of stress (Fig. 3A) in tolerant cultivar and 25 minutes after drought exposure in sensitive plants (Fig. 3 B).

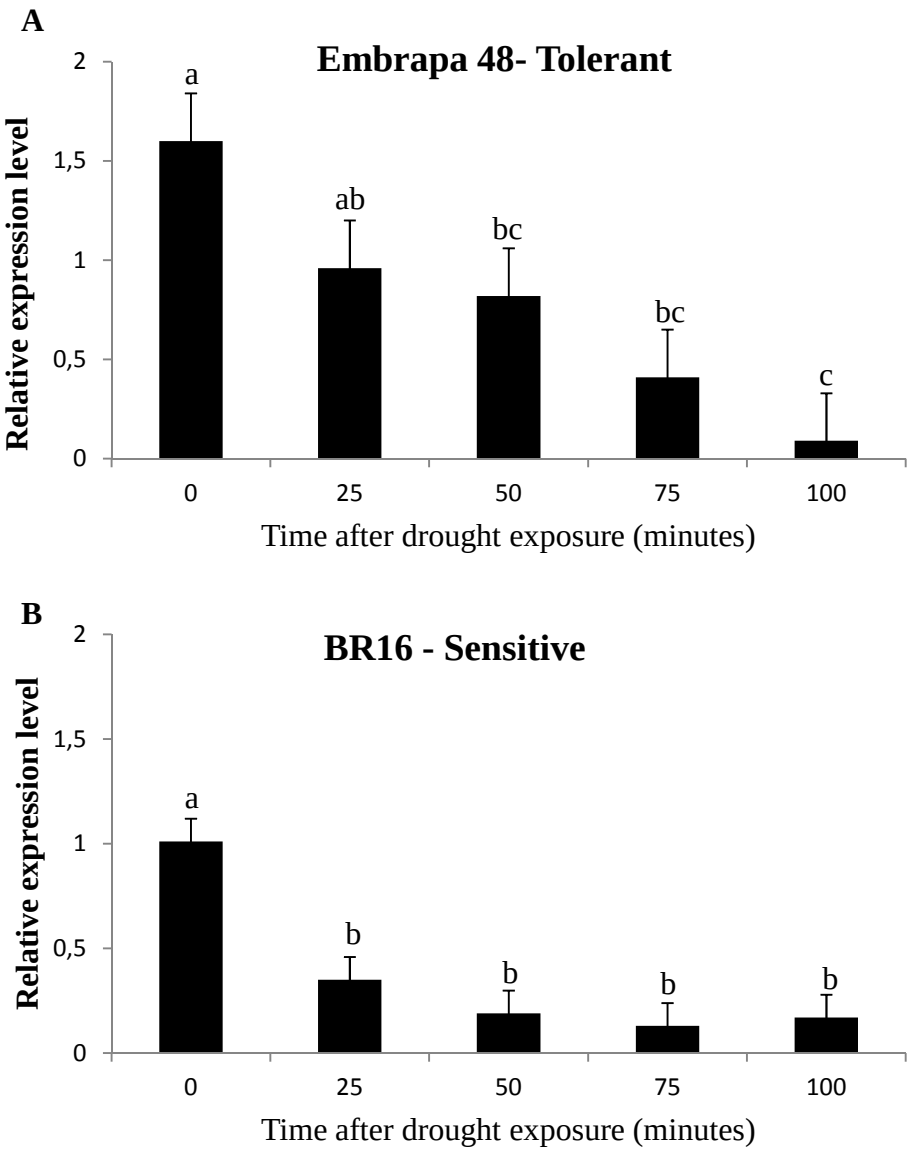

Figure 3. *GmbHLH63* gene expression in the leaves (A) EMBRAPA48 (tolerant) and (B) BR16 (sensitive) cultivars in response to dehydration stress. The relative expression levels were measured by RT-qPCR at 0 (control), 25, 50, 75 and 100 minutes of dehydration stress. The means that are labeled identically (with a letter) in the same cultivar do not differ significantly (Bonferroni multiple comparison test, $p<0.05$). The transcript levels from the plants at 0 minutes (control) were used to normalize the transcript levels from the plants subjected to dehydration stress.

Effect of the *GmbHLH63* overexpression in *Arabidopsis thaliana* plants

Arabidopsis transgenic plants were developmented in order to evaluate the phenotypic characterization in these transgene plants. Four different development stages in *GmbHLH63*-overexpressing *Arabidopsis thaliana* plants were evaluated in T3 stage (homozygous). Seven independent transgenic lines (#5, #8, #9, #10, #11, #12 and #16) were produced and analyzed (Fig. 4). The emission of roots was similar between wild type and transgenic plants. In all lineages, the root emission started about 3 days after sowing (Fig. 4A). The transgenic plants exhibited the cotyledon emission and openied one day before when compared to the wild type plants (Fig. 4B and 4C). Regarding leaf emission, five independents lines (#9, #10, #11, #12 and #16) presented emission about five days after sowing, whereas in the wild type plants leaf emission occurred six days after sowing (Fig. 4D).

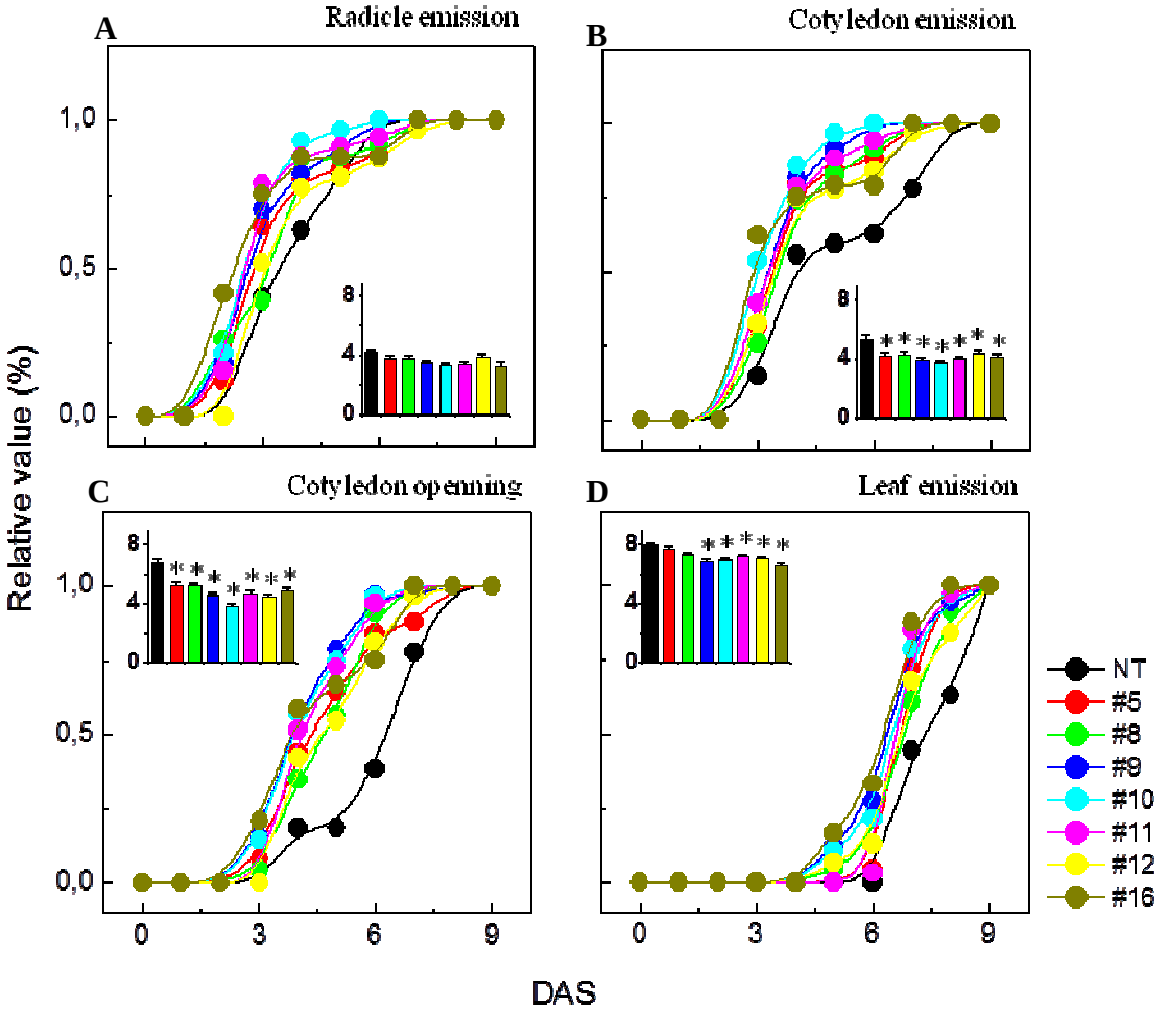

Figure 4. Characterization of transgenic *Arabidopsis thaliana* plants overexpressing the *GmbHLH63* gene. DAS: Days after sowing; NT: non transformated plant; #5 to #16: different lineages of transgenic plants.

GmbHLH63 has transcriptional activation capacity

We performed an assay to verify the capability of *GmbHLH63* to transcribe a reporter gene in yeast cell. The result of this analysis showed a high capacity to act as a transcriptional activator in selective media with addition until 100mM of the 3-AT.

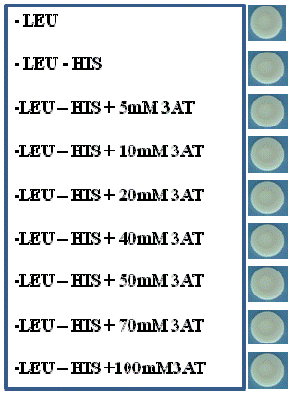

Figure 5. Self-activation of the transcription by the *GmbHLH63* in yeast cell. The self-activation was tested under increasing doses of the 3-AT (5 to 100mM 3-AT) in media with autotrophic selection (SD –LH).

Discussion

GmbHLH63 encodes a cryptochrome-interacting basic-helix-loop-helix (CIB1) transcription factor and is homologous to bHLH63 of *Arabidopsis thaliana* (accession number: AT4G34530) according to the Plant Transcription Factor Database v3.0 (Jin et al., 2014: http://planttfdb.cbi.pku.edu.cn/). *CIB1* is a MYC like gene and it is known that positively regulates *FLOWERING LOCUS T* gene expression via CRY protein interaction (Jin et al., 2014: http://planttfdb.cbi.pku.edu.cn/). The overexpression of *AthCIB1* in *Arabidopsis thaliana,* anticipated the flowering, indicating that CIB1 promotes floral initiation in a CRY-dependent model (Liu et al., 2008). Interestingly, in a two-hybrid assay, using soybean CIBs proteins it was demonstrated that GmbHLH63 does not interact

with CRY2a in response to blue light in yeast cells (Meng et al., 2013). So, possibly GmbHLH63 does not participates of the CRY-dependent signaling mechanism.

Several works have been done to investigate the role of bHLH transcription factor in adverse conditions due its capacity to regulate a range of stress response pathways. MYC and others bHLH transcription factors have been described as negative regulator of pathogen responses in *Arabidopsis thaliana* (Song et al., 2013; Malinovsky et al., 2014). In *Arabidopsis thaliana*, HOMOLOG OF BEE2 INTERACTING WITH IBH1 (HBI1) bHLH is a negative regulator of the bacterium immunity responses resulting in more than two fold inhibition in *HBI1* transcript levels after 1 hour of stress leading to sensibility response in *Arabidopsis thaliana* seedlings (Malinovsky et al., 2014). In addition, *AthCIB1* over-expression also negatively regulates immunity but not increase susceptibility response to bacterium *Pseudomonas syringae* (Malinovsky et al., 2014).

Our results suggest a significant increase on the transcription level of *GmbHLH63* in a susceptible cultivar at 12 hours under *Phakopsora pachyrhizi* infection (Fig. 2). *GmbHLH63* does not seem to be related to the response to susceptibility since the level of transcripts did not change with the advancement of stress in a susceptible cultivar under *Phakopsora pachyrhizi* infection (Fig 2). This data of the expression level pattern could indicate a possible role for *GmbHLH63* as a positive regulator in defense responses to *Phakopsora pachyrhizi* fungus in soybean. Regarding abiotic stress, previous *in silico* analysis of the SuperSage data available in LGE database revealed that *GmbHLH63* expression was not modulated by drought (annex 1); however, our RT-qPCR data were conflicting with the *in silico* analyzes. The *GmbHLH63* expression level reduced with the exposure to drought stress with a similar pattern in tolerant and sensitive soybean cultivars (Fig. 3).

GmbHLH63 is a transcription factor with high self-activation activity in yeast assay (Fig. 5) and is ubiquitously express in vegetative and reproductive stages of the development in soybean (Fig. 1). There is the possibility that *GmbHLH63* controls development related genes in steam, leaves, flower, pods and seeds because its transcripts had been detected constitutively in all these organs (Fig. 1). To further characterize the function of *GmbHLH63* we obtained and characterized *Arabidopsis thaliana* transgenic plants carrying the construct *35S::GmbHLH63*. *Arabidopsis thaliana* plants overexpressing *GmbHLH63* presented early development compared to the wild type in some parameters

such as emission and opening cotyledons and leaf emission (Fig. 4). These results suggest that *GmbHLH63* positively regulates the plant growth in early development stages.

Few studies have reported the relationship between bHLH transcription factor and early stages of the plant development and the role of these transcription factors is still unknown. MYC2 bHLH transcription factor is a growth regulator and its overexpression in tomato transgenic lines led to an increase in the lateral root number and the root length was longer than the wild type (Gupta et al., 2014). A recent study showed that *GmCIB1*, homologous of *GmbHLH63*, promotes leaf senescence in soybean transgenic plants suggesting that *GmCIB1* activates transcription of senescence-associated genes, such as WRKY DNA BINDING PROTEIN53b (WRKY53b) (Meng et al., 2013).

Ultimately, the GmbHLH63 is a bHLH transcription factor with high self-activation activity. The role of GmbHLH63 in the stresses responses is not well understood. Our data suggest that *GmbHLH63* probably is a positive regulator to *Phakopsora pachyrhizi* defense response in soybean. The function of *GmbHLH63* in drought stress does not seem to be relevant since the transcription level were reduced either on the tolerant or sensitive cultivar. The data presented here indicated that *GmbHLH63* is a gene regulator involved in plant growth pathway and the *GmbHLH63* overexpression anticipates cotyledon opening and cotyledon and leaves emission in *Arabidopsis thaliana* transgenic plants. A detailed analysis of the plant development and physiology is necessary to conclude if this gene is able to achieve the tolerance to stresses. Further studies are in progress in our laboratory.

GmbHLH63 is a good target gene to genetic engineering since it was observed an increasing in its transcription level in a resistant cultivar under pathogen stress. In addition the overexpression of this gene in transgenic *Arabidopsis thaliana* promoted early plant development. Due to the economic importance of soybean, these traits should be considered and the GmbHLH63 genetic manipulation could be evaluated as useful strategy in crop improvement.

Materials and Methods

In silico analysis of *GmbHLH63*

Expression profiles of the bHLH sequences in response to both biotic and abiotic stresses were obtained from subtractive libraries and superSAGE data available in LGE Soybean

Genome database (http://bioinfo03.ibi.unicamp.br/soja/) according to Osorio et al. (2012) (Annex I). The expression patterns were searched in roots and leaves from susceptible cultivar (BR16) submitted to drought stress (25 to 150 min), leaves from resistant genotype (PI561356) infected with *Phakopsora pachyrhizi* (12 to 192 h) and roots from MG/BR46 inoculated with *Bradyrhizobium japonicum*.

Stress related experiments

Phakopsora pachyrhizi Infection

This experiment was conducted at Embrapa Soja, Londrina, PR, Brazil. Soybean plants were grown in a pot-based system and maintained in a greenhouse at 28±1ºC with 16/ h light/dark at a light intensity of 22.5 $\mu Em^{-2}s^{-1}$. Two genotypes were evaluated: susceptible (Embrapa-48) and resistant (PI561356). The Embrapa-48 genotype develops a Tan lesion in response to the fungal infection (Van de Mortel et al., 2007), and the PI561356 genotype is a resistant standard, which carries the resistance to soybean rust. One trifoliolate leaf from each genotype previously inoculated with *Phakopsora pachyrhizi* spores was collected at 1, 12, 24, 48h after inoculation, frozen in liquid nitrogen, and stored at -80 °C. Three biological replicates from each genotype were analyzed for both treatments.

Drought stress

This experiment was performed at Embrapa Soja, Londrina, PR, Brazil using a highly sensitive (BR16) cultivar and a tolerant (EMBRAPA48) cultivar (Oya et al. 2004), according described by Kulcheski et al. (2011). To dehydration stress, seedlings in the V2 stage were removed from a hydroponic solution and kept in a tray in the dark without nutrients. Leaves and roots were collected at 0 (control), 25, 50, 75 and 100 min after the initiation of dehydration stress and were froze in liquid nitrogen at − 80 °C until RNA extraction (Martins et al., 2008). Three biological replicates (three plants/replicate) were sampled for each organ/genotype/treatment point.

Organs

Leaves, roots, and stems of plants in the vegetative (V) phase and seeds, flowers before and after fertilization and pods in reproductive (R) phase were harvested and immediately

frozen in liquid nitrogen before being stored at -80º C until RNA extraction. For this experiment it was used the Conquista Brazilian soybean cultivar (MGBR-46) grown under greenhouse conditions. Three biological replicates, with three plants per replicate, were sampled for each plant organ.

Quantitative real-time PCR (RT-qPCR)

The RT-qPCR reactions were performed with specific primers for *GmbHLH63*. *F-Box* and *Metalloprotease* (Libault et al., 2008) sequences were used as reference genes in the *Phakopsora pachyrhizi* infection analyses. To the drought stress, the *f-box, ACTII* and *ELF* (Jian et al. 2008) genes were used as internal controls to normalize the amount of mRNA present in each sample. To the analyses of the expression in different organs, the normalizer genes ACTII, CYP2 (Jian et al. 2008), and *metalloprotease* were used. Reactions were performed with 2.5 ng/µL cDNA, 0.2 µM forward and reverse primers, 0.1 mM of dNTPs, 1x PCR Buffer, 1.5 mM $MgCl_2$, 0.1x Sybr Green and 0.25 U Platinum Taq DNA polymerase (Invitrogen). The PCR conditions included a initial step of desnaturation of 5 minutes at 94ºC, followed by 40 cycles of 15 seconds at 94ºC, 10 seconds at 60ºC and 15 seconds at 72 ºC. Melting curve was performed by 15 seconds at 95ºC for desnaturation, 1 minute at 60ºC for annealing, and measurement each 0.4ºC up to 95ºC. The method for evaluating the relative expression of *Glyma06g01430* genes were $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001)**.**

Statistical analyses

To statistically analyze the *GmbHLH63* expression pattern under biotic and abiotic stress a variance analysis was performed a variance analysis to compare the gene relative expression level considering stress exposure time and cultivar. Data were transformed using the weighted least squares method and the means were compared using the Bonferroni multiple comparison test. All experiments related to the expression patterns were analyzed with Statistical Analysis System (SAS) 9.2 and the Statistical Package for the Social Sciences (SPSS/PASWSTAT) 18 programs.

***GmbHLH63* over-expression vector construction**

The *GmbHLH63* coding sequence was amplified (1180 bp) by PCR using soybean cDNA as template. The PCR fragment was inserted into the entry vector pENTR/D-TOPO (Invitrogen) and then recombinated using LR Clonase II (Invitrogen) into the plant expression binary vector pH7WG2-D, which contains the steptomicine/spectinomicine bacterium resistance gene and hygromycin plant resistence gene.

Yeast two-hybrid *Screening*

cDNA Library Construction

The cDNA library of soybean plants (Conquista cultivar) was kindly provided by Professor Dr. Luciano Fietto of the Universidade Federal de Viçosa (UFV). The library was prepared with cDNA of soybean plants submitted to tunicamycin- and PEG-treated simulating two stress conditions, according Alves et al. (2011). cDNAs were synthesized and cloned into pEXP-AD502 yeast expression plasmids, transformed into *Escherichia coli* DH5α, allowing the expression of the fusion library with the activation domain of the GAL4 transcription factor.

GmbHLH63 Cloning and yeast transformation

A coding sequence of 1180 bp of the *GmbHLH63* was amplified by PCR from cDNA of soybean and inserted, by gateway technology, into the yeast expression vector pDEST32, which contain the sequence encoding the DNA binding domain of GAL4. The bacterial transformations were carried out by heat shock with termocompetent DH5α bacteria. The yeast strain used was AH109 (Clontech). Yeasts were transformed by the LiAc-mediated, ssDNA and PEG (Gietz and Woods, 2005) method. The transformants clones were selected by auxotrophic markers (tryptophan for pEXP-AD502, leucine for pDEST32, and was evaluated HIS3 reporter gene) and plated on synthetic dropout (SD) medium lacking leucine, tryptophan and histidine (SD-LTH) in different 3-Amino-1, 2, 4-triazol (3-AT) concentrations. The ability to self-activation transcription of the GmbHLH63 protein was tested by increasing concentrations from 5mM to 100mM of the 3-AT.

***Arabidopsis thaliana* transformation**

Transgenic *Arabidopsis thaliana* plants were obtained by *Agrobacterium*-mediated floral-dip method according Zhang et al., 2006. The transformation was accomplished by dipping developing *Arabidopsis thaliana* inflorescences for a few seconds into a 5% sucrose solution containing 0.01–0.05% (vol/vol) Silwet L-77 and resuspended *Agrobacterium* cells carrying the *35S:: GmbHLH63* to be transferred. The dipped plants were covered with plastic films to maintain high humidity for 16–24 h. After this, plastic covers were removed and plants were maintained in a growth chamber for 1 month. Dry seeds were collected and submitted to plant selection in hygromycin (25 mg L^{-1}) according to Zhang et al., 2006.

Analysis of transgenic Arabidopsis thaliana

The hygromicin-resistent *Arabidopsis thaliana* seeds were placed on half MS agar medium without hygromycin (30 seeds per plate in a total of 10 plates). The radicule emission, cotyledon emission, cotyledon opening and leaf emission were evaluated 0, 3, 6 and 9 days after sowing the *35S:: GmbHLH63* and wild type seeds.

Table 1. Primers used in the experiments

GmbHLH63	**Forward sequence**	**Reverse sequence**
Quantitative real-time PCR	CCCGAGTCCGAGTCCAAG	CTCCATCATCAGAGCCGAA
Yeast two-hybrid Screening	**CACC**ATGTTGCACTGCGCCAACACG	TTGCAGAAGGAAATAGAAGCTC
Arabidopsis thaliana transformation	**CACC**ATGTTGCACTGCGCCAACACG	TTGCAGAAGGAAATAGAAGCTC

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Identificação de Fatores de Transcrição que regulam o gene bHLH *Os 01g06640* em arroz (*Oryza sativa*)

Os experimentos descritos neste capítulo foram realizados durante estágio Sanduíche no Instituto de Tecnologia Química e Biológica em Oeiras, Portugal, sob a orientação do Dr. Nelson Saibo. Os resultados apresentados neste trabalho são preliminares e estão em andamento em nosso laboratório.

Introdução

O sistema antioxidante tem sido estudado como mecanismo de defesa a estresses devido a sua capacidade de aumentar a tolerância da planta, diminuindo os efeitos causados pelas espécies reativas de oxigênio (ERO). A redução da peroxidação de lipídeos, bem como o controle dos níveis das ERO na utilização dos mesmos como mensageiros secundários em rotas de transdução de sinal, são exemplos conhecidos de ação do sistema antioxidante. As plantas desenvolveram mecanismos enzimáticos e não enzimáticos que as permitem detoxificar as ERO. Estes mecanismos são requeridos para a remoção destas espécies reativas nos diferentes compartimentos celulares, sendo essenciais para a sobrevivência da planta ao estresse (Moller *et al*., 2007).

O mecanismo enzimático de defesa antioxidante compreende enzimas que catalisam as reações de neutralização das espécies ativas de oxigênio como, por exemplo, a Superóxido Dismutase (SOD), Catalase (CAT), Ascorbato Peroxidase (APX), Glutationa Redutase (GR) e Ascorbato Oxidase (AOX). O mecanismo não enzimático abrange moléculas pequenas como o ascorbato (AsA) e o grupo de tióis não protéicos (NPSH) (Noctor & Foyer, 1998).

A fim de investigar o papel de fatores de transcrição bHLH envolvidos na regulação redox da célula, nós selecionamos o gene LOC_Os01g06640, o qual codifica um FT do tipo bHLH ainda não caracterizado em arroz, que apresentou aumento no seu padrão

de expressão em resposta ao silenciamento de ascorbato peroxidases citoplasmáticas de arroz, APX1 e APX2 (Riberio et al, 2012). Estas plantas silenciadas para estas APXs mostraram alteração na homestase redox (Rosa et al, 2010). Análises anteriores permitiram a identificação de uma interação da proteína bHLH (Glyma05g02110) e poliamina oxidase (PAO) (capítulo 2), utilizando a técnica de duplo híbrido em levedura. As PAOs de plantas estão envolvidas nas respostas de defesa a estresses bióticos e abióticos. Muitos genes apresentam elementos responsivos a poliaminas e a transcrição destes parece estar regulada através do status redox da célula (Pegg, 2009). Nós especulamos que esta interação com a PAO pode estar modulando o estado redox das proteínas, alterando a atividade do fator de transcrição bHLH.

Há indicações que esses fatores de transcriçao bHLH respondem às alterações do estado redox da célula em diferentes espécies de plantas. Para identificar quais são as vias que levam à ativação da transcrição das bHLH em resposta ao estado redox, nós buscamos identificar os prováveis fatores de transcrição que se ligam na região promotora do gene codificado por LOC_Os01g06640 através da técnica de monohíbrido em levedura (Yeast one Hybrid- Y1H). Mais estudos devem ser feitos a fim de elucidar a relação destes fatores de transcrição encontrados através de *screening* por Y1H com estresses em geral.

Resultados e discussão

Visando identificar os prováveis fatores de transcrição que se ligam na região promotora do gene LOC_Os01g06640 foram utilizadas duas bibliotecas de cDNA de expressão de arroz, uma induzida pela salinidade e outra pelo frio, disponibilizadas pelo Instituto de Tecnologia Química e Biológica - Universidade Nova de Lisboa (ITQB). Essas bibliotecas foram utilizadas para experimentos de Y1H. A pesquisa na biblioteca de salinidade não permitiu identificar qualquer fator de transcrição. Por outro lado, foram identificados dois fatores de transcrição que se ligam à região promotora do gene LOC_Os01g06640, utilizando uma biblioteca de cDNA de plantas submetidas ao estresse por frio. Os fatores de transcrição identificados no *screening* de mono-híbrido foram: LOC_Os08g01090, o qual foi identificado 20 vezes utilizando como isca o fragmento 1.2 (próximo ao ATG) e LOC_Os04g51190, o qual foi identificado 2 vezes utilizando o fragmento 2 como isca (Tabela 1).

O fator de transcrição LOC_Os08g01090, que possui o domínio B3 de ligação ao DNA, é também denominado IRON DEFICIENCY-RESPONSIVE ELEMENT FACTOR 1 (IDEF1), envolvido na resposta e tolerância à deficiência de ferro. IDEF1 foi encontrado como uma proteína que se liga ao promotor do gene IDS2, o qual é induzido por deficiência de ferro em cevada (http://www.uniprot.org/uniprot/Q6Z1Z3). O gene fator de transcrição LOC_Os08g01090 possui homologia ao gene AT3G24650 de *Arabidopsis thaliana*, o qual pertence à família de fatores transcricionais AP2/B3 (http://www.phytozome.net). De acordo com Kobayashi et al. (2009) IDEF1 pertence à família de fatores de transcrição ABI3/VP1, reconhece a sequência de cis-elementos CATGC e regula vários genes de resposta à tolerância à deficiência de ferro. O fator de transcrição IDEF1 é constitutivamente expresso em folhas e raízes de arroz e está relacionado com a indução de genes envolvidos na maturação da semente (Kobayashi et al., 2009). Plantas transgênicas de arroz superexpressando *IDEF1* apresentaram aumento no número de transcritos do gene *OsIRO2*, o qual é um fator de transcrição bHLH induzido por deficiência de Fe (Kobayashi et al., 2007). Vários genes bHLH envolvidos na aquisição de Fe têm sido induzidos em condições de deficiência de Fe, como o gene FER em tomate e o ortólogo (FIT FIT1/FRU/AtbHLH29) em *Arabidopsis thaliana* (Kobayashi et al., 2009).

O gene LOC_Os04g51190, é um fator de transcrição relacionado com a regulação do crescimento da planta, conhecido como o ativador transcricional Growth-regulating factor 3 (GRF3), possui dois domínios: QLQ envolvido na interação proteína-proteína e o domínio WRC responsável pela ligação ao DNA. Este fator de transcrição possui papel regulatório na elongação do caule induzida por giberelina (http://www.uniprot.org/uniprot/Q6AWY6). Possui homologia ao gene AT3G13960 de *Arabidopsis thaliana*, conhecido como growth-regulating factor 5 (GRF5) (http://www.phytozome.net).

Material e Métodos

A sequência de gene *Os01g06640* foi obtida do *Rice Genome Annotation Project* (http://rice.plantbiology.msu.edu).

Identificação de fatores de transcrição utilizando a técnica de Yeast One hybrid

O promotor do gene LOC_Os01g06640 foi dividido em quatro fragmentos sobrepostos (até 1500 pb antes do ATG), os quais foram usados como iscas (baits) para o *screening* de Y1H. Desta forma, foram obtidas quatro construções diferentes (4 íscas): fragmento 1.1 (próximo ao ATG) com 200pb; fragmento1. 2 com 332pb; fragmento 2 com 441pb e fragmento 3 com 557pb (Fig.1). Cada fragmento foi clonado no vetor pHIS3/pINT1 (Ouwerkerk and Meijer, 2001) e integrado na cepa de levedura Y187 (Clontech). Estas estirpes foram transformadas com a biblioteca de cDNA de expressão e crescidas em meio seletivo (CM-His + 5mM 3AT) de modo a identificar interações fator de transcrição-DNA (Ouwerkerk and Meijer, 2001). Para cada fragmento do promotor foram pesquisados mais de três milhões de clones em meio seletivo (CM-His + 5mM3-AT). Os plasmídeos dos clones de levedura que cresceram em meio seletivo foram extraídos e o inserto contendo o cDNA foi sequenciado. A sequência foi identificada por homologia no genoma do arroz usando BLAST.

As bibliotecas de cDNA de plantas tratadas com estresses de frio e salinidade foram disponibilizadas pelo laboratório de Genômica de Plantas em Stress no ITQB. As plântulas de arroz foram crescidas em solução hidropônica em um meio de crescimento de arroz (Yoshida et al., 1976), a 28ºC, 700 lmol photons $m^{-2}s^{-1}$, 70% de umidade, fotoperíodo de 12/12h (luz/escuro) durante 14 dias. Para o tratamento de frio as plântulas foram transferidas para câmara de crescimento a 5ºC ou 10ºC. Para o estresse de salinidade, as plântulas foram transferidas para um meio contendo 200 mM NaCl. Para o tratamento controle as plântulas foram transferidas para solução contendo água conforme Figueiredo et al. (2012).

Tabela1. Genes que codificam proteínas que se ligam na região promotora do gene *Os01g06640*.

Nome do gene	Locus	Função	Referência	Isca utilizada	Nº de vezes encontrado
IDEF1	LOC_Os08g01090	Deficiência de ferro.	Kobayashi et al., 2007	1.2	20
GRF3	LOC_Os04g51190	Crescimento da planta	Choi et al., 2004	2	2

IDEF1: Iron Deficiency-Responsive Element Factor 1; GRF3: Growth-regulating factor 3.

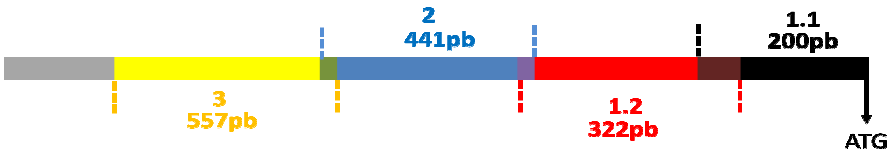

Figura 1. Promotor do gene *Os01g06640*. As iscas foram construídas a partir de fragmentos sobrepostos da sequência promotora de gene *Os01g06640*. As iscas foram denominadas de 1.1; 1.2; 2 e 3.

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DISCUSSÃO GERAL

A soja tem sido recomendada como planta modelo para estudos genéticos e moleculares (Gepts et al., 2005). Em 2010, foi publicado o sequenciamento do genoma inteiro da soja e dentre as famílias gênicas, 63 famílias codificam fatores de transcrição, o que representa 12,2% nos lócus que codificam proteínas no genoma da soja (Schmutz et al., 2010). Os fatores de transcrição têm sido considerados genes alvos potenciais para o uso em engenharia genética devido a sua capacidade regulatória em diversas rotas de sinalização. Os fatores de transcrição bHLH representam a maior família de fatores de transcrição com 480 membros em soja (Jin et al., 2014). O emprego de fatores de transcrição bHLH tem sido considerado muito promissor devido a sua participação na regulação da expressão de genes downstream envolvidos no controle de loops feed-forward e feed-backs que podem resultar em possíveis resposta de tolerância a condições adversas.

Neste trabalho foi analisado o perfil de expressão de dois genes bHLH de soja submetidas a estresses bióticos e abióticos. Além disso, foram utilizadas diferentes técnicas moleculares para tentar esclarecer a participação de fatores de transcrição bHLH em rotas metabólicas.

O gene *Glyma05g02110* foi nomeado *GmbHLH162* devido a homologia ao *bHLH162* de *Arabidopsis thaliana* (Jin et al., 2014). A análise do perfil de expressão de *GmbHLH162* em plantas de soja infectadas com o fungo *Phakopsora pachyrhizi* sugeriu que este gene pode estar envolvido na modulação de genes alvos relacionados a estresses levando a resposta de susceptibilidade ao fungo. Por outro lado, o gene *GmbHLH162* pode ser um regulador positivo relacionado com a resposta de estresse por seca, uma vez que os níveis de transcritos foram aumentados na raiz da cultivar tolerante analisada, sugerindo que este gene pode estar envolvido na resposta de adaptação da planta.

GmbHLH162 é um fator de transcrição bHLH do tipo MYC (Jin et al., 2014), e diversos trabalhos têm classificado as proteínas MYC como ativadoras ou repressoras envolvidas em diferentes rotas metabólicas (Song et al., 2013; Chen et al., 2011). A fim de identificar qual a possível rota metabólica em que *GmbHLH162* atua, foi conduzido um *screening* de duplo-híbrido em leveduras, utilizando uma biblioteca de cDNA de plantas de soja submetidas ao estresse osmótico ocasionado por tunicamicina. Nossos resultados

permitiram a identificação de uma interação da proteína GmbHLH162 com a proteína poliamina oxidase 2 (PAO2) no citoplasma e no núcleo, e esta interação pode ser confirmada pela técnica de BiFC. Nós especulamos que esta interação possa estar alterando o status redox da célula e modulando a atividade do fator de transcrição bHLH. Ainda, na tentativa de verificar o possível papel desta interação, foi realizado um ensaio de transativação em protoplastos de *Arabidopsis thaliana* para verificar se *GmbHLH162* está envolvido na ativação ou repressão de genes alvos e qual o efeito desta interação com a PAO2 na modulação da expressão gênica. Os resultados de transativação mostraram que *GmbHLH162* provavelmente liga-se a G-box, no entanto a utilização de um promotor sintético não permitiu verificar qual o efeito na modulação gênica desta interação. Novos experimentos com plantas transgênicas de *Arabidopsis thaliana* superexpressando *GmbHLH162*, estão em andamento. Estas plantas estão sendo selecionadas em higromicina com o intuito de realizarmos um experimento de RNA seq para então identificar os possíveis genes alvos e por fim conduzir o ensaio de transativação utilizando um promotor específico.

O gene *Glyma06g01430* codifica um fator de transcrição que apresenta alta homologia com o gene *bHLH63* de *Arabidopsis thaliana* (Jin et al., 2014), por este motivo, este gene foi nomeado *GmbHLH63*. O perfil de expressão do gene *GmbHLH63* também foi analisado em plantas de soja submetidas a infecção com *Phakopsora pachyrhizi* e os resultados mostraram um aumento no nível de transcritos deste gene na cultivar resistente analisada. Estes resultados sugerem que *GmbHLH63* pode ser um regulador positivo relacionado com a resposta ao fungo.

Além disso, tem sido relatado o envolvimento de genes AthCIB1 ao longo do desenvolvimento da planta (Meng et al., 2013). GmbHLH63 é um fator de transcrição relacionado a CIB1, o qual pertence a família MYC de fatores de transcrição (Gupta et al., 2014). O gene MYC2 é conhecido como regulador de crescimento em tomate, aumentando o numero de raízes laterais de plantas de tomate superexpressando MYC2 (Gupta et al., 2014). Visando avaliar o papel de bHLH no desenvolvimento da planta, foram geradas plantas de *Arabidopsis thaliana* superexpressando o gene *GmbHLH63*. Estas plantas transgênicas apresentaram um crescimento antecipado da planta em estágios iniciais de desenvolvimento, como a emissão e abertura de cotilédones e a emissão de folhas. Estes

dados indicam uma possível relação do gene *GmbHLH63* com a regulação do crescimento em *Arabidopsis thaliana.*

Além de verificar o envolvimento de genes bHLH de soja em diferentes situações de estresses, neste trabalho nós avaliamos o papel de fatores de transcrição bHLH de arroz envolvidos na regulação redox da célula. Para tal, foi selecionado o gene LOC_Os01g06640, que apresentou alteração no nível de transcritos de plantas de arroz com homeostase redox alterada (Ribeiro et al., 2012). Na tentativa de identificar em qual rota metabólica associada a estresse o gene bHLH *Os01g06640* está envolvido, realizou-se um screnning de mono-híbrido em levedura a partir de uma biblioteca de cDNA de plantas de arroz submetidas ao estresse por frio. Este estudo permitiu identificar dois fatores de transcrição que se ligam a região promotora do gene *Os01g06640*: IDEF1 e GRF3. O fator de transcrição IDEF1 está envolvido na resposta de deficiência de ferro e, outros trabalhos mostram que IDEF1 liga-se a sequência promotora de outros genes bHLH como por exemplo o gene FER (Kobayashi et al., 2009). Além disso, os resultados sugerem que *Os01g06640* pode estar relacionado à regulação do crescimento da planta de arroz via rota de GRF3.

Por fim, existe um grande potencial para a expansão da utilização de fatores de transcrição em biotecnologia devido a participação destes em diferentes vias regulatórias, visando o aumento da tolerância a estresses. Neste sentido, o melhoramento genético por transgenia vem ao encontro desta necessidade, possibilitando o aumento na produtividade e, assim, aumentando a eficiência da produção. Os resultados de expressão dos genes bHLH estudados mostraram uma relação destes com respostas a estresses bióticos e abióticos, indicando que estes genes poderiam ser alvos potenciais para o desenvolvimento de novas estratégias para o melhoramento vegetal.

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Anexo I

Artigo Publicado

Título do trabalho: Identification and in silico characterization of soybean trihelix-GT and bHLH transcription factors involved in stress responses

Autores: Marina Borges Osorio, Lauro Bücker-Neto, Graciela Castilhos, Andreia Carina Turchetto-Zolet, Beatriz Wiebke-Strohm, Maria Helena Bodanese-Zanettini and Márcia Margis-Pinheiro.

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