GISELLE CAMARGO MENDES

CARACTERIZAÇÃO FISIOLÓGICA DE PLANTAS SUPEREXPRESSANDO GmNAC6 EM SOJA E SEUS EFEITOS NA MORTE CELULAR PROGRAMADA

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fisiologia Vegetal, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 04 de abril de 2013.

_____	_____
Wagner Luiz Araújo	Adriano Nunes Nessi (Coorientador)
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_____

Elizabeth Pacheco Batista Fontes
(Orientadora)

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BIOGRAFIA

GISELLE CAMARGO MENDES, filha de José Mendes Vieira e Marlene Camargo Mendes, nasceu em 26 de agosto de 1983 em Juiz de Fora, Minas Gerais.

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RESUMO

MENDES, Giselle Camargo, D.Sc., Universidade Federal de Viçosa, abril de 2013. **Caracterização fisiológica de plantas superexpressando GmNAC6 em soja e seus efeitos na morte celular programada.** Orientadora: Elizabeth Pacheco Batista Fontes. Coorientadores: Adriano Nunes Nesi e Marcelo Ehlers Loureiro.

O estresse no RE e o estresse osmótico prolongado convergem sinergicamente para a indução das proteínas N-Rich (NRP), as quais ativam o sinal de morte celular mediado pelas proteínas GmNAC6 em soja. Para identificar novos componentes da via de sinalização induzida por estresses que geram a resposta de morte celular programada (*Programmed cell death* - PCD) foi realizado um ensaio de duplo híbrido para identificar os parceiros de GmNAC6. Foi descoberto outro membro da família NAC, GmNAC30, capaz de interagir com GmNAC6 no núcleo de células de plantas para regular coordenadamente promotores dos genes alvos comuns, que possuem o cis elemento comum TGTG [TGC]. Nós descobrimos que GmNAC6 e GmNAC30 podem funcionar como ativadores ou repressores da transcrição e cooperam entre si para melhorar a regulação da transcrição de promotores alvo comuns, sugerindo que a heterodimerização pode ser necessária para a completa regulação da expressão do gene. Assim, GmNAC6 e GmNAC30 apresentam um perfil de expressão que se sobrepõe em resposta a vários estímulos ambientais e durante o desenvolvimento vegetal. Consistente com o papel na morte celular programada, GmNAC6 e GmNAC30 se ligam *in vivo* e transativam os promotores de enzimas hidrolíticas em protoplastos de soja. Um cis-elemento onde GmNAC6/GmNAC30 se ligam foi encontrado no promotor do gene que codifica uma enzima de processamento vacuolar (*Vacuolar processing enzyme* - VPE), a qual tem atividade de caspase-1 e é um executor da morte celular programada em plantas. Nós demonstramos que a expressão de GmNAC6 juntamente com GmNAC30 transativa o gene VPE em protoplastos de soja. Coletivamente, nossos resultados indicam que GmNAC30 coopera com GmNAC6 para induzir o evento de PCD pela ativação de VPE. A interpretação que GmNAC6 funciona como regulador da PCD induzida por estresse via indução de VPE, uma enzima chave envolvida na morte celular programada pelo colapso do vacúolo, levantou a possibilidade que GmNAC6 funcione como um regulador da senescência foliar. Para elucidar esta questão, plantas de soja variedade BR16 foram transformadas com o gene GmNAC6 sob o controle do promotor 35S, e a análise da incorporação do transgene foi realizada por PCR até a terceira geração. Três linhagens transgênicas homozigotas (geração T3) foram selecionadas e

apresentaram níveis semelhantes de expressão de GmNAC6. As três linhagens transgênicas (GmNAC6.1, GmNAC6.2 e GmNAC6.3) foram fenotipicamente idênticas à linhagem controle (não transformada) durante a fase vegetativa de desenvolvimento e durante o florescimento. No entanto, na fase reprodutiva de desenvolvimento R3, a senescência foliar das linhagens transgênicas foi acelerada em comparação com as plantas controle. Em todas as três linhagens transgênicas, a expressão ectópica de GmNAC6 acelerou o amarelecimento foliar, o qual está associado a uma maior perda de pigmentos (clorofila-a e b, e os carotenóides) e ao maior acumúmulo de ROS em comparação com as plantas controle. Além disso, a senescência precoce das plantas transgênicas foi associada a uma maior indução de genes marcadores de senescência nas folhas na fase R3 de desenvolvimento em comparação com os níveis nas folhas das plantas controle. Consistente com o papel como um regulador da transcrição da expressão do gene VPE, a superexpressão de GmNAC6 induziu um acúmulo muito maior dos transcritos VPE nas linhas transgênicas quando comparado as plantas controle no mesmo estádio de desenvolvimento R3. Sabendo que VPE media a morte celular pelo colapso do vacúolo, que GmNAC6 induz a expressão de VPE e que o aceleramento da senescência nas plantas transgênicas esta associado a expressão temporal e espacial de VPE, é razoável propor que GmNAC6 regula a senescência foliar via indução da VPE, um tipo de PCD que é mediada pelo colapso vacuolar. Como mais uma evidência de que GmNAC6 pode regular a senescência foliar, as linhagens transgênicas super expressando GmNAC6 exibem uma maior sensibilidade ao estresse abiótico, ao estresse osmótico, ao estresse do RE e a seca. Portanto, os resultados da presente investigação suportam ainda mais a noção de que a tolerância das plantas ao estresse abiótico está geneticamente ligada à longevidade da folha.

ABSTRACT

MENDES, Giselle Camargo, D.Sc., Universidade Federal de Viçosa, April, 2013. **Physiological characterization of plants over expressing GmNAC6 in soybean and its effects on programmed cell death.** Adviser: Elizabeth Pacheco Batista Fontes. Co-advisers: Adriano Nunes Nesi and Marcelo Ehlers Loureiro.

Prolonged ER stress and osmotic stress synergistically activate the stress-induced N-rich protein (NRP)-mediated signaling that transduces a cell death signal by inducing GmNAC6 in soybean. To identify novel regulators of the stress-induced programmed cell death (PCD) response, we screened a two-hybrid library for partners of GmNAC6. Here we discovered another member of the NAC family, GmNAC30, which binds to GmNAC6 in the nucleus of plant cells to coordinately regulate common target promoters, which harbor the core cis-regulatory element TGTG[TGC]. We found that GmNAC6 and GmNAC30 can function either as transcriptional repressors or activators and cooperate to each other to enhance transcriptional regulation of common target promoters, suggesting that heterodimerization may be required for full regulation of gene expression. Accordingly, GmNAC6 and GmNAC30 display an overlapped expression profile in response to multiple environmental and developmental stimuli. Consistent with a role in programmed cell death, GmNAC6 and GmNAC30 bind *in vivo* to and transactivate hydrolytic enzyme promoters in soybean protoplasts. A GmNAC6/GmNAC30 binding site is found in the promoter of the caspase-1 like vacuolar processing enzyme (VPE) gene, an executioner of programmed cell death in plants. We showed that expression of GmNAC6 along with GmNAC30 fully transactivate VPE gene in soybean protoplasts. Collectively our results indicate that GmNAC30 cooperates with GmNAC6 to activate PCD through induction of the cell death executioner VPE. The interpretation that GmNAC6 functions as a regulator of stress-induced PCD, likely via induction of VPE, a key enzyme involved in vacuole-mediated PCD, raised the possibility that GmNAC6 would function as a regulator of leaf senescence as well. To address this issue, soybean plants, variety BR16, were transformed with GmNAC6 under the control of the 35S promoter and analysis of transgene incorporation was carried out by PCR for three generations. Three homozygous transgenic lines (T3 generation) were selected as they display similar levels of GmNAC6 expression. All three transgenic lines (GmNAC6.1, GmNAC6.2 and GmNAC6.3) were phenotypically identical to the control, untransformed line during the vegetative phase of development and flowered with similar rate and period. However, at the

R3 stage of development, the leaf senescence in the transgenic lines was accelerated as compared to control leaves. In all three transgenic lines, ectopic expression of GmNAC6 accelerated leaf yellowing that was associated with a greater loss of pigments (Chlorophyll a and b, and carotenoids) and a higher accumulation of ROS, as compared to the control plants. Furthermore the early senescence phenotype displayed by the transgenic lines was associated with a higher induction of senescence marker genes in their leaves at the R3 developmental stage in comparison with the levels in the control leaves. Consistent with a role as a transcriptional regulator of VPE gene expression, the overexpression of GmNAC6 induced VPE transcript accumulation in the transgenic lines to a much higher extent than those in the control lines at the R3 developmental stage. Given that VPE is an executioner of vacuole-mediated PCD, GmNAC6 induces VPE expression and GmNAC6-mediated leaf senescence was directly correlated with temporal and spatial expression of VPE, it is reasonable to propose that GmNAC6 regulates leaf senescence via induction of VPE, a kind of PCD that is mediated by the vacuolar collapse. As further evidence that GmNAC6 may regulate developmentally programmed leaf senescence, the GmNAC6-overexpressing transgenic lines displayed an enhanced sensitivity to the abiotic stresses, osmotic stress, RE stress and drought. Therefore, the results of the present investigation further support the notion that plant tolerance to abiotic stresses is genetically linked to leaf longevity.

INTRODUÇÃO GERAL

As plantas, por serem organismos sésseis, estão constantemente expostas a estresses abióticos e bióticos que causam um impacto negativo no crescimento e desenvolvimento vegetal. Para minimizar os efeitos causados por estresses do meio ambiente, as plantas desenvolveram durante a evolução respostas rápidas e coordenadas às condições adversas de desenvolvimento. Alterações morfológicas, fisiológicas, bioquímicas e moleculares são responsáveis pelo aumento de tolerância das plantas a condições restritivas (Hu *et al*., 2006); (Mahajan & Tuteja, 2005).

Os sinais de estresses no meio ambiente são percebidos e transduzidos pela planta por meio de vias de sinalização que permitem uma comunicação das células vegetais com seu meio exterior. O entendimento da percepção de sinais de estresses pelas plantas, bem como a transdução destes sinais que levará a uma resposta adaptativa, tem sido fundamental para o desenvolvimento de ferramentas biotecnológicas que minimizem os danos causados por estresse nas plantas. Os fatores transcricionais, que muitas vezes são ativados na via de sinalização por estresses, são os principais alvos da engenharia genética, pois controlam a expressão de muitos genes responsáveis pela manutenção da homeostase vegetal. De acordo com Tran & Mochida (2010) foram identificados cerca de 5000 fatores transcricionais no genoma da soja, agrupados em 61 subfamílias (Yamaguchi-Shinozaki & Shinozaki, 2005; Hu *et al.*, 2006; Tran & Mochida, 2010).

Os membros da família de fatores de transcrição NAC (acrônimo de NAM, ATAF e CUC) são específicos de plantas e foram descritos primeiramente por Souer *et al.* (1996). Esta família está representada por 151 membros em *Oryza sativa*, 126 membros em *Arabidopsis thaliana* (http://plntfdb.bio.uni-potsdam.de) e 153 membros em *Glycine max (*Lee *et al.*, 2011) Em geral, os membros da família NAC se caracterizam por terem um domínio conservado na porção N-terminal, denominado domínio NAC, no qual é responsável pela ligação ao DNA. Este domínio é codificado por dois éxons e compreende cerca de 150 aminoácidos, e estes estão agrupados em cinco regiões conservadas (A-E). Entretanto, a porção C-terminal se caracteriza por alta divergência tanto em seqüência quanto em tamanho, e é responsável pelo domínio de ativação ou repressão da transcrição *(Ren et al., 2000; Xie et al.*, 2000; Duval *et al.*, 2002; Ooka *et al.*, 2003; Nakashima *et al.*, 2012). O domínio C-terminal exibe atividade de ligação entre proteínas e, algumas proteínas NACs contem um domínio adicional transmembrana (Seo *et al.*, 2009 ; Tran *et al*., 2009). Assim sendo, embora o grande conjunto de proteínas NACs se localiza no núcleo, existem um sub-conjunto de

proteínas NACs que estão inseridos na membrana plasmática (Kim *et al*., 2007; Seo *et al.*, 2009).

Os membros desta família de transfatores estão envolvidos em processos importantes do desenvolvimento vegetal, como manutenção do meristema apical (Souer *et al*., 1996; Aida *et al.*, 1997); participação no controle do florescimento (Yoo *et al.*, 2007), embriogênese (Duval *et al.*, 2002; Kunieda *et al.*, 2008); sinalização hormonal (Xie *et al.*, 2000; Fujita *et al*., 2004); formação da parede secundaria (Zhong *et al.*, 2006; Mitsuda *et al*., 2007) e no processo de senescencia foliar (Uauy *et al.*, 2006; Guo & Gan, 2006; Kim *et al.*, 2009). Além disso, diversos genes NACs têm sido associados com os mecanismos de respostas das plantas contra diversas situações de estresses.

Entre os diversos genes NACs de soja envolvidos em respostas contras estresses bióticos e abióticos já identificados (Pinheiro *et al.*, 2009, Lee *et al.*, 2011), destaca-se o gene *GmNAC6*, recentemente identificado e caracterizado como um componente de uma via de sinalização adaptativa que transduz um sinal de morte celular em uma resposta integrada aos estresses osmótico e do retículo endoplasmático (Faria *et al.*, 2011). O modelo atual desssa via de sinalização, que integra a transdução de múltiplos sinais de estresses em uma resposta de morte celular programada (PCD), preconiza que as vias de respostas a uma diversidade de estresses abióticos, como seca, estresse osmótico e estresse do retículo endoplasmático, convergem no nível transcricional para ativar a expressão dos genes NRPs que, por sua vez, acionam a via de transdução de um sinal de PCD (Reis e Fontes, 2012). Mecanisticamente, os sinais de estresses induzem a expressão do transfator GmERD15 (*Glycine max early responsive to dehydrion stress 15*) que, por sua vez, liga ao promotor dos genes NRPs (*N-rich proteins*) para ativar a expressão desses genes. Acúmulo aumentado de NRPs leva a indução da expressão do gene *GmNAC6*, como efetor de PCD. A relevância dessa via de sinalização para adaptação de plantas tem sido demonstrada por meio de manipulação da atividade de seus componentes em plantas transgênicas (Valente *et al.*, 2009). Superexpressão do chaperone molecular BiP (*Binding Protein*), residente no retículo endoplasmático (RE), altera a indução dos genes NRPs e GmNAC6 em reposta a estresses no RE, a estresse osmótico e à seca, e atenua o processo de PCD-mediada por GmNAC6. Atenuação dessa via de sinalizaçao de PCD por BiP causa tolerância a deficit hídrico e proteção contra estresses osmótico e do RE (Reis *et al.*, 2011; Valente *et al.*, 2009).

Além da relevância da via integrativa de respostas a múltiplos sinais de estresses como alvo para engenharia genética de plantas, a natureza da resposta em um evento de morte

celular programada induzido por um transativador transcricional específico de plantas, encerra uma importância biológica singular em fisiologia vegetal. Apesar disso, o avanço de conhecimento quanto ao mecanismo de ativação da via e dos eventos downstream a GmNAC6 que causam PCD tem sido limitado. Assim sendo, esta investigação teve como objetivos primordiais identificar novos componentes da via de sinalização de estresses integrativos e caracterizar o evento de PCD mediado por GmNAC6. O primeiro objetivo é explorado no capítulo I intitulado *Interações entre GmNAC6 e GmNAC30 integra as respostas de morte celular induzidas por estresse no retículo endoplasmático e por estresse osmótico via VPE (vacuolar processing enzyme*). O segundo objetivo, ou seja, a caracterização do evento de PCD mediado por GmNAC6 é também abordada no capítulo I e expandida para o capítulo II, intitulado *Expressão ectópica de GmNAC6 acelera senescência foliar e aumenta suscetibilidade de plantas transgênicas a estresses abióticos.*

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CAPÍTULO 1

Interaction between GmNAC6 and GmNAC30 integrates ER stress- and osmotic stress-induced cell death responses through vacuolar processing enzyme

Giselle Camargo Mendes, Pedro Augusto Braga dos Reis, Humberto Henrique Carvalho, José Francisco Aragão and Elizabeth Batista Pacheco Fontes. Interaction between GmNAC6 and GmNAC30 integrates ER stress- and osmotic stress-induced cell death responses through vacuolar processing enzyme. The Plant Cell, *in preparation*.

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Interaction between GmNAC6 and GmNAC30 integrates ER stress- and osmotic stress-induced cell death responses through vacuolar processing enzyme

ABSTRACT

Prolonged ER stress and osmotic stress synergistically activate the stress-induced N-rich protein (NRP)-mediated signaling that transduces a cell death signal by inducing GmNAC6 in soybean. To identify novel regulators of the stress-induced programmed cell death (PCD) response, we screened a two-hybrid library for partners of GmNAC6. Here we discovered another member of the NAC family, GmNAC30, which binds to GmNAC6 in the nucleus of plant cells to coordinately regulate common target promoters, which harbor the core cis-regulatory element TGTG[TGC]. We found that GmNAC6 and GmNAC30 can function either as transcriptional repressors or activators and cooperate to each other to enhance transcriptional regulation of common target promoters, suggesting that heterodimerization may be required for full regulation of gene expression. Accordingly, GmNAC6 and GmNAC30 display an overlapped expression profile in response to multiple environmental and developmental stimuli. Consistent with a role in programmed cell death, GmNAC6 and GmNAC30 bind *in vivo* to and transactivate hydrolytic enzyme promoters in soybean protoplasts. A GmNAC6/GmNAC30 binding site is found in the promoter of the caspase-1 like vacuolar processing enzyme (VPE) gene, an executioner of programmed cell death in plants. We showed that expression of GmNAC6 along with GmNAC30 fully transactivate VPE gene in soybean protoplasts. Collectively our results indicate that GmNAC30 cooperates with GmNAC6 to activate PCD through induction of the cell death executioner VPE.

INTRODUCTION

The NAC (NAM, ATAF1, 2, CUC2) domain-containing proteins make up a large family of plant-specific transcription factors (TF), which is represented by at least 151 members in *Oryza sativa*, 126 members in *Arabidopsis thaliana* (http://plntfdb.bio.uni-potsdam.de) and 153 members in *Glycine max* (Nuruzzaman et al., 2010; Le et al., 2011). The NAC proteins are highly conserved at their N-terminus, which binds specifically to target DNA through a new-type of TF fold consisting of a twisted six-stranded β-sheet that is surrounded by a few helical elements (Ernst et al., 2004; Chen et al., 2011). Their C-terminal regions are rather divergent in sequence and length and function as activators or repressors of transcription (Duval et al., 2002; Fujita et al., 2004; Tran et al., 2004). Biological functions have been assigned to members of this family in development and stress response (Olsen et al., 2005; Nakashima et al., 2012). As transcriptional regulators, several NAC proteins have been shown to control a series of plant developmental programs, including biogenesis of lateral roots, apical shoots, floral organs and secondary walls (Aida et al., 1997; Balazadeh et al., 2011; Kim et al., 2009; Li et al., 2012; Matallana-Ramirez et al., 2013; Souer et al., 1996; Mitsuda et al., 2007). NAC proteins have also been shown to play a major role in both biotic and abiotic stress responses (Collinge and Boller, 2001; Fujita et al., 2004; Hegedus et al., 2003; Nakashima et al., 2007; Seo et al., 2010; Sun et al., 2013; Wu et al., 2009) Yamaguchi and Nakashima, 2010). These NAC transfactors are rapidly induced by a variety of stresses, and modulate defense or abiotic responses when inactivated or ectopically expressed in transgenic lines.

A growing body of evidence has demonstrated a pivotal role of NAC genes in the regulation of developmental programmed leaf senescence and programmed cell death. Several NAC genes from different species have been shown to be highly expressed during leaf senescence (John et al., 1997; Andersson et al., 2004; Lin and Wu, 2004; Guo et al., 2004; Buchanan-Wollaston et al., 2005) and, in Arabidopsis, 20 of 151 *AtNAC* genes are up-regulated by senescence (Guo et al., 2004). Several of these Arabidopsis senescence-induced NAC genes, including *AtNAP* (ANAC029: Guo and Gan, 2006), ORE1 (ANAC092, AtNAC2; Kim et al., 2009; Balazadeh et al., 2010), *ORS1 (ANAC059;* Balazadeh et al., 2011), *VNI2* (*ANAC083*, Yang et al., 2011) and JUB1 (ANAC049; Wu et al., 2012), have been shown, through reverse genetics and overexpression studies, to play a pivotal role in leaf senesence. NAM-BI, a NAC transfactor from wheat, has been shown to stimulate leaf

senescence and increase the remobilization of nutrients from the leaves to the developing grains (Uauy et al., 2006). In soybean, at least three NAC genes are associated with senescence (Pinheiro et al., 2009). A sub-set of these senescence-induced NAC genes has emerged as regulators of stress-induced senescence and/or cell death (Balazadeh et al., 2010; Faria et al., 2011; Lee et al., 2012; Yang et al., 2011). Among them, the GmNAC6 (Glyma12g02540.1; recently designated GmNAC081, Le et al., 2011) from soybean potentially integrates multiple stress signaling pathways into a programmed cell death response (Faria et al., 2011).

GmNAC6 (GmNAC081) has been identified as a component of the endoplasmic reticulum (ER) stress- and osmotic stress-induced cell death response that is mediated by the N-rich proteins in soybean (Faria et al., 2011). The N-rich mediated signaling pathway integrates a cell death signal generated from prolonged ER stress and osmotic stress into a synergistic and convergent response (Irsigler et al., 2007; Costa et al., 2008). The current model for this integrative pathway holds that, under stress conditions, GmERD15, an ER stress- and osmotic stress- induced transcriptional activator, up-regulates *NRP* expression that in turn induces *GmNAC6* to promote a cell death response, which resembles a programmed cell death event (Alves et al., 2011; Costa et al., 2008; Faria et al., 2011). Accordingly, the overexpression of either NRPs or GmNAC6 in soybean protoplasts induces caspase-3-like activity and promotes extensive DNA fragmentation. Furthermore, the transient expression of NRPs or NACs *in planta* causes leaf yellowing, chlorophyll loss, malondialdehyde production, and the induction of senescence marker genes, which are hallmarks of leaf senescence.

ER stress results from any condition that disrupts ER homeostasis and causes the accumulation of unfolded proteins in the lumen of the organelle. To cope with these perturbations, the ER stress signal triggers a cytoprotective signaling pathway, designated as the unfolded protein response (UPR), which has been described in details in mammalian cells (for a review, see Malhotra and Kaufman, 2007). Recent progress in elucidating UPR in plants has demonstrated that the ER stress signal is transduced by two classes of ER transmembrane receptors, the bZIP class (bZIP28 and bZIP17) and the inositol requiring kinase 1 (IRE1) homologs, Ire1a and Ire1b in Arabidopsis (Iwata and Koizumi, 2012; Reis and Fontes, 2013). Upon activation, these ER transducers act in concert to restore ER homeostasis by up-regulating ER resident chaperones genes and the ER-associated protein degradation system. In addition to the cytoprotective role of the unfolded protein response,

ER stress also generates a cell death signal that is transduced by ER stress-specific signaling pathways and also by shared pathways, connecting the ER stress response to other stress response pathways (Shore et al., 2011). For instance, in mammalian cells, the ASK1/JNK stress-activated kinase pathway can be induced by both ER stress and oxidative stress to promote apoptosis (Urano et al., 2000). One plant-specific, ER stress shared response is the ER stress- and osmotic stress-integrating signaling that converges on N-rich proteins (NRPs) to transduce a cell death signal through activation of GmNAC6 gene expression (Reis and Fontes, 2012).

As a branch of the ER stress response that connects with other environmentally induced responses, the stress-induced NRP-mediated cell death signaling has the potential to allow versatile adaptation of cells to different stresses. Accordingly, modulation of this pathway by constitutive expression of the ER molecular chaperone BiP has been shown to promote a better adaptation of transgenic lines to drought (Valente et al., 2009; Reis et al., 2011). Despite its conceptual relevance in plant adaptation to adverse conditions, several key players of this stress-induced signaling pathway are unknown and molecular events downstream of GmNAC6 remain to be determined. Here, we isolated a new NAC domain-containing protein from soybean, GmNAC30, by its capacity to bind to GmNAC6 in yeast. We showed that GmNAC6 and GmNAC30 interact with each other in vitro and in vivo, bind to common cis-regulatory sequences in target promoters and synergistically regulate these target genes. One such target gene, the vacuolar processing enzyme (VPE), may be responsible for execution of the cell death program induced by ER stress and osmotic stress.

RESULTS

Identification of GmNAC30, a new NAC-domain containing protein from soybean, as a GmNAC6-specific interactor

To identify potential targets of GmNAC6 in the N-rich mediated cell death response, we screened a soybean cDNA library (Alves et al., 2011) with the GmNAC6 full-length ORF as bait. Several clones showed His protrotophy and lacZ expression on X-gal indicator plates following secondary screening. The characterization of a NAC-domain containing protein, designated GmNAC30 [previously designated GmNAC32 by Hao et al., 2010], is reported here. From about 5 x 10^5 clones screened, 3 positive clones harbored cDNA fragments from

the soybean gene GmNAC30. We next examined whether GmNAC6 was able to interact with the full-length GmNAC30 protein in yeast (Supplemental Figure 1A and 1B online). Co-expression of BD-NAC6 and AD-NAC30 fusion proteins promoted growth of yeast in the selective medium (Supplemental Figure 1A online) and an activity of β-galactosidase (2.38 ± 0,25) significantly higher than that of control cells transformed with pBD + pAD empty vectors (0.4 ± 0,01) or pBD-NAC6 + pAD empty vector (0.26 ± 0,01) (Supplemental Figure 1B online). These results confirmed that GmNAC6 interacted specifically with full-length GmNAC30, as the HIS3 marker gene and lacZ reporter were activated in yeast cells co-transformed with BD-NAC6 and AD-NAC30 fusions but not with the negative controls.

There are six homologs of GmNAC30 predicted in the soybean genome, as designated by GmNAC018 (76% identity), GmNAC039 (74% identity; previously designated as GmNAC15 by Hao et al., 2010); GmNAC022 (64% identity; previously designated as GmNAC8 by Hao et al., 2010); GmNAC035 (65% identity; previously designated as GmNAC2 by Meng et al., 2007; and Pinheiro et al., 2009); GmNAC109 (63% identity; previously designated as GmNAC12 by Tran et al., 2009 or GmNAC21 by Hao et al., 2010), GmNAC011 (65% identity), which cluster together in a phylogenetic tree of NAC domain-containing protein from soybean (Figures 1A) and are most closely related to the ATAF group of the NAC family from Arabidopsis (Supplemental Figure 2A online). The deduced protein sequence of GmNAC30 displays the highly conserved NAC domain at the N-terminus which is divided into the five NAC subdomains (A–E) of conserved blocks intercalated with heterogeneous blocks or gaps (Supplemental Figure 2B online; Ooka et al., 2003). A predicted nuclear localization signal (^{105}-IKKALVFYAGKAPKGVKTN-^{123}) is found within the NAC D subdomain of GmNAC30, which was similar in sequence and position to the NLS predicted on AtNAC1 and ANAC (Xie et al., 2000; Greve et al., 2003; Xie et al., 2013). Consistent with this finding, the fluorescence of a GmNAC30-GFP fusion concentrated in the nucleus of electroporated soybean protoplasts, which was also stained with the nuclear marker DAPI (Figure 1B). GmNAC6 has also been shown to be a nuclear localized protein (Pinheiro et al., 2009).

We next examined whether GmNAC6 and GmNAC30 would interact in the nucleus of plant cells by the BiFC (bimolecular fluorescence complementation) assay. The complementation of the fluorescent YFP fragments mediated by interaction between GmNAC30 and GmNAC6 was detected in the nucleus of transfected leaf protoplasts as it was coincident with the DAPI signal (see Figure 1Ca and 1Cb). In addition, the formation of a

GmNAC6-GmNAC30 complex occurred in vivo independently on the orientation of GmNAC6 or GmNAC30 fusions (N-terminus or C-terminus of YFP; Figure 1Cc and 1Cd) and the reconstituted fluorescent signal was much higher than the background levels (control panels with combination of the protein fusions with empty vectors; Figures 1Ce to 1Cl). Accumulation of GmNAC6 and GmNAC030 transcripts in the protoplasts electroporated with the different combinations of the constructs was confirmed by qRT-PCR (Supplemental Figure 3 online). Collectively, these results indicate that GmNAC6 interacts with GmNAC30 in the nucleus of plant cells.

As a GmNAC6-specific partner, GmNAC30 may be a downstream target of the osmotic and ER stress-induced N-rich mediated cell death signaling

The NRP-mediated signaling has been shown to integrate and transduce a cell death signal derived from prolonged ER stress and osmotic-stress (Reis and Fontes, 2012). Upon activation, NRPs trigger a signaling cascade that culminates with the enhanced expression of GmNAC6 to promote a programmed cell death response (Faria et al., 2011). As a putative downstream component of the ER stress- and osmotic stress-integrating signaling pathway, we compared the expression of GmNAC30 and GmNAC6 under conditions that activate this specific cell death program (Figure 2). We also monitored expression of NRP-B, another member of the ER stress- and osmotic stress-induced cell death signaling, as an additional readout for activation of the cell death program. Controls for effectiveness of the osmotic stress treatment, such as seed maturation protein SMP, and the ER stress treatment, such as calnexin (CNX) and binding protein (BiP), were also included in the assay. Like GmNAC6 and NRP-B, treatment of soybean seedlings with the osmotic stress inducer PEG and the ER stress inducer tunicamycin (that blocks protein glycosylation in the organelle) induced GmaNAC30 transcript accumulation (Figures 2A and 2B). Likewise, treatment of soybean cells with cycloheximide, a potent inducer of cell death in soybean protoplast, strongly up-regulated GmNAC30 gene expression, indicating that GmNAC30 may be involved in cell death events, like NRP-B and GmNAC6 (Figure 2C; Costa et al., 2008; Faria et al., 2011). These results establish that GmNAC30 displays similar pattern of expression as GmNAC6 and NRPs, which are components of the osmotic stress- and ER stress-induced cell death signaling.

We have recently demonstrated that transient expression of NRP-A or NRP-B in leaf protoplasts activates the GmNAC6 promoter and induces accumulation of GmNAC6 transcripts (Faria et al., 2011). Here we showed that overexpression of GmNRP-A or GmNRP-B in leaf protoplasts also caused a significant increase in GmNAC30 transcript accumulation (Figure 3D). These results favors the argument that GmNAC30, as a GmNAC6 partner, acts downstream of NRPs in the stress-induced cell death signaling.

GmNAC6 and GmNAC30 display overlapping pattern of expression in response to distinct stimuli

As classified by Pinheiro et al., (2009), GmNAC30 belongs to the subfamily StNAC of NAC domain-containing proteins that also encompasses drought- and ABA-induced representatives, such as OsNAC6 from *Oryza sativa* (Ohnishi et al., 2005) and SsNAC23 from *Saccharum* (Nogueira et al., 2005). GmNAC30 also clustered together with the ATAF clade from Arabidopsis (Supplemental Figure 2A), which has been shown to be involved in response to biotic and abiotic stresses (Colling e Boller, 2001; Hegedus 2003). The stress-responsive profile of NAC gene expression has been used to assign functional divergence among the members of the NAC superfamily in a given plant species (Pinheiro et al., 2009; Le et al., 2011). To examine whether expression of GmNAC30 is coordinately regulated with GmNAC6 as it would be expected from functional partners, the expression profile of these transfactors was examined under a variety of stimuli.

The hormones abscisic acid (ABA), salicylic acid (SA) and methyl jasmonate (MeJA) and the stress treatments, drought, cold and wounding, induced both GmNAC30 and GmNAC6 although to a different extent (Figure 3). Except for SA (Figure 3E) and wounding (Figure 3G) that caused a delayed response of GmNAC6 in comparison with GmNAC30 induction, all the other stimuli up-regulated GmNAC30 and GmNAC6 expression with similar kinetics (Figures 3A, 3B, 3C, 3D, 3F). GmNAC35 (previously designated GmNAC2), a closely related member of GmNAC30 (Figure 1A) was also included in the assay as a control for stress-induced NAC gene expression (Meng et al., 2007; Pinheiro et al., 2009). As an ATAF1 homolog, GmNAC35 is strongly induced by drought, and wounding, moderately induced by NaCl, SA and MeJA and displays an early kinetics of induction by cold (Figure 3; Pinheiro et al., 2009). The induction of GmNAC35 by drought occurred with a delayed response kinetics (full induction at 12-h post-treatment) in an ABA-independent

manner, which was in contrast with the rapid ABA-dependent up-regulation of GmNAC6 and GmNAC30 that reaches full induction at 1-h post-treatment (Figures 3A and 3B). These results confirmed that stress-induced NAC genes maintain a functional divergence of gene expression and hence the coordinated up-regulation of GmNAC6 and GmNAC30 under exposition to different stimuli may be a further indicative that interactions between them are relevant for function.

We also examined whether GmNAC30 and GmNAC6 expression could be coordinately regulated in distinct organs and developmental stages (Figures 3H and 3I). GmNAC30 and GmNAC6 are ubiquitously expressed in all organs analyzed at different developmental programs, vegetative phase (Figure 3H) and reproductive phase (Figure 3I). In the vegetative stage, the transcripts levels of GmNAC6 and GmNAC30 were lower in roots as compared to the other organs analyzed, whereas in the reproductive phase GmNAC6 and GmNAC30 are expressed to higher levels in roots and leaves and lower levels in seeds.

Full-length GmNAC30 exhibits transactivation activity in yeast

The full-length GmNAC6 protein is not able to transactivate the expression of reporter genes in yeast (Pinheiro et al., 2009), but it has been shown to harbor an active transactivation domain at the carboxyl terminus (Tran et al., 2009). To investigate whether GmNAC30 harbors a functional transactivator domain, the full-length GmNAC30 coding region was fused to the binding domain of GAL4 (BD-GAL4) and expressed as a fusion protein in a yeast strain harboring the reporter genes *HIS3* and *lacZ* under the control of the GAL1 promoter. We also included in the assay GmNAC35 (GmNAC2 in Pinheiro et al., 2009) and NIG (an Arabidopsis unrelated protein that exhibits transactivation activity in yeast, Carvalho et al., 2008) as positive controls and full-length GmNAC6 as a negative control (Pinheiro et al., 2009). Expression of BDGAL4 fused to GmNAC30 promoted growth of yeast in the absence of histidine and presence of 3-aminotriazole (Figure 4A) and induced *lacZ* expression, as measured by β-galactosidase activity (Figure 4B). These results demonstrate that GmNAC30 exhibits transactivation activity in yeast and are consistent with the presence of the v conserved transactivation motif EVQSDEPKW in its transcriptional activation region (TAR; Supplemental Figure 2B online). As previously demonstrated, the full-length GmNAC6 fused to BD-GAL4 was not capable to activate histidine/adenine auxotrophy (Figure 4A) and high levels of β-galactosidase expression (Figure 4B), but expression of its

carboxyl domain promoted transactivation of the reporter genes in yeast (Figure 4A). These results also confirmed previous observations that the N-terminal region of some NAC domain-containing proteins interferes negatively with the transactivation activity in their carboxyl-terminus, as deletion of their N-terminus renders the BD-fused truncated protein a potent transactivator in yeast (Hegedus et al., 2003; Tran et al., 2009; Xie et al., 2000). With such a potential conformational hindrance, GmNAC6 may depend on specific interactions with other transfactors to activate their target genes as heterodimers, which favors the argument that interaction of GmNAC6 and GmNAC30 may be biologically relevant.

GmNAC6 and GmNAC30 bind to common target promoters in vivo and regulate expression of the target genes

To identify potential target promoters for GmNAC30 and GmNAC6 we used a small scale ChIP-sequencing assay, in which the precipitated DNA from individual GmNAC6- or GmNAC30-expressing tissues were cloned and sequenced. From 25 sequences targeted by each transcriptional factor, we found 17 common sequences, which were mapped to 5' transcriptional regulatory regions of soybean genes (Tables 1 and 2). The expression of these genes was monitored by quantitative RT-PCR in soybean protoplasts electroporated with 35S:GmNAC30 or 35S:GmNAC6. Both GmNAC30 and GmNAC6 genes were efficiently expressed in transfected soybean protoplasts (Figures 5A and 5B). Transient expression of either GmNAC6 or GmNAC30 promoted a significant induction of genes encoding a dual specificity protein phosphatase, predicted endo 1,3 beta glucanase, D-Alanyl-d alanine carboxipeptidase, Cdc2-related protein kinase in addition to inducing Gm20g22140 expression (Figures 5C-5G). For another sub-set of the selected genes, GmNAC6 and GmNAC30 expression caused a significant down-regulation of their expression (Figure 6A-6L), implicating that GmNAC6 and GmNAC30 may coordinately function as both activators and repressors of gene expression in soybean. The expression of the remaining common target genes, Serine-Threonine Protein Kinase and Non-specific serine/threonine Protein Kinase was not significantly altered by overexpression of GmNAC6 or GmNAC30 in soybean protoplasts and hence it was not considered further (Supplemental Figure 4 online).

Interaction between GmNAC6 and GmNAC30 is required for full transactivation or repression of target promoters

To provide further evidence for regulation of these target genes by GmNAC6 or GmNAC30, we performed a GUS-transactivation assay in soybean protoplasts using 1-kb to 2-kb 5'flanking sequences of an up-regulated (D-alanyl-D-alanine carboxypeptidase) gene and a down-regulated (ACC synthase) gene fused to the GUS reporter (Figure 7). Accumulation of GmNAC6 and GmNAC030 transcripts in the electroporated protoplasts was confirmed by qRT-PCR (Supplemental Figure 5 online). The results of transactivation or repression are shown by β-galactosidase activity (Figures 7A and 7B) as well as quantitation of reporter GUS transcript accumulation (Figure 7C). Consistent with the gene expression profile, GmNAC6 or GmNAC30 specifically activated and repressed the ACPase promoter and ACC promoter, respectively. Expression of an unrelated transcription factor, from the MyB family, At5G05800, did not target either NAC-regulated promoter. Co-transfection of soybean protoplasts with both GmNAC6 and GmNAC30 promoted an enhanced activation of the ACPase promoter (Figures 7A and 7C) as well as an increased repression of the ACC promoter (Figures 7B and 7C) as compared to the regulation of the gene reporters by individual expression of the transfactors. Collectively, these results indicated that GmNAC6 and GmNAC30 function as transcriptional activators or repressors of common target genes that, nevertheless, require both transfactors for full regulation. Therefore, GmNAC6 and GmNAC30 may form heterodimer to coordinately regulate common target promoters.

GmNAC6 and GmNAC30 bind to the common consensus sequence element TGTGT[T/C/G] in a cooperative manner

The co-immunoprecipitated sequences by the ChIP assay share the consensus sequence TGTGTT, which was found as a derivative form (TGTGT[TGC]) in GmNAC6- and GmNAC30-regulated promoters. Except for Serine-Threonine Protein Kinase and Non-specific serine/threonine Protein Kinase promoters that do not harbor a derivative consensus sequence, in all other common target genes, the consensus sequence was found within 1-kb to 2-kb 5'flanking sequences (Tables 1 and 2). The relative proximity of the putative cis-element to the promoter core of the NAC-regulated genes suggests that the consensus sequence functions as a specific binding site for GmNAC6 and GmNAC30. To confirm this hypothesis

we examined whether *E.coli*-produced and purified GmNAC6 and GmNAC30 were able to bind to the consensus sequence in vitro (Figure 8). Both purified GST-GmNAC6 and GST-GmNAC30 bound to the labeled probe harboring directly repeated TGTGTT core sequences (Figure 8A) but not to a mutated sequence, in which both Gs were replaced by Cs (Figure 8B). Although a combination of GST-GmNAC6 and GST-GmNAC30 in the binding reaction seems to improve site occupancy and binding affinity, as shown in Figure 8A, these results were not 100% reproducible (see Figure 8C). A GST purified protein did not bind to the core consensus sequence, confirming that the in vitro DNA:protein complex formation was due to interactions between the NAC TFs and the core consensus sequence. These interactions were specific as a 100 fold molar excess of unlabeled probe efficiently competed for binding (Figure 8C). Collectively, these results establish that both GmNAC6 and GmNAC30 bind specifically to the core consensus sequence TGTGTT.

Repression of ACC synthase (Glyma17g16990) by GmaNAC6 and GmNAC30 does not lead to inhibition of ethylene synthesis under prolonged ER stress

We have previously demonstrated that NRPs induce GmNAC6 expression, programmed cell death (PCD) and evolution of ethylene in leaves (Costa et al., 2008; Faria et al., 2011). Our present data revealed that GmNAC6 down-regulates an ACC synthase homolog, the enzyme involved in last step of ethylene biosynthesis. The next set of experiments was designed to address the apparent contradiction between GmNAC6-mediated induction of cell death and down-regulation of an ACC synthase gene. Because NRPs/GmNAC6-mediated PCD can be induced by ER stress (Reis et al., 2011), we first performed a time course experiment to monitor ethylene synthesis in soybean seedlings in response to the ER stress inducer tunicamycin. Our data showed that tunicamycin promotes ethylene evolution with a significantly enhanced synthesis of the hormone as the glycosylation block stress persists (Figure 9A). The effectiveness of tunicamycin treatment was monitored by examining induction of the ER stress markers BiP and CNX (Figure 9B). GmNAC6 and GmNAC30 were slightly but significantly induced after 8h of treatment, when ethylene synthesis was already significantly higher in tunicamycin-treated than in control seedlings. Then, we examined the expression profile of the ACC gene in response to prolonged ER stress, a condition that induces ethylene synthesis and parallels the induction of cell death (Figure 9C). Consistent with repression of the ACC gene by GmNAC6 and

GmNAC30, the ACC synthase gene is down-regulated by tunicamycin in an inverse correlation to ethylene evolution as the ER stress progresses. These results indicated that the gene Glyma17g16990 (found in the ChIP assay and annotated as ACC synthase) is not involved in ethylene synthesis during ER stress. In addition they illustrated a perfect correlation between induction of the GmNAC6 and GmNAC30 TFs and down-regulation of the ACC synthase gene under stress conditions, further substantiating our results of transient expression in protoplasts.

Because ER stress induced ethylene synthesis and ACC synthase activity has been used to monitor ethylene accumulation, we examined the expression of other ACC synthase annotated genes from soybean under ER stress in order to sort out which ACC synthase could be involved in ethylene biosynthesis during ER stress progression. The soybean genome is represented by nine copies of annotated ACC synthase genes, which can be grouped into five clusters based on sequence comparison (Figure Supplemental 6 online). We selected a member of each cluster for a functional representation of the ACC synthase family. Only the ACC homologs Glyma05g36250 and Glyma16g03600 were induced by tunicamycin treatment although to a different extent. In contrast to the modest induction of Glyma05g36250 transcript by tunicamycin, Glyma16g03600 transcripts accumulated to a much higher level in response to ER stress and displayed an induction kinetics that paralleled the evolution of ethylene synthesis. Very likely the Glyma16g03600 ACC synthase is the major enzyme involved in ER stress-induced ethylene synthesis, but it is not a direct target of GmNAC6 and GmNAC30.

GmNAC6 and/or GmNAC30 transactivate the expression of vacuolar processing enzyme (VPE)

The repertoire of up-regulated genes by GmNAC6 and GmNAC30 detected by ChIP includes predominantly hydrolytic enzymes (carboxypeptidase, phosphatase and glucanase) that may be involved in plant PCD. A plant-specific strategy for PCD consists on the up-regulation of a variety of vacuolar hydrolytic enzymes, which might then be released into the cytosol to hydrolyze organelles and nuclear DNA, leading to cell death (Fukuda, 2004; Hara-Nishimura et al., 2005). In ethylene-treated leaves, glucanase has been shown to accumulate predominantly in vacuoles (Mauch and Staehelin, 1989). The vacuolar processing enzyme (VPE) has been shown to trigger vacuolar collapse-mediated PCD in pathogenesis and

development (Hatsugai et al., 2004; Kuroyanagi et al., 2005; Nakaune et al., 2005; Zhang et al., 2010). The VPE family in soybean is represented by five homologs. Except for the Glyma17g34900 promoter that does not harbor a GmNAC6/GmNAC30 binding site; the remaining VPE promoters contain one to three copies of the cis-regulatory element. These observations prompted us to examine whether GmNAC6 and GmNAC30 would control VPE expression in soybean protoplasts. Ectopic expression of either GmNAC6 or GmNAC30 transactivated strongly the expression of VPE genes (Figure 10). Given that VPE is a key executioner of cell death in plant cells, the presence of GmNAC6/GmNAC30 binding site in VPE promoter along with the capacity of the TFs to directly transactivate its expression suggest that GmNCA6/GmNAC30 and VPE constitutes a regulatory cascade for stress-induced cell death in soybean.

DISCUSSION

In contrast to the UPR, the NRP-mediated cell death signaling pathway is a plant-specific ER-stress cell death response that communicates with other environmental stimuli through shared components. In fact, osmotic stress and drought stress also activate the transduction of a cell death signal through NRPs. This cell death integrated pathway has emerged as a relevant adaptive response of plant cells to multiple environmental stimuli. NRP promoters are activated by the stress-induced TF GmERD5 to induce the expression of NRP genes (NRP-A and NRP-B) that in turn induce the expression of GmNAC6 to promote a cell death response. Here we discovered another possible component of this integrated circuit of stress-induced cell death, the GmNAC30 TF, which binds to GmNAC6 in the nucleus of plant cells to coordinately regulate the expression of common target promoters. We found that genes encoding hydrolytic enzymes are coordinately up-regulated by GmNAC6 and GmNAC30. Among them, the plant-specific executioner of programmed cell death, VPE, is transcriptionally regulated by the TFs. We propose that GmNAC6/GmNAC30-mediated activation of VPE expression links the activation of the stress-induced NRP-mediated signaling to the programmed cell death response.

GmNAC30 as a downstream component of the NRP-mediated cell death signaling

This investigation has characterized another member of the NAC domain-containing proteins of plant-specific transfactors, GmNAC30, as a possible component of the ER stress- and osmotic stress-induced NRP-mediated cell death signaling. Our hypothesis that GmNAC30 functions in this stress-induced cell death integrated circuit is supported by the following observations. Firstly, GmNAC30 was identified by its capacity to bind to GmNAC6, a downstream component of the signaling pathway, in the nucleus of plant cells. Secondly, like GmNAC6, GmNAC30 is induced by ER and osmotic stress with similar kinetics and is also induced by ectopic expression of NRP-A and NRP-B. Thirdly, as judged by ChIP assay, GmNAC30 and GmNAC6 were found to bind in vivo to common target promoters and regulate their expression in a coordinated manner. The target promoters were regulated by the individual expression of each TF, but all of them required both TFs for full induction or repression. Finally, GmNAC6 and GmNAC30 are coordinately regulated by multiple environmental and developmental stimuli, as it would be expected for TFs that act in concert as heterodimers to regulate gene expression.

GmNAC30 and GMNAC6 bind in vivo to common target promotes and function as repressors or activators of gene expression

In general, NAC domain-containing proteins have been described as repressor or activator of gene expression (Duval et al., 2002; Fujita et al., 2004, Tran et al., 2004, Kim et al., 2007). For instance, ATAF1 and ATAF2 from Arabidopsis act as transcriptional repressors of genes involved in pathogenesis, whereas *OsNAC6,* a rice ortholog of *ATAF1*, has been shown to be a positive contributor to disease resistance and salinity tolerance (Delessert et al., 2005; Nakashima et al., 2007; Wu et al., 2009). Contrasting results have been reported for ATAF1 function in drought tolerance as positive or negative regulator (Lu et al., 2007; Wu et al., 2009). Our data revealed that GmNAC6 and GmNAC30 are capable of suppressing or activating transcription though coordinated action on common target promoters. They specifically bind to the core DNA binding element TGTG[TGC] *in vitro*, and to TGTG[TGC]-containing promoters *in vivo*. Furthermore GmNAC6- or GmNAC30-mediated transactivation or repression of target promoters is enhanced when both TFs are

provided in trans. These results indicate that the TFs may bind to their target promoters as heterodimers.

As a further support for this hypothesis, we showed that GmNAC6 interacts with GmNAC30 in the nucleus of transfected cells. The heterodimerization of these TFs may provide an explanation for their ability to repress or activate gene expression through the same core DNA binding sequence. It has been shown in yeast one-hybrid assay that conformational interactions between the N-terminal NAC domain and the C-terminal transcriptional activation region (TAR) of NAC proteins are crucial for their activating or repressing activity (Tran et al., 2004; Kim et al., 2007). Here we showed that an intact, full-length GmNAC30 protein was able to transactivate expression of reporter genes in a yeast system, whereas GmNAC6 was impaired in transactivation due to the inhibitory effect of the N-terminal binding domain that imposes conformational constraints to its carboxyl activation domain. Consistent with these findings, GmNAC30 harbors the conserved motif EVQSDPKW in its TAR, which has been categorized as the motif v found in ATAF sub-group members of the NAC family, whereas GmNAC6 lacks a TAR conserved motif (Ooka et al., 2003). It rather possesses a NAC repression domain (NARD), which has been characterized as a highly hydrophobic motif with the core LVFY, located in the N-terminal region of NAC proteins and can efficiently repress the activation capacity of transcriptional factors. Therefore, the ability of the carboxyl terminal domain of GmNAC6 to activate transcription of reporter genes in the yeast system is highly dependent on the deletion of its NARD domain. Homo and heterodimerization of GmNAC6 and GmNAC30 may provide the means for different interactions between the NARD and TAR domains. The current model for transcriptional repressing or activating function of NAC proteins holds that the strength of a NARD function over a TAR function or vice versus renders the NAC protein as repressor or activator. According to this model, the capacity of GmNAC6 and GmNAC30 to function as transcriptional repressor or activator would depend on the conformational assembly of these TFs on their binding site. Different assemblies could be driven by homo or heterodimerization of GmNAC30 and GmNAC6 as well as by promoter context that would provide different stabilization contacts for the DNA:protein interaction.

In vivo binding of GmNAC6 and GmNAC30 to target promoters was assayed by ChIP assay. About 90% of the target promoters isolated in the GmNAC6 and GmNAC30 driven co-immunoprecipitation assays were identical, suggesting that GmNAC6 and GmNAC30 regulate similar genes. Among the shared promoters, only two were not transactivated by

GmNAC6 and GmNAC30 and they did not harbor the core consensus sequence TGTGT[CGT], which was present in all promoter regulated by GmNAC6 and GmNAC30. Furthermore, both GmNAC6 and GmNAC30 were capable to bind specifically to an oligonucleotide contained two copies in tandem of the core sequence, but not to the mutated version TcTcTTGGGTcTcTT. Therefore, we identified a core minimal sequence capable of supporting stable specific binding of GmNAC6 and GmNAC30, which was present in all promoters that displayed GmNAC30 and GmNAC6-mediated repression or transactivation. This TGTGT[GCT] core sequence does not resemble previously characterized NAC family DNA binding elements. For instance, the 25-bp ATAF2 binding site is A/T rich and contains repeats of a [CG]AAA motif either consecutively or in reverse orientation (Wang and Culver, 2012). A core binding element of CGT[GA] was identified as the binding motif for two functionally diverse NAC domain-containing proteins, ANAC019, involved in stress response and ANAC092, implicated in morphogenesis (Olsen et al., 2005). ANAC019, ANAC055, and ANAC072 recognize a 63-bp sequence harboring CACG as the core DNA binding site, which is the reverse complement of the core NAC binding element GGTG (Tran et al., 2004). In contrast, the NAC1 protein binds to a 25-pb sequence harboring an as-1 core element, TGACG (Xie et al., 2000). These previous results indicate that functionally diverse NAC proteins can share similar core DNA binding motives, whereas other NAC TFs bind to quite diverse DNA sequences. This diversity of sequence recognition is likely a consequence from the large family of NAC domain-containing proteins but also may reflect the capacity of heterodimerization among NAC members that would increase the possibility of site specific contacts with different DNA sequences.

GmNAC30 cooperates with GmNAC6 to induce PCD via activation of VPE

Since the discovery of the stress-induced NRPs-mediated cell death signaling which integrates a PCD signal derived from prolonged ER stress and osmotic stress, limited progress has been achieved towards elucidation of components and activation mechanisms of the pathway (Reis and Fontes, 2012). The stress-induced NRP-mediated cell death signaling has been shown to induce a cell death response with the appearance of hallmarks of leaf senescence and PCD (Costa et al., 2008; Faria et al., 2011). Either expression of NRPs or GmNAC6 induce leaf senescence *in planta* and a PCD-like response in protoplasts. Apart from the knowledge that NRPs induce GmNAC6 expression, a regulator of the cell death

response, the events downstream of GmNAC6 that could account for the execution of the cell death program are totally unknown. In the present investigation, in addition to describing GmNAC30 as a molecular partner of GmNAC6, we identified downstream targets of this interaction. Very importantly we showed that GmNAC6 and GmNAC30 induce the expression of caspase1-like VPE underlying a mechanism for execution of the ER stress and osmotic-stress induced cell death program (Figure 11). In this model prolonged ER stress and osmotic stress induce the expression of the transcriptional activator GmERD15 to target the NRP promoter. Up-regulation of NRPs leads to induction of GmNAC6 and GmNAC30, which cooperate to each other to activate VPE promoter and expression. VPE was originally identified as a vacuolar enzyme responsible for maturation of seed storage proteins and various vacuolar proteins (Hara-Nishimura et al., 1991, 1993; Hiraiwa et al., 1997). VPE was then associated with Tobacco mosaic virus–induced hypersensitive cell death (Hatsugai et al., 2004) and more recently with developmental PCD (Nakaune et al., 2005). VPE is a cysteine protease that exhibits caspase-1 like activity and cleaves a peptide bond at the C-terminal side of asparagine and aspartic acid (Hara-Nishimura et al., 2005). Because VPE acts as a processing enzyme to activate various vacuolar proteins, it has been proposed that VPE might also convert the inactive hydrolytic enzymes to the active forms, which are involved in the disintegration of vacuoles, to initiate the proteolytic cascade in plant PCD. As vacuole-triggered PCD is unique to plants, it is not surprise the identification of a stress-induced plant-specific signal transduction pathway as a regulatory circuit that leads to activation of VPE.

Modulation of the stress-induced NRP-mediated cell death signaling under stress conditions has been shown to confer a better adaptation to abiotic stresses. Recent data show that BiP, an ER molecular chaperone, acts as a negative regulator of NRP-mediated cell death signaling and that the manipulation of BiP expression protects plants against drought (Valente et al., 2009; Reis et al., 2011). Therefore, the underlying mechanism of BiP-mediated increase in water stress tolerance has been proposed to be associated, at least in part, with its capacity to modulate the ER stress- and osmotic stress-induced NRP-mediated cell death response (Reis et al., 2011, 2012). Overexpression of BiP in transgenic lines delays and attenuates drought induction of components of the NRP-mediated cell death signaling, such as NRP-A, NRP-B and GmNAC6. The overexpression of BiP in mammals and plants attenuates ER stress and suppresses the activation of the UPR (Morris et al., 1997; Leborgne-Castel et al., 1999; Costa et al., 2008). As a regulator of the UPR, BiP may regulate NRP and GmNAC6 expression through modulation of the UPR. Both GmNAC6 and NRP-B promoters

harbor the unfolded protein response element (UPRE) ccnnnnnnnnnnnccacg (positions -200 on NRP-B promoter and -383 on GmNAC6 promoter), which has the potential to be activated by UPR and hence be modulated by BiP. However, BiP has also been shown to inhibit the cell death activity of NRPs and GmNAC6, as manipulation of BiP levels affected the cell death response mediated by expression of either NRPs or GmNAC6 under the control of 35S promoter (Reis et al., 2011). The results of the present investigation shed light into possible mechanisms for BiP modulation of cell death activity of intermediate components of the stress-induced PCD response. We showed that VPE, which encodes a plant-specific cell death executioner, is transcriptionally regulated by GmNAC6 and GmNAC30. As a vacuolar protein that is targeted to the secretory apparatus, maturation of VPE in the lumen of ER is likely to involve binding to BiP. Therefore, BiP modulation of cell death activities of GmNAC6 and NRPs may be associated with its molecular chaperone function acting upon their target protein VPE. It would be interesting to examine whether VPE is a BiP client protein and whether overexpression of BiP would compromise the secretion of VPE to the vacuoles.

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Author Contributions

G.C.M. co-wrote the manuscript and performed almost all the experiments. P.A.A.R. performed the GmNAC6-dirven CHiP experiment. H.H.C. performed the physiological measurements. J.F.A. transformed the soybean plants. E. P. B. F. co-wrote the manuscript and designed all the experiments

METHODS

Plant growth and stress treatments

Soybean (Glycine max) seeds (cultivar Conquista) were germinated in soil and grown under greenhouse conditions (an average temperature of 21°C, max. 31°C, min. 15°C) under natural light, 70% relative humidity, and approximately equal day and night length. When the seedlings had developed two fully-open trifoliate leaves, the roots, the cotyledons, the leaves and stems were collected as for the vegetative stage of plants. For the reproductive stage of soybean plants, the roots, the leaves, the stems and the seeds were harvested at 80 days after sowing.

For the stress treatments, seven-day-old soil-germinated seedlings were first transferred to ½ Hoagland solution and grown under greenhouse conditions. Then, 21-day-old plants were transferred to 200 mM NaCl, 100 µM abscisic acid (ABA), 100µM jasmonic acid (JA) and 5mM salicylic acid (SA), respectively, for different periods of time, as indicated in the figure legends. For dehydration treatment, 21-day-old plants were transferred and left onto filter paper for 12 h. Low temperature treatment was conducted by transferring 21-day-old plants to 4°C for 12h. Mechanical wounding was induced by cutting leaves of 21-day-old plants with a spatula; the apical blades of all leaves were pressed several times. This methodology resulted in a wound area corresponding to 50% of leaf area (Oh et al., 2005).

To induce ER stress, osmotic stress and cell death, three-week-old plants were excised below the cotyledons and directly treated with 10 µg/mL tunicamycin (Sigma; DMSO, as control), 10% (w/v) polyethylene glycol (PEG; MW 8000, Sigma) or 0.5 µg/ml cycloheximide for the periods indicated in the figure legends, as described (Costa et al., 2008; Irsigler et al., 2007). After all the treatments, the plant material was harvested, immediately frozen in liquid N2 and stored at -80°C until use. Each stress treatment and RNA extraction were replicated in three independent samples.

Quantitative Real-Time PCR (qRT-PCR)

For quantitative RT-PCR, total RNA was extracted from frozen leaves or cells with TRIzol (Invitrogen), according to the instructions from the manufacturer, and treated with RNase-free DNase I,. First-strand cDNA was synthesized from 3 µg of total RNA using oligo-dT(18) and Transcriptase Reversa M-MLV (Invitrogen), according to the manufacturer's instructions. To confirm the quality and primer specificity, we monitored the size of amplification products

by electrophoresis through a 1.5% agarose gel and analysed the TM of amplification products in a dissociation curve. Real-time PCR procedures including pilot tests, validations and experiments were carried out according to the information supplied by the Applied Biosystems manual. Real-time RT-PCR assays were performed on an ABI 7500 instrument (Applied Biosystems), using SYBR® Green PCR Master Mix (Applied Biosystems) with gene-specific primers (Table Supplemental 1 online). The conditions for amplification reactions were as follows: 10 min at 95°C followed by 40 cycles of 94°C for 15 sec and 60°C for 1 min. The variation in gene expression in seedlings and soybean protoplast was quantified using the comparative Ct method ($2^{-\Delta\Delta Ct}$) and absolute gene expression was quantified using the $2^{-\Delta Ct}$ method. The values were normalized to a RNA helicase endogenous control gene (Irsigler et al., 2007). The data was subjected to statistical analysis and the means were compared using confidence Interval by the t test at ($P \leq 0.05$).

Yeast two-hybrid screening

The soybean cDNA library, which was prepared from tunicamycin and PEG-treated leaf mRNA, has been previously described (Alves et al., 2011). The clone pBD-GmNAC6, which harbors GmNAC6 cDNA fused to the GAL4 binding domain, has already been described (Pinheiro et al., 2009) and was used as bait. The yeast reporter strain AH109 ((MATa, Trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, LYS2::GAL1UAS-GAL1TATAHIS3, MEL1 GAL2UAS-GALTATA::MELUAS-MEL1TATA-lacZ) was transformed with pBD-NAC6 along with 2 mg of salmon sperm carrier DNA, using the lithium acetate/polyethylene glycol method. After transformation, the transformants were plated on synthetic dropout medium lacking leucine (Leu) and grown for 3 days at 28ºC. Yeast cells carrying pDN-NAC6 were co-transformed with 12.5 µg of cDNA library along with 400 µl of salmon sperm carrier DNA, according with the *Matchmaker Yeast Two-Hybrid System* (Clontech) prot006Fcol. Transformants were plated on synthetic dropout medium lacking leucine, tryptophan (Trp) and histidine (His), but supplemented with 15 mM 3-aminotriazole and cultured for 5 days at 28°C. The interactions were further confirmed by repicating the positive colonies in the same selective medium for His protrophy and measuring β-galactosidase activity. In this case, positive colonies were transferred to a nylon membrane, lysed with liquid nitrogen and incubated with a β-galactosidase substrate by overlaying the colonies with a 3MM filter paper soaked with Z buffer (16.1 g/L $Na_2HPO_4.7H_2O$, 5.5 g/L $NaH_2PO_4.H_2O$, 0.75 g/L KCl and 0.246 g/l $MgSO_4.7H_2O$), β-

mercaptoetanol and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Approximately 5 x 10^6 transformants were screened as estimated by the number of colonies growing on synthetic-dropout Trp and Leu minus plates. Plasmid DNA isolated from positive yeast transformants was propagated in *E. coli* DH5α and sequenced.

Plasmid Construction

The coding region of GmNAC30 (GenBank accession no. EU661926.1) was isolated by PCR. All the recombinant plasmids were obtained through the GATEWAY system (Invitrogen). To clone the complete GmNAC30 ORF sequence into different expression vectors, the full-length coding region was amplified from leaf cDNA using the primers Fw-GmNAC30 and GmNAC30st-Rvs or GmNAC30ns-Rvs (Supplemental Table 2 online) and inserted by recombination into the entry vector pDONR201 and pDONR207 (Invitrogen) to yield pDONR201St-GmNAC30 (pUFV1389.1), pDONR201Ns-GmNAC30 (pUFV1401.1) and pDONR207Ns-GmNAC30 (pUFV1671.1). Then GmNAC30 cDNA was transferred from the entry vectors to different expression vectors (pDEST22, pDEST32, pK7FWG2, pK7WG2, pDEST15, pSPYCEGW and pSPYNEGW), by recombination, using the enzyme LR clonase (Invitrogen) to generate the clones listed below. For expression in yeast, GmNAC30 cDNA was fused to the GAL4 binding domain yielding pBD-GmNAC30 (pUFV1417) and to the GAL4 activating domain generating pAD-GmNAC30 (pUFV1449). For expression in bacteria, GmNAC30 cDNA was fused to GST resulting in pGST-GmNAC30 (pUFV1479). For transient expression in protoplast, GmNAC30 cDNA was fused to GFP [pGFP-GmNAC30 (pUFV1403)], or not [pK7WG2-GmNAC30 (pUFV1542)] and placed under the control of the 35S promoter into the respective binary vector for plant transformation. For bimolecular fluorescence complementation (BiFC) assay, the full-length coding sequences of GmNAC30 and GmNAC6 were fused to the N-terminal region of YFP to generate pSPYNE-GmNAC30 (pUFV1693) and pSPYNE-GmNAC6 (pUFV1730), respectively and to the YFP C-terminus, producing pSPYCE-GmNAC30 (pUFV1694) and pSPYCE-GmNACE6 (pUFV 1731), respectively.

Two Hybrid assay (Y2H)

Yeast two-hybrid assays were based on Matchmarker GAL4 two-hybrid systems (Clontech). To confirm the interactions, the yeast strain Sacchatomyces cerevisae AH109 was co-transformed with pBD-GmNAC6 and pAD-GmNAC30, which harbors full-length coding

sequences of the respective NAC genes fused to GAL-4 domains (see above). The presence of both expression plasmids was confirmed by growth on SD/-Leu/-Trp plate. To assess protein interaction, the transformed yeasts were grown on SD/-Leu/-Trp/-His + 5mM 3AT (3-amino 1, 2, 4 triazol). The interaction was observed after 3 days of incubation at 28 °C and confirmed by measuring β-Galactosidase activity, which was monitored colorimetrically according to Amberg (2005). The experiments were repeated twice with similar results.

Transactivation assays in yeast cells

The presence of a functional transactivation domain in GmNAC30 was monitored through a yeast transactivation assay, as described previously (Pinheiro et al., 2009). The GmNAC30 coding region was fused to the GAL4-binding domain (pBD-GmNAC30), and the transactivation of the reporter genes was assayed in *Saccharomyces cerevisiae* strain AH109. AH109 competent cells were transformed with pBD-GmNAC32 using the lithium acetate method (R. Daniel Gietz, 1993). Transformants were plated on synthetic dropout medium lacking leucine and histidine, but supplemented with 7,5 mm 3-aminotriazole, and cultured for 3 days at 28 °C. β-Galactosidase activity was monitored colorimetrically, as described by Amberg (2005).

Transient expression in protoplasts

Protoplasts were prepared directly from soybean leaves as described (Franceschi, 1984), with some modifications. Briefly, the protoplasts were isolated by digestion for 7 h, under agitation at 50 rpm, with 2% cellulase, 0.1% pectolyase Y23, 0.6 M mannitol and 20 mM MES, pH 5.5. The extent of digestion was monitored by examining the cells microscopically every 30 min. After filtration through nylon mesh of 65 μm, the protoplasts were recovered by centrifugation, resuspended in 2 mL of 0.6 M mannitol plus 20 mM MES, pH 5.5, separated by centrifugation in a sucrose gradient (20% [w/v] sucrose, 0.6 M mannitol and 20 mM MES, pH 5.5) and diluted with 2 mL of electroporation buffer (25 mM HEPES-KOH, pH 7.2, 10 mM KCl, 15 mM MgCl2 and 0.6 M mannitol). Transient expression assays were performed by electroporation (250 V, 250 μF) of 10 μg of the expression cassette DNA and 30 μg of sheared salmon sperm DNA into 2×10^5 - 5×10^6 protoplasts in a final volume of 0.8 mL. Protoplasts were diluted into 8 ml of MS medium supplemented with 0.2 mg/ml 2, 4-dichlorophenoxyacetic acid and 0.6 M mannitol, pH 5.5. After 36 h of incubation in the dark,

the protoplasts were washed with 0.6 M mannitol plus 20 mM MES, pH 5.5 and frozen in liquid N2 until use.

Subcellular Localization Assay

Soybean leaf protoplasts were electroporated with the plasmid pGFP-GmNAC30, as described above. The subcellular distribution of GmNAC30 protein was visualized by fluorescence microscopy. After incubation for 36 h, the protoplasts were collected and treated with 2 µg/mL 4',6-diamidino-2-phenylindole (DAPI) for 45 min and visualized with a Olympus BX61 fluorescence microscope coupled to a digital camera. For fluorescence detection, the excitation wavelength and the emission wavelength were 514 nm and 535-590 nm, respectively, for YFP, and 358 nm and 461 nm, respectively, for DAPI (nuclear staining).

Bimolecular Fluorescence Complementation (BiFC)

Soybean leaf protoplasts were co-transformed, as described above, with the combinations of the recombinant plasmids: pSPYNE-GmNAC30 + pSPYCE-GmNAC6; pSPYCE-GmNAC6 + pSPYNE-GmNAC6; pSPYCE-GmNAC30 + pSPYNE empty vector and pSPYNE-GmNAC6 + pSPYCE empty vector. After incubation for 36h, the protoplasts were collected and treated with 2 µg/mL DAPI for 45 min and visualized with a Olympus BX61 fluorescence microscope coupled to a digital camera. For fluorescence detection, the excitation wavelength and the emission wavelength were 514 nm and 535-590 nm, respectively, for YFP, and 358 nm and 461 nm, respectively, for DAPI. The experiments were repeated twice with similar results.

Chromatin immunoprecipitation (ChIP) Assay

CHiP assay was performed using a chromatin immunoprecipitation kit (Imprint ChIP kit; SIGMA), as recommended by the manufacturers. Soybean protoplasts prepared from leaves were electroporated with 35S::GmNAC30-GFP (pGFP-GmNAC30) and 35S::YFP-GmNAC6 (Pinheiro et al., 2009). After 36-h of induction, DNA-bound proteins were cross-linked with 1% (v/v) formaldehyde, resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS), followed by sonication. The protein extracts were incubated with monoclonal antibodies anti-GFP, anti-human RNA polymerase II, or normal mouse IgG. The immunecomplexes were washed and the DNA was recovered by using a chromatography column and eluted in 50 µL of 10 mM Tris-HCl, pH 8.5. The DNA was used as template for

PCR amplification with a set of degenerated primers (Whole Genome Amplification Kit, Sigma). The amplified fragments were cloned in the entry vector pCR-TOPO8, sequenced and mapped in the soybean genome using the soybean database at Phytozome (www.phytozome.net/soybean).

Induction, purification and quantitation of recombinant proteins.

E. coli strain BL21 was transformed with GST-GmNAC6 or GST-GmNAC30 and 5 mL of overnight-grown transformed cells were used to inoculate 500 ml of LB medium, which were incubated under shaking at 37^0C. When the cells reached an A600nm of 0.4–0.6, the expression of the NAC genes were induced by 2 mM isopropyl-β-D-thiogalactopyranoside and growth continued for 8 h at 28^0C. After the incubation period, the cells were centrifuged at 5.000 x *g* for 10 min, and re-suspended in 10 mL of PBS 1X (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH7,3) supplemented with 100 µL of a protease inhibitor solution containing 100 mM PMSF, 50 mM Thiourea and 50 mM bnezamidine. Then, lysozyme (100 $\mu g.mL^{-1}$) was added to the mixture, which was incubated on ice for 30 min. The cells were disrupted by sonication and cell debris was removed by centrifugation at 10.000 x *g* for 20 min. The induced proteins were affinity-purified using a Glutathione Sepharose 4B resin (*GE Healthcare* Life Sciences), previously equilibrated with lysis buffer, and after 4 h under gentle agitation, the bound proteins were eluted as recommended by the manufacturers. Purified proteins were quantified according to Bradford (1976), using BSA as a standard.

Electrophoretic Mobility Shift Assay

Double-stranded consensus sequences 5´ TGTGTTGGGTGTGTTTGG 3´, which was derived from analysis of the promoters isolated by ChIP, were biotinylated using the 3′ OH biotin labeling kit from Pierce. Electrophoretic mobility shift assays were performed using a LightShift Chemiluminescent EMSA kit (Pierce), according to the manufacturer's protocol. Each 20-µl binding reaction contained 20 mM HEPES at pH 7.9, 100 mM KCl, 5% (v/v) glycerol, 1 mM MgCl2, 1 mMDTT, 0.1% (v/v) Tween 20, 25 ng/ml poly(dI-dC), 20 fmol biotin-labeled probe, and 2 µg of GST-GmNAC6 and/or GST-GmNAC32. Unlabeled DNA was used as a competitor. The binding reactions were incubated at room temperature for 20 min. A 6% polyacrylamide minigel (37.5:1 acrylamide: bisacrylamide in 0.5 mm Tris borate-EDTA containing 3% glycerol) was prerun for 1 h at 100 V with 0.5 mm Tris borate-EDTA

(TB buffer).5 µl of loading buffer was added to the binding reaction and 20 µL of the reaction were separated by gel electrophoresis at 80 V for 90 min. The gel was then transferred to a charged nylon membrane (Amersham Biosciences Bioscience) for 1 h at 320 mA, UV cross-linked to the membrane at 120 mJ/cm2 for 1 min, and visualized by chemiluminescence.

GUS activity assays

A 2000-pb fragment of 5' flanking sequences of D-alanyl-d-alanine carboxypeptidase (ACPase) gene and a 1000-pb fragment of 5' flanking sequences of 1-aminocyclopropane-1-carboxylase synthase (ACC) gene (http://www.phytozome.net/soybean), relative to the translational initiation codon, were amplified from soybean DNA with the primers as listed in Supplemental Table 3. The amplified fragments were cloned into the TOPO-pCR8 entry vector (Invitrogen), and then transferred to pMDC162 by recombination with LR clonase to yield $pACPase_{pro}$:GUS (pUFV2066) and $pACC_{pro}$:GUS (pUFV2067).

The protein extraction and fluorometric assays for GUS activity were performed essentially as described by Jefferson et al. (1987) with methylumbelliferone (MU) as a standard. For the standard assay, protoplasts were ground in 0.5 mL of GUS assay buffer (100 mM NaH2PO4 ·H2O [pH 7.0], 10 mM EDTA, 0.1% [w/v] sarcosyl, and 0.1% [v/v] Triton X-100), and 25 µL of this extract were mixed with 25 µL of GUS assay buffer containing 2 mM of the fluorescent 4-methylumbelliferone β-D glucuronide (MUG) as a substrate (Freitas et al., 2007)). The mixture was incubated at 37°C in the dark for 30 min, and GUS activity was measured using a Lector Multi-Modal de Microplacas Synergy HT (BioTek). The total protein concentration was determinate by Bradford method (Bradford, 1976). The experiments were repeated three times with similar results.

Ethylene determination

Ethylene measurements were carried out as described by Costa et al. (2008).

Phylogenetic analysis

Aminoacid and (nucleotide) sequences used in phylogenetic analyses were aligned using the MUSCLE (Edgar, 2004) module in MEGA v. 5 software. Phylogenetic analysis were performed with 152 NAC sequences obtained from Le et al., (2011). Unrooted phylogenetic trees were constructed by the neighbor-joining method implemented in MEGA v. 5 with model Tamura 3-parameter. The robustness of each internal branch was estimated from 5 000 bootstrap replications.

Accession number

Sequence data from this article can be found in the Soybean Genome initiative under the following accession numbers: GmNAC30 (Glyma05g32850.1), GmNAC018 (Glyma04g38560), GmNAC039 (Glyma06g16440), GmNAC022 (Glyma04g42800), GmNAC035 (Glyma06g11970), GmNAC109 (Glyma14g24220), GmNAC011 (Glyma02g26480), SMP (AW397921), CNX (AW508066), BiP (AF031241), Serine-Threonine Protein Kinase (Glyma18g44930), Non-specific serine/threonine protein Kinase (Glyma17g17520), ACC (Glyma17g16990); ACC (Glyma05g36250); ACC (Glyma16g03600), ACC (Glyma11g04890); ACC (Glyma05g23020); ACC (Glyma06g05240), ACC (Glyma04g05150); ACC (Glyma01g00700); ACC (Glyma08g03400), ACC (Glyma07g15380), VPE (Glyma14g10620), VPE (Glyma17g34900), VPE (Glyma01g05135), VPE (Glyma04g05250), VPE (Glyma06g05350). Sequence data from this article can be found in the Arabidopsis Genome initiative under the following accession numbers: ATAF1 (At1g01720), ANAC032 (At1g77450), ATAF2 (At5g08790), ANAC102 (At5g63790),
MYB (At5g05800), ACC8 (AT4G37770), ACC9 (AT3G49700), ACC4 (AT2G22810), ACC5 (AT5G65800), ACC11 (AT4G08040), ACC7 (AT4G26200), ACC1 (AT3G61510), ACC2 (AT1G01480), ACC6 (AT4G11280), ACC12 (AT5G51690.), ACC10 (AT1G62960), ACC (AT5G36160), ACC (AT1G23310), ACC (AT1G77670), ACC (AT2G20610). ACC (AT1G17290), ACC (AT1G70580), ACC (AT2G24850), ACC (AT1G72330), ACC (AT4G28420), ACC (AT4G23600), ACC (AT4G23590),ACC (AT4G28410).

Supplemental Data

Supplemental Figure 1. GmNAC6 interacts with GmNAC30 by the yeast two-hybrid system.

Supplemental Figure 2. Structural organization of GmNAC30 as a member of the soybean NAC domain-containing protein family.

Supplemental Figure 3. Expression analysis of GmNAC6 and GmNAC30 fusions in soybean protoplasts electroporated with the BiFC DNA constructs.

Supplemental Figure 4. **GmNAC6 and GmNAC30 do not regulate the ChIP-identified genes harboring a TGTGGT[TCG]-less promoter.**

Supplemental Figure 5. Expression analysis of GmNAC30, GmNAC6 and At5g05800 genes in the GmNAC30- and GmNAC6-dependent promoter regulation assay in soybean protoplasts.

Supplemental Figure 6. Phylogenetic analysis-based on ACC synthase genes from soybean and Arabidopsis

Supplemental Table1. Primers used for expression analysis by quantitative real time RT-PCR (qRT-PCR)

Supplemental Table 2. Primers used for cloning GmNAC30 coding region

Supplemental Table 3. Primers used to cloned promoters

Table 1. GmNAC030 target genes indentified via ChIP assay

Gene	Locus	Gene locus distance from ATG	Frequency of positive colonies	Consensus
Predicted small molecule transport	Glyma20g03960	5 Kb	1	TGTGT(T)
Adenylosuccinate lyase	Glyma20g28590	2 Kb	1	TGTGT(G)
Permease of the major facilitator superfamily	Glyma05g27410	20 Kb	1	TGTGT(T)
MYB family	Glyma17g17560	5 Kb	1	TGTGT(C)
Microtubule-associated proteins 1A/1B light chain-3 related	Glyma0504560	1 Kb	1	TGTGT(T)
Alpha/Beta hydrolase	Glyma17g05080	1 Kb	1	TGTGT(T)
Iron/ascorbate family oxidoreductases	Glyma08g41980	20 Kb	1	TGTGT(T)
1-aminocyclopropane-1-carboxylase synthase	Glyma17g16990	1 Kb	1	TGTGT(G)
d-alanyl-d alanine carboxipeptidase	Glyma18g02260	2 Kb	6	TGTGT(T)
S-adenosylhomocysteine hidrolase	Glyma05g28480	1 Kb	1	TGTGT(T)
Dual specificity protein phosphatase	Glyma06g15000	20 Kb	1	TGTGT(G)
Predicted endo 1,3 beta glucanase	Glyma18g50180	10 Kb	1	TGTGT(T)
Protein of unknown function	Glyma17g35070	2 Kb	1	TGTGT(G)
FE(II)/ Ascorbate Oxidase Superfamily	Glyma01g33350	2 Kb	1	TGTGT(C)
Protein of unknown function	Glyma05g25260	5Kb	1	TGTGT(C)
Protein of unknown function	Glyma20g22140	5 Kb	1	TGTGT(C)
CDC2-Related Kinase	Glyma06g17460	5Kb	2	TGTGT(T)
Non-specific serine/threonine protein kinase	Glyma17g17520	20 Kb	1	-
Serine-Threonine Potein Kinase	Glyma18g44930	2 Kb	1	-
60S Ribosomal Protein L9 Family Member	Glyma04g40470	10Kb	1	TGTGT(A)
30S Ribosomal Protein S3	Glyma20g21190	10 Kb	1	TGTGT(A)
Replication Factor C / DNA Polymerase III	Glyma17g13560	5 Kb	1	TGTGT(A)
Mitochondrial Carrier Protein Related	Glyma17g34240	2 Kb	1	TGTGT(G)
Cytochrome P450 CYP2 subfamily	Glyma06g21920	1 Kb	1	TGTGT(A)
Kinesin Heavy Chain	Glyma06g41600	20 Kb	1	TGTGT(T)
Glycerol -3-Phosphate Dehydrogenase	Glyma05g24070	2 Kb	1	TGTGT(G)
ATP Dependent Protease Family member	Glyma05g05820	20 Kb	1	TGTGT(G)

Table 2. GmNAC6 target genes indentified via ChIP assay

Gene	Locus	Gene locus distance from ATG	Frequency of positive colonies	Consensus
Predicted small molecule transporter	Glyma20g03960	5 Kb	1	TGTGT(T)
Adenylosuccinate lyase	Glyma20g28590	2 Kb	1	TGTGT(G)
		20 Kb	1	TGTGT(T)
Permease of the major facilitador superfamily	Glyma05g27410			
MYB family	Glyma17g17560	5 Kb	1	TGTGT(C)
Microtubule-associated proteins 1A/1B light chain-3 reletated	Glyma05g04560	1 Kb	1	TGTGT(T)
Alpha/Beta hidrolase	Glyma17g05080	1 Kb	1	TGTGT(T)
Iron/ascorbate family oxidoreductases	Glyma08g41980	20 Kb	1	TGTGT(T)
1-aminocyclopropane-1-carboxylase synthase	Glyma17g16990	1 Kb	1	TGTGT(G)
d-alanyl-d alanine carboxipeptidase	Glyma18g02260	2 Kb	4	TGTGT(T)
S-adenosylhomocysteine hidrolase	Glyma05g28480	1 Kb	1	TGTGT(T)
Dual specificity protein phosphatase	Glyma06g15000	20 Kb	1	TGTGT(G)
Predicted endo 1,3 beta glucanase	Glyma18g50180	10 Kb	1	TGTGT(T)
Protein of unknown function	Glyma17g35070	2 Kb	1	TGTGT(G)
FE(II)/ Ascorbate Oxidase Superfamily	Glyma01g33350	2Kb	1	TGTGT(T)
Protein of unknown function	Glyma05g25260	5 Kb	1	TGTGT(T)
Protein of unknown function	Glyma20g22140	5 Kb	1	TGTGT(C)
CDC2-Related Kinase	Glyma06g17460	5Kb	2	TGTGT(T)
Non-specific serine/threonine protein kinase	Glyma17g17520	20 Kb	1	-
Serine-Threonine Potein Kinase	Glyma18g44930	2Kb	1	-
EF-1 guanine nucleotide Exchange domain Elongation factor 1beta/delta chain	Glyma02g44460	20Kb	1	TGTGT(A)
Mitochondrial transcription termination factor, mTERF	Glyma15g16400	2Kb	1	-
Protein of unknown function	Glyma13g02220	5Kb	1	TGTGT(C)
Protein of unknown function	Glyma07g36130	2Kb	1	TGTGT(G)
CAF1 family ribonuclease	Glyma07g02730	2Kb	1	TGTGT(T)

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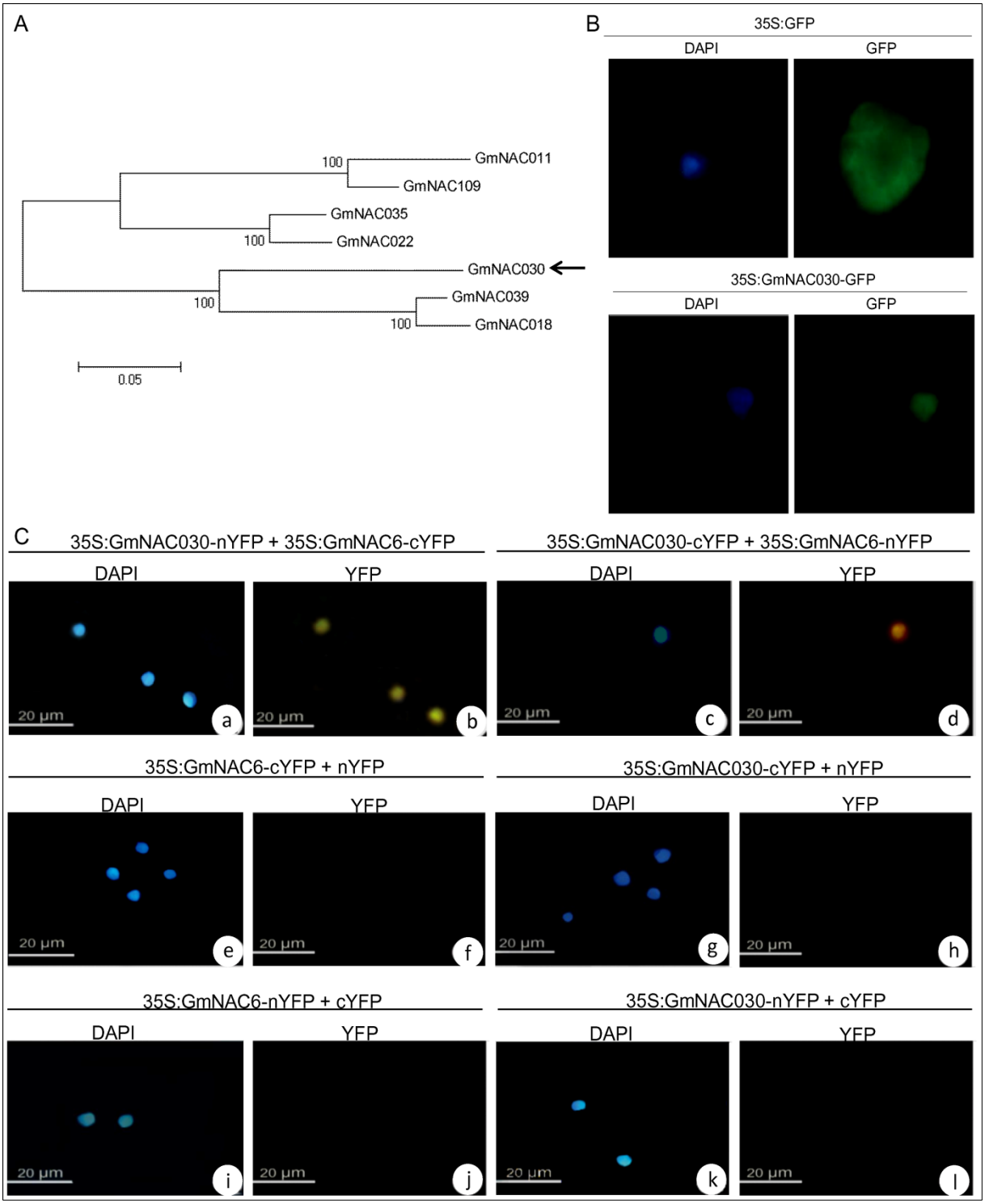


Figure 1. GmNAC30 is a nuclear protein that binds to GmNAC6 in the nucleus of plant cells.

(A) GmNAC30 homologs in soybean. The dendogram is an illustration of sequence relatedness of the GmNAC30 closest members of the soybean NAC domain-containing protein family. The arrow indicates the position of GmNAC30. **(B)** GmNAC30 is localized in the nucleus. The 35S::GFP and 35S:GmNAC030-GFP constructs were expressed transiently

in soybean leaf protoplasts and the subcellular localization of GFP and fusion protein was visualized by fluorescence microscopy 36-h after electroporation of protoplasts. Nuclei were stained with DAPI. **(C)** In vivo interaction between GmNAC6 and GmNAC30 by BiFC analysis. Fluorescence (YFP) images were taken from soybean leaf protoplasts co-expressing 35S:GmNAC030-nYFP + 35S:GmNAC6-cYFP, 35S:GmNAC030-cYFP + 35S:GmNAC6-nYFP fusion proteins 36 h after electroporation of protoplasts with the indicated DNA constructs. Negative controls are: 35S:GmNAC6-cYFP + nYFP, 35S:GmNAC030-cYFP + nYFP, 35S:GmNAC6-nYFP + cYFP, 35S:GmNAC030-nYFP + cYFP, as indicated in the figure. Scale bars = 20 µm

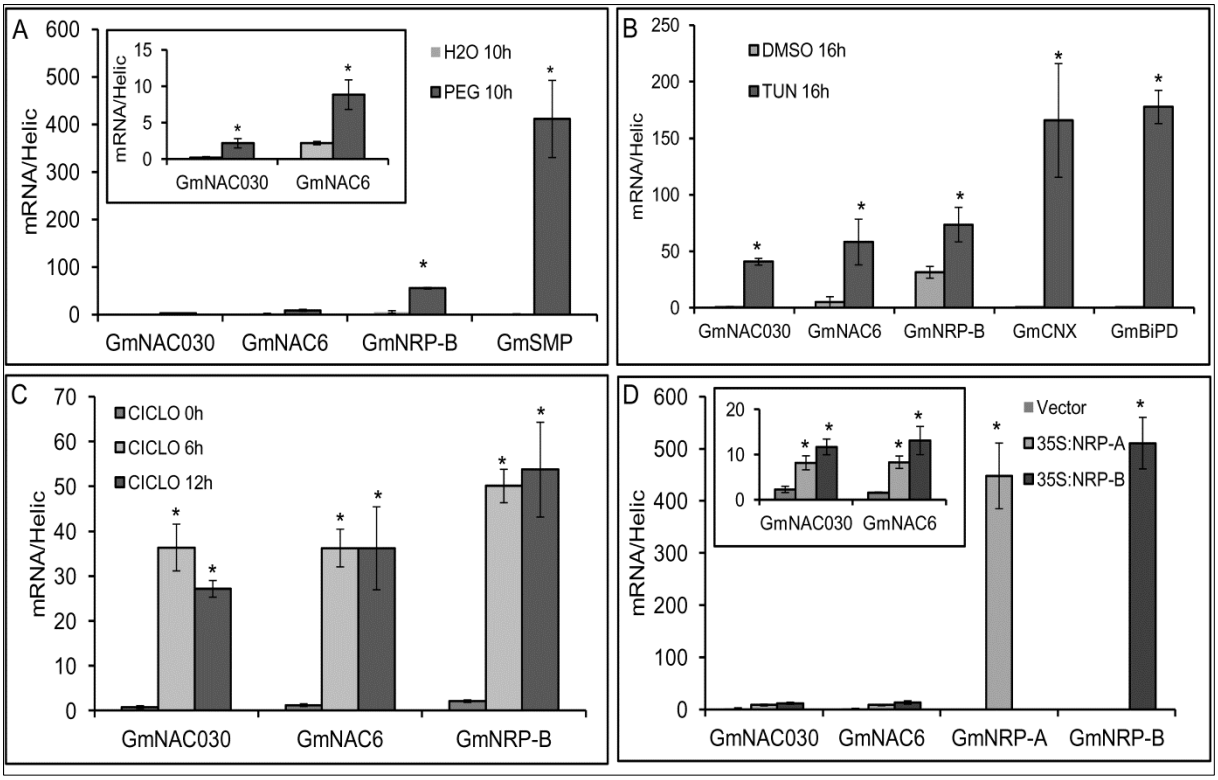


Figure 2. **GmNAC30 may be a downstream component of the ER stress- and osmotic stress-induced NRP-mediated cell death signaling**.

(A,B,C) GmNAC30 is induced by osmotic stress, ER stress and cell death inducers. Soybean seedlings were treated with the osmotic stress inducer PEG for 10 h **(A)**, the ER stress inducer tunicamycin for 16 h **(B)** or the cell death inducer cycloheximide for 6h and 12 h. After exposition to the stress treatments the expression of the indicated genes was monitored by qRT-PCR. Gene expression was calculated using the $2^{-\Delta CT}$ method and RNA helicase as an endogenous control. GmSMP is seed maturation protein used as control for PEG treatment. GmCNX (calnexin) and GmBiPD (binding protein) are ER stress markers. GmNAC6 and GmNRP-B are components of the ER stress- and osmotic stress-integrating cell death pathway. Values are given as mean ± I.C of three independent determinations. Asterisks indicate significant differences from the controls by the t test at $P \leq 0.05$. **(D)** GmNRP-A and GmNRP-B induce the expression of the GmNAC30 gene. Plasmids containing GmNRP-A and GmNRP-B expression cassettes or empty vector were electroporated into soybean protoplasts and the gene expression of GmNAC genes was monitored by qRT-PCR as above.

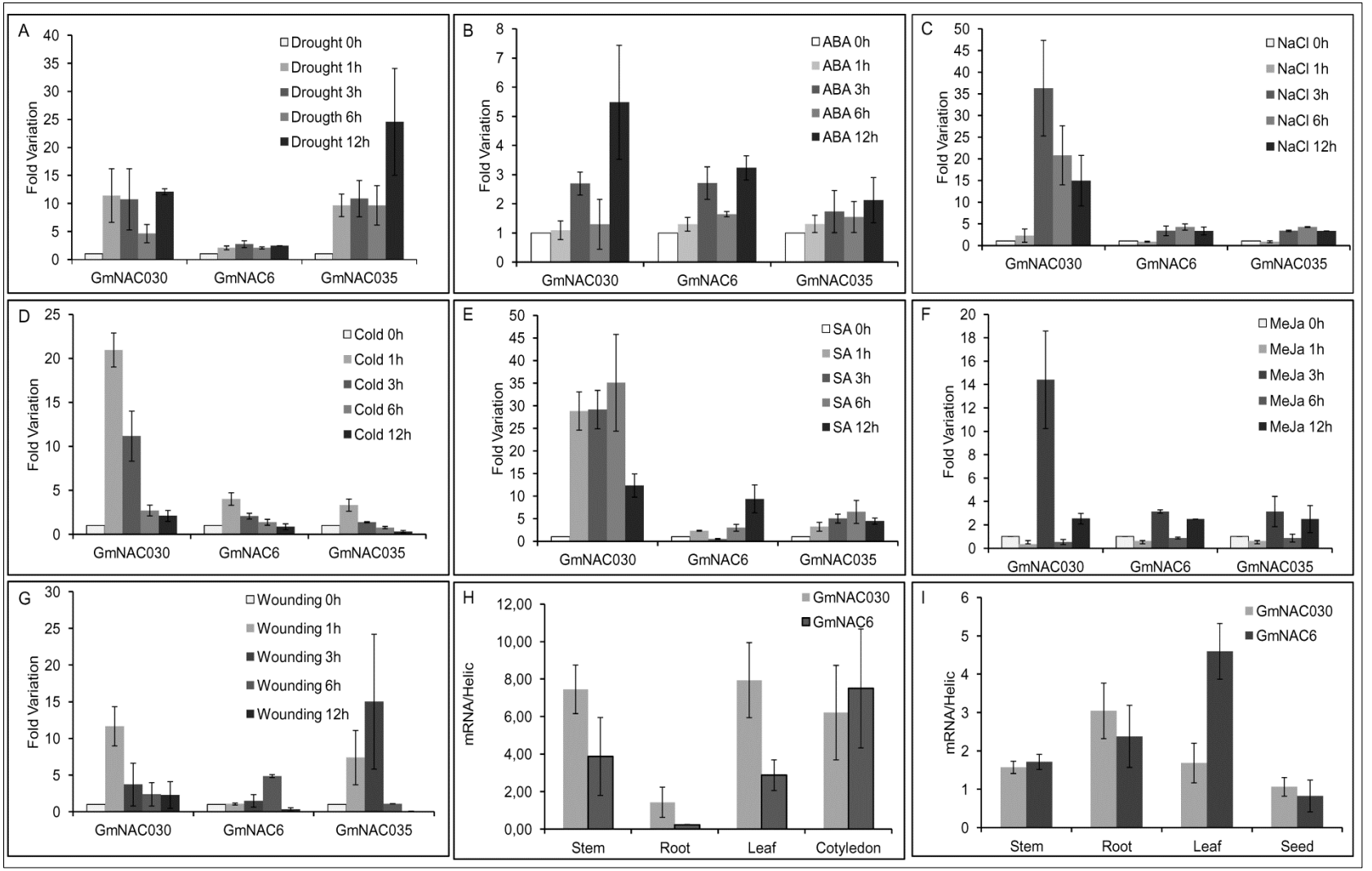

A
Fold Variation
Drought 0h
Drought 1h
Drought 3h
Drough 6h
Drought 12h
GmNAC030
GmNAC6
GmNAC035
B
Fold Variation
ABA 0h
ABA 1h
ABA 3h
ABA 6h
ABA 12h
GmNAC030
GmNAC6
GmNAC035
C
Fold Variation
NaCl 0h
NaCl 1h
NaCl 3h
NaCl 6h
NaCl 12h
GmNAC030
GmNAC6
GmNAC035
D
Fold Variation
Cold 0h
Cold 1h
Cold 3h
Cold 6h
Cold 12h
GmNAC030
GmNAC6
GmNAC035
E
Fold Variation
SA 0h
SA 1h
SA 3h
SA 6h
SA 12h
GmNAC030
GmNAC6
GmNAC035
F
Fold Variation
MeJa 0h
MeJa 1h
MeJa 3h
MeJa 6h
MeJa 12h
GmNAC030
GmNAC6
GmNAC035
G
Fold Variation
Wounding 0h
Wounding 1h
Wounding 3h
Wounding 6h
Wounding 12h
GmNAC030
GmNAC6
GmNAC035
H
mRNA/Helic
GmNAC030
GmNAC6
Stem
Root
Leaf
Cotyledon
I
mRNA/Helic
GmNAC030
GmNAC6
Stem
Root
Leaf
Seed

Figure 3. **Expression profile of GmNAC6 and GmNAC30 genes exposed to different environmental and developmental stimuli**.

Time course of GmNAC30, GmNAC6 and GmNAC35 induction by exposition of soybean plants at the VC developmental stage to a drought regime **(A),** abscisic acid (ABA) treatment **(B),** NaCl **(C)**, Cold **(D)**, salicylic acid (SA) treatment **(E),** methyl jasmonate (MeJa) treatment **(F)** and wounding **(G**) for the indicated period of time. The fold variation of gene expression (in relation to control treatments) was calculated using $2^{-\Delta\Delta Ct}$ method and helicase as an endogenous control. Error bars represent the confidence interval ($\alpha = 0.05$) of three biological replicates. GmNAC35 is closely related to GmNAC30 and was used as control. **(H and I)** GmNAC6 and GmNAC30 expression in different organs during the vegetative (H) and reproductive (I) phases of development. The transcript levels of the indicated genes were quantified by qRT-PCR. Gene expression was calculated using the $2^{-\Delta CT}$ method and RNA helicase as an endogenous control. Error bars represent the confidence interval ($\alpha = 0.05$) of three biological replicates.

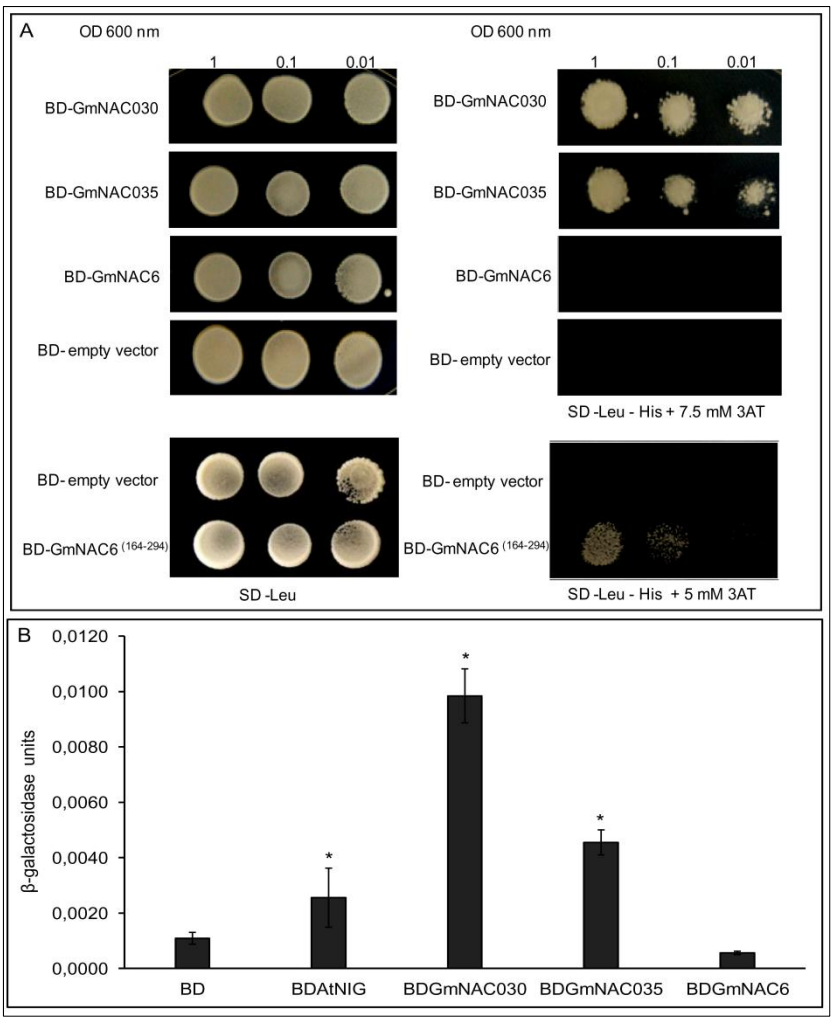


Figure 4. Transcriptional activation activity of GmNAC30.

(A) Transactivation assay for histidine prototrophy. The plasmids containing the BD-Gal4 fusions, as indicated in the Figure, and the empty vector (pDEST32) were introduced separately into yeast strain AH109. The transformants were incubated for 3 days at 28 °C in SD media lacking leucine (SD-Leu) or lacking leucine and histidine, but supplemented with 7.5 mM 3-aminotriazol (3AT; top panel) or 5 mM 3AT (bottom panel). BD-GmNAC6$^{(164-294)}$ indicates the C-terminal region of GmNAC6 fused to the binding domain (BD) of GAL4, whereas all the other constructs are fusions of full-length NAC proteins with BD-GAL4**. (B)** Transactivation of the β-Galactosidase reporter gene. expression._ β -Galactosidase activity was determined from total protein extracts of yeast strains carrying the indicated plasmids or empty vector (pBD). AtNIG is an unrelated protein from Arabidopsis used as positive control. Error bars represent the confidence interval ($\alpha = 0.05$) of three biological replicates and asterisks indicate values significantly different from the negative control pBD ($P \leq 0.05$, t test).

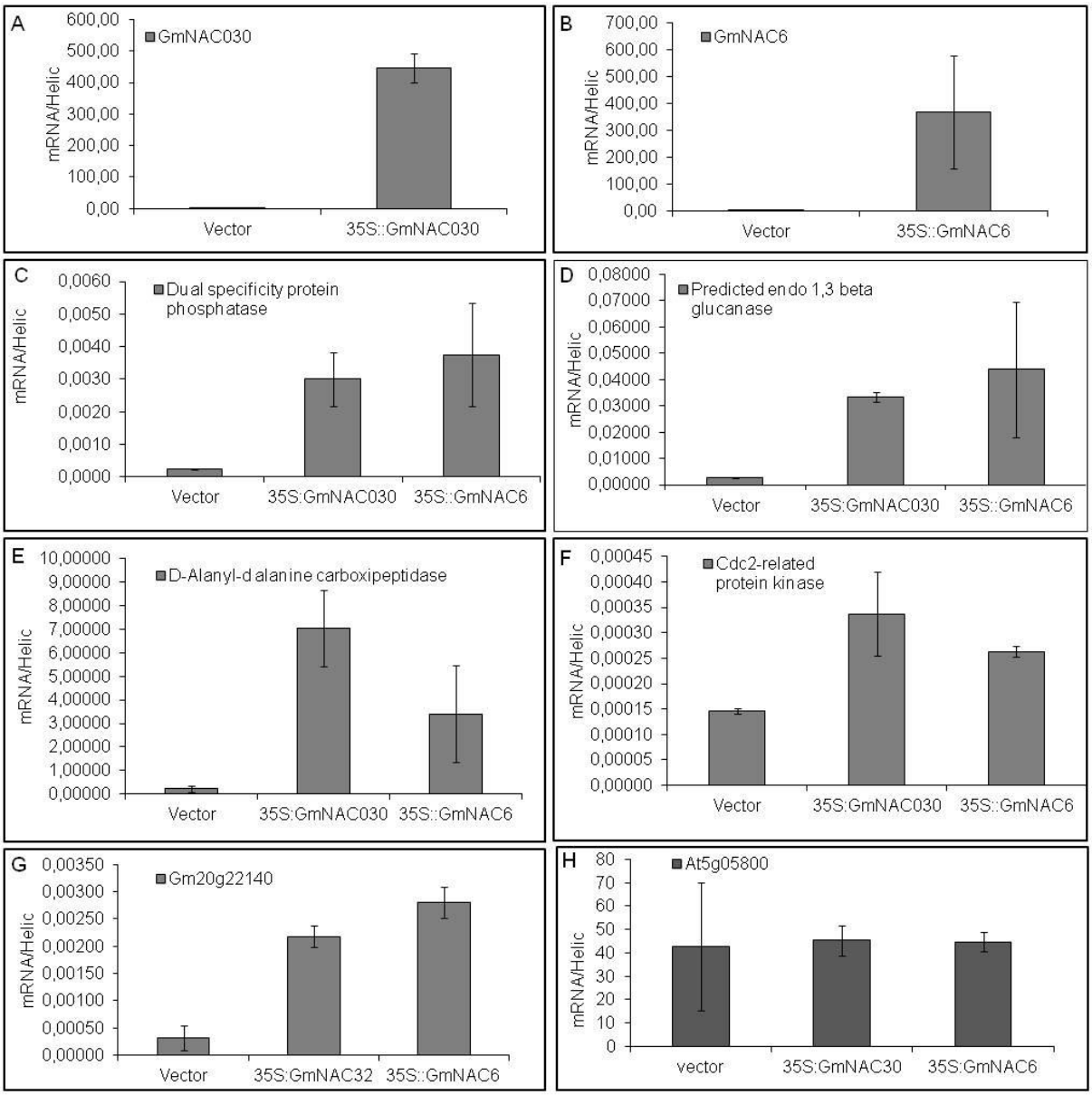


Figure 5. Common target genes up-regulated by GmNAC6 and GmNAC30.

(A and B) Ectopic expression of GmNAC6 (A) and GmNAC30 (B) in soybean protoplasts. Soybean protoplasts were electroporated with the 35S:YFP-NAC6 construct or the empty vector, as well as with 35S::GmNAC30 construct or the empty vector and the expression of GmNAC6 and GmNAC30 was monitored by qRT-PCR. Gene expression was calculated using the $2^{-\Delta CT}$ method and RNA helicase as an endogenous control. Error bars represent the confidence interval ($\alpha = 0.05$) of three biological replicates**. (C-G)** Up-regulation of the indicated genes by transient expression of GmNAC6 and GmNAC30 in soybean protoplasts. After 36-h electroporation of soybean protoplasts with GmNAC6, GmNAC30 or the empty vector, the transcript levels of the indicated genes were quantified by qRT-PCR. Gene expression was calculated using the $2^{-\Delta CT}$ method and RNA helicase as an endogenous

control. Error bars represent the confidence interval ($\alpha = 0.05$) of three biological replicates. Phytozome accession of each gene is given in Tables 1 and 2.

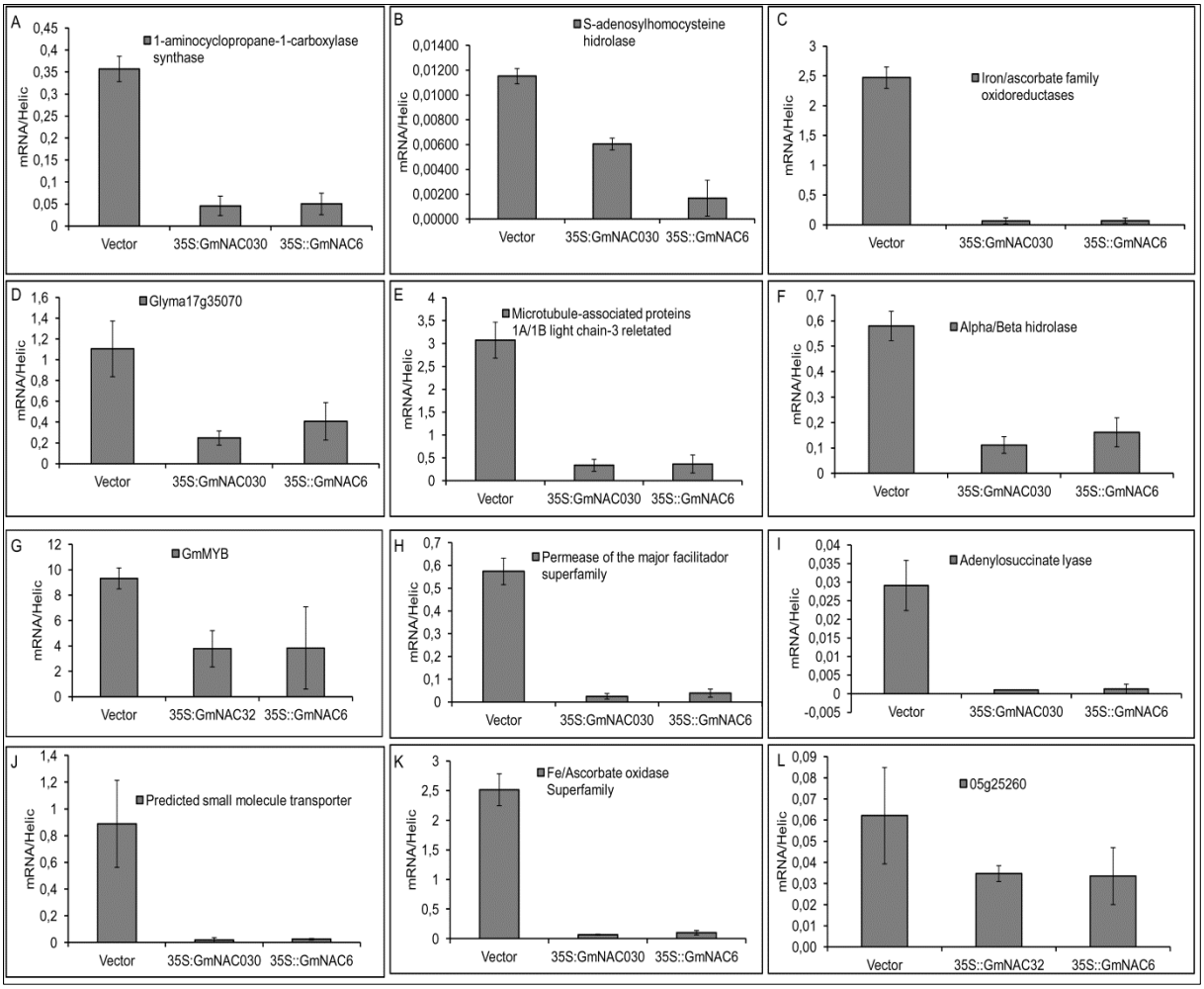


Figure 6. Common target genes down-regulated by GmNAC6 and GmNAC30.

(A-L) 36-h post-electroporation of soybean protoplasts with GmNAC6, GmNAC30 or the empty vector, the transcript levels of the indicated genes were quantified by qRT-PCR. Gene expression was calculated using the $2^{-\Delta CT}$ method and RNA helicase as an endogenous control. Error bars represent the confidence interval ($\alpha = 0.05$) of three biological replicates. Phytozome accession of each gene is given in Tables 1 and 2.

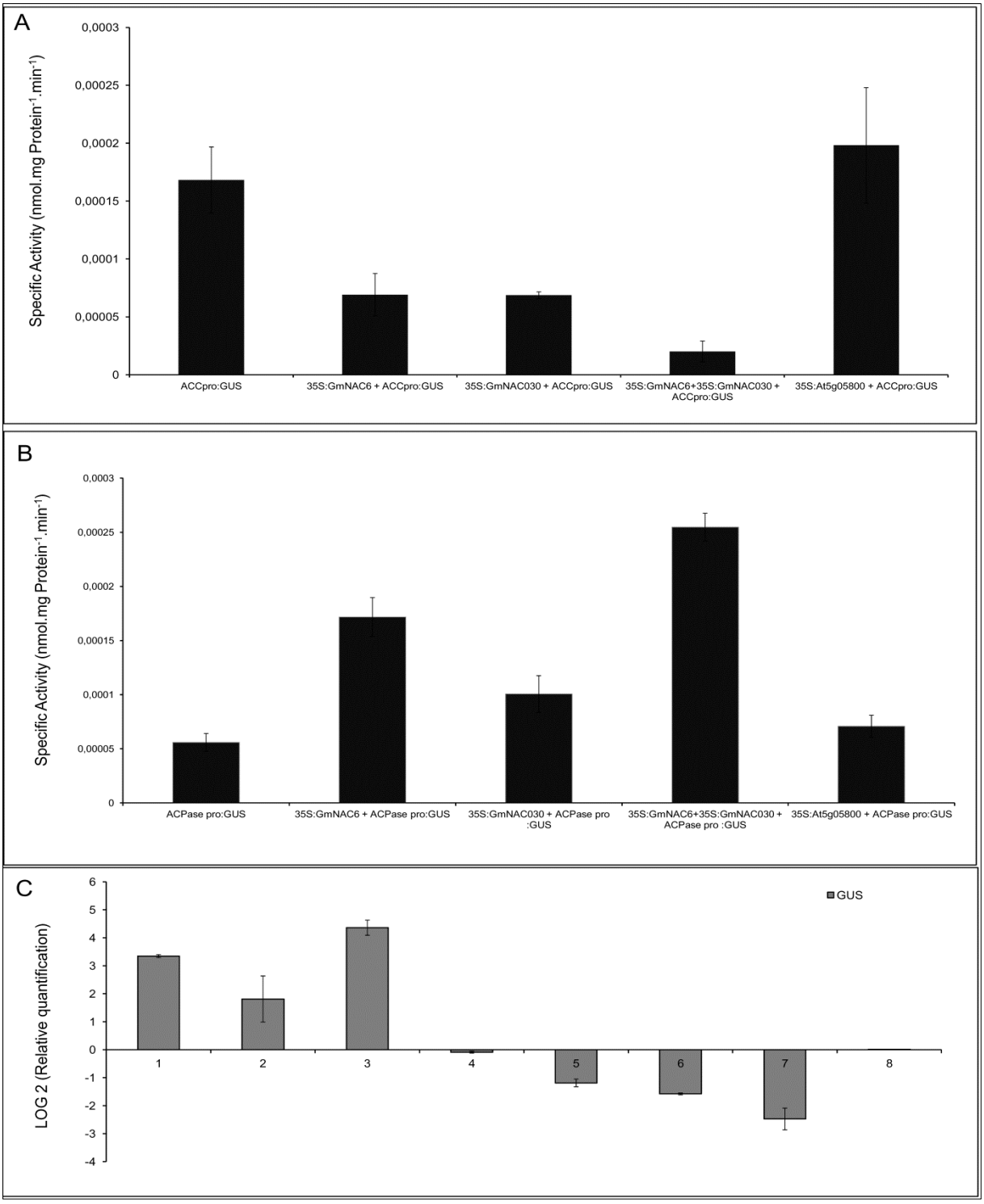


Figure 7. GmNAC6 along with GmNAC30 determine full activation or repression of target promoters.

(A) GmNAC30 and GmNAC6 repress an ACC synthase promoter. Soybean protoplasts were co-electroporated with plasmids carrying 1000pACC$_{pro}$:β-glucuronidase (GUS) gene and either 35S:GmNAC6 DNA constructs or 35S:GmNAC30 DNA constructs or a combination of both DNA constructs. After 48 h, β-glucuronidase activity (nmol/min/mg protein) was

measured from the total protein extracts of transfected soybean cells. An unrelated Myb transfactor, 35S:At5g05800, was used as negative control. Error bars represent the confidence interval ($\alpha = 0.05$) of three biological replicates. **(B)** Full activation of the carboxypeptidase (ACPase) promoter requires both GmNAC6 and GmNAC30. Soybean protoplasts were co-electroporated with plasmids carrying 2000pACPase$_{pro}$:β-GUS gene and the same combinations of DNA constructs as described in A and samples were processed for β-glucuronidase activity as in A. **(C)** Quantitation of GUS reporter gene expression by quantitative RT-PCR. Soybean protoplasts were electroporated with the same DNA constructs as described in A and B, and GUS transcript levels were monitored by quantitative RT-PCR. The relative quantitation showed in log2 scale of gene expression was calculated using $2^{-\Delta\Delta Ct}$ method and helicase as endogenous control. The values are relative to the control treatment (empty vector), and error bars represent the confidence interval ($\alpha = 0.05$) of three biological replicates. An unrelated Myb transfactor, 35S::At5g05800, was used as negative control. The X axis numbers are the results of co-electroporation with the following DNA constructs: 1-35S:GmNAC6 + 2000pACPase$_{pro}$:β-GUS; 2- 35S:GmNAC30 + 2000pACPase$_{pro}$:β-GUS; 3.-35S:GmNAC6+ 35S:GmNAC30 2000pACPase$_{pro}$:β-GUS; 4. 35S:At5g05800 + 2000pACPase$_{pro}$:β-GUS; 5. 35S:GmNAC6+ 1000pACC$_{pro}$:GUS; 6. 35S:GmNAC30 + 1000pACC$_{pro}$:GUS; 7. 35S:GmNAC6+ 35S:GmNAC30 + 1000pACC$_{pro}$:β-GUS; 8-35S:At5g05800 + 1000pACC$_{pro}$: β -GUS

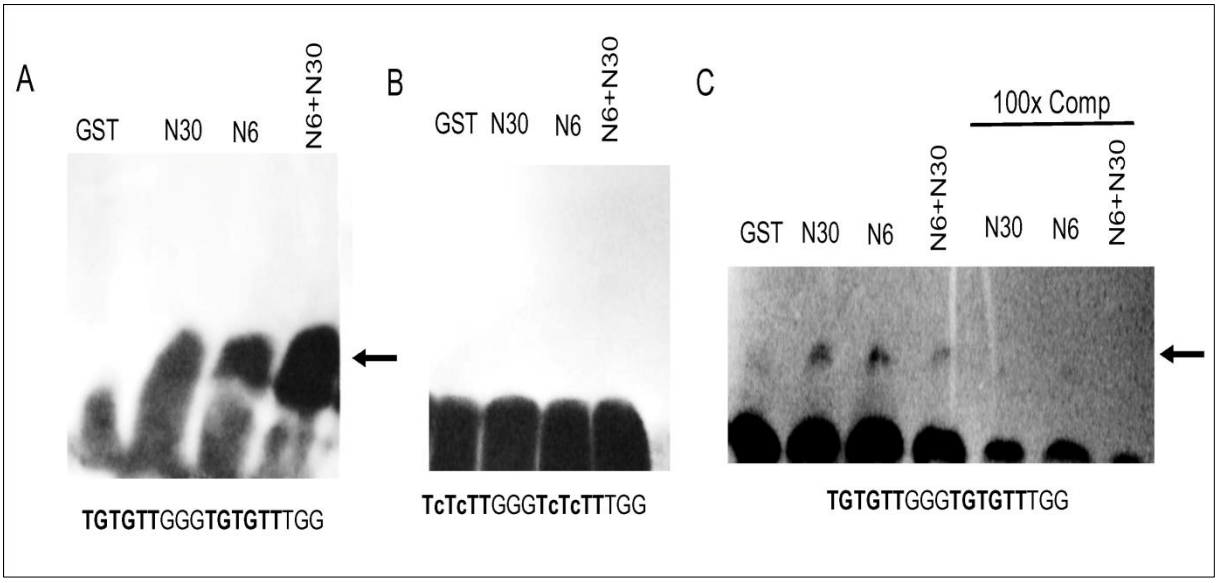


Figure 8. GmNAC6 and GmNAC32 bind specifically to the core sequence TGTGTT *in vitro*.

(A) Binding of GmNAC6 and GmNAC30 to the directly repeated core sequence (TGTGTTGGGTGTGTTTGG). An 18-bp biotin-labeled fragment harboring the directly repeated core sequence was incubated with E coli produced and purified GST, GST-tagged GmNAC30 (N30), GST-GmNAC6 (N6) and both NAC proteins (N30+N6) for 20 min at RT. The products were separated by electrophoresis in a 4% polyacrylamide gel in TB buffer. The arrow indicates the DNA:Protein complexes. **(B)** Replacement of G with C in the core DNA binding sequence disrupts protein-DNA complex formation. The condition of the gel shift experiment was exactly as in A, except that the 18-bp biotin-labeled fragment was TcTcTTGGGTcTcTTGG. **(C)** Specific binding of GmNAC6 and GmNAC30 to their core DNA binding site. The conditions of the gel shift was exactly as in A, but binding to the core element was also assayed in the presence of 100 fold molar excess of unlabeled probe, as indicated in the Figure.

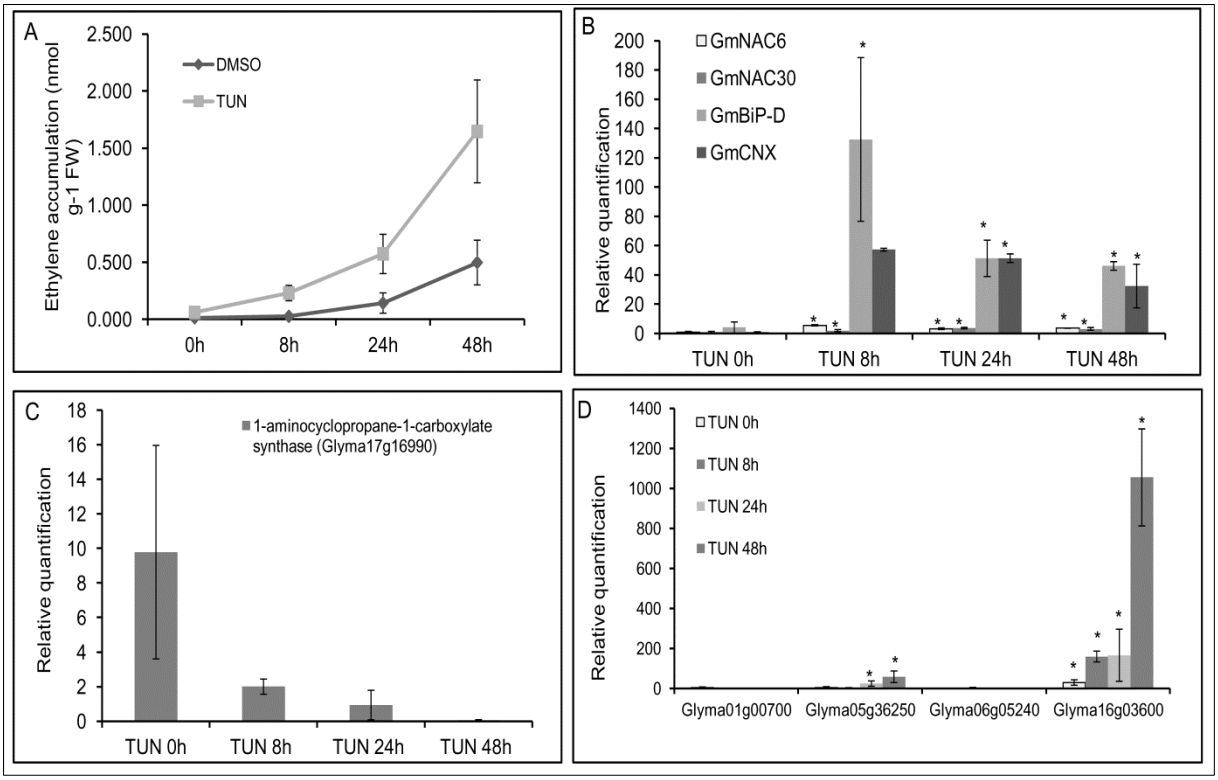


Figure 9. The **GmNAC6- and GmNAC30-down-regulated ACC synthase gene is not involved in ethylene synthesis during ER stress.**

(A) Time course of ethylene evolution by tunicamycin treatment. Soybean plants at the VC developmental stage were treated with 5 µg/mL of tunicamycin for 24 h. The plants were transferred to a sealed container, and ethylene evolution was monitored by gas chromatography at the time points indicated in the figure. **(B)** Time course of GmNAC6, GmNAC30 and ER stress markers by tunicamycin treatment. Total RNA was isolated from tunicamycin-treated leaves at the period indicated in the figure, and the relative expression of GmNAC6, GmNAC30 and UPR-specific gene markers (CNX and BiP) was determined by quantitative RT-PCR. The fold variation of gene expression (in relation to control treatments) was calculated using $2^{-\Delta\Delta Ct}$ method and helicase as endogenous control. Values are relative to the control treatment (DMSO) and asterisks indicate statistic differences ($p < 0.05$, t test). **(C)** Down-regulation of the ACC synthase gene (Glyma17g16990) by ER stress. The repression of the GmNAC6- and GmNAC30-down-regulated ACC synthase gene by ER stress was monitored by quantitative RT-PCR, as described in B. **(D)** Induction of ACC synthase homolog genes by ER stress. The gene induction was monitored by quantitative RT-PCR using gene-specific primers. Values are relative to the control treatment (DMSO) and asterisks indicate statistic differences ($p < 0.05$, t test).

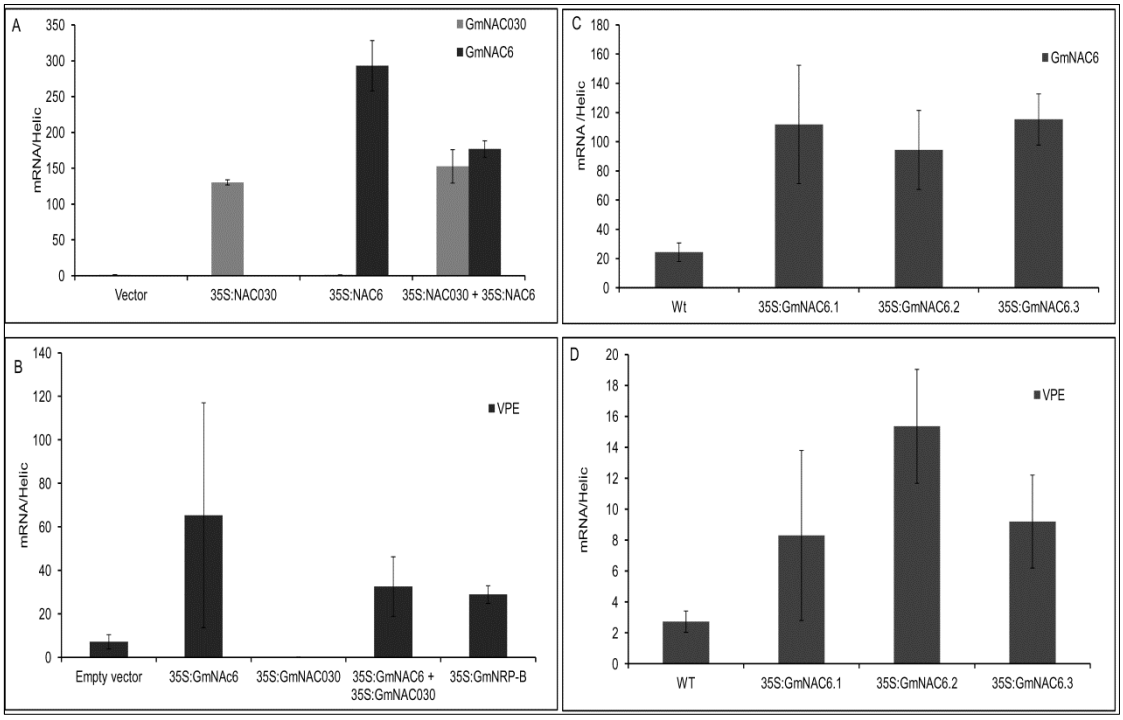


Figure 10. GmNAC6 transactivates VPE expression. A. Transient expression of GmNAC6 and GmNAC30 in soybean protoplasts.

(A) Soybean protoplasts were electroporated with the 35S:YFP-NAC6 construct or the empty vector, as well as with 35S:GmNAC30 construct or the empty vector and the expression of GmNAC6 and GmNAC30 was monitored by qRT-PCR. Gene expression was calculated using the $2^{-\Delta CT}$ method and RNA helicase as an endogenous control. Error bars represent the confidence interval ($\alpha = 0.05$) of three biological replicates. **(B)** Transient expression of GmNAC6 transactivates VPE expression in soybean protoplasts. The transcript levels of VPE were quantified by qRT-PCR 36-h post-electroporation of soybean protoplasts with GmNAC6, GmNAC30, GmNRP-B or the empty vector, using a set of primers that anneals to sequences present in all five homologs of the soybean VPE family. Gene expression was calculated using the $2^{-\Delta CT}$ method and RNA helicase as an endogenous control. Error bars represent the confidence interval ($\alpha = 0.05$) of three biological replicates. **(C)** Ectopic expression of GmNAC6 in soybean transgenic lines. The expression of GmNAC6 in the leaves of three independent transgenic lines was monitored by quantitative RT-PCR. Values of expression were calculated using the $2^{-\Delta Ct}$ method with helicase as an endogenous control. Error bars represent the confidence interval ($\alpha = 0.05$) of three replicates. **(D)** Enhanced accumulation of VPE by overexpression of NAC6 in transgenic lines. The expression of VPE in the leaves of GmNAC6-overexpressing transgenic lines was monitored by RT-PCR, as described in C.

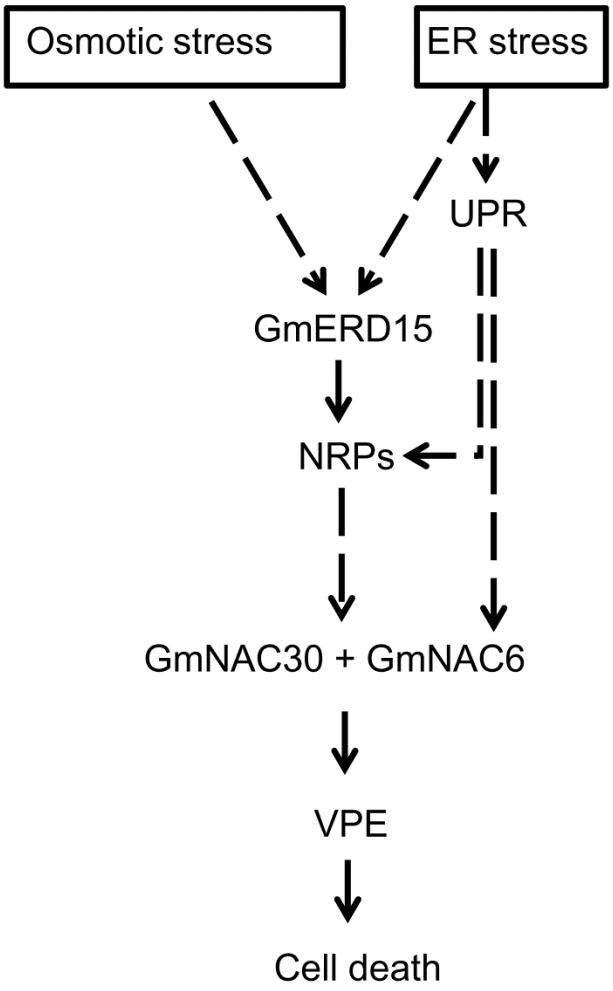
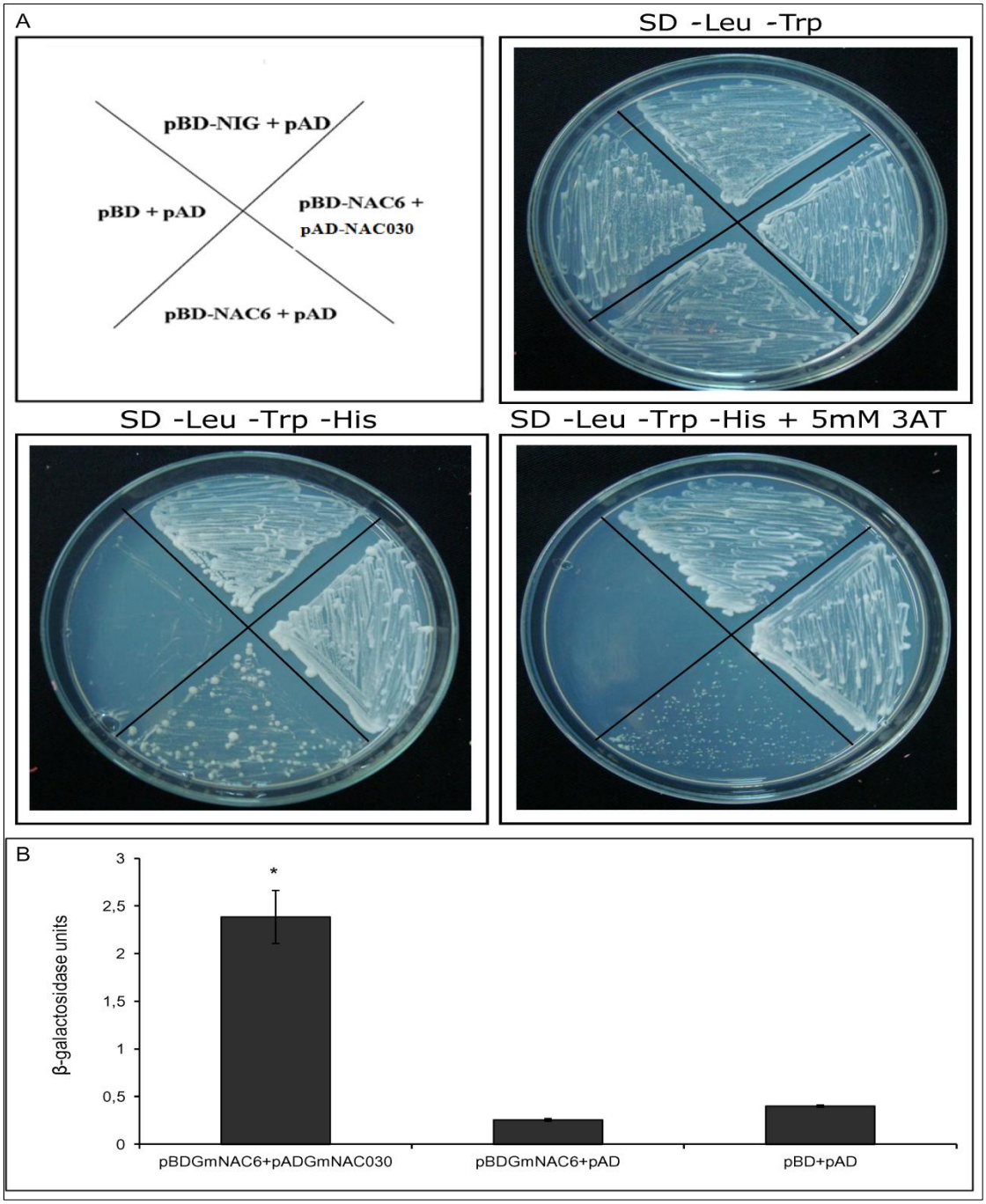
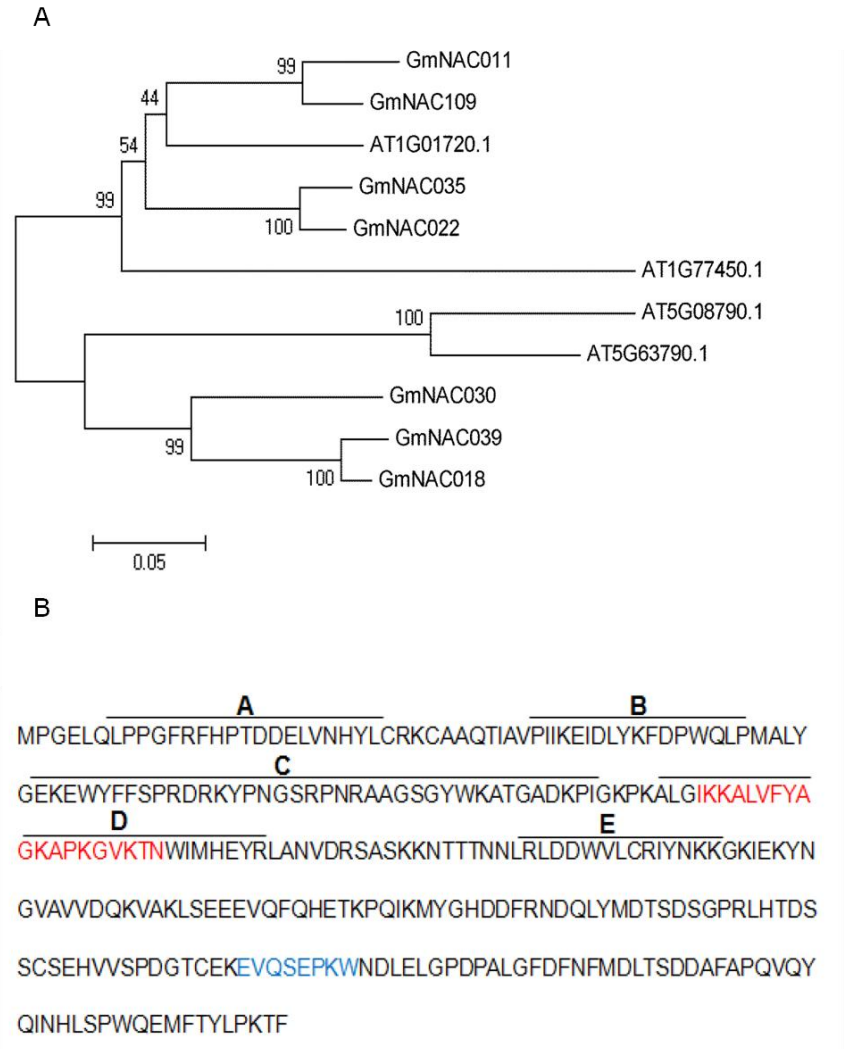


Figure 11. ER stress and osmotic stress-induced NRP-mediated cell death signaling.
The scheme illustrates the propagation of a cell death signal derived from prolonged ER stress and osmotic stress through the NRP-mediated PCD signaling. A broken arrow indicates an effect on gene expression, whereas a solid arrow indicates that the gene is an immediate downstream target. Specifically in this study we show that interaction between the NRP-induced GmNAC6 and GmNAC30 target the VPE promoter to induce VPE expression and cell death. Abbreviation: GmERD15, Glycine max early responsive to dehydration stress 15; NRP, N-rich protein; VPE, vacuolar processing enzyme; UPR, unfoldd protein response.



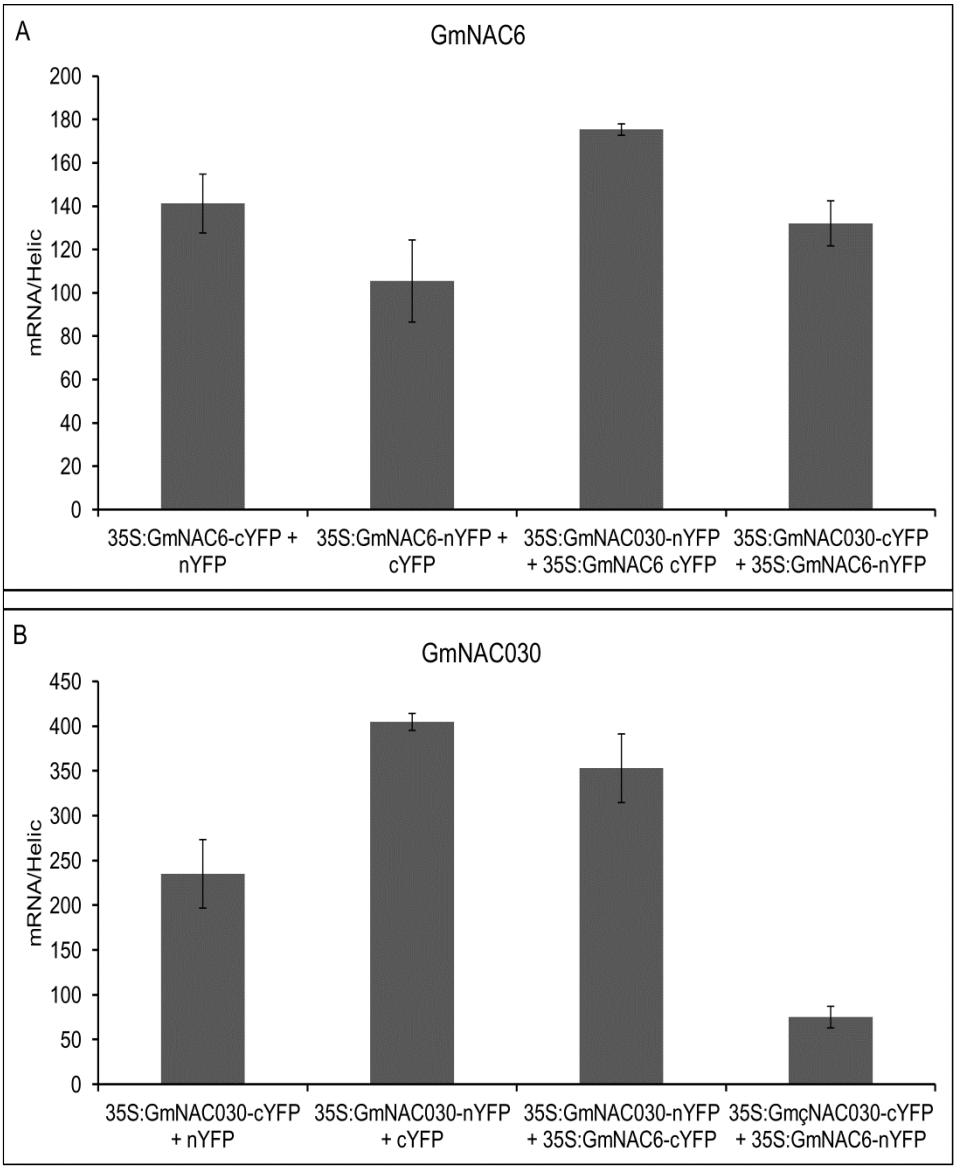
Supplemental Figure 1. GmNAC6 interacts with GmNAC30 by the yeast two-hybrid system.

(A and B) GmNAC30 was expressed in yeast as GAL4 activation domain (AD) fusion (pAD-NAC30), and GmNAC6 was expressed as a GAL4 binding domain (BD) fusion (pBD-NAC6). Interactions between the tested proteins were examined by monitoring His prototrophy **(A)** and confirmed by measuring the activity (mean ± SD, n=3) of the β-galactosidase reporter enzyme expression of the second reporter gene β-Gal **(B).** Asterisks indicate statistic differences ($p < 0.05$, t test, n=3).



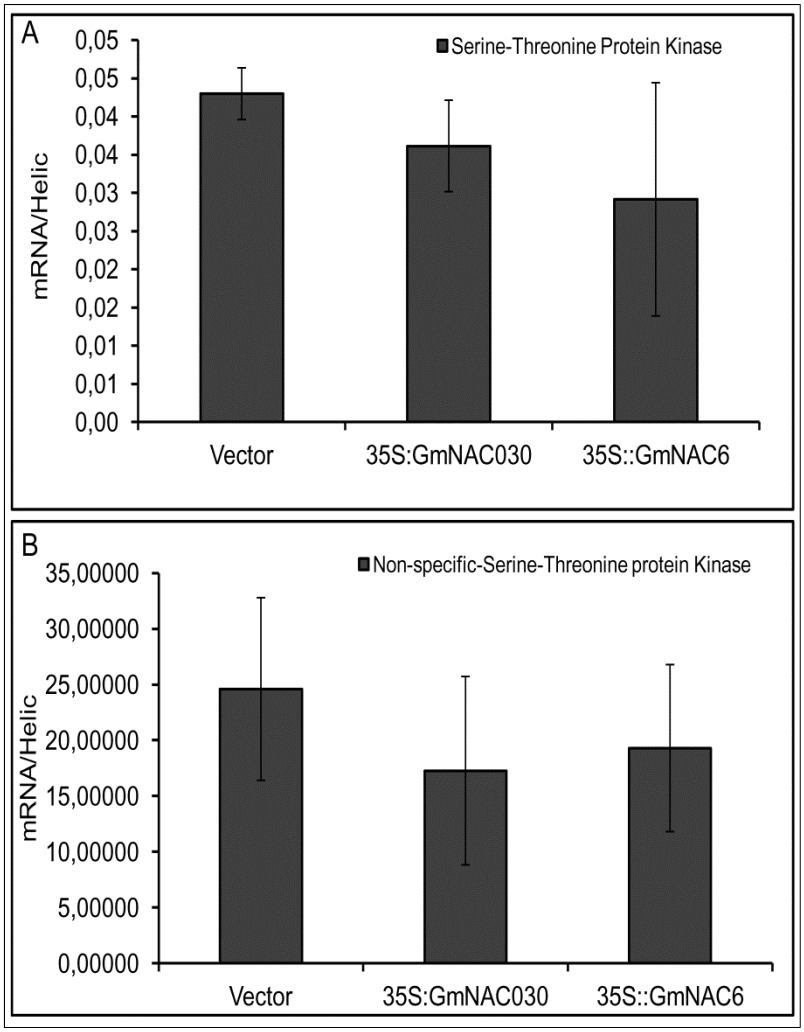
Supplemental Figure 2. Structural organization of GmNAC30 as a member of the soybean NAC domain-containing protein family.

(A) GmNAC30 belongs to the ATAF family of NAC-domain containing protein family. For the phylogenetic analysis, the sequences were obtained from the database provided by Le et al, 2011. The Arabidopsis sequences were At1g01720 (ATAF1), At1g77450 (ANAC032) and At5g08790 (ATAF2), which represent the GmNAC30 closest related homologs in this plant species. The alignment was constructed using MUSCLE (Edgar, 2004) from the program MEGA 5.0, and the method neighbor-joining with 5000 bootstrap, and the model Tamura 3-parameter. **(B)** Amino acid sequence of GmNAC30 and domain structure. The N-terminal NAC domain sub-divided into the five motives (A to E) is indicated. The nuclear localization signal is show in red and overlaps with the D motif. The conserved transcriptional activator motif found in members of the ATAF group is shown in blue in the C-terminal activation region (TAR).



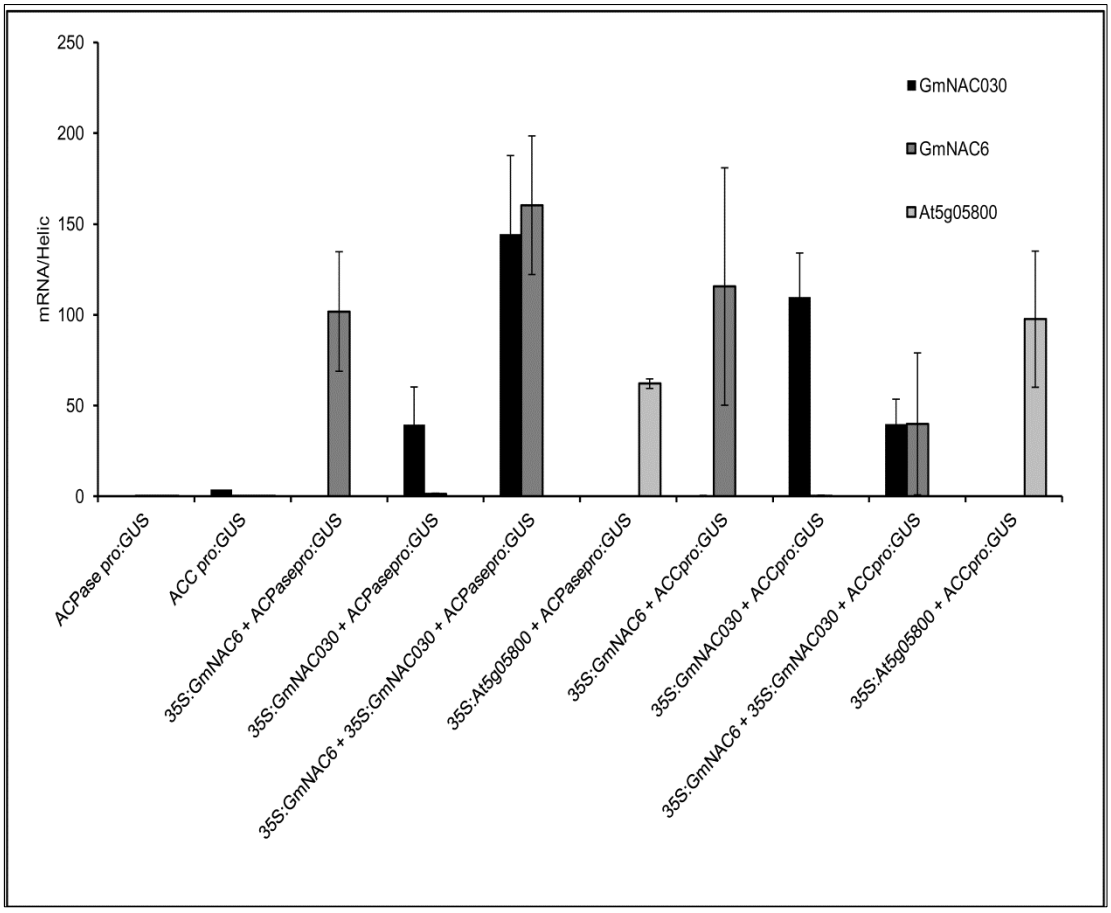
Supplemental Figure 3. Expression analysis of GmNAC6 and GmNAC30 fusions in soybean protoplasts electroporated with the BiFC DNA constructs.

Soybean protoplasts were electroporated with combinations of the DNA constructs, as indicated in the figures, and expression of GmNAC6 (A) and GmNAC30 (B) fusions was monitored by quantitative RT-PCR 36-h post electroporation. Gene expression was calculated using the $2^{-\Delta CT}$ method and RNA helicase as an endogenous control. Error bars represent the confidence interval ($\alpha = 0.05$) of three biological replicates.



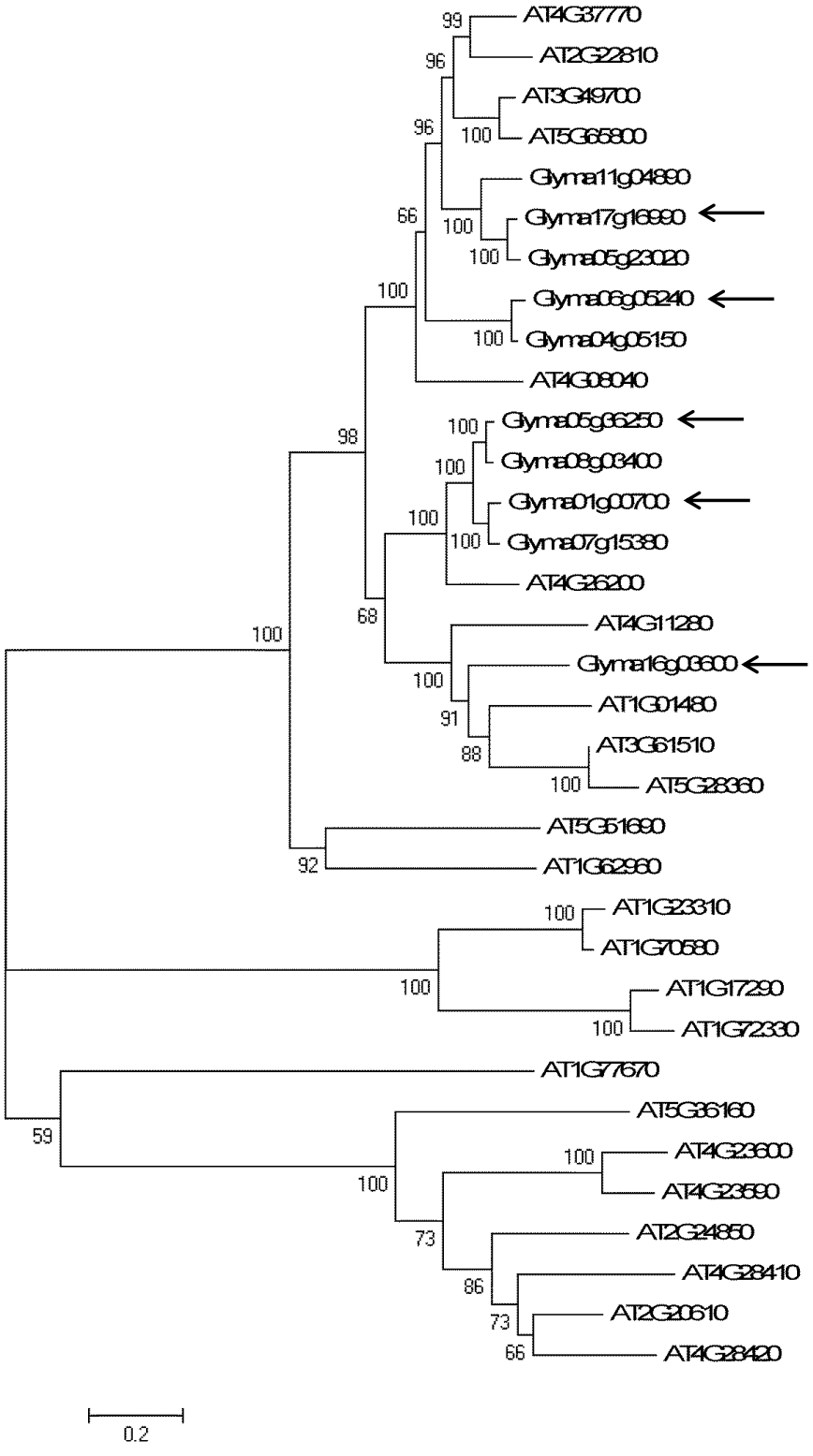
Supplemental Figure 4. **GmNAC6 and GmNAC30 do not regulate the ChIP-identified genes harboring a TGTGGT[TCG]-less promoter.**

(A and B) The transcript levels of the indicated genes were quantified by qRT-PCR, 36 h after electroporation of soybean protoplasts with GmNAC6, GmNAC30 or the empty vector. Gene expression was calculated using the $2^{-\Delta CT}$ method and RNA helicase as an endogenous control. Error bars represent the confidence interval ($\alpha = 0.05$) of three biological replicates. Phytozome accession of each gene is given in Tables 1 and 2.



Supplemental Figure 5. Expression analysis of GmNAC30, GmNAC6 and At5g05800 genes in the GmNAC30- and GmNAC6-dependent promoter regulation assay in soybean protoplasts.

Soybean protoplasts were electroporated with DNA constructs carrying GmNAC6, GmNAC30 or At5g05800 under the control of the 35S promoter as indicated in the figure. The expression of the transgenes was monitored by quantitative RT-PCR.



Supplemental Figure 6. Phylogenetic analysis-based on ACC synthase genes from soybean and Arabidopsis. The unrooted plylgenetic tree was constructed by the neighbor-joining method implemented in MEGA v.5 with model Tamura 3-parameter and 5000 bootstrap replications, using the full-length ORFs of ACC synthase proteins found in the

databases of Glycine max (Phytozome) and Arabidopsis thaliana (TAIR). The soybean sequences were: ACC (Glyma17g16990); ACC (Glyma05g36250); ACC (Glyma16g03600), ACC (Glyma11g04890); ACC (Glyma05g23020); ACC (Glyma06g05240), ACC (Glyma04g05150); ACC (Glyma01g00700); ACC (Glyma08g03400), ACC (Glyma07g15380) and Arabidopsis sequence were ACC8 (AT4G37770), ACC9 (AT3G49700), ACC4 (AT2G22810), ACC5 (AT5G65800), ACC11 (AT4G08040), ACC7 (AT4G26200), ACC1 (AT3G61510), ACC2 (AT1G01480), ACC6 (AT4G11280), ACC12 (AT5G51690.), ACC10 (AT1G62960), ACC (AT5G36160), ACC (AT1G23310), ACC (AT1G77670), ACC (AT2G20610). ACC (AT1G17290), ACC (AT1G70580), ACC (AT2G24850), ACC (AT1G72330), ACC (AT4G28420), ACC (AT4G23600), ACC (AT4G23590),ACC (AT4G28410).

Supplemental Table 1. Primers used for expression analysis by quantitative real time RT-PCR (qRT-PCR).

Oligonucleotide	Sequence (5`→3´)	Gene	Access number
NAC030 fwd	TGCCGGAAGCGGGTACT	NAC30	EU661926
NAC030 rev	CGGATTTCCGATCGGTTTG		
NAC6 fwd	CCAACAAAAGCACTTGTGGCA	NAC6	AY974354
NAC6 rev	GGACTATTCAACTGAGCCCAAAAG		
GUS fwd	AGTGAAGCGCGAACAGTTCCT	GUS	GUS
GUS rev	GCAAGTCCGCATCTTCATGA		
Helic fwd	TAACCCTAGCCCCTTCGCCT	HELICASE	AI736067
Helic rev	GCCTTGTCGTCTTCCTCCTCG		
NAC2 fwd	GGGTGCTTTGCCGTATTTACAA	NAC2	AY974350
NAC2 rev	CTCCTCCGCTTTTCAGAATCTC		
BIP-D fwd	ATCTGGAGGAGCCCCAGGCGGTGG	BIPD	AF031241
BIP-D rev	CTTGAAGAAGCTTCGTCGTAAAACTAAG		
CALN fwd	TGATGGGGAGGAGAAGAAAAAGGC	CNX	AW508066
CALN rev	CACTTGGGTTTGGGATCTTGGCTC		
SMP fwd	GCCGAACTGAGGAAAAGACGAACC	SMP	AW397921
SMP rev	CTTGGGCTGTTTGTTGGGTGTTC		
Nrich fwd	TACAGGCATCCAATTTGGCGAACC	NRP-B	AI973541
Nrich rev	TGACTTGAAAGAGTTGATCTCACCCC		
Glyma18g02260 fwd	GGATCCATGGTCCCCTTCTC	D-alanyl-d alanine carboxipeptidase	Glyma18g02260
Glyma18g02260 rev	TCCATGCCTTGCTGACGTT		
Glyma08g41980fwd	GTTGGCCATGAACCTTTATCCA	Iron/ascorbate family oxidoreductases	Glyma08g41980
Glyma08g41980 rev	CGAAGCCAGGGTCAGTGTGT		
Glyma20g03960 fwd	CACGCACCACCACAGACACT	Predicted small molecule transport	Glyma20g03960
Glyma20g03960 rev	GCACCGAATGCACCAAAAT		
Glyma17g05080 fwd	CTGTCGCCGTAGGCTCAAAA	Alpha/Beta hydrolase	Glyma17g05080
Glyma17g05080 rev	TGTGGAAGAGATTCTCGAAAGGA		
Glyma17g16990 fwd	GGAACGAAACGACGTCACTGA	1-aminocyclopropane-1-carboxylase synthase	Glyma17g16990
Glyma17g16990 rev	CCGGCAAACCCAAGTCTTTT		
Glyma06g15000 fwd	AGGAACCCTTATGAGTATCACCATGA	Dual specificity protein phosphatase	Glyma06g15000
Glyma06g15000 rev	GGTTGAGAGCCCACAATCAAG		
Glyma05g27410 fwd	TGGCCTTGGTGTTGGAATG	Permease of the major facilitator superfamily	Glyma05g27410
Glyma05g27410 rev	AAGCACCTCGAACTCTAGTTGGA		
Glyma18g50180 fwd	TTGGTGGGTCTTGCATATGGT	Predicted endo 1,3 beta glucanase	Glyma18g50180
Glyma18g50180 rev	TGTGCGGCTAGAATTTCCAAT		
Glyma20g28590 fwd	TGCTTTGTTTCCATTCTTCCAA	Adenylosuccinate lyase	Glyma20g28590
Glyma20g28590 rev	CCCTATACCTACACCCATGTTCCT		
Glyma17g17520 fwd	GGAATCATGCATCGTGATGTG	Non-specific serine/threonine protein kinase	Glyma17g17520
Glyma17g17520 rev	CCCCAATCTATAAGGCGAAGCT		
Glyma17g17560 fwd	GCATTGTGAAGGCAGATGGAA	MYB family	Glyma17g17560
Glyma17g17560 rev	CCCATCTCAATCTGCAGCTCTT		
Glyma05g04560 fwd	CGTCTATGTTATCCGCAAAAGGA	Microtubule-associated proteins 1A/1B light chain-3 related	Glyma05g04560
Glyma05g04560 rev	TCGGTGGAAGGACGTTGTC		
Glyma05g28480 fwd	GGTCATTGCTCAGCTTGAGTTG	S-adenosylhomocysteine hidrolase	Glyma05g28480
Glyma05g28480 rev	GGTGCTTGGGCAAAACGTAA		
17g3507fwd	GAGCACTGCGAGCATTAAAGG	unknown function	Glyma17g35070
17g3507rev	CTCTGCCGTCCGTTTTCTCT		
At5g05800fwd	GTCCGGTATTCGTTGAGATTGAA	unknown function	At5g05800
At5g05800rev	TTCAATTGCGTTGAGGCTTTT		

Glyma20g22140 fwd	ACTCAGGAGGTCCTTCAAATGC	Protein of unknown function	Glyma20g22140
Glyma20g22140 rev	TTTTGCGGTTGTTGCTGTGT		
Glyma05g25260 fwd	TTAATCCCTGTGACACCATCCA	Protein of unknown function	Glyma05g25260
Glyma05g25260 rev	TGCTGCCCTGTCTTGACCTT		
Glyma06g17460 fwd	AAGCAAGAAGGCAAAAGGCTTT	CDC2-Related Kinase	Glyma06g17460
Glyma06g17460 rev	GCCACGCTCACGTACTCTGA		
Glyma05g36250 fwd	TTGCCATTGTTCTGAACC	1-aminocyclopropane-1-carboxylase synthase	Glyma05g36250
Glyma05g36250 rev	TTCCATGAAGTTACGTATTCTC		
Glyma01g00700 fwd	TGCGGGGATTGAGTGCTTGAA	1-aminocyclopropane-1-carboxylase synthase	Glyma01g00700
Glyma01g00700 rvs	TCCTTTTGGCTTATTCTTCTCC		
Glyma16g03600 fwd	AACGATGGCAACCGAATCTT	1-aminocyclopropane-1-carboxylase synthase	Glyma16g03600
Glyma16g03600 rvs	TCAATTGGCTCCAACAAGTG		
Glyma06g05240 fwd	CATTCTTTGCAAAGTTGGG	1-aminocyclopropane-1-carboxylase synthase	Glyma06g05240
Glyma06g05240 rvs	TCCGCAAAAGCCTTTATGC		
GmVPE fwd	GTTGGGGAACATATTGCC	Vacuolar Processing enzime	VPE
GmVPE rev	TCTTCCATCCAAGCAACACT		

Supplemental Table 2. Primers used for cloning GmNAC30 coding region

Oligonucleotide	**Sequence (5`→3´)**	**Gene**	**Access number**
NAC30 fwd	AAAAAGCAGGCTTCACAATGCCAGGAGAACTCCA	NAC30	EU661926
NAC30 rvs	AGAAAGCTGGGTCTCAAAATGTCTTTGGTAGGTA		
NAC30 nsRvs	AGAAAGCTGGGTCAAATGTCTTTGGTAGGTACGT		

Supplemental Table 3. Primers used to cloned promoters

Oligonucleotide	Sequence (5`→3´)	Gene	Access number
pACPase fwd	GGCAAATTAAACCGAACCC	D-alanyl-d alanine carboxipeptidase	Glyma18g02260
pACPase rev	GCCTTGATTGGGTTTGTTGG		
pACC fwd	CGACACAAAATTGGACTG GG	1-aminocyclopropane-1-carboxylase synthase	Glyma17g16990
pACC rev	TTTGTGTGTTTGGGATGG		

CAPÍTULO 2

Expressão ectópica de GmNAC6 acelera senescência foliar e aumenta suscetibilidade de plantas transgênicas a estresses abióticos

RESUMO

Os fatores de transcrição da família NAC são específicos de plantas e atuam em eventos no desenvolvimento vegetal. Membros desta família são induzidos durante o evento de senescência. GmNAC6 é um efetor de morte celular na via de sinalização mediada pelas proteínas NRPs. Para avaliar uma possível função de GmNAC6 como regulador de senescência foliar, plantas de soja, variedade BR16, foram transformadas com o gene *NAC6*, sob o controle do promotor 35S, e a análise da incorporação do transgene foi realizada por PCR. Tres linhagens transgênicas em homozigose (geração T3) foram selecionadas por apresentaram níveis similares de expressão de NAC6. As três linhagens transgênicas (GmNAC6.1, GmNAC6.2 e GmNAC6.3) foram fenotipicamente similares às plantas não transformadas na fase vegetativa de desenvolvimento, porém exibiram um fenótipo de senescência precoce no estádio R3 de desenvolvimento. Em todas as três linhagens transgênicas, expressão ectópica de GmNAC6 acelerou o amarelecimento foliar que foi associado com maior perda de pigmentos e maior acúmulo de ROS, como comparado com as plantas controles. Além disso, este fenótipo de senescência foliar precose nas linhagens transgênicas foi associado com um maior nível de indução de genes marcadores de senescência nas folhas no estágio R3 de desenvolvimento quando comparado com os níveis exibidos pelas plantas controles. Consistente com o seu papel regulador na expressão do gene VPE (vacuolar processing enzyme), a superexpressão de GmNAC6 nas linhagens transgênicas induziu a expressão do gene VPE em níveis muito superiores do que aqueles observados em folhas das plantas controles no estádio R3 de desenvolvimento. Considerando que VPE está diretamente envolvida no processe de PCD mediado pelo vacúolo, que GmNAC6 induz a expressão de VPE e que senescência foliar mediada por GmNAC6 foi positivamente correlacionado com a expressão de VPE em tempo e espaço, propõe-se que GmNAC6 regula senescência foliar via indução de VPE, um processo de PCD que deve ser mediado pelo colapso do vacúolo. Como evidência adicional de que GmNAC6 regula o processo de senescência foliar, foi demonstrado que as linhagens transgênicas superexpressando GmNAC6 foram mais suscetívies aos estresses abióticos, estresse osmótico, estresse do retículo endoplasmático e seca. Consequentemente, os resultados da presente investigação reforçam a hipótese de que um dos mecanismos de tolerância de plantas a estreses abióticos está conectado ao controle de longevidade foliar.

INTRODUÇÃO

Os estresses abióticos e bióticos são as principais causam que limitam a produtividade das espécies vegetais. Por serem organismos sésseis, as plantas apresentam um amplo mecanismo de defesa contra estas adversidades. As plantas percebem os sinais ambientais e sinalizam a nível celular, fisiológico, bioquímico e molecular, a fim de ativarem uma resposta adaptativa aos estresses (Hu *et al.*, 2006; Nakashima *et al.*, 2012). Os fatores de transcrição, em sua maioria, medeiam esta resposta de estresse e são responsáveis por controlarem a expressão de vários genes, e assim são capazes de acionar os mecanismos de defesa vegetal (Yamaguchi-Shinozaki & Shinozaki, 2005).

A família de fatores de transcrição NAC (acrônimo NAM, ATAF1,2 e CUC) são específicos de plantas e atuam em diversos processos importantes ao longo do ciclo de vida da planta (Olsen *et al.*, 2005). De acordo com http://plntfdb.bio.uni-potsdam.de 151 membros desta família de fatores de transcrição foram anotadas em *Oryza sativa,* e 126 membros foram anotados em *Arabidopsis thaliana*. Em soja foi recentemente anotados 152 membros de fatores de transcrição NAC (Pinheiro *et al.*, 2009; Nuruzzaman *et al*., 2010).

As proteínas pertencentes a esta família de transfatores se caracterizam por terem um domínio conservado na porção N-terminal, denominado domínio NAC, e este é responsável pela ligação ao DNA. Este domínio é correspondido por dois éxons e compreende cerca de 150 aminoácidos, e estes estão agrupados em cinco regiões conservadas (A-E). Entretanto, a porção C-terminal se caracteriza por alta divergência tanto em sequencia quanto em tamanho, e é responsável pelo domínio de ativação ou repressão da transcrição (Ren *et al*., 2000; Xie *et al.*, 2000; Duval *et al.*, 2002; Ooka *et al.*, 2003; Nakashima *et al.*, 2012). O domínio C-terminal exibe atividade de ligação entre proteínas e, algumas proteínas NACs contem um domínio adicional transmembrana (Seo *et a*l., 2009; Tran *et al.*, 2009). Assim sendo, embora o grande conjunto de proteínas NACs se localiza no núcleo, onde exercem a função de fatores transcricionais, existem um sub-conjunto de proteínas NACs que estão inseridos na membrana plasmática (Kim *et al*., 2007; Seo *et al.*, 2009).

Os representantes desta família de fatores de transcrição participam de eventos importantes no desenvolvimento vegetal, como manutenção do meristema apical (Souer *et al*., 1996; Aida *et al*., 1997; Weir *et al.*, 2004), sinalização hormonal (Aida *et al.*, 1997; Xie *et al*., 2000; Greve *et al.*, 2003; Chen *et al.*, 2011), nas respostas a infecções por patógenos *(Xie et al.*, 1999; Ren *et al*., 2000; Collinge & Boller, 2001; Selth *et al.*, 2005), na senescência de

folhas (John *et al.*, 1997), e nas respostas a diferentes estresses abióticos (Hegedus *et al*., 2003; Tran *et al.*, 2004).

Como fatores transcricionais que são regulados por estresses bióticos e abióticos, diversos membros da família de proteínas contendo o dominio NAC têm sido alvos para a manipulação gênica em plantas. Por exemplo, expressão ecotopica dos genes *SNAC1, SNAC2 (OsNAC6)* confere tolerância à condição de estresses bióticos e resistência a patógenos (Tran *et al.*, 2004; Hu *et al.*, 2006; Nakashima *et al.*, 2007). Similarmente, foi observado que plantas transgênicas superexpressando três diferentes genes ANAC de *Arabidopsis* (ANAC019, ANAC055 e ANAC072) mostraram um significante aumento na tolerância à seca (Tran *et al.*, 2004). ANAC072 (*RD26-responsive to desiccation*) participa em uma via de sinalização de estresse dependente de ácido abscísico, e a sua expressão eh induzida por espécies reativas de oxigênio (Fujita *et al.*, 2004). Em arroz, foi demonstrado que a super-expressao do gene *SNAC1 e OsNAC6* causa aumento de tolerância `a seca e estresse salino, respectivamente (Hu *et al.*, 2006; Nakashima *et al.*, 2007). Entretanto, Kaneda *et al.*, (2009) mostraram que plantas super-expressando o gene *OsNAC4* atua como regulador positivo no evento de morte celular causado pela resposta hipersensível. Conclusivamente, estes dados indicam o potencial das proteínas NACs como alvos na resposta ao estresse abiótico e biótico.

O papel de transatores da família NAC em resposta a senescência é pouco estudada (Nakashima *et al*., 2012). Entretanto, a participação de alguns membros desta família de fatores de transcrição tem sido relatada. Como exemplo, o gene *ERD1* em Arabidopsis, é por senescência (Nakashima *et al.*, 1997) e assim como o gene *AtNAP*. Associado a alta expressão de AtNAP em folhas senescentes, foi demonstrado que a inativação do gene AtNAP retarda senescência, enquanto que a indução da super-expressão do gene *AtNAP* causa a senescência precoce. Guo and Gan, (2006) sugeriram que o fator NAP pode ser um fator de regulação universal de senescência de folhas. Uma vez que a ausência da expressão de NAP retarda a queda das folhas, a manipulação dos níveis de AtNAP pode ser considerada uma nova estratégia para o controle intensional da senescência de folhas de importantes culturas agrícolas. Recentemente, têm sido identificatos uma série de genes induzidos pelo processo de senescência em outras espécies vegetais. Em arroz, experimentos de super-expressão descreveu o gene *OsNAC5* como associado ao processo de senescência natural (Sperotto *et al.*, 2009). Similarmente, o gene *VND Interacting 2* (VND2), também um membro da família de fatores de transcrição NAC, foi descrito com uma conecção molecular associada à comunicação cruzada existente entra a via de estresse salino e a via de senescência natural da

folha (Yang *et al.,* 2011). *VND2* se liga à região promotora dos genes *COLD REGULATED* (COR) e RESPONSIVE *TO DEHYGRATION* (RD) para aumentar a expressão destes genes, resultando em um aumento da longevidade foliar. Embora o repertório de fatores de transcrição induzidos durante a senescencia tem aumentado consideravelmente (Buchanan-Wollaston *et al.,* 2005; Balazadeh *et al.,* 2008; Breeze *et al.,* 2011), poucos destes fatores de transcrição têm sido relatados até o momento, como reguladores da senescência (Matallana-Ramirez *et al.,* 2013).

Em soja, três genes envolvidos no processo de senescencia tem sido descritos (Pinheiro *et al.*, 2009). O gene *GmNAC6* de soja mostrou integrar uma resposta de sinalização de estresse osmótico e estresse no retículo endoplasmático que conduz ao evento de morte celular (Faria *et al.*, 2011). Dentro deste contexto, o sinal de estresse osmótico e do retículo endoplasmático induz a expressão do fator de transcrição *GmERD15*, este se liga ao promotor do gene NRP para aumentar sua expressão, resultando na indução da expressão de *GmNAC6* que, por sua vez, promove a morte celular (Alves *et al.*, 2011; Faria *et al.,* 2011). Expressão ectópica tanto de NPRs quanto de GmNAC6 em protoplastos promove fragmentação do DNA, bem como o aumento na expressão da atividade de *caspase 3-like*. Em adição, plantas de tabacco super expressando estes genes apresentaram um fenótipo de clorose, associado à diminuição do pelo teor de clorofila, além de exibirem um aumento na peroxidação de lipídeo e na expressão de genes marcadores de senescência (Costa *et al.,* 2008; Faria *et al.,* 2011; Reis & Fontes, 2012).

O efeito primário do estresse osmótico, sentido pelas plantas, desencadeia uma série de respostas moleculares que por sua vez ativam genes que são responsáveis pelo fechamento estomático, visando ao controle da perda de água, bem como a manutenção do aparato fotossintético (Xiong *et al.,* 2002). O estresse do retículo endoplasmático acontece por uma alteração de sua homeostase, e esta alteração interfere no dobramento correto das proteínas (Schroder & Kaufman, 2005; Ron & Walter, 2007). Diante disso, as plantas desenvolveram mecanismos para tentar minimizar estes danos causados por estresses associados ao retículo endoplasmático. Entre eles, a via UPR (unfolded protein response), acionada em resposta ao acúmulo de proteínas mal dobradas no lúmen do RE, é um dos mecanismos protetores mais bem caracterizados em células de mamíferos e leveduras. Em Arabidopsis, genes responsáveis pela sinalização da via UPR incluem dois receptores transmembrana no RE, *bZIP28 e bZIP17, além de IRE1a e IRE1b* (Iwata & Koizumi, 2012; Reis & Fontes, 2013). Esta sinalização tem como objetivo restaurar a homeostase no RE. Estresse no RE também induz a

expressão de proteínas NRPs e de GmNAC6, que sinaliza um sinal de morte celular, resultando em morte celular programada (Faria *et al.*, 2011). Com a finalidade de identificar o evento de morte celular associado ao gene GmNAC6, foram preparadas plantas transgências super-expressando NAC6 e e o fenótipo associado ao aumento dos níveis de transcritos do referido foram avaliados. Nossos dados demonstram que plantas super-expressando NAC6 são mais sensíveis ao estresse hídrico, bem como ao estresse osmótico e o estresse no retículo endoplasmático e apresentam um fenótipo precoce de senescência.

MATERIAL E MÉTODOS

Construção do vetor e transformação da soja

Uma sequencia de cDNA de *GmNAC6* (acesso n.° AY974354 Genbank) foram re-amplificadas com os pares de primers e NAC6XBAF (5' ATCTCTAGACAACCATGGAAGACATG 3') e NAC6KPNR (5' CGCGGTACCTCAGTAGAAGTTTAG 3') para a inserção do sítio de restrição. Os fragmentos com os sítios de restrição XbaI e KpnI foram inseridos no vetor pUC19-35SAMVNOS sob controle do promotor *CaMV35S* (vírus do mosaico da couve-flor) com a seqüência *enhancer* do vírus do mosaico da alfafa e do sinal de poliadenilação 3' do gene da nopalina sintase *(nos)* (Dias *et al.*, 2006), dando origem ao vetor p35SNAC6. Em seguida, o cassete de expressão de NAC6 foi clonado no vetor pAC321 (Rech *et al.*, 2008), neste vetor está presente o gene *ahas* (que confere tolerância ao herbicida *imazapyr)* de *Arabidopsis thaliana*, onde foi removido do vetor pAC321 (Aragão *et al.*, 2000) e gerando, por fim, o vetor pNAC6ahas. A construção foi usada para transformar a soja da variedade 'BR16', via biobalística, de acordo com Aragão *et al.* (2000).

Confirmação das plantas Transgênicas

O DNA foi isolado de folhas de soja (*Glycine max)* variedade BR16 e das plantas transformantes do gene *NAC6* sob controle do promotor 35S C*auliflower mosaic virus*, de acordo com o protocolo do CTAB (1M Tris-HCl (pH 8,0); 1,4 M NaCl; 500 mM EDTA (pH 8,0) β.mercaptoetanol). Reações de PCR, a partir do DNA genômico extraído, com o volume de reação de 50 µl foram feitas para confirmar a transgenia. Para a reação de PCR foi utilizada *GoTaq*® DNA Polymerase (Promega). A mistura foi aquecida a 94°C (3 mim) e submetidas a 35 ciclos de amplificação (94°C por 45 seg, 53°C por 45 seg, 72°C por 1 min),

com um ciclo final de 72°C por 10 min, no termociclador PCR System 9700 (Applied Biosystem). Os oligonucleotídeos 35SAMVF (5` CCACTATCCTTCGCAAGAC 3´) e NAC6R (5' TCTCTCTCTTCCTCTAGTGCTCG 3´) foi usados para amplificar uma sequencia de 700 pb (Tabela 1).

Tabela 1. Listagem dos oligonucleotídeos para confirmar a transgenia em soja

Primers	**Sequence (5' – 3')**	**Gene**
35SAMVF	CCACTATCCTTCGCAAGAC	NAC6 transgene
NAC6R	TCTCTCTCTTCCTCTAGTGCTCG	

Material Vegetal

Sementes de soja WT e plantas super-expressando o gene *NAC6* foram crescidas em vasos de 3L contendo uma mistura de solo e esterco, na proporção de 3:1. Os vasos foram mantidos na casa de vegetação sob condições naturais de luz, com temperatura de 35°C de dia e 15°C à noite. Foram analisadas a soja controle (BR16) e as três transformantes independentes (35S::NAC6.1; 35S::NAC6.2 e 35S::NAC6.3). Após o período de experimento, como indicado nas figuras, o material de soja foi coletado em nitrogênio liquido e armazenado em *freezers* a -80°C até o processamento das amostras.

Tratamento de estresse

Sementes de soja WT e plantas transformantes foram germinadas em subtrato (Bioplant). Após uma semana (estádio VC de desenvolvimento) as plantas foram transferidas para 15 ml de solução, suplementadas com 0,5% (p/v) de polyethylene glycol (PEG; MW 8000, Sigma) e 2,5 μg/ml tunicamicina (Sigma). Água foi utilizada como controle do tratamento de PEG, e DMSO foi utilizada como controle do tratamento de tunicamicina. Os tratamentos de estresses foram realizados por 8h, 24h e 48h. As folhas foram coletadas e imediatamente congeladas no nitrogênio liquido e armazenadas em *freezers* a -80°C até o processamento das amostras.

Extração de RNA e síntese de cDNA

O RNA total foi extraído utilizando o reagente TRIzol (Invitrogen) de acordo com as instruções do fabricante. Para a eliminação de contaminantes de DNA, o RNA foi tratado com

2 unidades de DNase livre de RNase (Invitrogen). Após a extração, o RNA foi quantificado no espetrofotometro (Evolution 60 Thermo Scientific) e sua integridade foi monitorada em um gel de agarose desnaturante a 1,5% (p/v) corado com brometo de etideo a 0,1µg/ml. A síntese de cDNA foi realizada utilizando 3 µg de RNA total 5 µM de oligo-dT, 0,5 mM de dNTPs e 1U de Trancriptase Reversa M-MLV (Life Tecnologies Inc.), de acordo com as instruções do fabricante.

PCR em tempo real

Todos os procedimentos incluindo testes, validações e experimentos foram conduzidos de acordo com as informações no manual da Applied Biosystems. As reações de Real time PCR (qRT-PCR) foram realizadas no aparelho ABI7500 (Applied Biosystems), e foi utilizado o corante SYBR® Green PCR Master Mix (Applied Biosystems) com primers específicos (Tabela 2).

Tabela 2. Listagem dos oligonucleotídeos para qRT-PCR em soja

Primers	**Sequence (5' – 3')**	**Gene**
HELIC Fw	TAACCCTAGCCCCTTCGCCT	HELICASE
HELIC Rv	GCCTTGTCGTCTTCCTCCTCG	
GmCYSTP Fw	TGGAAAGCAACTCAATCATGGT	CystP
GmCYSTP Rv	CCCCATGAGTTCTTCACAATCC	
Nac6 Fw	CCAACAAAAGCACTTGTGGCA	NAC6
Nac6 Rv	GGACTATTCAACTGAGCCCAAAAG	
GmPR1 Fw	AACTATGCTCCCCCTGGCAACTATATTG	PR1
GmPR1 Rv	TCTGAAGTGGCTTCTACATCGAAACAA	
GmVPE Fw	GTTGGGGAACATATTGCC	VPE
GmVPE RVs	TCTTCCATCCAAGCAACACT	
GmDID Fw	TCGGGTGTGAGCTTGGTAAAA	DiD
GmDID Rv	GCCATGTCTGTGAGTCCAGTGA	

As condições de amplificação da reação foram realizadas a 10 min at 95°C, and 40 ciclos de 94°C por 15 sec e 60°C por 1 min. A variação na expressão gênica foi quantificada usando o método de expressão gênica $2^{-\Delta Ct}$. Para a quantificação da expressão gênica foi usado o RNA da helicase (Irsigler *et al.,* 2007) como controle endogeno para a normalização dos dados de real time RT-PCR.

Medições fisiológicas

A caracterização será feita em plantas WT e transformadas sob condições normais e sob déficit hídrico em folhas completamente expandidas. Essas determinações permitem avaliar a extensão das alterações no controle da abertura estomática em resposta ao status hídrico do solo, e suas conseqüências no metabolismo fotossintético da folha. A análise dos parâmetros ligados à fotossíntese foi realizada utilizando um sistema de determinações da concentração de gases no infravermelho (IRGA, Li-Cor - Li6400 *XT*). Parâmetros como taxa fotossintética líquida (*A*, µmol CO_2 m^{-2} s^{-1}), condutância estomática (g_s, mol H_2O m^{-2} s^{-1}), concentração interna de CO_2 (C_i, µmol CO_2 mol^{-1}) e transpiração (*E*, mmol m^{-2} s^{-1}) serão determinados em todos os genótipos. Foram utilizados 1000 µmol m^{-2} s^{-1} de irradiancia durante todo o experimento. Todas as medições foram realizadas no período de 8:00 h as 11:00h.

Para o estresse hídrico as plantas de soja foram irrigadas, diariamente. Após o período de aclimatação, as plantas foram separadas em dois tratamentos, irrigadas e não irrigadas (n=5). Os indivíduos do tratamento das plantas irrigadas receberam irrigação diariamente durante todo o experimento, com duração de 10 dias. As medições foram realizadas em dias alternados. No tratamento das plantas não irrigadas, a irrigação foi suspensa. O potencial hídrico foliar (Ψw) foi medido às 4h da manha, utilizando-se uma bomba de pressão tipo Scholander. A determinação consistiu na coleta de amostras de folhas completamente expandidas e em seguida foram colocadas na câmara da bomba de pressão, onde, em seguida, foi aplicada pressão até ocorrer à exsudação pelo corte feito no pecíolo da folha, para a leitura da pressão aplicada (Turner, 1981).

Determinação conteúdo de clorofila

O conteúdo de carotenoides, clorofila a e b foram determinadas espectrofotometricamente a 480 nm, 649,1 nm e 665,1 nm, respectivamente, após a extração dos pigmentos, a partir de um disco foliar 0,5 cm com 5ml de CaCO3 saturado em DMSO a

temperatura ambiente (Wellburn, 1994), os discos permaneceram na solução por 16 horas. Os valores foram transformados para teores de clorofilas a, b e totais nas folhas, expressos em unidades de área ($\mu g\ cm^{-2}$).

Detecção de H_2O_2 por DAB (Diaminobenzidina)

A produção de peróxido de hidrogênio (H2O2) em plântulas de WT e dos transformantes de NAC6 foi avaliada usando o reagente 3,3-diaminobenzidina (DAB - Sigma) segundo Weigel & Glazebrook (2002). As folhas foram destacadas após 40 dias de germinação e imediatamente submersas em solução contendo 1mg.mL-1 de DAB, pH 3,8, e foram mantidas nessa solução por 8 horas. Após esse período, foram descoradas com etanol 100% por 3 horas e observadas em esterioscópio (Zeizz, Stemi 2000-C).

Análise Estatística

Os resultados entre os genótipos serão comparados por teste-t com nível de significância de 5%, com base no intervalo de confiança.

RESULTADOS

Obtenção da linhagem transgênica de soja

Para a obtenção das linhagens transgênicas, o cDNA de *GmNAC6* na orientação senso, sob o controle do promotor CaMV35S com uma sequencia enhancer e do sinal de poliadenilação 3' do gene da nopalina sintase (*nos*), foi introduzido na região apical do eixo embriogênico de soja da variedade 'BR16', por biobalística, de acordo com Aragão *et al.* (2000). Após o bombardeamento, os transformantes primários (T0) obtidos foram selecionados com base em sua resistência ao herbicida imazapyr. Esses transformantes foram mantidos em casa-de-vegetação e analisados quanto à incorporação do transgene via PCR. Dos transformantes confirmados, foram obtidas sementes (T1) para análises posteriores.

As análises de segregação do transgene foram realizadas em plantas da geração T2 e as linhagens GmNAC6.1, GmNAC6.2 e GmNAC6.3 foram selecionadas pela aparente homozigose (frequencia de 100% de incorporação do transgene nas plantas originadas das sementes T2; Figura 1). As três linhagens independentes apresentaram níveis de transcritos de NAC6 superiores, quando comparados aos níveis de plantas não transformantes, conforme analisado por PCR em tempo real (Figura 2). Estes resultados de RT-PCR quantitativos,

avaliados em plantas de soja na geração T3, confirmam a maior expressão de NAC6 em plantas de soja transgênica.

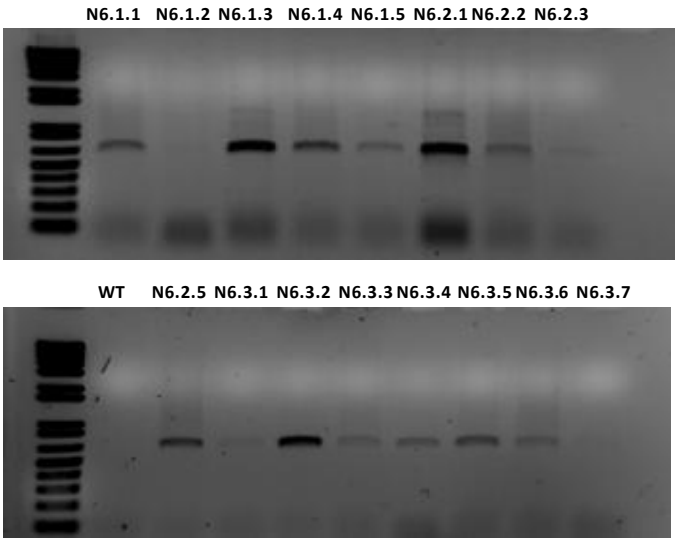


Figura 1. Diagnóstico molecular de transgenia (35S::*GmNAC6)* em plantas de soja na geração T2. RNA foi isolado de plantas individuais na geração T2 provenientes das linhagenes transgências 35S::GmNAC6.1, 35S::GmNAC6.2 e 35S::GmNAC6.3 e analisados por PCR utilizando primers espcíficos para o transgene.

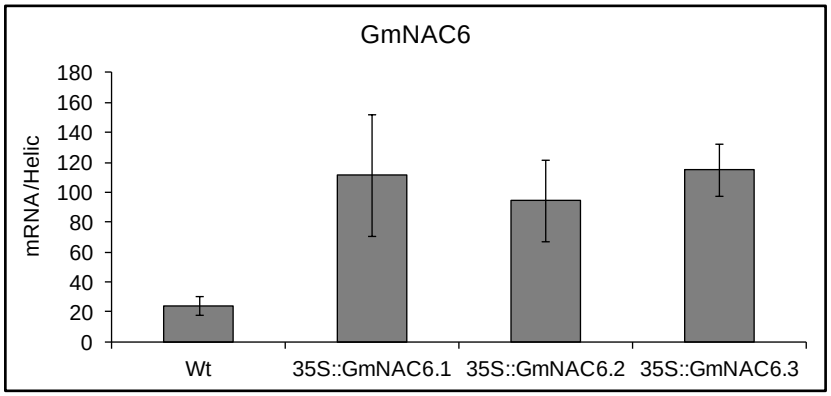


Figura 2. Acúmulo dos transcritos de GmNAC6 em plantas de soja na geração T3. O nível dos transcritos GmNAC6 em plantas transgênicas foi avaliado por meio de PCR em tempo real. O RNA total foi isolado de plantas WT e dos três transformantes independentes (35S::GmNAC6.1; 35S::GmNAC6.2; 35S::GmNAC6.3). O Valor da expressão foi obtido usando o método do $2^{-\Delta Ct}$ e utilizando como controle endógeno a *helicase.* As barras indicam o intervalo de confiança com base no teste t ($p<0,05$, n=3).

Avaliação do fenótipo das plantas de soja super-expressando *GmNAC6*

As plantas de soja não transformadas (WT) e as plantas transformantes (35S::GmNAC6.1; 35S::GmNAC6.2; 35S::GmNAC6.3) na geração T3 foram mantidas na casa de vegetação e no estádio V3 de desenvolvimento, as plantas foram fotografadas (Figura 3A). Além disso, durante cinco semanas da fase vegetativa foi monitorada a altura das plantas (Figura 3B). As medições foram feitas do caule, com auxílio de uma régua, desde emersão do solo até o ápice. Sob condições normais, plantas transgênicas superexpressando GmNAC6 foram fenotipicamente similar às plantas controles, nao transformadas, durante a fase vegetativa de desenvolvimento (Figura 3). Estes dados são contrastantes com os obtidos para plantas super expressando o gene *ANAC036* em *Arabidopsis*, (homologo ao gene *NAC6* de soja, as quais apresentavam altura inferior às plantas não transformadas (Kato *et al.*, 2010). A dinâmica similar de taxa de crescimento entre plantas transgenicas, linhagens GmNAC6.1, GmNAC6.2 e GmNAC6.3, e plantas controles levou a um idêntico período de florescimento.

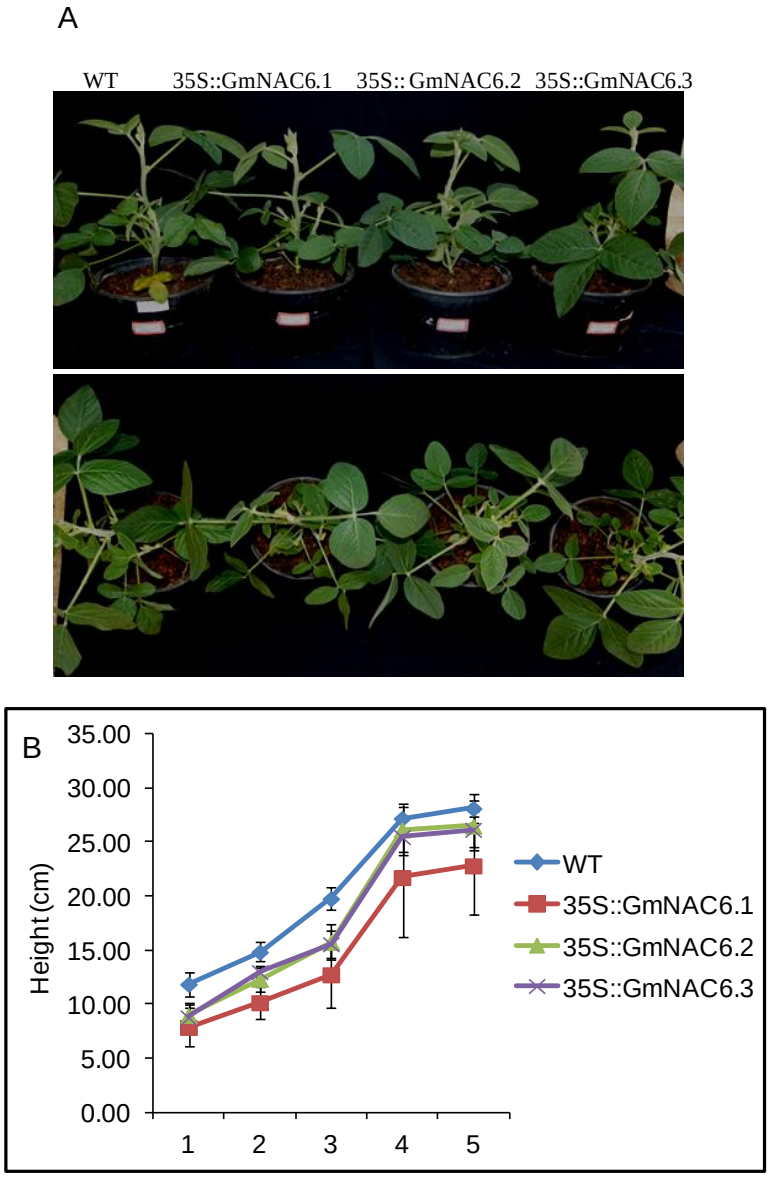


Figura 3. Fenótipo das plantas transgênicas. A) Plantas de soja não transformadas (WT) e transformadas (35S::GmNAC6.1; 35S::GmNAC6.2; 35S::GmNAC6.3) foram crescidas em condições normais na casa de vegetação. As fotografias foram tiradas no estádio V3 de desenvolvimento. B) A altura das plantas de soja foi avaliada semanalmente durante cinco semanas. Os números correspondem às semanas. As barras indicam o intervalo de confiança com base no teste t ($p<0,05$, $n=6$).

Super expressão de *GmNAC6* acelera o fenótipo de senescência foliar nas linhagens transgênicas.

Recentemente foi demonstrado que a proteína GmNAC6 atua como efetor de morte celular programada na via de sinalização mediada pelas proteínas NRPs induzida por estresses osmótico e no retículo endoplasmático (Faria *et al.*, 2011). Uma vez que GmNAC6 foi previamente descrito como regulador de morte celular programada, sob condições de

estresses, foi de nosso interesse avaliar o fenótipo das plantas de soja, durante as condições de desenvolvimento normal, durante todo o ciclo de desenvolvimento das linhagens transgênicas. Enquanto no estágio vegetativo, as plantas transgências foram fenotipicamnete similares às plantas controle (Figure 3), no estádio R3 de desenvolvimento foi verificado que as plantas transformantes exibiam uma aceleração da senescência quando comparado a plantas não transformadas (Figura 4). Este fenótipo foi associado a um acelerado amarelecimento das folhas e confirmado por uma série de marcadores fisiológicos e moleculares de senesncência, conforme a seguir.

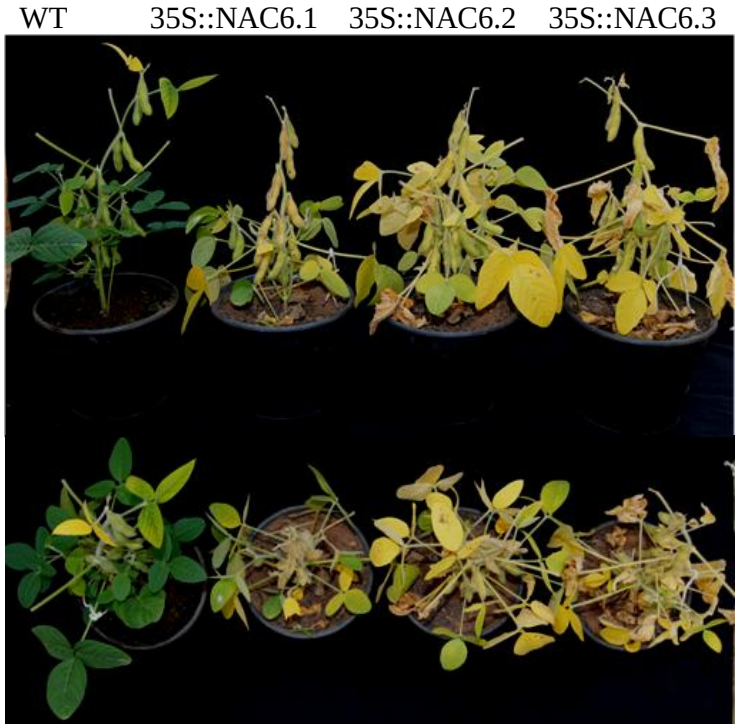


Figura 4. Plantas de soja não transformadas (WT) e transformadas (35S::GmNAC6.1; 35S::GmNAC6.2; 35S::GmNAC6.3) foram crescidas em condições normais na casa de vegetação. As fotografias foram tiradas no estádio R3 de desenvolvimento.

O processo de senescência foliar é iniciado pela degradação dos cloroplastos, seguindo por uma degradação de macromoléculas, como ácidos nucleicos, lipídeos e proteínas (Gepstein, 2004; Ulker *et al.*, 2007). Assim, a fim de comprovar o fenótipo de senescência precoce nas plantas super-expressando NAC6 foi avaliado o teor de pigmentos presentes nas plantas em estudo, durante dois meses de crescimento das plantas (Figure 5). As plantas não transformantes exibiram maior teor de clorofila a, b, total e maior teor de carotenoides a partir do estádio R3 de desenvolvimento, comprovando assim o fenótipo de clorose das plantas transformantes.

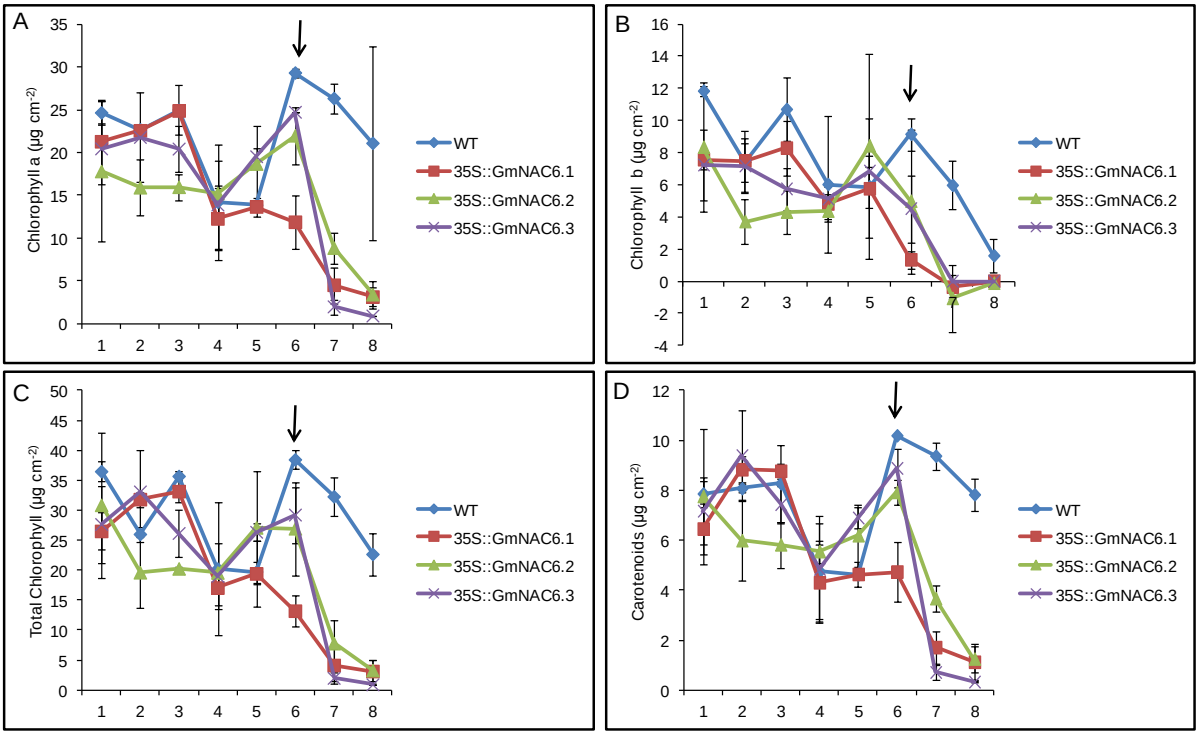


Figura 5. Teor de clorofila e carotenoides avaliados em plantas de soja transgênicas e controle. Os números correspondem a semanas de realização do experimento, em um total de oito semanas. As barras correspondem ao valor do intervalo de confiança pelo teste t ($p < 0,05$; n=3). A seta indica a transição para o período R3 de desenvolvimento.

Além de clorose, também foi avaliado o efeito da superexpressão do gene *GmNAC6* na produção de H_2O_2. Espécies reativas de oxigênio, ROS, têm sido associadas com funções relevantes na fase inicial de diversos eventos de morte celular programada em plantas pelo declínio da atividade de enzimas como SOD e catalase (Gechev *et al.*, 2006)). A peroxidação de lipídeos associado à senescência resulta em um aumento na produção de ROS, além de perda de clorofila (Dhindsa, 1981). Folhas de plantas não transformadas e transformadas (35S::NAC6.1; 35S::NAC6.2 e 35S::NAC6.3) no estádio de desenvolvimento R3 foram submetidas à coloração histoquímica com DAB (3,3‟ Diaminobenzidina), para avaliar o teor de H_2O_2. Conforme demonstrado na Figura 6, o acúmulo de H_2O_2 em folhas das plantas transgênicas foi superior do que em folhas de plantas não transformadas, como evidência adicional de que GmNAC6 é um regulador positivo do processo de senescência natural (ou envelhecimento) em plantas sob condições normais de desenvolvimento.

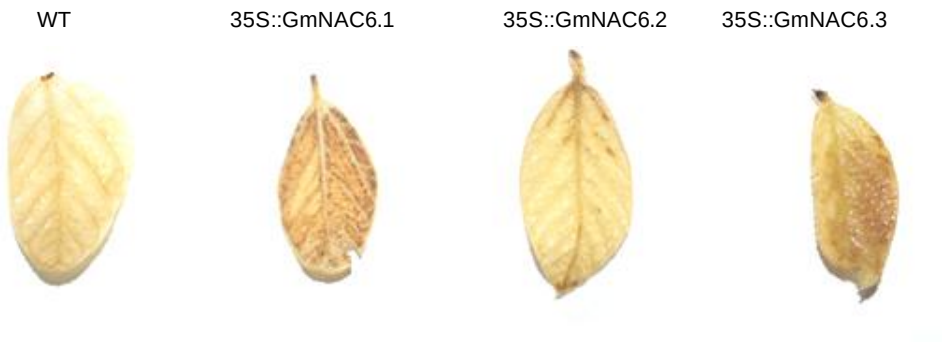


Figura 6. Acúmulo de ROS em folhas de plantas transgênicas. Folhas de soja foram destacadas no estádio R3 de desenvolvimento e coradas com o reagente DAB, e em seguida foram observadas em esterioscópio.

A senescência constitui o estádio final de desenvolvimento da folha caracterizado pela translocação de nutrientes das folhas para as sementes. Como consequência, os genes pertencentes à família das cisteíno proteases são induzidos para a remobilização de nutrientes (Lim *et al.*, 2007). Além de cisteino proteases, os genes PRs relacionados à patogênesis são induzidos em eventos de morte celular e pela expressão transiente de GmNAC6 em folhas de tabaco (Faria *et al.*, 2011). Para substanciar a hipótese de que GmNAC6 está associado à senescência natural em condições normais de desenvolvimento, o acúmulo dos transcritos de marcadores moleculares associados à senescencia natural, CystP (Ueda *et al.*, 2000), *did A9* (AAZ23261) que é homologo ao gene *SEN1* de *Arabidopsis* e o gene *PR1* (genes relacionados a patogênese), foi avaliado em folhas de plantas transgênicas no estádio R3 de desenvolvimento por RT-PCR quantitativo (Figure 7A). Na linhagem transgênica GmNAC6.2, a expressão ectópica de GmNAC6 causou um aumento significativo na expressão do gene Cyst, porém apresentou o mesmo nível de expressão dos genes DiDi e PR1 em relação à linhagem controle. Em contraste, a expressão ectópica de GmNAC6 nas demais plantas transgênicas causou um aumento no acúmulo dos transcritos correspondentes aos três genes marcadores de senescência, quando comparado com a linhagem controle. Coletivamente, este resultados indicam que GmNAC6 induz o processo de senescência em plantas sob condições normais de desenvolvimento. Apesar de diferenças sutis observadas quanto à intensidade de fenótipos associados à senescência entre as linhagens transgênicas, o nível de expressão de GmNAC6 parece ser similar entre as linhagens GmNAC6.1, GmNAC6.2 e G,NAC6.3 (Figure 2).

Recentemente, foi descoberta uma cisteíno protease, denominada *Vacuolar processing enzime (VPE*), no qual exibe atividade de caspase-1 em plantas, sendo crucial para execução do processo de morte celular mediada pelo vacúolo (Hara-Nishimur*a et al.*, 2011). Sendo alvo

direto do fator de transcrição GmNAC6 (resultados do capítulo I), foi importante monitorar a expressão de VPE como resultado da overexpressão de GmNAC6 (Figure 7B). Os resultados claramente indicam que overexpressão do gene GmNAC6 causa um aumento na expressão do gene VPE, sugerindo um mecanismo pelo qual GmNAC6 intermedia o processo de senescência.

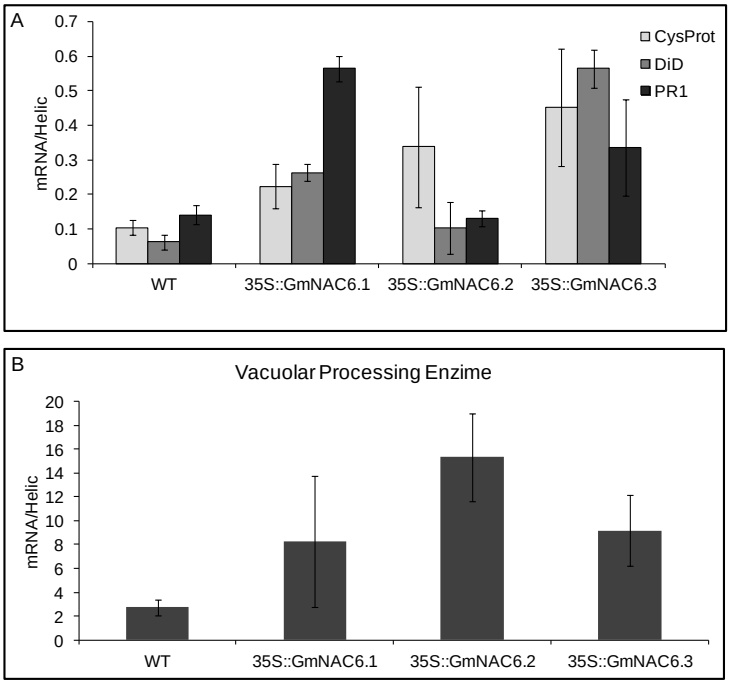


Figura 7. Análise de expressão gênica dos genes relacionados à senescência e morte celular em plantas transgênicas durante condições normais de desenvolvimento. A. Acúmulo de transccritos de genes associados com a senescência em plantas transgênicas. O RNA total foi isolado de plantas WT e dos três transformantes independentes (35S::GmNAC6.1; 35S::GmNAC6.2; 35S::GmNAC6.3). Valores de expressão foram obtidos usando o método do 2^-(DCt) e utilizando como controle endógeno a *helicase*. As barras indicam o intervalo de confiança com base no teste t ($p<0,05$, $n=3$). B. Análise da expressão da protease VPE como resultado da overexpressão de GmNAC6. O acúmulo dos transcritos de VPE foram determinados por qRT-CRP, conforme descrito acima, exceto que o conjunto de oligonucleotídeos utilizado nao discrimina entre membros da família de VPEs em soja.

As plantas superexpressando GmNAC6 são mais sensíveis ao tratamento com PEG

A proteína GmNAC6 é sinergicamente induzida quando submetido ao estresse osmótico e no reticulo endoplasmático induzindo uma resposta de de morte celular (Faria et *al.*, 2011). Com isso o nosso interesse foi verificar o fenótipo das plantas transformantes quando submetidas à PEG, indutor do estresse osmótico, e a Tunicamicina, indutor do estresse no retículo endoplasmático. As plantas transformantes e não transformantes foram tradadas com PEG em um período de 48 horas, e o efeito do estresse foi avaliado pela alteração fenotípica. Em todas as plantas avaliadas sob estresse por PEG uma murcha

acentuada nas folhas unifoliadas foi detectada (Figura 8). Porém, nas linhagens transgênicas foi verificado um murchamento e um aceleramento da senescência nas folhas cotiledonares, quando comparados ao fenótipo das plantas não transformadas tratadas com PEG em todos os tempos avaliados (Figura 8). Embora o fenótipo de morte celular induzido por tratamento por PEG em plântulas de soja seja similar àquele descrito por Reis *et al.* (2011), uma avaliação quantitativa da intensidade desse fenótipo por meio do monitoramento da expressão de genes marcadores de estresse hídrico e genes marcadores de senescencia, torna-se necessária para confirmar estes resultados.

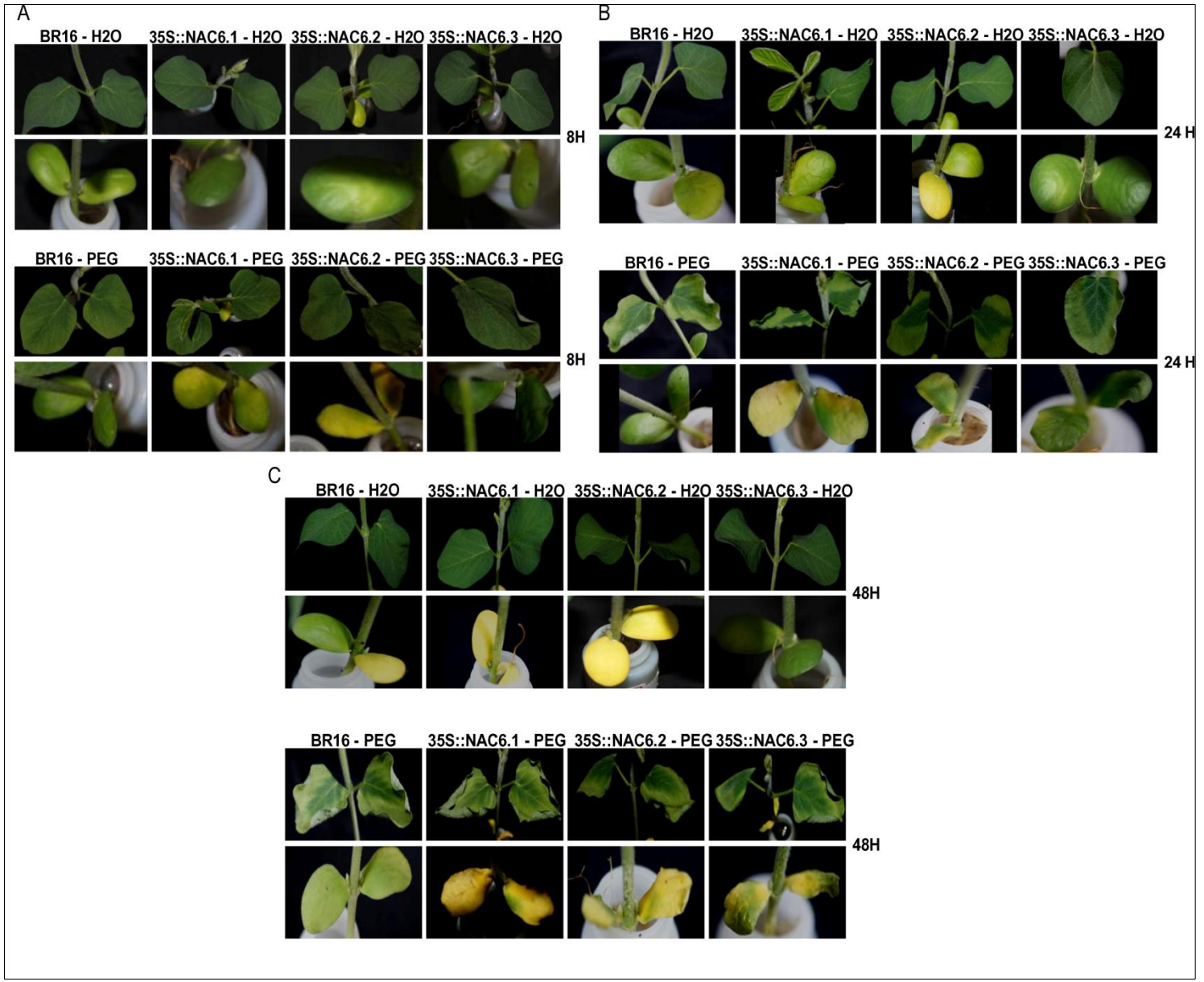

A
BR16 - H2O
35S::NAC6.1 - H2O
35S::NAC6.2 - H2O
35S::NAC6.3 - H2O
8H
BR16 - PEG
35S::NAC6.1 - PEG
35S::NAC6.2 - PEG
35S::NAC6.3 - PEG
8H
B
BR16 - H2O
35S::NAC6.1 - H2O
35S::NAC6.2 - H2O
35S::NAC6.3 - H2O
24 H
BR16 - PEG
35S::NAC6.1 - PEG
35S::NAC6.2 - PEG
35S::NAC6.3 - PEG
24 H
C
BR16 - H2O
35S::NAC6.1 - H2O
35S::NAC6.2 - H2O
35S::NAC6.3 - H2O
48H
BR16 - PEG
35S::NAC6.1 - PEG
35S::NAC6.2 - PEG
35S::NAC6.3 - PEG
48H

Figura 8. A super expressão de GmNAC6 acelera a senescencia e murcha, nas folhas cotiledonares, sob tratamento de PEG. Plântulas de soja não transformadas (WT) e transformadas (35S::GmNAC6.1; 35S::GmNAC6.2; 35S::GmNAC6.3) foram expostas ao tratamento de PEG e avaliadas a 8h, 24h e 48h. As plantas transformantes exibiram um aceleramento de clorose e murcha com a evolução do estresse nas folhas cotiledonares.

As plantas superexpressando GmNAC6 são mais sensíveis ao tratamento com tunicamicina

Plantas de soja transformantes e não transformantes foram submetidas ao estresse por tunicamicina por 48 horas, e as avaliações fenotípicas foram realizadas as 8h, 24h e 48 horas (Figura 9). Em todas as plantas tratadas com tunicamicina não foi verificada uma alteração fenotípica nas folhas unifoliadas, porém, foi verificado que as plantas transformantes apresentavam uma senescência acentuada nas folhas cotiledonares, quando comparado a plantas não transformantes (Figura 9). Experimento futuros para verificar a expressão de genes associados com senescencia, (Costa *et al.*, 2008; Pinheiro *et al.*, 2009; Faria *et al.*, 2011; Reis *et al.*, 2011) devem ser monitorados para comprovar o efeito da super expressão de NAC6 no evento de senescencia.

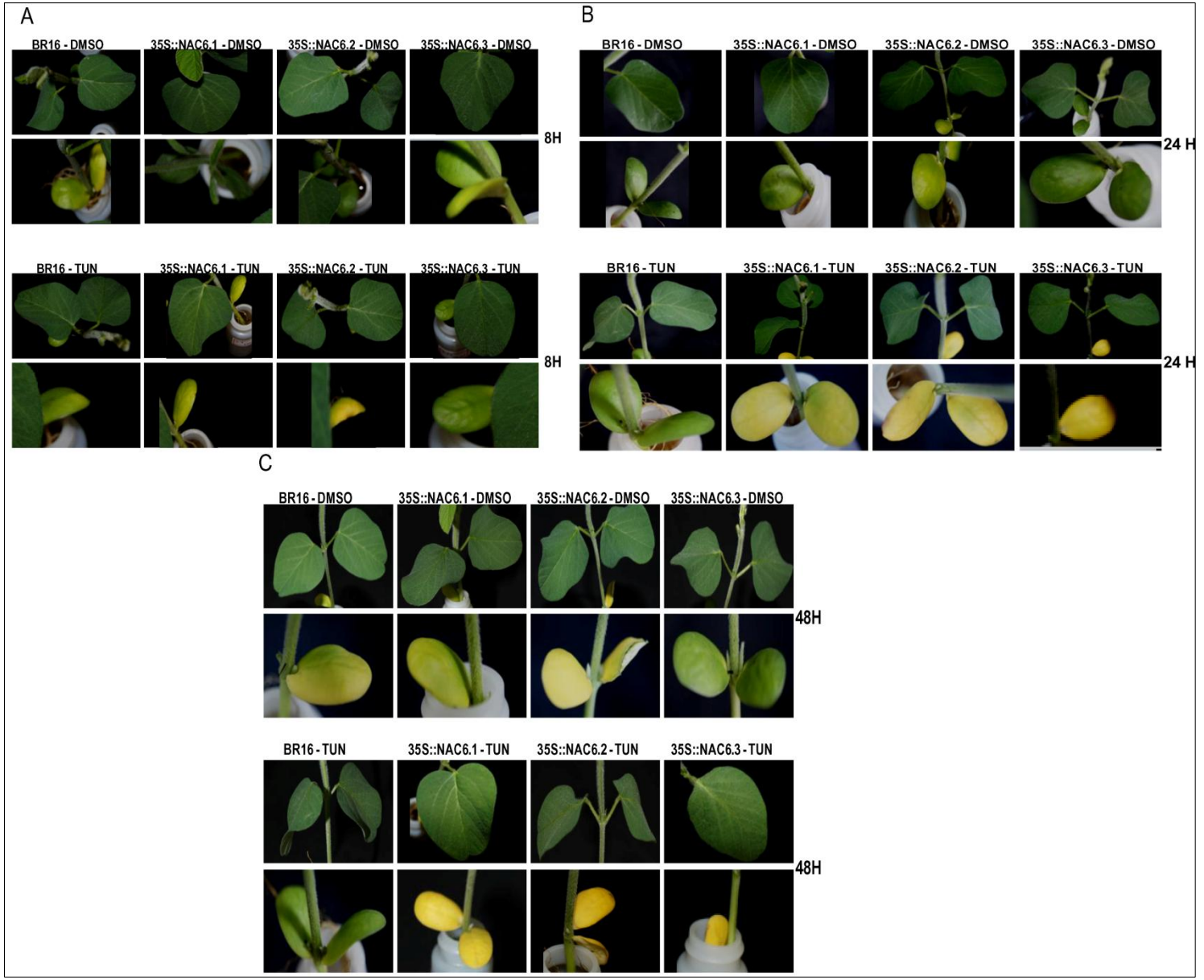

A
BR16 - DMSO
35S::NAC6.1 - DMSO
35S::NAC6.2 - DMSO
35S::NAC6.3 - DMSO
8H
BR16 - TUN
35S::NAC6.1 - TUN
35S::NAC6.2 - TUN
35S::NAC6.3 - TUN
8H
B
BR16 - DMSO
35S::NAC6.1 - DMSO
35S::NAC6.2 - DMSO
35S::NAC6.3 - DMSO
24 H
BR16 - TUN
35S::NAC6.1 - TUN
35S::NAC6.2 - TUN
35S::NAC6.3 - TUN
24 H
C
BR16 - DMSO
35S::NAC6.1 - DMSO
35S::NAC6.2 - DMSO
35S::NAC6.3 - DMSO
48H
BR16 - TUN
35S::NAC6.1 - TUN
35S::NAC6.2 - TUN
35S::NAC6.3 - TUN
48H

Figura 9. A super expressão de GmNAC6 acelera a senescencia sob tratamento de tunicamicina. Plântulas de soja não transformadas e transformadas (35S::GmNAC6.1; 35S::GmNAC6.2; 35S::GmNAC6.3) foram expostas ao tratamento de tunicamicina e avaliadas a 8h, 24h e 48h. As plantas transformantes exibiram um aceleramento de clorose com a evolução do estresse.

O estresse hídrico causa uma maior suscetibilidade em plantas super expressando GmNAC6

Como verificado no item anterior, as plantas de soja super-expressando GmNAC6 apresentaram uma murcha acentuada quando tratadas com PEG. Assim foi de nosso interesse investigar o comportamento destas plantas sob déficit hídrico. Para isto, as linhagens de soja da geração T4 e no estádio V2 de desenvolvimento foram submetidas a dez dias de estresse com redução total de água. Durante este período análises fisiológicas utilizando o IRGA, e o potencial hidrico (ψw) foram examinados.

O fenótipo das plantas foi observado de acordo com a progressão do estresse em 3, 7 e 10 dias de estresse. Nas condições normais de irrigação, não houve diferença entre o fenótipo das plantas transformantes e não transformantes (Figura 10). Porém, sob condições de déficit hídrico as linhagens transgênicas foram aparentemente mais susceptíveis à desidratação exibindo um nível de clorose induzida por estresse mais acentudo e aparente, conforme visualizado nas folhas unifoliadas ao longo do estresse (Figura 10). Estes resultados indicam que o gene NAC6 deve estar envolvido no processo de senescência prematura induzida por déficit hídrico.

Todas as plantas transformantes e não transformantes em condições de défict hídrico exibiram um murchamento acentuado. Este fenótipo pode ser característico da variedade BR16 considerada uma cultivar de alta sensibilidade à seca (Embrapa, 2009).

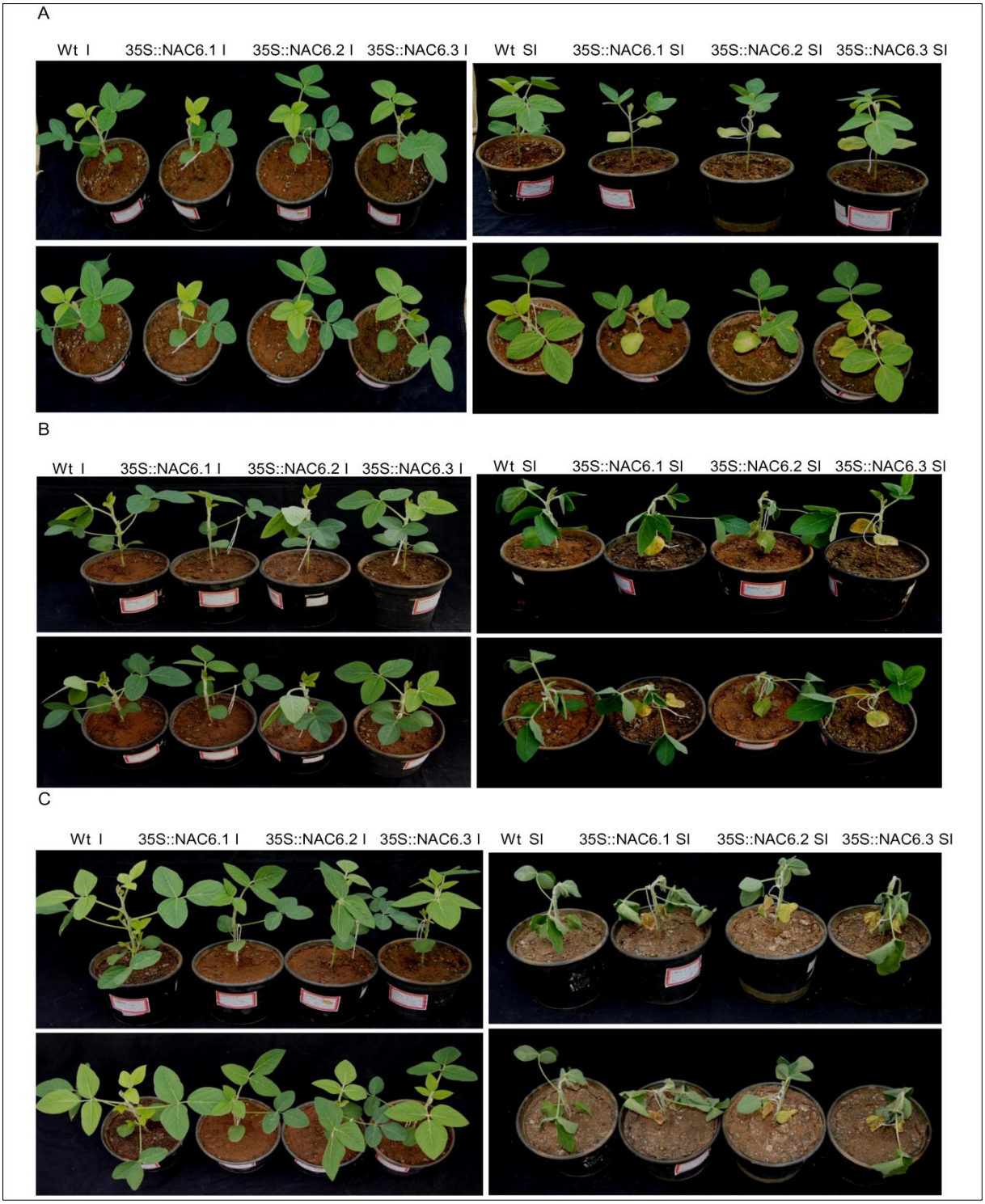


Figura 10. Fenótipo das plantas de soja não transformadas e transformadas sob déficit hídrico. A) Plantas com três dias de estresse hídrico. B) Plantas com 7 dias de déficit hídrico. C) Plantas com 10 dias de deficit hídrico. A letra (I) indica irrigação e a letra (SI) indica sem irrigação.

Os dados de balanços hídricos e fotossintéticos das plantas não transformadas e transformadas sob condições normais de irrigação permaneceram iguais, sem alterações estatísticas entre os valores, em todos os tempos observados (Figura 11). A diferença entre as médias foi observada praticamente no terceiro dia de análise, sob condições de restrições hídricas.

Enquanto que o potencial hídrico das folhas (ψw) das linhagens transgênicas sob condições de estresse foram drasticamente reduzidos até -2,2 Mpa, o potencial hídrico das folhas das plantas de soja não transformadas reduziram ao máximo até -1 MPa no ultimo dia de estresse (Figura 11 D). O status hídrico das folhas nas plantas transformantes sob estresse hídrico indica que elas são mais sensíveis à desidratação. Estes dados de potencial hídrico foram correlacionados positivamente com os dados de condutância estomática (gs),transpiração (E) e taxa fotossintética (A) (Figuras 11A, 11B e 11C). Maior redução do potencial hídrido nas folhas de linhagens transgênicas, sob condições de estresse hídrico, refletiu em menores índices de *gs*, *E* e *A*, quando comparadas com os índices exibidos pelas plantas controles submetidas ao mesmo regime de déficit hídrico.

A queda abrupta na condutância estomática para 0,05 e 0,1 mol H_20 m^{-2} s^{-1} com o prolongamento do déficit hídrico indica que as plantas no ultimo dia de estresse sofreram um estresse severo pela falta de água. Queda de *gs* sob estresse severo corresponde ao primeiro evento fisiológico de resposta à redução total de água, levando a uma queda consequente na taxa fotossintética e transpiração (Figura 11 A-C). Para o caso da soja, já foi demonstrado à existência de correlação positiva entre gs, taxa transpiratória e assimilação de CO2 (Isoda *et al.*, 2005). Uma vez que as plantas não transformadas, exibiram uma maior *gs* com o prolongamento do regime de déficit hídrico, quando comparado às plantas transformantes, provavelmente uma maior difusão de CO_2 para o mesófilo das folhas acarretou em uma maior taxa fotossintética observada, dentro da disponibilidade hídrica de cada planta. Os valores significativamente maiores de *gs* observados nas plantas não transformadas sob condições de desidratação podem estar relacionado ao maior turgor celular destas plantas. Coletivamente, estes resultados indicam que as linhagenes transgênicas são mais suscetíveis à seca, sendo pouco eficiente no controle do estado hídrico foliar. Este balanço hídrico pode ser reflexo do sistema radicular menos desenvolvido, porém este possível fenótipo não foi avaliado.

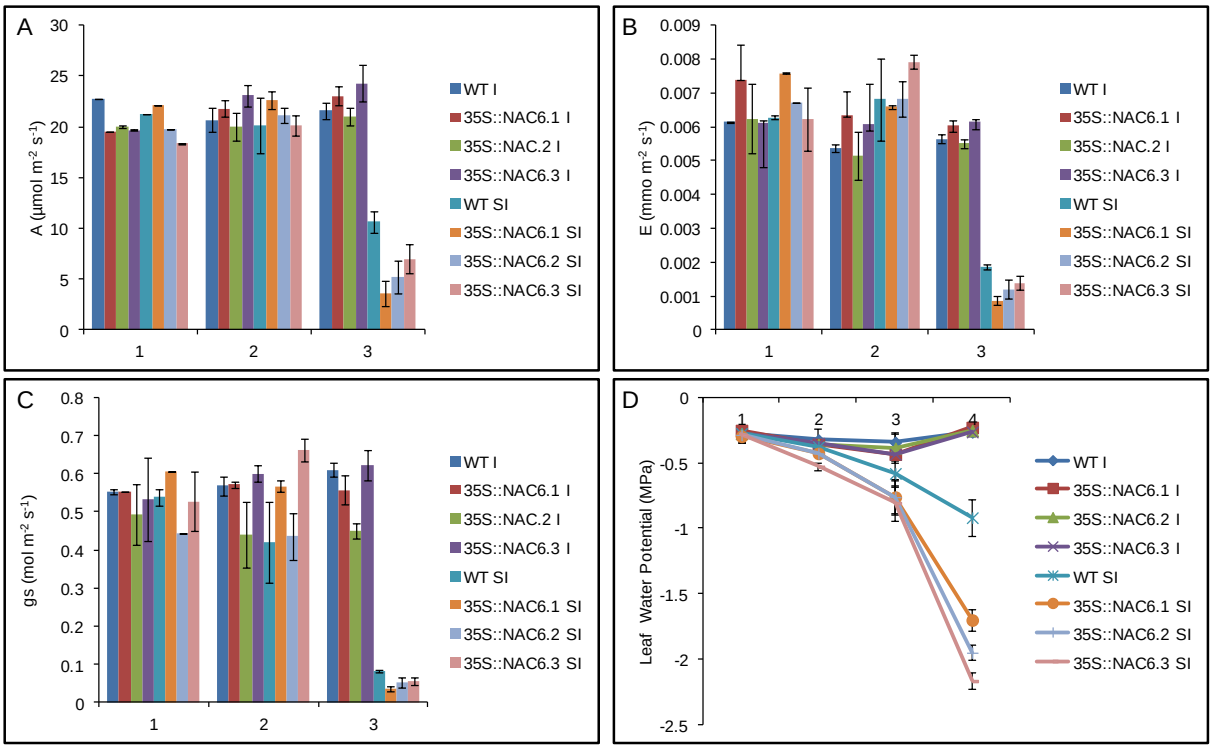


Figura 11. Expressão ecotópica de GmNAC6 confere uma maior sensibilidade ao déficit hídrico. O déficit hídrico foi induzido por restrição total de água por dez dias de experimento. A, B e C) Dados fotossintéticos e ralação do balanço hídrico entre as plantas não transformantes e transformantes. Fotossíntese (A), Transpiração (E) e condutância estomática (gs) foram avaliadas no segundo trifólio completamente expandido mensurado pelo IRGA. D) Potencial hídrico das plantas em condições normais de irrigação e submetidas ao estresse severo. Os números 1, 2, 3 e 4 no eixo x correspondem ao primeiro, quinto, sétimo e décimo dia de estresse, respectivamente. As barras correspondem ao valor do intervalo de confiança pelo teste t ($p < 0,05$; n=5).

DISCUSSÃO

GmNAC6 é um componente downstream da via de sinalização, que conecta um sinal de morte celular gerado pelos estresses osmótico e no retículo endoplasmático via indução das proteínas NRPs (Reis e Fontes, 2012). GmNAC6 tem sido descrito como o efetor da resposta de morte celular programada induzida por estes estresses (Faria *et al.*, 2011).Nesta investigação, foi demonstrado que a expressão ectópica do gene GmNAC6 em linhagens transgênicas de soja acelera o processo de senescência natural nas follas de soja e de senescência induzida por estresses abióticos como seca, estresse osmótico e estresse do retículo endoplasmático.

O evento de senescência pode ser acelerado em respostas a estresses abióticos, como seca, frio, estresse osmótico, e ataque de patógenos (Buchanan-Wollaston *et al.*, 2005). Por sua vez, o prolongamento deste processo de senescência leva ao sinal de morte celular

programada. Recentemente, foi demonstrado que a enzima VPE exerce um papel chave no processo de morte celular em plantas mediado pelo colapso dos vacúolos (Hara-Nishimur*a et al.*, 2011). Uma vez que GmNAC6 regula a expressão de VPE, nesta investigação, é proposto que GmNAC6 regula o processo de senescência natural via controle da expressão do genes VPE. Será relevante avaliar a atividade de caspase-1, exibida por VPE, nas plantas transgênicas, além de monitorar a estrutura dos vacúolos durante o processo acelerado de senescência foliar mediado pela super expressão de GmNAC6.

GmNAC6 é um regulador positivo do processo de senescência natural em condições normais de desenvolvimento

GmNAC6 é um componente de uma via de sinalização que transduz um sinal de morte celular induzido por estresses osmótico e do RE. A expressão transiente de GmNAC6 induz senescência em folhas de tabaco e morte celular programada em protoplastos de folhas de soja (Faria *et al.*, 2011). Na presente investigação, foram obtidas linhagens de soja superexpressando o gene GmNAC6 a fim de confirmar o papel regulador de GmNAC6 em eventos de morte celular programada. Consistente com um envolvimento no processo de morte celular programada, a expressão ectópica de GmNAC6 em linhagens transgênicas acelerou o processo de senescência foliar. Esta interpretação foi baseada na quantificação de uma série de marcadores fisiológicos e moleculares de eventos de senescência controlada pelo desenvolvimento. Foi inicialmente observado um amarelecimento precoce das folhas nas linhagens transgênicas que foi associado com maior perda de pigmentos nas folhas de linhagens transgênicas do que folhas controles. Este menor teor de pigmento pode estar associado à destruição de pigmentos para a realocação de nutrientes para o grão (Hayati R., 1995). Além disso, as folhas de linhagens transgênicas exibiram um aumento do acúmulo de ROS em relação a linhagens controle A senescência foliar está associada a crescentes níveis de peroxidação de lipídeos que levam a produção de espécies reativas de oxigênio (Dhindsa, 1981). A interpretação de que GmNAC6 induz senescênica foliar foi ainda reforçada pela observação de que o acúmulo de transcriptos correspondentes a genes maracadores de senescência em soja (CysP, DiD e PR; Ueda, *et al.*, 2000) foi superior em folhas transgênicas no estádio de desenvolvimento R3 do que em folhas das plantas controles coletadas no mesmo período de crescimento.

Além da indução de marcadores de senescência mediada pela proteína GmNAC6, foi demonstrado que a expressão ectópica de GmNAC6 leva a indução da enzima VPE em folhas

no estádio de desenvolvimento R3. VPE é considerada uma molécula chave no processo de morte celular programada mediada pelo colapso de vacúolos em células de plantas (Hara-Nishimura *et al.*, 2011). Esta enzima é diretamente envolvida no rompimento programado do vacúolo nas células vegetais, que leva ao extravazamento de enzimas hidroliticas para o meio citoplasmastico, culminando no processo de morte celular programada (Nakaune *et al.*, 2005). Os resultados da presente investigação associados a evidências na literatura sobre a função de VPE no processo de morte celular programada permitem inferir que GmNAC6 provoca morte celular via ativação do gene VPE e subsequente colapso do vacúolo, um mecanismo de PCD singular de células vegetais.

Uma vez que a expressão do gene GmNAC6 tenha sido dirigida pelo promotor 35S e assim constitutivamente aumentada em linhagens transgênicas, pode-se argumentar que a indução de PCD mediada por GmNAC6 nao está diretamente associado ao processo de senescência natural programada pelo desenvolvimento, mas sim um resultado direto da expressão ectópica de um efetor de PCD. Entretanto, a análise do efeito da superexpressão de NAC6 ao longo do desenvolvimento das linhagens transgênicas demonstraram que a superexpressão de GmNAC6 não alterou o crescimento dessas linhagens na fase vegetativa de desenvolvimento e, tampouco, o período de floresciemento. Alterações fenótipicas nas linhagens transgênicas só foram observadas na fase R3 de desenvolvimento, durante o processo natural de senescência foliar. Estes resultados indicam que o processo acelerado de senescência foliar observado nas linhagens transgênicas nao é o resultado de uma dinâmica de aceleração do desenvolvimento global mediado por GmNAC6, mas sim o resultado da atuação do transgene durante senescência. Estas observações favorecem a hipótese de que GmNAC6 possa atuar como regulador positivo do processo de senescência foliar, sob condições normais de desenvolvimento.

A maior sensibilidade das plantas overexpressando GmNAC6 a estresses abióticos está associada a sua tendência em acelerar o processo de morte celular programada

O processo de desenvolvimento vegetal está diretamente relacionado a sinais ambientais que alteram o crescimento e desenvolvimento. Um exemplo desta relação é a comunicação existente entre os estresses abióticos com a resposta de senescência. Em plantas, o atraso na senescência tem uma relação direta com a tolerância ao estresse. Recentemente, foi demonstrado de uma grande fração dos genes da rede regulatória do transfactor AtNAC2, que controla senescência foliar em Arabidopsis, são também regulados por estresse salino

(Balzadeh *et al.,* 2010). Inativação do gene *AtNAC2* leva a um atraso no envelhecimento foliar, sendo associado a uma maior tolerância ao estresse salino. Em uma correlação inversa, overexpressão de AtNAC diminui a longevidade foliar e aumenta suscetibilidade ao estresse salino. Similarmente, uma correlação entre níveis de expressão gênica, indução de senesencência e suscetibilidade a estresse salino foi obervada para o gene a super gene VNI2 (*VND Interacting 2*), também um membro da família de fatores de transcrição NAC (Yang *et al.,* 2011). Em Arabidopsis, foi demonstrado que a superexpressao do gene VNI2 aumenta a longevidade foliar em resposta a estresse salino, por coordenar os genes *COR e RD* (*Responsive to Dehydratation*) (Yang *et al.*, 2011), enquanto que inativação deVNI2 acelera o processo de senescência e aumenta suscetibilidade ao estresse salino . Assim também, a super expressão do gene SAG29 de soja em Arabidopsis acelera senescência e as linhagens transgências exibem hipersensibilidade ao estresse salino, enquanto que inativação de SAG29 promove uma correlação inversa de efeitos (Seo *et al.,* 2010).

Os resultados da presente investigação reforçam a hipótese de que um dos mecanismos de tolerância a estresses abióticos está associado ao controle de longevidade foliar em plantas. Em uma correlação direta entre suscetibilidade a estresses abióticos e aceleração de senescência, foi demonstrado que a superexpressão do gene GmNAC6, que controla sensescência foliar, acelera o processo de senescência e aumenta a suscetibilidade aos estresses abióticos, estresse osmótico, estresse do retículo endoplasmático e seca. A maior suscetibilidade das linhagens transgênicas ao estresse osmótico e do RE foi observada através do amaralecimento precoce das folhas cotiledonares submetidas ao tratamento com PEG e com tunicamicina, quando comparadas com as linhagens controles. Similarmente, o deficit hídrico severo acelerou o proceso, de senescência nas folhas unifoliadas das linhagenes transgênicas quando comparadas com as folhas das plantas controles. Uma maior sensibilidade dessas linhagens transgênicas ao deficit hídrico foi comprovada medindo as mudanças no desempenho fisiológico das mesmas sob condições de estresses. Com a indução de estresse hídrico, o portential hídrico foliar das linhagens transgênicas teve um declínio muito superior do que as plantas controle. Assim também *gs, A e E* destas linhagens transgênicas foram significativamente menores do que aqueles das plantas controles. O fenótipo de senescência foi observado nas folhas mais velhas, nas quais são mais sensíveis ao estresse. Este amarelecimento precoce esta relacionado à morte celular programada, e provavelmente uma translocação de nutrientes das folhas senescentes para as folhas mais jovens. O estresse de maneira geral acelera o processo de senescência e abscição,

principalmente pela produção do hormônio etileno. Durante a senescência, os cloroplastos são os primeiros a se deteriorarem, degradando a clorofila, e a cor amarela das folhas esta relacionada aos maiores níveis de carotenoides, além disso, uma redução do volume do citoplasma e rompimento do vacúolo é observada (Buchanan-Wollaston, 2005; Hara-Nishimura *et al.,* 2011**).** Uma vez que GmNAC6 regula positivamene a senescência foliar via ativação do gene VPE, envolvido na execução de PCR mediada pelo colapso de vacúolo, é plausível prever que o mecanismo pelo qual GmNAC6 acelera o processo de senescência sob condições de estresses abióticos deve estar relacionado com PCD mediada pelo vacúolo em plantas.

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CONCLUSÕES GERAIS

Recentemente, GmNAC6 foi identificado como componente da via de sinalização de morte celular mediada pelas proteínas NRPs e induzida por estresse osmótico e estresse do reticulo endoplasmático. Apesar da relevância dessa via de sinlização que integra as respostas a múltiplos sinais de estresses em plantas, o conhecimento do mecanismo pelo qual GmNAC6 provoca morte celular é limitado.

Nesta investigação, foi identificado um novo componente desta via de sinalização de morte celular, denominado GmNAC30 que pertence à família de fatores de transcrição NAC, especifico em plantas. GmNAC30 foi isolado pela sua capacidade de interagir com GmNAC6 em leveduras. Evidencias foram obtidas que sugerem que GmNAC30 e GmNAC6 dimerizam no núcleo de células vegetais para induzir o evento de morte celular programada. Foi demostrado que GmNAC6 e GmNAC30 interagem no núcleo de células de soja e são capazes de se ligarem *in vivo* na região promotora dos mesmos genes alvos, que contem uma seqüência similar caracterizada como TGTG[TGC]. Foi demonstrado in vitro que tanto GmNAC6 quanto GmNAC30 ligam especificamente a esta sequência consenso. Este dado é de suma importância, pois identifica uma região para sitio de ligação de membros da família de transcrição NAC ainda não relatada na literatura. Expressão transiente de GmNAC6 ou GmNAC30 em céulas de soja induz ou reprime a expressão de seus genes alvos; porém, a presença dos dois transfatores aumenta o efeito regulatório da ação individual de cada um sob a expressão gênica. Além disso, GmNAC6 and GmNAC30 são regulados de uma forma coordenada durante o desenvolvimento e em resposta a diferentes estímulos, conforme esperado para proteínas que atuam como dímeros.

A enzima *Vacuololar processing enzyme* (VPE) foi descoberta, recentemente, estar envolvida no proceso de morte celular em plantas. Esta enzima é caracterizada como uma protease que exibe atividade de caspase-1 em plantas, e está associada ao rompimento do vacúolo, apresentando como consequência a liberação de enzimas hidrolíticas no meio citoplasmático, levando a morte celular programada. Análise de sequência da regiao promotora do gene VPE mostrou a presenca de cis elemento para o sitio de ligação GmNAC30 e GmNAC6, sugerindo que VPE seja regulada por ambas as proteínas. Os resultados apresentados nesta investigacao confirmaram uma correlação positiva entre a co-expressao de GmNAC6 e GmNAC30 e a expressão de VPE. Este resultado caracteriza a importancia biológica da interação entre ambas as proteínas para aumentar o sinal de morte celular via VPE.

A função de GmNAC6 como efetor de morte celular foi confirmada em plantas de soja transgênicas expressando GmNAC6 constitutivamente. Um fenótipo de senescência foliar precoce resultante da expressão etópica de GmNAC6 foi observado apenas na fase reprodutiva. O fenótipo de senescencia precoce foi acompanhado do aumento da expressão de VPE nas plantas transgênicas e foi intensificado pela exposição das plantas transgênicas a estresses abíoticos. Consequentemente, as plantas transgênicas foram mais suscetíveis aos estreses. Os resultados dessa investigação substanciam a hipótese de que tolerância a estreses em plantas está geneticamente ligado a longevidade foliar. Além disso, confirmam que GmNAC6 deve promove morte celular programada via indução da enzima VPE.

Coletivamente, os resultados apresentados nesta investigação indicaram que as proteínas, responsivas ao estresse, GmNAC6 e GmNAC30 atuam como ativadoras transcricionais para aumentar a resposta de morte celular programada pelo aumento de expressao do gene VPE. Em adição, plantas super expressando NAC6 são mais sucepticeis a condições normais de desenvolvimento, e em condições de estresse, pois aceleram a senescencia foliar via VPE. Provavelmente a modulação desta via de sinalização pode ser uma forma de aumentar a tolerância das plantas a estes estresses. Experimentos adicionais serão necessários para elucidar o mecanismo pelo qual VPE causa a morte celular.