PEDRO AUGUSTO BRAGA DOS REIS

INTEGRATION OF OSMOTIC- AND ER-STRESS INTO A CELL DEATH RESPONSE: POSITIVE AND NEGATIVE REGULATORS OF THE SIGNALING PATHWAY ARE CONSERVED IN PLANTS

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

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Gilberto Sachetto Martins	Adriano Nunes Nesi
Wagner Luiz Araujo	Humberto Josué de Oliveira Ramos

Elizabeth Pacheco Batista Fontes
(Orientadora)

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SUMÁRIO

RESUMO

REIS, Pedro Augusto Braga dos, D.Sc., Universidade Federal de Viçosa, fevereiro de 2014. **Integração dos estresses osmótico e do RE no processo de morte celular: reguladores positivos e negativos da via de sinalização são conservados em plantas.** Orientadora: Elizabeth Pacheco Batista Fontes. Coorientadores: Joanne Chory e Gregory Vert.

A via de resposta mediada por proteinas ricas em asparagina (NRPs), as quais apresentam o dominio de desenvolvimento e morte celular (DCD), foi primeiramente identificadas em soja (Glycine max), pela capacidade de mediar o processo de morte celular derivado de um prolongado estresse no retículo endoplasmatico (RE), estresse osmótico, seca ou senescencia foliar associada ao desenvolvimento. Como um braço da via de resposta de estresse no RE que se conecta com outras respostas induzidas pelo ambiente, a via de morte celular mediada por NRP/DCD permite a celula uma adaptação versatil a diferentes estresses. Assim, a modulação desta via pela expressão constitutiva do chaperone molecular BiP promove uma melhor adaptação de linhagens transgenicas à seca. BiP alivia a propagação do sinal de morte celular pela modulação da expressão e atividade de componentes da via de morte celular GmNRP-A, GmNRP-B and GmNAC81.. Neste trabalho, nós apresentamos varias linhas de evidencia que a via de morte celular mediada por NRP/DCD também se propaga em arabidopsis com caracteristicas de morte celular programada. Primeiramente, o genoma de arabidopsis apresenta sequencias conservadas de GmNRPs e GmNAC81, denominadas AtNRP-1, AtNRP-2, ANAC036 e VPE-γ, as quais compartilham analogia funcional com os genes de soja. Como os ortologos em soja, tanto AtNRPs quanto ANAC036 induzem morte celular programada quando expressos ectopicamente em folhas ou protoplastos de tabaco. Além disso, a linhagem mutante nula de AtNRP1 apresenta hipersensibilidade ao estresse osmotico induzido por PEG, um fenótipo que pode ser complementado pela expressão ectopica de ou GmNRP-A ou GmNRP-B. Segundo, AtNRPs e ANAC036 são induzidos por estresses osmotico e do RE em nivel que foi modulado, similarmente em soja , pela superexpressão de BiP. Terceiro, como componente da via de morte celular mediada por NRP/DCD, a indução induzida por estresse de ANAC036 foi relacionada à função de AtNRPs. Finalmente, a superexpressão de BiP também confere tolerancia a estresse hídrico em arabidopsis, provavelmente devido à capacidade de inibir a via de morte celular mediada por NRP/DCD induzida por seca. Nós mostramos que soyBiPD modula tanto a expressão quanto a tividade dos componentes da via de morte celular

mediada por NRP/DCD. Por serem componentes upstream da via, NRP-B em soja e AtNRP1 em arabidopsis, são prováveis alvos da UPR, devido a presença de cis-elementos regulatorios em seus promotores. Assim, nós propomos que BiP pode inibir a expressão da via de sinalização mediada por NRP/DCD por meio de sua capacidade de regular a UPR. A regulação da atividade de componentes da via mediada por BiP pode ser associada com a interação do mesmo com VPE. Nós encontramos que BiP interage com Caspase-1-like VPE (Vacuolar Processing Enzyme), a qual tem sido proposta ser executora o processo de morte. A interação entre VPE-BiP foi modulada por ATP, uma caracteristica de interações produtivas entre BiP e proteinas alvo. Além disso, a deficiencia na atividade de chaperone de BiP, que retêm a atividade de ligação foram capazes de modular a via de morte celular mediada por NRP/DCD e conferir tolerancia a déficit hídrico. Estes resultados indiam que a atividade de ligação, ao contrario da atividade chaperone, pode ser o mecanismo para atenuação de via de NRP/DCD por BiP. Nós também identificamos que a subunidade B da proteína G heterotrimérica, AGB1, pode modular a via de resposta mediada por NRP/DCD. O mutante nulo para agb1 apresenta menor expressão dos genes NRP/DCD em condições de estresse osmótico e do RE, além de ser mais tolerante ao estresse hídrico. Coletivamente nossos resultados indicam que a via de morte celular mediada por NRP/DCD é conservada em plantas. Baseado nos resultados nós propomos que o modelo de mecanismo para atenuação de morte celular mediada por BiP atua regulando a expressão da via de morte celular mediada por NRP/DCD como um regulador da UPR. Uma vez que genes upstream da via podem ser alvos diretos da UPR. Assim, BiP pode modular a atividade dos componentes da via de morte celular por meio da retenção de VPE no interior do RE. Usando o organismo modelo arabidopsis podemos decifrar as ligações que conectam estresse no RE, níveis de BiP e sua modulação no processo de morte celular.

ABSTRACT

REIS, Pedro Augusto Braga dos, D.Sc., Universidade Federal de Viçosa, February of 2014. **Integration of osmotic- and ER-stress into a cell death response: positive and negative regulators of the signaling pathway are conserved in plants**. Adviser: Elizabeth Pacheco Batista Fontes. Co-advisers: Joanne Chory and Gregory Vert.

The stress-induced asparagine-rich proteins (NRPs), which harbor a developmental and cell death (DCD) domain, were first identified in soybean (Glycine max) by their capacity to mediate programmed cell death derived from prolonged endoplasmic reticulum (ER) stress, osmotic stress, drought or developmentally-programmed leaf senescence. As a branch of the ER stress response that connects with other environmentally induced responses, the NRP-mediated cell death signaling pathway may allow for the versatile adaptation of cells to different stresses. Accordingly, we showed in this investigation that the modulation of this pathway by the constitutive expression of the ER molecular chaperone BiP promotes a better adaptation of transgenic lines to drought. BiP overexpression increased tolerance of soybean transgenic seedlings to tunicamycin, an inducer of ER stress, and to PEG, which induces osmotic stress. We also showed that BiP attenuates the propagation of the cell death signal in soybean by modulating the expression and activity of the components of the cell death pathway GmNRP-A, GmNRP-B and GmNAC81. We also provided several lines of evidence revealing that the NRP-mediated cell death pathway also propagates a stress-induced cell death signal in Arabidopsis with features of a PCD response First, the Arabidopsis genome harbors GmNRPs GmNAC81and VPE conserved sequences, designated as AtNRP-1, AtNRP-2, ANAC036 and VPE-γ, which share functional analogy with the soybean counterparts. Like the soybean orthologs, both AtNRPs and ANAC036 induced PCD when ectopically expressed in tobacco leaves and tobacco protoplast. Furthermore, AtNRP-1 knockout mutants displayed enhanced sensitivity to PEG-induced osmotic stress, a phenotype that could be complemented with ectopic expression of either GmNRP-A or GmNRP-B. Second, AtNRPs and ANAC36 were induced by osmotic and ER stress to an extent that was modulated by BiP overexpression similarly as in soybean. Third, as putative downstream components of the NRP-mediated cell death signaling, the stress induction of AtNRP2, ANAC36 and VPE-γ was dependent on the AtNRP1 function. Finally, BiP overexpression also conferred tolerance to water stress in Arabidopsis, most likely due to its capacity to inhibit the drought-induced NRP-mediated cell death response. We showed that soyBiPD modulates both the expression and activity

of the NRP/DCD cell death signaling components. Because the upstream components of the pathway, NRP-B in soybean and AtNRP1 in Arabidopsis, are likely UPR targets, due to the presence of conserved UPR cis-regulatory elements in their promoter, we proposed that BiP may inhibit the expression of the NRP/DCD cell death signaling genes through its capacity to regulate the UPR. The BiP-mediated regulation of the cell death activity of the NRP/DCD cell death signaling components may be associated with the BiP interaction with VPE. We found that BiP interacts with the Caspase-1-like VPE (Vacoular Processing Enzyme), which has been proposed to be the executioner of the cell death process. The VPE-BiP interaction was modulated by ATP, a feature of productive interactions between BiP and client proteins. Furthermore, chaperone activity deficient mutants that retain ligand binding activity were capable of modulating the NRP/DCD cell death response and of conferring tolerance to water deficit. These results indicated that the ligand binding activity rather than the chaperone activity may underlie the mechanism for BiP attenuation of the NRP/DCDC cell death signaling response. We also identified that the subunit B of G heterotrimeric protein, AGB1, can modulate the NRP/DCD-mediated cell death response. The agb1 null mutant line displayed lower expression of NRP/DCD genes upon osmotic and ER stress and also was more tolerant to water stress. Collectively, our results indicated that the plant-specific NRP-mediated cell death signaling is conserved in other plant species. Based on our results we propose a mechanistic model for the BiP-mediated attenuation of stress cell death response. We propose that BiP regulates the expression of NRP/DCD cell death signaling genes as a regulator of the UPR, since upstream genes of the pathway may be UPR direct targets. BiP may also modulate the activity of the cell death pathway components by controlling the retention of VPE into the ER lumen. We are currently using the plant model system Arabidopsis to decipher the molecular links connecting ER stress, BiP levels and modulation of this programmed cell death response.

INTRODUÇÃO GERAL

A agricultura mundial sofre grandes perdas todos os anos devido a condições adversas que as plantas enfrentam. Seca, salinidade e variações de temperatura são os principais causadores destes prejuízos. Mesmo em condições não favoráveis as plantas tentam se adaptar e criar condições de se desenvolverem. Para conseguir tal feito, elas desenvolveram mecanismos de respostas moleculares que visam minimizar ou retardar os danos causados por estresses de maneira que possam se recuperar. Os mecanismos bioquímicos utilizados pelas plantas em resposta a estas condições ainda não foram totalmente elucidados, mas avanços significativos no conhecimento sobre as bases moleculares de respostas adaptativas das células vegetais foram alcançados na última década. Alguns tipos de estresses podem causar um acúmulo de proteínas que não adquirem uma conformação necessária para desempenhar uma função bioquímica. Assim, uma resposta molecular que visa diminuir este acúmulo e manter a homeostase celular deve ser ativada. A via de resposta a proteínas mal dobradas (UPR) é uma via de sinalização desencadeada no reticulo endoplasmático que é ativada quando ocorre o acúmulo de proteínas que não adquiriram a conformação adequada e que seriam danosas às células (Urade., 2007). Outros tipos de estresses podem desencadear vias de sinalização diferentes, como as vias de sinalização em resposta ao déficit hídrico e à salinidade, que culminam em mecanismos de manutenção do equilíbrio osmótico celular (Shinozaki, Yamaguchi-Shinozaki and Seki., 2003). Uma possível via que conecta estresse no reticulo endoplasmático e osmótico foi evidenciada por meio de análises de microarranjo de DNA de soja (Irsigler et al., 2007). Neste experimento, foram identificados 10 genes que tinham a expressão aumentada quando submetidos a ambos os estresses. Dentre estes genes, destacam-se os da família NAC (NAM, ATAF e CUC) e da família NRP (N rich protein) por apresentarem uma indução sinergística quanto submetidos à combinação de tratamentos com indutores de estresse osmótico e do RE.

O papel de defesa celular da UPR está relacionado ao aumento coordenado de chaperones residentes do RE, que atuam tanto no enovelamento de proteínas quanto na ativação de vias de sinalização (Malhotra and Kaufman, 2007). BiP é um chaperone residente do RE responsável pelo desencadeamento da resposta ao acumulo de proteínas mal dobradas. A superexpressão deste chaperone tem mostrado uma atenuação às condições de estresses no RE de células de mamíferos. Em plantas, tem sido relatado que a superexpressão de BiP atenua os efeitos do estresse hídrico (Cascardo et al., 2000,

Alvim et al., 2001, Valente et al., 2009). Este efeito tem sido relacionado ao papel de BiP em atrasar o processo de morte celular causado pelo defict hídrico (Valente et al., 2009). Como residente do reticulo endoplasmático, BiP deve regular coordenadamente vias de sinalização de morte celular que integra sinais de estresses no retículo endoplasmático e resultantes de desidratação. A via de sinalização de morte celular derivada de sinais de estresses do retículo e que integra os sinais osmótico convergindo nas proteínas NRPs em nível de expressão gênica (Isrigler et al., 2007; Costa et al., 2008) representa um alvo em potencial para controle coordenado de BiP. NRPs são proteínas, cuja expressão é aumentada por indutores de estresse no reticulo endoplasmático (RE) e estresse osmótico. NRP é codificada por uma pequena família gênica, sendo representada por três cópias no genoma de soja, NRP-A, NRP-B, NRP-C. Tem sido demonstrado que a expressão de genes NRP-A e NRP-B é aumentada sinergisticamente em resposta à combinação dos dois estresses osmótico e RE (Irsigler et al., 2007; Costa., 2008). As proteínas NRP apresentam um papel importante nesta possível via integrativa, pois elas são capazes de induzir morte celular programada (Costa., 2008). Recentemente, foi identificado a proteína GmERD15 que ativa o promotor e a expressão de NRPs (Alves et al., 2011). Além disso, foi mostrado também que a superexpressão de NRPs induz a expressão de GmNAC81, um fator de transcrição da família NAC (Faria et al., 2011). Além, de ser induzido por NRPs, a expressão de GmNAC81 aumenta sinergisticamente em resposta aos tratamentos com indutores de estresse no RE e osmótico. GmNAC30, um membro da familia NAC, foi identificado por interagir com GmNAC81 e atuar na via de morte celular mediada por NRP/DCD. GmNAC30, assim como GmNAC81, promove morte celular quando transientemente expresso em protoplastos de soja. Além disso, ambos são capazes de se ligarem em sequencias especificas e ativar genes de resposta à morte celular (Mendes et al., 2013). Dentre os genes identificados, destaca-se o gene de VPE (Vacuolar Processing Enzyme) que apresenta atividade Caspase-1-like e atua como executor no processo de morte celular programada. O modelo atual da via que integra os sinais de estresses no RE e osmótico em uma resposta de morte celular preconiza que os estresses osmótico e do RE induzem a expressão de GmERD15 que, por sua vez, ativa o promotor e a expressão de NRPs. A indução de NRPs leva a ativação de uma cascata de sinais, resultando na indução de GmNAC81 e GmNAC30 que por conseguinte, a expressão de VPE, um efetor de morte celular programada (Mendes et al., 2013).

Além da relevância da via de sinalização de morte celular mediada pelas proteínas NRPs/DCDs como alvo para aumento de tolerância a múltiplos estresses, a referida via culmina com um processo de morte celular programada específico de plantas e, portanto, encerra uma importância biológica singular em fisiologia vegetal. Ainda assim, os componentes upstream de percepção do sinal de morte celular da via de sinalização são desconhecidos e as conecções moleculares que ligam a regulação do molecular chaperone BiP à resposta de morte celular permanecem para serem identificados. Assim assim, esta investigação teve como objetivos principais (i) identificar os mecanismos pelos quais BiP retarda o processo de morte celular em plantas desidratadas, (ii) identificar ferrramentas moleculares para acelerar a elucidação da via de sinalização de morte celular induzida por estresses osmótico e do retículo endoplasmático e (iii) avaliar a conservação da referida via em outras espécies de plantas. O primeiro objeto é explorado no capítulo II intitulado "The binding protein BiP attenuates stress-induced cell death in soybean via modulation of the N-rich protein-mediated signaling pathway" que foi publicado em Plant Physiology, 2011 (Reis et al., 2011). O segundo e terceiro objetivos são desenvolvidos no capítulo III intitulado "BiP may control the ER stress and osmotic stress-mediated cell death through interaction with VPE".O capítulo I consiste em uma revisão de literatura sobre o tema principal da tese e foi publicado como capítulo 12 do livro "A Comprehensive Survey of International Soybean Research - Genetics, Physiology, Agronomy and Nitrogen Relationships" (Pedro A.B. Reis and Elizabeth P. B. Fontes; Edited by James E. Board, Publisher: InTech, Chapters published J, 2013)

CAPITULO I

Cell death signaling from the endoplasmic reticulum in soybean

Pedro A.B. Reis and Elizabeth P. B. Fontes
Departamento de Bioquímica e Biologia Molecular/ Bioagro, Universidade Federal de Viçosa, Viçosa, MG, Brazil.

1.Introduction

Plants are constantly subjected to adverse environmental conditions, such as extreme of temperature, cold, salinity and drought. As a consequence, plant cells have developed coordinated and integrated mechanisms that respond these injuries and are immediately activated upon stresses. To cope with the stress, cell signaling pathways are activated and promote up or down regulation of specific genes, which minimize the deleterious effect of stresses within the cell. The endoplasmic reticulum (ER) is a key signaling organelle involved in the activation of cellular stress responses in eukaryotic cells. One such well-characterized signaling event is the unfolded protein response (UPR), which is activated to cope with the disruption of ER homeostasis that results in the accumulation of unfolded or misfolded proteins in the lumen of the organelle. Upon disruption of ER homeostasis, plant cells activate at least two branches of the unfolded protein response (UPR) through IRE1-like and ATAF6-like transducers, resulting in the up-regulation of ER-resident molecular chaperones and the activation of the ER-associated degradation protein system. However, if ER stress is sustained, an apoptotic pathway is activated. Persistent ER stress has been shown to trigger both ER-stress specific apoptotic pathways and shared PCD (programmed cell death) signaling pathways elicited by other death stimuli. One plant-specific, ER stress-shared response is the ER and osmotic stress-integrated signaling, which converges on N-rich proteins (NRPs) to transduce a cell death signal. NRP-mediated cell death signaling is a distinct, plant-specific branch of the ER stress pathway that has been shown to integrate the ER and osmotic stress signals into a full response. This ER- and osmotic-stress induced cell death signaling pathway has been uncovered in soybean and constitutes the major focus of this chapter. A second cell death pathway induced by ER stress has been shown to be mediated by the G protein in Arabidopsis, but it remains to be determined whether it operates in soybean as well.

2. ER stress response: the cytoprotective unfolded protein response

The ER is a highly dynamic organelle, which mediates several cellular functions, such as the folding and post-translational modification of secretory proteins and protein quality control in addition to maintaining Ca^{2+} homeostasis (Schröder 2008). The loading of unfolded protein in the lumen of ER for maturation is tightly controlled and dependent on the cellular requirements. Under stress conditions, the folding capacity of the ER can be overloaded causing the accumulation of unfolded proteins and disruption of cellular homeostasis (Xu, Bailly-Maitre & Reed 2005). To cope with this stress condition, eukaryotic cells evolved a sophisticated signaling mechanism referred to as unfolded protein response (UPR; Malhotra and Kaufman., 2007). In mammalian cells, the UPR is transduced through three distinct ER-transmembrane sensors: PERK (protein kinase RNA-like ER kinase), Ire1 (inositol-requiring protein-1) and the basic leucine zipper transcription factor ATF6 (activating transcription factor-6; Ron and Walter, 2007; Malhotra and Kaufman, 2007, Kapoor and Sanyal, 2009). The activation of the UPR allows the ER processing and folding capacities to be balanced with protein loading into the lumen of the organelle under conditions of ER stress (Malhotra and Kaufman, 2007). This balance is achieved by (i) shutting down protein synthesis via PERK activation, (ii) up-regulating the expression of ER-resident processing proteins, such as molecular chaperones and foldases, via activation of Ire1 and ATF6, and (iii) inducing the ER-associated protein degradation (ERAD) machinery, through activation of Ire1, which mediates the targeting and subsequent degradation of unfolded proteins by the proteosome. However, if the ER stress is sustained, multiple apoptotic pathways can be activated in mammalian cells.

In plants, the UPR seems to operate as a bipartite module, as the ER stress signal is transduced through homologs of the Ire1 and ATF6 transducers, but a PERK-mediated branch of the UPR has not been shown (Urade 2009; Chen and Brandizzi., 2012). Two components of the Ire1-mediaded branch of the UPR is known. The first one is the Ire1 ortholog that is represented by two copies in the Arabidopsis genome, Ire1a and Ire1b, and one copy, OsIre1, in the rice genome. Like the mammalian counterpart, plant Ire1 is associated with the ER membrane and exhibits ribonuclease activity and autophosphorylation activities, as shown for Ire1a, Ire1b and OsIre1 (Koizumi et al., 2001; Okushima et al., 2002). The second component is the the ER membrane-associated transcription factor bZIP60. Upon ER stress, bZIP60 mRNA is spliced in an IRE1-mediated process to generate an alternatively spliced transcript that lacks the

transmembrane domain-encoding sequences (Liu et al., 2007 e 2008 ; Deng et al., 2011; Nagashima et al., 2011). This splicing leads to the synthesis of a soluble and functional bZIP60 transfactor that can be translocated to the nucleus, where it activates ER stress inducible promoters, such as the BiP3 promoter. Likewise, OsbZIP74 or OsbZIP50 from rice, an ortholog of Arabidopsis AtbZIP60, is regulated through the IRE1-mediated splicing of its RNA to render the activation of ER stress-inducible promoters (Hayashi et al., 2011; Lu et al., 2011).

The second branch of UPR in plants mechanistically resembles the ATF6-mediated transduction of the ER stress signal. Upon ER stress, the membrane-associated Arabidopsis ATF6 homologs bZIP17 and bZIP28 are relocated to the Golgi, where their transcriptional domains are proteolytically released from the membrane by SP2 (Tajima et al., 2008: Che et al., 2010). The released bZIP domain of these transfactors is then translocated to the nucleus, where it acts in concert with the heterotrimeric NF-Y complex to activate UPR genes (Liu e Howel., 2010). The NF-Y complex is composed the transcriptional factors NF-YA4, NF-YB3 and NF-YC2.

Comprehensive genome-wide evaluations of ER stress-induced changes in gene expression have provided evidence that the UPR operates in a similar fashion in both soybean and Arabidopsis (Irsigler et al., 2007). Inducers of ER stress, such as tunicamycin and AZC, promote the up-regulation of a class of genes that functions in protein folding and ERAD. In the protein folding category, the up-regulated genes include ER-resident molecular chaperones such as BiP, calreticulin, calnexin, and the folding catalyst protein disulfide isomerase (PDI). ERAD-associated genes that are up-regulated by ER stress in soybean include those encoding polyubiquitin, ubiquitin conjugating enzyme, the alpha subunit of the proteasome, CDC48 and Derlin. These genomic analyses suggested that soybean, like Arabidopsis, have evolved at least two different mechanisms that mediate UPR: (i) transcriptional induction of genes encoding chaperones and vesicle trafficking proteins and (ii) upregulation of the ER-associated protein degradation (ERAD) system for rapid disposal of unfolded proteins in the ER as a protective measure.

In addition to the cytoprotective bipartite response to ER stress in plants, two apparently distinct branches of the ER stress-induced pathways have been shown to transduce a cell death signal: (i) the ER membrane associated Gβ-Gγ heterodimer-mediated signaling events that trigger UPR-associated cell death in Arabidopsis (Wang

et al., 2007) and (ii) the ER stress-induced NRP-mediated cell death response that has been uncovered in soybean (Reis and Fontes, 2012).

3.The ER-stress-induced NRP-mediated cell death response

NRP-mediated cell death signaling is a distinct, plant-specific branch of the ER stress pathway that has been uncovered in soybean and has been shown to integrate the ER and osmotic stress signals into a full response. This integrative pathway was first identified through genome-wide approaches and expression profiling, which revealed the existence of a modest overlap of the ER and osmotic stress-induced transcriptomes in soybean seedlings treated with PEG (an inducer of osmotic stress) or tunicamycin and AZC (potent inducers of ER stress; Irsigler et al., 2007). The co-regulated genes were first considered to be downstream targets of the integrated pathway based on similar induction kinetics and a synergistic response to the combination of osmotic and ER stress-inducing treatments. Based on these criteria, the selected downstream components of this ER and osmotic stress response-integrating pathway encode proteins with diverse roles, such as plant-specific development and cell death (DCD) domain-containing proteins (NRP-A and NRP-B), an ubiquitin-associated (UBA) protein homolog and NAC domain-containing proteins (GmNAC6). Among them, NRP-A and NRP-B were the first ones to be characterized and to show to induce a cell death response when ectopically expressed in tobacco leaves or soybean protoplasts (Costa et al., 2008). As a consequence, the ER and osmotic stress response-integrating pathway has been designated as the NRP-mediated cell death response.

An upstream component of the NRP-mediated cell death response, GmERD15 (Glycine max Early Responsive to Dehydration 15), has been recently identified using one-hybrid screening that targeted the NRP-B promoter in yeast (Alves et al., 2011). GmERD15 is induced by ER and osmotic stress to activate the expression of NRP genes (NRP-A and NRP-B). Up-regulation of NRP-B leads to the induction of an NAC domain-containing protein, GmNAC6, which is a critical mediator of stress-induced cell death in plants (Faria et al., 2011). These components of the ER stress-induced NRP-mediated cell death signaling pathway, GmERD15, NRPs and GmNAC6, have been further characterized.

3.1.GmERD15 is a ssDNA binding transcriptional activator

The Early responsive Dehydration (ERD) genes are rapidly induced in response to water deficit and form a family comprised by ERD1 to ERD16 representatives. The ERD encoded proteins exhibit diverse and heterogeneous biochemical functions and fall into different classes of proteins, such as chloroplast ATP-dependent protease (ERD1), cytosolic HSP70 (ERD2), glutationa-S-transferases (ERD9,ERD11, ERD13) among others (Soitano et al., 2008; Kiyosue et al., 1994; Kiyosue et al., 1993). ERD15 was first identified in Arabidopsis as a hydrophilic protein that possesses a PAM2 domain that interacts with polyA-binding proteins (PABP11; Kiyosue et al., 1994; Kariola et al., 2006)., ERD15 has been shown to function as a negative regulator of the abcisic acid (ABA)-mediated response (Kariola et al., 2006). Overexpression of ERD15 reduces the ABA sensitivity of Arabidopsis, whereas silencing of ERD15 by RNAi promotes hypersensitivity to the hormone. The negative effect of ERD15 on ABA signaling enhances salicylic acid-dependent defense because overexpression of ERD15 was associated with increased resistance to the bacterial necrotroph Erwinia carotovora, and the enhanced induction of marker genes for systemic acquired resistance. These results are consistent with the observed antagonistic effect of ABA on salicylic acid-mediated defense and may implicate ERD15 as a shared component of these responses.

The soybean GmERD15 homolog has been described as a new ER stress- and osmotic stress-induced transcription factor that binds to the promoter and induces the expression of the NRP-B gene. In fact, GmERD15 was isolated by its capacity to associate stably with the promoter of NRP-B in yeast cells using the one-hybrid system (Alves et al., 2011). The GmERD15 binding site in the NRP-B promoter was mapped to a 12-bp palindromic sequence (511 AGCAnnnnTGCT -500) that resembles binding sites for ssDNA binding proteins, such as NF1C and PBF2 that recognize the sequences -TTGGCnnnnnGCCAA-3' and 5- TGACAnnnnTGTCA-3', respectively (Wang and Kiledjian., 2000). Furthermore, GmERD15 is located in the nucleus, and chromatin immunoprecipitation (ChIP) assays revealed that it binds to the NRP-B promoter in vivo (Alves et al., 2011). The ectopic expression of GmERD15 in soybean cells activates the NRP-B promoter and induces NRP-B expression. Collectively, these results indicate that GmERD15 functions as an upstream component of the NRP-mediated cell death signaling pathway that is induced by ER stress and osmotic stress

3.2.NRPs: molecular and functional characterization

The N Rich Protein (NRP) gene was first identified by its rapid induction in response to pathogen incompatible interactions in soybean (Ludwig and Tenhaken, 2001). The NRP designation was derived from its high content of asparagine residue, about 25 %. NRP is represented in the soybean genome by a small family of three genes: NRP-A, NRP-B and NRP-C. The encoded proteins share a highly conserved development and cell death (DCD) domain at the C-terminal portion in addition to a high content of asparagine residues at their more divergent N termini. The N-rich asparagine domain is not well characterized but harbors putative glycosylation and myristoylation sites that may be relevant for function. The DCD domain is found exclusively in plant proteins and it is composed of about 130 amino acid residues, organized into several conserved motifs: FGLP and LFL in the N-treminal region of the domain, PAQV and PLxE at its C-terminus (Tenhaken et al; 2005). DCD domain-containing proteins may be subdivided into four groups, according to the localization of the DCD domain in the primary structure. NRPs belong to the the subgroup I of DCD domain-containing family of proteins, as their domains are located at the C-terminal portion of the protein (Tenhaken et al; 2005).

NRPs are critical mediators of ER and osmotic stress-induced cell death in soybeans (Costa et al., 2008). The cell death response mediated by NRPs resembles a programmed cell death event. The overexpression of NRPs in soybean protoplasts induces caspase-3-like activity and promotes extensive DNA fragmentation. Furthermore, the transient expression of NRPs in plants causes leaf yellowing, chlorophyll loss, malondialdehyde production, ethylene evolution and the induction of senescence marker genes, which are hallmarks of leaf senescence.

NRPs are up-regulated by ER or osmotic stress but need both stress signals for full induction (Isrigler et al., 2007). This synergistic interaction of both signals upon NRP induction indicates that the ER stress and osmotic stress responses converge at the level of gene expression to potentiate a NRP-mediated cell death response (Costa et al., 2008). NRPs are also up-regulated by other abiotic and biotic signals, such as salt stress, oxidaive stress and pathogens. Because the NRP-mediated cell death signaling pathway represents a shared response to multiple stress signals in plants, it might permit coordinate adaptive cellular responses under a large array of stress conditions

3.3.GmNAC6 as a downstream component of the NRP-mediated cell death response

NAC domain-containing proteins are plant-specific transcriptional factors that are expressed in several tissues and developmental stages. The NAC transfactors are organized into a general structure that consists of a highly conserved N-terminal domain involved in DNA binding (called NAC domain) and a C-terminal region highly divergent in sequence and length that functions as the activation domain. The NAC domain was derived from comparison of consensus sequences among NAM from Petunia, ATAF1/2 and CUC2 from Arabidopsis (Souer et al., 1996.). It comprises nearly 160 amino acid residues, divided into five subdomains (A–E) exhibiting a negative net charge and a nuclear localization signal (Xie et al., 1999; Seo et al., 2008). The subdomains A, C and D are conserved among plant species whereas B and E subdomains are variable (Ooka et al., 2003). The C- terminus harbors a protein-protein interaction domain in some NAC-containing proteins while a transmembrane domain is present in other transcriptional factors (Seo et al., 2008). Therefore, the NAC family is comprised by both soluble, nuclear transactivators and membrane proteins.

The members of the NAC gene family are involved in a variety of developmental events and defense responses, such as shoot apical meristem formation and maintenance (SAM; Aida et al., 1997; Souer et al.,1996; Weir et al., 2004), hormone signaling (Fujita et al., 2004; Xie et al., 2000), response to pathogen infection (Ren et al., 2000; Selth et al., 2005; Xie et al., 1999), leaf senescence (John et al., 1997) and response to different abiotic stresses (Hegedus et al., 2003; Tran et al.,2004).

The soybean NAC family is comprised by 180 putative sequences of NAC domain-containing proteins, which display different expression profiles in response to distinct environmental stress conditions and developmental signals (Mochida et al.,2009; Mochida et al., 2010; Wang et al., 2010). Frequently, the stress-induced expression profile of the soybean NAC genes reflects the functional profile of the encoded protein (Pinheiro et al., 2009). GmNAC6 was identified by its synergistic induction in response to a combined treatment of inducers of osmotic stress (polyethylene glycol) and ER stress (tunicamycin) and was functionally linked to the NRP-mediated cell death response (Faria et al., 2011). Transient expression of GmNAC6 promotes cell death and hypersensitive-like responses in planta. GmNAC6 and NRPs also share overlapping responses to biotic signals, but the induction of NRPs peaks before the increased accumulation of GmNAC6 transcripts. Consistent with the delayed kinetics of GmNAC6 induction, increased levels of NRP-A and NRP-B transcripts induce promoter activation

and the expression of the GmNAC6 gene. Therefore, GmNAC6 is biochemical and functionally linked to the ER stress- and osmotic stress-integrating cell death response, in which it acts downstream of the NRPs.

GmNAC6 encodes a 33kDa protein that belongs to the TERN (Tobacco elicitor-responsive gene encoding NAC domain protein) group of the NAC family, which is induced by elicitors of the pathogen response (Ooka et al.,2003). Likewise, GmNAC6 is induced by the pathogenic bacteria Pseudomonas syringae patovar tomato which elicits an incompatible interaction in soybean and by cell wall-degrading enzymes, which mimic bacterial pathogen attack. Like GmNAC6, the other components of the ER- and osmotic-stress induced cell death signaling pathway, GmERD15 and NRPs, are also induced by other biotic and abiotic signals, such drought and pathogen incompatible interactions. Therefore, the activation of the NRP-mediated senescence-like response is not specific to ER stress or osmotic stress but is, rather, a shared branch of general environmental adaptive pathways.

4.Acnowledgements

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Capitulo II

The binding protein BiP attenuates stress-induced cell death in soybean via modulation of the N-rich protein-mediated signaling pathway

Running title: BiP modulates a cell death response

* Corresponding author: E. P. B. Fontes

Departamento de Bioquímica e Biologia Molecular/BIOAGRO, Universidade Federal de Viçosa, 36571.000 Viçosa, MG, Brazil. Phone: +55 31 3899 2949, Fax: +55 31 3899 2863, E-mail address: bbfontes@ufv.br

Environmental Stress and Adaptation

The binding protein BiP attenuates stress-induced cell death in soybean via modulation of the N-rich protein-mediated signaling pathway

Pedro A. A. Reis[a,b], Gustavo L. Rosado[a,b], Lucas A. C. Silva[a,,b], Luciana C. Oliveira[a], Lucas B. Oliveira[a], Maximiller D. L. Costa[a], Fátima C. Alvim[c], Elizabeth P. B. Fontes[a,b*a]Departamento de Bioquímica e Biologia Molecular/BIOAGRO, [b]National Institute of Science and Technology in Plant-Pest Interactions, Universidade Federal de Viçosa, 36570.000, Viçosa, Minas Gerais, Brazil. [c]Centro de Biotecnologia e Genética, Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, Salobrinho, Ilhéus, BA, Brasil.

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* Corresponding author: Fontes E.P.B., e-mail: bbfontes@ufv.br

ABSTRACT

The molecular chaperone binding protein (BiP) participates in the constitutive endoplasmic reticulum (ER) function and protects the cell against stresses. In this study, we investigated the underlying mechanism by which BiP protects plant cells from stress-induced cell death. We found that enhanced expression of BiP in soybean (Glycine max) attenuated ER-stress- and osmotic-stress-mediated cell death. Ectopic expression of BiP in transgenic lines attenuated the leaf necrotic lesions that are caused by the ER stress inducer tunicamycin and also maintained shoot turgidity upon polyethylene glycol (PEG)-induced dehydration. BiP-mediated attenuation of stress-induced cell death was confirmed by the decreased percentage of dead cell, the reduced induction of the senescence-associated marker gene GmCystP and reduced DNA fragmentation in BiP-overexpressing lines. These phenotypes were accompanied by a delay in the induction of the cell death marker genes NRP-A, NRP-B and GmNAC6, which are involved in transducing a cell death signal generated by ER stress and osmotic stress through the N-rich protein (NRP)-mediated signaling pathway. The prosurvival effect of BiP was associated with modulation of the ER-stress- and osmotic- stress-induced NRP-mediated cell death signaling, as determined in transgenic tobacco lines with enhanced (sense) and suppressed (antisense) BiP levels. Enhanced expression of BiP prevented NRP- and NAC6-mediated chlorosis and the appearance of senescence-associated markers, whereas silencing of endogenous BiP accelerated the onset of leaf senescence mediated by NRPs and GmNAC6. Collectively, these results implicate BiP as a negative regulator of the stress-induced NRP-mediated cell death mediated.

INTRODUCTION

Exposing cells to environmental stress induces the expression of stress proteins in various intracellular compartments including the endoplasmic reticulum (ER). The ER mediates several cellular functions, such as the folding and post-translational modification of secretory proteins and protein quality control in addition to maintaining Ca^{2+} homeostasis (Naidoo 2009). The ER also plays a major role in the signaling response to conditions that disrupt ER homeostasis and promote the accumulation of misfolded or unfolded proteins in the lumen of the organelle. This ER stress signal is transduced through the unfolded protein

response (UPR) pathway. In mammalian cells, the UPR is trasnduced through three distinct ER-transmembrane sensors: an Ire1 homolog, the basic leucine zipper transcription factor ATF6, and the PKR-like ER kinase (PERK) (reviewed in Schroder and Kaufman, 2005; Malhotra and Kaufman, 2007; Kapoor and Sanyal, 2009). In plants, there is evidence that the UPR operates through a signaling response with at least two components; this bipartite response is mediated by IreI-like receptors and ATAF6 analog transducers (reviewed in Urade, 2009; Liu and Howell, 2010). The activation of the UPR allows the ER processing and folding capacities to be balanced with cell secretory activities under conditions of ER stress (Malhotra and Kaufman, 2007). This balance is achieved by shutting down protein synthesis, activating the expression of ER-resident processing proteins, such as molecular chaperones and foldases, and inducing the ER-associated protein degradation machinery (Schroder and Kaufman, 2005).

However, if the ER stress is sustained, an apoptotic pathway is activated. In mammalian cells, this involves the ER-localized caspase-12 enzyme, which is highly specific to the UPR pathway (Nakagawa et al., 2000). Multiple additional pathways can also contribute to ER-stress induced apoptosis. IRE1 can activate the ASK1/JNK mitogen- activated protein kinase pathway (Urano et al., 2000; Xu et al., 2005) or p53 (Li et al., 2006), promoting apoptosis via the classical mitochondrial apoptosis pathway. Furthermore, the proapoptotic BCL-2 family proteins, BAX and BAK, have been found to interact with IRE1 directly on the ER membrane surface (Hetz et al., 2006). In plants, a Gβ-Gγ heterodimer protein associated with the ER membrane is involved in the signaling events that trigger UPR-associated cell death

in Arabidopsis (Wang et al., 2007). In addition, we recently identified a novel branch of the ER-stress signaling pathway that diverges from the molecular chaperone-inducing branch of the UPR and transduces a programmed cell death (PCD) signal. This pathway integrates the ER-stress and osmotic-stress signals to synergistically increase the expression of N-rich proteins (NRP-A and NRP-B) and an NAC domain-containing protein, GmNAC6, which are critical mediators of stress-induced cell death in plants (Irsigler et al., 2007; Costa et al., 2008, Pinheiro et al., 2009). These roles indicate that the integrated pathway mediated by NRP upregulation transduces a PCD signal that is generated by prolonged ER stress and osmotic stress. More recently, we showed that NRP-B expression is controlled by a novel ER-stress- and osmotic-stress-induced transcriptional factor, GmERD15 (Glycine max Early Responsive to Dehydration 15; Alves et al., 2011). ERD15 was first described in Arabidopsis as a dehydration-induced gene (Kiyosue et al., 1994) that functions as a negative regulator of the abscisic acid (ABA)-mediated response and a positive regulator of the salicylic acid (SA)-dependent defense pathway (Kariola et al., 2006). It is very likely that the NRP-mediated cell death signaling pathway represents a common response of plant cells to a variety of different stimuli.

The cytoprotective role of the UPR has been linked to the coordinated upregulation of ER-resident molecular chaperones, which are involved in controlling the major functions of the ER (Malhotra and Kaufman, 2007). The ER-resident molecular chaperone BiP plays a central role in ER stress signaling by sensing alterations in the ER environment that affect protein folding and assembly (Hendershot, 2004; Malhotra and Kaufman, 2007). In addition to its role as molecular chaperone, in mammalian cells, BiP directly regulates the UPR by controlling the activation status of the three transducers, IRE1, PERK, and ATF6 (Hendershot, 2004). Because BiP is the sole molecular chaperone involved in the activation of the UPR, its level may be monitored by the cell as an indicator of changes in the folding environment and ER processing capacity. Accordingly, 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). Mammalian BiP also exhibits protective properties that prevent oxidative stress, Ca^{2+} disturbances and cell death (Liu et al., 1997; 1998; Gething, 1999; Kishi et al., 2010).

In addition to alleviating ER stress, the overexpression of BiP in plants has also been shown to increase their tolerance to water deficits (Cascardo et al., 2000, Alvim et al., 2001, Valente et al., 2009). The apparent increase in drought tolerance mediated by BiP has not been associated with typical short-term and long-term avoidance responses or with other known tolerance mechanisms (Valente et al., 2009). The only variations observed in BiP- overexpressing (OE) lines are a delay in drought-induced leaf senescence and an inhibition of the drought-mediated downregulation of ER molecular chaperone transcripts that occurs under prolonged osmotic stress. However, BiP's mode of action is still unclear. The major goal of this study was to address the role of a plant BiP in stress-induced cell death in relation to ER function. Our results provide new insights into the protective properties of plant BiP against stresses and indicate that BIP may play an important role in protecting cells from cell death by modulating the NRP-mediated stress response.

RESULTS

BiP-overexpressing transgenic lines show increased resistance to the cell death-promoting effect of tunicamycin

Little is known about the mechanism of ER stress-induced PCD in plants relative to that in animal systems. We recently reported that the manipulation of BiP levels in transgenic plant lines affects drought-induced senescence in leaves (Valente et al., 2009). However, it remained unclear whether the delay in leaf senescence was a direct effect of BiP overexpression or the result of an absence of stress in OE transgenic lines. In this work, we directly addressed this issue by testing the effect of BiP overexpression on a typical ER stress- induced cell-death event. We treated OE soybean (Glycine max) seedlings with tunicamycin, a potent inducer of ER stress and cell death (Crosti et al., 2001; Fontes et al., 2001; Zuppini et al., 2004), and examined the evolution of senescence-associated morphological and molecular markers over a period of 48 h. A reproducible pattern was observed (Figure 1). In wild-type seedlings at 24 h, tunicamycin-induced leaf yellowing became visible and cotyledon leaves became senescent with necrotic lesions. At 48 h, the chlorotic phenotype was more intense and necrotic lesions were observed over the entire surface of the cotyledons. In contrast, the OE seedlings were more resistant to these changes as shown by an attenuated leaf yellowing that only became visible at 48 h in cotyledon leaves.

This phenotype was confirmed by assessing cell viability with Evans Blue staining (Levine et al., 1996) as evidenced by lethally damaged cells that were unable to exclude the dye (Figure 2A). In wild-type (WT) seedlings, a decrease in cell viability was evident after 24 h of tunicamycin exposure. At 48 h post- treatment, the difference in cell viability between WT and OE lines was further enhanced, indicating that OE lines were more resistant to tunicamycin-induced cell death.

To determine whether the differences in the leaf phenotypes displayed by the two genotypes upon tunicamycin exposure reflected similar differences in the onset of leaf senescence, we monitored the expression of the senescence-associated marker gene GmCystP (cysteine protease 1; Valente et al., 2009) over 48 h of tunicamycin treatment by quantitative RT-PCR (Figure 2B). GmCystP expression was strongly induced by tunicamycin at 48 h in WT seedlings but not in OE seedlings, further supporting the hypothesis that BiP increases the resistance of leaf cells to ER stress-induced senescence. The late induction kinetics of the senescence-associated gene paralleled the tunicamycin-induced morphological changes in WT leaves.

Figure 2C shows the levels of the BiP transgene transcripts during the experiment, which also served as an endogenous control for the qRT-PCR analysis of transgenic lines. The effectiveness of the tunicamycin treatment was evaluated by monitoring the expression of the ER-stress marker genes soyBiPD and calnexin (CNX) over a period of 48 h (Figure 2D). At 8 h post-treatment, both calnexin and endogenous BiP were strongly induced by tunicamycin, but the level of induction progressively declined over time. However, in OE lines, the UPR activation was inhibited as the variation in endogenous BiP gene expression was lower in OE seedlings than in WT seedlings treated with tunicamycin. These results are consistent with previous studies showing that BiP overexpression suppresses the activation of the UPR (Costa et al., 2008). Nevertheless, the BiP-mediated inhibition of UPR activation appears to be relieved as ER stress persists, because at 24 and 48 h post-treatment, the levels of BiP and CNX transcripts were higher in OE seedlings than in WT seedlings by both absolute and relative measurements of gene expression. In OE lines, BiP and CNX induction were barely detectable at 8 h; their expression plateaued at 24 h and then declined after 48 h of tunicamycin treatment although not to basal levels, which it did in WT seedlings.

We also examined the expression of the cell death-associated genes NRP-A and NRP- B (Costa et al., 2008) and GmNAC6 (Pinheiro et al., 2009) in response to

prolonged ER stress (Figure 2E). These genes have previously been shown to be induced by both tunicamycin and polyethylene glycol (PEG) (Irsigler et al., 2007; Costa et al., 2008; Pinheiro et al., 2009; Faria et al, in press), and the NRP-mediated signaling pathway transduces a cell death signal that originates from ER stress and osmotic stress. NRP-A and NRP-B are functional analogs that display early induction kinetics and upregulate GmNAC6 expression (Costa et al., 2008; Faria et al., in press). The induction of GmNAC6 in turn promotes cell death in soybean cells and a senescence-like response in planta (Pinheiro et al., 2009; Faria et al., in press). Here, we showed first that induction of NRPs and GmNAC6 transcript levels by ER stress reflected an enhanced accumulation of the proteins. Then, we performed a time course experiment to monitor transcript accumulation in wild type and BiP-overexpressing lines in response to tunicamycin treatment. At the protein level, the accumulation of NRP was enhanced at 8h and 24h after tunicamycin treatment in the same fashion as the ER-stress molecular markers, BiP and Calnexin (Supplemental Figure 1A). We also detected enhanced accumulation of GmNAC6 protein in nuclei of tunicamycin-treated cells (Supplemental Figure 1B). As an ER-stress induced nuclear protein, ATAF2 was included in the immunoblotting to monitor enrichment of nuclear extracts (Irsigler et al., 2007, Pinheiro et al., 2009). In both WT and OE soybean seedlings, prolonged ER stress enhanced the expression of the NRP and GmNAC6 genes, albeit with different kinetics (Figure 2E). In WT seedlings, the induction of the NRP and GmNAC6 genes was observed at 8 h after treatment and increases to reach a maximum level at 48 h, whereas the induction of the cell death-associated genes was delayed and occurred to a lesser extent in OE lines. These results may indicate that the overexpression of BiP delays the induction of cell death-associated genes due to ER stress, consistent with the attenuated tunicamycin-induced cell death phenotype displayed by OE seedlings.

To confirm that BiP would be modulating an ER stress-induced event of cell death that has features of PCD, we applied the terminal deoxyribonucleotidyl transferase (TDT)- mediated dUTP-digoxigenin nick end labeling (TUNEL) assay for in situ detection of DNA fragmentation in protoplasts of tunicamycin-treated soybean leaves (Figure 3). A negative control was performed without terminal deoxynucleotidyl transferase enzyme, and a positive control was performed with DNase1 (data not shown). The extensive cleavage of nuclear DNA into oligonucleosome-sized fragments is one of the features of active cell death (PCD). For the TUNEL assay, semi-protoplasted leaf cells were counterstained with propidium iodide (PI). Under the

conditions of our assay, the PI fluorescence signal concentrates in the nucleus as we treated the samples with RNase. In some field, we also observed PI signal in the outlines of agglomerated cells, most likely due to the presence of cell wall, indicating that the protoplasting procedure was not completed (Figure 3). At 8 h post-treatment, semi- protoplasted wild-type cells had mostly TUNEL-positive nuclei, whereas BiP overexpressing semi-protoplasts exhibited TUNEL-negative nuclei. At 48h, the TUNEL-positive signal is intensified in wild type semi-protoplasts and is slightly visible in BiP-overexpressing semi- protoplasted cells. In some cells, we could not distinguish labeling of nuclear DNA from cytoplasmic RNA, but the green fluorescent TUNEL signal was always absent in semi- protoplasted BiP-overexpressing cells at 8h post-treatment and almost undetectable at 48 h post-treatment. Collectively, our results suggest that BiP may protect cells against active cell death promoted by tunicamycin-induced ER stress. Cell death in response to osmotic stress is attenuated in BiP-overexpressing soybean seedlings WT and transgenic soybean seedlings were exposed to PEG treatment over a period of 48 h (Figure 4) and the effect of the stress was monitored by measuring the expression of the osmotic-stress-induced SMP and NAC3 genes (Costa et al., 2008; Figure 5A). Consistent with the phenotype of drought tolerance mediated by BiP in tobacco and soybean (Alvim et al., 2001; Valente et al., 2009), OE seedlings maintained leaf turgidity under dehydration conditions caused by PEG (Compare WT PEG with 35S-BiP PEG at 8 h, 24 h and 48 h). In contrast, at 48 h of PEG-induced osmotic stress, the leaves of the WT plants had completely wilted. Furthermore, while PEG accelerated the senescence of cotyledon leaves in WT seedlings, the overexpression of BiP in transgenic seedlings delayed PEG-induced leaf senescence, as judged by the degree of yellowing of the leaves (Figure 4) and the expression of the senescence-associated gene CystP (Figure 5B). The differences in shoot turgidity observed between the two genotypes following PEG treatment reflected differences in cell viability (Figure 5C). Under our experimental conditions, cell viability was monitored by the Evans blue dye method, which measures membrane integrity. The percentage of dead cells induced by PEG was lower in OE seedlings than in WT seedlings. This indicates that BiP overexpression is associated with increased viability of cells exposed to PEG. The TUNEL assay was applied to compare DNA fragmentation in semi-protoplasted cells of PEG-treated wild-type and BiP-overexpressing leaves. A representative sample is showed in Supplemental Figure 2. At 8 h post-treatment, semi-protoplasted wild-type cells exhibited TUNEL-positive nuclei

(see arrow in merged), whereas the semi-protoplasts from overexpressing lines lacked the TUNEL green fluorescent signal. These results further substantiate the notion that BiP protects cells against osmotic stress-induced cell death. We also monitored BiP transgene expression in all replicates (Figure 5D).

Because drought- and PEG-induced dehydration have been shown to downregulate genes that encode ER-resident molecular chaperones, such as BiP and calnexin (Irsigler et al.,

2007; Valente et al., 2009), we also examined the variations in endogenous BiP levels mediated by PEG in WT and OE lines (Figure 5E). Consistent with previous observations, the levels of endogenous BiP and CNX transcripts declined to below detectable levels with persistent osmotic stress. In contrast, the OE lines displayed higher levels of endogenous BiP than the WT lines under normal conditions and also retained higher levels of transcript under PEG treatment. These results indicate that the overexpression of BiP suppresses the osmotic- stress-induced downregulation of endogenous BiP.

BiP also interferes with the induction of the cell death-associated NRP-A and NRP-B genes by osmotic stress

Previously, we showed that ER and osmotic stress signals converge on NRP gene expression to potentiate a cell death response. This crosstalk between the UPR and osmotic stress signaling may provide a molecular link that permits the flow of this integrated information to be controlled by a regulator of either of the stress signals. This hypothesis prompted us to examine whether BiP, as a regulator of the UPR, also modulated NRP-A and NRP-B upregulation in response to PEG-induced cell dehydration (Figure 5F). The cell death marker genes NRP-A, NRP-B and GmNAC6 followed a similar pattern of early kinetic induction in WT seedlings and late induction in OE seedlings (Figure 5F). In WT seedlings, a robust induction of NRP-A and NRP-B and a weaker induction of GmNAC6 was observed as early as 8 h after PEG treatment. This preceded the late induction of the senescence- associated gene GmCystP (Figure 5B) and the appearance of the PEG-induced chlorotic phenotype of cotyledon leaves (Figure 4). In OE seedlings, NRP-A, NRP-B and GmNAC6 transcript levels reached maximum induction at 48 h. Collectively, these results suggest

that the modulation of osmotic stress-induced cell death by BiP may be linked to NRP-mediated signaling, at least in part.

BiP attenuates NRP-A- and NRP-B-induced cell death in agroinfiltrated tobacco leaves

We have previously shown that NRP-A and NRP-B promote cell death with PCD -like features (Costa et al., 2008). Here, we have demonstrated that BiP attenuation of cell death is accompanied by delayed induction kinetics and downregulation of NRP-B and NRP-A. To determine whether BiP directly modulates NRP-A and NRP-B PCD signaling, we used transgenic tobacco lines in which BiP was overexpressed (sense lines) or suppressed (antisense lines; Alvim et al., 2001) to transiently express the NRP genes (Figure 6A). Leaves from sense, antisense and untransformed tobacco lines were inoculated with Agrobacterium carrying NRP-A or NRP-B expression DNA cassettes; the accumulation of these proteins was monitored by immunoblotting microsomal fractions (Figure 6B; Supplemental Figure 3). The transient expression of either protein promoted leaf yellowing in untransformed lines (NRP-B infiltrated sectors are shown in Figure 6C; NRP-A agroinfiltrated sectors are shown in Supplemental Figure 4), which was associated with decreased chlorophyll and carotenoid content compared with WT sectors that were infiltrated with Agrobacterium alone (Figure 6D, WT). However, the transient expression of NRP-B and NRP-A in the leaves of the transgenic sense lines did not cause leaf yellowing (Figure 6C and Supplemental Figure 4), and chlorophyll and carotenoid content were maintained at normal levels (Figure 6D, S). In contrast, in antisense-expressing tobacco leaves, the development of chlorosis as a result of NRP-A and NRP-B expression in infiltrated sectors was enhanced (Figures 6C and Supplemental Figure 4, see antisense), with greater chlorophyll and carotenoid loss (Figure 6D, AS). The onset of senescence was also accelerated relative to the WT control. These phenotypes paralleled the induction of the senescence-associated cysteine-protease genes NTCP-23 (Costa et al., 2008) and CystP (Figure 6E). In fact, the induction of the cysteine protease genes by NRP expression was greater in antisense leaves than in WT leaves and barely detectable in sense leaves. These results demonstrate an inverse correlation between the level of BiP expression and NRP-induced leaf senescence and suggest that BIP is directly involved in modulating the NRP-mediated cell death response.

BiP also modulates the cell death response resulted from expression of GmNAC6 in tobacco leaves.

To determine the position of BiP action in the ER-stress- and osmotic-stress-induced NRP-mediated cell death response pathway, we examined whether altered levels of BiP would modulate the cell death response promoted by expression of GmNAC6, a downstream component of the NRP-mediated signaling pathway (Faria et al., in press). Tobacco leaves from sense, antisense and untransformed lines were inoculated with Agrobacterium carrying a GmNAC6 DNA cassette, and the expression of the transgene was monitored by RT-PCR (Figure 7C). Transient expression of GmNAC6 caused leaf yellowing in untransformed lines (Figure 7A, WT), which was associated with a decreased chlorophyll content relative to the control WT sectors agroinfiltrated with Agrobacterium alone (Figure 7B, blue bars). Transient expression of GmNAC6 in the leaves of transgenic sense lines (S) did not cause leaf yellowing, and the chlorophyll content was maintained at normal levels (Figure 7B, green bars). In antisense tobacco leaves (AS), the development of chlorosis as a result of GmNAC6 expression in infiltrated sectors was accentuated (Figure 7A, antisense) with greater chlorophyll loss (Figure 7B, yellow bars), and the onset of senescence was accelerated relative to the WT control. This observation was confirmed by monitoring the accumulation of transcripts of the senescence-associated genes NTCP-23 and CystP in the GmNAC6- agroinfiltrated sectors. The expression of these genes was higher in antisense lines than in the WT control and was barely detectable in sense lines (Figure 7C). These data indicate that BiP interferes with GmNAC6-induced leaf senescence; therefore, the suppression of the NRP- mediated signaling pathway by BiP may occur downstream of GmNAC6.

DISCUSSION

The ER chaperone BiP is a multifunctional protein that is involved in the major functions of the ER. In mammalian cells, BiP is responsible for maintaining the permeability barrier of the ER during protein translocation, facilitating protein folding and assembly, targeting misfolded proteins for degradation, contributing to ER calcium stores, and sensing stress conditions that activate the mammalian unfolded protein response (for a review, see Hendershot, 2004). Although translocon sealing activity and

involvement in calcium storage have not been demonstrated for BiP in planta, the other BiP functions have also been described in plant cells (Foresti et al., 2003; Leborgne-Castel et al., 1999; Li et al., 1993; Pimpl et al., 2006). As a major regulator of ER function, the concentration of BiP may be constantly monitored by the cell as an indicator of changes in the folding environment and ER processing capacity. Mammalian BiP also exhibits protective properties that prevent oxidative stress, disturbances in Ca^{2+} concentration and cell death (Liu et al., 1997; 1998; Gething, 1999; Kishi et al., 2010). None of these protective functions of BiP has been directly addressed in plant cells, despite the clear potential for plant BiP level fluctuations to play a role in sensing stresses and promoting adaptive responses.

Herein, we provide several lines of evidence showing that BiP prevents stress-induced cell death in plant cells. The prosurvival role of BiP is linked to the modulation of the NRP- mediated cell death response. Ectopic expression of BiP in transgenic lines attenuated the leaf necrotic lesions that are caused by tunicamycin, a potent inducer of ER stress and cell death in soybean, and promoted the maintenance of shoot turgidity under PEG-induced dehydration conditions, whereas untransformed control leaves wilted under similar PEG treatment conditions. These phenotypes were coordinated with the lower (and in some cases delayed) expression of senescence-associated marker genes. These results are also consistent with our data showing that the content of dead cells and DNA fragmentation measured by TUNEL assay were lower in OE transgenic lines exposed to ER stress and osmotic stress than in WT lines under the same conditions. Furthermore, the attenuation of stress-induced cell death by BiP paralleled a delay in the induction of the cell death marker genes NRP-A, NRP-B and GmNAC6, which are all involved in transducing a cell death signal that is generated by ER stress and osmotic stress. Finally, by manipulating BiP levels in sense and antisense transgenic tobacco lines, we showed an inverse correlation between BiP protein level and NRP-induced leaf senescence. We discovered that enhanced expression of BiP in sense tobacco transgenic lines prevented NRP-mediated PCD, whereas silencing of endogenous BiP in antisense lines accelerated the onset of leaf senescence induced by NRP-mediated signaling. These results indicate the direct involvement of BiP in modulating the NRP- mediated cell death response. Although the protective function of BiP against cell death is well known in mammalian cells, the role of BiP in protecting plant cells against stress- induced cell death has not

been previously shown. Thus, our data shed new light on the protective functions of BiP against stresses in plants along with the underlying mechanism of this protection. In our model, BiP modulates the NRP-mediated cell death response, a plant- specific pathway that is synergistically induced by a combination of ER stress and osmotic stress signals (Figure 8).

We previously reported that overexpression of BiP in soybean does not affect the induction of NRP genes by the ER stress inducer tunicamycin (Costa et al., 2008). In this study, we clearly observed a delay in the induction of the NRP-mediated signaling genes NRP-A, NRP-B and GmNAC6 by both tunicamycin and PEG treatments in OE lines compared with WT lines (Figures 2 and 5). It is very likely that this delay accounted for the lower levels of induction of these ER-stress- and osmotic-stress-integrating pathway genes in OE lines. Therefore, it is reasonable to suggest that, at any given time point, interference in stress- induced NRP expression due to BiP overexpression could be masked by differences in the kinetics of NRP induction between WT and overexpressing lines. This would explain the apparent contradiction between the results of the present report and those reported by Costa et al. (2008). However, whether the delay in the PEG- and tunicamycin-mediated induction of the NRP-A, NRP-B and GmNAC6 displayed by the OE lines is a primary response to BiP overexpression or a consequence of a downstream impairment of the NRP-mediated cell death response (or both) remains to be determined. Our data favor the second hypothesis because BiP directly inhibited cell death mediated by 35S promoter-driven ectopic expression of NRP-A, NRP-B and GmNAC6 in tobacco leaves (Figures 5,6 and 7).

In mammalian cells, ER stress has been shown to trigger both ER-stress-specific apoptotic pathways and shared PCD signaling pathways that are also elicited by other proapoptotic stimuli (Malhotra and Kaufman, 2007). NRP-mediated PCD signaling appears to be a shared pathway that integrates ER stress with other stress signals, providing a molecular link that allows BiP, a regulator of the UPR, to control cell death induced by various stimuli, such as tunicamycin and PEG (this work) as well as drought (Valente et al., 2009). Thus, the control of BiP over distinct events in the cell death program may be linked to a BiP-mediated modulation of the NRP-mediated cell death signaling. In addition to interfering with NRP- induced senescence, BiP prevented GmNAC6-mediated cell death. Although this resul tsuggests that BiP acts downstream of GmNAC6, collectively our data do not rule out the hypothesis that BiP may regulate

multiple targets in the pathway (Figure 8). This model may explain the phenotypes of OE lines observed under drought conditions (see below).

We recently showed that the overexpression of BiP in soybean and tobacco delays drought-induced senescence and confers tolerance to water deficit (Valente et al., 2009). The underlying mechanism of BiP-mediated increases in water stress tolerance is very likely associated, at least in part, with its capacity to modulate the osmotic stress-induced NRP- mediated cell death response. Evidence in the literature has linked drought tolerance with the suppression of stress-induced senescence. Ectopic expression of an isopentenyl transferase (IPT) gene suppresses leaf senescence and results in drought tolerance in transgenic tobacco lines (Rivero et al., 2007). In the case of the stress-induced NRP-mediated cell death response, we recently showed that this pathway may also be part of the hypersensitive response elicited by pathogen incompatibility interactions. The expression of both NRPs and GmNAC6 is induced by incompatibility interactions as well as by salicylic acid (Ludwig and Tenhaken, 2001; Faria et al., in press). Furthermore, we found that the prolonged expression of GmNAC6 and NRPs caused necrotic lesions resembling those of the hypersensitive response phenotype and induced pathogenesis-related genes (Faria et al., in press). Finally, a novel ER-stress- and osmotic-stress-induced transcriptional factor, GmERD15, which is a positive regulator of the SA-dependent defense in Arabidopsis (Kariola et al., 2006), was shown to activate the promoter and induce the expression of the NRP-B gene (Figure 8; Alves et al., 2011). Arabidopsis ERD15 has also been shown to function as a negative regulator of ABA signaling (Kariola et al., 2006), which is consistent with the observed antagonistic effect of ABA on SA-mediated defense and may implicate ERD15 as a shared component of these responses. In fact, the overexpression of ERD15 reduces ABA sensitivity and improves resistance to bacterial pathogens in Arabidopsis, whereas RNAi silencing of ERD15 promotes hypersensitivity to the hormone and improved tolerance to drought. Thus, the activation of the SA-mediated defense and NRP-mediated cell death signaling may be coupled through ERD15, which acts antagonistically to suppress the ABA-mediated response. As the NRP- mediated cell death response shares at least one component with and displays several features of the SA-mediated defense (Faria et al., in press), the activation of NRP signaling may also negatively impact ABA signaling. In this case, given that ABA is a central regulator of plant adaptation to drought (Yamaguchi-Shinozaki and Shinozaki, 2006; Zhu, 2002), one may envision a scenario in which the

inhibition of the NRP-mediated cell death response by BiP overexpression relieves the repression of ABA signaling and hence promotes stress tolerance.

We found that BiP-mediated attenuation of ER-stress- and osmotic-stress-induced cell death occurred as a direct result of the inhibition of the NRP-mediated cell death response that integrates ER stress and osmotic stress signals. It remains unknown whether the stimulus that is received or generated by BiP could lead to the inhibition of NRP-mediated senescence-like responses. PEG-induced dehydration and drought have been demonstrated to promote a coordinated downregulation of ER molecular chaperones and folding catalysts in young soybean plants (Irsigler et al., 2007, Valente et al., 2009). The overexpression of BiP, however, prevents the drought-mediated downregulation of UPR genes (Valente et al., 2009). We showed here that BiP also alleviated PEG-mediated downregulation of the UPR genes BiP and CNX (Figure 5E). We also found an inverse correlation between the levels of UPR gene transcripts and the onset of senescence induced by tunicamycin (Figure 2D). Together, these results suggest that changes in BiP levels may serve as a sensing mechanism for the cell death machinery. Thus, BiP overexpression may prevent the cell from sensing ER-stress- and osmotic-stress-induced variations in ER function by maintaining basic ER functions at a normal level under persistent stress conditions.

METHODS

Plant growth and stress treatments

Soybean seeds from WT soybean (Glycine max cv. Conquista) and the OE transgenic line 35S::BiP-4 (Valente et al., 2009) were germinated in organic soil (Bioplant) containing a mixture of soil, sand and composted manure (3:1:1). One-week-old seedlings were transferred to 2 mL of hydroponic medium supplemented with 0.5% (w/v) polyethylene glycol (PEG; MW 8,000, Sigma) or 2.5 μg/mL tunicamycin (Sigma). DMSO was used as a control for tunicamycin treatment. Stressed leaves were harvested at 8, 24 and 48 h post-treatment, immediately frozen in liquid nitrogen and stored at -80°C until processing.

Real-time PCR

All 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 reactions were performed on an ABI 7500 instrument (Applied Biosystems), using SYBR® Green PCR Master Mix (Applied Biosystems) with gene-specific primers (Supplemental Table 1). 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 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 endogenous control genes, namely RNA helicase in soybean seedlings (Irsigler et al., 2007) and actin in tobacco leaves (Costa et al., 2008).

Protein production and antibody preparation

The developmental and cell death (DCD) domain was amplified from NRP-B (Costa et al., 2008) with the primers DCD-Fwd (aaaaagcaggcttcacactttggtaacctccgatata) and NRP-B- Rvs (agaaagctgggtcatttgccggcaaagcct) and introduced by recombination into the entry vector pDONR201 to yield pDON-DCD (pUFV1199). The DCD fragment, encoding amino acids 187–330 at the C-terminus of NRP-B, was transferred to pDEST17 (His-tagged bacterial protein expression vector) to generate pHis-DCD (pUFV1337). The clone pDON-NAC6 harboring the cDNA of GmNAC6 has been previously described (Pinheiro et al., 2011) and was used to transfer the GmNAC6 insert to pDEST17 yielding pHis-NAC6. The plasmids, pHis-DCD (pUFV1337) and pHis-NAC6, were transformed into E. coli strain BL21, and the synthesis of the recombinant proteins was induced with 0.4 mM isopropyl-β-D- thiogalactopyranoside (IPTG) for 4 h at 37°C. His-tagged DCD protein and His-tagged GmNAC6 protein were affinity purified using Ni^{2+}-agarose (Qiagen) according to manufacturer's instructions and used as an antigen for antibody production in rabbits, which were immunized with three subcutaneous injections at two-week intervals.

Immunoblot analysis

A membrane-enriched fraction from agroinfiltrated tobacco leaves was prepared as described by Pirovani et al. (2002). Total protein extract from transgenic tobacco leaves was prepared as described by Alvim et al. (2001). Equivalent amounts of membrane proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane using a blotting apparatus (BioRad) according to the manufacturer's instructions. The membrane was blocked with Blotting Grade Blocker, i.e., nonfat dry milk (BioRad) in TBST (100 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.05 % (v/v) Tween-20) for 1 h at room temperature. NRP-A and NRP-B were detected using a polyclonal anti-DCD antibody at a 1:1,000 dilution followed by a goat anti-rabbit IgG conjugated to alkaline phosphatase at a 1:5,000 dilution. Alkaline phosphatase activity was assayed using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and p-nitroblue tetrazolium (NBT). BiP was detected with an anti-carboxyl BiP antibody (Buzeli et al., 2003) at a 1:1,000 dilution. GmNAC6 was probed with and anti-GmNAC6 serum at a 1:1,000 dilution and the detection was performed with the WesternBreeze® Chemiluminescent kit (Invitrogen).

In situ labeling of DNA fragmentation (TUNEL)

Free 3´OH in the DNA was labeled by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay using the ApoAlert DNA Fragmentation Assay Kit (Clontech) as instructed by the manufacturer. Samples were observed with a Zeiss LSM 410 inverted confocal laser scanning microscope fitted with the configuration: excitation at 488 nm and emission at 515 nm. As positive control, samples were treated with DNase1.

Transient overexpression in Nicotiana tabacum by Agrobacterium tumefaciens infiltration

The DNA expression cassettes pYFP-NRP-A, pYFP-NRP-B and pYFP-NAC6, in which NRP-A, NRP-B or GmNAC6 cDNAs, respectively, are fused to YFP under the control of the 35S promoter, have been described elsewhere (Costa et al., 2008; Pinheiro et al., 2009). Clones pK7-NRP-A and pK7-NRP-B harbor each respective cDNA under the control of the 35S promoter (Costa et al., 2008). To obtain a vector containing the DCD domain under the control of the 35S promoter, the DCD fragment was transferred from pDON-DCD to the plant transformation binary vector

pK7WG2 to generate pK7-DCD. Agrobacterium strain GV3101 carrying the appropriate DNA constructs was grown for 12 h and subsequently centrifuged for 5 min at 5,000 x g. Pelleted cells were resuspended in 1 mL of infiltration medium (10 mM $MgCl_2$, 10 mM MES pH 5.6, and 100 µM acetosyringone), recentrifuged and resuspended in infiltration medium to an OD of 0.05-0.2. Nicotiana tabacum plants were grown in a greenhouse with natural day-length illumination. Agrobacterium infiltration was performed in the leaves of three-week-old WT, untransformed control, transgenic sense (35S::BiPS), and transgenic antisense (35S::BiPAS) tobacco lines (Alvim et al., 2001) with sterile syringes, with perfusion of the Agrobacterium cell suspension under manually controlled pressure, as previously described (Carvalho et al., 2008).

Determination of chlorophyll and carotenoid contents

Carotenoid, chlorophyll a and chlorophyll b contents were determined spectrophotometrically at 480 nm, 649.1 nm and 665.1 nm, respectively, after quantitative extraction of pigments from individual 1 cm leaf discs with 5 mL of $CaCO_3$- saturated DMSO for 24 h at room temperature.

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Figure Legends

Figure 1. BiP overexpression increases resistance against tunicamycin-induced cell death. Overexpressing (OE) soybean seedlings (35S::BiP) and untransformed WT seedlings (WT) were exposed to tunicamycin and monitored for the development of chlorosis and necrotic lesions. Photographs were taken at 8, 24 and 48 h after treatment.

Figure 2. BiP overexpression attenuates the ER stress-mediated induction of cell-death- and senescence-associated markers. A. The extent of tunicamycin-induced cell death is inversely correlated with BiP levels. Soybean seedlings were exposed to tunicamycin, as described in Figure 1, and cell viability was measured by the Evans blue dye method. Abs_{600} reflects the dead cell content. The values represent the average of four replicates (±S.D.). B, C, D, E. Variations in the expression of senescence-associated genes and ER-stress molecular markers in OE lines in response to tunicamycin. Total RNA was isolated from OE (35S::BiP) and WT soybean seedlings harvested at 8, 24 and 48 h after tunicamycin treatment. DMSO was used as a control. The transcript levels of selected genes (as indicated) were quantified by qRT-PCR. Gene expression was calculated using the $2^{-\Delta Ct}$ method using RNA helicase as an endogenous control. cDNAs were obtained from three biological replicates and validated individually (±S.D., n = 3 technical replicates). GmCystP is a senescence-associated gene in soybean. 35S::BiP represents the transgene. BiP and CNX (calnexin) are ER stress markers. NRP-A, NRP-B and GmNAC6 are components of the ER-stress- and osmotic-stress- integrating cell death pathway.

Figure 3. Reduced TUNEL green fluorescent signal in nuclei of BiP overexpressing leaf cells. Wild type (WT) and BiP-overexpressiong (35S:BiP) soybean seedlings were treated with tunicamycin for the indicated number of hours. Then, protoplasts were prepared from tunicamycin-treated leaves, submitted to TUNEL labeling and examined by confocal microscopy. The semi-propoplasted cells were also counterstained with propidium iodide (PI) and sorted based on red fluorescence at 632 nm. Merged is an overlay of the fluorescent image of TUNEL labeling with PI staining cells that facilitates the identification of TUNEL- positive nuclei as the protoplasts were not completely formed.

Figure 4. Phenotypes of soybean seedlings exposed to PEG treatment. Seedlings of OE (35S- BiP) and untransformed WT lines were exposed to treatment with PEG and examined for leaf wilting, chlorosis and necrotic lesions. Photographs were taken at 8, 24 and 48 h after treatment.

Figure 5. BiP overexpression delays cell death promoted by PEG-induced dehydration. A. Induction of osmotic stress control genes by PEG treatment. Total RNA was isolated from OE (35S::BiP) and WT soybean seedlings harvested at 8, 24 and 48 h after PEG treatment. The transcript levels of the treatment control genes NAC3 and SMP were monitored by qRT-PCR. Gene expression was calculated using the $2^{-\Delta Ct}$ method using RNA helicase as an endogenous control. cDNAs were obtained from three biological replicates and validated individually (±S.D., n=3 technical replicates). B. BiP overexpression prevents PEG induction of the senescence-associated gene GmCystP. Variations in gene expression were determined as described in A. C. Osmotic stress induction of cell death. Soybean seedlings were exposed to PEG treatment, as described in Figure 4, and cell viability was measured by the Evans blue dye method. Abs_{600} reflects the dead cell content. The values represent the average ±S.D. of three replicates. In PEG-treated WT seedlings, massive cell death at 48 h prevented the accurate measurement of dead cells. D. Transgene expression of OE lines exposed to PEG treatment. Total RNA was isolated from OE (35S::BiP) and WT soybean seedlings harvested at 8, 24 and 48 h after PEG treatment. The transcript levels of the 35S::BiP transgene were monitored by qRT-PCR, as described in A. E. BiP overexpression attenuates PEG-induced downregulation of UPR marker genes. Transcript levels of the BiP and CNX (calnexin) genes were monitored by qRT-PCR, as described in A. F. BiP overexpression delays the induction of genes from the ER-stress and osmotic-stress-integrating cell death pathway. NRP-A, NRP- B and GmNAC6 expression was monitored over 48 h of PEG treatment, as described in A.

Figure 6. Increased levels of BiP prevent the NRP-B-induced cell death response. A. Levels of BiP protein in tobacco transgenic lines. Equal amounts of total protein extracted from the leaves of untransformed tobacco (WT) lines and transgenic lines expressing a BiP cDNA in either the sense or antisense orientations were separated by SDS-PAGE and immunoblotted with anti-carboxyl BiP antibody. B. NRP-B is correctly

located in microsomal fractions when transiently expressed in tobacco leaves. Two-week-old sense (S), antisense (AS) and wild- type (WT) tobacco leaves were infiltrated with Agrobacterium cells carrying a pYFP-NRP-B expression vector. At approximately 36 h postinfiltration, microsomal fractions were prepared from agroinfiltrated sectors and immunoblotted with an anti-DCD serum (polyclonal antibody raised against the DCD domain of NRP-B). C. Leaf yellowing and necrosis caused by the expression of NRP-B. Leaf sectors from sense, antisense and WT lines were agroinfiltrated with the indicated agroinoculum, and photographs were taken seven days after infiltration. D. Chlorophyll and carotenoid contents of agroinoculated sectors. The contents of chlorophyll a and b and carotenoids were determined in leaf sectors taken from untransformed wild-type (WT) lines and from sense (S) and antisense (AS) transgenic lines that were infiltrated with Agrobacterium carrying NRP-A or NRP-B expression cassettes or Agrobacterium cells (LBA) alone. The values represent the average ± S.D. of three independent experiments. E. NRP-mediated induction of senescence-associated genes in agroinoculated tobacco sense and antisense leaves. Two-week-old sense (S), antisense (S) and wild-type (WT) tobacco leaves were infiltrated with untransformed Agrobacterium cells (LBA 4404) or cells carrying the pYFP-NRP-B expression vector. The transcript levels of the senescence-associated gene NTCP-23 and CystP were monitored by qRT-PCR. Gene expression was calculated with the $2^{-\Delta Ct}$ method using actin as an endogenous control. cDNAs were obtained from three biological replicates and individually validated (±S.D., n=3 biological replicates).

Figure 7. The GmNAC6-mediated senescence-like response is correlated with the levels of BiP expression in transgenic tobacco lines. Two-week-old untransformed tobacco (WT) lines and transgenic lines expressing a BiP cDNA in either the sense or antisense orientations were infiltrated with untransformed Agrobacterium cells (LBA 4404) or cells carrying the pYFP- NAC6 expression vector. A. Leaf yellowing and chlorotic symptoms caused by the expression of GmNAC6. Leaf sectors from sense, antisense and WT lines were agroinfiltrated with the indicated agroinoculum, and photographs were taken seven days after infiltration. B. Chlorophyll contents of agroinoculated sectors. Chlorophyll a and b contents were measured in leaf sectors from untransformed wild-type (WT) lines and from sense (S) and antisense (AS) transgenic lines that were infiltrated with the indicated agroinoculum (pYFP-NAC6 or

LBA alone). The values represent the average ± S.D. of three independent experiments. Asterisks indicate values that are significantly different from the control treatment ($p<0.05$, Tukey HSD test). C. GmNAC6-mediated induction of senescence-associated genes in sense- and antisense-agroinoculated tobacco leaves. The expression levels of GmNAC6 (NAC6) and the senescence-associated genes NTCP-23 and CystP were monitored by qRT-PCR from infiltrated sectors as in A. Gene expression was calculated with the $2^{-\Delta Ct}$ method using actinas the endogenous control. cDNAs were obtained from three biological replicates and validated individually (±S.D., n=3 biological replicates).

Figure 8. Model of BiP action in the ER-stress and osmotic-stress-integrating NRP-mediated cell death response. ER-stress and osmotic-stress-mediated upregulation of GmERD15 leads to the transcription of NRP genes and in turn induces GmNAC6 expression and a cell death response with senescence-like and apoptotic-like features. In the integrated pathway, BiP negatively regulates the transduction of the cell death signal.

Supplemental Data

Supplemental Figure 1. Enhanced accumulation of ER-stress molecular markers and cell death molecular markers in response to tunicamycin treatment. A. Time course of BiP, calnexin (CNX) and NRP-B induction by the ER stress inducer, tunicamycin. Soybean seedlings were treated with tunicamycin for the indicated number of hours. After the treatment, total protein was isolated from the treated leaves, fractionated in SDS-PAGE and immunoblotted with an anti-BiP, an anti-CNX or an anti-NRP-B serum. B. Enhanced accumulation of GmNAC6 by tunicamycin. Nuclear extracts from soybean leaves treated with tunicamycin for 0 (-) or 24 hours (+) were immunoblotted with an anti-GmNAC6 or an anti-ATAF2 serum.

Supplemental Figure S2. BiP overexpression attenuates osmotic stress- induced DNA fragmentation. Protoplats were prepared from leaves of wild-type (WT) and BiP-overexpressiong (35S:BiP) soybean seedlings that were treated with PEG for 8 hours,

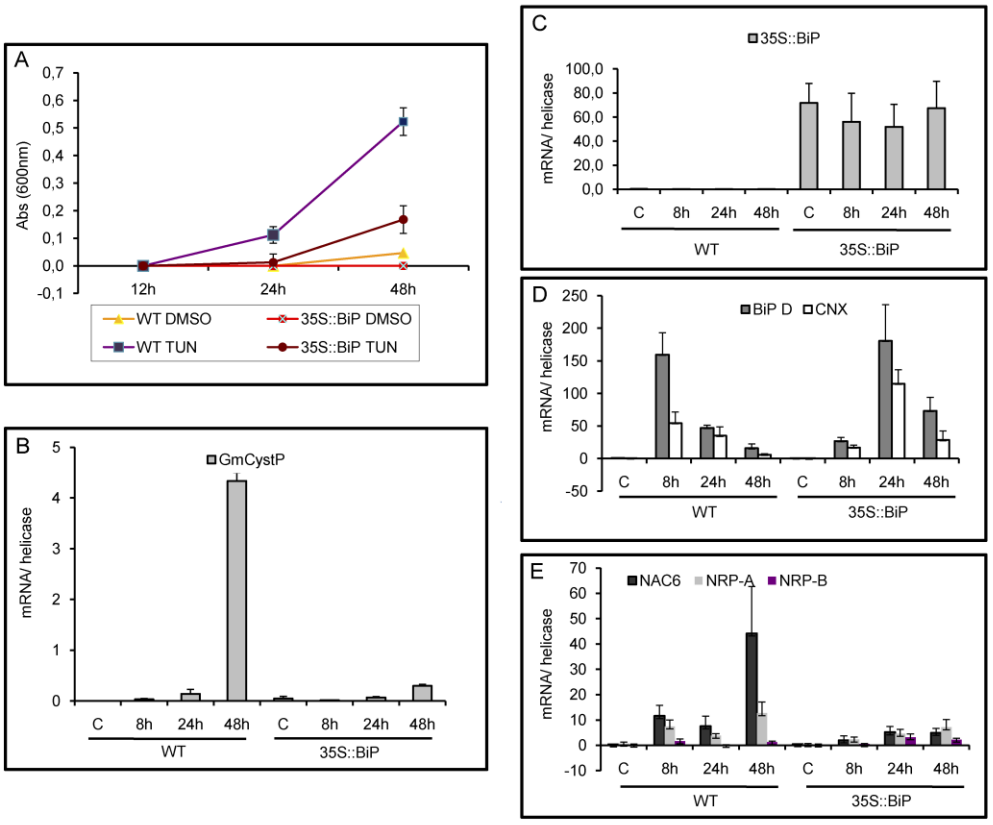
submitted to TUNEL labeling and examined by confocal microscopy. The semi-propoplasted cells were also counterstained with propidium iodide (PI) and sorted based on red fluorescence at 632 nm. Merged is an overlay of the fluorescent image of TUNEL labeling with PI staining cells that facilitates the identification of TUNEL-positive nuclei (see arrow) as the protoplasts were not completely formed and in some cells, we could not distinguish labeling of nuclear DNA from cytoplasmic RNA.

Supplemental Figure 3. A. The anti-DCD antibody recognizes NRP-B in soybean cells. Equal amounts of total protein (30 µg) extracted from untreated soybean cells (lane 2) and cells treated with tunicamycin (lane 1) and PEG (lane 3) were separated by SDS-PAGE and immunoblotted with anti-DCD antibody (polyclonal antibody raised against the DCD domain of NRP-B). Lane 4 shows the purified DCD domain of NRP-B. B. NRP-A and NRP-B are located in microsomal fractions when transiently expressed in tobacco leaves. Two-week-old wild-type tobacco leaves were infiltrated with Agrobacterium cells carrying a pNRP-A (A) or pNRP-B (B) expression vector. At approximately 36 h postinfiltration, soluble fractions (S) and microsomal fractions (M) were prepared from agroinfiltrated sectors and immunoblotted with an anti-DCD serum.

Supplemental Figure 4. NRP-A-induced leaf yellowing and necrotic symptoms are modulated by BiP. Leaves from two-week-old untransformed tobacco (WT) lines and transgenic lines expressing BiP cDNA either in the sense or antisense orientations were infiltrated with Agrobacterium carrying a pYFP-NRP-A expression vector. Photographs were taken seven days after infiltration.

Figure 1

Figure 2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35S::BiP TUN
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Figure 3

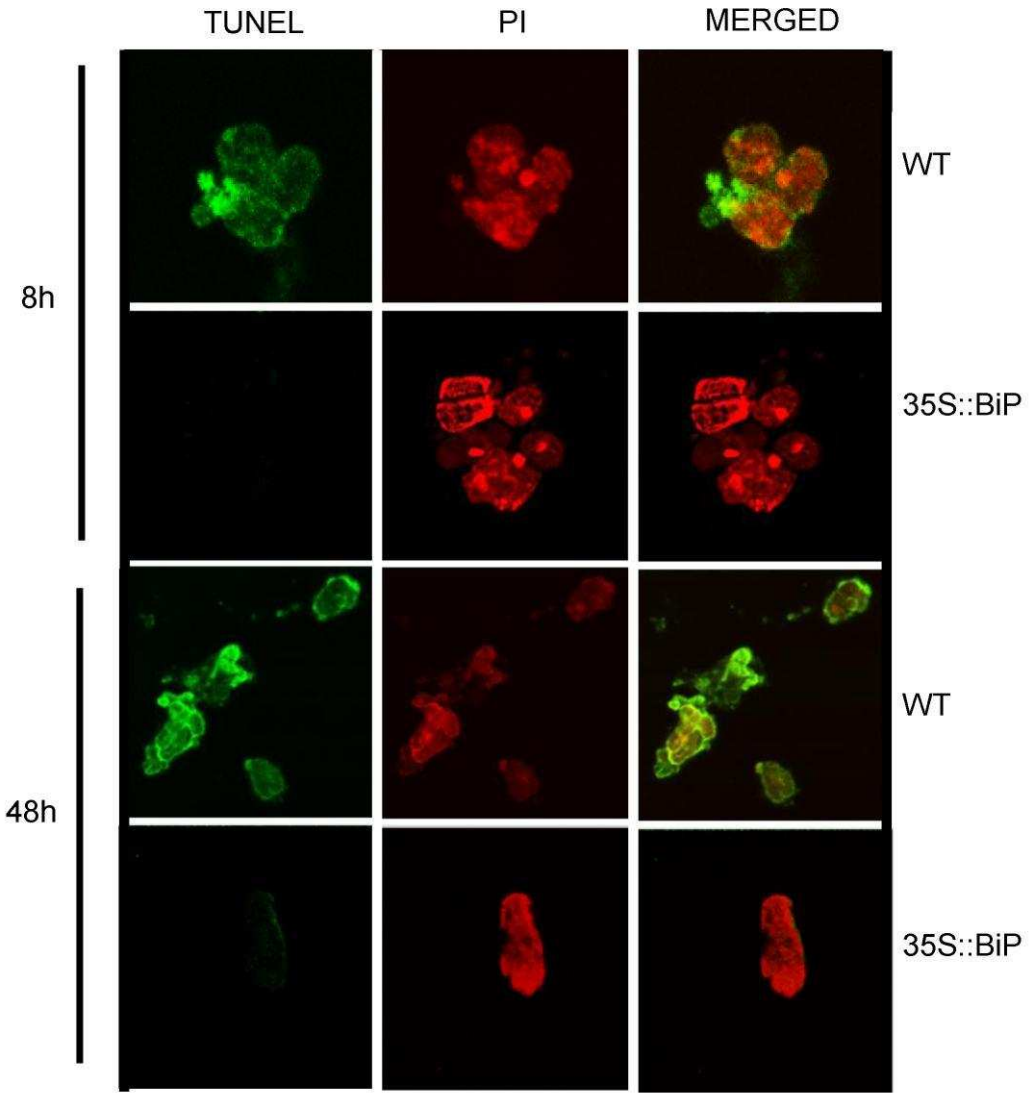

Figure 4

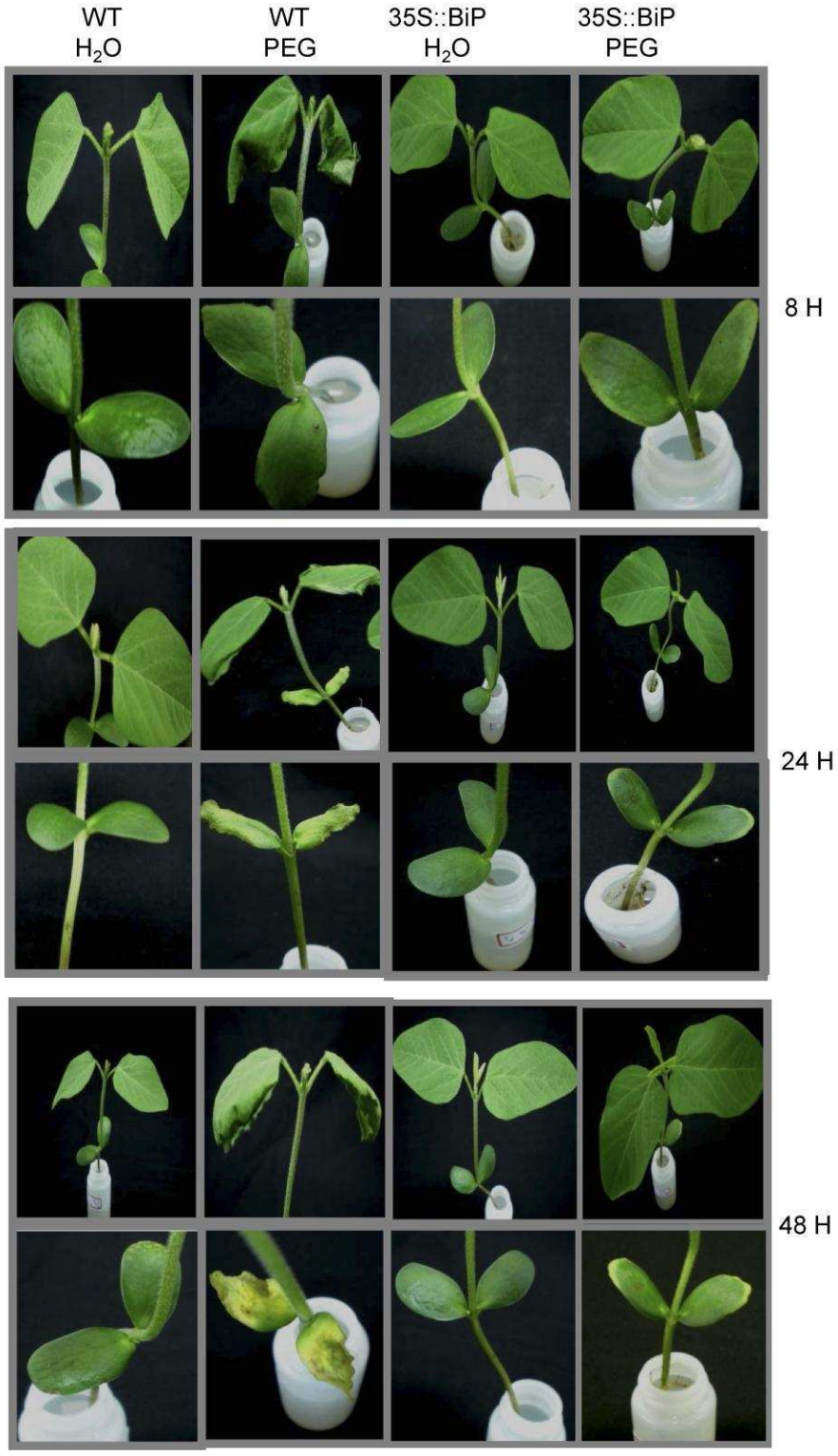

Figure 5

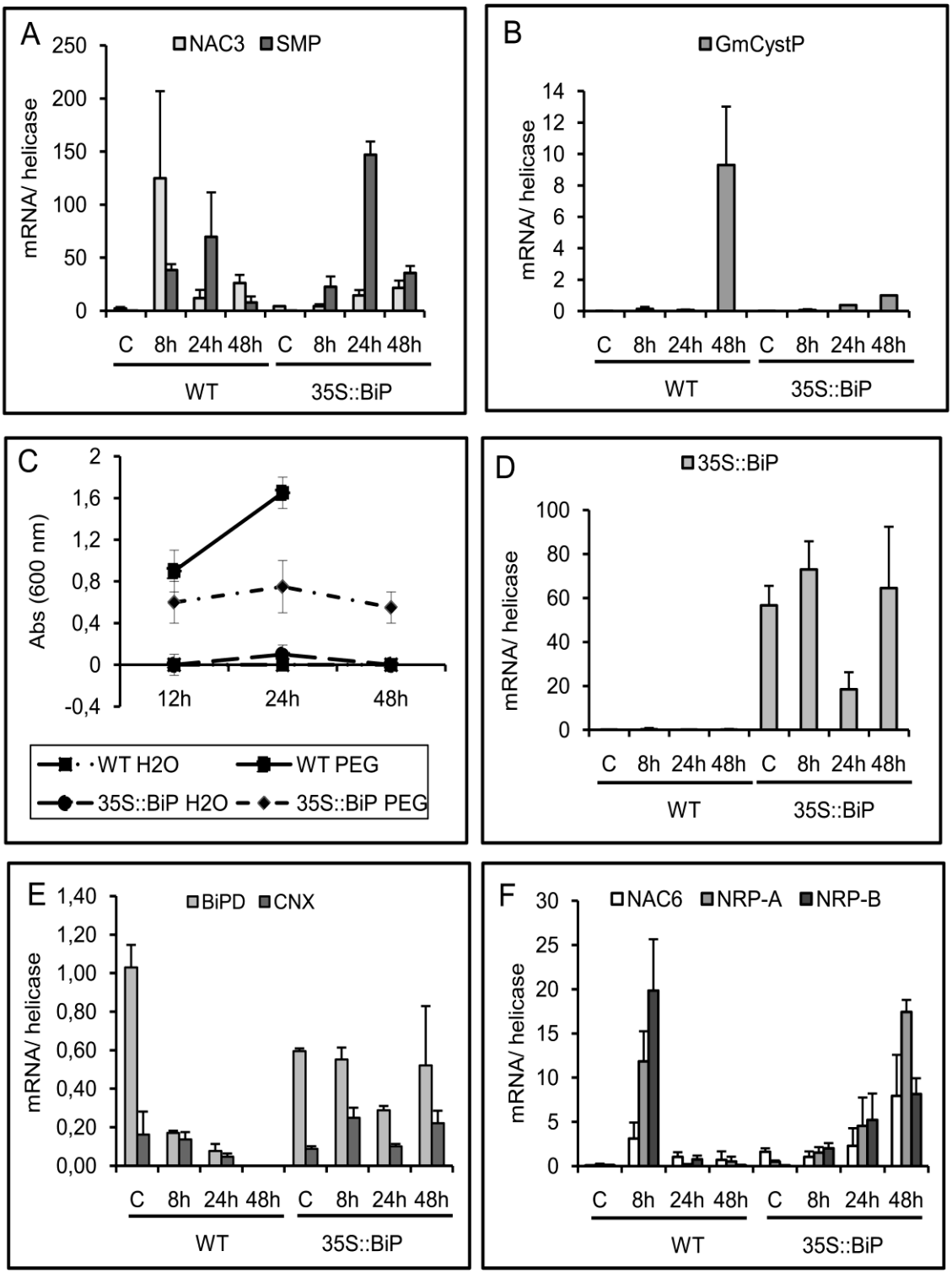
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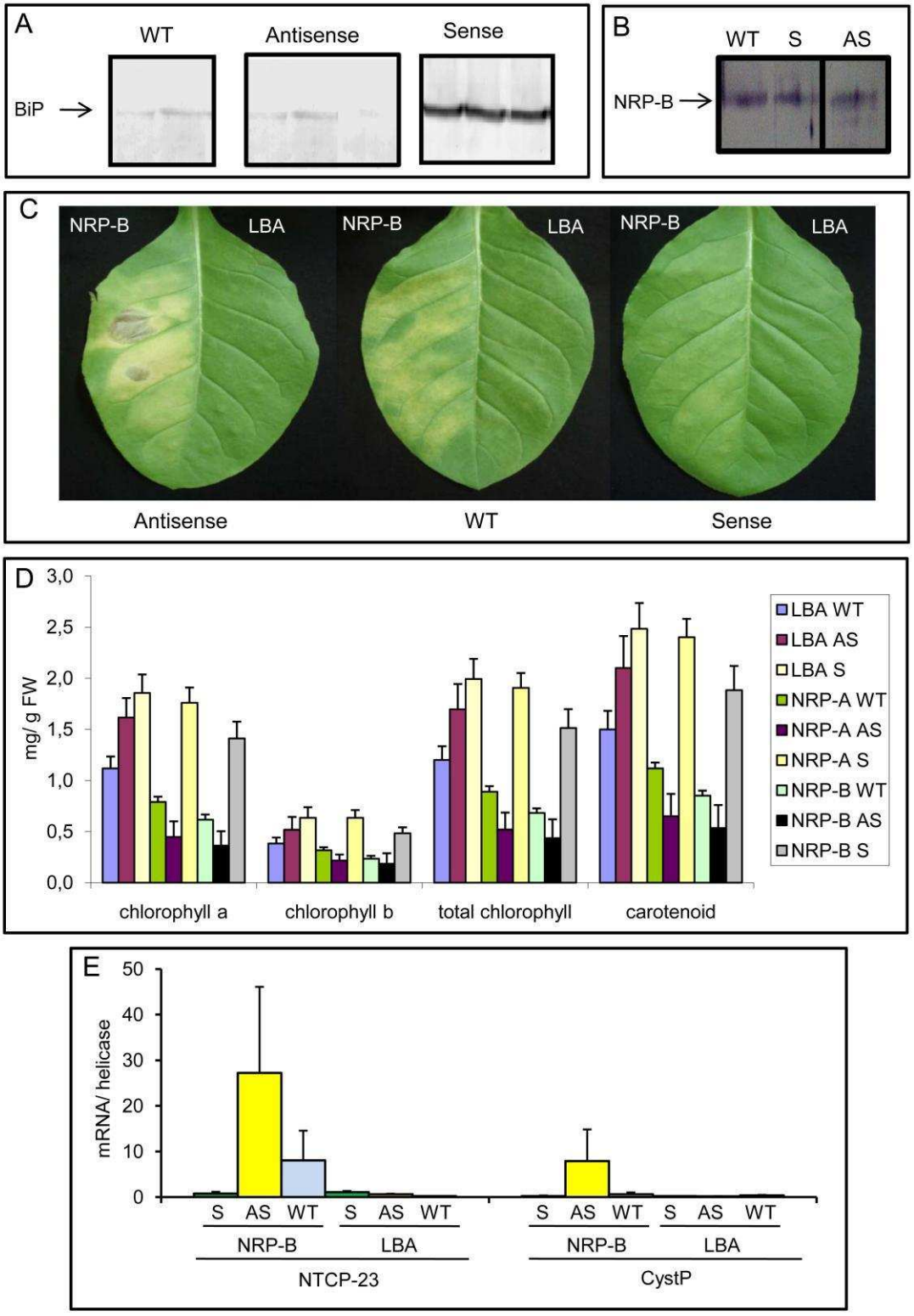
Figure 6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NRP-A S
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Figure 7

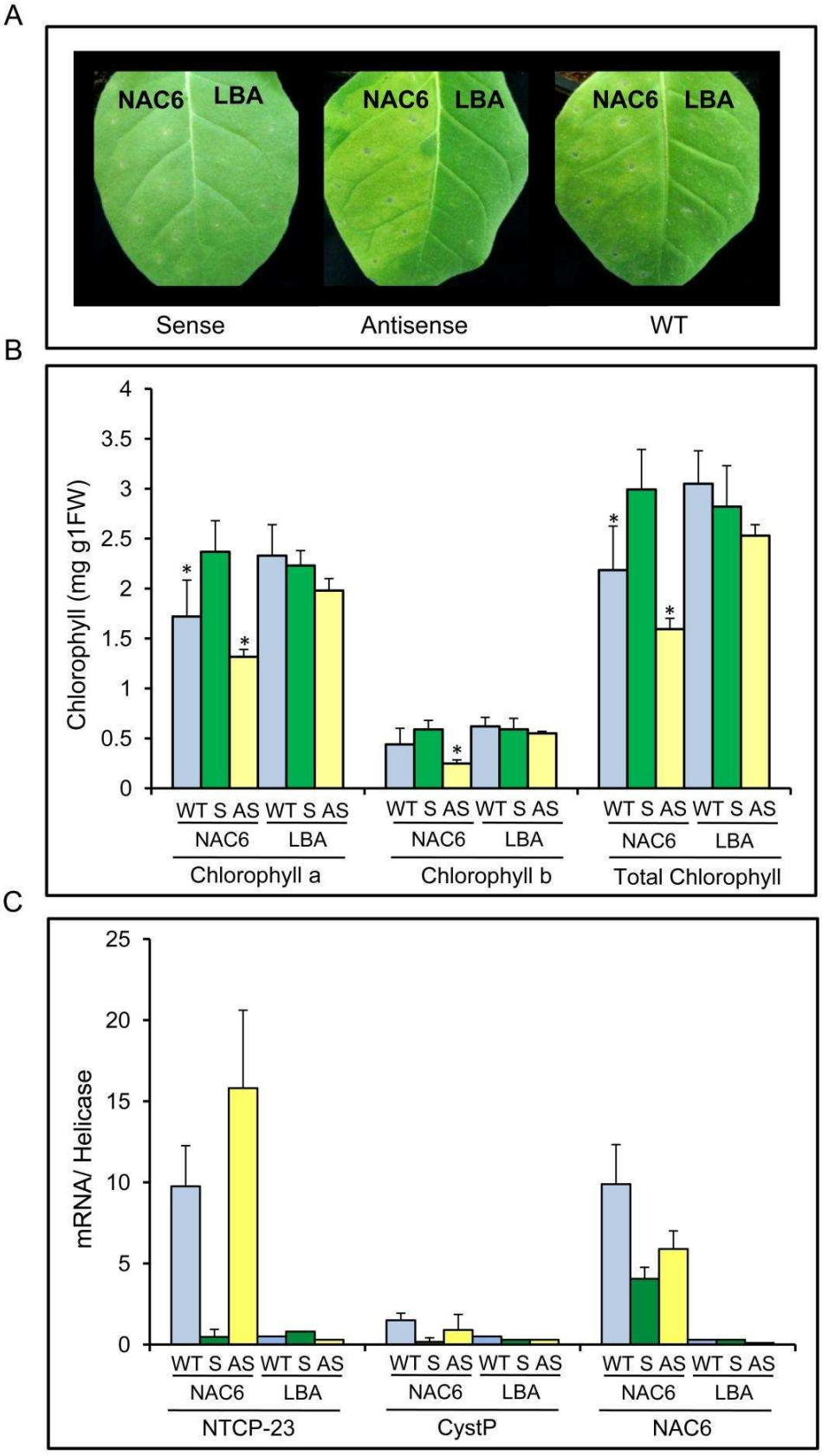
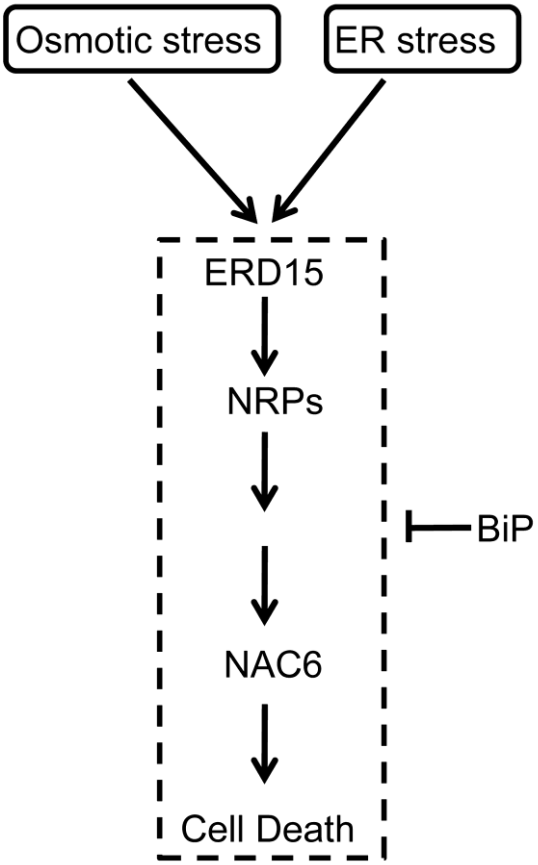


Figure 8


Osmotic stress
ER stress
ERD15
NRPs
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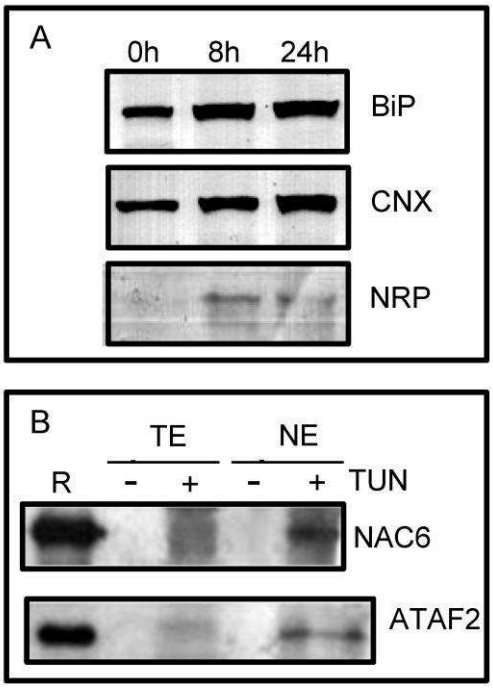
NAC6
Cell Death

Supplemental figure 1



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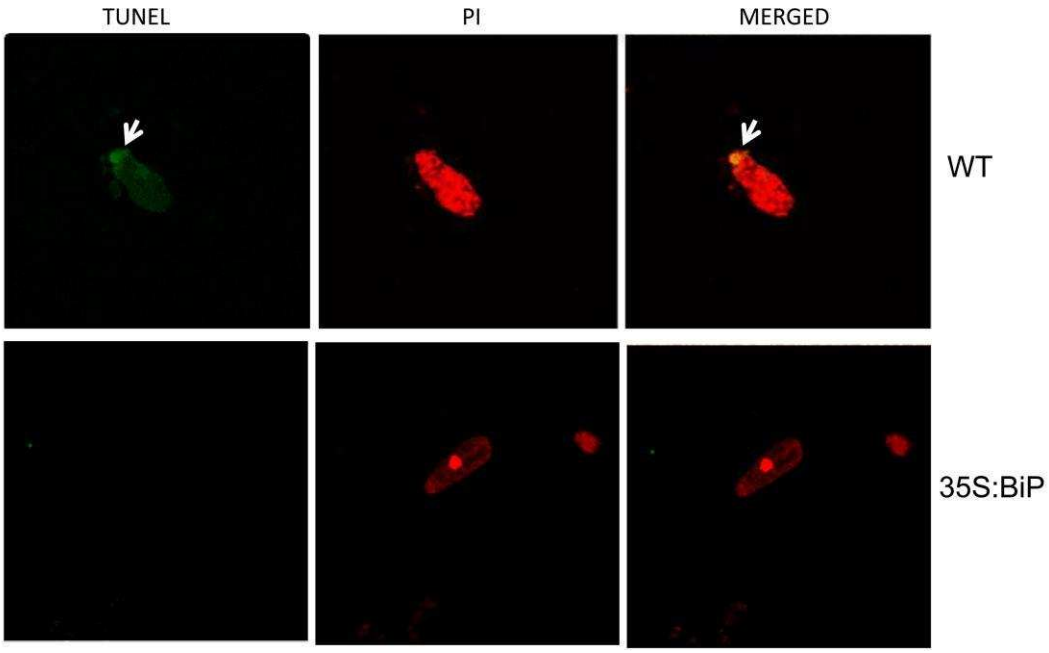

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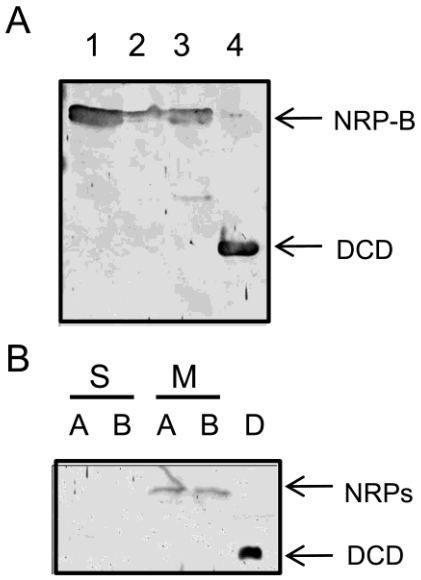

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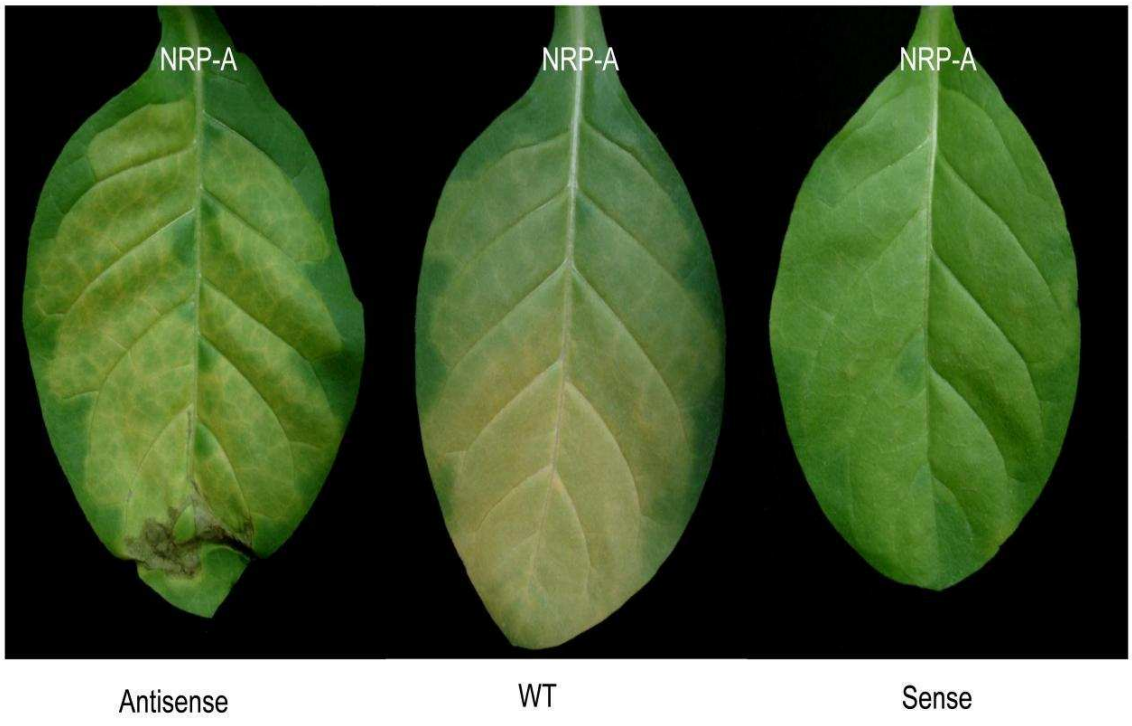

CAPÍTULO III

The binding protein BiP attenuates stress-induced cell death in soybean via modulation of the N-rich protein-mediated signaling pathway

Abstract

Biotic and abiotic stresses are responsible for lost in crops around the world. Drought, high levels of salinity, temperature, fungus and virus infection are some examples of important damage crops. As a consequence, plant cells have developed coordinated and integrated mechanisms that respond these injuries and are immediately activated upon stresses. To cope with the stress, cell signaling pathways are activated and promote up or down regulation of specific genes, which minimize the deleterious effect of stresses within the cell. In this way a new pathway has been shown to connect osmotic and endoplasmatic reticulum (ER) stress and promote cell death. This pathway is mediated by asparagine-rich proteins (NRPs). Both ER and osmotic stress induce the expression of the novel transcription factor GmERD15, which binds and activates NRPs promoters to induce NRP expression and cause the up-regulation of GmNAC6, an effector of the cell death response. In contrast to this activation mechanism, the ER-resident molecular chaperone binding protein (BiP) attenuates the propagation of the cell death signal by modulating the expression and activity of components of the ER and osmotic stress-induced NRP-mediated cell death signaling. This new integrative pathway was first identified in soybean and to understand how NRPs are involved in it, we have moved to a model system, Arabidopsis thaliana. In this work we have provided several lines of evidence showing that NRP-mediated cell death operate in arabidopsis as well. First of all, GmNRPs and GmNAC6 have homologs in arabidopsis with high level of identity. Secondly, arabidopsis homologs are induced by osmotic and ER stress and those genes might promote cell death response. Thirdly, AtNRP null line plant has shown sensitivity to osmotic stress when it grow in PEG conditions, but the expression GmNRPs in that background could rescue the wild type phenotype. As a master regulator of NRP-mediated cell death in soybean, BiP has shown the same function in arabidopsis. Once BiP overexpressing lines delay the expression of AtNRPs and ANAC036 genes upon osmotic and ER stress conditions. Moreover, BiP overexpressing lines can promote water stress tolerance and can modulate the expression and interacting

with a Caspase-1-like, VPE (Vacoular Processing Enzyme) an executor of cell death process. Furthermore, we have shown that chaperone activity may not required to modulate the NRP/DCD-mediated cell death pathway and the tolerance to water stress. We also identified that the subunit B of G heterotrimeric protein, AGB1, can modulate the NRP/DCD-mediated cell death response. The agb1 null mutant line displayed lower expression of NRP/DCD genes upon osmotic and ER stress and also was more tolerant to water stress. Thus, we suggest that NRP/DCD pathway is conserved in other plant species.

Introduction

Environmental changes and extreme conditions, such as temperature variations, drought and salinity, cause major crop losses around the world. However, plants do not passively accept these abiotic stresses and, therefore, have developed mechanisms for perception, transduction and adaptive responses to cope with the environmental stressors. Any stress conditions that cause homeostasis changes activate specific cellular responses, which promote a reprogramming of the gene expression profile towards adaptation. Thus, plants try to adapt to stress conditions and minimize damage in the cellular structure by regulating adaptive responses under adverse conditions of growth.

The endoplasmic reticulum (ER) is a key signaling organelle involved in the activation of cellular stress responses in eukaryotic cells. One such well-characterized signaling event is the unfolded protein response (UPR), which is activated to cope with the disruption of ER homeostasis that results in the accumulation of unfolded or misfolded proteins in the lumen of the organelle. Upon disruption of ER homeostasis, plant cells activate the unfolded protein response (UPR) through IRE1-like, ATAF6-like transducers and subunit of the heterotrimeric GTP-binding protein family (AGB1) resulting in the up-regulation of ER-resident molecular chaperones and the activation of the ER-associated degradation protein system. However, if ER stress is sustained, an apoptotic pathway is activated. Persistent ER stress has been shown to trigger both ER-stress specific apoptotic pathways and shared PCD (programmed cell death) signaling pathways elicited by other death stimuli.

Genome-wide approaches and expression profiling revealed the existence of a modest overlap of the ER and osmotic stress-induced transcriptomes in soybean seedlings treated with PEG (an inducer of osmotic stress) or tunicamycin and AZC

(potent inducers of ER stress). Different genes showed strong induction in both stress and synergistic response in that profiling, these are involved in diverse roles, such as plant- specific development and cell death (DCD) domain-containing proteins (NRP-A and NRP-B) (Irsigler et al., 2007) and NAC domain-containing proteins (GmNAC81). NRPs are mediators of ER and osmotic stress-induced cell death in soybeans. (Costa et al., 2008). which shows correspondence to programmed cell death event. Additionally, GmNAC81 is induced by osmotic and ER stress, with synergistic response and can promote cell death response in tobacco and also induces caspase-3-like activity and DNA fragmentation in soybean protoplasts (Faria et al., 2011). As a downstream to NRPs in the ER and osmotic stress-induced cell death response, GmNAC81 is induced by NRPs and display latter induction kinetic compared to them (Faria et al., 2011). An upstream component of ER stress-induced NRP-mediated signaling, GmERD15 connects the response of the ER and osmotic stress to activate the expression of NRP genes (Alves et al., 2011). Recently we identified a new component of NRP/DCD mediated cell death. GmNAC30, an another NAC member, interacts with GmNAC81 in vitro and in vivo. As GmNAC81, GmNAC30 is induced by osmotic and ER stress and also induced by NRPs. The overexpression of GmNAC can promote cell death response (Mendes et al., 2013). Furthermore, GmNAC30 and GmNAC81 bind in vivo to a member of VPE (Vacuolar Processing Enzyme), a caspase-1-like protein. In a model proposed, this protein can act as one executor of the ER stress- and osmotic-stress induced cell death response.

Molecular chaperone BiP is an endoplasmatic reticulum molecular chaperone responsible for correct folding and assembly of secretory proteins. Furthermore, BiP is also involved in the regulation of ER and osmotic stress responses (Ron e Walter., 2007; Urade 2009; Leborgne-Castel et al., 1999; Valente et al., 2009; Reis et al., 2011). BiP-overexpressing lines have been shown to display tolerance to water deficit and ER stress (Valente et al., 2009; Reis et al., 2011). The mechanism of BiP-mediated increases in water stress tolerance has been associated with the BiP capacity to modulate the expression and activity of components of the stress-induced NRP/DCD-mediated cell death response. Accordingly, BiP- overexpressing lines displayed lower and delayed expression of NRP/DCD- mediated cell death pathway genes when plants were submitted to ER and osmotic stress. Additionally, transient expression of NRPs and GmNAC81 in tobacco transgenic plants with overexpressed (sense lines) or suppressed (antisense lines) levels of BiP showed that the extend of the induced cell death response correlated with BiP levels. In BiP-overexpressing line, the cell death response was

delayed compared to WT, whereas in the BiP suppressed line, the NRP- or GmNAC81-induced cell death response was accelerated (Reis et al., 2011). However, the mechanism of BiP attenuation of the NRP/DCD-mediated cell death response is still unclear and the molecular link between BiP and the signaling pathway is missing.

The NRP/DCD-mediated cell death response has been uncovered in soybean and the extent to which this pathway may operate in the plant kingdom has not been investigated. Since the discovery of the stress-induced cell death pathway through genome-wide approaches and expression profiling, several other components of the pathway have been reported. Nevertheless, the lack of an efficient reverse genetic system in soybean has limited the progress toward deciphering this branch of the ER stress response that integrates an osmotic signal into a programmed cell death response. In spite of the relevance of the NRP/DCD-mediated signaling as a versatile adaptive response to multiple stresses, mechanistic knowledge of the pathway is lacking and molecular components linking BiP to the modulation of the cell death response remains to be identified. Here we show first that the DCD/NRP-mediated cell death components are replicated in the Arabdopsis genome and function to propagate a cell death signal in response to ER and osmotic stress. Using reverse genetic, the characterized components were hierarchically ordered in the signaling pathway and an upstream component of NRP/DCD was discovered. Furthermore, we showed that both an intact BiP and a chaperone activity deficient BiP mutant attenuate the DCD/NRP-mediated cell death signaling in Arabidopsis, suggesting that a regulatory function separated from its chaperone activity may account for the mechanism for BiP-mediated increases in water stress tolerance.

Materials and Methods

Plant Growth

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild-type control. The Arabidopsis T-DNA mutants agb1-3 (SALK_061896, insertion in the exon) and atnrp1 (SALK_041306, insertion in the exon) were obtained from the Arabidopsis Biological Resource Center. The primers used for genotyping are listed in Table 2. Surface-sterilized seeds were plated directly onto square Petri dishes containing 1/2 Linsmaier and Skoog (LS) medium, and 0.7% agar. For normal growth conditions, plants were grown at 21°C under a 16 h light/8 h dark cycle. Agrobacterium-mediated

transformation was performed using the floral-dip method (Bechtold et al., 1993). Agrobacterium strain GV3101 was used in all transformation experiments.

PEG and tunicamycin treatment

In the plate system, tunicamycin (Tm) (T7765, Sigma, dissolved in DMSO) or PEG (MW 8000, Sigma) were directly added to 1/2 LS medium containing 0.7% agar, at the concentrations indicated. Seeds were directly germinated in Tm-containing medium or PEG-containing medium for observation of ER stress tolerance and osmotic stress, respectively. To harvest tissue for osmotic or ER gene expression analysis, the seeds were germinated in 1/2 LS medium for 2 weeks, and then transferred to Tm-containing medium or PEG-containing medium.

Drought tolerance

Drought tolerance assay were performed on 5-week-old seedlings. After germination on 1/2 LS plates, 7-d-old seedlings of transgenic lines were planted in sieve-like rectangular plates (3 cm deep) filled with a mixed soil that had been well-watered. The seedlings were cultured in a greenhouse (22 uC, 70% humidity, 120 mmol. m22.s21, 12 h light/12 h dark cycle) without watering for 15 days.

Glucuronidase Activity Assays.

A 2,000-bp fragment of the 5′flanking sequences of the AtNRP1, AtNRP2 genes, relative to the translational initiation codon, were amplified from arabidopsis DNA with the primers listed in Table S2. The amplified fragments were cloned into the pDONR221 entry vector (Invitrogen) and then transferred to pGWB203 by recombination with LR clonase to yield pAtNRP1pro:GUS (glucuronidase) and pAtNRP2pro: GUS. Tobacco leaves were agroinfiltrated with the constructions carrying YFP-AtNRP1, YFP-AtNRP2 and YFP-ANAC036. After three days the protein extraction and fluorometric assays for GUS activity were performed essentially as described by Jefferson et al, with methylumbelliferone as a standard. For the standard assay, the leaves were ground in 0.5 mL GUS assay buffer [100 mM $NaH_2PO_4 \cdot H_2O$ (pH 7.0), 10 mM EDTA, 0.1% (w/v) sarkosyl, and 0.1% (v/v) Triton X-100], and 25 μL of this extract were mixed with 25 μL GUS assay buffer containing 2 mM fluorescent4-methylumbelliferyl β-D glucuronide as a substrate. The mixture was incubated at 37 °C in the dark for 30 min, and the GUS activity was measured using a Lector Multi-Mode Microplate Reader Synergy HT

(BioTek). The total protein concentration was determined by the Bradford method .The experiments were repeated three times with similar results

Real-time RT-PCR Analysis

For quantitative RT-PCR, total RNA was extracted from frozen leaves or cells with TRIzol (Invitrogen) according to the instructions from the manufacturer. The RNA was treated with 2 units of RNase-free DNase (Promega) and further purified through RNeasy Mini kit (Qiagen) columns. First-strand cDNA was synthesized from 4μg of total RNA using oligo-dT(18) and Transcriptase Reversa M-MLV (Invitrogen), according to the manufacturer's instructions. Real-time RT-PCR reactions were performed on an ABI7500 instrument (Applied Biosystems), using SYBR_ Green PCR Master Mix (Applied Biosystems). The amplification reactions were performed as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 94 °C for 15 s and 60 °C for 1 min. To confirm quality and primer specificity, we verified the size of amplification products after electrophoresis through a 1.5% agarose gel, and analyzed the Tm (melting temperature) of amplification products in a dissociation curve, performed by the ABI7500 instrument. The primers used are listed in Table 1. For quantitation of gene expression in arabidopsis seedlings, we used actin 2 (At3g18780) (Liu and Howell., 2010) or UBQ5 (At3g62250) (Moreno et al., 2012) as the endogenous control gene for data normalization in real-time RT-PCR analysis. Fold variation, which is based on the comparison of the target gene expression (normalized to the endogenous control) between experimental and control samples, was quantified using the comparative Ct method: $2^{\wedge\ \Delta CtTreatment-\Delta CtControl}$). The absolute gene expression was quantified using the $2^{-\Delta CT}$ method, and values were normalized to the endogenous control.

Plasmid Construction

For transient expression in protoplasts, tobacco leaves and arabidopsis transformation AtNRP1, AtNRP2 and ANAC036, BiPD, BiP1 and VPE-γ were amplified from arabidopsis and soybean cDNA using specific primers (AtNRP1 Fwd and AtNRP1 Rvs; AtNRP2 Fwd and AtNRP2 Rvs; ANAC036 Fwd and ANAC036 Rvs, BiP1Fwd and BiP1 Rvs, BiPD Fwd and BiPD Rvs, VPE-γ Fwd and VPE-γ Rvs) (Table 2) and inserted by recombination into the entry vectors pDONR201 and pDONR221 (Invitrogen) to yield pDONR201Ns-AtNRP1, pDONR201Ns-AtNRP2, pDONR201-

ANAC036, pDONR221BiPD, pDONR201BiP1 and pDONR201VPE-γ. These genes were then transferred from the entry vectors to different expression vector (pK7WG2, pEarleygate100 and pEarleygate 104) by recombination using the enzyme LR clonase (Invitrogen) to generate the clones described here: p35S:AtNRP1, p35S:AtNRP2, p35S:ANAC036, p35S:BiPD, p35S:BiPD-T46G, p35S:BiPD-G235D, p35S:BiP1, p35S:VPE-γ. For the transient expression in the protoplasts and tobacco AtNRP1, AtNRP2, ANAC036, BiPD, BiP1 and VPE- γ were fused to YFP (pYFP-AtNRP1,pYFP-AtNRP2, pYFP-ANAC036, pYFP-VPE- γ) or not (BiPD, BiP1) and placed under the control of the 35S promoter in the respective binary vector for plant transformation. For the BiPD mutations, pDONR221 containing BiPD sequence was used to amplify the DNA using BiPD mutated primer (T46G and G235D). The vectors amplified with BiPD mutations (T46G or G235D) were purified and then used at ligase reaction (Promega). The ligase reaction was used to transform E.coli DH5a, to generate pDONR221-BiPD-T46G and pDONR221-BiPD-G235D. These entry vectors were used to transfer, by LR reactio, to the destination vector pK7WG2, generating pK7WG2- BiPD-T46G and pK7WG2- BiPD-G235D.

Transient Expression in tobacco Protoplasts

Protoplasts were prepared from soybean suspension cells, as essentially described by Fontes et al (1993). The protoplasts were isolated from tobacco leaves by digestion for 3 h, under agitation at 40 rpm, with 0.5% (w/v) cellulase, 0,5% 9w/v) macerozyme R-10, 0.1% (w/v) pectolyase Y23, 0.6 M mannitol, 20 mM MES, pH 5.5. The extent of digestion was monitored by examining the cells microscopically at each 30-min interval. After filtration through nylon mesh of 65 µm, protoplasts were recovered by centrifugation, resuspended in 2 mL of 0.6 M mannitol, 20 mM MES, pH 5.5, separated by centrifugation in a sucrose gradient (20% (w/v) sucrose, 0.6 M mannitol, 20 mM MES, pH 5.5), and diluted into 2 mL of electroporation buffer (25 mM Hepes-KOH, pH 7.2, 10 mM KCl, 15 mM MgCl2, 0.6 M mannitol). Transient expression assays were performed by electroporation (250 V, 250 µF) of 10 µg of expression cassette DNA, and 30 µg of sheared salmon sperm DNA into $2x10^5$-$5x10^6$ 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, 20 mM MES, pH 5.5.

Determination of Chlorophyll Content

Total chlorophyll content was determined spectrophotometrically at 663 and 646 nm after quantitative extraction from individual leaves with 80% (v/v) acetone in the presence of 1 mg of Na_2CO_3

in Situ Labeling of DNA Fragmentation (TUNEL)

Free 3`-OH in the DNA was labeled by the terminal deoxynucleotidyl transferase-mediated dUTP nickend labeling (TUNEL) assay using the ApoAlert DNA Fragmentation Assay kit (Clontech), as instructed by the manufacturer. Samples were observed with a Zeiss LSM 410 inverted confocal laser scanning microscope fitted with the configuration: excitation at 488 nm and emission at 515 nm. As a positive control, samples were treated with DNase1.

Results

Functional conservation of the stress-induced NRP/DCD-mediated cell death response in Arabidopsis

The NRP-mediated cell death pathway was originally identified in soybean (Costa et al., 2008); however, the molecular tools for the characterization of this pathway in soybean are still limited. In contrast, in the Arabidopsis model system, reverse genetic studies are possible to assign function and hierarchical order to components of signal transduction pathways. Therefore, we first examined whether the stress-induced DCD/NRP-mediated signaling would function in Arabidopsis, integrating multiple stress signals into a cell death response, as described in soybean. An Arabidopsis NRP/DCD homolog, At5g42050, has been described previously (Hoepflinger et al., 2011) and it was designated as AtNRP1. We have also used the GmNRP-A and GmNRP-B sequences against the arabidopsis database to search for additional NRP homologs. Like AtNRP1, AT3G27090, which was referred to as AtNRP2, contains N-rich and DCD domains and belongs to the group I of DCD domain-containing proteins (Tenhaken et al., 2005). The AtNRP1 gene shared 65% sequence identity with GmNRP-B and 60% with GmNRP-A, whereas AtNRP2 was 52% identical to both GmNRP-A and GmNRP-B (Supplemental Figure 1A).

We also used the GmNAC81 sequence to search for homologs in the Arabidopsis genome. Among the NAC-domain containing proteins of the plant-specific transcriptional factor superfamily, GmNAC81 was most related to AT2G17040.1, also designated GmNAC036 (59.7% sequence identity;Supplemental Figure 1B). GmNAC81 shared about 30% sequence identity with AT2G02450 (ANAC035), AT3G10480 (ANAC50), AT1G26870 (ANAC009) and AT5G39820 (ANAC094). Based on sequence comparison, GmNAC36 was selected for expression analysis and functional studies.

Soybean NRPs and GmNAC81 are induced by the osmotic stress inducer PEG, and the inducer of ER stress, tunicamycin (Costa el al., 2008 e Faria et al., 2011). Among a series of other stress inducers, the gene AtNRP1 has also been shown to be induced by osmotic stress, but the expression of AtNRP1 in response to ER stress inducers has not been evaluated (Hoepflinger et al., 2011). As putative components of the stress-induced DCD/NRP-mediated signaling that integrates a cell death signal in response to ER stress and osmotic stress, we examined whether AtNRP1, AtNRP2 and ANAC036 would respond to these stresses. Fifteen days-old Arabidopsis seedlings (columbia background) were treated with PEG (10% w/v) and tunicamycin (2,5 µg/mL) during 24 hours and the gene expression was analyzed by qRT-PCR. The effectiveness of the stress treatments was monitored by analyzing the expression of the osmotic-stress marker Rab18 gene and the ER stress marker calnexin (CNX) gene (Figures 1A and 1B). Under these conditions, AtNRP1, AtNRP2 and ANAC036 were induced by osmotic stress (Figure 1C) and ER stress (Figure 1D), although with differences in their induction kinetics. AtNRP1 displayed higher level of induction at 24 hours after PEG treatment and at 6 hours after tunicamycin treatment. AtNRP2 was also induced by both treatments, although to a lower extent as compared to the expression of AtNRP1 and exhibited a late kinetic of induction in response to PEG. This result may be interpreted with caution because primer efficiency may account for the difference in expression of the analyzed genes. ANAC036 was induced with different kinetic from AtNRPs, reaching maximum induction at 24 hours after PEG and tunicamycin treatment. We also monitored the tunicamycin and PEG induction of a vacuolar processing enzyme (VPE-γ) that has been shown to be the downstream component of the pathway that acts as the executioner of the cell death program (Figure S2; Mendes et al., 2013).

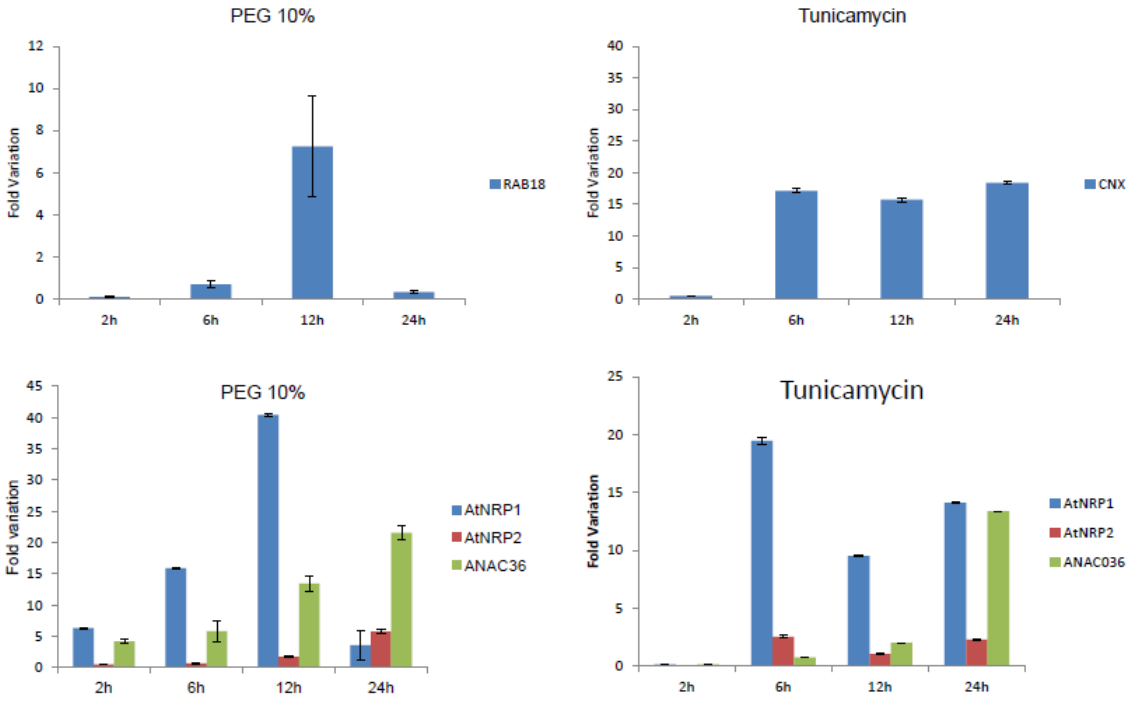

Figure 1. AtNRP1, AtNRP2 and ANAC036 are induced by osmotic and ER stresses. Total RNA was isolated from 15 days-old Arabidopsis seedlings that had been treated with PEG (10% w/v) or Tunicamycin (2,5 μg/mL) for 2h, 6h, 12h and 24h. H2O was used as control for PEG and DMSO for Tunicamycin. The transcript levels of selected genes were quantified by qRT-PCR. Gene expression was calculated using the $2^{-\Delta Ct}$ method and UBQ5 as endogenous control. cDNAs were obtained from five biological replicates and validated individually. RAB18 and CNX are osmotic stress and ER stress gene markers, respectively. (S.D, n=5 biological replicates)

As putative components of the ER stress- and osmotic stress-integrating signaling pathway, we examined whether transient expression of AtNRP1, AtNRP2 and ANAC036 would activate a cell death program in tobacco leaves. After 7 days post-infiltration, the leaf sectors expressing AtNRP1, AtNRP2 and ANAC036 displayed a chlorotic phenotype with necrotic lesions as a result of massive cell death (Figures 2A, 2B ad 2C). The positive controls GmNRP-A and GmNRP-B also induced a chlorotic phenotype (Figures 2D and 2E), contrasting with the remaining green phenotype displayed by the expression of a soybean BiP gene, used as negative control (Figure 2F). These phenotypes correlated with the chlorophyll loss in the agroinfiltrated sectors (Figure 2G) and suggest a role for AtNRP1, AtNRP2 and ANAC036 as effectors of cell

death response. This interpretation was further confirmed by applying the terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay for the in situ detection of DNA fragmentation in the AtNRP1-, AtNRP2- and ANAC036-expressing leaf sectors (Figure 2H).. The extensive cleavage of nuclear DNA into oligonucleosome-sized fragments is one feature of active cell death (PCD). The nuclei of the leaf sectors that were transformed with the empty vector fluoresced intensely with DAPI and exhibited only TUNEL-negative nuclei. In contrast, the AtNRP1-, AtNRP2- and ANAC036-expressing samples had TUNEL-positive nuclei that showed the same degree of staining as the NRP-B-expressing leaf sectors (Figure 2E). These results suggest that AtNRP1, AtNRP2 and ANAC036 promote cell death when they are transiently expressed in tobacco leaves, a functional role reminiscent of the components of the osmotic stress- and ER stress-induced cell death signaling pathway.

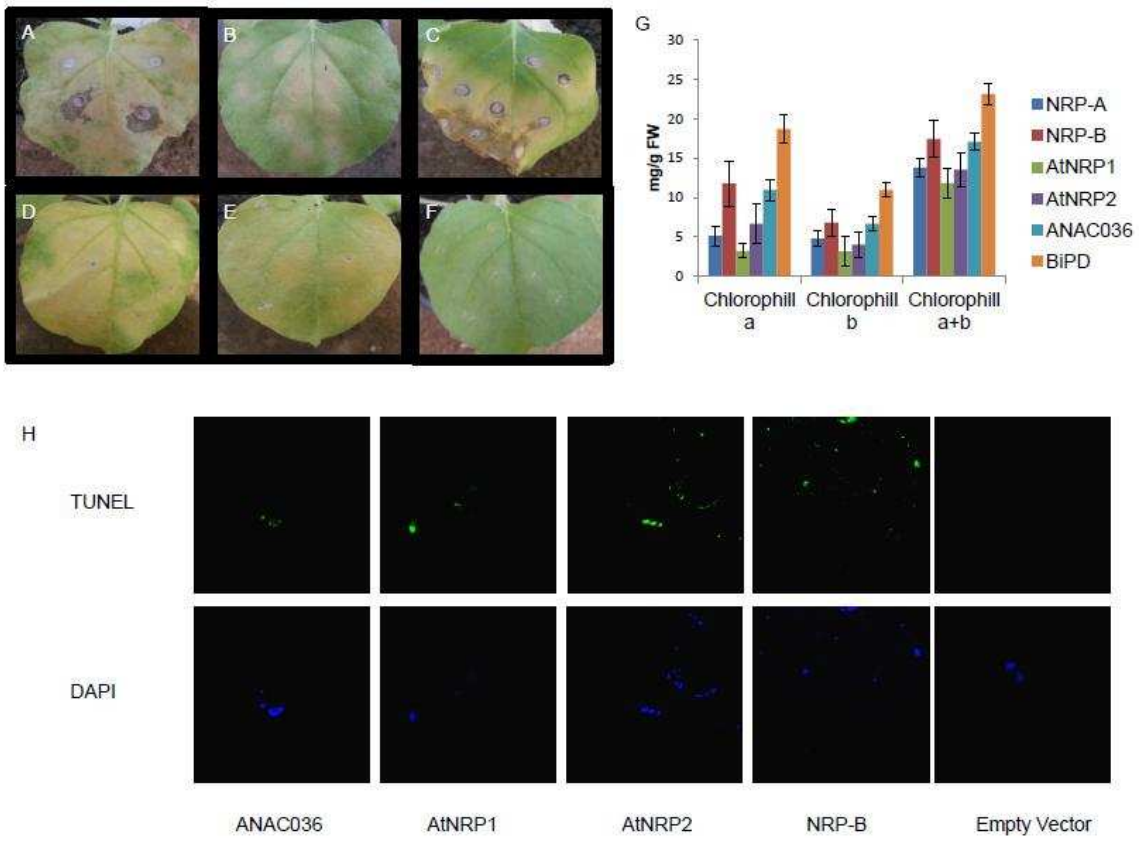

Figure 2. Arabidopsis NRPs and ANAC036 cause cell death in planta. Leaves from 3 weeks-old N. benthamiana were infiltrated with agrobacterium cells transformed with p35S:AtNRP1 (A), p35S:AtNRP2 (B), p35S:ANAC036 (C) p35S:NRP-A (D),

p35S:NRP-B (E) expression vectors or with control binary expression vectors harboring the BiPD (p35S:BIPD) gene from soybean (F). Pictures were taken 6 days after infiltration. (G) Chlorophyll loss induced by AtNRP1, AtNRP2, ANAC036, NRP-A and NRP-B expression. Total chlorophyll, chlorophyll a and b were determined from the leaf sectors agroinfiltrated with the described DNA constructions. Values are given as mean ± S.D. from three biologicals replicates. (H) Transient expression of NRP/DCD-mediated cell death genes from Arabidopsis can induce DNA fragmentation. Tobacco protoplast were electroporated with the constructions carrying AtNRP1, AtNRP2, ANAC036, NRP-B, under control of 35S promoter or the empty vector, as a negative control. After 36 h pos-agroinfiltration, protoplasts from leaf sectors were submitted to TUNEL labeling. The nuclei were stained with DAPI.

To further examine the functional analogy between soybean and Arabidopsis NRPs, we took advantage of the stress hypersensitive phenotype of atnrp1 null alleles (Salk_041306) for complementation assays. RT-PCR on RNA from atnrp1 leaves detected no accumulation of the ATNRP1 transcript in the homozygous T-DNA insertion mutant, confirming it is null alleles (Figure S3). Inactivation of AtNRP1 gene has been shown to cause a higher inhibition of seedling root growth under osmotic stress as compared to wild type seedlings (Hoepflinger et al., 2011). Likewise, we found that PEG inhibited root growth to a higher extent in atnrp1 knockout seedlings than in wild-type seedlings (Figures 3A and 3C). This phenotype was linked to the inactivation of the AtNRP1 gene because expression of AtNRP1 in the atnrp1 restored the wild type phenotype (Figure 3B). Furthermore, the enhanced inhibition phenotype of root growth by PEG displayed by the atnrp1 knockout line was replicated by overexpression of BiP, a modulator of the NRP-mediated cell death response (Figure 3C; Reis et al., 2011). In order to determine whether GmNRPs would replace the AtNRP1 function, we transformed the knockout line with GmNRP-A, GmNRP-B and the Arabidopsis homolog AtNRP2, under the control of 35S promoter. Ectopic expression of AtNRP2, NRP-A and NRP-B reversed the atnrp1 phenotype upon osmotic stress as the complemented transgenic lines displayed wilt type root growth under PEG.. Collectively, these results further indicated that Arabidopsis and soybean NRPs are functionally analogs.

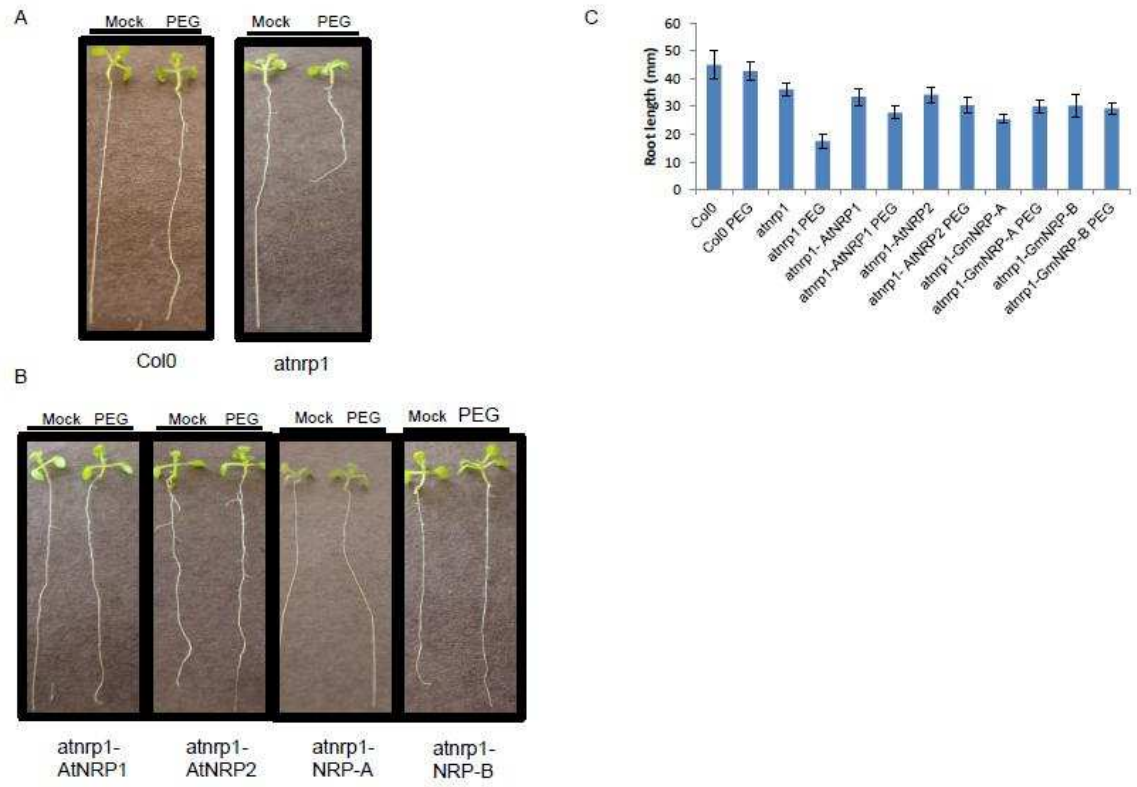

Figure 3. AtNRP2, NRP-A and NRP-B complement the enhanced sensitivity phenotype of root growth to osmotic stress displayed by the atnrp1 knockout line. Seeds of Col0 and atnrp1 lines were germinated in LS-agar plates with and without PEG (0,5%) and root length was measured at 6 days post-germination (A) Loss of Atnrp1 function increases sensitivity of root growth to osmotic stress. Photography was taken 6 days after germination of Col-0 and atnrp1 seeds under osmotic stress. (B) Complementation assays of the atnrp1 function. The knockout line atnrp1 was transformed with p35S:AtNRP1, p35S:AtNRP2, p35S:NRP-A and p35S:NRP-B and germinated in LS-agar plates with and without PEG (0,5%) during 6 days . (C) Measurement of root length. Values represent the means (±S.D.) of root length from 15 biological replicates.

GmNAC81 has been placed downstream of NRPs in the stress-induced NRP-mediated cell death signaling based on expression analysis and kinetics of the cell death activities of the pahtway components (Faria et al., 2011). Ectopic expression of NRP-A or NRP-B has been shown to activate the GmNAC81 promoter and induce GmNAC81 expression. Furthermore, stress induction of NRP-B and NRP-A genes precedes the induction of GmNAC81 and GmNAC81-mediated cell death in tobacco leaves occurs with early kinetics, as expected from a downstream effector in the pathway. This

hierarchical order of the components in the transduction signal pathway was confirmed in the Arabidopsis system by a reverse genetic approach and promoter transactivation assay (Figure 4). Both atnrp1 and Col0 lines were treated with PEG and tunicamycin for 12 hours and the extent of AtNRP2 and ANAC036 induction was determined by qRT-PCR (Figures 4A and 4B). The expression levels of AtNRP2 and ANAC036 induced by tunicamycin or PEG were remarkably lower in atnrp1 line compared to Col0. Furthermore, in the atnrp1 line, the stress induction of ANAC036 gene was delayed. These results indicate that the full induction of AtNRP2 and ANAC036 by osmotic or ER stress requires the AtNRP1 function. To confirm that AtNRP2 acts downstream of AtNRP1, we performed a GUS transactivation assay in tobacco leaves using the 2-kb 5' flanking sequences of AtNRP1 and AtNRP2 genes fused to the GUS reporter. Transient expression of AtNRP1, AtNRP2 or ANAC036 (Figure S4) did not activate the AtNRP1 promoter (Figure 4C), whereas expression of AtNRP1 and AtNRP2, but not ANAC036, activated the AtNRP2 promoter (Figure 4D). Collectively, these results placed AtNRP1 upstream of AtNRP2 and confirmed that ANAC36 is downstream of AtNRPs in the pathway. The VPE-γ was also genetically linked to the stress-induced NRP-mediated cell death signaling because induction of VPE-γ by ER stress and osmotic stress was dependent on the AtNRP1 function (Figure S2). This result confirmed the biochemical data that identified VPE as a downstream component in the NRP-mediated cell death response in soybean (Mendes et al., 2013).

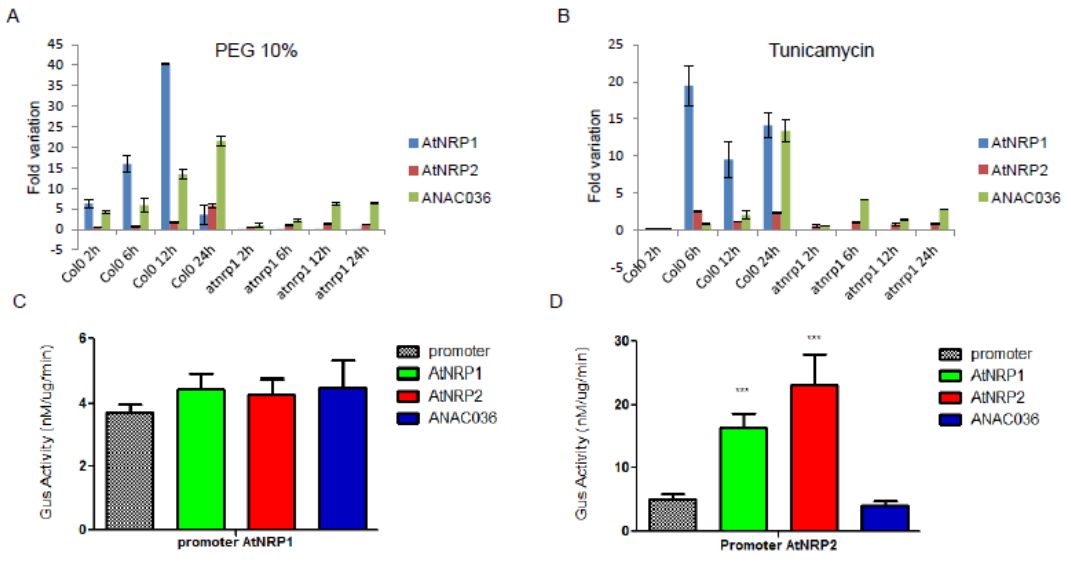

Figure 4. AtNRP1 is upstream of AtNRP2 and ANA036 in the stress-induced cell death response. (A) AtNRP1, AtNRP2 and ANAC036 expression in Col0 and atnrp1 line. Total RNA was isolated from 15 days-old Arabidopsis seedlings treated with (A) PEG (10%) and (B) Tunicamycin (2,5 μg/mL) for 2h, 6h, 12h and 24h. H2O was used as control for PEG and DMSO for Tunicamycin. The transcript levels of selected genes were quantified by qRT-PCR. Gene expression was calculated using the $2^{-\Delta Ct}$ method using UBQ5 as endogenous control. cDNAs were obtained from five biological replicates and validated individually. (C, D) Ectopic expression of AtNRP1 activated the AtNRP2 promoter. Tobacco leaves were co-infiltrated with Agrobacterium carrying AtNRP1pro:β–GUS, AtNRP2pro:β–GUS in combination with YFP-AtNRP1, AtNRP2 and YFP-ANAC036. Values represent β-Glucuronidase activity of three biological replicates ns asterisks indicate statistical differences by the test T ($p<0,05$).

BiPD overexpression attenuates the expression of NRP/DCD-mediated cell death genes and promotes water stress tolerance in Arabidopsis

The stress-induced NRP-mediated cell death response has been shown to be modulated by the ER-resident molecular chaperone BiP (Costa et al; 2008; Reis et al., 2011). Overexpression of soybean BiPD delays the cell death response induced by osmotic stress, ER stress and drought, a phenotype that has been linked to the BiP-mediated increases in the water stress tolerance (Valente et al., 2009; Reis et al., 2011). To examine whether the NRP-mediated cell death response in Arabidopsis would share similar regulatory mechanisms as in soybean, we transformed Arabidopsis Col-0 with soyBiPD and also with the Arabidopsis BiP gene, AtBiP1 (Figure S5) and monitored the BiP attenuation of the stress-induced expression of pathway components. The induction of AtNRP1, AtNRP2 and ANAC036 by tunicamycin was lower in all BiP-overexpressing lines than in Col-0 (Figures 5 and S6). Likewise, PEG treatment induced the expression of AtNRP1, AtNRP2 and ANAC036 to a lower extent in BiPDox T07 line and BiP1-overexpressing line than in Col-0. These results confirmed that BiP also modulates the NRP-mediated cell death response in Arabidopsis. (Figure S6).The BiP-mediated attenuation of the stress-induced NRP-mediated cell death response has been linked to its capacity to confer tolerance to drought (Alvim et al., 2001; Valente et al., 2009). We next examined whether BiP overexpression in Arabidopsis conferred tolerance to drought. For the drought treatment, water was withheld from 5-week-old

plants for 15 days (Figure 6A). A water stress tolerant phenotype was clearly developed by the transgenic lines overexpressing soyBiPD (6A) and the AtBiP1 (Figure S7). This phenotype was typical of tobacco and soybean BiP-overexpressing lines, such as maintenance of leaf turgicity (Figure 6A and S7), higher relative water content (data not shown), higher photosynthetic rate (data not shown) under a water deficit regime and attenuation of drought-mediated induction of the AtNRP1 gene (Figure 6B).However the induction of AtNRP2 is lower upon water stress and it seems to be dalayed in relation to AtNRP1. These results indicate that conservative regulatory mechanisms may account for the BiP modulation of drought tolerance and NRP-mediated cell death signaling in different plant species.

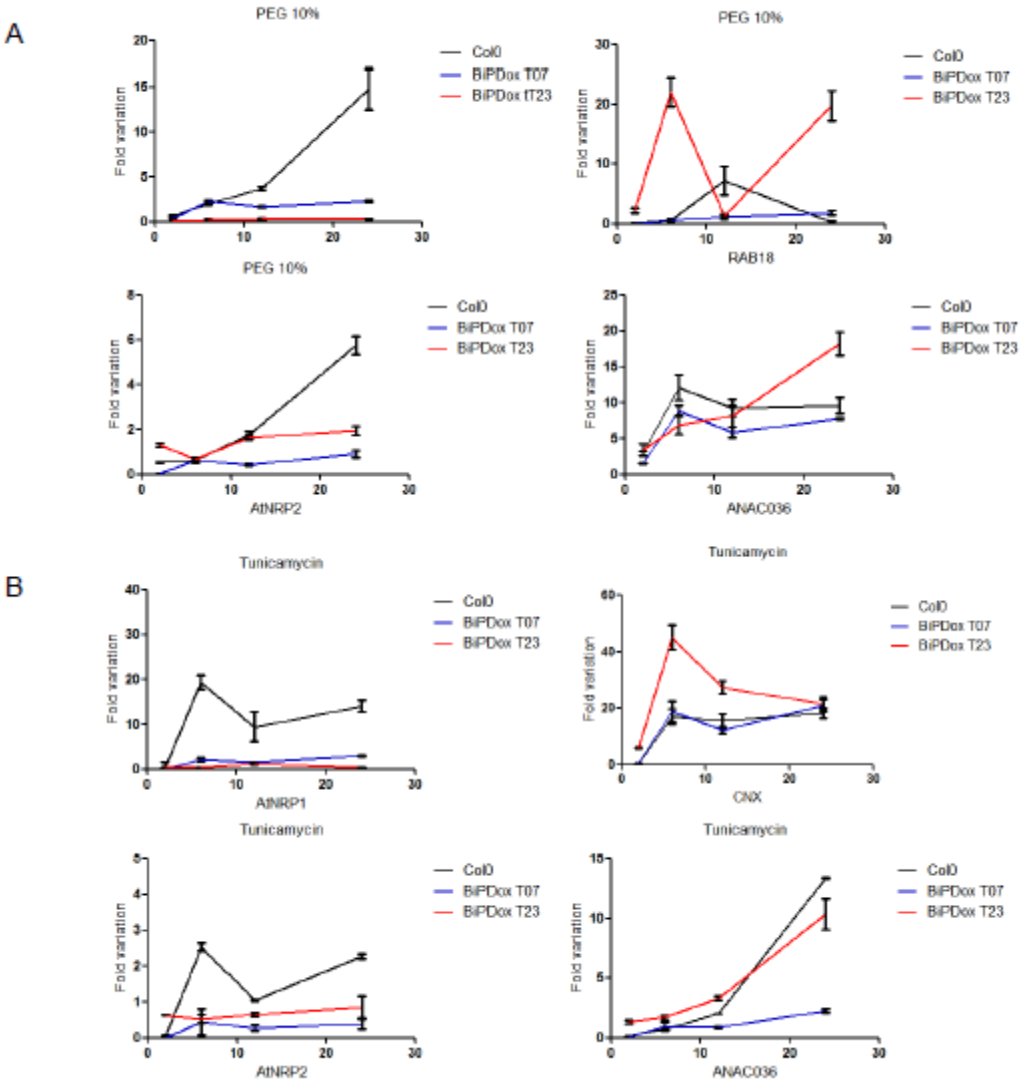

Figura 5. Overexpression of soyBiPD in Arabidopsis attenuates the PEG and tuncamycin-mediated induction of NRPP/DCD-mediated cell death genes. AtNRP1,

AtNRP2 and ANAC036 expression in Col0 and BiPD overexpressing lines, BiPox T07 and BiPox T23. Total RNA was isolated from 15 days-old Arabidopsis plants treated with (A) PEG (10% w/v) and (B)Tunicamycin (2,5µg/mL) for 2h, 6h, 12h and 24h. H2O was used as control for PEG and DMSO for Tunicamycin. The transcript levels of selected genes were quantified by qRT-PCR . Gene expression was calculated using the $2^{-\Delta Ct}$ method using UBQ5 as endogenous control. cDNAs were obtained from four biological replicates and validated individually. (S.D, n=4 biological replicates).

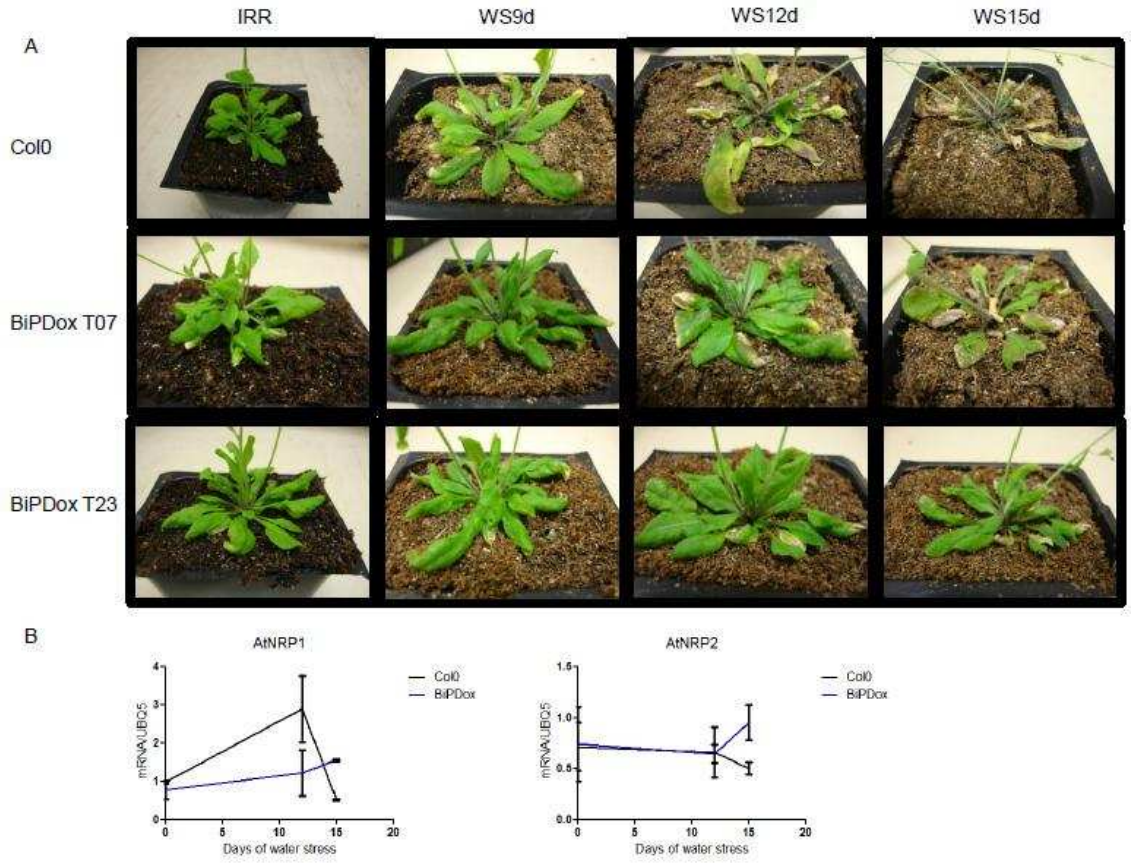

Figure 6. BiPD-overexpressing lines are more tolerant to water stress. (A) Transgenic lines under a water deficit regime. Arabidopsis plants, genotypes Col0, BiPDox line T07 and BiPDox line T23, were grown in soil and water stress was induced in 40 days-old plants by withholding irrigation for 35 days. Photography was taken 9, 12 and 15days after withholding irrigation (B) Expression of AtNRP1 and AtNRP2 during the water stress treatment. Total RNA was isolated from leaves of Col0 and BiPDox line T07 at the indicated time points during the water stress treatment. The transcript levels of selected genes were quantified by qRT-PCR . Gene expression was calculated using the

$2^{-\Delta Ct}$ method using UBQ5 as endogenous control. cDNAs were obtained from five biological replicates and validated individually. Bars represent standard deviation

AGB1 gene may be an upstream component of the NRP/DCD-mediated cell death response

Having established that the stress-induced DCD/NRP-mediated cell death signaling operates and is modulated by BiP in Arabidopsis, we isolated T-DNA insertion mutants of typical ER signaling receptors to search for upstream components of the pathway. While loss of bZIP60, bZIP28 or bZIP17 function increased sensitivity to water stress (data not shown), inactivation of the AGB1 function increased tolerance to water deficit, a phenotype that paralleled the attenuation of the NRP-mediated cell death signaling by BiP overexpression (Figure 7). AGB encodes the Gβ subunit of the heterotrimeric G protein, which has been demonstrated to associate partially with ER membranes and to be involved in UPR-associated cell death signaling (Wang et al., 2007; Chen et al., 2011). Because AGB1 may be involved in transducing a cell death signal derived from ER stress and antagonizes the BiP drought tolerant phenotype, which has been associated with attenuation of the NRP-mediated cell death response, we examined whether AGB1 would function in the stress-induced cell death signaling. RT-PCR on RNA from agb1-3 leaves detected no accumulation of the AGB1 transcript in the homozygous T-DNA insertion mutant confirming it is a null mutant (Figure S8). Similarly to BiP overexpression, loss of AGB1 function impaired the PEG- and tunicamycin-mediated induction of ArNRP1, AtNRP2 and ANAC036 (Figure 8). Likewise, AtNRP1 induction by drought conditions was compromised in the agb1-3 null alleles (Figure 9B). Collectively, these results indicate that AGB1 may act upstream of AtNRP1 in the cell death signaling pathway as mutation in AGB1 gene suppresses the osmotic and ER stress-induction of the NRP-mediated cell death components.

As a positive regulator of the stress-induced NRP-mediated cell death response, we examined whether AGB1 would be directly involved in the activation of a cell death program radiated from prolonged osmotic stress or ER stress. Col-0 and agb1-3 mutant lines were treated with PEG and tunicamycin for 72 h (Figure 8). We also included the BiP-overexpressing lines as a control for negative regulation of the cell death pathway. Accordingly, loss of AGB1 function in the agb1-3 null mutant enhanced tolerance to PEG and to tunicamycin, a phenotype that resembled the overexpression of the negative

regulator BiP. While prolonged PEG treatment promoted leaf wilting in Col-0, BiPox and agb1-3 lines maintained leaf turgidity under osmotic stress. Prolonged ER stress induced by tunicamycin treatment caused intense leaf yellowing and some chlorotic and necrotic lesions in the leaves of Col-0, whereas in the BiPox and agb1-3 mutant lines the tunicamycin-induced cell death phenotype was attenuated. Collectively, these results further support the notion that the stress-induced NRP-mediated cell death response is positively regulated by AGB1.

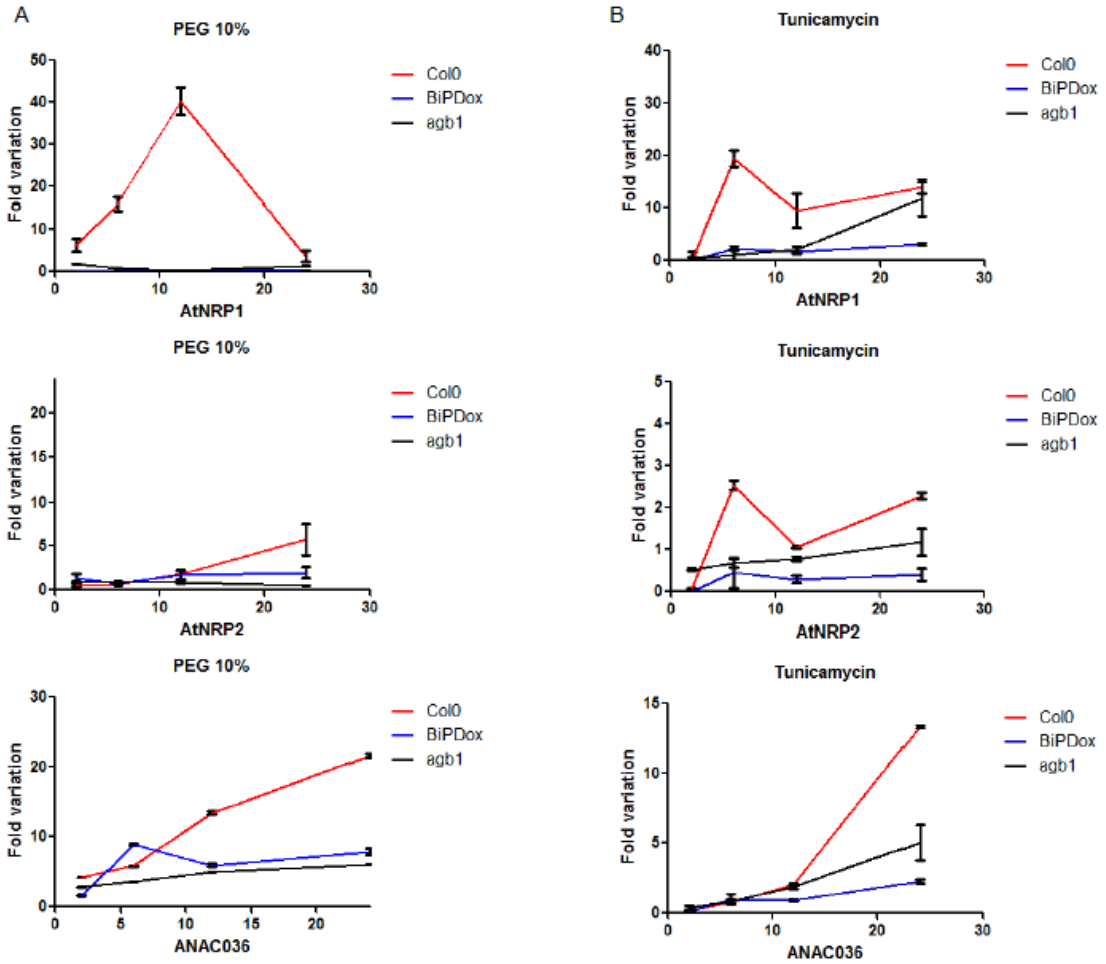

Figure 7. AtNRP1, AtNRP2 and ANAC036 require the AGB1 function for PEG- and tunicamycin-mediated induction of gene expression. Total RNA was isolated from 15 days-old Arabidopsis plants treated with (A) PEG (10% w/v) and tunicamycin (2,5 µg/mL) for 2h, 6h, 12h and 24h. H2O was used as control for PEG and DMSO for Tunicamycin. The transcript levels of selected genes were quantified by qRT-PCR . Gene expression was calculated using the $2^{-\Delta Ct}$ method and UBQ5 as endogenous control. cDNAs were obtained from five biological replicates and validated individually. Bars represent standard deviation.

Figure 8. agb1-3 null mutant line is more tolerant to osmotic and ER stress than Col0. Col0, BiPDox line and agb1-3 null mutant line were treated with PEG (10% w/v) and tunicamycin (2,5 μg/mL) for 72 hours when the photographies were taken.

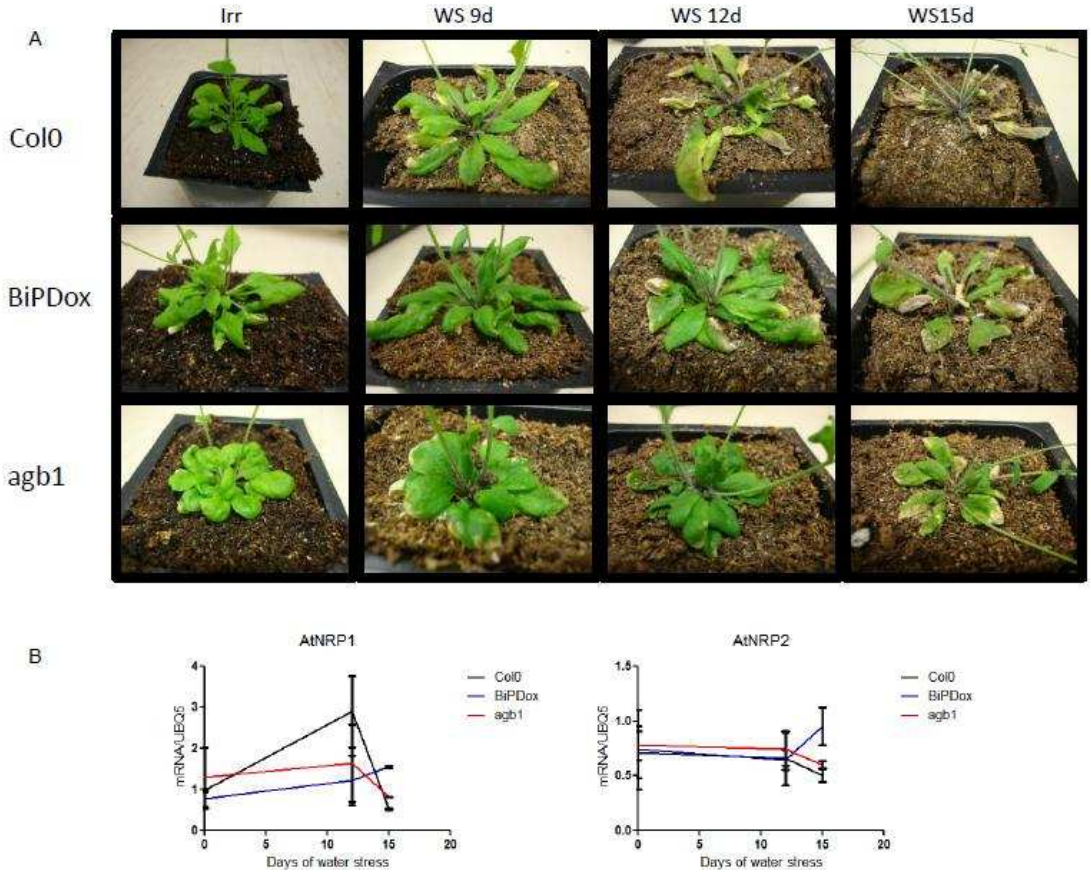

Figure 9. Loss of AGB1 function confers tolerance to water deficit and impairs drought-mediated induction of AtNRP1. (A) Drought tolerant phenotype of agb1-3 mutant line. Col0, BiPDox line and agb1-3 null mutant line were grown in soil and water stress was induced in 40 daysold Arabidopsis plants bu withholding irrigation. (B) Attenuation of drought-induced expression of AtNRP1 in agb1-3 null mutant line and BiPDox line. Total RNA was isolated from leaves of Col0, agb1-3 and BiPDox line during the water stress. The transcript levels of selected genes were quantified by qRT-PCR . Gene expression was calculated using the $2^{-\Delta Ct}$ method and UBQ5 as endogenous control. cDNAs were obtained from five biological replicates and validated individually. Bars represent standard deviation.

BiP interacts with VPE-γ protein and regulates the osmotic stress- and ER stress-induced expression of the VPE-γ gene.

BiP modulates the propagation of an ER stress and osmotic stress-induced cell death signal by negatively regulating the expression and activity of NRP-mediated cell death signaling components (Reis et al., 2011). BiP-mediated negative modulation of this pathway is likely associated with its capacity to regulate the UPR activation

(Srivastava et al., 2013). The AtNRP1 promoter harbors the conserved motif I of the UPR cis-acting element at position -362 and may be a direct UPR target. However, in addition to controlling the expression, BiP also inhibits the cell death activity of pathway components (Reis et al., 2011). This BiP property may be linked to its chaperone activity. As a vacuolar enzyme, the downstream component of the cell death pathway VPE enters to the ER lumen for translocation to the vacuole. Retention of VPE in the lumen of ER would prevent VPE trafficking to the vacuole and thereby the activation of the cell death program. This hypothesis raised the possibility that BiP could bind to VPE to control folding and translocation. To address whether BiP is involved in posttranslational regulation of VPEs, we transiently expressed YFP-VPE-γ (Figure S9) in tobacco plants and immunoprecipitated it using anti-GFP matrix. The coimmunoprecipitated was detected using anti-BiP antibody (Figure 10). The characteristic of the interaction between BiPD and VPE-γ appeared to be conventional, because BiPD was released from VPE-γ in the presence of ATP. Fnctional interactions between the molecular chaperone BiP and secretory proteins have been shown to occur in an ATP-dependent manner. Binding of ATP to BiP induces a conformational change, which releases BiP from the complex (Wei et al., 1995). This result indicates that BiP may modulate cell death activities of the signaling components through interactions with the cell death executioner VPE. The determination of VPE caspase-1 like activity in BiPDox lines will be necessary to complement this interpretation.

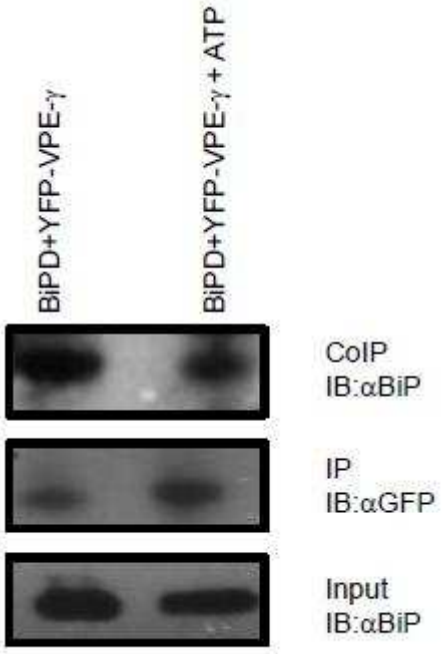

Figure 10. In vivo interaction between BiPD and VPE-γ. Immunoprecipitation of transiently expressed proteins was performed using anti-GFP matrix, and coimmunoprecipitated BiP was detected using anti-BiP antibody. Immunoblots of coimmunoprecipitated BiPD and BiPD +ATP (CoIP), iImmunoprecipitated YFP- VPE-γ (IP) and from total protein extract (input BiPD) from transiently expressed proteins in N. benthamiana leaves.

Chaperone activity is not essential for BiP tolerance mechanism in plants

BiP as a molecular chaperone is an important protein that assists the folding and assembly of nascent secretory proteins. BiP consists of a C- terminal protein binding domain and an N-terminal ATP binding domain. The mechanism of BiP binding to and release from nascent polypeptide is regulated by cycles of ADP/ATP binding and hydrolysis. The binding domain function is dependent on conformational changes caused by ATP/ADP affinity that promote cycles of client protein binding and release (Blond-Elguindi et al., 1993; Zhu et al., 1996). Therefore, ATPase activity and ATP binding are critical for the chaperone activity of BiP as they regulate the cycles of substrate protein binding and release. As we have shown that BiP binds to VPE-γ in planta, the BiP modulation of VPE-γ activity could be driven by its chaperone activity or solely by retention of VPE-γ into the ER through ligand binding. Mutations of conservative residues in the ATPase domain of tobacco BiP have separated the chaperone function from the ligand binding function in the mutant protein. The resulting ATP-hydrolysis defective (T46G) or nucleotide-binding deficient (G235D) mutant proteins bind strongly to client proteins due to a block in ligand release. We introduced the same conservative mutations in soyBiPD (Figure S10) and asked whether overexpression of BiPD-G235Dox and BiPD-T46G mutants which are impaired for chaperone activity but display stronger and more permanent binding to client protein, would negatively impact stress-induced expression of NRP-mediated pathway components. We treated Arabidopis seedlings with PEG and tunicamycin for 24 hours and compared the gene expression of selected genes in Col0, BiPDox and the mutants lines (Figure 11). For tunicamycin treatment, the expression profile of NRP/DCD-mediated cell death genes in BiPD-G235D ox line was similar compared to BiPD ox line, with a delayed expression of those genes during the treatment. Under PEG conditions, the expression pattern during the

treatment was different. BiPD-G235D ox showed higher levels of AtNRP1 and ANAC036 when compared with BiPD and Col0, but AtNRP2 expression in BiPD-G235D ox was similar to BiPDox.

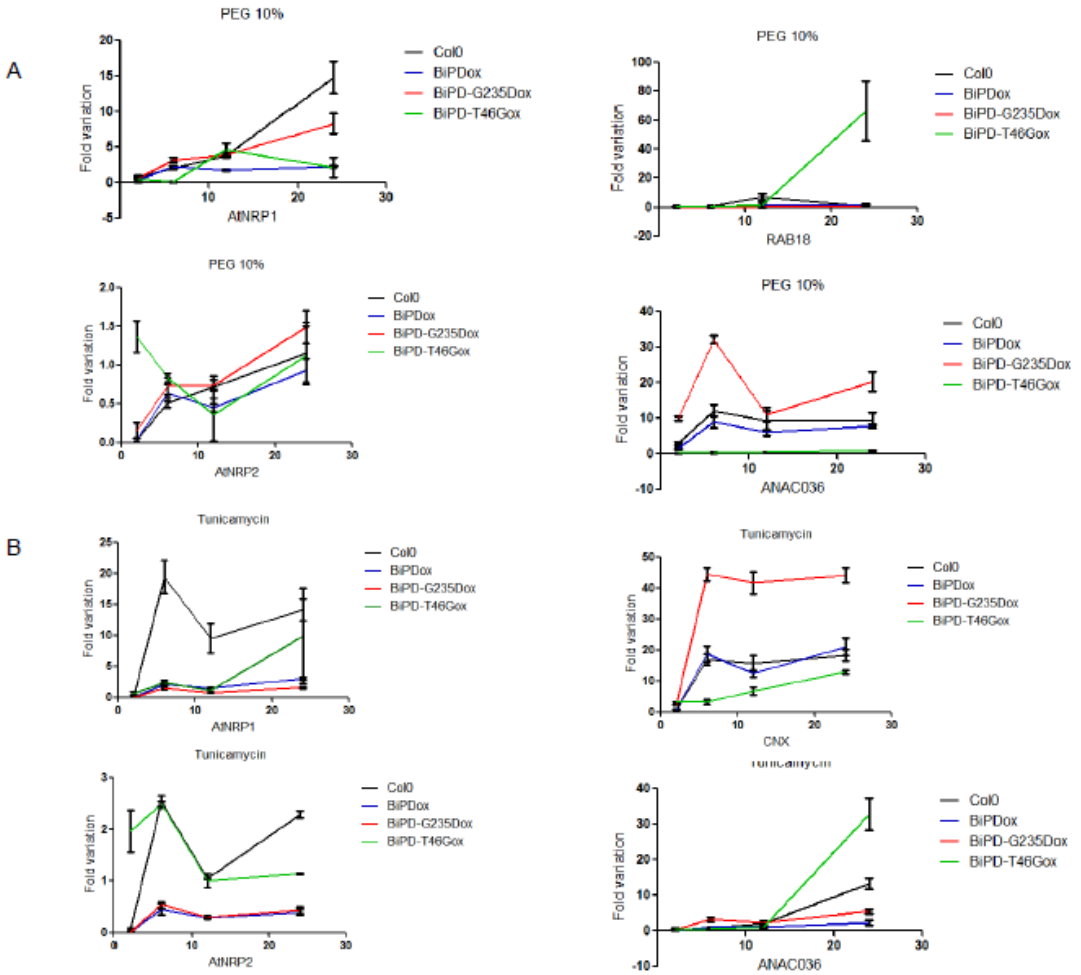

Figure 11. BiPD- T46Gox and BiPD-G235Dox lines delay the expression of NRP/DCD-mediated cell death upon tunicamycin treatment, but not under osmotic stress. Total RNA was isolated from 15 days-old arabidopsis plants treated with PEG (10% w/v) and tunicamycin (2,5 µg/mL) for 2h, 6h, 12h and 24h. H2O was used as control for PEG and DMSO for tunicamycin. The transcript levels of selected genes were quantified by qRT-PCR . Gene expression was calculated using the $2^{-\Delta Ct}$ method and UBQ5 as endogenous control. cDNAs were obtained from five biological replicates and validated individually. RAB18 and CNX are stress markers for osmotic and ER stress, respectively.

BiPDox lines have been shown to be more tolerant to water stress in tobacco and soybean (Alvin et al., 2001; Valente et al., 2009). To determine whether chaperone activity deficient mutants were able to confer tolerance to water deficit, we performed water stress experiment using BiPDox lines and mutated lines, BiPD-T46G ox and BiPD-G235D ox, and Col0. BiPD ox lines and mutated lines were more tolerance to water stress during the period of stress (Figure 12), indicating that stronger ligand binding rather than chaperone activity may account for the underlying mechanism for BiP-mediated increases in water stress tolerance.

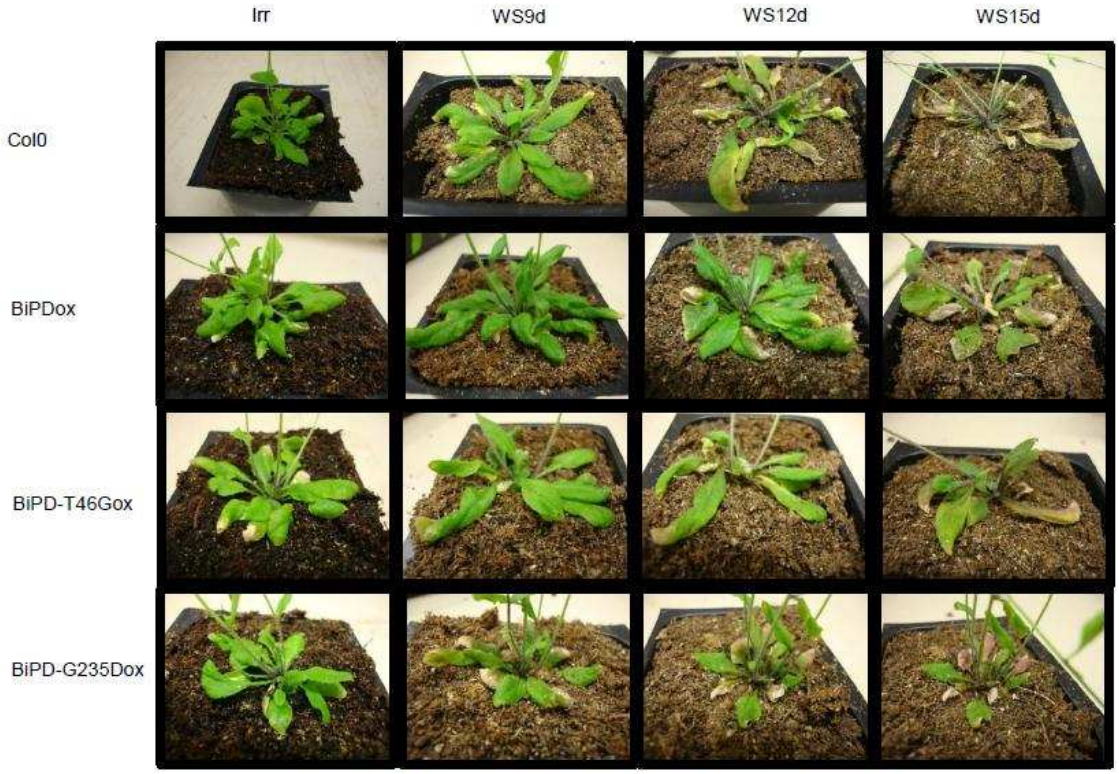

Figure 12. Mutations on the ATPase domain of BiP does not impair BiP-mediated increases in water stress tolerance. BiPDox BiPD-T46Gox, BiPD-G235Dox and Col0 lines were exposed to a water defict regime for 15 days. Water stress was induced in 40 days-old Arabidopsis plants grown in soil by suspending irrigation.

Discussion

NRP/DCD-mediated cell death response is functionally conserved in arabidopsis

NRP/DCD-mediated cell death pathway connects osmotic and ER stress on NRP genes to activate a cell death program. This pathway was originally identified in soybean (Irsigler et al., 2007), and circumstantial evidence indicates that it also operates in tobacco (Valente et al., 2009). In this investigation we extended the characterization of the cell death pathway by demonstrating that it is also functionally conserved in other plant species. In fact, we provided several lines of evidence revealing that the NRP-mediated cell death pathway also propagates a stress-induced cell death signal in Arabidopsis with features of a PCD response. First, homologs for all identified components of the pathway are present in the Arabdiopsis genome Among the asparagine-rich (N-rich) and DCD domain-containing proteins from Arabidopsis, AtNRP1 and AtNRP2 as designated in this investigation, share high conservation of sequence with NRP-A and NRP-B from soybean and they cluster together as subgroup I of the DCD domain-containing protein family (Tenhaken et al; 2005). Members of this subgroup contain a highly conserved DCD domain at C-terminus and a more divergent N-rich domain at the N-terminus and they are plant-specific proteins. Likewise, another component of the NRP/DCD-mediated cell death signaling, the GmNAC81, belongs to a plant-specific family of transcriptional factors, the NAC (NAM, ATAF1/2 and CUC2) domain-containing superfamily of transcription factors. It is is a member of the subgroup TERN (Tobacco elicitor-responsive gene-encoding NAC domain protein) which is induced by elicitors of the pathogen response (Ooaka et al., 2003; Pinheiro et al., 2009). GmNAC81 shares 62% of identity with Arabidopsis protein ANAC036. In addition to the NAC conserved domain at the N-terminus, they share the conserved domains C1 and C2 at C-terminus regions The C1 domain consisted from 27 amino acids and contained the putative NAC subdomain E and its immediately downstream region. The C2 domain consisted of approximately 40 amino acids and it was located near the C-terminal end (Kato et al., 2010). We also found four homologs of the plant-specific vacuolar processing enzyme VPE encoded by the genome of Arabidopsis. Second, like the soybean counterparts, AtNRP1, AtNRP2, ANAC036 and VPE-γ are induced by osmotic and ER stress. We did not observe, however, a synergistic induction of the Arabidopsis homologs by a combined treatment of both stresses, as already reported for the soybean cell death pathway components (Costa et al., 2008; Faria et al., 2011). These differences in the expression profile may be explained by differences in the experimental conditions of the experiment, It has not been well established for Arabidopsis in which conditions the treatment with the osmotic stress inducer PEG would not interfere with

tunicamycin uptake during the combined treatments. In soybean, the plants arepre-treated with tunicamycin for six hours, and then PEG is added for an additional ten hours (Irsigler et al., 2007). Third, as expected for components of a cell death signaling, the Arabidopsis NRP/DCD-mediated cell death genes can promote cell death in planta. Transient expression of AtNRP1, AtNRP2 and ANAC036 promotes causes leaf yellowing, which was associated with chlorophyll loss in agroinfiltrated leaf sectors that evolved to necrotic lesions (Figure 2), a phenotype similar to that developed by e NRP-A and NRP-B expression. This process of accelerated leaf yellowing induced by expression of NRP/DCD cell death pathway components has been shown to be associated with programmed cell death in soybean (Costa et al, 2008; Faria et al., 2011; Reis et al., 2011). Finally, functional homology between AtNRP1, AtNRP2, GmNRP-A and GmNRP-B were further demonstrated in complementation assays of the hypersensitive phenotype to osmotic stress displayed by the atnrp1 null mutants. In osmotic conditions a further enhancement of root growth inhibition is clearly observed in the atnrp1 line (Figure 3A). Stable expression of AtNRP1, AtNRP2, NRP-A or NRP-B in the atnrp1 null mutant rescued the wild-type phenotype and the root growth of transgenic lines under PEG treatment was compared to the untransformed wild-type line (Figure 3B and 3C).

The function of NRPs could be related to ROS response in the root. Water stress increases ROS levels and their distribution in the root tip is necessary to define the transition from proliferation to differentiation of the root cells. Superoxide accumulates in meristematic zone, where the new cells are generated, and H_2O_2 accumulates in the elongation zone. This partitioning of the accumulation of ROS in root zones implicates that superoxide may be associated with activation of the proliferative state while H_2O_2 mayarrest cell proliferation (Tsukagoshi et al., 2010; Tsukagoshi., 2012). In addition, root growth is determined by the rates of cell division in the meristematic zone and cell expansion in the elongation zone. As meristem activity and cell cycle progression are strongly correlated, cell cycle progression in the meristematic zone regulates the rate of cell division in the root meristem. Therefore, ROS levels in the root tip might affect the expression of genes involved in cell cycle progression. As NRPs respond to H_2O_2 (Ludwig and Tenhaken., 2001; Costa et al., 2008), the lack of AtNRP1 in the cell can interfere with H_2O_2 response and regulates the root growth. However, more experiments should be done to prove this hypothesis.

The components of the DCD/NRP cell death signaling pathway have been identified in soybean mostly as a result of biochemical and forward genetic approaches and their hierarchical order in the pathway has been assigned based on expression and kinetic studies (Isrigler et al., 2007, Faria et al., 2009, Mendes et al., 2013). In the Arabidopsis system, however, the characterization of the atnrp1 null allele confirmed that AtNRP1 is upstream of GmNAC36 and indicated that AtNRP2 and AtNRP1 may not be functionally redundant in the pathway, as AtNRP2 expression depends on AtNRP1 function. In fact, loss of AtNRP1 function prevents osmotic and ER stress-mediated induction of AtNRP2 and the other downstream components of the pathway, ANAC036 and VPE. Consistent with this interpretation, AtNRP1 and AtNRP2 transactivate the AtNRP2 promoter, but not the AtNRP1 promoter. As a downstream component, the transcriptional factor GmNAC36 does not feedback regulate the AtNRP1 and AtNRP2 upstream components. Recently, we demonstrated that the ANAC36 soybean homolog, GmNAC81, interacts with another member of the NAC family, GmNAC30, to fully activate the expression of the downstream component VPE. It would be very interesting to investigate whether ANAC36 transactivates the Arabidopsis VPE homolog gene and whether it would require another member of the NAC family for full transactivation activity, as for GmNAC81. (Faria et al. 2011)

BiPD overexpression promotes water stress tolerance and modulates NRP/DCD-mediated cell death response in arabidopsis

BiP overexpressing plants have been demonstrated to respond to abiotic and biotic stress conditions either through gene expression and posttranslational regulation (Costa et al., 2008; Alvim et al., 2001; Leborgne-Castel et al., 1999; Valente et al., 2009; Wang et al., 2005; Reis et al., 2011; Carvalho et al., 2013). As a molecular chaperone, BiP attenuates the ER stress response through the UPR (unfolded protein response) (Costa et al., 2008; Leborgne-Castel et al., 1999) by regulating the stress transducer bZiP28 activity (Srivastava et al., 2012). BiP binds to C-terminal tail of bZiP28, and upon ER stress conditions the interaction is released, allowing the translocation of bZiP28 to the Golgi where it is proteolytically cleaved to be transported into to nucleus. In addition to the ER stress response, BiP plays an important role in a plant-specific stress response, which connects osmotic stress and ER stress and promotes cell death through NRPs genes (Irsigler et al., 2007; Costa et al., 208; Faria et al., 2011; Reis et al., 2011). BiP has been shown to attenuate the NRP/DCD-mediated cell death response in

soybean, Here, we demonstrated that the NRP-mediated cell death signaling pathway is also negatively controlled by BiP in Arabidopsis thaliana. Overexpression of the soyBiPD and Aradidopsis BiP1 gene attenuated the osmotic and ER stress induction of NRP/DCD genes (Figure 6). The BiP attenuation of the NRP-mediated cell death response has been associated with the BiP-mediated increases in water stress tolerance. Accordingly, in Arabdidopsis, BiP-overexpressing lines were more tolerant to drought. The BiPDox lines maintained leaf turgidity under dehydration conditions caused by drought stress and photosynthesis, stomatal condutance and transpiration in BiPDox were higher than Col0 under drought, a typical phenotype mediated by BiP overexpression in soybean and tobacco (Alvim et al., 2001; Valente et al., 2009). Our current results confirmed the conservation for a BiP negative regulation of NRP-mediated cell death response in the plant kingdom and implicate BiP as potential target for engineering water stress tolerance in other crops.

AGB1 protein can modulate NRP/DCD-mediated cell death response

G proteins are GTP binding proteins, which are important signaling molecules in eukaryotes. Heterotrimeric G proteins, which consist of three subunits a, b and g are the major class of G proteins (Cabrera-Vera et al., 2003). Arabidopsis have one single G α gene, one G β and two G γ gene, AGB1 and AGB2. AGB1 is one subunit of arabidopsis G protein, which is associated to ER membrane and may be involved in transducing a cell death signal radiated from the ER stress (Wang et al., 2007). Here, we we provided evidence that AGB1 may function as an upstream component of the DCD/NRP-mediated cell death signaling pathway. . Upon ER and osmotic stress conditions, the expression of NRP/DCD-mediated cell death genes was lower in agb1-3 than Col0. This implicates that the downstream components of the stress-induced cell death response requires AGB1 function for full induction. As a putative upstream component of the pathway that relays the cell death signal to the downstream components, AGB1 is expected to function as a positive regulator of the NRP/DCD-mediated signaling pathway. Accordingly, inactivation of AGB1 function increases tolerance to cell death inducers, such as tunicamuycin and PEG. The cell death tolerant phenotype of the agb1-e null mutant lines resembles the cell death tolerance induced by overexpression of BiP, a negative modulator of the NRP/DCD-mediated cell death signaling. In the agb1-3 line and in the BiP-overexpressing lines the stress-induced expression of the NRP/DCD cell death

pathway genes is attenuated as a common denominator. Collectively, these results genetically link AGB1 to the DCD/NRP-mediated cell death response. Nevertheless, evidence of AGB1 as a cell death transducer is quite controversial in the literature. Our results are consistent with those reported by Wang et al. (2007), which showed that loss of AGB1 function enhanced resistance to teh ER stressor tunicamycin.The leaves of agb1 line displayed less cell death when they were infiltrated with tunicamycin and lower expression of UPR marker genes (Wang et al., 2007). However, more recently, Chen and Brandizzi (2011) reported opposite results, as agb1 were more sensitive to growth inhibition by tunimycin and displayed a higher expression of UPR marker genes. In our results we found agb1-3 line more sensitive to growth inhibition by tunicamycin when compared to Col0 and BiPDox line (Supplemental figure 11). A possible explanation for the controversial results may be related to a phenotypic plasticity of agb1 null mutant line, the ability to display different phenotypes under different environmental conditions. A model has been proposed that G proteins may actuate as signal modulator instead of being a direct transducer of signals, and through cross-talk could fit a phenotype or physiological response based on multiples signals (Nilson and Assmann., 2009). Arabidopsis G proteins have been shown to play diverse roles in germination, development, phytohormone response, stress response and stomatal aperture regulation (Ullah et al., 2001, 2002, 2003; Wang et al., 2001, 2007; Pandey & Assmann, 2004; Joo et al., 2005; Llorente et al., 2005; Pandey et al., 2006; Fan et al., 2008; Zhang et al., 2008a; Zhang et al., 2008b). As AGB1 is involved in plant germination, the agb1-3 mutant line is more sensitive to growth inhibition by tunicamycin than Col0 and seedlings are more resistant to osmotic and ER stress. Likewise, agb1 mutants are hypersensitive to ABA inhibition during germination and root growth (Pandey et al., 2006), and ABA signaling regulates the response to drought and contributes to plants survival in drought conditions. Nevertheless, agb1 mutants are more tolerant than Col0 when submitted to drought conditions (Figure 10 and Nilson and Assmann., 2009), a phenotype that is associated to a delay or impairment of the NRP/DCD cell death signaling rather than modulation of ABA signaling.

BiP can modulate NRP/DCD-mediated cell death through VPE gene

NRP/DCD-mediated cell death pathway is activated by either osmotic or ER stress but requires both stress for full activation (Costa et al., 2008; Faria et al., 2011; Reis et al., 2011). The constitutive expression of molecular chaperone BiP can modulate

this pathway and promote better adaptation of transgenic lines to drought. In this pathway, NRPs genes are induced by osmotic or ER stress and promote the induction of GmNAC81 gene. GmNAC81 interacts with GmNAC30 and both can induce caspase-1-like VPE gene (Glyma14g10620), which is involved in PCD in plants (Mendes et al., 2013). GmNAC30 and GmNAC81 recognize the consensus sequence TGTG[T/G/C] in the promoter region for the activation of VPE gene. As the NRP/DCD mediated cell death operates in Arabidopsis, we analyzed the existence of VPE (Glyma14g10620) homologs. Arabidopsis have four VPE genes, VPE-α, VPE-β, VPE-γ and VPE-δ, and through sequence alignment we identified VPE-γ with higher homology (82%) to Glyma14g10620 gene, which has been shown to be regulated by GmNAC81 and GmNAC30. Furthermore, we found the presence of three GmNAC81-binding consensus sequence TGTG[T/G/C] in the promoter region of can VPE-γ, TGTGTT at position -1110, TGTGTC at -1215 and TGTGTG at -872. As for the other components of the NRP/DCD cell death signaling, VPE-γ is induced by either PEG or tunicamycin (Figure 12) to an extent modulated by the negative regulator BiP. Furthermore, stress induction of VPE depends on the AtNRP1 function, as the atnrp1 null mutant line displayed a lower expression of VPE-γ compared to Col0 under osmotic and ER stress conditions (Supplemental figure1). BiPD modulation in the NRP/DCD pathway can be either transcriptional, delaying the expression of NRPs genes, or posttranscriptional, modulating the response of NRP/DCD mediated cell after the expression of these genes. As a new component of this pathway and because VPE is a secretory protein, we examined whether BiPD could regulate the function of VPEs. VPEs enzymes are synthesized in an inactive form and start the maturation process in the ER. An unglycosylated pre-protein precursor (ppVPE) is translationally co-translocated to the lumen of ER mediated SRP recognition of a 22-aminoacid N-terminal signal peptide, where the N-terminal sequence is proteolytically cleaved to produce pVPE, a glycolylated pro-protein precursor (Kuroyanagi et al., 2002). During the maturation process, the ER resident molecular chaperones are expected to assist proper folding by transiently binding to the nascent VPE. We provided evidence that BiP may modulate the VPE activity. We showed first that BiP interacts with VPE in planta in an ATP-dependent manner. BiP dissociates from VPE in the presence of ATP indicating that BiP binding to VPE is biochemically relevant. It is very likely that BiP overexpression may modulate VPE activity by increasing the retention of the protein in the ER lumen and,

thereby, delaying fully maturation for activation in the vacuole. Consistent with this hypothesis, mutations in BiP ATPase domain that impairs chaperone activity but not ligand binding modulate negatively the NRP/DCD cell death signaling and the mutant BiPs maintain the property to confer drought tolerance to transgenic lines. Experiments to confirm VPE-BiP interaction by BiFC and enhanced ER retention of VPE in BiP and mutant BiP-overexpressing lines are under progress.

BiP mutations in the ATP binding domain don`t change the BiP modulation in NRP/DCD-mediated cell death response.

As a molecular chaperone, member of HSP70 family, BiP protein has two domains, an N-terminal nucleotide binding domain and a C-terminal peptide-binding domain (Bukau and Horwich., 1998; Gething., 1999). C-terminal domain is conformationally regulated by ATP hydrolysis at N-terminal domain. With the interaction of ATP at the N-terminal domain, the binding domain assumes the open conformation, which has low affinity to any ligand peptide. ATP hydrolysis promotes a conformational change of the binding domain to the closed conformation, which have high affinity to any ligand peptide (Blond-Elguindi et al., 1993; Zhu et al., 1996). Therefore, mutations in the ATPase domain that block ATPase activity and ATP binding have been shown to impair chaperone activity and to strengthen ligand binding by blocking substrate peptide release (Snowden et al., 2007). We found that the ATP-hydrolysis defective (T46G) or nucleotide-binding deficient (G235D) mutants maintain the capacity to modulate negatively the expression of the NRP/DCD cell death pathway genes in response to ER stress conditions and to increase drought stress tolerance to the transgenic lines. These results indicate that the protective function of BiP against dehydration is likely linked to its ligand binding property rather than its chaperone activity.

General conclusion

As plants are subjected to environmental changes and extreme conditions, they have developed coordinated and integrated mechanisms that respond these injuries and are immediately activated upon stresses. To cope with the stress, plant cells activate molecular response to rescue the homeostase and keep themselves alive. One important mechanism activated by plants is the NRP/DCD-mediated cell death response. Ectopic

expression of BIP in transgenic lines attenuates the leaf necrotic lesions caused by treatments with tunicamycin, a potent inducer of ER-stress, and maintaing shoot turgidity under PEG-induced dehydration conditions, whereas control, untransformed leaves wilted under the same PEG conditions. These phenotypes were coordinated with lower and in some cases delayed expression of senescence-associated marker genes. These results are also consistent with previous data showing that the relative content of dead cells is lower in BIP-overexpressing transgenic lines exposed to ER-stress and osmotic stress (Costa, 2008). Furthermore, attenuation of stress-induced cell death by BiP paralleled a delay in the induction of the cell death marker genes NRP-A and NRP-B that are involved in transducing a PCD signal emanated from the ER stress and osmotic stress. The NRP/DCD-mediated PCD signaling fits the concept of a shared pathway induced by ER stress and other stimuli, providing a molecular link that would allow BiP, a regulator of UPR, to control cell death induced by different stimuli. To directly assess the BIP involvement in modulating NRP/DCD-mediated cell death signaling response, we use an Agrobacterium-mediated transient expression assay to monitor NRP-mediated PCD in tobacco transgenic lines with increased (sense) and suppressed (antisense) BiP expression. Enhanced expression of BiP in sense transgenic lines prevented NRP-induced chlorosis in agroinfiltrated sectors, whereas silencing of endogenous BiP in antisense lines accelerated the onset of leaf senescence. These results show a direct co-relation between BiP mRNA abundance and NRP-induced leaf senescence and argue favorably for a direct involvement of BIP in modulating NRP-mediated cell death response. This pathway has been shown conserved in plants. It was first identified in soybean and here we prove that the NRP/DCD-mediated cell death response can operates in arabidopsis with conserved mechanisms. We also presented that the molecular chaperone BiP can modulate the NRP/DCD pathway and promote water stress tolerance. The mechanism of this response may not depend on chaperone activity, because the overexpression of mutated BiP proteins at ATP binding domain still can modulate the NRP/DCD mediated cell death. Moreover, the role of BiP can be implied that regulates the expression and activation of VPE gene as an executor of cell death process. AGB1 was also identified as a modulator of NRP/DCD-mediated cell death, the absence of AGB1 in the cells promotes more tolerance to water stress and lower expression of NRP/DCD-mediated cell death genes. Our results have demonstrated that this pathway is

conserved in plant kingdom, although we need more studies to understand the mechanism of the NRP/DCD-mediated cell death response.

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Supplemental figures

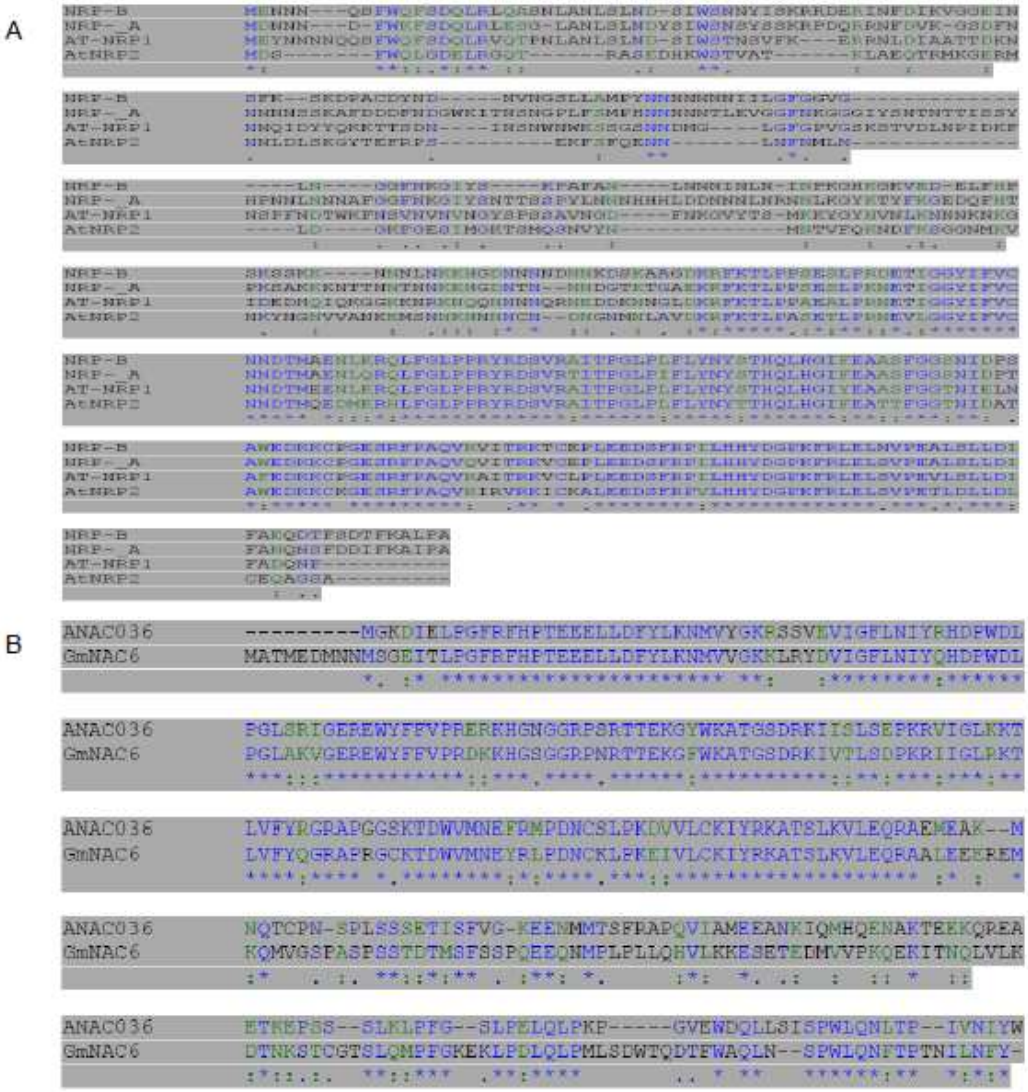

Figure S1. Sequence alignment among soybean NRP genes and arabidopsis genes (A) and GmNAC81 and ANAC036 (B). The sequence comparison was performed using ClustalW2.

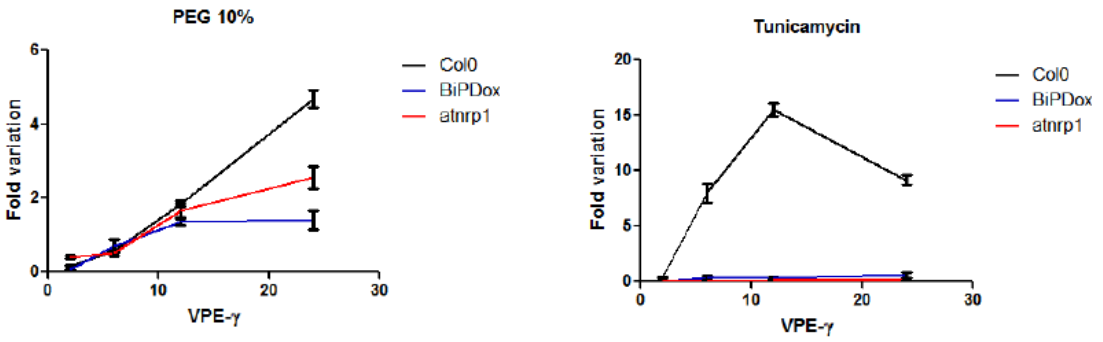

Figure S2. Induction of the VPE-γ gene by PEG and tunicamycin requires the AtNRP1 function. Total RNA was isolated from 15 days-old Arabidopsis plants treated with PEG (10% w/v) and tunicamycin (2,5 μg/mL) for 2h, 6h, 12h and 24h. H2O was used as control for PEG and DMSO for tunicamycin. The transcript levels of selected genes were quantified by qRT-PCR. Gene expression was calculated using the $2^{-\Delta Ct}$ method and UBQ5 as endogenous control. cDNAs were obtained from five biological replicates and validated individually. Barrs represent standard deviation.

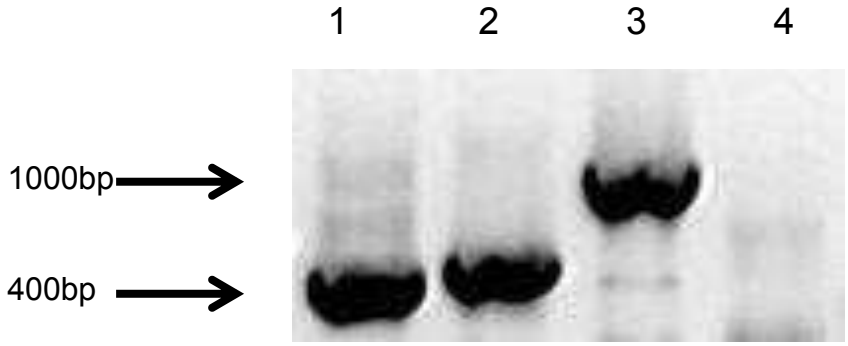

Figure S3. atnrp1 null mutant line confirmation. RT-PCR of plants Col0 (lines 1 and 3) and atnrp1 null mutant line (lines 2 and 4) using primers of AtERD15 (lines 1 and 2) as control, and primers of AtNRP1 (lines 3 and 4).

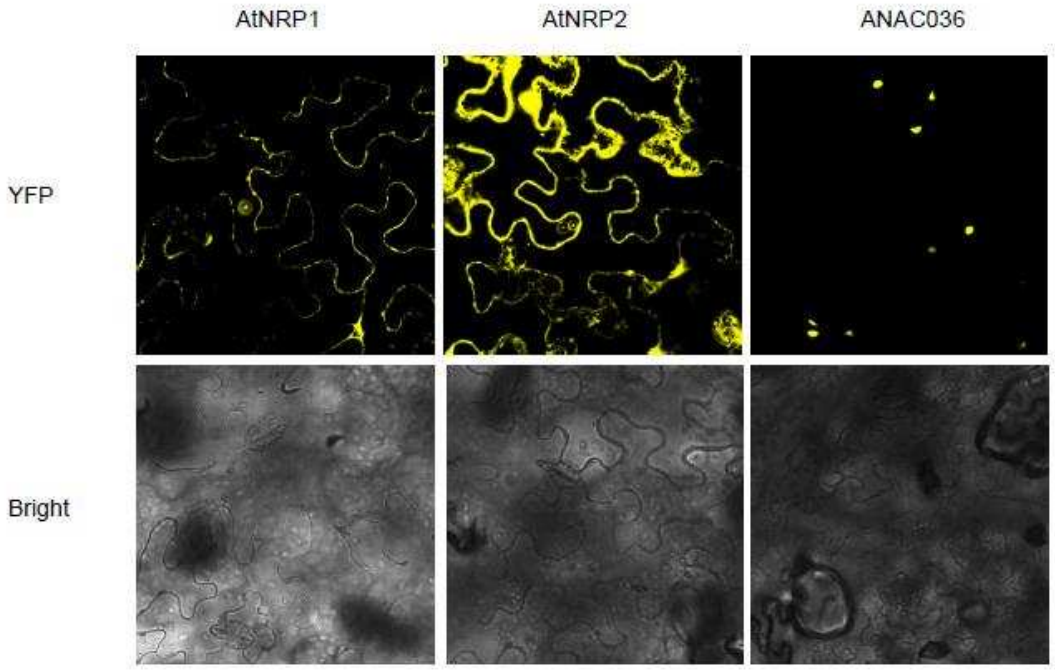

Figure S4. Confocal microscopy analyses of AtNRP1,AtNRP2 and ANAC036 fused to YFP. Tobacco leaves were infiltrated with A. tumefaciens carrying the indicated DNA construct, and images were taken by confocal laser scanning microscopy 72-h post-transfection. GUS:reporter activity was determined from leaves expressing the indicated recombinant proteins.

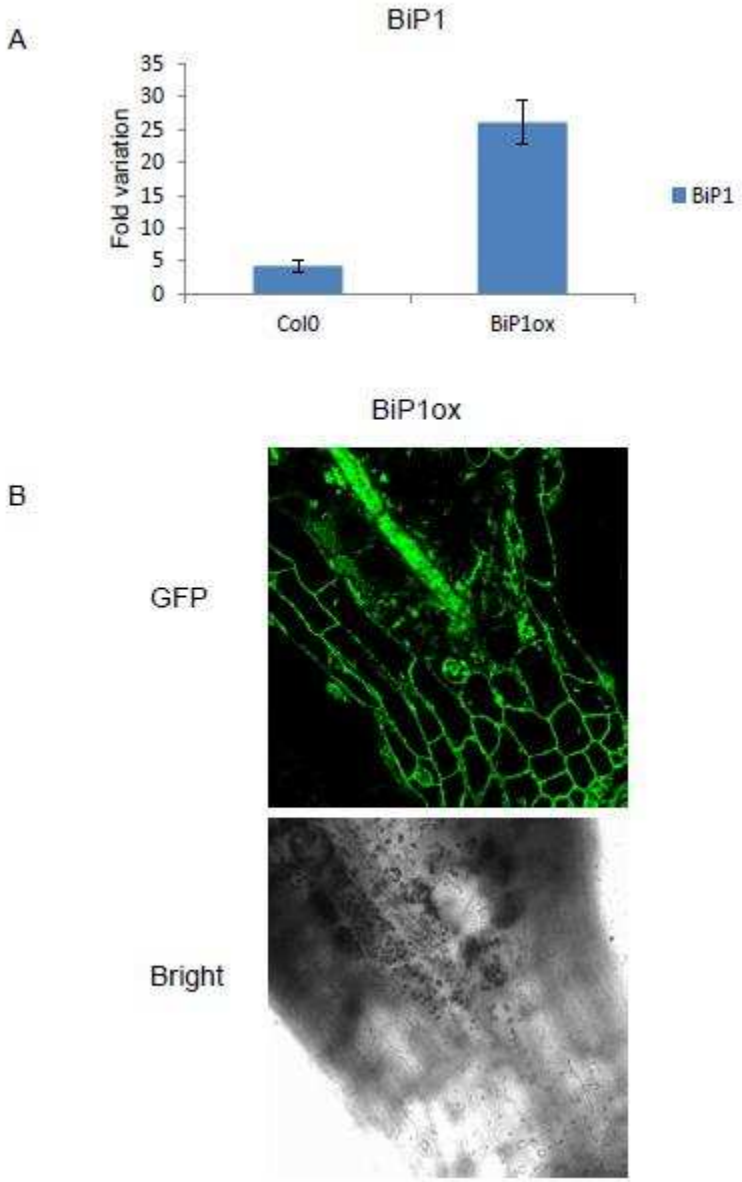

Figure S5. Confirmation of BiP1 overexpressing line. Transcripts levels of seedlings of Col0, BiP1ox (A). Confocal analyses of BiP1ox (B).

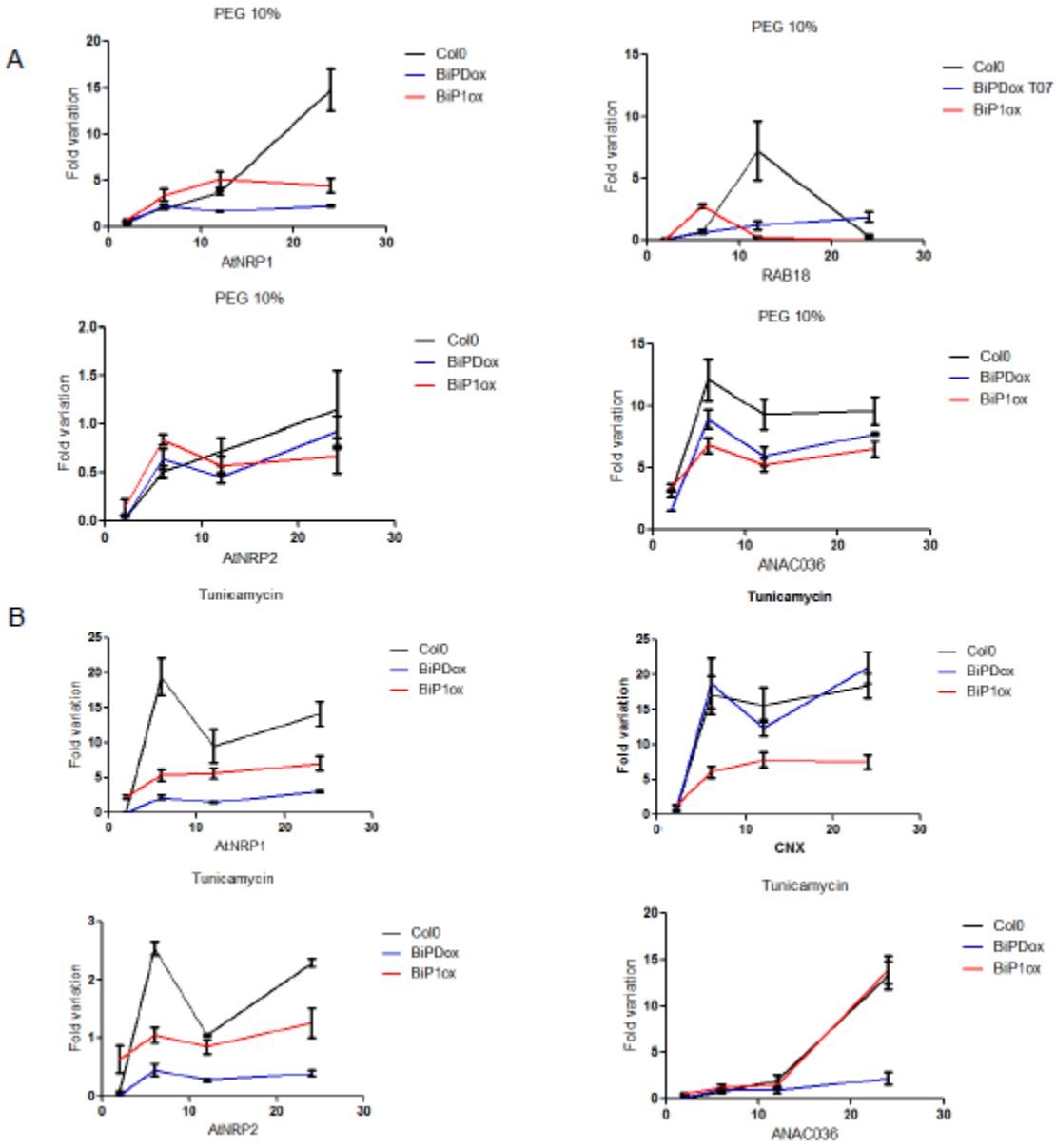

Figure S6. Expression of NRP/DCD-mediated cell death genes in BiP1ox. under osmotic and ER stress. Total RNA was isolated from 15 days-old Arabidopsis seedlings treated with PEG (10% w/v) and Tunicamycin (2,5 µg/mL) for 2h, 6h, 12h and 24h. H2O was used as control for PEG and DMSO for Tunicamycin. The transcript levels of selected genes were quantified by qRT-PCR . Gene expression was calculated using the $2^{-\Delta Ct}$ method using UBQ5 as endogenous control. cDNAs were obtained from five biological replicates and validated individually.

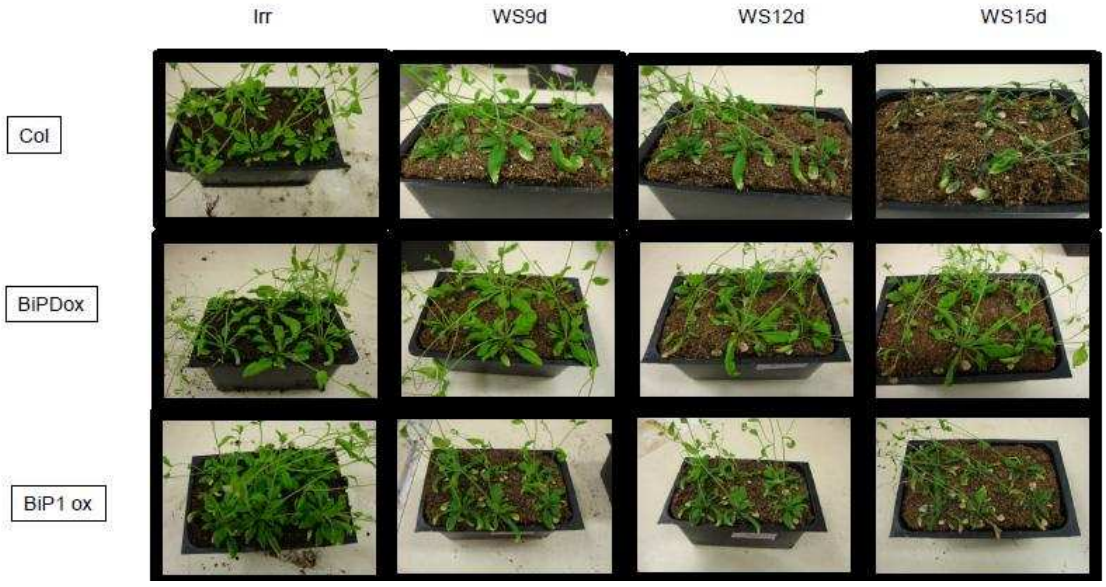

Figure S7. BiP1ox is tolerant to water stress. Col0, BiPD ox line, BiP1ox were submitted to water stress by withholding irrigation for 15 days from 40 days-old soil grown Arabidopsis plants.

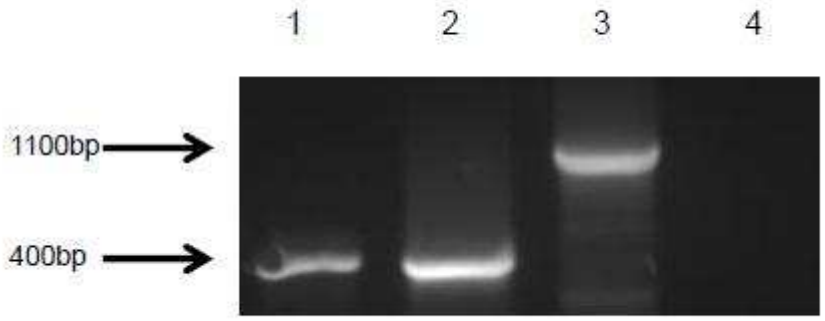

Figure S8. agb1-3 null mutant line confirmation. RT-PCR of plants Col0 (lines 1 and 3) and agb1-3 null mutant line (lines 2 and 4) using AtERD15-specific primers (lines 1 and 2) as control, and AGB1-specific primers (lines 3 and 4)

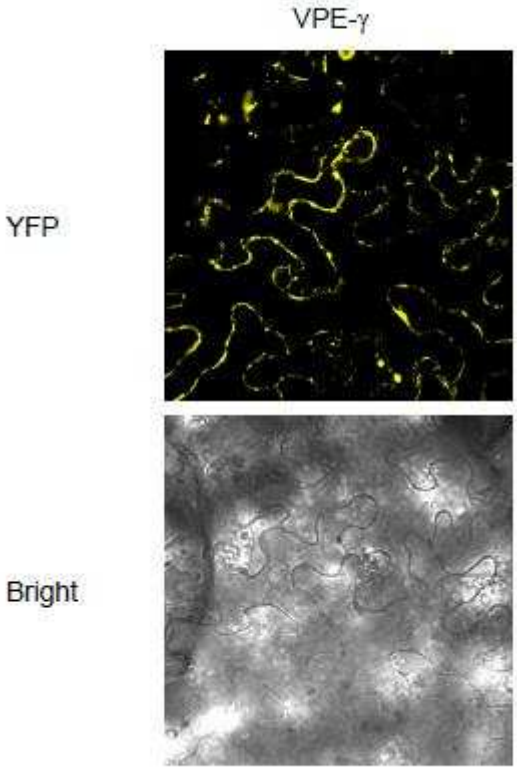

Figure S9. Confocal microscopy analyses of VPE-γ to YFP. A, subcellular localization of 35S:YFP- VPE-γ . Tobacco leaves were infiltrated with A. tumefaciens carrying the indicated DNA construct, and images were taken by confocal laser scanning microscopy 72-h post-transfection

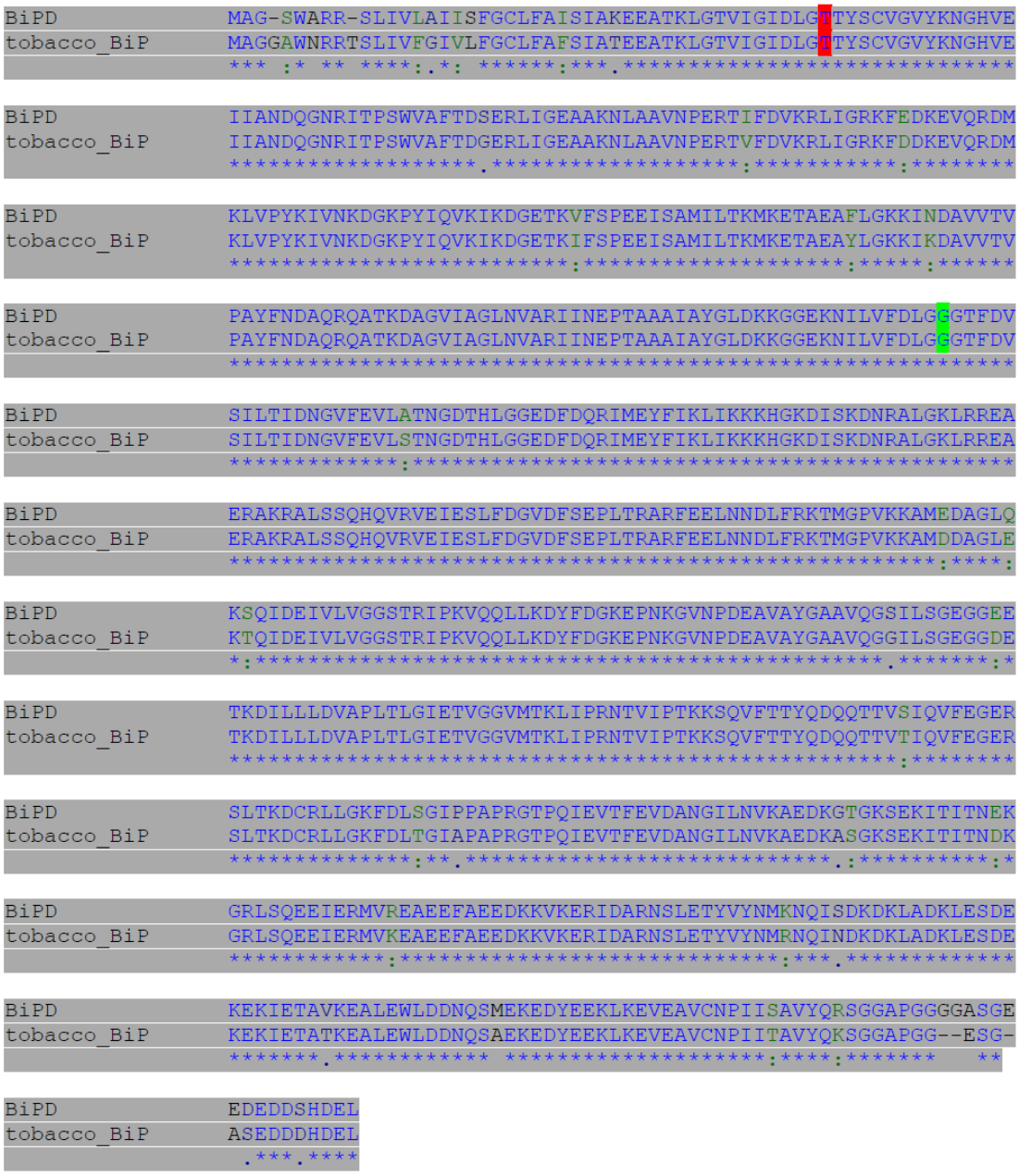

Figure S10. BiPD alignment with BiP from tobacco. The mutated amino acid residues are filled in red (mutation T46G) and in green (G235D).

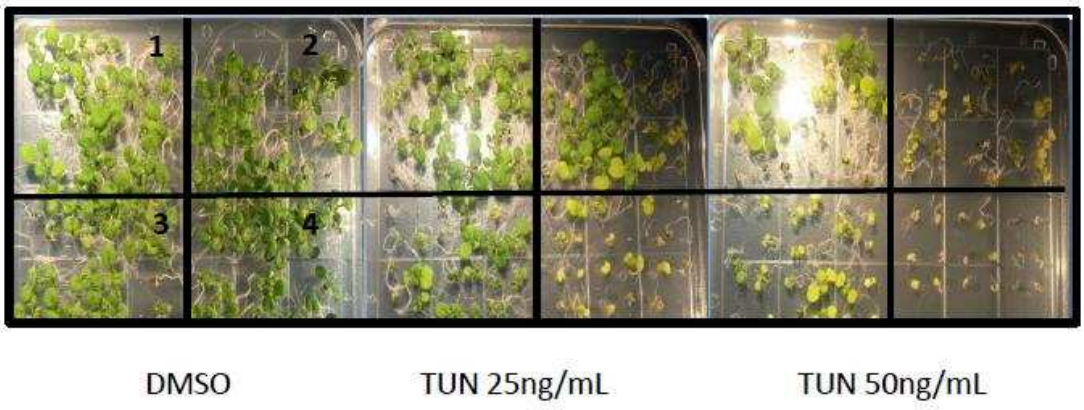

Figure S11. agb1-3 is more sensitive to germination in ER stress conditions. Seeds of BiPDox line (1), Col0 (2), At3g58560 (3) and agb1-3 (4) were germinated in 1/2LS with either DMSO (Control) or 25 and 50 ng/mL of tunicamycin. Upon tunicamycin germination agb1-3 didn`t grow in concentration of 25 ng/mL of tunicamycin, whereas Col0, BiPDox and At3g58560 grew.

Supplemental table 1

Primers used for qRT-PCR analysis			
Name	Sequence 5'->3'	Target	Locus
qRT- Actin Fwd	GGTAACATTGTGCTCAGTGGTGG	Actin 2	At3g18780
qRT-Actin Rvs	AACGACCTTAATCTTCATGCTGC		
qRT-UBQ5 Fwd	GACGCTTCATCTCGTCC	UBQ5	At3g62250
qRT-UBQ5 Rvs	GTAAACGTAGGTGAGTCCA		
qRT-AtNRP1 Fwd	CAAACGCCAGCTTTTCGGATTG	AtNRP1	At5g42050
qRT-AtNRP1 Rvs	TGAGCACGCTCTTCTTGCTTTCA		
qRT-ANAC036 Fwd	TCCTCTTTCGTCTTCCGAGA	ANAC036	At2g17040
qRT-ANAC036 Rvs:	TGCATTTGGATCTTGTTTGC		

qRT-CNX1 Fwd	ATGAGACAACGGCAACTATTTTCC	CNX1	At5g61790
qRT-CNX1 Rvs	CCATAATCCTCATGTCCTTCACT		
qRT-BiP3 Fwd	CACGGTTCCAGCGTATTTCAAT	BiP3	At1g09080
qRT-BiP3 Rvs	ATAAGCTATGGCAGCACCCGTT		
qRT-VPE–γ Fwd	CTGCTGGGCAACCTCTAGTC	VPE–γ	
qRT-VPE–γ Rvs	CGTACTGAGACAGCGATCCA		

Supplemental table 2

Primers used for cloning			
Name	Sequence 5'->3'	Target	Locus
NRP-B Fwd:	AAAAAGCAGGCTTCACAATGGAGAATAATAAT	NRP-B	Glyma08g02840
NRP-B Rvs:	AGAAAGCTGGGTCTTTATGCCGGCAAAGCCTT		
NRP-A Fwd	AAAAAGCAGGCTTCACAATGGACAACAACAATG	NRP-A	Glyma20g16100
NRP-A Rvs	AGAAAGCTGGGTCTTCTATGCTGGAATGGCTTT		
BiPD Fwd	AAAAAGCAGGCTTCACAATGGCTGGCTCGTGG	BiPD	Glyma05g36620

BiPD Rvs	AGAAAGCTGGGTCTTCTAGAGCTCATCGTGAGA		
ANAC036 Fwd	AAAAAGCAGGCTTCACAATGGTTTACGGTAAGAG	ANAC036	At2g17040
ANAC036 Rvs	AGAAAGCTGGGTCTTCCA		
AtNRP1 Fwd	AAAAAGCAGGCTTCACAATGGAGTATAATAAC	AtNRP1	At5g42050
AtNRP1 Rvs	AGAAAGCTGGGTCTTTCAAGGGTTTTGGTCAGC		
AtNRP2 Fwd	AAAAAGCAGGCTTCACAAGCTTCTGGCAATTA	AtNRP2	At3g27090
AtNRP2 Rvs	AGAAAGCTGGGTCTTCCAATATATGTTAACTATTGG		
BiP1 Fwd	AAAAAGCAGGCTTCATGGCTCGCTCGTTTGG	BiP1	At5g28540
BiP1 Rvs	AGAAAGCTGGGTCCGAGCTCATCGTGAGACTCATG		
BIP2 Fwd	AAAAAGCAGGCTTCATGGCTCGCTCGTTTGG	BiP2	At5g42020
BiP2 Rvs	AGAAAGCTGGGTCCTAGAGCTCATCGTGAGACTCATG		
promAtNRP1 Fwd	GGGGACAACTTTGTATAGAAAAGTTGCCTCGTCATGATTGGAAGATTG	AtNRP1	At5g42050
promAtNR	GGGGACTGCTTTTTTGTA		

P1 Rvs	CAAACTTGCCTCTAACTC TCTGATTGATCTG		
promAtNR P2 Fwd	AAAAAGCAGGCTTCACA CTATACACTAGAGTATG ACTTTTG	AtNRP2	At3g27090
promAtNR P2 Rvs	AGAAAGCTGGGTCTTTC TAGTTTGCTCAGCTAGCT TC		
promANAC 036 Fwd	AAAAAGCAGGCTTCACA AGATGACACTTCAACTT GAATG	ANAC036	At2g17040
promANAC 036 Rvs	AGAAAGCTGGGTCTTGT TCTTGAGGTAGAAATCA AGAAG		
VPE-gama Fwd	AAAAAGCAGGCTTCACA ATGGCCACAACGATGAC ACGTG	VPE-gama	At4g32940
VPE-gama Rvs	AGAAAGCTGGGTCTTTG CACTGAATCCACGGTTA AGC		
BiPD T46G Fwd	GGGACCTATTCATGTGTT GGTG	BiPD	Glyma05g3662 0
BiPD T46G Rvs	TCCAAGATCAATCCCGA TGACCG		
BiPD G235D Fwd	ATGGGACCTTTGATGTC AGTATC	BiPD	Glyma05g3662 0
BiPD G235D Rvs	CACCAAGATCAAAGACT AGAATG		
AGB1 Fwd	AAAAAGCAGGCTTCACA ATGTCTGTCTCCGAG	AGB1	At4G34460

AGB1 Rvs	AGAAAGCTGGGTCTTCT CTCCTGGTCCTCCAAA		