The Binding Protein BiP Attenuates Stress-Induced Cell Death in Soybean via Modulation of the N-Rich Protein-Mediated Signaling Pathway[1][C][W][OA]

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The molecular chaperone binding protein (BiP) participates in the constitutive function of the endoplasmic reticulum (ER) 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-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 *N-RICH PROTEIN-A* (*NRP-A*), *NRP-B*, and *GmNAC6*, which are involved in transducing a cell death signal generated by ER stress and osmotic stress through the 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 (*Nicotiana tabacum*) 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 response.

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 posttranslational 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 transduced through three distinct ER transmembrane sensors: an Ire1 homolog, the basic Leu zipper transcription factor ATF6, and the PKR-like ER kinase (PERK; for review, see Schröder 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 (for review, see 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 (Schröder and Kaufman, 2005).

However, if the ER stress is sustained, an apoptotic pathway is activated. In mammalian cells, this in-

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volves 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 MAPK 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 (*Arabidopsis thaliana*; 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 a 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 up-regulation 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 Dehydration15; 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 up-regulation 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 binding protein (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 down-regulation 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-OE 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 (Fontes et al., 1991; Crosti 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 (Fig. 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 (Fig. 2A). In wild-type seedlings, a decrease in cell

Figure 1. BiP overexpression increases resistance against tunicamycin (TUN)-induced cell death. OE soybean seedlings (35S::BiP) and untransformed wild-type (WT) seedlings 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.

viability was evident after 24 h of tunicamycin exposure. At 48 h posttreatment, the difference in cell viability between wild-type 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* (for Cys protease 1; Valente et al., 2009) over 48 h of tunicamycin treatment by quantitative reverse transcription (qRT)-PCR (Fig. 2B). *GmCystP* expression was strongly induced by tunicamycin at 48 h in wild-type 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 wild-type 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 (Fig. 2D). At 8 h posttreatment, 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 wild-type 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 posttreatment, the levels of BiP and CNX transcripts were higher in OE seedlings than in wild-type 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 wild-type 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 (Fig. 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., 2011), 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 up-regulate *GmNAC6* expression (Costa et al., 2008; Faria et al., 2011). 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., 2011). 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-OE lines in response to tunicamycin treatment.

At the protein level, the accumulation of NRP was enhanced at 8 and 24 h after tunicamycin treatment in the same fashion as the ER stress molecular markers, BiP and calnexin (Supplemental Fig. S1A). We also detected enhanced accumulation of GmNAC6 protein in nuclei of tunicamycin-treated cells (Supplemental Fig. S1B). As an ER stress-induced nuclear protein, ATAF2 was included

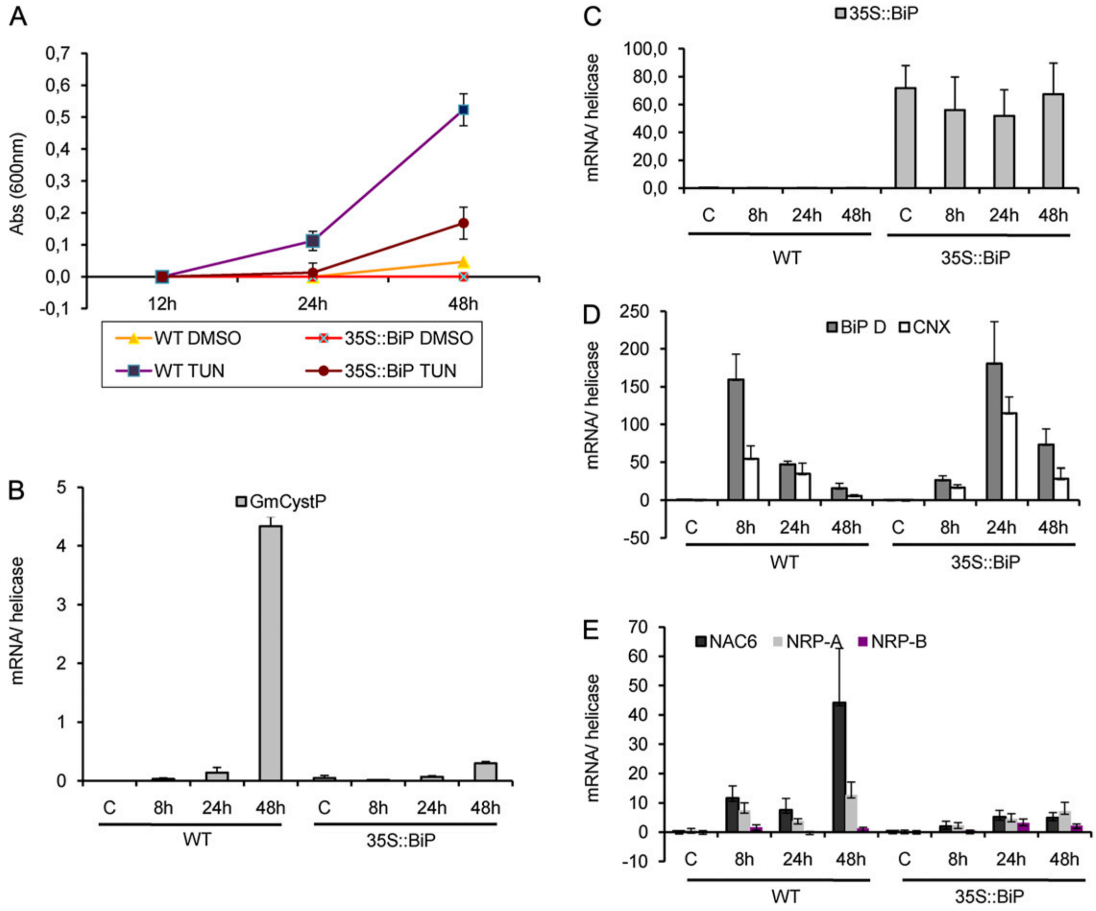

Figure 2. BiP overexpression attenuates the ER stress-mediated induction of cell death- and senescence-associated markers. A, The extent of tunicamycin (TUN)-induced cell death is inversely correlated with BiP levels. Soybean seedlings were exposed to tunicamycin, as described in Figure 1, and cell viability was measured by the Evans blue dye method. Abs_{600} reflects the dead cell content. The values represent the average of four replicates (±SD). B to 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 wild-type (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 (±SD, $n = 3$ technical replicates). *GmCystP* is a senescence-associated gene in soybean. 35S::BiP represents the transgene. *BiP* and *CNX* are ER stress markers. *NRP-A*, *NRP-B*, and *GmNAC6* are components of the ER stress- and osmotic stress-integrating cell death pathway. [See online article for color version of this figure.]

in the immunoblotting to monitor enrichment of nuclear extracts (Irsigler et al., 2007; Pinheiro et al., 2009). In both wild-type and OE soybean seedlings, prolonged ER stress enhanced the expression of the *NRP* and *GmNAC6* genes, albeit with different kinetics (Fig. 2E). In wild-type 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-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay for in situ detection of DNA fragmentation in protoplasts of tunicamycin-treated soybean leaves (Fig. 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, semiprotoplasted 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

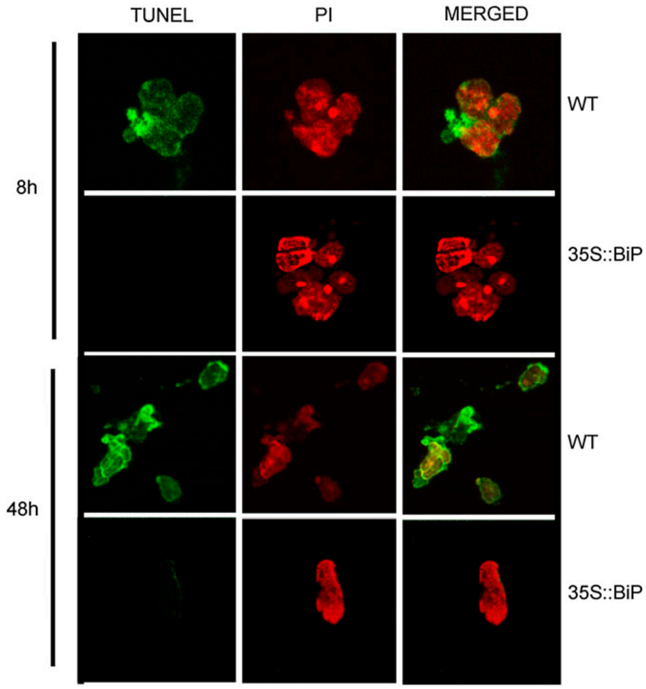

Figure 3. 2Reduced TUNEL green fluorescent signal in nuclei of BiP-OE leaf cells. Wild-type (WT) and BiP-OE (35S::BiP) soybean seedlings were treated with tunicamycin for the indicated number of hours. Then, protoplasts were prepared from tunicamycin-treated leaves, submitted to TUNEL labeling, and examined by confocal microscopy. The semipropoplasted cells were also counterstained with 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.

to the presence of cell wall, indicating that the protoplasting procedure was not completed (Fig. 3). At 8 h posttreatment, semiprotoplasted wild-type cells had mostly TUNEL-positive nuclei, whereas BiP-OE semiprotoplasts exhibited TUNEL-negative nuclei. At 48 h, the TUNEL-positive signal is intensified in wild-type semiprotoplasts and is slightly visible in BiP-OE semiprotoplasted 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 semiprotoplasted BiP-OE cells at 8 h posttreatment and almost undetectable at 48 h posttreatment. 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-OE Soybean Seedlings

Wild-type and transgenic soybean seedlings were exposed to PEG treatment over a period of 48 h (Fig. 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; Fig. 5A). Consistent with the phenotype of drought tolerance mediated by BiP in tobacco (*Nicotiana tabacum*) and soybean (Alvim et al., 2001; Valente et al., 2009), OE seedlings maintained leaf turgidity under dehydration conditions caused by PEG (compare wild-type PEG with 35S-BiP PEG at 8, 24, and 48 h). In contrast, at 48 h of PEG-induced osmotic stress, the leaves of the wild-type plants had completely wilted. Furthermore, while PEG accelerated the senescence of cotyledon leaves in wild-type seedlings, the overexpression of BiP in transgenic seedlings delayed PEG-induced leaf senescence, as judged by the degree of yellowing of the leaves (Fig. 4) and the expression of the senescence-associated gene *CystP* (Fig. 5B). The differences in shoot turgidity observed between the two genotypes following PEG treatment reflected differences in cell

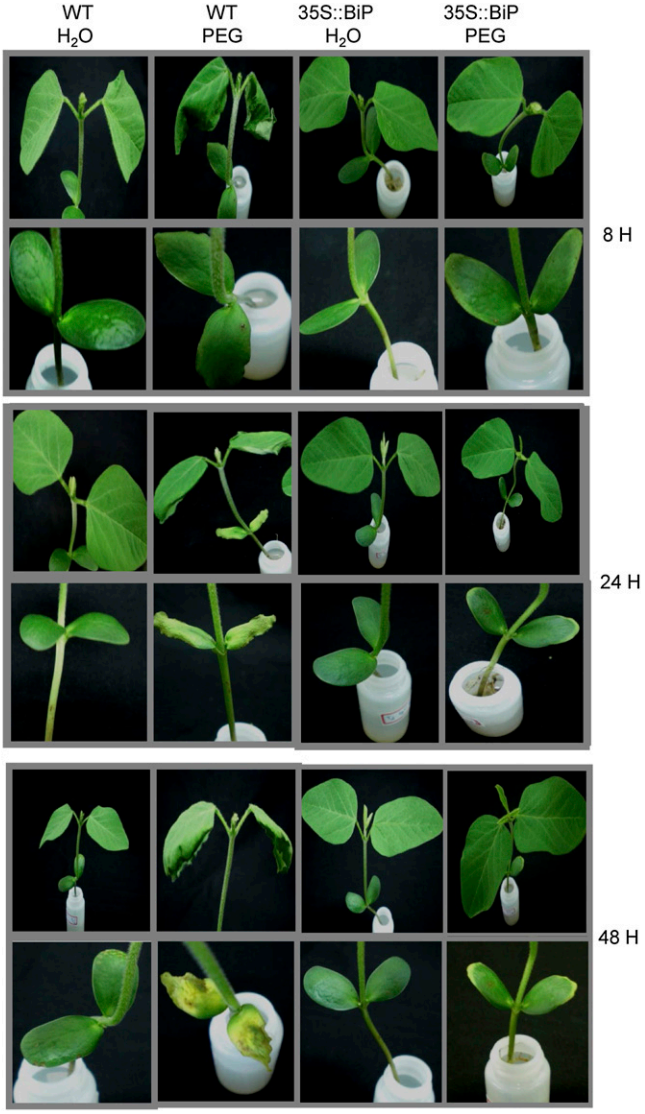

Figure 4. Phenotypes of soybean seedlings exposed to PEG treatment. Seedlings of OE (35S-BiP) and untransformed wild-type (WT) lines were exposed to treatment with PEG and examined for leaf wilting, chlorosis, and necrotic lesions. Photographs were taken at 8, 24, and 48 h after treatment.

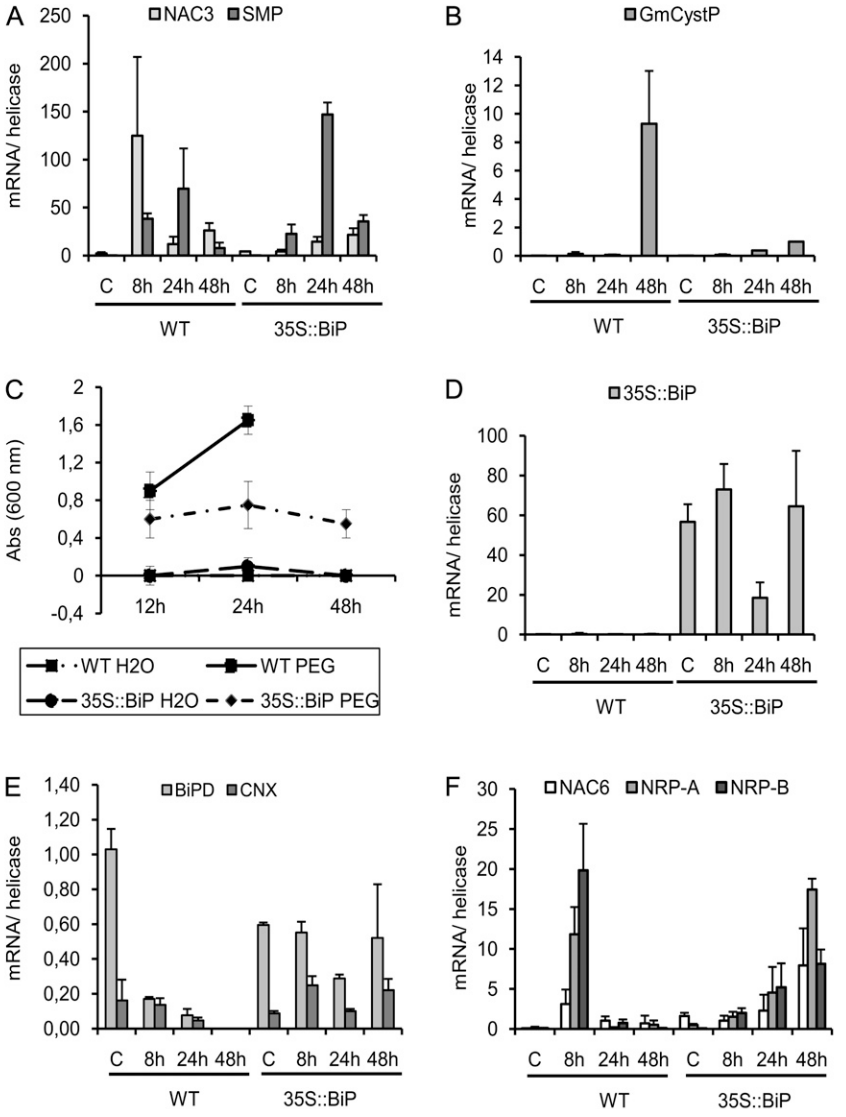

Figure 5. BiP overexpression delays cell death promoted by PEG-induced dehydration. A, Induction of osmotic stress control genes by PEG treatment. Total RNA was isolated from OE (35S::BiP) and wild-type (WT) soybean seedlings harvested at 8, 24, and 48 h after PEG treatment. The transcript levels of 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 (±SD, *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 ± SD of three replicates. In PEG-treated wild-type 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 wild-type 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 down-regulation of UPR marker genes. Transcript levels of the *BiP* and *CNX* 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.

viability (Fig. 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 wild-type 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 semiprotoplasted cells of PEG-treated wild-type and BiP-OE leaves. A representative sample is showed in Supplemental Figure S2. At 8 h posttreatment, semiprotoplasted wild-type cells exhibited TUNEL-positive nuclei (see arrow in merged), whereas the semiprotoplasts from OE 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 (Fig. 5D).

Because drought- and PEG-induced dehydration have been shown to down-regulate 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 wild-type and OE lines (Fig. 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 wild-type 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 down-regulation 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 cross talk 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* up-regulation in response to PEG-induced cell dehydration (Fig. 5F). The cell death marker genes *NRP-A*, *NRP-B*, and *GmNAC6* followed a similar pattern of early kinetic induction in wild-type seedlings and late induction in OE seedlings (Fig. 5F). In wild-type 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* (Fig. 5B) and the appearance of the PEG-induced chlorotic phenotype of cotyledon leaves (Fig. 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 down-regulation 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 (Fig. 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 (Fig. 6B; Supplemental Fig. S3). The transient expression of either protein promoted leaf yellowing in untransformed lines (NRP-B-infiltrated sectors are shown in Fig. 6C; NRP-A-agroinfiltrated sectors are shown in Supplemental Fig. S4), which was associated with decreased chlorophyll and carotenoid content compared with wild-type sectors that were infiltrated with *Agrobacterium* alone (Fig. 6D, wild type). However, the transient expression of *NRP-B* and *NRP-A* in the leaves of the transgenic sense lines did not cause leaf yellowing (Fig. 6C; Supplemental Fig. S4), and chlorophyll and carotenoid content were maintained at normal levels (Fig. 6D, sense). 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 (Fig. 6C; Supplemental Fig. S4, see antisense), with greater chlorophyll and carotenoid loss (Fig. 6D, antisense). The onset of senescence was also accelerated relative to the wild-type control. These phenotypes paralleled the induction of the senescence-associated Cys protease genes *NTCP-23* (Costa et al., 2008) and *CystP* (Fig. 6E). In fact, the induction of the Cys protease genes by *NRP* expression was greater in antisense leaves than in wild-type 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 Resulting 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., 2011). 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 (Fig. 7C). Transient expression of GmNAC6 caused leaf yellowing in untransformed lines (Fig. 7A, wild type), which was associated with a decreased chlorophyll content relative to the control wild-type sectors agroinfiltrated with *Agrobacterium* alone (Fig. 7B, blue bars). Transient expression of *GmNAC6* in the leaves of transgenic sense lines did not cause leaf yellowing, and the chlorophyll content was maintained at normal levels (Fig. 7B, green bars). In antisense tobacco leaves, the development of chlorosis as a result of *GmNAC6* expression in infiltrated sectors was accentuated (Fig. 7A, antisense) with greater chlorophyll loss (Fig. 7B, yellow bars), and the onset of senescence was accelerated relative to the wild-type 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 wild-type control and was barely detectable in sense lines (Fig. 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 UPR (for

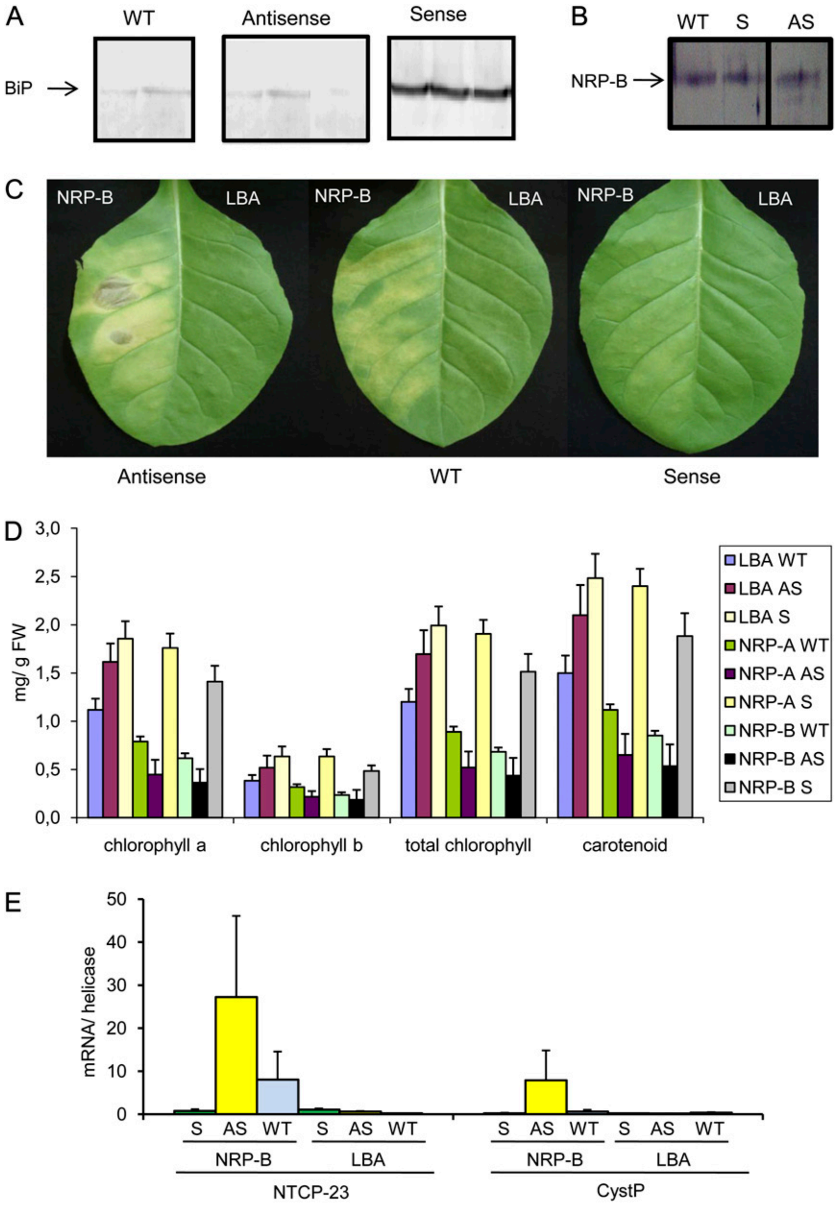

Figure 6. Increased levels of BiP prevent the NRP-B-induced cell death response. A, Levels of BiP protein in tobacco transgenic lines. Equal amounts of total protein extracted from the leaves of untransformed tobacco (wild-type [WT]) lines and transgenic lines expressing a BiP cDNA in either the sense or antisense orientation were separated by SDS-PAGE and immunoblotted with anticarboxyl 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 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 wild-type lines were agroinfiltrated with the indicated agroinoculum, and photographs were taken 7 d 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 lines and from sense and antisense 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 ± SD of three independent experiments. E, NRP-mediated induction of senescence-associated genes in agroinoculated tobacco sense and antisense leaves. Two-week-old sense, antisense, and wild-type 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 genes *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 (±SD, $n = 3$ biological replicates).

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 (Li et al., 1993; Leborgne-Castel et al., 1999; Foresti et al., 2003; 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 wild-type 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

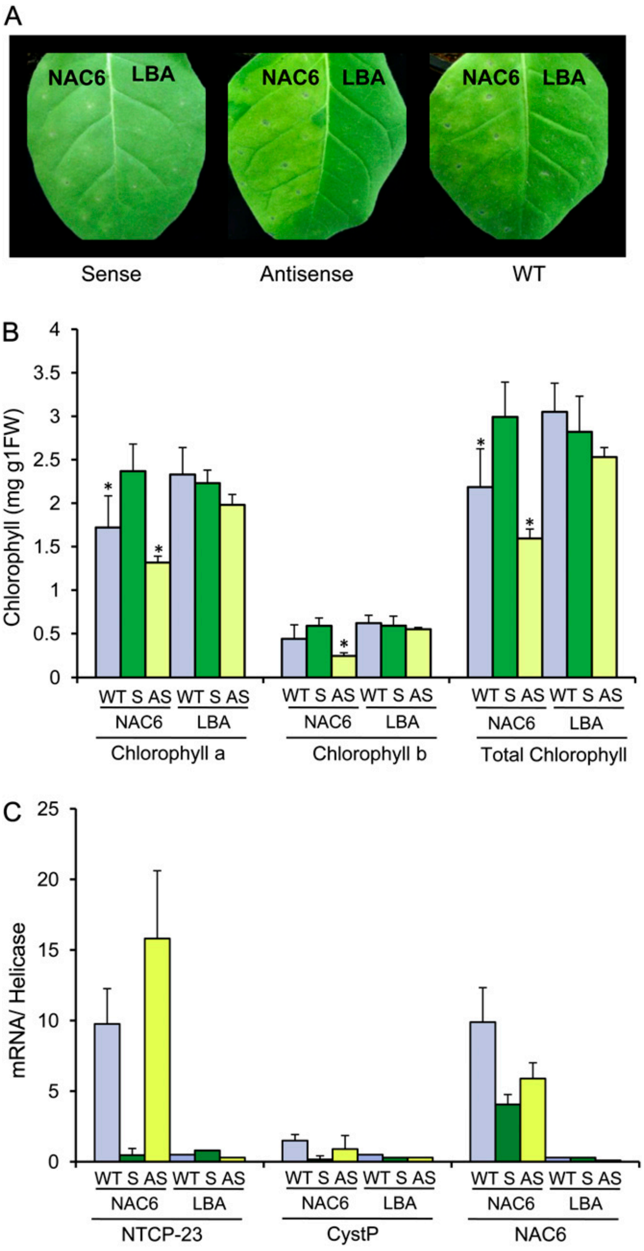

Figure 7. The GmNAC6-mediated senescence-like response is correlated with the levels of BiP expression in transgenic tobacco lines. Two-week-old untransformed tobacco (wild-type [WT]) lines and transgenic lines expressing a BiP cDNA in either the sense or antisense orientation were infiltrated with untransformed *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 wild-type lines were agroinfiltrated with the indicated agroinoculum, and photographs were taken 7 d 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 ± SD of three independent experiments. Asterisks indicate values that are significantly different from the control treatment ($P < 0.05$, Tukey's honestly significant difference mean separation 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 actin as the endogenous control. cDNAs were obtained from three biological replicates and validated individually (±SD, $n = 3$ biological replicates).

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 (Fig. 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 wild-type lines (Figs. 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 wild-type and OE lines. This would explain the apparent contradiction between the results of this 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 (Figs. 5–7).

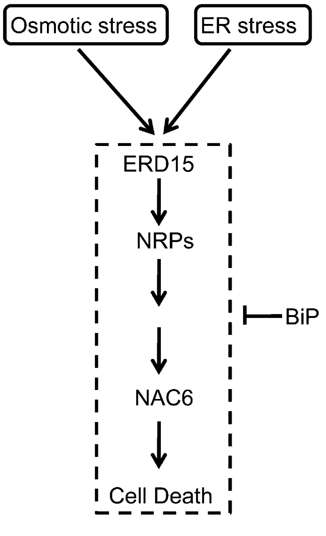

Figure 8. Model of BiP action in the ER stress- and osmotic stress-integrating NRP-mediated cell death response. ER stress- and osmotic stress-mediated up-regulation of *GmERD15* leads to the transcription of *NRP* genes and in turn induces *GmNAC6* expression and a cell death response with senescence-like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

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 result suggests that BiP acts downstream of GmNAC6, collectively our data do not rule out the hypothesis that BiP may regulate multiple targets in the pathway (Fig. 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* 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 *NRP*s and *GmNAC6* is induced by incompatibility interactions as well as by SA (Ludwig and Tenhaken, 2001; Faria et al., 2011). Furthermore, we found that the prolonged expression of *GmNAC6* and *NRP*s caused necrotic lesions resembling those of the hypersensitive response phenotype and induced pathogenesis-related genes (Faria et al., 2011). 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 (Fig. 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., 2011), 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 (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006), 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 down-regulation 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 down-regulation of UPR genes (Valente et al., 2009). We showed here that BiP also alleviated PEG-mediated down-regulation of the UPR genes *BiP* and *CNX* (Fig. 5E). We also found an inverse correlation between the levels of UPR gene transcripts and the onset of senescence induced by tunicamycin (Fig. 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.

MATERIALS AND METHODS

Plant Growth and Stress Treatments

Soybean (*Glycine max*) seeds from wild-type soybean (*Glycine max* '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) PEG (molecular weight 8,000, Sigma) or 2.5 μg/mL tunicamycin (Sigma). Dimethyl sulfoxide (DMSO) was used as a control for tunicamycin treatment. Stressed leaves were harvested at 8, 24, and 48 h posttreatment, 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 S1). The conditions for amplification reactions were as follows: 10 min at 95°C followed by 40 cycles of 94°C for 15 s 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 (*Nicotiana tabacum*) 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 to 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., 2009) and was used to transfer the GmNAC6 insert to pDEST17 yielding pHis-NAC6. The plasmids, pHis-DCD (pUFV1337) and pHis-NAC6, were transformed into *Escherichia coli* strain BL21, and the synthesis of the recombinant proteins was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside 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 2-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 Tris-buffered saline plus Tween 20 (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 and *p*-NBT. BiP was detected with an anti-carboxyl BiP antibody (Buzeli et al., 2002) at a 1:1,000 dilution. GmNAC6 was probed with an 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 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 Tobacco 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*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 to 0.2. Tobacco plants were grown in a greenhouse with natural daylength illumination. *Agrobacterium* infiltration was performed in the leaves of 3-week-old wild-type, 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, 649.1, 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.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Enhanced accumulation of ER stress molecular markers and cell death molecular markers in response to tunicamycin treatment.

Supplemental Figure S2. BiP overexpression attenuates osmotic stress-induced DNA fragmentation.

Supplemental Figure S3. A, The anti-DCD antibody recognizes NRP-B in soybean cells.

Supplemental Figure S4. NRP-A-induced leaf yellowing and necrotic symptoms are modulated by BiP.

Supplemental Table S1. Primers used for expression analysis by real-time PCR.

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