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# N-rich protein (NRP)-mediated cell death signaling

## A new branch of the ER stress response with implications for plant biotechnology

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Upon disruption of ER homeostasis, plant cells activate at least two branches of the unfolded protein response (UPR) through IRE1-like and ATF6-like transducers, resulting in the upregulation of ER-resident molecular chaperones and the activation of the ER-associated degradation protein system. Here, we discuss a new ER stress response pathway in plants that is associated with an osmotic stress response in transducing a cell death signal. Both ER and osmotic stress induce the expression of the novel transcription factor GmERD15, which binds and activates N-rich protein (NRP) promoters to induce NRP expression and cause the upregulation 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 interaction attenuates dehydration-induced cell death and promotes a better adaptation of BiP-overexpressing transgenic lines to drought.

### ER Stress Response and NRP-mediated Cell Death Signaling

Any condition that disrupts ER homeostasis to induce the accumulation of unfolded or misfolded proteins in the ER lumen activates a signal transduction system that connects the ER lumen with the cytoplasm and nucleus, a process called the unfolded protein response (UPR). In mammalian cells, this signaling pathway is transduced through three branches of ER stress sensors: the ER-associated receptors PERK (protein kinase RNA-like ER kinase), IRE1 (inositol-requiring protein-1) and ATF6 (activating transcription factor-6).<sup>1,5</sup> The activation mechanism of these proteins has not been completely resolved, but it is known that certain molecular chaperones in the ER lumen, e.g., BiP (Grp78), are involved in the activation of these transmembrane transducers. Under normal conditions, the molecular chaperone BiP is bound to the luminal domain of these receptors, rendering them inactive. The accumulation of misfolded proteins in the ER increases the need for the molecular chaperone activity of BiP, and thus, upon ER stress, BiP is released from these receptors. BiP dissociation causes the activation of the three transducers. The activation of PERK suppresses protein synthesis through the phosphorylation of translation initiation factor 2 $\alpha$  (IF2 $\alpha$ ). Upon BiP release, Ire1 undergoes dimerization, which sequentially activates its kinase and ribonuclease activity to induce the spliceosome-independent splicing of XBP1 (X-box binding protein-1) mRNA to generate mature mRNA that encode an active transcription factor. The activation of ATF6 promotes its translocation to the Golgi, where it is specifically cleaved by the proteolytic enzymes SP1 and SP2. Upon proteolytic hydrolysis of the transmembrane, the transcriptional activation domain of ATF6 detaches from the membrane and is directed to the nucleus. In the nucleus, XBP1 and ATF6 act in concert to activate the expression of ER-resident molecular chaperones, foldases and components of the ER-associated degradation (ERAD) system to increase the protein folding and processing capacity of the ER under stress conditions. Thus, on a physiological level, the activation of UPR triggers three protective cellular responses: (i) attenuation of protein translation through the PERK-mediated phosphorylation of

The endoplasmic reticulum 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. In mammalian cells, the UPR has been associated with other stress response pathways through shared components.<sup>1</sup> In plants, the potential of the ER stress response to accommodate adaptive pathways and its connection with other environmentally induced responses have been the subjects of studies in recent years.<sup>2,3</sup> One plant-specific, ER stress-shared response is ER and osmotic stress-integrated signaling, which converges on N-rich proteins (NRPs) to transduce a cell death signal.<sup>4</sup> As an integrated pathway, NRP-mediated cell death signaling is activated by either ER or osmotic stress but requires both signals for full activation.

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IF2 $\alpha$ , (ii) upregulation of ER chaperones and (iii) degradation of misfolded proteins by the proteasome (ERAD). However, the failure to restore ER homeostasis under stress conditions results in the activation of programmed cell death signaling pathways as an ultimate attempt for survival. 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.

In Arabidopsis, two IRE1-related genes, IRE1a and IRE1b, encode transmembrane domain-containing proteins that function as ER stress sensors or transducers. The GFP fusions of these receptors have been localized to the perinuclear ER, and the luminal sensor domain of these receptors can functionally replace the yeast IRE1 gene in complementation assays.<sup>6</sup> Furthermore, both IRE1a and IRE1b display ribonuclease activity *in vitro*,<sup>7</sup> and the functional characterization of an *atire1a atire1b* double mutant has revealed an essential and overlapping role for AtIRE1A and AtIRE1B as plant UPR regulators.<sup>8</sup> Recently, a second component of this branch of ER stress signaling has been identified as the ER membrane-associated transcription factor bZIP60.<sup>7,9</sup> Under normal conditions, the transcription factor bZIP60 is associated with microsomes, but under stress conditions, a truncated version of bZIP60 concentrates in the nucleus and activates the stress-inducible BiP3 promoter.<sup>10</sup> More recently, it has been demonstrated that upon ER stress, bZIP60 mRNA is spliced in an IRE1b-mediated process to generate an alternatively spliced transcript that lacks the transmembrane domain-encoding sequences.<sup>7,9</sup> 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.<sup>11,12</sup>

The second branch of UPR in plants mechanistically resembles the ATF6-mediated transduction of the ER stress signal. The membrane-associated Arabidopsis ATF6 homologs are bZIP17 and bZIP28, and they have canonical S1P sites in their C-terminal tails that face the ER lumen and appear to be processed by S1P and S2P. Upon ER stress, bZIP17 and zZIP28 are relocated to the Golgi, where their transcriptional domains are proteolytically released from the membrane by SP2.<sup>13</sup> The transcriptional activator components of these transmembrane transducers are then translocated to the nucleus, where they promote the expression of stress response genes.<sup>2,14-16</sup> Thus, the bipartite cellular response to ER stress in plants is mediated through Ire1-like receptors and ATAF6 analog transducers to induce ER-resident molecular chaperones and the ER-associated protein degradation machinery as a protective measure. However, if ER stress is sustained, an apoptotic pathway is activated. Nevertheless, little is known about the pathways that can contribute to ER stress-induced cell death in plants. Recently, an ER membrane-associated G $\beta$ -G $\gamma$  heterodimer protein has been shown to be involved in the signaling events that trigger UPR-associated cell death in Arabidopsis.<sup>17</sup> The study demonstrates that inactivation of the G $\beta$  subunit (AGB1) of the heterotrimeric G protein protects

plant cells against cell death induced by tunicamycin (Tm), a potent inducer of ER stress. Discrepant results were later reported with the demonstration that an AGB1 loss-of-function mutation causes over-sensitivity to ER stress and enhances the Tm-sensitive phenotype in the *atire1a atire1b* double mutant.<sup>8</sup> Although the involvement of the GTP-binding protein  $\beta$ 1 (AGB1) in mediating ER stress-induced cell death is still a matter of debate, the latter study clearly demonstrated through reverse genetics that AtIRE1 and AGB1 independently and antagonistically control two essential plant UPR pathways.

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.<sup>18</sup> 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).<sup>18</sup> 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 demonstrated the strongest synergistic induction, encoding 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). NRP-A and NRP-B share a highly conserved C-terminal DCD (development and cell death) domain and possess a high number of asparagine residues at their more divergent N terminus.<sup>19</sup> NRPs are critical mediators of ER and osmotic stress-induced cell death in soybeans.<sup>4</sup> 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.

Similar to NRPs, GmNAC6 is another target of the integrated pathway that is strongly induced by cycloheximide, a potent inducer of cell death in soybean suspension cells. It is synergistically activated by a combination of ER and osmotic stress signals and induces a senescence-like response in plants and cell death in soybean protoplasts.<sup>20,21</sup> GmNAC6 belongs to a class of NAC (NAM, ATAF1, ATAF2 and CUC2) domain-containing proteins, which make up a large family of plant-specific transcription factor genes represented by at least 101 sequences in the soybean genome that are clustered into 15 different sub-families.<sup>20</sup> Members of this family are involved in the development and stress response. The NAC transducers are organized into a general structure that consists of a highly conserved N-terminal domain involved in DNA binding (called the NAC domain) and a C-terminal region that is highly divergent in sequence and

length, which functions as the activation domain.<sup>22</sup> NRPs and GmNAC6 are coordinately regulated through a variety of biotic and abiotic stresses, but induction of NRPs precedes the upregulation of GmNAC6. Consistent with this early induction kinetics, the expression of NRPs activates the GmNAC6 promoter and induces GmNAC6 expression, suggesting that GmNAC6 is located downstream of NRP in the ER and osmotic stress-induced cell death response.<sup>21</sup>

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.<sup>23</sup> GmERD15 is induced by ER and osmotic stress to activate the expression of *NRP* genes. GmERD15 binds to and activates the NRP-B promoter *in vitro* and *in vivo*, exhibits transcriptional activity, is localized in the nucleus and induces the expression of the NRP-B gene when transiently expressed in soybean protoplasts. GmERD15 belongs to a class of ssDNA binding proteins and specifically recognizes the 12-bp palindromic sequence-511AGCAnnnnTGCT-500 in both SS (single-stranded) and DS (double-stranded) configurations of the NRP-B promoter. As an upstream component of ER stress-induced NRP-mediated signaling, GmERD15 associates stress in the ER with an osmotic stress-induced cell death signal.

### The Binding Protein Inhibits NRP-mediated Cell Death Signaling and Confers Tolerance to Drought

Although the activation mechanism of the three UPR transducers has not been totally deciphered in mammalian cells, the molecular chaperone BiP has been shown to play a pivotal role in controlling the activation status of IRE1, PERK and ATF6.<sup>1,24</sup> Because BiP is the sole molecular chaperone involved in the activation of UPR, changes in its expression might indicate changes in the folding environment and ER processing capacity. Accordingly, the overexpression of BiP in mammals attenuates ER stress and suppresses the activation of UPR.<sup>25</sup> Mammalian BiP also exhibits protective properties that prevent oxidative stress, Ca<sup>2+</sup> disturbances and cell death.<sup>26-29</sup> In plants, there is no compelling evidence that BiP functions as a regulator of UPR transducers. Nevertheless, the overexpression of BiP in tobacco and soybean attenuates the activation of the UPR, suggesting a role for BiP in controlling the activation of UPR.<sup>4,30</sup> More recently, it has been shown that the BiP-mediated inhibition of UPR activation appears to be dependent on the duration and intensity of the ER stress, such that BiP inhibition seems to be relieved if the stress persists.<sup>31</sup> This observation might explain the controversy surrounding a potential role for BiP as regulator of UPR in plants.

In addition to alleviating ER stress,<sup>30</sup> the overexpression of BiP in plants has also been shown to increase their tolerance to water deficits.<sup>32-34</sup> The apparent increase in BiP-mediated drought tolerance was not associated with typical short and long-term avoidance responses or with other known tolerance mechanisms.<sup>34</sup> The only variations observed in the 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. Recently, it has been shown that the enhanced expression of *BiP* in soybean (*Glycine max*) attenuated ER and osmotic-stress-mediated cell death.<sup>31</sup> In transgenic lines, BiP overexpression attenuates ER and osmotic stress-induced cell death phenotypes, such as foliar necrotic lesions and wilting, percentage of dead cells, induction of senescence-associated gene markers, DNA fragmentation, caspase activity, lipid peroxidation and induction of the cell death marker genes *NRP-A*, *NRP-B* and *GmNAC6*. Accordingly, the prosurvival effect of BiP was associated with the modulation of the ER and osmotic stress-induced NRP-mediated cell death signaling, as determined in transgenic tobacco lines with enhanced and suppressed BiP levels. The enhanced expression of BiP prevented NRP- and NAC6-mediated cell death, whereas the silencing of endogenous *BiP* accelerated the onset of leaf senescence mediated through the ectopic expression of NRPs and GmNAC6 in tobacco leaves. These results implicate BiP as a negative regulator of the stress-induced NRP-mediated cell death response. Thus, it is not surprising that the overexpression of BiP delays drought-induced senescence in tobacco and soybean plants and confers the increased adaptation of these transgenic lines under water deprivation conditions compared with wild-type controls.

### NRP-mediated Cell Death Signaling Represents a General Plant Response to Multiple Stresses

The components of the NRP-mediated cell death response, which include GmERD15, NRP-A, NRP-B and GmNAC6, are coordinately induced by several biotic and abiotic stimuli.<sup>20,21</sup> It is likely that the NRP-mediated cell death signaling pathway represents a common response of plant cells to a variety of different stimuli. At least three lines of evidence implicate the stress-induced NRP-mediated cell death response as part of the hypersensitive response elicited by pathogen incompatibility interactions. First, the expression of both NRPs and *GmNAC6* is induced through incompatibility interactions and salicylic acid.<sup>21,35</sup> Second, the prolonged expression of *GmNAC6* and NRPs causes necrotic lesions, resembling those of the hypersensitive response phenotype, and induces the expression of pathogenesis-related genes.<sup>21</sup> Finally, *ERD15*, the transcriptional activator of NRP expression, was first described in Arabidopsis as a dehydration-induced gene<sup>36</sup> 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.<sup>37</sup>

### Conclusions

Since the discovery of the stress-induced NRP-mediated cell death response, many aspects of this signaling pathway induced by prolonged ER and osmotic stress have been elucidated. We now know that this pathway integrates the ER and osmotic-stress signals to synergistically increase the expression of N-rich proteins (NRP-A and NRP-B) and the NAC domain-containing protein GmNAC6, which are critical mediators of stress-induced cell death in plants. We also know that *NRP-B* expression is controlled by the novel ER and osmotic stress-induced transcriptional

factor GmERD15 (*Glycine max* Early Responsive to Dehydration 15). However, several key players of this stress-induced signaling pathway are unknown, and many questions remain unanswered. What is the ER receptor that molecularly links the ER stress signal with the cell death response? How is the cell death signal propagated from induction of the plasma membrane-associated NRPs to the activation of the GmNAC6 promoter? What are the molecular events downstream of GmNAC6 that promote the cell death response? The synergistic induction of NRPs and NAC6 expression through the combination of ER and osmotic stress inducers indicates that the osmotic and ER stress-mediated cell death signals are transduced through separate pathways that converge on NRP expression. Which upstream components of NRPs are activated by the osmotic stress signal? The identification of NRP-interacting partners and downstream targets of the GmNAC6 transcriptional factor combined with the use of reverse

genetics to examine plant ER stress transducers will be crucial to decipher this stress-induced cell death signaling response.

Recent data show that BiP, an ER molecular chaperone, acts as a negative regulator of NRP-mediated cell death signaling and that the manipulation of BiP expression protects plants against drought. We propose that the underlying mechanism of BiP-mediated increase in water stress tolerance is likely associated, at least in part, with its capacity to modulate the osmotic stress-induced NRP-mediated cell death response. The positive effect of the modulation of the NRP-mediated cell death pathway on plant adaptation to stress might implicate this pathway as an excellent target for engineering superior crops. 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.

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