Chapter

A Regulatory Circuit Integrating Stress-Induced with Natural Leaf Senescence

Otto Teixeira Fraga, Bruno Paes de Melo, Luiz Fernando de Camargos, Debora Pellanda Fagundes, Celio Cabral Oliveira, Eduardo Bassi Simoni, Pedro Augusto Braga dos Reis and Elizabeth Pacheco Batista Fontes

Abstract

Any condition that disrupts the ER homeostasis activates a cytoprotective signaling cascade, designated as the unfolded protein response (UPR), which is transduced in plant cells by a bipartite signaling module. Activation of IRE1/bZIP60 and bZIP28/bZIP17, which represent the bipartite signaling arms and serve as ER stress sensors and transducers, results in the upregulation of ER protein processing machinery-related genes to recover from stress. However, if the ER stress persists and the cell is unable to restore ER homeostasis, programmed cell death signaling pathways are activated for survival. Here, we describe an ER stress-induced plant-specific cell death program, which is a shared response to multiple stress signals. This signaling pathway was first identified through genome-wide expression profile of differentially expressed genes in response to combined ER stress and osmotic stress. Among them, the development and cell death domain-containing N-rich proteins (DCD/NRPs), *NRP-A* and *NRP-B*, and the transcriptional factor *GmNAC81* were selected as mediators of cell death in plants. These genes were used as targets to identify additional components of the cell death pathway, which is described here as a regulatory circuit that integrates a stress-induced cell death program with leaf senescence via the NRP-A/NRP-B/GmNAC81:GmNAC30/VPE signaling module.

Keywords: senescence, stress, NRP, DCD, BiP, NAC, VPE, ER, osmotic stress, drought

1. Introduction

The onset of leaf senescence is a highly regulated developmental program that is controlled by both genetics and the environment. Multiple stresses in plants induce programmed cell death, and the underlying regulatory mechanisms are often associated with molecular links of developmentally programmed senescence. The transcriptome changes induced by different environmental stressors are not

entirely overlapping, but functional analysis of genes commonly induced as shared responses can give clues on signaling integration. This approach has been used to select for overlapping genes as candidate regulatory components that integrate the ER stress and osmotic stress responses, which were shown later to participate also in natural leaf senescence. Among genes identified as components of the ER and osmotic stress shared response, the developmental and cell death (DCD) domain-containing asparagine-rich proteins (NRP-A and NRP-B) were the first ones to be characterized as cell death-promoting proteins, and hence this multiple stress-integrating signaling was designated as stress-induced DCD/NRP-mediated cell death response. Further characterization of the cell death pathway implicated in the discovery of the signaling module ERD15/NRPs/GmNAC81:GmNAC30/VPE that also has been shown to operate in developmentally programmed leaf senescence. This plant-specific cell death signaling module, which operates in both stress-induced and natural leaf senescence, constitutes the primary focus of this chapter.

2. Modest overlapping of ER stress and osmotic stress response identifies NRPs and NACs as cell death-promoting genes

2.1 Osmotic stress responses

Organisms, in general, are continually adapting to internal and external stimuli, which activate sensor proteins to subsequently transmit the signal to downstream effectors responsible for the assembly of adaptive cellular responses [1]. Abiotic stresses consist of a set of adverse environmental conditions that limits plant development. Cold, high temperature, salinity, water availability (drought or overflow), radiation, pollution, and chemical exposure are the most common examples of types of abiotic stresses [2].

Generally, a signaling sensor network connects internal and external stimuli to adaptive responses leading to molecular modifications that allow physiological adjustments, which ultimately cause susceptibility or tolerance to the exposed conditions. Molecular responses to abiotic stress conditions in plants are crucial for survival and productivity as these stresses often limit yield. Among abiotic stresses, drought and excess salinity conditions induce sophisticated adaptive responses in plants to cope with or acclimate to these adverse environmental conditions [3, 4]. Some types of abiotic stress responses are better understood than others. In plants, for example, the molecular mechanisms of perception and responses to drought, high salinity, and endoplasmic reticulum stress are well characterized, and many stress-related cell signaling pathways are completely elucidated, revealing some convergence points between them.

The osmotic stress in plants, caused by water deprivation or high salinity, for example, undergoes a set of characteristic morphological, molecular, and physiological changes. One of the most notorious symptoms in plants under low water availability is the ABA-mediated stomatal closure [5]. This hormone-mediated morphological change affects plant physiology. The stomatal closure prevents the evapotranspiration, optimizing the cell water use, but it also compromises carbon dioxide uptake, causing imbalances on photosynthetic apparatus, which culminates on reactive oxygen species (ROS) production [6, 7]. The ROS accumulation acts as a signal to the cell, which triggers mechanisms of ROS-associated detoxification, including upregulation of antioxidant enzymes, osmolyte, and electron-carrier synthesis [8]. There is evidence that osmotic stress and temperature changes are capable of generating lipid-derived signal transducers, including the phosphatidic acid, phosphoinositides, sphingolipids, lysophospholipids, oxylipins,

N-acylethanolamines, and others. Water deprivation causes a collapse on the organization of membrane lipids, disrupting its permeability and some significant molecular interactions between lipids and proteins, which act as a cell signal to stress-mediated physiological changes. The mechanisms of how stress responses are connected with membrane lipid transducer generation are still unclear, but lipid messengers can alter protein and enzymatic functions [9].

2.2 ER stress responses

The endoplasmic reticulum is one of the most dynamics organelles in cell machinery. It is the gateway for the synthesis of secretory proteins and contains the necessary apparatus to ensure quality protein synthesis, protein maturation, and secretion in eukaryotic cells [10]. Furthermore, the ER can modulate some chronic stress-related pathways, promoting oxidative stress, autophagy, and apoptotic cell death in mammals and plant cells [11–13].

Several adverse environmental conditions can affect the ER quality control machinery, causing unfolded/misfolded protein accumulation in the ER lumen. The secretory proteins are synthesized in ER membrane-bound polysomes, and, as soon as they enter the organelle, they are processed by the ER processing machinery. Under normal conditions, there is a perfect balance between the rate of protein synthesis and ER processing capacity. Any conditions that disrupt this balance promote unfolded/misfolded protein accumulation in the ER lumen. As a consequence, the perturbation on ER function triggers a sophisticated and coordinated signal cascade, perceived by ER membrane-associated sensors, which activate the expression of ER-resident chaperones, foldases, and components of the ER quality control machinery. Collectively, these cytoprotective mechanisms are known as the unfolded protein response pathway (UPR, **Figure 1**) [14].

The detection of ER stress is mediated by membrane-associated sensors, identified both in mammals and plants. In mammals, there are three of these sensors: kinase/endoribonuclease inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase RNA-like ER kinase (PERK) [15], which are regulated by the ER-resident molecular chaperone BiP (binding protein). The ER sensors initiate the UPR to restore ER homeostasis under stress condition. If the adverse physiological status is prolonged, they can initiate some alternative routes leading to cell death.

Under normal conditions, BiP is bound to the luminal domain of these receptors, keeping them inactive. With the stress progression and consequent misfolded protein accumulation, the BiP molecular chaperone function is required to prevent aggregation of the unfolded proteins. Therefore, under these stress conditions, BiP is released from the ER receptors, which leads to their activation. The three ER signal transducers act in different ways, but in convergent stress-responsive pathways. IRE1 (IRE1a and IRE1b) displays a dual biochemical activity. It harbors a ribonuclease and kinase activity at the C-terminus, responsible for the unconventional spliceosome-independent splicing of X-box binding protein 1 (XBP1) mRNA. Stress-mediated BiP release from the IRE1 N-terminus promotes IRE1 homodimerization, which sequentially activates its kinase via autophosphorylation and endoribonuclease activity, culminating on spliceosome-independent splicing of XBP1, a bZIP transcriptional factor. Under normal conditions, the XBP1u (unspliced form) is constitutively translated into a low-functional transcription factor, which is rapidly degraded by the proteasome and does not effectively activate UPR. The IRE1-mediated mRNA splicing removes an unconventional intron of 26 nucleotides, which causes a shifting frame in XBP1 mRNA translation, generating a protein of 376 amino acids instead of 261 amino acids when unprocessed.

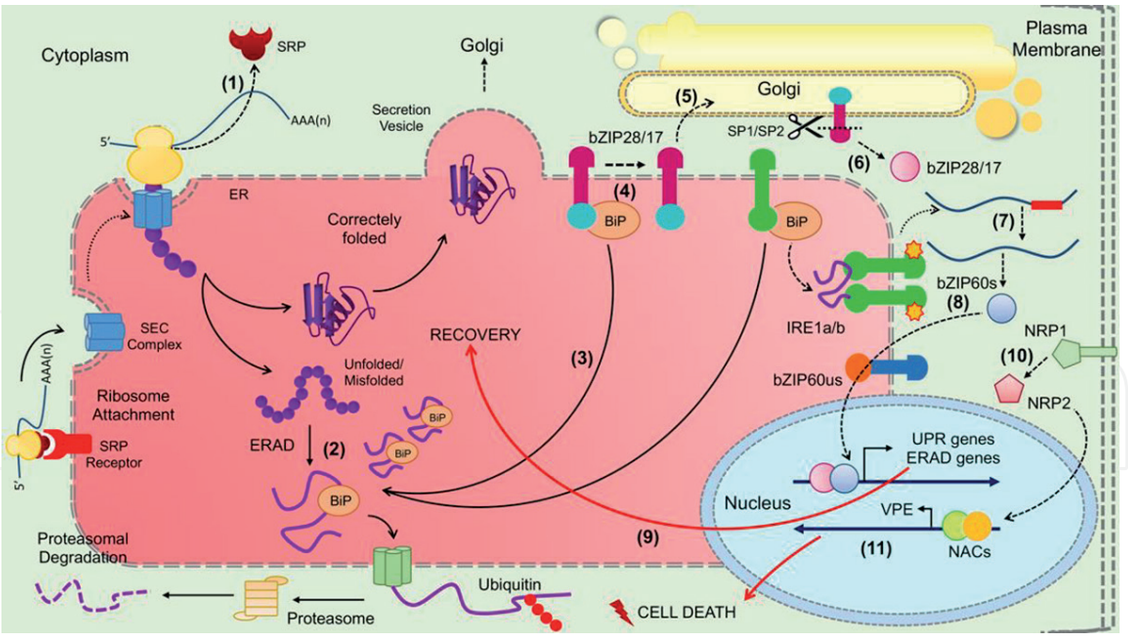

Figure 1.
The endoplasmic reticulum stress response in Arabidopsis. *The secretory proteins are synthesized in ER-bound polysomes (1) attached to the ER membrane through the interaction of signal recognition particle (SRP) and membrane receptor. As soon as they enter the lumen of the organelle, they are bound to a series of molecular chaperones, including BiP, to assist correct folding (2). Upon ER stress, the accumulation of unfolded protein (UP) activates a protective signaling cascade, designated as unfolded protein response, which allows communication of ER with the nucleus via a bipartite signaling module: the bZIP28/bZIP17 and IRE1a/IRE1b-bZiP60 signaling modules. Under normal conditions, BiP is bound to bZIP28/17, keeping the transducer in an inactive configuration (4). Upon ER stress, UP causes the dissociation of BiP from bZIP28/bZIP17, which is, then, translocated to the Golgi (5), where it is proteolytically cleaved to release the bZIP28/bZIP17 domain from the membrane that, in turn, is translocated to the nucleus (6). UP accumulation also causes the oligomerization of IRE1a/IRE1b, subsequent activation of its kinase domain by phosphorylation, and the endonuclease activity (6). The activated IRE1a/IRE1b endonuclease domain promotes unconventional splicing of bZIP60 mRNA to remove a transmembrane motif-encoding fragment, generating bZIP60 spliced mRNA that is translated into a soluble bZIP60 protein (bZIP60s) (7), which otherwise would be translated into the membrane-associated bZIP60us as it occurs under normal conditions. bZIP60s is, then, translocated to the nucleus (8), where it cooperates with bZIP28/bZIP17 to upregulate UPR genes and ERAD-related genes, increasing the ER protein processing capacity under ER stress to promote recovery (9). However, if the stress persists, and ER homeostasis cannot be restored, cell death signaling pathways are activated. among them, the DCD/NRP-mediated cell death signaling is initiated with activation of AtNRP1 (10) that leads to the induction of AtNRP2 and activation of a signaling cascade that culminates with the induction of ANAC36 that binds to the* VPE *promoter (11) and induces the expression of* VPE, *the executioner of the cell death program via collapse of the vacuole. These ER stress signaling pathways are conserved in other plant species.*

This unconventional splicing seems to prevent the degradation of XBP1s (spliced form) product by the proteasome and increase its transactivation activity, causing activation of UPR-related genes [16, 17]. Thus, the XBP1s is a soluble and functional transcription factor, which is reallocated to the cell nucleus to activate genes involved in cytoprotective pathways, such as some members of ER quality control or programmed cell death-related genes, including the apoptotic signaling kinase 1 (ASK1) and Jun-N-terminal kinase (JNK) [16–19].

The ER signal transducer ATF6 is anchored to the ER membrane and harbors an N-terminal sensor domain facing the ER lumen and a C-terminal bZIP domain facing the cytosolic side. Under normal conditions, ATF6 is inactivated by BiP binding to the ER stress sensor domain. ER stress conditions promote the BiP disassociation and reallocation of ATF6 to the Golgi apparatus, where it is specifically processed by SP1 and SP2 proteolytic enzymes. The limited proteolysis of ATF6 transmembrane domain allows that the bZIP domain of ATF6 be directed to the nucleus, where it acts in concert with XBP1 to induce genes involved in ER protein processing, ER quality control, and ER-associated protein degradation (ERAD) pathway.

Finally, the PERK activation upon BiP release by stress conditions promotes global translation suppression through the phosphorylation of the translation initiation factor IF2α [20]. PERK also activates the transcription factor CHOP, involved in the regulation of apoptosis-related genes [10, 21].

In plants, the UPR pathway has, at least, two arms (**Figure 1**). The first one activates IRE1 (IRE1a–AT2G17520 and IRE1b–AT5G24360, in *Arabidopsis thaliana*), and the other is transduced through bZIP membrane-associated transcription factors (bZIP17–AT2G40950 and bZIP28–AT3G10800, in *Arabidopsis thaliana*) [22, 23]. In the first arm of plant UPR, like in mammals, the accumulation of misfolded proteins leads to the activation of IRE1, which promotes unconventional cytosolic splicing of bZIP60 mRNA [24]. The unspliced bZIP60 mRNA, called bZIP60us, is translated into an ER membrane-associated transcription factor and does not exhibit transcriptional activity. Upon IRE1 activation by UPR, the spliced bZIP60 mRNA, called bZIP60s, does not display the transmembrane domain coding region, and its translation generates an active transcription factor, which is reallocated to the nucleus to activate UPR and cytoprotective genes, such as *BiP3*, *CNX* (calnexin), *CRT* (calreticulin), etc. [24–26]. This mechanism is conserved among plants, as the rice (*Oryza sativa*) bZIP60 orthologs, OsbZIP74 or OsbZIP50, display similar IRE-mediated mRNA splicing to render the activation of ER stress-inducible promoters [27, 28]. Likewise, in maize (*Zea mays*), ZmbZIP60 mRNA splicing leads to the activation of ER stress-inducible promoters [29], and, in soybean (*Glycine max*), the ZIP60 ortholog GmbZIP68 harbors a canonical site for IRE1 endonuclease activity and is efficiently spliced under ER stress conditions to activate UPR genes [30].

The second arm of plant UPR pathway is mediated by posttranslational modification of bZIP17 and bZIP28 transcription factors, the functional analogs of ATF6. Both bZIP17 and bZIP28 display a canonical SP1 site in their C-terminal domain, facing the ER lumen [31]. Upon stress conditions, BIP is released from the bZIP28 and bZIP17 ER sensor domain, and the transcription factors are reallocated from the ER to the Golgi apparatus, where they are processed by SP1 and SP2 proteases. These proteases remove the transmembrane domain of bZIP17 and bZIP28, exposing their cytosolic regions, which will activate UPR-related genes in the nucleus [31–34]. Like the IRE1/bZIP60 signaling module of plant UPR, the bZIP28/bZIP17 arm triggers the evolutionarily conservative UPR but also accommodates cross-talk with several other adaptive signaling responses [24, 30, 31]. In summary, upon ER stress, bZIP60s and bZIP28 use a different mechanism to be translocated to the nucleus where they act in concert to induce the expression of UPR genes and ERAD-related genes to increase the ER protein processing capacity for recovery from stress.

2.3 Convergence of ER stress and osmotic stress responses into a cell death signaling pathway

At a physiological level, the UPR encompasses three protective mechanisms: (i) global translation suppression by PERK-mediated IF2α phosphorylation; (ii) upregulation of ER-resident molecular chaperones, and (iii) proteasome-mediated protein degradation by ERAD pathway. However, if the stress conditions are sustained and the UPR pathway fails to restore ER homeostasis, apoptotic pathways are triggered as an ultimate attempt to survive. In plants, there is a specific branch of ER stress that integrates the osmotic stress and leads to programmed cell death (PCD), the development and cell death domain-containing N-rich protein (DCD/NRP)-mediated cell death signaling (**Figure 1**) [12]. This cell death pathway was

first identified via genome-wide and expression profiling approaches, which revealed a modest overlapping between ER and osmotic stress-induced transcriptomes of soybean seedlings treated with PEG (an osmotic stress inducer) and tunicamycin and AZC (ER stress inducers). Several genes displayed similar kinetics and a synergistic induction under combined ER and osmotic stresses, indicating that the ER stress response integrates the osmotic signal to potentiate transcription of shared target genes. Among them, two plant-specific DCD/N-rich proteins, NRP-A and NRP-B, an ubiquitin-associated protein homolog (UBA), and a NAC domain-containing protein, GmNAC81, displayed the most robust synergistic upregulation by the combination of both stresses [35]. Transient expression of NRPs or GmNAC81 in soybean protoplasts and *Nicotiana benthamiana* leaves demonstrated that they are critical mediators of ER stress- and osmotic stress-induced cell death in plants [36–38].

The NRP-A and NRP-B display a highly conserved DCD domain at their C-terminal protein region and a high number of asparagine residues at their more divergent N-terminus (**Figure 2**) [39]. Consistent with the presence of a DCD domain, overexpression of *NRPs* in soybean protoplasts induces caspase-3-like activity and promotes extensive DNA fragmentation. Furthermore, transient

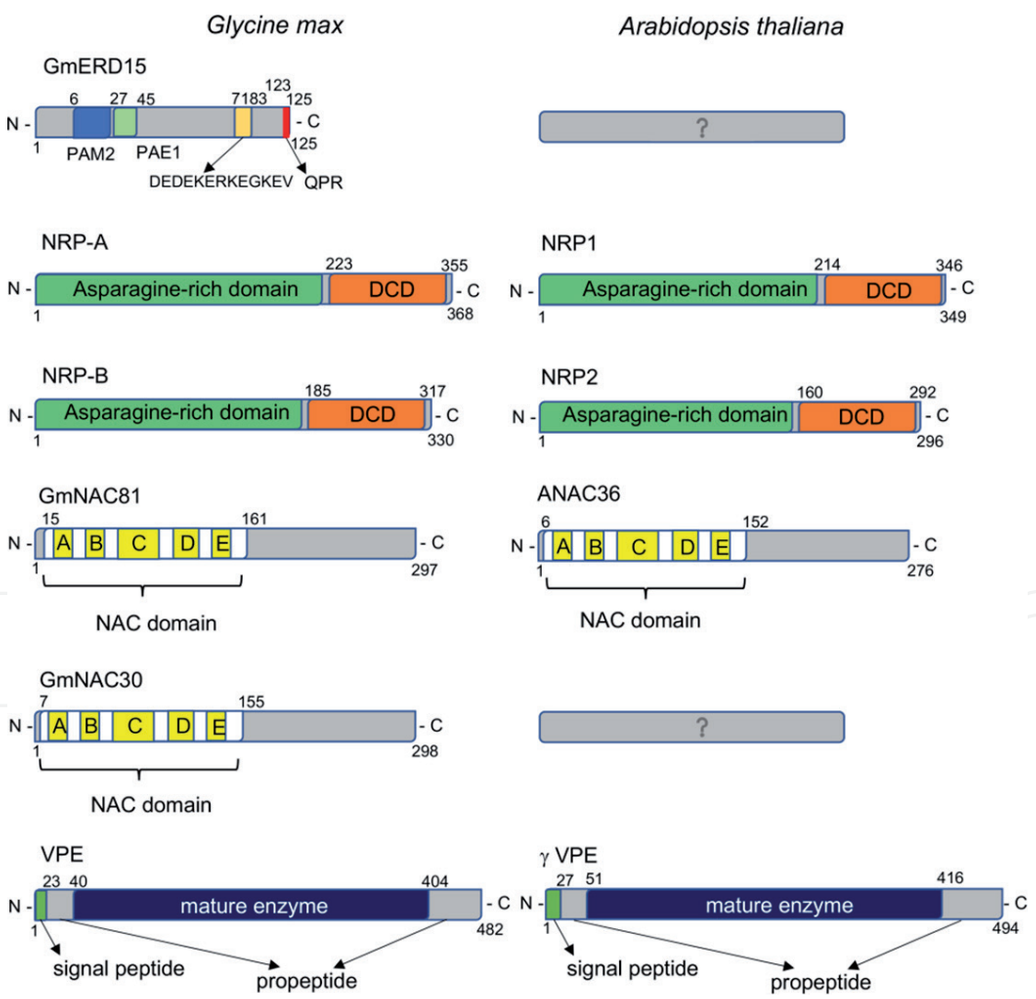

Figure 2.
Schematic representation of the cell death pathway components. The predicted domains of each protein are highlighted. The indicated domains are delimited by the amino acid positions in the primary structure shown by the numbers. For ERD-15, PAM2 is a PABP-interacting motif, PAE2 is PAM2-associated element 1 motif, DEDEKERKEGKEV is a conserved sequence representing a putative motif of ssDNA-binding transcriptional regulators, and QPR is a highly conserved C-terminal QPR motif. As for GmNAC81, GmNAC30, and ANAC36, the N-terminal NAC domain is subdivided into five conserved motifs (A to E) as indicated. In the AtNRP1, AtNRP2, NRP-A, and NRP-B schemes, DCD is development and cell death domain.

expression of NRPs *in planta* causes leaf yellowing, chlorophyll loss, malondialdehyde production, ethylene evolution, and induction of the senescence marker genes, which are hallmarks of leaf senescence and cell death [36, 38, 40]. The cell death response mediated by NRPs resembles a programmed cell death event. Because NRPs were the first components of the ER stress and osmotic stress-integrating cell death response to be characterized, this signaling pathway is commonly referred to as the DCD/NRP-mediated cell death response.

Similar to NRPs, GmNAC81 (*Glycine max* NAC81, formerly designated as GmNAC6) is another target of the ER stress- and osmotic stress-integrating pathway that induces a senescence-like response *in planta* and cell death in soybean protoplasts [37, 41]. GmNAC81 belongs to the plant-specific transcriptional factor superfamily of domain-containing proteins, represented by 111 members in *Arabidopsis*, 151 in rice, 152 in maize, and 180 in soybean [42, 43]. Members of this family function in development and stress response. The NAC transcriptional factors display a highly conserved N-terminal domain, called NAC domain, responsible for recognition of cis-regulatory elements on target promoters and DNA binding (**Figure 2**). The C-terminal domain is more divergent in sequence but is undoubtedly responsible for transcriptional activity [44, 45]. In addition, a subset of NAC proteins, which also exhibits protein binding activity, harbors an additional transmembrane domain present in the membrane-tethered NAC proteins [43, 46, 47].

NRPs and *GmNAC81* are induced by several different abiotic and biotic stresses in a coordinated manner, but induction of *NRPs* precedes the upregulation of *GmNAC81*. This early induction kinetics of *NRPs* is consistent with its capacity to activate the promoter and induce the expression of *GmNAC81*. These data placed GmNAC81 downstream of NRPs in the ER and osmotic stress-induced cell death pathway [37]. More recently, using reverse genetics in *Arabidopsis*, NRPs were confirmed to be upstream of ANAC36, the *Arabidopsis* ortholog of GmNAC81, in the DCD/NRP-mediated cell death signaling [40].

3. Early dehydration responsive gene 15, ERD15-like, controls NRP expression

The early dehydration responsive (*ERD*) genes were first identified due to their rapid induction in response to drought stress. The ERD genes (*ERD1* to *ERD16*) encode a set of proteins that differ in biological functions and cell localization [48]. Among them, ERD15 is a small acidic and hydrophilic protein that belongs to the PAM2 domain-containing protein family (**Figure 2**). The PAM2 domain is a well-characterized protein–protein interaction domain, which allows ERD15 to interact with polyA-binding proteins (PABP) regulating mRNA stability and protein translation [49]. In addition to PAM2, ERD15 contains two other domains with unknown function, designated as PAM2-associated element 1 (PAE1) and QPR.

ERD15 is a multiple stress-responsive gene that is involved in adaptation to abiotic and biotic stress. Light treatment, cold stress, and high salinity trigger *ERD15* expression [50, 51]. ERD15 functions as a negative regulator of the abscisic acid (ABA)-mediated response and a positive regulator of the salicylic acid (SA)-dependent defense pathway. *ERD15*-overexpressing transgenic lines are less sensitive to ABA and display enhanced salicylic acid-dependent defense pathway, which was associated with increased resistance to the bacterial *Erwinia carotovora* of the transgenic lines [52].

Consistent with the multiple stress-responsive expression profiles, the soybean *ERD15* ortholog (*GmERD15*) is also induced by ER and osmotic stress. *GmERD15*

was identified using one hybrid screening that targeted the NRP-B promoter in yeast. As an upstream member of the NRP-mediated cell death response, GmERD15 binds the *NRP-B* promoter region in vivo and in vitro and induces the *NRP-B* expression [53]. Despite its role as a transcription factor, GmERD15 does not harbor a typical DNA-binding motif, but instead, it contains a conserved sequence of 13 amino acids at positions 71–83 (DEDEKERKEgKEv), which is a part of a tripartite motif domain derived from ssDNA-binding transcriptional regulators [54]. Accordingly, the GmERD15 binding site was mapped to a 12-bp palindromic sequence $^{-511}$AGCAnnnnnTGCT^{-500} on the *NRP-B* promotor in both single-stranded and double-stranded configurations [53].

4. The stress-induced NRP/NAC081/VPE module transduces a cell death signal

As components of the DCD/NPR-mediated cell death signaling, NRPs and GmNAC81 are critical mediators of cell death derived from ER stress and osmotic stress signals. More recent progress toward deciphering this branch of stress-induced cell death signaling includes the identification of two additional downstream components, the NAC transcriptional factor (GmNAC30) and the vacuolar processing enzyme (VPE) [55].

GmNAC30 was identified as a nuclear partner of GmNAC81 via two-hybrid screening using GmNAC81 as a bait. *GmNAC30* and *GmNAC81* exhibit similar expression profiles and cell death activity. They are upregulated by ER stress, osmotic stress, and by the cell death-inducer cycloheximide. Consistently, GmNAC30 promotes cell death when transiently expressed in soybean protoplasts and, as a downstream component of the cell death signaling, is induced by expression of NRP-A and NRP-B.

GmNAC30 interacts with GmNAC81 in vitro and in vivo, the complex formed binds to common cis-regulatory sequences in target promoters and synergistically regulates hydrolytic enzyme promoters, including the caspase-1-like vacuolar processing enzyme (*VPE*) gene, which is involved in PCD in plants [55]. Consistent with their transcriptional function as a heterodimer, *GmNAC81* and *GmNAC30* display overlapping and coordinate expression profiles in response to multiple environmental and developmental stimuli. Therefore, the stress-induced *GmNAC30* cooperates with *GmNAC81* to activate PCD through the upregulation of the cell death executioner VPE.

VPE is a vacuole-localized cysteine protease that exhibits caspase-1-like activity and hydrolyzes a peptide bond at the C-terminal side of aspartate and asparagine residues [56]. It is synthesized as an inactive preprotein precursor, which is self-catalytically converted into the active mature form, under a processing step that resembles the activation of caspase 1 (**Figure 2**). It has been associated with *Tobacco mosaic virus*-induced hypersensitive cell death and developmental PCD [57, 58]. As an executioner of a cell death program, VPE is self-activated by hydrolytic cleavage and, in turn, mediates the initial activation of vacuolar enzymes, which degrade the vacuolar membrane and initiate the proteolytic cascade leading to PCD. Therefore, VPE activation may result in vacuolar collapse-mediated cell death, a type of plant-specific programmed cell death.

The discovery of VPE as a downstream target of the coordinate action of GmNAC81 and GmNAC30 underlies a mechanism for the execution of the ER and osmotic stress-induced cell death program (**Figure 1**). This model holds that prolonged ER and osmotic stresses induce the expression of the transcriptional activator GmERD15 to target the NRP promoter. The upregulation of NRPs initiates

a transduction signaling that leads to the induction of GmNAC81 and GmNAC30, which cooperate to activate the VPE promoter and expression. Activation of VPE promotes the disintegration of vacuoles, initiating the proteolytic cascade in plant PCD. As vacuole-triggered PCD is unique to plants, the regulatory circuit linking the stress signal to activation of VPE is fundamentally composed of plant-specific signaling components.

The DCD/NRP-mediated programmed cell death pathway is conserved and operates with similar regulatory mechanisms in plants [40]. Soybean prototypes of each component of the cell death pathway were used to search for orthologs in the *Arabidopsis* genome (**Figure 3**) [30]. Arabidopsis AtNRP1 is most closely related to GmNRP-A and GmNRP-B, whereas a third homolog GmNRP-C was

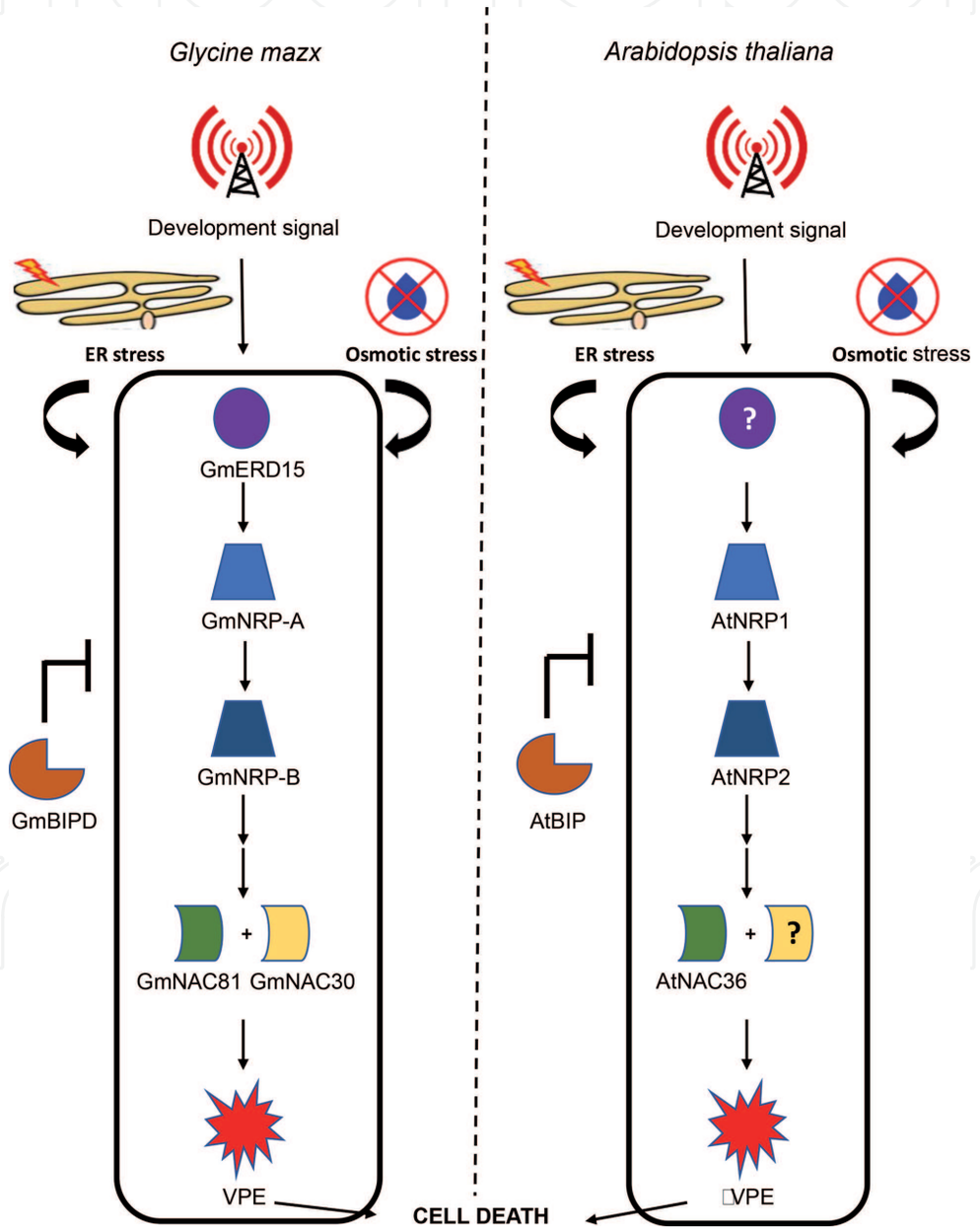

Figure 3.
Integration of developmental signal and stress signals into the DCD/NRP-mediated cell death response. Leaf senescence, ER stress, and osmotic stress induce the expression of ERD15-regulated NRP-A that in turn upregulates NRP-B to initiate a signaling cascade that culminates with the induction of GmNAC30 *and* GmNAC81 *expression. The NAC transcription factors form a heterodimer to fully induce the activation of* VPE *promoter, which leads to* VPE *upregulation and subsequent execution of a cell death program. The ER-resident molecular chaperone BiP acts as a negative regulator of cell death by modulating the expression and activity of the cell death pathway components. The DCD/NRP-mediated cell death signaling is conserved in other plant species, and the* Arabidopsis *orthologs are shown on the right.*

related to AtNRP-2. GmNAC81 and its paralog share sequence conservation with the *Arabidopsis* ortholog ANAC36 (At2G17040), whereas the predicted *Arabidopsis* ortholog of soybean VPE was identified as At4G32940/γVPE. Transient expression of the selected *Arabidopsis* orthologs of pathway components (*AtNRP-1*, *AtNRP-2*, *ANAC36*, and *γVPE)* induces cell death in *Nicotiana benthamiana* leaves with the appearance of hallmarks of PCD and leaf senescence, including DNA fragmentation, leaf yellowing, chlorophyll loss, and lipid peroxidation [38]. In addition, knockout lines for each one of pathway genes in *Arabidopsis* display enhanced tolerance to ER stress-mediated cell death induction. Very importantly, the stress induction of *AtNRP2*, *ANAC36*, and *γVPE* was dependent on the AtNRP1 function, confirming the upstream position of AtNRP1 in the cell death pathway. Therefore, in *Arabidopsis*, the execution of the cell death program has been proposed to occur through AtNRP1-mediated induction of the AtNRP2-ANAC36-γVPE signaling module. Nevertheless, functional information about the GmERD15 and GmNAC30 orthologs in *Arabidopsis* is lacking, and these pathway components have not been identified yet in *Arabidopsis*. Both in soybean and *Arabidopsis*, the DCD/NRP-mediated cell death pathway is modulated by the ER-resident molecular chaperone BiP, which negatively regulates the gene expression and activity of these cell death-inducing genes [13, 40].

5. A negative regulator of the NRP/NAC081/VPE signaling module confers tolerance to drought

Plants can negatively modulate the NRP/DCD-mediated cell death response to suit the cellular balance during the stress conditions. Moreover, this modulation improves the cellular stableness and consequently increases the plant tolerance to stress conditions in an essential process that is required for plant acclimatization and development. The molecular chaperone BiP plays a crucial role as a negative regulator of NRP/DCD-mediated cell death response. BiP belongs to the HSP70 family, which is essential to protect the cells against environmental stresses and to restore the cell homeostasis [59].

The molecular chaperone BiP has a catalytic site at the amino-terminal region and a substrate-binding site at the carboxy-terminal region [60]. BiP is involved in the regulation of several processes in the endoplasmic reticulum, a critical organelle that is related to responses to abiotic and biotic stress in plants. In the ER, BiP acts as a sensor that responds to quantitative and qualitative changes in the ER by regulating the activity of ER stress transducers [61]. Furthermore, BiP coordinately regulates the cell death signaling, which connects the signals from osmotic and ER stress in a DCD/NRP-dependent manner [35, 36, 38].

BiP attenuates the NRP/DCD-mediated cell death signal propagation by the modulation of expression and activity of the pathway signaling components (**Figure 3**). BiP overexpression in soybean attenuates ER stress- and osmotic stress-mediated cell death, a phenotype that is linked to a delay in the induction of *GmNRP-A*, *GmNRP-B*, and *GmNAC81* under ER stress and osmotic stress [38]. Furthermore, enhanced accumulation of BiP in tobacco (*Nicotiana tabacum*) prevents the GmNRP- and GmNAC81-mediated induction of cell death-associated physiological and molecular markers, whereas silencing of endogenous BiP enhances the cell death response.

In addition to alleviating ER and osmotic stress-mediated cell death, the *BiP* overexpression in plants has also been shown to increase their tolerance to water deficits [62–64]. Enhanced accumulation of BiP in soybean, tobacco, and *Arabidopsis* promotes a delay in drought-induced senescence and wilting of leaves

leading to a higher survival rate of overexpressing lines under water-deficit regimes [12, 38, 40, 63–64]. The BiP-mediated tolerance mechanism is not associated with conventional mechanisms of drought tolerance and avoidance, as the BiP-overexpressing lines do not display lower photosynthesis and transpiration rates than untransformed lines under drought, and the stomata closure and root growth are not stimulated under water deprivation. Furthermore, the *BiP*-overexpressing lines exhibit a lower induction of drought-related genes than WT under water-deficit conditions, and the abscisic acid content in *BiP*-overexpressing plants is similar to untransformed lines, indicating that the BiP-mediated drought tolerance mechanism is independent on ABA [59, 64, 65]. Under drought conditions, the only variations observed in *BiP*-overexpressing lines are a delay in drought-induced leaf senescence and an attenuation in the drought induction of PCD-associated marker genes, which is associated with the protective function of BiP as a negative modulator of the DCD/NRP-mediated cell death response. A metabolomic approach was used to detect the metabolite profile of *BiP*-overexpressing lines under drought conditions [65]. Due to a higher osmolyte accumulation, mainly amino acids, the *BiP*-overexpressing plants can maintain the leaf turgidity upon drought stress, which is a phenotypic hallmark of the BiP-mediated tolerance to drought. The *BiP*-overexpressing lines also display a higher accumulation of salicylic acid and upregulation of SA-responsive genes, which is associated with accelerated hypersensitive response triggered by *Pseudomonas syringae pv tomato* in soybean and tobacco [59, 65]. The SA signaling also activates the antioxidative metabolism, which may be linked to the BiP protective function to drought. Very importantly, the BiP modulation of the DCD/NRP-mediated cell death response does not impair the plant growth and development.

6. The stress-induced DCD/NRP-mediated cell death signaling positively regulates leaf senescence

Leaf senescence is a natural process in plant development, which begins with a physiological transition between active photosynthetic leaves to degenerative and nutrient-recycling leaves. The classical age senescence-related symptom is the leaf yellowing caused by generalized chlorophyll loss. The age-induced senescence or naturally programmed leaf senescence, hereafter referred to as leaf senescence, occurs by plant aging and is precisely regulated by senescence-associated genes (SAGs) [66, 67].

Many SAGs are environmental- and stress-responsive genes, integrating a convergent regulatory cascade between natural plant development and stress-induced PCD [68]. At the molecular level, the onset of senescence is accompanied by a massive reprogramming of gene expression, probably controlled by senescence-associated transcription factors. Among these, several NAC transcription factors have been associated with senescence regulation based on high-resolution temporal expression profiles [69].

In soybean, a transcriptomic analysis of senescing leaves reveals that 44% of the *GmNAC* genes were differentially expressed at the onset of leaf senescence. The most representative subfamilies of soybean senescence-associated *NAC* genes were the abiotic stress-induced SNAC-A (ATAF) subfamily, in which 90% of the members were differentially expressed during senescence, followed by the biotic stress-induced TERN subfamily, displaying 80% of the members differentially expressed during leaf senescence [43]. *GmNAC30* and *GmNAC81*, which belong to the SNAC-A and TERN subfamilies, respectively, are among the upregulated genes by leaf senescence [43, 59]. These results raise the hypotheses that the (i)

DCD-NRP/NAC/VPE signaling module may integrate stress-induced with natural leaf senescence and (ii) other NAC genes may be involved in integrated circuits between age- and stress-induced cell death pathways.

Regarding the first hypothesis, several lines of evidence indicate that the regulatory circuit NRPs/GmNAC81:GmNAC30/VPE integrates osmotic stress- and ER stress-induced PCD response with natural leaf senescence. First, not only *GmNAC30* and *GmNAC81* but also the other cell death pathway components, *NRP-A*, *NRP-B*, and *VPE*, are induced by leaf senescence [43, 59, 70]. Second, the activity of VPE is also induced during the onset of leaf senescence [59]. Third, transient expression of the soybean components of ER stress- and osmotic stress-induced cell death response, NRP-A, NRP-B, GmNAC81, and GmNAC30, as well as the *Arabidopsis* orthologs AtNRP1, AtNRP2, ANAC36, and γVPE, in protoplasts and *in planta* induce a cell death response bearing the hallmarks of leaf senescence and PCD. These symptoms include the induction of caspase 1-like activity and DNA fragmentation, chlorophyll loss, protein degradation, enhanced lipid peroxidation, and the induction of senescence-associated marker genes [36–38, 40, 55]. Fourth, enhanced accumulation of BiP, which negatively regulates the NRPs/GmNAC81:GmNAC30/VPE signaling module, also promotes a delay in leaf senescence in transgenic plants [59]. Finally, GmNAC81 is a positive regulator of naturally programmed leaf senescence [70]. Although leaf senescence is genetically programmed in an age-dependent manner, it can be triggered by environmental cues and is also positively and negatively regulated by various plant hormones. *GmNAC81* and *GmNAC30* are induced by the phytohormones ABA, jasmonic acid (JA) and salicylic acid (SA), which are positive regulators of senescence, and GmNAC81-overexpressing lines display high levels of ABA, mimicking the enhanced endogenous levels of this hormone during leaf senescence [70, 71]. Consistent with a role in leaf senescence, the overexpression of *GmNAC81* in soybean plants accelerates leaf senescence, a phenotype associated with extensive leaf yellowing, increased chlorophyll loss, faster photosynthetic decay, and enhanced expression and activity of the GmNAC81 direct target VPE, than untransformed, wild-type plants. Conversely, suppressing *GmNAC81* expression delays leaf senescence and decreases the expression of GmNAC81 direct target genes, including *VPE* [70]. Therefore, GmNAC81 is involved in developmentally programmed leaf senescence. Furthermore, ER stress- and osmotic stress-induced PCD is integrated with natural leaf senescence through the NRPs/NACs/VPE regulatory circuit.

7. Conclusion

Since the discovery of the ER stress- and osmotic stress-induced DCD/NRP-mediated cell death response, considerable progress has been achieved toward deciphering the components and regulation of the pathway (**Figure 3**). We now know that the combination of multiple stresses synergistically activates a plant-specific PCD response that is initiated by induction of the stress-responsive transcription factor GmERD15, which, in turn, binds and activates the DCD/NRP promoter. Induction of the DCD/NRP genes *NRP-A* and *NRP-B* leads to the activation of a signal cascade that culminates with the upregulation of the transcription factors GmNAC81 and GmNAC30. The NAC transcription factors form a heterodimer to activate the expression of hydrolytic enzymes, including VPE, an executioner of vacuole-triggered programmed cell death. The stress-induced DCD/NRP-mediated cell death response is conserved in plants with similar regulatory mechanisms and represents a shared response to multiple stress signals. As a negative regulator of the stress-induced DCD/NRP-mediated cell death response, overexpression of the

ER-resident molecular chaperone BiP delays drought-induced senescence in tobacco and soybean plants and confers the increased adaptation of these transgenic lines under water deprivation conditions. This DCD/NNP-mediated stress-induced cell death program is also activated during age-dependent leaf senescence and contributes positively for the progression of the developmentally programmed senescence. Therefore, the plant-specific NRPs/NACs/VPE signaling module represents a regulatory circuit integrating stress-induced with natural leaf senescence.

Acknowledgements

We thank the Brazilian government agencies, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), for financial support.

Conflict of interest

The authors declare no conflict of interest.

Author details

Otto Teixeira Fraga[1,2], Bruno Paes de Melo[1,2], Luiz Fernando de Camargos[1,2], Debora Pellanda Fagundes[1], Celio Cabral Oliveira[1,2], Eduardo Bassi Simoni[1,2], Pedro Augusto Braga dos Reis[1,2] and Elizabeth Pacheco Batista Fontes[1,2*]

1 National Institute of Science and Technology in Plant-Pest Interactions, Bioagro, Universidade Federal de Viçosa, Viçosa, Brazil

2 Department of Biochemistry and Molecular Biology/BIOAGRO, Universidade Federal de Viçosa, Viçosa, Brazil

*Address all correspondence to: bbfontes@ufv.br

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