OTTO TEIXEIRA FRAGA NETTO

FUNCTIONAL AND *IN SILICO* ANALYSES OF NRP-MEDIATED CELL DEATH PATHWAY COMPONENTS IN STRESS AND SENESCENCE

Thesis submitted to the Applied Biochemistry Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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RESUMO

NETTO, Otto Teixeira Fraga, D.Sc. Universidade Federal de Viçosa, janeiro de 2022. **Análises funcionais e *in silico* dos componentes da via de morte celular mediada por NRP no estresse e senescência.** Orientadora: Elizabeth Pacheco Batista Fontes. Coorientadores: Pedro Augusto Braga dos Reis e Virgilio Adriano Pereira Loriato.

As plantas estão continuamente expostas às condições ambientais e uma complexa via de sinalização celular é formada para lidar com condições desfavoráveis. A persistência dessas condições pode ativar vias de morte celular. A via de morte celular mediada por NRP tem sido relatada como uma importante via de sinalização celular em plantas. Aqui, fornecemos evidências de que os componentes de morte celular mediados por NRP representam uma resposta geral da planta a múltiplos estresses e senescência. No primeiro capítulo, revisamos a rede de genes reguladores GmNAC-SAGs, incluindo a regulação positiva de GmNAC081 e GmNAC030 na senescência foliar e descrevemos o circuito regulatório, que integra a morte celular programada induzida por estresse com a senescência foliar através do modulo de sinalização NRP-NAC-VPE. No segundo capítulo, desvendamos a superfamília e a subfamília de GmERD15, um membro a montante da resposta de morte celular mediada por NRP. Fornecemos uma extensa análise *in silico* da superfamília PAM2 e da subfamília ERD15 em Arabidopsis, soja e arroz. A superfamília PAM2 foi subdividida em sete subfamílias (A-G), com base na conservação e estrutura da sequência. Entre eles, a subfamília ERD15 ou grupo A contendo 3 domínios conservados, PAM2, PAE1 e QPR. Demonstramos o diferente perfil de expressão dos membros da subfamília ERD15 em resposta à seca, senescência e indução hormonal. Os membros da subfamília ERD15 exibiram uma sobreposição funcional em nossos ensaios de complementação funcional sob condições de seca, estresse osmótico e senescência. Identificamos o perfil de expressão dos membros da subfamília ERD15 na senescência e AtERD15 foi identificado como um modulador da senescência induzida pelo escuro. A localização subcelular dos membros foi elucidada, observamos uma localização nuclear de AtERD15 sob todas as condições de estresse, assim como OsERD15 sob estresse no reticulo endoplasmatico e Gm03ERD15 permanecendo no núcleo em todas as condições. Coletivamente, nossos dados indicam que os componentes da via de morte celular mediada por NRP e a subfamília ERD15 são moduladores da senescência e da sinalização de estresses.

Palavras-chave: Senescência. Estresse hídrico. ERD15. NAC.

ABSTRACT

NETTO, Otto Teixeira Fraga, D.Sc. Universidade Federal de Viçosa, January, 2022. **Functional and *in silico* analyses of NRP-mediated cell death pathway components in stress and senescence.** Adviser: Elizabeth Pacheco Batista Fontes. Co-advisers: Pedro Augusto Braga dos Reis and Virgilio Adriano Pereira Loriato.

Plants are continually exposed to environmental conditions, and complex cell signaling pathways are assembled to deal with unfavorable conditions. The persistence of these environmental stress conditions often activates cell death pathways. The NRP-mediated cell death pathway, first described in soybean, has been implicated as an essential cell signaling in plants induced by multiple stress conditions. The cell death signal is transduced via the GmNRPs/GmNAC81-GmNAC30/VPE signaling module. Here, we provide evidence that the NRP-mediated cell death represents a general plant response to multiple stress and senescence. In the first chapter, we review the regulatory gene networks underlying the GmNAC-SAGs, including GmNAC081 and GmNAC030-positive regulation in leaf senescence, and describe the regulatory circuit that integrates a stress-induced cell death program with developmental leaf senescence via the NRP-NAC-VPE signaling module. In the second chapter, we unveil the superfamily and subfamily of Gm02ERD15, an upstream member of the NRP-mediated cell death response. We provide an extensive *in silico* analysis of the PAM2 superfamily and the ERD15-like subfamily in Arabidopsis, soybean, and rice. The PAM2 superfamily was subdivided into seven subfamilies (A-G) based on sequence conservation, structure, and domain organization. The ERD15-like subfamily or group A contains 3 conserved domains, PAM2, PAE1, and QPR. We demonstrated the differential expression profile of ERD15-like subfamily members in response to drought, senescence, and hormone induction. The ERD15-like subfamily members exhibited functional redundancy in response to drought, osmotic, and senescence conditions, as judged by complementation assays of an aterd15 mutant. We identified the senescence induction expression profile of GmERD15-like subfamily members, and AtERD15 was described as a modulator of the dark-induced senescence. The subcellular localization of the subfamily members was elucidated; we observed nuclear relocalization of AtERD15 under ER stress, osmotic stress, and ABA treatment; OsERD15 was redistributed to the nucleus under ER stress, whereas Gm03ERD15 fractionated between the nucleus and cytoplasm under all conditions. Collectively, our data indicate that NRP-mediated cell death pathway components and the ERD15-like subfamily represent multiple stress-induced

modulators of senescence and stress signaling response.

Keywords: Senescence. Drought stress. ERD15. NAC.

LIST OF ILLUSTRATIONS

LIST OF TABLES

SUMMARY

GENERAL INTRODUCTION

As sessile organisms, plants evolved a sophisticated mechanism to deal with adverse environmental conditions. These mechanisms activate signaling pathways that perceive the stress conditions by sensor proteins, which transmit the signal to downstream effectors to promote physiological adjustments (Huang et al., 2012). Despite the strategies developed by plants to resist the different types of stress, in cases of persistence of stress, programmed cell death pathways are activated to prevent all intracellular content from being disorderly released into the extracellular environment, thus damaging adjacent cells. The cell death response mediated by developmental and cell death domain (DCD)-containing asparagine-rich proteins (NRPs), which integrates signals from osmotic stress and prolonged endoplasmic reticulum (ER) stress, has been characterized as a relevant adaptive response of plants to stress (Costa et al., 2008; Pimenta et al., 2016).

Initially, the osmotic and ER stress induces *Gm02ERD15* (Early Dehydration Responsive gene), a transcription factor that binds the NRP promoter region and upregulates its expression (Alves et al., 2011). The NRP protein is localized in the cytoplasm and activates a signaling cascade, leading to *GmNAC081* and *GmNAC030* induction (Faria et al., 2011). In the nucleus of stressed plant cells, GmNAC081 interacts with GmNAC030 to bind to promoters and activate the expression of genes that encode hydrolytic enzymes, especially VPE (Vacuolar Processing Enzyme) (Mendes et al., 2013). VPE has a caspase-1 activity and performs a plant-specific cell death program through the collapse of the vacuole (Hara-Nishimura et al., 2005). This pathway was first identified in soybean but conservation of the cell death program and pathway components was extended to include other plant species. In the Arabidopsis genome, the identified pathway orthologs include AtNRP1, AtNRP2, ANAC036, and γVPE (Reis et al., 2016). 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 (Reis et al., 2011). In addition, the BiP-mediated phenotype has been linked to drought stress tolerance. Under drought conditions, the BiP overexpression attenuates the induction of cell death pathway signaling components, delaying cell death and leaf senescence (Reis et al., 2011).

Several lines of evidence indicate that the NRP-mediated cell death pathway represents a general plant response to multiple stress and senescence. GmNAC081 not only positively modulates leaf senescence through the NRP/GmNAC081/VPE signaling module (Pimenta et al., 2016), but also negatively modulates drought stress by directly regulating drought-responsive genes (Ferreira et al., 2020). Furthermore, leaf senescence can trigger the expression

of other NRPs/NAC/VPE module components and induce VPE activity (Pimenta et al., 2016; Carvalho et al., 2014; Melo et al., 2018). The NRP-mediated cell death components have been associated with hormonal signaling cascades. The phytohormones abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) induce *GmNAC081* and *GmNAC030* expression, and the GmNAC081-overexpressing lines display high levels of ABA (Pimenta et al., 2016). Accordingly, the upstream component of the NRP-mediated signaling, Gm02ERD15, was first described in Arabidopsis as a dehydration-induced gene (Kyouse et al., 1994). AtERD15 functions as a negative regulator of the ABA-mediated response and a positive regulator of the SA-dependent defense pathway (Kariola et al., 2006). Recently, a Cd2+-mediated cell death response has been shown to induce the expression of *NRPs*, *GmNAC081*, *GmNAC030*, and *VPE* in soybean at the vegetative and reproductive developmental stages (Quadros et al., 2022).

Integrating signaling with overlapping responses provides plant cells with a strategy to optimize cell signaling processes. Multiple stresses induce programmed cell death in plants, a situation that may persist on field conditions and underscore the relevance of the NRP-mediated cell death response as a target for engineering stress tolerance. This study aimed to decipher how the NRP-mediated cell death pathway components respond to different stresses and natural senescence; a topic split into three chapters. The regulatory gene networks underlying the GmNAC-SAGs, including GmNAC81 and GmNAC30, were explored in chapter I entitled: Senescence-Associated Glycine max (Gm)NAC Genes: Integration of Natural and Stress-Induced Leaf Senescence, which was published in International Journal of Molecular Science, 2021 (Fraga et al., 2021). The characterization of the ERD15-like subfamily members was described in chapter II entitled: Expansion and diversification of the Glycine max (Gm) ERD15-like subfamily of the PAM2-like superfamily. The ER stress-induced plant-specific cell death signaling, which integrates a stress-induced cell death program with leaf senescence was described in chapter III entitled: A regulatory circuit integrating stress-induced with natural leaf senescence, which was published in Plant Science - Structure, Anatomy and Morphogenesis in Plants Cultured in Vivo and in Vitro. 1ed.: IntechOpen, 2019 (Fraga et al., 2019).

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CHAPTER I

SENESCENCE-ASSOCIATED *GLYCINE MAX* (GM) NAC GENES: INTEGRATION OF NATURAL AND STRESS-INDUCED LEAF SENESCENCE

Published article

Otto Teixeira Fraga, Bruno Paes de Melo, Iana Pedro Silva Quadros, Pedro Augusto Braga Reis, Elizabeth Pacheco Batista Fontes (2021). Senescence-Associated Glycine max (Gm) NAC Genes: Integration of Natural and Stress-Induced Leaf Senescence. International Journal of Molecular Science. 1;22(15):8287. doi: 10.3390/ijms22158287.

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Review

Senescence-Associated *Glycine max* (*Gm*)*NAC* Genes: Integration of Natural and Stress-Induced Leaf Senescence

Otto Teixeira Fraga [1,2,†], Bruno Paes de Melo [1,3,†], Iana Pedro Silva Quadros [1,2], Pedro Augusto Braga Reis [1,2] and Elizabeth Pacheco Batista Fontes [1,2,*]

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Abstract: Leaf senescence is a genetically regulated developmental process that can be triggered by a variety of internal and external signals, including hormones and environmental stimuli. Among the senescence-associated genes controlling leaf senescence, the transcriptional factors (TFs) comprise a functional class that is highly active at the onset and during the progression of leaf senescence. The plant-specific NAC (NAM, ATAF, and CUC) TFs are essential for controlling leaf senescence. Several members of Arabidopsis *AtNAC-SAGs* are well characterized as players in elucidated regulatory networks. However, only a few soybean members of this class display well-known functions; knowledge about their regulatory circuits is still rudimentary. Here, we describe the expression profile of soybean *GmNAC-SAGs* upregulated by natural senescence and their functional correlation with putative *AtNAC-SAGs* orthologs. The mechanisms and the regulatory gene networks underlying *GmNAC081*- and *GmNAC030*-positive regulation in leaf senescence are discussed. Furthermore, new insights into the role of *GmNAC065* as a negative senescence regulator are presented, demonstrating extraordinary functional conservation with the Arabidopsis counterpart. Finally, we describe a regulatory circuit which integrates a stress-induced cell death program with developmental leaf senescence via the NRP-NAC-VPE signaling module.

Keywords: leaf senescence; GmNAC; abiotic stresses; programmed cell death; PCD; NAC transcription factors; soybean; regulatory gene networks

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1. Introduction

Leaf senescence is a developmentally programmed or environmentally induced process by which cells activate the programmed cell death response (PCD), resulting in the relocation of nutrients towards different organs. Leaf senescence is not an unregulated cell death process initiated by aging; it is rather a sophisticated biochemical and genetic mechanism that controls cell/tissue/organism development through dynamic modulation of gene expression, metabolic reprogramming, and structural changes [1–6]. At the cellular level, leaf senescence can be modulated by environmental stimuli, signaling components, metabolism regulators, and phytohormones, although hormones are the major players in the different stages of leaf senescence.

The dismantling of chloroplasts generates the first visible indication of leaf senescence, leaf yellowing. During this process, carbon assimilation is succeeded by the catabolism of chlorophyll, proteins, and lipids; thereby, their metabolized products are relocated to different organs [1,2]. Chlorophyll catabolism occurs in three different cell compartments: chloroplasts, endoplasmic reticulum (ER)/cytosol, and vacuole. Furthermore, this process

requires expression and activation of a group of genes related to chlorophyll catabolism (chlorophyll catabolic genes; CCGs) [1]. In the chloroplasts, chlorophyll a is converted to chlorophyll b by chlorophyll b reductase, and the tetrapyrrole ring is then cleaved to generate the fluorescent chlorophyll catabolite (FCC). FCC is exported to the cytosol/ER and then to the vacuole [2]. The chloroplast dismantling steps do not occur simultaneously, as chlorophyll catabolism precedes protein degradation and structural changes [1]. This entire process is tightly regulated by gene expression control and hormone signaling. Thus, several transcription factors have been identified as modulating the expression of CCG genes upon hormone triggering and stress/environmental conditions [4–9].

A complex signaling network is triggered during the leaf senescence process; it is activated by aging or environmental factors, but the mechanisms and players that control the whole process are not fully understood. Nevertheless, forward and reverse genetic studies, next-generation sequencing (NGS), and omics technologies have enabled identification of components, signaling molecules, and different genes involved in several steps of the leaf senescence process [3–6]. To date, the leaf senescence database (LSD) has broadly listed over 5000 SAGs (senescence-associated genes) and 600 mutants from 68 species [7], and the use of these different tools has provided deep knowledge regarding the leaf senescence mechanism.

Hormones play significant roles over a plant's lifespan; they are involved in distinct developmental and environmental responses. Regarding leaf senescence, tight hormone control and balance allow the correct onset and progress of senescence signaling pathways [8]. Different hormones have specific functions in the activation/repression and control of leaf senescence. Ethylene, abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) accelerate senescence, while auxin, gibberellic acid (GA), and cytokinins act by delaying the process [8,9]. However, it is worth noting that activation/repression or acceleration/delaying of processes by hormones might depend on the organism and the environmental conditions. Moreover, the role of hormones in the modulation of leaf senescence relies on different signaling transducers, transcription factors, and metabolic and structural enzymes [3,10–14]. Over 5000 genes upregulated by senescence have been identified in plants [7], and some of these have been functionally characterized. Among the different classes of SAGs, the transcription factors (TF) play crucial roles in controlling leaf senescence activation, inhibition, and progress. Leaf senescence is regulated through dynamic crosstalk among the different hormone signaling and TF families, including NAC, WRKY, and MYB [15–17].

NAC (an acronym for **N**AM, **A**TAF1,2, and **C**UC2) is a plant-specific family of transcription factors, one of the largest found in the plant kingdom. The NAC transcription factors modulate several cell signaling responses and play significant roles in developmental, hormonal, and stress events [5,18–21]. The NAC proteins display a well-conserved N-terminal NAC domain, comprising the DNA-binding domain and a variable domain at the C-terminus, harboring the transcriptional regulatory region [20,21]. A genome-wide analysis identified 180 NAC genes in the soybean genome, which were clustered into 15 phylogenetic subfamilies [5]. Roughly 40% of the *Glycine max (Gm)NAC* genes were differentially expressed (DE) during the natural leaf senescence process, and the majority of DE genes were upregulated at the initial phase of leaf senescence. This large proportion of senescence-associated NAC genes has also been observed in Arabidopsis and rice [3,22,23]. Several SAG-NACs from Arabidopsis have been functionally characterized and key senescence regulatory networks elucidated [9,24–26]. Despite the longstanding interest in crop leaf senescence, few similar studies have been conducted in soybean, although some mechanisms underlying GmNAC-mediated leaf senescence have been partially elucidated.

This review focuses on the roles of senescence-associated *GmNAC* genes in developmentally programmed leaf senescence and their integration with signaling modules of stress-induced leaf senescence. We discuss possible GmNAC-derived senescence regulatory networks and the underlying mechanism of GmNAC-mediated leaf senescence.

Furthermore, we highlight the profiles of the senescence-associated *GmNAC* genes under different stress conditions and their possible connections with the leaf senescence process.

2. Leaf Senescence-Associated *GmNAC* Genes: Expression Profile and In Silico Analyses

The main feature of leaf senescence is the extensive gene expression reprogramming and subsequent physiological changes, which transform leaves from naturally nutrient-accumulating organs into nutrient-recycling organs [27]. Senescence is typically one of the natural developmental processes; however, it is also triggered by adverse environmental conditions [27–32].

The aging process that culminates senescence is designated developmental programmed cell death (dPCD), whereas senescence as a stress-escape response is designated environmental programmed cell death (ePCD) [31]. dPCD and ePCD share the same molecular basis, resulting in ROS accumulation, chlorophyll loss, and tissue and organ disassembly until abscission [30,33,34], but exhibit different regulatory gene networks [31]. However, several signals for plant physiology adjustment, including the synthesis of phytohormones such as ethylene (ETH), abscisic acid (ABA), jasmonate (JA), auxins (AUX), and salicylic acid (SA), also promote senescence [29]. Regarding the biochemical crosstalk between dPCD and ePCD, some genes conceptually classified as SAGs are also stress-responsive genes (Figure 1).

In *Arabidopsis thaliana*, more than 20% of encoded genes have their expression profiles changed by senescence [35]. A temporal profile of global gene expression variation reveals gene sets that are highly ordered in the control of molecular events associated with leaf senescence [36], and many of these encode transcription factors (TFs) [3,24,29,30,36]. TFs are involved in different developmental processes and act as nodes in signaling processes, connecting signal-sensing and morphophysiological changes [5,24,37–40]. Therefore, the identification of senescence-associated TFs and dissection of their hormone-controlled regulatory networks represent valuable tools for molecular breeding and the generation of superior crops.

Different TF families participate in the ongoing senescence, mainly the NAC [5,25,28,38,41–48], MYB [13,16], AP2/ERF [49,50], and WRKY [17,51–53] families. In Arabidopsis, several regulatory gene networks have already been characterized and the crosstalk regulation between phytohormones and TFs is extensively understood [54]. However, few SAGs have been described in crop plants, including soybean, maize, cotton, sunflower, barley, rice, and wheat, and their regulatory networks in such plant species remain unclear.

Approximately 44% (79 genes) of the GmNAC genes were recently shown to be differentially expressed (DE) at the senescence onset. These DE *GmNACs* predominate in the upregulated (54 genes; 68%) changes over the downregulated (25 genes; 32%) changes (Figure 1) [5]. We classified the *GmNAC* genes upregulated by natural leaf senescence as SAGs. Along with their putative orthologs from *A. thaliana*, *GmNAC-SAGs* were distributed into the already described NAC subfamilies (Table 1; [5,55]). The most representative subfamilies, harboring the higher number of functionally characterized soybean and Arabidopsis related-genes, are SNAC-A/B, NAM, TERN, TIP, Senu5, and ONAC022. Almost half of the *GmNAC* genes upregulated by natural senescence also respond to at least one type of environmental stress, demonstrating the functional plasticity of NAC TFs. The partial overlapping of GmNAC functions in dPCD and ePCD is also shared by hormone signaling branches controlling these phenomena, as described by well-characterized Arabidopsis gene regulatory networks [24,29,54].

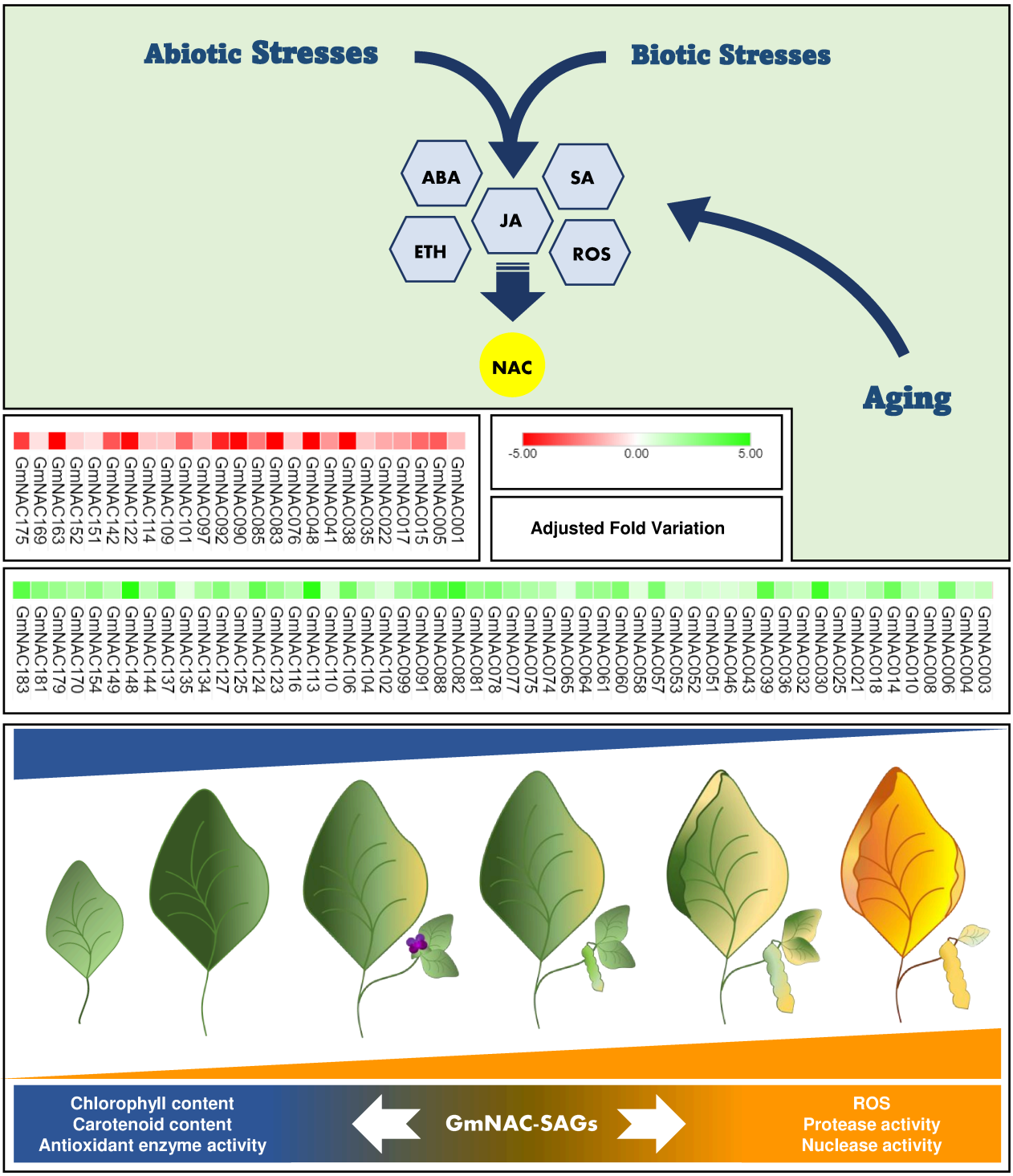

Figure 1. Integrated environmental and developmental senescence mechanisms in soybean and *GmNAC-SAGs* involved in this process. Senescence is a naturally controlled program that culminates in nutrient recycling before plant reproduction. The biological event is complex and finely regulated by several gene networks integrating different TF families, including NAC-TFs. During ongoing senescence, plant metabolism undergoes drastic changes imposed by the extensive gene expression reprogramming coordinated by phytohormones, especially ABA, ETH, JA, and SA. However, these hormones also serve as stress signalers in plants subjected to multiple stresses, overlapping the developmental and stress-responsive mechanisms that converge to programmed cell death (PCD). Conceptually, PCD is classified as developmental PCD (dPCD) and environmental PCD (ePCD), according to the signal's origin.

Since the transcription factors, mainly those from the GmNAC family, are hormone-responsive, dPCD and ePCD might be temporally controlled and partially integrated by *GmNAC-SAGs* responsive to stress. The genes upregulated in senescence are conceptually designated SAGs (senescence-associated genes). Several of these are also stress-responsive and participate in regulatory gene networks that confer stress tolerance or trigger cell death as a stress-evasive program. The *GmNACs* differentially regulated by developmental senescence are shown. The genes upregulated (54) by senescence are shown in green, and the downregulated genes are shown in red. GmNAC TFs may integrate these programs and, as protein nodes, regulate the balance between the plant antioxidant system and aging, the molecular and phenotypical readouts of which are ROS accumulation and chlorophyll and biomolecule breakdown until leaf abscission.

2.1. SNAC-A (ATAF) and SNAC-B (NAP) Subfamilies Harbor an Expressive Set of Putative Positive Regulators of Senescence in Soybean

Phylogenetically, the NAC superfamily in soybean is divided into 15 subfamilies, according to sequence and functional conservation between soybean and *A. thaliana* genes [5]. Only two subfamilies, OsNAC8 and ANAC011, do not include a member upregulated by aging (Table 1). SNAC-A (ATAF) is the most represented subfamily in senescence; 90% of the genes are differentially expressed in leaf senescence (10 of 11 members). Within this family, *GmNAC018*, *GmNAC030*, *GmNAC039*, and *GmNAC043* are SAGs displaying stress-responsiveness [5,41] (Table 1). *GmNAC018* and *GmNAC039* are putative paralogs and display similar induction patterns, with expressive upregulation by simulated drought, ER, and biotic stresses [41]. Similarly, *GmNAC030* belongs to a partially overlapped regulatory circuit integrating drought, ER, and biotic stresses with natural senescence in *planta*, and thus is considered a *GmNAC-SAG*. GmNAC030 interacts with GmNAC081 (TERN subfamily), which is involved in both dPCD and ePCD [43,56,57].

Table 1. *GmNAC-SAGs* and their putative *AtNAC-SAG* orthologs are responsive to multiple stresses.

Subfamily *	*GmNAC* *	ID	Features **	References	Putative Ortholog in Arabidopsis ***	ID	Features **	References
SNAC-A (ATAF)	*GmNAC018*	Glyma.04G208300	Responsive to drought, ER, and biotic stresses in soybean seedlings, respectively elicited by PEG, tunicamycin, and salicylic acid treatments.	Ferreira et al., 2020 [41]	*ANAC002 (ATAF1)*	AT1G01720	It is induced by ABA and H_2O_2 treatments in Arabidopsis. It belongs to a regulatory network involving ABA-triggered senescence, targeting other SAGs such as *ORE1*.	Wu et al., 2009 [58] Jensen et al., 2013 [59] Garapati et al., 2015 [60] Qiu et al., 2015 [15]
	GmNAC030	Glyma.05G195000	Responsive to ER and drought stresses. It belongs to NAC-VPE circuit promoting cell death in natural and stress-induced senescence.	Irsigler et al., 2007 [61] Pinheiro et al., 2009 [18] Mendes et al., 2013 [56]				
	GmNAC039	Glyma.06G157400	Phylogenetically grouped with the *GmNAC018* putative paralogue. It displays the same stress responsiveness.	Ferreira et al., 2020 [41]				
	GmNAC043	Glyma.06G248900	Upregulated by drought and oxidative stresses. It responds to ABA and air-drying treatments in soybean seedlings.	Melo et al., 2021 [28] Thu et al., 2014 [62] Hussain et al., 2017 [63]	*ANAC055*	AT3G15500	Responsive to drought, salt, ABA, and JA. It integrates a stress-responsive and senescence-promoting circuit together with *ANAC019* and *ANAC072*.	Bu et al., 2008 [64] Hickman et al., 2013 [65] Zhu et al., 2015 [66]

Table 1. *Cont.*

Subfamily *	*GmNAC* *	ID	Features **	References	Putative Ortholog in Arabidopsis ***	ID	Features **	References
SNAC-B (NAP)	*GmNAC003*	Glyma.01G051300	It responds to ABA treatment in soybean. Highly induced by drought, mainly in roots.	Tran et al., 2009 [67] Quach et al., 2014 [68]	*ANAC029 (AtNAP)*	AT1G69490	Upregulated by ABA-treatment, drought, and osmotic stresses in Arabidopsis. Also responsive to ethylene.	Guo and Gan, 2006 [48] Jensen et al., 2013 [59]
	GmNAC010	Glyma.02G109800	Responsive to dehydration in shoots.	Tran et al., 2009 [67]				
	GmNAC052	Glyma.07G229100	Key player in cold responses and flowering-time coordination.	Hussain et al., 2017 [63]				
	GmNAC148	Glyma.20G033300	Drought-responsive gene.	Hussain et al., 2017 [63]				
	GmNAC006	Glyma.02G070000	Highly responsive to drought in the sensitive soybean cultivar MDT720.	Thu et al., 2014 [62]	*ANAC047*	AT3G04070	Responsive to salt and osmotic stress in Arabidopsis. Also responsive to bacterial infection.	Mito et al., 2010 [69] Shaik and Ramakr-ishna, 2013 [70]
	GmNAC127	Glyma.16G151500	-	-				
	GmNAC124	Glyma.16G043200	Responsive to abiotic stresses, conferring salt tolerance in transgenic soybean hairy-roots.	Hao et al., 2011 [71]				
	GmNAC181	Glyma.19G108800	Responsive to multiple stresses and plant hormones. It confers salt tolerance in transgenic plants. Regulatory gene network analyses suggest it regulates DREB1A and other stress-related genes.	Hao et al., 2011 [71]				
	GmNAC091	Glyma.12G221400	-	-	*ANAC056*	AT3G15510	Downregulated by ABA treatment.	Kleinow et al., 2009 [72] Aslam et al., 2012 [73]
	GmNAC102	Glyma.13G280000	Responsive to dehydration in soybean roots during vegetative and reproductive stages.	Le et al., 2012 [74]				
	GmNAC099	Glyma.13G243200	-	-	*ANAC025*	AT1G61110	-	-
	GmNAC113	Glyma.15G070300	-	-				
NAM	*GmNAC149*	Glyma.20G172100	Slightly induced by severe drought.	Carvalho et al., 2014 [75] Melo et al., 2021 [28]	*ANAC017*	AT1G34190	Not responsive to classical abiotic stresses. Associated with mitochondrial stresses and consequent H_2O_2 accumulation.	Ng et al., 2013 [76] Meng et al., 2019 [77]
	GmNAC074	Glyma.10G219600						
	GmNAC182	Glyma.19G165600	-	-	*ANAC074*	AT4G28530	-	-
	GmNAC058	Glyma.08G156500	Upregulated by persistent water stress conditions.	Carvalho et al., 2014 [75] Silva et al., 2015 [78]	*ANAC103*	AT5G64060	-	-

Table 1. *Cont.*

Subfamily *	*GmNAC* *	ID	Features **	References	Putative Ortholog in Arabidopsis ***	ID	Features **	References
	GmNAC061	Glyma.08G173400	-	-	*ANAC022*	AT1G56010.2	-	-
	GmNAC125	Glyma.16G051800	-	-	*ANAC083 (VNI2)*	AT5G13180	Upregulated by salt and ABA treatments. Negative regulator of natural senescence in Arabidopsis.	Yang et al., 2011 [26]
	GmNAC123	Glyma.16G042900	Responsive to drought in late vegetative stages.	Le et al., 2012 [74]	*ANAC087*	AT5G18270	-	-
	GmNAC144	Glyma.19G109100	-	-				
TERN	*GmNAC077*	Glyma.11G096600	Downregulated by severe drought.	Carvalho et al., 2014 [75] Melo et al., 2021 [28]	*ANAC036*	AT2G17040	-	-
	GmNAC081	Glyma.12G022700	Upregulated by drought and ER stresses. Transgenic plants overexpressing GmNAC081 display accentuated drought sensitivity and accelerated senescence phenotypes.	Irsigler et al., 2007 [61] Pinheiro et al., 2009 [18] Faria et al., 2011 [19] Pimenta et al., 2016 [43] Ferreira et al., 2020 [41]				
	GmNAC078	Glyma.11G182000	-	-	*ANAC090*	AT5G22380	Expression remains unaltered during stress responses but the protein acts as a negative regulator of ROS and SA pathways.	Kim et al., 2018 [42]
	GmNAC082	Glyma.12G091200	Highly responsive to mild–severe and severe drought stresses in the tolerant Jindou soybean cultivars.	Hussain et al., 2017 [63]				
	GmNAC088	Glyma.12G186200	-	-				
	GmNAC104	Glyma.13G315300	-	-				
	GmNAC014	Glyma.03G197900	-	-				
	GmNAC053	Glyma.07G271100	Key player in cold responses and flowering-time coordination.	Hussain et al., 2017 [63]	*ANAC035*	AT2G02450	-	-
ONAC022	*GmNAC060*	Glyma.08G169400	-	-	-	-	-	-
	GmNAC116	Glyma.15G257700	-	-	-	AT3G12910	-	-
	GmNAC106	Glyma.14G030700	Negatively regulated by bleomycin (cell death inducer) treatment.	Melo et al., 2021 [28]	*ANAC042 (JUB1)*	AT2G43000	Responsive to H_2O_2 accumulation. Negative regulator of natural senescence. Also responsive to flagellin-PAMP.	Wu et al., 2012 [79] Saga et al., 2012 [80]
	GmNAC154	Glyma.02G284300	Slightly induced by bleomycin treatment.	Melo et al., 2021 [28]				
	GmNAC064	Glyma.08G307100	-	-				
	GmNAC137	Glyma.18G110700	-	-				
	GmNAC134	Glyma.17G185000	-	-	*ANAC083 (VNI2)*	AT5G13180	Upregulated by salt and ABA treatments. Negative regulator of natural senescence in Arabidopsis.	Yang et al., 2011 [26]

Table 1. *Cont.*

Subfamily *	*GmNAC* *	ID	Features **	References	Putative Ortholog in Arabidopsis ***	ID	Features **	References
ANAC063	*GmNAC004*	Glyma.01G088200	Upregulated by drought stress in soybean. Arabidopsis ectopically expressing the soybean gene displays a hallmarked lateral root formation under drought.	Quach et al., 2014 [68] Hussain et al., 2017 [63]	*ANAC008*	AT1G25580	-	-
	GmNAC008	Glyma.02G100200	-	-				
	GmNAC170	Glyma.10G204700	-	-				
	GmNAC025	Glyma.05G002700	-	-	*ANAC044*	AT3G01600	Responsive to genotoxic stresses: bleomycin, hydroxyurea, mitomycin C, and methanesulfonate.	Takahashi et al., 2019 [81]
TIP	*GmNAC021*	Glyma.04G226700	Highly responsive to mild–severe and severe drought stresses in the tolerant Jindou soybean cultivars.	Hussain et al., 2017 [63]	*NTL9*	AT4G35580	Responsive to osmotic stress.	Yoon et al., 2008 [82]
	GmNAC110	Glyma.14G189300	*pGmNAC110* harbors a UPR-cis regulatory element, suggesting some responsiveness in ER stress.	Sun et al., 2013 [83] Silva et al., 2015 [78]				
	GmNAC036	Glyma.06G138100	Predicted *GmNAC062* ortholog. Its promoter harbors a UPR-cis regulatory element.		*ANAC090*	AT5G22380	Expression remains unaltered during stress responses but the protein acts as a negative regulator of ROS and SA pathways.	Kim et al., 2018 [42]
ANAC001	*GmNAC051*	Glyma.07G201800	-	-	*ANAC073*	AT4G28500	-	-
	GmNAC032	Glyma.05G225100	-	-	*ANAC099*	AT5G56620	-	-
Senu5	*GmNAC065*	Glyma.08G360200	Differentially responsive to PEG, tunicamycin, and salicylic acid treatments in soybean. Highly responsive to ABA. Ectopically expressing Arabidopsis transgenic lines display delayed senescence and an enhanced antioxidant system when subjected to abiotic and biotic stresses. *GmNAC065* is also upregulated in drought-tolerant soybean plants.	Hussain et al., 2017 [63] Melo et al., 2018 [5] Melo et al., 2021 [28]	*ANAC083 (VNI2)*	AT5G13180	Upregulated by salt and ABA treatments. Negative regulator of natural senescence in Arabidopsis.	Yang et al., 2011 [26]
	GmNAC179	Glyma.18G301500	Predicted *GmNAC065* paralog. It displays a similar stress-induction pattern. Slightly induced by bleomycin treatment.	Melo et al., 2021 [28]				

Table 1. *Cont.*

Subfamily *	*GmNAC* *	ID	Features **	References	Putative Ortholog in Arabidopsis ***	ID	Features **	References
VND-NAC	*GmNAC075*	Glyma.11G030600	-	-	*ANAC007*	AT1G12260	-	-
Unnamed Group	*GmNAC135*	Glyma.17G240700	-	-	*ANAC011*	AT1G32510	Responsive to wounding.	Matsuoka et al., 2021 [84]
	GmNAC057	Glyma.08G075300	Upregulated by water stress. The drought-susceptible cultivar MDT777-2 displays higher gene expression than tolerant cultivars. In reproductive stages, under drought, the gene appears to be downregulated.	Le et al., 2012 [74] Thu et al., 2014 [62]	*ANAC104*	AT5G64530	-	-
	GmNAC046	Glyma.07G048000	Cold-responsiveness. Possibly associated with the control of flowering.	Hussain et al., 2017 [63]	*NTL9*	AT4G35580	Responsive to osmotic stress.	Yoon et al., 2008 [82]

(*) According to the last investigation of GmNAC superfamily in the soybean genome (Melo et al., 2018) [5]. (**) Reports associating NAC TFs with different stresses. (***) According to deduced amino acid sequence homology, defined by Phytozome and Soybase BALSTp in-house algorithms (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Gmax and https://www.soybase.org/GlycineBlastPages/).

Finally, *GmNAC043* is also referred to as a putative *GmNAC-SAG*, as previously reported by Melo et al. in 2021 [28]. *GmNAC043* is responsive to different stresses, such as drought, oxidative stress, and insect and fungus attack, and is slightly upregulated by natural senescence (Table 1). Furthermore, *GmNAC043* (*ANAC055* ortholog) is significantly induced by bleomycin treatment in soybean, reinforcing its role as a gene involved in programmed cell death control [28].

The putative orthologs of SNAC-A (ATAF) soybean genes identified in Arabidopsis belong to very well-described regulatory circuits integrating multiple stress responses and senescence (Table 1). *ATAF1* (*ANAC002*) integrates ABA-hormone signaling and senescence by simultaneously suppressing the expression of photosynthesis-associated genes and stimulating the expression of other SAGs [24,85]. Since it is a hormone-responsive TF, several stressful conditions that culminate in ABA and ROS production activate *ATAF1* expression [15,58–60]. Additionally, *ATAF1* represents a classical *AtSAG* since it promotes dark and developmental senescence, which is remarkably delayed in *ataf1* mutants [60].

The *GLK1* and *ORE1* promoters harbor *cis*-acting elements directly targeted by ATAF1. Accordingly, ATAF1 upregulates the expression of *ORE1*, which shares a similar expression pattern as *ATAF1* [54]. Conversely, ATAF1 downregulates the expression of chloroplast-maintenance genes including *GLK1*, which delays senescence when overexpressed in plants. Interestingly, ORE1 directly interacts with GLK1 to inhibit its activity [86]. Therefore, *ATAF1* is activated by ABA during natural and stress-induced senescence and in turn activates the expression of ORE1 which, together with ATAF1, regulates the chloroplast-maintenance function of GLK1, providing a feedforward loop to promote photosynthesis decay and leaf yellowing leading up to abscission. Not surprisingly, ATAF1 targets genes involved in ABA biosynthesis and transport, *NCED3* and *ABCG40*, respectively, which are essential for ABA signaling [59]. Despite the high structural conservation of SNAC-A (ATAF)-SAGs, a soybean ortholog of ATAF1, GmNAC030, forms a heterodimer with GmNAC081 to repress the expression of *ABCG40* and regulators of ABA signaling [41]. These results indicate that the underlying mechanism of NAC-SAG-mediated senescence may differ across species.

Additionally, in the SNAC-A (ATAF) subfamily, GmNAC085 forms a divergent and distant clade with GmNAC101, GmNAC092, and GmNAC043, which differ from their Arabidopsis orthologs in expression profiles in response to natural leaf senescence [5,28]. With the exception of GmNAC043-SAG, which shares a similar expression profile with the orthologs in Arabidopsis ANAC072, ANAC019, and ANAC055, the other soybean

members of this clade are downregulated by developmentally programmed leaf senescence [5,28,65]. Despite the divergent expression profile, there is a lack of information about the molecular functions of these GmNACs and the redundant functional aspects of their paralogs remains unclear. Ectopic expression of GmNAC085 in *Nicotiana benthamiana* leaves leads to classic symptoms of senescence, including chlorophyll loss, H_2O_2 accumulation, and leaf yellowing [5]. Similar phenotypes are also observed in transgenic Arabidopsis lines expressing the soybean gene. In addition, these plants display an imbalanced enzymatic and metabolic antioxidant system, with low expression and activity of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), associated with low concentrations of soluble sugars, carotenoids, and anthocyanins [28]. Although *GmNAC085* is not considered a conceptual SAG, as it is downregulated during senescence, these findings strongly suggest that it may be associated with the control of ePCD in soybean. Consistent with a role in ePCD, co-expression analyses revealed that *GmNAC085* positively correlates with ABA-responsive genes, genes involved in aromatic amino acid metabolism, and genes associated with protein and metabolite breakdown, mainly the subunits of proteasome complex, endopeptidases, and peroxidases [28].

The SNAC-B (NAP) subfamily also harbors an expressive number of senescence-upregulated genes. Among 23 genes, 12 genes are induced by the onset of senescence, representing almost 50% of soybean SNAC-B (NAP) genes. *GmNAC006*, *GmNAC010*, *GmNAC102*, *GmNAC148*, and *GmNAC181* are responsive to drought [62,63,67,68,71,74] (Table 1). *GmNAC052* is upregulated by cold stimuli and may control the ongoing senescence in flowers [63]. Likewise, *GmNAC046* (unnamed group) is positively regulated by senescence. Displaying a similar expression pattern as *GmNAC181*, *GmNAC124* might be involved in salt-stress responses and soybean hairy-root development; overexpression of these GmNAC-SAGs confers tolerance to osmotic stresses [71]. *GmNAC181*-overexpressing Arabidopsis lines (previously referred to as *GmNAC11*) show a more than 60% survival rate after recovery from 15 days exposure to salt stress. The *GmNAC181*-mediated increases in osmotic stress tolerance have been associated with the DNA-binding activity of GmNAC181, which directly targets *cis*-elements in the DREB-1A promoter. Gene expression analyses in *GmNAC085*-overexpressing lines revealed enhanced expression of *DREB-1A*, *ERD11*, *COR15A*, *ERF5*, *RAB18,* and *KAT2*, frequently associated with multiple stress-tolerance phenotypes [71].

The putative orthologs from the SNAC-B (NAP) subfamily in Arabidopsis also respond to multiple stresses (Table 1). *ANAC047* (*GmNAC006*, *GmNAC124*, *GmNAC127,* and *GmNAC181*) respond to salt- and mannitol-induced osmotic stresses with late to intermediate kinetics, with higher transcript accumulation after 12 h of treatment [69]. Transgenic *A. thaliana* lines expressing the chimeric repressor ANAC047-SRDX display high salt tolerance, implicating *ANAC047* as a positive regulator of stress-induced senescence [69]. Interestingly, *ANAC047* also responds to bacterial infection [70]. Bacterial and fungal infections trigger SA-responsive pathways, stimulating oxidative burst and leading to cell death in hypersensitive reactions (HR) [87]. Lipid peroxides accumulate during HR because of ROS production and membrane degeneration, the most common features during ePCD [88].

Another *AtNAC* ortholog from the SNAC-B (NAP), *ANAC029 (AtNAP)*, integrates a senescence regulatory network with *ANAC055* and *ANAC072*, SNAC-A subfamily gene members [55,89] (reviewed by [29,54]). These genes are phylogenetically related and display the same expression patterns induced by ABA and different abiotic stresses [38,55,90] (Table 1). The senescence circuit integrated by ANAC019 and ANAC055 encompasses components of the EIN2 (ETHYLENE INSENSITIVE 2) senescence-regulatory pathway [12,91]: ANAC019 and ANAC055 are direct targets of EIN3 TF, a downstream component of the *EIN2* cascade in ETH-mediated signaling [92]. EIN3 can bind *cis*-elements in the promoter of several *AtNAC-SAGs*, including *ANAC019*, *ANAC055,* and *ANAC059 (ORS1)* [93]. For *ANAC072*, the activation pathway diverges in some aspects from its closely related genes. The promoter region of *ANAC072* harbors *cis*-elements recognized by CBF (C-

REPEAT/DEHYDRATION RESPONSIVE BINDING FACTORS), which is frequently associated with senescence progression [65,94].

2.2. Putative GmNAC-SAGs also Share Structural Conservation with Negative Regulators of Senescence Progression from Arabidopsis

Few soybean and Arabidopsis NAC TFs from the NAM subfamily have been associated with stress-responsive and/or senescence-regulatory gene networks. A total of 16 of 42 *GmNAC* genes (38%) of the NAM members are upregulated in soybean during natural senescence. The NAM subfamily encompasses gene members typically related to growth and developmental processes [5]; however, the putative *GmNAC125* ortholog, *ANAC083 (VNI2)*, belongs to a negative regulatory cascade of senescence in Arabidopsis [26] (Table 1). *ANAC083* integrates ABA signaling and controls xylem vessel specification [26,95]. *ANAC083*-overexpressing plants are remarkably tolerant to salt and drought, as well as displaying a delayed senescence phenotype. The higher expression levels of *ANAC083* lead to a significantly higher expression of *COR/RD* genes [26], shared with the AREB-1 drought-tolerance pathway. Multiple physiological events upregulate *RD29A*, *RD29B*, *RD22*, and *RD20* genes as plants try to cope with long-standing adverse conditions before activating ePCD [96–100], culminating in improved plant performance under adverse conditions [101]. Similarly, *GmNAC065* (Senu5), a putative *ANAC083* ortholog, also acts as a negative regulator of natural and stress-induced senescence [28].

Curiously, all of the ONAC022 subfamily members in soybean (except *GmNAC006* and *GmNAC116*, for which putative orthologous genes have not been identified; Table 1) are phylogenetically related to negative regulators of natural and stress-induced senescence in Arabidopsis. *GmNAC134* is a putative ortholog of *ANAC083*. *GmNAC064*, *GmNAC137*, *GmNAC106*, and *GmNAC154* are phylogenetically grouped with *ANAC042 (JUB1)*. *GmNAC106* and *GmNAC154* are considered putative *GmNAC-SAGs* and are involved in aging but not in environmental stresses, consistent with their slight induction in bleomycin-treated soybean seedlings [28].

Like *ANAC083 (VNI2)*, *ANAC042 (JUB1)* is a negative regulator of senescence and increases plant longevity in Arabidopsis. Its expression is promptly induced by H_2O_2 accumulation, and overexpression of *ANAC042* (*JUB1*) confers tolerance to abiotic stresses, including heat and salt, and delays natural senescence. The opposite effect is observed in *jub1* mutants [79]. As described for the soybean TF GmNAC181, JUB1 directly activates DREB-2A, which targets RD29A from the COR/RD stress evading circuit [26,71,102]. Accordingly, DREB-2A activates the *Hsf-A3* gene [103], which takes part in a feedforward regulatory loop that upregulates genes of heat-shock proteins (Hsps) and ROS-scavenging-related proteins [104–107].

A large proportion of the GmNAC-SAGs respond to an array of biotic and abiotic stresses (Table 1), but functional information is restricted to GmNAC030, GmNAC065, GmNAC181, and GmNAC118. Remarkably, these characterized GmNAC-SAGs are functionally similar to the Arabidopsis orthologs ATAF1, ANAC083, ANAC036, and ANAC047, respectively. Table 1, which compiles information about structural conservation, phylogenetic relationships, and expression profiles of GmNAC-SAGs and AtNAC-SAGs, provides reliable information to rationalize functional studies of GmNAC-SAGs.

2.3. GmNAC-SAGs as Positive and Negative Regulators of Leaf Senescence

Despite the large fraction of *GmNACs* upregulated during leaf senescence, functional information is restricted to a few members of the *GmNAC-SAG* class. One such example is *GmNAC081* (previously designated *GmNAC6*), one of the first *GmNACs* to be identified and cloned [108], which has been shown to be induced by abiotic stress [61] and cell death inducers, is repressed by senescence inhibitors [18], and is classified as a TERN subfamily member [18,108]. It induces necrotic lesions when expressed in tobacco leaves [18] with hallmarks of PCD [19,109]. Full-length GmNAC081 does not transactivate transcription in yeast, yet a truncated GmNAC081 protein, expressing the carboxyl region alone in yeast, functions as a transactivator domain [18,67]. The lack of transactivation activity of

GmNAC081 may be due to inhibitory interactions between the C-terminal transactivator domain and the N-terminal NAC DNA-binding domain, which are supposed to be relieved by expressing the C-terminal region alone. The negative interactions between GmNAC081 domains may also be relieved by interactions with other TFs upon heterodimer formation. Accordingly, GmNAC081 has been shown to interact in yeast and in plants with another GmNAC-SAG, GmNAC030, which exhibits transactivation activity in yeast and induces PCD in GmNAC030-expressing soybean protoplasts [56]. The GmNAC081-GmNAC030 heterodimer enhances the transcriptional regulation of shared target promoters, indicating that heterodimerization is required to fully regulate gene expression. Furthermore, as partners, GmNAC081 and GmNAC030 are coordinately regulated in response to multiple environmental and developmental stimuli [43,56]. Among the GmNAC081–GmNAC030 target genes, the vacuolar processing enzyme (VPE) may be responsible for executing a cell death program induced by activation of these TFs [43,56,75]. VPE is a caspase-like 1 protein that executes plant-specific cell death via vacuole collapse, resulting in extensive biomolecule degradation [110].

GmNAC081 has also been functionally characterized as a downstream component of the DCD/NRP-mediated cell death response [19,56] and as a positive regulator of leaf senescence [5,28,41,43]. Transcript expression analysis and *GmNAC081* promoter-GUS assay indicated that GmNAC081 transcriptional control is correlated with leaf senescence events [41,43]. Both soybean and tobacco leaves display low GmNAC081 promoter activity and expression at the early developmental stages, while GmNAC081 transcript level and GUS activity increase in the late stages. Similarly, *GmNAC081*-overexpressing plants show accelerated senescence, a phenotype associated with accelerated leaf yellowing, decreased photosynthesis rate, and enhanced upregulation of SAG marker genes. Additionally, the expression of GmNAC081 target genes encoding cell-death-inducing hydrolytic enzymes are coordinately upregulated with GmNAC081 expression in the late stages of development [43,56]. Conversely, silencing of *GmNAC081* by VIGS (virus-induced gene silencing) in soybean plants causes a delay in leaf senescence. Leaf yellowing and expression of the GmNAC081 target genes during leaf senescence are significantly lower in silenced leaves than in wild-type leaves [43].

Consistent with the role of *GmNA0C81* in senescence, a subset of DEGs (differentially expressed genes) in natural leaf senescence in wild-type leaves is significantly overrepresented at the early developmental stage in GmNAC081-overexpressing soybean lines [41]. Therefore, GmNAC081 regulates leaf senescence by modulating the expression of senescence-, cell-death-, and hormone-signaling-related genes [41,43]. GmNAC081 functions either as a transcriptional activator or repressor of TGTG(T/G/C) cis-element-containing target promoters [56]. Analysis of the GmNAC081-induced transcriptome in leaves at early developmental stages uncovered both downregulated senescence-inhibiting and upregulated senescence-promoting genes harboring GmNAC081-binding sites on their promoters [41]. As a positive regulator of leaf senescence, GmNAC081 activates the expression of senescence-inducing genes and negatively modulates the expression of senescence-suppressing genes.

In contrast to GmNAC081 and GmNAC030, positive regulators of leaf senescence, GmNAC065 negatively regulates natural and stress-induced senescence [74]. *GmNAC065* was first identified as a drought-induced gene in soybean leaves at the late vegetative V6 stage and reproductive R2 stage [74]. In contrast, an analysis of drought-responsive genes in soybean leaves at the V3 developmental stage revealed that *GmNAC065* was strongly downregulated by gradually declining the leaf water potential to a maximum stress of −2.0 MPa [75]. The apparent contradiction between these earlier studies may be due to drought induction at different developmental stages, suggesting that specific pathways regulating the drought response might operate in different stages of plant development. As a negative regulator of leaf senescence, the induction of *GmNAC065* by drought may modulate leaf longevity and the stay-green phenotype under stress conditions. Accordingly, a comparison of *GmNAC065* expression in drought-tolerant and drought-

sensitive soybean genotypes demonstrated that *GmNAC065* is induced by drought to a greater extent in drought-resistant genotypes, suggesting the potential of *GmNAC065* as a target for molecular breeding or genetic engineering of drought tolerance in soybean [63].

GmNAC065 has also been demonstrated to be widely responsive to biotic and abiotic stimuli in soybean seedlings treated with the osmotic stress inducer PEG, the ER stress inducer tunicamycin, and the pathogen defense hormone salicylic acid (SA), displaying early induction kinetics [5]. Nevertheless, ectopic expression of GmNAC065 in *N. benthamiana* leaves induces weaker cell death phenotypes compared to those displayed by the stress-induced GmNAC085 and the senescence-associated GmNAC081 expression [5].

GmNAC065 and its paralog *GmNAC179* are phylogenetically most related to the negative regulator of senescence in Arabidopsis *ANAC083 (VNI2)*, and together they form a closely related branch with another negative regulator of senescence, *ANAC042 (JUB1)*, and its putative orthologous SAGs in soybean, *GmNAC106* and *GmNAC154* [5,28]. These findings support a negative role of *GmNAC065* in leaf senescence. Accordingly, in silico co-expression analyses revealed a strong positive correlation of *GmNAC065* expression profile with stress-sensing- and signal-transduction-associated genes, besides those associated with cell survival under multiple stresses, including LEA proteins, β-carotene hydroxylase, acyl-CoA oxidase, and ubiquinol oxidase. Not surprisingly, these proteins and enzymes are frequently associated with ROS-avoiding mechanisms, photosynthetic apparatus preservation, jasmonate-mediated tolerance to biotic stresses, and drought tolerance [111–113]. Overall, *GmNAC065* is co-expressed with genes involved in plant maintenance and redox homeostasis, possibly sharing a redox-balanced environment that results in senescence delay since low ROS levels activate signal pathways that mediate stress escape. However, at higher concentrations, ROS induces PCD [88,114].

Further evidence that *GmNAC065* negatively regulates leaf senescence was provided by overexpressing *GmNAC065* in Arabidopsis [28]. The Arabidopsis transgenic lines ectopically expressing *GmNAC065* exhibited stunted growth compared to wild-type plants during the vegetative stage and a decelerated progression throughout the reproductive stage with delayed leaf senescence, a phenotype similar to that displayed by *ANAC083 (VNI2)*-overexpressing lines [26,28]. Additionally, *GmNAC065*-overexpressing transgenic plants exhibited enhanced expression of *COR/RD* genes, the targets of ANAC083 (VNI2), the Arabidopsis *GmNAC065* ortholog [26], as well as reduced expression of genes associated with the catabolism of proteins and pigments [28]. Furthermore, in the *GmNAC065*-overexpressing lines, the expression and activity of the SOD, CAT, and APX, components of the antioxidant system were increased compared to the wild type. Moreover, the transgenic lines displayed higher contents of soluble sugar, carotenoids, and anthocyanin, as parts of the nonenzymatic plant antioxidant system acting in water retention, UV protection, and ROS scavenging, respectively [115]. Finally, the extent of stress-induced cell death in leaves and roots of *GmNAC065*-overexpressing lines was lower compared to the wild-type control. Remarkably, *GmNAC065* overexpression in Arabidopsis affected the expression of different SAGs and their downstream targets, predominantly as downregulation. *AtNAP*, *ATAF1*, *ORE1*, *ANAC016*, *ANAC019*, and *ANAC055* were slightly induced by *GmNAC065* overexpression, and the downstream targets *NYC1 (NONYELLOW COLORING 1)*, *SINA1 (SEVEN IN ABSENTIA)*, *BFN1 (BIFUNCTIONA NUCLEASE1)*, *BSMT1 (S-ADENOSYLMETHIONINE-DEPENDENT METHYL-TRANSFERASE)*, *GLK1 (GOLDEN2-LIKE 1)*, and *SAG113* were suppressed.

Collectively, these findings suggest that GmNAC065 integrates a negative senescence-regulatory pathway via activation of *RD* genes (upregulated in GmNAC065-overexpressing lines) and, consequently, downregulation of *AtNAC-SAGs* and their downstream targets, executors of stress-triggered programmed cell death. Besides the robust enzymatic and nonenzymatic antioxidant systems and the attenuated senescence phenotype under multiple stresses and normal development, the functional characterization of *GmNAC065* provides new insights into SAGs in soybean and highlights the relevance of these genes as hotspots for biotechnological soybean breeding.

3. A Regulatory Circuit Integrating Stress-Induced with Natural Leaf Senescence

Both developmental and stress stimuli have been shown to trigger leaf senescence. Our knowledge about the crosstalk between stress-induced and developmentally programmed leaf senescence has advanced considerably through reverse/forward genetics, multiple omics-based technologies, and expression profiling studies under different stress conditions in Arabidopsis [9]. A consensus theme that has emerged from studies of regulatory networks controlling leaf senescence is the existence of a signaling module in plants integrating the transduction of environmental and developmental signals at the onset and progression of leaf senescence. In soybean, the developmental cell death (DCD) domain-containing asparagine-rich protein (NRP)-mediated cell death response has been characterized as a regulatory circuit that integrates stress-induced with developmentally programmed leaf senescence [57,116].

The DCD/NRP-mediated cell death signaling pathway, which has been uncovered in soybean, transduces a cell death signal derived from multiple stress conditions and late stages of leaf development [116]. The first components of this cell death pathway identified were DCD/NRPs, designated GmNRP-A and GmNRP-B, which gave the pathway its name (NRP signaling; Figure 2). NRPs were isolated in a wide-genome transcriptional screening for commonly induced genes as candidate regulatory components that integrate the ER and osmotic stress responses [61]. GmNRP-A and GmNRP-B belong to the subgroup I of plant-specific DCD-containing proteins [117]. They share a highly conserved C-terminal DCD domain in addition to a high content of asparagine residues at their more divergent N-terminus. The screening for genes synergistically induced by combined stress conditions also identified a NAC transcription factor, GmNA0C81, which was later characterized as a downstream component of the DCD/NRP-mediated cell death pathway [19,61]. The overexpression of NRPs or GmNAC081 in plants induces cell death and apoptotic-like phenotypes such as foliar necrotic lesions, DNA fragmentation, caspase activity, chlorophyll loss, and lipid peroxidation [19,109,118].

Further progress in deciphering the NRP signaling identified GmNAC030 as the GmNAC081 cellular partner [56] (Figure 2). GmNAC030 cooperates with GmNAC081 to bind to target promoters, and the transcriptional complex either induces the expression of activators or represses the expression of inhibitors of the cell death program [41,56]. Among the target hydrolytic enzymes, the expression of VPE is fully induced by the GmNAC081–GmNAC030 heterodimer. The vacuole-localized cysteine protease VPE has caspase-1 activity and is synthesized as an inactive proprotein precursor [119]. The precursor is self-catalytically converted into the active mature form, which in turn mediates the activation of vacuolar enzymes that degrade the vacuolar membrane, resulting in vacuolar-collapse-mediated cell death [120]. Therefore, the NRP/GmNAC081–GmNAC030/VPE signaling module transduces a stress-induced cell death signal into a PCD response.

The multiple-stress responsive gene GmERD15 is the most upstream component of the DCD/NRP-mediated cell death signaling that has been identified to date. ERD15 is a small acidic and hydrophilic protein that belongs to the PAM2-domain-containing protein family, induced by diverse biotic and abiotic stress such as light, cold, high salinity, and drought, osmotic, and ER stress [121–123]. Its crucial role as a negative regulator of the abscisic acid (ABA)-mediated response and a positive regulator of the salicylic acid (SA)-dependent defense pathway is consistent with its plasticity in response to multiple environmental stressors [124]. GmERD15 was isolated via its capacity to associate stably with the GmNRP-B promoter in vivo and in vitro to induce the *GmNRP-B* expression [125]. Although ERD15 from Arabidopsis does not bind to NRP promoters, the Arabidopsis orthologs of the NRP/GmNAC081–GmNAC030/VPE signaling module components have been shown to transduce a cell death signal in Arabidopsis with similar regulatory mechanisms [44,125]. The functional orthologs of the soybean pathway components were identified in Arabidopsis as *AtNRP-1* and *AtNRP-2* (GmNRPs), *ANAC036* (*GmNAC081*), *ATAF2*/ATAF1 (*GmNAC030*), and *gVPE;* they have been shown to be induced by ER and osmotic stress and to induce cell death when transiently expressed in *N. benthamiana* leaves. Importantly,

knockout lines of the selected Arabidopsis orthologs display enhanced tolerance to ER stress-mediated cell death [44,109]. Conserved sequences of the pathway components are present in several plant genomes; they represent a plant-specific signaling pathway widely distributed in the plant kingdom.

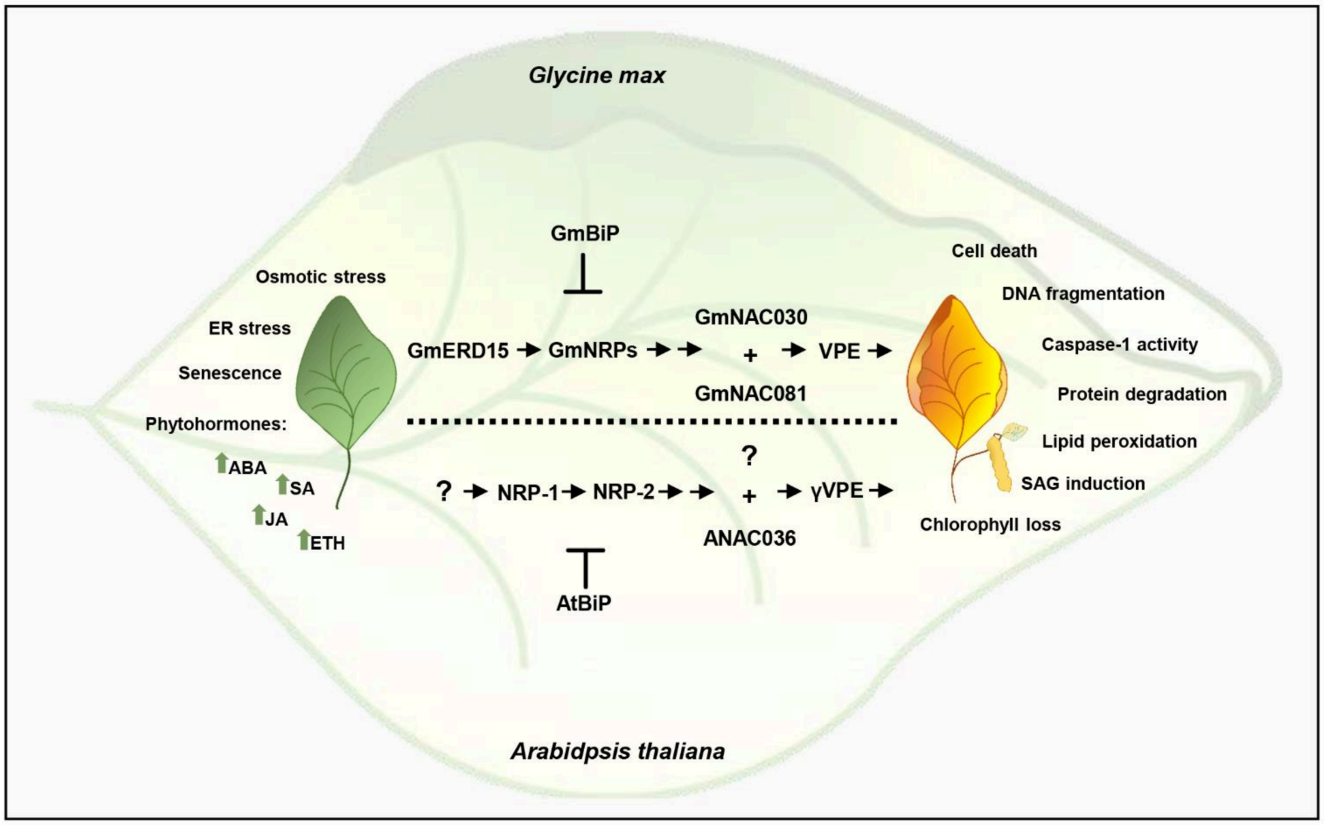

Figure 2. Integration of senescence signals and stress signals in the DCD/NRP-mediated cell death response. Leaf senescence, ER stress, and osmotic stress induce the expression of GmERD15, which upregulates GmNRPs to initiate a signaling cascade that culminates in the induction of GmNAC030 and GmNAC081 expression. The NAC transcription factors form a heterodimer to fully induce VPE promoter activation, leading to VPE expression and subsequent execution of a cell death program. The phytohormones positively regulate the signaling pathway, and 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. This DCD/NRP-mediated cell death signaling is conserved in other plant species, and the Arabidopsis orthologs are shown at the bottom of the figure.

Besides being synergistically activated by multiple stresses conditions, the DCD/NRP-mediated programmed cell death pathway is also associated with developmentally programmed leaf senescence. The expression of cell death pathway components NRP-A, NRP-B, GmNAC081, GmNAC0030, and VPE is associated with the onset and progression of leaf senescence. Furthermore, caspase-1 activity of the executioner of the DCD/NRP-mediated cell death VPE is highly increased in leaf senescence [5,43,75]. The transient expression in plants of soybean and Arabidopsis components of the cell death pathway induces a cell death response with hallmarks of leaf senescence, including induction of caspase-1-like activity and DNA fragmentation, chlorophyll loss, protein degradation, enhanced lipid peroxidation, and the induction of senescence-associated marker genes [44,56,109,118]. Furthermore, the phytohormones ABA, jasmonic acid (JA), and salicylic acid (SA), which positively regulate senescence, also induce the expression of DCD/NRP-mediated cell death pathway components [43]. More strictly, GmNAC081 overexpression accelerates leaf senescence, associated with enhanced chlorophyll degradation, faster photosynthetic decay, and higher expression of hydrolytic-enzyme-encoding GmNAC081/GmNAC030 target genes

including VPE, the executioner of programmed cell death [43]. Unlike the accelerated leaf senescence phenotype displayed by the GmNAC081-overexpressing lines, VIGS-mediated suppression of GmNAC081 expression delays leaf senescence and decreases the expression of GmNAC081 direct target genes, including VPE [43]. Thus, GmNAC081 emerges as a positive regulator of naturally programmed leaf senescence, which in turn may be integrated with multiple-stress-induced PCD via the NRPs/NACs/VPE regulatory circuit.

As a PCD-mediated phenomenon, senescence may provide a stress-escape mechanism. However, the regulatory signaling modules that integrate natural and stress-induced senescence very often inversely modulate senescence and stress tolerance. In the case of the NRPs/GmNACs/VPE signaling module, overexpression of GmNAC081 has been shown to accelerate leaf senescence and decrease drought tolerance considerably [41,43]. In the GmNAC081-induced transcriptome, SAGs and cell death inducers predominate in the upregulated changes, whereas regulators of ABA signaling are predominantly downregulated. This transcriptional landscape derived from GmNAC081 overexpression further supports the argument that leaf senescence is molecularly linked to drought tolerance. Compelling evidence in the literature has linked drought tolerance with the impairment of stressed leaves' ability to undergo senescence [126]. Further supporting this interpretation, the overexpression of a negative modulator of the DCD/NRP-mediated cell death signaling, the molecular chaperone binding protein (BiP), has been shown to delay leaf senescence and enhance drought tolerance [75,127].

The binding protein (BiP) belongs to the HSP70 family and plays a crucial role in the unfolded protein response (UPR) pathway. The chaperone BiP is an ER-resident protein that acts as a sensor of changes in ER homeostasis and accumulation of unfolded proteins in the organelle lumen [128,129]. Furthermore, BiP has been shown to attenuate ER-stress- and osmotic-stress-mediated cell death by downregulating GmNRP-A, GmNRP-B, and GmNAC081 expression under stress conditions. In contrast, the silencing of endogenous BiP enhances the stress-induced cell death response [109]. Therefore, the BiP cytoprotective function is associated with the inhibition of the DCD/NRP-mediated cell death response. As multiple stresses trigger DCD/NRP-mediated cell death signaling, the engineered control of this pathway might permit the coordination of adaptive cellular responses under a large array of stress conditions.

4. Conclusions

The NAC proteins comprise a large family of plant-specific transcription factors involved in stress responses and development. In general, approximately 40% of the NAC genes are upregulated in leaf senescence in flowering plants. Most of the knowledge about NAC-SAGs is derived from Arabidopsis studies and may not apply to all flowering plants. However, the high conservation among NACs from different crops and plant model systems suggests that biological information regarding Arabidopsis NAC-SAGs may be transferable to the studies of crops. In soybean, among 54 GmNAC-SAGs identified by functional genomic approaches, only three of them, GmNAC030, GmNAC065, and GmNAC081, have been functionally characterized. Interestingly, the functional characterization of GmNAC065 demonstrated that it plays a similar function as its Arabidopsis ortholog AtNAC083 (VNI2). Both are negative regulators of leaf senescence and control similar regulatory networks, targeting similar SAGs. These findings further substantiate the notion that similarity-based clustering of the NAC superfamily members along with expression profile correlates with their function, providing a reliable means to rationalize functional studies of the NAC gene family. GmNAC065 expression is correlated with drought tolerance, as it is highly expressed in drought-tolerant genotypes. These findings may implicate GmNAC065 as a potential target for molecular breeding and engineering towards drought tolerance.

GmNAC030 and GmNAC081 were first identified and characterized in soybean, but their orthologs from Arabidopsis show similar conserved functions. Both GmNACs are positive regulators of leaf senescence and are downstream components of a signaling

pathway that integrates multiple environmental signals into the plant's internal senescence information, culminating in PCD. Many aspects of this cell death pathway have been elucidated in the last years. We now know that multiple stress conditions activate the upstream component NRP, which is controlled by the ERD15 TF. NRPs induce GmNAC030 and GmNAC081, which form a dimer to fully induce the expression of VPE, the executioner of the cell death program via vacuole collapse. GmNAC081 and GmNAC030 also target the promoter of other senescence-promoting hydrolytic enzymes and repress the expression of senescence suppressors. However, several crucial players of this pathway are missing, and relevant questions remained unanswered. For example, we still do not know about the most upstream component of the pathway that senses external and internal signals of leaf senescence. How the signal is relayed from NRPs to GmNACs is unclear. We know, however, that modulation of this cell death pathway may confer tolerance to drought and other abiotic stresses. In fact, the protective function of BiP against water dehydration has been associated with its capacity to negatively modulate the extent of the cell death response resulting from the activation of DCD/NRP-mediated signaling. These findings might implicate this pathway as an excellent target for engineering superior crops.

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CHAPTER II

EXPANSION AND DIVERSIFICATION OF THE *GLYCINE MAX* (GM) ERD15-LIKE SUBFAMILY OF THE PAM2-LIKE SUPERFAMILY.

ABSTRACT

Expansion and diversification of the *Glycine max* (Gm)ERD15-like subfamily of the PAM2-like superfamily

The PAM2 motif represents a binding site for poly (A) binding proteins (PABP), often associated with RNA metabolism regulation. In plants, the PAM2-containing protein ERD15 stands out as a critical regulator of diverse stress responses. Despite the relevance of the PAM2 motif, a comprehensive analysis of the PAM2 superfamily and ERD15-like subfamily in the plant kingdom is lacking. Here, we provide an extensive in silico analysis of the PAM2 superfamily and the ERD15-like subfamily in Arabidopsis, soybean, and rice. The Glycine max ERD15-like subfamily members were clustered in pairs, originating from DNA-based gene duplication, as the paralogs display high sequence conservation, similar exon/intron genome organization, and are undergoing purifying selection. The complementation assays on an *aterd15* mutant demonstrated that the plant ERD15-like subfamily members are functionally redundant in response to drought, osmotic stress, and dark-induced senescence. Nevertheless, the soybean members display differential expression profile, biochemical activity, and subcellular localization, consistent with functional diversification. The *Gm04ERD15* expression profile under SA and ABA treatments differed from the other *GmERD15*-like genes oppositely. Furthermore, abiotic stress-induced co-expression analysis with soybean PABPs showed that *Gm04ERD15* was clustered separately from other *GmERD15s*. In contrast to the AtERD15 stress-induced nuclear redistribution, Gm04ERD15 and Gm02ERD15 localized in the cytoplasm while Gm03ERD15 fractionated between the cytoplasm and nucleus under normal and stressful conditions. These data collectively indicate that GmERD15-like subfamily members, in spite of modulating senescence and drought responses, are differentially induced by multiple stresses and may diverge partially in stress signaling functions.

Keywords: Senescence. drought. ERD15. soybean.

INTRODUCTION

The PAM2 motif is the second of two PABC domain-binding motifs PAM1 and PAM2, present in the C-terminus of poly(A)-binding proteins (PABPs), primarily involved in RNA metabolism (Albrecht & Lengauer, 2004). PAM1 and PAM2 motifs were first described in the human polyadenylate-binding protein-interacting protein 1 (Paip1) protein, a coactivator in translation initiation regulation of 3' polyadenylated mRNAs (Roy, 2002). The PAM2 motif is usually present in proteins related to mRNA metabolism or the formation of the scaffold for assembly of the ribonucleoprotein (RNP) complex on the poly-A tail of mRNAs (Albrecht & Lengauer, 2004). The PAM2 domain is often associated with other accessory domains, including the RRMs (RNA recognition motifs) that are known to bind single-stranded RNAs (Maris et al., 2005), the LsmAD (Like Sm associated domains) reported as a RNA Helicase binding motif (Jiménez and Guzmán, 2014), the TPRs (Tetratricopeptide repeats) that form scaffolds for protein interactions (D'Andrea and Regan, 2003), and the endonuclease Smr (Small MutS-related) domain involved in nucleic acid mismatch repair (Moreira and Philippe, 1999). Despite being extensively studied in the animal kingdom, a more comprehensive analysis of the PAM2 superfamily in the plant kingdom is still lacking. Among PAM2 superfamily members in plants, the PAM2 motif-containing protein ERD15 has emerged as an important modulator of plant cell signaling (Kariola et al., 2006; Alves et al., 2011a).

The Arabidopsis ERD15 (Early responsive to dehydration 15) (AT2G41430) was initially identified in screening genes rapidly induced by dehydration stress. The *ERD* genes (ERD1 to ERD16) were induced by a 1h dehydration treatment, and encode proteins with different biological functions and cell localization (Kiyosue et al., 1994). The members of the ERD15-like family are small acidic proteins with 99 to 177 amino acids. The ERD15-like family is confined to the plant kingdom, and orthologs can be found in photosynthetic organisms from green algae (Zygnemophyceae) to higher plants (Embryophyta). The ERD15-like subfamily members have some protein domains in common, the adjacent N-terminal PAM2 (PABP-interacting motif 2) and PAE1 (PAM2 associated element 1) motifs, followed by an acidic region and a highly conserved C-terminal QPR motif (Aalto et al., 2012).

Despite being predominantly found in the C-terminal portion of animal and yeast proteins, the PAM2 motif is located in the N-terminal portion of the ERD15-like proteins. All members of the ERD15 subfamily have the highly conserved PAE1 motif adjacent to PAM2. However, its function remains unknown. The non-conserved acidic region is present in all

family members and possibly is a remnant of the PAM1 motif, present in the human Paip1 and Paip2 proteins (Aalto et al., 2012). The QPR motif has a conserved amino acid sequence (isoleucine-glutamine/histidine-glutamine-proline-arginine) and is present in most ERD15-like subfamily members but absent in two of the six Glycine max homologs, Glyma03g131900 and Glyma19g133800 (Aalto et al., 2012).

In addition to its rapid induction in response to dehydration, AtERD15 (AT2G41430) has been implicated as a negative modulator of drought tolerance. While *ERD15* overexpression decreases drought tolerance, the RNAi silencing of *ERD15* confers tolerance to drought (Kariola et al., 2006). Recently, the apple Zinc-finger protein MdBBX7 has been shown to bind the promoter and activate the expression of *MdERD15* under drought stress (Chen et al., 2022). Under drought stress, *ERD15* was reported to be considerably induced in different species, including *MiERD15* from Morus indica (Saeed and Khurana, 2017), *IbERD15* from Ipomoea batatas (Shao et al., 2014), and *SpERD15* from Solanum pennellii (Ziaf et al., 2011), implicating *ERD15* as a drought stress marker gene. Furthermore, AtERD15 has been described as a negative regulator of the abscisic acid response, which controls the mRNAs encoding proteins involved in ABA core signaling, regulating stomatal closure (Aalto et al., 2012). ERD15 has also been reported to be induced by pathogen elicitors and modulate positively SA-dependent defense pathways (Brader et al., 2001; Kariola et al., 2006). The AtERD15-overexpressing lines display enhanced resistance to Erwinia carotovorum, associated with the upregulation of SA-mediated systemic acquired resistance (SAR) marker gene PR2 (Kariola et al., 2006).

The ERD15-like genes are classified as multiple stress-induced regulators due to their functional plasticity in response to a wide range of stresses. High salinity, cold, wounding, and hipergravity have been shown to stimulate *ERD15* expression in Arabidopsis (Park et al., 2009; Kariola et al., 2006; Walley et al., 2007; Yoshioka et al., 2003). *AtERD15* (*Lsr1*) has been characterized as one of the light stress-responsive (*Lsr1–5*) genes, which is highly induced by light stress (Dunaeva and Adamska, 2001). Solanum pennellii *SpERD15* is induced by drought (dehydration), salinity, cold, ABA, gibberellic acid, and ethylene (Ziaf et al., 2011). VaERD15 has been shown to improve cold tolerance in Chinese Wild Vitis amurensis (Yu et al., 2017). The soybean ERD15 ortholog (*Gm02ERD15*) is induced by endoplasmic reticulum stress (ER) and osmotic stress (Alves et al., 2011a) and acts as an upstream member of the NRP-mediated cell death response, binding to the NRP-B promoter region in vivo and in vitro. Although Gm02ERD15 does not harbor a typical DNA-binding motif, a conserved sequence of 13 amino acids (-71DEDEKERKEgKEv83-) at the acidic region binds to the 12-bp palindromic sequence

−511AGCAnnnnnTGCT−500 on the NRP-B promotor to activate *NRP-B* expression (Alves et al., 2011a). A recent genetic study using 34-salt tolerant accessions of Glycine soja has identified a naturally variant motif consisting of a 7-bp insertion/deletion in the promotor of *GsERD15B*, linked to enhanced salt tolerance of soybean and upregulation of the *GsERD15B* gene (Jin et al., 2021). The *GsERD15B* overexpression was also associated with increased expression of genes involved in ABA signaling, cation transport, proline content and dehydration-responsive genes, as a probable cause for the salt tolerance of the soybean accessions harboring the naturally variant GsERD15B promoter. In the soybean accession LY01-10 (Hap2), the 7-bp deletion in the GsERD15B promoter footprinted the formation of an 8-bp inverted sequence creating a possible binding site for autoregulation or for another transcription factor to enhance the expression of GsERD15B in response to salt stress. Therefore, like Gm02ERD15, the salt-tolerant function of GsERD15B may be associated with its transcriptional activity. In addition to transcriptional activity, GsERD15B interacts with PABPs, as expected from a PAM2.

ERD15-like subfamily members from different species have been shown to display diverse subcellular localizations and transactivation activity. The Gm02ERD15 exhibits cytoplasm/nuclear localization and transactivation activity, while the Arabidopsis thaliana ERD15 protein does not exhibit DNA binding activity in yeast (Alves et al., 2011b). The mulberry MiERD15 exhibits transactivation activity, mapped to a non-conserved acidic region before the C-terminal QPR motif, also present in Gm02ERD15 (Saeed and Khurana, 2017). The SpERD15 from Solanum pennellii is a nuclear protein (Ziaf et al., 2011), whereas GsERD15B fractionates in the nucleus and cytoplasm (Jin et al., 2021).

Despite the importance of the PAM2 motif and ERD15-like proteins in response to multiple stress, a comprehensive analysis of PAM2 superfamily and ERD15-like subfamily in Arabidopsis, soybean, and rice is lacking. Here, we performed in silico analyses showing the expansion of ERD15-like subfamily of the PAM2 superfamily in soybean. Our functional complementation assay demonstrated that the members of the GmERD15-like subfamily are functionally redundant in response to drought and dark-induced senescence but display differential expression profiles in response to the hormones SA and ABA. Finally, we provide comprehensive subcellular localization data of the ERD15-like subfamily members in Arabidopsis, soybean, and rice under different stress inducers.

MATERIAL AND METHODS

Construction of Plasmids

All recombinant constructs were generated from the GATEWAY cloning system. AtERD15 (AT2G41430), Gm02ERD15 (Glyma02g260800), Gm03ERD15 (Glyma03g131900), Gm04ERD15 (Glyma04g138600), and OsERD15 (Os07g46670) ORFs were amplified from Arabidopsis, soybean, and rice cDNA using specific primers (Table 1) and inserted by recombination into the entry vector pDONR207 (Invitrogen). These genes were then transferred from the entry vector to the expression vector 35S-YFP-cassetteA-Nos-pCAMBIA1300 by recombination, using the enzyme LR clonase (Invitrogen) to generate the clones pUFV2129 (YFP-AtERD15), pUFV2130 (YFP-OsERD15), pUFV1470 (YFP-Gm02ERD15), pUFV2537 (YFP-Gm03ERD15) and pUFV2538 (YFP-Gm04ERD15). The resulting clones harbor the specified ERD15 fused in-frame to the C-terminus of YFP under the control of the 35S promoter.

Plant Material, Growth Conditions and Stress Treatment

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild-type control. The Arabidopsis T-DNA mutant erd15 (SALK_073116) was obtained from Arabidopsis Biological Resource Center. The primers used for genotyping are listed in Table 1. The erd15 knockout line was complemented with AtERD15 (AT2G41430), Gm02ERD15 (Glyma02g260800), Gm03ERD15 (Glyma03g131900), Gm04ERD15 (Glyma04g138600), and OsERD15 (Os07g46670) by transformation via the *Agrobacterium tumefaciens* floral-dip method (Zhang et al., 2006). Agrobacterium strain GV3101 was used in all transformation experiments. Homozygous transformants were selected by hygromycin resistance. Three independent homozygous lines were selected for subsequent experiments.

For normal growth conditions, Arabidopsis plants were grown at 21◦C under a 12h light/12h dark cycle. For dark-induced senescence, the third and fourth rosette leaves of 4 week-old plants were enveloped with aluminum for 7 days in the dark. For seedling root growth under osmotic stress assay, seeds were germinated in MS-phytagel plates for 4 days and transferred to MS-phytagel plates supplemented with mannitol 200 uM for 6 days. The root length was measured using a pachymeter.

Soybean (Glycine max BR16) seeds were germinated and grown under greenhouse conditions (12h of light, 15–30◦C). For hormone induction, the roots at the V3 developmental stage were immersed in Clark's nutrient solution (Clark, 1975) supplemented with 100 uM

abscisic acid (ABA) or 5 mM salicylic acid (SA). Leaves from stressed and control plants were collected at 1h, 3h, 6h, and 12h post-treatment, immediately frozen in liquid nitrogen, and stored at −80◦C until processing. For the analysis of soybean ERD15-like subfamily members in senescence, leaf samples were collected at two different developmental stages, vegetative stage three (V3; 20 days after germination, DAG) and reproductive stage six (R6; 80 DAG) and frozen in liquid nitrogen.

For the drought treatment, wild-type soybean plants (Glycine max cv. Conquista) were germinated in a greenhouse in pots containing a mixture of soil and fertilizer (3:1) under natural light conditions, relative humidity of 65-85% and temperature ranging from 35ºC during the day to 15ºC at night. The plants were stressed for 25 days and different points were collected at different water potentials: 19, 23 and 25 days, ψW = 1.0, ψW = 1.7 and ψW = 2.0, respectively (Carvalho et al.; 2013). The collected samples were immediately frozen in nitrogen and stored at -80ºC.

For the drought treatment, *Arabidopsis thaliana* (ecotype Columbia) seeds were sown in moistened peat pellets (Jiffy Products), stratified at 4°C for 2 days, and then transferred to a growth room kept at 10h of light (100 µmol m−2 s−1) and 22°C. Two types of drought treatment were conducted, moderate drought (mDr) and progressive drought (pDr), as described by Harb et al. (2010). For moderate drought, controlled mDr was maintained by keeping the soil moisture level at 30% of field capacity, which is 200% or 2 g water g−1 dry soil, for 10 days. For pDr, water was withheld at 35 days after sowing, and peat pellets were kept to dry and monitored by weighing the peat pellets until the required pDr level was reached.

In Silico Analyses

For microarray data analysis, we retrieved the soybean data obtained by Carvalho et al. (2013) (GSE50408) representing soybean, and Harb et al. (2010) (GSE24177) representing Arabidopsis. For heatmap construction, the cell intensity reading files (CEL) were analyzed with the R/Bductor package (Li & Wong, 2001), normalized by the robust multiarray analysis (RMA) method (Irizarry et al., 2003), which is available in the R/Bioconductor package for Affymetrix arrays (Kerr et al., 2000). The Bioconductor 'limma' library was used to analyze the expression data, the 'mclust' package for data clustering and the 'gplot' package for heatmap plotting. The value of log2fc (log base 2 of the contrast between treatment and control) was used for data comparison.

For the characterization of the PAM2 superfamily of proteins and the ERD15-like

subfamily in Arabidopsis, soybean and rice, databases of the respective proteomes (www.phytozome.com) were used for comparison with PAM2 motif databases (pfam.sanger.ac.uk/). After the recovered of protein sequences that contained an annotated PAM2 domain, a new search and re-annotation was performed using the HMMER program (Finn et al., 2011) to find those that had the domain, but not the annotation. The phylogenetic analysis was performed using the MEGA6 program (Tamura et al., 2013), and the trees were inferred by the Neighbor-Joining methodology (Saitou & Nei, 1987). The motifs and conserved domains among the identified groups were found with the HMMER software (Finn et al., 2011), through the HMMsearch suite, and compared to the Phytozome database (www.phytozome.org). Only domains with an e-value less than or equal to 0.01 were considered to compare between the amino acid sequence and the domains.

For molecular evolution analyses, the R package seqinr (http://seqinr.r-forge.r-project.org) was used to calculate the non-synonymous (Ka) and synonymous (Ks) rate ratio of the paralog genes. The formula applied by Zhang et al. (2018) was used to calculate the divergence time [$T = Ks/(2 \times 6.1 \times 10{-}9) \times 10{-}6$ Mya].

For coexpression analysis, *Glycine max* RNA-seq samples were used in EXPath expression database under abiotic conditions: cold (GSE117686), dehydration (GSE112584), drought (GSE69469), low pH (GSE112584), Pi deficiency (GSE104286), salt (GSE57960/GSE69571), and zinc deficiency (GSE111799) and biotic conditions: Fusarium oxysporum (GSE66861), and weedy (GSE59875). The Pearson's correlation coefficient was used to measure the linear correlation between the coexpressed genes. Correlation coefficient >0.7 were selected.

Drought Stress

Drought stress was performed on 4-week-old plants. Seeds were germinated for 2 weeks and then transplanted to individual pots filled with soil that had been well-watered for 2 weeks. To induce drought stress, the plants were grown in a greenhouse (at 21◦C under a 12 h light/12 h dark cycle) without water for 18 days. The relative water content was measured during this period, and the survival rate was measured after 2 days of re-watering. The relative water content was calculated as described by Barrs and Weatherley (1962): RWC (%) = [(FW - DW)/(TW -DW)] x100, where FW is initial fresh weight, TW is turgid fresh weight, DW is dry weight, and RWC is the relative water content.

Chlorophyll Content

Total chlorophyll content was determined spectrophotometrically at 645 nm, and 663 nm after quantitative extraction from individual leaf disc with ethanol. The total chlorophyll content was calculated by the expression: Clf(a+b) = (20.2·A645nm) + (8.02·A663nm), and expressed as µg/leaf disc.

RNA Extraction, cDNA Synthesis, and Quantitative RT-PCR

Total RNA was extracted from 100 mg of powdered leaves using Trizol (ThermoFisher) and quantified using NanoDrop spectrophotometer. cDNA synthesis was performed with 4 µg of total RNA (previously treated with DNase I Amplification Grade Thermo Fisher Scientific), 10 µM oligoDT (18T), 10 mM dNTPs, and 1 U of MMLV reverse transcriptase, according to the manufacturer's instructions (ThermoFischer).

Quantitative RT-PCR assays were performed with an ABI 7500 instrument (ThermoFisher) using SYBR Green PCR Master Mix (Life Technologies), gene-specific primers (Table 1), and cDNA from three biological replicates with two technical replicates. Relative gene expression was quantified using the 2-ΔCt method. The endogenous control gene UKN-2 (Glyma.06G041800) (Libault et al., 2008) and Actin 2 (At3g18780) (Liu e Howell, 2010) were chosen as the normalizer.

Subcellular Localization

The subcellular localization of the Arabidopsis, soybean, and rice ERD15-like subfamily members was observed by confocal microscopy in Arabidopsis complemented lines. Roots of 3 days-old seedlings were transferred to 12 wells plates filled with MS ¼ strength medium. After 24h, the MS medium was replaced by MS ¼ strength supplemented with polyethylene glycol (PEG 10%), Tunicamycin 5 ug/ml, and ABA 10 uM for 16h and analyzed under Zeiss LSM510 META Laser Confocal Microscopy. YFP was excited by argon/helium-neon laser system in 514 nm wavelength, and emission was collected in 530–600 nm band-pass filter. Images were captured and treated in LSM Image Browser 4 (Carl-Zeiss) software.

Nuclear Fractionation

The nuclear fractionation protocol was adapted from Yamaguchi et al. (2014). The seeds were germinated in MS medium for 2 weeks and then transferred to 12-well plates filled with MS medium. After 24h, the MS medium was replaced by MS supplemented with polyethylene glycol (PEG 10%), Tunicamycin 5 ug/ml, and ABA 10 uM for 16h. The samples were weighted to 400 mg, pulverized in liquid nitrogen and ground in 600 uL of Nuclear Extraction Buffer

[100 mM MOPS pH 7,6, 10 mM MgCl2 , 0,25 M sucrose, 5% dextran T-40, 2,5% Ficoll 400,40 mM 2-Mercaptoetanol] supplemented with protease inhibitor cOmplete. The extract was filtered in 100 µm nylon and centrifuged. The supernatant (cytosol fraction) was removed and quantified via the Bradford method. The pellet (nuclear fraction) was resuspended in 60 µL of Nuclei Lysis Buffer (50 mM Tris-HCl pH 8,0, 10 mM EDTA pH 8,0, 1% SDS). Samples were kept on ice for 30 min and agitated at every 10 min interval. The samples were then resuspended in 120 µL of Protein Extraction Buffer [50 mM Tris-HCl pH 8,0,1% (v/v) NP-40, 2mM PMSF, 2 mM Benzamidine].

Equivalent amounts of nuclear and cytosol fractions were resolved on 10% (w/v) SDS-PAGE gel, transferred to a nitrocellulose membrane and immunoblotted with Rabbit anti-HA (Invitrogen, 71-5500) and Rabbit anti-GFP (Invitrogen, A11122). As for loading and contamination control between fractions, Rabbit anti-UGPase (Agrisera, AS05 086) and mouse Anti-RNApolII (Sigma, 087K0498) antibodies were deployed. The membranes were washed and incubated with Goat Anti-Rabbit-HRP (Invitrogen, 65-6120) and goat anti-mouse-HRP (Santa Cruz Biotechnology, sc-2005) conjugated antibodies. The detection was performed with the SuperSignal™ West Dura Extended Duration Substrate, according to the manufacturer's instructions via the ChemiDoc® photodocumentator (Bio-Rad™).

RESULTS

ERD15-like is a subfamily of the PAM2 superfamily expanded in soybean.

The protein sequences of the PAM2 superfamily in Arabidopsis, soybean, and rice were recovered from databases of the respective proteomes, and 72 proteins with the PAM2 motif were identified (Figure 1a). Among them, 19 were identified in Arabidopsis, 31 in soybean, and 22 in rice. The phylogenetic analysis clustered the proteins into 7 functionally related subgroups sharing similar domain architectures (Figure 1b).

Group A represents the ERD15-like subfamily, which is characterized by the presence of 3 conserved regions, the N-terminal PAM2 motif, PAE1 (PAM2 associated element 1) motif, and a highly conserved C-terminal QPR motif. Group A has 11 proteins, 2 from Arabidopsis, 6 from soybean, and 3 from rice. Interestingly, the Gm03ERD15 (Glyma03G131900) and Gm19ERD15 (Glyma19G133800) lack the QPR motif in the C-terminal. Moreover, Gm03ERD15 is the only ERD15 subfamily member with an additional domain, the vacuolar sorting protein 39 domain 2 (Vps39_2), responsible for protein-protein interaction and

localization of VPS39 (Wurmser et al., 2000).

Group B is composed of the largest proteins of the PAM2 superfamily, ranging from 813 to 1859 amino acids with 3 conserved regions, the N-terminal CLUstered mitochondrial (CLU) domain, the eukaryotic initiation factor 3, subunit 153 (eIF3_p135) domain, the tetratricopeptide repeat (TPR), and the C-terminal PAM2 domain. In addition, 3 members (Glyma.07G028800, Glyma.08G214000, and LOC_Os04g55230) present the Anaphase-promoting complex/cyclosome, subunit 3 (Apc3) domain mapped between the eIF3_p135 and the TPRs domains. The CLU domain has been reported as crucial for the mitochondria's correct functioning transport (Zhu et al., 1997; Fields et al., 2002). The eIF3_p135 is part of the 135 subunits of the eIF3 protein complex required for translation initiation in eukaryotic cells (Vornlocher et al., 1999). TPR motifs are characterized by having 3-16 tandem repeats of 34 amino acids residues, which serve as a protein-protein interaction-promoting scaffold (Van Bibber et al., 2020). The Apc3 domain has been reported as a subunit present in the E3 ligase complex that controls mitosis by ubiquitination (Yamaguchi et al., 2015). Group B has 7 proteins, 2 from Arabidopsis, 4 from soybean, and 1 from rice. The Arabidopsis members were described as reduced chloroplast coverage REC1 (AT1G15290) and REC2 (AT4G28080), which regulates the size of the chloroplast compartment (Larkin et al., 2016).

Group C proteins range from 419 to 624 amino acids with 2 conserved regions, the N-terminal Ataxin 2 SM (SM-ATX) domain and a PAM2 motif located in the middle of the protein. The SM-ATX domain has been shown to interact with RNA helicase DDX6, modulating mRNA degradation, stability, and translation (Nonhoff et al., 2007). Group C has 7 proteins, 2 from Arabidopsis, 2 from soybean, and 3 from rice. The Arabidopsis members were identified as a polyadenylate-binding protein-interacting protein with an unknown function (Arabidopsis Interactome Mapping Consortium, 2011).

Group D proteins range from 495 to 718 amino acids with 3 conserved regions, the N-terminal SM-ATX, the like-Sm-associated domain (LsmAD) in the central portion, and the C-terminal PAM2 domain. The LsmAD and SM-ATX domains can be found in eukaryotic ataxin-2, which regulates RNA metabolism (Ralser et al., 2002). Group D has 7 proteins, 2 from Arabidopsis, 4 from soybean, and 1 from rice. The Arabidopsis members CTC-interacting domain CID3 (AT1G54170) and CID4 (AT3G14010) have been reported to act redundantly in developmental pathways throughout the life cycle of plants (López-Juárez et al., 2021).

Group E proteins range from 399 to 545 amino acids with 3 conserved regions, the N-terminal PAM2 domain, followed by two domains in the central portion; an RNA recognition

motif (RRM) and the La domain. In ribonucleoprotein particles (RNPs), the RRM and La domains are responsible for regulating transcription in the nucleus and translation in the cytoplasm (Kenan and Keene, 2004). Group E has 9 proteins, 2 from Arabidopsis, 5 from soybean, and 2 from rice. The Arabidopsis members la and related proteins LARP6B (AT2G43970) and LARP6C (AT3G19090) have been implicated in translation control and mRNA transport (Bousquet-Antonelli, 2021).

Group F proteins range from 513 to 693 amino acids with 3 conserved regions, the N-terminal PAM2, and two C-terminal domains, unknown function (DUF1771), and Small MutS-related (Smr) domains. The DUF1771 domain represents a highly charged extension of the Smr domain, which has an endoribonuclease function (Glover et al., 2020). Group F has 8 members, 3 from Arabidopsis, 3 from soybean, and 2 from rice. Only one Arabidopsis member has been described in the literature, CID7 (AT2G26280), identified as an interaction partner of Arabidopsis PAB2 and PAB5 (Bravo, 2005).

Group G was divided into three subgroups. Group G1 proteins range from 296 to 362 amino acids, with 3 conserved regions, N-terminal PAM2 domain, and two RRM domains at the central portion. Group G1 has 7 members, 2 from Arabidopsis, 2 from soybean, and 3 from rice. The Arabidopsis member CID8 (AT1G53650) was identified as an interaction partner of Arabidopsis PAB2, and PAB5, as CID7 of group F (Bravo, 2005). Group G2 protein range from 302 to 406 amino acids, with 3 conserved regions, a central PAM2 domain, followed by two C-terminal RRM domains. Group G2 has 9 members, 4 from Arabidopsis, 4 from soybean, and 1 from rice. The Arabidopsis members have been described as the RNA-binding proteins CID10 (AT3G49390), CID11 (AT1G32790), CID12 (AT4G10610), and CID13 (AT5G24440) (Bach-Pages et al., 2020). Group G3 proteins range from 144 to 185 amino acids, with 2 conserved regions, an N-terminal PAM2 domain and a C-terminal CUE domain. The CUE domain is required for intramolecular monoubiquitylation, a post-translational modification (Shi et al., 2003). The group G3 has 3 members; all are from rice. Only 4 proteins remained isolated from the 7 main groups, 3 from rice and 1 from soybean.

Compelling evidence in the literature has implicated the ERD15-like subfamily members in drought-stress, cell-death responses, and hormonal regulation (Kyouse et al., 1994; Alves et al., 2011a; Kariola et al., 2006). Nevertheless, the already described functional divergence between Arabidopsis and soybean members (Alves et al., 2012) prompted a comprehensive analysis of the ERD15-like subfamily to uncover further related or divergent functions in soybean, Arabidopsis, and rice. The phylogenetic analysis of the ERD15-like subfamily in Arabidopsis, soybean, and rice demonstrated two different clades (Figure 2a). The

upstream component of the NRP-mediated signaling *Gm02ERD15* (Glyma02G260800) clustered separately from the other members of soybean and Arabidopsis but grouped with the rice members (Os03g36900), (Os07g01260), and (Os05g27780). The other clade was split into two subgroups. In the first subgroup, *AtERD15* (AT2G41430) is clustered with *Gm03ERD15* (Glyma03G131900) and *Gm19ERD15* (Glyma19G133800). In the second subgroup, *AtERL15* (AT4G14270) clustered with *Gm04ERD15* (Glyma04G138600) and *Gm11ERD15* (Glyma11G149900). The six members of the GmERD15-like subfamily are grouped in pairs in the phylogenetic tree, as closely related duplicated genes, and the sibling paralogs have similar exon/intron organization. *Gm02ERD15* and *Gm14ERD15* have a small 5'UTR region, followed by a CDS region separated by an intron and a large 3'UTR region. *Gm04ERD15* and *Gm11ERD15* have a 5'UTR region separated by an intron, followed by a CDS region separated by an intron, and a normal-sized 3'UTR region. *Gm03ERD15* and *Gm19ERD15* have a 5'UTR region separated by a large intron, followed by a large CDS region, an intron, a small-sized CDS region, and a 3'UTR region.

Therefore, the duplication of ERD15-like family members in soybean is evident through phylogenetic analysis (Figure 2a) and exon/intron organization (Figure 2b). However, the GmERD15-like sibling paralogs possessed identical numbers, localization, and lengths of exons, in addition to high conservation of amino acid sequence and genomic regulatory sequences. In fact, conserved cis-regulatory elements were found in 5' flanking sequences of all GmERD15-like genes (Table 2). The lack of significant divergence in regulatory regions and changes in the coding regions between the paralogs is indicative that shifts in expression patterns and neofunctionalization of the pairs may not have occurred yet.

Polyploidization events have been considered essential for increasing biodiversity. Two large-scale duplication events were found in soybean, 15 and 44 Mya (Schlueter et al., 2014). The duplication of these genes indicates that 3 GmERD15-like genes had diverged in soybean before a duplication event, subsequently leading to the existence of 6 GmERD15-like genes in the plant genome. The molecular evolution of the duplicated pairs was investigated by calculating the ratio of non-synonymous to synonymous substitutions (Ka/Ks). The three pairs were formed by the second event of the whole-genome duplication of Glycine max, with the divergence period ranging from 7 to 14 Mya (Figure 2c). Our molecular evolution analysis also indicates that all GmERD15-like pairs are under a purifying selection with a ratio Ka/Ks < 1.

Coordinated regulation of the NRP/GmNACs/VPE signaling module components with ERD15-like in soybean

In addition to being first identified as an early responsive gene to dehydration, ERD15 is very well-characterized as a drought marker gene in several species (Kiyosue et al., 1994, Saeed and Khurana, 2017, Shao et al., 2014, Ziaf et al., 2011). However, an expression profile analysis of the ERD15 subfamily members at different drought intensities in Arabidopsis and soybean is lacking.

To analyze the Arabidopsis ERD15-like subfamily members in drought conditions, we retrieve the gene expression data obtained by Harb et al. (2010) (GSE24177) under two drought treatments, and a heatmap was built allocating genes with similar expression profiles in the same clades (Figure 3a). The progressive drought (pDr) treatment causes a severe level of stress, confirmed by the induction of marker genes related to senescence and programmed cell death (PCD), such as *VPEγ* (AT4G32940), *VPEα* (AT2G25940), *AtNRP1* (AT5G42050), and *AtNRP2* (AT3G27090), which does not occur with the same intensity in the adaptation to moderate drought (mDr10). The Arabidopsis ERD15-like members were clustered into different groups; *AtERD15* (AT2G41430) displayed a profile more responsive to moderate drought, whereas *AtERL15* (AT4G14270) exhibited a profile more responsive to progressive drought, coordinately clustered with the NRP/NACs/VPE signaling module components. The divergent response profile of the Arabidopsis ERD15-like subfamily shows that these genes may diverge in function or play complementary roles at different stress intensities.

The soybean ERD15-like subfamily members were also evaluated in drought conditions. The soybean data obtained by Carvalho et al. (2013) (GSE50408), under different water potentials, were retrieved to evaluate the expression profile of the Glycine max (Gm) ERD15-like subfamily members, at different stages of drought (Figure 3b). The heatmap highlights the functional diversity of the PAM2 superfamily, which has members in all clusters with different expression profiles. The NRP/GmNACs/VPE signaling module components are concentrated in two clusters. *GmNAC030* and *GmNRP-A* are associated with the third cluster, upregulated under all stress conditions. *GmNRP-B* and *VPE* with the sixth cluster are strongly induced by high water deprivation. Interestingly, the ERD15-like subfamily members are also grouped into these two clusters. *Gm04ERD15* and *Gm11ERD15* had a moderate induction profile under all stress conditions and were associated with the third cluster. *Gm02ERD15* and *Gm14ERD15* are also induced in high water deprivation and were associated with the sixth cluster. Like in Arabidopsis, the Glycine max ERD15-like subfamily members are differently induced by drought stress, which may indicate they are triggered at different stress severity. In order to decipher if ERD15-like subfamily members have functional redundancy or divergence, the *erd15* knockout line (SALK_073116) was transformed with *AtERD15* (AT2G41430),

Gm02ERD15 (Glyma02g260800), *Gm03ERD15* (Glyma03g131900), *Gm04ERD15* (Glyma04g138600), and *OsERD15* (Os07g46670) for further functional complementation analyses (Figure 4b).

Functional overlap of the GmERD15-like subfamily members in dark-induced senescence

The NRP/GmNACs/VPE signaling module has been associated with drought stress and leaf senescence (Pimenta et al., 2016; Ferreira et al., 2020; Fraga et al., 2021). However, the upstream component of this programmed cell death pathway, Gm02ERD15, and other ERD15-like members were not evaluated under senescence conditions. Accordingly, we demonstrated that *Gm02ERD15*, *Gm03ERD15*, and *Gm04ERD15* are strongly induced under natural leaf senescence, reinforcing its functional plasticity as a multiple stress-induced regulator (Figure 4a). To further assess the function of GmERD15 in the dark-induced senescence, we first confirmed the involvement of AtERD15 in leaf senescence by examining typical dark-induced senescence phenotypes in *erd15* knockouts. We selected an *AtERD15* T-DNA insertional mutant line from Arabidopsis, which barely expressed *AtERD15* transcripts (Figure 4b), and prepared three complemented lines (*erd15/AtERD15*). Interestingly, in addition to enhancing drought and cold tolerance (Kariola et al., 2006), silencing *ERD15* expression enhanced dark-induced senescence tolerance (Figure 4c and 4d). The *erd15* knockout lines conserved a greenish phenotype during the dark treatment, a phenotype associated with higher chlorophyll content than the wild-type leaves, and was reversed by reconstructing *ERD15* expression in the complemented line (Figure 4c and 4d). All the complemented lines, expressing ectopically the *ERD15* gene from Arabidopsis, three *ERD15-like* genes from soybean, and the *OsERD15-like* from rice, exhibited a sensitivity phenotype to dark-induced senescence, complementing the knockout tolerance phenotype. Here, we provide evidence that AtERD15 negatively regulates leaf senescence, and the GmERD15-like subfamily members are functionally redundant in dark-induced senescence.

The members of the GmERD-15 like subfamily are functionally redundant in response to drought.

The silencing of the drought marker gene *AtERD15* has been shown to increase drought tolerance, while the *ERD15* overexpression enhances the plant sensitivity to drought (Kariola et al., 2006). Taking advantage of the drought tolerance phenotype of *aterd15* lines, a drought stress assay was conducted to assess the function of the ERD15-like members under water

deprivation. To induce drought stress, 4 week-old-plants were left without water for 18 days monitoring the relative water content; after that, the plants were re-watered for the survival rate analyses (Figure 5a). The *erd15* mutant maintained a high percentage of water content during all assays (50% at 18d) and displayed a high survival rate (70%), indicating a drought tolerance phenotype as previously described (Figure 5b and 5c). Expression of *AtERD15* in the *erd15* genetic background recapitulated the wild-type drought susceptible phenotype, and the complemented transgenic line displayed an enhanced drought sensitivity phenotype: with a significant loss of water content (20% at 18d) through the experiment and a low survival rate (20%). Likewise, the *GmERD-15* like genes and *OsERD15-like* gene were capable of complementing the *erd15* knockout line and exhibited similar loss of water content (20%) as *erd15/AtERD15* line and a lower survival rate varying from 18 to 3%, which correlated with the higher expression of the transgene in these complemented lines (Figure 3 and Figure 5c). To complement the drought stress assay, we measure the root growth of the transgenic lines under the osmotic stress inducer mannitol (Figure 5d). Under osmotic stress, the Col-0 (wt) display 65% relative root growth compared to normal growth conditions, while the *erd15* knockout line displays a tolerance to root growth inhibition phenotype, with 90% relative root growth under osmotic conditions. The ERD15-like complemented lines exhibited a range of 60% to 80% relative root growth, partially complementing the knockout tolerance phenotype. Under drought and osmotic stress, the ERD15-like subfamily members display a functional redundancy, despite the differential expression profile observed in Figure 3.

The members of the GmERD15-like subfamily display differential expression profile in response to the hormones ABA and SA

Consistent with its role as a multiple stress-induced regulator, the Arabidopsis ERD15 has been reported to be rapidly induced by abscisic acid (ABA) and slower induced by salicylic acid (SA). Furthermore, AtERD15 has been described as a negative modulator of ABA-response and a positive modulator of SA-response (Kariola et al., 2006). To assess the expression profile of the GmERD15-like subfamily in response to the hormones ABA and SA, Glycine max BR16 roots were immersed in Clark's nutrient solution supplemented with ABA (100 uM) and SA (5 mM) for 1h, 3h, 6h, and 12h (Figure 6). As previously described, *Gm02ERD15* was rapidly induced after 1h of ABA treatment, followed by *Gm03ERD15* induction, which showed a small peak at 1h post-treatment but not statistically significant (Figure 6a). *Gm04ERD15* seems to display a differential expression profile from other

subfamily members, keeping its expression constant throughout the ABA induction. *Gm02ERD15* and *Gm03ERD15* displayed a similar rapid induction by SA as observed by ABA, although the SA induction was more persistent until 3 hours of treatment (Figure 6b). Curiously, *Gm04ERD15* was downregulated by the long SA induction, indicating that despite being functionally redundant in senescence, drought, and osmotic stress, the ERD15-like subfamily members may trigger differential responses to hormone induction.

Only Gm03ERD15-like displays similar nuclear localization as AtERD15 in response to stress conditions

The characterization of the subcellular protein localization is an essential clue to its cellular functions. The ERD15 proteins from different species have been described as nuclear and/or cytosolic proteins, which are consistent with their role as a PABP-binding protein and mRNA regulator (Alves et al., 2011a; Ziaf et al., 2011; Jin et al., 2021). Despite the presence of the PABP-interacting motif (PAM2) in all members of the ERD15-like subfamily and the reported interaction between AtERD15 and AtPAB2/AtPAB4/AtPAB8, the correlation between Glycine max ERD15-like members and Glycine max PABs has not been explored yet (Aalto et al., 2012). Then, we analyze the co-expression correlation between (Gm)ERD15-like members and (Gm)PABs under abiotic and biotic stress (Figure 7). Under abiotic conditions, two *GmPAB2* ortologs (Glyma05g31026 and Glyma08g14240) and *GmPAB8* (Glyma02g11580) represent the largest number of correlationship, with a positive co-expression correlation with *Gm03ERD15*, *Gm04ERD15* and *Gm19ERD15* (Figure 7a and 7c). Under biotic conditions, other *GmPAB2* (Glyma20g14240) and *GmPAB8* (Glyma06g04460) stand out as the largest number of correlationship, with a positive co-expression correlation with *Gm02ERD15*, *Gm04ERD15*, *Gm11ERD15*, *Gm14ERD15* and *Gm19ERD15* (Figure 7b and 7c). The expansion of both the Glycine max ERD15-like subfamily and the Glycine max PABs family raises the possibility of complementary or divergent functions in different conditions. Consistent with that, we provide evidence that different PABs orthologs are co-expressed with distinct ERD15-like members under diverse conditions, which may indicate the expression/translation regulation of different sets of mRNAs.

Here, we further evaluated the cellular localization of members, not yet described, under normal conditions and in the presence of stress inducers (Figure 8). Under normal conditions, Gm03ERD15 is the only one that displays nuclear localization (Figure 8a). Interestingly, Gm03ERD15 is the only subfamily member that has an extra conserved domain (Vps39_2),

which is described as responsible for cellular localization and a surface-protein interaction in VPS39 protein (Wurmser et al., 2000). Under stress induction, only AtERD15, OsERD15, and Gm03ERD15 present nuclear localization. AtERD15 was relocated to the nucleus under all tested conditions: osmotic stress induced by PEG 10%, ER stress induced by tunicamycin 5 ug/ml, and ABA induction response by ABA 10 uM for 16h. The OsERD15 was found in the nucleus only under ER stress, and Gm03ERD15 remained in the nucleus under all tested conditions. Curiously, the Gm02ERD15, described as a nuclear protein, which binds the NRP-B promoter were not observed here in the nucleus (Alves et al., 2011a). However, this difference may be that Gm02ERD15 localization was first described in tobacco leaf cells by a transient expression, and here we provide a subcellular localization in Arabidopsis roots by a stable expression. To confirm the stress-induced AtERD15 subcellular relocation to the nucleus, a nuclear fractionation was performed using Anti-GFP for AtERD15 detection, Anti-RNApolII for nuclear fraction control, and anti-UGPase as a cytoplasmic fraction marker (Figure 8b). In response to the treatments, the predominant accumulation of AtERD15 protein in the nucleus confirmed the confocal microscopy results (Figure 8a). Therefore, the ERD15-like subfamily members in Arabidopsis, soybean, and rice are predominantly located in the cytoplasm. Under certain conditions, ATERD15 and OsERD15 relocate to the nucleus, and Gm03ERD15 remains in the nucleus under all tested conditions. The differential subcellular localization of the GmERD15-like subfamily members may be indicative of partial functional divergence among GmERD15s.

DISCUSSION

The PAM2 motif is largely distributed in eukaryotic proteins and is found at the carboxyl terminal of PABP and ubiquitin ligases (Roy et al., 2002; Deo et al., 2001). Despite its relevance, a comprehensive analysis of the PAM2 domain in the plant kingdom is missing. This investigation describes an updated characterization of the PAM2-like superfamily in Arabidopsis, soybean, and rice (Figure 1a). A phylogenetic analysis clustered the plant PAM2-containing proteins in seven groups (A-G), each with distinct conserved domains (Figure 1b). We suggest that the functional diversity of the PAM2-like subfamilies are associated with their conserved accessory domains, which can dictate distinct protein interactions or confers additional function.

Interestingly, group A or the ERD15-like subfamily members were identified as a subfamily of multiple stress-induced regulators with functional plasticity in response to diverse

environmental conditions. Here, we provide pieces of evidence for duplication and expansion of ERD15-like subfamily members in soybean through robust phylogenetic analysis, exon/intron organization, molecular evolution, and conserved cis-regulatory elements (Figure 2). Accordingly, the absence of a conserved QPR motif at the C-terminus of a highly conserved GmERD15-like gene pair indicates gene duplication after initial diversification into three genes. Furthermore, we found that the duplicated genes are under a purifying selection, which prevents evolutionary mutations at critical functional sites (Figure 2c).

Several lines of evidence indicate that the members of the soybean ERD15-like subfamily diverge in expression profile and possibly in protein-protein interactions. The contrast between the expression pattern of *Gm04ERD15/Gm11ERD15* gene pair and other (Gm)ERD15-like members is evident in our microarray, hormone-induced, and coexpression analysis. The *Gm04ERD15/Gm11ERD15* gene pairs are induced by moderated drought stress, whereas the *GmERD15-like* members are strongly induced by severe drought stress (Figure 3b). *Gm04ERD15* also exhibits an expression pattern different from the other subfamily members in response to ABA and SA hormones. *Gm04ERD15* is not induced by ABA but is repressed during prolonged SA treatment (Figure 6a and 6b). Under abiotic stress, a coexpression analysis clustered *Gm04ERD15* separately from the other subfamily members and the main correlated PABPs (Figure 7a). The *Gm11ERD15* paralog displayed the same isolated profile from the main coexpression correlations between PABs and GmERD15-like members under biotic stress (Figure 7b). Our results indicate that the differential expression profile of *Gm04ERD15/Gm11ERD15* gene pairs may have functional implications under different conditions.

The expression profile and coexpression analysis correlated well with the phylogenetic analysis that placed the *Gm04ERD15/Gm11ERD15* gene pairs separately from *AtERD15*. In contrast, functional complementation analyses of an *aterd15* knockout line demonstrated that representatives of all three GmERD15-like clusters function redundantly during dark-induced leaf senescence and in response to drought stress (Figures 4 and 5).

Our results implicated AtERD15 as a positive modulator of dark-induced senescence (Figure 4). As a transcriptional activator of the *NRP-B* gene, which regulates leaf senescence (Pimenta et al., 2016; Alves et al., 2011a), Gm02ERD15 is expected to function in leaf senescence, which is consistent with its capacity to complement *aterd15* function in senescence. Nevertheless, AtERD15 does not seem to be involved in the NRPs/NACs/VPE cell death

signaling module because AtERD15 neither binds to the NRP-B promoter nor transactivates a target promoter in yeast (Alves et al., 2011b). Therefore, Gm02ERD15 and AtERD15 may regulate dark-induced senescence by a different shared pathway. Although SA modulates leaf senescence and AtERD15 induces SA signaling (Kariola et al., 2006), the GmERD15-like members may diverge functionally in SA signaling because SA represses *Gm04ERD15*, which is in marked contrast with the SA-mediated induction of the other GmERD15 members (Figure 6). The *Solanum pennellii SpERD15* is induced by several phytohormones, including ethylene (Ziaf et al., 2011), an essential modulator of leaf senescence (Wang et al., 2021). The ethylene signal transduction positively regulates several essential targets in the leaf senescence progression as transcription factors and chlorophyll catabolic genes (Chang et al., 2013; Ren et al., 2010). Accordingly, cis-regulatory motifs recognized by the ethylene signaling transcription factors are conserved in all promoter regions of (Gm)ERD15-like subfamily members (Table 2). Further studies are necessary to decipher the role of the ERD15-like subfamily in ethylene signaling response and its relationship with senescence.

Consistent with its transactivation activity, Gm02ERD15-GFP transiently expressed in tobacco leaves has been previously shown to be nuclear localized (Alves et al., 2011a). In contrast, a stably expressed Gm02ERD15-GFP in Arabidopsis roots was localized in the cytoplasm of root cells (Figure 8). As the ERD15-like subfamily members do not harbor a genuine NSL (nuclear location signal), their subcellular localization might vary depending on the cellular status. Stressful conditions, such as wounding induced by agroinfiltration, may activate the expression of putative interactors of ERD15, leading to this ectopic localization into the nucleus. Curiously, ERD15-like members are associated with the wounding response (Kariola et al., 2006). However, the stable expression of *Gm02ERD15* in Arabidopsis roots may require that stimuli be severe enough to activate the expression of its interactors. Therefore, Gm02ERD15 would remain in cytosol until a strong intrinsic signal is built up to move it to the nucleus. Alternatively, Gm02ERD15 may display functional tissue-specificity and remains in the cytosol in roots, a characteristic that may evoke neofunctionalization of a duplicated paralog gene. Accordingly, in vivo association of Gm02ERD15 with the NRP-B promoter was only assayed in soybean leaf protoplasts (Alves et al., 2011a). In contrast, AtERD15 was strongly relocated to the nucleus under diverse stress conditions, a pattern that was only replicated by OsERD15 under ER stress. Among the soybean members, Gm03ERD15 fractionated between the nucleus and cytoplasm under normal conditions, a pattern of subcellular localization which did not change by ER-, osmotic-stress, and ABA. The distinct subcellular localization of

Gm03ERD15 may be due to its additional conserved domain VSP39_2 that may render specific protein-protein interactions in different localizations.

Our results provide evidence for expanding the functional role of the *Glycine max* ERD15-like subfamily members. As multiple stress-induced regulators, we demonstrated that the *Glycine max* ERD15-like members are intrinsically involved with conserved pathways in mediating senescence and drought response. Nevertheless, we provided several lines of evidence for the diversification of the GmERD15-like family regarding differential stress-induced expression patterns, coexpression correlations with putative protein interactors, and differential subcellular localization that may define specific functions. However, further analyses are necessary to understand the functional correlation between the stress-responsive role of ERD15-like members and the PABP interactions. Elucidating the ERD15 underlying mechanism in RNA metabolism and response to various stresses may be crucial to understanding the convergent and divergent signaling responses.

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FIGURES

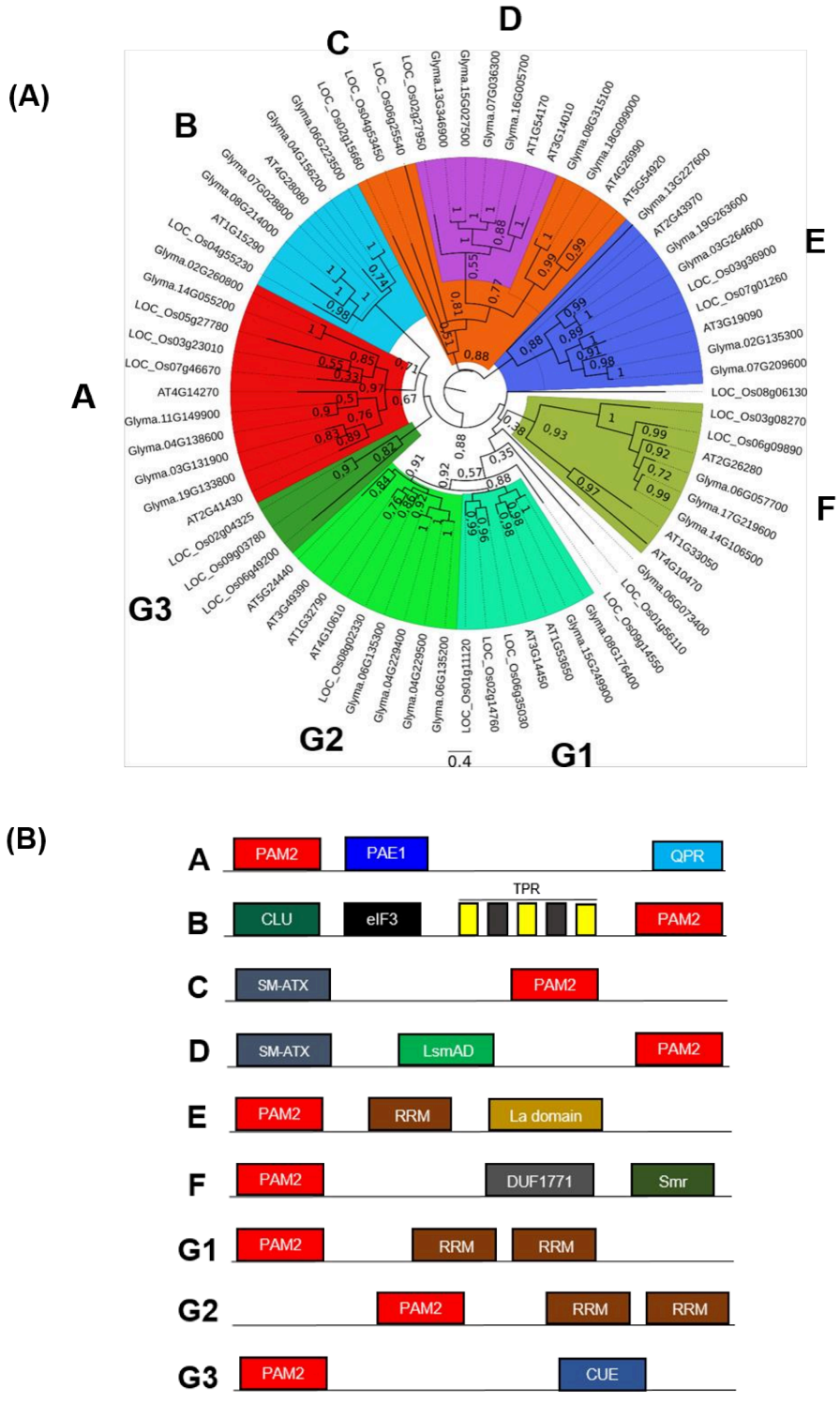
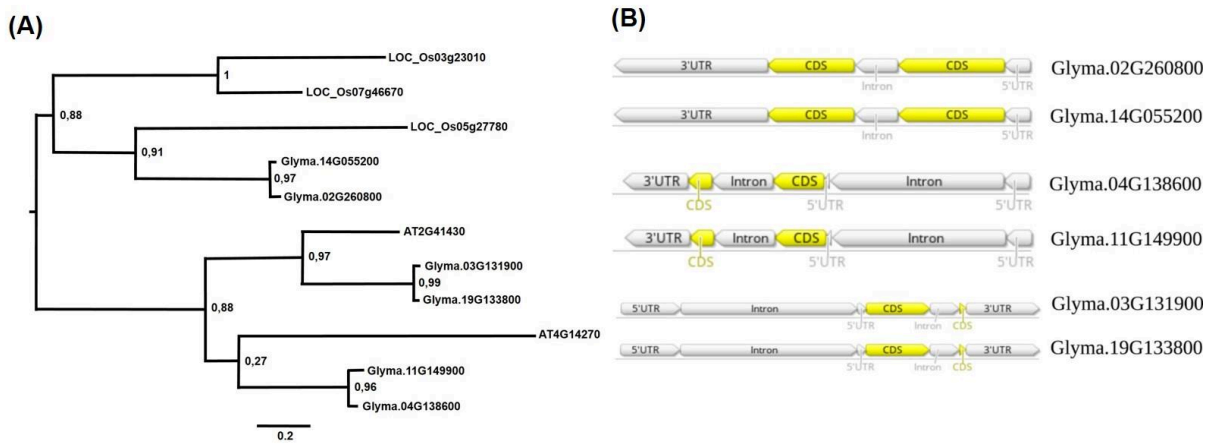


Figure 1. ***In silico*** **analysis of the plant PAM2-like protein family. (A)** Phylogenetic tree of the PAM2-like protein family in Arabidopsis, soybean, and rice. All PAM2 deduced amino acid sequences from *Arabidopsis thaliana* genome (19), soybean genome (31), and rice genome (22) were used to proceed to the phylogenetic analysis. PAM2-like genes were grouped from A to G. The phylogenetic tree was built using the maximum likelihood statistic method in a 10.000 bootstrap consensus. **(B)** Organization of the putative motifs identified 7 groups of the PAM2-like superfamily. The motifs were predicted with HMMER software using the sequences recovered from to the database of Phytozome. PAM2 denotes PABP-interacting motif 2; PAE1,

PAM2-associated element 1 motif; QPR, highly conserved C-terminal QPR motif; CLU, CLUstered mitochondrial domain; eIF3, eukaryotic initiation factor 3; TPR, tetratricopeptide repeat; SM-ATX, Ataxin 2 SM domain; LsmAD, like-Sm-associated domain; RRM, RNA recognition motif; La domain, La conserved domain; DUF1771, unknown function domain 1771; Smr, Small MutS-related domain; CUE, similar to yeast Cue1 protein domain.



(C)

Gene name	Gene name	ka	SD	Ks	SD	Ka/ks	Date(Mya)**	Selection
Glyma.02G260800	Glyma.14G055200	0,029479446	0,0151486138	0,17194613	0,08490239101	0,1714458243	14,09394508	Purifying
Glyma.03G131900	Glyma.19G133800	0,009952751	0,007125280345	0,15810552	0,05408582069	0,06295005386	12,95946885	Purifying
Glyma.11G149900	Glyma.04G138600	0,019612247	0,01413893914	0,09574732	0,06754910066	0,2048333781	7,848140984	Purifying

Figure 2. ***In silico*** **analysis of the plant ERD15-like protein subfamily.** **(A)** Phylogenetic tree of the ERD15-like subfamily in Arabidopsis, soybean, and rice. The phylogenetic tree was built from 11 sequences identified as ERD15-like by the presence of PAM2 and PAE1 motifs using the maximum likelihood statistic method in a 10.000 bootstrap consensus. **(B)** The exon/intron organization of the ERD15-like subfamily in soybean. **(C)** Molecular evolution analyses of duplications/paralogous pairs.

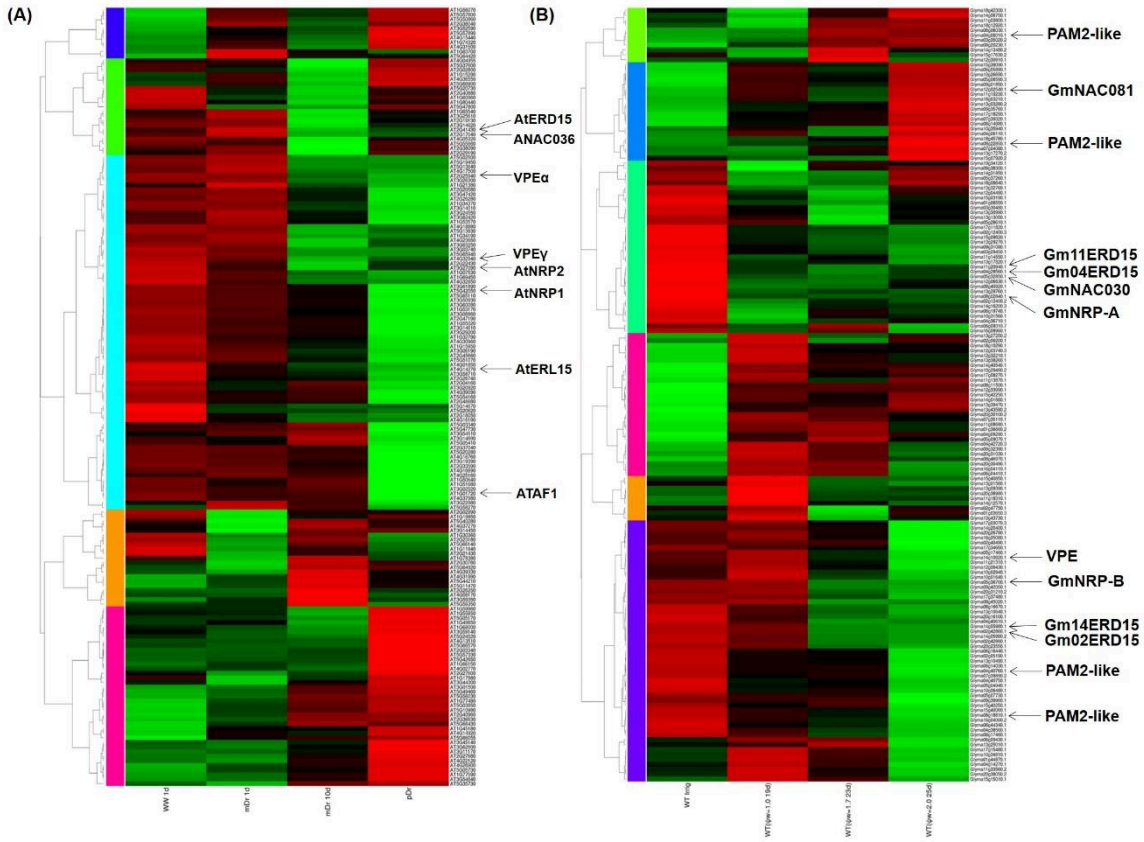


Figure 3. Expression of cell death pathway components under drought in Arabidopsis and soybean leaves. (A) The heatmap represents the relative expression of 169 genes in Arabidopsis wild-type plants, control (WW 1d), moderate drought (mDr) 1d, and progressive drought (10d) (Harbet al., 2010). **(B)** The heatmap represents the relative expression of 157 genes in soybean wild type plants, irrigated control (WT irrig), and under 3 different water potential, ψW = 1,0 (19d), ψW = 1,7 (23d) and ψW = 2,0 (25d).

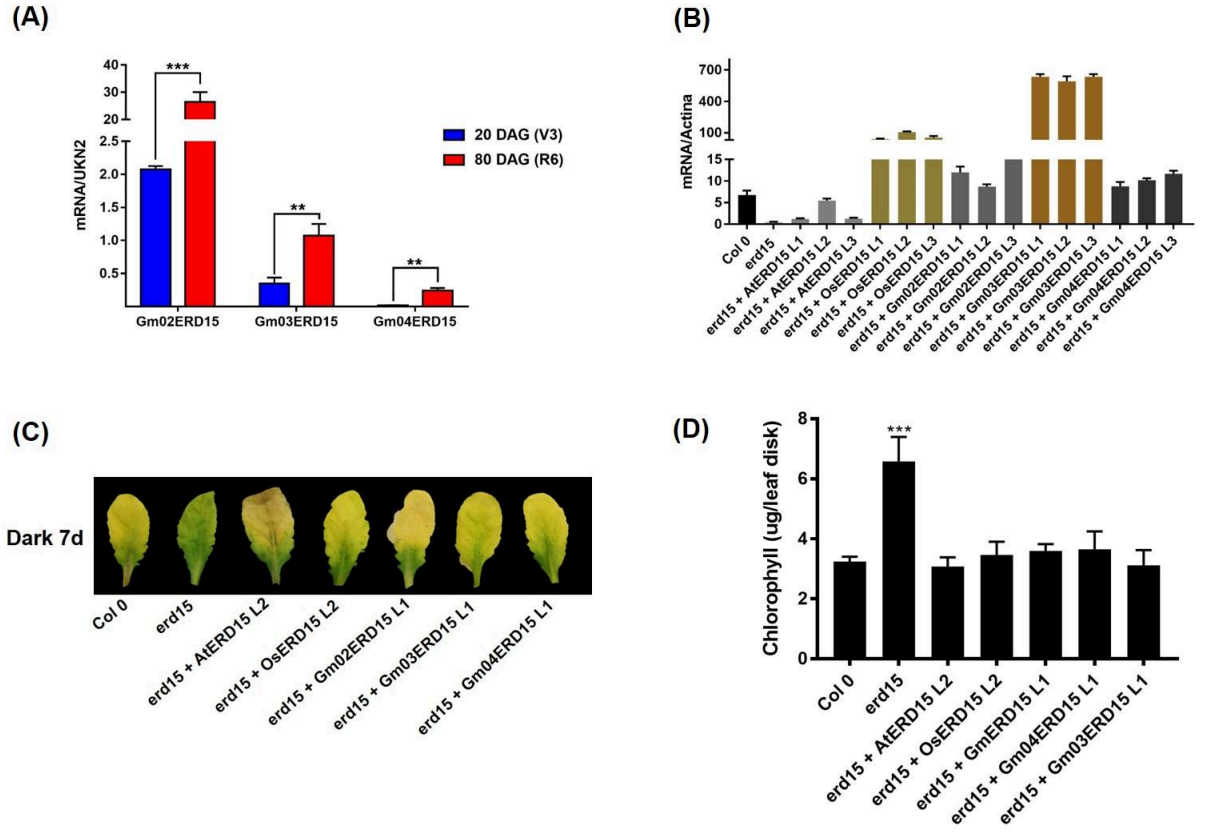


Figure 4. ERD15-like subfamily under senescence conditions. **(A)** Expression of GmERD15-like genes in soybean plants at two different developmental stages: vegetative stage three, 20 days after germination (V3/20DAG), and reproductive stage six, 80 days after germination (R6/80DAG). The UNK-2 gene was used as an endogenous control. **(B)** Transgene expression in complemented lines. The expression levels of ERD15 genes were measured in seedlings under normal growth conditions by qRT-PCR. **(C)** Dark-induced senescence phenotype of ERD15-like subfamily members after 7 days under dark treatment **(D)** Total chlorophyll content after 7 days under dark treatment. Asterisks indicate statistically significant differences by the test-t (*p<0.05, **p<0.01, ***p<0.001).

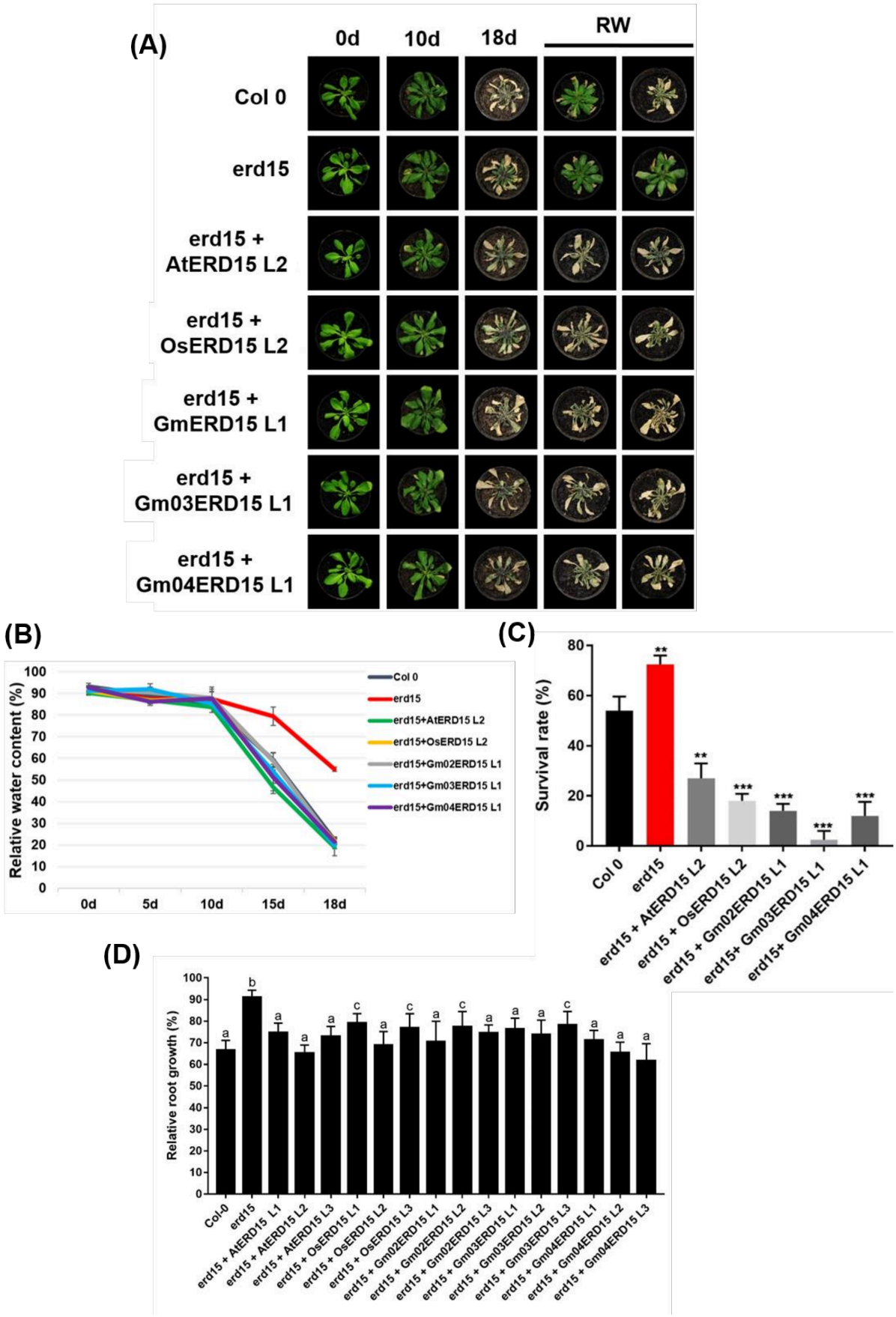


Figure 5. ERD15-like subfamily under drought stress and root growth inhibition under osmotic stress. **(A)** Phenotype of Col 0, erd15 (Salk_073116), erd15 + AtERD15, erd15 +

OsERD15, erd15 + Gm02ERD15, erd15 + Gm03ERD15, and erd15 + Gm04ERD15 lines before and after drought, and after re-watering. **(B)** The relative water content of leaves during 18 days of water deprivation. **(C)** The survival rate of the different genotypes after re-watering. The survival rate was scored based on plants that had survived after re-watering. Data are mean values SD. Asterisks indicate statistically significant differences by the test T (*p<0.05, **p<0.01, ***p<0.001). **(D)** Phenotypes of Col 0, erd15 (Salk_073116), and complemented lines germinated in MS medium for 4 days and transferred to MS medium supplemented with mannitol 200 uM for 6 days. Different letters denote statistically significant differences by the test T.

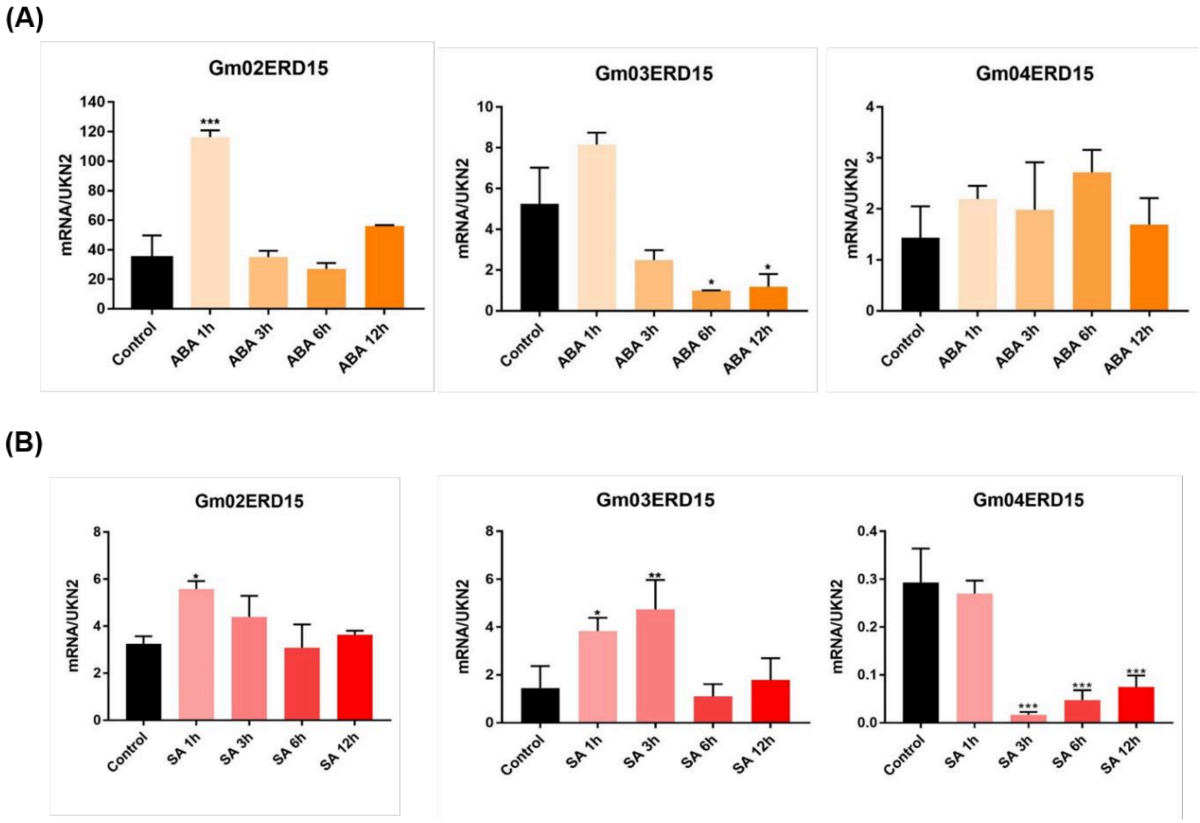
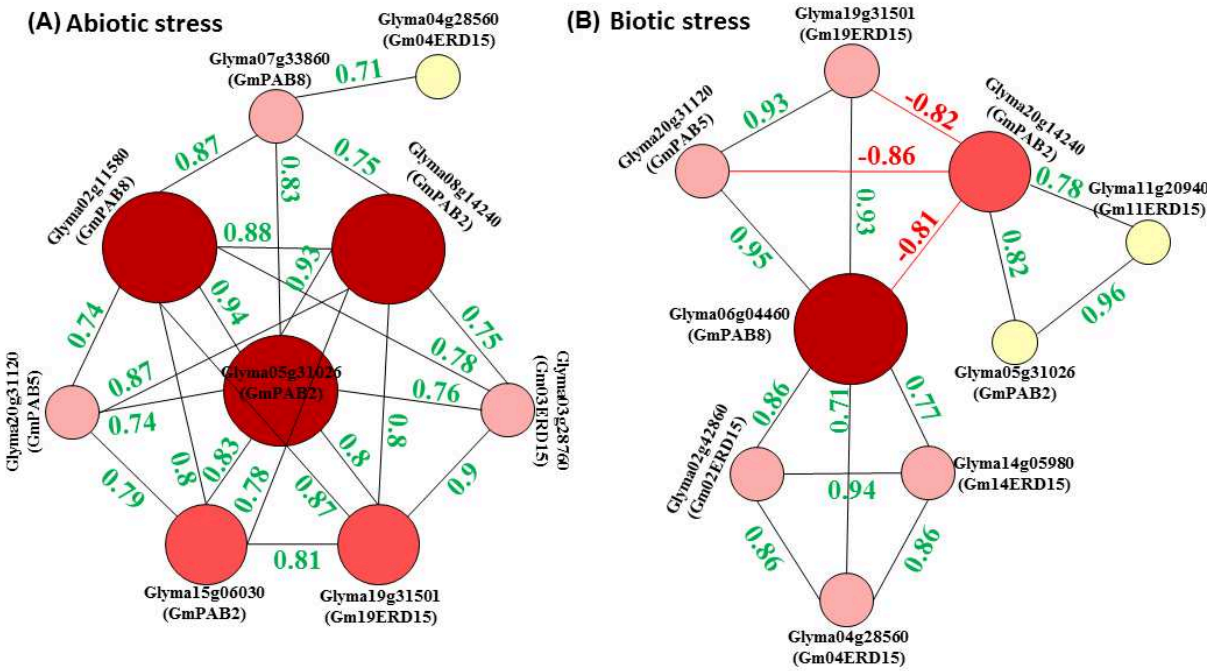


Figure 6. Expression patterns of GmERD15-like subfamily under hormone treatment. Soybean (wild-type BR16) seedlings were stressed with **(A)** 100 uM abscisic acid (ABA) and **(B)** 5 mM salicylic acid (SA). Leaves from stressed and control plants were collected at 1h, 3h, 6h, and 12h post-treatment. The UNK-2 gene was used as endogenous control**,** asterisks indicate statistically significant differences by the test T (*p<0.05, **p<0.01, ***p<0.001).



(C)

Abiotic stess			Biotic stress				
Gm03ERD15	GmPAB8/PAB2	0.78/0.76	Gm02ERD15	GmPAB8	0.86	Gm14ERD15 GmPAB8	0.77
Gm04ERD15	GmPAB8	0.71	Gm04ERD15	GmPAB8	0.71	Gm19ERD15 GmPAB8/PAB5	0.93
Gm19ERD15	GmPAB2/PAB8	0.8/0.77	Gm11ERD15	GmPAB2	0.96		

Figure 7. Coexpression network modules of *Glycine max* ERD15-like subfamily members and GmPABs under abiotic and biotic conditions. (A) Coexpression analysis using *Glycine max* RNA-seq samples in EXPath expression database under abiotic conditions, such as cold (GSE117686), dehydration (GSE112584), drought (GSE69469), low pH (GSE112584), Pi deficiency (GSE104286), salt (GSE57960/GSE69571), and zinc deficiency (GSE111799). **(B)** Coexpression analysis using *Glycine max* RNA-seq samples in EXPath expression database under biotic conditions, such as *Fusarium oxysporum* (GSE66861), and weedy (GSE59875) **(C)** Correlation coefficient of the coexpressed GmERD15-like genes and GmPAB-like genes under abiotic and biotic conditions. The Pearson's correlation coefficient was used to measure the linear correlation between the co-expressed genes. Correlation coefficients >0.7 were selected.

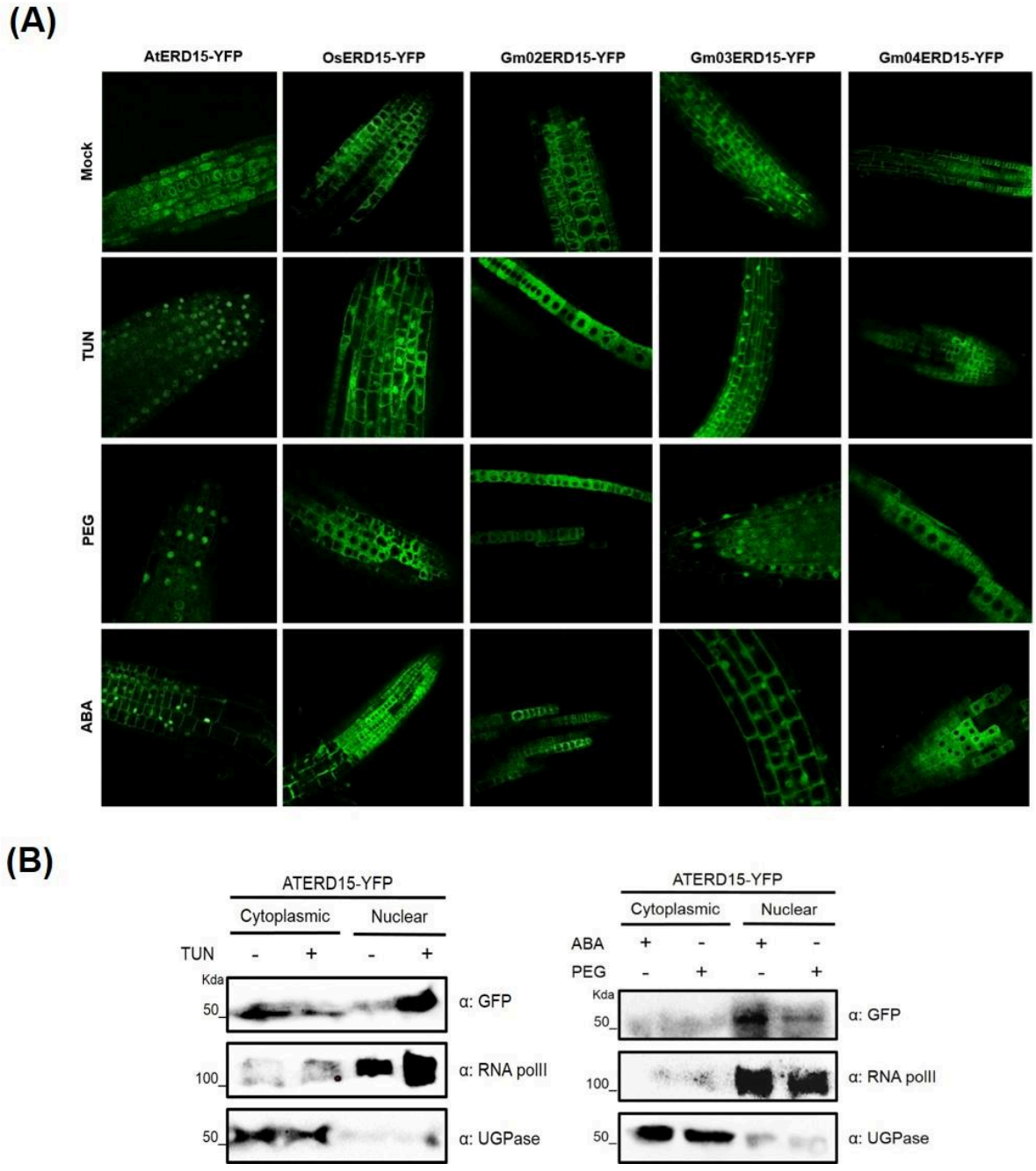


Figure 8. Subcellular localization of ERD15-like family members. (A) Arabidopsis knockdown seeds for AtERD15 (Salk_073116) transformed with the construct 35S::YFP::AtERD15, 35S::YFP::OsERD15, 35S::YFP::Gm02ERD15, 35S::YFP::Gm03ERD15 and 35S::YFP::Gm04ERD15 via Agrobacterium were germinated in MS medium or MS medium supplemented with tunicamycin (TUN) 5 ug/ml, polyethylene glycol (PEG) 10%, 10 uM abscisic acid (ABA) for 16h. Roots from transformed lines were examined by confocal microscopy. **(B)** Nuclear fractionation of 35S::YFP-AtERD15 under normal and stress conditions. The cytosolic protein UGPase and the nuclear protein RNApolII were used as controls for the cytoplasmic and nuclear fractions, respectively.

TABLES

Table 1	Primers used for cloning, genotyping and qRT-PCR	
Name	**Sequence 5' → 3'**	**Locus**
AtERD15-Fwd	AAAAAGCAGGCTTCACAATGGCGATGGTATCAGG	AT2G41430
AtERD15-Rvs	AGAAAGCTGGGTCTCAGCGAGGCTGGTGGATG	AT2G41430
OsERD15-Fwd	AAAAAGCAGGCTTCACAATGAGTGCTATGGCG	Os07g46670
OsERD15-Rvs	AGAAAGCTGGGTCCTAGCGAGGCTGGTGGATG	Os07g46670
ProBiP-ERDFwd	AAAAAGCAGGCTTCACAATGGAAGTCATTTCTGGC	Glyma02g260800
ProBiP-ERDRvs	AGAAAGCTGGGTCCTACCTGGGCTGGTGAATAGC	Glyma02g260800
GmERL1503GW-fwd	AAAAAGCAGGCTTCACAATGGCACTAGT	Glyma03g131900
GmERL1503GW-rvs	AGAAAGCTGGGTCTCATGCAGTTACTTTG	Glyma03g131900
GmERL1504GW-fwd	AAAAAGCAGGCTTCACAATGGCATTGGT	Glyma04g138600
GmERL1504GW-rvs	AGAAAGCTGGGTCCCTCAACGAGGTTGG	Glyma04g138600
Salk_073116LP	CGGTGAGAAATCTTCCACTTG	AT2G41430
Salk_073116RP	CCGTTTTGCAACATAACAATG	AT2G41430
qRT_ACTIN-FWD	ATGTCGTGAGCCATCCTGTC	AT3G18780
qRT_ACTIN-RVS	ACACCGGATTCGTGCGGCAT	AT3G18780
GmUKN2FWD	TGTGCTCTGTGAAGAGATTG	Glyma06g038500
GmUKN2RVS	TCATAATCTGTGTGCAGTTC	Glyma06g038500
qRTGmERD15_02G_fwd	CGCAAATGACTTCAGAGCTT	Glyma02g260800
qRTGmERD15_02G_rvs	AGATCTGTAACACGGACACAA	Glyma02g260800
qRTGmERD15_03G_fwd	TGTGACTGTTTCATGTACTGAT	Glyma03g131900
qRTGmERD15_03G_rvs	AGTTCAGGACGGTGGTGAAA	Glyma03g131900
qRTGmERD15_04G_fwd	TCAAGCCTTGTGATTTGCATT	Glyma04g138600
qRTGmERD15_04G_rvs	CATGAGGCGCACAAATGTG	Glyma04g138600

Table 2. Conserved binding sites of TFs in promoter regions of (*Glycine max*) ERD15-like subfamily members

TF-binding group	Binding site	Transcription factor	Involved condition
AP2	CAACA	RAV2	Dehydration and floral development
	TCACCG	DRE2F	Dehydration stress
AT-Hook	TTTAT	AHL1	Root development
B3	TGTCG	ARF8	Auxin signaling
C2H2	AGTGT	ZAT11	Stress responses and root growth
EIN3	GGTACAT	EIN3	Ethylene response, root elongation and salt stress
GATA zinc finger	(A/T)GATA(A/G)	GATA8	Light response and circadian signaling
Homeobox-leucine zipper	CATTAATT	ANL2	Root development and anthocyanin accumulation
MADF	GGTTAA	GT-1	Light signaling response
MADS-box	CCAAAAATG	AGL42	Flower organ senescence and ethylene response
Myb/SANT	GTTAA	CDC5	mRNA splicing and cell cycle control
NAC; NAM	TTGAC	SAG113	Senescence, ABA and drought
		SNAC49	JA, wounding and drought
SBP	GTACA	SPL14	plant development

WRKY	TTGAC	WRKY27	Gibberellin signaling and defense response
bHLH	ACTTG	bHLH128	Stomatal movement
bZIP	ACGTC	CPRF2	light-induction and hormone control

CHAPTER III

A REGULATORY CIRCUIT INTEGRATING STRESS-INDUCED WITH NATURAL LEAF SENESCENCE

Published chapter

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Pages 75 to 92

Chapter

A Regulatory Circuit Integrating Stress-Induced with Natural LeafSenescence

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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), whichis 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 stresspersists and the cell is unable to restore ER homeostasis, programmed cell death sig-naling 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 expres- sion 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, whichis 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 thediscovery 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 identifiesNRPs 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 devel-opment. 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 physiologicaladjustments, 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 physi- ological 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 culminateson 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 phos-phatidic acid, phosphoinositides, sphingolipids, lysophospholipids, oxylipins,

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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 areconnected 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 thenecessary 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. Thesecretory proteins are synthesized in ER membrane-bound polysomes, and, as soonas 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 conse- quence, 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 theunfolded protein response pathway (UPR, **Figure 1**) [14].

The detection of ER stress is mediated by membrane-associated sensors, identi- fied both in mammals and plants. In mammals, there are three of these sensors: kinase/endoribonuclease inositol-requiring enzyme 1 (IRE1), activating transcrip- tion 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 theadverse 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 recep- tors, keeping them inactive. With the stress progression and consequent misfoldedprotein accumulation, the BiP molecular chaperone function is required to preventaggregation 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 harborsa ribonuclease and kinase activity at the C-terminus, responsible for the uncon- ventional 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 autophosphorylationand endoribonuclease activity, culminating on spliceosome-independent splic-

ing 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 acti- vate UPR. The IRE1-mediated mRNA splicing removes an unconventional intron of26 nucleotides, which causes a shifting frame in XBP1 mRNA translation, generat- ing a protein of 376 amino acids instead of 261 amino acids when unprocessed.

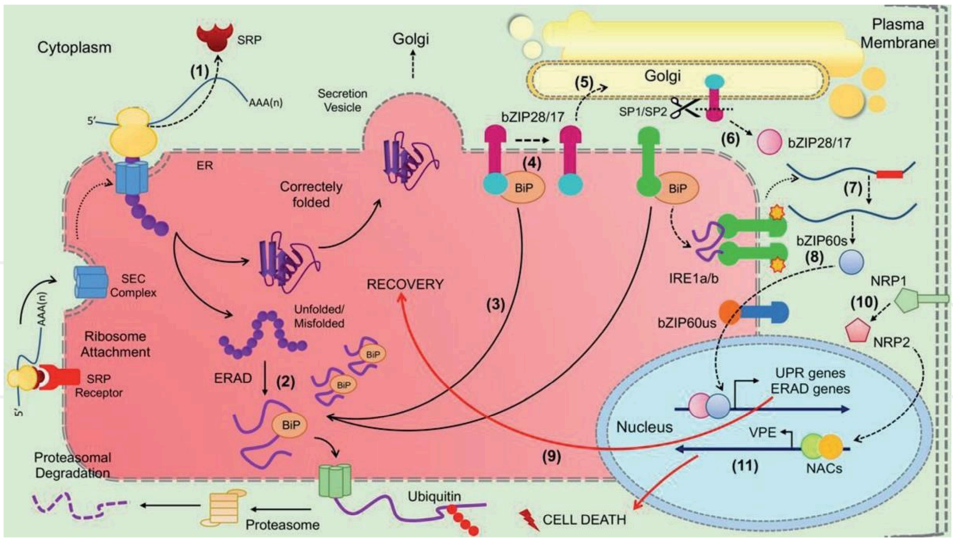

Figure 1.
The endoplasmic reticulum stress response in Arabidopsis. *The secretory proteins are synthesized in ER-bound polysomes (1) attached to the ER membrane through the interaction of signal recognition particle (SRP) and membrane receptor. As soon as they enter the lumen of the organelle, they 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 fromthe membrane that, in turn, is translocated to the nucleus (6). UP accumulation also causes the oligomerizationof 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 homeostasiscannot 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 thevacuole. 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 func- tional transcription factor, which is reallocated to the cell nucleus to activate genesinvolved 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 anN-terminal sensor domain facing the ER lumen and a C-terminal bZIP domain fac- ing the cytosolic side. Under normal conditions, ATF6 is inactivated by BiP bindingto the ER stress sensor domain. ER stress conditions promote the BiP disassociationand reallocation of ATF6 to the Golgi apparatus, where it is specifically processed by SP1 and SP2 proteolytic enzymes. The limited proteolysis of ATF6 transmem- brane 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 process- ing, ER quality control, and ER-associated protein degradation (ERAD) pathway.

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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 theregulation 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 unconventionalcytosolic 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 modifi- cation 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, expos- ing 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-talkwith 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 andERAD-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 deathsignaling 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 pathwaysare 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 transcrip- tomes of soybean seedlings treated with PEG (an osmotic stress inducer) and tunicamycin and AZC (ER stress inducers). Several genes displayed similar kineticsand a synergistic induction under combined ER and osmotic stresses, indicating that the ER stress response integrates the osmotic signal to potentiate transcriptionof 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 dem-onstrated 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 moredivergent 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

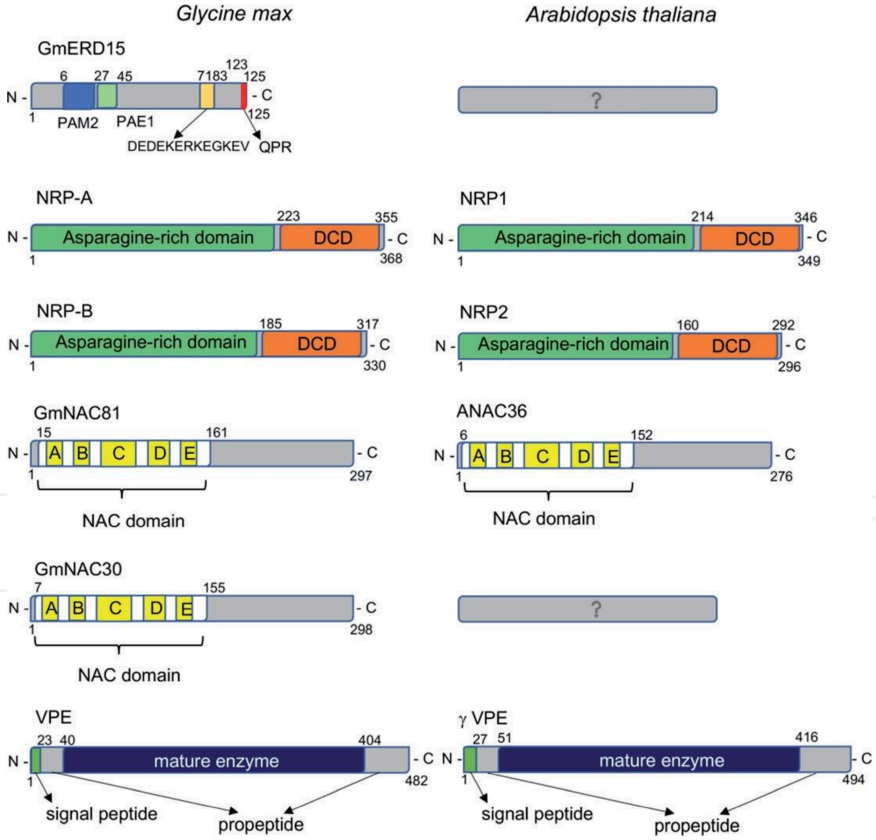

Figure 2.
Schematic representation of the cell death pathway components. The predicted domains of each protein are highlighted. The indicated domains are delimited by the amino acid positions in the primary structure shown by the numbers. For ERD-15, PAM2 is a PABP-interacting motif, PAE2 is PAM2-associated element 1 motif, DEDEKERKEGKEV is a conserved sequence representing a putative motif of ssDNA-binding transcriptionalregulators, 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.

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expression of NRPs *in planta* causes leaf yellowing, chlorophyll loss, malondial- dehyde 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-inte- grating cell death response to be characterized, this signaling pathway is commonlyreferred 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 soy- bean 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 transcriptionalfactors 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 stressesin a coordinated manner, but induction of *NRPs* precedes the upregulation of *GmNAC81*. This early induction kinetics of *NRPs* is consistent with its capacity toactivate 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 NRPexpression

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 trans-lation [49]. In addition to PAM2, ERD15 contains two other domains with unknownfunction, 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 sensi- tive to ABA and display enhanced salicylic acid-dependent defense pathway, whichwas 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, GmERD15binds 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 sequenceof 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 deathsignal

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 down- stream components, the NAC transcriptional factor (GmNAC30) and the vacuolarprocessing 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 protoplastsand, as a downstream component of the cell death signaling, is induced by expres-sion 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 envi-ronmental 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 *Tobaccomosaic 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 thevacuolar 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 thatprolonged ER and osmotic stresses induce the expression of the transcriptional
activator GmERD15 to target the NRP promoter. The upregulation of NRPs initiates

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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 plantPCD. As vacuole-triggered PCD is unique to plants, the regulatory circuit linkingthe 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 prototypesof 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

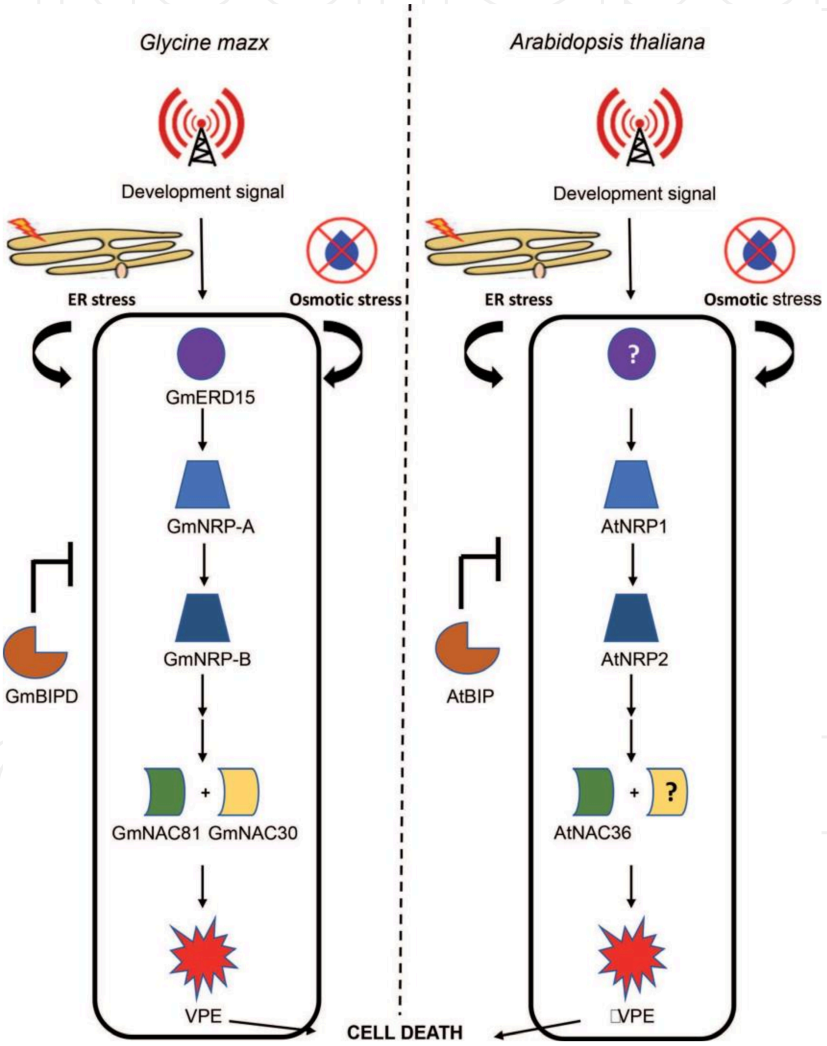

Figure 3.
Integration of developmental signal and stress signals into the DCD/NRP-mediated cell death response. Leaf senescence, ER stress, and osmotic stress induce the expression of ERD15-regulated NRP-A that in turnupregulates NRP-B to initiate a signaling cascade that culminates with the induction 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 expressionof 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 fragmenta- tion, 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 occurthrough 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 chaperoneBiP, 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 moduleconfers 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 torestore 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 organ- elle 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-associatedphysiological 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

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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 modula- tor 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 upregu-lation 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 modu- lation 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 signalingpositively regulates leaf senescence

Leaf senescence is a natural process in plant development, which begins with aphysiological transition between active photosynthetic leaves to degenerative andnutrient-recycling leaves. The classical age senescence-related symptom is the leafyellowing 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 con- vergent 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 temporalexpression 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* geneswere 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 naturalleaf 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 regu-latory 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, theactivity 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 protoplastsand *in planta* induce a cell death response bearing the hallmarks of leaf senes-cence 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 leafsenescence in transgenic plants [59]. Finally, GmNAC81 is a positive regulator of naturally programmed leaf senescence [70]. Although leaf senescence is geneticallyprogrammed 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 soy-bean 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 senes- cence 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 integratedwith 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 deci-phering 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-mediatedcell 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

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ER-resident molecular chaperone BiP delays drought-induced senescence in tobaccoand 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 contrib- utes 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.

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Conflict of interest

The authors declare no conflict of interest.

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GENERAL CONCLUSION

In response to environmental stress, signaling pathways are activated as a form of plant defense against adverse conditions. In order to optimize the cellular response, some signaling pathways have been shown to share responses to different conditions. Recent findings have shown that the DCD/NRP-mediated cell death signaling is activated not only in response to multiple stresses but also to developmental and stress-induced senescence. Accordingly, the cell death signaling components GmNAC081 and GmNAC030 play positive roles in senescence regulation. In addition, the multiple stress responsiveness of senescence-associated GmNAC genes (GmSAGs) has been observed in recent studies, which highlights the importance of the NAC superfamily in shared responses. The expression profile of GmNAC-SAGs and their putative AtNAC-SAG orthologs in response to multiple stresses unveil that these transcription factors are involved in the integration of the senescence response with stress, and a conserved funcional correlation between species was found in most cases.

Furthermore, another component of the NRP-mediated cell death pathway was revealed here as a positive regulator of senescence. The PAM2 motif-containing protein, ERD15 was identified as a senescence regulator that may control senescence through phythormone signaling regulation. The phylogenetic, exon/intron organization and molecular evolution analysis of the ERD15-like subfamily of the PAM2 superfamily reveal a duplication event and expansion of the members in soybean, which matches the date of the duplication of the soybean genome. However, it has been observed that these gene pairs are under a negative selection, which prevents evolutionary mutations at critical functional sites. Besides the functional complementation in drought stress, osmotic stress and dark-induced senescence of the ERD15-like subfamily members, the coexpression analyses in abiotic and biotic conditions, the expression profile in response to phytorhormones and drought stress suggest a different response at different times of stress. Curiously, the ERD15 members displays different subcellular localization in different conditions, which should modulate their molecular interactions and functions. The correlation between the response to various stresses and the interaction with PABPs should be explored in future studies to understand whether their function in multiple stresses is correlated with the mRNA or translation regulation. Collectively, the data obtained here allowed a better understanding of the multiple roles of the NRP mediated cell death pathway components in shared responses.