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## Unfolded protein response in plants: one master, many questions

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### Abstract

To overcome endoplasmic reticulum (ER) stress, ER-localized stress sensors actuate distinct downstream organelle-nucleus signaling pathways to invoke a cytoprotective response, known as the unfolded protein response (UPR). Compared to yeast and metazoans, plant UPR studies are more recent but nevertheless fascinating. Here we discuss recent discoveries in plant UPR, highlight conserved and unique features of the plant UPR as well as critical yet-open questions whose answers will likely make significant contributions to the understanding plant ER stress management.

### Introduction

In growth and stress situations, cell homeostasis depends on the functional integrity of the major biosynthetic organelle, the ER, which synthesizes one third of the cellular proteome and essential lipids, and assembles complex-protein associated carbohydrates. When the cell's biosynthetic demands overcome the ER biosynthetic capacity, a potentially lethal condition, known as ER stress, is initiated. In multicellular eukaryotes if the UPR fails, ER stress-induced apoptosis occurs. Along eukaryotic lineages, specialized sensors have evolved to manage the UPR and control life-or-death outcomes in ER-stress conditions [1,2]. For ER-stress protection, yeast relies exclusively on the ER-associated ribonuclease and kinase IRE1. Plants and metazoans have evolved a complex signaling network based on membrane-tethered transcription factors (MTTFs) (Box 1), some of which are plant-specific (Figure 1). In metazoans, the ER-associated PERK also operates in UPR signaling (Box 1). Although PERK-like activity has been detected in plants [3], plant PERK-like proteins have not been identified yet (Box 1).

How the sensors input stress to modulate an effective output in life-or-death decisions is largely unknown, especially in plants. Here we discuss plant UPR strategies that recent exciting results have brought to light in support of convergence of signaling strategies among multicellular eukaryotes as well as plant-specific features of the UPR.

### Diversification of IRE1 signaling in the UPR: activation of a transcription factor

Plants encode one or more IRE1 isoforms (Figure 1a). IRE1-mediated splicing of a transcription factor (TF) mRNA is a conserved strategy for UPR gene regulation (Figure 2). Among eukaryotes the IRE1-TF targets are members of the large bZIP-TF family: Hac1 in yeast and Xbp1 in metazoans. In flowering plants, *AtbZIP60*, *Osb-ZIP74/50* and *ZmbZIP60* are the targets identified in *Arabidopsis thaliana* (dicot), *Oryza sativa* and *Zea mays* (monocots), respectively [4–9] (Figure 1b). The consensus mRNA structure that is recognized by IRE1 (i.e. twin-stem loops with conserved bases in each loop) is largely conserved across eukaryotes. The excised intron sequence is slightly different among monocots but conserved in dicots [8]. However, there are noticeable differences in the splicing output among eukaryotes. In plants, the IRE1-mediated mRNA splicing causes an intron-excising frameshift that occurs closely upstream of the region encoding a putative transmembrane domain and leads to elimination of the membrane anchor in the translated active TF [4,7,8]. In metazoans, the IRE1-spliced Xbp1 loses the ER membrane anchor and gains the TF domain located in the new C-terminal tail [10,11]. In yeast, the splicing of Hac1 mRNA is followed by transcriptional joint of the DNA-binding domain to the newly encoded TF transcription activation domain [12]. The lack of a close conservation of the sequence of the mature TFs and the diversification of the splicing outputs among eukaryotes support that, despite IRE1 being an ancient sensor, IRE1-TF targets may have evolved independently. Under this light, the notion that Hac1 and Xbp1 are the sole direct TF targets of IRE1 may not apply in plants where IRE1 may have other direct TF targets that may fine-tune IRE1 signaling in the UPR management in specific stress situations. This possibility is predicated by the evidence that even in the simultaneous absence of the *Arabidopsis* ER-stress activated TFs (*AtbZIP28* and *AtbZIP60*), one of the major UPR gene markers, *AtBiP3* is mildly induced in conditions of ER stress [13••].

### Diversification of IRE1 signaling in the UPR: RIDD

In addition to TF-mRNA splicing, metazoan IRE1 degrades mRNAs through a site-specific cleavage process, known as regulated IRE1-dependent decay (RIDD) [14], which is uncoupled from the site-specific splicing activity of IRE1 [15]. RIDD activity is required for ER homeostasis in non ER-stress conditions and increases progressively with ER-stress intensity. RIDD also occurs in *Arabidopsis* [16••], but its physiological relevance without ER-stress induction is yet to be defined [16••]. In normal conditions of growth, loss of *AtIRE1* function causes defects in root elongation [17••], independently of *Atb-ZIP60* [18••]. It is possible therefore that in the absence of induced ER stress RIDD may be important to maintain growth homeostasis in specific plant organs by controlling the levels of mRNA of proteins involved in cell growth.

### Diversification of IRE1 signaling in the UPR: miRNA cleavage

In mammalian cells, IRE1 also cleaves sequences of specific miRNAs [19]. miRNAs operate to translationally repress gene expression [20]. Whether plant IRE1 has a similar control of miRNA levels is yet unknown. Selective miRNA cleavage could be a strategy to either overcome stress, modulate tissue growth, or both. Addressing this question may lead to new insights into the functions of plant IRE1, and to establishing whether IRE1 activity is

conserved among eukaryotes. Indeed the findings that loss of IRE1 is lethal in conditions of prolonged ER stress support a function as ER-stress protector [17••]. The recent evidence that the mammalian IRE1 may not activate caspases to induce cell death in prolonged ER stress, contrarily to a previous notion [19], but rather delay the onset of apoptosis via decay of the pro-apoptotic Death Receptor 5 (DR5) [21], poses that plant and animal IRE1s may be functionally close in protecting the organism from death.

### How may multiple IRE1 help plants in stress defense?

An intriguing question relates to the diversification of the ER-stress sensors in plants both qualitatively and quantitatively. In Arabidopsis there are two IRE1 isoforms, AtIRE1a and AtIRE1b [22]. Whether IRE1 isoforms may be functionally equivalent is a matter of debate. Both genes are expressed throughout the plant, but AtIRE1a is more abundant in embryos and seeds [22,23]. It has been proposed that unlikely AtIRE1a, AtIRE1b is relevant to *AtbZIP60* splicing in conditions of DTT-induced stress [4], supporting the possibility of functional diversification of AtIRE1 proteins. This is further reinforced by the observations that while AtIRE1b has a preponderant role in *AtbZIP60* splicing upon tunicamycin-induced ER-stress, AtIRE1a seems preferentially required for *AtbZIP60* splicing upon pathogen and salicylic acid (SA) infection [5]. Although functional diversification of the SA response has been recently questioned [24], the evidence that AtIRE1b but not AtIRE1a is required for *AtbZIP60* splicing [4] supports that AtIRE1 proteins may have undergone functional specialization. These observations raise the question how the multiple IRE1-functions may be executed in species with one IRE1 isoform. The observation that in Arabidopsis AtIRE1b but not *AtbZIP60* is required for ER stress-induced autophagy [25••] hints to the possibility that, in species containing only one IRE1 gene, some of the IRE1-roles may be executed by other proteins. Nonetheless the evidence in Arabidopsis that in induced-stress conditions, unlike a double *atire1a/atire1b* mutant, single IRE1 loss-of-function mutants survive prolonged ER stress supports that at least the pro-survival role of AtIRE1 is shared by the two proteins. Whether the roles of the IRE1 isoforms are spatially and/or temporally regulated is yet to be established. It is possible that in certain tissues IRE1 may have a specific relevance and other ER stress sensors might operate predominately in other tissues.

### How may multiple bZIPs help plants in stress defense?

Loss of either *AtbZIP60* or *AtbZIP28* (Box 1) attenuates the expression of some common transcriptional targets such as BiP3 and calnexin, an effect that is additive when both genes are simultaneously deleted [26•]. This evidence supports overlapping functions of these two TFs for which a precise extent of overlap is yet to be completely understood both at a temporal resolution as well as target identity. Specifically, it is yet to be defined whether at the onset of ER stress, activation of *AtbZIP28* and *AtbZIP60* occurs simultaneously or sequentially. One may predict that mild stress might be resolved by the function of one of the two TFs and that the activation of the other TF may be actuated in situations of unresolved ER stress to potentiate an ER stress-mitigating response. It is also known however that activation of bZIP-TFs may lead to responses that are qualitatively distinct. For example, in conditions of salt stress, *AtbZIP17* modulates only the expression of *BiP3* among the known ER stress markers [27], which, on the other hand, is controlled by both *AtbZIP28* and *AtbZIP60* in conditions of ER stress induced by heat or by drugs such as

tunicamycin and DTT. Furthermore, heat, tunicamycin and DTT but not salt stress can induce activation of AtbZIP60 and AtbZIP28, and a role of AtIRE1-AtbZIP60 but not AtbZIP28 has been demonstrated in virus-plant interactions [28]. These findings support that plant stress response relies on functional specialization of the UPR effectors for which the underlying mechanisms remain poorly understood. In this view the UPR sensors might be specialized in perceiving specific classes of unfolded proteins, which may be more sensitive than others to specific stresses.

### Requirements for the UPR sensors

In yeast and metazoans, a model has emerged that the binding of unfolded proteins to IRE1 dimers allows further oligomerization and trigger the IRE1 activation, while the BiP binding to IRE1 may have a modulatory role in buffering the pool of IRE1 monomers [2,29]. In plants the mechanisms of activation of IRE1 are largely unknown. Nonetheless, binding to AtBiP is a mechanism that constrains AtbZIP28 to the ER and therefore modulates AtbZIP28 traffic to the Golgi for activation [30]. Such a mechanism, which is similar to the activation mechanism of ATF6 [31] could suggest also conservation of IRE1 activation. Similarly, AtbZIP17 may be retained in the ER via interactions with other proteins that specifically respond to salt stress. One alternative that cannot be excluded is that one branch of the UPR may be activated as consequence to the operation of another ER-stress response, such as production of radicals or unfolded proteins, which could lead to the activation of other ER stress defenses.

Interaction with unfolded proteins might not be the only upstream requirement for plant IRE1 activation. Functional and genetic evidence have shown that the ER-shaping protein AtRHD3 is a critical factor that acts upstream of AtIRE1 in the *AtbZIP60* splicing in conditions of ER stress [32•], but the underlying causes are not known. In conditions of ER stress yeast and metazoan IRE1s are known to cluster in ER subdomains where stress signaling is believed to occur [2]. In the absence of induced ER stress, fluorescent protein fusions to plant IRE1s have been localized to the perinuclear ER [22]. It is possible that, if plant IRE1 is also mobilized into signaling clusters in ER-stress conditions, then alteration of the ER network structure or lipid composition, known to occur with RHD3 deletions [33,34], may affect mobilization of plant IRE1 into signaling hubs.

Besides factors acting upstream the UPR sensors in plants, we know very little about downstream regulating factors. Unlike metazoan IRE1, which is known to interact with multiple proteins which may or may not regulate its function [35], the identity of plant IRE1-interacting proteins is still unknown. The situation is markedly different for the bZIP-TFs. *In vitro* AtbZIP28 can homodimerize but also heterodimerize with AtbZIP60, AtbZIP49 or AtbZIP17 [36]. Validation of these findings *in vivo* coupled with functional analyses may provide insights into the relevance of the TF interactions in the UPR. AtbZIP28 has also been found to interact with NF-Y subunits to form a transcriptional complex that upregulates the expression of ER stress-induced genes [36]. Furthermore, AtbZIP28 and AtbZIP60 interact with AtAsh2 and AtWDR5a, which are core components of the COMPASS-like complex that regulates gene expression through histone H3K4 trimethylation [13••]. These findings support that the protein-protein interactions of the UPR

bZIP-TFs may have important roles in the regulation of gene expression and that a diversification of such interactions may be needed to modulate responsive outputs that are different both at quantitative and qualitative levels. One critical yet unanswered question is how the activated sensors are restored to basal levels once the ER stress is resolved. In other words, how is the plant UPR tamed once ER homeostasis is obtained?

## Conclusions

In the last few years, numerous significant studies have contributed to the understanding of the UPR; this trend is likely to keep steady. Numerous fundamental questions are still open (Box 2). Understanding the mechanisms of the plant UPR is important to define conserved and unique strategies for ER stress responses, and it offers exciting perspectives to improve plant productivity. While we are beginning to understand how the UPR intersects with hormone-regulated growth [37•,38], defense [5,24,39], and resistance to hostile environments [4,40], further insights into the temporal and spatial complexity of the UPR regulation will support biotechnological applications. For example, overexpression of rice IRE1 may lead to suppression of endogenous IRE1 expression, which may compromise endogenous defense [41]; on the other hand, overexpression of BiP proteins renders them more resistant to stress [42,43]. Exploration of plant ER-stress responses in the field using multiple species and ecotypes adapted to different environmental conditions could provide further insights into the design of novel strategies for plant ER stress management outside standard laboratory conditions.

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membrane-tethered transcription factor NAC062 is upregulated by ER stress through AtbZIP60-TF and in response to ER stress it relocated from the plasma membrane to the nucleus, where it plays important roles in regulating UPR downstream gene expression. [PubMed: 24961665]

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**Box 1****Multicellular UPR signaling strategy in multicellular eukaryotes****Membrane-tethered bZIP TFs**

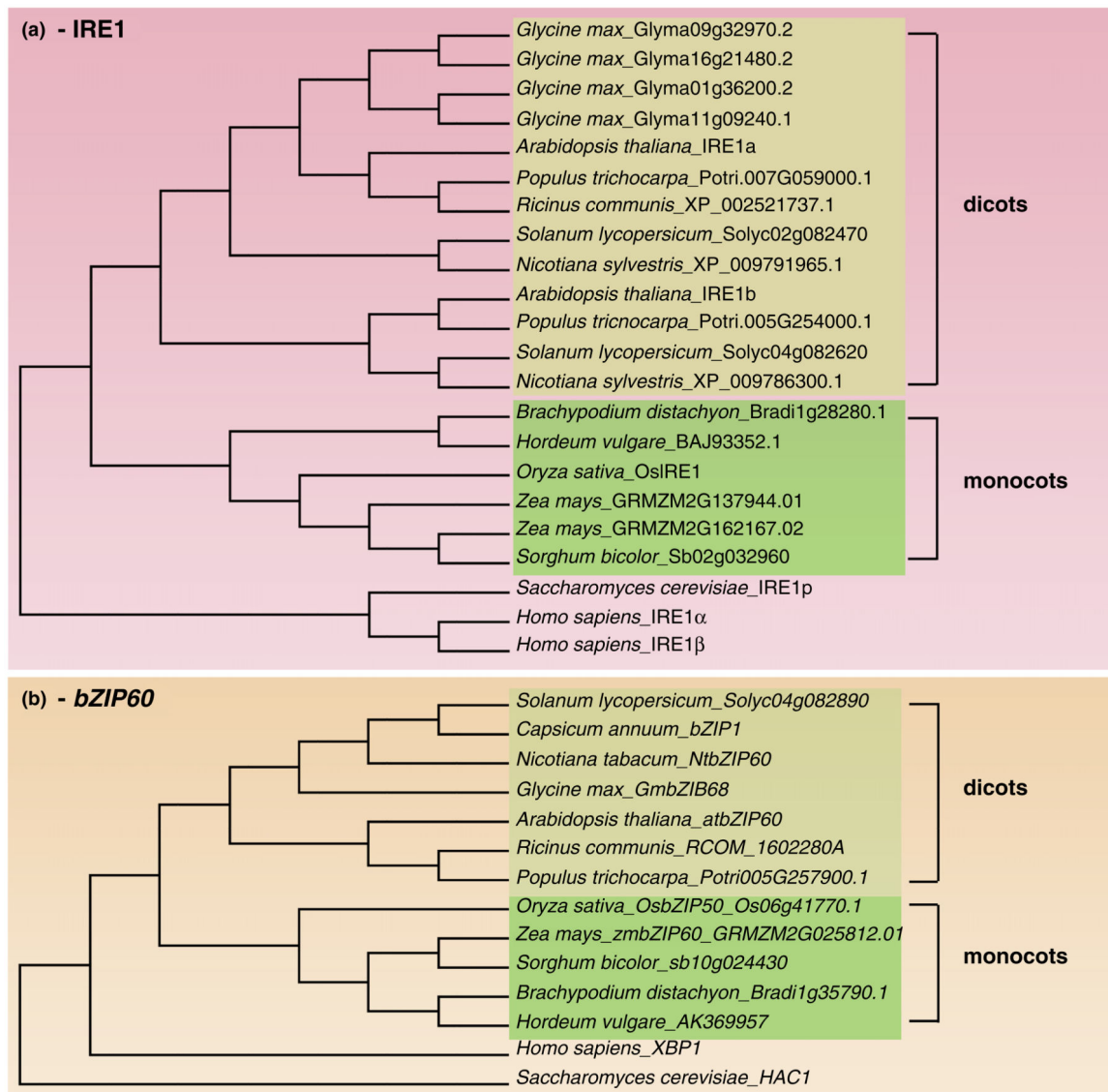
In response to ER stress in metazoans, the ER membrane-tethered activating transcription factor 6 (ATF6) moves to the Golgi where the site 1 and site 2 proteases (S1P, S2P) release the TF domain from the membrane anchor. This process is followed by nuclear translocation of the TF domain to modulate gene transcription. The Arabidopsis genome encodes functional homologs of ATF6: AtbZIP28 [44,45] and AtbZIP17 [40]. Functional equivalent TFs have been reported in other plant species [7,46]. The mechanisms of activation of AtbZIP28 are similar to those of ATF6 with COPII-dependent ER-to-Golgi translocation [47] and subsequent activation through proteolysis at the Golgi by S1P [48] and S2P [38], which recognize cleavage sites in the luminal face and in the transmembrane domain of AtbZIP28, respectively [47,48]. Activation of AtbZIP17 relies on S2P [38], but reliance on S1P is yet unknown.

**PERK**

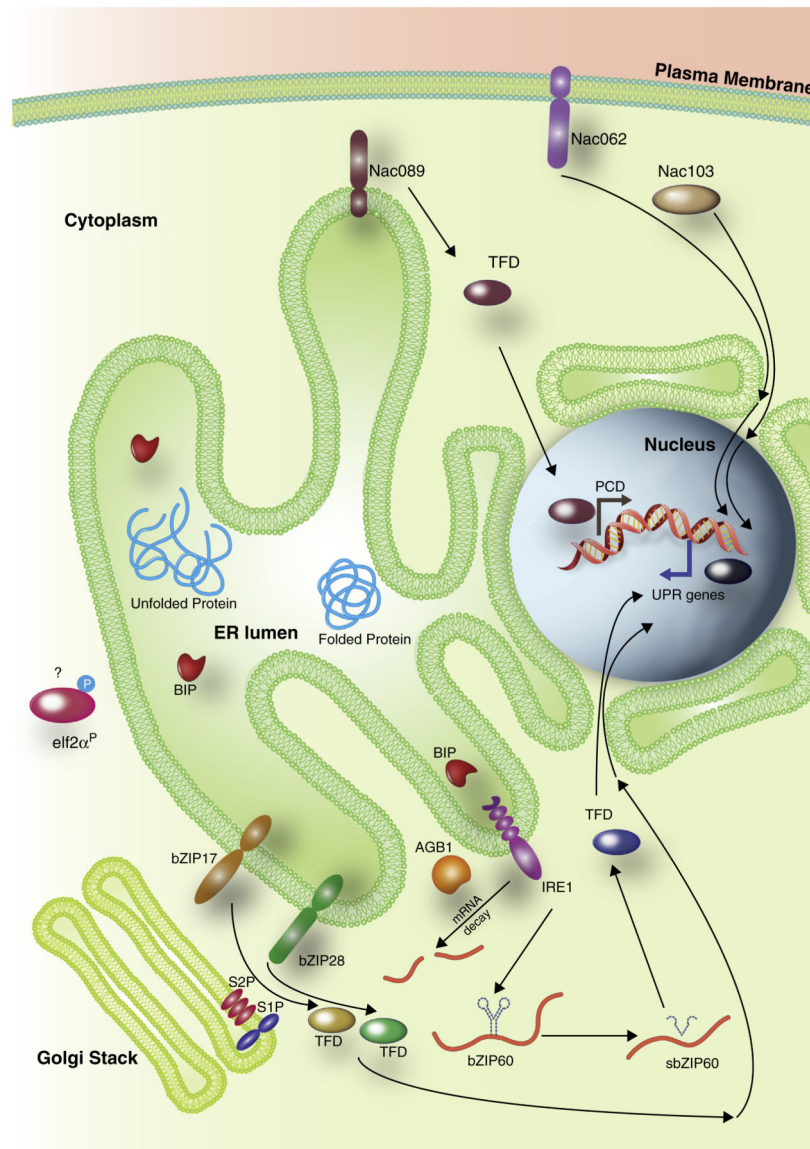
In metazoans, the protein kinase RNA-like ER kinase (PERK) attenuates general translation but favors selective translation of the ATF4 TF through selective phosphorylation of the eukaryotic elongation factor eIF2 $\alpha$  [2,49]. In plants a functional homologue of PERK has not been identified yet, despite the evidence that a PERK-like phosphorylation of transcription initiation factors (eIF) occurs in ER stress. AteIF2 $\alpha$  hyper-phosphorylation occurs in a loss-of-function mutant background of the Arabidopsis SPIKE1 (SPK1), a DOCK family GEF involved in cell morphogenesis [3,50]. Intriguingly, *spk1* has constitutively elevated expression levels of UPR target genes, and shows hypersensitivity to tunicamycin [3]. The evidence that simultaneous loss of AtIRE1 and AtbZIP28 is lethal at embryonic level [18] supports that if PERK-like proteins were involved in the UPR in plant cells, they would likely function in specific stress situations. Yet it cannot be excluded that the kinase activity of AtIRE1, which has been experimentally validated [18], may be responsible for the observed AteIF2 $\alpha$  hyper-phosphorylation.

**Box 2****Major unsolved questions in the plant UPR**

1. What is the trigger for a life-to-apoptosis switch in ER stress?
2. What are the triggers of ER-stress induced autophagy?
3. Is plant age a contributing factor to the amplitude of the UPR?
4. Do the UPR sensors have specific functions in different cell types?
5. Do the various UPR sensors respond to the levels and/or duration of the accumulation of specific unfolded proteins?
6. How is plant IRE1 activated and inactivated?
7. What terminates the activity of activated bZIP-factors once ER stress is resolved and cell homeostasis is restored?
8. Does plant IRE1 cluster similar to the non-plant IRE1 proteins? And how does the ER shape specifically affect the UPR response?
9. Are there interacting proteins on the cytosolic region of plant IRE1?
10. What is the role of RIDD in normal conditions of growth in plants?
11. Does plant IRE1 cleave specific miRNAs as occurs in mammals?
12. What are the downstream factors that IRE1 utilizes to control organ development?
13. Does AtIRE1 have non-self-phosphorylation targets?
14. Does unspliced bZIP60 protein have a functional role in the absence of ER stress?
15. Are PERK-like proteins functional in plant UPR?

**Figure 1.**

Phylogenetic analysis of IRE1-like proteins and *AtbZIP60*-like transcripts from different plant species, yeast and human. **(a)** Phylogenetic tree analysis of IRE1-like proteins from Plantae (eudicots and monocots), Metazoa (*Homo sapiens*) and Fungi (*Saccharomyces cerevisiae*) was generated with MEGA6 program with 1000 replicates in the bootstrap test [51] using the neighbor-joining method [52]. The IRE1-like amino acid sequences were collected from NCBI database. Branch length is not to scale. **(b)** Phylogenetic tree analysis of *AtbZIP60*-like transcripts from Plantae (dicots and monocots), Metazoan (*Homo sapiens*) and Fungi (*Saccharomyces cerevisiae*) was generated with MEGA6 program with 1000 replicates in the bootstrap test [51] using the neighbor-joining method [52]. The *AtbZIP60*-like transcripts were collected from NCBI database. Branch length is not to scale.



**Figure 2.**

Players of the plant UPR known to date. The diagram illustrates that in addition to IRE1, bZIP28 and bZIP60, also bZIP17 plays a role in the control of UPR target genes in Arabidopsis, as recently proposed [27]. A role for the beta subunit of the heterotrimeric G-protein complex, AGB1, in modulating the UPR has also been reported [17•,53]. Furthermore, recent work has shown that NAC TFs participate to the plant UPR. NAC are plant-specific transcription factors, among which at least 13 members are predicted to be membrane-anchored in Arabidopsis. In condition of stress, the plasma membrane NAC062/ANAC062/NTL6 relocates to the nucleus, and the overexpression of a protein deletion devoid of the membrane anchor leads to activation of UPR genes [54•]. Another membrane-anchored NAC TF, NAC089, participates to ER stress responses by relocating from the ER to the nucleus and regulating PCD-related gene expression [55•]. *NAC089* expression is regulated by bZIP28 and bZIP60; bZIP60 also regulates directly the expression

of *NAC062*. Finally the cytosolic *NAC103*, whose expression is directly regulated by *bZIP60*, is involved in controlling UPR gene expression [26•]. The evidence that plant-specific NAC TFs have a role in stress responses support that in addition to common routes plants have evolved unique strategies for ER stress defense that, as in the case of *NAC062*, may also depend on the activity of TFs that are localized to the membrane organelles other than the ER. Interestingly also, it has been recently suggested that unspliced *AtbZIP60* may have a role in modulating the expression of *BiP3* in conditions of ER stress [27]. Phosphorylation of the *elf2α* may occur in a hypothetical PERK-like pathway for ER stress responses. See also the main text for additional details. TFD: transcription factor domain

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