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Plant J. Author manuscript; available in PMC 2015 January 01.

Published in final edited form as:
Plant J. 2014 January ; 77(1): 97–107. doi:10.1111/tpj.12373.

INTER-REGULATION OF THE UNFOLDED PROTEIN RESPONSE AND AUXIN SIGNALING

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SUMMARY

The unfolded protein response (UPR) is a signaling network triggered by overload of protein-folding demand in the endoplasmic reticulum (ER), a condition termed ER stress. The UPR is critical for growth and development; nonetheless, connections between the UPR and other cellular regulatory processes remain largely unknown. Here, we identify a link between the UPR and the phytohormone auxin, a master regulator of plant physiology. We show that ER stress triggers down-regulation of auxin sensors and transporters in *Arabidopsis thaliana*. We also demonstrate that an *Arabidopsis* mutant of a conserved ER stress sensor IRE1 exhibits defects in the auxin response and levels. These data not only support that the plant IRE1 is required for auxin homeostasis, they also reveal a species-specific feature of IRE1 in multicellular eukaryotes. Furthermore, by establishing that UPR activation is reduced in mutants of ER-localized auxin transporters, including PIN5, we define a long-neglected biological significance of ER-based auxin regulation. We further examine the functional relationship of IRE1 and PIN5 by showing that an *ire1 pin5* triple mutant enhances defects of UPR activation and auxin homeostasis in *ire1* or *pin5*. Our results imply that the plant UPR has evolved a hormone-dependent strategy for coordinating ER function with physiological processes.

Keywords

ER stress; unfolded protein response; auxin response; IRE1; PIN5; *Arabidopsis thaliana*

INTRODUCTION

The UPR adjusts the ER protein folding capacity to cope with the dynamic secretory protein demands in cells (Kozutsumi *et al.* 1988, Schroder and Kaufman 2005). When the ER protein folding machinery is competent, stress sensors are restrained in the ER by ER-resident chaperones (Bertolotti *et al.* 2000, Kimata *et al.* 2003). Accumulation of unfolded proteins in the ER activates ER stress sensors either by causing them to dissociate from protein chaperones or to associate with unfolded proteins (Bertolotti, *et al*. 2000, Credle *et*

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al. 2005, Gardner and Walter 2011, Kimata *et al*. 2003). Activated ER stress sensors transmit signals to the nucleus for transcriptional regulation of UPR target genes (Kozutsumi *et al*. 1988, Schroder and Kaufman 2005). If ER stress is not resolved, the UPR triggers the activation of cell death (Lin *et al.* 2007). IRE1, the only identified ER stress sensor in yeast, is conserved in multicellular eukaryotes (Cox *et al.* 1993, Mori *et al.* 1993). Two IRE1 homologues, AtIRE1A and AtIRE1B, have been proven to be functional ER stress sensors in *Arabidopsis* (Chen and Brandizzi 2012, Nagashima *et al.* 2011). The activation of IRE1 relies on auto-phosphorylation, conformational modification, and oligomerization. Activated IRE1 splices an intron from the mRNA of a UPR-specific bZIP transcription factor (Cox and Walter 1996). The spliced transcription factor enters the nucleus to control UPR target genes (Cox and Walter 1996).

The UPR is critical for numerous fundamental cellular processes (Wu and Kaufman 2006). IRE1 alpha knockout mice exhibit embryonic lethality (Iwawaki *et al.* 2009). Dysregulation of the UPR contributes to the pathology of several significant diseases, including diabetes, neurodegeneration, and cancer (Marciniak and Ron 2006). In *Arabidopsis*, mutations of IRE1 lead to a short primary root phenotype (Chen and Brandizzi 2012). Despite the high significance of the UPR in growth and development in multicellular eukaryotes, the regulatory connections between the UPR and other cellular responses remain unclear.

Because the hormone auxin has profound roles in most plant developmental processes, nucleus-based auxin signaling and plasma membrane (PM)-based intercellular auxin transport have been intensively studied. Three major classes of auxin signaling regulators exist in the nucleus: TIR1/AFB auxin co-receptors (Dharmasiri *et al.* 2005, Gray *et al.* 1999, Kepinski and Leyser 2005), AUX/IAA transcriptional repressors (Gray *et al.* 2001), and ARF transcription factors (Ulmasov *et al.* 1997). To initiate the auxin response in the nucleus, TIR1/AFBs and auxin coordinately promote degradation of AUX/IAA transcriptional repressors. Consequently, ARFs are released from repression and activate the transcription of auxin responsive genes (Dharmasiri *et al*. 2005, Gray, del Pozo *et al*. 1999, Kepinski and Leyser 2005).

Directional (polar) transport between cells is another crucial regulatory aspect of the auxin response. The auxin efflux carriers of the PIN family are the principal components of the polar auxin transport machinery (Petrasek *et al.* 2006, Wisniewska *et al.* 2006). Based on protein topology and subcellular localization, PINs can be classified into PM- or ER-localized types (Dal Bosco *et al.* 2012, Ding *et al.* 2012, Mravec *et al.* 2009). While PM-based intercellular auxin transport has been considered the most critical point of regulation in the auxin response, it has recently been revealed that ER-based auxin regulation is also important. A putative auxin receptor, ABP1, and several auxin transporters (PIN5, PIN6, PIN8, and PILSs) have been shown to localize to the ER. The requirement of the ER-localized regulators in the auxin response underscores the existence of ER-based auxin biology (Barbez *et al.* 2012, Dal Bosco *et al*. 2012, Mravec *et al*. 2009). Despite accumulating evidence that the ER is crucial for auxin regulation (Friml and Jones 2010), the physiological impact of ER-based auxin signaling is largely unknown.

As the UPR is critical for growth and development, we sought to identify the regulatory connection between the UPR and other cellular regulatory processes. Given the central roles of auxin in numerous aspects of plant physiology, we hypothesized that the UPR regulates auxin signaling for coordinating secretory activities and physiological responses. Through biochemical, molecular biology, and genetic analyses, we demonstrate a connection between the UPR and auxin biology. Specifically, we show that ER stress negatively influences auxin signaling and that the ER-based auxin homoeostasis is important for UPR activation, supporting that the plant UPR alters auxin signaling to cope with ER stress. On the contrary,

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by establishing that IRE1 is required for the auxin responses, our work reveals that IRE1 has a specific role in hormonal signaling. The regulatory connections between the UPR and auxin biology revealed here hint that plants have evolved an organism-specific strategy to maintain balance between stress adaption and growth regulation.

RESULTS

ER stress alters the expression of auxin regulators

To examine whether ER stress modulates the transcription of auxin regulators, we monitored the expression of four auxin co-receptors: *TIR1*, *AFB1*, *AFB2*, and *AFB3* (*TIR1*/*AFBs*), under ER stress. The UPR was activated by inhibiting protein *N*-glycosylation using a classical ER stress inducer termed tunicamycin (Tm). *Arabidopsis* seedlings were subjected to Tm for various periods of time, as adopted in established protocols (Koizumi *et al.* 2001, Mishiba *et al.* 2013, Noh *et al.* 2002). The transcriptional induction of UPR target genes is a molecular indication of UPR activation. To quantify the UPR activation levels, we monitored the transcription of classical UPR activation indicators, *BiP1/2* and *PDI6* over a 4-h time course of Tm treatment using quantitative reverse transcription–polymerase chain reaction (RT-qPCR) analyses (Kamauchi *et al.* 2005). *BiP1/2* is an ER chaperone essential for the UPR and a primary UPR target gene. *PDI6* encodes protein disulfide isomerase. Similar to BiP proteins, upregulation of PDI6 under ER stress contributes to increasing protein-folding capacity in the ER. RT-qPCR showed that both *BiP1/2* and *PDI6* were induced more than 2-fold at 0.5 h of Tm treatment and their levels increased over the time course of treatment (Figure S1). Interestingly, we found that there was a 20 to 55% percent reduction in the level of *TIR1*/*AFB* transcripts 4 h after Tm treatment in wild-type Col-0 seedlings (Figure 1a). These results imply that ER stress negatively influences auxin signaling by repressing *TIR1*/*AFB* transcripts. Since *TIR1*/*AFBs* activate the auxin response by promoting degradation of the AUX/IAA transcriptional repressors, we sought to determine whether the ER stress-induced repression of *TIR1*/*AFB* transcripts resulted in the stabilization of AUX/IAA proteins. To do so, we conducted western blot analyses using transgenic *Arabidopsis* plants expressing DII-VENUS, a fluorescently tagged auxin-interaction domain (DII) of AUX/IAAs that contains the canonical degron responsible for auxin- and TIR1/AFB-mediated protein degradation (Brunoud *et al.* 2012). As the stabilization of AUX/IAAs is a downstream response of TIR1/AFB reduction, we examined DII-VENUS protein levels 6 h after Tm treatment. Indeed, immunoblot analyses showed that the protein levels of DII-VENUS increased in wild-type Col-0 roots under ER stress treatment (Figure 1b). Consistent with the western blot analysis results, a confocal microscopy approach revealed that DII-VENUS fluorescence levels, and therefore AUX/IAA protein levels, were consistently greater in roots challenged by the ER stress inducer than in mock-treated ones (Figure 1c). Together these observations support that ER stress leads to an increase in AUX/IAA levels, which is most likely a consequence of protein stabilization resulting from the down-regulation of *TIR1*/*AFBs* (Figure 1a).

Next, we investigated whether ER stress could control the transcription of auxin transporters. Using RT-qPCR analyses, we detected a 30–80% decrease in the mRNA levels of *PIN1*, *PIN2*, *PIN3*, *PIN4*, *PIN5*, *PIN6*, and *PIN7* in wild-type Col-0 seedlings during ER stress treatment (Figure 1d). In contrast, the transcriptional levels of an ER-associated ethylene receptor (ETR1), two ER-localized cytokinin receptors (AHK2 and AHK3), three nuclear protein (RAN2, ABH1, and FIB1), and two secretory proteins (VSR1 and SCAMP3) (Ahmed *et al.* 1997, Chang *et al.* 1993, Kanneganti *et al.* 2007, Kierzkowski *et al.* 2009, Law *et al.* 2012, Ma *et al.* 2007, Wulfetange *et al.* 2011) remained unchanged in ER stress conditions (Figure S2). Thus, we conclude that the Tm-induced decrease in the abundance of *TIR1*/*AFB* and *PIN* transcripts is a specific cellular response. When ER stress was triggered

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by reduction of disulfide bond formation using dithiotreitol (DTT) treatment, similar down-regulation of *TIR1/AFB* and *PIN* transcripts was observed (Figure S3). Overall, these results show that ER stress specifically modulates the auxin response by repressing the transcription of auxin co-receptors and transporters.

Next, we examined whether either IRE1 or TIR1/AFBs is essential for ER stress-induced down-regulation of auxin regulators. To do so, we performed the same ER stress treatment coupled with RT-qPCR analyses in an *atire1a atire1b* double mutant and a *tir1 afb1 afb2 afb3* mutant (Chen and Brandizzi 2012, Dharmasiri *et al.* 2005). In *atire1a atire1b*, both *TIR1*/*AFB* and *PIN* transcripts were still reduced under ER stress conditions (Figure 2a, b), similar to the decreased *TIR1*/*AFB* and *PIN* transcripts pattern seen in wild-type Col-0 (Figure 1a). The *PIN* transcripts also decreased under ER stress conditions in *tir1 afb1 afb2 afb3* mutant backgrounds (Figure 2b). However, with the exception of *PIN7*, in *atire1a atire1b* the *PIN* and *TIR1*/*AFB* transcription levels were further slightly reduced compared to wild-type Col-0 (Figure 2c and S4). In contrast, the reduction of *PIN1*, *PIN2*, and *PIN4* transcript levels was larger in the *tir1 afb1 afb2 afb3* mutant compared to wild-type Col-0 (Figure 2c). These data indicate that down-regulation of *PIN* transcripts on ER stress is partially and slightly affected by mutations of either IRE1 or TIR1/AFBs. These results further suggest that the *IRE1* and *TIR1*/*AFBs* play unessential but fine-tuning roles in ER stress-mediated repression of auxin regulators.

IRE1 is required for auxin response and homeostasis

While the UPR is necessary for growth and development, the manner by which the UPR influences other cellular regulatory processes is largely unknown. Mammalian IRE1 controls multiple physiological responses under normal growth conditions. Our findings that auxin signaling is altered under ER stress hint that the UPR participates in the auxin response in plants. Although IRE1 is unessential for ER stress-triggered down-regulation of auxin regulators, we aimed to determine whether IRE1-dependent UPR is required for the auxin response without chemical induction of ER stress. Thus, we performed root inhibition assays to test the sensitivity of exogenous auxin application in *atire1a atire1b*. To do so, *atire1a atire1b* and wild-type Col-0 were germinated on medium containing a synthetic auxin analog, 1-naphthaleneacetic-acid (NAA), a naturally occurring auxin, indole-3-acetic acid (IAA), or an auxin transport inhibitor, 1-N-Naphthylphthalamic-acid (NPA). Interestingly, we found that *atire1a atire1b* was significantly less sensitive to exogenously applied NAA, IAA, or NPA than wild-type Col-0 (Figure 3a-c). The findings that *atire1a atire1b* and wild-type Col-0 displayed comparable root-inhibition responses to three other plant hormone, jasmonic acid (JA), abscisic acid (ABA) and ethylene, indicate that the plant *IRE1* has a role specifically in the auxin response, as opposed to general hormones responses or growth regulation (Figure S5). To further confirm that AtIRE1A/AtIRE1B is involved in the auxin response, we investigated the transcriptional activation of auxin-responsive genes in *atire1a atire1b* upon external application of auxin. RT-qPCR analyses showed that the transcriptional induction of five auxin-responsive genes, *IAA3*, *IAA5*, *IAA19*, *IAA20*, and *GH3.6*, was compromised after 2 and 4 h of NAA treatment in *atire1a atire1b* relative to wild-type Col-0 (Figure 3d and Figure S6a). The lower induction of auxin-responsive genes was repeatedly observed after both 1 and 2 h of IAA treatment (Figure S6b). These data further indicate that *atire1a atire1b* exhibits an impaired response to exogenously applied auxin. The transcription of *BiP1/2* or *PDI6* was not significantly altered under IAA or NPA treatment in wild-type Col-0, suggesting that IAA or NPA treatment does not trigger ER stress like Tm treatment (Figure S7). Furthermore, we found that there was a 30% reduction in the free auxin level in roots of ten-day-old *atire1a atire1b* plants compared to wild-type Col-0 (Figure 3e). While IRE1 is required to maintain the free auxin level without ER stress treatment, the free auxin level remained unaffected within 4 h after Tm treatment (Figure

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S8). All together, these data support that plant *IRE1* is required for auxin response and homeostasis.

ER-localized auxin regulators are involved in UPR activation

Our observations that auxin signaling is regulated by ER stress led us to test whether auxin homeostasis influences UPR activation. To this end, we examined whether mutations in auxin signaling, polar transport, or biosynthesis affected the induction of UPR target genes. Intriguingly, we found that a mutation in *PIN5* or *PIN6*, two ER-localized auxin transporters, compromised UPR activation under ER stress. Compared to wild type, *pin5-5* (Mravec *et al*. 2009) and *pin6-4* (Cazzonelli *et al.* 2013) exhibited a 30–40% reduction in the level of *BiP1/2* and *PDI6* transcripts during ER stress treatment (Figure 4a and S9). These data imply that ER-based auxin homeostasis contributes to UPR regulation in plants. This hypothesis was supported by the observation that mutants of other types of ER-localized putative auxin transporters, *pils2-2*, *pils5-2*, and *pils2-2 pils5-2* (Barbez *et al.* 2012) exhibited similar defects in UPR activation (Figure 4a and S9). We found that the expression levels of *BiP1/2* and *PDI6* were comparable among wild-type Col-0 and the auxin mutants without ER stress treatment (Figure S10), supporting that the ER-localized auxin transporters are involved in ER stress-triggered UPR activation. In contrast, we found no significant differences in the induction of UPR target genes in mutants defective in either PM-localized auxin exporters, *pin1-1* (*pin1*), *eir1-1* (*pin2*), *pin3-4* (*pin3*), *pin4-3* (*pin4*), *pin7-2* (*pin7*), *pin3-5 pin4-3 pin7-1* (*pin3 pin4 pin7*), an overexpressor of *PIN1* (*OxPIN1*), or the auxin importer *aux1-22* (*aux1*) compared to wild-type Col-0 under the same ER stress treatment conditions (Okada *et al.* 1991, Roman *et al.*, 1995, Zádníková *et al.*, 2010, Friml *et al.* 2002, Friml *et al.* 2003, Sauer *et al.* 2006, Swarup *et al.* 2004) (Figure 4b). These results show that ER-based intracellular auxin transport, but not intercellular auxin transport, is required for the optimal UPR activation in plants.

We next investigated whether the ER-localized putative auxin receptor ABP1 and the auxin biosynthesis enzyme YUC were also involved in the UPR activation. Similar to the auxin mutants defective in ER-based transport, the *abp1-5* and *YUC* (Xu *et al.* 2010, Zhao *et al.* 2001) mutants also exhibited reduced levels in the activation of UPR target genes under ER stress (Figure 4a and S9). Conversely, the transcription levels of UPR target genes were higher in the *tir1 afb1 afb2 afb3* auxin co-receptor mutant than in wild-type Col-0 (Figure 3a and S9), suggesting that TIR1/AFBs plays a negative role in UPR target gene induction. Altogether, these data highlight that the ER-based regulation of auxin homeostasis may operate as a molecular link between the UPR and other cellular processes.

pin5 enhances the *ire1* phenotype in auxin responses and UPR activation

To investigate a functional relationship of IRE1 and PIN5 in the UPR and auxin response, we generated an *atire1a atire1b pin5* triple mutant and performed phenotypic analyses. Consistent with previous reports, *atire1a atire1b* and *pin5-5* displayed a short primary root phenotype (Chen and Brandizzi 2012, Mravec *et al.* 2009). We found that the roots of the *atire1a atire1b pin5* triple mutant were significantly shorter than those of *atire1a atire1b* or *pin5-5* (Figure 5a). However, *atire1a atire1b*, *pin5-5*, and *atire1a atire1b pin5* showed comparable lateral root density and hypocotyl length to wild-type Col-0 (Figure S11), suggesting that the IRE1 and PIN5 have a role specifically in regulation of primary root elongation. In addition, *atire1a atire1b pin5* also displayed lower free auxin levels compared to *atire1a atire1b* or *pin5-5* (Fig 5b). Specifically, compared to wild-type Col-0, the roots of *pin5-5* and *atire1a atire1b* exhibited a 15 and 30% reduction in free auxin levels, respectively. Nonetheless, the roots of *atire1a atire1b pin5* displayed a 45% reduction in free auxin level (Figure 5b). Finally, in agreement with previous findings (Mravec *et al.* 2009), root inhibition assays showed that *pin5-5* was less sensitive than wild-type Col-0 to

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low concentrations of IAA but displayed a normal response to NAA, NPA, or high concentrations of IAA (Mravec *et al.* 2009). Intriguingly, the *atire1a atire1b pin5* mutant was significantly less sensitive than *atire1a atire1b* to all three treatments (Figure 5c-e). A comparable root-inhibition response to JA, ABA, and ethylene in *atire1a atire1b pin5* indicated that the genetic interaction between IRE1 and PIN5 is specific to the auxin response (Figure S5).

We next tested the functional relationship of IRE1 and PIN5 under ER stress. RT-qPCR revealed that the induction of *BiP1/2* and *PDI6* was also reduced in *atire1a atire1b pin5* compared to *atire1a atire1b* or *pin5-5*, supporting that PIN5 participates in the UPR activation in a manner not entirely dependent on *IRE1*. Altogether, our results imply that regulation of ER-based auxin homeostasis is part of ER stress adaptive mechanisms that plants have evolved to parallel the classical UPR signaling pathways.

DISCUSSION

Our findings uncover an unpredicted but critical regulatory relationship between two fundamental signaling pathways in plants, the UPR and auxin response. Studies of the mammalian UPR indicate that distinct UPR signaling pathways mediate specific physiological processes (Wu and Kaufman 2006). While the *IRE1*-dependent mRNA splicing event is the most evolutionarily conserved UPR pathway in eukaryotes, *IRE1* has also evolved specific functions in multicellular organisms to associate the UPR with more complex physiological processes (Hetz *et al.* 2006, Urano *et al.* 2000). Nevertheless, our understanding of the connection between the UPR and other cellular processes is still in its infancy. Here, we have defined a plant-specific regulatory role for *IRE1* in the auxin response. Although we cannot exclude the possibility that IRE1 regulates auxin response independently from the classical UPR, it is plausible that IRE1-dependent UPR signaling is involved in auxin transport. The auxin transport is one of the most crucial regulatory mechanisms in the auxin biology. As most regulatory components of the auxin transport system are secretory proteins, we speculate that the *IRE1*-dependent UPR signaling maintains a robust and efficient membrane trafficking system for the supply of functional auxin regulators. The identification of auxin regulators directly controlled by IRE1 will elucidate how IRE1 modulates specific aspects of auxin biology to coordinate the secretory pathway with physiological responses. Together with the involvement of UPR-specific membrane tethered transcription factors in brassinosteroid signaling (Che *et al.* 2010), our results support the significance of the plant UPR in hormone signaling.

IRE1 regulates the UPR through various mechanisms including unconventional splicing, RNA decay, and protein-protein interaction. It has recently been reported that similar to its mammalian counterpart, plant IRE1 controls gene expression through RNA decay in addition of splicing bZIP60 transcription factor (Mishiba *et al.* 2013). Mammalian IRE1 operates RNA decay to trigger diverse UPR signaling pathways. It would be interesting to test whether plant IRE1 also relies on its RNA decay function for the auxin response. If IRE1-dependent RNA decay contributes at least partially to the regulation of auxin signaling on ER stress, it would represent a specific regulatory event of the UPR as opposed to random RNA decay under stress. Notably, we have established that IRE1 is required for the optimal auxin response under exogenously applied auxin (Figure 3 and 6a), but plays only partial role in ER stress-induced down-regulation of auxin regulators (Figure 2). These findings support that distinct mechanisms regulate auxin signaling under various conditions to achieve context-specific auxin responses.

We have established that only ER-localized auxin transporters, but not PM-localized auxin exporters or importers, are required for the optimal UPR activation (Figure 6b). Studies of

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ER-localized auxin regulators suggest that a distinct auxin signaling pathway exists in the ER (Friml and Jones 2010). Accordingly, we propose that ER-based auxin signaling actively transports free auxin through the ER membranes to modulate the signaling response in the nucleus. More specifically, plant cells can transmit signals between sub-cellular compartments by adjusting the free auxin level in the ER, cytosol, and nucleus. We thus propose a previously uncharacterized cellular function for auxin as a signaling molecule that connects subcellular compartments and maintains cellular homeostasis in plants. It has long been believed that intercellular polar auxin transport is the key regulatory component of the auxin response; however, the biological significance of intracellular auxin transport has been overlooked. Our findings support a specific cellular function of ER-based intracellular auxin distribution in the UPR activation, and thus emphasize the importance of ER auxin biology in plant physiology.

We have shown that ER-localized auxin transporters have a role in the UPR activation. A plausible hypothesis to explain this is that the auxin levels in the ER lumen contribute to UPR activation on ER stress. Namely, ER-localized auxin transporters or their associated proteins might sense ER stress and rapidly adjust auxin levels in the ER lumen. The consequent fluctuation of auxin levels in the ER could in turn affect the magnitude of UPR activation. Nevertheless, because a mutation of PIN5 enhances the *atire1a atire1b* mutant phenotype in the UPR activation (Figure 5f), PIN5-dependent regulation of auxin levels under ER stress does not completely rely on IRE1. Whether ER-localized auxin transporters can directly monitor ER stress or indirectly sense ER stress-related cellular homeostasis is yet to be established; however, the findings presented here support that ER-localized transporters play a role in the UPR activation. Once a reliable system to monitor auxin levels in the ER lumen is developed, it will be interesting to experimentally confirm that ER-localized transporters mediate auxin transport between ER lumen and cytosol on ER stress.

PIN5 has been proposed to play a unique role in the auxin response since its transcriptional regulation and regulatory mechanisms appear to be different from PM-localized PINs. It was reported that the transcription of PIN5 is decreased under exogenous application of auxin although PIN5 is required for the auxin response (Mravec *et al.* 2009). Likewise, our study also showed that ER stress induces a decrease in the transcription of PIN5 while PIN5 is required for the optimal induction of UPR activation. As the PIN5 protein levels have not been monitored under auxin or ER stress treatment, one possibility is that the down-regulation of PIN5 transcript represents a feedback regulatory mechanism. Namely, the cellular availability or the activity of PIN5 may be increased in response to ER stress (e.g. by protein stabilization events or post-translational modifications). This in turn may cause reduction of PIN5 transcriptional levels to safeguard cellular auxin homeostasis. Another possibility is that ER stress represses general auxin responses including inter- and intra-cellular auxin transport to optimize cellular responses to cope with stress. Thus, both PM- and ER-localized transporter are down-regulated under ER stress; however, a basal level of ER-localized transporters may be still required for optimal induction of UPR target gene as they might be involved in stress signal transmission through transport the auxin between subcellular compartments. Thus, mutants of ER-localized auxin regulators would display a compromised UPR activation. Further experimental evidences are needed to verify the possibilities. Nonetheless, our data support that regulation of PIN5 transcripts is a mechanism to maintain PIN5-related cellular homeostasis. Also, *pin5-5* was shown to have a higher free auxin levels (Mravec *et al.* 2009) but we found that *pin5-5* displayed lower free auxin level. This is possibly because unlike Mravec *et al*, which used intact seedlings, we used only root tissues in the free auxin level assay. Future comprehensive quantification analyses of free auxin levels in various tissues will likely reveal whether PIN5 regulates auxin distribution among tissues.

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The molecular mechanisms underlying transcriptional down-regulation of auxin receptors and transporters on ER stress are still unknown. Whether UPR regulators can directly control transcription of auxin receptors and transporters or ER stress-dependent cellular responses mediate auxin homeostasis in a manner independent of classical UPR regulation awaits further validation.

In contrast to animals, plants, as sessile organisms, have an extraordinary plasticity in post-embryonic development, responding to both internal and external cues. Nonetheless, our understanding of how plants integrate developmental and environmental signals to balance growth and adaptive regulation is limited. The inter-regulation between the UPR and auxin response demonstrated in this study opens a new area of investigation in plant physiology. Given the essential roles of the UPR in multiple stresses adaptation, the integrated action of the UPR and auxin response highlights a plant-specific strategy that evolved to maintain the crucial balance between stress response and growth regulation for ultimate fitness.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) plants were used. Surface-sterilized seeds were plated directly onto petri dishes containing half-strength Linsmaier and Skoog (LS) medium, 1.5% w/v sucrose, and 0.4% Phytagel (Sigma). For normal growth conditions, plants were grown at 21°C under a 16-h light/8-h dark cycle.

Tm treatment

Seeds were germinated and grown on half-strength LS medium for 10 days, and then transferred to half-strength LS medium containing 5 μg/ml Tm (Sigma) for the indicated periods of time.

RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted from whole seedlings using an RNeasy Plant Mini Kit (Qiagen) and treated with DNase I (Qiagen). All samples within an experiment were reverse-transcribed simultaneously using SuperScript® VILO™ Master Mix, (Invitrogen). A no-RT reaction, in which RNA was subjected to the same conditions of cDNA synthesis but without reverse transcriptase, was included as a negative control in all real-time quantitative PCR (RT-qPCR) assays. RT-qPCR with SYBR Green detection using a relative standard curve method was performed in triplicate using the Applied Biosystem 7500 Fast Real-Time 7500 PCR system. Data were analyzed by the summary of efficiency (DDCT) method. The values presented are the mean of three independent biological replicates. Primers used are listed in Supplementary Table S1.

Phenotypic analysis

Root length and hypocotyl elongation measurements were averaged from 30 plants for each genotype. Data were analyzed by Student's two-tailed *t*-test, assuming equal variance; differences with a P-value < 0.05 were considered significant.

Immunoblotting and confocal microscopy analyses

Fifty milligrams of fresh root tissues was ground in plastic tubes with plastic pestles using liquid nitrogen and 500 ml of SDS-containing extraction buffer (60 mM Tris-HCL (pH 8.8), 2% SDS, 2.5% glycerol, 0.13 mM EDTA (pH 8.0), and 1X Protease Inhibitor Cocktail Complete (Roche)). The tissue lysates were vortexed for 30 s, heated at 70°C for 10 minutes, and then centrifuged at 13,000 g twice for 5 minutes at room temperature. The

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supernatants were transferred to new tubes. For SDS-PAGE analysis, 5 ml of the extract in 1x NuPAGE LDS Sample Buffer (Invitrogen) was separated on 4–12% NuPage gel (Invitrogen) and transferred to PVDF (polyvinyl difluoride) membrane. The membrane was incubated with 3% BSA in 1x TBST (50 mM Tris-base, 150 mM NaCl, 0.05% Tween 20, pH 8.0) overnight at 4°C, and was probed with antibody (α-GFP, 1:20,000; Abcam) diluted in the blocking buffer (1:20,000) at room temperature for 1 h. The probed membrane was washed three times with 1x TBST for 5 min and then incubated with secondary antibody (goat anti-rabbit IgG for α-GFP, 1:20,000; Abcam) at room temperature for 1 h. The membrane was further washed four times with 1x TBST for 10 min before the signals were visualized with SuperSignal® West Dura Extended Duration Substrate (Pierce Biotechnology). To visualize YFP fluorescence, an inverted laser scanning confocal microscope Zeiss LSM510 was used to detect the DII expression.

Free IAA analysis

Approximately 20 roots were cut from ten-day-old seedlings and trasferred into an Eppendorf tube containing 1 ml of methanol. Internal standard of [2H5] IAA was added to the sample at amount of 100 fmol per root.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Teva Vernoux for sharing the DII-VENUS seeds and Jürgen Kleine-Vehn for sharing the *pils2-2*, *pils5-2*, and *pils2-2pils5-2* seeds and the Arabidopsis Biological Resource Center (ABRC) for seed stocks. This study was supported by grants from the Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, Office of Science, U.S. DOE (DE-FG02-91ER20021) for the infrastructure, National Institutes of Health (R01 GM101038-01), and NASA (NNX12AN71G).

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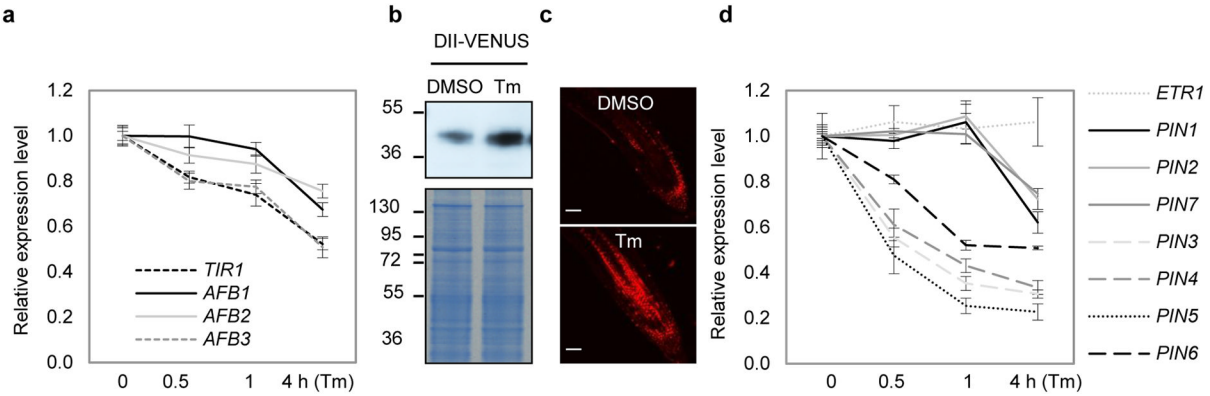

Figure 1. ER stress alters the expression of auxin regulators

(a) RT-qPCR analyses of *TIR1*, *AFB1*, *AFB2*, and *AFB3* expression in ten-day-old Col-0 *Arabidopsis* seedlings after treatment with 5 μg/ml Tm for 0.5, 1, or 4 h. Error bars represent standard error of the mean (SEM) from three independent biological replicates. *P*-values were calculated by Student's two-tailed t test against expression levels at 4 h relative to 0 h: *TIR1* (*P* = 0.00036), *AFB1* (*P* = 0.00041), *AFB2* (*P* = 0.00048), *AFB3* (*P* = 0.00032).

(b) The levels of DII-VENUS fusion proteins increase upon exposure to ER stress. Ten-day-old DII-VENUS transgenic plants were treated with 5 μg/ml Tm or DMSO for 6 h. Proteins were extracted from root tissues and the fusion proteins were detected by immunoblot analysis using anti-GFP serum (upper panel). Coomassie blue staining gel used as loading control (lower panel).

(c) Ten-day-old transgenic plants expressing DII-VENUS were treated with 5 μg/ml Tm or DMSO for 6 h. Primary root tips were subjected to confocal microscopy analyses. Scale bars = 50 μm.

(d) *PIN* mRNA levels decrease upon exposure to ER stress. RT-qPCR analyses of *PIN* family transcripts in ten-day-old wild-type Col-0 seedlings during treatment with 5 μg/ml Tm for 0.5, 1, or 4 h. Error bars represent SEM from three independent biological replicates. *P*-values were calculated against expression levels at 4 h relative to 0 h: *PIN1* (*P* = 0.00221), *PIN2* (*P* = 0.00316), *PIN3* (*P* = 5.4E-05), *PIN4* (*P* = 4.9E-05), *PIN5* (*P* = 6.4E-05), *PIN6* (*P* = 0.00012), *PIN7* (*P* = 0.00353). The transcriptional level of ETR1, an ER-associated ethylene receptor, was unchanged after treatment with Tm for 0.5, 1, or 4 h.

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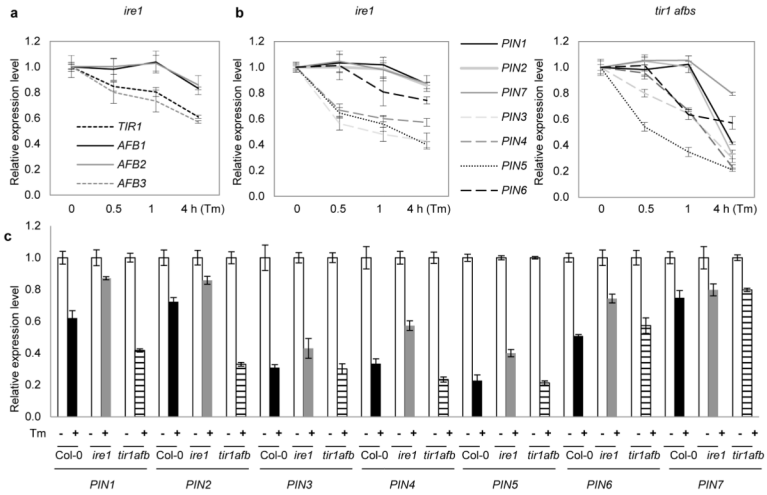

Figure 2. IRE1 and TIR1/AFBs play fine-tuning roles in ER stress-induced down-regulation of auxin regulators

(a) RT-qPCR analyses of *TIR1*, *AFB1*, *AFB2*, and *AFB3* expression in ten-day-old *atire1a atire1b* (*ire1*) *Arabidopsis* seedlings after treatment with 5 μg/ml Tm for 0.5, 1, or 4 h.

(b) *PIN* mRNA levels decrease upon exposure to ER stress in ten-day-old *ire1* or *tir1 afb1 afb2 afb3* (*tir1 afb*). RT-qPCR analyses of *PIN* family transcripts in ten-day-old *ire1* or *tir1 afb Arabidopsis* seedlings after treatment with 5 μg/ml Tm for 0.5, 1, or 4 h.

(c) Transcriptional repression of *PIN*s after treatment with 5 μg/ml Tm for 4 h in ten-day-old Col-0, *ire1*, or *tir1 afb Arabidopsis* seedlings.

Error bars represent SEM from three independent biological replicates.

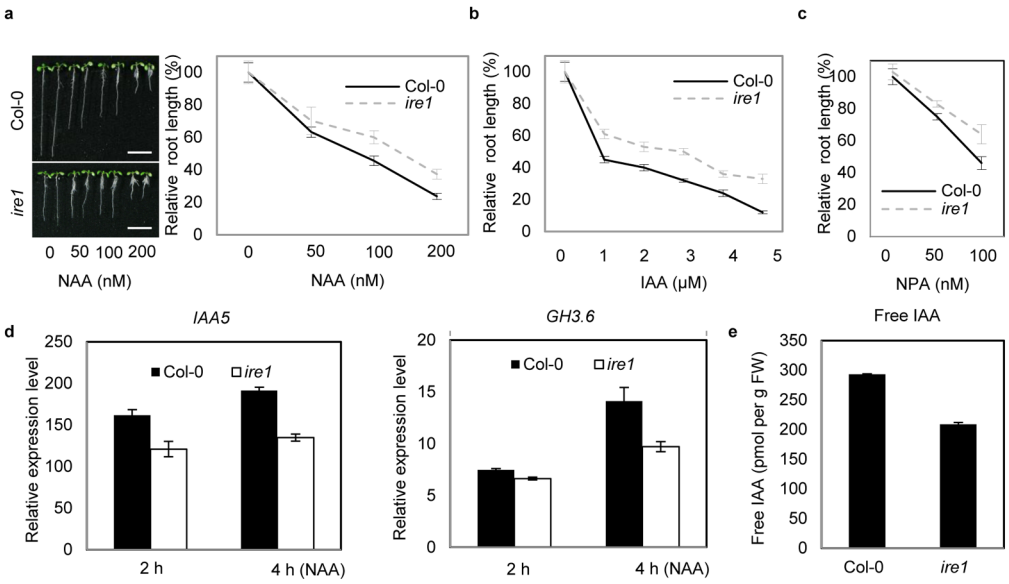

Figure 3. *atire1a atire1b* (*ire1*) exhibits compromised auxin responses

(a–c) In *ire1*, root growth is largely resistant to treatments with auxin (NAA and IAA) or an auxin transport inhibitor (NPA). Relative primary root length of ten-day-old Col-0 and *ire1 Arabidopsis* seedlings grown in the presence 50, 100, 200 nM NAA (a), or 1, 2, 3, 4, 5 μM IAA (b), or 50, 100 nM NPA (c) compared to those grown in the absence of the chemicals. Error bars represent standard error of the mean (SEM), n > 30. Scale bars = 1 cm. *P*-values are relative to Col-0: 100, 200 nM NAA ($P < 0.00078$), 1, 2, 3, 4, 5 μM IAA ($P < 0.00050$), 50, 100 nM NPA ($P < 0.00344$).

(d) RT-qPCR analyses of *IAA5* and *GH3.6* expression in ten-day-old Col-0 and *ire1 Arabidopsis* seedlings after a 2- or 4-h treatment with 10 μM NAA. Error bars represent SEM from three independent biological replicates. *P*-values are relative to Col-0: *IAA5* ($P < 0.00016$), *GH3.6* ($P < 0.00391$).

(e) Free IAA concentration in ten-day-old Col-0 and *ire1* roots. Error bars represent SEM from three independent biological replicates. *P*-value is relative to Col-0: $P = 4.9E-05$.

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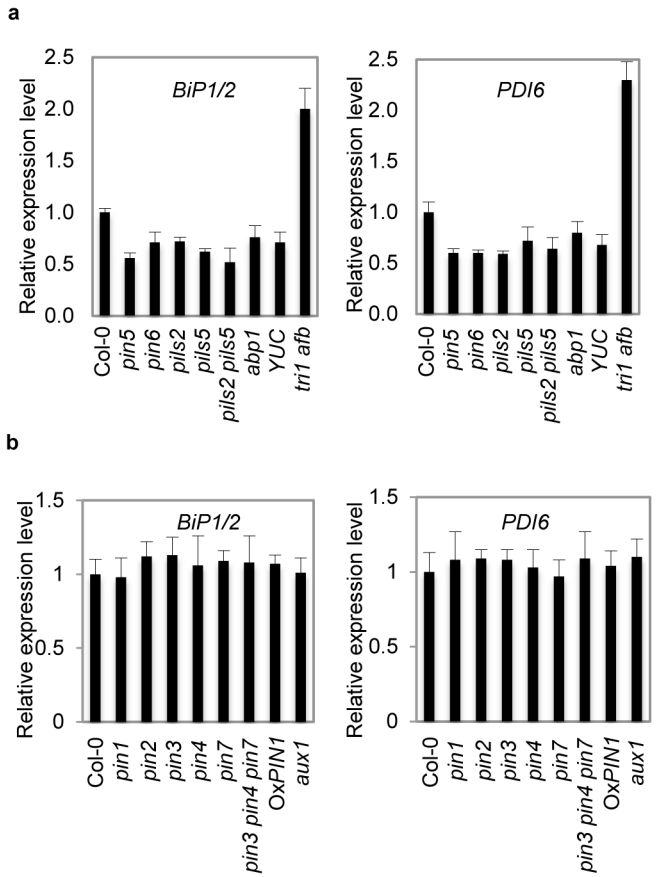

Figure 4. Mutants impaired in intracellular auxin transport display a defective UPR phenotype

(a) RT-qPCR analyses of *BiP1/2* and *PDI6* in ten-day-old *pin5-5* (*pin5*), *pin6-4* (*pin6*), *pils2-2* (*pils2*), *pils5-2* (*pils5*), *pils2-2 pils5-2* (*pils2 pils5*), *abp1-5* (*abp1*), *YUC*, and *tir1 afb1 afb2 afb3* (*tir1 afb*) relative to wild-type Col-0 *Arabidopsis* seedlings after a 1-h treatment with 5 μg/ml Tm. Error bars represent standard error of the mean (SEM) from three independent biological replicates. *P*-values are relative to Col-0: *pin5* ($P = 0.00029$), *pin6* ($P = 0.00095$), *pils2* ($P = 0.00093$), *pils5* ($P = 0.00067$), *pils2 pils5* ($P = 0.00089$), *abp1* ($P = 0.00215$), *YUC* ($P = 0.00014$), *tir1 afb* ($P = 0.00026$).

(b) RT-qPCR analyses of *BiP1/2* and *PDI6* in ten-day-old *pin1-1* (*pin1*), *eir1-1* (*pin2*), *pin3-4* (*pin3*), *pin4-3* (*pin4*), *pin7-2* (*pin7*), *pin3-4 pin4-3 pin7-2* (*pin3 pin4 pin7*), *OxPIN1*, and *aux1-22* (*aux1*) relative to wild-type Col-0 *Arabidopsis* seedlings after a 1-h treatment with 5 μg/ml Tm. Error bars represent SEM from three independent biological replicates.

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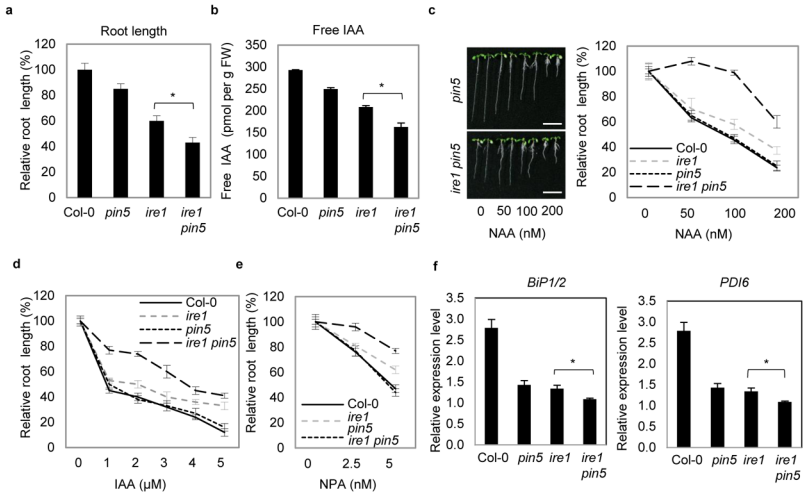

Figure 5. *pin5* enhances the auxin and ER stress response phenotype in *atire1a atire1b* (*ire1*)

(a) *pin5-5* (*pin5*) enhances the short root phenotype of *ire1*. Relative primary root length of *pin5*, *ire1*, and *ire1 pin5* compared to Col-0 under unstressed conditions. Error bars represent standard error of the mean (SEM), n > 30. *P*-value is *ire1 pin5* relative to *ire1*: **P* = 0.00226.

(b) Free IAA measurement in the roots of ten-day-old Col-0, *pin5*, *ire1*, and *ire1 pin5* seedlings. Error bars represent SEM from three independent biological replicates. *P*-value is *ire1 pin5* relative to *ire1*: **P* = 0.00182.

(c–e) Relative primary root length of ten-day-old Col-0, *pin5*, *ire1*, and *ire1 pin5* *Arabidopsis* seedlings grown in the presence 50, 100, 200 nM NAA (c), or 1, 2, 3, 4, 5 μM IAA (d), or 50, 100 nM NPA (e) compared to those grown in the absence of the chemicals. Error bars represent SEM, n > 30. *P*-values are *ire1 pin5* relative to *ire1*: 50, 100 or 200 nM NAA (*P* < 0.00032), 1, 2, 3, 4, or 5 μM IAA (*P* < 0.00075), 50 or 100 nM NPA (*P* < 0.00149). Scale bars = 1 cm.

(f) *pin5* enhances the UPR defects in *ire1* under ER stress. RT-qPCR analyses of *BiP1/2* and *PDI6* in ten-day-old Col-0, *pin5*, *ire1*, and *ire1 pin5* relative to DMSO mock control after a 1-h treatment with 5 μg/ml Tm. Error bars represent SEM from three independent biological replicates. *P*-value is *ire1 pin5* relative to *ire1*: **P* = 0.01856.

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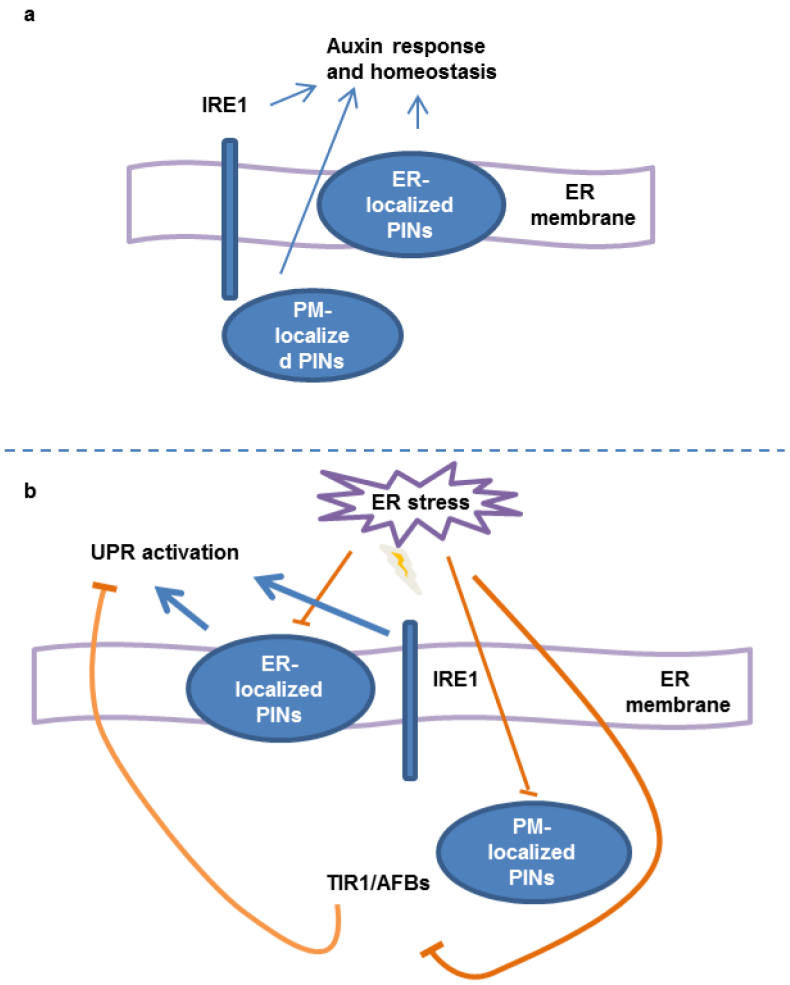

Figure 6. Working model

(a) IRE1 is required for the auxin responses upon external auxin application. IRE1, ER- and PM-localized PINs are involved in the maintenance of auxin homeostasis without chemical induction of ER stress.

(b) ER stress triggers down-regulation of auxin receptors TIR1/AFBs, ER- and PM-localized PINs. IRE1 and ER-localized PINs are required for the optimal induction of UPR target genes.