Systemic signaling contributes to the unfolded protein response of the plant endoplasmic reticulum

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The unfolded protein response (UPR) of the endoplasmic reticulum constitutes a conserved and essential cytoprotective pathway designed to survive biotic and abiotic stresses that alter the proteostasis of the endoplasmic reticulum. The UPR is typically considered cell-autonomous and it is yet unclear whether it can also act systemically through non-cell autonomous signaling. We have addressed this question using a genetic approach coupled with micro-grafting and a suite of molecular reporters in the model plant species Arabidopsis thaliana. We show that the UPR has a non-cell autonomous component, and we demonstrate that this is partially mediated by the intercellular movement of the UPR transcription factor bZIP60 facilitating systemic UPR signaling. Therefore, in multicellular eukaryotes such as plants, non-cell autonomous UPR signaling relies on the systemic movement of at least a UPR transcriptional modulator.
n physiological conditions of growth and in disease, eukaryotic life depends on the biosynthetic ability of the endoplasmic reticulum (ER) to synthesize correctly folded secretory proteins. Conditions that alter the ER proteostasis and induce accrual of misfolded proteins in the ER lead to a potentially lethal condition known as ER stress. At the onset of ER stress, cells activate cell-intrinsic UPR signaling pathways mediated by specialized ER stress sensors whose function is to reprogram gene expression for the synthesis of effectors that attenuate ER stress and restore the biosynthetic ability of the ER. If the UPR is ineffective to initiate proper cytoprotective mechanisms to attenuate ER stress, it induces programmed cell death.

The main branch of the UPR is mediated by the ER-associated kinase and ribonuclease inositol-requiring protein 1 (IRE1) through largely conserved mechanisms. During ER stress, upon oligomerization and trans-autophosphorylation for self-activation, IRE1 splices the mRNA of a basic leucine zipper (bZIP) transcription factor, namely HAC1 in yeast, XB1 in maize, and bZIP60 in plants. This step removes the coding region for a transmembrane domain (TMD), releasing the translational inhibition of a potent UPR transcriptional factor. The newly synthesized transcription factor is translocated to the nucleus where it modulates the expression of nuclear UPR target genes for the restoration of ER proteostasis. Multicellular eukaryotes also harbor another UPR branch, which is mediated by ER membrane tethered transcription factors (MTTFs), such as ATF6 in maize and bZIP28 in plants. Upon ER stress sensing, these MTTFs translocate to the Golgi where the transcription factor domain is cleaved off the transmembrane anchor and is then transported to the nucleus to regulate transcription of UPR target genes.

In addition to cell-intrinsic signaling, the metazoan UPR may actuate non-cell autonomous signaling for the activation of stress responses in tissues and cell types that are different from those where the ER stress signal is originated. Specifically, in *C. elegans* intercellular signaling of the UPR has been induced through the overexpression of spliced (i.e., active) XB1 in neuron cells, which elicits UPR activation in non-stressed intestine cells. Similarly, in mice overexpression of active XB1 in hypothalamic proopiomelanocortin (POMC) neurons is followed by non-cell autonomous signaling for the activation of stress responses in tissues and cell types that are different from those where the ER stress signal is originated. Specifically, in *C. elegans* intercellular signaling of the UPR has been induced through the overexpression of spliced (i.e., active) XB1 in neuron cells, which elicits UPR activation in non-stressed intestine cells. Similarly, in mice overexpression of active XB1 in hypothalamic proopiomelanocortin (POMC) neurons is followed by non-cell autonomous splicing of XB1 and UPR activation in the liver. Although the existence of secreted stress signals to actuate transcellular UPR has been hypothesized, the identity of the effectors that act downstream XB1 in intercellular communication of the UPR in metazoans is currently unknown. It is yet also unknown whether the systemic UPR signaling occurs in experimental conditions that do not rely on tissue-specific overexpression of XB1.

Plants show cell-intrinsic UPR signaling; however, whether they also execute non-cell autonomous UPR signaling is still an open question. Here, we demonstrate that in plants, in addition to cell-autonomous signaling, the UPR extends to systemic tissues by non-cell autonomous signaling through the contribution of the mobile UPR transcription factor bZIP60. Our findings indicate that in eukaryotes non-cell autonomous UPR signaling can directly rely on the translocation of at least one UPR transcriptional regulator.

**Results**

**Spliced bZIP60 translocates transcellularly.** To test whether systemic UPR signaling may take place in plants, we first adopted a cell-type specific expression assay in *Arabidopsis* transgenic roots. We used the short-root (SHR) promoter, which is exclusively active in the stele, the central tissue of the root, and drives the expression of SHR. The latter is a nucleus-localized transcription factor that moves from the stele, where it is synthesized, to the endodermis, a tissue layer surrounding the stele; notoriously, SHR does not reach the cortex and epidermis, which envelope the endodermis. We used the SHR promoter to drive expression of cytosolic green fluorescent protein (GFP) (pSHR-GFP) and GFP fused either to SHR (pSHR-SHR-GFP) or to a constitutively active form of bZIP60, spliced bZIP60-GFP (pSHR-bZIP60-GFP). We used wild-type Col-0 (hereafter Col-0), an SHR knockout (shr-2, hereafter shr), and bzip28/60-18 (hereafter bzip28/60). In bzip28/60 both UPR branches (i.e., bZIP60 and bZIP28) are inactive in conditions inducing ER stress (i.e., Tunicamycin (Tm) treatment), and the expression of UPR genes, including *BIP3*, the major target of sbZIP60, is not actuated. This setup was therefore designed to test movement of bZIP60 across tissues. A GFP fusion of bZIP60 driven by the native promoter in a bzip60 mutant is localized throughout the root tissues in control conditions and in conditions of ER stress (Supplementary Fig. 1) hampering the possibility to assess systemic movement of this transcription factor. In our experimental setup, we expected that cytosolic GFP would be detected exclusively in the stele, while SHR-GFP would be localized in the stele and the endodermis. Conversely, if sbZIP60 moved transcellularly, then sbZIP60-GFP expression in the stele would result in the accumulation of sbZIP60-GFP in the stele as well as in other cell layers. Confocal imaging of cytosolic GFP and SHR-GFP in the root of the respective Col-0 and *shr* transgenic lines showed a diffuse distribution of cytosolic GFP in the stele, and a localization of SHR-GFP in the nuclei of the stele and endodermis (Fig. 1a). These results are consistent with earlier findings and indicate that stele-expressed cytosolic GFP accumulates only in the stele, while SHR-GFP, which is produced in the stele, moves to the endodermis. When we analyzed bzip28/60; pSHR-bZIP60-GFP roots, we found accumulation of sbZIP60-GFP in the nuclei and cytoplasm of cells in the stele and endodermis, as well as cortex and epidermis (Fig. 1a), which is comparable with the localization of GFP-bZIP60 driven by the bZIP60 native promoter in conditions of ER stress (see also Supplementary Fig. 1). In addition, such distribution pattern was visible throughout the division, elongation and differentiation zones of roots with graded level of fluorescence from the younger regions of the root upward (Supplementary Fig. 2). In light of the restricted accumulation of cytosolic GFP to the stele and of SHR-GFP to the stele and endodermis, these results strongly support that sbZIP60 can move transcellularly from the stele to the epidermis through the endodermis and cortex.

Next, we tested whether the transcellular movement of sbZIP60 could play a role in UPR signaling in the tissues in which it is translocated. We developed a genetically encoded reporter for UPR activation by sbZIP60 in systemic tissues by expressing β-glucuronidase (GUS) under the control of the *Bip3* promoter (pBip3-GUS) in either Col-0 (Col-0; pBip3-GUS; positive control) or bzip28/60. In the latter background we introduced pBip3-GUS alone (bzip28/60; pBip3-GUS; negative control) or in combination with pSHR-bZIP60-GFP (bzip28/60; pSHR-bZIP60-GFP/pBip3-GUS). We then tested where the UPR could be activated systemically in the root by stele-expressed sbZIP60. We expected that if sbZIP60 activated the Bip3 promoter in a systemic manner, then the tissue labeling by GUS would mirror the verified tissue distribution of sbZIP60-GFP (Fig. 1a) and would show staining intensity levels above background. As expected, we found no expression of pBip3-GUS throughout the bzip28/60 roots (Fig. 1b, c), owing to the lack of functional bZIP60 and bZIP28. In wild type, Bip3 is generally expressed in the absence of induced ER stress. Consistently with this, in Col-0; pBip3-GUS we verified GUS expression, which was visible in the endodermis of the upper maturation zone, and predominantly in the stele and cortex.
endodermis of the middle maturation and lower maturation zones (Fig. 1b, c), in agreement with tissue-specific transcriptomics analyses of the root24. In conditions of ER stress, the BiP3 expression was robustly enhanced in the stele, endodermis, cortex, and epidermis layers throughout all the root zones (Supplementary Fig. 3), consistently with previous findings25. We then analyzed bzip28/60; pSHR-sbZIP60-GFP/pBiP3-GUS transgenic plants and found a strong GUS expression in the stele and endodermis but also in the cortex and epidermis of all the root zones under analysis (Fig. 1b, c). The strong GUS activity in the bzip28/60; pSHR-sbZIP60-GFP/pBiP3-GUS line is likely linked to the overabundance of sbZIP60 driven by pSHR in these tissues compared to Col-0; pBiP3-GUS. Importantly also, the GUS activity in bzip28/60; pSHR-sbZIP60-GFP/pBiP3-GUS mirrors the verified distribution of pSHR-sbZIP60-GFP in the cortex and epidermis as well as BiP3 expression pattern under stress condition (Fig. 1a, Supplementary Fig. 3). These results support our original observations that sbZIP60-GFP can translocate systemically from a tissue where it is specifically expressed and activate transcription of a target gene in systemic tissues. Taken together, these data indicate that, in conditions of overexpression of a constitutively active UPR modulator, the UPR signaling is executed systemically in plants. The results also indicate that overexpressed sbZIP60 can act as transcellular mobile transcription factor, triggering UPR gene expression in a systemic manner.

**Systemic induction of UPR genes.** To test a systemic signaling of sbZIP60 from the root to a different organ, we assessed the expression of UPR marker genes in the shoot and root of bzip28/60; pSHR-sbZIP60-GFP/pBiP3-GUS seedlings grown vertically on half LS agar medium for 11 days. X-Gluc was used for histochemical staining to monitor GUS activity. Scale bar: 100 μm.

**c** Longitudinal confocal optical sections of the regions along the primary root shown in b. Epidermis: Ep; Co: cortex; En: endodermis; St: stele. The indications upper, middle and lower refer to the a, b, and c zones indicated in panel b. Scale bar: 20 μm.

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**Fig. 1** Intercellular translocation of sbZIP60 induces BiP3 expression in systemic tissues. a Confocal laser scanning microscopy of Col-0; pSHR-GFP, shr; pSHR-SHR-GFP, and bzip28/60; pSHR-sbZIP60-GFP at the primary root tips of 5-day-old transgenics reveals stele (St) accumulation of GFP, and stele and endodermis (En) distribution of SHR-GFP; noticeably, sbZIP60-GFP is localized in the stele, endodermis, cortex (Co) and epidermis (Ep). Similarly to SHR-GFP, sbZIP60-GFP is localized in nuclei (arrows). As also reported earlier22, we did not find SHR-GFP localization in the nuclei of the cortex and epidermis. Propidium iodide (PI) was used for counterstaining. Scale bar: 50 μm. b Expression of pBIP3:GUS in bzip28/60, Col-0 and bzip28/60; pSHR-sbZIP60-GFP seedlings grown vertically on half LS agar medium for 11 days. X-Gluc was used for histochemical staining to monitor GUS activity. Scale bar: 100 μm. c Longitudinal confocal optical sections of the regions along the primary root shown in b. Epidermis: Ep; Co: cortex; En: endodermis; St: stele. The indications upper, middle and lower refer to the a, b, and c zones indicated in panel b. Scale bar: 20 μm.

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Root-expressed sbZIP60 transcripts are translocated to the shoot. a, b Quantitative RT-PCR analyses of sbZIP60 (a) and BiP3 (b) in 14-day-old wild type (Col-0), bzip28/60, and bzip28/60; pRoot::sbZIP60 seedlings treated with DMSO or 0.5 μM Tm (Tunicamycin) at the root in the shoot-root split system for 24 h. Transcription of UBQ10 was used as internal control. Error bars represent s.e.m among three biological replicates. Data significantly different from the corresponding control are indicated by asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; Unpaired t-test). Black asterisks designate differences between bzip28/60 and bzip28/60; pRoot::sbZIP60 lines under Tm non-treatment condition. Red asterisks designate differences between bzip28/60 and bzip28/60; pRoot::sbZIP60 under Tm treatment condition

Systemic UPR acts systematically in a shoot-ward direction. We next tested the occurrence of endogenous systemic UPR using the shoot–root split culture system in order to apply Tm for 24 h either to the medium at the shoot or the root of 2-week old wild-type Col-0 seedlings (Fig. 3a); we then monitored the UPR signaling in each portion of the seedling by qRT-PCR (Fig. 3b). We first monitored the sbZIP60 and BiP3 mRNA levels in seedlings treated with Tm at the roots and found increased levels compared to the root of a mock control (Fig. 3b; D/T vs 0 h). When we analyzed the untreated shoot of the root-treated seedlings, we observed a significant increase in the transcript levels of sbZIP60 and BiP3 compared to mock control (Fig. 3b; D/T vs 0 h). These results indicate that Tm-treatment of the root leads to UPR signaling activation both in the Tm-treated root as well as in the untreated shoot. Next, we analyzed the UPR gene transcripts in seedlings treated with Tm at the shoots (Fig. 3a; T/D). We found that both sbZIP60 and BiP3 transcript levels increased in the shoot compared to mock control (Fig. 3b; T/D vs 0 h). These results point towards the possibility of systemic actuation of ER stress responses mainly in a shoot-ward direction. We next tested the kinetic profiles of bZIP60 transcription and splicing as well as the subsequent activation of BiP3 expression in response to ER stress within 24 h. We found that in the Tm-treated roots, a significant induction of unspliced bZIP60 (unbZIP60) occurred at 3 h (Supplementary Fig. 6A). Consistently, the emergence of sbZIP60 transcripts started at 3 h and reached peak expression at 6 h post treatment followed by a
The BiP3 expression displayed a similar trend like sbZIP60 but reached the peak at 12 h post treatment (Supplementary Fig. 6C), in line with a time correlation of the sbZIP60-driven BiP3 activation in response to the ER stress. In the untreated shoots, a significant induction of unbZIP60 and sbZIP60 was detected at 3 h post treatment and BiP3 expression was predominantly detected at 6 h post treatment (Supplementary Fig. 6A–C) supporting a roughly 3 h-requisite for systemic UPR signals, including bZIP60 transcripts translocation, from treated roots to shoots to elicit the downstream UPR gene BiP3 in our experimental conditions. We then monitored Tm levels in the shoot and root of seedlings treated with Tm in the shoot–root split culture system, using HPLC/MS. As controls, we used seedlings exposed to either DMSO (D/D) or Tm (T/T) at both the shoot and the root. As expected, we observed a significant Tm accumulation in the treated shoot and root compared to the respective DMSO (mock control)-only treated tissues (Fig. 3c; D/T and T/D vs D/D), indicating that the seedlings absorbed Tm from the growth medium. Furthermore, the root of seedlings with Tm-treated shoot showed accumulation of Tm (Fig. 3c; T/D vs D/D), indicating that Tm can translocate from the shoot to the root. Importantly however, we found no significant increase of Tm levels in the DMSO only-treated shoot of seedlings with Tm-treated root compared to mock seedlings (Fig. 3c; D/T vs D/D) indicating that Tm is not transported from the root to the shoot in the shoot–root split culture system to a detectable level. Taken together, these results support that an

**Fig. 3** Local ER stress ignites the UPR systemically, mostly in a shoot-ward direction. a Diagrams illustrating the Arabidopsis shoot–root split culture system in which the shoot and root of an intact seedling are exposed to separate growth media with different chemical conditions: mock DMSO (D) or 0.5 µM Tm (T, Tunicamycin). T/D denotes shoot on Tm-containing medium and root on DMSO-containing medium; conversely, D/T denotes shoot on DMSO-containing medium and root on Tm-containing medium. b Quantitative RT-PCR analyses of UPR markers in 14-day-old wild-type seedlings treated with DMSO or 0.5 µM Tm for 24 h as described in a. Values are presented relative to non-treated control (0 h), which was set to 1. Transcription of UBQ10 was used as internal control. Error bars represent s.e.m among three biological replicates. Data significantly different from the corresponding control are indicated by asterisks (*P < 0.05, ****P < 0.0001, NS, nonsignificant; Unpaired t-test). c Quantitative HPLC/MS analyses of Tm content in shoot and root of seedlings after treatments as described in a in a shoot–root split culture system. The numbers over the histograms express ng g−1 fresh weight (F.W.). Data significantly different from the corresponding control are indicated by asterisks (**P < 0.01, NS, nonsignificant; Unpaired t-test).
endogenous UPR signaling acts systemically mainly in a shootward direction that is independent of Tm transport from the root to the shoot.

Root signals induce UPR genes in unchallenged tissues. We next performed reciprocal micro-grafting analyses in which the aerial tissue (scion) is grafted onto the root (rootstock) of a different plant, using wild type (Col-0) and bzip28/60 (Fig. 4). We expected that if an endogenous transcellular UPR signal existed as so far supported by our experiments (Fig. 3, Supplementary Fig. 6), we would observe UPR gene transcripts in the bzip28/60 tissues grafted with Col-0 tissues. Because our results indicate that a UPR signaling moves mainly in a shoot-ward direction (Fig. 3b, Supplementary Fig. 6), to monitor the UPR signaling at tissue-specific level we compared the abundance of sbZIP60, BiP3 and bZIP28 transcripts in self-grafts (same genetic background) and hetero-grafts (different genetic background) of scion and rootstock from independent seedlings treated with Tm at the rootstock. As a reference, we used untreated (i.e., DMSO only) micro-grafted seedlings with the same genetic combination as the stock. As expected, upon grafting with Col-0 as the scion and in the rootstock of Col-0 self-grafts, the UPR gene transcript levels were not significantly different compared to the scions of bzip28/60 self-grafts (Supplementary Fig. 7A). These controls indicate that the micro-grafting approach does not hamper the response of the grafted unions to ER stress. We then tested the bzip28/60 scion grafted on Col-0 rootstock (bzip28/60/Col-0). Compared to Col-0 self-grafts, in the rootstock of bzip28/60/Col-0 we found similar levels of UPR gene transcripts (Fig. 4a–c), indicating that the Col-0 rootstock in the bzip28/60/Col-0 grafts can respond to ER stress as the self-grafted Col-0/Col-0 rootstock. In the scion of bzip28/60/Col-0 hetero-grafts, the UPR gene transcript levels were significantly higher compared to the scions of bzip28/60 self-grafts albeit lower when compared to the Col-0/Col-0 scion (Fig. 4a–c). Because the bzip28/60 background is unable to evoke the UPR, these results indicate that the scions of bzip28/60/Col-0 hetero-grafts contain UPR gene transcripts originated from the Col-0 rootstock. These results are consistent with our observations that a shoot-ward signal originated from a Tm-treated tissue can induce the UPR in an unchallenged systemic tissue (Fig. 3b) and findings that the bZIP28 mRNA can move intracellularly in unstressed conditions.

Next, we tested whether UPR signaling other than the canonical bZIP60 and bZIP28 arms could be involved in the systemic UPR response. We conducted a separated reciprocal grafting with Col-0 as the scion and bzip28/60 as the rootstock (Col-0/bzip28/60). We expected that, if other shoot-ward UPR signaling were in place beside the bZIP28 and bZIP60 arms, then the levels of BiP3 would be affected in the scion of the Col-0/bzip28/60 hetero-graft. Similar to bzip28/60 self-grafts, there was no significant induction of sbZIP60, BiP3, and bZIP28 in the rootstocks of Col-0/bzip28/60 hetero-grafts upon Tm treatment (Supplementary Fig. 7A–C). Also, in the scions of the Col-0/bzip28/60 hetero-grafts the sbZIP60, BiP3, and bZIP28 were not induced and their respective mRNA levels were not significantly different compared to the scions of bzip28/60 self-grafts (Supplementary Fig. 7A–C). The lack of BiP3 induction in the scions is likely due to the absence of a functional UPR machinery in the rootstocks of Col-0/bzip28/60 hetero-grafts, these data indicate that the systemic UPR signaling requires the function of the canonical UPR bZIP-arms.

Systemic UPR signaling is plasmodesmata dependent. Direct intercellular communication between plant cells, including cells of the stellae and the endodermis, occurs via plasmodesmata (PD), which are connecting micro-channels between adjacent cells and the main route for certain signaling molecules in cell-to-cell trafficking. To establish if the systemic UPR relies on PD-mediated traffic, we tested whether sbZIP60 could target the PD. We generated a YFP fusion to sbZIP60 driven by a constitutive promoter for confocal microscopy analyses in leaf epidermal cells, which are more suitable for imaging analyses of PD compared to root tip cells. We verified a nuclear localization of YFP-sbZIP60 as well as a diffused distribution with conspicuous punctate reminiscent of PD (Supplementary Fig. 8A). The PD localization of sbZIP60 was confirmed through co-localization analyses with CFP fused to the established PD-localized receptor-like transmembrane protein, PDL1 (Supplementary Fig. 8A). In contrast, a cytosolic YFP (cYFP) control did not show marked co-localization with PDL1-CFP (Supplementary Fig. 8A). Integrated density measurements of the YFP signal at the PD using Aniline Blue (AB), a vital PD dye, and Pearson correlation coefficient estimation, as a measure of the association of fluorescence intensity between the YFP and AB signals, indicated that YFP-sbZIP60 co-localized at PD at significantly higher levels compared to cYFP (Supplementary Fig. 8B). The evidence that sbZIP60-GFP moves away from the stele (Fig. 1a, Supplementary Fig. 2), a process that is mediated exclusively by PD, and the coincidental distribution of YFP-sbZIP60 at PD (Supplementary Fig. 8) support that sbZIP60 is translocated via PD in conditions of ectopic expression. A PD dependency of endogenous systemic UPR response was tested in the shoot-root split culture system using the conditional PD blockage mutant, pMDC7-ical3m, in which the PD passage is reduced by an enhanced accumulation of callose throughout whole seedling upon gene induction by estrogen. In Col-0, in the presence of estrogen, the expression of sbZIP60, and BiP3 was similar to the mock condition, indicating that exogenous estrogen does not elicit ER stress (Fig. 5a). Both UPR markers exhibited similar expression profiles in shoots and roots in conditions of Tm-treatment only, or in condition of Tm-treatment in conjunction with estrogen application to the roots (Fig. 5a), further supporting that the addition of exogenous estrogen does not affect the systemic UPR response. In the conditional PD blockage mutant, there were no significant differences in the transcript levels of UPR markers in Tm+estrogen treated roots compared to Tm only treated roots, indicating no effect of estrogen on the UPR in this mutant (Fig. 5b). Most importantly, the expression of both UPR markers was significantly reduced in the shoots of seedlings treated with Tm+estrogen at the roots compared to the shoots of seedlings with Tm only treated roots (Fig. 5b). Therefore, an induction of PD closure compromises the systemic UPR signaling likely mediated by a translocation of signaling molecules such as sbZIP60 to elicit UPR in the distal tissues.

Discussion

Long-distance signaling is required for plants to actuate physiological processes and thrive in response to environmental challenges. Here we demonstrate that during a 24 h-time course upon local application of Tm to wild-type seedlings, the UPR markers can be detected in systemic tissues, indicating that ER stress in plants evokes a long-distance signal transduction of the UPR. Our conclusions are further corroborated by reciprocal...
grafting and ectopic expression assays showing significant transcript levels of sbZIP60 and BiP3 distally from the Tm-treated tissues. Therefore, our work demonstrates that, in addition to the well-established existence of a cell-intrinsic UPR signaling, plants harness long-distance signaling to communicate the occurrence of ER stress in a tissue to systemic tissues. We also show that sbZIP60 moves transcellularly and that its movement causes downstream UPR activation in distal tissues. Based on these results, we propose that sbZIP60 participates as a non-cell autonomous factor to actuate distal UPR signaling directly through its movement across cells.

Previous studies in C. elegans and mice relied on overexpression of transcriptionally active XBP1 to infer the existence of systemic UPR signaling11,12. Similarly, local expression of

Fig. 4 Micro-grafting analyses support the existence of endogenous systemic UPR signals. a–c Plots of the mean values (dots) of the quantitative RT-PCR analyses of the transcripts of sbZIP60 (a) BiP3 (b) and bZIP28 (c) in scion and rootstock of 4-week-old grafted seedlings of wild-type and bzip28/60 backgrounds treated with DMSO or 0.5 μM Tm (Tunicamycin) for 48 h as described in Fig. 3a. The average of all the values is indicated by a black line. Error bars represent mean with 95% confidence interval among all grafts. Error bars above and below indicate the 95th and 5th percentiles. Average values significantly different from the values of wild-type (Col-0) and bzip28/60 self-grafts are indicated by red asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, NS, nonsignificant; Mann-Whitney test). n, total number of grafted unions.
sbZIP60 caused the activation of a downstream target gene. However, an endogenous signal is actuated to evoke a systemic UPR, as supported by the detection of endogenous sbZIP60 and BiP3 transcripts in the shoot of wild-type seedlings exposed to Tm at the root in the split-plate system and the micro-grafting experiments using the bzip28/60/Col-0 hetero-grafts in which we detected the endogenous sbZIP60 and BiP3 transcripts (i.e., Col-0-originated) in the scion. The lack of genetic information in the bzip28/60/Col-0 scions that is necessary to express normally sbZIP60 and BiP3 in the aerial tissues supports that the presence of transcripts of these genes in the scion is the result of endogenous systemic UPR signaling. The induction level of BiP3 in bzip28/60/Col-0 scion was lower than that in Col-0 shoots under shoot–root split split treatment, which may be a consequence of a lack of UPR amplification in bzip28/60 scions.

In plants, several types of mobile RNA have been identified\(^\text{41}\). Transcriptomic analyses in Arabidopsis phloem also reported on the existence of cellular mRNA, suggesting the potential role of these mRNA as signaling molecules in the long-distance trafficking\(^\text{42}\). However, also proteins can contribute to systemic signaling, as it occurs for the well-established floral systemic signaling mediared not only by FT proteins but also by the FT mRNA\(^\text{43}\). This may be also the case for sbZIP60 protein and sbZIP60 mRNA, which both could function as transposable molecules eliciting systemic UPR signaling. The evidence that bZIP60 is localized at the PD supports the possibility that the bZIP60 protein moves systemically, but it also possible that the intercellular translocation of bZIP60 mRNA leads to translation of an active transcription factor in systemic tissues.

bZIP28 is another master UPR modulator contributing to the activation of ER stress response genes, including BiP3, ERDJ3A, and TIN1 in the plant UPR\(^\text{19,43}\). While the results obtained using bZIP60 as transgene in the bzip28/60 background provide evidence for a role of bZIP60 in systemic UPR signaling, the absence of bZIP28 in the wild-type background may facilitate to some extent the expression of BiP3 in the wild-type shoot of seedlings challenged with Tm at the root on the split-plate system. The bZIP28 transcripts are transposable between cells, at least in conditions different from ER stress\(^\text{29}\). Therefore, bZIP28 mRNA or its gene product may be involved in the systemic UPR regulation in parallel to the bZIP60 arm. Indeed, the lack of induction of UPR transcripts in the scions of Col-0/bzip28/60 hetero-grafts exposed to Tm at the roots argues that the systemic UPR signaling relies on the canonical UPR arms.

In plants, cell-to-cell communication takes place through pathways that involve the apoplast via the continuum of the cell wall, symplastic-driven cytoplasmic transport between different cells within the same tissue or among tissues connected by PD, and vascular-driven transport between different groups of cells or tissues utilizing conducting system composed of phloem and xylem\(^\text{44}\). Using subcellular localization analyses and transcriptional analyses, we have found sbZIP60 protein and sbZIP60

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**Fig. 5** Long-distance of UPR signaling relies on the PD availability. a, b Quantitative RT-PCR analyses of sbZIP60 and BiP3 in 14-day-old wild type (Col-0) (a) and a conditional PD block mutant (pMDC7-icals3m) (b) seedlings treated with DMSO (Mock), 10 μM 17–estradiol (estrogen), 0.5 μM Tm (Tunicamycin), and 10 μM 17–estradiol (estrogen) in combination with 0.5 μM Tm (Tunicamycin) at the root in the shoot-root split system for 24 h. Transcription of UBO10 was used as internal control. Error bars represent s.e.m among three biological replicates. Data significantly different from the corresponding control are indicated by asterisks (*P < 0.05, NS, nonsignificant; Unpaired t-test)
mRNA in ectopic tissues, respectively; we also found that the sbZIP60 protein can associate with PD and enter the nucleus, implying that the movement of bZIP60 in systemic signaling may occur through PD. A requirement for PD availability for long-distance UPR signaling is supported by the evidence that in a conditional PD mutant the systemic UPR is attenuated when PD closure is induced. Although we have verified the presence of sbZIP60 protein at the PD, the sbZIP60 mRNA may translocate through the PD, as it occurs for the mRNA of other proteins. Additionally, the reported existence of unspliced bZIP60 (unbZIP60) mRNA and likely co-localization with ER-associated unbZIP60 protein does not exclude a potential mobility of unbZIP60 mRNA for involvement in the systemic UPR through PD where a modified ER is present. The visualization of sbZIP60 protein at the PD may be facilitated by expression of sbZIP60 by the CaMV 35S promoter. However, as PD protein targeting is a highly specific process, the subcellular localization of sbZIP60 at PD is unlikely a result of overexpression. This is further supported by a relatively lower frequency of localization of cytosolic YFP at the PD in the same experimental conditions. In plants, some systemic response regulators that target PD do not act directly on systemic response effectors. The evidence provided in our work that transcellularly translocated sbZIP60 protein can induce the activity of the promoter of a target gene indicates that the systemic movement of sbZIP60 is functional in evoking UPR gene expression.

Biotic stress like pathogen attack induces SAR through which the signals generated from infected sites are translocated to distal plant tissues priming the defense response and eliciting immunity for subsequent infections. Based on the results that sbZIP60 traffics across cells, we propose that, similar to SAR, sbZIP60 participates in long-distance stress signaling to modulate the UPR in cells distally from the site where ER stress occurs. In nature, ER stress is caused by a variety of challenges including pathogens, heat, and salinity. A long-distance signaling of ER stress from challenged tissues may help cells anticipate incoming stress to yet-unchallenged tissues. For example, the UPR is required for plant defense response by modulating secretion of antimicrobial proteins. Therefore, a systemic signaling of ER stress may prepare cells of systemic tissues for responding to a potentially-upcoming ER stress by inducing the accumulation of transcripts of ER stress-attenuating proteins.

In our work, we have verified significant levels of sbZIP60 and BiP3 transcript accumulation in the shoot when Tm was applied to the root; conversely, when applied to the shoot, Tm induced sbZIP60 and BiP3 transcript accumulation in the roots to much lower levels. Based on these results, we propose that a root-originated transcriptional signal may operate mainly in a shoot-ward direction for ER stress signaling. However, a shoot-originated signal that moves in a root-ward direction may also exist. If such a signal were in place it would operate to lower levels than the root-originated signal, which would prevent its detection by qRT-PCR.

Long-distance stress signaling in plants is mediated by a number of inducers. The role of these inducers in long-distance ER stress signaling is yet to be evaluated and it is conceivable that long-distance ER stress signaling may overlay a bZIP60-mediated signaling with the action of other stress transducers. Nonetheless, it has been shown in seedlings that ER stress responses are independent from endogenous SA and that Tm does not induce accumulation of SA. Therefore, long-distance ER stress signaling is likely independent from SA. The evidence that micro-grafts with a bizp28/60 rootstock fail to evoke UPR signaling in a Col-0 scion further supports that the systemic UPR signaling mainly relies on the canonical UPR arms. Our findings address the long-standing question—whether plant UPR constitutes an endogenous systemic signal. From our results, we conclude that this is the case. The identification of bZIP60 as a component of the long-distance UPR signal transduction is a significant step forward in the understanding of the mechanisms underlying systemic signaling transduction of ER stress responses in intact organisms.

Methods

Lines and plant growth condition. Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used as a wild-type genotype in this study. The mutant lines used in this work are: bizp28 (Col-0; SALK_132285), bizp60-1 (Col-0; SALK_050203). Sterilized seeds were stratified at 4 °C for 2 days and plated on half-strength Linsmaier Skoog (LS) medium (1/2x LS salts, 1.5% sucrose, 1.2% Agar). Plants were grown in a 16-h-light/8-h-dark cycle at 21 °C. Agrobacterium (GV3101)-mediated transformation of Arabidopsis thaliana and Nicotiana tabacum plants was performed by the floral dip and infiltration methods, respectively.

Shoot-root split culture system. Plants were grown vertically on the half-strength LS medium for 14 days and transferred onto a 9 cm Petri dish equipped with two compartments (Kord-Valmark #2903, USA) containing medium with either DMSO in one side or 0.5 μM Tm, 10 μM 17-β-estradiol and 0.5 μM Tm in combination with 10 μM 17-β-estradiol in the other side and cultured horizontally for an additional 1 day. Data were acquired on at least three biological replicates.

qRT-PCR for gene expression analyses. Total RNA was extracted using Machery-Nagel Nucleospin RNA Plant kit (www.mn-net.com). All samples within an experiment were reverse transcribed at the same time using iScript cDNA synthesis Kit (BIO-RAD 1708891). Real-time qRT-PCR with SYBR green detection was performed in triplicate using the Applied Biosystems 7500 Fast Real-Time PCR System. URRQ0 was utilized as an internal control in normalization of qRT-PCR, unless otherwise stated in Supplementary Fig. 4. Similar patterns of expression were observed in the three independent biological replicates. Primers used in this study are listed in Supplementary Table 1.

Tunicamycin measurements. Two-week-old plate-grown seedlings (~ 100 mg) exposed to the shoot–root split culture system as detailed above were harvested and ground in liquid nitrogen. The samples were extracted at 4 °C overnight using 1 ml of ice cold methanol:water (80:20 v/v) containing 0.1% formic acid; 0.1 g L−1 tunicamycin spiked with propyl 4-hydroxybenzoate as an internal standard for quantification of Tm levels. Then samples were vortexed and centrifuged at 12,000 g × 4 °C for 10 min, after which the flow-through samples were transferred to HPLC vials for the measurement of endogenous concentration of Tm by LC-MS with Quattro Premier XE (Waters company) instrument according to an established protocol. The experiments were repeated at least two times with three biological replicates each time showing similar results.

Grafting experiment. Hypocotyl micro-reciprocal grafting was performed as described in Marsch-Martinez et al., with minor modifications. Briefly, micro-grafting was conducted using ten-day-old seedlings grown vertically that were first grown in 16h-light/8h-dark 120 μmoles.m−2.s−1 for 6 days and then in dark for 4 additional days. Grafts were generated by transverse sectioning of the aerial and root portions followed by conjunction on growth medium. After the grafts were made, samples were grown in dark vertically for 2 days, moved to light but covered with fine mesh for 1 day and then uncovered to grow in light. Adventitious roots were removed every 2–4 days. Samples were harvested by removing the whole hypocotyl portion avoiding the potential contamination at grafted junction and processed 4 weeks after micro-grafting for further analysis. Lack of contamination of adventitious roots in grafted plants was confirmed by genotyping with PCR. Analyses were performed on at least three biological replicates.

Plasmid construction. The Phusion high-fidelity DNA polymerase (NEB, USA) was employed to amplify all DNA sequences using the primer sets in Supplementary Table 1; the Gateway system (Invitrogen, USA) was used to generate expression plasmids. The promoter sequences of GLYT, SHR, and BiP3 and the coding sequences of spliced bZIP60 and POPL1 were amplified using Arabidopsis genomic and cDNA as templates, respectively. The amplified coding sequences were recombined into indicated Gateway vectors via LR reaction (Invitrogen, USA) and confirmed by sequencing.

Confocal laser scanning microscopy imaging. Confocal imaging was performed with an inverted laser scanning confocal microscope Nikon A1RSi. For subcellular localization assays, leaf tissues were mounted on a slide in a drop of tap water and covered with confocal microscope. GFP fluorescence was monitored at excitation wavelength of 488 nm and a bandpass 500–550 nm emission filter. Propidium iodide was monitored with a 560 nm excitation wavelength and a 570–620 nm
bandpass emission filter. YFP fluorescence was monitored with a 513 nm excitation wavelength and 520–550 nm bandpass emission filter. CFP was monitored with a 443 nm excitation wavelength and a 465–505 nm emission filter.

Quantification of protein co-localization at PD. Agrobacterium cultures at a cell density A600 0.025 and 0.5, containing the constructs for cytosolic YFP and YFP-abZIP60, respectively, were infiltrated through stomata into Nicotiana tabacum leaves using needleless syringe. The images were acquired 48 h after infiltration. Pearson correlation coefficient (PCC) values were calculated using the co-localization tool with default settings of the Nikon microscope software (NIS-Element AR 4.30). Areas with CFP and/or YFP signals at PD puncta were selected for the coefficient calculations. The coefficient calculations were performed on 170 of 3.4 μm² areas.

Propidium staining. Arabidopsis transgenic seedlings were stained in the 10 μg ml⁻¹ working solution of propidium iodide which was diluted with half LS liquid medium from 1 mg ml⁻¹ stock solution (Sigma-Aldrich P4864).

GUS staining. β-Glucuronidase activity in transgenic roots carrying pBIP3-GUS was visualized by X-Gluc as substrate using a conventional protocol. Staining was performed three times in three independent lines with consistent results.

Aniline Blue staining. The vital dye Aniline Blue diaminonitro salt (Sigma-Aldrich 415049) dissolved in 1 M Glycine (pH 1 – 9.5) (0.1% working solution) was adopted for PD-associated callose staining upon stoma infiltration with a needleless syringe into Nicotiana tabacum leaves for confocal microscopy analyses. Staining was performed three times in three independent lines with consistent results.

Data availability

The authors declare that the data supporting the findings of this study are available within the article and its supplementary files. All relevant data are available from the authors upon request.

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Author contributions

Y.-S.L. and F.B. designed research; Y.-S.L., G.S., S.Z-D and L.G. performed the research; Y.-S.L., C.R., and F.B. interpreted the results; Y.-S.L and F.B. wrote the manuscript.

Additional information

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