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In *Brachypodium* a complex signaling is actuated to protect cells from proteotoxic stress and facilitate seed filling

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Abstract

A conserved UPR machinery is required for *Brachypodium* ER stress resistance and grain filling.

Human and livestock diets depend on the accumulation of cereal storage proteins and carbohydrates, including mixed-linkage glucan (MLG), in the endosperm during seed development. Storage proteins and proteins responsible for the production of carbohydrates are synthesized in the endoplasmic reticulum (ER). Unfavorable conditions during growth that hamper the ER biosynthetic capacity, such as heat, can cause a potentially lethal condition known as ER stress, which activates the unfolded protein response (UPR), a signaling response designed to mitigate ER stress. The UPR relies primarily on a conserved ER-associated kinase and ribonuclease, IRE1, which splices the mRNA of a transcription factor (TF), such as *bZIP60* in plants, to produce an active TF that controls the expression of ER resident chaperones. Here, we investigated activation of the UPR in *Brachypodium*, as a model to study the UPR in seeds of a monocotyledon species, as well as the consequences of heat stress on MLG deposition in seeds. We identified a *Brachypodium bZIP60* orthologue and determined a positive correlation between *bZIP60* splicing and ER stress induced by chemicals and heat. Each stress condition led to transcriptional modulation of several *BiP* genes, supporting the existence of condition-specific *BiP* regulation. Finally, we found that the UPR is elevated at the early stage of seed development and that MLG production is negatively affected by heat stress via modulation of MLG synthase accumulation. We propose that successful accomplishment of seed filling is strongly correlated with the ability of the plant to sustain ER stress via the UPR.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Keywords

bZIP60; Endosperm; ER stress; Heat stress; Mixed-linkage glucan

Introduction

As the principal component of human diets, cereal grains are the most important food staple in the world. Cereal seed storage proteins are produced in the endoplasmic reticulum (ER) and deposited into protein bodies (Shewry and Halford 2002). The ER is also responsible for the synthesis and folding of the proteins responsible for the production of storage carbohydrates, such as mixed-linkage glucan (MLG) (Kim et al. 2015; Wilson et al. 2015). Therefore, production of storage proteins and carbohydrates relies on the biosynthetic capacity of the ER, which is monitored by a surveilling signaling pathway, known as the unfolded protein response (UPR). The UPR is designed to sense accumulation of unfolded or misfolded proteins in the ER, which can cause a potentially lethal condition known as ER stress. The UPR also operates to alleviate ER stress through the modulation of the expression of proteins involved in protein folding and quality control in the ER, as well as in selective degradation of mRNA transcripts of proteins destined to the secretory pathway (Walter and Ron 2011). In multicellular eukaryotes, the UPR is partially conserved. Three ER stress sensors have been identified in metazoans: the protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol requiring enzyme-1 (IRE1), and the membrane-tethered transcription factor (TF) ATF6/ bZIP28. While a PERK-mediated pathway (Harding et al. 2000) has not been identified in plants, the IRE1 and ATF6/ bZIP28 signaling pathways appear to be functionally and mechanistically conserved in metazoans and plants (Ruberti et al. 2015). ATF6 in metazoans and bZIP28 in *Arabidopsis* have been found to be activated by dissociation from binding protein (BiP) in the ER for relocation to the Golgi under ER stress (Shen et al. 2002; Srivastava et al. 2013). The Golgi-translocated ATF6/bZIP28 is cleaved by two Golgi resident proteases, and the resulting truncated N-terminal TF domain activates UPR genes, such as *BiP*, to increase protein folding capacity in the ER (Ye et al. 2000; Liu et al. 2007). The other conserved UPR signaling pathway is mediated by IRE1, which has three distinct domains: an ER luminal domain for sensing ER stress, cytosolic kinase domain for auto-phosphorylation and formation of dimer or oligomers, and a cytosolic ribonuclease domain for unconventional splicing of the mRNA of a UPR transcription factor and site-specific RNA degradation (RIDD) (Ruberti et al. 2015). In mammalian cells experiencing ER stress, activation of IRE1 occurs by dissociation of BiP from the ER luminal domain, causing multimerization and auto-phosphorylation of IRE1 mediated by its kinase domain (Li et al. 2010). The activated form of IRE1 mediates unconventional splicing of an intron from the mRNA of mammalian *Xbp1* and plant *bZIP60* (Yoshida et al. 2001; Nagashima et al. 2011). Splicing of *bZIP60* leads to the removal of an intron in the mRNA transcript and to the translation of a potent transcription factor (TF) that is translocated to the nucleus to modulate UPR-related gene expression.

In addition to *Arabidopsis bZIP60*, *bZIP60* orthologues have recently been identified in rice and maize with slightly different length of the intron (Hayashi et al. 2012; Li et al. 2012; Lu et al. 2012). The intron of *Arabidopsis bZIP60* is 23 bp long, while rice and maize *bZIP60*

introns are 20 bp long. The different length of the intron seems to be conserved among other dicot and monocot species (Ruberti et al. 2015). Nonetheless, at the amino-acid level, bZIP60 orthologues are not closely conserved between dicots and monocots. Despite these differences, the function of the activated form of bZIP60 in the UPR seems to be conserved in plants (Iwata et al. 2008; Hayashi et al. 2012).

The UPR has been shown to be activated by chemical ER stress inducers, such as tunicamycin (Tm) and dithiothreitol (DTT), as well as abiotic and biotic stresses (Moreno et al. 2012; Deng et al. 2013). As an abiotic stress, heat has been shown to induce the UPR in plants (Gao et al. 2008; Deng et al. 2011; Qu et al. 2013). Heat stress during seed development also affects crop yield significantly (Hatfield and Prueger 2015). Therefore, a better understanding of the UPR during seed development may provide new knowledge likely to be useful in increasing crop yield and improving crop quality.

Brachypodium distachyon is an important monocot model for its rapid life cycle and genetic proximity to cereal crops, such as wheat and barley. Here, we aimed to gain insights on the relationship between the UPR, heat stress, and MLG accumulation in seeds. To do so, we identified a *bZIP60* orthologue in *Brachypodium* (*BdbZIP60*) and established the conditions leading to unconventional splicing during ER stress with chemicals or heat treatment. We also determined that ER stress-inducing chemicals and heat modulate the expression of diverse UPR-responsive genes and found that the regulation of such genes differs depending on the developmental stage of the seeds. Finally, we have established that the abundance of MLG and cellulose synthase-like F6 (CSLF6), the enzyme primarily responsible for the synthesis of this polysaccharide, is differentially controlled during seed development and is negatively affected by heat stress. Together, these results provide new insights into the UPR signaling of a monocot model species and indicate the existence of a complex regulation of the UPR during seed development, which likely supports successful seed filling.

Materials and methods

Plant materials and growth conditions

Brachypodium distachyon (Bd21-3) obtained from the Great Lakes Bioenergy Research Center (GLBRC) was used in this study. Surface-sterilized seeds were plated onto 1× Murashige and Skoog media with 1% sucrose and 0.7% Agar (MS + 1% suc) and grown under 16 h light/8 h dark photoperiod at 21–22 °C. One week-old seedlings were transferred to MS + 1% sucrose with 3 mM DTT or 50 ng/ml of Tm up to 6 h. For heat treatment, 1-week-old seedlings were transferred to 42 °C up to 6 h. Control set of developing endosperm and seeds was collected from 8-week-old plants grown at 20 h (22 °C)/4 h (18 °C) light period. For heat treatment, 7-week-old plants were transferred to a growth chamber (30 °C) with the same light conditions and grown for 1 week before collecting heat-treated endosperm and developing seeds. For the cycloheximide (CHX) treatment, 1-week-old seedlings were transferred to MS + 1% sucrose media with 100 µg/ml CHX. After 30 min CHX treatment, 3 mM DTT (final) was added to the media. Only roots were collected after treatments.

Reverse transcription-polymerase chain reaction (RT-PCR)

After treating *Brachypodium* seedlings with Tm, DTT, or heat to induce UPR, seedlings were collected in liquid nitrogen and total RNA was extracted from samples. RT-PCR and qRT-PCR were performed as previously described using primers listed in the Table S1 (Lai et al. 2014). Relative expression levels were normalized to that of ubiquitin-conjugating enzyme E2 (Bradi5g25450).

Preparation of alcohol insoluble residue (AIR) and quantification of mixed-linkage glucan

AIR was prepared from developing seeds as described previously (York et al. 1985). MLG content in the AIR was quantified using the mixed-linkage β -glucan assay kit from Megazyme (www.megazyme.com) as reported previously (Vega-Sánchez et al. 2012; Kim et al. 2015).

Western blot analysis

Dehulled seeds were ground in liquid nitrogen using mortar and pestle. A pre-warmed (70 °C) protein extraction buffer (0.1 M Tris-HCl pH 7.5, 2% SDS, and 5% 2-mercaptoethanol) was added to the ground powder and incubated for 5 min at 75 °C. The mixture was centrifuged at 20,000g for 5 min. The supernatant was collected, and the protein concentration was measured using Bio-Rad protein assay reagent (www.bio-rad.com). Forty micrograms of proteins were separated on 9% SDS-PAGE gel, and CSLF6 was detected by western blotting using anti-CSLF6 serum (1:2000 dilution; gift from Dr. Doblin, University of Melbourne, Melbourne).

Results

Unconventional splicing of *BdbZIP60*

To identify the *Brachypodium* bZIP60 orthologue (BdbZIP60), we used OsbZIP50, the rice ortholog of *Arabidopsis* bZIP60 (Hayashi et al. 2012), and the maize bZIP60 (ZmbZIP60) (Li et al. 2012), as protein sequence reference. By scanning the *Brachypodium* genome, we identified a putative BdbZIP60 (Bradi1g35790) gene, and predicted to encode a gene product that shares ~54% identity to OsbZIP50 and ~67% identity to ZmbZIP60 at amino-acid level, as estimated by a blast search and a pairwise comparison using Clustal-O (Altschul et al. 1990; Sievers et al. 2011). BdbZIP60 shares ~29% amino-acid identity to *Arabidopsis* bZIP60, supporting a separate evolutionary clade of monocot bZIP60 (Fig. 1a) (Li et al. 2012). The unconventional splicing of the *bZIP60* intron in *Arabidopsis*, maize, and rice occurs at a specific mRNA structure composed of two adjacent (kissing) hairpins (Nagashima et al. 2011). Modeling of mRNA secondary structure with mfold (Zuker 2003) indicated the presence of adjacent hairpins also in the *BdbZIP60* mRNA similar to other *bZIP60* orthologues (Fig. 1b). Splicing of the *OsbZIP50* intron leads to frameshift and generation of a nuclear localization domain which contains a lysine and arginine-rich region at the C-terminus of the mature OsbZIP50 form (Hayashi et al. 2012; Li et al. 2012). Through protein sequence scanning analyses (TMpred) (Hofmann and Stoffel 1993), we found that unspliced *BdbZIP60* mRNA (Fig. 1c, d) is predicted to encode a sequence with a

transmembrane domain and that removal of the putative *BdbZIP60* intron results in a nuclear localization domain enriched with lysine and arginine in the translated protein (Fig. 1c, d).

We next validated our bioinformatics searches by testing for the splicing of *BdbZIP60* in conditions leading to the UPR activation. We utilized primers designed to amplify the unspliced and spliced mRNA forms of *BdbZIP60* by RT-PCR (Fig. 2a). To induce the UPR, we treated *Brachypodium* seedlings with 3 mM DTT for a series of time intervals. The levels of spliced or unspliced forms of *BdbZIP60* were assayed using RT-PCR analyses, and the amplified bands were isolated and sequenced to confirm their identity. We found that the unspliced form of *BdbZIP60* was detected in all the samples regardless of the DTT treatment, and that the amount of unspliced *BdbZIP60* increased according to the duration of the DTT treatment (Fig. 2b). The spliced form of *BdbZIP60* was detected only in the seedlings treated with DTT (Fig. 2b). Similar to unspliced *BdbZIP60*, the amount of spliced *BdbZIP60* increased with the duration of DTT treatment (Fig. 2b). Indeed, we observed both unspliced and spliced forms of *BdbZIP60* when we used primers to amplify both forms of *BdbZIP60* after treating with DTT. Interestingly, we noticed an extra band (hybrid) that is presumably generated by unspliced *BdbZIP60* and spliced *BdbZIP60* PCR products as observed in *XBPI* and *Arabidopsis bZIP60* processing (Shang and Lehrman 2004; Moreno et al. 2012). These results indicate that *BdbZIP60* is spliced similar to other *bZIP60* orthologues under ER stress conditions.

The splicing of *BdbZIP60* parallels the UPR activation

We next tested whether splicing of *BdbZIP60* caused UPR activation in *Brachypodium*. A hallmark of the UPR activation is the transcriptional induction of the UPR marker genes encoding ER protein chaperones, such as BiP and PDI (Chen and Brandizzi 2012). To test for UPR activation in *Brachypodium*, we treated seedlings with DTT or Tm, and investigated the transcriptional induction of *Brachypodium* UPR genes using quantitative RT-PCR (qRT-PCR) with specific primers to detect UPR genes as well as spliced (*spbZIP60*) or the unspliced form of *bZIP60* (*unbZIP60*). Upon Tm treatment, we observed occurrence of *spbZIP60* at 2 h of treatment; the *spbZIP60* levels increased along the duration of the treatment (i.e., 4 and 6 h) (Fig. 3a), but we did not observe substantial increase of *unbZIP60* expression up to 4 h Tm treatment (Fig. 3a). Similar to the Tm treatment, we observed an increase in *spbZIP60* levels at all three time points of DTT treatment (Fig. 3c). In addition, accumulation of *unbZIP60* mRNA was observed at 4 and 6 h of treatment.

Next, we tested whether the accumulation of *BdbZIP60* splicing was correlated with the induction of the UPR. Therefore, we investigated the accumulation of UPR marker genes in *Brachypodium*, such as *BdBiP1* and *BdPDIL1-1*, that are known to be upregulated under stress conditions (Zhu et al. 2014). Analogously to the splicing of *BdbZIP60*, we observed an increase of *BdBiP1* and *BdPDIL1-1* levels upon Tm and DTT treatments (Fig. 3b, d), supporting our hypothesis that the production of the spliced form of *BdbZIP60* is required to activate the UPR under Tm and DTT treatments. These results demonstrate that both Tm and DTT are effective in inducing UPR genes in *Brachypodium* along with stimulating *BdbZIP60* splicing.

Heat stress induces the UPR in *Brachypodium*

As shown above, Tm and DTT are effective reagents to induce ER stress and activate the UPR in *Brachypodium*. To define a natural UPR-inducing stress, we tested the effect of heat on UPR activation. Previously, heat stress has been shown to effectively induce the UPR in *Arabidopsis* as well as in maize (Gao et al. 2008; Deng et al. 2011; Li et al. 2012). Therefore, we grew *Brachypodium* seedlings at temperatures known to activate the UPR in maize (42 °C; Li et al. 2012) and monitored *BdbZIP60* splicing. Similar to Tm and DTT treatments (Fig. 3), we observed an increase of *spbZIP60* at 2 h of heat treatment (Fig. 4a). We also noticed a strong increase of *unbZIP60* accumulation (Fig. 4a) to levels similar to those induced by Tm or DTT. Accordingly, we observed an increase in the expression of *BdBiP1* but only a slight increase in the expression of *BdPDIL1-1* (<twofold) at 2 h heat treatment (Fig. 4b). Expression of both *spbZIP60* and *unbZIP60* decreased at 4 h but increased again at 6 h, indicating a more complex regulation of *BdbZIP60* during heat stress compared to a drug-induced transcriptional induction (Fig. 3a, c). However, the expression levels of *BdBiP1* and *BdPDIL1-1* were decreased after 4 h heat treatment, supporting that either other UPR players functionally replace *BdBiP1* and *BdPDIL1-1* during ER stress mitigation or *Brachypodium* may initiate other signaling mechanisms, such as programmed cell death, under prolonged heat stress. Nonetheless, the verified pattern of the UPR gene transcript accumulation is different between chemical and heat-induced ER stresses. Together, the heat treatment experiments support a similarity to chemically induced UPR in *Brachypodium* where the IRE1-bZIP60-mediated UPR is activated by heat stress; however, the two types of UPR-inducing stresses lead to responses that are not completely overlapping.

Expression of *BdBiP* genes under ER stress

To establish whether an overlap in gene regulatory responses could exist between the UPR generated by chemical ER stress inducers and heat stress, we monitored the expression levels of *Brachypodium* homologues of the UPR marker BiP (Lu et al. 2012; Ruiz-May et al. 2012). Based on protein homology searches, we identified 13 putative BiP homologues in addition to *BdBiP1*. We selected *BdBiP1*, *BdBiP2*, and *BdBiP3*, as they are closely clustered with *Arabidopsis* BiP proteins and also contain signal peptides and ER retention peptides (Fig. S1). In addition, UPR-responsive *cis*-elements (UPRE-I, UPRE-III, ERSE-I, and ERSE-II) (Buzeli et al. 2002; Hayashi et al. 2013; Sun et al. 2013) were identified in the promoter region for all three *BdBiP* genes (*BdBiP1*, *BdBiP2*, and *BdBiP3*; Table 1).

Next, we tested whether these genes respond to ER stress. We monitored their expression levels through qRT-PCR analyses in a time course of DTT treatment of *Brachypodium* seedlings. We found that *BdBiP2* was largely induced (up to 160-fold) (Fig. 5a), and the expression levels of *BdBiP1* and *BdBiP3* increased at 6 h as well, albeit to a lesser extent than compared to *BdBiP2* (Fig. 5b). These results indicate that three *BdBiP* homologues are responsive to DTT-induced ER stress admittedly at different levels. Then, we compared the expression profile among *BdBiPs* in heat stress by investigating their expression levels in a time-course treatment of *Brachypodium* seedlings with heat (42 °C). *BdBiP2* and *BdBiP3* were induced at 2 h similar to *BdBiP1*, but their expression levels remained elevated at 6 h in contrast to *BdBiP1* (Fig. 5c). In addition, we found that the fold change of *BdBiP3*

(~seven fold increase at 6 h) was higher than that of *BdBiP2* (~two fold increase at 6 h) under heat stress. These results support that different effectors, such as chaperones and transcriptional modulators, likely facilitate the UPR independent of the type and duration of stress that alters the homeostasis of the ER proteome.

***BdbZIP60* regulates expression of *BdBiP1* and *BdBiP2* during the UPR**

Because we observed distinct expression patterns of *BdBiPs* during DTT and heat treatment, we tested which *BdBiP* members were directly regulated by *BdbZIP60* using cycloheximide (CHX), a protein synthesis inhibitor, in combination with DTT. We first pre-treated *Brachypodium* seedlings with CHX for 30 min to block protein synthesis, including that of the active form of spbZIP60. Then, we added 3 mM of DTT to the incubation medium to induce the UPR. After treatments, only roots were collected to ensure effective drug treatment. We hypothesized that because of the CHX treatment, the bZIP28-dependent UPR (presumably the arm controlled by a *Brachypodium* homolog of AtbZIP28) would be active, while the bZIP60-dependent UPR would be blocked due to the absence of spbZIP60 protein synthesis. First, we examined the expression pattern of *unbZIP60* and *spbZIP60* (Fig. 6a, b). In a time course of only DTT treatment, we observed transcriptional induction of *spbZIP60* at 2, 4, and 6 h with *unbZIP60* at 6 h, consistent with our experiments (Fig. 3b). Interestingly, under the DTT treatment, we also found induction of *spbZIP60* regardless of the CHX treatment, and we established a moderate increase of *spbZIP60* transcript levels under the CHX treatment alone (Fig. 6b). Under the CHX or DTT/CHX treatment, we found increased levels of *unbZIP60*, while we observed only a slight increase of *unbZIP60* at 6 h DTT treatment (Fig. 6a). These results indicate that both DTT and CHX treatments likely activate IRE1-mediated unconventional splicing, and that the CHX treatment is responsible for the verified accumulation of *unbZIP60*.

Next, we examined the expression of *BdBiPs* and *BdPDIL-1* under the DTT and CHX treatments. Upregulation of *BdBiP1* and *BdBiP2* under the DTT treatment was observed (Fig. 6c, d) as also verified earlier (Figs. 3b, d, 5). However, increased expression of *BdBiP1* and *BdBiP2* was not observed in the seedlings co-treated with DTT and CHX (Fig. 6c, d). These results together with the evidence that *BdPDIL-1* was not induced under the DTT and CHX co-treatment (Fig. 6f) imply that the expression of *BdBiP1*, *BdBiP2*, and *BdPDIL-1* is likely controlled by spbZIP60 rather than bZIP28, although we cannot rule out a possible involvement of CHX in inhibiting gene expression or translation of other factors working with bZIP28 (Liu and Howell 2010). Although we have seen an increase of *BdBiP3* expression in whole seedlings treated with DTT (Fig. 5b), we have not observed any specific expression pattern of *BdBiP3* in the root under the conditions used for these analyses (Fig. 6e). These results may indicate tissue-specific regulation of *BdBiP3* expression in *Brachypodium* under stress.

The UPR is differentially modulated during endosperm development and stress responses

In *Brachypodium*, endosperm cellularization begins at 3–5 days after anthesis (DAA) and grains reach full length by 6 DAA (Opanowicz et al. 2011). Grain filling begins at 5 DAA and it is completed by 18 DAA. To characterize the UPR during endosperm development, we analyzed the levels of UPR-responsive markers at two different stages of endosperm

development: early at 5–8 DAA, and late 9–12 DAA before the endosperm is fully filled (Opanowicz et al. 2011). First, we followed the transcriptional induction of *BdbZIP60*, *BdBiP1*, and *BdPDIL1-1* in seeds from plants grown in physiological conditions (22 °C). We found that the expression levels of *unbZIP60*, *spbZIP60*, *BdBiP1*, and *BdPDIL1-1* were elevated at the early stages of development and that they decreased in the late stage of development (Fig. 7a–d). Therefore, we hypothesized that the UPR may be required during the early endosperm development rather than at the late stages. To test this hypothesis, we analyzed the expression level of *unbZIP60*, *spbZIP60*, *BdBiP1*, and *BdPDIL1-1* in leaf, root, stem, and endosperm. We did not observe marked differences in *unbZIP60* expression levels in the tissues tested, while we observed elevated levels of *spbZIP60*, *BdBiP1*, and *BdPDIL1-1* in the endosperm compared to other tissues (Fig. S2). These results suggest that the UPR is activated during endosperm development in physiological conditions of growth. To assay the UPR during endosperm development under heat-stress conditions, we treated *Brachypodium* plants with heat for one week at 30 °C, and then, we analyzed transcript levels in the endosperm at the two developmental stages (early, 5–8 DAA; late, 9–12 DAA). We found that the expression pattern of *spbZIP60* and *unbZIP60* at 30 °C mirrored that of physiological condition (22 °C) (Fig. 7a, b). Compared to normal conditions, the expression levels of *BdBiP1* and *BdPDIL1-1* in seeds grown at 30 °C were decreased significantly at the early stages of development (Fig. 7c, d), suggesting that the early seed developmental stage is more vulnerable to the heat stress than later stages. Based on the elevated expression levels of *spbZIP60*, *BdBiP1*, and *BdPDIL1-1* in the endosperm, we deduce that the UPR may be developmentally induced during endosperm development. Therefore, heat stress during endosperm development may not cause further induction of the UPR; alternatively, the UPR may become attenuated during seed development in conditions of prolonged heat stress, or the heat-stress condition (30 °C) could be too low to induce detectable level of the UPR during endosperm development when developmental UPR is active.

Heat stress reduces MLG filling of seeds

MLG, a chain of glucose linked by $\beta(1-3)$ and $\beta(1-4)$ bonds, is a storage carbohydrate in cereal grains that can reach ~40% (w of MLG/w of the endosperm) (Guillon et al. 2011). MLG is a soluble dietary fiber beneficial to human health, and its amount in the endosperm is an important factor determining the quality of grain. To understand the effect of heat stress on grain development, we aimed to examine seed quality by measuring seed weight and MLG content in *Brachypodium* seeds of two different stages from plants grown either in standard conditions of growth or subjected to heat stress. We found that heat stress affected seed weight (Fig. 8a) and MLG accumulation (Fig. 8b) of both early and late developmental stages. Because MLG is produced primarily by the cellulose synthase-like F6 (BdCSLF6) (Burton et al. 2006), we monitored the abundance of the enzyme in seeds under heat-stress conditions. First, we monitored response of the *BdCSLF6* gene in *Brachypodium* seedling during a time course of heat stress (42 °C) using qRT-PCR. We found that the expression levels of *BdCSLF6* were progressively reduced over time (Fig. 8c), indicating that the expression of *BdCSLF6* is sensitive to elevated temperature. Based on this result and the reduced levels of MLG accumulation observed in seeds of heat-challenged plants (Fig. 8b), we hypothesized that BdCSLF6 transcript and protein levels would be reduced also in seeds after heat treatment. To test this hypothesis, we analyzed the BdCSLF6 transcriptional and

protein levels in seeds from plants grown in normal conditions of growth or in heat-stress conditions. Both the transcript and protein levels of BdCSLF6 were decreased at late stage of seed development regardless of the growth conditions (Fig. 8d, e). Noticeably, we also observed decreased levels of BdCSLF6 transcript and protein in the heat-treated developing seeds compared with the levels of BdCSLF6 in the normal condition, especially at the early developmental stage (Fig. 8d, e). These results establish that the UPR is active during grain filling and that heat stress negatively influences the abundance of BdCSLF6 and MLG at the stage of seed development when MLG synthesis is elevated in physiological growth conditions.

Discussion

The UPR is largely conserved in plants, metazoans, and yeast, and it provides protection from proteotoxic stress caused by the accumulation of misfolded and unfolded proteins in the ER in unfavorable conditions of growth. Here, we aimed to assay the UPR in a model monocot species. We established that at least one branch of the UPR signaling in *Brachypodium* is conserved. Similar to other monocot *bZIP60* orthologues, *BdbZIP60* also has a 20 bp intron for unconventional splicing by BdIRE1. The increase of spliced form of *BdbZIP60* is well correlated with the level of *BdBiP1* under ER stress regardless of the type of stresses tested in this work. To identify the downstream players during ER stress and understand the overall UPR mechanism in *Brachypodium*, we have investigated the expression pattern of *Brachypodium* *BiP* genes using qRT-PCR and analyzed their promoter sequences. Interestingly, we found that different expression patterns of *Brachypodium* *BiP* genes depend on the specific type of stress. In addition, we have found that prolonged heat stress during endosperm development clearly affected seed quality and the biosynthesis of MLG synthase. These results demonstrated that *Brachypodium* has a conserved UPR activation mechanism, and the UPR in *Brachypodium* is regulated with high complexity.

In our work, we have shown that *BdbZIP60* splicing occurs upon ER stress. When we compared the relative levels of *BdbZIP60* splicing and expression level of *BdBiP1* in Tm and DTT experiments, we found a high correlation between splicing of *BdbZIP60* and expression of *BdBiP1* (Fig. S3). Furthermore, we found UPRE-I, UPRE-III, and ERSE-I elements in the promoter of *BdBiP1* (Table 1). *Xbp1*, a mammalian orthologue of *bZIP60*, has been shown to bind preferentially UPRE elements (Yamamoto et al. 2004). Although we cannot rule out the regulation of *BdBiP1* expression by *bZIP28* homologues through the UPR-responsive cis-element ERSE-I, these results along with the results after DTT/CHX treatment (Fig. 6c) pose that the spliced form of *BdbZIP60* may be the preponderant factor for induction of *BdBiP1* in *Brachypodium* under ER stress. However, it is also possible that inhibition of the synthesis of analogous factors to the NF-Y transcription factors known to work with *bZIP28* in *Arabidopsis* could be responsible for the low expression level of *BdBiP1* under DTT/CHX co-treatment (Liu and Howell 2010).

When we compared the expression pattern of all three *BdBiP* genes with respect to *BdbZIP60*, we observed distinct expression patterns for each *BiP*. Under heat-stress conditions, we verified a distinctive pattern of *BdbZIP60* splicing that was maximal at 2 h, and then slightly reduced at 4 and 6 h of treatment. Such pattern was mirrored by the

expression levels of unspliced *BdbZIP60* and *BdBiP1* (Fig. 4). However, *BdBiP2* and *BdBiP3* did not match the expression pattern of spliced form of *BdbZIP60*, and showed specific expression patterns depending on the type of stress. Specifically, *BdBiP2* was highly upregulated by DTT treatment (~160-fold increase at 6 h DTT treatment), while only about a twofold increase of *BdBiP2* expression was observed under heat stress (Fig. 5). Different from the upregulation of *BdBiP2* under DTT treatment, *BdBiP3* was highly expressed under heat stress rather than DTT treatment. The expression pattern of *BdBiP3* under heat stress was also different from that of *BdBiP1*, which showed a slight decrease at 4 and 6 h time points of drug treatment (Fig. 5). These results indicate that the regulatory elements in the *BdBiP3* promoter may respond to transcriptional regulators that are different from those of the other *BdBiP* genes.

We established that ER stress leads to the accumulation of unspliced and spliced forms of *BdbZIP60*. While the spliced *BdbZIP60* form is likely involved in modulating UPR gene expression, the accumulation of unspliced *BdbZIP60* is enigmatic. Although we cannot exclude that the mRNA of *BdbZIP60* may not be translated, in metazoans, the unspliced form of *Xbp1* is translated and is required to recruit *Xbp1* mRNA to the ER membrane likely to facilitate IRE1-mediated splicing (Yanagitani et al. 2009). One possible explanation for the accumulation of unspliced *BdbZIP60* in ER stress conditions in *Brachypodium* is that ER stress may induce *BdbZIP60* transcription to levels that overcome the RNase activity of IRE1 to lead to a translated form of *BdbZIP60* with specific roles in stress responses. For example, it has been suggested that the unspliced form of *AtbZIP60* in *Arabidopsis* may have a role in salt-stress response (Henriquez-Valencia et al. 2015; Parra-Rojas et al. 2015). Analogously it is possible that the *Brachypodium* UPR also relies on the translated unspliced *BdbZIP60*. In addition, we found that CHX treatment accumulated *unbZIP60* regardless of DTT treatment (Fig. 6a). This result suggests that CHX treatment may activate a signaling pathway that requires *BdbZIP60*. However, we have not observed any remarkable expression of UPR downstream effectors in this specific condition. These results indicate that, in conditions of inhibition of protein synthesis, either the unspliced form of *bZIP60* has a physiological role or that degradation of *BdbZIP60* transcripts may be inefficient.

UPR during seed development and heat stress

Similar to other cereals, *Brachypodium* seeds produce endosperm with high content of storage molecules such as proteins, carbohydrates, and lipids (Larré et al. 2010; Hands and Drea 2012), most of which are synthesized in the secretory pathway. Consequently, synthesis and delivery of the storage products in developing seeds require elevated production of the relevant protein machinery during endosperm development. Therefore, it is likely that the UPR is necessary to meet the needs of protein synthesis during endosperm development. To gain new insights into the UPR during seed development, we have analyzed the expression profile of *BdbZIP60*, *BdBiP1*, and *BdPDIL1-1* at the early and late stages of endosperm development and other *Brachypodium* tissues. We observed that the UPR is activated during endosperm development, especially at the early stages of endosperm development when seed filling is taking place rather than at later stages when the filling is concluding. Our data are, therefore, consistent with a need of signaling mechanisms to favor elevated synthesis of proteins and carbohydrates in the secretory pathway of seeds.

Crop production is largely dependent on environmental factors (Boyer 1982). Among abiotic stresses, high temperatures during grain development can reduce crop yield (Bita and Gerats 2013; Hatfield and Prueger 2015), and the elevated temperatures have been shown to induce the UPR signaling during development in plants (Gao et al. 2008; Deng et al. 2011; Li et al. 2012; Fragkostefanakis et al. 2016). We expected, therefore, that the UPR could also be mounted in seeds during heat stress, although we cannot exclude the possibility that the temperature (30 °C) may be too low to induce strong UPR. Contrary to our expectations, we have observed overall decreased expression of *BdBiP1* and *BdPDIL1-1* in the endosperm during heat stress, although the expression levels of *BdbZIP60* were largely unchanged (Fig. 7). Although the type of tissue and temperature of heat treatment is different, we have seen a high increase of *BdBiP1* and *spbZIP60* expression level after 2 h heat treatment in Fig. 4. However, 6 h heat-treated samples showed reduced levels of *BdBiP1* compared to 4 h heat-treated sample, although 6 h sample has higher levels of *spbZIP60*. This suggests that either extended heat or over-heating treatment may initiate cellular response other than UPR, or that expression of UPR genes in the heat-treated endosperm may require other transcription factors besides *BdbZIP60* according to a developmental program. An alternative explanation, however, may reside in the possibility that the UPR may be attenuated in conditions of prolonged heat stress. It has been established in metazoans that the three UPR signaling pathways mediated by IRE1, ATF6, and PERK are differently regulated after the first stages of response to ER stress (Lin et al. 2007). For example, the IRE1 and ATF6-mediated signaling pathways are attenuated at 8 and 20 h after initiation of ER stress, respectively. Therefore, it is possible that the decreased expression of UPR-related genes examined in the heat-treated endosperm may represent a cellular response postattenuation of the UPR in combination with developmental regulation during endosperm development.

With decreased levels of UPR gene expression in the endosperm during heat stress conditions, we also observed decreased MLG accumulation and corresponding reduction of the levels of transcripts and protein of the MLG synthase *BdCSLF6* in the developing seeds. These results support that heat stress reduces the synthesis of *BdCSLF6*. Although we cannot exclude the existence of post-translational regulation of *BdCSLF6*, our results implicate that the abundance of *BdCSLF6* in seeds is highly controlled transcriptionally under environmental stress conditions. The observed large reduction of MLG synthase levels in the heat stress-treated seeds in the early development stage supports that MLG accumulation in the early stage of seed development is important for improving the grain quality. Based on our results that the UPR is elevated at the early stages of seed development in physiological normal conditions of growth, but it is comparatively lower in heat-stress conditions, we propose that heat stress causes a general reduction of the seed filling with a consequently reduced necessity of the UPR to monitor correct folding and synthesis of the machinery needed for synthesis and deposition of MLG and other storage compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

MLG	Mixed-linkage glucan
UPR	Unfolded protein response
RIDD	Regulated Ire1-dependent decay

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Fig. 1. Conserved features of BdbZIP60. **a** Amino-acid sequences of unspliced and spliced forms of bZIP60 homologs in *Arabidopsis*, maize, rice, and *Brachypodium* were compared by pair-wise alignment using clustal-O. The sequence identity between species is presented. **b** Predicted RNA secondary structure of *BdbZIP60* presents the characteristic two kissing loop hairpin structures, as shown in *Arabidopsis* (*AtbZIP60*) and rice (*OsbZIP50*). **c** Unspliced and spliced sequences of *BdbZIP60*. The red characters indicate a 20 bp intron for unconventional splicing. Green characters indicate new amino-acid sequence after splicing,

likely by *Brachypodium* IRE1. **d** Protein domain structures encoded from unspliced and spliced forms of *BdbZIP60* are shown. A frameshift by unconventional splicing replaces the transmembrane domain (TM) for a new domain containing a putative nuclear localization signal (NLS)

Fig. 2.

Unconventional splicing of *BdbZIP60*. **a** Schematic representation of the primer design to amplify unspliced and spliced *BdbZIP60*. A reverse primer to detect unspliced *BdbZIP60* was designed using the sequence covering both the spliced intron and the preceding exon. A reverse primer to detect spliced *BdbZIP60* was generated using the exon sequences connected with intron after splicing. **b** RT-PCR analyses using primers specific to unspliced, spliced, and both forms of *BdbZIP60*. Spliced form of *BdbZIP60* was only detected after DTT treatment. A hybrid was formed from unspliced and spliced forms of *BdbZIP60*. UbiE2 primers were used for control

Fig. 3.

ER stress caused by either Tunicamycin (Tm) or DTT induces UPR in *Brachypodium*.

BdbZIP60 splicing and induction of UPR genes were analyzed by qRT-PCR after Tm or DTT treatment. Tm treatment induces *BdbZIP60* splicing (a) and expression of *BdbiP1* and *BdpDIL1-1* (b). DTT treatment induces *BdbZIP60* splicing (c) and expression of *BdbiP1* and *BdpDIL1-1* (d)

Fig. 4.

Heat stress induces the UPR in *Brachypodium*. *BdbZIP60* splicing and induction of downstream UPR genes were analyzed by qRT-PCR after heat treatment. Heat stress induces *BdbZIP60* splicing (a) as well as expression of *BdbiP1* and *BdpDIL1-1* (b). Statistical significance was analyzed by Student's *t* test (* $P < 0.01$)

Fig. 5.

Expression of *Brachypodium BdBiPs* under ER stress and during endosperm development.

Expression of 3 *BdBiP* homologues in *Brachypodium* were analyzed by qRT-PCR. **a, b**

Expression of *BdBiPs* in the *Brachypodium* seedlings after DTT treatment. **c** Expression of

BdBiPs in the *Brachypodium* seedlings after heat treatment (42 °C)

Fig. 6.

Expression of *BdBiP1* and *BdBiP2* is regulated by *BdbZIP60*. One week-old *Brachypodium* seedlings were treated with DTT (3 mM), DTT/cycloheximide (CHX), or CHX only. Seedlings treated with DTT/CHX and CHX only were pre-treated with CHX for 30 min. 0 h DTT is the sample pre-incubated in the 1/2MS + 1% sucrose for 30 min, and 0 h CHX is the sample pre-incubated in the 1/2MS + 1% sucrose with CHX for 30 min. Expression levels of unspliced *BdbZIP60* (a), spliced *BdbZIP60* (b), *BdBiP1* (c), *BdBiP2* (d), *BdBiP3* (e), and *BdPDIL-1* (f) were assayed by qRT-PCR

Fig. 7.

UPR during seed development. **a–d** Detection of UPR marker genes in physiological conditions and in heat stress. Seven-week-old *Brachypodium* plants were heat treated (30 °C) for 1 week. The developing seeds were grouped by their development stages (Early, 5–8 DAA; Late 9–12 DAA). Expression of unspliced *BdbZIP60* (**a**), spliced *BdbZIP60* (**b**), *BdBiP1* (**c**), and *BdPDIL1-1* (**d**) was assayed by qRT-PCR. Statistical significance was analyzed by Student's *t* test (** $P < 0.01$)

Fig. 8.

Heat stress decreases expression of the MLG synthase, *BdCSLF6*, in *Brachypodium*. **a** Weight of developing seeds from 8-week-old *Brachypodium* in normal conditions of growth or subjected to heat treatment at two developmental stages (Early, 5–8 DAA; Late, 9–12 DAA). **b** Amount of MLG in the seeds was quantified after AIR production from seeds at the early and late developmental stages. Heat-treated seeds contained less MLG compared to non-treated seeds. **c** Quantification of the expression level of *BdCSLF6* in seedlings subjected to heat treatment shows that the expression of *BdCSLF6* is negatively affected by heat. **d** Quantification of the expression of *BdCSLF6* shows that the enzyme abundance is regulated during development and that heat stress decreased the expression of *CSLF6* at the early stages of seed development. Statistical significance (**a–d**) was analyzed by Student's *t*

test (* $P < 0.05$; ** $P < 0.01$). **e** Decreased protein level of BdCSLF6 was observed by western blot analyses using anti-CSLF6 serum at the later stage of seed development as well as heat treated developing seeds at the early stage. Protein band intensity is indicated by number directly below each band calculated via Image Lab™ software normalized to early seed stage under normal condition. **f** Ponceau stained membrane shows protein loading

Table 1

List of UPR-related cis-elements identified in the promoter of *Brachypodium BIPs*

Name of element	Sequence	BdBIP1	BdBIP2	BdBIP3	AtBIP3
UPRE-III	TCATCG	1(+)	1(+)	1(+), 1(-)	1(-)
ERSE-I	CCAAT-N9-CCACG	1(+)	na	na	2(-)
ERSE-II	ATTGG-N2-CACG	na	na	na	na
UPRE	TGACGTG	1(-)	3(-)	1(-)	na

UPR-related *cis*-elements were searched up to 1 k bp upstream from initiation codon of the *BIP* genes. Number and direction of UPR *cis*-elements are presented in the table. (+) indicates sense, and (-) indicates anti-sense direction in the promoter region