bZIP17 and bZIP60 Regulate the Expression of BiP3 and Other Salt Stress Responsive Genes in an UPR–Independent Manner in Arabidopsis thaliana

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ABSTRACT

Plants can be severely affected by salt stress. Since these are sessile organisms, they have developed different cellular responses to cope with this problem. Recently, it has been described that bZIP17 and bZIP60, two ER-located transcription factors, are involved in the cellular response to salt stress. On the other hand, bZIP60 is also involved in the unfolded protein response (UPR), a signaling pathway that up-regulates the expression of ER-chaperones. Coincidentally, salt stress produces the up-regulation of BiP, one of the main chaperones located in this organelle. Then, it has been proposed that UPR is associated to salt stress. Here, by using insertional mutant plants on bZIP17 and bZIP60, we show that bZIP17 regulate the accumulation of the transcript for the chaperone BiP3 under salt stress conditions, but does not lead to the accumulation of UPR-responding genes such as the chaperones Calnexin, Calreticulin, and PDIL under salt treatments. In contrast, DTT, a known inducer of UPR, leads to the up-regulation of all these chaperones. On the other hand, we found that bZIP60 regulates the expression of some bZIP17 target genes under conditions were splicing of bZIP60 does not occur, suggesting that the spliced and unspliced forms of bZIP60 play different roles in the physiological response of the plant. Our results indicate that the ER-located transcription factors bZIP17 and bZIP60 play a role in salt stress but this response goes through a signaling pathway that is different to that triggered by the unfolded protein response. J. Cell. Biochem. 116: 1638–1645, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: Arabidopsis thaliana; SALINITY STRESS; UPR; bZIP17; bZIP60; TRANSCRIPTION FACTOR

As sessile organism, plants are daily exposed to abiotic stress conditions, which affects plant growth, limiting the production of economically important species. Of these factors, drought and high salinity represent a major problem for agricultural development [Flowers, 2004; Tester and Langridge, 2010; Golldack et al., 2011; Agarwal et al., 2013].

High salinity can affect plants in several ways such as water stress, ion toxicity, alteration of metabolic processes, oxidative stress, membrane disorganization and reduction of cell division [Hasegawa et al., 2000; Munns, 2002; Zhu, 2007]. Furthermore, several signaling pathways related to salt stress have been reported in the model plant Arabidopsis thaliana. Those pathways include the signaling mediated by the hormone abscisic acid (ABA) [Ben-Ari, 2012; Xu et al., 2013], the activation of second messengers (including calcium and reactive oxygen species (ROS) [Abogadallah, 2010; Miller et al., 2010; Wurzinger et al., 2011], and the activation of transcription factors [Golldack et al., 2014]. Besides, the participation of subcellular organelles such as mitochondria, chloroplasts, Golgi apparatus and endoplasmic reticulum (ER) have been proposed to be important factors in the transduction and integration of the signaling under abiotic stress [Rhoads et al., 2006; Kang et al., 2008; Zhang et al., 2009; Jin and Daniell, 2014].

The ER is a main site of synthesis and processing of all secretory and membrane proteins in eukaryotic cells. When the protein folding process is perturbed, a signaling network called unfolded protein response (UPR) is activated to recover ER homeostasis [Ron and Walter, 2007]. This signaling network requires the action of ER associated transcription factors, such as bZIP60 and bZIP28, to...
restore the ER capabilities of synthesis, folding, and degradation of proteins. These capabilities are restored by the transcriptional activation of chaperones like Calnexin, Calreticulin, and BiP proteins [Vitale and Boston, 2008; Che et al., 2010]. Interestingly, some authors have suggested the participation of the ER in the salt response through the endoplasmic reticulum-associated degradation (ERAD) and the ER-bound transcription factor bZIP17 [Liu et al., 2007a, 2011; Cui et al., 2012]. bZIP17 is an ER-membrane anchored transcription factor that responds to salt stress. Upon treatment with salt, bZIP17 moves to the Golgi apparatus where it gets processed by SIP and S2P proteases, producing a soluble form of the transcription factor, that is then translocated to the nucleus [Che et al., 2010]. Overexpression of a soluble form of bZIP17 (bZIP17ΔC) leads to salt resistant phenotypes [Liu et al., 2008] while bzip17 mutant plants display salt sensitive phenotypes [Liu et al., 2007a]. Further analyses had shown that salt stress responding genes such as NAM-LIKE, ATHB7, PP2C-LIKE, RD20, and RD29b are direct targets of bZIP17 [Liu et al., 2007a, 2008]. Interestingly, the overexpression of bZIP17 under non-stress conditions leads to the up-regulation of the BiP chaperone, suggesting that bZIP17 may participate in UPR [Che et al., 2010]. On the other hand, bZIP60, a transcription factor involved in UPR signaling, also seems to be involved in the plant response to salt stress. The overexpression of the full-length cDNA of this gene confers resistant phenotypes when plants are exposed to high salt conditions [Fujita et al., 2007]. Furthermore, the expression of bZIP60 in culture cells also increases their tolerance to salt stress [Tang et al., 2012; Tang and Page, 2013].

It is well known that activation of UPR leads to the unconventional splicing of the bZIP60 mRNA, mediated by the ER anchored protein Ire1 [Deng et al., 2011; Nagashima et al., 2011]. The processed mRNA encodes for an active transcription factor that binds to P-UPRE sequences in the DNA, regulating the transcription of chaperones involved in folding and degradation of proteins in the ER [Nagashima et al., 2011; Humbert et al., 2012]. Despite the fact that the transcripts of bZIP60 and BiP chaperones accumulate under salt treatment [Wang et al., 2011], recently published data demonstrate that the mRNA of bZIP60 is not processed under salt stress conditions [Moreno et al., 2012]. Furthermore, bZIP28 is not active under salinity stress [Liu et al., 2007b], indicating that none of these two transcription factors are being activated. These results pose a question regarding the role of UPR in the plant response to salt stress. Therefore, we decided to further evaluate the effects of salt stress on the accumulation of bZIP17, bZIP60, and UPR related gene transcripts, and compare this effect with the one of a known UPR inducer such as DTT. Our results indicate that bZIP17 regulate the accumulation of the transcript for the chaperone BiP3 under salt stress conditions, but does not show any effect on the accumulation of UPR genes such as the chaperones Calnexin, Calreticulin, and PDIL. On the other hand, we found that bZIP60 regulates the expression of some bZIP17 target genes under conditions of splicing bZIP60 does not occur. Then, our results suggest that the ER-located transcription factors bZIP17 and bZIP60 play a role in salt stress but this response goes through a signaling pathway that is different to that triggered by the unfolded protein response. Furthermore, our results also indicate that the spliced and unspliced forms of bZIP60 play different roles in the physiology of the plant.

MATERIAL AND METHODS

PLANT MATERIAL AND GROWTH CONDITION

Arabidopsis (A. thaliana) seeds ecotypes Col-0 and Col-3 qrt1-2 and T-DNA insertion mutant lines bzip17 (SALK_104326) [Liu et al., 2007a] and bzip60 (SAIL_283_B03) [Deng et al., 2011] were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, OH). The T-DNA lines were confirmed by PCR using gene-specific primers and left border T-DNA primer and RT-PCR was performed to examine the mRNA levels of bZIP17 and bZIP60 (Fig. S1). The primers are listed in supporting information. Seed were grown at 21°C and 16/8 h light/dark cycle under white light (~63 μE m⁻² s⁻¹) in liquid ½ Murashige and Skoog (MS) medium. For salt and DTT treatments, 7-day-old seedlings were incubated in MS medium supplemented with 150 mM NaCl or 2.5 mM DTT (Dithiothreitol) for the indicated periods of time.

RNA ISOLATION AND cDNA SYNTHESIS

Total RNA was isolated from 7-day-old seedling using TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA) according to manufacturer instructions and treated with DNase I (Fermentas, Thermo Scientific, Waltham, MA). cDNA was synthesized from total RNA using the SuperScript II first-strand RT-PCR kit (Invitrogen, Life Technologies).

ANALYSIS OF THE bZIP60 SPLICING

PCR amplification of cDNA was performed using specific primers to selectively amplify genes. To amplify both unspliced and spliced bZIP60 forms, specific primers were used. EF-1a gene was amplified as control. PCR reactions conditions for amplification of EF-1a and unspliced-spliced bZIP60 forms were as follow: 96°C for 1 min, (98°C for 5 s, 55°C for 5 s and 72°C for 5 s) for 30 cycles, and 72°C for 2 min. PCR performed with Sapphire Amp Fast PCR Master Mix (Takara Bio Inc, Otsu, Shiga, Japan). PCR products were resolved by electrophoresis in agarose gel (3%). The primers are listed in the supporting information.

QUANTITATIVE ASSESSMENT OF DIFFERENT TRANSCRIPTS

cDNA was evaluated by real-time quantitative PCR (qPCR) using Fast EvaGreen Master Mix qPCR (Biotium, Hayward, CA) reagent in Stratagene Mx3000P qPCR system according to manufactures instructions. Transcript levels were normalized using the expression of EF-1a and clathrin adaptor genes as the controls. The primers for all genes analyzed are listed in the supporting information.

RESULTS

THE bZIP60 mRNA IS UP-REGULATED UNDER SALT STRESS BUT DOES NOT UNDERGOES SPLICING

In order to evaluate what is the transcriptional response of bZIP60 and bZIP17 to salt stress, we measured the transcript levels for both genes using quantitative PCR. Figure 1A shows that bZIP60 mRNA is up-regulated nearly fivefold after 4 h of treatment with 150 mM NaCl, while the transcript levels for bZIP17 showed a twofold increase (Fig. 1B). Furthermore, when DTT, a known inducer of UPR was
Arabidopsis. Since bZIP28 it is also not affected by salt stress, it appears that these two branches of the UPR are not activated during salt stress.

**SALT REGULATES BiP3 BUT NOT OTHER UPR RESPONDING GENE TRANSCRIPTS**

Some reports have suggested that UPR is induced by salt stress because the transcript levels of BiP are up-regulated [Wang et al., 2011; Ozgur et al., 2014]. In order to further analyze this phenomenon, we compared the extent of the up-regulation of chaperones that respond to UPR when plants were treated with either 150 mM NaCl or 2.5 mM DTT. BiP3 was up-regulated about 10-fold in the presence of salt (Fig. 2 inset). However, the up-regulation observed in the presence of DTT was much more significant, reaching an increment in the range of 2,000-fold (Fig. 2). Interestingly, the transcript levels of other ER chaperones that respond to UPR such as calreticulin 1 (CRT1), calreticulin 2 (CRT2) and protein disulfide isomerase like-1 (PDIL-1) did not change in the presence of salt (Fig. 2). Furthermore, as expected, they did respond to DTT. As control we analyzed the expression of a salt-responding gene, RD29b which was highly expressed in the presence of salt (around 1,500-fold), but it was not activated by DTT (Fig. 2). This data indicate that, with the exception of BiP3, ER chaperones do not seem to be regulated by NaCl, posing a question about the activation of UPR under salt stress.

**UP-REGULATION OF BiP3 DEPENDS ON bZIP17 UNDER SALT STRESS CONDITIONS**

BiP3 is a classical chaperone marker that is up-regulated during UPR. However, during salt stress bZIP28 is not active and the bZIP60 mRNA is not processed. Therefore, since the two known transcription factors involved in the activation of UPR are not active, we wonder how BiP3 can be regulated under these conditions.

Since bZIP17 is active during salt stress and its transcript is up-regulated, we evaluated if this transcription factor could be responsible for the up-regulation in the content of the BiP3 mRNA. Hence, we measured the BiP3 transcript levels in bZIP17 mutant plants Figure 3A shows that, as expected, BiP3 transcript levels were up-regulated during salt treatment. In contrast, BiP3 transcript levels were not different from the control in bZIP17 mutant plants treated for 4 h with 150 mM NaCl. Even more, a slight decrease, in comparison to the control, was observed after 6 h of treatment. These results suggest that bZIP17 plays a major role on the up-regulation of BiP3 during salt stress. In order to analyze whether bZIP60 plays a role on the up-regulation of the BiP3 transcript levels during salt treatment, we assessed the mRNA level of this chaperone in wild type and bZIP60 mutant plants. Since the background of the bZIP60 mutant utilized in this analyses corresponds to Col-3, we used this plant as wild type. BiP3 was also up-regulated upon the addition of salt, but to a lower extent in comparison to Col-0. When bzip60 mutants were treated with salt, a slight decrease in the BiP3 transcript levels was observed in comparison to wild type plants treated under the same conditions. However, no up-regulation of the BiP3 transcript was observed at 6 h and the transcript levels were as the controls. These results indicate that bZIP60 also plays a role in the up-regulation of BiP3. Since bZIP60 is up-regulated during salt stress, the increment in the expression of bZIP60 rose to 12-fold, whereas the bZIP17 showed no significant change to the control. Since exposure of plants to salt produced an increase in the expression of bZIP60 we decided to investigate whether this change was associated to the splicing of bZIP60. Figure 1C shows that no splicing was observed at any time of the incubation of plants with 150 mM NaCl. This observation confirms the findings made by other authors [Deng et al., 2011; Moreno et al., 2012] and suggests that the IRE1/bZIP60 branch of the UPR is not active under salt stress in Arabidopsis.
treatment (Fig. 1B), but no splicing takes place during salt treatment (Fig. 1C), the effect of bZIP60 on the mRNA levels of BiP3 should be due to the unspliced form of this transcription factor.

THE EXPRESSION OF GENES THAT DEPEND ON bZIP17 IS ALTERED ON bZIP60 MUTANTS UNDER SALT STRESS CONDITIONS

To get further insights on the role of bZIP17 and bZIP60 in the plant response to salt stress, we evaluated the expression of salt-responding genes, which have been proposed to depend on bZIP17, in bzip60 mutant plants. Figure 4 shows that the mRNA levels of LTP3, RD20, HB7, and NAM-like genes, as expected, were up-regulated upon exposure of plants to salt. Furthermore, the transcript levels of these genes decreased in bzip17 mutants in comparison to wild type plants exposed to salt, confirming the findings of Liu et al. [2007a]. When transcript levels of these genes were measured in bzip60 mutant plants interestingly, the mRNA levels of LTP3 were lower in comparison to wild type plants, resembling the observation made in bzip17 mutants. In contrast, the transcript levels of HB7 and RD20 showed a different behavior in bzip60 mutant plants in comparison to bzip17 mutant plants treated with salt. Finally, the mRNA levels of NAM-like showed no significant differences between wild type and bzip60 mutants exposed to salinity conditions.

**Fig. 2.** Salt stress up-regulates BiP3 but not other ER chaperone transcripts. Seven-day-old wild type (Col-0) seedlings were grown in liquid MS medium and treated with 150 mM NaCl or 2.5 mM DTT for 0, 4, and 6 h. BiP3, CRT1, CRT2 and PDIL-1 ER chaperone transcript levels were analyzed by quantitative RT-PCR. The obtained data were normalized against the expression of EF-1a and clathrin adaptor genes. The means and standard errors were calculated from three independent replicates and compared to no treatment condition (0 h) (**P < 0.001, t-student). RD29b gene expression served as control of salt stress.
Due to their sessile nature, plants are continuously exposed to environmental stress conditions caused by abiotic and biotic factors. This results in a high demand of protein synthesis, folding and degradation at the ER, which in turn triggers a mechanism called UPR [Moreno and Orellana, 2011; Howell, 2013]. This response is characterized by the transcriptional activation of a specific set of UPR responding genes like ER-chaperones involved in the protein folding assistance [CNX, CRT, BiP, and PDIL] in this organelle [Iwata and Koizumi, 2012]. To date, two UPR signal transduction pathways have been described in plants. The first involves two ER membrane-associated transcription factors (bZIP17 and bZIP28) and the second, composed by a protein with dual RNase-kinase function (IRE1) and its target RNA (bZIP60) [Liu et al., 2007a; Liu et al., 2007b; Che et al., 2010; Deng et al., 2011]. During UPR, the mRNA of bZIP60 is processed by IRE1 [Deng et al., 2011; Nagashima et al., 2011] and the spliced form encodes for an active transcription factor that regulates the activation of UPR responding genes like the chaperones CNX, CRT, BiP, and PDIL [Moreno et al., 2012; Howell, 2013].

It has been proposed that salt stress induces an ER signal transduction pathway that involves the processing in the Golgi apparatus and translocation to the nucleus of bZIP17 and the up-regulation of salt stress responding genes such as ATHB7, NAM-LIKE, PP2C-LIKE, RD20, and RD29a [Liu et al., 2007a, 2008]. In addition, bZIP60 has also been involved in salt stress since its overexpression confers resistant phenotypes to plants exposed to this stress [Fujita et al., 2007]. Moreover, the transcript accumulation of bZIP60 and BiP after salt treatments has also been reported [Wang et al., 2011], while the overexpression in cell cultures of AtbZIP60 lacking the transmembrane domain leads to salt resistant phenotypes [Tang et al., 2012; Tang and Page, 2013], suggesting the participation of UPR in the salt stress response. Despite these observations, the mRNA of bZIP60 is not processed under salt stress conditions as Tunicamycin (Tm) and DTT [Iwata et al., 2008] are not up-regulated by salt (Fig. 2). This was also recently observed by Ozgur et al. [2014], where the authors described that salinity treatment had a slight effect on the classical unfolded protein response (UPR), where chaperones are up-regulated, is not activated by salt stress. The following evidences support this statement. The splicing of bZIP60, one of the classical pathways involved in triggering UPR, does not occur in plants treated with salt (Fig. 1C). Additionally, UPR marker genes such as CRT1, CRT2, and PDIL-1 that are activated by chemical UPR inducers such as Tunicamycin (Tm) and DTT [Iwata et al., 2008] are not up-regulated by salt (Fig. 2). This was also recently observed by Ozgur et al. [2014], where the authors described that salinity treatment had a slight effect on the expression of UPR marker genes compared to Tm treatment even though ER stress induces the antioxidant system in a similar way to salinity. On the other hand, genes that respond to salt and are dependent on bZIP17 [Liu et al., 2007a] do not respond to DTT (Fig. 5). These results indicate that although the plant response to salt and to inducers of UPR is mediated by transcription factors located in the ER, the responses triggered on both conditions involve different signaling pathways.

The aim of this study was to further analyze the role of bZIP17 and bZIP60 in the activation of salt stress genes. Our results indicate that the classical unfolded protein response (UPR), where chaperones are up-regulated, is not activated by salt stress. The following evidences support this statement. The splicing of bZIP60, one of the classical pathways involved in triggering UPR, does not occur in plants treated with salt (Fig. 1C). Additionally, UPR marker genes such as CRT1, CRT2, and PDIL-1 that are activated by chemical UPR inducers such as Tunicamycin (Tm) and DTT [Iwata et al., 2008] are not up-regulated by salt (Fig. 2). This was also recently observed by Ozgur et al. [2014], where the authors described that salinity treatment had a slight effect on the expression of UPR marker genes compared to Tm treatment even though ER stress induces the antioxidant system in a similar way to salinity. On the other hand, genes that respond to salt and are dependent on bZIP17 [Liu et al., 2007a] do not respond to DTT (Fig. 5). These results indicate that although the plant response to salt and to inducers of UPR is mediated by transcription factors located in the ER, the responses triggered on both conditions involve different signaling pathways.

The present study also revealed that even though the expression of the chaperone BiP3 is observed under salt treatment and during UPR, the fold induction of BiP3 are very different when the treatment with salt and DTT are compared (Fig. 2). Interestingly, the up-regulation of...
BiP3 in plants exposed to salt is strongly dependent on bZIP17, indicating that this chaperone responds by different mechanisms to salinity conditions and UPR.

According to our data, bZIP60 seems to play a role in the expression of some genes that are up-regulated by salt stress and are depending on bZIP17 (Fig. 4). However, the analyses of their expression pattern show some differences suggesting that bZIP17 and bZIP60 play complementary but not identical roles. Since the bZIP60 mRNA does not undergo splicing, one of the questions that arise from this study is how bZIP60 plays a role during salt stress? One
The possibility is that the unspliced form of bZIP60 (bZIP60u) is playing a role by itself within this process, providing evidence that the protein derived from bZIP60u plays a different role to the protein derived from the spliced form. On this regard, it has been described that the unspliced form of XBP1 (XBP1u), the orthologue of bZIP60 in mammals, participates in the regulation of autophagy through the interaction with another transcription factor leading to its degradation [Zhao et al., 2013]. It is important to mention that XBP1u differs from the spliced form of XBP1 by its inability to migrate to the nucleus; instead XBP1u is degraded by the proteasome [Navon et al., 2010]. The existence in A. thaliana of a similar mechanism, involving the unspliced form of bZIP60 under salt stress conditions, cannot be discarded. Despite the mechanism that regulates the gene expression mediated by bZIP60 under salt stress conditions, our work provides evidence that bZIP17 and bZIP60 participate in the salt stress response where bZIP17 can regulate the expression of BiP3 under these conditions, an observation that has not been reported elsewhere. Also, our work indicates that a mechanism involving bZIP60, but independent of its splicing, might be involved in the plant response to salt stress. Further work should provide further insights on this mechanism.

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