Salt stress responses in Arabidopsis utilize a signal transduction pathway related to endoplasmic reticulum stress signaling

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Summary

We describe a signaling pathway that mediates salt stress responses in Arabidopsis. The response is mechanistically related to endoplasmic reticulum (ER) stress responses described in mammalian systems. Such responses involve processing and relocation to the nucleus of ER membrane-associated transcription factors to activate stress response genes. The salt stress response in Arabidopsis requires a subtilisin-like serine protease (AtS1P), related to mammalian S1P and a membrane-localized b-ZIP transcription factor, AtbZIP17, a predicted type-II membrane protein with a canonical S1P cleavage site on its lumen-facing side and a b-ZIP domain on its cytoplasmic side. In response to salt stress, it was found that myc-tagged AtbZIP17 was cleaved in an AtS1P-dependent process. To show that AtS1P directly targets AtbZIP17, cleavage was also demonstrated in an in vitro pull-down assay with agarose bead-immobilized AtS1P. Under salt stress conditions, the N-terminal fragment of AtbZIP17 tagged with GFP was translocated to the nucleus. The N-terminal fragment bearing the bZIP DNA binding domain was also found to possess transcriptional activity that functions in yeast. In Arabidopsis, AtbZIP17 activation directly or indirectly upregulated the expression of several salt stress response genes, including the homeodomain transcription factor ATHB-7. Upregulation of these genes by salt stress was blocked by T-DNA insertion mutations in AtS1P and AtbZIP17. Thus, salt stress induces a signaling cascade involving the processing of AtbZIP17, its translocation to the nucleus and the upregulation of salt stress genes.

Keywords: salt stress, transcription factor, unfolded protein response, subtilase, regulated intramembrane proteolysis, proteolytic processing.

Introduction

Salinization is a widespread agricultural problem affecting 20% of the world’s irrigated cropland, and many other regions of the earth designated as arid and desert lands (Yamaguchi and Blumwald, 2005). Salinization of croplands will present challenges for the future given the predictions of climate change, population growth and the greater demand for more intensive agriculture (Yeo, 1999). Some of these challenges may be met by the development of crops tolerant to salt stress (Wang et al., 2003; Yamaguchi and Blumwald, 2005). Salt stress, drought and cold elicit a broad range of physiological and gene expression responses in plants (Thomashow, 1999; Xiong and Zhu, 2001; Zhu, 2002; Seki et al., 2003; Bray, 2004; Chinnusamy et al., 2004; Zhang et al., 2004; Fujita et al., 2006; Shinozaki and Yamaguchi-Shinozaki, 2006; Yamaguchi-Shinozaki and Shinozaki, 2006).

Much progress has been made in recent years in understanding the primary signaling pathway in plants responding to salt stress (see, for example, Zhu, 2002). From the analysis of Arabidopsis salt overly sensitive (sos) mutants, it has been learned that the signaling pathway involves a salt-elicited Ca$^{2+}$ signal, which is sensed by SOS3 (Liu and Zhu, 1998; Ishitani et al., 2000) and activates SOS2, a serine/threonine protein kinase (Haltier et al., 2000; Liu et al., 2000). Together SOS2 and SOS3 regulate the activity and
expression level of SOS1, a plasma membrane Na⁺/H⁺ antiporter (Qiu et al., 2002). SOS2 and possibly other Ca²⁺-activated protein kinases initiate a protein phosphorylation cascade channeled downstream through mitogen-activated protein (MAP) kinases (Chinnusamy et al., 2004). Teige et al. (2004) implicated an MAP kinase kinase (MKK2) and two MAP kinases (MPK4 and 6) in salt stress responses.

Salinity, drought and cold elicit many common and interactive downstream effects. Drought and salt stresses activate dehydration response element binding factor 2 (DREB2), members of the ethylene response factor (ERF)/APETALA2 (AP2) transcription factors family. DREB2 binds CRT/DRE promoter elements in stress response genes (Gosti et al., 1995; Yamaguchi-Shinozaki and Shinozaki, 2006). Many of these same genes are activated by cold stress, through the action of DREB1/C-repeat binding element factors (CBFs), as are members of the ERF/AP2 transcription factor family that bind to CRT/DRE promoter elements (Thomashow, 1999; Chinnusamy et al., 2004). In addition, salt and drought stress, but not cold stress, activate NAC and homeodomain-ZIP transcription factors involved in the expression of ERD1, a Clp protease regulatory subunit.

Salt stress, drought and, to a lesser extent, cold stress elevate abscisic acid (ABA) levels (Nambara and Marion-Poll, 2005). ABA upregulates the expression of many, but not all, drought-response genes (Krep et al., 2002; Seki et al., 2002; Leonhardt et al., 2004; Li et al., 2006), indicating that there are ABA-dependent and ABA-independent salt and drought stress responses in plants (Liu et al., 1998; Kizis et al., 2001; Shinozaki and Yamaguchi-Shinozaki, 2006). ABA-dependent gene expression involves several classes of transcription factors, including members of the bZIP, MYC/MYB and NAC families (Fujita et al., 2004; Satoh et al., 2004; Zhang et al., 2004; Shinozaki and Yamaguchi-Shinozaki, 2006; Yamaguchi-Shinozaki and Shinozaki, 2006). Some of the best-studied transcription factors mediating ABA responses are bZIP transcription factors, AREB/ABF, which bind to ABA response elements (ABREs) in the promoters of drought-responsive genes (Marcotte et al., 1988, 1992; Iwasaki et al., 1995; Li et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2006).

In this study, we have observed that salt stress generates an ER stress response in Arabidopsis. ER stress occurs when normal protein folding or secretory processes are inhibited, and unfolded or misfolded proteins accumulate in the ER (Urade, 2007). ER stress activates signaling pathways, collectively referred to as the unfolded protein response (UPR), which mitigate the accumulation of unfolded or misfolded proteins by upregulating the expression of genes encoding ER-folding proteins (Rutkowski and Kaufman, 2004). UPR is highly conserved in eukaryotic organisms, and has been extensively described in yeast and mammalian cells (Wellhinda et al., 1999; Patil and Walter, 2001). In yeast, ER stress activates inositol-requiring kinase 1 (Ire1), an ER-localized type-I transmembrane protein with a C-terminal RNase domain (Cox et al., 1993; Mori et al., 1993). Activated Ire1 splices a precursor mRNA encoding HAC1 (Cox and Walter, 1996; Sidrauski and Walter, 1997), a transcription factor that targets stress response genes possessing UPR promoter elements (Mori et al., 1992; Kohno et al., 1993; Mori et al., 1996). Plants that undergo ER stress responses upregulate the expression of a broad range of genes (Jelitto-Van Dooren et al., 1999; Koizumi et al., 1999; Leborgne-Castel et al., 1999; Martinez and Chrispeels, 2003), including those encoding proteins that create a more optimal protein folding environment in the ER (Martinez and Chrispeels, 2003).

UPR is more elaborate in mammals than in yeast (Schroder and Kaufman, 2005a). The accumulation of unfolded or misfolded proteins in mammalian cells activates three types of ER stress sensor/transducer proteins: activating transcription factor 6 (ATF6), IRE1α or IRE1β, and dsRNA activated protein kinase-like ER kinase (PERK; Schroder and Kaufman, 2005b). ATF6 is a type-II transmembrane protein normally retained in the ER by its association with binding protein BiP/GRP78 (Chen et al., 2002; Shen et al., 2002). In response to stress, ATF6 dissociates from BiP/GRP78 and is transported to the Golgi where it is subjected to proteolytic processing (Chen et al., 2002; Shen et al., 2002). Relocated ATF6 is cut on its luminal side in the Golgi by a site-1 protease (S1P), and the N-terminal, cytosol-facing domain of the protein is released by cleavage within the membrane by a site-2 protease (S2P) (Ye et al., 2000). The released N-terminal domain contains a basic leucine zipper motif and is translocated to the nucleus, where it activates the transcription of genes with ER stress promoter elements (ERSE), such as BiP (Wang et al., 2000). Membrane-associated transcription factors have been recently described for a number of different transcription factor families in plants (Kim et al., 2007). Membrane-associated NAC transcription factors, in particular, have been implicated in cytokinin and stress signaling (Kim et al., 2006).

In this paper we identified and characterized a salt stress signaling pathway that resembles an ER stress response. In this pathway AtS1P, a plant subtilisin-like serine protease (subtilase), targets a membrane-associated bZIP factor, AtbZIP17, which functions as a stress sensor/transducer. In response to salt stress, AtbZIP17 is cleaved and the N-terminal bZIP component is translocated to the nucleus, where it activates the expression of salt stress response genes.

Results

A T-DNA mutation in AtS1P demonstrates a role for this subtilisin-like serine protease gene in salt stress responses

Some of the important components of the ATF6-related ER stress response in mammalian cells are the stress sensor
ATF6, chaperones such as BiP, and Golgi-located protein processing factors (Chen et al., 2002; Shen et al., 2002). A key processing factor is S1P, a subtilisin-like serine protease or subtilase that initiates the processing of ATF6. Arabidopsis encodes a large family of subtilases (Rautengarten et al., 2005), and one of them, AtS1P, At5g19660, is surprisingly similar to human S1P (P = 4.4e–231; Figure S1). Like most other Arabidopsis subtilases, AtS1P has a preprodomain structure, in that it has an N-terminal signal peptide targeting it to the secretory pathway, and a subterminal prodomain that is processed upon activation of the proenzyme. However, AtS1P differs from other Arabidopsis subtilases by the presence of a long C-terminal tail with a transmembrane domain (TMD) near its C-terminus (Figure 1a).

To investigate the function of AtS1P, T-DNA insertion mutations in AtS1P (s1p-1 through s1p-5) were analyzed (Figure 1b). The mutant s1p-3, with a T-DNA insertion in the 7th exon, was considered a knockout mutation as no AtS1P transcripts were detected in the line (Figure 1c). None of the T-DNA mutants in AtS1P had observable seedling or mature plant phenotypes under normal conditions. However, it was found that s1p-3 was more sensitive to salt stress. In the absence of salt stress, s1p-3 and wild-type roots grew at about the same rate (Figure 1d), but s1p-3 root growth was reduced in comparison with wild type on 50 and 100 mM NaCl (Figure 1e,f). The mutant s1p-3 was also sensitive to other monovalent salts, such as KCl and LiCl, and to mannitol (Figure 1g–i). Thus, s1p-3 is sensitive to salt-induced osmotic stress. (For convenience we use the term salt stress throughout the paper.)

To determine whether the T-DNA in s1p-3 was responsible for the salt sensitivity, the mutant was crossed with wild type, and salt sensitivity in the F2 generation co-segregated with the T-DNA (χ² = 1.68, P = 0.20; Fig. S2a). A cauliflower mosaic virus (CaMV) 35S promoter:AtS1P cDNA construct rescued s1p-3 salt sensitivity in line S1P-9, confirming that AtS1P is responsible for the salt-sensitive phenotype (Fig. S2b). From these findings, we concluded that AtS1P functions in salt stress tolerance in Arabidopsis.

We examined other properties of AtS1P to better understand its role in salt tolerance, and to determine if this subtilase functions in ER stress responses in a manner comparable with mammalian S1P. An essential feature about the function of mammalian S1P in ER stress is its localization in the Golgi. To determine if AtS1P is similarly located, we fused the yellow fluorescent protein (YFP) to the C-terminus of AtS1P and expressed it in transgenic Arabidopsis using the 35S promoter. We found that the YFP signal was largely located in punctate structures of leaf epidermal cells co-localizing with a fluorescent marker, BODIPY TR ceramide, which has been used by others as a Golgi marker (Figure 2a–c; Vadaie et al., 2002; Egelund et al., 2006). From this, we concluded that AtS1P is also likely to be a Golgi-resident protein in Arabidopsis. To determine where AtS1P is expressed in Arabidopsis plants, AtS1P promoter:GUS constructs were developed and found to be expressed in the vasculature of roots, cotyledons and leaves of seedlings (Figure 2d, e).

A membrane-associated bZIP transcription factor, AtbZIP17, mediates salt stress responses and is processed by AtS1P

The bZIP transcription factor, ATF6, is a target of S1P processing in ER stress responses in mammalian cells, and the transcription factor has a characteristic structure of a type-II membrane protein with an N-terminal bZIP domain, a transmembrane domain and a canonical (RXXL or RXLX)
S1P cleavage site on the luminal side of the membrane (Figure 3a). We looked for bZIP factors with similar structures in the Arabidopsis genome and identified three, AtbZIP17 (At2g40950), AtbZIP28 (At3g10800) and AtbZIP49 (At3g56660), which we compared with AtbZIP60 (At1g42990) (Figure 3b). To determine whether any of these bZIP factors might be involved in salt tolerance, available T-DNA insertions in AtbZIP17 and AtbZIP28 were screened for salt sensitivity, and it was found that $\text{zip17}$ (SALK_104326, T-DNA insertion in the first exon of AtbZIP17; Figure 4a) was modestly salt sensitive (Figure 4b,c). The mutant was considered to be a knockout mutation because AtbZIP17 transcripts were not detected by RT-PCR (Figure 4d). The salt sensitivity trait co-segregated with the T-DNA ($v^2 = 0.79, P = 0.37$) in backcrosses to wild type (Figure S2c), and a 35S promoter:AtbZIP17 cDNA construct, used in developing line ZIP-4, rescued the salt-sensitive phenotype (Figure 4b,c; Figure S2d). These findings confirmed that the T-DNA insertion was responsible for the salt-sensitive phenotype.

To determine whether AtbZIP17 is processed in response to salt stress, an N-terminal myc-tagged AtbZIP17 construct was developed and introduced into transgenic seedlings. It was found that the 84-kDa construct was processed in vivo to a 46-kDa protein in salt-treated seedlings in a wild-type background (Figure 5a), consistent with cleavage in or near the TMD of AtbZIP17 (Figure 3a). The processing of AtbZIP17 did not occur in similarly treated $s1p-3$ seedlings (Figure 5a).

We did not observe an intermediate corresponding to processing at the S1P site in vivo. However, processing intermediates of ATF6 have not been directly observed in mammalian cells either, suggesting that the S1P-cleaved intermediate is short lived (Ye et al., 2000). We did consistently observe that more 84-kDa precursor accumulated after salt treatment than before, and that more 46-kDa product was produced than expected from the quantity of precursor (Figure 5a). One explanation is that the precursor turns over in the ER membrane under normal conditions, and that both the precursor and product are stabilized following stress. An examination of that possibility is underway.
Processing of ATF6 in mammalian cells is a regulated intramembrane proteolysis (RIP) process involving cleavage of the protein by S1P, followed by S2P digestion and release from the ER of the cytosolic component of the protein (Ye et al., 2000). RIP processing of ATF6 is thought to be dependent on prior S1P cleavage to remove the bulky lumenal domain, making the protein accessible to S2P digestion (Shen and Prywes, 2004). Although we did not directly observe S1P cleavage of AtbZIP17 in vivo, we wanted to determine whether AtbZIP17 is indeed a substrate for proteolysis by AtS1P. To do so, we tested whether AtbZIP17 could be cleaved by affinity-purified AtS1P in an in vitro pull-down assay. A myc-tagged form of AtS1P (AtS1P-myc-HDEL) produced in transgenic Arabidopsis was pulled down out of plant extracts with anti-myc agarose beads. (The AtS1P-myc-HDEL construct lacks a transmembrane domain to aid in solubilizing the protein and has an HDEL element to retain the protein in the ER.) The beads were washed extensively and incubated with a C-terminal His-tagged, lumen-facing fragment of AtbZIP17 (40 kDa; Figure 5b). The products of the in vitro reaction were analyzed on western blots, and a 10-kDa protein product consistent with cleavage at the canonical S1P site in AtbZIP17 was found. The product was not formed in controls when plant extracts from transgenic plants bearing an empty vector were used, or when the serine protease inhibitor phenylmethylsulphonyl fluoride (PMSF) or the calcium chelator EGTA was added to the reaction. From this we posit that AtS1P directly targets AtbZIP17 for proteolysis, which makes the protein further accessible for cleavage by S2P-like processing activities. If AtS1P does indeed process AtbZIP17 in response to salt stress, then one would expect the two gene products to interact and be expressed in the same tissues. The expression pattern of AtbZIP17 promoter:GUS demonstrated that AtbZIP17 is expressed mainly in the vasculature (Figure 2f,g), similar to the tissue expression pattern for AtS1P (Figure 2d,e).
overnight on 100 mM NaCl, mGFP fluorescence from the N-terminal labeled fusion protein appeared in nuclei (Figure 6g–i). Nuclear relocalization happened on a time scale consistent with the observations on in vivo processing (Figure 5a). We observed substantial nuclear relocation of fluorescence 2 h after salt stress, and nearly maximal nuclear relocation after 4 h (Figure 6j–k). We interpret this to mean that the N-terminal component of AtbZIP17 relocates from the ER to the nucleus in response to salt stress. We have not yet observed intermediate stages in the nuclear relocalization process, particularly whether AtbZIP17 moves through the Golgi. We found that AtbZIP17 is predicted to be Golgi-localized based on the attributes of its TMD (Yuan and Teasdale, 2002). However, further investigation will be needed to determine whether AtbZIP17 transiently relocates to the Golgi following salt treatment.

The cytosolic-facing component of AtbZIP17 is a transcription factor that activates the expression of salt stress genes

To determine whether AtbZIP17 has transcription factor activity, and, if so, to identify where the activity is located, various elements of the N-terminal cytosolic-facing component of AtbZIP17 were fused to a GAL4 DNA binding domain, and tested for transcriptional activation in yeast (Figure 7). Those constructs bearing the N-terminal 138 amino acids of AtbZIP17 activated the LacZ reporter gene in yeast, indicating that the N-terminal domain of AtbZIP17 is a transcriptional activator. Thus, the N-terminal cytoplasmic-facing segment of AtbZIP17, which is translocated to the nucleus during salt stress, bears bZIP DNA binding and transcriptional activation domains.

To identify genes activated by AtbZIP17, we looked for salt stress responsive genes with altered expression in s1p-3. To do so, 1-week-old wild-type and s1p-3 seedlings were transferred to liquid media with or without 100 mM NaCl, and were harvested 4 h later for RNA extraction. Affymetrix Arabidopsis gene chips (ATH1) were used to profile gene expression, and significant changes in the estimated means of gene expression were determined while controlling for a false discovery rate of 1.00E⁻⁰² (Storey and Tibshirani, 2003). Genes were rank ordered by increases in expression under salt stress conditions in wild type compared with s1p-3, and those that differed in expression by more than 2.5-fold are shown (Table 1). To determine whether AtbZIP17 exercises control over these genes during salt stress, their expression was assessed in zip17 (T-DNA mutant) using semiquantitative RT-PCR (Figure 8). Exposure to 100 mM NaCl elevates the expression of these genes in wild type, but much less so in zip17. These findings are consistent with a model in which AtS1P initiates the activation of AtbZIP17, which either directly or indirectly upregulates genes such as ATHB-7. It is interesting that genes such as BiP1, BiP2 and BiP3, which are considered to be ER stress markers, were not significantly induced by salt stress.
**Figure 7.** Identification of the transcriptional activation domain in AtbZIP17.

Transcriptional activation domain in AtbZIP17 was identified by fusing various segments of the cytoplasm-facing component of the gene in yeast with the GAL4 DNA-binding domain. Indicated on the map of AtbZIP17 are the transcriptional activation domain (TAD), bZIP DNA binding domain (bZIP), proline-rich domain (PRD), transmembrane domain (TMD) and canonical S1P site. Transcriptional activity was demonstrated by X-Gal staining.

**Table 1** Salt-induced genes dependent on AtS1P functiona

| Locus    | Salt induced level/control in wild type | Salt induced level/control in s1p-3 | Wild type/s1p-3 | TGACG element present in promoter | Gene descriptions                      |
|----------|----------------------------------------|-------------------------------------|-----------------|----------------------------------|----------------------------------------|
| At5g05390| 26.8                                   | 3.3                                 | 8.2             | +                                | Laccase                                |
| At3g09090| 23.0                                   | 3.3                                 | 7.0             | +                                | Ras-like protein                       |
| At2g46880| 6.6                                    | 1.6                                 | 4.2             | +                                | ATHB-7                                 |
| At2g47780| 6.6                                    | 1.6                                 | 4.0             |                                  | Rubber elongation factor               |
| At1g51460| 7.6                                    | 1.9                                 | 4.0             | +                                | CCAAT-binding factor                   |
| At5g62420| 14.4                                   | 3.9                                 | 3.7             |                                  | Aldose reductase                       |
| At5g06760| 17.5                                   | 5.3                                 | 3.3             | +                                | LEA protein                            |
| At1g48100| 10.4                                   | 3.4                                 | 3.1             | +                                | Polygalacturonase PG1                  |
| At1g52890| 6.5                                    | 2.2                                 | 3.0             | +                                | NAM-like protein                       |
| At1g68480| 6.5                                    | 2.1                                 | 3.0             | +                                | EXS family protein                     |
| At1g52690| 13.7                                   | 4.5                                 | 3.0             | +                                | LEA protein                            |
| At2g33380| 22.4                                   | 7.4                                 | 3.0             |                                  | RD20                                   |
| At2g40435| 9.0                                    | 3.0                                 | 3.0             | +                                | Expressed protein                      |
| At2g34610| 5.5                                    | 1.9                                 | 2.9             | +                                | Expressed protein                      |
| At5g59320| 13.4                                   | 4.8                                 | 2.8             | +                                | Lipid-transfer protein                 |
| At1g04220| 11.3                                   | 4.2                                 | 2.7             |                                  | Beta-ketoacyl-CoA synthase             |
| At4g36720| 6.9                                    | 2.6                                 | 2.7             | +                                | Expressed protein                      |
| At5g04370| 5.4                                    | 2.0                                 | 2.7             | +                                | SAM methyltransferase                 |
| At2g47770| 14.3                                   | 5.4                                 | 2.7             | +                                | Benzodiazepine receptor-related        |
| At5g59220| 6.7                                    | 2.5                                 | 2.6             | +                                | Protein phosphatase 2C                |
| At1g56650| 6.8                                    | 2.6                                 | 2.6             | +                                | Anthocyanin2                           |
| At2g43580| 5.1                                    | 2.0                                 | 2.6             |                                  | Endochnitinase                         |

aGenes from microarray analyses involving two biological replicates in which the salt-induced expression level of genes in wt type (wt) was compared with the s1p-3 mutant. The table shows genes with differences in salt-induced expression comparing wt and s1p-3 > 2.5 while controlling for a false discovery rate (FDR) of 10%.

bGenes analyzed for dependence on AtbZIP17 function in Figure 8.
that ER stress responses can be activated by different stressors, such as glucose starvation, treatment with drugs that inhibit cellular glycosylation, treatment with calcium ionophores or treatment with amino-acid analogues (Kozutsumi et al., 1988). In mammalian cells, low cholesterol levels elicit an ER stress response by activating sterol regulatory element binding proteins (SREBPs) via an RIP mechanism (for a recent review, see Goldstein et al., 2006). SREBP precursor proteins form a complex with another protein, SREBP cleavage activating protein (SCAP), which contains a sterol sensory domain. When cellular cholesterol concentrations drop, SCAP escorts SREBP to the Golgi, where it is activated by RIP, a two-step process involving S1P and then S2P.

AtbZIP17 activation involves RIP and nuclear relocation of an ER resident protein

We observed in this study that AtbZIP17 is activated by salt stress in a manner similar to the activation of mammalian ATF6: by proteolytic processing and translocation of the cytosol-facing component to the nucleus (Schroder and Kaufman, 2005a). Iwata and Koizumi (2005) proposed that the signaling mechanisms mediating ER stress responses in Arabidopsis might be unconventional, based on the structure of AtbZIP60, a bZIP transcription factor upregulated in response to stress agents. Although AtbZIP60 is predicted to be a transmembrane protein on the secretory pathway, it did not have a conserved S1P protease cleavage site, and its luminal sequence, which would interact with chaperones, is much shorter than ATF6. The bZIP factors we have identified have a structure similar to ATF6, and AtbZIP17, in particular, appears to be processed by a mechanism similar to RIP, as is ATF6.

Regulated intramembrane proteolysis (RIP) is conserved from bacteria to humans, and in other systems RIP plays an important role in cellular differentiation, lipid metabolism and response to unfolded protein stress (Brown et al., 2000). RIP involves intramembrane-cleaving proteases (ICLiPs), for which there are four major families in animals: the S2Ps, presenilins, SPP family and rhomboid family (Weihofen and Martoglio, 2003). Kanaoka et al. (2005) showed RIP activity of an Arabidopsis rhomboid homolog with Drosophila substrates. The substrate for the Arabidopsis rhomboid homolog and the function of other ICLiPs have not yet been identified in plants. Recently, an RIP mechanism was reported in Arabidopsis that involves the activation of another membrane-bound transcription factor, NTM1 (Kim et al., 2006). However, in this case NTM1 is an NAC transcription factor that mediates cytokinin signaling during cell division, and is likely to be released from the membrane by a calpain protease. We have demonstrated that AtS1P is involved in the processing of AtbZIP17, and our data support the idea that another protease, such as an S2P protease

Discussion

Is salt stress a specific ER stress response?

This report describes a salt stress response in plants that involves a membrane-associated bZIP transcription factor, which serves as a stress sensor/transducer. The response has some of the features of a classical ER stress response, but not all of those associated with UPR. The AtS1P-dependent salt stress gene differ from those reported for UPR when induced by stress agents such as tunicamycin and DTT (Martinez and Chrispeels, 2003; Kamauchi et al., 2005). The molecular signature of UPR in plants is dominated by BiP and ER-associated calcium binding proteins, and these genes are not significantly upregulated by salt stress.

Nonetheless, our findings raise an important issue as to whether salt stress is a specific ER stress response or if it is just another way to generate misfolded proteins. It is possible that salt stress simply promotes the misfolding of a different group of proteins than those affected by tunicamycin and DTT treatment. Koiva et al. (2003) argued that the salt/osmotic stress response in Arabidopsis is, in fact, a UPR based on the salt sensitivity of a mutation in a gene encoding a component of the protein N-glycosylation machinery. However, we have not observed significant upregulation of BiP1, BiP2 or BiP3 by salt stress.

Of course, we do not know whether salt is the only stressor to which this system responds, or whether this system in Arabidopsis exists in nature to cope with salt-stressing environments. Findings in other systems confirm
Salt stress genes upregulated following AtbZIP17 activation

We have described a group of salt stress induced genes in Arabidopsis that depend on AtS1P and AtbZIP17 for upregulation in response to salt stress. In yeast, UPR genes are characterized by the presence of 22-bp promoter elements called UPR elements (UPREs) (Mori et al., 1992). The bZIP transcription factor, Hac1p, binds to UPREs and upregulates the expression of UPR genes (Cox and Walter, 1996; Mori et al., 1996). Likewise, in animal systems, ER stress elements (ERSEs) have been identified that bind bZIP factors, such as ATF6 and XBP1 (Yoshida et al., 1998). In Arabidopsis, Oh et al. (2003) analyzed the AtBiP2 promoter and found a 24-bp sequence element that conferred tunicamycin-induced expression on a minimal 35S promoter and that was similar to an ERSEII and an XBP1 binding site. In Arabidopsis microarray experiments, no single promoter element motif was recognized from among the genes induced by tunicamycin and DTT; however, motifs similar to various yeast and mammalian stress response elements were over-represented in the group of upregulated genes (Martinez and Chrispeels, 2003). In our study, a blast search revealed that AtbZIP17 is most similar to tobacco TGA1b, a TGACG-specific bZIP binding protein (Katagiri et al., 1989; Schindler et al., 1992). We found that 16/22 of the top S1P-dependent salt stress induced genes have at least one TGACG element in the 1-kb upstream regions of these genes (Table 1). On a random basis one might expect to find only five genes with TGACG elements. We have not yet shown that the group of salt stress upregulated genes in Arabidopsis are direct transcriptional targets of AtbZIP17, but those studies are underway. The genes that do not bear promoter elements recognized by AtbZIP17 might be indirectly upregulated.

Some of the genes we found in our study to be the most dependent on AtS1P and AtbZIP17 function are genes that had been shown by others to be associated with salt stress. They include a homeobox-leucine zipper protein ATHB-7 (At2g46680) and a calcium-binding EF hand protein (RD20 or At2g33380) reported to be upregulated by salt and drought stress, or by ABA treatment (Soderman et al., 1996; Lee and Chun, 1998; Takahashi et al., 2000). Some of these genes still show some low level salt stress induced response in the s1p-3 background, and, therefore, it will be important for us to determine whether the regulation of expression of these genes involves interaction with other salt stress signaling pathways, or whether these genes are targets of one or more independent stress signaling pathways.

In conclusion, we identified a signaling pathway in plants that is exquisitely simple, but highly effective in responding to salt stress. This pathway involves an ER membrane-bound transcription factor AtbZIP17 that is cleaved by AtS1P, and the cystosolic-facing component, bearing bZIP DNA binding and transcription activation domains, is translocated into the nucleus where it activates stress response genes.

Experimental procedures

Plant material and growth conditions

Five T-DNA insertion mutant lines for AtS1P and one line for AtbZIP17 were obtained from the Arabidopsis Biological Resource Center (ABRC; http://www.biosci.ohio-state.edu/pcbmb/Facilities/abrc/abrchome.htm). The lines were screened for homozygous segregants by PCR using gene-specific primers and left border (LB) T-DNA primers (Table S1). To test for salt sensitivity, seeds were surface sterilized, rinsed with sterile water and stratified at 4°C for at least 2 days in 0.1% agar. Seeds were germinated on agar plates containing half-strength MS (½ MS) salts, 1% sucrose and 0.05% 2-(N-morpholine)-ethanesulphonic acid (MES; pH 5.7) for 2 days, then transferred to ½ MS plates containing mannitol or various salts. Seedlings were grown vertically on agar plates in an illuminated growth chamber at 23°C for 1 week. For microarray and RT-PCR analysis, 1-week-old seedlings grown on ½ MS plates were transferred to liquid MS medium plus 100 mM NaCl for 4 h, and whole seedlings were harvested for RNA. For GFP-AtbZIP17 localization experiments, 1-week-old transgenic seedlings grown on normal ½ MS plates were transferred to ½ MS plates ± 100 mM NaCl for further incubation, and roots were harvested for confocal microscope examination.

Plasmid construction

See Table S1.

GUS staining and confocal microscopy

Whole transgenic seedlings grown on MS plates or organs from mature plants grown in soil were harvested and incubated (6 h) in GUS staining solution (100 mM Tris/NaCl buffer, pH 7, 2 mM ferricyanide, 2 mM X-gluc (5-bromo-4-chloro-3-indolyl-D-glucuronide), 2 mM ferrocyanide, 10 mM EDTA and 0.1% Triton) at 37°C in
the dark. The staining solution was removed, and the tissues were dehydrated in an ethanol series from 70% (v/v) to absolute ethanol. Samples were visualized using a light microscope. For subcellular localization, leaf epidermis was stained with Golgi marker BODIPY TR ceramide (5 μM; Invitrogen, http://www.invitrogen.com), roots were stained with 4′,6-diamidino-2-phenylindole (DAPI, 5 μg ml⁻¹; Invitrogen) or the ER marker ER-tracker Blue-White DPX (1 μM; Invitrogen) and observed under a laser confocal microscope (Olympus Fluo View™ FL1000; Olympus, http://www.olympus.com). A sequential scanning mode was used when DAPI or ER marker staining was combined with GFP to minimize the crosstalk between the two partially overlapped emission spectra.

In vivo and in vitro AtS1P reactions

For in vivo cleavage studies, total protein was extracted from transgenic plants (with wild-type or s1p-3 mutant backgrounds) with extraction buffer [0.1 M HEPES–KOH, pH 7.0, 20 mM 2-mercaptoethanol, 0.1 mg ml⁻¹ PMSF, 0.1% (v/w) Triton X-100, 1 mM EDTA, 20% (v/w) glycerol and 0.5% (v/w) SDS]. An aliquot of total protein was precipitated with trichloroacetic acid and quantified by a Bicinchoninic acid (BCA) protein assay. Reaction products were resolved on 8% SDS-PAGE gels and visualized by western blotting using c-myc antibody (9E10; Santa Cruz Biotechnology, http://www.scbt.com) and ECL kit (GE Healthcare, http://www.gehealthcare.com/worldwide.html).

To characterize AtS1P activity in vitro, AtS1P-myc-HDEL was semi-purified from transgenic Arabidopsis plants. A 500-g sample of seedlings was ground in liquid nitrogen and suspended in 25 mM Tris-HCl, 25 mM MES, 25 mM acetic acid and 1 mM CaCl₂. The reaction was carried out at 32°C with continuous rotation. The agarose beads with bound AtS1P-myc-HDEL were recovered by centrifugation at 1000 g and then with 25 mM Tris-HCl, 25 mM MES, 25 mM acetic acid (pH 8.0). Parallel purification was performed using transgenic plants transformed with the empty vector to obtain material for control reactions.

The His-tagged luminal fragment of AtbZIP17 was used as substrate for AtS1P cleavage reactions. AtbZIP17 was purified from control reactions. Background activity was estimated by SDS-PAGE and western blot analysis using anti-c-myc antibody (9E10; Santa Cruz Biotechnology, http://www.scbt.com) and ECL kit (GE Healthcare, http://www.gehealthcare.com/worldwide.html).

Microarray analysis and semi-quantitative RT-PCR

Total RNA was isolated from ground plant tissues using an RNeasy kit according to the manufacturer’s instructions (Qiagen), and quantified by 260/280-nm UV light absorption. Microarray and semi-quantitative RT-PCR procedures were carried out according to the method described by Che et al. (2002, 2006). Affymetrix Arabidopsis gene chips (ATH1) were used to profile gene expression in wild-type and s1p-3 seedlings in a randomized complete block design with two independent replications. Increases in the estimated mean expression of genes significantly upregulated on NaCl were determined while controlling for a false discovery rate of 1.00E⁻² according to Storey and Tibshirani (2003). P- and Q-values for the comparison of genotypes were calculated using EDGE software (http://edge.oncology.wisc.edu). Genes were ranked ordered by the ratio of expression increases in the wild-type samples compared with s1p-3 samples to give a measure of the salt stress genes most highly affected by the s1p-3 mutation.

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Supplementary material

The following supplementary material is available for this article online:

Figure S1. Alignment of Arabidopsis and mammalian S1P proteins. Rat (locus O97ZT32), mouse (locus O97ZT32), hamster (locus O97Z58), human (locus O14703) and Arabidopsis (AtS1P, locus At6g19660) protein sequences were aligned using CLUSTAL W (Chenna et al., 2003). N-terminal signal sequences predicted by SIGNALP are underlined, proenzyme sequences are shaded and the predicted cleavage sites are marked with *. Catalytic DHS residues are shaded and marked with \( \text{\()\}, \) catalytic DHH residues are shaded and marked with \( \text{\()\}, \) and C-terminal transmembrane segments predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) are shaded and marked ‘transmembrane’. In the line below each set of sequences: *, identical residues across all sequences; :, conserved residues; ., semiconserved residues.

Figure S2. Segregation of salt-sensitive phenotype with T-DNA, and rescue of mutants with cDNAs.

(a) Segregation of salt-sensitive phenotype in an F2 population of s1p-3 x wt. Seedlings were grown for 7 days on ½ MS media with 150 mM NaCl. Root growth was scored, and DNA was extracted from each seedling and genotyped. The salt-sensitive seedlings were confirmed to be homozygous s1p-3. Arrows indicate homozygous s1p-3 progeny.

(b) Rescue of s1p-3 by 35S:AtS1P cDNA in line S1P9. Seedlings were grown for 7 days on normal ½ MS media or ½ MS media with 150 mM NaCl added. Ten seedlings were scored in each of three replications, and mean root lengths were plotted (error bars are ±SE).

(c) Segregation of salt-sensitive phenotype in a F2 population of zip17 x wt. Seedlings were grown for 7 days on ½ MS media with 150 mM NaCl. The salt-sensitive seedlings indicated by arrows were confirmed to be homozygous zip17. (d) Rescue of zip17 by 35S:AtbZIP17 cDNA in line ZIP4. As above, seedlings were grown for 7 days on normal ½ MS media or ½ MS media with 150 mM NaCl added and root length was scored. Mean root lengths are plotted and error bars are ±SE.

Table S1. Primers used for PCR and RT-PCR analysis.

This material is available as part of the online article from http://www.blackwell-synergy.com
Salt stress elicits ER stress response

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