



Functional integration between defence and IRE1-mediated ER stress response in rice

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Conditions within the endoplasmic reticulum (ER) influence most secretory proteins that pass through the ER. Therefore, eukaryotic cells must strike a balance between the ER stress response, which changes the conditions in the ER, and other considerations associated with protein secretion. Here, an interaction between the ER stress and defence responses in rice is described. Expression of *OsWRKY45*, which encodes a transcription factor that plays a central role in defence mediated by salicylic acid (SA), is induced by ER stress. Additionally, expression of some genes encoding pathogenesis-related (PR) secretory proteins is reduced by the ER stress response mediated by the stress sensor IRE1. Concomitant activation of the SA and ER stress responses suppresses the induction of ER stress-responsive genes, with the exception of *OsWRKY45*, and the reduction of PR gene expression. These findings demonstrate a functional integration between the defence and ER stress responses in plants.

The ER stress response is triggered by the accumulation of unfolded proteins in the ER lumen. ER stress activates intracellular signal transduction pathways, such as the unfolded protein response, that contribute to the relief of the stress¹. In plants, the signal transduction pathways involved in the ER stress response have been identified². However, the relationships between the ER stress response and other plant cellular responses remain poorly understood. In particular, because plants are simultaneously subjected to biotic and abiotic stresses in their natural environments, understanding interference among multiple stress responses represents a major challenge in plant science.

In many eukaryotes including plants, ER stress is detected by the transmembrane protein IRE1. The N-terminal portion of IRE1 resides in the ER lumen; the C-terminal portion resides in the cytosol and contains a serine/threonine kinase domain and an endonuclease domain^{1,3,4}. When activated by ER stress, IRE1 mediates an unconventional splicing of the mRNA encoding a key bZIP transcription factor, HAC1 (in yeast), XBP1 (animals), AtbZIP60 (Arabidopsis) or OsbZIP50 (rice); the spliced forms of these mRNAs are translated as active forms⁵⁻⁹. In animals, ER stress is also sensed by ATF6, a type II transmembrane protein activated by proteolysis by the site 1 and 2 proteases. Cleavage of ATF6 liberates its cytosolic domain, which contains a bZIP transcription factor that, like XBP1/AtZIP60/OsZIP50, activates transcription of ER stress response genes^{10,11}. In plants, AtbZIP17 and AtbZIP28 (Arabidopsis) and OsbZIP39 and OsbZIP60 (rice) are the counterparts of ATF6^{9,12-15}. In the case of rice, the activated forms of OsbZIP39, OsbZIP60 and OsbZIP50 induce the expression of genes that encode ER quality control (ERQC)-related factors such as the ER chaperone BiP^{9,15}.

In the life cycle of higher plants, massive amounts of seed storage proteins are produced during seed maturation according to a developmental program, resulting in induction of the ER stress response in response to the large load of secretory protein^{16,17}. Defects in secretion of storage proteins are associated with expression of ERQC-related genes in maturing seeds. Furthermore, when plants are attacked by pathogens, multiple types of proteins including pathogenesis-related (PR) proteins are secreted through the ER as part of the defence response. Consistent with this, defects in ERQC disturb the expression of secretory proteins required for defence responses^{18,19}. Furthermore, in Arabidopsis, some ER chaperones transiently induced by transcription factor NPR1, which plays a central role in the SA response, promote efficient secretion of PR proteins²⁰. These findings suggest that ER function and defence in plants are interrelated.

The mechanisms of the defence response differ between rice and Arabidopsis. For example, the level of SA, a phytohormone that plays a central role in the defence response, is several orders of magnitude higher in rice than in Arabidopsis²¹. Additionally, the SA response in rice involves both an ortholog of Arabidopsis NPR1 as well as



an SA-regulated transcription factor, OsWRKY45, which is absent from *Arabidopsis*²². Although overexpression of the *OsWRKY45* gene alone is not sufficient to activate the defence response fully, the resulting accumulation of OsWRKY45 contributes to efficient activation of the defence response genes when the plant is attacked by pathogens²². Therefore, the biological activities of OsWRKY45 are predicted to enhance disease resistance in rice²². Understanding the specific mechanisms of the rice defence response is an important challenge in agriculture.

In this study, in order to understand the relationship between the ER stress response and other cellular responses, we measured the expression patterns of many ER stress-responsive genes that are closely associated with other responses. From data obtained by DNA microarray analysis of ER-stressed rice plants, we found that expression of *OsWRKY45* is also induced by ER stress. Additionally, we investigated the interactions between the ER stress and SA responses. We close by discussing the implications of the ER stress-responsive induction of *OsWRKY45*.

Results

Expression of *OsWRKY45* induced by ER stress response in an OsbZIP50-dependent manner. Our first DNA microarray screen for ER stress-responsive genes revealed that the expression of the SA-responsive gene *OsWRKY45* is also markedly induced by ER stress. Quantitative reverse transcription-PCR (RT-PCR) analysis confirmed that expression of *OsWRKY45* was induced by tunicamycin (Tm) treatment, which induces ER stress by inhibiting N-linked glycosylation. The induction by Tm was suppressed by 4-phenylbutyric acid (PBA), which acts as a chemical chaperone by masking unfolded proteins (Figure 1a). Additionally, the induction of *OsWRKY45* expression by ER stress was markedly decreased in *OsbZIP50* knockdown (KD) lines, indicating that the induction depended strongly on OsbZIP50 (Figure 1b). To determine whether OsbZIP50 interacts directly with the promoter region of *OsWRKY45*, we performed chromatin immunoprecipitation (ChIP) assays. Quantitative PCR analysis of ChIP output material demonstrated that DNA fragments derived from upstream regions of *OsWRKY45* gene were specifically precipitated with anti-OsbZIP50 antibodies⁹ (Figure 1c). This result indicates that OsbZIP50 is directly involved in the induction of *OsWRKY45* expression in response to ER stress. By contrast, the induction of *OsWRKY45* expression by SA was similar between wild type and *OsbZIP50* KD, indicating that induction by SA does not depend on OsbZIP50 (Figure 1d).

ER stress-induced gene expression is generally suppressed by SA. When Tm was co-administered with SA, the effects on *OsWRKY45* expression were additive (Figure 2). By contrast, high-level expression of many ER stress-inducible genes by Tm was significantly suppressed by SA treatment, although induction of *OsWRKY45* itself was not suppressed (Figure 2). This suppression was also observed upon addition of benzothiadiazole (BTH), a functional analogue of SA that activates the SA signalling pathway (Supplementary Figure S1), indicating that the suppression is a result of SA signalling. Furthermore, the same effect was observed when DTT was used in place of Tm (Supplementary Figure S2), indicating that the SA pathway suppresses expression of genes that are responsive to ER stress per se, rather than to some specific consequence of Tm treatment. Inversely, in order to investigate whether expression of SA-inducible genes is suppressed by ER stress, we investigated expression levels of several SA-inducible genes (*Os07g0418500* encoding P450 and *Os07g0129200* encoding PR1a) using the same samples. However, induction of these genes by SA was not suppressed by Tm treatment (Figure 2). The induction of *Os07g0418500* expression by the SA response depends on *OsWRKY45*²². However, in plants treated with Tm alone, although transcripts of *OsWRKY45* accumulated to high levels, the expression level of

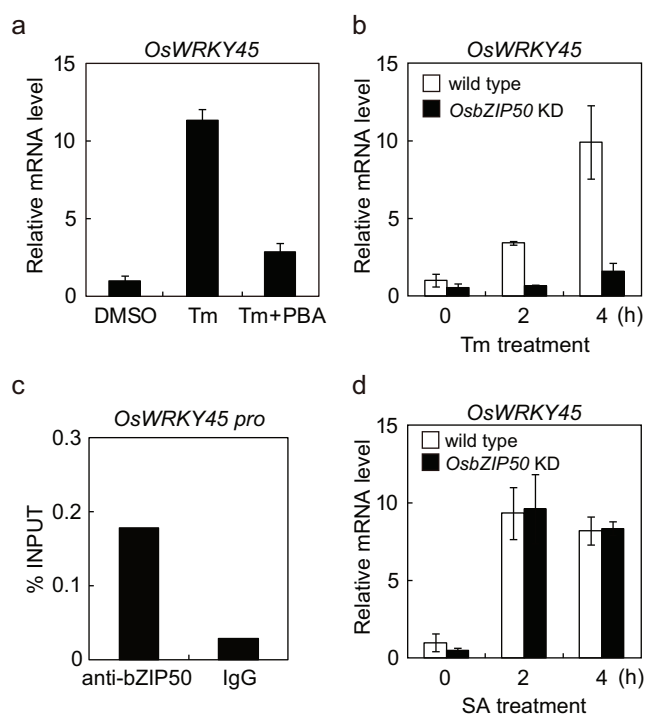


Figure 1 | Expression of *OsWRKY45* is induced by the ER stress response in an OsbZIP50-dependent manner. (a, b, d) Relative mRNA levels of *OsWRKY45*. Wild type plants after treatment for 4 h with DMSO, tunicamycin (Tm) or Tm and 4-phenylbutyric acid (Tm+PBA) (a). Wild type (open bars) and *OsbZIP50* knockdown (KD) plants (filled bars) after treatment with Tm (b) or SA (d). Quantitative RT-PCR analyses. Data are expressed as means \pm s.d. of three or four biological replicates. (c) ChIP-PCR analysis of DNA fragments precipitated with anti-OsbZIP50 antibodies. A short region upstream of the transcription initiation site of *OsWRKY45* gene was analysed by quantitative PCR. DNA samples before ChIP were used as INPUT. IgG that does not react with OsbZIP50 was used as a control. Similar results were observed in two independent experiments (c).

Os07g0418500 was not elevated (Figure 2). This data suggests that *OsWRKY45* induced by ER stress confers a “priming” effect, in which the *OsWRKY45* target genes are potentiated for expression but must await activation of the SA response in order to be expressed at high levels.

ER stress response reduces expression of some PR genes in an OsIRE1-dependent manner. In order to examine in more detail the relationship between the ER stress response and the defence response against pathogens, we screened for defence-related genes in DNA microarray data obtained from *OsIRE1* KD plants⁹. The first such screen revealed that after treatment with DTT, expression levels of some genes were higher in *OsIRE1* KD plants than in wild type. Many of these genes encoded pathogenesis-related (PR) proteins (e.g., *Os03g0663400*, a thaumatin-like protein; *Os050477900*, a lipid-transfer protein; *Os06g0726100*, endochitinase; and *Os11g0645400*, a disease-resistance response protein) that are putative secretory proteins expressed in root tissue even under non-stressed conditions. Quantitative RT-PCR analysis confirmed that expression levels of these PR genes were severely reduced upon DTT treatment, and that some of these effects depended on expression of *OsIRE1* (Figures 3a and b). Decreased expression levels of these genes were also observed when DTT was replaced with Tm, although the effects of Tm at the doses we used were slightly milder than those of DTT (Figure 3c). These results strongly suggest that these effects are caused by the ER stress response. Furthermore, the

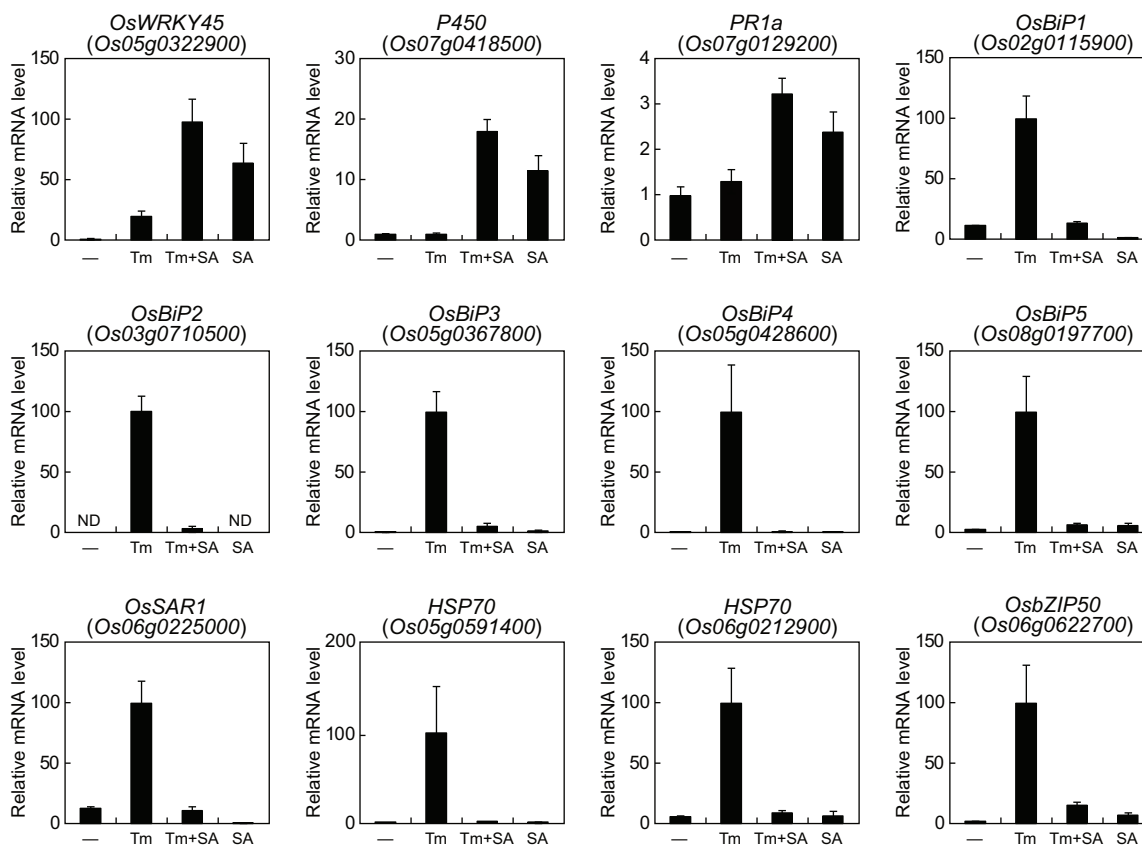


Figure 2 | Gene expression induced by ER stress is generally suppressed by SA. mRNA levels, determined by Quantitative RT-PCR, of representative SA-responsive and ER stress-responsive genes in wild type seedlings after treatment for 4 h with DMSO (–), tunicamycin (Tm), Tm and SA (Tm+SA) or SA and DMSO (SA). ND, not detected. Data are expressed as means \pm s.d. of four biological replicates.

reduction in expression of PR genes by ER stress was suppressed by SA treatment, although expression of the PR genes was not induced by SA alone (Figure 3c).

SA does not relieve or mask ER stress. As described above, the SA response suppressed multiple effects of the ER stress response, raising the possibility that the SA response relieves or masks ER stress. However, ER stress-responsive ERQC-related factors such as BiPs, which contribute to relief or masking of ER stress, were not induced in SA-treated plants (Figure 2). Additionally, SA treatment slightly induced expression of *OsbZIP50* and OsIRE1-mediated splicing of *OsbZIP50* mRNA, but the induction of gene expression was suppressed by a chemical chaperone (Supplementary Figure S3). These data suggest that SA acts to induce, rather than relieve, ER stress. These observations strongly suggest that the SA response suppresses ER stress-inducible gene expression by affecting the functions of the signalling components of the ER stress response.

In summary, our results demonstrate that ER stress up-regulated the expression of genes encoding ERQC-related chaperones (such as BiPs) and *OsWRKY45*, but down-regulated expression of a subset of PR genes. This regulation of gene expression by ER stress response was widely suppressed by the SA response. Additionally, it is possible that the SA response utilises the *OsWRKY45* protein accumulated during ER stress response to activate defence responses.

Discussion

Comparison of the gene expression patterns induced by distinct signalling pathways can provide new insight into the relationship between the responses. In this study, we found that *OsWRKY45* was induced by both the ER stress and SA responses. Consequently, we

investigated the possibility that interference between the ER stress and defence responses might influence the regulation of gene expression. Our findings reveal a functional integration between these two stress responses, and suggest that the ER stress response has a close relationship with the defence systems that are unique to plants.

Based on our data, we propose a model (Figure 4) of the relationship between the ER stress and defence responses, in particular the SA response (Figure 4). Some PR genes are expressed even under normal conditions. For example, according to the Rice XPro microarray expression database (<http://ricexpro.dna.affrc.go.jp>), a thaumatin-encoding gene (*Os03g0663400*) analysed in this study (Figure 3) is highly expressed in root tissue. These PR proteins, which confer antibiotic resistance, were secreted through the ER (Figure 4a). When ER stress arose, ERQC-related genes were induced by activation of the OsIRE1-OsbZIP50, OsbZIP39, and OsbZIP60 pathways in order to relieve the stress (Figure 4b). In addition, ER stress down-regulated the expression of some PR genes in an OsIRE1-dependent manner (Figures 3 and 4b). This decrease in gene expression levels reduced secretion of the PR proteins, lowering the secretory burden on the ER and thereby preventing the stress condition from worsening further. Concomitantly, the ER stress response induced expression of *OsWRKY45* in an OsbZIP50-dependent manner (Figures 1 and 4b). Resistance to some diseases is improved by overexpression of *OsWRKY45*²². Therefore, the *OsWRKY45* protein accumulated in response to ER stress might offset the risk associated with decreased expression of PR genes (Figure 4b). When the SA response was activated at the same time as the ER stress response, most of the aforementioned effects of the ER stress response were suppressed, although *OsWRKY45* was still induced (Figures 2, 3c, and 4c). As a result of this suppression, the ER stress response refrains from

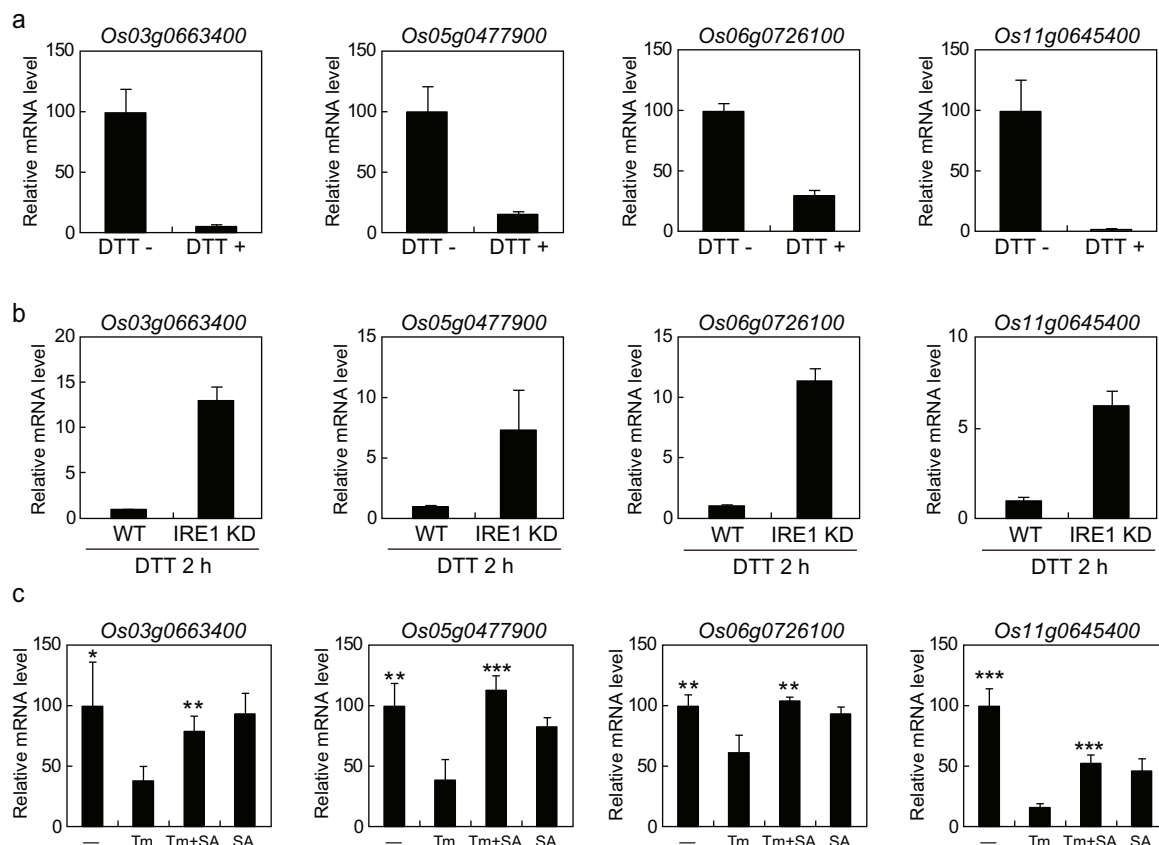


Figure 3 | ER stress response reduces expression of some PR genes in an *OsIRE1*-dependent manner. mRNA levels, determined by Quantitative RT-PCR, of representative PR protein-encoding genes that exhibit ER stress-responsive changes in expression. Data are expressed as means \pm s.d. of three or four biological replicates. Wild type plants after treatment for 2 h with or without DTT (a). Wild type plants (WT) and *OsIRE1* knockdown (*IRE1* KD) plants after treatment for 2 h with DTT (b). Wild type plants after treatment for 4 h with DMSO (–), tunicamycin (Tm), Tm and SA (Tm+SA) or SA and DMSO (SA) (c). Asterisks indicate significant differences relative to treatment with Tm (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *t*-test).

consuming energy that might be used to mount the SA response, and defence-related secretory proteins are permitted to pass through the ER. Additionally, accumulated *OsWRKY45* protein can be subsequently activated by the SA response, resulting in rapid induction

of SA-responsive gene expression (Figure 4c). Although the molecular mechanism underlying the suppression of the ER stress response by the SA response remains unknown, this finding is nonetheless important to the understanding of the environment adaptability of plants.

Our results showed that *OsIRE1* is involved in down-regulation of some PR genes under conditions of ER stress (Figure 3a). Additionally, our previous studies showed that expression levels of some seed storage proteins, secretory proteins that are produced at high levels during seed maturation, are significantly down-regulated in transgenic rice seeds under severe ER stress conditions^{17,23}. Thus, ER stress may be responsible for regulating expression of secretory proteins other than PR proteins. In animals, *IRE1* directly degrades mRNAs encoding secretory proteins²⁴; some of the reduction in PR gene expression mediated by *OsIRE1* may occur by a similar mechanism. Further study will be required to elucidate the involvement of *OsIRE1* in such phenomena.

In our first DNA microarray screen for ER stress-responsive genes, we observed that *OsWRKY45* was induced to a greater extent than other WRKY family members, suggesting that the transcription factor *OsWRKY45* plays a specialised role. Induction of *OsWRKY45* expression during ER stress is dependent on the *OsIRE1*-*OsZIP50* pathway (Figure 1). Taken together, these observations indicate that *OsIRE1* is involved not only in expression of various ERQC-related genes, but also in expression of defence-related genes (*OsWRKY45* and some PR genes), as observed in this study. Thus, over the course of evolution, *IRE1* can be incorporated into species-specific pathways such as the plant defence response.

In *Arabidopsis*, *IRE1* is also involved in immunity²⁵. Additionally, *Arabidopsis WRKY33* is induced by ER stress²⁶, implying that the

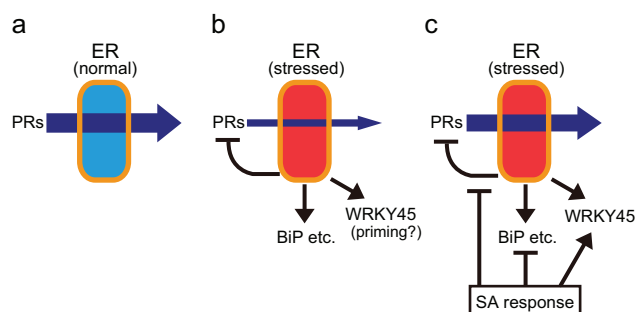


Figure 4 | Model of the relationship between ER stress and defence responses. Under normal conditions (a), some PR proteins are synthesised and secreted through the ER. When ER stress conditions arise (b), the *OsIRE1*-*OsZIP50*, *OsZIP39* and *OsZIP60* pathways induce genes encoding ERQC-related factors (such as *BiPs*) in order to relieve the stress. Furthermore, the ER stress response reduces expression of some PR genes in an *OsIRE1*-dependent manner and induces expression of *OsWRKY45* in an *OsZIP50*-dependent manner. ER stress-induced *OsWRKY45* is not fully activated. When the SA response is activated concomitantly with the ER stress response (c), most of the aforementioned effects of the ER stress response are suppressed. Accumulated *OsWRKY45* are subsequently activated by the SA response and contribute to SA-responsive gene expression.



relationship between ER stress and SA responses in rice may also exist in to Arabidopsis to some extent. However, because the SA responses of the two species differ in specific detail, individual analysis will be necessary to evaluate the role of IRE1 in the Arabidopsis response.

The plant defence response is very complicated, with several distinct phytohormones participating in regulation of defence response genes. Therefore, the results presented here cannot fully explain all aspects of the defence response; nonetheless, our findings contribute significantly to our understanding of these pathways. Further studies of the relationships between ER stress and other phenomena will be required in order to elucidate plant stress responses in full detail.

Methods

Oligonucleotides. The oligonucleotides used in this study are listed in Supplementary Table S1.

Plant material and growth conditions. *Oryza sativa* L. cv. Kita-ake plants were grown on MS medium [1× Murashige and Skoog salt mix, 3% sucrose, B5 vitamin, 2.5 mM MES (pH 5.8), and 0.25% Gelrite] at 25 °C using a 16 h light/8 h dark cycle. Seedlings (8 days old) were incubated in liquid MS medium containing 2 mM DTT, 5 µg/ml tunicamycin, 0.5 mM SA (salicylic acid, sodium salt), 0.2 mM BTH or 2 mM PBA, unless otherwise indicated. As negative controls for DTT, SA and PBA, equal volumes of water were added. As negative controls for tunicamycin and BTH, equal volumes of DMSO (final concentration of 0.1%) were added.

RNA extraction and gene expression analysis. Total RNA was extracted from root tissues using the RNeasy Plant Mini Kit (QIAGEN). For RT-PCR analysis, first-strand cDNA was synthesised from 0.8 µg of total RNA using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen), which includes both oligo(dT) and random hexamers. Quantitative RT-PCR was performed using 1/80 of the prepared cDNA, specific primers (Supplementary Table S1), and SYBR® *Premix Ex Taq*TM (Takara). The ubiquitin-encoding gene (*Os06g0681400*) was used as an internal reference.

ChIP-PCR. ChIP analysis was performed as described by Haring et al. (2007) with modifications²⁷. Chromatin samples were prepared from root tissues of wild type seedlings after treatment with DTT for 2 h and fixed by submerging the root tissues in 1% (w/v) formaldehyde solution under vacuum for 10 minutes. The fragmentation of chromatin was performed by sonication (TOMY UR-20P, output setting 8, for about 7 minutes). For immunoprecipitation, anti-OsbZIP50 antibody⁹ bound to protein A-Sepharose 4 Fast Flow (GE Healthcare Bio-Sciences) was pre-incubated with salmon sperm DNA and BSA. For a control IgG that does not react with OsbZIP50, we used purified anti-OsBiP4&5 antibody²⁸. Quantitative PCR analysis of immunoprecipitated DNA fragments was performed using SYBR® *Premix Ex Taq*TM (Takara).

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Author contributions

SH, YW and FT designed the experiments. SH and YW executed the experiments. SH and YW analysed the data. SH and FT wrote the manuscript. All authors reviewed the manuscript.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

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