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Multiple roles of the ER stress sensor IRE1 demonstrated by gene targeting in rice

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The endoplasmic reticulum (ER) stress sensor, IRE1, contains a kinase domain and a ribonuclease domain. Ribonuclease mediates the unconventional splicing of mRNA encoding the transcription factor AtbZIP60 in Arabidopsis, or OsbZIP50 in rice, and thereby transduces signals from stressed ER. Here, we demonstrate the additional roles of plant IRE1 using genetically modified rice plants. Using a gene targeting system based on homologous recombination, genomic *IRE1* was replaced with two types of missense alleles, leading to a defect in the kinase or ribonuclease activity of IRE1. Genetic analysis of these alleles demonstrated that the kinase activity of IRE1 plays a vital role independent of ribonuclease activity. Furthermore, the existence of ribonuclease substrates other than *OsbZIP50* mRNA is demonstrated for the first time. This study provides new insights into higher plant signalling using a gene targeting approach.

Endoplasmic reticulum (ER) stress is triggered by the accumulation of unfolded proteins in the ER lumen. ER stress activates intracellular signal transduction (called ER stress response or unfolded protein response) pathways that contribute to relief of the stress[1]. In many eukaryotes, including plants, ER stress is sensed by the transmembrane protein IRE1. The N-terminal portion of IRE1 resides in the ER lumen, and the C-terminal portion resides in the cytosol and contains a serine/threonine kinase domain and an endo-ribonuclease (RNase) domain[2,3]. Accumulation of unfolded proteins in the ER lumen leads to the clustering of IRE1, autophosphorylation of the kinase domain, and consequent activation of the RNase domain[2]. The RNase activity mediates an unconventional splicing of the mRNA encoding a key transcription factor, HAC1 (yeast), XBP1 (animals), AtbZIP60 (Arabidopsis) or OsbZIP50 (rice); the spliced forms of these mRNAs are translated as active forms[4–8]. In the case of rice, the spliced form of OsbZIP50 is involved in the expression of genes that encode ER quality control-related factors such as the ER chaperone, BiP[8]. The expression of some of these genes, such as *OsBiP4*, are almost completely dependent on the spliced form of OsbZIP50, indicating that the IRE1-OsbZIP50 pathway is particularly important for the ER stress response in rice[8,9].

In animals, IRE1 plays multiple roles other than the cleavage of *XBP1* mRNA and serves as a branch point for ER stress response signalling[2]. Therefore, plant IRE1 orthologues are also expected to play multiple roles[10]. However, such roles have not been clarified in plants. In our previous study[8], transgenic plants were generated in which *OsbZIP50* or rice *IRE1* (*OsIRE1*) were knocked down by RNA interference. The *OsIRE1* knockdown (KD) lines exhibited severe defects in growth and so were difficult to maintain. In contrast, *OsbZIP50* KD lines did not exhibit a similar phenotype even though *OsbZIP50* expression was almost completely suppressed. This difference suggests that OsIRE1 plays roles other than the cleavage of *OsbZIP50* mRNA.

One strategy that can be employed to clarify the additional roles of OsIRE1 is the analysis of rice plants that are defective in the kinase or RNase activity of OsIRE1. However, such mutants have not been isolated, and the creation of intended plants by T-DNA insertion has not been possible because a disruption mutant of *OsIRE1* is not available. To overcome this problem, an alternative plan for obtaining such mutants was considered.

Gene targeting (GT) based on homologous recombination (HR) enables the modification of desired genomic DNA sequences. This technique has greatly contributed to the functional analysis of many genes in various organisms and has been established as an indispensable tool for functional genomics. In plant species, the GT technique is routinely used in moss[11]. However, practical examples of the use of such a technique in higher plants, which includes most agricultural crops, have been extremely limited due to technical difficulties. Therefore, gene modification in higher plants has generally depended on the occurrence of incidental mutations or random insertions of designed T-DNA.

Recently, experimental techniques for performing *Agrobacterium*-mediated GT based on HR were improved, and some GT rice plants have become available[12,13]. Thus, using this improved GT system, we attempted to

generate rice plants that are selectively defective in the kinase or RNase activities of OsIRE1 and investigated whether OsIRE1 plays additional roles in plant signalling.

Results

Based on previous data regarding IRE1 homologues in yeast, mammals and plants[3,14,15], amino acids required for the enzymatic activities of OsIRE1 were predicted. Multiple alignments of amino acid sequences of IRE1 orthologues indicate that K519 and K833 are essential for the kinase and RNase activities of OsIRE1, respectively (Fig. 1a). Indeed, a mutation of K519A was found to lead to a defect in the autophosphorylation activity of recombinant OsIRE1 proteins produced in *Escherichia coli*[14]. The genomic region containing the *OsIRE1* locus was cloned, and K519A or K833A mutations were introduced. To easily detect these mutations, restriction enzyme recognition sites were incorporated close to the mutation sites (Fig. 1b). Plasmid vectors for HR-based GT were constructed as shown in Fig. 1c; these vectors were then introduced into rice via *Agrobacterium*-mediated transformation (Fig. 1c and d). Using PCR and sequencing analysis, we selected GT plants and confirmed that the *OsIRE1* loci were modified as designed. At least four T0 plants in which *OsIRE1* was replaced with the K519A allele and two T0 plants in which *OsIRE1* was replaced with the K833A allele were independently obtained. All of the T0 plants were heterozygous for the alleles of *OsIRE1*.

Genotyping showed that 7 of the 39 T1 progeny derived from the K833A heterozygous T0 plants were homozygous for the K833A mutation (Fig. 2a–c). To confirm the deficiency of RNase activity in the K833A homozygous lines (K833A lines), the response of these lines to ER stress was investigated. Dithiothreitol (DTT) treatment, which induces ER stress, led to OsIRE1-mediated splicing of *OsbZIP50* mRNA and marked accumulation of *OsBiP4* transcripts in wild-type plants, whereas this activity was not detected in the K833A lines (Supplementary Fig. S1). This result indicates that OsIRE1 RNase activity was defective in the K833A lines.

In contrast to K833A plants, transgenic plants homozygous for the K519A mutation could not be obtained from the selfed progeny of T0 plants, although the growth conditions were similar to those of the K833A plants (Fig. 2a–c, Table 1). A similar result was also obtained from the progeny of several independent lines, indicating that this phenomenon was not the result of other mutations. These data genetically demonstrate that the deficiency of OsIRE1 kinase activity is lethal and that kinase activity plays a vital role that is independent of RNase activity. The lethality of homozygotes for the K519A mutation is consistent with the growth defect of the *OsIRE1* KD line (Supplementary Fig. S2). Additionally, K833A lines did not show a growth phenotype similar to that of the *OsIRE1* KD lines (Fig. 2d), again supporting the notion that OsIRE1 plays roles that are independent of RNase activity.

In our previous study[8], genes that are up-regulated or down-regulated by OsIRE1 were screened by DNA microarray analysis of
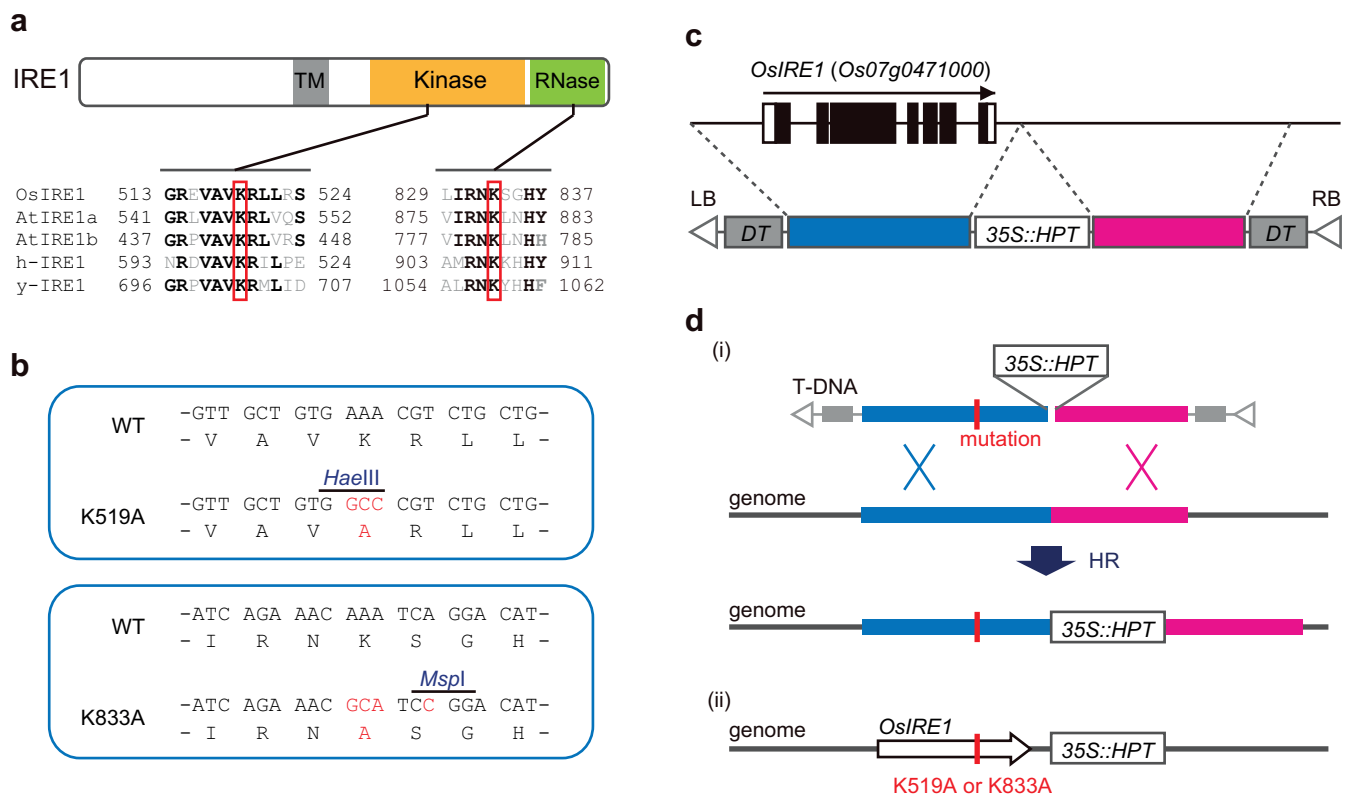
Figure 1 | Introduction of missense mutations into the *OsIRE1* locus by HR-based GT. (a) Basic structure of IRE1. Transmembrane (TM), kinase and RNase domains are indicated. Partial amino acid sequence alignments of IRE1 orthologues (lower part of figure: At, Arabidopsis; h-, human; y-, yeast). Amino acids identical in at least three sequences are highlighted in boldface type. Red boxes indicate residues that are essential for kinase or RNase activities of IRE1. (b) Missense mutations introduced into *OsIRE1*. Recognition sites of the restriction enzymes *Hae*III or *Msp*I were incorporated into these mutation sites. (c) T-DNA used for GT. White and black boxes indicate untranslated and coding regions of genomic *OsIRE1*, respectively. A genomic region containing full-length *OsIRE1* (blue) and the immediate downstream region (magenta) were cloned into the binary vector for *Agrobacterium*-mediated GT. K519A or K833A mutations were introduced into the cloned *OsIRE1*. Genes encoding diphtheria toxin (DT) and hygromycin phosphotransferase (HPT) in the T-DNA were used as negative and positive selection markers, respectively. (d) Schematic representation of GT. The HR event via double crossovers at the *HPT*-flanking homologous regions in the T-DNA resulted in an insertion of the *HPT* gene and partial replacements of the homologous regions (i). The successfully modified genomic region containing *OsIRE1* is shown (ii).
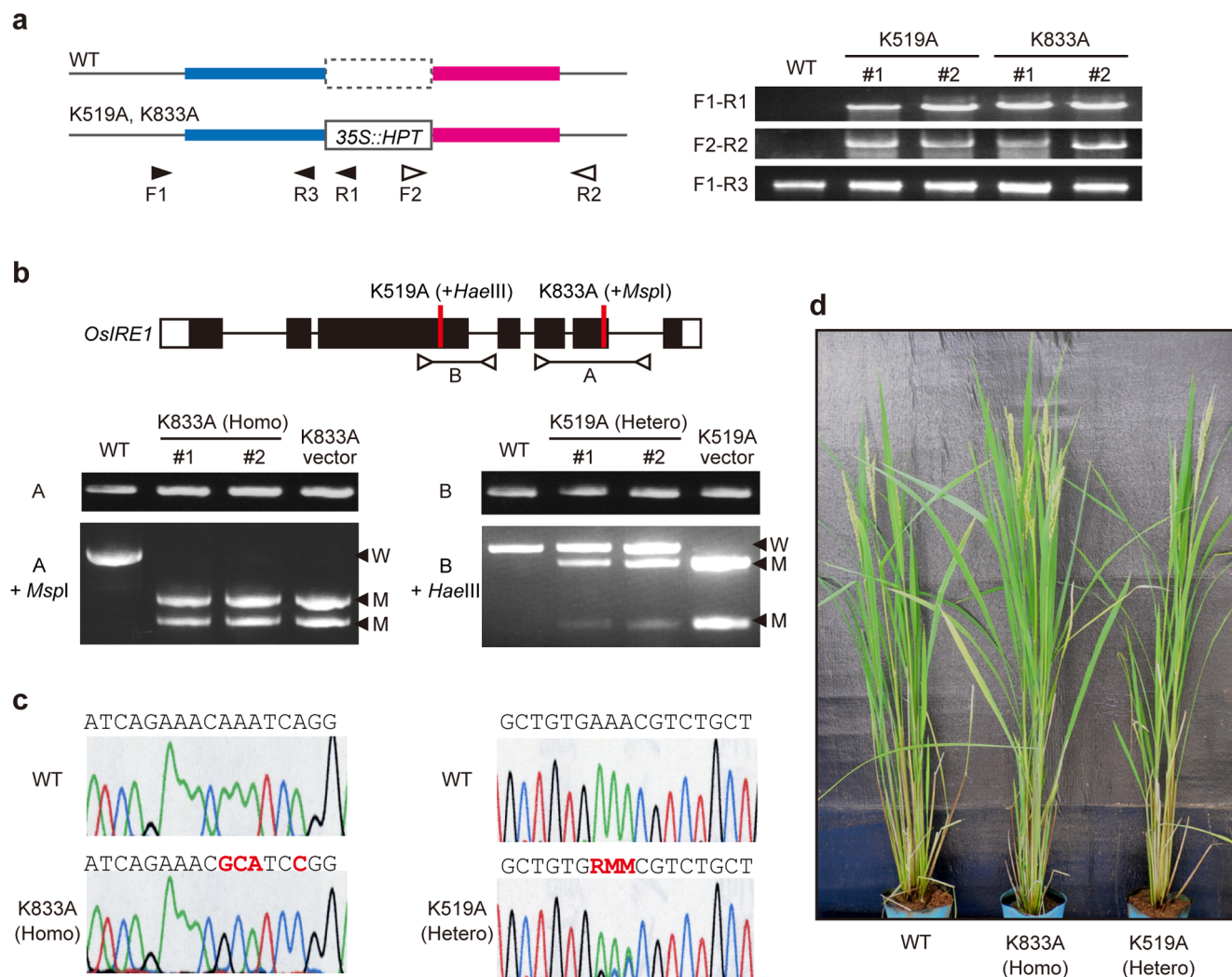
Figure 2 | Confirmation of GT plants. (a) PCR analysis for confirmation of HR events at the target locus. Wild-type and modified (K519A and K833A) genomic regions are shown (left). Blue and magenta bars indicate regions homologous to *HPT*-flanking regions in the T-DNA used for GT. *OsIRE1* is included in the blue region. Arrowheads indicate PCR primers. PCR analysis of genomic DNA extracted from K519A and K833A lines (two independent lines each) is shown (right). (b) Confirmation of genotypes in the progeny of the GT plants. Genotyping of K833A homozygous T1 plants and K519A heterozygous T1 plants (two independent lines each) is shown. K519A homozygous plants were not obtained due to lethality (see Table 1). Open arrowheads and lines (A and B) indicate PCR primers and amplified regions for genotyping (upper panel). Regions A or B were amplified from genomic DNA and were digested with *Msp*I or *Hae*III, respectively. Fragments originating from mutant type (M) and wild-type (W) *OsIRE1* are indicated by closed arrowheads. (c) Sequencing chromatograms of mutation sites. K833A homozygous plants (left) and K519A heterozygous plants (right). (d) Growth of adult GT plants. Wild-type (left), K833A homozygous (middle) and K519A heterozygous (right) plants germinated at approximately the same time and grown under similar conditions.
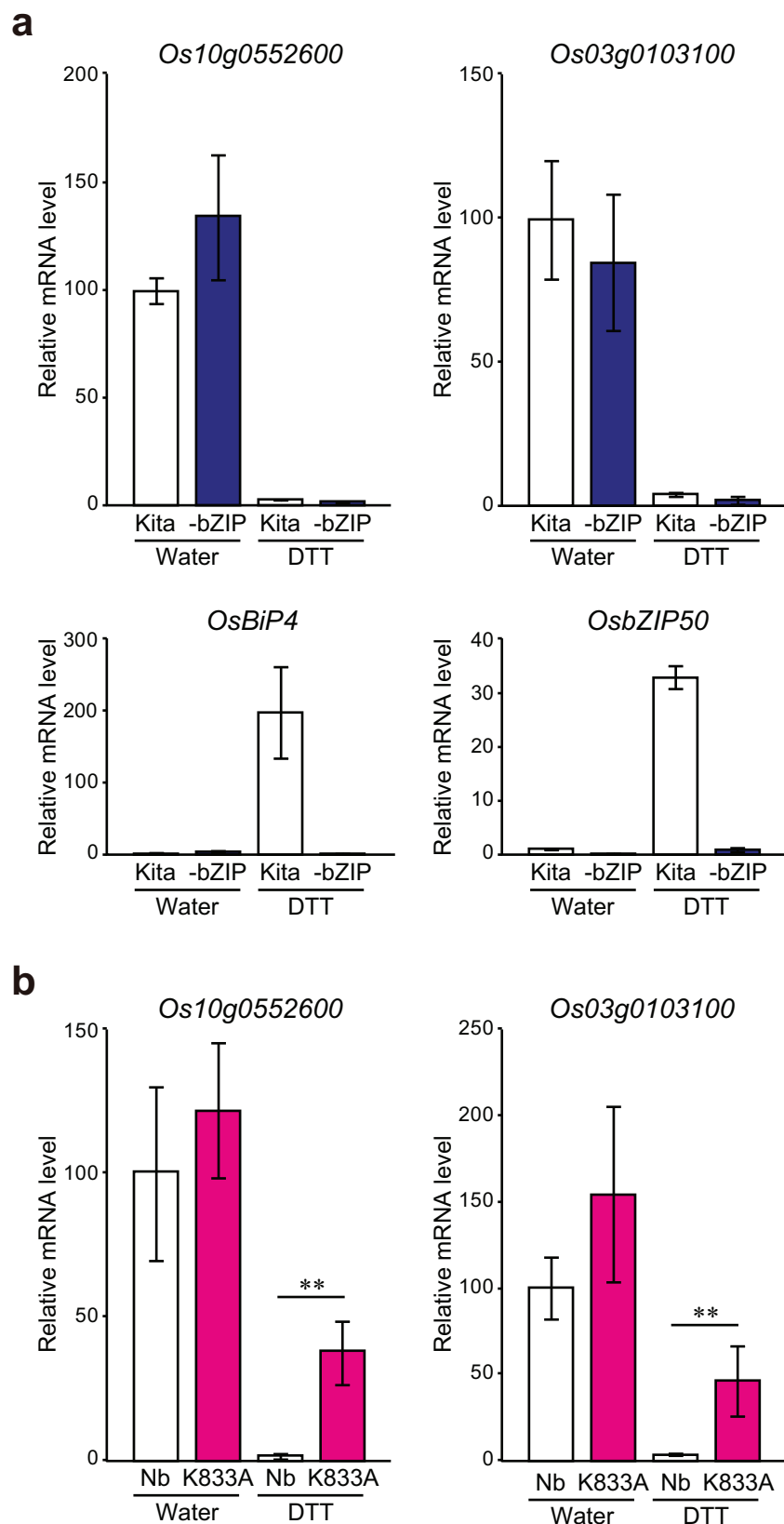
OsIRE1 KD plants after treatment with DTT. Detailed analysis demonstrated that, in response to ER stress, OsIRE1 up-regulates the expression of various genes (e.g., *OsBiP4*) by mediating mRNA

Table 1	Segregation analysis of *OsIRE1* K519A mutation					
	Number of plants					
Genotype	Line A	Line B	Line C	Line D	Total	Expected ratio
K519A/ K519A	0	0	0	0	0	1
K519A/WT	28	7	5	8	48	2
WT/WT	14	1	3	3	21	1

Genotypes of *OsIRE1* loci in the selfed progeny of four K519A heterozygous plants derived from independent lines (Lines A–D). Genotyping was performed by genomic PCR, followed by restriction digestion with *Hae*III.

splicing of OsbZIP50[8]. In this study, to investigate whether the down-regulation of some genes by OsIRE1 is also dependent on OsbZIP50, the expression levels of these down-regulated genes were analysed in detail. Consistent with the result of the first screening, the mRNA levels of some genes, such as *Os10g0552600* and *Os03g0103100* (putative lipid transfer proteins), were strikingly reduced following two hours of DTT treatment (Fig. 3a). As shown in Fig. 3a, there was little difference in the level of reduction between the wild-type and *OsbZIP50* KD plants, indicating that the down-regulation of these genes is independent of OsbZIP50 function. Next, to investigate whether the down-regulation of these genes is also independent of OsIRE1 RNase activity, we analysed the mRNA levels of these genes in the K833A lines. As shown in Fig. 3b, the reduction of the mRNA levels of these genes was significantly less pronounced in the K833A lines. These results genetically demonstrate that the RNase activity of OsIRE1 is partly implicated in the reduction of mRNA levels of *Os10g0552600* and *Os03g0103100*, independent of OsbZIP50.

Figure 3 | OsIRE1 RNase activity is involved in the reduction of mRNA levels of some genes independent of OsbZIP50. (a) mRNA levels, determined by quantitative RT-PCR, of *Os10g0552600* and *Os03g0103200* in *OsbZIP50* KD (-bZIP) and wild-type plants (Kita, Kitaake) after treatment for 2 h with or without DTT. mRNA levels of *OsBiP4* and *OsbZIP50* reflect the activity of OsbZIP50. Data are expressed as the means ± s.d. of four or six biological replicates. (b) mRNA levels, determined by quantitative RT-PCR, of *Os10g0552600* and *Os03g0103100* in K833A (K833A) and wild-type plants (Nb, Nipponbare) after treatment for 2 h with or without DTT. Data are expressed as the means ± s.d. of four or five biological replicates. Asterisks indicate significant differences (**, $P < 0.01$; *t*-test).

Furthermore, these results strongly suggest the existence of substrates of OsIRE1 RNase other than *OsbZIP50* mRNA, which is currently the only known OsIRE1 substrate.

Discussion

We generated GT rice plants in which the *OsIRE1* gene was replaced with missense alleles, and we genetically demonstrated that OsIRE1 plays multiple roles and serves as a branch point in signalling. As reported previously[8], one of the multiple roles of this gene is the cleavage of *OsbZIP50* mRNA through RNase activity. This leads to transcription of various genes, including those related to ER quality control. In this study, we demonstrated that RNase plays an additional role in the reduction of the mRNA levels of some genes in an OsbZIP50-independent manner. Previously, OsIRE1 kinase activity was thought to activate RNase activity, and other roles for OsIRE1 kinase had not been identified[14]. However, we demonstrated that kinase activity plays a vital role that is independent of RNase activity.

In animals, it was proposed that the RNase activity of IRE1 directly degrades various mRNAs[16]. Additionally, many of the degraded mRNAs encode membrane or secretory proteins that pass through the ER lumen. *Os10g0552600* and *Os03g0103100*, which were analysed in this study, are also predicted to encode membrane proteins. Although it remains undetermined whether the mRNAs of these rice genes are directly catalysed by OsIRE1 RNase, there is no doubt that OsIRE1 RNase has various substrates other than *OsbZIP50* mRNA.

IRE1 is encoded by a single gene in rice, whereas Arabidopsis has two IRE1 paralogues[3]. Additionally, a double disruption mutant of the Arabidopsis *IRE1* paralogues did not show lethality[7]. These differences between rice and Arabidopsis suggest that IRE1 plays species-specific roles in plants. In mammals, adaptor proteins that bind to the cytosolic domain of IRE1 have been isolated and suggest the existence of cellular signalling pathways mediated by the adaptors[2]. It is possible that OsIRE1 also interacts with such signalling components in a kinase activity-dependent manner. Such a pathway may be revealed in the future by isolating factors that genetically or physically interact with plant IRE1.

Gene disruption in higher plants has generally depended on incidental mutations or random insertions of transgenes such as T-DNA and transposons. Additionally, *in planta* analysis of modified genes in the absence of wild-type genes requires corresponding disruption mutants. Therefore, studies of gene function in higher plants have been significantly influenced by the availability of disruption mutants. Unfortunately, an *OsIRE1* disruption mutant was not available for the current study. However, the HR-based GT approach successfully overcame this problem and readily demonstrated the existence of additional roles of OsIRE1, as described above. Furthermore, the GT technique is advantageous because mutagenesis by gene replacement based on HR enables functional analysis of the mutated gene in plants with the same genetic background as the wild type. Indeed, the mRNA level of the *OsIRE1* gene was similar in wild-type and GT plants (Supplementary Fig. S3).

In this study, HR-based GT rice plants were obtained by employing exogenous selection markers and highly efficient *Agrobacterium*-mediated transformation. Some GT rice plants obtained using fundamentally the same selection system have already been reported. In these studies, the genomic *Waxy*, *Adh2* and *OsDRM2* genes were disrupted by the insertion of a positive selection marker gene[11,17,18]. Additionally, several rice plants modified by HR-based GT systems using endogenous selection markers have also been reported[19,20]. In systems using endogenous markers, the target genes and the types of mutations that were induced were restricted because specific phenotypes of modified target genes were used for selection. In contrast to previous studies, we introduced desired missense mutations into genomic *OsIRE1* to investigate several functions of the OsIRE1 protein. Additionally, our study is the first to provide new insights into higher plant signalling using such an approach. This investigation will encourage the use of similar techniques in other studies of higher plants.

Methods

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

Plant material and growth conditions. *Oryza sativa* L. (cv. Nipponbare or Kita-ake) were used in this study. Plants were grown on MS medium (1x 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. For ER stress treatment, seedlings (8-days-old) were incubated in liquid MS medium containing 2 mM DTT. The DTT was substituted with equal volumes of water in the negative controls.

Generation of transgenic rice lines. To generate GT plants, an approximately 7.0 kb DNA fragment containing a promoter (2.0 kb), a cistron (4.5 kb) and a terminator (0.5 kb) of *OsIRE1* was cloned by PCR, and mutations that led to a defect in kinase activity (K519A, Fig. 1a and b) or RNase activity (K833A, Fig. 1a and b) were introduced into the fragments. Additionally, 4.5 kb of the region downstream of the *OsIRE1* terminator was cloned. As shown in Figure 1c, these fragments were inserted into the binary vector for *Agrobacterium*-mediated GT[12,21]. *Agrobacterium*-mediated transformation and selection of candidate cells (gene-targeted calli) were performed as described by Ozawa et al. 2012[12]. In this GT system, Nipponbare calli were used. To select gene-targeted calli, genomic DNA was extracted from calli growing on selection medium and subjected to PCR using specific primer sets (Fig. 2a, F1 and R1 or F2 and R2). Gene-targeted calli (approximately 0.7% [K519A] and 2.2% [K833A] of transformants) were regenerated on MS regeneration medium. Regenerated plantlets were grown in a closed glasshouse at constant temperature (28°C). Genotyping of the *OsIRE1* loci was performed by genomic PCR followed by restriction digestion, as shown in Figure 2b. The absence of undesirable mutations in the *OsIRE1* loci was confirmed by sequencing analysis.

OsIRE1 KD and *ObZIP50* KD lines (background, Kita-ake) were obtained as described by Hayashi et al. 2012[8].

RNA extraction and gene expression analysis. Total RNA was extracted from root tissues using an RNeasy® Plant Mini Kit (Qiagen). For RT-PCR analysis, first-strand cDNA was synthesised from 1.0 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 analysis was performed using 1/80 of the prepared cDNA, SYBR® Premix Ex Taq™ (Takara) and specific primer sets (Supplementary Table S1). The ubiquitin-encoding gene (*Os06g0681400*) was used as an internal reference.

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Author contributions

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

Additional information

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