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Short title: OsDER1 is linked to the ERAD pathway.

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OsDER1 is an ER-associated protein degradation factor that responds to ER stress

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One sentence summary:

Overexpression or suppression of OsDER1 in rice leads to unfolded protein response and ER stress hypersensitivity, and suppression results in floury, shrunken seeds.

Author contributions

D.Q. and L.Q.Q. designed the research, analyzed the data, and wrote the paper. D.Q. performed experiments. G.C. and L.T. provided technical assistance to D.Q.

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ABSTRACT

Endoplasmic reticulum-associated protein degradation (ERAD) plays an important role in endoplasmic reticulum (ER) quality control. To date, little is known about the retrotranslocation machinery in the plant ERAD pathway. We obtained a DERLIN-like protein (OsDER1) through a SWATH-based quantitative proteomic analysis of ER membrane proteins extracted from ER-stressed rice (*Oryza sativa*) seeds. OsDER1, a homolog of yeast and mammal DER1, is localized in the ER and accumulates significantly under ER stress. Overexpression or suppression of OsDER1 in rice leads to activation of the unfolded protein response and hypersensitivity to ER stress, and suppression results in floury, shrunken seeds. In addition, the expression levels of polyubiquitinated proteins increased markedly in *OsDER1* overexpression or suppression transgenic rice. Coimmunoprecipitation experiments demonstrated that OsDER1 interacted with OsHRD1, OsHRD3, and OsCDC48, the essential components of the canonical ERAD pathway. Furthermore, OsDER1 associated with the signal peptide peptidase (SPP), a homologue of a component of the alternative ERAD pathway recently identified in yeast and mammals. Our data suggest that OsDER1 is linked to the ERAD pathway.

INTRODUCTION

Secretory and membrane proteins are synthesized on the endoplasmic reticulum (ER) membrane and translocated into the ER lumen for folding and modification, which is under the control of the ER-mediated protein quality control (ERQC) system in eukaryotes. In the ER, accumulation of unfolded, unassembled, or damaged proteins causes ER stress, which activates the unfolded protein response (UPR) signaling pathway to increase the expression of ER chaperones to facilitate protein folding. Another process, called endoplasmic reticulum-associated protein degradation (ERAD), also responds to stimulate the degradation of unfolded proteins; ERAD is an integral part of the ERQC and can also regulate the turnover of ER-resident proteins (Liu et al., 2011, 2015; van den Boomen et al., 2014).

To date, most ERAD studies have focused on yeast and mammalian systems.

Currently, the commonly accepted view of ERAD is that CDC48 (an AAA-ATPase motor) mediates protein retrotranslocation across the ER membrane, and is ultimately degraded by the ubiquitin-proteasome system in the cytosol (Ye et al., 2001, 2003; Avci and Lemberg, 2015). In this process, there are two distinct degradation routes according to the substrate in yeast. Membrane proteins with lesions in their cytosolic domains are recognized by the E3 ubiquitin ligase DOA10 complex (Habeck et al., 2015), whereas proteins with folding problems in their transmembrane or luminal domains are targeted to the E3 ubiquitin ligase HRD1 complex (Sato et al., 2009; Ruggiano et al., 2014). The yeast HRD1 was identified as a retro-translocation channel for the movement of misfolded polypeptides through the ER membrane (Baldridge and Rapoport, 2016; Schoebel et al., 2017). The HRD1 E3 ligase complex includes multiple receptors for ERAD substrates, such as HRD3, YOS9, and USA1, and another subunit, DER1, is recruited to HRD1 through USA1 (Knop et al., 1996; Kim et al., 2005; Denic et al., 2006; Gauss et al., 2006a, b; Horn et al., 2009). Notably, the DER1 subunit is linked to substrate recognition, ubiquitination, and extraction of ERAD substrates (Mehnert et al., 2014). A high activation barrier must be overcome to extract membrane integral proteins from the ER, and CDC48 can provide the pulling force needed for extraction (Cymer et al., 2015). However, an alternative CDC48-independent retrotranslocation pathway has been reported (Carlson et al., 2006; Hampton and Sommer, 2012; Avci et al., 2014). In this pathway, aberrant proteins are recognized by a complex composed of signal peptide peptidase (SPP); an E3 ubiquitin ligase, TRC8; and DER1 and released from the ER by intramembrane proteolysis (Stagg et al., 2009; Chen et al., 2014). SPPs cleave the aberrant proteins in the plane of the membrane (Lemberg et al., 2001) and release peptide fragments from the lipid bilayer by catalyzing the cleavage of tail-anchored proteins (Boname et al., 2014; Hsu et al., 2015), while DER1 acts as a substrate receptor, recognizing the misfolded proteins (Chen et al., 2014).

Multiple studies have demonstrated that plants have ERAD machinery to degrade aberrant proteins. A number of ERAD-related genes similar to those in yeast and mammals have been identified in plants. *Arabidopsis thaliana* HRD3 is involved in

the degradation of mutated brassinosteroid receptor BRI (Su et al., 2011). OsHRD3 plays an important role in ER quality control by interacting with OsOS-9 and OsHRD1 in rice (*Oryza sativa*) (Ohta and Takaiwa, 2015). Plant CDC48 is involved in the retrotranslocation of the mutated mildew resistance O (MLO) protein and ricin (Müller et al., 2005). Ubiquitin conjugase UBC32, an ERAD component, is involved in brassinosteroid-mediated salt stress tolerance in *Arabidopsis* (Cui et al., 2012). However, compared to the knowledge gained in yeast and mammalian studies, our understanding of the retrotranslocation machinery of the plant ERAD pathway is still limited, especially about its core component, DERLIN. Kirst et al. (2005) reported the discovery of four *DERLIN* genes in maize (*Zea mays*), and these *ZmDERLINs* functionally complemented a yeast *DER1* deletion mutant. Kamauchi et al. (2005) found that *AtDER1* was upregulated under ER stress in a fluid microarray analysis of tunicamycin-treated plantlets. These studies suggested a potential role for a plant DER1 homolog in an ERAD pathway; however, no biochemical and genetic evidence exists to support this hypothesis. In fact, the precise functions of DERLINs in yeast and mammals remain unclear. It has been suggested they are a factor that links the recognition, ubiquitination, and extraction of ERAD substrates. Due to their functional role in retrotranslocation, DERLINs have also been suggested to form a protein-conducting channel (Lilley and Ploegh, 2004), but recent studies have demonstrated that DER1 is an inactive rhomboid intramembrane protease (Greenblatt et al., 2011) and acts in the SPP-TRC8-DER1 complex as a substrate receptor by recognizing misfolded substrates (Avci et al., 2014; Chen et al., 2014). The current lack of a crystal structure suitable for modeling the structure of DERLIN has hindered understanding the structure-function relationships underlying DERLIN-mediated biological processes.

Adverse environmental conditions during seed development induce ER stress. Since ERAD serves to remove unfolded proteins, it is crucial for maintaining functional and healthy proteostasis in the ER. Previously, we developed seed-specific ER stress transgenic plants by endosperm-specific knockdown of *OsSAR1* (secretion-associated, Ras-related protein 1) (Tian et al., 2013). SAR1, a small

GTPase, acts as a molecular switch to regulate the assembly of coat protein complex II, which exports secretory protein from the ER to the Golgi apparatus. Suppression of *OsSAR1* induced ER stress by blocking the exit of secretory proteins from the ER (Tian et al., 2013). The differentially expressed proteins in the *OsSAR1* RNAi transgenic rice seed were analyzed via proteomic strategies (Qian et al., 2015). However, due to their low abundance in low salt-soluble protein extraction, ER-localized membrane proteins were almost undetectable. In this study, we isolated the ER from developing seeds of *OsSAR1* RNAi transgenic rice and performed proteomic analysis of the ER to identify differentially expressed membrane proteins. Focusing on the ERAD pathway, we found that 14 proteins that may be involved in protein processing in the ER pathway were differentially expressed. Among these differentially expressed proteins, OsDER1, a homolog of yeast DER1, was dramatically upregulated in the ER of stressed seeds. Consequently, we analyzed the function of OsDER1 and illustrated its role in ERAD. The present study sheds light on the ERAD machinery in plants.

RESULTS

Mass spectrometric analysis of the ER enrichment fraction in rice seeds

A previous study found that knockdown of *OsSAR1* led to ER stress in rice seeds, and the resulting seeds had reduced levels of the glutelin acidic and basic subunits and increased levels of glutelin precursors (Qian et al., 2015). The ER-stressed rice seeds were generated as reported previously (Tian et al., 2013; Qian et al., 2015). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the seeds showed that the glutelin precursors had accumulated (Fig. 1A), indicating that we had obtained ER-stressed rice seeds as reported (Tian et al., 2013). The ER was extracted from the developing seeds by grinding, differential centrifugation, and sucrose density gradient centrifugation (Fig. 1B). To estimate the enrichment of the ER membrane fraction, some ER-localized proteins were detected by antibodies, such as CNX (calnexin), BIP, and PDI. Compared to the total protein (marked as “seeds”), the enriched ER also contained CNX, BIP, and PDI (marked as “ER”) (Fig. 1C).

153 Glutelins are initially synthesized on the ER as 57 kDa precursors and then cleaved to
154 37-39 kDa acidic and 19-20 kDa basic subunits and stored in the protein storage
155 vacuole (PSV) (Yamagata et al., 1982; Yamagata and Tanaka, 1986). Glutelin acidic
156 subunits or basic subunits can serve to evaluate the purity of the ER in ER-enriched
157 fractions and to demonstrate the separation of ER from PSVs, which tend to sediment
158 along with the ER. No glutelin acidic subunits were detected in our samples (Fig. 1C)
159 suggesting that we obtained the ER enrichment fraction without vacuole
160 contamination.

161 To identify the ER membrane proteins differentially expressed in the stressed
162 condition, we performed a quantitative proteomics analysis of the ER fractions using
163 a SWATH strategy, which combines the advantages of both shotgun and targeted
164 proteomics (Tang et al., 2015). Each biological sample was composed of four
165 individual extractions of ER enrichment fractions. After searching the NCBI *O. sativa*
166 database using ProteinPilot 5.0 at a 1% critical false discovery rate (FDR), we
167 identified 1270 proteins (Supplemental dataset S1). Of these, 1129 proteins had
168 quantitative information, of which only the peptides with confidence >99% and
169 without modification could be used for quantitative analysis.

170 Among the 1129 proteins, 403, 549, and 879 proteins were predicted to be
171 membrane proteins after analysis with the Transmembrane Hidden Markov Model
172 (TMHMM) Server version 2.0 algorithm, Blast2GO, and TopPred II (topology
173 prediction of membrane proteins), respectively. Based on the consideration that the
174 proteins present in at least two analyses were reliable, 581 proteins were predicted as
175 membrane proteins (Fig. 2A). These predicated membrane proteins accounted for
176 51.5% of the total proteins identified, indicating that this method was more effective
177 for identifying ER membrane proteins than the conventional proteomics strategies
178 using total seed proteins (Qian et al., 2015).

179

180 **Membrane proteins differentially expressed in *OsSAR1* RNAi rice seeds**

181 A total of 263 differentially expressed proteins were identified by a greater than
182 1.5-fold difference in quantity ($p < 0.05$), with 103 proteins upregulated and 160

proteins downregulated (Supplemental dataset S2). Many of the upregulated proteins were storage proteins, reflecting the fact that these proteins were blocked in the ER due to repression of SAR1 (Tian et al., 2013).

These differentially expressed proteins were subsequently used for bioinformatics and pathway analyses. Based on a hypergeometric distribution, the 263 differentially expressed proteins were predicted to be involved in mainly five pathways ($p < 0.05$), including carbon fixation in photosynthetic organisms (11 proteins), carbon metabolism (15 proteins), protein processing in ER (14 proteins), glyoxylate and dicarboxylate metabolism (six proteins), and biosynthesis of amino acids (13 proteins) (Fig. 2B). Fourteen differentially expressed proteins were involved in protein processing in the ER pathway, two of which were upregulated: BIP1 (Os02g02410) and DER1-like protein (Os05g09550) (OsDER1 hereafter). The others were downregulated, including OsPDIL2-1 (Os05g06430), calreticulin (Os03g61670), HSP70 (Os03g16920, Os11g47760, Os03g16860), ribophorin II precursor (Os01g68324), HSP20 (Os03g16030, Os03g14180, Os03g16020), OsPDIL5-2 (Os04g35290), SAR1 (Os12g37360), and DNAK family protein (Os05g23740). Most proteins are chaperons that facilitate folding, sorting, or degradation, relieving ER stress. To investigate whether these genes responded to ER stress, their expressions in DTT- and Tm-induced ER-stressed seedlings were analyzed by RT-qPCR. The results showed that the expression of OsDER1 increased more than 20-fold in the ER-stressed seedlings, which was comparable to the change in BIP1 expression (Supplemental Fig. S1). Thus, we focused on the study of OsDER1.

OsDER1 is an ER-localized membrane protein and *OsDER1* gene shows significant increase under ER stress

A survey of the rice (*O. sativa*) genome revealed two *DERLIN* homologues, named *OsDER1* and *OsDER2*. *OsDER1* and *OsDER2* encode polypeptides of 242 and 249 amino acids, respectively. The amino acid sequence of OsDER1 shares only 36% identity with the OsDER2 sequence, whereas it is nearly 64% and 81% identical to those of AtDER1 and ZmDER1, respectively. Based on a sequence alignment,

OsDER1 and OsDER2 showed high similarity to the rhomboid intramembrane protease GlpG. Like mammalian DERLINs (Greenblatt et al., 2011), OsDER1 is a rhomboid pseudoprotease without the active site Ser-His dyad but with the conserved WR motif in loop L1, which connects transmembrane domain 1 (TM1) and TM2, and the conserved GxxxG transmembrane dimerization motif in TM6 (Fig. 3, A and B). The sequence similarity between the TM3 regions of OsDER1 and *E. coli* GlpG is extremely low, but this region is highly conserved among other species (Fig. 3A). The hydropathy of TM3 of OsDER1 was further subjected to Kyte-Doolittle hydropathy analysis, and the results suggested that this region is a possible transmembrane region (Supplemental Fig. S2). In addition, it has been demonstrated that the human DER1 (HsDER1) spans the ER membrane six times (Greenblatt et al., 2011); the TM3 of OsDER1 is highly similar to that of HsDER1, and thus, OsDER1 was also proposed to have a six-pass transmembrane topology (Fig. 3B). There is also a possibility that it has four or five TMs instead of six due to their length; a 3D crystal structure is needed to clarify this issue.

DERLIN genes in plants are grouped into two subfamilies (Supplemental Fig. S3). To test which gene in rice has the same function as the yeast and mammalian DERLINs, we analyzed the expression of the two genes under ER stress. The expression level of *OsDER1* markedly increased under ER stress, while no significant change was observed in *OsDER2*, indicating that OsDER1 is part of the UPR in rice (Fig. 3, C and D). An RT-qPCR analysis revealed that *OsDER1* was expressed in all tissues and has the highest expression level in leaves (Fig. 4A). In addition, the specific expression pattern of the promoter of *OsDER1* was analyzed using GUS as a reporter gene. GUS expression was detected in the root, stem, leaf, flower, sheath, pulvinus, and seed, and the leaf showed the highest expression (Fig. 4B), which was consistent with the RT-qPCR result. The subcellular localization of OsDER1 was examined by confocal microscopy using a transient expression system in rice protoplasts. The result indicated that OsDER1-GFP showed typical ER localization. The ER localization of OsDER1-GFP was further confirmed by coexpression with the ER marker mCherry-HDEL; the green fluorescence of OsOsDER1-GFP merged well

with the ER marker (Fig. 4C). These results confirmed that OsDER1 is localized to the ER.

Endosperm-specific knockdown of *OsDER1* induced UPR and resulted in an abnormal phenotype

To further elucidate the function of OsDER1, transgenic rice was produced in which *OsDER1* was knocked down (KD) by RNAi under the control of the endosperm-specific promoter *GluA-2*. The *OsDER1* mRNA levels in *OsDER1* KD were lower than those in the wild type (Fig. 5A). The seeds of the *OsDER1* KD lines were longer and thinner than those of the wild-type control (Fig. 5B; Supplemental Fig. S4). Furthermore, opaque, floury, and shrunken seeds were found in *OsDER1* KD. The starch granules were observed by scanning electron microscopy (SEM). In the wild type, the starch granules were densely packed and similar in size and had irregular polyhedral shapes with sharp edges. In the *OsDER1* KD lines, the starch granules were loosely packed, round and heterogeneous in size (Fig. 5C). To verify whether *OsDER1* KD induced UPR, the levels of ER-resident proteins in mature seeds were investigated by western-blotting analysis. The results showed that the expression levels of BIP1, PDI1-1, PDI2-3 and CNX increased obviously in *OsDER1* KD compared to those in WT (Fig. 5D). It is noteworthy that OsBIP4, which is only expressed under ER stress conditions (Wakasa et al., 2012), was detected in *OsDER1* KD. These results showed that knockdown of *OsDER1* induced UPR and resulted in an abnormal phenotype, indicating that OsDER1 plays an essential role in maintaining protein homeostasis in the ER.

Overexpression or suppression of *OsDER1* affects steady-state polyubiquitylation and leads to hypersensitivity to ER stress

Transgenic rice was generated with overexpression (*OsDER1* OE) and suppression (*OsDER1* Ri) of *OsDER1* under the control of the maize *ubiquitin* promoter. The expression of *OsDER1* was measured by RT-qPCR. The mRNA levels of *OsDER1* were lower in *OsDER1* Ri leaves and higher in *OsDER1* OE leaves than in the leaves

of the wild type (Supplemental Fig. S5, A and B), indicating that the *OsDER1* was successfully overexpressed or suppressed, respectively. To investigate whether OsDER1 is involved in the ubiquitination of unfolded proteins in rice, the levels of polyubiquitinated proteins in the transgenic plants were analyzed. Total proteins were extracted from *OsDER1* OE and *OsDER1* Ri seeds and subjected to immunoblot analysis using anti-α-ubiquitin as an antibody. The results showed that the levels of ubiquitinated proteins in the *OsDER1* OE and *OsDER1* Ri lines were both higher than those in the wild type (Fig. 6A). These results suggested that OsDER1 affects steady-state polyubiquitination, hinting that OsDER1 is related to the ubiquitination of unfolded proteins.

To further investigate the effect of overexpressed or suppressed *OsDER1* on the UPR signaling pathway, the seedling of *OsDER1* OE, *OsDER1* Ri, and WT were treated with 2 mM DTT for 4 h, and the expression levels of BIP1, CNX and PDI2-3 were detected. Their expression was higher in *OsDER1* OE and *OsDER1* Ri seedling than in WT seedling (Fig. 6B). These results demonstrated that the alteration of the expression of *OsDER1* enhanced the UPR signaling in response to ER stress. In addition, we also observed the growth of *OsDER1* OE and *OsDER1* Ri rice under ER stress. Under normal conditions, the lengths of the shoot and root were not significantly different among the seedlings of WT, *OsDER1* OE, and *OsDER1* Ri (Fig. 6, C and D). After treatment with 1 mM DTT for 14 d, the shoot lengths of *OsDER1* OE and *OsDER1* Ri were significantly (OE: $p = 0.0065$, n = 30; Ri: $p = 0.02$, n = 30) shorter than those of the wild type. The root length of *OsDER1* Ri was also shorter than that of the wild type (Fig. 6, E and F). These results indicated that alteration of the expression of *OsDER1* led to hypersensitivity to ER stress and affected the growth of rice. Overall, the expression level of *OsDER1* should be precisely controlled in rice.

OsDER1 is linked to the ERAD pathway

To search for proteins interacting with OsDER1 in rice, a series of coimmunoprecipitation assays was performed to uncover OsDER1 interacting

proteins. Transgenic calli were generated with OsDER1-3×FLAG tag under the control of the *Ubiquitin* promoter. After detergent extraction, the supernatants from the transgenic calli were incubated with anti-FLAG resin; the WT calli acted as a negative control. After SDS-PAGE and staining, an intense band and a faint band were detected, with no band observed at the equivalent positions in the control lane. The different bands were identified by mass spectrometry. The results showed that the tryptic peptides of the intense band aligned well with the primary sequences deduced from OsCDC48-1 (Os03g05730), OsCDC48-2 (Os10g30580) and OsHRD3 (Os03g15350), while that from the faint band aligned with OsSPP1 (Os02g02530) and OsSPP2 (Os05g36070) (Fig. 7A).

To confirm the interaction between OsDER1 and these proteins, we performed a coimmunoprecipitation assay in *Spodoptera frugiperda* (Sf9) insect cells. His-tagged OsDER1 was separately coexpressed with FLAG-epitope-tagged OsHRD1, OsHRD3, OsCDC48-1, and OsCDC48-2. The results showed that OsDER1 was immunoprecipitated with each of these proteins (Fig. 7, B and C). OsHRD1, OsHRD3, OsCDC48-1, and OsCDC48-2 are essential components of the HRD1 complex; their interaction with OsDER1 demonstrated that the HRD1-mediated ERAD pathway is conserved in rice and involves OsDER1.

SPP1 and SPP2 are components of the Der1-SPP-TRC8 complex involved in the alternative ERAD pathway reported in yeast and mammalian systems (Avci et al., 2014; Chen et al., 2014). Our data suggested that OsDER1 was coimmunoprecipitated with OsSPP1 and OsSPP2 (Fig. 7D), indicating that the alternative DER1-SPP-TRC8-mediated proteolysis pathway might also be conserved in rice.

DISCUSSION

The ERQC system recognizes and eliminates misfolded, unassembled, or damaged proteins. To date, most insights into ERQC have been obtained through analysis of plants and plantlets in which ER stress was induced by treating them with chemical agent tunicamycin or dithiothreitol (DTT), which interfere with N-glycosylation or disulfide bond formation, respectively (Martínez and Chrispeels, 2003; Kamauchi et

al., 2005). However, the use of these chemical agents to induce ER stress is not possible in seeds. Previously, we reported that endosperm-specific knockdown of OsSAR1 blocked the seed storage proteins (SSP) glutelin and α-globulin in the ER and accumulated in the ER lumen, eliciting UPR (Tian et al., 2013; Qian et al., 2015). The UPR mitigates ER stress by upregulating the expression of genes encoding components of the protein-folding machinery or the ER-associated degradation system. BIP1, BIP4, PDIL1-1, PDIL2-3, PDIL5-1, calreticulin, and ERO1 were upregulated in *OsSAR1* RNAi seeds based on iTRAQ analysis (Qian et al., 2015). However, many factors involved in protein processing and modification identified previously were not detected, or the expression levels of these proteins were downregulated in this study (Supplemental dataset S2). These results might be due to the fact that chaperones mainly reside in the ER lumen but are not ER membrane proteins, which might leak out during the extraction of ER membranes. It is also possible that this phenomenon is caused by an attenuation in bulk protein synthesis which happens in mammalian cells to alleviate ER stress (Rutkowski and Kaufman, 2004), but has not been reported in plant cells.

In this study, we isolated ER from ER-stressed developing rice seeds and performed a SWATH-based proteomic analysis aiming to identify global differentially expressed ER membrane proteins. Through this strategy, we identified 581 putative ER membrane proteins (Fig. 2). Many more membrane proteins were identified in this study compared to those obtained in a previously reported proteomics study of total soluble proteins extracted from the same *OsSAR1* RNAi seed samples (Qian et al., 2015), indicating that it is effective to identify organelle-resident proteins by proteomic analysis of the organelle. Among the differentially expressed proteins, an obviously upregulated protein, OsDER1, was observed. In contrast to the diverse biological functions of DER1 in yeast or mammalian systems, relatively little is known about DER1 in plants. In this study, we found that endosperm-specific knockdown of OsDER1 induced UPR, which is similar to a result in OsHRD3 knockdown rice seeds (Ohta and Takaiwa, 2015), and resulted in opaque and floury rice seeds (Fig. 5, A and B). In addition, the overexpression or

repression of *OsDER1* led to higher expression of ubiquitinated proteins. Proteins that fail to fold correctly are labeled with ubiquitin and escorted by a series of chaperones to the proteasome. Ubiquitination in the Ri lines was stronger than in the OE lines (Fig. 6A). Ubiquitination in the OE2 line was stronger than that in the OE1 line (Fig. 6A), corresponding to the higher expression level of OsDER1 mRNA in the OE2 line (Supplemental Fig. S5B). These results suggest that OsDER1 plays a key role in the ER stress response in rice, and the stoichiometry of OsDER1 relative to other components is important to the HRD complex. Loss of OsDER1 promotes ubiquitination, while the increase of ubiquitination in the OE line could be caused by some non-specific effect on the UPR signaling pathway. Similar effects have been reported for BIP1 in rice, in which either under- or overexpression of BIP1 activates an ER stress response (Wakasa et al., 2011).

HRD3 is a component of the HRD1 ubiquitin ligase complex in yeast. OsHRD3 is involved in the ubiquitination of RM1 in rice endosperm, and the aberrant distribution of RM1 leads to deformation of PB-I in *OsHRD3* KD seeds (Ohta and Takaiwa, 2015). To investigate whether protein body formation was altered in *OsDER1* KD seeds, we performed an immunohistochemical analysis using transmission electron microscopy. Rice endosperm has two types of PBs: the spherical protein body PB-I is formed by prolamin, and the irregularly shaped, electron-dense PB-II contains glutelin and α-globulin (Tian et al., 2013). Therefore, the anti-prolamin antibody and anti-glutelin antibody were used to label PB-I and PB-II, respectively. In *OsDER1* KD endosperm, PB-II was identical to that in the wild type. In addition to the normal PB-I in *OsDER1* KD endosperm, some abnormal PB-Is with venation or cracks were observed (Supplemental Fig. S6). However, the storage proteins of *OsDER1* KD were not significantly different from those in the wild type, and the proportion of abnormal PB-I was not large. The regulatory mechanism of OsDER1 on PB-I formation remains to be investigated.

Recent studies revealed that, in addition to the canonical ERAD pathway mediated by the HRD1 complex, an alternative ERAD pathway mediated by DER1-SPP-TRC8 complex exists in yeast and mammals. However, the existence of this alternative

ERAD pathway in plants was unconfirmed. In this study, we found that OsDER1 interacted with OsSPP1 and OsSPP2 (Fig. 7E). These results indicated that the alternative ERAD pathway may be conserved in plants. It is noteworthy that no ortholog of TRC8 was found in rice. Whether an E3 ubiquitin ligase in rice has functional roles akin to those of TRC8, and whether the entire alternative pathway is present in plants, remains to be demonstrated.

MATERIALS AND METHODS

Generation of transgenic plants

ER-stressed transgenic rice was generated by repressing *OsSAR1a/b/c* using RNA interference (RNAi), as previously described. Developing seeds at 10 to 15 days after fertilization (DAF) were used for the SWATH analysis. Endosperm-specific knockdown transformants for *OsDER1* (*OsDER1* KD) were generated by RNAi. The inserted gene fragment was 309 bp (base pairs 229-538). This fragment was inserted as positive and negative into a modified GluA2-pTCK303 vector with the endosperm-specific *GluA2* promoter replacing the ubiquitin promoter. Transgenic rice plants (*Oryza sativa* cv. Zhonghua 11) were generated by *Agrobacterium*-mediated transformation (Qu et al., 2005).

To produce the gene overexpression transformants (*OsDER1* OE), the coding region of *OsDER1* was amplified using rice cDNA as a template and connected with a 3×FLAG sequence by PCR. The DNA fragment was inserted into the pCAMBIA1301 vector with the ubiquitin promoter. To produce the RNA interference transformants (*OsDER1* Ri), the same fragment was used for insertion into the pTCK303 vector containing the ubiquitin promoter. Transgenic rice plants (*Oryza sativa* cv. Kitaake) were generated by Agrobacterium-mediated transformation (Qu et al., 2005). The calli of *OsDER1* OE and *OsDER1* Ri were analyzed on selective medium. To construct the *OsDER1* pro-GUS vector, the promoter of *OsDER1* was amplified using rice genomic DNA as a template. The DNA fragment was introduced into the pCAMBIA1301 vector, which contains the gusA gene encoding β-glucuronidase (GUS). All primer sequences are listed in Supplemental Table S1. The constructs are

423 listed in Supplemental Table S2.

424

425 **Endoplasmic reticulum membrane protein isolation**

426 Developing seeds were dehusked and then milled in a mortar for 30 min with
427 homogenization buffer (500 mM sucrose; 10 mM KCl; 1 mM EDTA; 1 mM $MgCl_2$; 2
428 mM dithiothreitol; 150 mM Tricine-KOH, pH 7.5). The crude homogenate was
429 filtered through a 100-μm mesh, and the filtrate was centrifuged at 1,000 ×g and 4°C
430 for 15 min. The supernatant was then centrifuged at 10,000 ×g and 4°C for 15 min.
431 Discontinuous sucrose density gradients were prepared with 30% and 20% sucrose
432 and the supernatant of differential centrifugation with a ratio of 7:4:2 and then
433 centrifuged at 100,000 ×g for 2 h at 4°C. The membrane at the 20-30% sucrose
434 interface was removed with a homemade pipette and mixed with an equal volume of
435 60% sucrose. Then, discontinuous sucrose density gradients were prepared with the
436 diluted membrane fractions using 40%, 30%, and 20% sucrose with the ratio 3.5:4:3:2
437 and centrifuged at 250,000 ×g for 22 h at 4°C. The purified ER was carefully removed
438 from the 20-30% sucrose interface and diluted with ice-cold water. Then, the
439 membranes were pelleted by centrifugation, followed by snap freezing in liquid
440 nitrogen and storage at -80°C. All operations were performed on ice, and all sucrose
441 solutions contained a protease inhibitor cocktail (Roche).

442

443 **Membrane proteomics analysis**

444 The ER membrane proteins were extracted with buffer (0.125 M Tris-HCl, 4 M
445 urea, 4% SDS and 2% β-mercaptoethanol, pH 6.8) from WT and the OsSAR1 RNAi
446 and quantified using a 2-D Quant Kit (GE Healthcare); trypsin digestion was
447 performed as previously described (Qian et al., 2015). Because the yield of extraction
448 of the endoplasmic reticulum was very low, the digestion peptides from four
449 independent experiments were pooled together as one sample, and then subjected to
450 SWATH analysis performed on a TripleTOF 5600 system (AB SCIEX) (Gillet et al.,
451 2012). A total of three technical replicates were designed and conducted in the study.
452 To analyze the membrane proteins identified, the TMHMM Server version 2.0

(http://www.cbs.dtu.dk/services/TMHMM-2.0/) and TopPred II (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::toppred) were used to predict the number of transmembrane helices, and the Blast2GO software was used to categorize their subcellular categories. A protein pathway analysis was performed using KOBAS 2.0.

DTT treatment, RNA isolation and reverse transcription quantitative PCR (RT-qPCR)

The seedlings of Kitaake were incubated in liquid MS medium that contained 2 mM DTT and 5 μg/ml of tunicamycin (Tm) for 4 h. Equal volumes of water and DMSO were added as negative controls. The treated materials with developing seeds at 10 DAF acted as samples and were used to extract total RNA using TRIpure Reagent (Bioteke Corp). RT-qPCR was performed on a LightCycler system (Roche Diagnostics) as previously described (Qian et al., 2015).The primers were designed by Beacon Designer 8.0 (Supplemental Table S3).

Seeds of *OsDER1* OE, *OsDER1* Ri, and wild-type rice were germinated in water for 5 d and then transferred to half-strength liquid MS medium with or without 1 mM DTT and cultured for 14 d. The lengths of the shoots and roots of the rice plants were calculated by ImageJ.

SDS-PAGE and immunoblot analysis

Total protein was extracted with buffer (0.125 M Tris-Cl, 4% SDS, 4 M urea and 2% β-mercaptoethanol, pH 6.8) from seeds and leaves. After centrifugation at 12000 ×g for 5 min, the protein samples were subjected to 13.6% SDS-PAGE and then for immunoblot analysis using a PVDF membrane (Millipore, USA) and immunodetection with HRP-conjugated secondary antibodies. Antibodies against α-tubulin and FLAG were generated by Sigma, and antibodies against α-ubiquitin were generated by the Beijing Genomics Institute.

Transient expression in rice protoplasts

Green fluorescent protein (GFP) was fused to the C-terminus of OsDER1. The signal peptide of AtWAK2 and the ER retention signal His-Asp-Glu-Leu were combined to create the ER marker (mCherry-HDEL). Then, the chimeric genes were subcloned into vector pBI221 containing the cauliflower mosaic virus (CaMV) 35S promoter and cotransformed into rice protoplasts as described by Chen et al. (Chen et al., 2006). The transformed cells were observed through a confocal microscope (Olympus FV1000 MPE).

Analysis of GUS expression

Different tissues of OsDER1 pro-GUS transgenic rice were incubated with GUS staining buffer (Real-Times, RTU4032) at 37 °C for 12 h and then destained in 70% ethanol until the chlorophyll was removed.

Transmission electron microscopy

Transverse sections of mature seeds were used for SEM analyses as described (Fujita et al., 2003) and then observed by transmission electron microscopy (Hitachi S-4800 SEM).

Coimmunoprecipitation assays

Transgenic calli containing the OsDER1-3×FLAG tag under the control of the ubiquitin promoter were generated (Wakasa et al., 2011). Successful transgenic calli were screened by anti-FLAG antibodies. In the Co-IP assay, WT served as a negative control; the transgenic calli and WT calli were ground and extracted by lysis buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.0% (w/v) n-dodecyl-β-D-maltopyranoside (DDM, Anatrace), and a protease inhibitor cocktail (Roche)). Briefly, after centrifugation, the supernatant was incubated with anti-FLAG resin for 2 h at 4°C, the immunoprecipitation complexes were washed three times using wash buffer (25 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.02% (w/v) DDM), and the protein complexes were eluted by 2× SDS/PAGE loading buffer and subjected to immunoblot analysis. To assess coexpression with OsHRD1, OsHRD3, OsSPPs, or

OsCDC48s, the recombinant proteins were expressed using the pFastBac baculovirus system (Invitrogen) (Sokolenko et al., 2012). Briefly, recombinant bacmid DNAs were generated in DH10Bac cells and then transfected and amplified in Sf9 insect cells (Invitrogen). At 72 h after viral infection, the cells were harvested and extracted by lysis buffer, and the subsequent procedure was the same as that mentioned above.

Accession Numbers

Sequence data from this article can be found in the Rice Genome Annotation Project Database under accession numbers Os03g15350 (OsHRD3), Os03g05730 (OsCDC48-1), Os10g30580 (OsCDC48-2), Os05g09550 (OsDER1), Os03g63520 (OsDER2), Os02g02530 (OsSPP1), Os05g36070 (OsSPP2).

Supplemental Data

Supplemental Fig. S1: RT-qPCR analysis of the mRNA expression of 9 differentially expressed genes.

Supplemental Fig. S2: Kyte-Doolittle hydropathy analysis of the OsDER1 transmembrane domain 3.

Supplemental Fig. S3: Phylogenetic tree of OsDERs with homologues in other species.

Supplemental Fig. S4: Statistical analysis of the length and width of *OsDER1* knock-down transgenic rice seeds.

Supplemental Fig. S5: Analysis of the mRNA levels of *OsDER1* in overexpressed and suppressed *OsDER1* transgenic rice seeds.

Supplemental Fig. S6: Immunohistochemical analysis of the alteration of protein body formation in *OsDER1* KD seeds using transmission electron microscopy.

Supplemental Table S1: Primers used for vector construction.

Supplemental Table S2: Details for construction.

Supplemental Table S3: Primers used for RT-qPCR.

Supplemental dataset S1: List of the total proteins identified by mass spectra.

Supplemental dataset S2: List of the differentially expressed proteins identified by

mass spectra.

Acknowledgments

We thank Dr. Xiangbai Dong for his valuable suggestions throughout this project.

Figure legends

Figure 1 Extraction of the ER enrichment fraction from rice seeds.

A. Coomassie blue stained gel showing the increase in glutelin precursors in ER-stressed rice seeds. M: marker. WT: wild-type rice seeds. Ri: *OsSAR1* RNAi rice seeds; the numbers represent different transgenic rice lines. B. A brief workflow of the extraction of the ER enrichment fraction from rice seeds. C. CNX, BIP1, PDI2-3, and glutelin subunits were detected by Coomassie blue staining and immunoblotting. OE23 (the 23-kDa subunits of the oxygen-evolving complex) serves as a marker protein of chloroplasts. “seeds” indicates total proteins from rice seeds; “ER” indicates proteins of the ER enrichment fraction.

Figure 2 Membrane association and functional analysis of differentially expressed proteins.

A. Venn diagram of the predicted numbers of membrane proteins using Blast2Go, TMHMM, and TopPred2. B. KEGG pathway analysis of differentially expressed proteins. All of differentially expressed proteins were subjected to the KEGG pathway analysis.

Figure 3 *OsDER1* gene expression markedly increased under ER stress.

A. Sequence alignment of OsDER1 with other homologues. Blue box indicates the highly conserved motif. Red star indicates the active sites in GlpG. The hydropathy of TM3 of OsDER1 was analyzed by Kyte-Doolittle software, and the results suggested that this region is a possible transmembrane region. There is also a possibility that this protein has four TMs instead of six TMs due to the lengths of some TMs. B. Transmembrane topology of OsDER1. Red indicates the highly conserved motif in

GlpG; the active site Ser-His dyad is shown. Blue indicates the highly conserved motif in OsDER1. C and D. RT-qPCR of *OsDER1* and *OsDER2* mRNA level under ER stress, respectively. The expression of the *OsDERs* was normalized to that of *ACTIN*. All data are the average of three independent experiments and error bars represent SD. WT: wild-type rice seeds. Ri: *OsSAR1* RNAi rice seeds. TM: tunicamycin treatment.

Figure 4 Tissue-specific expression of *OsDER1*.

A. RT-qPCR of *OsDER1* mRNA levels in different tissues. The expression of *OsDER1* was normalized to that of *ACTIN*. All data are the average of three independent experiments and error bars represent SD. B. Histochemical GUS staining of different tissues. α: stem; β: leaf; γ: sheath; δ: shell; ε: seed; ζ: pulvinus; η: flower; θ: root. C. Subcellular localization of OsDER1 analyzed by confocal microscopy. OsDER1-GFP colocalized with mCherry-HEDL.

Figure 5 Analysis of the changes in *OsDER1* knock-down transgenic rice.

A. RT-PCR of *OsDER1* mRNA levels in transgenic rice plants with knocked-down expression of *OsDER1* by RNAi, under the control of the endosperm-specific promoter *GluA-2*. B. Phenotypic analysis of *OsDER1* knock-down transgenic rice seeds. C. The starch granules of transgenic rice seeds were observed by scanning electron microscopy D. Changes in the expression of BIP1, BIP4, PDI1-1, PDI2-3, and CNX in transgenic rice seeds detected by SDS-PAGE and immunoblotting.

Figure 6 The effects of overexpression and repression of *OsDER1*.

A. Changes in the levels of ubiquitinated proteins in transgenic rice seeds with increased and repressed expression of *OsDER1*. Immunoblot analysis of the ubiquitinated proteins by anti-α-ubiquitin (α-Ub). The loading control is shown in Supplemental Fig. S5C. B. Changes in the expression of BIP1, PDI2-3, and CNX in seedlings (WT, OE and Ri) treated with 2 mM DTT for 4 h. Equal volumes of water were added as negative controls. C-F. The growth of *OsDER1* overexpressing and *OsDER1* repressed rice (*OsDER1* OE and *OsDER1* Ri) in liquid MS medium with

and without 1 mM DTT refreshed daily was observed over a period of 2 weeks; wild-type rice served as a control. C and E. Representative images of wild-type, *OsDER1* OE, and *OsDER1* Ri rice in the presence and absence of 1 mM DTT after 2 weeks. D and F. Quantitative measurements of the lengths of shoots and roots of wild-type, *OsDER1* OE, and *OsDER1* Ri seedlings in three trials and error bars represent SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (Student's *t*-test).

Figure 7. Interactions of OsDER1 with OsHRD1, OsHRD3, OsCDC48s, and OsSPPs.

A. Co-immunoprecipitation assay of transgenic OsDER1-FLAG and endogenous interacting proteins. B. Overexpression-based co-immunoprecipitation assay of Sf9 cells co-expressing FLAG-epitope-tagged OsHRD1 or OsHRD3 and His-tagged OsDER1. C. Overexpression-based co-immunoprecipitation assay in Sf9 cells co-expressing FLAG-epitope-tagged OsCDC48-1 or OsCDC48-2 and His-tagged OsDER1. D. Overexpression-based co-immunoprecipitation assay of Sf9 cells co-expressing FLAG-epitope-tagged OsSPP1 or OsSPP2 and His-tagged OsDER1. E. Schematic diagram of the interactions found in the present study. Two-way arrows indicate interactions.

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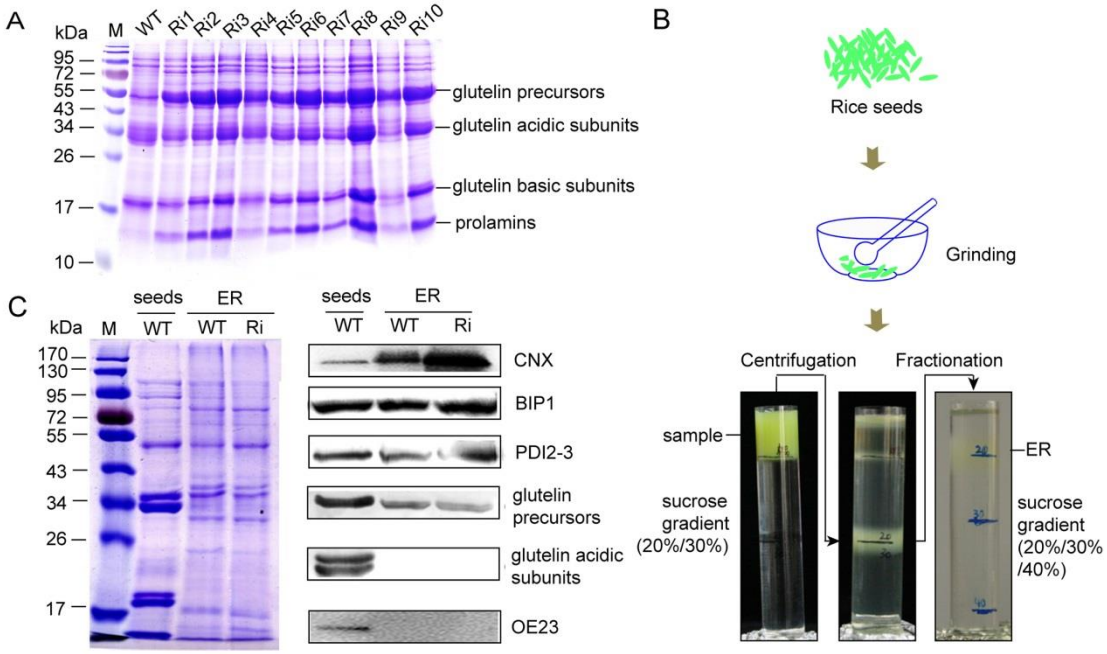
774

Figure 1 Extraction of the ER enrichment fraction from rice seeds.

A. Coomassie blue stained gel showing the increase in glutelin precursors in ER-stressed rice seeds. M: marker. WT: wild-type rice seeds. Ri: *OsSAR1* RNAi rice seeds; the numbers represent different transgenic rice lines. B. A brief workflow of the extraction of the ER enrichment fraction from rice seeds. C. CNX, BIP1, PDI2-3, and glutelin subunits were detected by Coomassie blue staining and immunoblotting. OE23 (the 23-kDa subunits of the oxygen-evolving complex) serves as a marker protein of chloroplasts. "seeds" indicates total proteins from rice seeds; "ER" indicates proteins of the ER enrichment fraction.



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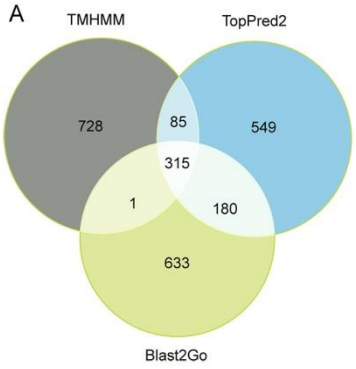
A. Venn diagram of the predicted numbers of membrane proteins using Blast2Go, TMHMM, and TopPred2. B. KEGG pathway analysis of differentially expressed proteins. All of differentially expressed proteins were subjected to the KEGG pathway analysis.

A

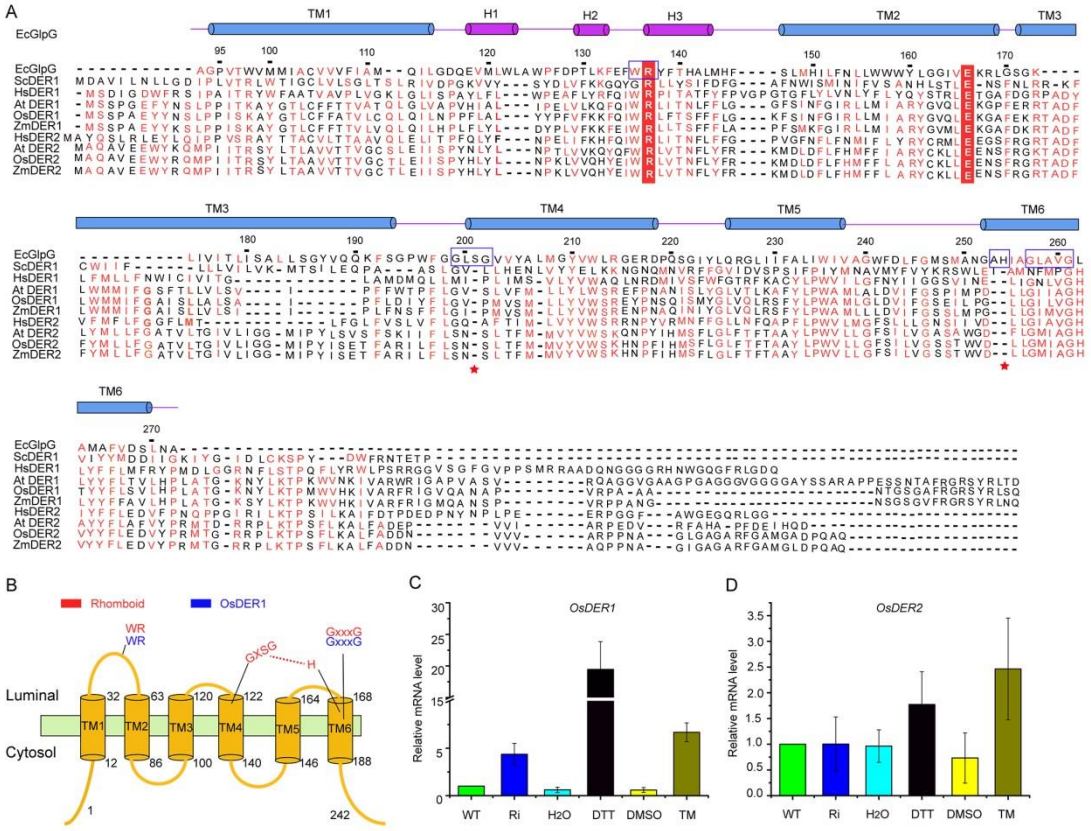


B

KEGG pathway	KEGG ID	Background number	Input number	p-Value	FDR-corrected p-Value
Carbon fixation in photosynthetic organisms	osa00710	76	11	0.000113	0.015447
Carbon metabolism	osa01200	203	15	0.005066	0.115667
Protein processing in endoplasmic reticulum	osa04141	201	14	0.010615	0.207744
Glyoxylate and dicarboxylate metabolism	osa00630	57	6	0.016347	0.279936
Biosynthsis of amino acids	osa01230	206	13	0.026902	0.409515

Figure 3 *OsDER1* gene expression markedly increased under ER stress.

A. Sequence alignment of OsDER1 with other homologues. Blue box indicates the highly conserved motif. Red star indicates the active sites in GlpG. The hydropathy of TM3 of OsDER1 was analyzed by Kyte-Doolittle software, and the results suggested that this region is a possible transmembrane region. There is also a possibility that this protein has four TMs instead of six TMs due to the lengths of some TMs. B. Transmembrane topology of OsDER1. Red indicates the highly conserved motif in GlpG; the active site Ser-His dyad is shown. Blue indicates the highly conserved motif in OsDER1. C and D. RT-qPCR of *OsDER1* and *OsDER2* mRNA level under ER stress, respectively. The expression of the *OsDERs* was normalized to that of *ACTIN*. All data are the average of three independent experiments and error bars represent SD. WT: wild-type rice seeds. Ri: *OsSAR1* RNAi rice seeds. TM: tunicamycin treatment.



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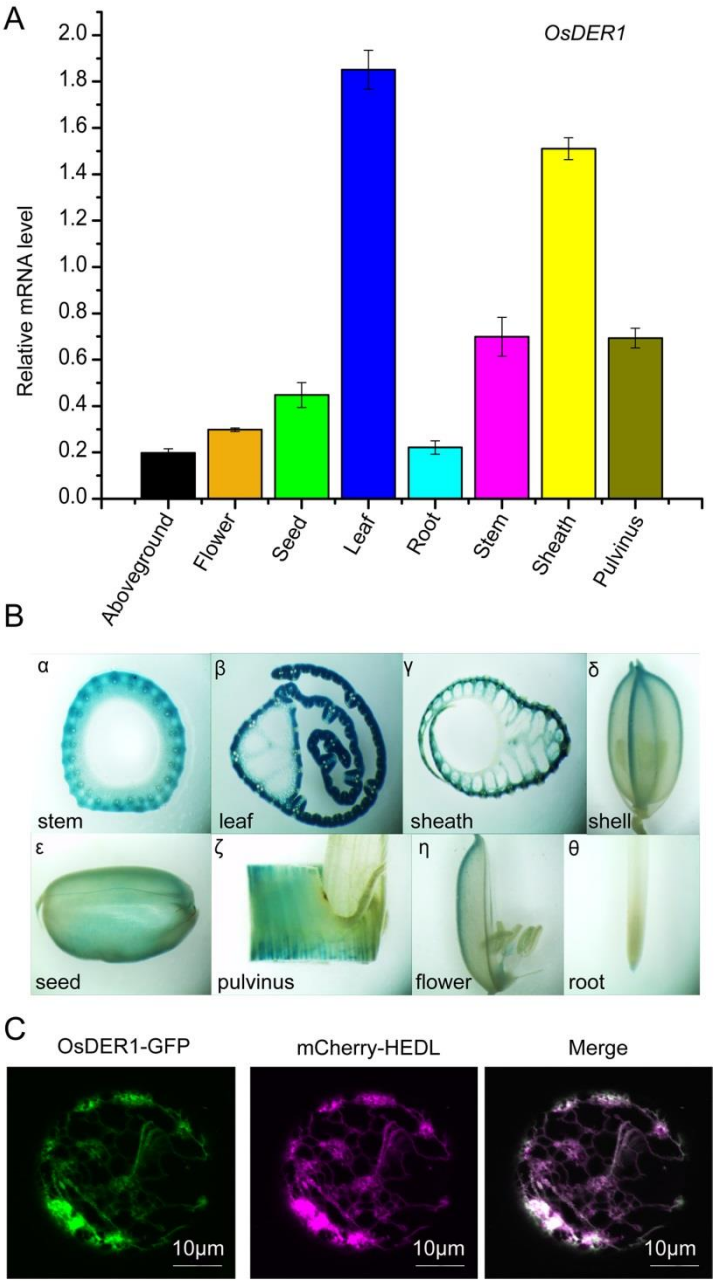

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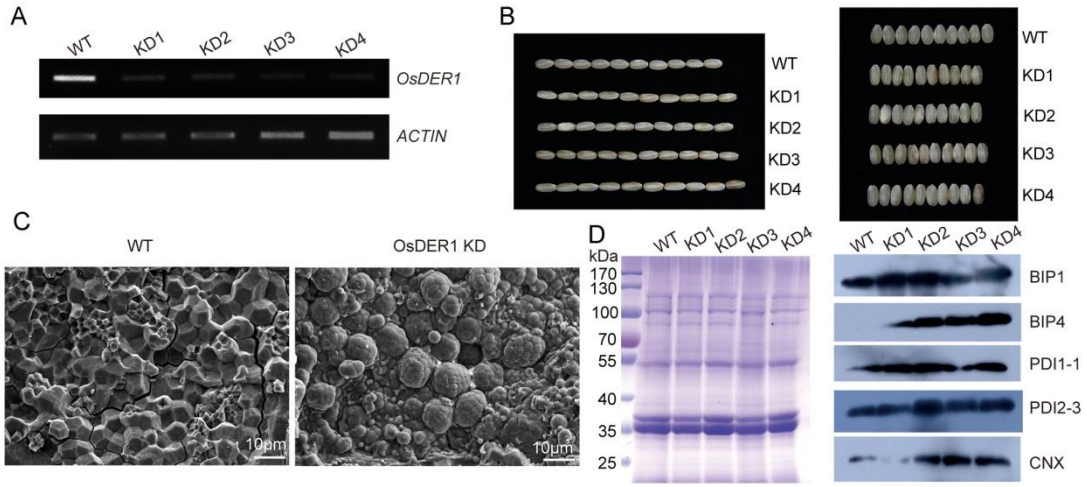

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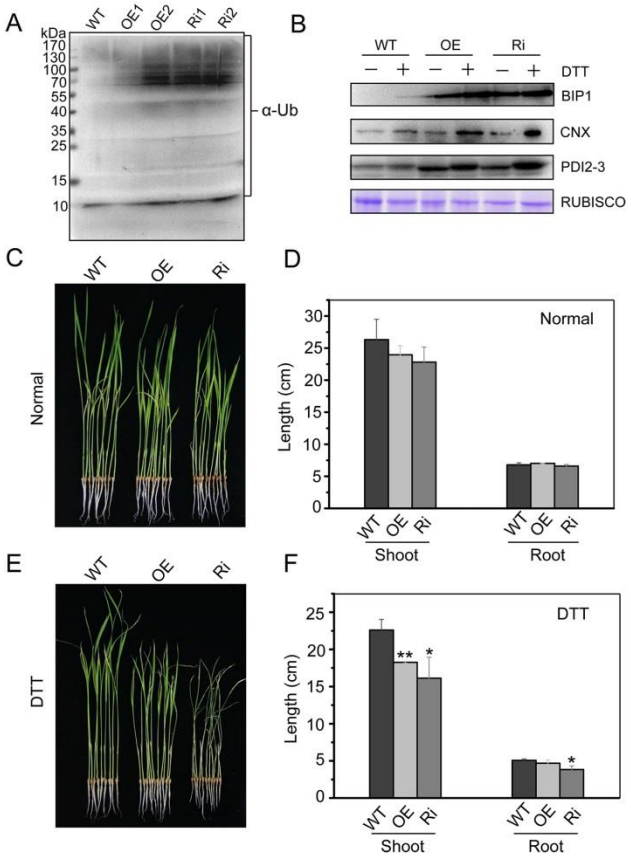
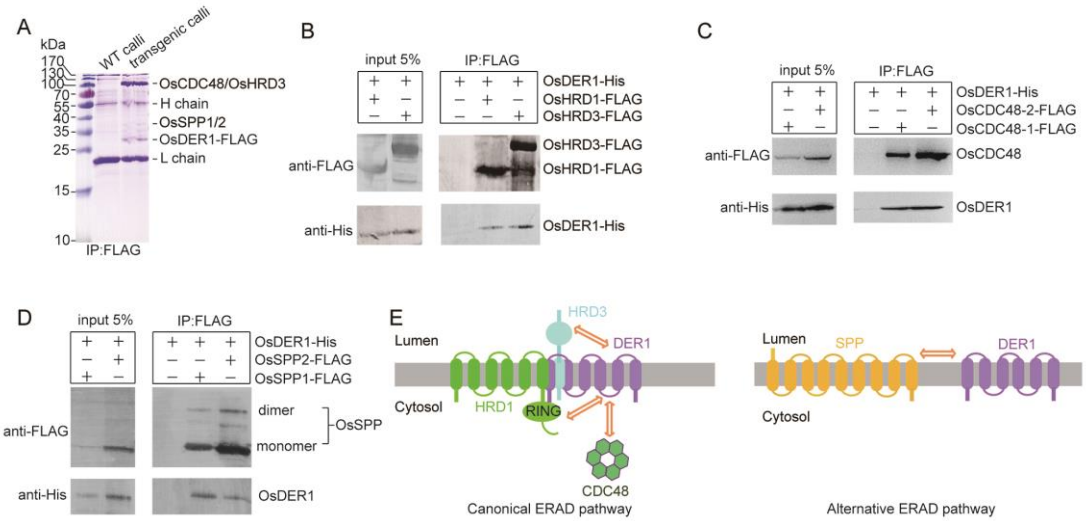


Figure 7. Interactions of OsDER1 with OsHRD1, OsHRD3, OsCDC48s, and OsSPPs.

A. Co-immunoprecipitation assay of transgenic OsDER1-FLAG and endogenous interacting proteins. B. Overexpression-based co-immunoprecipitation assay of Sf9 cells co-expressing FLAG-epitope-tagged OsHRD1 or OsHRD3 and His-tagged OsDER1. C. Overexpression-based co-immunoprecipitation assay in Sf9 cells co-expressing FLAG-epitope-tagged OsCDC48-1 or OsCDC48-2 and His-tagged OsDER1. D. Overexpression-based co-immunoprecipitation assay of Sf9 cells co-expressing FLAG-epitope-tagged OsSPP1 or OsSPP2 and His-tagged OsDER1. E. Schematic diagram of the interactions found in the present study. Two-way arrows indicate interactions.



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