Unfolded protein-independent IRE1 activation contributes to multifaceted developmental processes in Arabidopsis

Kei-ichiro Mishiba, Yuji Iwata, Tomofumi Mochizuki, Atsushi Matsumura, Nanami Nishioka, Rikako Hirata, Nozomu Koizumi

In Arabidopsis, the IRE1A and IRE1B double mutant (ire1a/b) is unable to activate cytoplasmic splicing of bZIP60 mRNA and regulated IRE1-dependent decay under ER stress, whereas the mutant does not exhibit severe developmental defects under normal conditions. In this study, we focused on the Arabidopsis IRE1C gene, whose product lacks a sensor domain. We found that the ire1a/b/c triple mutant is lethal, and heterozygous IRE1C (ire1c/+ ) mutation in the ireta/b mutants resulted in growth defects and reduction of the number of pollen grains. Genetic analysis revealed that IRE1C is required for male gametophyte development in the ireta/b mutant background. Expression of a mutant form of IRE1B that lacks the luminal sensor domain (ΔLD) complemented a developmental defect in the male gametophyte in ireta/b/c haplotype. In vivo, the ΔLD protein was activated by glycerol treatment that increases the composition of saturated lipid and was able to activate regulated IRE1-dependent decay but not bZIP60 splicing. These observations suggest that IRE1 contributes to plant development, especially male gametogenesis, using an alternative activation mechanism that bypasses the unfolded protein-sensing luminal domain.

Introduction

The ER in eukaryotes copes with an accumulation of unfolded proteins by activating the unfolded protein response (UPR), which increases protein folding capacity and attenuates protein synthesis in the ER (Walter & Ron, 2011). Inositol-requiring enzyme 1 (IRE1) is the primary transducer of the UPR. IRE1 consists of an N-terminal sensor domain facing the ER lumen, a single transmembrane helix embedded in the ER membrane, and kinase and RNase domains at its C terminus on the cytosolic side (Nikawa & Yamashita, 1992; Sidrauski & Walter, 1997). Under ER stress, IRE1 senses ER luminal unfolded proteins, ultimately leading to IRE1 dimerization, autophosphorylation, and RNase activation, which catalyze cytoplasmic splicing. Targets of the cytoplasmic splicing are mRNAs encoding UPR-specific transcription factors, such as HAC1 in yeasts (Sidrauski & Walter, 1997), XBP1 in metazoans (Yoshida et al, 2001), and bZIP60 in Arabidopsis (Deng et al, 2011; Nagashima et al, 2011). Activated IRE1 also degrades mRNAs encoding secretory pathway proteins, designated as the regulated IRE1-dependent decay (RIDD) of mRNAs in fission yeast (Kimmig et al, 2012), metazoans (Hollien & Weissman, 2006; Iqbal et al, 2008; Han et al, 2009; Hollien et al, 2009), and plants (Mishiba et al, 2013; Hayashi et al, 2016). Although distinct catalytic mechanisms between cytoplasmic splicing and RIDD has been reported (Tam et al, 2014), how IRE1 outputs these two modules during physiological and developmental processes is still unclear (Maurel et al, 2014).

Although IRE1-deficient mice (Zhang et al, 2005) and flies (Ryoo et al, 2013) cause embryonic lethality, IRE1-deficient yeast (Nikawa & Yamashita, 1992; Kimmig et al, 2012) and worms (Shen et al, 2001) are viable. In plants, Arabidopsis IRE1A- and IRE1B-defective mutants do not exhibit severe developmental phenotypes under normal conditions (Nagashima et al, 2011; Chen & Brandizzi, 2012), whereas rice homozygotes that express kinase-defective IRE1 is lethal (Wakasa et al, 2012; note that rice has one IRE1 gene). The disparate phenotypic consequences of IRE1 mutation between Arabidopsis and rice prompted us to investigate the degree of contribution that IRE1 makes to plant development.

In recent years, activations of IRE1 caused by lipid perturbation or inositol depletion were observed in yeast (Pineau et al, 2009; Promlek et al, 2011; Lajoie et al, 2012), human cells (Ariyama et al, 2010), and mouse cells (Volmer et al, 2013). These IRE1 activations do not require sensing of unfolded proteins by the luminal domain of IRE1 (Snapp, 2012) but does require an amphipathic helix adjacent to the transmembrane helix to sense ER membrane aberrancies (Halbleib et al, 2017). Although physiological functions of the alternative IRE1 activation are less well known, it has been presumed that unfolded protein-independent mechanisms allow cells to preemptively adapt their ER folding capacity (Volmer & Ron, 2015).
For instance, mutant worms with decreased membrane phospholipid desaturation activate IRE1 without promoting unfolded protein aggregates (Hou et al, 2016). However, there are no studies directly addressing the importance of the unfolded protein-independent IRE1 activation in developmental processes in multicellular organisms.

In this report, we investigated the contribution of IRE1 lacking its sensor domain to Arabidopsis development. We found that a third Arabidopsis IRE1 gene, encoding sensor domain-lacking IRE1, is functional and that the triple mutant of the three IRE1 (IRE1-Δ) genes is lethal. Our analyses with plants that express mutant IRE1B proteins without the sensor domain suggest contribution of unfolded protein-independent IRE1 activation to multifaceted developmental processes in Arabidopsis.

Results

Loss of function of IRE1C does not alter ER stress response

In addition to the IRE1A and IRE1B genes, Arabidopsis contains an IRE1-like gene (AT3G11870; designated as IRE1C hereafter), whose product lacks a sensor domain (Fig 1A). The sensor domain–lacking IRE1 was also found in some other Brassicaceae species, such as Camelina sativa, and phylogenetic analysis showed that the IRE1C forms an independent cluster from IRE1A and IRE1B groups in dicotyledonous plants (Fig 1B). A T-DNA insertion mutant of ire1c (SALK_204405; Fig S1A) and ire1a ire1c (designated as ire1a/c hereafter) double mutants did not exhibit any visible phenotypic alterations in normal growth conditions (Fig 1C).

Consistent with the previous studies (Nagashima et al, 2011; Mishiba et al, 2013), susceptibilities to ER stress inducers, DTT, and tunicamycin (Tm) were more apparent in ire1a/b mutant than those in WT, ire1a, and ire1b mutants (Figs 1D and S1B and C). The susceptibilities to DTT and Tm in ire1c and ire1a/c mutants were same as that in WT (Figs 1D and S1B and C). To detect bZIP60 splicing and RIDD in the IRE1 mutants under ER stress, expressions of BiP3 and PR-4 mRNA, which are the typical targets of bZIP60 (Iwata & Koizumi, 2005) and RIDD (Mishiba et al, 2013), respectively, and the spliced form of bZIP60 (bZIP60s) mRNA were analyzed by qPCR. Up-regulation of BiP3 and bZIP60s mRNA and down-regulation of PR-4 mRNA by Tm and DTT treatments were observed in ire1c and ire1a/c mutants as well as WT, ire1a, and ire1b mutants, but not in ire1a/b mutant (Fig 1E). These results indicate that IRE1C, which lacks a sensor domain, does not contribute to ER stress response in Arabidopsis.

A triple mutant of IRE1A, IRE1B, and IRE1C is lethal

We tried to produce ire1a/b/c triple mutant by crossing ire1a/b with ire1c mutants. We obtained three F2 plants heterozygous for ire1c (designated as ire1c+/+) and homozygous for ire1a and ire1b. Genotyping of their self-pollinated progenies showed that no plants homozygous for ire1c were obtained among 108 plants analyzed (Table 1 and Fig S1D). In addition, unexpected segregation of homozygotes and heterozygotes for ire1c (+/+;ire1c+/) = 1.0.0:3 was observed. These results indicate that ire1a/b/c triple mutant is lethal. The ire1a/b ire1c+/ plants exhibited growth retardation (Fig 2A) and reduced seed set (Fig 2B and C) compared with the ire1a/b/+ siblings. Pollen development was especially impaired in the ire1a/b ire1c/+ plants, whereas ire1c and ire1a/c mutants did not affect pollen development and seed set (Fig 2C). No transmission of the ire1c allele through male gametophyte was shown after reciprocal crossing between ire1a/b and ire1a/b ire1c+ mutants (Table 1). Transgenic plants carrying IRE1C promoter-driven GUS (β-glucuronidase) reporter gene construct (Fig S2A) showed that IRE1C is expressed in the anther (Fig S2A) and embryo (Fig S2B). No visible GUS staining was observed in vegetative tissues (root, leaf, and stem) of young seedlings with or without stress treatments (Fig S2C). These observations are qualitatively consistent with the microarray database (Arabidopsis eFP browser; http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), which shows that the IRE1C gene is scarcely expressed in vegetative tissues. Growth retardation of ire1a/b ire1c/+ plants was also observed under in vitro culture containing 1% sucrose (Fig S2D), under which IRE1C mRNA was slightly decreased in ire1a/b ire1c/+ compared with ire1a/b (Fig S2E). The decrease in IRE1C mRNA level in ire1a/b ire1c/+ was prominent in flower bud tissues (Fig S2F). Taken together, IRE1C, which lacks a sensor domain, contributes to male gametophyte development and acts redundantly with IRE1A and IRE1B.

Mutant IRE1B lacking the sensor domain is not responsible for UPR signal transduction

Because ire1a/c mutants retain fertility and IRE1-dependent UPR signal transduction, IRE1B possibly plays a role in both UPR and developmental processes. To investigate an unknown IRE1 function, we generated a construct expressing FLAG-tagged wild-type (WT) form of IRE1B as well as those with kinase (K487A), RNase (K821A), and luminal sensor deletion (ΔLD) mutants under the control of its native promoter (Figs 3A and S2B). For comparison, we generated constructs expressing FLAG-tagged wild-type (WT) IRE1A as well as mutant IRE1A with kinase (K442A) and RNase (K781A) mutation under the IRE1A native promoter (Figs 3A and S2B). These constructs were introduced into the ire1a/b mutant using Agrobacterium-mediated transformation and T1 homozygous transgenic plants were used for further analyses. All of the WT and mutant IRE1 constructs expressed expected sizes of IRE1 proteins in seedlings (Fig 3B). Up- and down-regulation of BiP3 and PR-4 mRNA, respectively, by Tm treatment were restored in the FLAG–IRE1A(WT) and FLAG–IRE1B(WT) transgenic plants, but not in kinase-, RNase-, and ΔLD-expressing transgenic plants (Fig 3C). We next analyzed in vivo phosphorylation of FLAG–IRE1B under ER stress by Phos-tag–based Western blot (Yang et al, 2010). A slower migrating, phosphorylated form of IRE1B was detected in Tm- and DTT-treated FLAG–IRE1B(WT) plants but not in the DTT-treated K487A plants (Fig 3D). Consistent with the results of BiP3 and PR-4 expressions, expression of FLAG–IRE1B(WT) restored hypersensitivity of the ire1a/b mutant to DTT and Tm to the level observed in WT, but expression of ΔLD did not (Fig 3E). These results indicate that the mutant IRE1B lacking the sensor domain does not contribute to the ER stress response.
Figure 1. Arabidopsis IRE1C does not contribute to the UPR.

(A) Structures of Arabidopsis IRE1A, IRE1B, and IRE1C proteins. (B) Phylogenetic tree of IRE1 proteins from mammals (Homo sapiens), fungi (Saccharomyces cerevisiae and Saccharomyces pombe) and plants was constructed by UPGMA using MEGA 6 (Tamura et al., 2013). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. (C) Wild-type (WT) and ire1 mutant plants 40 d after germination (DAG). Bar = 10 mm. (D) DTT sensitivity of the ire1 mutants. Seedlings at 15 DAG of the indicated lines were treated with or without 1 mM DTT. (E) The relative mRNA levels of BiP3 (upper), bZIP60s (middle), and PR-4 (lower).
Table 1. Transmission of the iretc allele through the male and female gametophyte in the progenies of the ireta iretb iretc/+ mutants crossed with the ireta iretb mutant or self-pollination.

| Female Genotype | Male Genotype | Genotypes of Progeny | Observed Ratio | Expected Ratio |
|-----------------|---------------|----------------------|----------------|---------------|
|                 |               | +/+                  | c/+            | c/c           | +/+<c/+<c/c    |
| a/a b/b c/+     | a/a b/b c/+   | 83                   | 25             | 0             | 108           | 1.0:0.30:**  |
| a/a b/b c/+     | a/a b/b +/+   | 186                  | 39             | 0             | 225           | 1.0:0.21:**  |
| a/a b/b +/+     | a/a b/b c/+   | 119                  | 0              | 0             | 119           | 119:0:**     |

**Significantly different from the Mendelian segregation ratio (χ², P < 0.01).

+, wild-type allele; a, ireta allele; b, iretb allele; c, iretc allele.

Flag-IRE1B(WT) and ΔLD restore the developmental defects in ireta/b iretc/+ mutant

We crossed the ireta/b mutant plants expressing FLAG-IRE1B(WT) or ΔLD with ireta/b iretc/+ mutant plants. F1 plants heterozygous for IRE1C (ireta/b iretc/+), were selected and self-pollinated. Among F2 plants, those that are homozygous for the transgenes and heterozygous for IRE1C were selected for further analyses. Growth defects (Fig 4A–F) and the reduction of seed set (Fig 4G–I) in the ireta/b iretc/+ mutant were restored by expression of FLAG-IRE1B(WT) and ΔLD. Abortion of pollen development in ireta/b iretc/+ mutant was restored, as well (Fig 4J–L). At the completion of meiosis, the ireta/b iretc/+ mutant plants expressing FLAG-IRE1B(WT) or ΔLD produced four viable microspores in each tetrad (Fig 4M and N), whereas the ireta/b iretc/+ mutant frequently produced abnormal tetrads (Fig 4O).

Impaired pollen development in ireta/b/c gametophyte is restored by ΔLD

Unexpectedly, self-pollinated progenies of the ireta/b iretc/+ plants expressing FLAG-IRE1B(WT) or ΔLD segregated with the IRE1C allele in ratios of 1.0:1.9:0 (+/+:c/+<c/c; n = 154) and 1.0:1.4:0 (n = 237), respectively (Table 2). Nevertheless, their occurrence ratios of the heterozygous allele were higher than that in the ireta/b iretc/+ mutant (1.0:0.30; n = 108; Table 1). To determine whether the impaired transmission of the iretc allele through male gametophyte in ireta/b iretc/+ mutant was restored by FLAG-IRE1B(WT) and ΔLD, we performed reciprocal crosses between the ireta/b iretc/+ plants with homozygous FLAG-IRE1B(WT) or ΔLD transgene and wild-type plants. Consistent with the results of the reciprocal crossing between ireta/b and ireta/b iretc/+ (Table 1), control reciprocal crossing between wild-type and ireta/b iretc/+ showed no transmission of the iretc allele through male gametophyte (Table 2). In the case of the crossing between the wild-type plants as female parents and the ireta/b iretc/+ plants with FLAG-IRE1B(WT) or ΔLD as male parents, progenies having iretc/+ allele were obtained in the ratios of 1.0:0.33 (+/+<c/+; n = 57) and 1.0:0.57 (n = 94), respectively (Table 2). These results indicate that not only FLAG-IRE1B(WT) but also ΔLD can compensate for impaired male gametogenesis in the ireta/b/c haplotype.

To investigate the defect of male gametogenesis in the ireta/b/c haplotype, we observed cross sections prepared from inflorescences of wild-type, ireta/b iretc/+ and ireta/b iretc/+ expressing ΔLD at different developmental stages. The anther size and the number of pollen grains were reduced in ireta/b iretc/+ compared with wild-type (Fig 5). Regarding pollen development, no obvious differences were observed between ireta/b iretc/+ and wild-type at stage 8 and 9 (Fig 5). However, a part of pollen grains collapsed in ireta/b iretc/+ at

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**Figure 2. ireta iretb homozygous and iretc heterozygous plants show developmental defects.**

(A) Self-pollinated progenies of ireta iretb iretc/+ mutant plants at 40 DAG. Genotypes are shown on the left. Bar = 10 mm. (B) Siliques of ireta iretb iretc/+ (left) and ireta iretb +/+ (right) plants. Bar = 10 mm. (C) Reproductive development of ire1 mutants. Flowers at stage 14 (Smyth et al, 1990; upper; bar = 1 mm), siliques (middle; bar = 3 mm), and anthers at stage 12 stained with Alexander’s stain (lower; bar = 100 μm) from wild-type (WT) and ire1 mutants are shown.

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PR-4 (lower) in WT and ire1 mutants. RNA from seedlings at 10 DAG were treated with 5 mg/l Tm, 2 mM DTT, or mock for 5 h and subjected to qPCR. Data are means ± SEM of three independent experiments. Different letters within each treatment indicate significant differences (P < 0.05) by the Tukey–Kramer Honestly Significant Difference (HSD) test. SS, signal sequence; TM, transmembrane domain.
stage 11 (Fig 5; indicated by arrowheads). The collapsing pollen grains were also shown in ire1a/b iretc/+ expressing ΔLD, but the frequency was very low (Fig 5; arrowhead). In ire1a/b iretc/+ expressing ΔLD, the anther size was restored to the wild-type level (Fig 5). Results of the crossing between ire1a/b iretc/+ as female parents and ire1a/b (Table 1) or wild-type (Table 2) as male parents suggest incomplete female gametogenesis in the ire1a/b/c haplotype. However, FLAG-IRE1B(WT) and ΔLD transgenes did not affect the occurrence ratios of heterogeneous iretc/+ allele through the female gametophyte (Table 2).

Different IRE1 activation states by saturated fatty acids in the presence or absence of sensor domain

Growing evidence suggests that yeast and metazoan IRE1 have lipid-dependent activation machinery (Volmer & Ron, 2015). Because exogenous application of glycerol is known to reduce oleic acid (18:1) level in Arabidopsis (Kachroo et al, 2004), we applied glycerol treatment to Arabidopsis seedlings to increase saturated fatty acid level in Arabidopsis (Kachroo et al, 2004), we applied glycerol treatment (Fig S4). As expected, levels of palmitic acid (16:0) and stearic acid (18:0) were increased after 3 d of glycerol treatment in wild-type seedlings (Fig 6A). Glycerol treatment induced impaired mRNA degradation in ire1a/biretc/+ compared with wild-type, FLAG-IRE1B(WT), and ΔLD plants under glycerol treatment (Fig S4D). Accumulation of ΔLD proteins was observed under glycerol and DTT treatments (Fig 6G). Phos-tag Western blot of ΔLD protein showed two slower migrating bands in wild-type plants treated with Tm or DTT. Samples were resolved on Phos-tag SDS–PAGE to detect the phosphorylated FLAG-IRE1B (p-IRE1B).

CRISPR/Cas9-induced deletion corresponding to IRE1B sensor region in ire1a/c mutant

To gain insight into the contribution of the sensor domain-independent IRE1 activation to the developmental process, we tried to induce deletion in the sensor domain–coding region of the
IRE1B gene in the ire1a/c mutant using CRISPR/Cas9 system. Agrobacterium harbouring pKIR1.0 binary vector (Tsutsui & Higashiyama, 2017) containing two gRNAs targeting the 5’- and 3’-ends of the IRE1B’s sensor domain–coding region (Figs 7A and S2C) was used to transform the ire1a/c mutant. We selected T3 lines showing 3:1 segregation ratio for the presence and absence of RFP fluorescence (see Fig S2C) in seeds and picked up some seeds with no fluorescence, indicative of no T-DNA insertion for further analyses (Tsutsui & Higashiyama, 2017). When we amplify an IRE1B-coding genomic region by PCR, we obtained smaller bands than that would be expected from intact IRE1B in some T3 plants, indicating that deletion is successfully introduced. Therefore, their self-progenies (T4) were used for further analyses. In lines #2–5 and #9–6, all T3 plants analyzed showed a single, smaller IRE1B fragment, indicating homozygous deletion of IRE1B locus (Fig S5A). Sequence analysis showed deletion of 981- and 1,216-bp regions, each corresponding to part of the sensor domain in #2–5 and #9–6, respectively, and 1-bp deletion at the gRNA2 target site was also detected in #2–5 (Fig 7A). Consistently, RT-PCR showed expression of shorter IRE1B mRNA in #2–5 and #9–6 seedlings (Fig S5B). We expected that two isolated lines express N-terminally truncated IRE1B proteins by illegitimate translation (Makino et al, 2016) because translation from the original ATG produces premature N-terminal peptides (52 and 76 aa, respectively). These predicted ORFs in #2–5 and #9–6 have a truncated and an intact transmembrane domain, respectively (Fig 7A). High sensitivity to Tm and DTT equivalent to ire1a/b was found in #2–5 and #9–6 lines compared with that in ire1a/c (Figs 7B and S5C). Like the ire1a/b mutant, up- and down-regulation of Bip3 and PR-4 mRNA, respectively, by Tm treatment were diminished in #2–5 and #9–6 lines (Fig 7C). Glycerol treatment–dependent bZIP60 splicing as shown in ire1a/c was also diminished in #2–5 and #9–6 lines (Fig 7D). Nevertheless, mRNA expression of RIDD target genes in these lines was decreased as compared with those in ire1a/b under glycerol treatment (Figs 7E and S6A). Growth defects and the reduction in seed set, which occurred in ire1a/b ire1c/+ mutant, were not observed in the #2–5 and #9–6 plants (Fig S6B and C).

Discussion

IRE1 is known as the most conserved and sole UPR signal transducer in lower eukaryotes (Mori, 2009). Evolution of multicellular organisms adapt IRE1 functions not only to environmental conditions but also to developmental conditions, as in the fact that IRE1 deficiency causes embryonic lethality in some organisms. In developmental processes, specific cells producing a large amount of secretory proteins, such as β-cells of pancreas (Lee et al, 2011), goblet cells (Tsuru et al, 2013), and dendritic cells (Ostorù et al, 2014), activate IRE1 in normal condition. These findings raise a question of whether production of unfolded proteins is prerequisite for the IRE1 activation in these specific cells. The present study showed that IRE1 activation without sensing unfolded protein is required for multifaceted developmental processes in Arabidopsis. We speculate that unfolded protein-independent IRE1 activation is a feature of anticipatory UPR (Vitale & Boston, 2008; Rutkowski & Hegde, 2010) to avoid producing “unprofitable”
Table 2. Transmission of the ire1c allele through the male and female gametophyte in the progenies of the ire1a ire1b ire1c/+ mutants having IRE1B or ΔLD transgenes crossed with wild-type or self-pollination.

| Parental Genotype | Genotypes of Progeny | Observed Ratio | Expected Ratio |
|-------------------|----------------------|----------------|----------------|
| Female            | Male                 | +/+ | c/+ | c/c | Total | +/+|c/+|c/c | +/+|c/+|c/c |
| +/+ +/+ +/+ +/+    | a/b a/b c/c          | 202 | 0   | 0   | 202   | 202.0 + | 1:0 | 1:0 | 1:0 |
| +/+ +/+ +/+ +/+    | a/b a/b c/+ IRE1B    | 43  | 14  | 0   | 57    | 1:0:0.3:0 + | 1:0 | 1:0 | 1:0 |
| +/+ +/+ +/+ +/+    | a/b a/b c/+ ΔLD      | 60  | 34  | 0   | 94    | 1:0:0.57:0 + | 1:0 | 1:0 | 1:0 |
| a/a a/b c/+       | +/+ +/+ +/+           | 266 | 81  | 0   | 347   | 1:0:0.3:0 + | 1:0 | 1:0 | 1:0 |
| a/a a/b c/+ IRE1B | +/+ +/+ +/+           | 130 | 32  | 0   | 162   | 1:0:0.25:0 + | 1:0 | 1:0 | 1:0 |
| a/a a/b c/+ ΔLD   | +/+ +/+ +/+           | 135 | 25  | 0   | 160   | 1:0:0.19:0 + | 1:0 | 1:0 | 1:0 |
| a/a a/b c/+ IRE1B | a/a a/b c/+ IRE1B     | 53  | 101 | 0   | 154   | 1:0:0.9:0 + | 1:2 | 1:2 | 1:2 |
| a/a a/b c/+ ΔLD   | a/a a/b c/+ ΔLD      | 97  | 140 | 0   | 237   | 1:0:1.4:0 + | 1:2 | 1:2 | 1:2 |

*Significantly different from the Mendelian segregation ratio (χ², P < 0.01).
+ wild-type allele; a, ire1a allele; b, ire1b allele; c, ire1c allele; IRE1B, FLAG-IRE1B(WT) transgene (homozygote); ΔLD, ΔLD transgene (homozygote).

We found that plants heterozygous for the IRE1C allele (ire1c+/+) in ire1a/b mutant background display developmental defects of male (and also probably female) gametogenesis, incomplete floral organ formation, and retardation of vegetative growth (Figs 2 and S3D). The incomplete dominance of the IRE1C allele is probably due to low expression of the IRE1C transcript (Fig S3C and E). IRE1C expression is strongest in the anther (Fig S3A) and embryo (Fig S3B), which is somewhat similar to that of IRE1B (Koizumi et al, 2001). Expression of IRE1A alone may be insufficient for proper Arabidopsis development because we could not obtain ire1b ire1c double mutant. Functions and physiological significance of the Arabidopsis IRE1C is still unclear. A closely related species, Arabidopsis lyrata, has four IRE1 genes, IRE1A (XP_002884063), IRE1B (EFH48369), IRE1C (XP_002884871), and IRE1C-like (EFH64123). Amino acid sequence similarities between those in Arabidopsis thaliana and A. lyrata are high in IRE1A (93%) and IRE1B (91%), whereas IRE1C proteins are more diversified (66%). A. lyrata also has IRE1C-like gene with low sequence similarity. These results suggest that these IRE1C genes probably arose through gene duplication during evolution of Brassicaceae species.

Poollen is known to be particularly sensitive to environmental conditions that disturb protein homeostasis (Fragkostefanakis et al, 2016). In high temperature, ire1a/b mutant displays male sterility (Deng et al, 2016), suggesting that pollen development is sensitive to heat stress and that IRE1-dependent UPR pathway is required for protecting male fertility from heat stress. This feature seems to be distinct from the requirement of the UPR-independent IRE1 activation for pollen development in unstressed condition observed in the present study. IRE1 activation in pollen without stress conditions was suggested by detection of bZIP60s in anther (Iwata et al, 2008), whereas ire1a/b mutant does not compromise pollen development under normal conditions (Deng et al, 2013, 2016). In the present study, we demonstrate that IRE1C, which lacks a sensor domain, acts redundantly with IRE1A and IRE1B in pollen development. Observation of the pollen development in ire1a/b ire1c/+ mutant showed reduced number of pollen (Fig 5) and abnormal tetrad (Fig 4O), suggesting that male gametogenesis in the ire1a/b/c haplotype is defective in meiosis. In addition, the ire1a/b ire1c/+ mutant showed collapsed pollen grains at stage 11 (Fig 5), which is somewhat similar to that observed in RNAi-mediated suppression of ER- and Golgi-located phospholipase A2 transgenic plants (Kim et al, 2011). Together with the results that no transmission of ire1c allele was found through ire1a/b male gametophyte and that ΔLD can restore the transmission in the ire1a/b/c haplotype (Tables 1 and 2), the unfolded protein-independent IRE1 activation is required for the male gametogenesis in unstressed conditions. Given the fact that lipids (Piffanelli et al, 1997) and proteins (Mascarénhas, 1975; Holmes-Davis et al, 2005) are largely produced during pollen development, Arabidopsis IRE1 may sense and maintain protein-to-lipid ratio in cellular membranes as suggested in other organisms (Covino et al, 2018; Michell, 2018).

Genetic analysis also showed distorted segregation ratios in progenies of crosses between ire1a/b ire1c/+ females and ire1a/b or wild-type males (Tables 1 and 2), suggesting that the unfolded protein-independent IRE1 activation may be involved in embryogenesis or...
female gametogenesis. This hypothesis is supported by the observations that the sensor domain-lacking IRE1B mutant lines (i.e., #2–5 and #9–6) in ire1a/b background set seeds normally (Fig S6C) and that IRE1B (Koizumi et al., 2001) and IRE1C (Fig S3B) express in the ovule and embryo, respectively. Inconsistent with the result of the #2–5 and #9–6 lines, we could not obtain homozygous irec mutant plants from selfed progenies of the irea/b irec/+ plants expressing FLAG-IRE1B(WT) or ΔLD plants. A possible reconciliation could be that the IRE1B transgene promoter is not expressed in embryo at a level equivalent to endogenous IRE1B because of epigenetic modifications of the promoter or to insufficiency in the length of the transgene promoter.

By co-expression of two gRNAs and Cas9, part of the sensor domain-lacking IRE1B (Fig 7A). IRE1B mRNA may be compatible with illegitimate translation because its 5'UTR contains uORF and IRE1B mRNA degradation by premature stop codon (Garneau et al., 2007) was not observed in #2–5 and #9–6 (Fig S5B). Therefore, it is most conceivable that expression of sensor domain-lacking IRE1B confers normal seed set in #2–5 and #9–6. This unexpected IRE1 translation may also occur in known mutants in other organisms, such as Caenorhabditis elegans ire1(v33) mutant (Shen et al., 2001). Whether a shorter TM domain in ire1a ire1b mutants in other organisms, such as C. elegans, is functional at a transmembrane domain needs to be elucidated. TM domain of IRE1 is known to sense biophysical properties of the ER membrane (Volmer et al., 2013; Halbleib et al., 2017). In Arabidopsis, both IRE1A and IRE1B cause bZIP60 splicing by glycerol treatment (Fig 6C), although there is no homology between their TM domains. Further studies focusing on the properties of the TM domains should be conducted.

The present study shows distinct modes of IRE1 activation by saturated fatty acid in vivo. Compared with Tm or DTT treatments (Mishiba et al., 2013), the level of IRE1 activation (i.e., fold induction of bZIP60s mRNA and fold reduction of PR-4 mRNA) by glycerol treatment was low. Whereas full-length IRE1B activates both bZIP60 splicing and RIDD under glycerol treatment, sensor domain–lacking IRE1B activates RIDD but not bZIP60 splicing (Figs 6, 7, S4, and S6). Biochemical studies in other model systems showed that oligomerization of IRE1 is required for XBP1/HAC1 cleavage but not RIDD (Tam et al., 2014). If plant IRE1 also acts in the same way, sensor
domain–lacking IRE1 may be less likely to undergo oligomerization by saturated fatty acid. In vivo phosphorylation of FLAG–IRE1B(WT) was found under glycerol treatment (Fig 6D), whereas phosphorylation of ΔLD is rather complicated. Because ΔLD exhibits multiple phosphorylation states and one of the state is stable regardless of stress (Fig 6H), basal RIDD activity (Maurel et al, 2014) may exist in unstressed tissues. This hypothesis may explain growth retardation of ire1a/b ire1c/+ plants (Fig 2A) in unstressed conditions. Considering our current observations that ΔLD restores developmental defects found in the ire1a/b ire1c/+ mutant and that deletion of the IRE1B’s sensor domain in #2–5 and #9–6 does not prevent their development, RIDD activity may play an important role in the developmental processes. This hypothesis is partially supported by the findings of Deng et al (2013) that ire1a/b bzip28 but not bzip60 bzip28 mutant haplotypes impaired male gametogenesis (note that bZIP28 is another UPR arm in Arabidopsis).

In conclusion, this study shows that the unfolded protein-independent IRE1 activation is involved in multifaceted developmental processes, especially pollen development, in Arabidopsis. We hypothesize that the alternative IRE1 activation pathway may be conserved in multicellular organisms as an “anticipatory” mode (Walter & Ron, 2011; Shapiro et al, 2016) of the UPR to avoid producing unfolded proteins in differentiated cells synthesizing a large amount of secretory proteins.

Materials and Methods

Plant materials and stress treatments

A. thaliana Col-0 ecotype and T-DNA insertion mutants in the Col-0 background were used in this study. Plants were grown on soil or half-strength Murashige and Skoog (1/2 MS) medium containing 0.8% agar and 1% sucrose under 16 h light and 8 h dark conditions at 22°C. T-DNA insertion mutants of ire1a, ire1b, and ire1a/b were described previously (Nagashima et al, 2011). A T-DNA insertion mutant of IRE1C (SALK_204405) was obtained from the Arabidopsis Biological Resource Center. T-DNA insertions were confirmed by genomic PCR as shown in Fig S1A and D using primers listed in Table S1. Extraction of DNA for genotyping was carried out as described by Kasajima et al (2004). Genotyping PCR was performed using KAPA Taq Extra PCR kit (Kapa Biosystems) according to the manufacturer’s protocol. To test the sensitivity of seedlings to Tm and DTT, sterilized seeds were sown on a 1/2 MS plate containing Tm (0.1 mg/l) or DTT (1 mM). Relative shoot fresh weight of seedlings was calculated as described by Meng et al (2017). For stress treatments, 10-d-old seedlings in 1/2 MS liquid medium (Nagashima et al, 2011)
were treated with 5 mg/l Tm, 2 mM DTT, or DMSO (mock) for 1–5 h. For glycerol treatment, 7-d-old seedlings in liquid medium were treated with 50 mM glycerol for 3 d followed by treatment with 0.6 mM cordycepin (Wako) for 0–5 h.

**Production of transgenic Arabidopsis plants**

For IRE1C promoter–GUS fusion construct, a 1,577-bp fragment of the IRE1C promoter was cloned into pENTR/D-TOPO (Thermo Fisher Scientific) and transferred into pSMAB-GW-GUS (Fig S2A) binary vector by Gateway LR reaction (Thermo Fisher Scientific). For FLAG-tagged IRE1 constructs, 5,126- and 5,081-bp fragments of IRE1A and IRE1B genes, respectively, comprising ~0.9 and 1.2 kb of their promoter regions, respectively, were amplified by PCR with the primers listed in Table S1 and cloned into pENTR/D-TOPO. Triple FLAG-tag and mutations in the kinase and RNase domains were introduced by PCR. These pENTR vectors were transferred into pSMAB-GW destination binary vector (Fig S2B) by Gateway LR reaction. The ΔLD-expressing vector was made by partial MluI digestion of pSMAB-FLAG-IRE1B(WT) followed by Nhel digestion, blunt end formation by T4 DNA polymerase, and self-ligation. For CRISPR/Cas9, we used pKIR1.0 binary vector (Tsutsui & Higashiyama, 2017) comprising AtU6 promoter-driven gRNA1 and AtU6 promoter-driven gRNA2 (Fig S2C). The target sequences of the gRNA1 and gRNA2 are listed in Table S1. The binary vectors were introduced into strain EHA101 (Hood et al, 1986) and transformed into ire1a/b (for IRE1A and IRE1B constructs), ire1a/c (for CRISPR/Cas9) mutants, and wild-type (for IRE1C promoter–GUS) by the floral dip method (Clough & Bent, 1998).

**RNA analysis**

Total RNA was extracted using a NucleoSpin RNA kit (TaKaRa) according to the manufacturer’s protocol. For RT-PCR and qPCR, 500 ng of RNA was subjected to RT with random primers using High Capacity CDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. qPCR was performed with an ABI 7300 and QuantStudio 3 Real-Time PCR System (Applied Biosystems) using Thunderbird SYBR qPCR Mix (Toyobo), and the transcript abundance of the target genes were normalized to that of 18S rRNA (Zoschke et al, 2007). Primers used for RT-PCR and qPCR are listed in Table S1. RNA gel blot analysis was conducted using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) according to the manufacturer’s protocol. Primers used to generate probes are listed in Table S1.

**Protein analysis**

Total protein extraction from Arabidopsis seedlings was performed as described by Liu et al (2011). Protein extracts were fractionated by SDS–PAGE followed by Western blotting with HRP-conjugated anti-FLAG antibody (PM020-7; MBL; 1:10,000) and chemiluminescent detection using Chemi-Lumi One Ultra (Nacalai Tesque). To reveal Rubisco large subunit, Coomassie Brilliant Blue (CBB) staining of the gel or Ponceau-S staining of the membrane were performed. For Phos-tag SDS–PAGE (Kinoshita & Kinoshita-Kikuta, 2011), 6% polyacrylamide gels containing 5–15 μM Phos-tag acrylamide (Wako) and 10–30 μM ZnCl2 were run according to the manufacturer’s protocol. For Phos-tag SDS–PAGE sample preparation, Arabidopsis seedlings were ground in liquid nitrogen and homogenized in an extraction buffer (100 mM Tris–HCl, pH 7.5, 0.25 M sucrose, and 5 mM PMSF). The homogenate was centrifuged at 2,000g for 2 min (4°C) and the supernatant was centrifuged at 10,000g for 2 min (4°C). The supernatant was further centrifuged at 100,000g for 30 min (4°C). The crude microsomal fraction pellet was subjected to the protein extraction as described above.

**Histological analysis**

Anther samples were fixed with 4% glutaraldehyde in 60 mM Hepes (pH 7.0) containing 0.125 M sucrose. After dehydration in a graded series of ethanol/water mixtures, the samples were embedded in Quetol 651 resin (Nishin EM) with formulation for plant material (Ellis, 2016). Semi-thin (2 μm) transverse sections were prepared from at least eight resin blocks per sample and stained with 0.2% toluidine blue. Stained sections were examined using a BX-9000 microscope (Keyence). Histochemical GUS staining of IRE1C promoter–GUS plants was performed as previously described (Iwata et al, 2008).

**Fatty acid analysis**

Arabidopsis seedlings (~150 mg FW) at 10 DAG was used for the analysis of fatty acids, most of which are components of membrane lipids (mainly phospholipids and glycolipids) (Ohlrogge & Browse, 1995). The fatty acids were methylated and extracted using fatty acid methylation kit (Nacalai Tesque) following the manufacturer’s instructions. The fatty acid compositions were determined using an Agilent 6890 gas chromatograph (Agilent Technologies) equipped with a DB-23 column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies). Nonadecanoic methyl ester (C19:0) was used as the internal standard.

**Supplementary Information**

Supplementary information is available at https://doi.org/10.26508/lsa.201900459.

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**Author Contributions**

K-i Mishiba: conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation,
visualization, methodology, project administration, and writing—original draft, review, and editing. Y Iwata: resources, formal analysis, investigation, methodology, and writing—original draft, review, and editing.

T Mochizuki: formal analysis, investigation, and visualization. A Matsumura: formal analysis and investigation. N Nishioka: resources, formal analysis, validation, and investigation. R Hirata: formal analysis, validation, and investigation. N Koizumi: supervision, writing—original draft, and project administration.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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