Short Title: ERdj3B functions in anther development

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ERdj3B-mediated quality control maintains anther development at high temperatures

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One sentence summary:

ER quality control mediated by ERdj3B is important for anther development at high temperature in \textit{Arabidopsis thaliana}. 

Footnotes:

Author Contributions
M.Y., S.U., T. Su., T. Sa., S. K., and S. N. designed and performed the experiments. M. Y., S. U., T. Su., T. Sa., S. K., T. E., and S. N. analyzed the data. M. Y., S. K., T. E., and S. N. wrote the article.

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Abstract

Pollen development is highly sensitive to heat stress, which impairs cellular proteostasis by causing misfolded proteins to accumulate. Therefore, each cellular compartment possesses a dedicated protein quality control system. An elaborate quality control system involving molecular chaperones, including immunoglobulin binding protein (BiP), heat shock protein 70, and regulatory J domain-containing co-chaperones (J proteins), allows the endoplasmic reticulum (ER) to withstand a large influx of proteins. Here, we found that *Arabidopsis thaliana* mutants of ER-localized DnaJ family 3B (ERdj3B), one of three ER-resident J proteins involved in ER quality control, produced few seeds at high temperatures (29°C) due to defects in anther development. This temperature-sensitive fertility defect is specific to the defective interactions of BiP with ERdj3B, but not with the other two J proteins, indicating functional differences between ERdj3B and the other J proteins. RNA-seq analysis revealed that heat stress affects pollen development in both wild-type and mutant buds, but the *erdj3b* mutant is more susceptible, possibly due to defects in ER quality control. Our results highlight the importance of a specific ER quality control factor, ERdj3B, for plant reproduction, particularly anther development, at high temperatures.
INTRODUCTION

Plants are exposed to various environmental stresses including high temperature, drought, and salinity. Plant reproduction, particularly the development and functioning of the male gametophyte, pollen, is the most temperature-sensitive process during a plant’s lifecycle. Exposure to both short-term high temperatures and long-term mildly elevated temperatures leads to defects in pollen development (Rieu et al. 2017). High-temperature stress affects protein structures and can lead to the accumulation of misfolded proteins. These misfolded proteins, which have a serious impact on cellular proteostasis, must be handled by the protein quality control systems that operate in each cellular compartment.

The endoplasmic reticulum (ER) is the entry site of the secretory pathway for approximately 30% of cellular proteins and is thus exposed to large protein influxes (Vembar and Brodsky 2008). The ER is therefore equipped with an elaborate quality control system to constantly monitor the folding states of newly synthesized and imported proteins and to prevent their potential misfolding in the ER (Ellgaard and Helenius 2003). ER quality control is one of the most important mechanisms for thermotolerance during pollen development (Fragkostefanakis et al. 2016; Rieu et al. 2017). The accumulation of unfolded or misfolded proteins in the ER lumen leads to increased expression of the components of the ER quality control system via a mechanism known as the unfolded protein response (UPR) (Iwata and Koizumi 2012), involving the ER-localized sensor protein, inositol-requiring enzyme 1 (IRE1). The UPR is affected by heat stress in plants,
and the *A. thaliana* *ire1* mutant shows defects in male reproduction at high temperatures (Deng et al. 2016).

Molecular chaperones in the ER, including immunoglobulin binding protein (BiP), calnexin, and calreticulin play important roles in ER quality control. BiP, a major heat shock protein 70 (Hsp70) molecular chaperone in the ER, plays key roles in ER quality control (Nishikawa et al. 2005). Hsp70 chaperones bind to and dissociate from their client proteins via an ATP-regulated cycle. This chaperone cycle is regulated by cofactors of Hsp70 (Bukau et al. 2006). J-domain-containing co-chaperones (J proteins) are a major class of cofactors of Hsp70, which interact with Hsp70 through the well-conserved J domain (Kampinga and Craig 2010). *A. thaliana* has three luminal ER-resident J proteins, ERdj3A, ERdj3B, and P58IPK, which are thought to function in ER quality control as cofactors of BiP (Yamamoto et al. 2008).

In the present study, we analyze *A. thaliana* T-DNA mutants of the *ERDJ3A*, *ERDJ3B*, and *P58IPK* genes. The *erdj3b* mutant showed defects in anther development, which resulted in reduced seed production at an elevated temperature of 29°C. This seed production defect was observed in the *erdj3b* mutant but not in *erdj3a* or *p58ipk*, suggesting that ER quality control mediated by a specific cofactor of BiP is involved in thermotolerance during pollen development. RNA-seq analysis showed that a high temperature caused reduced expression of pollen development-related genes in both wild-type and *erdj3b* anthers, but the *erdj3b* anthers were highly susceptible to heat stress. Therefore, the heat-induced low seed production phenotype of the *erdj3b* mutant is
most likely caused by a combination of heat injury during pollen development and the increased vulnerability of \textit{erdj3b} anthers to heat due to defects in ER quality control.
RESULTS

The erdj3b Mutants Exhibit Reduced Seed Production at a High Temperature

Pollen development and functioning are among the most heat-sensitive processes in the plant lifecycle (Rieu et al. 2017). High-temperature stress affects protein structures; therefore, we analyzed the reproductive growth phenotypes of A. thaliana mutants deficient in J proteins in the ER lumen at high temperature. We found that the erdj3b mutants produced small siliques with very reduced seed sets when they were exposed to the high temperature of 29°C during their reproductive growth phase (Figure 1A). While wild-type plants grown at 29°C produced 28.1 ± 6.1 seeds/silique (n = 8), the erdj3b-1 and erdj3b-2 mutants produced only 3.6 ± 1.5 and 6.3 ± 2.3 seeds/silique (n = 8), respectively (Supplemental Figure S1). The mutant plants did not show any obvious defects in flower development or fertility at 22°C (Supplemental Figure S1; Supplemental Figure S2A and S2B; Yamamoto et al., 2008; Maruyama et al., 2014). The seed production defect of the erdj3b-1 mutant at 29°C was alleviated by introducing a construct expressing the ERDJ3B gene from the ERDJ3B promoter (3Bpro:3B) (Supplemental Figure S1: 3Bpro:3B; Supplemental Figure S3A: 3Bpro:3B), indicating that the erdj3b mutation caused the low seed yield phenotype at 29°C.

Production of seeds in the erdj3a and p58ipk mutants, which were deficient in other ER-resident luminal J proteins, was similar to that in wild-type plants at 29°C (Supplemental Figure S3B). The erdj3b mutant lines harboring constructs that expressed ERdj3A or P58IPK under the control of the ERDJ3B promoter had short siliques and exhibited low seed production at 29°C (Supplemental Figure S1; Supplemental Figure
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Figure 1. Phenotypes of the erdj3b Mutants Grown at 29°C.
Phenotypes of wild-type, erdj3b-1 and erdj3b-2 plants grown at 29°C.
(A) Inflorescences of wild-type (WT), erdj3b-1 [3b-1] and erdj3b-2 [3b-2] plants grown at 29°C are shown. Seed numbers per siliquae are shown in Supplemental Figure 1. Bar, 10 mm.
(B) Flowers observed by stereomicroscopy. Bar, 0.5 mm.
(C) Mature anthers observed by stereomicroscopy. Note that erdj3b mutant anthers dehisce normally and pollen grains are observed on the anthers. Bar, 100 μm.
(D) Self-pollinated stigmas stained by DIOC2 and observed using differential interference contrast microscopy. Pollen grains were visualized by DIOC staining, which stains pollen exine. Arrows indicate pollen grains on the mutant stigmas. Bar, 100 μm.
(E) Number of pollen grains on stigmas of wild-type (n = 14), erdj3b-1 (n = 16), and erdj3b-2 (n = 15) plants grown at 29°C. Each dot shows the number of pollen grains on each stigma; the circle indicates an outlier. Statistical differences were calculated using the Tukey-Kramer method. Different letters indicate statistically significant differences between results at P < 0.05.
promoter in flower buds of the transgenic plants at both 22°C and 29°C (Supplemental Figure S3D). These results indicate that expression of ERdj3A or P58IPK did not suppress the reduced fertility phenotype of the \textit{erdj3b} mutant at 29°C; ERdj3B is functionally distinct from other J proteins in the ER lumen and is required to maintain fertility at 29°C.

\textit{erdj3b} Mutant Plants Grown at 29°C are Defective in Pollen Release and Accumulate Aberrant Materials on the Pollen Grain Surface

When grown at 29°C, the \textit{erdj3b} mutants exhibited normal floral development (Figure 1B and C). Although the mutant flowers contained dehisced anthers with pollen grains (Figure 1C), only a few pollen grains were found on the stigmas of self-pollinated mutant flowers grown at 29°C (Figure 1D); while 44.1 ± 14.4 pollen grains were found on the stigmas of wild-type flowers \((n = 14)\), only 4.4 ± 7.1 and 2.6 ± 3.2 pollen grains were found on the stigmas of \textit{erdj3b-1} \((n = 16)\) and \textit{erdj3b-2} flowers \((n = 15)\), respectively (Figure 1E). By contrast, when plants were grown at 22°C, the numbers of pollen grains found on the stigmas of self-pollinated \textit{erdj3b-1} and \textit{erdj3b-2} mutants were similar to those found on wild-type plants (Supplemental Figure S2C). These results strongly suggest that the low seed yield in the \textit{erdj3b} mutant at 29°C is due to pollination defects. Similar numbers of pollen grains were found on the stigmas of heterozygous \textit{erdj3b/+} and wild-type plants grown at 29°C (Supplemental Figure S4), indicating that lesions in sporophytic tissue(s) caused the pollination defect in \textit{erdj3b}.

Whereas pollen grains were released from wild-type anthers (Figure 2A), pollen grains in mutant anthers tightly adhered to anther tissues (Figure 2B), indicating
that the pollination defect seen at 29°C in the *erdj3b* mutant was mainly caused by
defective pollen release from the anthers. We used transmission electron microscopy to analyze dehisced anthers (anther stage 13) from wild-type and erdj3b mutant plants grown at 29°C. We observed collapsed pollen grains in anthers from the erdj3b mutant (Figure 2D; Supplemental Figure S5B and S5C, arrows) but not in wild-type anthers (Figure 2C; Supplemental Figure S5A). We sometimes observed pollen grains and collapsed pollen grains in contact with the anther wall in erdj3b mutants, suggesting pollen adhesion to anther tissue (Figure 2D; Supplemental Figure S5B and S5C).

Transmission electron microscopy also revealed abnormalities in the pollen coat of the erdj3b mutant grown at 29°C (Figure 2D and 2F); large clumps of electron-dense materials were deposited on the surfaces of pollen grains, which were noticeably absent from wild-type pollen grains (Figure 2C and 2E). These electron-dense materials were continuous with the pollen coat deposited within the cavities of the exine layer, suggesting that they were related to the pollen coat. However, these materials were markedly different from those of the wild-type pollen coat (Figure 2E). Electron lucent regions were occasionally observed inside the electron-dense clumps (Figure 2F, asterisk). The large clumps appeared to join pollen grains together and to join pollen grains to anther walls (Figure 2D and 2F). The surface of pollen grains in erdj3b anthers lacked parts of the pollen coat. We analyzed three independent sections of erdj3b anthers and observed such abnormal pollen grains and pollen coats in all cases (Figure 2D; Supplemental Figure S5B and S5C).

We next analyzed germination of the pollen grains found on the stigmas of erdj3b-1 and erdj3b-2 plants grown at 29°C. Pollen tubes were stained with aniline blue,
which binds specifically to β-1, 3-glucan (callose) (Kho and Bear, 1968). Pistils of self-pollinated flowers were collected from wild-type, erdj3b-1, and erdj3b-2 plants grown at 22°C or 29°C one day post-flowering, and fixed and stained with aniline blue. When plants were grown at 22°C, approximately 100 pollen tubes were observed on each stigma of wild-type, erdj3b-1, and erdj3b-2 plants (Supplemental Figure S6A and S6B). No significant differences were observed between wild-type, erdj3b-1, and erdj3b-2 pistils in the number of pollen tube per stigma (Supplemental Figure S6B). The number of pollen tubes observed on a pistil from a wild-type plant grown at 29°C was 56.1 ± 18.1 (Supplemental Figure S6A and S6B), which was similar to the number of pollen grains found on the stigma (Figure 1E). We also counted the number of pollen tubes developing on the pistils of mutant plants grown at 29°C (Supplemental Figure S6A) and found that the mean numbers of pollen tubes on the pistils of erdj3b-1 and erdj3b-2 plants were 3.1 ± 6.2 and 8.0 ± 8.7, respectively (Supplemental Figure S6B). Notably, these values were similar to the numbers of pollen grains found on the stigmas of mutant plants grown at 29°C (Figure 1E). These results indicate that pollen grains deposited on the stigmas of erdj3b-1 and erdj3b-2 plants retained their viability and could germinate.

ERdj3B is Important for Anther Development at 29°C

We analyzed anther development in wild-type and erdj3b plants grown at 29°C by examining semi-thin cross sections to detect the pollination defect of the erdj3b mutant (Figure 3). Anther development in wild-type *A. thaliana* can be divided into 14 stages (Sanders et al. 1999). Stage 5 anthers contain microspore mother cells surrounded
by four layers of different cell types, including (from inside to outside) the tapetum,
middle layer, endothecium, and epidermis (Figure 3A; Supplemental Figure S7A).

Meiosis occurs between stages 5 and 7, generating tetrads consisting of four haploid microspores surrounded by callose walls (Figure 3C; Supplemental Figure S7C). At stage
8 of anther development, the callose walls degenerate and microspores are released into the anther locule (Figure 3E). In anthers of wild-type plants grown at both 22°C and 29°C, tapetum cells were vacuolated from stage 6 onwards and started to collapse at stage 9 of anther development (Figure 3C, 3E, and 3G; Supplemental Figure S7A, C, and E; Sanders et al., 1999). Anther development in the erdj3b mutant grown at 22°C was indistinguishable from that in wild-type plants (Supplemental Figure S7B, D, and F). In stage 5–8 anthers of the erdj3b mutant grown at 29°C, tapetum cells became abnormally enlarged and vacuolated (Figure 3B, 3D, and 3F). The mutant tapetum layer in stage 9 anthers was even thicker than that of wild-type cells at the same stage (Figure 3G and 3H). The mutant anther walls at stages 5 and 7 consisted of a tapetum, endothecium, and epidermis, but lacked the middle layer (Figure 3B and 3D). We occasionally observed cells morphologically similar to those in the middle layer between the tapetum and endothecium layers (Figure 3B, arrowheads). However, the middle layer-like cells did not form a layer. Microspore mother cells underwent meiosis in mutant anthers, producing microspore tetrads at stage 7 (Figure 3D). Callose degeneration also took place at stage 8, releasing microspores into the anther locule (Figure 3F). In contrast to wild-type anthers, collapsed or shrunken microspores were visible in mutant anthers at stage 9 (Figure 3H, white arrows).

Since abnormal anther development was observed in the erdj3b mutant at 29°C, we compared the expression patterns of ERDJ3B in the developing anthers of plants grown at 22°C and 29°C using transgenic plants expressing β-glucuronidase (GUS) driven by the endogenous ERDJ3B promoter (Figure 4). A cross-section of
GUS-stained anthers from a plant grown at 22°C showed GUS signals in all anther
tissues, including tapetum cells, microspores, and anther locules, at anther stage 5 (Figure 4A). Strong GUS signals in tapetum cells were observed at anther stages 7 and 8 (Figure 4A). GUS signals were also observed in microspores at anther stages 7 and 8 (Figure 4A). Although abnormal phenotypes were observed in anthers at stages 5, 7, and 8 from erdj3b mutants grown at 29°C (Figure 3B, D, and F), the GUS staining patterns in anthers of transgenic plants grown at 29°C were indistinguishable from those in anthers of transgenic plants grown at 22°C (Figure 4A). We also analyzed the pattern of GUS expression in anthers at later stages of development. Strong GUS signals were observed in anthers at flower stage 10/11, which corresponds to anther stages 9–10, in plants grown at either 22°C or 29°C (Figure 4B, left-hand panels; Sanders et al. 1999). GUS signals were observed mainly in pollen grains at flower stage 13 (Figure 4B, middle panels), consistent with tapetum degeneration during flower stage 12 (Sanders et al., 1999). The patterns of GUS staining were similar in anthers of plants grown at 22°C and 29°C (Figure 4B), which suggested that plant growth at an elevated temperature did not affect ERDJ3B expression.

ERDJ3B was strongly expressed in tapetum cells at anther stages 8 and 9; therefore, we investigated whether the functions of ERdj3B in tapetum cells are important for fertility at 29°C. We constructed a fusion gene, ATA1prom:3B, which allowed ERDJ3B to be expressed under the control of the ATA1 promoter, which is active in the tapetum at anther stages 7 to 10 (Lebel-Hardenack et al., 1997). Immunoblotting revealed ERdj3B expression in flower buds at flower stage 12/13 and before flower stage 12 (Figure 4C). We analyzed the numbers of pollen grains on the stigmas of self-pollinated
plants grown at 29°C. A mean of 18.1 pollen grains were attached to the self-pollinated stigmas of erdj3b plants harboring the \( ATA1_{pro}:3B \) transgene (n = 7), whereas 2.5 pollen grains were found on stigmas of erdj3b (n = 8) (Figure 4D). This result indicates that \( ERDJ3B \) expression in tapetum cells partially suppresses the pollination defects of erdj3b at 29°C (Figure 4D, \( 3b(ATA1_{pro}:3B) \)). However, \( ERDJ3B \) expression from the \( ATA1 \) promoter was not sufficient to suppress the seed production defect of the erdj3b mutant grown at 29°C (Supplemental Figure S1).

Abrogation of ERdj3B-BiP Interactions Causes a Fertility Defect at High Temperature

We next asked whether the role of ERdj3B as a partner for BiP is required for pollination at 29°C. J proteins generally interact with Hsp70 molecular chaperones through the well-conserved J domain, and bind to client proteins of Hsp70 as well. To determine whether ERdj3B would exhibit such Hsp70-assisting activities, we expressed a fusion protein comprising glutathione S-transferase (GST) and ERdj3B lacking its signal sequence (residues 23–346) in \( E. coli \) cells, and purified it by affinity chromatography (Figure 5). Pull-down assays showed that BiP was recovered from detergent extracts of \( A. thaliana \) seedlings in the presence of ATP with GST-ERdj3B but not with GST (Figure 5A). A highly conserved His-Pro-Gln sequence in the J domain plays an essential role in the interaction between J proteins and Hsp70 (Walsh et al. 2004), and a His-to-Gln substitution of this sequence abolishes the interactions between J proteins and Hsp70, including yeast Ydj1/Ssa1 and Jem1/BiP (Tsai and Douglas 1996: Makio et al. 2008).
The H54Q substitution in the His-Pro-Asp sequence of GST-ERdj3B substantially reduced the amount of co-precipitated BiP (Figure 5A). In addition to binding to BiP, we

Figure 5. Interactions between ERdj3B and BiP are Responsible for Fertility at 29°C. (A) GST-ERdj3B protein (WT), GST-ERdj3B(H54Q) mutant protein (H54Q), or GST alone was immobilized on glutathione Sepharose beads and incubated with total lysate prepared from A. thaliana seedlings with (+) or without (-) 2 mM ATP. The beads were washed as described in the Methods, and the eluate was analyzed by SDS-PAGE and immunoblotting using anti-BiP antibodies. U indicates proteins recovered in the unbound (1%) fraction; B indicates proteins recovered in the glutathione Sepharose beads (100%) fraction.

(B) Rhoanase was denatured at 46 µM in 6 M guanidinium-HCl for 60 min at room temperature. Unfolded rhoanase was diluted 100-fold (0.46 µM final concentration) in 50 mM Tris-HCl, pH 7.4, 1 mM CaCl₂, and 150 mM NaCl at 23°C in the absence (black line) or presence of 2.3 µM GST-ERdj3B (blue line) or GST-ERdj3B(H54Q) (red line). Aggregation (turbidity) was measured at 320 nm.

(C) Inflorescences of wild-type [WT], erdj3b-1 [3b], and erdj3b-1 plants transformed with a construct expressing ERdj3B [3b(comp.]) and a construct expressing ERdj3B(H54Q) [3b(H54Q)] grown at 29°C are shown. Note that expression of ERdj3B(H54Q) failed to restore the temperature-sensitive low seed yield phenotype of the erdj3b-1 mutant. Seed numbers per silique are shown in Supplemental Figure 1. Bar, 10 mm.
investigated whether the recombinant GST-ERdj3B is functional as a co-chaperone by performing a chaperone assay with rhodanese. Chemically unfolded rhodanese produces aggregates after being diluted in buffer lacking a denaturant, and this aggregation is suppressed by the *E. coli* DnaJ protein (Langer et al. 1992). Purified GST-ERdj3B also suppressed the aggregation of chemically denatured rhodanese (Figure 5B). GST-ERdj3B containing the H54Q mutation also suppressed the aggregation of rhodanese. Therefore, the H54Q mutation impairs the interaction between ERdj3B and BiP, but does not affect its binding to client proteins. We next examined whether these observations are also relevant *in planta*. We found that ERDJ3B containing the H54Q mutation failed to complement the impaired fertility of *erdj3b* plants at 29°C (Figure 5C; Supplemental Figure S1). Therefore, the interaction of ERdj3B with BiP is essential for maintaining fertility at 29°C.

**Loss of ERdj3B Function Affects the Expression of Pollen Coat Protein Genes**

We used a transcriptomic approach to identify mechanisms underlying the anther developmental defect of the *erdj3b* mutant under elevated temperature. We analyzed the effects of elevated temperature on gene expression in wild-type and *erdj3b* mutant anthers during pollen development (Figure 6). Total RNA fractions were prepared from anthers from stage 8–13 flowers of wild-type and *erdj3b* mutant plants grown at 22°C and 29°C and subjected to RNA sequencing (RNA-seq). We included three biological replicates for each condition, and the results were highly reproducible (Supplemental Figure S8). When plants were grown at 22°C, only 35 genes were
identified as differentially expressed between wild-type and *erdj3b* plants with a false
discovery rate (FDR) < 0.01; when plants were grown at 29°C, this number increased to
100 (Figure 6A). By contrast, we identified 1685 genes as differentially expressed
between wild-type plants grown at 22°C and 29°C, and 2054 genes as differentially
expressed between erdj3b mutants grown at 22°C and 29°C, with an FDR < 0.01 (Figure
6A; Supplemental Data Set S1–S6). Since growth temperature had a greater effect on
gene expression than genotype, we conducted a further analysis of differential gene
expression in wild-type and erdj3b plants grown at 22°C and 29°C. In wild-type plants,
growth at 29°C resulted in 1158 and 527 genes being down- and upregulated,
respectively, relative to their expression in plants grown at 22°C. In erdj3b mutants,
growth at 29°C resulted in 1276 and 778 genes being down- and upregulated,
respectively, relative to expression in plants grown at 22°C (Figure 6B). Among these.
344 and 242 genes were down- and upregulated, respectively, specifically in wild-type
plants, while 462 and 493 genes were down- and upregulated, respectively, specifically in
erdj3b plants.

Gene ontology (GO) enrichment analysis of the downregulated genes showed
significant enrichment of GO terms associated with pollen formation and anther
development, such as ‘sporopollenin biosynthetic process’, ‘tapetal layer development’,
and ‘biosynthetic process of flavonoid’, in both wild-type and erdj3b plants (Figure 6C;
Supplemental Data Sets S7 and S8). Genes annotated with the GO term ‘tapetal layer
development’ include TDF1, AMS, MS188/MYB103/MYB80, and MS1, which encode
transcription factors required for this process (Li et al. 2017). These GO terms were not
enriched when we performed GO enrichment analysis of downregulated genes.
specifically in wild-type plants (Supplemental Data Set S9). GO-enrichment analysis of genes that were downregulated specifically in erdj3b plants showed no significant enrichment of GO terms. These results suggest that growth under temperature stress at 29°C generally affected pollen/anther development in A. thaliana. Genes annotated as ‘biosynthetic process of flavonoid’, an important pollen coat component, were enriched among downregulated genes in erdj3b but not the wild type, which is consistent with the pollen coat abnormality of the mutant (Figure 2D and F). Interestingly, genes annotated as ‘response to heat’, including several Hsp genes, were downregulated in both wild-type and erdj3b plants at 29°C (Supplemental Table S1). We selected four genes, ROF1, At1g54050, cpHSC70-2, and HSP17.8, which were annotated as ‘response to heat’, and analyzed their expression by real-time quantitative PCR. Expression of these four genes was confirmed to be lower in anthers of both wild-type and erdj3b plants grown at 29°C than of plants grown at 22°C in (Supplemental Table S2). We also analyzed expression of other Hsp genes, seven Hsp100-family genes, six Hsp90-family genes, 11 Hsp70-family genes, 36 Hsp20-family genes, and 18 Hsf-family genes, all of which had not been identified as differentially expressed genes with an FDR <0.01. RNA-seq showed that these genes were not significantly upregulated in either wild-type or erdj3b plants grown at 29°C (Supplemental Table S3). It is likely that the chronic exposure of male reproductive organs to heat stress does not result in a heat shock response, instead repressing the expression of heat shock genes. Genes annotated as ‘photosynthesis’ and ‘regulation of transcription DNA-dependent’ were enriched among downregulated genes in wild-type plants (Figure 6C).
GO-enrichment analysis of upregulated genes in wild-type plants showed no significant enrichment of GO terms. By contrast, GO terms related to phytohormone responses, including ‘response to abscisic acid’, ‘response to ethylene’, and ‘response to jasmonic acid’ were enriched among upregulated genes in the *erdj3b* mutant at 29°C (Figure 6C; Supplemental Data Set S10). The GO terms ‘glucosinolate metabolic process’, ‘response to water deprivation’, and ‘response to salt stress’ were also significantly enriched among the upregulated genes in *erdj3b*. Abscisic acid, ethylene, and glucosinolate contribute to high-temperature stress tolerance (Larkindale and Knight 2002), suggesting that strong heat- and drought-stress responses occurred in *erdj3b* anthers at 29°C. The GO terms ‘glycoside biosynthetic process’, ‘carotenoid catabolic process’, and ‘lipid transport’ were also significantly enriched among upregulated genes in *erdj3b* plants at 29°C (Figure 6C). Most of these GO terms were also enriched among genes upregulated specifically in the *erdj3b* mutant at 29°C (Supplemental Data Set S11).

Accumulation of misfolded proteins in the ER induces expression of genes targeted by the UPR, including BiP, ERDJ3A, ERDJ3B, and P58IPK. The *A. thaliana* ire1 mutant, which is defective in the UPR, is sterile at elevated temperatures (Deng et al. 2016). To examine whether the UPR was activated in the *erdj3b* mutant, we investigated whether expression of UPR-targeted genes was altered in *erdj3b* anthers. RNA-seq showed that genes encoding ER chaperones, including BiP, ERdj3A, and P58IPK, were not upregulated in the *erdj3b* mutant at 22°C or 29°C (Supplemental Table S4). No significant differences in levels of *IRE1A* and *IRE1B* expression were observed between wild-type and *erdj3b* plants at either 22°C or 29°C (Supplemental Table S4). When we
compared our RNA-seq results with data from the *ire1a ire1b* double mutant reported by Deng et al. (2016), in which RNA samples were prepared from flowers at stages 10 and 11, we found no substantially overlaps in down- or upregulated genes between the *erdj3b* mutant and *ire1a ire1b* (Supplemental Figure S9). Expression levels of *ERDJ3B* in *ire1a ire1b* or *bzip60* flowers resembled those in wild-type flowers (Deng et al., 2016), and it is therefore unlikely that the developmental defects of the anther seen in *erdj3b* are related to the UPR. Our results suggest that a different signaling pathway from the UPR operates in developing anthers to mitigate heat stress.
DISCUSSION

ER-resident J proteins function in various processes as functional partners for BiP, the major Hsp70 in the ER. Here, we found that ERdj3B, an ER-luminal resident J protein in *A. thaliana* involved in ER quality control, is required for maintaining fertility, especially pollination, at a high temperature (29°C). The number of pollen grains on self-pollinated stigmas of the *erdj3b* mutant was dramatically reduced compared to the wild type. Pollination and anther developmental defects were also observed in *male gametogenesis impaired anthers*, a loss-of-function mutant of a P5-ATPase in the ER and secretory vesicles (Jakobsen et al 2005). Cod1/Spf1, a yeast homolog of MALE GAMETOGENESIS IMPAIRED ANTHERS, functions in ER quality control (Cronin et al. 2000 and 2002; Vashist et al. 2002). Therefore, ER quality control plays an important role in anther development.

Among mutants of the three ER-luminal resident J proteins, ERdj3A, ERdj3B, and P58IPK, the temperature-sensitive seed production defect was observed only in *erdj3b* plants grown at 29°C. *ERDJ3A* or *P58IPK* expression driven by the *ERDJ3B* promoter did not suppress the reduced seed yield phenotype of *erdj3b* plants grown at 29°C, indicating that the functions of ERdj3B differ from those of ERdj3A and P58IPK. Mammalian cells contain four ER-luminal resident J proteins: ERdj3, ERdj4, ERdj5, and P58IPK (Gidalevitz et al. 2013). Although these J proteins function in protein folding and ER quality control, they exhibit differences in substrate recognition. For example, ERdj4 and ERdj5, but not ERdj3, bind to misfolded surfactant protein C (Dong et al. 2008). Differences in substrate recognition similar to those in mammalian cells might explain
the difference between the requirement for ERdj3B vs. ERdj3A and P58IPK in *A. thaliana* grown at 29°C.

Yang et al. (2009) reported that *tms1-1*, a mutant allele of *ERDJ3A*, shows male sterility at 30°C. However, we did not observe such a fertility defect in *erdj3a-1* or *erdj3a-2* grown at 29°C. The *erdj3a-1* mutant has a T-DNA insertion in the fourth exon of *ERDJ3A* (Yamamoto et al. 2008). Immunoblotting showed that no truncated ERdj3A protein is expressed in *erdj3a-1*, indicating that this mutant is a null allele (Maruyama et al., 2014). Thus, it is unlikely that *erdj3a-1* has retained partial ERdj3A activity and is fertile at 29°C. A possible explanation for this discrepancy is the difference in the *A. thaliana* ecotype backgrounds of the mutants; *tms1-1* is in the Landsberg erecta background and *erdj3a-1* and *erdj3a-2* are in the Columbia background, and recent genome-wide association analysis revealed that the heat sensitivity of male fertility in *A. thaliana* varies among ecotypes (Bac-Molenaar et al. 2015).

Analysis of the temperature-sensitive fertility defects of *erdj3b* revealed aberrant anther development in the mutant plants at 29°C. Both transmission electron microscopy and light microscopy revealed collapsed pollen grains in *erdj3b* mutants grown at 29°C, suggesting that pollen viability was affected. On the other hand, we observed pollen germination on stigmas of *erdj3b* mutants grown at 29°C, indicating some fraction of the pollen grains retained their viability at this temperature; consistent with this observation, the *erdj3b* mutant produced seeds at 29°C.

The observed pollen coat-related abnormality in *erdj3b* at 29°C was likely to have been caused by tapetum defects. Aberrant tapetum morphology, including
hypertrophy and vacuolation, was only observed in erdj3b anthers when plants were
grown at 29°C. ERDJ3B is expressed in the tapetum cells in developing anthers. Both the
pollination defects and the reduced seed yield phenotype seen in erdj3b plants grown at
29°C were partially suppressed by tapetum-specific expression of ERdj3B. This points to
an important role of ERdj3B in the tapetum. Growth at 29°C reduced seed production to
some extent, even in wild-type plants; by contrast, no significant differences in anther
developmental phenotypes were observed between plants grown at 29°C and 22°C. We
also observed reductions in the numbers of pollen grains and pollen tubes in the stigmas
of wild-type plants grown at 29°C compared with those grown at 22°C. RNA-seq
analysis revealed that expression of genes related to pollen formation and anther
development was reduced in wild-type plants grown at 29°C, indicating that chronic mild
heat stress affected anther development resulting in reduced pollination.

By contrast, RNA-seq analysis indicated that genes encoding major heat shock
proteins were not upregulated in anthers of either wild-type or erdj3b plants grown at
29°C relative to their expression in plants grown at 22°C, indicating the major heat shock
response was not induced by chronic heat stress at 29°C. The expression patterns of
ERDJ3B in developing anthers were similar in plants grown at 22°C and 29°C. RNA-seq
analysis also showed that the UPR did not occur in wild-type and erdj3b plants grown at
29°C. Heat stability varies from protein to protein, and it is possible that ERdj3B
functions to stabilize a fraction of heat-labile proteins under a mild heat stress of 29°C.
The severe pollination defect of erdj3b plants grown at 29°C most likely resulted from
Reduced crop yields at high temperature is a crucial problem in several crops (Hatfield et al. 2011). Heat-stress-induced male sterility is associated with disturbed tapetal development in several other plants (Dolferus et al. 2011). The erdj3b mutant also showed tapetum abnormalities at 29°C. Thus, it would be interesting to analyze whether ER quality control in the tapetum is related to thermotolerance in crop plants in the future.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

*A. thaliana* ecotype Columbia was used as the wild type. The T-DNA insertional mutants used in this study included erdj3a-1 (SALK_103280), erdj3a-2 (SALK_027193), erdj3b-1 (SALK_113364), erdj3b-2 (SALK_055599), p58iapk-1 (SALK_140273), and p58iapk-2 (SALK_080901) (Yamamoto et al., 2008). Seeds were either sown in soil or surface-sterilized with chlorine gas and sown on Murashige–Skoog medium (Wako, Osaka, Japan) containing 0.7% (w/v) agar and 1% (w/v) sucrose. The *A. thaliana* plants were grown at 22°C or 29°C under continuous light (50 μmol m⁻² s⁻¹).

Construction of Transgenic Plants

The oligonucleotide primers used in plasmid construction are listed in Supplemental Table S5. The plasmids used for the expression of ERDJ3B from the ERDJ3B or ATAI promoters were constructed as follows. A 3.3 kb genomic DNA
fragment containing the ERDJ3B coding region and 1 kb of the region downstream of the stop codon of ERDJ3B was amplified using primers 62600F2 and 62600R2, digested with Smal and HindIII, and inserted into the Smal and HindIII sites of pCAMBIA1300 (Cambia, Brisbane, Australia) to produce pMYP022. Gateway reading frame cassette A (Life Technologies Japan, Tokyo, Japan) was inserted into the Smal site of pMYP022 to produce pMYP023. DNA fragments corresponding to 2 kb of the region upstream of the start codon of ERDJ3B or 1.59 kb of the region upstream of the start codon of ATA1 were amplified from A. thaliana genomic DNA by PCR using primer sets 62600F3/62600R3 and ATA1F1/ATA1R1, respectively, and cloned into pENTER/D-TOPO (Life Technologies Japan). The promoter region was inserted upstream of ERDJ3B in pMYP023 using LR Clonase II (Life Technologies Japan). The ERDJ3B promoter:ERDJ3A fusion gene was constructed by nested PCR as follows. DNA fragments corresponding to the promoter of ERDJ3B and the coding region of ERDJ3A together with 1 kb of the region downstream of the stop codon of ERDJ3A were amplified by PCR using primer sets 62600F3/62600R4 and 08970F1/08970R1, respectively. The amplified DNA fragments were mixed and used as templates for PCR using primers 62600F3 and 08970R2. Similarly, the ERDJ3B promoter:P58IPK fusion gene was constructed by nested PCR using primer sets 62600F2/62600R5 and 03160F1/03160R1 for the first-round PCR and 62600F3/03160R2 for the second-round PCR. The amplified DNA fragments were cloned into pENTER/D-TOPO. The fusion genes were introduced into pGWB1 (Nakagawa et al. 2007a) using LR Clonase II. The plasmid used for the complementation test of ERDJ3B is described in Maruyama et al. (2014). The H54Q
mutation was introduced into ERDJ3B by PCR using primers 62600(HQ)F1 and 62600(HQ)R1. Plasmid construction for ERDJ3B promoter:GUS fusion genes is described in Maruyama et al. (2014). The resulting plasmids were transformed into A. thaliana plants by the floral dip method (Clough and Bent, 1998) using Agrobacterium tumefaciens strain GV3101.

Histochemical Analyses

The number of pollen grains that adhered to pistils was counted as described by Zinkl et al. (1999), except that self-pollinated pistils were washed with 200 μl of 50 mM potassium phosphate, pH 7.4, before staining. To observe semi-thin sections of anthers, bud clusters were fixed with 2% (w/v) glutaraldehyde in 20 mM sodium cacodylate, pH 7.4, overnight at 4°C. The samples were dehydrated through an ethanol series and embedded in Technovit 7100 resin (Kulzer and Co., Wehrheim, Germany) according to the manufacturer’s instructions. Sections were cut to a thickness of 2 μm using a Leica RM2255 microtome (Leica Biosystems, Tokyo, Japan), stained with 0.5% (w/v) toluidine blue O in 0.1% (w/v) Na2CO3, and observed under a BX51 microscope (Olympus) with bright-field illumination. Each sample was analyzed at least in two biological and two technical repeats with similar results.

Histochemical GUS staining was performed as described by Preuss et al. (1994). To prepare sections, GUS-stained samples were embedded in Technovit 7100 resin (Kulzer and Co., Wehrheim, Germany). Each sample was analyzed at least in two biological and two technical repeats with similar results.
Electron Microscopy

For transmission electron microscopy, anthers were fixed in 2% (w/v) glutaraldehyde, 2% (w/v) paraformaldehyde, and 50 mM sodium cacodylate, pH 7.4, for 3 days at 4°C. The tissue was washed with 50 mM sodium cacodylate, pH 7.4, and postfixed for 8 h in 2% (w/v) aqueous osmium tetroxide at 4°C. The tissue was then dehydrated in a graded acetone series, transferred into propylene oxide, infiltrated, and embedded in Quetol 651. Thin sections (70 nm) were stained with 2% (v/v) aqueous uranyl acetate and lead citrate and examined under a JEOL JEM 1200 EX electron microscope at 80 kV.

GST-ERdj3B Fusion Proteins

Plasmids used for the expression of wild-type or H54Q mutant GST-ERdj3B in E. coli were constructed as follows. A DNA fragment corresponding to residues 23–346 of ERdj3B was amplified by PCR using primers 62600F4 and 62600R6 from the ERDJ3B cDNA clone (Yamamoto et al. 2008) or the ERDJ3B cDNA clone containing the H54Q mutation and cloned into pGEX4T-2 (GE Healthcare, Piscataway, NJ) to produce pMYA010 and pMYA011, respectively. E. coli strain BL21(DE3) harboring pMYA010, pMYA011, or pGEX4T-2 was grown in LB medium containing 50 μg/ml ampicillin at 37°C to OD600 of 0.5–0.6. Isopropyl β-D-thiogalactopyranoside was added to the medium at a final concentration of 1 mM, and the culture was further incubated overnight at 18°C. Cells were harvested by centrifugation at 3,400 × g for 5 min at 4°C;
suspended in 540 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1 mM MgCl₂, 1 mM EDTA, 1 mM DTT, pH 7.4, and protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml chymostatin, 10 μg/ml N-tosyl-L-phenylmethyl-chloromethyl ketone, 200 μg/ml p-aminobenzamidine hydrochloride, 5 μg/ml pepstatin A, 5 μg/ml E-64, 1 mM 6-aminohexanoic acid, 2 μg/ml aprotinin, 2 μg/ml antipain, 2 μg/ml leupeptin, and 20 μg/ml 4-(2-aminoethyl) benzenesulfonfyl fluoride hydrochloride), and disrupted by sonication for 1 min (2 s on, 1 s off pulsed periods, 40% duty cycle) using an Astorason XL2020 sonicator with a microtip (Heat Systems-Ultrasonic, Misonix, Farmingdale, NY). The cell lysate was clarified by two sequential centrifugations at 13,000 × g for 10 min at 4°C and 48,000 × g for 1 h at 4°C. The resulting supernatant was applied onto a glutathione Sepharose 4B column (GE Healthcare; 2 ml bed volume). The column was washed with 30 ml of 540 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1 mM MgCl₂, 1 mM EDTA, and 0.1% (v/v) Tween 20, pH 7.4, and with 30 ml of 540 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.4. Proteins were eluted from the column with 50 mM Tris-HCl, pH 8.0, and 10 mM glutathione and dialyzed against 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM MgCl₂. Concentrations of the prepared proteins were determined by Coomassie brilliant blue staining after SDS-PAGE (Laemmli, 1970) using bovine serum albumin as a standard.

**Chaperone Activity Analysis**
Rhodanese from bovine liver (Sigma-Aldrich) was dissolved at 46 μM in 6 M guanidinium-HCl, 50 mM Tris-HCl, pH 7.4, and 5 mM DTT, and denatured by 60 min of incubation at room temperature. Denatured rhodanese was diluted 100-fold in 50 mM Tris-HCl, pH 7.4, 1 mM CaCl₂, and 150 mM NaCl containing 2.3 μM of GST-ERdj3B, GST-ERdj3B(H54Q), or GST. Protein aggregation was measured by monitoring the absorbance at 320 nm at 23°C. Experiment was repeated four times with similar results.

Total protein extracts were prepared from erdj3b-1 mutant seedlings by grinding the tissue in 100 mM HEPES-KOH, pH 7.4, 5 mM EGTA, 5 mM MgCl₂, 300 mM sucrose, and protease inhibitors. The homogenate was centrifuged at 13,000 × g for 20 min at 4°C to remove cell debris. The homogenate was diluted to 0.1 μg protein ml⁻¹ in 20 mM HEPES-KOH, pH 7.4, 400 mM KCl, 1 mM EDTA, 1.5 mM MgCl₂, 2 mM DTT, 15% (w/v) glycerol, and 0.65% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonate (CHAPS), and insoluble materials were removed by centrifugation at 100,000 × g for 1 h at 4°C. The supernatant was mixed with an equal volume of 20 mM HEPES-KOH, pH 7.4, 1 mM EDTA, 1.5 mM MgCl₂, 2 mM DTT, 15% (w/v) glycerol, and 0.65% (w/v) CHAPS. Glutathione Sepharose 4B beads (10 μl of slurry, GE Healthcare) prebound to 3 μg of GST-ERdj3B, GST-ERdj3B(H54Q), or GST proteins were incubated with 1 ml of protein extract (50 μg) in the presence (2 mM) or absence of ATP for 1 h at 4°C. The reaction mixtures were centrifuged at 21,000 × g for 20 sec at 4°C to remove unbound proteins. The glutathione Sepharose 4B beads were washed four times with 140 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1 mM MgCl₂, pH 7.4, with or without 2 mM ATP, and proteins retained on the beads were
eluted in SDS sampling buffer (Laemmli, 1970) at 95°C. Interaction between BiP and
GST-ERdj3B was reproducibly observed in two independent experiments.

Western Blot Analysis

Proteins were extracted from bud clusters or flowers from *A. thaliana*
inflorescences. Samples were frozen in liquid nitrogen, ground to a fine powder,
suspended in SDS-PAGE sampling buffer (Laemmli, 1970), heated at 94°C for 5 min, and
centrifuged to remove insoluble materials. The extracted proteins were subjected to
SDS-PAGE (Laemmli, 1970), followed by transfer to Immobilon-P membranes
(Millipore, Billerica, USA) as described by Towbin et al. (1979). Primary antibodies
were used at the following dilutions for immunoblotting: anti-ERdj3A 1:1,000;
anti-ERdj3B 1:1,000; anti-P58\(^{IPK}\) 1:1,000; anti-BiP 1:5,000 (Yamamoto et al. 2008); and
anti-actin 1:2000 (Affinity BioReagents, Golden, USA). HRP-labeled anti-rabbit IgG or
anti-mouse IgG (GE Healthcare, Piscataway, USA) was used as a secondary antibody at
1:5,000 dilution. Signals were detected using an ECL Prime western blotting detection kit
(GE Healthcare) and an LAS4000 mini image analyzer (Fujifilm, Tokyo, Japan).

RNA-seq Analysis

Total RNA was prepared from anthers of stage 8–13 flowers (~180 anthers)
using an RNeasy Plant Mini kit (Qiagen, Venlo, Netherlands). All samples were treated
with DNase I using a Qiagen RNase-Free DNase Set (Qiagen) and quantified. RNA
samples prepared from three independent plants were used for subsequent analysis. For
library preparation, 0.5 μg of total RNA from samples with a RNA Integrity Number value ≥ 8 determined by the Agilent 2100 Bioanalyzer were used. All libraries were prepared using a TruSeq Stranded mRNA LT Sample kit (Illumina) according to the manufacturer’s protocol. Multiplex sequencing was performed on a NextSeq 500 (Illumina), and 75 bp-long single-end reads were obtained. The reads were mapped to the reference *A. thaliana* genome (TAIR10) using TopHat2 (Kim et al., 2013), and counted using the htseq-counts script in the HTSeq library (Anders et al., 2015). Count data were subjected to trimmed mean of M-values normalization, and differentially expressed genes were defined using EdgeR (Robinson et al., 2010; McCarthy et al., 2012). Genes with FDRs < 0.01 were classified as differentially expressed genes. Principal component analysis (PCA) was performed on the differentially expressed genes (DEGs) dataset using the built-in R function (R Core Team, 2013). GO-enrichment analysis was performed using the Biological Networks Gene Ontology (BiNGO) tool (Maere et al. 2005). Over-represented GO terms of corrected *p*-values < 0.05 calculated from the Hypergeometric test and adjusted by Benjamini and Hochberg’s method were selected.

**Real-Time Reverse-Transcription Quantitative PCR (RT-qPCR) Analysis**

First-strand DNA was synthesized from the RNA sample used for RNA-seq analysis using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) according to the manufacturer’s protocol. The gene-specific primers used for RT-qPCR are listed in Supplemental Table S6. RT-qPCR was performed using an Applied Biosystems 7300 Real-Time PCR System (Life Technologies Japan) and THUNDERBIRD SYBR qPCR
mix (Toyobo) with 40 cycles of denaturation at 95°C for 15 s and extension at 60°C for 1 min. ABI sequence detection software (version 1.4; Life Technologies Japan) was used for quantification. *UBQ10* was used as an internal control to normalize the RNA quantity. Each sample was analyzed in three biological and three technical repeats.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NP_187509 (ERdj3A/At3g08970), NP_191819 (ERdj3B/At3g62600), and NP_195936 (P58IPK/At5g03160). The AGI codes of the genes analyzed by real-time PCR are provided in Supplemental Table S2. The RNA-seq data reported here are available in the DDBJ Sequenced Read Archive under the accession number DRA006470.

Supplemental Data

**Supplemental Figure S1.** Seed Number per Silique in *A. thaliana* Wild-type, *erdj3b*, and Transgenic Plants Grown at 22°C and 29°C.

**Supplemental Figure S2.** *erdj3b* Mutant Plants Show no Obvious Defect in Flower Development at 22°C.

**Supplemental Figure S3.** The Role of ERdj3B in the Fertility of *A. thaliana* Plants Grown at 29°C Cannot be Rescued by Either ERdj3A or P58IPK.
Supplemental Figure S4. The erdj3b Heterozygous Mutant does not Show Pollination Defects at 29°C.

Supplemental Figure S5. Transmission Electron Micrographs showing Anther Locules of Plants Grown at 29°C.

Supplemental Figure S6. Pollen Tubes of Stigmas of Wild-type and erdj3b Plants.

Supplemental Figure S7. Sections of Anther from Plants Grown at 22°C.

Supplemental Figure S8. Principal Component Analysis (PCA) for All of the RNA-seq Libraries.

Supplemental Figure S9. The Unfolded Protein Response (UPR) does not Occur in erdj3b at 29°C.

Supplemental Table S1. Genes Annotated as 'response to heat' which were Downregulated in Wild-type and erdj3b Plants Grown at 29°C.
Supplemental Table S2. Real-Time Reverse-Transcription Quantitative PCR Analysis of Downregulated Genes Annotated as ‘Response to Heat’ In Wild-Type and erdj3b Anthers.

Supplemental Table S3. Expression of HSP and HSF in Anthers of Wild-type and erdj3b Plants.

Supplemental Table S4. Genes Targeted by the Unfolded Protein Response (UPR) and IRE1 are not Upregulated in erdj3b Anthers.

Supplemental Table S5. PCR Primers Used for Plasmid Construction.

Supplemental Table S6. PCR Primers Used for Real-Time Reverse-Transcription Quantitative PCR.

Supplemental Data Set S1. Downregulated Genes in both Wild-type and erdj3b Anthers Under High-Temperature Conditions.

Supplemental Data Set S2. Downregulated Genes in Wild-type Anthers Under High-Temperature Conditions.
Supplemental Data Set S3. Downregulated Genes in *erdj3b* Anthers Under High-Temperature Conditions.

Supplemental Data Set S4. Upregulated Genes in Both Wild-type and *erdj3b* Anthers Under High-Temperature Conditions.

Supplemental Data Set S5. Upregulated Genes in Wild-type Anthers Under High-Temperature Conditions.

Supplemental Data Set S6. Upregulated Genes in *erdj3b* Anthers Under High-Temperature Conditions.

Supplemental Data Set S7. Selected GO Terms (Biological Process Category) Over-Represented among Downregulated Genes in *erdj3b* Anthers, as Identified Using the Biological Networks Gene Ontology (BiNGO) Tool.

Supplemental Data Set S8. Selected GO Terms (Biological Process Category) Over-Represented among Downregulated Genes in Wild-type Anthers, as Identified Using the Biological Networks Gene Ontology (BiNGO) Tool.
Supplemental Data Set S9. Selected GO Terms (Biological Process Category)
Over-Represented among Downregulated Genes in Wild-type but not in erdj3b Anthers as Identified Using the Biological Networks Gene Ontology (BiNGO) Tool.

Supplemental Data Set S10. Selected GO Terms (Biological Process Category)
Over-Represented among Upregulated Genes in erdj3b Anthers, as Identified Using the Biological Networks Gene Ontology (BiNGO) Tool.

Supplemental Data Set S11. Selected GO Terms (Biological Process Category)
Over-Represented among Upregulated Genes in erdj3b but not in Wild-type Anthers as Identified Using the Biological Networks Gene Ontology (BiNGO) Tool.

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FIGURE LEGENDS
Figure 1. Phenotypes of wild-type, erdj3b-1, and erdj3b-2 plants grown at 29°C.
(A) Inflorescences of wild-type [WT], erdj3b-1 [3b-1], and erdj3b-2 [3b-2] plants grown at 29°C are shown. Seed numbers per silique are shown in Supplemental Figure 1. Bar, 10 mm.
(B) Flowers observed by stereomicroscopy. Bar, 0.5 mm.

(C) Mature anthers observed by stereomicroscopy. Note that *erdj3b* mutant anthers dehisce normally and pollen grains are observed on the anthers. Bar, 100 μm.

(D) Self-pollinated stigmas stained by DiOC<sub>2</sub> and observed using differential interference contrast microscopy. Pollen grains were visualized by DiOC<sub>2</sub> staining, which stains pollen exine. Arrows indicate pollen grains on the mutant stigmas. Bar, 100 μm.

(E) Number of pollen grains on stigmas of wild-type (n = 14), *erdj3b-1* (n = 16), and *erdj3b-2* (n = 15) plants grown at 29°C. Each dot shows the number of pollen grains on each stigma; the circle indicates an outlier. Statistical differences were calculated using the Tukey-Kramer method. Different letters indicate statistically significant differences between results at P < 0.05.

Figure 2. *erdj3b* Anthers Show Defects in Pollen Release.

Mature anthers of wild-type ([A], [C], and [E]) and *erdj3b-1* ([B], [D], and [F]) plants grown at 29°C.

(A) and (B) Pollen grains released from an anther observed by light microscopy. Bar, 100 μm.

(C) to (F) Thin sections of mature anthers observed by transmission electron microscopy. The regions boxed in (C) and (D) are magnified in (E) and (F), respectively. In (D), arrows indicate collapsed pollen grains. In (F), asterisk indicates aberrant deposition. Bars, 10 μm ([C] and [D]) and 1 μm ([E] and [F]).
Figure 3. Developing Anthers of the erdj3b Mutant Grown at 29°C Show Tapetal Hypertrophy.

Semi-thin sections of anthers prepared from wild-type ([A], [C], [E], and [G]) and erdj3b-1 ([B], [D], [F], and [H]) plants grown at 29°C, stained with toluidine blue, and observed by differential interference contrast microscopy. Sections of anthers at stage 5 ([A] and [B]), stage 7 ([C] and [D]), stage 8 ([E] and [F]), and stage 9 ([G] and [H]) are shown. E, epidermis; En, endothecium; ML, middle layer; T, tapetum cell; MMC, microspore mother cells; Td, tetrads; Msp, microspores. Arrowheads in panel (B) indicate aberrant middle layer-like cells. In (H), arrows indicate collapsed pollen grains. Bars, 30 μm.

Figure 4. Expression of ERDJ3B in Tapetum Cells.

(A) Semi-thin sections of GUS-stained anthers at various stages of development from transformants grown at 22°C (upper panels) or 29°C (lower panels), observed using differential interference contrast microscopy. Anthers at anther stage 5 (A5), anther stage 7 (A7), and anther stage 8 (A8) are shown. T, tapetum cell; MMC, microspore mother cells; Td, tetrads; Msp, microspores. Bars, 20 μm.

(B) The expression patterns of anthers at flower stage 10/11 (F10/11), flower stage 12 (F12), and flower stage 13 (F13) in transformants expressing the GUS gene driven by the ERDJ3B promoter grown at 22°C (upper panels) or 29°C (lower panels). Bar, 100 μm.

(C) Immunoblot of total proteins extracted from samples collected 0 days after flowering (0 DAF) and 2 days after flowering (2 DAF), from flower buds at flower stages 12 and 13.
(12/13), and from flower buds prior to flower stage 12 (~12) from wild type (WT), erdj3b-1 [3b], and erdj3b-1 harboring the ATA1pro:3B transgene [ATA1pro:3B] grown at 22°C. Samples were analyzed by SDS-PAGE and immunoblotting with anti-ERdj3B and anti-actin antibodies.

(D) Number of pollen grains on stigmas of wild type [WT], erdj3b-1 [3b], and erdj3b-1 harboring the ATA1pro:3B transgene [ATA1pro:3B]; plants grown at 29°C (n = 8, 8, and 7, respectively). Dots represent the number of pollen grains on each stigma; circles indicate outliers. Statistical differences were calculated using the Tukey-Kramer method. P < 0.05 is indicated by different letters.

**Figure 5.** Interactions between ERdj3B and BiP are Responsible for Fertility at 29°C.

(A) GST-ERdj3B protein (WT), GST-ERdj3B(H54Q) mutant protein (H54Q), or GST alone was immobilized on glutathione Sepharose beads and incubated with total lysate prepared from *A. thaliana* seedlings with (+) or without (-) 2 mM ATP. The beads were washed as described in the Methods, and the eluate was analyzed by SDS-PAGE and immunoblotting using anti-BiP antibodies. U indicates proteins recovered in the unbound (1%) fraction; B indicates proteins recovered in the glutathione Sepharose beads (100%) fraction.

(B) Rhodanese was denatured at 46 μM in 6 M guanidinium-HCl for 60 min at room temperature. Unfolded rhodanese was diluted 100-fold (0.46 μM final concentration) in 50 mM Tris-HCl, pH 7.4, 1 mM CaCl₂, and 150 mM NaCl at 23°C in the absence (black
line) or presence of 2.3 μM GST-ERdj3B (blue line) or GST-ERdj3B(H54Q) (red line).

Aggregation (turbidity) was measured at 320 nm.

(C) Inflorescences of wild-type [WT], erdj3b-1 [3b], and erdj3b-1 plants transformed
with a construct expressing ERdj3B [3b(comp.)] and a construct expressing
ERdj3B(H54Q) [3b(H54Q)] grown at 29°C are shown. Note that expression of
ERdj3B(H54Q) failed to restore the temperature-sensitive low seed yield phenotype of
the erdj3b-1 mutant. Seed numbers per silique are shown in Supplemental Figure 1. Bar,
10 mm.

Figure 6. Functional Biological Processes Affected in the erdj3b Mutant at 29°C, as
Revealed by RNA-seq Analysis.

(A) Total RNA fractions prepared from anthers from stage 8–13 flowers of wild-type and
erdj3b-1 (3b-1) plants grown at 22°C or 29°C and subjected to RNA-seq analysis. The
Venn diagram indicates the numbers of genes differentially expressed between wild-type
and erdj3b plants grown at 22°C (WT; 22°C vs 3b; 22°C); wild-type and erdj3b plants
grown at 29°C (WT; 29°C vs 3b; 29°C); wild-type plants grown at 22°C and 29°C (WT;
22°C vs WT; 29°C); and erdj3b plants grown at 22°C and 29°C (3b; 22°C vs 3b; 29°C).

(B) Venn diagrams showing the numbers of genes down- and upregulated in wild-type
and erdj3b plants grown at 29°C compared with the corresponding plants grown at 22°C.

(C) Over-represented GO terms of downregulated (upper graph) and upregulated (lower
graph) genes in wild-type and erdj3b plants grown at 29°C compared with the
 corre...
Benjamini and Hochberg’s method $< 0.05$. Background means all genes in the *A. thaliana* genome.
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