Rice Floury Shrunken Endosperm 5 encodes a putative plant organelle RNA recognition protein that is required for cis-splicing of mitochondrial nad4 intron 1

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Original article
Abstract

Background: Some important mitochondrial-encoded genes for respiration in higher plants are interrupted by introns. Many nuclear-encoded factors are involved in the splicing of these introns, but the underlying mechanisms remain to be deciphered.

Results: Here, we isolated and characterized a rice mutant named *floury shrunken endosperm5* (*fse5*). In addition to floury shrunken endosperm seeds with mutant phenotype either failed to germinate or produced retarded lethal seedlings. The *Fse5* encodes a putative plant organelle RNA recognition (PORR) protein targeted to mitochondria. Mutation of *Fse5* hindered splicing of the first intron of *nad4*, that encodes an essential subunit of mitochondrial NADH dehydrogenase Complex I. The assembly and NADH dehydrogenase activity of Complex I were disrupted and the structure of the mitochondria was abnormal in the *fse5* mutant. *FSE5* protein was shown to interact with Mitochondrial Intron Splicing Factor 68 (MISF68), which is also a splicing factor for *nad4* intron 1 identified previously in a yeast two-hybrid assay.

Conclusion: *Fse5* encoding a PORR protein is essential for splicing of *nad4* intron 1, and loss of function affects seed development and seedling growth.

Background

According to the endosymbiosis theory, mitochondria originated from α-proteobacteria ancestors (Gray, 1999). During long-term evolution, most mitochondrial genes were transferred to the nucleus of the host cell (Timmis et al., 2004). Only a minor part of mitochondrial genes were retained in angiosperms and those genes are involved in the electron transport system or encode ribosomal proteins and tRNA (Kubo and Newton, 2008). After these mitochondrial genes are transcribed, their transcripts are processed mainly by nuclear-encoded proteins. The post-transcriptional processing generally includes intron splicing, RNA editing, cleavage, and maturation (Small et al., 2013; Hammani and Giege, 2014).

According to structural features and splicing mechanisms, mitochondrial introns in flowering plants were divided into group I and group II with the latter being more predominant (Bonen, 2008). Group II introns are involved in both *cis*-splicing and *trans*-splicing. *Cis*-splicing occurs within one pre-mRNA molecule, whereas the *trans*-splicing occurs between two pre-mRNA molecules (Sharp, 1987; Lasda and Blumenthal, 2011).

Pentatricopeptide repeat (PPR) proteins are the most studied RNA-binding proteins involved in splicing of numerous mitochondrial introns (Small and Peeters, 2000). PPRs can be divided into the major P and PLS subfamilies (Lurin et al., 2004). P-subfamily PPRs contain only canonical 35-amino-acid PPR (P) motifs whereas PLS-subfamily PPRs consist of ordered series of P, longer (L) and shorter (S) PPR motifs (Lurin et al., 2004; Shikanai and Fujii, 2013; Cheng et al., 2016). Many studies have shown that P-type PPRs are involved in splicing of mitochondrial introns. In Arabidopsis, ORGANELLE TRANSCRIPT PROCESSING43 (OTP43) (Falcon de Longevialle et al., 2007), OTP439, TANG2 (Colas des Francs-Small et
ABA OVERLY SENSITIVE 5 (ABO5) (Liu et al., 2010), ABO8 (Yang et al., 2014), BSO-INSENSITIVE ROOTS6 (BIR6) (Koprivova et al., 2010), MITOCHONDRIAL TRANSLATION FACTOR1 (MTL1) (Haïli et al., 2016), SLOW GROWTH3 (SLO3) (Hsieh et al., 2015). Mitochondrial Intron Splicing Factor 26 (MISF26), MISF68 and MISF74 (Wang et al., 2018) were identified to be required for splicing of mitochondrial introns. Mutations of the above genes generally lead to delayed germination and retarded growth. Mutations of P-type PPRs in maize usually lead to lethal embryos within defective kernels or empty pericarps, in mutants such as defective kernel2 (dek2) (Qi et al., 2017), dek35 (Chen et al., 2017), dek37 (Dai et al., 2018), dek41/dek43 (Zhu et al., 2019; Ren et al., 2020), empty pericarp8 (emp8) (Sun et al., 2018), emp10 (Cai et al., 2017), emp11 (Ren et al., 2017), emp12 (Sun et al., 2019), emp16 (Xiu et al., 2016), emp602 (Ren et al., 2019) and ppr20 (Yang et al., 2020). In contrast to numerous P-subfamily PPR proteins reported in Arabidopsis and maize, few such proteins are reported in rice although its genome has 477 PPRs (O'Toole et al., 2008). FLOURY ENDOSPERM10 (FLO10), a mitochondrion-targeted P-type PPR protein characterized in rice, functions in regulating the trans-splicing of nad1 intron 1. Like mutants in Arabidopsis and maize, the o10 mutant exhibited defective grain development evidenced by smaller opaque grains at maturity and obviously slower plant growth during the vegetative and reproductive stages (Wu et al., 2019).

Other proteins in addition to PPRs participate in splicing mitochondrial introns. ABO6 encodes a DEXH box containing RNA helicase that is involved in regulating the splicing of several genes of Complex I in mitochondria (He et al., 2012). Nuclear-encoded maturase nMAT1 (Nakagawa and Sakurai, 2006; Keren et al., 2012), nMAT2 (Keren et al., 2009), and nMAT4 (Cohen et al., 2014), DEAD-box protein PUTATIVE MITOCHONDRIAL RNA HELICASE2 (PMH2) (Köhler et al., 2010), RCC1 family protein RUG3 (Kühn et al., 2011), mitochondrial transcription termination factor mTERF15 (Hsu et al., 2014), and RAD52-like protein ODB1 (Gualberto et al., 2015) are also involved in splicing mitochondrial introns. Another protein family characterized by the plant organelle RNA recognition (PORR) domain also plays an important role in intron splicing in mitochondria and chloroplasts. The PORR domain was previously known as the “domain of unknown function 860” (DUF860) (http://pfam.xfam.org/family/PF11955) but was renamed as the PORR domain in 2009 (Kroeger et al., 2009). AtRPD1 (ROOT PRIMORDIUM DEFECTIVE 1), a member of PORR/DUF860 family, has a role in prearranging the maintenance of active cell proliferation during root primordial development. Disruption of the RPD1 gene caused embryogenesis arrest at the globular to transition stages. RPD1 is expressed in all organs of fourteen-day-old seedlings and the encoded protein is annotated to localize in mitochondria or plastids. In silico structural characterization of RPD1 and RPD1-like proteins suggested a possible involvement of RPD1 and RPD1-like proteins with winged helix proteins in various regulatory functions through DNA binding, RNA binding, and protein-protein interaction (Konishi and Sugiyama, 2006). Structural modeling suggests that PORR adopts a structure that has a surface reminiscent of helical repeat RNA-binding motifs such as the PPR motifs (Kroeger et al., 2009). At4g08940, encoding a PORR protein, responds to oxidative stress. The over-expressed transgenic plants of At4g08940 were more tolerant to paraquat and cold, and less tolerant to tert-butyl hydroperoxide and salinity, but the underlying mechanism remains unknown (Luhua et al., 2008). ZmWTF1 (“what's this factor?”), a chloroplast-targeted PORR protein, is required for the splicing of
chloroplast-encoded introns. \textit{wtf1-1}, a weaker mutant, showed a pale green phenotype whereas mutants \textit{wtf1-3} and \textit{wtf1-4} with null alleles were albinic (Kroeger et al., 2009). \textit{AtWTF9}, a mitochondrially localized PORR protein, is required for \textit{rpl2} and \textit{ccmF}_C intron splicing. T-DNA insertion alleles \textit{wtf9-1} and \textit{wtf9-2} caused severely stunted shoots and roots; both homozygous mutants survived to flowering, but the flowers were small and produced only a few “milky” aborted seeds (Colas des Francs-Small et al., 2012). A recent study showed that mitochondrial heat shock protein 60 s (HSP60s) interact with \textit{WTF9} to regulate the intron splicing of \textit{ccmF}_C and \textit{rpl2}. A retarded growth phenotype was observed in \textit{hsp60-3a-hsp60-3b-1} which had small cotyledons, reduced root length and small stature (Hsu et al., 2019).

\textit{ZmEMP6}, a PORR protein located in mitochondria, is required in both endosperm and embryo development, but the intron(s) splicing was unclear (Chettoor et al., 2015). Up to now, no PORR protein has been identified in rice.

Here, we reported the isolation of \textit{FSE5} though map-based cloning in rice. The \textit{Fse5} allele encodes a mitochondria-localized PORR protein that is expressed constitutively in various tissues. \textit{Cis}-splicing of \textit{nad4} intron 1 is abolished in the \textit{fse5} mutant and mitochondrial function and structure are disrupted consequently. Like other mutants causing defective splicing of mitochondrial intron(s) \textit{fse5} seed development and seedling growth were affected. Mature \textit{fse5} seeds showed a floury, shrunken phenotype, and either failed to germinate or produced weak seedlings that died within one month. These results indicated an essential role of \textit{FSE5} in seed development and subsequent seedling growth.

\section*{Results}

\subsection*{The \textit{fse5} seeds have a floury, shrunken phenotype}

A N-methyl-N-nitrosourea (MNU)-treated population of wild type (WT) \textit{japonica} cultivar (cv) W017 was created to study rice endosperm development. A mutant with floury shrunken endosperm was selected and named \textit{floury shrunken endosperm5} (\textit{fse5}) (Figure 1A-1D). In addition to the abnormal phenotype \textit{fse5} seeds failed to germinate or produced seedlings with retarded growth and death within one month. The homozygous \textit{fse5} seeds are reproduced presumably by heterozygous plants (+/\textit{fse5}) whose progenies have approximately one quarter seeds with mutant phenotype (Additional file 1: Figure S1 and Table S1). Compared with WT W017, both 1000-grain weight and grain thickness of mature \textit{fse5} seeds were significantly reduced, but there was no difference in grain length and grain width (Additional file 1: Figure S2A-S2D). Total starch and amylose contents of mature \textit{fse5} seeds were lower than WT (Additional file 1: Figure S2E-S2F), whereas protein and lipid contents were higher than WT (Additional file 1: Figure S2G-S2H). These data showed that synthesis of seed storage products, especially starch, was significantly affected in \textit{fse5} seeds.

To examine defects at the cellular level, observations were made on mature and immature endosperm cells of WT and \textit{fse5} seeds. Scanning electron microscopy showed that starch grains of mature \textit{fse5} seeds were smooth and loosely packed whereas those of WT were sharp-edged and tightly assembled (Figure 1E-1H). Compared with WT, starch grains in \textit{fse5} seeds were irregular and loosely assembled in
the developing endosperm cells (Figure 1I-1L). These data indicated that the fse5 mutation affected starch grain development.

**The fse5 mutation causes embryo and seedling lethality**

Detailed examination of germination and seedling growth of the mutant and WT seeds showed that fse5 seeds with hulls could not germinate, so dehulled seeds were used in further experiments. Compared with WT, the germination percentage of fse5 seeds was significantly lower (Additional file 1: Figure S2I) and their plumules and radicles grew more slowly (Figure 2A-2B). Only 35.7% mutant seeds had produced seedlings by 9 days after sowing (DAS) in soil compared to 94.0% for WT seeds (Additional file 1: Figure S2J). Moreover, the heights of fse5 seedlings reached only one-fourth of WT (Figure 2C, Additional file 1: Figure S2K). All fse5 seedlings were dead by 30 DAS (Figure 2D). These data showed that the fse5 mutant was seed- or seedling-lethal.

To determine the cause of seed or seedling lethality, observations were made on the embryo structure. Longitudinal sections of seeds after 24 h of imbibition with water showed clear differentiation of the WT embryo but little change in the fse5 mutant (Figure 2E-2F). Triphenyl tetrazolium chloride (TTC) staining that is often used to test seed vigor indicated strong red staining of the WT embryo region indicating active dehydrogenase activity (Brown et al., 1987), but comparatively little staining of the same tissues in fse5 seeds (Figure 2G-2H). These results indicated defective embryo differentiation in the mutant.

**Positional cloning of FSE5**

An F$_2$ population from a cross between a heterozygous (+/fse5) plant whose selfing progenies showing segregation of normal and floury grains and Nanjing 11 (indica) was used to map the FSE5 locus. Based on 92 F$_2$ individuals produced by F$_{1-2}$ floury seeds FSE5 was located in a 7.2 cM region flanked by RM242 and RM257 on the long arm of chromosome 9. Nine hundred and seventy-eight F$_2$ individuals further restricted FSE5 to a 54 kb region between insertion/deletion (In/Del) markers wi-10 and wi-11. Nine open reading frames (ORFs) were predicted in this region (Figure 3A) (http://rice.plantbiology.msu.edu). Sequence analysis revealed a 408 bp deletion in LOC_Os09g29760 in the fse5 mutant compared with WT. The deletion caused a truncation of 136 amino acids in the fse5 mutant transcript (Figure 3B). Thus LOC_Os09g29760 was considered to be the candidate gene for FSE5.

To further confirm these results a vector containing the native promoter and wild-type coding sequence (CDS) of LOC_Os09g29760 was constructed and introduced into the fse5 mutant. Quantitative RT-PCR (qRT-PCR) showed that the expression level of LOC_Os09g29760 was recovered in the positive transgenic lines (Figure 3C). Brown rice seeds of these plants were transparent and plump similar to WT (Figure 3D). Moreover, seedlings grown from those seeds had normal phenotypes (Figure 3E). A further validation was acquired from LOC_Os09g29760 knock-out lines produced by CRISPR/Cas9 (Additional file 1: Figure S3A-S3B). Brown rice seeds from four independent plants segregated for normal and mutant seeds similarly
to that for the original mutant (Additional file 1: Figure S3C). Therefore, LOC_Os09g29760 was demonstrated to be the Fse5 allele.

**FSE5 encodes a protein containing plant organelle RNA recognition domain**

Sequence analysis showed that the cloned Fse5 allele contains a single exon (Figure 4A) and encodes a putative protein of 399 amino acids (http://rice.plantbiology.msu.edu).

The protein was annotated as containing a plant organelle RNA recognition (PORR) domain (https://www.ncbi.nlm.nih.gov; https://ricexpro.dna.affrc.go.jp) and the truncation of 136 amino acids encoded by 408 bp fragment deleted in the fse5 allele were within this domain (Figure 4B). FSE5 was named OsPORR1 because it was the first PORR protein identified among the 17 putative PORR domain-containing proteins in rice (Kroeger et al., 2009; Colas des Francs-Small et al., 2012; Chettoor et al., 2015) (Figure 4C). Multiple sequence alignment showed that 6 amino acids (Leu^{172}, Glu^{305}, Leu^{307}, Phe^{344}, Tyr^{345}, and Leu^{357}) are highly conserved among all 17 members of the rice PORR domain family and all six amino acids are within the PORR domain suggesting they are essential for function of PORR domain proteins (Figure 4D). Five of these acids (except Leu^{172}) are present within the truncation of 136 amino acids (Figure 4B) and therefore likely affect the function of OsPORR1.

**Spatiotemporal expression and subcellular localization of OsPORR1**

qRT-PCR analysis showed that OsPORR1 was expressed in roots, stems, leaves, leaf sheaths, panicles, developing seeds and seedlings, was consistent with the multiple phenotypes affected in the fse5 mutant (Figure 5A). A vector constructed with the β-Glucuronidase (GUS) reporter gene driven by the OsPORR1 promoter was transformed into cv Nipponbare (japonica) to confirm expression of OsPORR1. GUS staining was observed in the above tissues (Additional file 1: Figure S4). Both experiments proved that OsPORR1 was constitutively expressed in different tissues and growth stages.

Localization of OsPORR1 was predicted to be the mitochondrion based on TargetP analysis (http://www.cbs.dtu.dk/services/TargetP). A vector was constructed whereby the GFP protein fused to full-length OsPORR1 was transformed into cv Nipponbare. The GFP signals in protoplasts isolated from positive transgenic seedlings showed a punctate pattern that merged with the orange fluorescence of the mitochondrial indicator MitoTracker Orange (Figure 5B). These data showed that OsPORR1 was a mitochondrially localized protein.

**OsPORR1 functions in splicing mitochondrial nad4 intron1**

Previous studies showed that PORR proteins usually function in intron splicing of chloroplast- or mitochondrial-encoded genes (Kroeger et al., 2009; Colas des Francs-Small et al., 2012). Based on that function and the subcellular localization, transcripts of 9 mitochondrially encoded genes with introns were analyzed. The results showed that compared with WT an absence of mature nad4 transcripts, but larger transcripts were detected in seedlings grown from fse5 seeds (Figure 6A), suggesting that an intron splicing defect had occurred in nad4. To further confirm abnormal splicing of nad4 in the fse5 mutant,
each intron of \textit{nad4} was amplified by specific primers. The results indicated that splicing of \textit{nad4} intron 1 was abolished and was no mature \textit{nad4} transcript was detected in the \textit{fse5} mutant (Figure 6B). All mitochondrial introns in the rice were examined by qRT-PCR using two groups of specific primers. One group was used to amplify the spliced introns whereas the other group was used for non-spliced introns. Only \textit{nad4} exon 1-exon 2 (spliced fragment) was not detected in the \textit{fse5} mutant although its precursor fragment was present (Additional file 1: Figure S5). These data indicated that OsPORR1 specifically affected the splicing of \textit{nad4} intron 1.

\textbf{OsPORR1 mutation affects mitochondrial structure and function}

NAD4 is an important subunit of Complex I in the electron transfer chain of mitochondria. Complex I assembly and activity in seedlings were analyzed by BN-PAGE (blue native polyacrylamide gel electrophoresis). The results showed that the accumulation of the Complex I in the \textit{fse5} mutant was much lower than WT and the NADH dehydrogenase activity was almost completely lost (Figure 7A). The ATP content and respiration rate were also reduced in the mutant relative to the WT (Figure 7B-7C). Complex I is an important component of mitochondrial inner membranes. Observations were made on mitochondrial inner structure in developing endosperm cells of WT and \textit{fse5} mutant. The inner envelope cristae of mitochondria were well organized and surrounded by a dense stroma in WT, whereas the cristae development was poor and the stroma was thin in the \textit{fse5} mutant (Figure 7D). Previous studies had shown that the compromised main respiratory chain caused changes in expression levels of alternative oxidase and NADH dehydrogenase genes (Toda et al., 2012; Li et al., 2013). Therefore the transcription levels of alternative oxidase genes (\textit{OsAOX1a}, \textit{OsAOX1b} and \textit{OsAOX1c}) and NADH dehydrogenase genes (\textit{OsNDA1}, \textit{OsNDA2}, \textit{OsNDB1}, \textit{OsNDB2}, \textit{OsNDB3}, \textit{OsNDC1}) were examined by qRT-PCR. Expression of \textit{OsAOX1a} and \textit{OsNDB2} was higher in the \textit{fse5} mutant than WT, but there were no differences in regard to the other genes (Figure 7E). These data showed that defective splicing of \textit{nad4} intron 1 was associated with changes in mitochondrial structure and function.

\textbf{Discussion}

The \textit{fse5} mutant was isolated artificial mutagenesis. Like other floury-endosperm mutants, such as \textit{floury endosperm2} (\textit{flo2}) (She et al., 2010), \textit{flo4} (Kang et al., 2005), \textit{flo6} (Peng et al., 2014), \textit{flo7} (Zhang et al., 2016), \textit{flo10} (Wu et al., 2019), \textit{flo11} (Zhu et al., 2018), \textit{flo15} (You et al., 2019), and \textit{flo16} (Teng et al., 2019), the \textit{fse5} mutant endosperm has loosely packed starch grains. Total starch and amylose contents and accumulation of major starch synthases GBSSI, SSIIa, SBEI, and SBEIib were reduced in \textit{fse5} seeds relative to the WT W017 (Additional file 1: Figure S6). Unlike most other floury-endosperm mutants the \textit{fse5} mutant showed embryo- or seedling-lethality. OsPORR1 is involved in the splicing of \textit{nad4} intron 1 which is indispensable for normal mitochondrial structure and function. Defects in the splicing of mitochondrially encoded introns, especially those in \textit{nad} genes usually cause aborted seeds, retarded seedlings or small plants (Falcon de Longevialle et al., 2007; Koprivova et al., 2010; Liu et al., 2010; Colas des Francs-Small et al., 2014; Yang et al., 2014; Hsieh et al., 2015; Haili et al., 2016; Xiu et al., 2016; Cai et al., 2017; Chen et al., 2017; Qi et al., 2017; Ren et al., 2017; Dai et al, 2018; Sun et al., 2018; Wang et al.,
EMP6 (EMPTY PERICARP6), the ortholog of OsPORR1 in maize, encodes a PORR protein also located in mitochondria, but the particular intron(s) affected was unknown (Chettoor et al., 2015). At4g08940, the ortholog of OsPORR1 in Arabidopsis, is response to oxidative stress, but again, the underlying mechanism remained to be addressed (Luhua et al., 2008). Both ZmWTF1 and its PORR domain have RNA-binding activity and ZmWTF1 promotes the splicing of group II introns in chloroplasts (Kroeger et al., 2009). AtWTF9, another PORR domain-containing protein, can also bind RNA and is required for the splicing of mitochondrially encoded introns (Colas des Francs-Small et al., 2012). Multiple sequence alignment showed that the PORR domain of OsPORR1 shares 49.24% and 38.23% similarity with that of ZmWTF1 and AtWTF9, respectively (Additional file 1: Figure S7). The way by which OsPORR1 is involved in the splicing of nad4 intron 1 is probably via binding a RNA fragment.

NAD4 is an essential subunit of Complex I in the mitochondrial respiratory chain. Splicing of nad4 intron 1 is essential for mitochondrial function. Like OsPORR1, ZmDEK35, a mitochondrially targeted PPR protein, affected the splicing of only nad4 intron 1. The Mutation in Dek35 affected mitochondrial Complex I assembly and NADH dehydrogenase activity and the dek35 mutant displayed defective seed development similar to the fse5 mutant (Chen et al., 2017). Other PPR proteins including AtMISF68 (Wang et al, 2018), ZmEMP8 (Sun et al., 2018), ZmEMP602 (Ren et al, 2019), ZmDEK43 (Ren et al., 2020), and DEXH box RNA helicase AtABO6 (He et al., 2012), also affect splicing of nad4 intron 1. Their rice orthologs were identified by phylogenetic analysis and applied in yeast-two hybrid (Y2H) analysis to verify interactions between them. Except for OsEMP602 which had preferential self-activated activity in this system OsMISF68 interacted with OsABO6, OsDEK35, OsDEK43, OsEMP8, OsPORR1 and itself (Additional file 1: Figure S8). These observations showed that the nad4 intron 1 was likely spliced by a complex centered on dimeric or multimeric MISF68. Whether and how these factors synchronously regulate nad4 intron 1 splicing is yet to be determined.

Conclusions

Fse5 encodes a PORR protein involved in the splicing of nad4 intron 1 which is indispensable for normal mitochondrial structure and function. Abnormal mitochondrial cristae and reduced respiration rate in the fse5 mutant led to a lower ATP content, and finally affected seed development and subsequent seedling growth. These research results provided valuable clues for revealing the roles of PORR proteins in rice seed development and seedling growth.

Methods

Plant materials and growth conditions
The fse5 mutant was isolated from an MNU-mutagenized population of *japonica* rice cv W017. The F$_2$ population for gene mapping was generated by a cross between a heterozygous plant (+/fse5) and *indica* cv Nanjing 11. Seed germination tests and seedling growth were carried out in a growth chamber (12 h light/12 h darkness at 30°C). Other materials were grown in a paddy field.

**Microscopy**

The transverse sections of mature seeds were observed by a HITACHI S-3400N scanning electron microscopy (http://www.hitachi-hitec.com). Scanning electron microscopy was performed as described previously (Kang et al., 2005). Semi-thin sections of developing seeds (12 DAF) were observed by light microscopy. Sample fixation and sectioning followed described previously procedures (Peng et al., 2014).

**Map-based cloning of OsPORR1**

*OsPORR1* was initially mapped using more than 160 polymorphic SSR and In/Del markers to genotype a small number of mutant F$_2$ individuals from the above cross. Fine mapping then followed with closely linked molecular markers designed from nucleotide polymorphisms between Nipponbare and 93-11 reference genomes. The molecular markers for fine mapping were listed in Additional file 1: Table S2.

**Genetic complementation**

A vector containing the full-length cDNA of *OsPORR1* driven by the native promoter was constructed (the primers were listed in Additional file 1: Table S3) and transformed into the fse5 mutant via the *Agrobacterium tumefaciens*. Positive transgenic plants were identified by the hygromycin resistance gene used as the selective marker in the pCUbi1390 vector.

**RNA extraction, Reverse transcription PCR (RT-PCR) and qRT-PCR**

Total RNA was extracted from various tissues using an RNA Prep Pure Plant kit (Tiangen Co., Beijing, http://www.tiangen.com/en/). First-strand cDNA was synthesized using random hexamer primers for mitochondrially encoded genes and oligo(dT) for nuclear-encoded genes, and PrimeScript reverse transcriptase (TaKaRa, http://www.takara-bio.com) was used for reverse transcription. *OsActin1* (*Os03g0718150*) was used as an internal control (Peng et al., 2014). Quantitative real-time PCR was performed and analyzed by an ABI 7500 real-time system. The primers were listed in Additional file 1: Table S4 for RT-PCR of nine mitochondrially encoded genes with introns and three introns of *nad4*, Additional file 1: Table S5 for qRT-PCR of *OsPORR1*, alternative oxidase and NADH dehydrogenase genes, introns and corresponding exons of mitochondrial-encoded genes.

**Subcellular localization**

The coding region of *OsPORR1* was cloned and fused to pCAMBIA1305-GFP vector (the primers were listed in Additional file 1: Table S3) and then the vector was introduced into cv Nipponbare via
Agrobacterium tumefaciens. Protoplasts of positive seedlings were isolated as described (Chen et al., 2006). GFP fluorescent signals were detected by a confocal laser scanning microscopy (Leica SP8). MitoTracker™ Orange CMTMRos (Invitrogen, Shanghai) was used to mark mitochondria.

**Blue native polyacrylamide gel electrophoresis (BN-PAGE) and Complex I activity assay**

The crude mitochondria were extracted from ten-day-old seedlings grown at 30°C in darkness (Wang et al., 2017; Wu et al., 2019). Then BN-PAGE and Complex I activity assays were performed as described (Wittig et al., 2006; Wu et al., 2019).

**Measurement of ATP content and respiration rate**

Twelve-day-old seedlings were grown at 30°C in darkness and used to measure the ATP content by the ATP assay kit (Beyotime, Shanghai). Nine-day-old seedlings were grown at 30°C and used to measure the respiration rate by a liquid-phase oxygen electrode (Hansatech, UK).

**Declarations**

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**Availability of Data and Materials**

All data supporting the conclusions of this article are provided within the article (and its additional files).

**Authors’ Contributions**

LW, WZ, YW and JW conceived and designed the experiments. WZ provided the fse5 mutant material. SL, YT and XL were responsible for field work. LW, HY, YC, XT, HD, RC and XJ performed the experiments and analyzed the data. LW wrote the paper. YW revised the paper. All authors read and approved the manuscript.

**Ethics Approval and Consent to Participate**
Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

Abbreviations

ABO: ABA OVERLY SENSITIVE; AGPL2: ADP-glucose pyrophosphorylase large subunit 2; AGPS2b: ADP-glucose pyrophosphorylase small subunit 2b; AOX: Alternative oxidase; BN-PAGE: Blue native polyacrylamide gel electrophoresis; CBB: Coomassie brilliant blue; ccmF_C: cytochrome c biogenesis F_C; CDS: Coding sequence; COM: Complemented lines; cox2: cytochrome c oxidase subunit II; cv: Cultivar; DAF: Days after flowering; DAG: Days after germination; DAS: Days after sowing; dek: defective kernel; EF-1α: Translation elongation factor 1α; emp: empty pericarp; flo: floury endosperm; FSE5: FLOURY SHRUNKEN ENDOSPERM5; GBSSI: Granule bound starch synthase I; GFP: Green fluorescent protein; GUS: β-Glucuronidase; In/Del: Insertion/Deletion; KO: Knock-out lines; MISF: Mitochondrial Intron Splicing Factor; MNU: N-methyl-N-nitrosourea; nad: NADH dehydrogenase subunit; ND: Not detected; NDA: Alternative NADH dehydrogenase A; NDB: Alternative NADH dehydrogenase B; NDC: Alternative NADH dehydrogenase C; ORFs: Open reading frames; PAM: Protospacer-adjacent motif; Porr: Plant organelle RNA recognition; PPR: Pentatricopeptide repeat; qRT-PCR: Quantitative RT-PCR; RPD1: ROOT PRIMORDIUM DEFECTIVE 1; rpl2: ribosomal protein L2; rps3: ribosomal protein S3; SBE: Starch branching enzyme; SSIIa: Starch synthase Ila; TTC: Triphenyl tetrazolium chloride; UTR: Untranslated region; WT: Wild type; WTF: What's this factor; Y2H: Yeast two-hybrid assays

References

1. Bonen L (2008) Cis- and trans-splicing of group II introns in plant mitochondria. Mitochondrion 8:26–34
2. Brown P, Welch R, Cary E (1987) Nickel: A micronutrient essential for higher plants. Plant Physiol 85:801–803
3. Cai M, Li S, Sun F, Sun Q, Zhao H, Ren X, Zhao Y, Tan B, Zhang Z, Qiu F (2017) Emp10 encodes a mitochondrial PPR protein that affects the cis-splicing of nad2 intron 1 and seed development in maize. Plant J 91:132–144
4. Chen S, Tao L, Zeng L, Vega-Sanchez ME, Umemura K, Wang GL (2006) A highly efficient transient protoplast system for analyzing defence gene expression and protein-protein interactions in rice. Mol Plant Pathol 7:417–427
5. Chen X, Feng F, Qi W, Xu L, Yao D, Wang Q, Song R (2017) Dek35 encodes a PPR protein that affects cis-splicing of mitochondrial nad4 intron 1 and seed development in maize. Mol Plant 10:427–441

6. Cheng S, Gutmann B, Zhong X, Ye Y, Fisher MF, Bai F, Castleden I, Song Y, Song B, Huang J, Liu X, Xu X, Lim BL, Bond CS, Yiu SM, Small I (2016) Redefining the structural motifs that determine RNA binding and RNA editing by pentatricopeptide repeat proteins in land plants. Plant J 85:532–547

7. Chettoor AM, Yi G, Gomez E, Hueros G, Meeley RB, Becraft PW (2015) A putative plant organelle RNA recognition protein gene is essential for maize kernel development. J Integr Plant Biol 57:236–346

8. Cohen S, Zmdjuk M, Colas des Francs-Small C, Malik S, Shaya F, Keren I, Belausov E, Many Y, Brown GG, Small I, Ostterset-Biran O (2014) nMAT4, a maturase factor required for nad1 pre-mRNA processing and maturation, is essential for holocomplex I biogenesis in Arabidopsis mitochondria. Plant J 78:253–268

9. Colas des Francs-Small C, Kroeger T, Zmdjuk M, Ostterset-Biran O, Rahimi N, Small I, Barkan A (2012) A PORR domain protein required for rpl2 and ccmFC intron splicing and for the biogenesis of c-type cytochromes in Arabidopsis mitochondria. Plant J 69:996–1005

10. Colas des Francs-Small C, Falcon de Longevialle A, Li Y, Lowe E, Tanz SK, Smith C, Bevan MW, Small I (2014) The pentatricopeptide repeat proteins TANG2 and ORGANELLE TRANSCRIPT PROCESSING439 are involved in the splicing of the multipartite nad5 transcript encoding a subunit of mitochondrial complex I. Plant Physiol 165:1409–1416

11. Dai D, Luan S, Chen X, Wang Q, Feng Y, Zhu C, Qi W, Song R (2018) Maize Dek37 encodes a P-type PPR protein that affects cis-splicing of mitochondrial nad2 intron 1 and seed development. Genetics 208:1069–1082

12. Falcon de Longevialle A, Meyer EH, Andrés C, Taylor NL, Lurin C, Millar AH, Small ID (2007) The pentatricopeptide repeat gene OTP43 is required for trans-splicing of the mitochondrial nad1 intron 1 in Arabidopsis thaliana. Plant Cell 19:3256–3265

13. Gray MW (1999) Evolution of organellar genomes. Curr Opin Genet Dev 9:678–687

14. Gualberto JM, Le Ret M, Beator B, Kühn K (2015) The RAD52-like protein ODB1 is required for the efficient excision of two mitochondrial introns spliced via first-step hydrolysis. Nucleic Acids Res 43:6500–6510

15. Haiili N, Planchard N, Amal N, Quadrado M, Vrielynck N, Dahan J, Colas des Francs-Small C, Mireau H (2016) The MTL1 pentatricopeptide repeat protein is required for both translation and splicing of the mitochondrial NADH DEHYDROGENASE SUBUNIT7 mRNA in Arabidopsis. Plant Physiol 170:354–366

16. Hammani K, Giege P (2014) RNA metabolism in plant mitochondria. Trends Plant Sci 19:380–389

17. He J, Duan Y, Hua D, Fan G, Wang L, Liu Y, Chen Z, Han L, Qu LJ, Gong Z (2012) DEXH box RNA helicase-mediated mitochondrial reactive oxygen species production in Arabidopsis mediates crosstalk between abscisic acid and auxin signaling. Plant Cell 24:1815–1833

18. Hsieh WY, Liao JC, Chang CY, Harrison T, Boucher C, Hsieh MH (2015) The SLOW GROWTH3 pentatricopeptide repeat protein is required for the splicing of mitochondrial NADH dehydrogenase
subunit7 intron 2 in Arabidopsis. Plant Physiol 168:490–501
19. Hsu YW, Wang HJ, Hsieh MH, Hsieh HL, Jauh GY (2014) Arabidopsis mTERF15 is required for
mitochondrial nad2 intron 3 splicing and functional complex I activity. PLoS One 9:e112360
20. Hsu YW, Juan CT, Wang CM, Jauh GY (2019) Mitochondrial heat shock protein 60 s interact with
what's this factor 9 to regulate RNA splicing of ccmFC and rpl2. Plant Cell Physiol 60:116–125
21. Kang HG, Park S, Matsuoka M, An G (2005) White-core endosperm floury endosperm-4 in rice is
generated by knockout mutations in the C4-type pyruvate orthophosphate dikinase gene (OsPPDKB).
Plant J 42:901–911
22. Keren I, Bezawork-Geleta A, Kolton M, Maayan I, Belausov E, Levy M, Mett A, Gidoni D, Shaya F,
Ostersetzer-Biran O (2009) AtnMat2, a nuclear-encoded maturase required for splicing of group-II
introns in Arabidopsis mitochondria. RNA 15:2299–2311
23. Keren I, Tal L, Colas des Francs-Small C, Araújo WL, Shevtsov S, Shaya F, Fernie AR, Small I,
Ostersetzer-Biran O (2012) nMAT1, a nuclear-encoded maturase involved in the trans-splicing of
nad1 intron 1, is essential for mitochondrial complex I assembly and function. Plant J 71:413–426
24. Köhler D, Schmidt-Gattung S, Binder S (2010) The DEAD-box protein PMH2 is required for efficient
group II intron splicing in mitochondria of Arabidopsis thaliana. Plant Mol Biol 72:459–467
25. Konishi M, Sugiyama M (2006) A novel plant-specific family gene, ROOT PRIMORDIUM DEFECTIVE 1,
is required for the maintenance of active cell proliferation. Plant Physiol 140:591–602
26. Koprivova A, Colas des Francs-Small C, Calder G, Mugford ST, Tanz S, Lee BR, Zechmann B, Small I,
Kopriva S (2010) Identification of a pentatricopeptide repeat protein implicated in splicing of intron 1
of mitochondrial nad7 transcripts. J Biol Chem 285:32192–32199
27. Kroeger TS, Watkins KP, Friso G, van Wijk KJ, Barkan A (2009) A plant-specific RNA-binding domain
revealed through analysis of chloroplast group II intron splicing. Proc Natl Acad Sci U S A 106:4537–
4542
28. Kubo T, Newton KJ (2008) Angiosperm mitochondrial genomes and mutations. Mitochondrion 8:5–14
29. Kühn K, Carrie C, Giraud E, Wang Y, Meyer EH, Narsai R, Colas des Francs-Small C, Zhang B, Murcha
MW, Whelan J (2011) The RCC1 family protein RUG3 is required for splicing of nad2 and complex I
biogenesis in mitochondria of Arabidopsis thaliana. Plant J 67:1067–1080
30. Lasda EL, Blumenthal T (2011) Trans-splicing. Wiley Interdiscip Rev-RNA 2:417–434
31. Li C, Liang D, Li J, Duan Y, Li H, Yang Y, Qin R, Li L, Wei P, Yang J (2013) Unravelling mitochondrial
retrograde regulation in the abiotic stress induction of rice ALTERNATIVE OXIDASE 1 genes. Plant Cell
Environ 36:775–788
32. Liu Y, He J, Chen Z, Ren X, Hong X, Gong Z (2010) ABA overly-sensitive 5 (ABO5), encoding a
pentatricopeptide repeat protein required for cis-splicing of mitochondrial nad2 intron 3, is involved in
the abscisic acid response in Arabidopsis. Plant J 63:749–765
33. Luhua S, Ciftci-Yilmaz S, Harper J, Cushman J, Mittler R (2008) Enhanced tolerance to oxidative stress in transgenic Arabidopsis plants expressing proteins of unknown function. Plant Physiol 148:280–292

34. Lurin C, Andrés C, Aubourg S, Bellaoui M, Bitton F, Bruyère C, Caboche M, Debast C, Gualberto J, Hoffmann B, Lecharny A, Le Ret M, Martin-Magniette ML, Mireau H, Peeters N, Renou JP, Szurek B, Taconnat L, Small I (2004) Genome-wide analysis of Arabidopsis pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell 16:2089–2103

35. Nakagawa N, Sakurai N (2006) A mutation in At-nMat1a, which encodes a nuclear gene having high similarity to group II intron maturase, causes impaired splicing of mitochondrial nad4 transcript and altered carbon metabolism in Arabidopsis thaliana. Plant Cell Physiol 47:772–783

36. O'Toole N, Hattori M, Andres C, Iida K, Lurin C, Schmitz-Linneweber C, Sugita M, Small I (2008) On the expansion of the pentatricopeptide repeat gene family in plants. Mol Biol Evol 25:1120–1128

37. Peng C, Wang Y, Liu F, Ren Y, Zhou K, Lv J, Zheng M, Zhao S, Zhang L, Wang C, Jiang L, Zhang X, Guo X, Bao Y, Wan J (2014) FLOURY ENDOSPERM6 encodes a CBM48 domain-containing protein involved in compound granule formation and starch synthesis in rice endosperm. Plant J 77:917–930

38. Qi W, Yang Y, Feng X, Zhang M, Song R (2017) Mitochondrial function and maize kernel development requires Dek2, a pentatricopeptide repeat protein involved in nad1 mRNA splicing. Genetics 205:239–249

39. Ren X, Pan Z, Zhao H, Zhao J, Cai M, Li J, Zhang Z, Qiu F (2017) EMPTY PERICARP11 serves as a factor for splicing of mitochondrial nad1 intron and is required to ensure proper seed development in maize. J Exp Bot 68:4571–4581

40. Ren Z, Fan K, Fang T, Zhang J, Yang L, Wang J, Wang G, Liu Y (2019) Maize Empty Pericarp602 encodes a P-Type PPR protein that is essential for seed development. Plant Cell Physiol 60:1734–1746

41. Ren R, Wang L, Zhang L, Zhao Y, Wu J, Wei Y, Zhang X, Zhao X (2020) DEK43 is a P-type PPR protein responsible for the cis-splicing of nad4 in maize mitochondria. J Integr Plant Biol 62:299–313

42. Sharp PA (1987) Trans splicing: variation on a familiar theme? Cell 50:147–148

43. She KC, Kusano H, Koizumi K, Yamakawa H, Hakata M, Imamura T, Fukuda M, Naito N, Tsurumaki Y, Yaeshima M, Tsuge T, Matsumoto K, Kudoh M, Itoh E, Kikuchi S, Kishimoto N, Yazaki J, Ando T, Yano M, Aoyama T, Sasaki T, Satoh H, Shimada H (2010) A novel factor FLOURY ENDOSPERM2 is involved in regulation of rice grain size and starch quality. Plant Cell 22:3280–3294

44. Shikanai T, Fujii S (2013) Function of PPR proteins in plastid gene expression. RNA Biol 10:1446–1456

45. Small ID, Peeters N (2000) The PPR motif: a TPR-related motif prevalent in plant organellar proteins. Trends Biochem Sci 25:46–47

46. Small ID, Rackham O, Filipovska A (2013) Organelle transcriptomes: products of a deconstructed genome. Curr Opin Microbiol 16:652–658
47. Sun F, Zhang X, Shen Y, Wang H, Liu R, Wang X, Gao D, Yang Y, Liu Y, Tan B (2018) EMPTY PERICARP8 is required for the splicing of three mitochondrial introns and seed development in maize. Plant J 95:919–932

48. Sun F, Xiu Z, Jiang R, Liu Y, Zhang X, Yang Y, Li X, Zhang X, Wang Y, Tan B (2019) The mitochondrial pentatricopeptide repeat protein EMP12 is involved in the splicing of three *nad2* introns and seed development in maize. J Exp Bot 70:963–972

49. Teng X, Zhong M, Zhu X, Wang C, Ren Y, Wang Y, Zhang H, Jiang L, Wang D, Hao Y, Wu M, Zhu J, Zhang X, Guo X, Wang Y, Wan J (2019) *FLOURY ENDOSPERM16* encoding a NAD-dependent cytosolic malate dehydrogenase plays an important role in starch synthesis and seed development in rice. Plant Biotechnol J 17:1914–1927

50. Timmis JN, Ayliffe MA, Huang CY, Martin W (2004) Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes. Nat Rev Genet 5:123–135

51. Toda T, Fujii S, Noguchi K, Kazama T, Toriyama K (2012) Rice *MPR25* encodes a pentatricopeptide repeat protein and is essential for RNA editing of *nad5* transcripts in mitochondria. Plant J 72:450–460

52. Wang C, Aubé F, Planchard N, Quadrado M, Dargel-Graffn C, Nogué F, Mireau H (2017) The pentatricopeptide repeat protein MTSF2 stabilizes a *nad1* precursor transcript and defines the 3 end of its 5-half intron. Nucleic Acids Res 45:6119–6134

53. Wang C, Aubé F, Quadrado M, Dargel-Graffn C, Mireau H (2018) Three new pentatricopeptide repeat proteins facilitate the splicing of mitochondrial transcripts and complex I biogenesis in Arabidopsis. J Exp Bot 69:5131–5140

54. Wittig I, Braun HP, Schägger H (2006) Blue native PAGE. Nat Protoc 1:418–428

55. Wu M, Ren Y, Cai M, Wang Y, Zhu S, Zhu J, Hao Y, Teng X, Zhu X, Jing R, Zhang H, Zhong M, Wang Y, Lei C, Zhang X, Guo X, Cheng Z, Lin Q, Wang J, Jiang L, Bao Y, Wang Y, Wan J (2019) Rice *FLOURY ENDOSPERM10* encodes a pentatricopeptide repeat protein that is essential for the *trans*-splicing of mitochondrial *nad1* intron 1 and endosperm development. New Phytol 223:736–750

56. Xiu Z, Sun F, Shen Y, Zhang X, Jiang R, Bonnard G, Zhang J, Tan B (2016) EMPTY PERICARP16 is required for mitochondrial *nad2* intron 4 *cis*-splicing, complex I assembly and seed development in maize. Plant J 85:507–519

57. Yang L, Zhang J, He J, Qin Y, Hua D, Duan Y, Chen Z, Gong Z (2014) ABA-mediated ROS in mitochondria regulate root meristem activity by controlling *PLETHORA* expression in Arabidopsis. PLoS Genet 10:e1004791

58. Yang Y, Ding S, Wang Y, Wang H, Liu X, Sun F, Xu C, Liu B, Tan B (2020) PPR20 is required for the *cis*-splicing of mitochondrial *nad2* intron 3 and seed development in maize. Plant Cell Physiol 61:370–380

59. You X, Zhang W, Hu J, Jing R, Cai Y, Feng Z, Kong F, Zhang J, Yan H, Chen W, Chen X, Ma J, Tang X, Wang P, Zhu S, Liu L, Jiang L, Wan J (2019) *FLOURY ENDOSPERM15* encodes a glyoxalase I involved in compound granule formation and starch synthesis in rice endosperm. Plant Cell Rep 38:345–359
60. Zhang L, Ren Y, Lu B, Yang C, Feng Z, Liu Z, Chen J, Ma W, Wang Y, Yu X, Wang Y, Zhang W, Wang Y, Liu S, Wu F, Zhang X, Guo X, Bao Y, Jiang L, Wan J (2016) FLOURY ENDOSPERM7 encodes a regulator of starch synthesis and amyloplast development essential for peripheral endosperm development in rice. J Exp Bot 67:633–647

61. Zhu X, Teng X, Wang Y, Hao Y, Jing R, Wang Y, Liu Y, Zhu J, Wu M, Zhong M, Chen X, Zhang Y, Zhang W, Wang C, Wang Y, Wan J (2018) FLOURY ENDOSPERM11 encoding a plastid heat shock protein 70 is essential for amyloplast development in rice. Plant Sci 277:89–99

62. Zhu C, Jin G, Fang P, Zhang Y, Feng X, Tang Y, Qi W, Song R (2019) Maize pentatricopeptide repeat protein DEK41 affects cis-splicing of mitochondrial nad4 intron 3 and seed development. J Exp Bot 70:3795–3808

Supplementary Information

Additional file 1:

Figure S1. Homozygous fse5 seeds were produced by a heterozygous plant (+/fse5). Segregation of grains with normal vitreous and floury (black, red arrows) kernels occurred among all seeds harvested from a single heterozygous plant (+/fse5) and was viewed by an X-ray viewer (PD-HA). Bar, 1 cm.

Figure S2. Phenotypes of seeds and seedlings of WT and fse5 mutant. A-D, 1000-grain weight (A), length (B), width (C) and thickness (D) of mature WT and fse5 grains. E-H, Total starch (E), amylose (F), protein (G) and lipid (H) contents of mature WT and fse5 seeds. I, Germination percentages for WT and fse5 seeds at 7 DAS in culture dishes. J, Percentages of seedlings grown from WT and fse5 seeds at 9 DAS in soil. K, Heights of 9-day-old seedlings grown from WT and fse5 seeds. Values are means ± SD. **P < 0.01, Student’s t tests.

Figure S3. OsPORR1 knock-out lines generated by CRISPR/Cas9. A, Knockout target site on the genomic sequence of OsPORR1. B, Target sequences of OsPORR1 allele in four independent positive lines in Nipponbare. Single nucleotide insertions (red letters, A, T and G) occurred in KO-140, KO-155 and KO-172 and a 32-nucleotide deletion (red dotted line) was present in KO-174. Black box indicates the protospacer-adjacent motif (PAM) sequence. C, Appearance (upper panel) and transverse sections (lower panel) of seeds from Nipponbare and knock-out lines. Bars, 1 mm.

Figure S4. GUS staining of various tissues from a ProOsPORR1:GUS transgenic plant. Left-to-right, young seedling, root, stem, leaf, leaf sheath, panicle and developing seed. The promoter of OsPORR1 was fused to vector pCAMBIA1381Z and introduced into Nipponbare via Agrobacterium tumefaciens transformation. GUS staining was performed as described previously (Zhang and Muench, 2015). Bars, 2 mm.

Figure S5. qRT-PCR analysis of 23 mitochondrial introns. Primers spanning adjacent exons were used to amplify fragments of mature mitochondrial transcripts (upper panel) and primers spanning adjacent exons and introns were used to amplify fragments of mitochondrial precursor mRNA (lower panel) (Cai et
The results of *nad4* exon 1-exon 2 (spliced fragment) and its precursor fragment are indicated by a black box. ND, not detected. Three biological replicates were performed in qRT-PCR and *OsActin1* was used as an internal control for data normalization. Values are means ± SD.

**Figure S6.** Immunoblot analysis of major starch synthases. Total proteins were extracted from developing WT and *fse5* seeds at 15 DAF and separated by SDS-PAGE (Takemoto et al., 2002; Wang et al., 2010). EF-1α, translation elongation factor 1α encoded by *Os03g0177400* served as the loading control. GBSSI, Granule bound starch synthase I. SSIIa, Starch synthase IIa. SBE, Starch branching enzyme; AGPS2b, ADP-glucose pyrophosphorylase 2b; AGPL2, ADP-glucose pyrophosphorylase large subunit 2.

**Figure S7.** Multiple sequence alignment of PORR domains in OsPORR1, ZmWTF1 and AtWTF9. ZmWTF1, *GRMZM2G403797*. AtWTF9, *At2g39120*.

**Figure S8.** Yeast two-hybrid assays showing that MISF68 interacts with the splicing factors of *nad4* intron 1. Empty-AD and Empty-BK were used as controls. Full-length cDNAs of *OsABO6* (*LOC_Os01g02884*), *OsDEK35* (*LOC_Os03g50500*), *OsDEK43* (*LOC_Os05g11700*), *OsEMP8* (*LOC_Os08g41380*), *OsMISF68* (*LOC_Os02g16650*) and *OsPORR1*, were cloned into pGADT7 or pGBK7 vectors. The yeast transformation and screening procedures were performed according to the manufacturer's instructions (TaKaRa Bio, Kusatsu, Japan).

**Table S1.** Segregation of vitreous and floury grains from seven heterozygous plants (*+/fse5*).

**Table S2.** Primers used for mapping.

**Table S3.** Primers used for vector construction.

**Table S4.** Primers used for splicing analysis.

**Table S5.** Primers used for qRT-PCR analysis.

**Figures**
Figure 1

Phenotypes of mature and developing seeds of WT and fse5 mutant. A and B, Mature WT (A) and fse5 (B) seeds. Bars, 1 mm. C and D, Transverse sections of mature WT (C) and fse5 (D) seeds. Bars, 0.5 mm. E-H, Scanning electron microscopy of transverse sections of mature WT (E, G) and fse5 (F, H) seeds. Bars, 100 μm in (E) and (F), 10 μm in (G) and (H). I-L, Iodine stained semi-thin endosperm sections of developing WT (I, K) and fse5 (J, L) seeds at 12 days after flowering (DAF). Bars, 15 μm in (I) and (J); 7.5 μm in (K) and (L).
Figure 2

Seed germination and seedling phenotypes. A and B, Germinating seedlings of WT (A) and fse5 mutant (B) at 3 days after sowing (DAS). Bars, 5 mm. C and D, Seedlings grown from WT (left) and fse5 (right) seeds at 9 (C) and 30 (D) days after sowing (DAS). Bars, 5 cm in (C) and 10 cm in (D). E and F, Longitudinal sections of mature WT (E) and fse5 (F) seeds after 24 h of imbibition. Bars, 1 mm. G and H, TTC staining of WT (G) and fse5 (H) seeds. Black arrows indicate embryos after staining. Bars, 1 cm.
Figure 3

Positional cloning of the FSE5 locus. A, The FSE5 locus was restricted to a 54 kb region flanked by markers wi-10 and wi-11 on chromosome 9 (Chr. 9) and included nine open reading frames (ORFs). Both markers and numbers of recombinants are shown. B, Mutation site in LOC_Os09g29760 in the fse5 mutant. A deleted 408 bp fragment near the end of the exon leads to a 136 aa truncation in the putative encoded protein in the fse5 mutant. Red letters and dotted lines represent the deletion in LOC_Os09g29760 or its encoded protein in the fse5 mutant. The yellow and orange boxes indicate the 5’ untranslated region (UTR) and single exon of LOC_Os09g29760 respectively. C, Expression of LOC_Os09g29760 in WT, fse5 mutant and complemented lines (COM). The primer pair designed within the 408 bp deletion was used in qRT-PCR. Three biological replicates were performed in qRT-PCR. OsActin1 was used as an internal control for data normalization. Values are means ± SD. ND, not
detected. D, Appearance of seeds (upper panel) and transverse sections (lower panel) of WT, fse5 mutant and COM. Bars, 1 mm. E, Nine-day-old seedlings of WT, fse5 mutant and COM. Bar, 5 cm.

Figure 4
OsPORR1 domain and its phylogenetic analysis. A, Representation of the deleted 408 bp fragment in the region 664-1071 bp of the OsPORR1 coding sequence CDS. B, The PORR domain is the region 65-385 aa of the OsPORR1 sequence. The deleted 136 aa fragment is in the region from the 222-357 aa, within the PORR domain. M, methionine. C, Phylogenetic analysis of all PORR family members in rice. Black asterisk shows OsPORR1. D, Multiple sequence alignment of 17 rice PORR proteins. The numbers over arrows represent the positions of six highly conserved amino acids (Leu172, Glu305, Leu307, Phe344, Tyr345, Leu357).
Figure 5

Spatiotemporal expression and subcellular localization of OsPORR1. A, Expression of OsPORR1 in various plant organs, developing seeds and seedlings at different times. DAG, days after germination. Three biological replicates were performed in qRT-PCR. OsActin1 was used as an internal control for data normalization. Values are means ± SD. B, Subcellular localization of OsPORR1. GFP was served as a control. GFP signals in protoplasts isolated from positive transgenic seedlings were observed by confocal laser scanning microscopy. Mitotracker, MitoTrackerTM Orange CMTMRos, was used to identify mitochondria. Bars, 10 μm.
Figure 6

Splicing of nad4 intron 1 was abolished in the fse5 mutant. Total RNA was extracted from nine-day-old seedlings and random hexamer primers were used to synthetize the first-strand cDNA. A, Transcript analysis of mitochondrial intron-containing genes in WT (left) and fse5 mutant (right). OsActin1 was used as the internal control. Specific primers were used to amplify 9 transcripts (Wu et al., 2019). B, Schematic of nad4 precursor mRNA and RT-PCR analysis of three introns of nad4. nad4 precursor mRNA includes 4 exons (E1-E4) and 3 introns. Black boxes indicate exons and curved lines represent introns. a, b, c, and m are fragments amplified by exon-exon flanking primers (Chen et al., 2017). Numbers of nucleic acids included by the introns and fragments are noted. M, DNA marker.
Figure 7

Mitochondrial function and structure disrupted in cells of the fse5 mutant. A, Mitochondrial complexes separated by blue native polyacrylamide gel electrophoresis (BN-PAGE) and stained by Coomassie Brilliant Blue (CBB) (left) and in-gel NADH dehydrogenase activity of Complex I (arrow) (right). B and C, Measurement of ATP contents (B) and respiration rates (C). Values are means ± SD. **P < 0.01, Student’s t tests. D, Mitochondria in developing endosperm cells of WT and fse5 mutant at 9 DAF. Bars, 0.5 μm. E, Expression of alternative oxidase genes (AOX1a, AOX1b and AOX1c) and NADH dehydrogenase genes (NDA1, NDA2, NDB1, NDB2, NDB3, NDC1) in nine-day-old seedlings. Specific primers were used in qRT-PCR (Toda et al., 2012; Li et al., 2013). Data are based on three biological replicates. OsActin1 was used as an internal control for data normalization. Values are means ± SD.

Supplementary Files

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