The nuclear-localized PPR protein OsNPPR1 is important for mitochondrial function and endosperm development in rice

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Abstract

Pentatricopeptide repeat (PPR) proteins constitute one of the largest protein families in land plants. Recent studies revealed the functions of PPR proteins in organellar RNA metabolism and plant development, but the functions of most PPR proteins, especially PPRs localized in the nucleus, remain largely unknown. Here, we report the isolation and characterization of a rice mutant named floury and growth retardation1 (fgr1). fgr1 showed floury endosperm with loosely arranged starch grains, decreased starch and amylose contents, and retarded seedling growth. Map-based cloning showed that the mutant phenotype was caused by a single nucleotide substitution in the coding region of Os08g0290000. This gene encodes a nuclear-localized PPR protein, which we named OsNPPR1, that affected mitochondrial function. In vitro SELEX and RNA-EMSAs showed that OsNPPR1 was an RNA protein that bound to the CUCAC motif. Moreover, a number of retained intron (RI) events were detected in fgr1. Thus, OsNPPR1 was involved in regulation of mitochondrial development and/or functions that are important for endosperm development. Our results provide novel insights into coordinated interaction between nuclear-localized PPR proteins and mitochondrial function.

Keywords: Floury endosperm, mitochondrial function, nuclear localization, pentatricopeptide repeat protein, retained intron, rice.

Introduction

PPR proteins are characterized by the presence of tandem arrays of degenerate 35 amino acid repeats. Most PPR proteins are present in eukaryotes, and only a few in bacteria via transfer from eukaryotic hosts (Small and Peeters, 2000; Lurin et al., 2004; O’Toole et al., 2008; Barkan and Small, 2014). PPR proteins are involved in post-transcriptional processes in organelles through sequence-specific recognition of their RNA substrates. They bind RNA in a parallel orientation via a modular recognition mechanism, with nucleotide specificity relying primarily on the amino acid identities at position 6 in
each repeat and the following repeat 1’ (Barkan et al., 2012; Yin et al., 2013; Cheng et al., 2016). The recognition mechanism has successfully predicted the binding sites of some PPR proteins (Takenaka et al., 2013; Yagi et al., 2013).

Proteins in the PPR family consist of two major subfamilies, defined as P and PLS. P-class proteins contain arrays of P motifs comprising 35 amino acids, whereas PL-class proteins are composed of repetitions of P–L–S motif triplets, where L motifs are 35–36 amino acids and S motifs are 31 amino acids. Each motif has a specific pattern of amino acid conservation (Lurin et al., 2004; O’Toole et al., 2008). P-class proteins are generally involved in RNA stabilization (Beick et al., 2008; Pfälz et al., 2009; Johnson et al., 2010; Cai et al., 2011; Zhang et al., 2017), RNA cleavage (Höhlze et al., 2011; Jonietz et al., 2011), translational activation (Schmitz-Linneweber et al., 2005; Zoschke et al., 2012), and RNA splicing (Schmitz-Linneweber et al., 2006; de Longeville et al., 2007, 2008; Khrouchchova et al., 2012; Chen et al., 2017; Dai et al., 2018). PL-class proteins mainly function in RNA editing (Barkan and Small, 2014). In addition, some P-class proteins contain a C-terminal small MutS-related (SMR) domain, which may confer RNA endonuclease activity (S. Liu et al., 2013; Zoschke et al., 2016; Zhou et al., 2017).

According to the assignment results of the TargetP and Predotar programs, most PPR proteins were predicted to be localized in mitochondria and/or plastids to affect multiple aspects of organellar gene expression (Lurin et al., 2004), which was subsequently confirmed experimentally (Schmitz-Linneweber and Small, 2008; Barkan and Small, 2014). PPR mutants express a range of different phenotypes. Mutants of chloroplast-localized PPRs show photosynthetic defects (Barkan et al., 1994; Hashimoto et al., 2003; Kotera et al., 2005; Cai et al., 2009; Johnson et al., 2010; Khrouchchova et al., 2012), aberrant leaf development (Petricka et al., 2008), and changes in leaf pigmentation in various crop species (Chatteignier-Bou tin et al., 2008; Yu et al., 2009; Zhou et al., 2009; Liu et al., 2010). In rice, this type of mutant generally develops lethal albino seedlings (Lin et al., 2015; D. Wang et al., 2016; Wu et al., 2016; Tang et al., 2017), or has white-striped leaves (Tan et al., 2014; Y. Wang et al., 2017). Mitochondrial-localized PPR mutants show restricted growth (Sung et al., 2010; Yuan and Liu, 2012), and defective seed development (Gutierrez-Marcos et al., 2007; Kim et al., 2009; Manavski et al., 2012; Sosso et al., 2012; Y. J. Liu et al., 2013). These mutants also generally result in floury/defective endosperm and retarded growth in rice (Kim et al., 2009; Toda et al., 2012; Y. J. Liu et al., 2013; Li et al., 2014).

In addition to mitochondrial and chloroplast localization, three PPR proteins (PNM1, GRP23, and SOAR1) in Arabidopsis have been localized in the nuclear (Ding et al., 2006; Hammani et al., 2011; Mei et al., 2014). PNM1 and GRP23 play essential roles in embryogenesis, whereas SOAR1 functions as a negative regulator of abscisic acid (ABA) signaling. The interactions of GRP23 with RNA polymerase II subunit III and of PNM1 with transcription factors TCP8 and NAP1 indicate that PPR proteins in the nuclear affect transcription and post-transcriptional processing of nuclear mRNA. Despite these studies, the functions of nuclear-localized PPR proteins remain largely unclear.

To identify novel factors involved in starch biosynthesis and/or endosperm development, the floury and growth retardation1 (fgr1) mutant was obtained. Map-based cloning revealed that a nuclear-localized PPR protein (OsNPPR1) was mutated in fgr1. OsNPPR1 was shown to regulate mitochondrial behavior. RNA sequencing (RNA-seq) analysis identified several genes with an altered splicing pattern. Our results provided evidence that nuclear-localized PPR proteins regulate normal functioning of organelles, especially mitochondria.

### Materials and methods

#### Plant materials and growth conditions

All plants were grown in a paddy field at Nanjing Agricultural University (118°46′E, 32°03′N) during the normal growing season or in a growth chamber (12 h of light/12 h of darkness at 30 °C). Developing seeds at 6–21 days after flowering (DAF) and mature seeds were harvested for biochemical and electron microscopic studies.

#### Microscopy

Mature seeds were prepared for SEM (Hitachi S-3000N, Tokyo) as described previously (Peng et al., 2014). Semi-thin sections of 6 DAF endosperm of wild-type, fgr1 mutant, and complementation lines were prepared as described (Peng et al., 2014). Samples were examined under a Nikon Eclipse 80i (Nikon, Tokyo) light microscope. TEM analysis of the mitochondrial structure of 6 DAF wild-type and fgr1 developing endosperm was performed as described previously (Takemoto et al., 2002; Wang et al., 2010). Observations were carried out with a Hitachi H-7650 microscope.

#### Mapping and complementation analysis

The fgr1 mutant was generated by 1 mM N-methyl-N-nitrosourea (MNU) treatment of N22 (Oryza sativa L., indica). An F2 population showing a floury endosperm phenotype for mapping was produced from a cross between fgr1 and Nipponbare (O. sativa L. ssp. japonica).

The OsNPPR1 coding sequence with a flag tag under the control of the Cauliflower mosaic virus (CaMV) 35S promoter (p35S:Os08g0290000-flag) was used for Agrobacterium tumefaciens- (strain EHA105) mediated transformation. Following sequence analysis, OsNPPR1 homologs were identified using the BLASTP search program of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). A Neighbor-Joining tree was constructed using MEGA 5.0 (http://www.megasoftware.net).

#### RNA isolation and real-time reverse transcription–PCR (RT–PCR)

Total RNA was extracted from roots, stems, leaves, sheaths, panicles, and 6–21 DAF endosperm using an RNAprep Pure Plant Kit (Tiangen Biotech, Beijing, China). First-strand cDNA was synthesized with 4 μg of total RNA by priming with a random primer (TaKaRa) for mitochondrial- and chloroplast-encoded genes or oligo(dT) (TaKaRa) for other genes in 20 μl reaction volumes with the PrimeScript Reverse Transcriptase Kit (TaKaRa, http://www.takara-bio.com). Real-time PCR was then performed. Each analysis was conducted with three biological replicates.

#### β-Glucuronidase (GUS) staining

A 2 kb promoter fragment upstream of the OsNPPR1 ATG start codon was introduced into the pCAMBIA1381Z vector which was then transformed into Nipponbare. GUS staining was performed as described (Zheng et al., 2015).
Subcellular localization of OsNPPR1 protein

The FGR1 sequence was cloned in-frame in the pAN580-GFP (green fluorescent protein) vector to create a fusion construct under control of the CaMV 35S promoter. Four GFP fusion constructs were generated and transformed into the rice protoplasts. FGR1–GFP and GFP–FGR1, containing a full-length FGR1 coding region in front of, and behind, the GFP, respectively, and N181aa–GFP containing only the N-terminal region (amino acids 1–181, in front of the GFP) repeat of FGR1 upstream of the CaMV 35S promoter were transiently expressed in rice protoplasts following Y. Wang et al. (2016). FGR1 was fused with the N-terminus of GFP in the binary vector pCAMBIA1305-GFP and the construct was introduced into the A. tumefaciens strain EHA105 and used to infiltrate Nigtitana benthamiana leaves as described previously (Peng et al., 2014). GFP alone was employed as the control, and the mCherry-tagged rice D53 (D53-mCherry) vector was used as a nuclear marker (Zhou et al., 2013). Confocal imaging was performed using a Zeiss LSM780 laser scanning confocal microscope.

Blue native-PAGE (BN-PAGE)

BN-PAGE was conducted as previously reported (Wittig et al., 2006). A 1 g aliquot of 6-day-old seedlings of the wild type and fgr1 were cut into pieces and ground into a paste in a mitochondrial extraction buffer (75 mM MOPS-KOH pH 7.6, 0.6 M sucrose, 4 mM EDTA, 0.2% polyvinylpyrrolidone 40, 8 mM cysteine, and 0.2% BSA). After centrifugation twice through Miracloth (Millipore), the filtrate was centrifuged at 20,000 g, 4 °C, 20 min, and resuspended in buffer (10 mM MOPS-KOH pH 7.2, 0.3 M sucrose). After centrifuging at 22,000 g for 30 min, the pellet was re-suspended in membrane extraction buffer (50 mM imidazole-HCl pH 7.0, 500 mM 6-aminohexanoic acid, 1 mM EDTA, and 1% Triton X-100), and gently mixed on ice for 30 min. After 15 min of centrifugation at 22,000 g at 4 °C, the supernatant was collected, and added to loading buffer (5% Coomassie blue G-250, 20 mM imidazole-HCl pH 7.0, 500 mM 6-aminohexanoic acid). A 25 μl aliquot of mitochondrial protein was loaded onto a 3–12% gradient gel to analyze NADH complex I activity in detection buffer [0.02 M NADH, 1 mM nitroblue tetrazolium (NBT), 0.05 M MOPS-KOH pH 7.6] in conjunction with Coomassie blue staining.

Measurement of ATP content, mitochondrial membrane potential, and respiratory rate

Mitochondrial membrane potential was measured using protoplasts isolated from 9-day-old seedlings of N22 and the fgr1 mutant grown in darkness. Intact protoplasts were suspended in JC-1 buffer supplied by the JC-1 kit (Beyotime, China) and analyzed on a Multifunctional Microplate Reader.

Total respiration rate was measured by a liquid-phase oxygen electrode (Hansatech, UK) using 9-day-old seedlings of N22 and the fgr1 mutant grown in darkness.

ATP contents were measured by the ATP assay kit (Beyotime, China) using 9-day-old seedlings grown in darkness and the 6 DAF endosperm of wild-type N22 and the fgr1 mutant.

Measurement of contents of metabolic components

The contents of metabolic components were measured using endosperm at ~6 DAF of wild-type N22 and the fgr1 mutant by Bioprofile (Shanghai, China), with three repeats.

RNA-SELEX

SELEX was performed as described by Zhang and Muench (2015) with minor modification. Oligonucleotides harboring a 40 bp random sequence surrounded by primer-binding sites [5'-GGGAAGATCTCG ACCAGAG(N)n-TATGTGCGTCTACATGGATCCTCA-3'] were synthesized and amplified by PCR using a forward primer containing the T7 promoter sequence and reverse primer (SEL-F, 5'-CGGAAATT CTAATACGACTCACTATAGGGGAAGATCTCGACCAGAG-3'; SEL-R, 5'-TGAGGATCCATGTAGACGCACATA-3') under the following conditions: 15 cycles of 10 s at 98 °C, 30 s at 57 °C, and 30 s at 72 °C. ddDNA was separated by electrophoresis on a 1% agarose gel and the 119 bp fragment was eluted. After removing RNase, the RNA pools were transcribed from 1 μg of ddDNA template using a T7 riboMAX™ express large-scale RNA production system (Promega). The RNA pools were combined with the purified OsNPPR1 repeat–maltose-binding protein (MBP) fusion protein together with MBP amylose resin (New England BioLabs) in a reaction buffer (20 mM Tris–HCl pH 7.4, 200 mM NaCl, 1 mM EDTA, and 1 mM DTT). After gentle shaking at 4 °C for 1 h, RNAs were extracted with 25:24:1 phenol:chloroform:i-soamyl and reverse transcribed with a PrimeScript Reverse Transcription Kit (TaKaRa) using the reverse primer. cDNA was used for PCR with SEL-F and SEL-R for 15 cycles. The PCR product was used for the next round in the selection procedure. This process was repeated 10 times before the final products were subcloned into the pEASY-Blunt Cloning vector (TransGen Biotech, Beijing, China). A consensus motif was generated by MEME (http://meme-suite.org/) after sequencing 42 unique clones in a tenth SELEX cycle.

RNA-EMSA

RNA-EMSA was performed using a Thermo Fisher LightShift™ Chemiluminescent RNA EMSA Kit (No. 20118). Probes were synthesized by Gene-script (http://www.genescrit.com.cn/) labeled with biotin at the 5′ end: probe, CUAACCUACCUCACCUCCUCACCUCCUCAC; control probe, GAUCAGAUCAGAUCAGAUCAGAUCA.

High-throughput RNA sequencing

RNA was extracted from 6 DAF endosperm of the wild type and fgr1 followed by sequencing by a BGISEQ-500 instrument (BGI). The raw sequence data were collected and filtered. After read filtering, 87.48% of reads were mapped, and the uniformity of the mapping result for each sample suggested that the samples were comparable. Differentially expressed genes (DEGs) between N22 and fgr1 were identified based on gene expression. Gene Ontology (GO) classification and functional enrichment were performed to classify the DEGs. GO has three ontologies: molecular biological function, cellular component, and biological processes. P-values <0.01 in each category are listed in Supplementary Table S1 at JXB online. Differently spliced genes (DSGs) are characterized by alternative splicing (AS), which allows the translation of a variety of isoforms from a single gene. Twenty-two genes with the retained intron (RI) in the fgr1 mutant compared with the wild type were identified and were selected from the RNA-seq results (Supplementary Table S3). The Integrative Genomics Viewer (IGV) tool was used to review the mapping results.

Results

The fgr1 mutant has opaque endosperm and slightly retarded seedling growth

As part of an effort to identify novel factors involved in starch biosynthesis and/or endosperm development, the fgr1 mutant was obtained. The fgr1 mutant exhibited significantly slower grain filling after fertilization (Fig. 1A) and the mature grain weight was dramatically reduced (Fig. 1B). The endosperm in the mutant produced an opaque grain appearance (Fig. 1D–G), suggesting abnormal starch biosynthesis during seed development. Compared with the wild type, the grain thickness of fgr1 was decreased, but the grain length and width were not affected (Fig. 1C). The germination of seeds from
the fgr1 mutant was 2 d later than those from the wild type (Fig. 1H, I), and juvenile leaves and roots were significantly shorter at 9 d post-germination (Fig. 1J, K). Mutant plants gradually recovered and grew into normal adult plants (data not shown). Thus, the mutation led to delayed seed germination, slightly retarded seedling growth, and abnormal endosperm development.

**Starch grain development is delayed in the fgr1 mutant**

Semi-thin sections from 6 DAF developing endosperm were prepared to investigate endosperm development. Consistent with the phenotypic characterization, numerous smaller, single starch grains (SGs) were present in endosperm cells with extra air spaces in the fgr1 mutant compared with the almost homogeneous compound SGs in the wild type (Fig. 2A, B). The reduction in SG number in endosperm cells led to decreased starch content in the fgr1 mutant endosperm (Fig. 2G). SEM analysis of mature grains further showed that fgr1 mutant endosperm cells were packed with loosely arranged starch granules that were much rounder (Fig. 2D, F) than the irregular polyhedral ones in the wild type (Fig. 2C, E). Amylose content was significantly lower in the mutant (Fig. 2H). Expression profiles of starch synthesis-related genes during seed development in the mutant were significantly changed compared with wild-type N22 (Supplementary Fig. S1), whereas total lipid contents were dramatically increased (Fig. 2I). Therefore, mutation of FGR1 affects starch grain development and accumulation of endosperm contents.

**Map-based cloning of FGR1**

Map-based cloning was undertaken to identify the FGR1 allele. Individuals with the fgr1 mutant phenotype were harvested from the F2 population of a cross of the fgr1 mutant and cv. Nipponbare (O. sativa ssp. japonica). Initial linkage analysis
placed the FGR1 locus between simple sequence repeat (SSR) markers I8-5 and N8-25 on chromosome 8. FGR1 was then narrowed down to a 273 kb region between markers HY8-61 and HY8-34 based on analysis of 2716 individuals with the recessive mutant phenotype. Fifteen candidate genes were predicted in the region (Fig. 3A). Compared with wild-type genomic sequences, Os08g0290000 in the fgr1 mutant contained a single nucleotide substitution, that led to transition of a highly conserved isoleucine (I) to threonine (T) (Fig. 3B).

Os08g0290000 contained a single exon that encoded a PPR protein with 16 PPR repeats predicted by the NCBI (https://www.ncbi.nlm.nih.gov/); it belonged to the P-type PPR protein group (Supplementary Fig. S6D, (Lurin et al., 2004)).

A quantitative RT–PCR (qRT–PCR) complementation test performed by introducing the 35S pro:1300-FGR1-flag vector into fgr1 plants verified elevated expression of the FGR1 allele in developing seeds of T2 transgenic lines (Fig. 3E). Fusion protein levels in these lines were confirmed by immunoblot analysis with anti-Flag antibody (Fig. 3F). Mature seeds from the transformed plants were similar to those of the wild type (Fig. 3C) and SGs from 6 DAF endosperm were also restored to wild-type appearance (Fig. 3D). We concluded that a mutation in Os08g0290000 was responsible for the fgr1 phenotype.

Subcellular localization and expression analysis

PPR proteins in plants are mostly targeted to mitochondrial and/or chloroplasts (Lurin et al., 2004). To determine subcellular localization, four GFP fusion constructs driven by the 35S promoter were transformed into rice protoplasts. FGR1-GFP and GFP-FGR1, which contain the full-length FGR1 coding region in front of, or behind, the GFP, respectively, both co-localized with nuclear maker signals. Since mitochondria and chloroplast localization signals are always in the N-terminus of proteins, the use of N181aa–GFP containing only the N-terminal region (amino acids 1–181, in front of the PPR repeat) in front of the GFP is to detect whether or not FGR1 is localized in mitochondria and chloroplasts; the result was the same as with the full-length fusion protein, localized in the nuclear (Fig. 4A). We verified this localization in leaf epidermal cells of N. benthamiana (Fig. 4B). Therefore, FGR1 is a nuclear-localized PPR protein, which we named Oryza sativa nuclear-localized PPR1 (OsNPPR1).

Phylogenetic analysis showed that homologs of the OsNPPR1-encoded protein were green plant specific, and the gene was widely distributed as a single copy in different species (Fig. 4C). qRT–PCR analysis showed that OsNPPR1 was constitutively expressed in various organs, including roots, stems, leaves, panicles, leaf sheaths, and endosperm at different developmental stages.
Higher expression levels were detected in leaves and at late stages in developing endosperm (Fig. 4D). Consistent with the real-time PCR results, GUS activity was detected in the above tissues as well as in glumes and embryos after germination (Supplementary Fig. S2). Immunoblotting of seed proteins using polyclonal antibodies against OsNPPR1 detected the continuous accumulation of OsNPPR1 at different stages of developing endosperm (Fig. 4E). The higher expression levels in leaf tissue and developing
endosperm suggested that OsNPPR1 is particularly important for seedling growth and seed development.

**Mitochondrial function is defective in fgr1 mutant**

Most PPR proteins function in mitochondrial and chloroplast gene expression (Barkan and Small, 2014). The phenotypes of opaque endosperm and growth retardation in fgr1 are very similar to those of mutants defective in mitochondrially localized PPR proteins in rice (Kim et al., 2009; Toda et al., 2012; Y.J. Liu et al., 2013; Li et al., 2014). Thus, we investigated the splicing and editing efficiency of mitochondrially encoded genes. No difference was detected between the wild type and fgr1 (Supplementary Fig. S3A, D), suggesting that mitochondrially encoded mRNA processing was not affected in the fgr1 mutant. Similar results were obtained for chloroplast-encoded genes (Supplementary Fig. S3B). Expression levels of large numbers of chloroplast-encoded genes showed no differences between wild-type N22 and fgr1 mutant (fold change <2; Supplementary Fig. S3C), but expression of mitochondrially encoded genes was significantly higher
in the fgr1 mutant (Fig. 5A). We then checked the expression levels of 20 DEGs in RNA-seq results related to mitochondrial function (Supplementary Table S2). All showed remarkable differences between the fgr1 mutant and wild type (Fig. 5B). Thus, mitochondrial function appeared to be affected in the fgr1 mutant. Moreover, mitochondrial membrane potential and respiration rate are both decreased in fgr1 (Fig. 5C, D). A defective cytochrome pathway always induces alternative pathways in the presence of inefficient mitochondrial oxidative phosphorylation (Chen et al., 2017). Accumulation levels of alternative oxidases (AOXs) in both mRNA and protein were highly increased in the fgr1 mutant (Fig. 5E). The elevated alternative pathway affects ATP production in the electron transport chain (Toda et al., 2012). Further measurement showed that ATP contents in young leaves and 6 DAF endosperm were both significantly decreased (Fig. 5E). Transmission electron micrographs of mitochondrial ultrastructure of 6 DAF endosperm cells indicated an incomplete structure of the cristae in the fgr1 mutant (Fig. 5H, I). Complex I, the first component of the cytochrome pathway, initiates electron transport. We performed BN-PAGE to analyze the activity of complex I. A slight but clear decrease in complex I activity was observed in NADH dehydrogenase activity staining; the protein content of complex I was also decreased as shown by Coomassie brilliant blue staining (Fig. 5J; Supplementary Fig. S4). Moreover, the tricarboxylic acid (TCA) cycle and oxidative phosphorylation are two important metabolic processes in mitochondria; contents of metabolic components were changed in fgr1 compared with N22 in these processes (Supplementary Fig. S5). Thus, despite its nuclear localization, OsNPPR1 still indirectly regulates mitochondrial function.

OsNPPR1 binds to the CUCAC motif

Identification of binding RNA substrates is essential for dissecting the molecular functions of PPR proteins. We attempted to predict where OsNPPR1 might bind in the transcriptome using the sequence recognition ‘code’ proposed by Barkan et al. (2012) (Fig. 6A; Supplementary Fig. S6D). An affinity elution-based RNA selection method was used to identify motifs bound by OsNPPR1 (Zhang and Muench, 2015). MBP-tagged OsNPPR1 fusion protein was expressed in Escherichia coli and purified. Full-length OsNPPR1 failed to be generated, whereas truncated protein with only PPR repeats was obtained (Supplementary Fig. S6B). OsNPPR1-repeat–MBP fusion protein bound in vitro with an initial RNA pool containing 40 nucleotide random RNA sequences synthesized by in vitro transcription using a PCR–amplified oligonucleotide library as the template (Supplementary Fig. S6C). In each round of selection, immunoblotting was adopted to verify the ability of recombination protein to bind to MBP beads (Fig. 6B). A CUCAC consensus motif was obtained (Fig. 6C, D). This motif is very similar to the predicted nucleotides bound by repeats 5–9 (Fig. 6A). We further verified the binding between the fusion protein and the selected motif through RNA-EMSA. A completely conservative probe synthesized to represent the CUCAC motif was bound by OsNPPR1 in vitro (Fig. 6E). Thus, CUCAC is the binding motif of OsNPPR1.

OsNPPR1 is required for a subset of RNA splicing events

OsNPPR1 belongs to the P-class family of PPR proteins, which generally function as RNA splicing factors (Barkan and Small, 2014). RNA-seq revealed many pre-mRNAs showing different AS patterns between the wild type and fgr1 (Supplementary Data S1). Combined with the binding motif identified by the SELEX assay, we analyzed the RI genes containing the CUCAC motif in their pre-mRNAs to ascertain whether OsNPPR1 is required for splicing of nuclear transcripts. The presumed function of the 22 most affected genes showing differences between N22 and the fgr1 mutant in RNA-seq analysis are listed in Supplementary Table S3. They contain transcription factors Flo2, BIM2, and bHLH056, the transcription regulator FNBP4, the transcriptional silencing protein MORC6, the splicing factor SCL33, and a transcription complex subunit. Nuclear proteins UBN1, PCO3, and RCD1 that can interact with transcription factors affecting gene expression in different metabolic pathways were also included. In addition, some of these genes were annotated to encode mitochondrial proteins or proteins involved in activities related to this organelle, such as pyruvate dehydrogenase ATG18b that decreases reactive oxygen species in mitochondria, and MICU1 that protects mitochondria from oxidative burden. Thus, proteins involved in transcription, RNA processes, and mitochondrial function were enriched.

We performed RT–PCR analysis on the 22 RI events to assess the validity of the sequencing data. Six genes showed obvious splicing differences between the wild type and fgr1 mutant (Fig. 7). Os10g0577800 encodes a nuclear RCD1, which has important roles in transcriptional regulation and participates in proinflammatory/cellular defense leading to cell death (Koh et al., 2005). Os04g0502900 encodes the ATP-binding protein MORC6, which needs ATP primarily produced in mitochondria and involved in transcriptional gene silencing (Brabbs et al., 2013). Os01g0907300 encodes the S subunit of the transamidase complex and participates in protein synthesis and embryonic development (Toh et al., 2011). Os04g0645100 encodes transcription factor FLO2, which regulates grain size and starch quality by affecting accumulation of storage materials in endosperm. An flo2 mutant showed floury endosperm (She et al., 2010). Os04g0670400 encodes an OTU domain–containing protein and affects the metabolic pathways associated with the mitochondria (Datta et al., 2017). It is worth noting that correctly spliced transcripts in the mutant continue to be the most abundant types in the above RI events, and could be the reason why splicing differences were not obvious in the other 16 genes assayed by RT–PCR analysis. Taken together, these results suggest that the mutation in OsNPPR1 affected splicing of a subset of nuclear genes, many of which are related to mitochondrial functions.
Fig. 5. Mitochondrial function is defective in the fgr1 mutant. (A) Transcript levels of mitochondrial encoded genes in the fgr1 mutant compared with N22. Total RNA was extracted from 6 DAF endosperm of N22 and the fgr1 mutant. Actin1 was used as an internal control. Error bars are means ±SD (n=3). (B) qRT–PCR verified increased and decreased transcription in the fgr1 mutant compared with the N22 from RNA-seq results for mitochondrial function. a–j, decreased transcripts; k–t, increased transcripts. Encoded proteins are listed in Supplementary Table S2. Actin1 was used as an internal control. Error bars are means ±SD (n=3). (C–E) Measurement of mitochondrial membrane potential ratio (C), total respiration rate (D), and the ATP contents (E). Error bars are means ±SD (n=3). Asterisks indicate significant difference between wild-type N22 and the fgr1 mutant, determined by a Student’s t-test (*P<0.05; **P<0.01). (F) Transcript levels of alternative oxidases (AOX1a–c) in the fgr1 mutant 6 DAF endosperm were relative to the N22 level. Actin1 was used as an internal control. Error bars are means ±SD (n=3). (G) Mitochondrial proteins extracted from 6 DAF endosperm were detected with AOX (containing AOX1a–c) antibodies. (H, I) Transmission electron micrographs of mitochondria in endosperm cells of N22 (H) and the fgr1 mutant (I) at 6 DAF. Arrowheads indicate incomplete cristae structure in fgr1. Scale bars: 400 nm. (J) Blue native-PAGE of mitochondrial proteins (25 μg) from 9-day-old N22 and the fgr1 mutant stained for complex I activity by NADH oxidase activity (left) and protein profile by Coomassie blue (right). Arrowheads indicate the complex I site.
Discussion

Endosperm development is delayed in the fgr1 mutant

Opaque endosperm, caused by loosely arranged starch grains and lower starch and amylose contents in the fgr1 mutant (Fig. 2), suggested that mutation of OsNPPR1 affected starch biosynthesis. Numerous single starch grains with extra spaces in developing endosperm cells characterized this defect. Expression levels of numerous starch synthesis-related genes were significantly changed in developing endosperm in the fgr1 mutant relative to the wild
type. Germination of seeds from the \textit{fgr1} mutant was 2 d slower than those from the wild type, accompanied by retarded seedling growth (Fig. 1H–K). Therefore, OsNPPR1 affected early seedling development and starch synthesis in the endosperm.
The phenotype of fgr1 mutant was very similar to rice mutants with defective mitochondrially localized PPR proteins, such as OGR1, SMK1, and EMP5. Mutants of these three proteins all show floury endosperm and retarded growth (Kim et al., 2009; Y.J. Liu et al., 2013; Li et al., 2014). In addition, mutations of many PPR proteins related to mitochondrial function, such as SLO4, PPR19, MTL1, AtGRS1, PPME, and MEF35 in Arabidopsis thaliana (Brehme et al., 2015; Hali et al., 2016; Leu et al., 2016; Xie et al., 2016; Lee et al., 2017; Weißenberger et al., 2017), and DEK1, Emp5, PPR78, Dek35, Dek36, Dek10, Dek37, and Dek39 in maize (Becraft et al., 2002; Y.J. Liu et al., 2013; Chen et al., 2017; Qi et al., 2017a; G. Wang et al., 2017; Zhang et al., 2017; Dai et al., 2018; Li et al., 2018), all affect seed development and/or seedling growth. Clearly, the fgr1 mutant has a very similar phenotype to mutants that are defective in mitochondrially coded PPR proteins.

**Mutation of OsNPPR1 leads to defective mitochondrial function**

Complex I is the major entrance to the cytochrome pathway and initiates electron transport. Complex I activity is visibly decreased in the fgr1 mutant (Fig. 5J). The inhibited mitochondrial complex I might change the redox state in the mitochondrial membrane and cause severe deficiency in mitochondrial function (Palmer, 1979; Qi et al., 2017b). Defects in the cytochrome pathway always initiate an alternative pathway when inefficient mitochondrial oxidative phosphorylation occurs, resulting in a remarkable increase in mRNA and protein levels of genes in the AOX pathway (Fig. 5E-G). The elevated alternative pathways lead to decreased ATP production in the electron transport chain (Fig. 5E; Toda et al., 2012). Nearly all mitochondrially encoded genes were increased in the fgr1 mutant, suggestive of feedback regulation that is ubiquitous in mitochondrially defective mutants (Fig. 5A; Chen et al., 2017; Lee et al., 2017; Zhang et al., 2017). Thus, the fgr1 mutation causes defects in mitochondrial function, leading to the floury endosperm and retarded seedling growth subsequently. This regulation mechanism is very similar to that of the mitochondrially localized PPR protein.

Mitochondria are the central coordinators of energy metabolism, and alterations in their function are associated with metabolic disorders. RNA-seq results of the cellular component GO category identified DEGs related to mitochondria showing significant differences between the fgr1 mutant and wild type (Supplementary Table S1). Moreover, abnormal mitochondrial function resulted in changes in other important biological processes (Supplementary Table S1). Nutrient reservoir activity in the endosperm affects the synthesis and transport of storage protein (Qi et al., 2017b). Purine ribonucleoside binding, ribonucleoside binding, nucleoside binding, and protein binding related to ribosome biogenesis are important for cellular protein expression and accumulation (Dai et al., 2018); all processes consume energy produced by the mitochondria. Anion binding, purine ribonucleoside triphosphate binding, and ATP binding all need the ATP primarily produced in mitochondria. The RNA-seq analysis provided evidence that mitochondrial dysfunction leads to other secondary biological defects.

**OsNPPR1 affects splicing of a subset of nuclear genes**

By transient expression in rice protoplasts and tobacco leaf epidermal cells, we showed that OsNPPR1 is localized in the nucleus. To our knowledge, OsNPPR1 is the first PPR protein found to be localized in the nuclear in rice (Fig. 4A, B). In addition to OsNPPR1, three other nuclear-localized PPR proteins were identified in plants; these were GRP23, PNM1, and SOAR1 in A. thaliana (Ding et al., 2006; Hammani et al., 2011; Mei et al., 2014). Each of these proteins participates in seed development. Interaction of GRP23 with RNA polymerase II subunit III and of PNM1 with transcription factors TCP8 and NAP1 suggests that PPR proteins in the nuclear affect transcription and post-transcription of nuclear mRNA. In this study, RNA-seq results for the biological process GO category indicated that genes involved in transcription and RNA biosynthesis showed significant differences between the fgr1 mutant and wild type (Supplementary Table S1). This suggests that as a nuclear-localized PPR protein, OsNPPR1 can participate in post-transcriptional regulation. Although we failed to identify protein partners of OsNPPR1 such as GRP23 and PNM1 through two-hybrid assays and immunoprecipitation–MS, the reason might be low expression of the PPR proteins and very short half-lives of this protein family (Morey and Van Dolah, 2013).

OsNPPR1 encodes a nuclear-localized PPR protein. Generally, PPR proteins have consistent roles in pre-mRNA editing or splicing in mitochondria and plastids (Small and Peeters, 2000; Lurin et al., 2004; Barkan and Small, 2014). Since there were no differences in mitochondrial mRNA splicing and editing (Supplementary Fig. S3), we tried other ways to find RNA substrates of OsNPPR1. Tandem arrays of the 16 incompletely conservative 35 amino acid repeats in the OsNPPR1 modular domain structure indicated its conventional RNA binding ability, which was verified by in vitro RNA–SELEX (Fig. 6C, D), and a CUCAC motif for its potential RNA substrate. This motif is very similar to the predicted nucleotides bound by repeats 5–9, but the modular recognition mechanism might not be appropriate for nuclear-localized PPR proteins. Proteins that participate in RNA processes in the nuclear are not always restricted to unique RNA substrates. UBL1 and Rgf3 are RNA splicing factors in the nuclear, and both affect a subset of RNA splicing events in nuclear genes, leading to defective endosperm and weak seedlings in maize (Fouquet et al., 2011; Li et al., 2017).

Twenty-two DSGs containing CUCAC motifs in the RNA-seq result (Supplementary Table S3) were verified by RT–PCR, of which six showed significant differences in splicing efficiency (Fig. 7). They are almost all annotated to encode either nuclear proteins such as transcription factors, mitochondrial proteins, or proteins involved in processes related to mitochondrial function. Thus, we propose that OsNPPR1 might regulate transcription and/or mRNA processing of nuclear genes that encode proteins related to mitochondrial function or are localized to mitochondria. Although we did not detect any splicing and editing deficiency in mitochondrial mRNA, it does not rule out the possibility that OsNPPR1 might indirectly regulate RNA processing of
mitochondrial genes. In our lab, other nuclear-localized PPR proteins were demonstrated to affect the splicing of the mitochondrial nad genes. These also led to defective mitochondrial function, compromised plant growth and floury endosperm, and even to a homozygous lethal embryo phenotype (J. Wan et al., unpublished). Moreover, interaction of nuclear-localized PPR protein OsNPPR1 with TCP8 was involved in the coordinated expression of nuclear-encoded subunits of the mitochondrial oxidative phosphorylation machinery, suggesting that OsNPPR1 might act in a similar way (Hammani et al., 2011). On the other hand, retrograde pathways could be triggered by the disorder of ATP production and activation of the alternative pathway, when the mitochondrial state is compromised in fgr1, and thus be signaled to the nuclear (Pesaresi et al., 2007; Yang et al., 2008; Zhu et al., 2011). It is possible that the splicing differences of nuclear genes may result from a mitochondrial dysfunction-associated retrograde signal. Therefore, the growth and developmental defects seen in fgr1 may be because of the dysfunction of mitochondria and mis-splicing of nuclear genes resulting from feedback of malfunctioning mitochondria.

In this study, we characterized a nuclear-localized PPR protein named OsNPPR1 in rice. Mutation of OsNPPR1 caused delayed seedling growth and endosperm development. We provide evidence showing that OsNPPR1 regulates mitochondrial function by a series of splicing events in the nucleus. Further identification of OsNPPR1-interacting proteins and its binding RNA substrates are needed to elucidate its molecular function.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Expression profiles of rice starch synthesis-related genes during seed development in the wild-type N22 and the fgr1 mutant.

Fig. S2. β-Glucuronidase (GUS) staining of various tissues in the ProOsNPPR1-GUS transgenic line.

Fig. S3. Splicing and editing efficiencies of mitochondrial genes are not affected in fgr1.

Fig. S4. Band intensity quantification of blue native-PAGE.

Fig. S5. Comparison of metabolic components contents in the TCA cycle and oxidative phosphorylation processes between fgr1 and N22.

Fig. S6. Protein and oligonucleotide library prepared for RNA-SELEX.

Table S1. Gene Ontology (GO) classification and functional enrichment of differentially expressed genes (DEGs) in wild-type and fgr1 from results of high-throughput RNA sequencing (RNA-seq).

Table S2. Twenty increased and decreased genes from RNA-seq results related to mitochondrial function.

Table S3. Twenty-two different splicing genes (DSGs) in RNA-seq analysis containing the CUCAC motif were selected for further verification by agarose gel electrophoresis.

Data S1. RNA-seq revealed many pre-mRNAs showing different AS patterns between the wild type and fgr1.

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