




RESEARCH PAPER

# 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-EMSA 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 PLS-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; Pfalz *et al.*, 2009; Johnson *et al.*, 2010; Cai *et al.*, 2011; Zhang *et al.*, 2017), RNA cleavage (Hölzle *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 Longevialle *et al.*, 2007, 2008; Khrouchtchova *et al.*, 2012; Chen *et al.*, 2017; Dai *et al.*, 2018). PLS-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; Khrouchtchova *et al.*, 2012), aberrant leaf development (Petricka *et al.*, 2008), and changes in leaf pigmentation in various crop species (Chateigner-Boutin *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 ECLIPSE80i (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 F<sub>2</sub> 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 PPR repeat) upstream of the GFP. The plasmid was 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 *Nicotiana 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 filtering twice through Miracloth (Millipore), the filtrate was centrifuged in a gradient to remove cell debris (600 g, 1300 g, 2100 g, 4 °C, 10 min each). The supernatant 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 resuspended 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 µg 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 ACCAGAAG(N)<sub>40</sub>TATGTGCGTCTACATGGATCCTCA-3'] were synthesized and amplified by PCR using a forward primer containing

the T7 promoter sequence and reverse primer (SEL-F, 5'-CGGAATTCTAATACGACTCACTATAGGGAAGATCTCGACCAGAAG-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. dsDNA 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 dsDNA template using a T7 riboMAX<sup>TM</sup> 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:isoamyl and reverse transcribed with a PrimeScript Reverse Transcriptase 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<sup>TM</sup> Chemiluminescent RNA EMSA Kit (No.20158). Probes were synthesized by Genscript (<http://www.genscript.com.cn/>) labeled with biotin at the 5' end: probe, CUCACCUCACCUCACCUCACCUCACCUCAC; control probe, GAUCAGAUCAGAUCAGAUCAGAUCAGAUC.

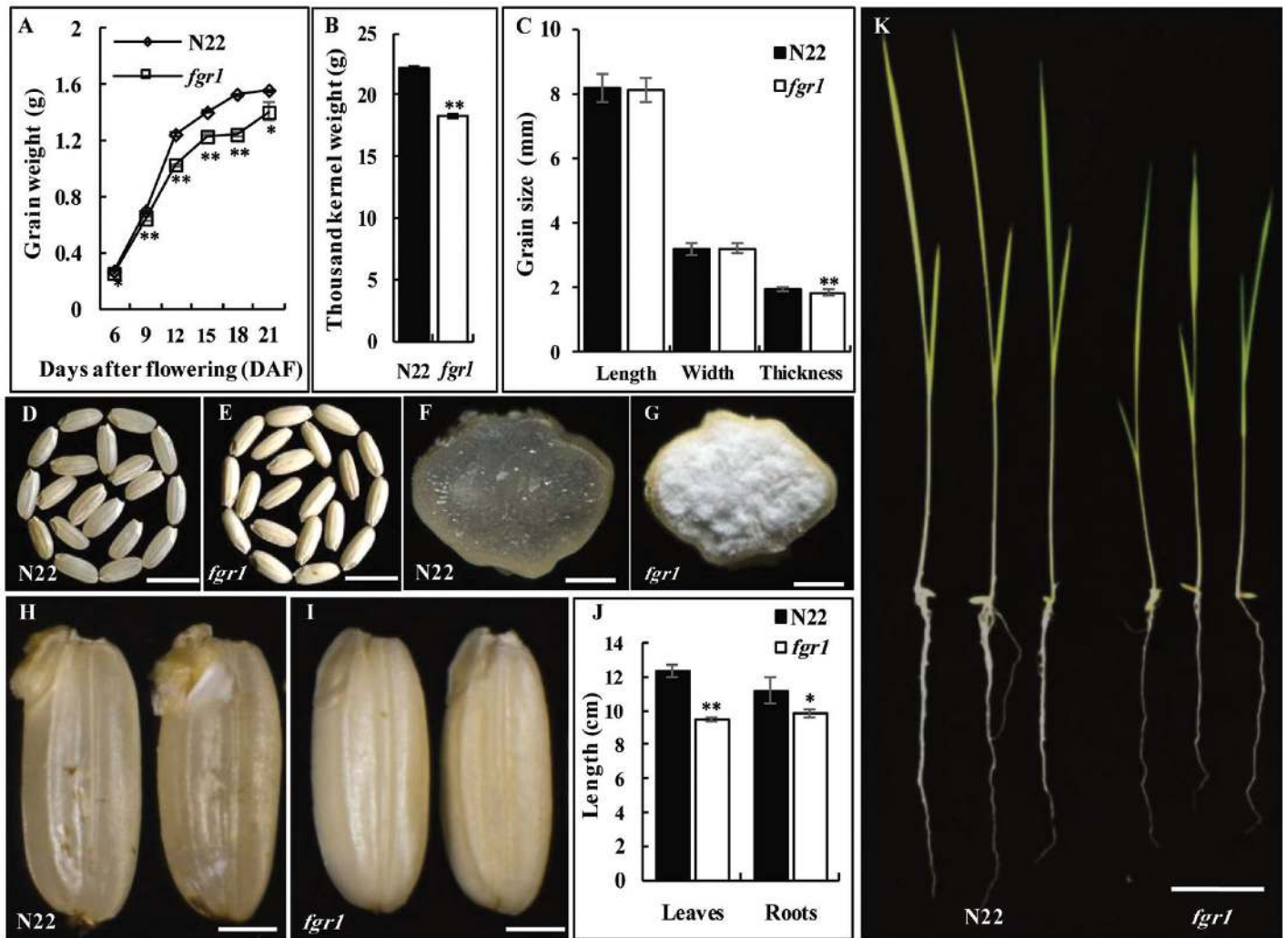
### High-throughput RNA sequencing

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



**Fig. 1.** The *fgr1* mutant displays opaque endosperm and retarded seedling growth. (A) Grain filling of wild-type N22 and *fgr1* at various developmental stages. Grain weight is the dry weight of 100 brown rice grains. (B) Thousand kernel weight of mature seeds of the wild type and *fgr1* mutant. (C) Grain size of the wild type and *fgr1* mutant. (D, E) Mature seeds from the wild type (D) and *fgr1* mutant (E). (F, G) Cross-sections of mature seeds of the wild type (F) and *fgr1* mutant (G). (H, I) Status of seed germination in the wild type (H) and *fgr1* (I) after 2 d of imbibition. (J) Analysis of leaf and root length of genotypes shown in (K). (K) Seedlings of the wild type (left) and *fgr1* mutant (right) at 9 d post-germination. Asterisks in (A–C) and (J) indicate statistical significance between the wild-type N22 and *fgr1*, determined by a Student's *t*-tests (\* $P < 0.05$ ; \*\* $P < 0.01$ ). Scale bars: 1 cm in (D, E); 0.1 cm in (F, G); 0.15 cm in (H, I); 2 cm in (K).

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.

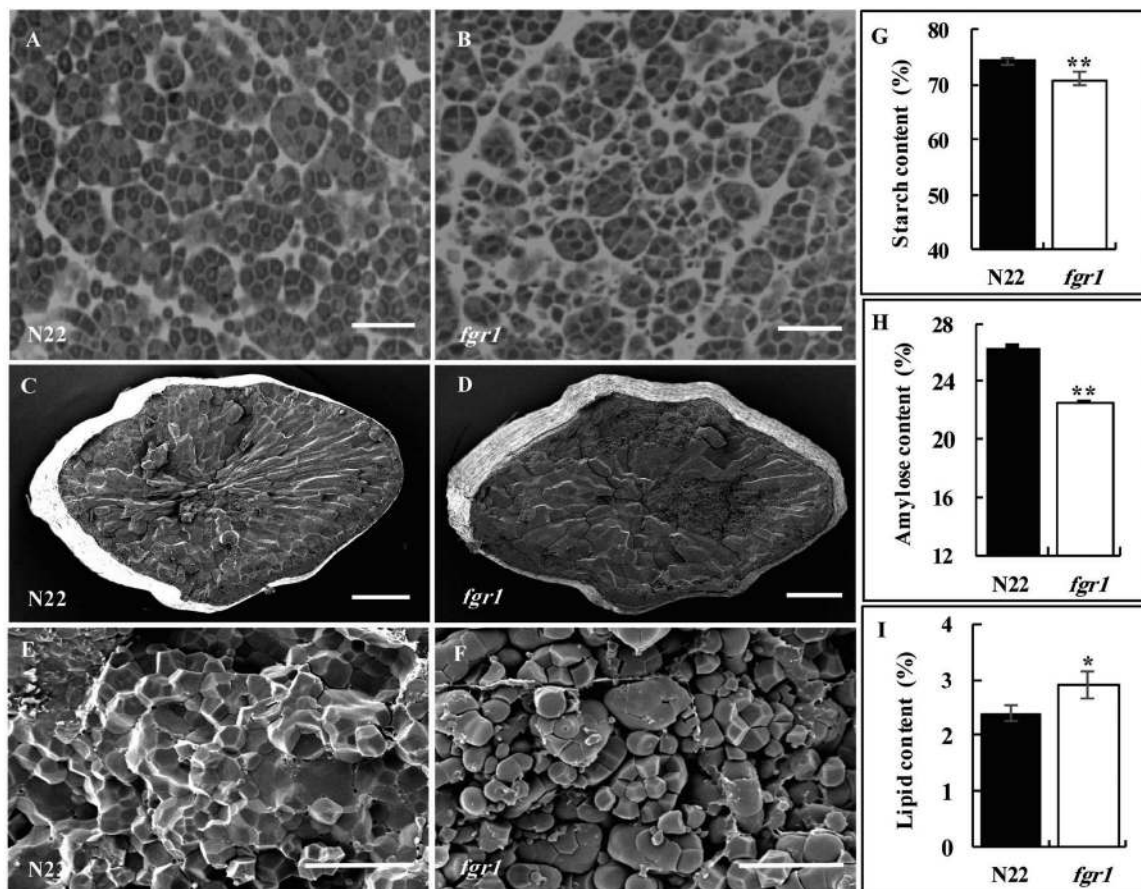
#### Starch grain development is delayed in the *fgr1* mutant

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 F<sub>2</sub> population of a cross of the *fgr1* mutant and cv. Nipponbare (*O. sativa* ssp. *japonica*). Initial linkage analysis



**Fig. 2.** Starch grain development is delayed in the *fgr1* mutant. (A, B) Semi-thin sections of wild-type (A) and *fgr1* mutant (B) endosperm at 6 DAF. (C–F) Scanning electron micrographs of mature endosperms from the wild type (C, E) and *fgr1* mutant (D, F). (G–I) Starch (G), amylose (H), and lipid (I) contents. Asterisks indicate statistical significance between the wild-type N22 and *fgr1* mutant, as determined by a Student's *t*-test (\* $P < 0.05$ ; \*\* $P < 0.01$ ). Scale bars: 10  $\mu\text{m}$  in (A, B); 0.5 mm in (C, D); 15  $\mu\text{m}$  in (E, F).

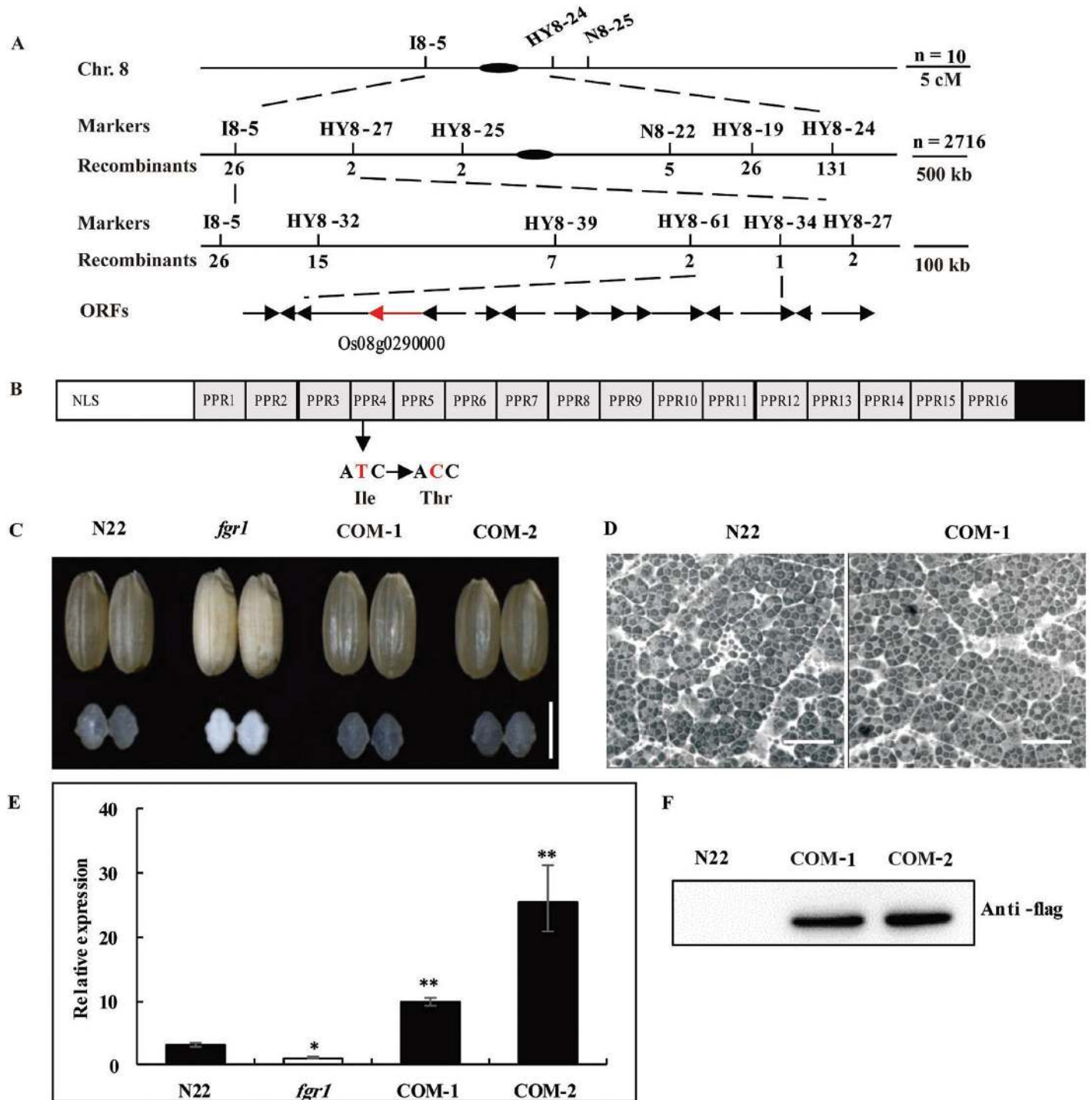
placed the *FGR1* locus between simple sequence repeat (SSR) markers I8–5 and N8–25 on chromosome 8. *FGR1* was then narrowed down to a 273 kb region between markers HY8–61 and HY8–34 based on analysis of 2716 individuals with the recessive mutant phenotype. Fifteen candidate genes were predicted in 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 T<sub>2</sub> 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 marker 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).

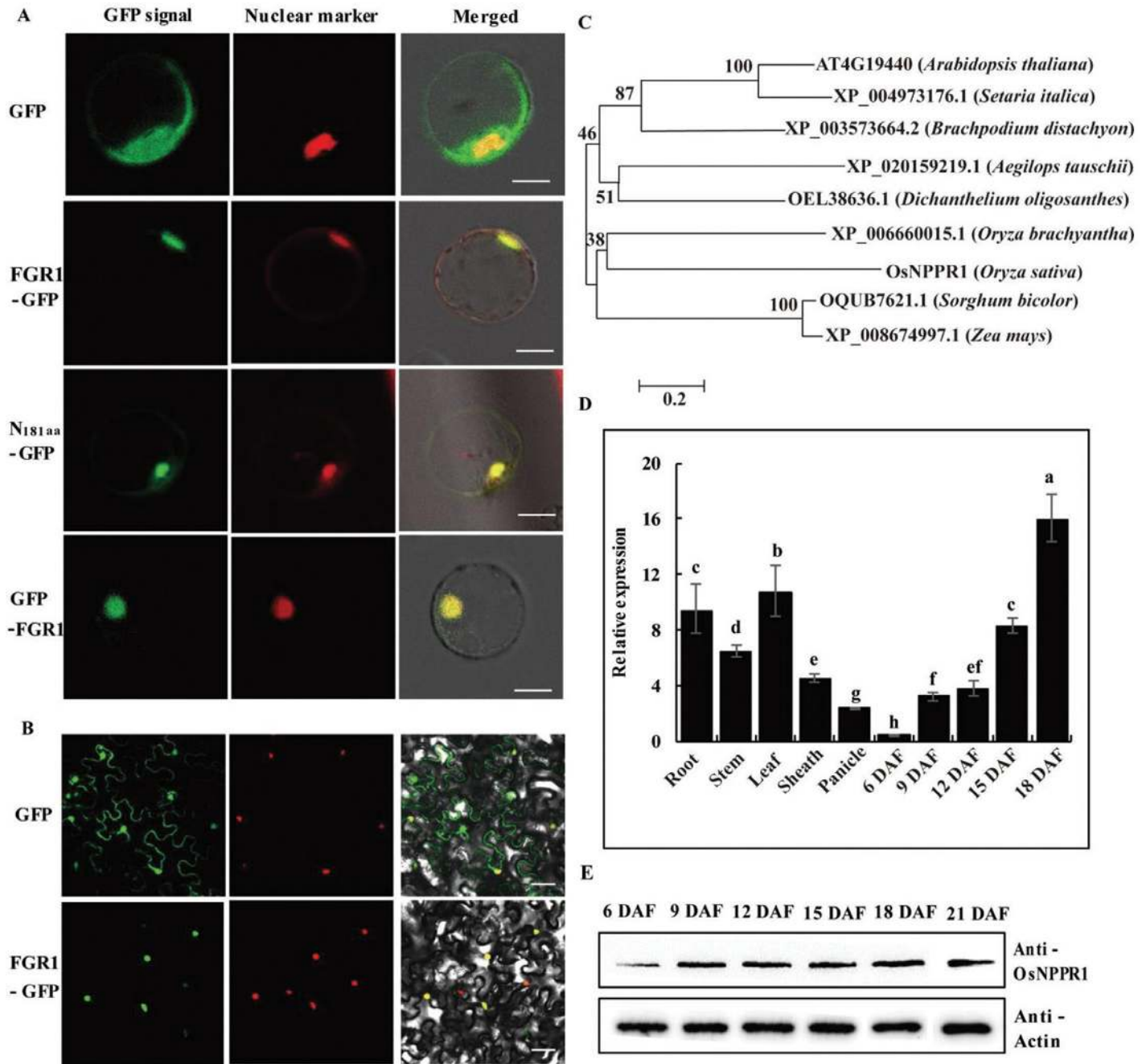
Phylogenetic analysis showed that homologs of the *OsNPPR1*-encoded protein were green plant specific, and the gene was widely distributed as a single copy in different species (Fig. 4C). qRT–PCR analysis showed that *OsNPPR1* was constitutively expressed in various organs, including roots, stems, leaves, panicles, leaf sheaths, and endosperm at different developmental stages.



**Fig. 3.** Map-based cloning of *FGR1* and complementation of the *fgr1* allele. (A) Fine mapping of the *FGR1* locus. The *FGR1* locus was mapped to a 273 kb region by markers HY8-61 and HY8-34 on chromosome 8 (Chr. 8); 15 ORFs were predicted (arrows). Numbers of recombinants are indicated below the map. (B) Schematic of the mutation in Os08g0290000 protein. White and gray boxes represent the putative N-terminus containing the nuclear localization signal (NLS) and 16 PPR repeats, respectively. The black box represents the C-terminal region. A T to C mutation in the exon led to an amino acid transition from isoleucine (Ile) to threonine (Thr). (C, D) Seed appearance (C) and starch grain development (D) were restored in the complementation lines. Scale bars: 5 mm in (C); 10  $\mu$ m in (D). (E) qRT-PCR confirmed higher expression of the *FGR1* allele in positive transgenic plants. *Actin1* was used as an internal control. Error bars are means  $\pm$ SD ( $n=3$ ). Asterisks indicate statistical significance of the difference between the wild-type N22 and *fgr1* mutant, COM-1 and COM-2, as determined by Student's *t*-tests (\* $P<0.05$ ; \*\* $P<0.01$ ). (F) Immunoblotting of total seed proteins of positive transgenic lines with monoclonal anti-flag antibody. COM-1 and COM-2 are independent complementation lines ( $T_2$  progeny).

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



**Fig. 4.** Expression analysis and subcellular localization. (A, B) Subcellular location of FGR1 protein in nuclei of a rice protoplast (A) and tobacco leaves (B). Scale bars: 10  $\mu$ m in (A); 40  $\mu$ m in (B). (C) Phylogenetic tree of OsNPPR1 and its homologs constructed using MEGA 5. (D) qRT-PCR analyses showing that *OsNPPR1* is expressed constitutively. *Actin1* was used as an internal control. Error bars are means  $\pm$ SD ( $n=3$ ). Different letters denote significant differences ( $P<0.05$ ) determined using Student's *t*-tests. (E) Immunoblotting of OsNPPR1 at different stages of endosperm development. Actin was used as a loading control.

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. 5F, G). 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

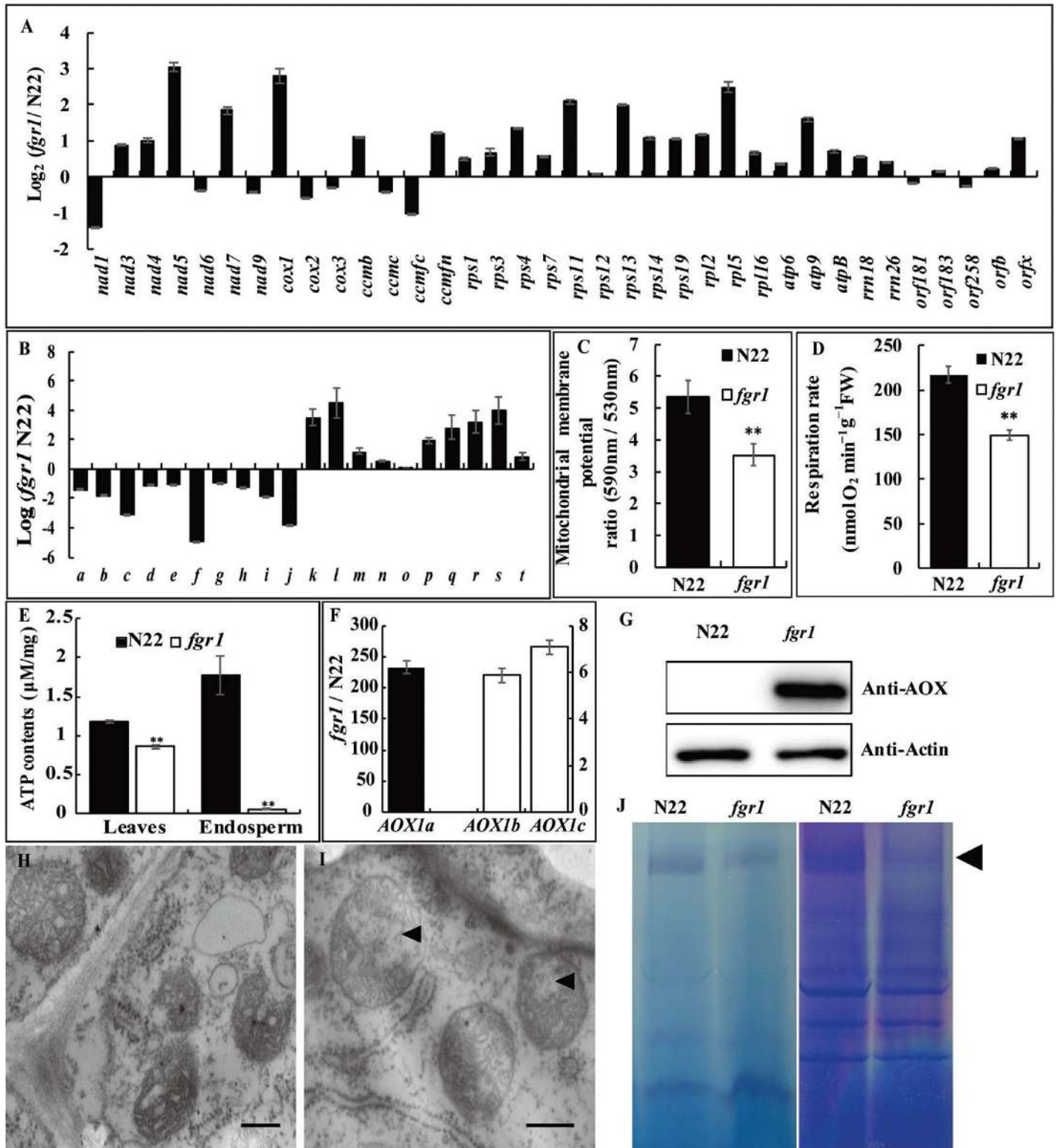
Identification of binding RNA substrates is essential for dissecting the molecular functions of PPR proteins. We attempted to predict where OsNPPR1 might bind in the transcriptome using the sequence recognition ‘code’ proposed by Barkan *et al.* (2012) (Fig. 6A; Supplementary Fig. S6D). An affinity elution-based 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 an 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

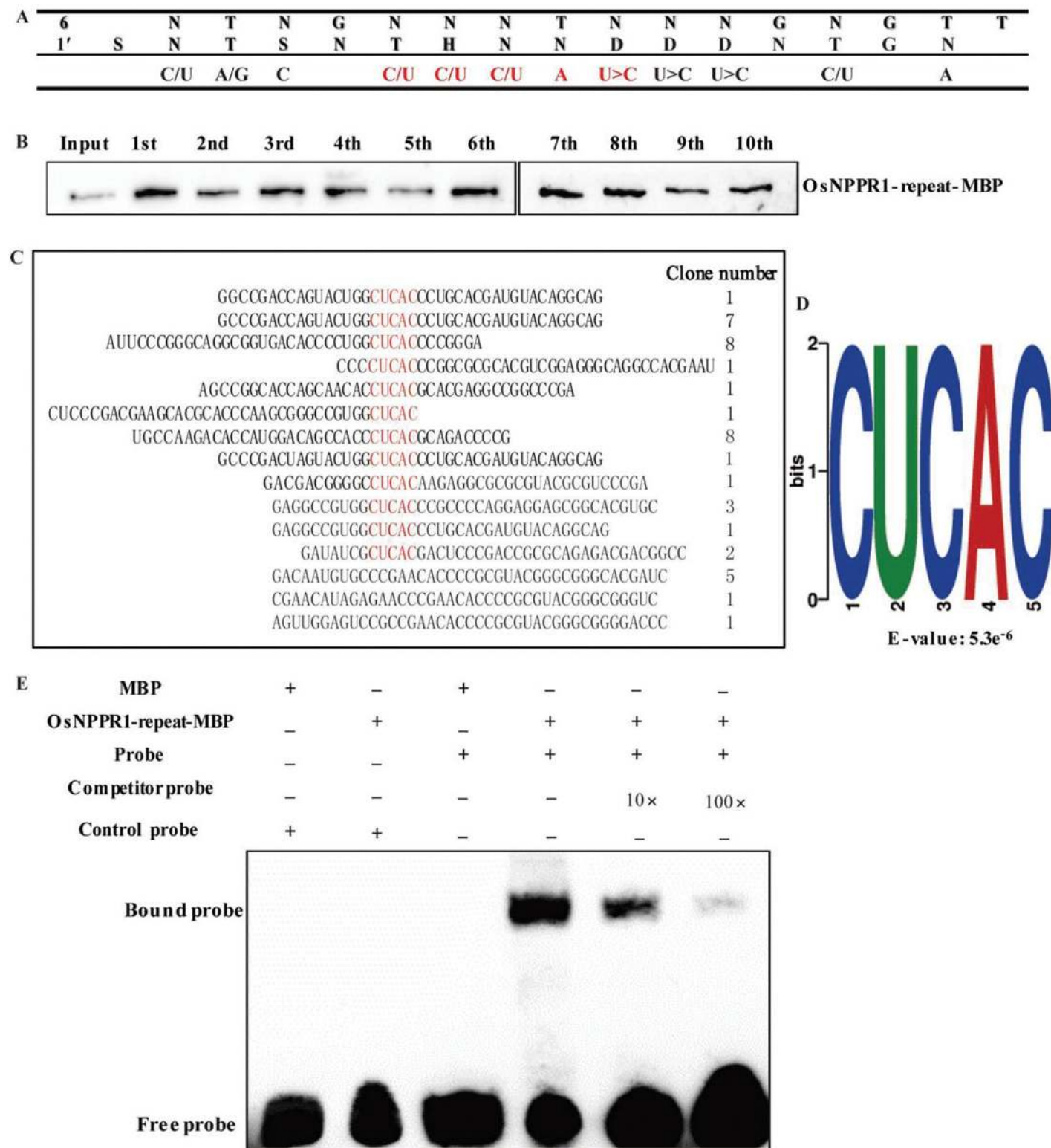
OsNPPR1 belongs to the P-class family of PPR proteins, which generally function as RNA splicing factors (Barkan and Small, 2014). RNA-seq revealed many pre-mRNAs showing different AS patterns between the wild type and *fgr1* (Supplementary Data S1). Combined with the binding motif identified by the SELEX 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 ATG18h 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 calcium uptake protein MICU1 with a predicted mitochondrial localization and is involved in cell physiology by stimulating ATP production, shaping cytosolic calcium transients, regulating cell death, and protecting cells from oxidative damage (Donato, 1999; Perocchi *et al.*, 2010; Datta *et al.*, 2017). *Os01g0549200* 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 mitochondrially 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  $\pm$ 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  $\pm$ 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  $\pm$ 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  $\pm$ 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  $\mu$ g) 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.



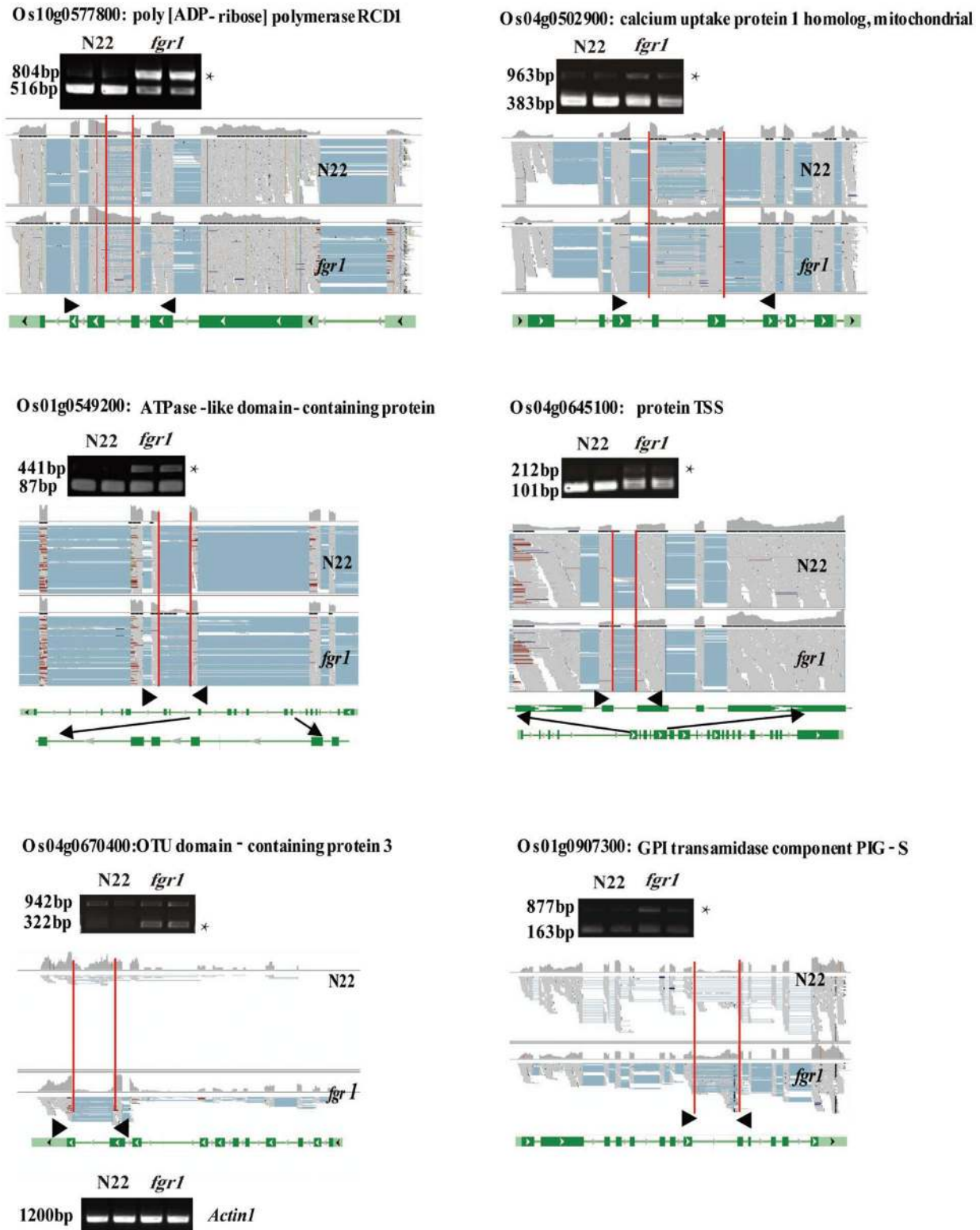
**Fig. 6.** OsNPPR1 binds to the CUCAC motif. (A) Schematic representation of predicted nucleotides for OsNPPR1 binding. (B) Immunoblotting showing OsNPPR1-repeat-MBP fusion protein eluted from MBP beads in each SELEX cycle. (C) Unique sequences from independent transformants (42 clones) in the 10th SELEX cycle including the binding site. Clone number represents the frequency of the corresponding sequences among sequencing results. Three sequences without CUCAC are listed at the bottom. (D) MEME (<http://meme-suite.org/>) predicted a consensus RNA-binding site. (E) RNA-EMSA of the OsNPPR1-repeat-MBP with the motif.

## 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



**Fig. 7.** OsNPPR1 is required for a subset of RNA splicing events. RNA-seq data showing alterations in intron splicing of selected genes in the *fgr1* mutant. RT-PCR validation for RI events. The RNA samples were extracted from two biological replications of N22 and *fgr1* mutant endosperm. There were 32 cycles of amplification. *Actin1* was used as the control. Primers for RT-PCR were designed according to the sequences of upstream and downstream exons of the detected RI events and are listed in [Supplementary Table S3](#). Asterisks indicate the intron-retained products. Figures were created using the Integrative Genomics Viewer program (<http://software.broadinstitute.org/software/igv>). Red lines indicate that the intron was retained and black arrowheads indicate the positions of forward and reverse primers used in agarose gel electrophoresis. The gene structure is listed at the bottom of each panel. Green boxes and lines indicate exons and introns, respectively.

type. Germination of seeds from the *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; Haili *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. 5F, 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 nuclear. 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 yeast 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 PNM1 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 nucleus (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.  $\beta$ -Glucuronidase (GUS) staining of various tissues in the *Pro<sub>OsNPPR1</sub>: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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## References

- Barkan A, Rojas M, Fujii S, Yap A, Chong YS, Bond CS, Small I. 2012. A combinatorial amino acid code for RNA recognition by pentatricopeptide repeat proteins. *PLoS Genetics* **8**, e1002910.
- Barkan A, Small I. 2014. Pentatricopeptide repeat proteins in plants. *Annual Review of Plant Biology* **65**, 415–442.
- Barkan A, Walker M, Nolasco M, Johnson D. 1994. A nuclear mutation in maize blocks the processing and translation of several chloroplast mRNAs and provides evidence for the differential translation of alternative mRNA forms. *The EMBO Journal* **13**, 3170–3181.
- Becraft PW, Li K, Dey N, Asuncion-Crabb Y. 2002. The maize *dek1* gene functions in embryonic pattern formation and cell fate specification. *Development* **129**, 5217–5225.
- Beick S, Schmitz-Linneweber C, Williams-Carrier R, Jensen B, Barkan A. 2008. The pentatricopeptide repeat protein PPR5 stabilizes a specific tRNA precursor in maize chloroplasts. *Molecular and Cellular Biology* **28**, 5337–5347.
- Brabbs TR, He Z, Hogg K, Kamenski A, Li Y, Paszkiewicz KH, Moore KA, O'Toole P, Graham IA, Jones L. 2013. The stochastic silencing phenotype of *Arabidopsis* *morc6* mutants reveals a role in efficient RNA-directed DNA methylation. *The Plant Journal* **75**, 836–846.
- Brehme N, Bayer-Császár E, Glass F, Takenaka M. 2015. The DYW subgroup PPR protein MEF35 targets RNA editing sites in the mitochondrial *rpl16*, *nad4* and *cob* mRNAs in *Arabidopsis thaliana*. *PLoS One* **10**, e0140680.
- Cai W, Ji D, Peng L, Guo J, Ma J, Zou M, Lu C, Zhang L. 2009. LPA66 is required for editing *psbF* chloroplast transcripts in *Arabidopsis*. *Plant Physiology* **150**, 1260–1271.
- Cai W, Okuda K, Peng L, Shikanai T. 2011. PROTON GRADIENT REGULATION 3 recognizes multiple targets with limited similarity and mediates translation and RNA stabilization in plastids. *The Plant Journal* **67**, 318–327.
- Chateigner-Boutin AL, Ramos-Vega M, Guevara-García A, *et al.* 2008. CLB19, a pentatricopeptide repeat protein required for editing of *rpoA* and *clpP* chloroplast transcripts. *The Plant Journal* **56**, 590–602.
- 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. *Molecular Plant* **10**, 427–441.
- Cheng S, Gutmann B, Zhong X, *et al.* 2016. Redefining the structural motifs that determine RNA binding and RNA editing by pentatricopeptide repeat proteins in land plants. *The Plant Journal* **85**, 532–547.
- 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.
- Datta G, Hossain ME, Asad M, Rathore S, Mohammed A. 2017. *Plasmodium falciparum* OTU-like cysteine protease (PfOTU) is essential for apicoplast homeostasis and associates with noncanonical role of Atg8. *Cellular Microbiology* **19**, e12748.
- de Longevialle AF, Hendrickson L, Taylor NL, Delannoy E, Lurin C, Badger M, Millar AH, Small I. 2008. The pentatricopeptide repeat gene OTP51 with two LAGLIDADG motifs is required for the cis-splicing of plastid *ycf3* intron 2 in *Arabidopsis thaliana*. *The Plant Journal* **56**, 157–168.

- de Longevialle AF, 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*. *The Plant Cell* **19**, 3256–3265.
- Ding YH, Liu NY, Tang ZS, Liu J, Yang WC.** 2006. Arabidopsis GLUTAMINE-RICH PROTEIN23 is essential for early embryogenesis and encodes a novel nuclear PPR motif protein that interacts with RNA polymerase II subunit III. *The Plant Cell* **18**, 815–830.
- Donato R.** 1999. Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type. *Biochimica et Biophysica Acta* **1450**, 191–231.
- Fouquet R, Martin F, Fajardo DS, Gault CM, Gómez E, Tseung CW, Pouchot T, Hueros G, Settles AM.** 2011. Maize rough endosperm3 encodes an RNA splicing factor required for endosperm cell differentiation and has a nonautonomous effect on embryo development. *The Plant Cell* **23**, 4280–4297.
- Gutiérrez-Marcos JF, Dal Prà M, Giulini A, et al.** 2007. *empty pericarp4* encodes a mitochondrion-targeted pentatricopeptide repeat protein necessary for seed development and plant growth in maize. *The Plant Cell* **19**, 196–210.
- Haïli N, Planchard N, Arnal N, Quadrado M, Vrielynck N, Dahan J, des Francs-Small CC, 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 Physiology* **170**, 354–366.
- Hammani K, Gobert A, Hleibieh K, Choulier L, Small I, Giegé P.** 2011. An Arabidopsis dual-localized pentatricopeptide repeat protein interacts with nuclear proteins involved in gene expression regulation. *The Plant Cell* **23**, 730–740.
- Hashimoto M, Endo T, Peltier G, Tasaka M, Shikanai T.** 2003. A nucleus-encoded factor, CRR2, is essential for the expression of chloroplast *ndhB* in *Arabidopsis*. *The Plant Journal* **36**, 541–549.
- Hölze A, Jonietz C, Törjek O, Altmann T, Binder S, Forner J.** 2011. A RESTORER OF FERTILITY-like PPR gene is required for 5'-end processing of the *nad4* mRNA in mitochondria of *Arabidopsis thaliana*. *The Plant Journal* **65**, 737–744.
- Johnson X, Wostrikoff K, Finazzi G, Kuras R, Schwarz C, Bujaldon S, Nickelsen J, Stern DB, Wollman FA, Vallon O.** 2010. MRL1, a conserved pentatricopeptide repeat protein, is required for stabilization of *rbcl* mRNA in *Chlamydomonas* and *Arabidopsis*. *The Plant Cell* **22**, 234–248.
- Jonietz C, Forner J, Hildebrandt T, Binder S.** 2011. RNA PROCESSING FACTOR3 is crucial for the accumulation of mature *ccmC* transcripts in mitochondria of *Arabidopsis* accession Columbia. *Plant Physiology* **157**, 1430–1439.
- Khrouchtchova A, Monde RA, Barkan A.** 2012. A short PPR protein required for the splicing of specific group II introns in angiosperm chloroplasts. *RNA* **18**, 1197–1209.
- Kim SR, Yang JI, Moon S, Ryu CH, An K, Kim KM, Yim J, An G.** 2009. Rice OGR1 encodes a pentatricopeptide repeat-DYW protein and is essential for RNA editing in mitochondria. *The Plant Journal* **59**, 738–749.
- Koh DW, Dawson TM, Dawson VL.** 2005. Mediation of cell death by poly(ADP-ribose) polymerase-1. *Pharmacological Research* **52**, 5–14.
- Kotera E, Tasaka M, Shikanai T.** 2005. A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. *Nature* **433**, 326–330.
- Lee K, Han JH, Park YI, Colas des Francs-Small C, Small I, Kang H.** 2017. The mitochondrial pentatricopeptide repeat protein PPR19 is involved in the stabilization of NADH dehydrogenase 1 transcripts and is crucial for mitochondrial function and *Arabidopsis thaliana* development. *New Phytologist* **215**, 202–216.
- Leu KC, Hsieh MH, Wang HJ, Hsieh HL, Jauh GY.** 2016. Distinct role of Arabidopsis mitochondrial P-type pentatricopeptide repeat protein-modulating editing protein, PPME, in *nad1* RNA editing. *RNA Biology* **13**, 593–604.
- Li J, Fu J, Chen Y, et al.** 2017. The U6 biogenesis-like 1 plays an important role in maize kernel and seedling development by affecting the 3' end processing of U6 snRNA. *Molecular Plant* **10**, 470–482.
- Li X, Gu W, Sun S, Chen Z, Chen J, Song W, Zhao H, Lai J.** 2018. Defective Kernel 39 encodes a PPR protein required for seed development in maize. *Journal of Integrative Plant Biology* **60**, 45–64.
- Li XJ, Zhang YF, Hou M, et al.** 2014. Small kernel 1 encodes a pentatricopeptide repeat protein required for mitochondrial *nad7* transcript editing and seed development in maize (*Zea mays*) and rice (*Oryza sativa*). *The Plant Journal* **79**, 797–809.
- Lin D, Gong X, Jiang Q, Zheng K, Zhou H, Xu J, Teng S, Dong Y.** 2015. The rice ALS3 encoding a novel pentatricopeptide repeat protein is required for chloroplast development and seedling growth. *Rice* **8**, 17.
- Liu S, Melonek J, Boykin LM, Small I, Howell KA.** 2013. PPR-SMRs: ancient proteins with enigmatic functions. *RNA Biology* **10**, 1501–1510.
- Liu X, Yu F, Rodermeil S.** 2010. An Arabidopsis pentatricopeptide repeat protein, SUPPRESSOR OF VARIATION7, is required for FtsH-mediated chloroplast biogenesis. *Plant Physiology* **154**, 1588–1601.
- Liu YJ, Xiu ZH, Meeley R, Tan BC.** 2013. Empty pericarp5 encodes a pentatricopeptide repeat protein that is required for mitochondrial RNA editing and seed development in maize. *The Plant Cell* **25**, 868–883.
- Lurin C, Andrés C, Aubourg S, et al.** 2004. Genome-wide analysis of Arabidopsis pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. *The Plant Cell* **16**, 2089–2103.
- Manavski N, Guyon V, Meurer J, Wienand U, Brettschneider R.** 2012. An essential pentatricopeptide repeat protein facilitates 5' maturation and translation initiation of *rps3* mRNA in maize mitochondria. *The Plant Cell* **24**, 3087–3105.
- Mei C, Jiang SC, Lu YF, et al.** 2014. Arabidopsis pentatricopeptide repeat protein SOAR1 plays a critical role in abscisic acid signalling. *Journal of Experimental Botany* **65**, 5317–5330.
- Morey JS, Van Dolah FM.** 2013. Global analysis of mRNA half-lives and de novo transcription in a dinoflagellate, *Karenia brevis*. *PLoS One* **8**, e66347.
- 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. *Molecular Biology and Evolution* **25**, 1120–1128.
- Palmer JM.** 1979. The 'uniqueness' of plant mitochondria. *Biochemical Society Transactions* **7**, 246–252.
- Peng C, Wang Y, Liu F, et al.** 2014. FLOURY ENDOSPERM6 encodes a CBM48 domain-containing protein involved in compound granule formation and starch synthesis in rice endosperm. *The Plant Journal* **77**, 917–930.
- Perocchi F, Gohil VM, Girgis HS, Bao XR, McCombs JE, Palmer AE, Mootha VK.** 2010. *MICU1* encodes a mitochondrial EF hand protein required for Ca<sup>2+</sup> uptake. *Nature* **467**, 291–296.
- Pesaresi P, Schneider A, Kleine T, Leister D.** 2007. Interorganellar communication. *Current Opinion in Plant Biology* **10**, 600–606.
- Patrick JJ, Clay NK, Nelson TM.** 2008. Vein patterning screens and the defectively organized tributaries mutants in *Arabidopsis thaliana*. *The Plant Journal* **56**, 251–263.
- Pfalz J, Bayraktar OA, Prikryl J, Barkan A.** 2009. Site-specific binding of a PPR protein defines and stabilizes 5' and 3' mRNA termini in chloroplasts. *The EMBO Journal* **28**, 2042–2052.
- Qi W, Tian Z, Lu L, Chen X, Chen X, Zhang W, Song R.** 2017a. Editing of mitochondrial transcripts *nad3* and *cox2* by Dek10 is essential for mitochondrial function and maize plant development. *Genetics* **205**, 1489–1501.
- Qi W, Yang Y, Feng X, Zhang M, Song R.** 2017b. Mitochondrial function and maize kernel development requires Dek2, a pentatricopeptide repeat protein involved in *nad1* mRNA splicing. *Genetics* **205**, 239–249.
- Schmitz-Linneweber C, Small I.** 2008. Pentatricopeptide repeat proteins: a socket set for organelle gene expression. *Trends in Plant Science* **13**, 663–670.
- Schmitz-Linneweber C, Williams-Carrier R, Barkan A.** 2005. RNA immunoprecipitation and microarray analysis show a chloroplast pentatricopeptide repeat protein to be associated with the 5' region of mRNAs whose translation it activates. *The Plant Cell* **17**, 2791–2804.
- Schmitz-Linneweber C, Williams-Carrier RE, Williams-Voelker PM, Kroeger TS, Vichas A, Barkan A.** 2006. A pentatricopeptide repeat protein facilitates the trans-splicing of the maize chloroplast *rps12* pre-mRNA. *The Plant Cell* **18**, 2650–2663.
- She KC, Kusano H, Koizumi K, et al.** 2010. A novel factor FLOURY ENDOSPERM2 is involved in regulation of rice grain size and starch quality. *The Plant Cell* **22**, 3280–3294.
- Small ID, Peeters N.** 2000. The PPR motif—a TPR-related motif prevalent in plant organellar proteins. *Trends in Biochemical Sciences* **25**, 46–47.
- Sosso D, Mbalo S, Vernoud V, et al.** 2012. PPR2263, a DYW-subgroup pentatricopeptide repeat protein, is required for mitochondrial *nad5* and *cob*

- transcript editing, mitochondrion biogenesis, and maize growth. *The Plant Cell* **24**, 676–691.
- Sung TY, Tseng CC, Hsieh MH.** 2010. The SLO1 PPR protein is required for RNA editing at multiple sites with similar upstream sequences in *Arabidopsis* mitochondria. *The Plant Journal* **63**, 499–511.
- Takemoto Y, Coughlan SJ, Okita TW, Satoh H, Ogawa M, Kumamaru T.** 2002. The rice mutant *esp2* greatly accumulates the glutelin precursor and deletes the protein disulfide isomerase. *Plant Physiology* **128**, 1212–1222.
- Takenaka M, Zehrmann A, Brennicke A, Graichen K.** 2013. Improved computational target site prediction for pentatricopeptide repeat RNA editing factors. *PLoS One* **8**, e65343.
- Tan J, Tan Z, Wu F, et al.** 2014. A novel chloroplast-localized pentatricopeptide repeat protein involved in splicing affects chloroplast development and abiotic stress response in rice. *Molecular Plant* **7**, 1329–1349.
- Tang J, Zhang W, Wen K, et al.** 2017. OsPPR6, a pentatricopeptide repeat protein involved in editing and splicing chloroplast RNA, is required for chloroplast biogenesis in rice. *Plant Molecular Biology* **95**, 345–357.
- 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. *The Plant Journal* **72**, 450–460.
- Toh YK, Kamariah N, Maurer-Stroh S, Roessle M, Eisenhaber F, Adhikari S, Eisenhaber B, Grüber G.** 2011. Structural insight into the glycosylphosphatidylinositol transamidase subunits PIG-K and PIG-S from yeast. *Journal of Structural Biology* **173**, 271–281.
- Wang D, Liu H, Zhai G, Wang L, Shao J, Tao Y.** 2016. OspTAC2 encodes a pentatricopeptide repeat protein and regulates rice chloroplast development. *Journal of Genetics and Genomics* **43**, 601–608.
- Wang G, Zhong M, Shuai B, Song J, Zhang J, Han L, Ling H, Tang Y, Wang G, Song R.** 2017. E+ subgroup PPR protein defective kernel 36 is required for multiple mitochondrial transcripts editing and seed development in maize and *Arabidopsis*. *New Phytologist* **214**, 1563–1578.
- Wang Y, Ren Y, Liu X, et al.** 2010. OsRab5a regulates endomembrane organization and storage protein trafficking in rice endosperm cells. *The Plant Journal* **64**, 812–824.
- Wang Y, Ren Y, Zhou K, et al.** 2017. WHITE STRIPE LEAF4 encodes a novel P-type PPR protein required for chloroplast biogenesis during early leaf development. *Frontiers in Plant Science* **8**, 1116.
- Wang Y, Wang C, Zheng M, et al.** 2016. WHITE PANICLE1, a Val-tRNA synthetase regulating chloroplast ribosome biogenesis in rice, is essential for early chloroplast development. *Plant Physiology* **170**, 2110–2123.
- Weißberger S, Soll J, Carrie C.** 2017. The PPR protein SLOW GROWTH 4 is involved in editing of *nad4* and affects the splicing of *nad2* intron 1. *Plant Molecular Biology* **93**, 355–368.
- Wittig I, Braun HP, Schägger H.** 2006. Blue native PAGE. *Nature Protocols* **1**, 418–428.
- Wu L, Wu J, Liu Y, Gong X, Xu J, Lin D, Dong Y.** 2016. The rice pentatricopeptide repeat gene *TCD10* is needed for chloroplast development under cold stress. *Rice* **9**, 67.
- Xie T, Chen D, Wu J, Huang X, Wang Y, Tang K, Li J, Sun M, Peng X.** 2016. Growing slowly 1 locus encodes a PLS-type PPR protein required for RNA editing and plant development in *Arabidopsis*. *Journal of Experimental Botany* **67**, 5687–5698.
- Yagi Y, Hayashi S, Kobayashi K, Hirayama T, Nakamura T.** 2013. Elucidation of the RNA recognition code for pentatricopeptide repeat proteins involved in organelle RNA editing in plants. *PLoS One* **8**, e57286.
- Yang J, Zhang M, Yu J.** 2008. Mitochondrial retrograde regulation tuning fork in nuclear genes expressions of higher plants. *Journal of Genetics and Genomics* **35**, 65–71.
- Yin P, Li Q, Yan C, et al.** 2013. Structural basis for the modular recognition of single-stranded RNA by PPR proteins. *Nature* **504**, 168–171.
- Yu QB, Jiang Y, Chong K, Yang ZN.** 2009. AtECB2, a pentatricopeptide repeat protein, is required for chloroplast transcript *accD* RNA editing and early chloroplast biogenesis in *Arabidopsis thaliana*. *The Plant Journal* **59**, 1011–1023.
- Yuan H, Liu D.** 2012. Functional disruption of the pentatricopeptide protein SLG1 affects mitochondrial RNA editing, plant development, and responses to abiotic stresses in *Arabidopsis*. *The Plant Journal* **70**, 432–444.
- Zhang C, Muench DG.** 2015. A nucleolar PUF RNA-binding protein with specificity for a unique RNA sequence. *Journal of Biological Chemistry* **290**, 30108–30118.
- Zhang YF, Suzuki M, Sun F, Tan BC.** 2017. The mitochondrion-targeted PENTATRICOPEPTIDE REPEAT78 protein is required for *nad5* mature mRNA stability and seed development in maize. *Molecular Plant* **10**, 1321–1333.
- Zheng M, Wang Y, Wang Y, et al.** 2015. DEFORMED FLORAL ORGAN1 (DFO1) regulates floral organ identity by epigenetically repressing the expression of OsMADS58 in rice (*Oryza sativa*). *New Phytologist* **206**, 1476–1490.
- Zhou F, Lin Q, Zhu L, et al.** 2013. D14-SCF(D3)-dependent degradation of D53 regulates strigolactone signalling. *Nature* **504**, 406–410.
- Zhou W, Cheng Y, Yap A, Chateigner-Boutin AL, Delannoy E, Hammani K, Small I, Huang J.** 2009. The *Arabidopsis* gene *YS1* encoding a DYW protein is required for editing of *rpoB* transcripts and the rapid development of chloroplasts during early growth. *The Plant Journal* **58**, 82–96.
- Zhou W, Lu Q, Li Q, Wang L, Ding S, Zhang A, Wen X, Zhang L, Lu C.** 2017. PPR-SMR protein SOT1 has RNA endonuclease activity. *Proceedings of the National Academy of Sciences, USA* **114**, E1554–E1563.
- Zhu Y, Lu J, Wang J, Chen F, Leng F, Li H.** 2011. Regulation of thermogenesis in plants: the interaction of alternative oxidase and plant uncoupling mitochondrial protein. *Journal of Integrative Plant Biology* **53**, 7–13.
- Zoschke R, Kroeger T, Belcher S, Schöttler MA, Barkan A, Schmitz-Linneweber C.** 2012. The pentatricopeptide repeat-SMR protein ATP4 promotes translation of the chloroplast *atpB/E* mRNA. *The Plant Journal* **72**, 547–558.
- Zoschke R, Watkins KP, Miranda RG, Barkan A.** 2016. The PPR-SMR protein PPR53 enhances the stability and translation of specific chloroplast RNAs in maize. *The Plant Journal* **85**, 594–606.