Running title: PPR756 coordinates with MORFs for RNA editing

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The rice PPR756 coordinates with MORFs for multiple RNA editing in mitochondria

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Author contributions

Q.Z, J.Hu and Y.Z designed the experiments. Q.Z carried out most experiments. Y.X performed the subcellular location and BiFC experiments. J.Huang contributed to the protein purification and field management. K.Z performed the mitochondria isolation and RNA Immunoprecipitation experiments. H.X and X.Q performed the data analysis. L.Z contributed to the RNAi transgenic lines. Q.Z and J.Hu wrote the paper with feedback from all authors.

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One sentence summary:

PPR756 takes part in the RNA editing of three various mitochondrial transcripts, cooperating with MORFs via editosome manner in rice.

ABSTRACT

In land plants, the pentatricopeptide repeat (PPR) proteins form a large family accounting for post-transcriptional process in mitochondria and chloroplasts. Although studies showed a number of PPR proteins generally influence the editing of organellar genes acting as a member of editing complex, few of them were characterized detailed in rice. Here, we reported a PLS-E subclass PPR protein in rice, PPR756, loss function of which lead to the abolishment of RNA editing events among three sites in mitochondrial genes including *atp6* (ATP synthase F0 subunit 6), *ccmC* (cytochrome c biogenesis C) and *nad7* (NADH dehydrogenase subunit 7). The defective C- to-U transformation of *atp6-368, ccmC-236* and *nad7-83* resulted in the retention of proline, proline and serine codon respectively to the right leucine in rice. Furthermore, PPR756 could bind to the three target genes directly and interacted with three OsMORFs (multiple organellar RNA editing factors), OsMORF1, OsMORF8-1 and OsMORF8-2. The knock-out plants of PPR756 exhibited retarded growth and greener leaves during the early stages, accompanied with sterile pollens at reproductive stage. These results established a role for PPR756 in the RNA editing of three various transcripts, cooperating with MORFs via editosome manner in rice.

Keywords: pentatricopeptide repeat (PPR) proteins; RNA editing; mitochondria; chloroplasts; sterility; OsMORFs

INTRODUCTION

The pentatricopeptide repeat (PPR) proteins constitute an interesting protein family widely spread in eukaryotes, from algae to human, although their number vary considerably in different organisms (Lurin et al., 2004). This protein family has been suggested to undergo great expansion when the terrestrial plants came into being, whose numbers ranged from ~100 (in *Physcomitrella patens*) to about over 1000 (in *Selaginella moellendorffii*) (O'Toole et al., 2008; Fujii and Small, 2011). However, except for the landplants, other eukaryotic species contain a few PPR members such as the animals, with 7 in human and 6 in mouse (Lightowlers and Chrzanowska-Lightowlers, 2008, 2013). Generally, PPR proteins constitute a kind of protein family which is generally characterized by 2 to about 30 degenerate tandem repeats about 35 amino acids (Lurin et al., 2004). Interestingly, there are three different types of PPR motifs, including P motif (35 amino acids), L motif (35 or 36 amino acids) and S motif (31 amino acids), according to which, the PPR proteins can be divided into two main classes: P-class and PLS-class. On account of the high sequence conservation of the C-terminal in different PPR proteins, the PLS-class proteins are then divided into three subclasses named PLS-E, E+ and DYW subclasses respectively (Small and Peeters, 2000; Lurin et al., 2004).

The mitochondria and chloroplasts in plants are semi-autonomous organelles, in which more than 2000 and 3000 proteins are respectively estimated, but only approximately 40 and 100 genes encoded by themselves, massive proteins are encoded by nuclei and imported into these organelles to play important roles in the regulation (Mullet, 1988; Sato et al., 1999; Millar et al., 2005). Accumulating researches established PPR proteins are almost exclusively targeted to the mitochondria or chloroplasts, where they take part in post-transcriptional processing of RNA by altering RNA sequence, stability, cleavage, splicing or translation to regulate the organellar gene expression (Dahan and Mireau, 2013; Shikanai and Fujii, 2013).

Different types of PPR proteins have been indicated to perform distinct functions, such as most P-class PPR proteins always take part in the progress of organelle transcripts cleavage, stability and translation, while the majority of PLS-class PPR proteins act as editing factors in the post-transcriptional process (Okuda et al., 2007; Saha et al., 2007). For example, PPR10 (Pfalz et al., 2009; Prikryl et al., 2011), ATP4 (Zoschke et al., 2012), CRP1 (Barkan et al., 1994), PGR3 (Yamazaki et al., 2004) could both stabilize the organelle gene transcripts and active their translation. Although almost all of the target transcripts are chloroplast ORFs, few is known to affect the mitochondria gene translation just except for that MPPR6 could influence the choice of start codon and the processing or stabilization of *rps5* 5' terminal (Manavski et al., 2012).

It has come to be known that some PPR-type *Rf* genes always belong to P-class and influence the cleavage of sterility-associated mitochondria RNAs (Dahan and Mireau, 2013). For instance, in rice ,RF1a and RF5 participated in the cleavage of toxic chimeric mitochondria transcripts contributing to fertility restoration (Wang et al., 2006; Hu et al., 2012). Not only the P-class PPR proteins take part in the splicing of Group II introns (such as THA8) but also several PLS-class proteins could (such as PpPPR43) (Ichinose et al., 2012; Khrouchtchova et al., 2012). However, when they were mentioned as editing factors, the PLS-class proteins may act as the main forces (Kotera et al., 2005). Up to now, a series of PLS-class proteins have been reported to play important roles in the editing of mitochondria or chloroplast genes. In rice, *OsOGR1*, *OsSMK1*, *OsMPR25* have been indicated to participate in the editing of organelle transcripts (Kim et al., 2009; Toda et al., 2012; Li et al., 2014). Previously, we also reported that *OsPGL1* and *OsPPS1* are responsible for the RNA editing of *ndhD*, *ccmFc* and *nad3*, respectively (Xiao et al., 2018; Xiao et al., 2018).

In this study, we characterized a novel PPR gene, *PPR756* (*Os12g19260*), encoding a PLS-E PPR protein, which is associated with the development of rice. PPR756 acted as an editing factor required for the editing of mitochondria genes, *atp6*, *ccmC* and *nad7*, loss function of which could influence the activities of mitochondrial electron transport chain complexes and result in the dysfunctional pollens.

RESULTS

PPR756 is essential for rice development

In previous reports, to identify the function of PPRs in rice, we generated a series of RNAi transgenic lines of several PPR genes under the background of ZhongHua11 (ZH11, *Oryza sativa*.L.*japanica*), and characterized OsPGL1 and OsPPS1 (Xiao et al., 2018; Xiao et al., 2018). In this study, we investigated a novel independent RNAi lines, in which the expression of *Os12g19260* was knocked down solidly (Supplemental Fig. S1C). Since, *Os12g19260* encodes a PPR protein with 756 amino acids, we named it as *PPR756* in this study. These lines displayed pleotropic phenotypes compared with WT, including delayed development, more green leaves, smaller leaf angle in early vegetative stage (Supplemental Fig. S1A to E).

According to these interesting phenotypes, we next applied the CRISPR-Cas9 system to create the knock-out (KO) mutant lines under the background of ZhongHua11 to confirm the function of PPR756. We selected three independent mutant lines for further detection in which two lines showed a premature translation termination, named *ppr756-1* and *ppr756-2* respectively (Fig. 1A). Meanwhile, in order to explore the function of *PPR756* in detail, we also generated the over-expression (OE) transgenic lines, in which the CDS of PPR756 was driven by the ubiquitin promoter and fused with the tags of Flag and cMyc (Supplemental Fig. S2A). We checked the expression of PPR756 in three independent OE lines, named PPR756-OE-1 to PPR756-OE-3. Results showed the relative expression of PPR756 was elevated in all OE lines, especially in PPR756-OE-2 (Supplemental Fig. S2B).

Moreover, the protein accumulation was conducted simultaneously. The signal of western blotting confirmed that PPR756 were successfully expressed in transgenic line (Supplemental Fig. S2C). Based on above detection and confirmation, we selected PPR756-OE-2 and *ppr756-1*for further analysis in this study.

Since the leaf colour was obvious a visible phenotypic differentiation, we first measured the chlorophyll contents among these lines. Data showed chlorophyll a (Chla), and chlorophyll b (Chlb) were highly accumulated in KO mutants while reduced in OE lines compared with WT, which well explained the change of leaf colour (Fig. 1B and C). Loss function of PPR756 also resulted in erect leaves, and lower seed-setting, while the OE line presented opposite phenotypes especially with larger panicle compared with WT (Fig. 1D and E) All of these results indicated that PPR756 played important roles in rice development and affected the yield in rice.

PPR756 especially influences pollen fertility

To make it clear whether the lower seed setting resulted from the male sterility or female sterility, we first tested the pollen fertility by I_2-KI staining. Results showed that the pollens became sterile when PPR756 was null (Fig. 2A). To explore the underlying mechanism, we further detected the morphological details of anther and pollen by SEM. The observations of anther showed the curly anther base in the KO mutant line compared with the WT and OE line (Fig. 2B). The observation of pollen further revealed that most of the pollen grains in the KO mutant were distorted and

shrunken, while those in the WT and OE lines were spherical and regular (Fig. 2C and D). All of these data indicated that the dysfunction development of pollen in KO mutant accounted for the reduced seed-setting rate, implied PPR756 is required for pollen development in rice.

PPR756 encodes a PPR-E subclass protein

Mostly, PPR proteins translated without introns (O'Toole et al., 2008), while PPR756 consisted of four exons and three introns based on the sequence analysis and database (Fig. 1A). PPR756 was predicted with 18 putative PPR motifs by the Pfam database (http://pfam.xfam.org.). In addition to the 18 PPR motifs (15 S motifs, 1 P motif and 2 L motifs), its C-terminal region (from 621 to 694 residues) also exhibited a strong similarity to the consensus sequence of E domain which attributed to classify this protein into the PLS-E subfamily (Fig. 3A). More interesting, most PPR proteins consisted of P, L, S motifs in turns, however, PPR756 consisted of numbers of S motifs (Fig. 3B).

To further explore the evolution of PPR756 in plants, a phylogenetic tree was constructed based on the protein sequence alignment from 22 other species, which exhibited over 55% similarity, including *Aegilops tauschii*, *Triticum turgidum*, *Brachypodium distachyon*, *Panicum hallii*, *Dichanthelium oligosanthes*, *Setaria italic*, *Eragrostis curvula*, *Setaria viridis*, *Sorghum bicolor*, *Zea mays*, *Panicum miliaceum*, *Phoenix dactylifera*, *Ananas comosus*, *Ensete ventricosum*, *Cinnamomum micranthum*

f. kanehirae, Musa balbisiana, Asparagus officinalis, Musa acuminata subsp. Malaccensis, Phalaenopsis equestris, Apostasia shenzhenica, Dendrobium catenatum. Results showed all of these homologous were vascular plants with most being monocots and only one dicot (Fig. 3C). Moreover, the homologous in top five species were further analyzed, results showing the extremely conservation in these monocots (Fig. 3D). Therefore, data suggested PPR756 may play a conservative role in different plant species, and might be more especially in monocots.

Subcellular localization of PPR756

The majority of PPR proteins are predicted to localize either in mitochondria or chloroplasts (Lurin et al., 2004). Up to now, the characterized PPR protein also confirmed this view, and some of them were dual-localized to mitochondria and chloroplasts. Bioinformatic analysis of the PPR756 protein sequence using the TargetP 1.1 server (http://www.cbs.dtu.dk/serivices/TargetP/) indicated that PPR756 is dual-localized to chloroplast and mitochondria. To determine the actual subcellular localization of PPR756 *in vivo*, we applied the p35S:PPR756-sGFP construct encompassing the full-length cDNA of PPR756 lacking the stop codon fused with the GFP (green fluorescent protein) driven by the constitutive CaMV 35S promoter. Then the recombinant vector and the empty p35S:sGFP vector (control) were transiently expressed in the rice protoplasts, respectively. The GFP fluorescent signals were detected by confocal microscopy, showing that the GFP signals were well overlapped

with the indicator signal of mitochondria (Fig. 4A).

Moreover, the Flag tag was used as a marker in the OE transgenic lines to observe the subcellular location of PPR756. Immunoblot assay with an anti-Flag antibody was then performed to detect the PPR756-Flag fusion protein in the chloroplasts and mitochondria extracted from the OE lines. An obvious signal detected in the mitochondria extractive confirmed that PPR756 was indeed targeted to mitochondria, while no signal was observed in the chloroplast (Fig. 4B). Therefore, these results demonstrated PPR756 was a mitochondria-localized protein.

Expression pattern of PPR756

To investigate the expression pattern of PPR756, a bioinformatic data was first conducted using the RiceXPro (http://ricexpro.dna.affrc.fo.jp), results indicated that PPR756 was constitutively expressed in all the rice tissues during the whole development process, but a higher level in the reproductive stage (Supplemental Fig. S3). To confirm the expression profile, we carried out the quantitative real-time PCR (qRT-PCR). Results demonstrated that PPR756 was expressed in all the tissues, including seedling, root, stem, leaf, young panicle, and mature spikelet. The relative expression of *PPR756* was much higher in mature panicle and seedling, implied it played essential roles in these tissues (Fig. 4C).

To characterize the expression pattern of PPR756 at tissue level, we also

generated the transgenic plants that harbor the p*PPR756*:GUS under the background of ZhongHua11. Different tissues of the transgenic plants were isolated for GUS histochemical staining. The staining results displayed PPR756 was preferentially expressed in shoot, seedling, stem and anther (Fig. 4D), which was consistent with the results of RT-PCR above. These findings demonstrated the significant function of *PPR756* in seedling which affected the chlorophyll contents in leaves and its indispensable roles in the reproductive organs which leaded to the sterile pollen.

PPR756 affects multiple C-to-U RNA editing in mitochondria

The PLS-PPR proteins have been reported to typically function as site-specificity factors in RNA editing (Barkan and Small, 2014). According to the sub-localization results of PPR756, we checked all well-known RNA editing sites, 21 in the chloroplasts and 491 in the mitochondria, respectively, (Corneille et al., 2000; Notsu et al., 2002) via RT-PCR and direct sequencing. In the chloroplast, results showed no obviously RNA editing efficiency difference between the KO mutant and WT were observed, which also implied PPR765 did not function in chloroplast directly. However, in the mitochondria, the RNA editing efficiency of three editing sites, *atp6-368*, *ccmC-236*, *nad7-83*, were significantly reduced in the KO mutant (Fig. 5A). All of these three genes were involved in mitochondrial electron transport chain (ETC) complexes. The editing defect caused by disabled PPR756 protein leaded to the amino acids transformation failure: CCA(Pro)-to-CTA(Leu), CCA(Pro)-to-CTA(Leu),

TCA(Ser)-to-TTA(Leu) in *atp6*, *ccmC*, *nad7* compared with the WT. The amino acids transformation failure generally influenced the function of the proteins as subunits of the corresponding complexes.

To investigate whether the transcript levels were affected by the editing deficiency, we took advantage of the qRT-PCR to detect the relative expression levels of these targets of PPR756. And the results demonstrated that there was almost no influence in the transcriptional expression of the three targets (Fig. 5B). Taken together, data suggested PPR756 is responsible for the three RNA editing sites in mitochondria.

PPR756 protein can directly bind to its targets both *in vitro* and *in vivo*

As previous studies reported, PPR protein could bind specifically to the surrounding RNA sequences of the editing sites as a trans-factor (Barkan et al., 2012; Nakamura et al., 2012). A series of experiments have also shown that the cis-element as a binding upholder were about 25 nucleotides of the upstream and 10 nucleotides of the downstream sequence surrounding the target editing sites (Okuda et al., 2006). To confirm PPR756 actually bind to its target transcripts, we generated a recombinant PPR756 proteins. We failed to express the complete PPR756 with 18 motifs for a long period. Then we removed the signal peptide and first S2 motif (residues 1-40) for expression, named MBP-PPR756$^{41\text{-}756}$-HIS protein which contains the MBP tag in its N-terminus, and 6 x HIS tag in the C-terminus (Supplemental Fig. S4A).

Recombinant tag protein containing the two tags (MBP-HIS) only was taken as a negative control. The two recombinant proteins were expressed in *Escherichia coli* and purified. The purified recombinant proteins were tested by western blot using His-tag antibody (Supplemental Fig. S4B). The binding cis-element sequences of PPR756's three targets were chosen by the -25 to +8 nucleotides surrounding the editing site according to the previous research, and labelled with fluorescent FAM. As a control, probe C (*nad5-1580*) was synthesized in our previous study and it has been reported as the target of MPR25, another PPR protein in rice. To carry out the RNA electrophoresis mobility shift assays (REMSA), the purified recombinant proteins were dialyzed to remove the RNase contamination, and incubated with the FAM-labelled RNA probes respectively. The binding efficiency could be detected by the shift difference between the protein-RNA complexes and the free RNA probes. The retarded bands were observed when the targets were incubated with MBP-PPR756$^{41\text{-}756}$ -HIS protein, while the Tag proteins could not bind to the RNA probes (Fig. 6A). Moreover, no electrophoresis shift was observed when the probe C was incubated with MBP-PPR756$^{41\text{-}756}$-HIS protein as a negative control, which suggested that PPR756 bound to its target RNAs specifically. We then performed the competitor assays using the corresponding unlabelled RNA probes respectively to further confirm the preference of PPR756 to its targets. The binding capacity to the labelled probes gradually decreased following the increased competitor concentration, suggested PPR756 bona fide bound to these targets directly (Fig. 6B). To validate the binding activity *in vivo*, we next conducted RNA Immunoprecipitation (RIP) in the

OE transgenic plants to check the transcripts incorporated to PPR756. In this procedure, we used the Flag antibody to obtain the RNAs bound to PPR756, and the extracted RNA were converted to cDNA by reverse-transcription (Fig. 6C). Several negative controls were designed, and the western blot was conducted to verify the RIP efficiency. Results showed the antibody anti-Flag could specifically capture PPR756-Flag and the outputs was appropriate for further analysis (Fig. 6D). Subsequently, we applied qRT-PCR by relevant primers to make sure the proportion of these three target transcripts. The results showed that *atp6*, *ccmC* and *nad7* transcripts displayed different binding efficiency to PPR756 nevertheless the negative control GAPDH did not (Fig. 6E). The percentage ratios of these three targets are 6.014, 0.210, 0.090, suggested that PPR756 preferentially bound to *atp6* compared with other two targets (Fig. 6F). It implied PPR756 might bind to these via different manner. Taken together, our results showed PPR756 could bind to the mitochondrial transcripts *atp6*, *ccmC* and *nad7* directly and specifically both *in vitro* and *in vivo*.

Dysfunction of PPR756 affects the activity of mitochondrial electron transport chain (ETC) complexes

Transcripts of *atp6* and *nad7* encode the subunit of respiratory complex V and I, respectively, and *ccmC* is involved in the biogenesis of cytochrome *c*, which transfers electrons from complex III to complex IV. According to the indispensable roles of *atp6*, *ccmC* and *nad7* in theses complexes, we attempted to detect the activities of

complexes of ETC in the WT, OE and KO mutant lines. The in-solution determination results showed that in the KO mutant, the activity of complex I, III, IV and V were all reduced significantly compared with the WT (Fig. 7A to D). The data from OE transgenic lines showed the activity of complex I, III, IV and V were all increased, which is consistent with the expected. Therefore, we speculated that these translated proteins without proper editing on RNA level might reduce their biological functions to varying degrees. Meanwhile, several representative antibodies for ETC complexes were next employed. The results suggested that except for the slight reduction of cytochrome c (Cyt C), the others such as NAD3, COXII and ATP6 all showed significant decrease in KO mutant compared with those in WT (Fig. 7E). It suggested that the decreased protein accumulation of these proteins in ETC complexes resulted in the reduction of the activities. The slight effect on Cyt C might resulted from the defective editing site was far away from its functional WWD domain (Ahuja and Thony-Meyer, 2003). Meanwhile, the coomassie brilliant blue staining of Blue-Native (BN) PAGE gel also showed all mitochondrial electron transport chain complexes were decreased in the KO mutant compared with the WT and OE line (Fig. 7F). All these data indicated that dysfunction of PPR756 leaded to the abortive editing and then induced the decrease of mitochondrial electron transport chain complexes activities.

PPR756 interacts with OsMORF1 and OsMORF8s

Previous studies reported that some PPR proteins function with MORFs as an editosome (Bentolila et al., 2012; Shi et al., 2016). Here, to make it clear whether PPR756 cooperated with MORFs, we investigated the interactions *in vitro* and *in vivo*. All of seven MORFs in rice database were cloned previously, including one MORF1-like (Os11g11020), two MORF2-like (Os04g51280, Os06g02600), one MORF3-like (Os03g38490), one MORF9-like (Os08g04450), and two MORF8-like (Os09g04670, Os09g33480). Yeast two-hybrid system was first used to check the interactions. Interactions between PPR756 and OsMORF1/8s were detected, while no interaction between PPR756 and OsMORF2/3/9 was observed, indicated that PPR756 interacted with OsMORF1/8s, but not with OsMORF2/3/9 (Fig. 8A). Studies showed OsMORF8s were dual-localized in the mitochondria and chloroplast, OsMORF1 and OsMORF3 were sublocalized in the mitochondria only. Therefore, the bimolecular fluorescence complementation (BiFC) was conducted to detect the interactions between PPR756 and all mitochondria-located OsMORFs *in vivo*. Yellow fluorescence signals were clearly observed when the PPR756 and MORF1 or MORF8s fusions were coexpressed in rice protoplast, whereas no fluorescence was detected in PPR756 and OsMORF3, as well as the negative control, indicating the special and physical interactions between them *in vivo* (Fig. 8B). Taken together, we demonstrated that PPR756 directly interacted with OsMORF1/8s in mitochondria, consistent with fact that the editing events of mitochondrial genes were affected rather than the chloroplast genes.

DISCUSSION

PPR756 is required for plant development

In plants, RNA editing is a familiar event occurred in organelle transcripts which is critical for gene repair and essential for the development of plants (Takenaka et al., 2013). There are several types of RNA editing in plants including C-to-U, U-to-C and A-to-I. The three editing events have different occurrences: C-to-U editing in mRNAs and tRNAs, A-to-I editing in tRNAs and U-to-C editing in mRNAs of a few non-flowering land plants (Chateigner-Boutin and Small, 2010). Previous study of the rice transcriptome revealed 21 and 491 C-to-U editing sites in the chloroplasts and mitochondria, respectively (Corneille et al., 2000; Notsu et al., 2002). Despite of the mechanism and evolutional significance of RNA editing is still obscure, several RNA editing factors have been characterized, such as PPRs, MORFs (also known as RIPs, RNA editing factor interacting protein), ORRM (organelle RNA recognition motif-containing), PPO1 (protoporphyrinogen IX oxidase 1) and OZ1 (organelle zinc finger 1) (Sun et al., 2016).

Up to now, approximately 65 nuclear encoded genes were characterized for RNA editing in mitochondrion and/or chloroplast in Arabidopsis, rice, maize and Physcomitrella patens, and so on. Only 5 of them, OGR1, OsSMK1, MPR25, OsPGL1 and OsPPS1 were identified in rice (Kim et al., 2009; Toda et al., 2012; Li et al., 2014; Xiao et al., 2018; Xiao et al., 2018). Here, we characterized a novel PPR gene, *PPR756*, which is involved in the RNA editing of *atp6-368*, *ccmC-236* and

nad7-83. NAD7 is the subunit of respiratory complex I and ATP6 is the subunit of respiratory complex V. Therefore, the activities of complex I and V are expected reduced, which may then influence the activities of complex III and complex IV. All of them are involved in the energy production, consequently, like most PPR mutants, loss of function of PPR756 leaded to the defects of plant pleiotropic phenotypes. Furthermore, in this study, we showed that PPR756 expressed in most tissues, but highly in seedling and anther, exhibiting a significant spatial expression pattern. The pollen was sterile and seed setting was extremely lower than WT, which was consistent with the high expression of PPR756 in anther. We speculated that the energy for seedling and anther is in great demand, any short of which could lead to the defects of the development in corresponding stage.

The majority of RNA editing events result in the conversion from proline/serine to leucine (Kim et al., 2009; Toda et al., 2012; Liu et al., 2013; Li et al., 2014). Besides of our previous studies on OsPGL1 and OsPPS1, findings of these editing sites of PPR756 also established that all of them contribute to convert the proline/serine to leucine, implied the function of leucine in proteins could be evolutionally conserved in plants.

PPR756 shares the PPR-RNA recognition model

A commonly held view is that PPR proteins can directly bind to target RNA with its specific manner, which is confirmed by bioinformatic and structural analysis (Barkan

et al., 2012; Yin et al., 2013). These analyses indicated that three positions of amino acids distributed in two adjacent PPR repeats were of great importance in recognizing its target RNA base (Takenaka et al., 2013). Position 6 in first helix motif is predicted more important for distinguishing between purines (A and G) and pyrimidines (U and C) while position 1 in the second helix motif directly recognizes amino (A and C) or keto (G and U) groups (Yagi et al., 2013). Position 3 is less important, but provides an additional constraint to binding nucleotides (Takenaka et al., 2013).

Most PPR proteins consisted of P, L, S motifs in turns, and usually end with a L2 linked to S2 motif ahead of the E domain (Takenaka et al., 2013). However, in this study, PPR756 was distinctive, including numbers of continuous S motifs in which several S2 motifs in the middle rather than the end. To evaluate the PPR-RNA recognition model, we analysed the associations between PPR756 and its three target genes on the basis of the reported general recognition mechanism. We found that the match index between PPR756 and its targets *atp6*, *ccmC* and *nad7* were 9/17, 11/17 and 12/17, respectively (Fig. 8C), such relative high match scores suggesting that PPR756 could bind its target genes directly in accordance with the PPR-RNA recognition model. More interesting founding in the match alignments, the highly matching score from the last 8 nucleotides (-5~-12) implied that the latter might be more important than the former during the recognition.

One of the most essential issue is the deaminase activities during RNA editing events. Taking the fact that DYW motif was dispensable for RNA editing activity *in*

vivo of CRR22 and CRR28, and some DYW motif of PLS-class members possess the RNA endonuclease activities *in vitro* (Okuda et al., 2009). Furthermore, given that PPR756 belongs to PLS-E group without DYW motif, we can speculate that other unknown factors deaminase activity is needed.

A coordination between mitochondrion and chloroplast

Recently research proposed that MORFs could enhance the interaction or recognition of PPR proteins with RNA targets *in vitro* (Yan et al., 2017). Although our REMSA results exhibited that PPR756 could bind to the three RNA targets specifically, the binding affinities among the threes targets were different. In view of the involvement of MORF1/8s, we speculated that the distinct binding activities could result from the interactions with the partners of PPR756. Of course, other unknown factors should be also considered *in vivo* (Fig. 9).

Meanwhile, PPR756, a mitochondria-located PPR protein, interacted with OsMORF8-1 and OsMORF8-2, two dual-localized proteins, which may also shed light on us that the amount of PPR756 in cell is quite critical. Since the overexpressed or absence of PPR756 could change the distribution of MORFs, we easily checked the alteration of the chlorophyll contents in the OE and mutant lines. Consequently, we believe that the dual-localized proteins such as MORF8 and other PPRs might play an essential role in regrade signal pathway or crosstalk between mitochondrion and chloroplast. Furthermore, mitochondrion and chloroplast are both semi-autonomous

organelles, part of whose functions rely on the nuclear-encoded proteins, a defects of ETC may have some retrograde signals to the nucleus to balance the associations among nucleus, mitochondrion and chloroplast in order to ensure the plant life.

MATERIALS AND METHODS

Plant materials and growth conditions

To construct the RNAi lines, a fragment (ranging from 13 to 354 bp) of *Os12g19260* cDNA was amplified with primers (Table S1) and cloned into the pH7GWIWG(II) vector to construct the RNAi vector. To construct the knock-out lines, in CRISPR-Cas9 system, we chose two target sites close to the start codon ATG to design the gRNAs, which were driven by OsU6a and OsU3. The overexpression lines were created by carrying the full-length of cDNA of PPR756 without stop codon fused to the N-terminus of tags, driven by ubiquitin promoter in pCAMBIA1301 backbone. All the constructs were transformed into the calli of *Oryza sativa* L. *japonica* Zhonghua 11 (ZH11) mediated by *Agrobacterium*. All the transgenic plants were grown in Hubei province and Hainan province, China and green chamber in Wuhan, Hubei province under proper management.

Subcellular localization of PPR756

For transient expression, the full-length encoding sequence of PPR756 was cloned

into HBT-sGFP vector under the control of CaMV 35S promoter. The protoplasts were extracted from 1-week old etiolated rice seedlings and then transformed with about 10~20 μg plasmids respectively according to a procedure described in previous study (Xiao et al., 2018). Mito Tracker Red (Invitrogen) was used as a mitochondrial specific dye (Invitrogen). The organelle and GFP signals were detected in the Leica microscope (DM4000 B, Germany) using different excitation wavelength. In addition, western blotting was performed to confirm the subcellular localization of PPR756, in which the mitochondria and chloroplasts were extracted from the PPR756 OE lines. Then the signals were detected by kinds of antibodies, including Flag (DIA-AN, Wuhan, China), VDAC, RbcL (Agrisera, Vannas, Sweden), Actin, and Histone (ABclonal).

RNA Extraction, reverse transcription PCR and Real-time PCR

The total RNA was extracted by Trizol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was resuspended in RNase-free water and treated with RNase-free DNase I (Thermo Fermentas). After digestion, a PCR was performed to confirm the elimination of DNA contamination. Then, approximately 5 μg of the digested total RNA was reverse-transcribed using the M-MLV reverse transcriptase (Invitrogen) and random primer (Thermo Fermentas) to obtain the first-strand cDNA. The real-time PCR was performed on a Lightcycler 480 (Roche) using the SYBR Green I Master PCR kit with gene-specific primers. Ubiquitin and

actin served as controls for gene expression.

Histochemical analysis of GUS activity

To further detect the temporal and spatial expression pattern of *PPR756*, a 1963 bp fragment including the promoter cassette of *PPR756* was amplified from ZH11 and cloned into the vector pCAMBIA1391 in order to drive the GUS report gene expression. The vector was transformed into the calli of ZH11 to obtain the transgenic plants. Then the GUS activity of various tissues in the transgenic plants were measured according to our previous study (Xiao et al., 2018).

Yeast two-hybrid assay

The full-length cDNA of *PPR756* was amplified and cloned into the bait vector pGBKT7, while *OsMORF1*, *OsMORF2*, *OsWSP1*, *OsMORF3*, *OsMORF8-1*, *OsMORF8-2* and *OsMORF9* were cloned respectively into the prey vector pGADT7. These constructs were then co-transformed into yeast strain AH109 in corresponding pairs as previously described method (Hu et al., 2012).

Bimolecular fluorescence complementation assays

For the bimolecular fluorescence complementation (BiFC) analysis, the full-length

cDNA of *PPR756* was fused into the C-terminus fragment of yellow fluorescent protein (YFP) in the pUC-SPYCE vector and OsMORF proteins were fused into the N-terminus fragment of yellow fluorescent protein (YFP) in the pUC-SPYNE vector. Constructs were co-transformed into rice protoplasts in pairs and the signals were observed using a Leica microscope (DM4000 B, Germany) in bright and fluorescent fields as described previously (Hu et al., 2012).

Determination of chlorophyll content

The acetone extraction technology was used with that extracting solution of chlorophyll was absolute ethyl alcohol : acetone : water as the ratio of 4.5 : 4.5 : 1. The 0.5 g fresh leaves derived from WT, OE line, KO line, were cut into splices about 1 cm and put into 10 ml extracting solution in a dark place for approximately 20h until the leave splices became white. Then, 200 μl pigment solution was taken out to measure the absorption values with the extracting solution as a control using the Tecan Infinite M200 (Switzerland) and the chlorophyll contents were calculated according to the formula described previously (Arnon, 1949).

Recombinant protein expression and RNA electrophoresis mobility shift assays

The recombinant protein was created with a fusion of MBP in the PPR756$^{41\text{-}756}$ N-terminus and 6 x His in the C-terminus which was purified across two columns

equipped with Ni^{2+} affinity resin (Ni-NTA Resin, GenScript) and MBP (PurKine MBP-Tag Dextrin Resin 6FF, Abbkine) in turn as well as another control fusion protein containing only MBP and His tags. RNA probes were synthesized and labeled with 6-FAM at the 5' end by GenScript (Nanjing, China). For the RNA electrophoresis mobility shift assays (REMSAs), the dialyzed recombinant protein was incubated with 100 fmol RNA probes in a 10 x binding buffer (100mM HEPES PH=7.3, 200mM KCl, 10mM $MgCl_2$, 10mM DTT) condition and reacted in a 20 μl system for 30 min at 25 ^{o}C with 10 units RNasin, followed with separation by 8% native acrylamide gels in a 0.5 × TBE buffer. After the electrophoresis, the gels were screened using a Typhoon Trio Imager (GE).

RNA Immunoprecipitation-PCR (RIP-PCR)

RIP is a well-developed technology to detect protein-RNA interactions *in vivo* and involves the immunoprecipitation (IP) of a target protein followed by purification of the associated RNA. Here, the cell lysate was obtained from 2-week-old OE line seedlings which were cross-linked in 0.1% formaldehyde in advance to strengthen protein-RNA interactions. The seedlings were grinded in liquid nitrogen and the powder was resuspended in extraction buffer (Tu et al., 2015). Then we used the anti-Flag antibody to IP the PPR756-Flag fusion protein from the supernatant, and the IgG as a negative control. After immunoprecipitation, the associated RNA was extracted by Trizol reagent and reverse-transcribed with random primers to obtain

cDNAs. The cDNAs were further detected by the quantitative PCR (qPCR).

Scanning electron microscopy assay

The samples for SEM assay were prepared according to previous study (Xiao et al., 2018). The seedlings were cut into 1 cm long and mature spikelets were immersed in 2.5% glutaraldehyde buffer for 18 h at 4 ^{o}C as quickly as possible when separated from the plants. The samples were dehydrated with the help of different concentrate ethyl alcohol at room temperature in every 1 hour and then the dehydrating agent was replaced in order to subsequently critical-point dried. At last, the samples were sputter coated with gold in an ion sputter (E-100, Japan), and observed with a scanning electron microscope (Hitachi S-3000N, Japan).

Blue native PAGE and activity examining of mitochondrial complex

The mitochondria were isolated from the calli of the WT, OE and KO lines, respectively, and the equivalent of 500 μg of total mitochondrial proteins were treated and loaded on BN-PAGE gel with continuous gradient according to the previous protocol (Liu et al., 2012). The gel was then stained with coomassie brilliant blue.

For further verification, the activity of mitochondrial electron transport chain complexes, complex I, III, IV and V, were measured using the electron transport chain complex assay kits from Solarbio Life Science according to the instructions (Beijing

Solarbio Science & Technology).

Data availability

Sequence data from this work can be found in the GenBank databases under the following accession numbers: PPR756 (Os12g19260), MORF1 (Os11g11020), WSP1(Os04g51280), MORF2 (Os06g02600), MORF3 (Os03g38490), MORF8-1 (Os09g33480), MORF8-2(Os09g04670), MORF9 (Os08g04450).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Phenotypic characterization of *PPR756* RNAi lines.

Supplemental Figure S2. Construction and validation of PPR756 OE lines.

Supplemental Figure S3. Predicted spatio-temporal expression pattern of PPR756.

Supplemental Figure S4. Expression and purification of recombinant protein .

Supplemental Table S1. Primers used in this study.

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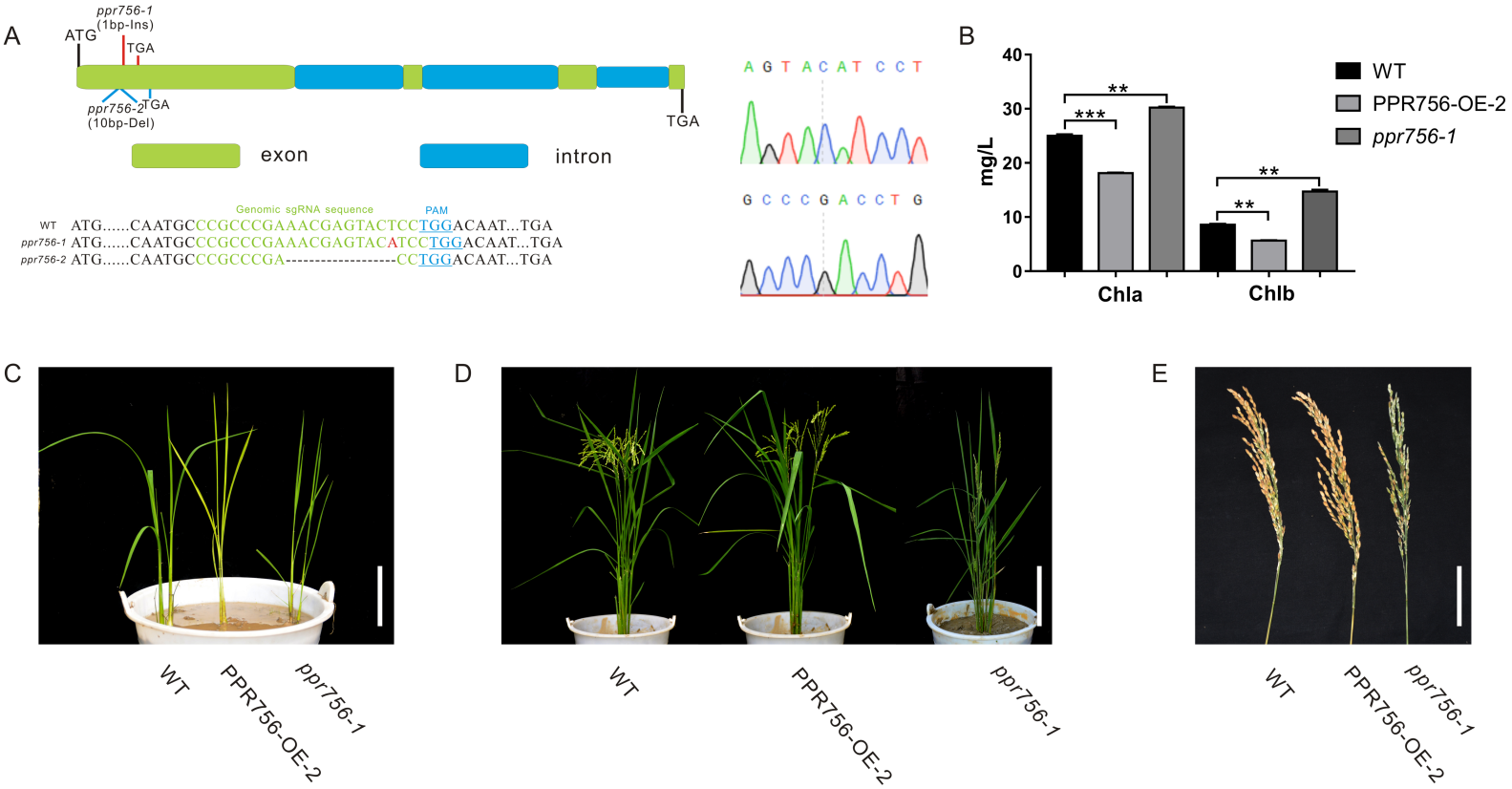


Figure 1. Phenotypic characterization of the PPR756 lines. A, The structure of PPR756 and ppr756 mutant. Two target sites were chosen and the obtained ppr756 mutant lines showed premature translation termination with 1-bp insertion in ppr756-1 line and 10-bp deletion in ppr756-2 line. B, The different chlorophyll contents of leaves in the wild-type (WT), the PPR756 OE line and ppr756 mutant. The bars represent the SD of three independent biological replicates and the unit of content is mg/L. C-D, Phenotypic comparison of the seedlings and mature plants of the wild-type (WT), the PPR756 OE line and ppr756 mutant. Bars, 5cm for c and 20cm for d, respectively. E, Comparison of the seed setting rate in wild-type (WT), the PPR756 OE line and ppr756 mutant. Bars, 5cm.

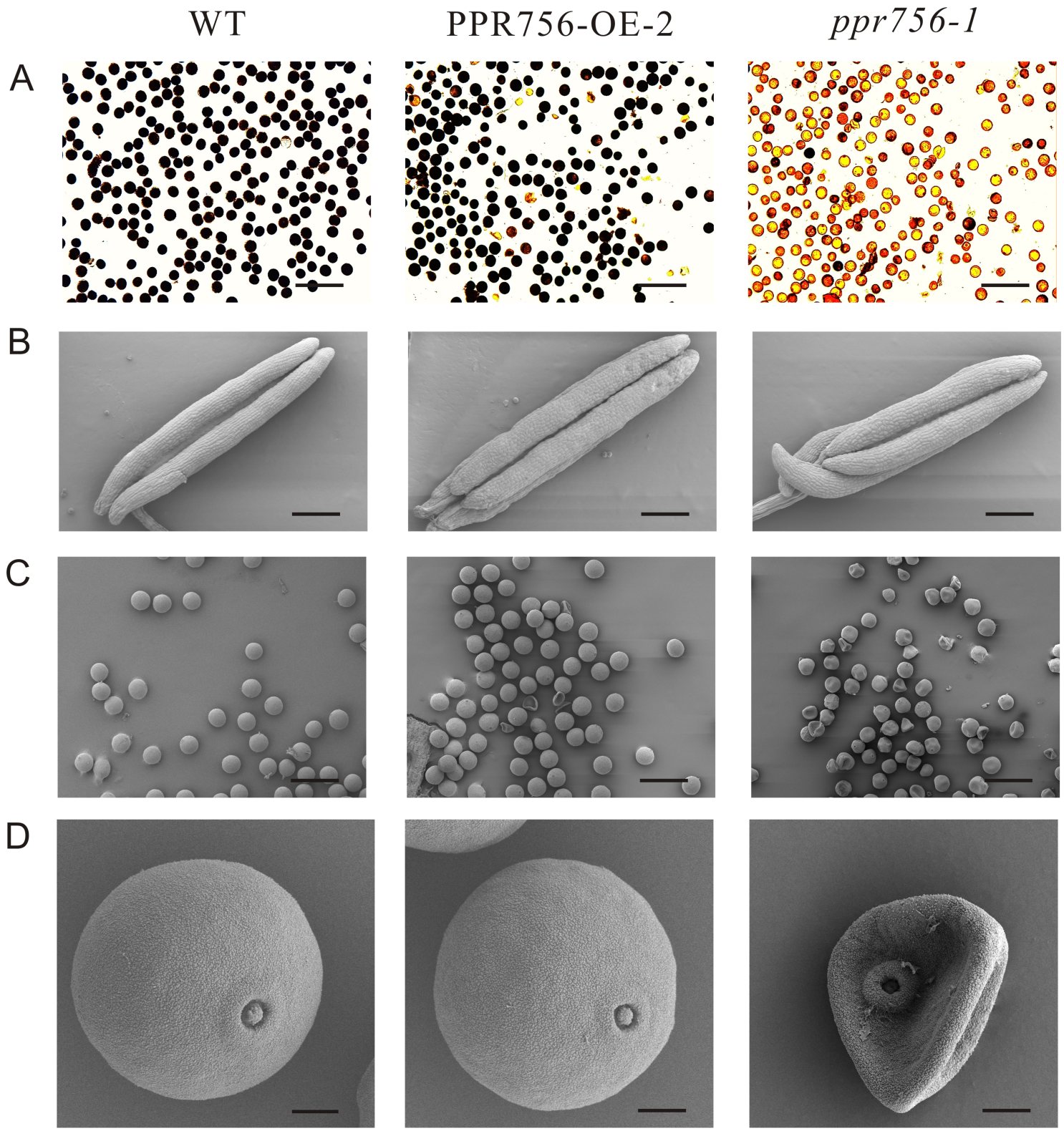


Figure 2. Fertility detection and scanning electron microscopy observations. A, Comparison of pollen fertility of WT, the PPR756 OE line and ppr756 mutant by potassium iodide (1% I2-KI) staining. The darkly stained pollen is fertile, whereas the lightly or non-stained pollen is sterile. Bars, 200 μm. B, Scanning electron microscopy (SEM) observation of anthers of WT, the PPR756 OE line and ppr756 mutant. Bars, 200 μm. C-D, Scanning electron microscopy (SEM) observation of pollens of the PPR756 OE line and ppr756 mutant. Bars, 100 μm for c and Bars, 10 μm for d, respectively.

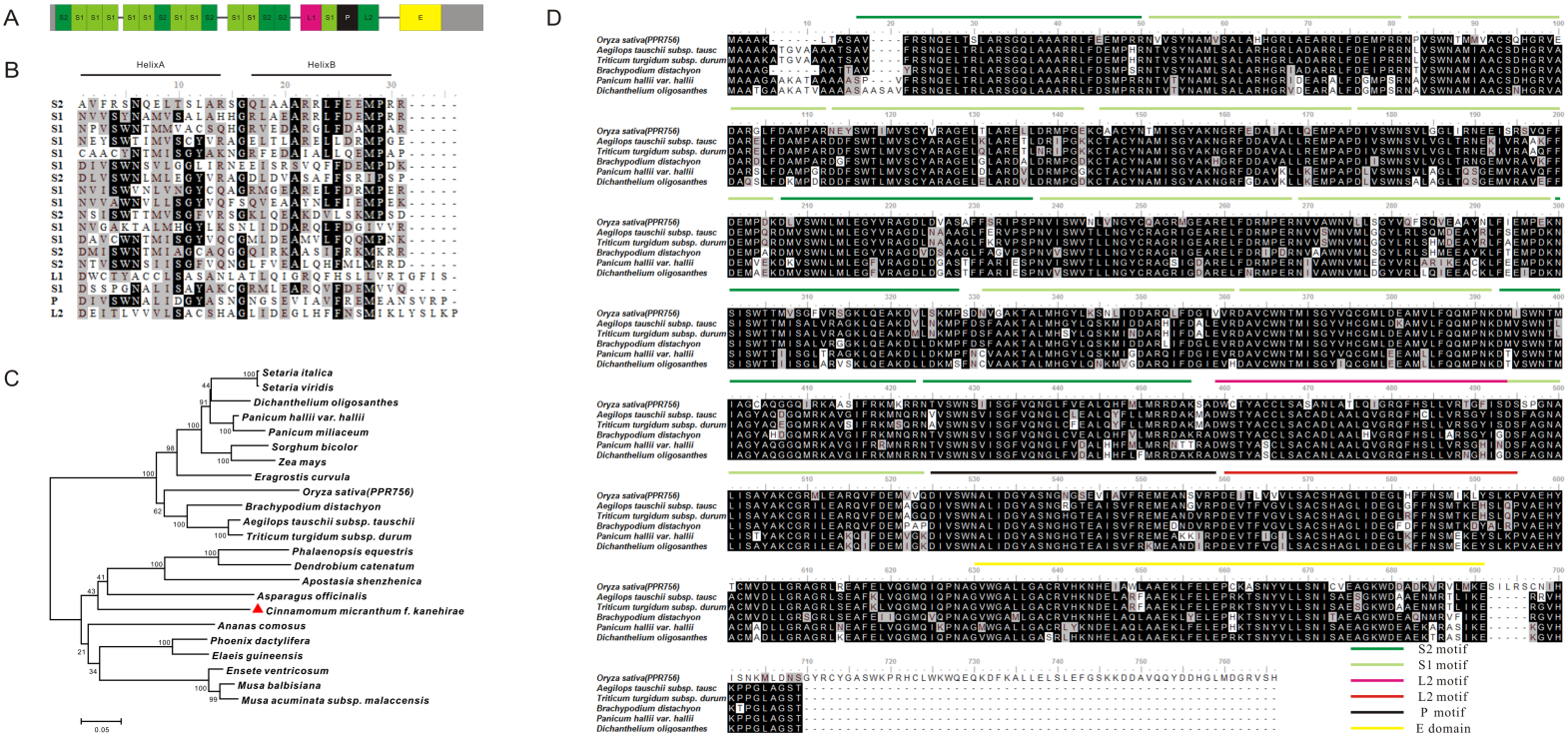


Figure 3. Structure and sequence alignment of PPR756 protein. A, PPR and E motifs of PPR756 protein were indicated and marked respectively. B, Amino acids sequence alignment of 18 PPR motifs of PPR756, highly conserved amino acids were marked in dark. C, Phylogenetic tree analysis of PPR756 homologs. D, Detailed sequence alignment of rice PPR756 protein with some highly conserved orthologs. The different motifs were marked out using various colors of straight lines.

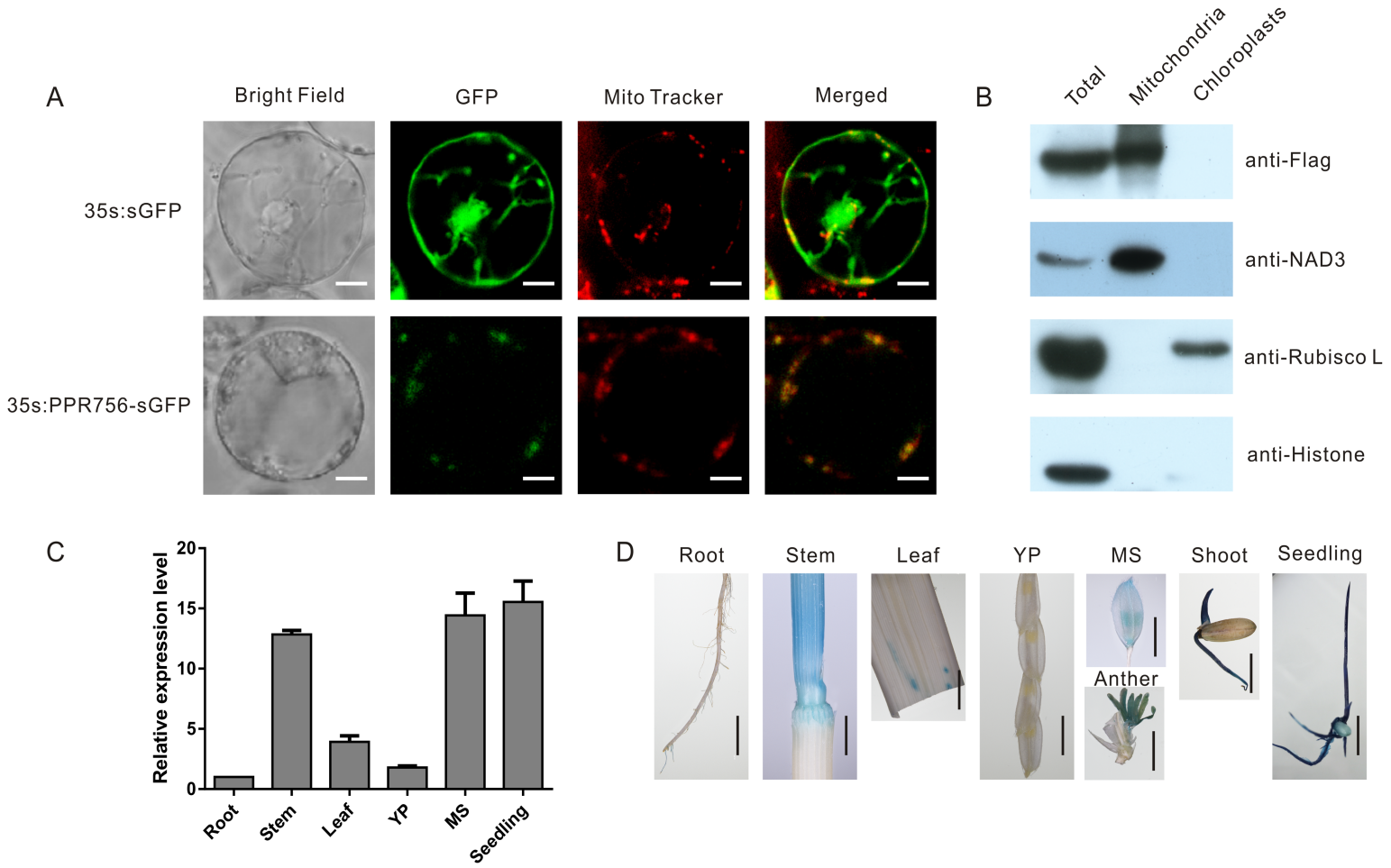
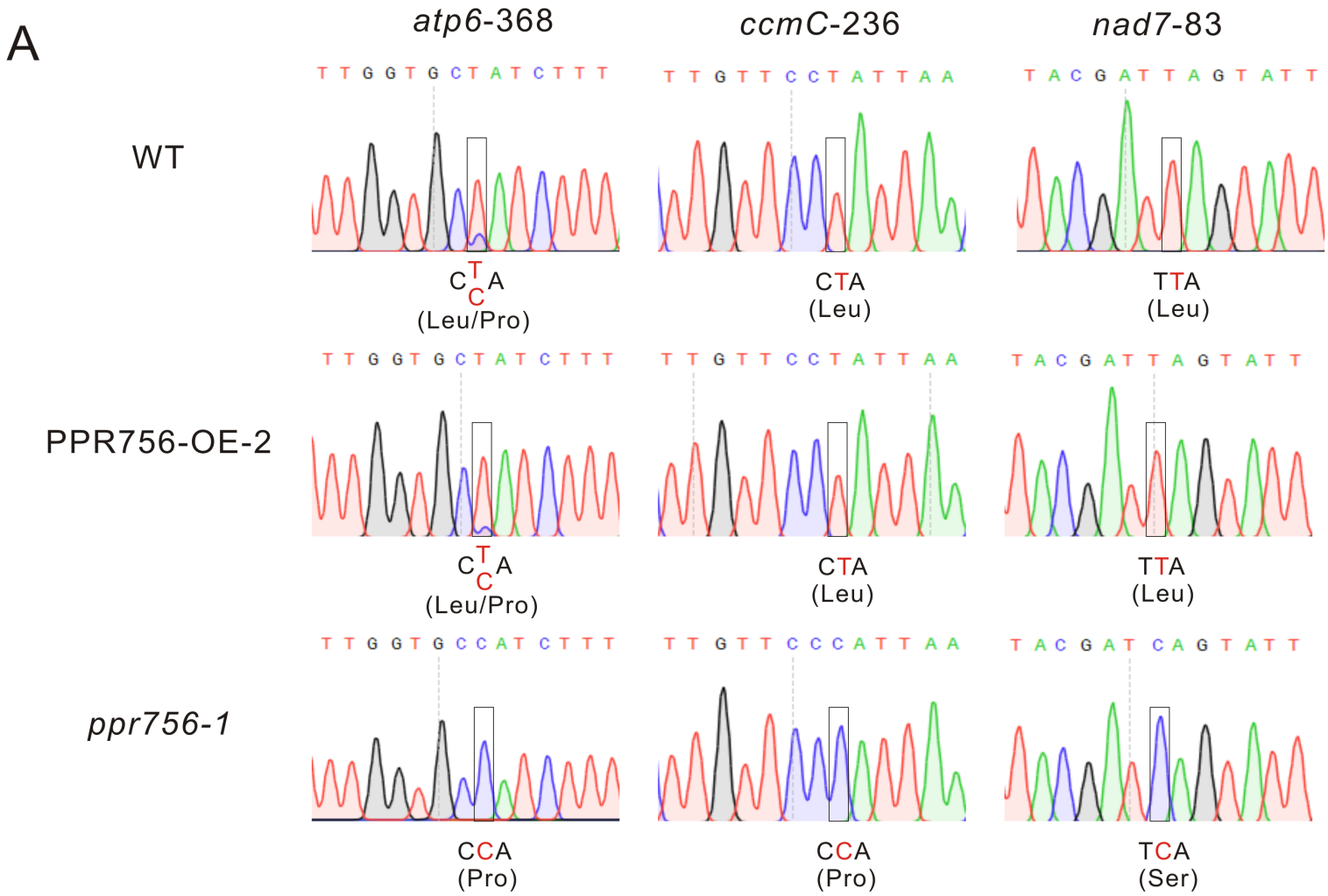


Figure 4. Subcellular localization and expression pattern of PPR756. A, The vectors 35s:sGFP and 35s:PPR756-sGFP were transferred into the rice protoplasts for transient expression. The green fluorescent protein (GFP) was detected and merged with the signal of MitoTracker, an mitochondria indicator. Bars, 5 μm. B, Total proteins, mitochondrial proteins and chloroplast proteins of transgenic plants were isolated for immunoblot assays to evaluate the subcellular localization of PPR756. Antibodies against NAD3, Rubisco L and Histone were used as indicators for the mitochondria, chloroplasts and nucleus, respectively. The Flag signal represented the fusion protein PPR756-Flag. C, Quantitative real-time PCR analysis of PPR756 in various WT tissues . Rice Actin gene was used as a control. D, Histochemical staining analysis of PPS1 promoter–GUS reporter gene in various tissues. YP, young panicle; MP, mature panicle; Bars, 0.5 cm.



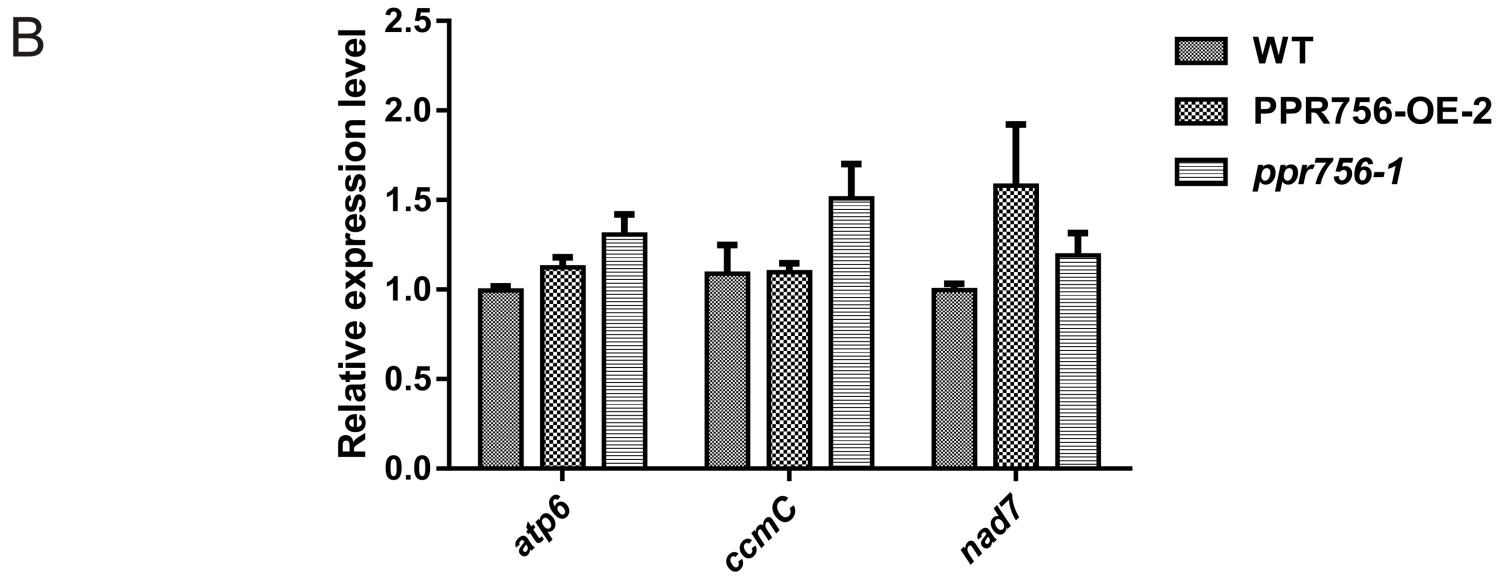
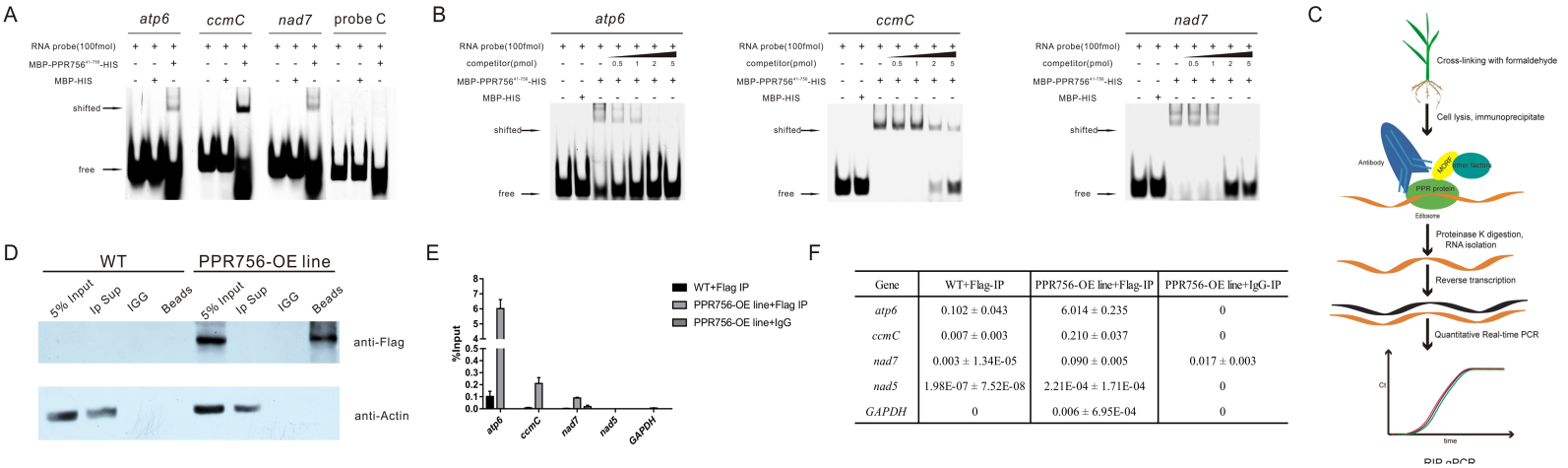


Figure 5. PPR756 is indispensable for mitochondrial gene editing. A, RNA editing analysis of atp6-368, ccmC-236, nad7-83 in the WT, PPR756 OE line and ppr756 mutant. B, Transcript level determination of the three target genes in the WT, PPR756 OE line and ppr756 mutant. Data are means from three independent biological replicates.



Gene	WT+Flag-IP	PPR756-OE line+Flag-IP	PPR756-OE line+IgG-IP
atp6	0.102 ± 0.043	6.014 ± 0.235	0
ccmC	0.007 ± 0.003	0.210 ± 0.037	0
nad7	0.003 ± 1.34E-05	0.090 ± 0.005	0.017 ± 0.003
nad5	1.98E-07 ± 7.52E-08	2.21E-04 ± 1.71E-04	0
GAPDH	0	0.006 ± 6.95E-04	0

Figure 6. PPR756 can directly bind to its target genes in vitro and in vivo. A, RNA electrophoresis mobility shift assays (REMSAs) of the recombinant MBP-PPR756$_{41\text{-}756}$-HIS protein and MBP-HIS tags with the FAM-labelled RNA probes. MBP-HIS tags and probe C served as a negative control. B, The corresponding non-labelled RNA probes in a concentration gradient were used as competitors for the REMSA of MBP-PPR756$_{41\text{-}756}$-HIS protein with its target probes, respectively. MBP-HIS tags served as a negative control. C, The flowchart of RIP-qPCR experiment. D, Western blots detection of the IP efficiency. The Actin was used as a control as well as the IgG served as negative control. E, Quantitative real-time PCR of atp6, ccmC and nad7. The nad5 and GAPDH were used as control genes. Bars represent the SDs from three independent biological replicates. F, The percentages of binding rates of three target genes and two control genes in the RIP-qPCR according to the input.

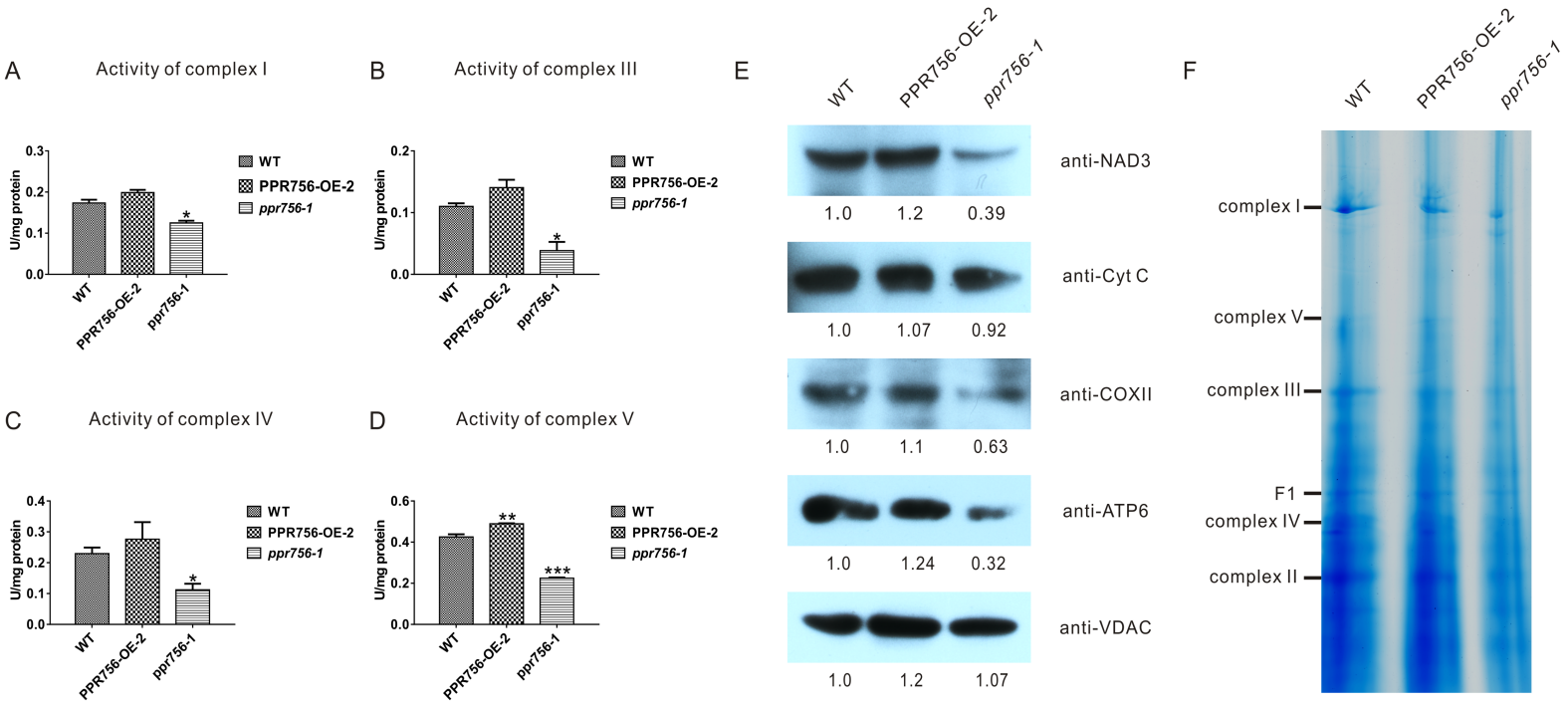


Figure 7. Detection of the activities and proteins in mitochondrial electron transport chain complexes. A-D, In-solution determination of the activities of complex I, III, IV and V in every 1mg protein to response to their substrate in 1 μmol per minute. Bars represented the SDs from three independent biological replicates. E, Western bolts detection of the components content in mitochondrial electron transport chain complexes. NAD3, Cyt C, COXII and ATP6 represented complex I, complex III, complex IV and complex V, respectively and VDAC served as a control.

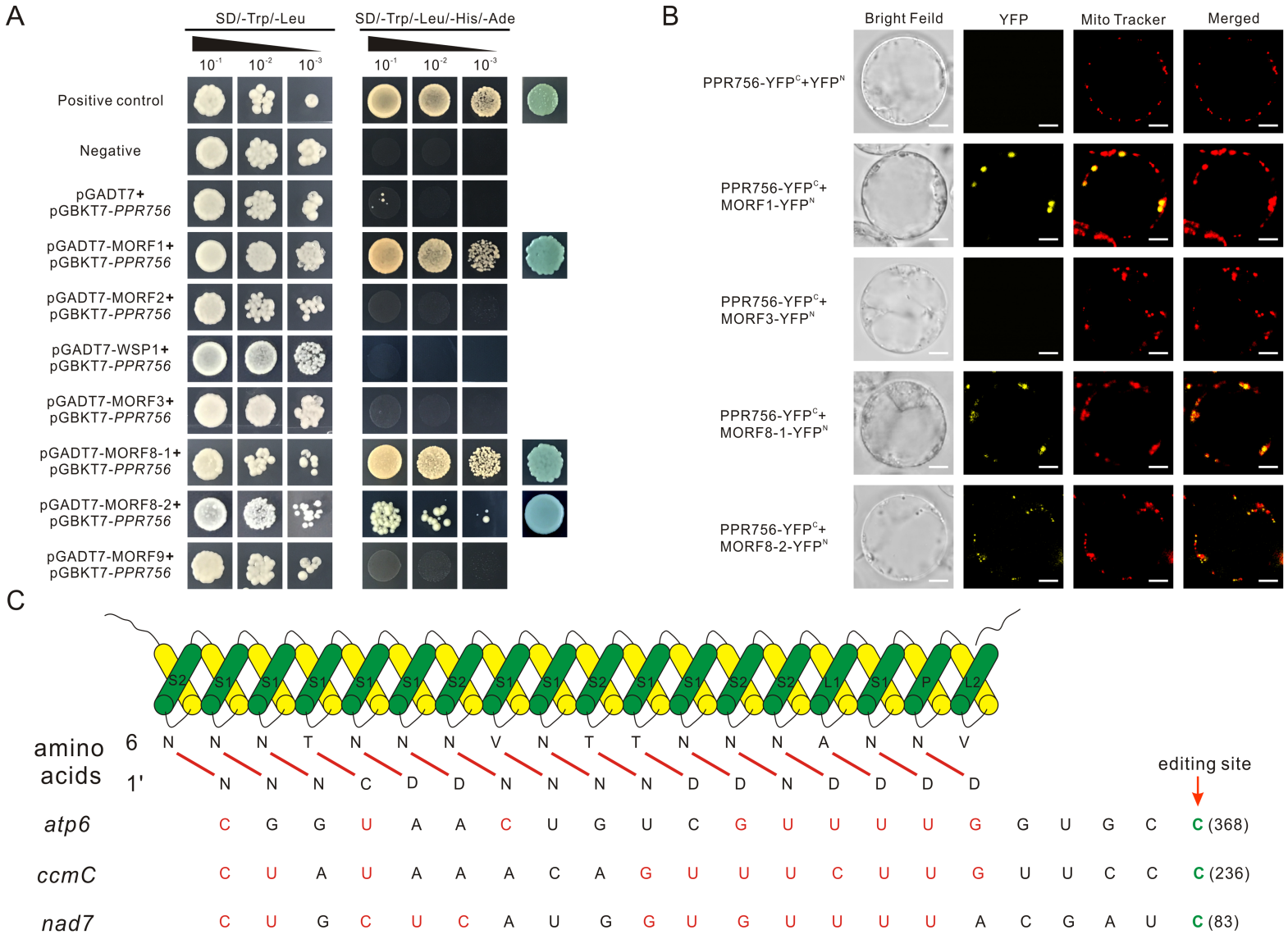


Figure 8. PPR756 directly interacts with OsMORF1 and OsMORF8s, and the PPR-RNA recognition model. A, Yeast two-hybrid assays. PPR756 was cloned into the bait vector pGBKT7 and OsMORFs were cloned into the prey vector pGADT7. Transformants were grown on SD/-Trp/-Leu and SD/-Trp/-His/-Leu-Ade dropout plates. The 53-T and 53-Lam interactions were used as positive and negative controls, respectively. B, Bimolecular fluorescence complementation (BiFC) assays were performed to confirm the interactions between PPR756 and OsMORFs in rice protoplasts. The mitochondria were indicated by MitoTracker Red. Bars, 5 μm. C, Potential recognition mechanism of PPR756 to its targets. The amino acid combination of 6 and 1'in each adjacent PPR motif of PPR756 were aligned to the nucleotide upstream of the editing site. The permissible matched nucleotides were colored in red, and the edited sites C were colored in green.

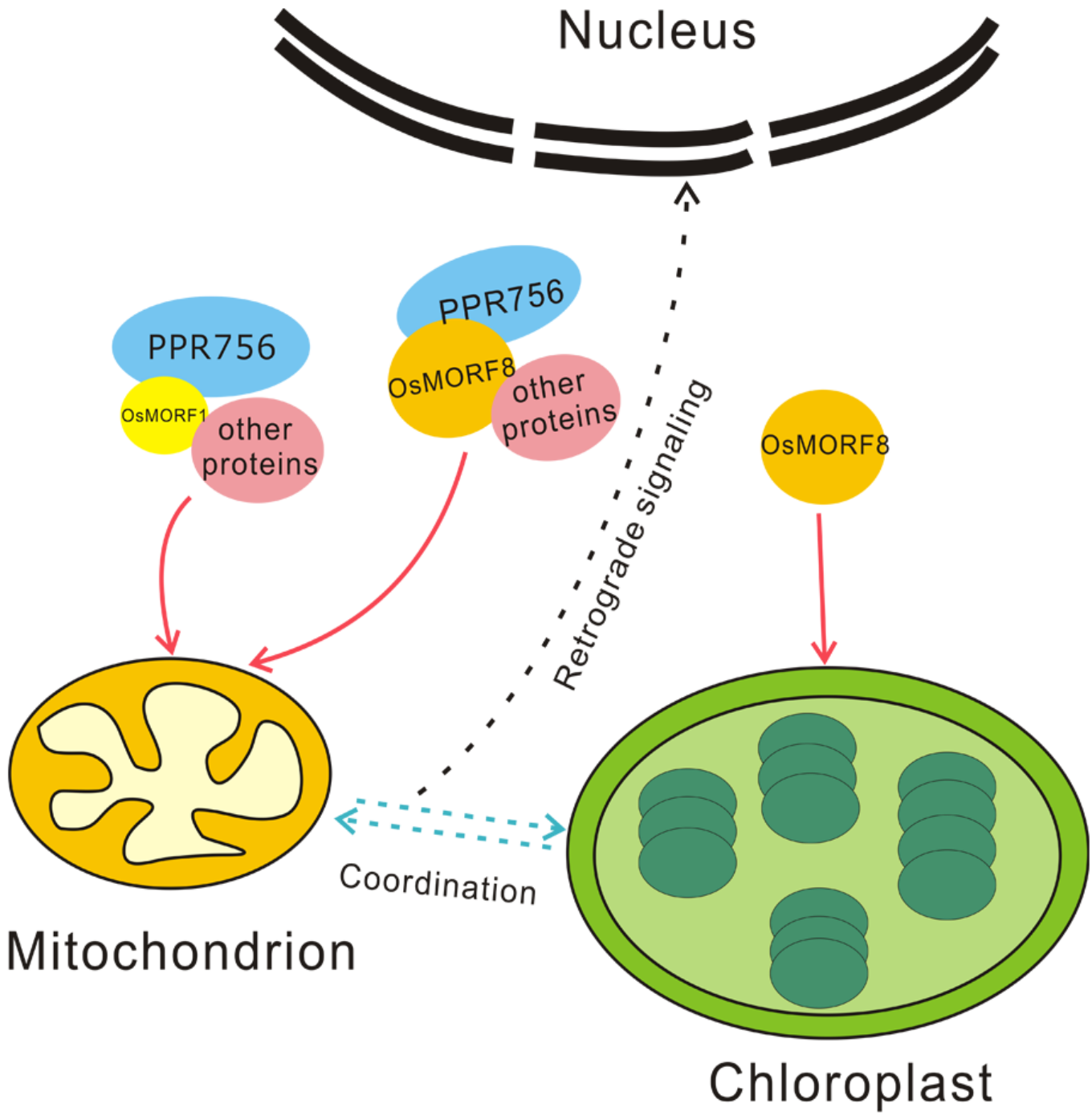


Figure 9. A working model for PPR756 function in the organelles . PPR756 interacted with OsMORF1, OsMORF8s and other proteins in mitochondria as an EDITOSOME for RNA editing. Because of the dual-localization of OsMORF8s and/or other unknown proteins, dysfunction of PPR756 could affect the protein distribution and the coordination between mitochondrion and chloroplast. Consequently, the editosome consisted by PPRs and MORFs can serve as a retrograde signaling factor in plant.

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