1 **A Rice Dual-localized Pentatricopeptide Repeat Protein is involved in Organellar**
 2 **RNA Editing with MORFs**

3

4 **Running Title** : A dual-localization PPR functions on RNA editing with MORFs

5

6 Haijun Xiao, Yanghong Xu, Chenzi Ni, Qiannan Zhang, Feiya Zhong, Jishuai Huang, Yingguo
 7 Zhu, and Jun Hu *

8

9 * Corresponding author: junhu@whu.edu.cn

10

11 State Key Laboratory of Hybrid Rice; Engineering Research Center for Plant Biotechnology and
 12 Germplasm Utilization of Ministry of Education; College of Life Sciences, Wuhan University,
 13 Wuhan 430072, China.

14

15 **Highlight**

16 We firstly characterized a dual-localized PPR protein which is required for RNA
 17 editing in mitochondrion and chloroplast simultaneously. OsPGL1 binds to two
 18 distinguish target transcripts directly and cooperated with MORFs.

19

20 **Abstract**

21 Flowering plants engage in diverse RNA editing events in mitochondrion and
 22 chloroplast on post-transcriptional process. Although several PPRs and MORFs were

23 identified as RNA editing factors, the underlying mechanism of PPRs and the
 24 cooperation among them are still obscure. Here, we identified a rice dual-localized
 25 PPR mutant *Ospgl1*. Loss-of-function of *OsPGL1* resulted in defect of chloroplast
 26 RNA editing at *ndhD*-878 and mitochondrial RNA editing at *ccmFc*-543, which can
 27 be restored via complementary validation. Despite the synonymous editing on
 28 *ccmFc*-543, loss of editing at *ndhD*-878 caused failure of conversion from serine to
 29 leucine, leading to the dysfunction of chloroplast and defective in photosynthetic
 30 complex, further studies demonstrated OsPGL1 directly bound to both two transcripts.
 31 The interaction between three MORFs (MORF2/8/9) and OsPGL1 were confirmed *in*
 32 *vitro* and *in vivo*, implied OsPGL1 functioned on RNA editing via an editosome. It
 33 also suggested MORFs assisted and contributed to the flexible PPR-RNA recognition
 34 model during RNA editing through the cooperation with PPRs. These results provide
 35 new insight into the relationship between RNA editing and plant development on
 36 chloroplast.

37

38 **Key words:** RNA editing, PPR, Chloroplast, Mitochondrial, MORF, Dual-localized

39

40 **Introduction**

41 RNA editing was broadly defined as a post-transcriptional process that changes the
 42 sequence of an RNA molecule from that of its DNA master (Covello and Gray, 1989).
 43 RNA editing is widely spread in eukaryotic cell and highlighted in plant mitochondria
 44 and chloroplasts. In animals, RNA editing was mediated by two mechanisms, one is
 45 C-to-U editing by apolipoprotein B mRNA editing enzyme, catalytic (APOBEC) and
 46 another is adenosine (A)-to-inosine (I) editing by adenosine deaminase acting on
 47 RNA (ADAR) (Kim *et al.*, 1994; Mehta and Driscoll, 2002; Melcher *et al.*, 1996;

48 Teng *et al.*, 1993). In plants, RNA editing includes C-to-U, U-to-C and A-to-I
 49 conversion (Takenaka *et al.*, 2013b). The majority of editing in plants occurs in
 50 mitochondrial and plastid transcripts, however, A-to-I editing also occurs in cytosolic
 51 tRNAs (Chateigner-Boutin and Small, 2010). RNA editing from C-to-U is the most
 52 frequent editing events in plants, in Arabidopsis and rice, 525 and 491 C-to-U editing
 53 sites in mitochondria, 34 and 21 C-to-U editing sites in chloroplast has been identified
 54 in previous study (Chateigner-Boutin and Small, 2010). In human, the APOBEC3
 55 proteins can deaminate cytidines to uridines in single-stranded DNA (ssDNA)
 56 (McDougall *et al.*, 2011). Recently, the fusion of APOBEC3 with catalytically dead
 57 Cas9 (dCas9) or other Cas9 variants in CRISPR system accomplished the genomic
 58 editing of single bases in mammalian, yeasts and plants (Hess *et al.*, 2016; Kim *et al.*,
 59 2017; Lu and Zhu, 2017; Ma *et al.*, 2016; Zong *et al.*, 2017). While in plants, the
 60 deaminase activity still need to be confirmed and elucidated. Researchers proposed
 61 RNA editing activity is governed by RNA-binding pentatricopeptide repeat (PPR)
 62 proteins, and DYW-subclass PPR proteins were considered as mainly RNA editing
 63 factors depend on its similarity with cytidine deaminases of the DYW motif (Salone
 64 *et al.*, 2007)

65 To date, several non-PPR editing factors, such as RNA editing factor interacting
 66 proteins (RIPs)/multiple organellar RNA editing factors (MORFs), organelle RNA
 67 recognition motif (ORRM) proteins, organelle zinc-finger (OZ) proteins, and
 68 protoporphyrinogen oxidase 1 (PPO1) have been identified as components of the
 69 plant RNA apparatus (Sun *et al.*, 2016). There were nine MORF proteins in
 70 Arabidopsis and five members in rice. MORF8/RIP1 was identified as an interacting
 71 factor of RARE1, a PPR protein required for RNA editing in Arabidopsis chloroplast
 72 (Bentolila *et al.*, 2012). MORF2 and MORF9 were both targeted exclusively in
 73 plastids and can affect most of RNA editing sites in chloroplast in Arabidopsis
 74 (Takenaka *et al.*, 2012). MORF2/8/9 can interact with ORRM6, which is involved in

75 RNA editing of psbF-C77 and accD-C794 in *Arabidopsis* (Hackett *et al.*, 2017).
 76 Furthermore, MORFs also interact with each other and form a heterodimeric or
 77 homodimeric complex, suggesting a more complicated regulation mechanism in
 78 plants (Takenaka *et al.*, 2012).

79 The PPR protein family was characterized by the degenerate motifs of 35 amino
 80 acids arranged as tandem repeats of 2–25 such elements, settled in a pair of
 81 antiparallel double alpha-helices, helices A and B (Small and Peeters, 2000; Yin *et al.*,
 82 2013). PPR proteins have been found to be in eukaryotic genomes and greatly
 83 expanded in the plants, study showed more than 400 members harbored into land
 84 plants (Cheng *et al.*, 2016). PPR protein can be divided into two sub-families: P and
 85 PLS subfamilies, the P sub-families proteins contain tandem arrays of canonical
 86 35-amino-acid (P) PPR repeats, whereas PLS sub-families characterized by triplets of
 87 P, L (i.e. 35 to 36 amino acids in length), and S (i.e. 31 amino acids) motifs (Lurin *et al.*,
 88 2004). Many post-transcriptional processes in these organelles were relevant to PPR
 89 proteins, including RNA editing, splicing, cleavage, RNA stability, and translation
 90 (Schmitz-Linneweber and Small, 2008). Most PPR proteins are targeted either
 91 mitochondria or chloroplasts, but few of them are dual-localization.

92 Here, we addressed a novel dual-localized PPR protein OsPGL1 in rice, which
 93 is required for RNA editing at two different *cis* elements. Loss of function of *OsPGL1*
 94 caused a pale green leaves phenotype, resulting from defective photosynthetic
 95 complex in chloroplast development. Loss of editing at *ndhD*-878 caused failure of
 96 conversion from serine to leucine, which an extremely conserved amino acid in plants.
 97 Further investigation showed OsPGL1 functions with MORF2/8/9 and directly binds
 98 to *ndhD* and *ccmFc* via its 9 PPR motifs. These results confirmed PPR proteins and
 99 MORFs are required for RNA editing, and function together via a complex editosome.

101 **Materials and Methods**

102

103 **Plasmid construction and transformation**

104 Two target sites at the 20 bp upstream of protospacer-adjacent motif sequence (PAM)
 105 according to the recognition principle of CRISPR/Cas9 were designed and analyzed
 106 the specificity by CAS-OFFinder (<http://www.rgenome.net/cas-offinder>) (Table S1).
 107 The target sequence joint was linked into gRNA-U3 and gRNA-U6 vector,
 108 respectively followed by two rounds of nest-PCR. PCR products were subsequently
 109 linked to CRISPR/Cas9 vector. The construction was identified through PCR and
 110 sequencing. Calli derived from ZhongHua 11 (*Oryza sativa*. *L. Japonica*) were used
 111 for *Agrobacterium*-mediated transformation. WT and CRISPR/Cas9 knockout lines
 112 were grown in a paddy field and greenhouse in Wuhan, China under proper
 113 management.

114

115 **Scanning electron microscopy and transmission electron microscopy assay**

116 For scanning electron microscopy assay, samples were prepared as described
 117 previously (Zhou *et al.*, 2011). Rice young leaves were cut into small section with a
 118 razor and immediately placed in 70% ethanol, 5% acetic acid, and 4% formaldehyde
 119 for 18 h. Samples were critical point dried, sputter coated with gold in an E-100 ion
 120 sputter, and observed with a scanning electron microscopy (Hitachi S-3000N, Japan).

121 For transmission electron microscopy, samples were fixed in 2.5% (w/v)
 122 paraformaldehyde and 0.25% glutaraldehyde in 0.2 N sodium phosphate buffer for
 123 2-4 h at 4 °C, pH 7.0, and were then post-fixed in 1% OsO₄ in PBS, pH 7.4.
 124 Following ethanol dehydration, samples were embedded in acrylic resin. Ultrathin

125 sections (50 to 70 nm) were double stained with 2% (w/v) uranyl acetate and 2.6%
126 (w/v) lead citrate aqueous solution and examined with a transmission electron
127 microscope at 200 kV (Tecnai G2 20 Twin, FEI, Netherlands).

128

129 **RNA Extraction and qRT-PCR**

130 Total RNA was extracted with 1 mL Trizol reagent according to the manufacturer's
131 instructions (Invitrogen). After isopropanol precipitation, the RNA was resuspended
132 in 30 µl RNase-free water and treated with RNase-free DNase I (New England
133 Biolabs). First-strand cDNA was reverse transcribed using random primers (Primers
134 were listed in Table S2). Ubiquitin was detected as control for gene expression.

135

136 **Analysis of RNA editing**

137 For RNA editing analysis in the wild type and the *Ospgll*, total RNAs were isolated
138 from the young leaves using the Trizol reagent as described before (Hu *et al.*, 2012).
139 RNA was treated with RNase-free DNase I (New England Biolabs), and confirmed by
140 PCR. Then, the RNAs were reverse transcribed with random primers and the
141 high-fidelity reverse transcriptase SuperScript III (Invitrogen). Primers were designed
142 to cover all 491 mitochondrial editing sites and 21 chloroplast editing sites (Table S2).
143 The RT-PCR products were sequenced directly.

144

145 **Subcellular localization of OsPGL1**

146 For transient expression in rice protoplast, 188 amino acid of the OsPGL1 N-terminal
147 were cloned into HBT-sGFP driven by the cauliflower mosaic virus 35S promoter to
148 construct the 35S:OsPGL1^{N1-188}:sGFP fusion protein. Protoplast preparation and

149 transformation procedures were as previously described (Yu *et al.*, 2014).
150 MitoTracker Red (Invitrogen) was used as a mitochondrial specific dye.

151

152 **RNA electrophoresis mobility shift assays (REMSA)**

153 The corresponding cDNA fragments of *OsPGL1* was amplified with specific primers
154 (Table S2), and cloned into the pGEX-6p-1 vector to create the fusion protein
155 GST-OsPGL1. Two RNA probes (probe 1 and probe 2) and negative control probe
156 (probe C) containing the target editing site were synthesized and labeled with biotin at
157 the 3' end by GenScript (Nanjing, China). For REMSA, the recombinant protein was
158 incubated with RNA probe in a 20 μ l reaction mixture including 10 μ l of 2 $\times$ binding
159 buffer (100 mM Na phosphate, pH 7.5, 10 units RNasin, 0.1 mg/mL BSA, 10 mM
160 DTT, 2.5 mg/ mL heparin, and 300 mM NaCl). The mixture was incubated at 25 $^{\circ}$ C
161 for 30 min, followed with separation by 5% native PAGE in 0.5 $\times$ TBE buffer and
162 transferred onto the nylon membrane (Roche). For the competitive REMSA, the
163 gradually increased concentration of the unlabeled probe was added into the reaction
164 mixture followed the procedure described above.

165

166 **Complementation of *Ospgll* mutants**

167 For complementation of the *Ospgll* mutant, a full-length (1815bp) cDNA fragment
168 was constructed into pCAMBIA-2300 vector driven by CMV-35S promoter and
169 transformed into *Ospgll-1* mutant background by *agrobacterium*-mediated method.
170 Independent transgenic lines were obtained and planted in Wuhan, China.

171

172 **Yeast two-hybrid assays**

173 The full-length cDNA of *OsPGL1* and *MORFs* were cloned into pGBKT7 and
174 pGADT7 vector. The constructs were co-transformed into yeast (AH109 strain) in
175 pairs according to previous study (Hu *et al.*, 2012).

176

177 **GST pull-down assays**

178 The purified recombinant proteins (GST tag, Trx-His tag, GST-OsPGL1,
179 Trx-MORF2-His, Trx-MORF8-His and Trx-MORF9-His) were dialyzed against
180 phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2
181 mM KH₂PO₄) for 24 h and quantified using the bicinchoninic acid (BCA) method. The
182 recombinant protein GST-OsPGL1 was incubated with glutathione sepharose for 1 h
183 on ice and washed with five volumes of PBS for five times. Trx-His, Trx-MORFs-His
184 proteins were subsequently added to detect the interaction. The binding proteins were
185 washed with five volumes of PBS for five times, eluted with glutathione reductase
186 and separated by 10% SDS-PAGE. Products were transferred onto a polyvinylidene
187 fluoride (PVDF) membrane (BioRad), and investigated with antibodies against GST
188 and His respectively.

189

190 **BiFC assays**

191 For bimolecular fluorescence complementation (BiFC) analysis, the full-length cDNA
192 of OsPGL1 without the stop codon was fused to the C-terminal fragment of yellow
193 fluorescent protein (YFP) in pUC-SPYCE (C-terminal), MORF2, MORF8 and
194 MORF9 were fused to the N-terminal fragment of YFP in pUC-SPYNE (N-terminal).
195 The two vectors were co-transformed into rice protoplasts in pairs and observed by
196 bright-field and fluorescent microscopy using a Leica DM4000B microscopy (Hu *et*
197 *al.*, 2012).

198

199 **Co-immunoprecipitation analysis**

200 Total proteins from transgenic plants fused with FLAG (UBI: OsPGL1-FLAG) and
 201 GFP (35S: MORFs-GFP) were extracted with extraction buffer (100 mM Tris-HCl,
 202 200mM NaCl, 5 mM EGTA, 5 mM EDTA, 10 mM DTT, 0.6% TritonX-100, 1 mM
 203 PMSF, pH 8.0) and incubated with 2µg anti-GFP antibody overnight at 4°C, 100 µl of
 204 protein A-Sepharose beads was added and incubated for a further 3 to 4 h.
 205 Immunoprecipitates were washed for five times with co-immunoprecipitation buffer,
 206 (150mM NaCl, 20mM Tris-HCl, 1mM EDTA, 0.2% NP-40, 1 mM PMSF, pH 7.4)
 207 and loaded in 6×SDS loading buffer by denaturing for 10 min. The proteins were
 208 separated on a 10% SD-SPAGE gel and detected by immunoblotting with anti-FLAG
 209 and anti-GFP antibodies.

210

211 **Immunoblot analysis**

212 Total proteins were extracted from young leaves and quantified with the BCA protein
 213 assay kit (Thermo scientific). 10 µg total proteins were separated by SDS-PAGE,
 214 transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-rad) and
 215 incubated with various primary antibodies against NdhD (Beijing Protein Innovation,
 216 China), PsaA, PsbA, PetA, AtpA, Lhca2 and Lhcb2 (Agrisera). Actin was used as a
 217 reference antibody. Detection was carried out by the ECL western blotting detection
 218 reagents (Bio-rad).

219

220 **Blue-native PAGE**

221 The equivalent of 500 µg of total mitochondrial proteins from WT and *Ospgll-1*

mutant were treated and loaded on BN-PAGE according to the previous protocol (Liu *et al.*, 2012). The gel was firstly stained with coomassie brilliant blue. For immunoblotting, the gel was transferred to the PVDF membranes, antibodies against Cyt c1 were used to detect the accumulation of complex III.

Accession numbers

Sequence for rice OsPGL1, chloroplast NdhD, and mitochondrial gene CcmFc can be found in the GenBank database under accession numbers XP_015618645.1 (OsPGL1), NP_039444 (NdhD), YP_002000589 (CcmFc), respectively.

Results

Phenotypic characterization of the *Ospgll* mutant

We used Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) system to generate several PPRs mutant under the background of the ZhongHua 11 (ZH11, *Oryza sativa*, *L. Japonica*) and obtained two independent transgenic knocked-out lines of LOC_Os12g06650. Further genome DNA sequencing revealed a 41 bp deletion from 288 to 328 and a 1 bp deletion at 530 in this PPR gene (Fig. 1A-C). Both two lines exhibited pale green leaves at all vegetative stages (Fig. 1D-F), thus we named this gene as *pale green leaves 1* (*Ospgll*) in this study. And we defined these two lines as *Ospgll-1* and *Ospgll-2*, respectively. The content of chlorophyll was drastically reduced than those in WT plants (Fig. 1G). This phenotype performed more pronounced in paddy fields (Fig. S1A). Despite of the distinct development on vegetative stage, the plant height, tiller number and seed setting were rarely impaired

(Fig. 1H-J). To confirm the phenotype of *Ospgll* knockout mutants, we also generated the transgenic RNAi lines. Results showed that suppression of *OsPGL1* expression recapitulated the knockout mutants phenotype (Fig. S2).

To dissect the morphologic details of the leaf, we performed scanning electron microscopy (SEM) and transmission electron microscopy (TEM) examinations. SEM results showed the leaf is complete and intact in WT (Fig. 2A), while some cracked holes were distributed in the leaf of *Ospgll-1* (Fig. 2F). Furthermore, TEM assays showed the starch granular stacks in WT plant were well-balanced (Fig. 2B), while the starch granular stacks in *Ospgll-1* were reduced and incompact (Fig. 2G). When we looked insight into the structure of thylakoid, results showed the density of well-organized thylakoid were not observed in *Ospgll-1*, which exhibited more hollow structures (Fig. 2C, 2H). In spite of the fact that chloroplasts were impaired, the ultrastructure of the mitochondria was undistinguishable between WT and *Ospgll-1* (Fig. 2D, 2E and 2I, 2J). These observations revealed that the *Ospgll* mutation primarily affects the development and morphology of chloroplasts rather than mitochondria.

***OsPGL1* encodes a DYW-motif containing protein**

OsPGL1 encodes a putative PPR protein consisting of 605 amino acids without intron. Motif prediction analysis by Pfam (<http://pfam.xfam.org/>) revealed that *OsPGL1* consists of 9 PPR motifs. 3 S motifs, 3 P motifs and 3 L motifs present staggered arrangement (Fig. S3A, 3B). The C-terminal region from residues 392 to 605 shows the consensus sequences of the extension domains (E, E+, and DYW domains) (Fig. S4). This data indicated that *OsPGL1* belongs to the typical DYW type of PLS subfamily. Alignment of *OsPGL1* with its orthologs in various plants showed 75%、

271 75%、46%、51%、51%、47% similarity with *Zea mays* (GRMZM2G001466), *Sorghum*
 272 *bicolor* (Sb08g003980), *Arabidopsis thaliana* (AT4G15720, REME2), *Theobroma*
 273 *cacao* (XP_017974392), *Glycine max* (XP_003529581), and *Brassica napus*
 274 (BnaC07g33170D) Fig. S4). Previous study reported that REME2 is involved in
 275 RNA editing of *rps3*-1534 and *rps4*-175 in mitochondria (Bentolila *et al.*, 2014). The
 276 high similarity among OsPGL1, GRMZM2G001466 and Sb08g003980 implied the
 277 function of this PPR gene might be conserved in monocots. Based on the PPR-RNA
 278 recognition mode, we analyzed the conservation of the 1' and 6 amino acid of each
 279 motif, which is essential for RNA recognition. Results showed these candidate
 280 orthologs could be divided into two subgroup, monocots and dicots, which suggested
 281 the functional conservation respectively (Fig. S5). It also implied the distinct function
 282 between OsPGL1 and AtREME2.

283

284 **Expression pattern of *OsPGL1***

285 To get insight into the expression pattern of *OsPGL1* in WT, RT-PCR and quantitative
 286 RT-PCR (qRT-PCR) were employed to detect various tissues. RT-PCR Results
 287 showed that *OsPGL1* was constitutively expressed in both vegetative and
 288 reproductive tissues, including root, stem, leaf, and panicle (Fig. 3A). Moreover,
 289 qRT-PCR data showed the expression level in root, stem and panicle, but
 290 preferentially accumulated in fresh leaf (Fig. 3B). These data are consistent with the
 291 results of the leaf phenotype, and slightly effects on plant height and seed setting.

292

293 **OsPGL1 is a novel dual-localized PPR protein**

294 Plenty of reports confirmed that most PPR proteins are targeted to plastids,
 295 chloroplast or mitochondrion. With bioinformatic analysis of TargetP

(<http://www.cbs.dtu.dk/services/TargetP/>), *OsPGL1* was predicted to localize into chloroplast, and also into mitochondrion with a low degree of confidence. To make sure of the subcellular localization of OsPGL1, the N-terminal region (amino acids from 1 to 188) were fused with green fluorescent protein (sGFP), driven by CaMV35S promoter and transiently expressed in rice protoplast. Results showed the GFP signals could overlap the red auto-fluorescent signals of chlorophyll (Fig. 3C). Interestingly, there were still some spots could not overlap the signals from chloroplast, implied OsPGL1 might target into mitochondria. Subsequently, mitotracker red were used to indicate mitochondrion, which signals were also overlapped with the signals of OsPGL1-GFP (Fig. 3D). Therefore, OsPGL1 is a novel dual-localized PPR protein in rice, both targets into mitochondria and chloroplasts.

307

308 **OsPGL1 is involved in C-to-U RNA editing of *ndhD* and *ccmFc* transcripts**

309 Studies showed PPR proteins were involved in the RNA editing of one or several
310 editing sites, especially in DYW subfamily (Sun *et al.*, 2016). Consideration of the
311 dual-localization of OsPGL1, we checked all 491 editing sites in mitochondria and 21
312 editing sites in chloroplasts respectively by RT-PCR. Sequencing results revealed that
313 the C-to-U editing efficiency of *ndhD*-878 and *ccmFc*-543 dramatically decreased to
314 zero in both two mutants expect for 8% at *ccmFc*-543 in *Ospgl1-2*, while *ndhD*-878
315 and *ccmFc*-543 were completely edited in WT (Fig. 4A). Consequently, abolishment
316 of editing in *ndhD*-878 recovered the codon from UUA to UCA, which resulted in the
317 amino acid substitution from leucine to serine. The loss of editing in another site
318 *ccmFc*-543, a mitochondrial RNA editing site converted the codon from GUU to
319 GUC, due to the degeneracy of codon, the change of the nucleotide at this position
320 does not cause any amino acid alteration, consistent with the results of no effects on
321 mitochondrion in *Ospgl1* (Fig. 2I, 2J).

To investigate the evolutionary conservation of the alteration from serine to leucine at the 293 position of NdhD protein sequence and valine at the 181 position of CcmFc protein sequence, the chloroplast NdhD orthologs and mitochondrial CcmFc orthologs from five representative species (*Oryza sativa*, *zea mays*, *Arabidopsis thaliana*, *Nicotiana tabacum* and *Brassica napus*) in plants were analyzed. Results showed these two residues are extremely conserved in the five tested species including monocots and dicots, implied that the leucine of NdhD and valine of CcmFc could important for plant development (Fig. 4B).

330

331 **OsPGL1 can bind to both *ndhD* and *ccmFc* transcripts**

To test the RNA binding activity of OsPGL1, we expressed recombinant OsPGL1 for further RNA electrophoresis mobility shift assays (REMSA). 9 PPR motifs from residues 46 to 605 was fused with a glutathione S-transferase (GST) tag for expression in *Escherichia coli*. The recombinant protein (GST-OsPGL1⁴⁶⁻⁶⁰⁵) was analyzed by western blot with anti-GST antibody to confirm the high purity (Fig. S7b). Subsequently, the recombinant protein was dialyzed to remove the contamination of RNase for REMSA and further quantified. The *ndhD* and *ccmFc* probes include 35 nucleotides surrounding the target editing sites were prepared and designated as probe 1 and probe 2. Probe C (*nad3*-155) was used as a negative control probe which has been reported as a specific target of another rice PPR protein (manuscript is preparing) (Fig. 5A). GST-OsPGL1⁴⁶⁻⁶⁰⁴ and GST tag were incubated with the biotin-labeled RNA probes, respectively. Both of the two protein–RNA complexes were detected as a shifted band that migrated more slowly than free RNA probe in the native gel, but no retarded band was observed when incubated with the GST (Fig. 5B, 5C). In addition, as negative control, no retarded band was observed when probe C was incubated with GST-OsPGL1⁴⁶⁻⁶⁰⁴. We next performed the competitor assay using

348 non-labeled RNA probe with the same sequence, the binding intensity of the band
349 were decreased accompanied with the increased concentration of competitors (Fig. 5B,
350 5C). These data validated the OsPGL1 binds to both *ndhD* and *ccmFc* transcripts
351 directed via the PPR motifs. *ndhD* and *ccmFc* derived from chloroplast and
352 mitochondrion also confirmed the dual-localization of OsPGL1.

353

354 **The transgenic complementation lines rescue the pale green phenotype**

355 To verify whether the pale green phenotype resulted from the dysfunction of *OsPGL1*
356 in deed, we performed the transgenic complementation assay in mutant lines.
357 Full-length coding sequences of *OsPGL1* was constructed into pCAMBIA-2300
358 vector driven by CaMV-35S promoter and transformed into *Ospgll-1* mutant by
359 *Agrobacterium*-mediated method. All 12 independent transgenic lines completely
360 rescued the mutant pale green leaves phenotype (Fig. 6A). Furthermore, we checked
361 the RNA editing efficiency of *ndhD-878* and *ccmFc-543* in all 12 independent
362 transgenic complementation lines, results showed 35S:OsPGL1 completely recovered
363 the RNA editing efficiency to 100% same as those in WT (Fig. 6B). Data indicates
364 that the pale green leaves phenotype was in deed caused by loss of RNA editing of
365 *ndhD-878*, which resulted from loss of function of *OsPGL1*.

366

367 **OsPGL1 interacts with three MORF proteins *in vitro* and *in vivo***

368 In addition to PPR proteins, another group of RNA editing factors in plant organelle
369 was identified, the multiple organelle RNA editing factor (MORF) proteins, which has
370 also been termed RNA editing factor interacting protein (RIP) proteins. There were
371 five MORF proteins in rice, two of which were targeted exclusively in plastids,
372 MORF2 and MORF9. MORF8 was a dual-localized protein which targeted to

mitochondria and plastids. The remaining two MORFs, MORF1 and MORF3 were targeted to mitochondria. Because of the dual-localization of OsPGL1, we further investigated the physical interactions between OsPGL1 and these MORF proteins in pairs. Firstly, we performed yeast two-hybrid assay, data showed that OsPGL1 can solid interact with three MORFs, MORF2, MORF8 and MORF9 in yeast. However, no interaction was observed between OsPGL1 and other two MORFs as well as the negative control (Fig. 7A). Interestingly, the interaction between OsPGL1 and MORF9 was much more stronger than those of other MORFs. Moreover, the interactions between OsPGL1 and MORF2/8/9 were also observed when we switched the bait and prey in a yeast two-hybrid system (Fig. S6). Data displayed the stronger interaction between OsPGL1 and MORF9, compared with others. Next, we performed GST pull-down assays to validate the interactions *in vitro*, the three MORF proteins were fused with His tag and OsPGL1 was fused with GST tag for expression (Fig. S8A). Recombinant proteins were further verified by western blots (Fig. S8B) and subjected for pull-down assay in pairs. Trx-MORF2-His, Trx-MORF8-His and Trx-MORF9-His were all pulled down by GST-OsPGL1, respectively, which demonstrated that OsPGL1 can interact with these three MORF proteins directly (Fig. 7B).

To test the interactions *in vivo*, we performed bimolecular fluorescence complementation (BiFC) assays in rice protoplast. OsPGL1 and three MORFs were fused to the C-terminal and N-terminal of yellow fluorescent protein (YFP), respectively. Results showed that co-expression of OsPGL1-YFP^C and MORF2-YFP^N/MORF8-YFP^N/MORF9-YFP^N exhibited strong signals overlapped with chlorophyll, while the negative combination OsPGL1-YFP^C and YFP^N did not produce any detectable fluorescence signal (Fig. 7C). Interestingly, the signals from OsPGL1-YFP^C and MORF8-YFP^N were much more than chlorophyll, suggesting the interaction in mitochondria, which consistent with the dual-localization of OsPGL1

400 and MORF8.

401 Moreover, we generated the transgenic plants carrying UBI:OsPGL1-FLAG,
402 35S:MORF2-GFP, 35S:MORF8-GFP and 35S:MORF9-GFP, respectively. The protein
403 crude extractions were incubated for co-immunoprecipitation assays with anti-GFP
404 antibodies. Results confirmed the interactions between OsPGL1 and MORFs (Fig.
405 7D). Taken together, the results solidly demonstrated that OsPGL1 interacted with
406 MORF2, MORF8 and MORF9 *in vitro* and *in vivo*.

407

408 ***Ospgll* exhibited defective in photosynthetic complex**

409 To make clear whether the photosynthetic and respiratory complex were impaired in
410 *Ospgll*, we detected the proteins involved in photosynthesis and electronic transfer
411 chains pathway. Firstly, we examined NdhD in WT, *Ospgll* mutants and the
412 complementation line. Results showed greatly reduced accumulation of NdhD,
413 suggesting the loss of editing at *ndhD*-878 generated an unstable NdhD in chloroplast
414 (Fig. 8A). Meanwhile, the accumulation of the photosystem I (PSI) subunits PsaA,
415 PSII subunits PsbA, cytochrome b6f (PetA), chloroplast ATP synthase subunit AtpA,
416 light harvesting complex of PSI (Lhca2) and light harvesting complex of PSII (Lhcb2)
417 were also examined. All of these proteins were dramatically decreased in both two
418 mutant lines (Fig. 8A). As expected, the levels of NdhD and other subunits in
419 photosynthetic complex were recovered in the transgenic complementation line (Fig.
420 8A).

421 We next examined the proteins involved in mitochondrial electron transport
422 chain pathway. Mitochondrial complexes isolated from calli of *Ospgll-1* and WT
423 were separated on blue-native gel. No obvious changes were observed in all of the
424 complexes via coomassie blue staining, implying *Ospgll* did not compromise the

function of mitochondria (Fig. 8B). Although the loss of RNA editing at *ccmFc-543* did not change the amino acid, we detected mitochondrial complex III as well, since CcmFc is a subunit of complex III. Protein immunoblotting with antibodies anti-CytC1 showed no difference at the amount of complex III in *Ospgll-1* compared with that in WT (Fig. 8B), consistent with the undistinguishable morphological structure of mitochondria between *Ospgll* and WT. Taken together, these results indicated photosynthetic complex was impaired while the respiratory complex was not affected in *Ospgll* mutants.

433

434 **Altered expression of chloroplast development related genes in *Ospgll*.**

The development of chloroplast in plant is related to the coordinated expression of both chloroplast and nuclear genes. Therefore, we first examined the transcript level of the chloroplast-encoded genes at 5-leaf stage of *Ospgll* and WT plants, most of which are mediated by two types of RNA polymerase: plastid-encoded polymerase (PEP) and nuclear-encoded polymerase (NEP). Results showed that the expression levels of PEP-dependent genes (*rbcL*, *psaA*, *psbA*, and *petB*) were reduced in *Ospgll*, which is consistent with results of the detection of protein level. Whereas, the NEP-dependent genes: RNA polymerase (*rpoA* and *rpoB*) were activated in *Ospgll* (Fig. 9A). Results implied that some retrograde signals from chloroplast might activate NEP-dependent genes transcription for compensation in the development of chloroplast.

Besides chloroplast-encoded genes, we also investigated the expression of some nuclear-encoded genes related to chloroplast development and photosynthesis in *Ospgll* and WT plants, including *RNRS* (encoding the large subunit of RNR), *RpoTp* (encoding NEP core subunits), *CAB1R* and *CAB2R* (light-harvesting Chl a/b-binding protein of PSII), *HEMA1* (encoding a glutamyl-tRNA reductase), *rbcS* (encoding a

451 Rubisco small subunit), *YGLI* (encoding a chlorophyll synthetase) and *CAOI*
 452 (encoding chlorophyll A oxygenase1). qRT-PCR analysis showed that the expression
 453 level of these genes was reduced in the *Ospgll* mutant (Fig. 9B). Taken together,
 454 these data indicated that *OsPGL1* plays an important role in regulating the chloroplast
 455 development and photosynthesis.

456

457 **OsPGL1 recognizes the target RNA sequence**

458 PPR protein binds target RNA via a modular recognition mechanism. To evaluate the
 459 conservation of the P, L, S motifs of OsPGL1, the sequence of each motif were
 460 analyzed. The alignments of these 9 motifs showed that residue Thr, Ala and Ser at
 461 position 6, Asn and Asp at position 1' was conserved polar residues (Fig. 10A). More
 462 interestingly, residue Gly is also highly conserved at position 16, especially
 463 completely conserved at position 33 in P and L motifs (Fig. 10A). Data implied Gly
 464 might be essential for the function of PPR motif. To evaluate the match degree of
 465 OsPGL1 protein binding to its two target transcripts, we did a computational
 466 prediction. Alignments of the target sites of OsPGL1 showed comparatively high
 467 matches of PPR-RNA recognition basis with these two editing sites (Fig. 10B). The
 468 alignments also suggested the combinations between PPR motifs and its targeted
 469 nucleotides might be very flexible.

470

471

472 **Discussion**

473

474 **OsPGL1 is a dual-localization PPR protein for RNA editing.**

PPR proteins were confirmed as a trans-acting factor for organelle RNA and acted as site specific RNA binding proteins (Okuda *et al.*, 2006). Most PPRs were involved in organelle RNA editing either mitochondrion or chloroplasts specifically, few of them are dual-localization. SOAR1 is a cytosol-nucleus dual-localized pentatricopeptide repeat (PPR) protein, which acting downstream of CHLH/ABAR and upstream of a nuclear ABA-responsive bZIP transcription factor ABI5 (Jiang *et al.*, 2015). PNM1 is dual localized to mitochondria and nuclei in Arabidopsis, which was only associated with polysomes and played a role in translation in mitochondria, and interacted with TCP8 in the nucleus (Hammani *et al.*, 2011). Both PPR2263 and MEF29 dually targeted to mitochondria and chloroplasts, and are required for RNA editing in maize and Arabidopsis, respectively (Sosso *et al.*, 2012). Here, we firstly reported a novel dual-localization PPR protein to mitochondria and chloroplasts in rice. Our data showed OsPGL1 is also required for RNA editing, and binds to target RNA directly. Both *ndhD*-878 (chloroplast) and *ccmFc*-543 (mitochondrion) were completely edited in WT, even though the editing of *ccmFc*-543 (mitochondrion) is synonymous. Loss of function of OsPGL1 leads to the editing efficiency decrease to zero, and resulted in the pale green leaf phenotype. The dual-localization of OsPGL1 suggested the signal peptide sequence of OsPGL1 could be applied for trans-locating an artificial protein for mitochondria and chloroplasts synchronously in future.

494

495 **OsPGL1 recognizes target RNAs and functions with MORF proteins**

The PPR motif consists of two anti-parallel α -helixes, the binding specificity depend on the first α -helix (Barkan *et al.*, 2012). Bioinformatic and structural analysis 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.*, 2013a). The alignment of OsPGL1 showed that high conservation of these positions in

monocots (Fig. S5), implying the conserved function of those orthologs genes in RNA editing of *ndhD* and *ccmFc* in monocots. OsPGL1 was a DYW subgroup PPR protein with 3 P, 3 L and 3S motifs. More interesting finding is that the highly conservation of Gly and Ala at position 16, and completely conservation of Gly at position 33 in P and L motifs, which could be important for the function of PPR proteins. In this study, the RNA recognition also follows the rules that the editing site is located at downstream of binding sites, and the interval space is 4 nucleotides. In a word, such modular mode can help us to deeply understand the RNA recognition of PPR proteins and its application in future.

Non-PPR editing factors RIPs/MORFs, ORRM, OZ, and PPO1 have been identified as components of the plant RNA editosome, which was required for RNA editing (Sun *et al.*, 2016). In Arabidopsis plastids, both of the two plastid-localized members, MORF2 and MORF9 were required for RNA editing for mostly sites (Takenaka *et al.*, 2012). Recent study showed the RNA-binding activity of an artificial (PLS)₃PPR could be sharply increased upon MORF9 binding, suggested the interaction between PPR and MORF9 could be more vital than that of others (Yan *et al.*, 2017). In Arabidopsis, CLB19 interacted with MORF2, PDM2 interacted with MORF2/9, all of PDM1/SEL1, PPO1 and ORRM6 interacted with MORF2/8/9 (Du *et al.*, 2017; Hackett *et al.*, 2017; Ramos-Vega *et al.*, 2015; Zhang *et al.*, 2014; Zhang *et al.*, 2015). All these data indicated the editosome in plant is quite complicated. Nevertheless, MORF proteins were rarely reported in rice. Recently, WSP1, a sequence similarity with MORF protein was identified in rice, which was involved in RNA editing and splicing of several plastid genes (Zhang *et al.*, 2017). In our study, we confirmed that OsPGL1 can also interact with MORF2, MORF8 and MORF9 *in vitro* and *in vivo*. The interaction between OsPGL1 and MORF9 was significant stronger than that of others, implying the RNA binding activities of OsPGL1 could be enhanced by cooperation with MORF9. According to our data, we believe that

528 OsPGL1 conducts the organelle RNA editing via an editosome coupled with MORF
529 proteins. Other factors or subunits of editosome will be further explored based on our
530 transgenic plants carrying UBI: OsPGL1-FLAG, 35S:MORF2-GFP,
531 35S:MORF8-GFP and 35S:MORF9-GFP.

532

533 **OsPGL1 plays an important role in rice chloroplast development**

534 Leaf is an important tissue for plant. In this study, we constructed CRISPR/Cas9
535 knockout mutants of PPR gene *OsPGL1*, *Ospgll-1* and *Ospgll-2*, both of which
536 exhibited pale green leaves during the whole vegetative stages. Further investigation
537 revealed the chloroplast development and photosynthesis were defective on RNA and
538 protein level in the mutant. NEP-dependent genes were activated in mutant implied
539 some unknown retrograde signals from chloroplast were involved in compensation
540 effects in the development of chloroplast. Complementation lines could rescue this
541 aberrant phenotype, suggested that the base deletion mutation in *OsPGL1* gene was
542 responsible for the PGL phenotype. OsPGL1 regulated chloroplast development by
543 organelle RNA editing of *ndhD*. The highly conserved leucine at NdhD-293 is
544 important for the structure or function of NdhD. We proposed the loss of RNA editing
545 might lead to unstable NdhD in chloroplast, based on the results of protein
546 immunoblotting, which showed the amount of photosynthetic complex subunits were
547 dramatically decreased in mutants. Results suggested that PPR genes play a vital role
548 in regulating chloroplast development via RNA editing in plant.

549

550 **Acknowledgements**

551 We thank Yaoguang Liu laboratory for providing us with pYLCRISPR/Cas9 vectors.
552 No conflict of interest declared

553

554 **Author contributions**

555 H.X., J.Hu. and Y.Z. designed the study. H.X. and Q.Z. contributed to constructed and
556 *Ospgll-1*, *Ospgll-2* mutants and RNAi lines. H.X. and Y.X. carried out most
557 experiments, H.X. and Q.Z. conducted SEM and TEM, F.Z. and C.N. performed
558 expression and purification of recombinant proteins, J.Huang. contributed to field
559 managements, H.X. and J.Hu. wrote the manuscript with feedback from all authors.

560

561 **Funding information**

562 This work is supported by funds from the National Key Research and Development
563 Program of China (2016YFD0100804), the National Natural Science Foundation of
564 China (31371698 and 31670310) and Suzhou Science and Technology Foundation
565 (SNG2017061).

566

567 **Supplemental data**

568

569 **Figure S1.** Phenotype of the *Ospgll* and WT in a paddy field.

570 **Figure S2.** Phenotype of WT and RNAi line.

571 **Figure S3.** Schematic structural sequence of OsPGL1.

572 **Figure S4.** Sequence alignment of *OsPGL1* with its orthologs in various plants.

573 **Figure S5.** Conservation of the amino acid at 6 and 1' position of each PPR motif.

574 **Figure S6.** Interaction of OsPGL1 with MORF2/MORF8/MORF9 by yeast

575 two-hybrid assay.

576 **Figure S7.** Expression and purification of GST-OsPGL1⁴⁶⁻⁶⁰⁴.

577 **Figure S8.** Expression and purification of Trx-MORF2-His, Trx-MORF8-His and
578 Trx-MORF9-His.

579 **Table S1.** Design of target adaptor for CRISPR/Cas9 system.

580 **Table S2.** Primers used for qRT-PCR, vector construction and RNA editing.

581

582 **References**

583

584 **Barkan A, Rojas M, Fujii S, Yap A, Chong YS, Bond CS, Small I.** 2012. A
585 combinatorial amino acid code for RNA recognition by pentatricopeptide repeat
586 proteins. *PLoS Genet* **8**, e1002910.

587 **Bentolila S, Babina AM, Germain A, Hanson MR.** 2014. Quantitative trait locus
588 mapping identifies REME2, a PPR-DYW protein required for editing of specific C
589 targets in Arabidopsis mitochondria. *RNA Biol* **10**, 1520-1525.

590 **Bentolila S, Heller WP, Sun T, Babina AM, Friso G, van Wijk KJ, Hanson MR.**
591 2012. RIP1, a member of an Arabidopsis protein family, interacts with the protein
592 RARE1 and broadly affects RNA editing. *Proc Natl Acad Sci U S A* **109**, E1453-1461.

593 **Chateigner-Boutin AL, Small I.** 2010. Plant RNA editing. *RNA Biol* **7**, 213-219.

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

598 **Covello PS, Gray MW.** 1989. RNA editing in plant mitochondria. *Nature* **341**,
599 662-666.

600 **Du L, Zhang J, Qu S, Zhao Y, Su B, Lv X, Li R, Wan Y, Xiao J.** 2017. The

- 601 Pentatricopeptide Repeat Protein Pigment-Defective Mutant2 is Involved in the
602 Regulation of Chloroplast Development and Chloroplast Gene Expression in
603 Arabidopsis. *Plant Cell Physiol* **58**, 747-759.
- 604 **Hackett JB, Shi X, Kobylarz AT, Lucas MK, Wessendorf RL, Hines KM,**
605 **Bentolila S, Hanson MR, Lu Y.** 2017. An Organelle RNA Recognition Motif Protein
606 Is Required for Photosystem II Subunit psbF Transcript Editing. *Plant Physiol* **173**,
607 2278-2293.
- 608 **Hammani K, Gobert A, Hleibieh K, Choulier L, Small I, Giege P.** 2011. An
609 Arabidopsis dual-localized pentatricopeptide repeat protein interacts with nuclear
610 proteins involved in gene expression regulation. *Plant Cell* **23**, 730-740.
- 611 **Hess GT, Fresard L, Han K, Lee CH, Li A, Cimprich KA, Montgomery SB,**
612 **Bassik MC.** 2016. Directed evolution using dCas9-targeted somatic hypermutation in
613 mammalian cells. *Nat Methods* **13**, 1036-1042.
- 614 **Hu J, Wang K, Huang W, Liu G, Gao Y, Wang J, Huang Q, Ji Y, Qin X, Wan L,**
615 **Zhu R, Li S, Yang D, Zhu Y.** 2012. The rice pentatricopeptide repeat protein RF5
616 restores fertility in Hong-Lian cytoplasmic male-sterile lines via a complex with the
617 glycine-rich protein GRP162. *Plant Cell* **24**, 109-122.
- 618 **Jiang SC, Mei C, Liang S, Yu YT, Lu K, Wu Z, Wang XF, Zhang DP.** 2015.
619 Crucial roles of the pentatricopeptide repeat protein SOAR1 in Arabidopsis response
620 to drought, salt and cold stresses. *Plant Mol Biol* **88**, 369-385.
- 621 **Kim D, Lim K, Kim ST, Yoon SH, Kim K, Ryu SM, Kim JS.** 2017. Genome-wide
622 target specificities of CRISPR RNA-guided programmable deaminases. *Nat*
623 *Biotechnol* **35**, 475-480.
- 624 **Kim U, Wang Y, Sanford T, Zeng Y, Nishikura K.** 1994. Molecular cloning of
625 cDNA for double-stranded RNA adenosine deaminase, a candidate enzyme for
626 nuclear RNA editing. *Proc Natl Acad Sci U S A* **91**, 11457-11461.
- 627 **Liu G, Tian H, Huang YQ, Hu J, Ji YX, Li SQ, Feng YQ, Guo L, Zhu YG.** 2012.
628 Alterations of mitochondrial protein assembly and jasmonic acid biosynthesis
629 pathway in Honglian (HL)-type cytoplasmic male sterility rice. *J Biol Chem* **287**,
630 40051-40060.
- 631 **Lu Y, Zhu JK.** 2017. Precise Editing of a Target Base in the Rice Genome Using a

632 Modified CRISPR/Cas9 System. *Mol Plant* **10**, 523-525.

633 **Lurin C, Andres C, Aubourg S, Bellaoui M, Bitton F, Bruyere C, Caboche M,**
634 **Debast C, Gualberto J, Hoffmann B, Lecharny A, Le Ret M, Martin-Magniette**
635 **ML, Mireau H, Peeters N, Renou JP, Szurek B, Taconnat L, Small I.** 2004.
636 Genome-wide analysis of Arabidopsis pentatricopeptide repeat proteins reveals their
637 essential role in organelle biogenesis. *Plant Cell* **16**, 2089-2103.

638 **Ma Y, Zhang J, Yin W, Zhang Z, Song Y, Chang X.** 2016. Targeted AID-mediated
639 mutagenesis (TAM) enables efficient genomic diversification in mammalian cells. *Nat*
640 *Methods* **13**, 1029-1035.

641 **McDougall WM, Okany C, Smith HC.** 2011. Deaminase activity on single-stranded
642 DNA (ssDNA) occurs in vitro when APOBEC3G cytidine deaminase forms
643 homotetramers and higher-order complexes. *J Biol Chem* **286**, 30655-30661.

644 **Mehta A, Driscoll DM.** 2002. Identification of domains in apobec-1
645 complementation factor required for RNA binding and apolipoprotein-B mRNA
646 editing. *RNA* **8**, 69-82.

647 **Melcher T, Maas S, Herb A, Sprengel R, Seeburg PH, Higuchi M.** 1996. A
648 mammalian RNA editing enzyme. *Nature* **379**, 460-464.

649 **Okuda K, Nakamura T, Sugita M, Shimizu T, Shikanai T.** 2006. A
650 pentatricopeptide repeat protein is a site recognition factor in chloroplast RNA editing.
651 *J Biol Chem* **281**, 37661-37667.

652 **Ramos-Vega M, Guevara-Garcia A, Llamas E, Sanchez-Leon N, Olmedo-Monfil**
653 **V, Vielle-Calzada JP, Leon P.** 2015. Functional analysis of the Arabidopsis thaliana
654 CHLOROPLAST BIOGENESIS 19 pentatricopeptide repeat editing protein. *New*
655 *Phytol* **208**, 430-441.

656 **Salone V, Rudinger M, Polsakiewicz M, Hoffmann B, Groth-Malonek M, Szurek**
657 **B, Small I, Knoop V, Lurin C.** 2007. A hypothesis on the identification of the editing
658 enzyme in plant organelles. *FEBS Lett* **581**, 4132-4138.

659 **Schmitz-Linneweber C, Small I.** 2008. Pentatricopeptide repeat proteins: a socket
660 set for organelle gene expression. *Trends Plant Sci* **13**, 663-670.

661 **Small ID, Peeters N.** 2000. The PPR motif - a TPR-related motif prevalent in plant

662 organellar proteins. *Trends Biochem Sci* **25**, 46-47.

663 **Sosso D, Mbello S, Vernoud V, Gendrot G, Dedieu A, Chambrier P, Dauzat M,**
664 **Heurtevin L, Guyon V, Takenaka M, Rogowsky PM.** 2012. PPR2263, a
665 DYW-Subgroup Pentatricopeptide repeat protein, is required for mitochondrial nad5
666 and cob transcript editing, mitochondrion biogenesis, and maize growth. *Plant Cell* **24**,
667 676-691.

668 **Sun T, Bentolila S, Hanson MR.** 2016. The Unexpected Diversity of Plant Organelle
669 RNA Editosomes. *Trends Plant Sci* **21**, 962-973.

670 **Takenaka M, Zehrmann A, Brennicke A, Graichen K.** 2013a. Improved
671 computational target site prediction for pentatricopeptide repeat RNA editing factors.
672 *PLoS One* **8**, e65343.

673 **Takenaka M, Zehrmann A, Verbitskiy D, Hartel B, Brennicke A.** 2013b. RNA
674 editing in plants and its evolution. *Annu Rev Genet* **47**, 335-352.

675 **Takenaka M, Zehrmann A, Verbitskiy D, Kugelmann M, Hartel B, Brennicke A.**
676 2012. Multiple organellar RNA editing factor (MORF) family proteins are required
677 for RNA editing in mitochondria and plastids of plants. *Proc Natl Acad Sci U S A* **109**,
678 5104-5109.

679 **Teng B, Burant CF, Davidson NO.** 1993. Molecular cloning of an apolipoprotein B
680 messenger RNA editing protein. *Science* **260**, 1816-1819.

681 **Yin P, Li Q, Yan C, Liu Y, Liu J, Yu F, Wang Z, Long J, He J, Wang HW, Wang J,**
682 **Zhu JK, Shi Y, Yan N.** 2013. Structural basis for the modular recognition of
683 single-stranded RNA by PPR proteins. *Nature* **504**, 168-171.

684 **Yu C, Wang L, Chen C, He C, Hu J, Zhu Y, Huang W.** 2014. Protoplast: a more
685 efficient system to study nucleo-cytoplasmic interactions. *Biochem Biophys Res*
686 *Commun* **450**, 1575-1580.

687 **Zhang F, Tang W, Hedtke B, Zhong L, Liu L, Peng L, Lu C, Grimm B, Lin R.**
688 2014. Tetrapyrrole biosynthetic enzyme protoporphyrinogen IX oxidase 1 is required
689 for plastid RNA editing. *Proc Natl Acad Sci U S A* **111**, 2023-2028.

690 **Zhang HD, Cui YL, Huang C, Yin QQ, Qin XM, Xu T, He XF, Zhang Y, Li ZR,**
691 **Yang ZN.** 2015. PPR protein PDM1/SEL1 is involved in RNA editing and splicing of

- 692 plastid genes in *Arabidopsis thaliana*. *Photosynth Res* **126**, 311-321.
- 693 **Zhang Z, Cui X, Wang Y, Wu J, Gu X, Lu T.** 2017. The RNA Editing Factor WSP1
694 Is Essential for Chloroplast Development in Rice. *Mol Plant* **10**, 86-98.
- 695 **Zhou S, Wang Y, Li W, Zhao Z, Ren Y, Wang Y, Gu S, Lin Q, Wang D, Jiang L,**
696 **Su N, Zhang X, Liu L, Cheng Z, Lei C, Wang J, Guo X, Wu F, Ikehashi H, Wang**
697 **H, Wan J.** 2011. Pollen semi-sterility1 encodes a kinesin-1-like protein important for
698 male meiosis, anther dehiscence, and fertility in rice. *Plant Cell* **23**, 111-129.
- 699 **Zong Y, Wang Y, Li C, Zhang R, Chen K, Ran Y, Qiu JL, Wang D, Gao C.** 2017.
700 Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion.
701 *Nat Biotechnol* **35**, 438-440.

702

703

704 **Figure legends**

705

706 **Figure 1. Mutant and phenotypic characterization of *Ospgll***

707 **(A)** Schematic drawing of the intronless gene *OsPGL1* attaching the position of
708 the deletion in *Ospgll*.

709 **(B)** Alignment of WT and mutant DNA sequences around the target sites highlighting
710 the deletion of 41bp in *Ospgll-1* and 1bp deletion in *Ospgll-2* marked with a pink
711 box.

712 **(C)** Sequencing results of *Ospgll-1* and *Ospgll-2* heterozygotes.

713 **(D)** Comparison of leaves from *Ospgll-1* and WT plant at the two-leaf stage, the red
714 box shows magnified view, bar = 2cm.

715 **(E)** Comparison of leaves from *Ospgll-1* and WT plant at the three-leaf stage, the red

- 716 box shows magnified view, bar = 2cm.
- 717 **(F)** Comparison of leaves from *Ospgll-1* and WT plant at the tillering stage, the red
718 box shows magnified view, bar = 5cm.
- 719 **(G)** Comparison of the chlorophyll content in *Ospgll-1* and WT. Bars represent mean
720 $\pm$ SD from three independent biological replicates. Asterisks indicate statistically
721 significant differences compared with the WT (Student's t-test: *** $P < 0.001$).
- 722 **(H)** Comparison of the plant height of *Ospgll-1* and WT plant.
- 723 **(I)** Comparison of the tiller number of *Ospgll-1* and WT plant.
- 724 **(J)** Comparison of the seed setting of *Ospgll-1* and WT plant.
- 725
- 726 **Figure 2. Scanning electron microscopy and transmission electron microscopy**
727 **examination of leaves at tillering stage of WT and *Ospgll-1***
- 728 **(A)** Scanning electron microscopy image of WT leaf, bar = 20 μ m
- 729 **(B)** Transmission electron microscopy image of WT chloroplasts, bar = 2 μ m
- 730 **(C)** A higher magnification image of WT chloroplast from **(B)**, bar = 0.3 μ m
- 731 **(D)** Transmission electron microscopy image of WT mitochondria, bar = 0.5 μ m
- 732 **(E)** A higher magnification image of WT mitochondrion from **(D)**, bar = 0.15 μ m
- 733 **(F)** Scanning electron microscopy image of mutant leaf, the image shows a defective
734 Leaf surface morphology with cracked, broken holes at the pale parts of *Ospgll-1*, bar
735 = 20 μ m
- 736 **(G)** Transmission electron microscopy image of chloroplasts in pale parts of *Ospgll-1*
737 mutant leaf, the image shows incompact granal stacks and lacked well-structured

738 thylakoid, bar = 1μm

739 **(H)** A higher magnification image of chloroplast in pale parts of *Ospgll-1* mutant leaf

740 from **(G)**, bar = 0.3μm

741 **(I)** Transmission electron microscopy image of mitochondria in *Ospgll-1* leaf, bar =

742 0.3μm

743 **(J)** A higher magnification image of *Ospgll-1* mitochondrion from **(I)**, bar = 0.15μm

744

745 **Figure 3. Expression and subcellular localization analysis of *OsPGL1***

746 **(A)** RT-PCR examination of *OsPGL1* in different tissues of WT. R, Root; S, Steam;

747 L3, three-leaf stage; L4, four-leaf stage; L5, three-leaf stage, five-leaf stage; P,

748 panicle.

749 **(B)** Real time RT-PCR examination of *OsPGL1* in different tissues of WT. Error bars

750 represent the SD.

751 **(C)** Transient expression of 35S:sGFP (top) and 35S:OsPGL1^{N1-188}-sGFP (bottom) in

752 rice protoplast. Bar = 5 μm.

753 **(D)** Transient expression of 35S:sGFP (top) and 35S:OsPGL1^{N1-188}-sGFP (bottom) in

754 rice protoplast. MitoTracker Red were used for mitochondria indicator. Bar = 5

755 μm.

757 **Figure 4. Organelle RNA editing analysis in different species**

758 **(A)** RNA editing analysis of the *ndhD*-878 and *ccmFc*-543 sites from WT and

759 *Ospgll-1* leaves. The black stars marks the editing site, the editing efficiency was

760 presented under the target sites.

761 **(B)** Alignment of the orthologous NdhD and CcmFc amino acids sequences in five
762 different species around the corresponding affected residues. Numbers and the red
763 rectangular box indicate the converted amino acid.

764

765 **Figure 5. OsPGL1 possesses specific RNA binding activities**

766 **(A)** Schematic sequences of RNA probes. Edited sites were indicated and marked in
767 red letter. Probe C is a negative control in this study.

768 **(B)** RNA electrophoresis mobility shift assays (REMSA) of GST-OsPGL1⁴⁶⁻⁶⁰⁵ and
769 GST-tag with RNA probe 1. Unlabeled probe 1 was used as competitor at a range
770 of the concentrations for competitive REMSA. GST-tag and probe C were used as
771 negative control.

772 **(C)** RNA electrophoresis mobility shift assays (REMSA) of GST-OsPGL1⁴⁶⁻⁶⁰⁵ and
773 GST-tag with RNA probe 2. Unlabeled probe 2 was used as competitor at a range
774 of the concentrations for competitive REMSA. GST-tag and probe C were used as
775 negative control.

776

777 **Figure 6. Complementation analysis of *Ospgll-1***

778 **(A)** The phenotypes of WT, *Ospgll-1* and complemented T₀ plant (com) at the
779 tillering stage. bars = 5cm.

780 **(B)** RNA editing efficiency of *ndhD*-878 and *ccmFc*-543 comparison among WT,
781 *Ospgll-1* and complemented T₀ plant (com), the stars show editing sites.

782

783 **Figure 7. OsPGL1 directly interacts with MORF2/8/9 proteins**

784 (A) Yeast-two hybrid assay, pGAD, GAL4 activation domain, used as prey vector,
 785 pGBK, GAL4 DNA binding domain, used as bait vector, SD/-TL and SD/-TLHA
 786 indicate SD/-Trp-Leu and SD/-Trp-Leu-His-Ade dropout plates, respectively.
 787 pGBK-53 and pGBK-lam was used as positive and negative control.
 788 Co-transcription of pGAD-T and pGBK-OsPGL1 used for self-activation
 789 detection.

790 (B) GST Pull-down assay, the interaction between OsPGL1 and MORF2/8/9 proteins
 791 with detected by GST Pull-down assay, respectively. GST and Trx-His tag protein
 792 was used as control. The eluates were immunoblotted with anti-GST and anti-His
 793 antibodies, respectively.

794 (C) Bimolecular fluorescence complementation assay showing that OsPGL1-YFP^C
 795 interacts with MORF2- YFP^N, MORF8- YFP^N and MORF9- YFP^N to produce
 796 YFP fluorescence in the chloroplasts. Bar = 5μm.

797 (D) Co-immunoprecipitation assay detection with anti-FLAG and anti-GFP antibodies,
 798 respectively.

799

800 **Figure 8. Immunoblotting analysis of the subunits of photosynthetic complexes**
 801 **and respiratory complex III**

802 (A) Immunoblot analysis of the subunits of photosynthetic complex in WT, *Ospgll*
 803 mutants, and complementation line at 4-leaf stage. Actin served as reference
 804 antibody, CBB staining indicated the loading control. Lanes were loaded with a
 805 series of dilutions as indicated.

806 (B) The level of major protein of mitochondrial respiratory complex III analysis by

807 BN-PAGE (left) and immunoblot (right). Cytc1 was used as a representative
808 subunit of complex III.

809

810 **Figure 9. Expression Analysis of chloroplast development related genes**

811 (A) Real time RT-PCR examination of PEP-dependent genes (*rbcL*, *psaA*, *psbA*, and
812 *petB*) and NEP-dependent genes (*rpoA* and *rpoB*) in WT and *Ospgll* at 5-leaf
813 stage. Error bars represent the SD. (Student's t-test: * P< 0.05, ** P< 0.01)

814 (B) Real time RT-PCR examination of chloroplast development and photosynthesis
815 related genes in WT and *Ospgll* at 5-leaf stage. Error bars represent the SD.
816 (Student's t-test: ** P< 0.01)

817

818 **Figure 10. Alignment of PPR motifs of OsPGL1 with the cis-elements**

819 (A) Sequence logo for PPR motifs in OsPGL1, the two positions that contribute to
820 RNA binding specificity was indicated by stars. Sequence logos were constructed
821 by Web-Logo.

822 (B) Alignment of amino acid residues at position 6 and 1' in each neighboring PPR
823 motif of OsPGL1 with the putative cis-elements surrounding the editing sites.
824 Nucleotides matching the PPR-RNA recognition combination were marked in
825 red.

826

WT

A

B

C

D

E

Ospgl1-1

F

G

H

I

J

A **B****Oryza sativa****Zea mays****Arabidopsis thaliana****Nicotiana tabacum****Brassica napus**

NdhD-293

G	A	M	Q	I	I	Y	A	A	L	T	S	L	G	Q	R	N	F	K
G	A	V	Q	I	I	Y	A	A	L	T	S	L	G	Q	R	N	F	K
G	T	I	Q	I	I	Y	A	A	L	T	S	L	G	Q	R	N	L	K
G	T	I	Q	I	I	Y	A	A	L	T	S	L	G	Q	R	N	L	K
G	T	I	Q	I	I	Y	A	A	L	T	S	P	G	Q	R	N	L	K

Oryza sativa**Zea mays****Arabidopsis thaliana****Nicotiana tabacum****Brassica napus**

ccmFc-181

H	P	H	I	-	-	E	R	R	V	E	G	F	G	P	V	A	F	P
H	P	H	I	-	-	E	R	R	V	E	G	F	G	P	V	A	F	P
H	P	H	L	D	L	E	R	R	V	E	G	F	G	P	L	A	F	P
H	P	H	L	-	-	E	R	R	V	E	G	F	G	P	V	A	F	P
H	P	H	L	D	L	E	R	R	V	E	G	F	G	P	L	A	F	P

ndhD-878
 ↓
 Probe1: AGUGAUGCAAUAAUCUAUGCAGCUU**C**AACUUCUC

ccmFc-543
 ↓
 Probe2: GGGCACCCCCAUAUAGAAAGAAGGGU**C**GAAGGUUU

nad3-155
 ↓
 ProbeC: GUAUCUAUUUAGUGAUCAGUC**C**GCUAGUUUCUUUG

	Probe1						ProbeC
RNA probe (1nmol)	+	+	+	+	+	+	+
GST-OsPGL1 ⁴⁶⁻⁶⁰⁵	-	-	+	+	+	+	+
Competitor (nmol)	-	-	-	10	50	100	-
GST	-	+	-	-	-	-	-

Shift →

Free →

	Probe2						ProbeC
RNA probe (1nmol)	+	+	+	+	+	+	+
GST-OsPGL1 ⁴⁶⁻⁶⁰⁵	-	-	+	+	+	+	+
Competitor (nmol)	-	-	-	10	50	100	-
GST	-	+	-	-	-	-	-

Shift →

Free →

A

B

A

B

C

D

A

bioRxiv preprint doi: <https://doi.org/10.1101/223115>; this version posted November 21, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

B

A

B

A

B