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Empty Pericarp5 Encodes a Pentatricopeptide Repeat Protein That Is Required for Mitochondrial RNA Editing and Seed Development in Maize[W]

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In flowering plants, RNA editing is a posttranscriptional mechanism that converts specific cytidines to uridines in both mitochondrial and plastidial transcripts, altering the information encoded by these genes. Here, we report the molecular characterization of the *empty pericarp5* (*emp5*) mutants in maize (*Zea mays*). Null mutation of *Emp5* results in abortion of embryo and endosperm development at early stages. *Emp5* encodes a mitochondrion-targeted DYW subgroup pentatricopeptide repeat (PPR) protein. Analysis of the mitochondrial transcripts revealed that loss of the EMP5 function abolishes the C-to-U editing of *ribosomal protein L16* at the *rpl16*-458 site (100% edited in the wild type), decreases the editing at nine sites in *NADH dehydrogenase9* (*nad9*), *cytochrome c oxidase3* (*cox3*), *and ribosomal protein S12* (*rps12*), and surprisingly increases the editing at five sites of *ATP synthase F0 subunit a* (*atp6*), *apocytochrome b* (*cob*), *nad1*, and *rpl16*. Mutant EMP5-4 lacking the E+ and DYW domains still retains the substrate specificity and editing function, only at reduced efficiency. This suggests that the E+ and DYW domains of EMP5 are not essential to the EMP5 editing function but are necessary for efficiency. Analysis of the ortholog in rice (*Oryza sativa*) indicates that rice EMP5 has a conserved function in C-to-U editing of the rice mitochondrial *rpl16*-458 site. EMP5 knockdown expression in transgenics resulted in slow growth and defective seeds. These results demonstrate that *Emp5* encodes a PPR-DYW protein that is required for the editing of multiple transcripts in mitochondria, and the editing events, particularly the C-to-U editing at the *rpl16*-458 site, are critical to the mitochondrial functions and, hence, to seed development in maize.

INTRODUCTION

RNA editing is a posttranscriptional mechanism that alters RNA sequences via insertion, deletion, and conversion of nucleotides, resulting in changes in the genetic information encoded by the DNA. In flowering plants, most RNA editing events are conversions of cytidines (C) to uridines (U), and all the reported C-to-U editing events occur in either mitochondria or plastids (Chateigner-Boutin and Small, 2010). C-to-U editing was first reported in mitochondrial mRNAs by three independent groups (Covello and Gray, 1989; Gualberto et al., 1989; Hiesel et al., 1989). The plastid genomes contain ~120 genes and fewer than 50 editing sites in the transcripts. By contrast, the mitochondrial genomes contain ~60 genes, and ~300 to 500 editing sites are predicted in the transcripts (Unseld et al., 1997; Giegé and Brennicke 1999; Notsu et al., 2002), implicating that RNA editing plays a major role in the expression of the mitochondrial genome. RNA editing is important for posttranscriptional regulation and in some cases critical to the functions of the encoded proteins. For example, editing can restore a conserved amino acid codon (Tillich et al., 2005), create an initiation or stop codon (Kotera et al., 2005), or remove a stop codon that leads to a functional larger protein (Grewe et al., 2009). Therefore, deficiency in editing may result in a compromised or complete loss of function for the encoded protein, leading to severe consequence in plant growth and development. Indeed, defects in mitochondrial RNA editing cause severe phenotypes, such as seed development defects, growth and development retardation (Kim et al., 2009; Sung et al., 2010; Hammani et al., 2011; Sosso et al., 2012; Yuan and Liu, 2012). Editing can also occur in introns and untranslated regions, potentially playing a role in regulating transcript stability (Börner et al., 1995; Chateigner-Boutin and Small, 2010).

Although RNA editing is important to the posttranscriptional regulation of organelle gene expression (Shikanai, 2006; Grennan, 2011), little is known about the mechanisms of C-to-U RNA editing in plants until 2005. The first insight came with the identification of CHLORORESPIRATORY REDUCTION4 (CRR4), a pentatricopeptide repeat (PPR) protein that is required for the C-to-U editing of a single site in the chloroplast transcript *ndhD* in maize (*Zea mays*) (Kotera et al., 2005). Since then, PPR proteins have been recognized as the *trans*-acting factors responsible for RNA editing in plastids and mitochondria in plants (Fujii and Small, 2011). All of the PPR proteins responsible for C-to-U editing are localized in either mitochondria or chloroplasts. Domain structure analysis indicated that they belong to E or DYW subclasses of the PPR protein family (Okuda et al., 2007; Schmitz-Linneweber and Small, 2008; Fujii and Small, 2011). The DYW domain shows a significant similarity to deaminase, raising the possibility of being a catalytic domain to convert C to

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U (Salone et al., 2007). Although this notion is not supported by the finding that truncated proteins lacking the DYW motifs can completely restore the RNA editing in vivo (Okuda et al., 2009), a recently identified protein DYW1, which contains a highly conserved DYW domain but no identifiable PPR motifs, can function in trans with the E subclass PPR protein CRR4 in the *ndhD-1* site editing (Boussardon et al., 2012). Recently, a new family of proteins essential for organelle RNA editing has been identified, the multiple organellar RNA editing factor (MORF), including RNA-EDITING FACTOR INTERACTING PROTEIN 1 (RIP1, =MORF8) (Bentolila et al., 2012; Takenaka et al., 2012). Loss-of-function mutations in MORFs alter editing efficiency at multiple sites, distinguishing them from PPR proteins typically affecting one or a few sites. RIP1 is localized in both chloroplasts and mitochondria, affecting numerous editing sites in the two organelles. MORF2 or MORF9 are required for almost all sites of RNA editing in the chloroplast, and mutations of MORF1 and MORF3 affect numerous editing sites in mitochondria. These MORF proteins can interact selectively with PPR proteins to establish a complex editosome, and they can also form hetero- and homodimers in RNA editing (Bentolila et al., 2012; Takenaka et al., 2012).

Increasing evidence indicates that PPR genes play an important role in embryo and endosperm development in flowering plants. In *Arabidopsis thaliana*, GLUTAMINE-RICH PROTEIN23 (GRP23) is shown to be essential for early embryo development (Ding et al., 2006). GRP23, an exceptional PPR protein that is localized in the nucleus, exerts its function by interacting with RNA Polymerase II Subunit III. Mutation of this gene leads to embryo arrest at 16-cell dermatogen stage (Ding et al., 2006). Similarly, mutation of *Arabidopsis PPR2*, a gene probably responsible for translation process in chloroplasts, causes defects in cell proliferation during embryogenesis (Lu et al., 2011). A systematic analysis of *Arabidopsis* embryo-defective mutants (*emb*) revealed that 17 PPR genes among 250 *Emb* genes were essential to embryogenesis (Tzafrir et al., 2004; Cushing et al., 2005). In rice (*Oryza sativa*), OPAQUE AND GROWTH RETARDATION1 (OGR1) is found to be important for seed development (Kim et al., 2009). OGR1 is a PPR-DYW protein responsible for the RNA editing of mitochondrial transcripts *nad2*, *nad4*, *cox2*, *cox3*, and *ABC transporter subunit C* (*ccmC*). In maize, three mitochondrial PPR proteins are shown to be essential for seed development. EMPTY PERICARP4 (EMP4), a PPR protein that is required for the correct expression of a small group of mitochondrial genes, is essential for early embryo and endosperm development (Gutiérrez-Marcos et al., 2007). Recently, PPR2263, which affects kernel size by affecting both the embryo and endosperm development, was reported to be required for *nad5* and *cob* transcript editing in maize mitochondria (Sosso et al., 2012). PPR protein MPPR6 is reported to be directly involved in the 5′ maturation and translation initiation of *rps3* mRNA in mitochondria. Mutation of MPPR6 affects both embryo and endosperm development in maize (Manavski et al., 2012).

In this study, we report the molecular characterization of a nuclear gene *Emp5*, which affects the embryo and endosperm development in maize. *Emp5* encodes a mitochondrion-targeted DYW subgroup PPR protein. Functional analysis indicates that EMP5 is responsible for the editing of 10 sites in four genes (*rpl16*, *nad9*, *cox3*, and *rps12*), of which the *rpl16*-458 site editing is completely abolished due to the *emp5* mutation. Molecular analysis of the *emp5-4* allele, which carried a *Mu* insertion disrupting the E+ and DYW domains, provides genetic evidence that E+ and DYW domains are not essential for the EMP5 editing function. Further analysis of the *Emp5* ortholog in rice by RNA interference (RNAi) transgenics confirmed that the editing function, and its role in affecting seed development, is conserved between the two species.

RESULTS

Phenotypic and Genetic Characterization of *emp5-1*

The *emp5-1* mutant was isolated from the UniformMu population where the active *Mu* lines were introgressed into the inbred W22 genetic background (McCarty et al., 2005). When this mutant was isolated, six backcrosses to W22 had been performed. The isolated *emp5-1* allele was backcrossed to W22 twice afterward to reduce *Mu* copy numbers. Therefore, the mutant was considered in nearly isogenic W22 background (99.6%). The selfed progeny of the *emp5-1* heterozygotes segregated *emp* kernels in a 3:1 ratio (WT:*emp*, 455:167, $P > 0.95$), indicating that *Emp5* is a monogenic, recessive, and nuclear gene. In contrast with the wild type, the mutant *emp5-1* kernels at maturity typically were small, containing a white pericarp that was often wrinkled (Figure 1A). During sectioning of the kernels, some residual tissues could be found, but not recognizable embryo or endosperm structures (Figure 1B). This result indicated that the *emp5* mutation blocks the development of both the embryo and the endosperm. The *emp5-1* allele is an embryo-lethal mutation that is maintained in heterozygotes.

To examine the developmental arrest in *emp5-1*, we analyzed the seed development process by light microscopy. Maize embryo development is characterized by three stages: transition, coleoptilar, and late embryogenesis. Endosperm development includes coenocytic, cellularization, differentiation, and maturation stages (Olsen, 2001). A close comparison was made by analyzing both the wild type and the *emp5-1* mutant in the same segregating ear. The *emp5-1* mutant kernel can be clearly distinguished from the wild-type siblings as early as 8 d after pollination (DAP), with characteristics of a small size and translucent appearance resulted from arrested embryo and endosperm development. The identification was confirmed by PCR genotyping. Dissection of the *emp5-1* mutant seeds at 8 and 13 DAP revealed that the mutant embryogenesis was arrested at the transition stage, characterized by the establishment of radial asymmetry that was introduced by the formation of an internal wedge-shape meristematic region in the upper part of the embryo. The endosperm development was blocked at the differentiation stage, characterized by the formation of distinct starchy endosperm and aleurone layer (Figure 1E, 1F, and 1H). By contrast, the wild-type embryo at 8 DAP already differentiated a scutellum and shoot apical meristem, and the endosperm was much larger than the mutant (Figures 1C and 1G).

Besides the dramatic difference in embryo and endosperm, we noticed that *emp5-1* appeared lacking basal transfer cells. Basal transfer cell layer is responsible for the uptake of solutes critical to seed development (Pate and Gunning, 1972). To investigate the

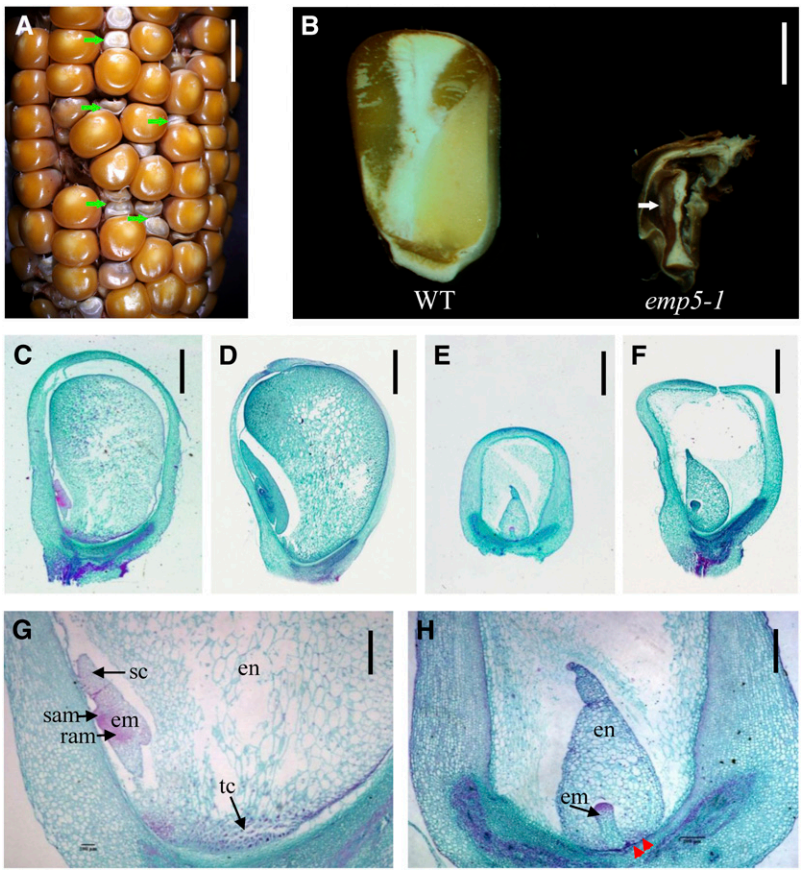

Figure 1. Mutant *emp5-1* Kernels Abort Early in Seed Development.

(A) The ear segregates 3:1 for wild-type and *emp5-1* mutant kernels (arrows).
(B) Dissection of mature wild-type (WT; left) and *emp5-1* (right) kernels.
(C) to **(H)** Developmental comparisons of wild-type and *emp5-1* kernels at 8 and 13 DAP. Wild-type kernels at 8 DAP (**[C]** and **[G]**) and 13 DAP **(D)**; *emp5-1* kernels at 8 DAP (**[E]** and **[H]**) and 13 DAP **(F)**. en, endosperm; em, embryo; ram, root apical meristem; sam, shoot apical meristem; sc, scutellum; tc, transfer cells. Red arrows point to positions where transfer cells were not clearly formed in the mutant.
Bars = 1 cm in **(A)**, 2.5 mm in **(B)**, 1 mm in **(C)** to **(F)**, and 500 μm in **(G)** and **(H)**.

development of basal transfer cell layer in the *emp5-1* mutant seeds, we performed the immunohistochemistry analysis using BETL-2 antibody. BETL-2 is a basal endosperm transfer layer–specific protein expressed in early and mid-term endosperm development (Hueros et al., 1999). Therefore, it is a marker of basal transfer cell formation. The wild-type kernels at 13 DAP differentiated more than three layers of transfer cells, and the BETL-2 protein was expressed in the whole basal transfer cell layer region (Figure 2A). By contrast, the *emp5-1* mutant kernels from the same ear only formed a single layer of transfer cells, and BETL-2 was detected in these cells (Figure 2B). During subsequent microscopy analyses, we did not observe the formation of multiple cell layers of transfer cells in the mutant. This result indicated that the basal transfer cell development in the *emp5-1* kernels is arrested.

Cloning of *Emp5*

Because the *emp5-1* allele was potentially tagged by *Mu* transposons, we performed DNA gel blot analysis to identify whether a *Mu* insertion was linked to the mutation. A segregating F2 population was created by self-crossing a heterozygous *emp5-1*/*Emp5* plant. Genomic DNA was isolated from the individual F2 plants, and the genotype was determined by selfing the plant and checking for *emp5-1* mutant segregation. Because homozygous *emp5-1* is not viable, only heterozygotes (segregating) or the wild type (nonsegregating) were available for this analysis. The blots were hybridized with several *Mu* elements, including *Mu1*/*Mu2*, *Mu3*, *Mu4*, *Mu8*, *MuDR*, and *Mu13* (Tan et al., 2011), and only the hybridization with a *Mu1*/*Mu2*-specific probe identified a 3.4-kb *Hin*dIII fragment that cosegregated with the *emp5* mutation (i.e., the presence of this fragment in *emp5-1* heterozygote and its absence in the wild type) (Figure 3A). No recombination was detected in the initial 22 F2 individuals tested. Increasing the population to 120 individuals still did not produce any recombination, suggesting a tight linkage between this *Mu* insertion and the *emp5-1* mutation. Because the *Mu1*/*Mu2* probe hybridizes to both *Mu1* and *Mu2*, we could not tell which one was inserted in this fragment at this stage.

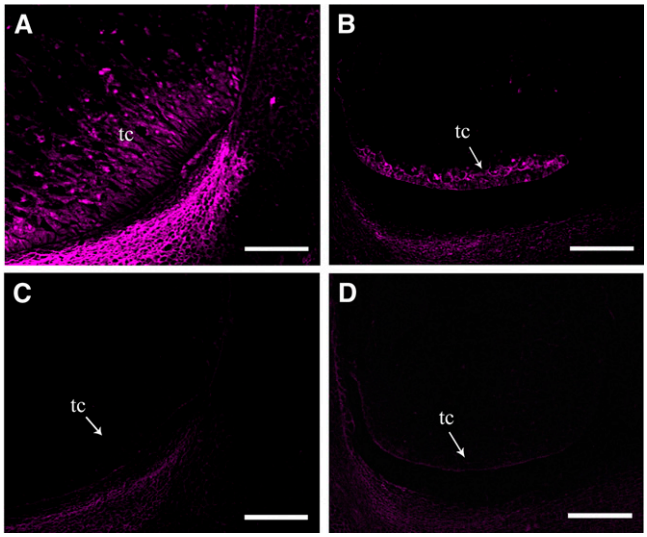

Figure 2. The Basal Transfer Cell Development in the *emp5-1* Kernels Is Arrested.

Confocal fluorescent microscopy visualization of BETL-2 by immunofluorescence in 13-DAP wild-type (**[A]** and **[C]**) and *emp5-1* mutant (**[B]** and **[D]**) kernels. To visualize the basal endosperm transfer layer, specific antibody BETL-2 was used (**[A]** and **[B]**), and no BETL-2 antibody PBS buffer was used as control (**[C]** and **[D]**). tc, transfer cell. Bars = 200 μm.

The *Mu1* or *Mu2* flanking sequence in the 3.4-kb *Hin*dIII fragment was amplified by inverse PCR using *Mu* TIR primers. The size of the product was 1.7 kb with TIR sequences on both ends and a *Hin*dIII site in the middle. A 9-bp target site duplication was found flanking the *Mu* insertion. This suggests that the *Mu* element is likely a *Mu2* (1.7 kb), not a *Mu1* (1.4 kb). BLAST search of the National Center for Biotechnology Information GenBank with the 1.7-kb *Mu2* flanking sequence identified an expression mRNA in maize (accession number EU956937). This gene is located on chromosome 3. This gene appears to be a single-copy gene based on the analysis of B73 whole genomic sequence draft AGPv2 (Schnable et al., 2009). Using two gene-specific primers designed according to this clone, the full genomic sequence of this *Emp5* candidate gene was cloned from W22. And the cDNA was amplified by RT-PCR from leaf RNAs.

To confirm that the cloned gene is the bona fide causative gene for the *emp5-1* phenotype, we isolated additional *Mu* insertional alleles from the Pioneer Hi-Bred International Trait Utility System for Corn (TUSC) population using *Emp5* gene-specific primers in combination with Mu-TIR primers (Bensen et al., 1995). Three independent *Mu* insertions in the *Emp5* candidate gene were identified, named *emp5-2*, *emp5-3*, and *emp5-4* (Figure 3B). The insertion sites were confirmed by sequencing the PCR products that were amplified with TIR8 primers and *Emp5*-specific primers. The selfed progeny of heterozygote *emp5-2* and *emp5-3* produced *empty pericarp* kernels segregation at a ratio of 1:3 (*emp*:WT), but that of the *emp5-4* produced all normal kernels. Crosses between *emp5-1* heterozygotes with heterozygotes for *emp5-2* and *emp5-3* alleles produced ears segregating *empty pericarp kernels* at a 1:3 ratio (*emp*:WT), whereas crosses between *emp5-1* and *emp5-4* produced all wild-type kernels. Genotyping using gene-specific primers indicated that homozygous *emp5-4* seeds were viable and contained a normal embryo and endosperm. Later analysis indicated that the *emp5-4* mutation was leaky and only partially abolished the *Emp5* function. Because three independent alleles carried Mu insertions in the *Emp5* gene conditioned the empty pericarp phenotype, we concluded that the *Emp5* locus was cloned.

Emp5 Encodes a Mitochondrion-Targeted PPR-DYW Subclass Protein

Sequence analysis indicated that the *Emp5* gene consists of two exons, and in *emp5-1*, a *Mu2* was inserted in the first exon (Figure 3B).This *Emp5* gene encodes a 776–amino acid protein. Motif prediction analysis by algorithm TPRpred (http://tprpred.tuebingen.mpg.de/tprpred) revealed that EMP5 contains 11 PPR motifs, classifying this protein as a member of the PPR protein family (Figures 4A and 4B). The C-terminal region between residues 590 and 776 shows a strong similarity to the consensus sequences of the E, E+, and DYW domains (Lurin et al., 2004), indicating that EMP5 is a DYW subclass PPR protein. Analysis of the rice and sorghum (*Sorghum bicolor*) genome identified an ortholog in each genome, named Os EMP5 and Sb EMP5, respectively. EMP5

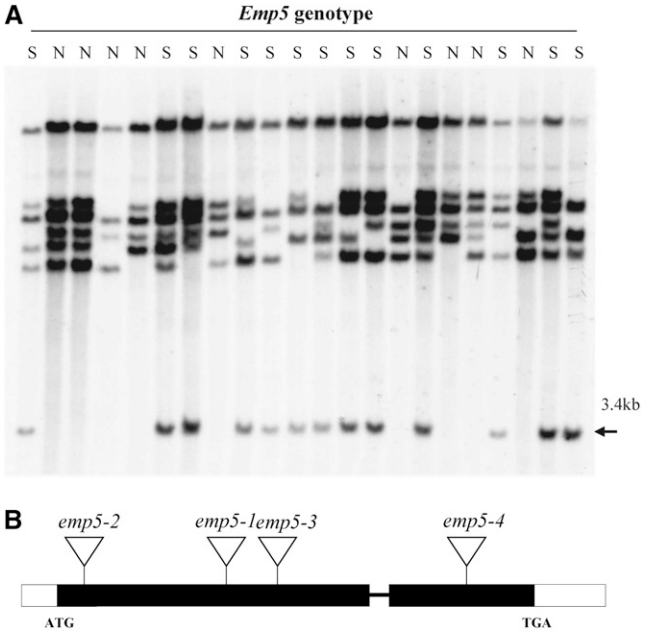

Figure 3. *Emp5* Gene Cloning.

(A) DNA gel blot cosegregation analysis of an *emp5-1* segregation population using the internal sequence of *Mu1* as a probe. The *Mu2* transposon-tagged 3.4-kb *Hin*dIII fragment (arrowhead) cosegregated with the *emp5-1* mutation. N, nonsegregating (the wild type); S, segregating (heterozygous).

(B) Gene structure of *Emp5* and locations of the *Mu* insertions in four independent alleles. Exons are closed boxes and introns are lines. *Mu* insertion sites of *emp5* alleles were confirmed by sequencing and marked by triangles.

showed clear divergence between monocots and dicots. Among the closely related proteins, it shares a high degree of similarity with sorghum (91%), rice (84%), and barley (*Hordeum vulgare*) (83%) but a low degree of similarity with grape (*Vitis vinifera*) (69%) and *Arabidopsis* (64%). Functions of these proteins are not known yet.

Analysis of EMP5 with the TargetP algorithm predicted a putative mitochondrial localization, but with marginal confidence (http://www.cbs.dtu.dk/services/TargetP/). To experimentally determine the subcellular localization of EMP5, we fused full-length EMP5 with green fluorescent protein (GFP) and transformed *Arabidopsis*. Ten lines of transgenic *Arabidopsis* were generated, and among these transgenics, GFP signals were either absent or low. We suspected that the length of the EMP5-GFP fusion may be the cause for this problem. So, the N-terminal 469 amino acids of EMP5 that contains eight PPR repeats was fused with GFP, resulting in fusion construct EMP5^{N469}-GFP. Fourteen lines of transgenic *Arabidopsis* were produced and analyzed. Confocal laser scanning microscopy analysis of the transgenic *Arabidopsis* leaf samples and protoplasts showed in vivo colocalization of the GFP signal of EMP5^{N469}-GFP with MitoTracker Red in mitochondria (Figure 4C). To independently test the possibility of chloroplast localization, we also performed pea (*Pisum sativum*) chloroplast import assays in which the EMP5 protein was labeled with ^{3}H-Leu and tested its capability in importing to live chloroplasts. The result showed that EMP5 was not imported into the chloroplasts as protease thermolysin treatment of the imported chloroplasts eliminated all the radioactive ^{3}H-EMP5 protein (Figure 4D). Thermolysin protease treatment degrades proteins unprotected by the chloroplast envelopes. Together, these results indicated that EMP5 is a PPR-DYW protein localized in maize mitochondria.

Expression of *Emp5*

BLAST analysis of EMP5 identified an EST from maize cDNA library of mix tissues, indicating that *Emp5* may be expressed in multiple tissues. Analysis of Os *Emp5*, the ortholog in rice, also identified two ESTs derived from rice panicle and callus. However, the *Emp5* mRNA could not be detected by conventional RNA gel blot analysis, suggesting that it may be expressed at low levels. Similarly, the maize mitochondrial PPR gene *Emp4*

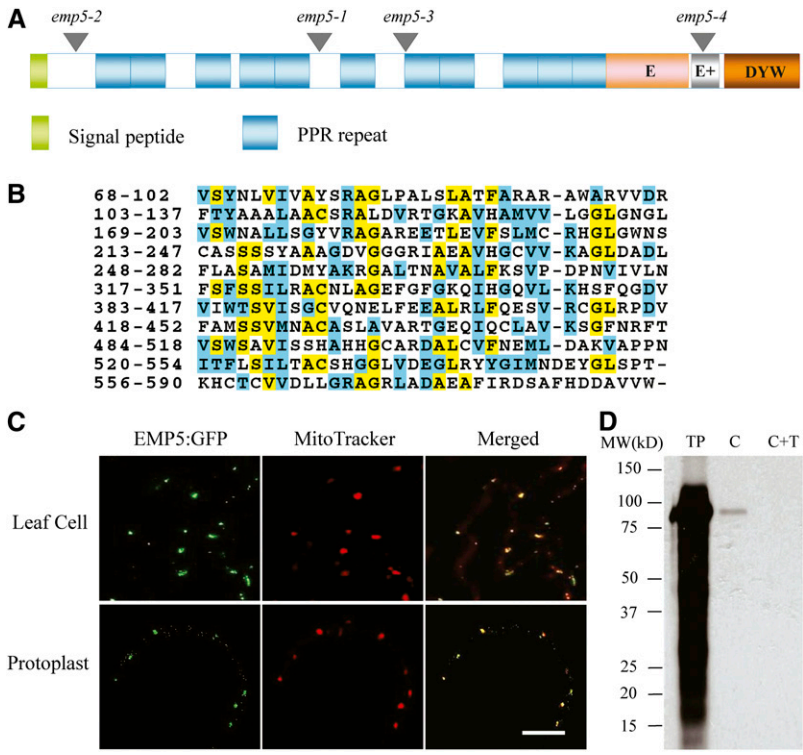

Figure 4. EMP5 Is a Mitochondrion-Localized PPR-DYW Protein.

(A) The EMP5 protein contains 11 PPR motifs and E, E+, and DYW motifs at the C terminus. Locations of four *Mu* insertion alleles are marked with triangles.

(B) Alignment of 11 PPR motifs found in EMP5 proteins. Identical residues are shaded in yellow and similar residues in light blue.

(C) A EMP5^{N469}-GFP fusion protein that carried the N terminus 469 amino acids fused with GFP was expressed in transgenic *Arabidopsis* leaves. The leaf samples and protoplasts were used for GFP signal detection by confocal microscopy. Fluorescent signals from EMP5^{N469}-GFP are green and MitoTracker stained mitochondria are red. Bar = 10 μm.

(D) Chloroplast import of full-length EMP5 protein into pea chloroplasts. C, chloroplasts after imported with ^{3}H-EMP5; C+T, ^{3}H-EMP5 imported chloroplasts treated with thermolysin to remove surface adhered proteins; TP, translated precursor EMP5 labeled with ^{3}H-Leu.

was also reported having a low expression level that could not be detected by RNA gel blot analysis (Gutiérrez-Marcos et al., 2007). The *Emp5* expression could be detected by RT-PCR, and the results confirmed that *Emp5* was expressed in all vegetative and reproductive tissues tested (Figure 5). Relative high mRNA expression was in stem, leaf, root, and ear and weak expression in tassel and kernels at developmental stages. This suggests that the EMP5 function may not be limited to embryo and endosperm development. Rather, it may have functions in other vegetative tissues during plant growth and development.

EMP5 Is Required for Mitochondrial RNA Editing

Up to now, DYW1, MORF, and PPR proteins are the only identified plant RNA editing *trans*-factors, and most of the PPR proteins belong to DYW subclass (Fujii and Small, 2011). Since EMP5 is a typical mitochondria-targeted DYW subclass PPR protein, it is highly likely that EMP5 functions in RNA editing in maize mitochondria.

A direct comparison of the mitochondrial transcripts between the *emp5* mutant and the wild type was performed by amplifying the transcripts and analyzing their sequences. Based on the maize mitochondrial genome (Clifton et al., 2004), 35 sets of primers were designed to cover the predicted 35 mitochondrial protein-coding genes (see Supplemental Table 1 online). These protein-coding mitochondrial genes include 22 genes of the electron transport chain, 11 ribosomal proteins genes, a maturase gene (*mat-r*), and a transporter gene (*mttB*). The reference allele *emp5*-1 was chosen for its near-isogenic W22 genetic background. To eliminate possible contamination of mitochondrial DNA (most mitochondrial genes are intronless), RNAs were treated with RNase-free DNase and confirmed to be DNA free by PCR amplification without reverse transcription. The RNA was isolated from the 13-DAP kernels as the mutant was very distinct from the wild type. The pericarp was carefully removed to prevent contamination by the heterozygous maternal tissues. RT-PCR was performed using proofreading DNA polymerase Phusion (New England Biolabs). The PCR products were purified from the gel and sequenced without cloning into a vector. This allows detection of both edited and unedited sites in one sequencing reaction and also eliminates cloning bias and random DNA polymerase errors. We amplified the mitochondrial genes in the *emp5-1*, and the sequences indicated that the W22 contains a NB-type mitochondrial genome (Clifton et al., 2004). As shown in Figure 6A, the C-to-U editing of *rpl16*-458 was completely abolished in the *emp5-1* allele, whereas it was completely edited in the wild type. The unedited sequence codes for a Pro, whereas the edited sequence codes for a Leu. Therefore, the lack of C-to-U editing in *rpl16*-458 resulted in an amino acid change from Leu to Pro in RPL16 protein in the *emp5-1* mutant. In addition, 100% C-to-U editing was found at *nad9*-190, *nad9*-356, *cox3*-245, and *cox3*-257 sites in the wild type, whereas it was dramatically diminished in *emp5-1*, especially for *nad9*-190 and *nad9*-356 sites (Figure 6A). Editing at five sites of the *rps12* transcript (*rps12*-71, *rps12*-196, *rps12*-221, *rps12*-269, and *rps12*-284) was also reduced in the *emp5-1* mutant (Figure 6A). All this editing lead to a change of the encoded amino acid as indicated in Figure 6A.

Interestingly, we also found that the editing of several sites was increased in the *emp5-1* mutant. Editing of *rpl16*-444, *atp6*-953, *nad1*-536, *nad1*-832, and *cob*-1098 was dramatically increased in the *emp5-1* mutant, comparing with weaker editing of these sites in the wild type (Figure 6B). However, this editing, except *atp6*-953, does not change the encoded amino acids. Increased editing was also reported in the *required for efficiency of mitochondrial editing1* (*reme1*) mutants encoding a PPR protein and the *rip1* T-DNA insertional mutants (Bentolila et al., 2010, 2012). However, the underlying mechanism is not clear.

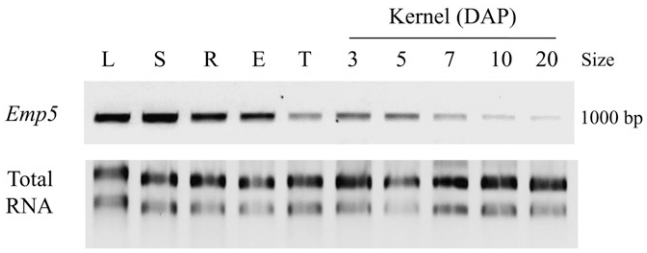

Figure 5. RT-PCR Analysis Indicates Expression of *Emp5* in Multiple Organs and during Seed Development in Maize.

Primers Emp5-F2 and Emp5-R2 were used in RT-PCR. E, ear; L, leaf; S, stem; R, root; T, tassel. Kernel at 3, 5, 7, 10, and 20 DAP.

Molecular Characterization of the *emp5-4* Allele

As described previously, homozygous *emp5-4* produced viable seeds. Genotyping with primers Emp5-F4 and Emp5-R4 and sequencing of the amplicon identified that the insertion is a 1.4-kb *Mu1* element in the middle of the E+ motif, thereby disrupting the E + and DYW domain (Figure 7A). However, homozygous *emp5-4* seedlings and adult plants were macroscopically indistinguishable from the wild type (Figure7B). In contrast with the use of transgenes, this allele provided excellent genetic material for studying the effect of disrupted E+ and DYW domains on the function of EMP5. First, we examined the expression using different *Emp5* primers anchored on different regions of the *emp5-4* allele (Figure 7A). As indicated in Figure 7C, RT-PCR results of the seedling leaf transcripts indicated that the region 5′ of the *Mu1* insertion in *emp5-4* was expressed at roughly the similar level as the wild type. The region including *Mu1* insertion was only expressed in *emp5-4*, as indicated in the RT-PCR using *Mu* primer TIR8 and Emp5-R1 in RT-PCR (Figure 7C). However, PCR with primers Emp5-F6/Emp5-R5 as well as Emp5-F4/Emp5-R4 across the *Mu1* insertion did not produce any products in *emp5-4* (Figure 7C). To address whether this was caused by a difficulty in PCR amplification across the *Mu1* element, we used wild-type and homozygous *emp5-4* genomic DNA as template. PCR amplification with primers Emp5-F4 and Emp5-R4 reliably produced a 1.7-kb fragment in *emp5-4* (predicted 1736 bp) and a 350-bp fragment in the wild type (predicted 349 bp). Sequencing confirmed that the 1.7-kb amplicon contains the *Mu1* insertion in the *emp5-4* allele (Figure 7C), indicating the primer set worked well in amplifying across the *Mu1* insertion. We did not detect any other alternatively spliced transcripts as some *Mu* inserted alleles showed splicing of the element. The spliced transcripts should be favored in amplification because of a shorter size. RT-PCR analysis indicated that the *emp5-4* allele does not produce a detectable level of *Emp5*

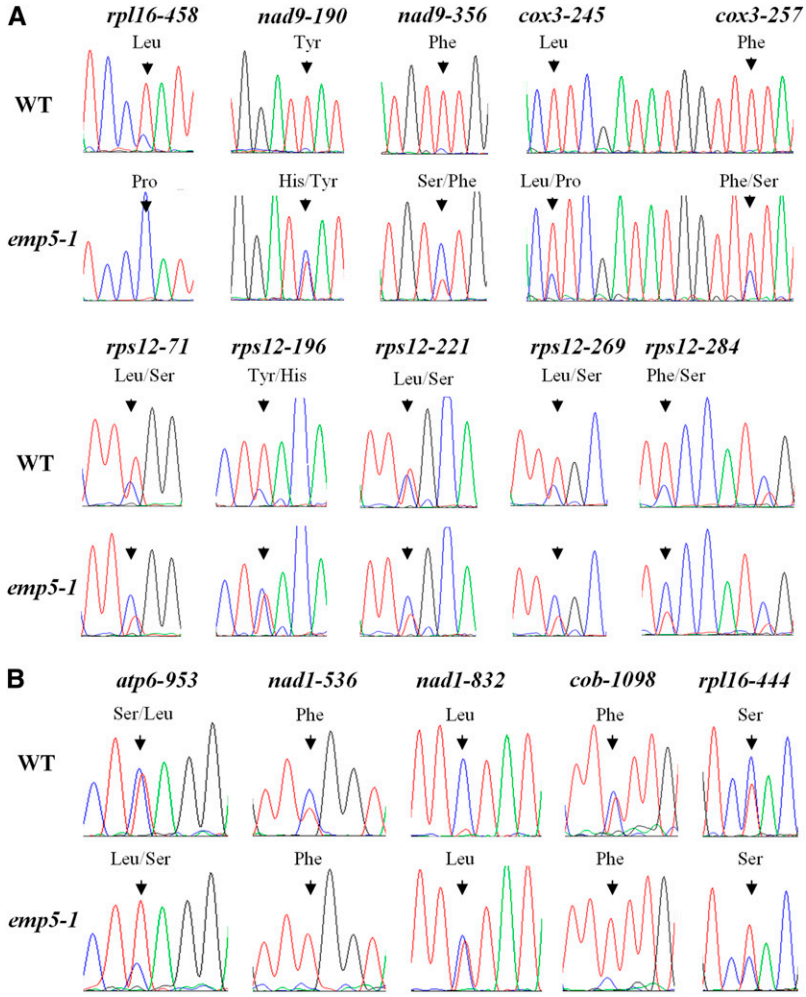

Figure 6. RNA Editing Defects of Mitochondria Genes in the *emp5-1* Mutant.

(A) RNA editing of 10 sites in four mitochondrial gene transcripts decreased in *emp5-1*. WT, the wild type.
(B) RNA editing of five sites in four mitochondrial gene transcripts increased in *emp5-1*. Sequence chromatogram of PCR-amplified wild-type cDNA or *emp5-1* cDNA of the editing sites is shown. The position of RNA editing represents the name of transcripts and the edited C position. The amino acid changes are indicated at the top, and the prevalent amino acids are put in front of the less prevalent ones. Arrowheads indicate the editing sites.

transcripts that is likely to be translated into a wild-type protein, although the gene including the *Mu1* element can produce two transcripts: One contains the 5′ region of the *Emp5* and ends in the *Mu1* insertion, and the second starts from somewhere inside the *Mu1* and ends probably where wild-type *Emp5* ends.

To determine the impact of this mutation on editing, we amplified and sequenced the mitochondrial transcripts using the same strategy as in *emp5-1*. The templates were the seedling RNAs from the wild type and the mutants from the same ear. The genotype was confirmed by PCR analysis. The results revealed that the *emp5-4* homozygotes showed similar levels of editing in most transcripts targeted by EMP5 in comparison to the wild type. These include *nad9*-356, *cox3*-245, *cox3*-257, *cob*-1098, *atp6*-953, *nad1*-536, and *nad1*-832 and five editing sites in *rps12* (*rps12*-71, *rps12*-196, *rps12*-221, *rps12*-269, and *rps12*-284). However, the *rpl16*-458 editing was diminished but clearly detectable in the *emp5-4* allele. This editing converts a Pro to a Leu residue, which is edited completely in the wild type, but the editing was completely abolished in *emp5-1*. Similarly, editing of *nad9*-190 site was increased in *emp5-4* in comparison to *emp5-1*. This site is also edited completely in the wild type, which converts a His to a Tyr residue (Figures 6A and 7D). Even in the same transcript, the other EMP5-targeted site (*nad9*-356) showed the same editing level as in the wild type. Similar to *emp5-1*, the editing of *rpl16*-444 was also increased in *emp5-4*, but this editing does not change in coded amino acid (Figure 7D). These results indicated that the mutation in *emp5-4* does not affect the editing of most EMP5 targeted sites but does decrease the editing in *rpl16*-458 and *nad9*-190.

Functional Analysis of the Rice *Emp5* Gene

Rice EMP5 protein is a putative ortholog of EMP5 in maize (Zm EMP5), sharing a high degree of similarity (84%) with EMP5 and containing 11 PPR repeats in the N terminus and E/E+/DYW

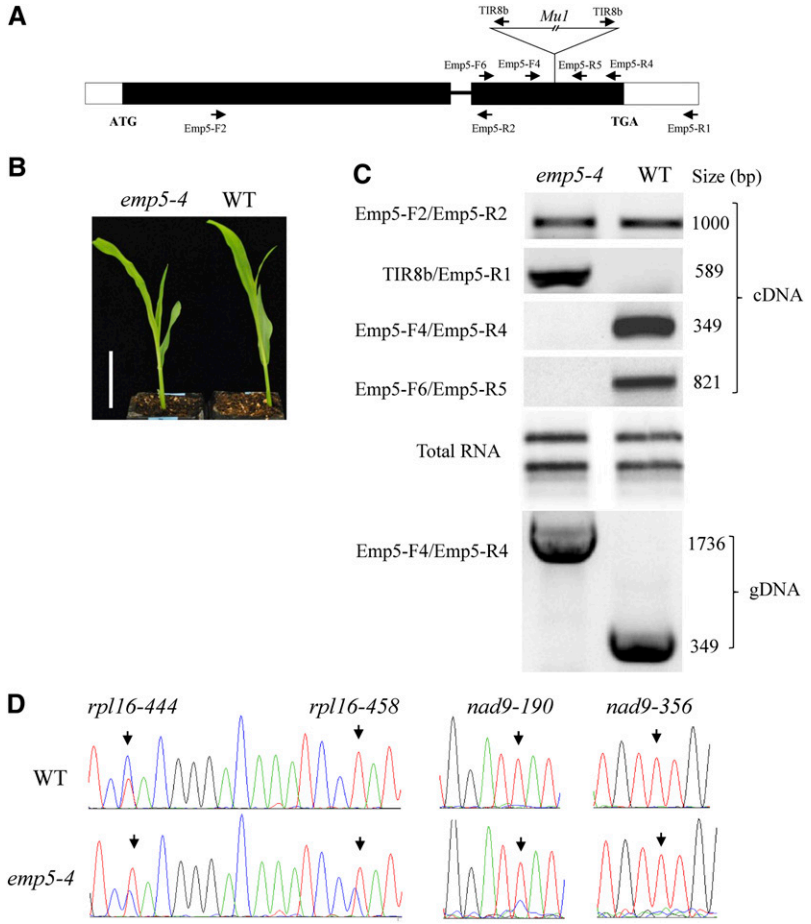

Figure 7. Insertion of a *Mu1* Element in the E+ Domain in the *emp5-4* Allele Reduces Editing Efficiency.

(A) Gene structure of *Emp5* and location of the *Mu1* insertion in the *emp5-4* allele. The positions of primers used in RT-PCR analysis are shown as arrows.

(B) Seedling phenotype comparison between the homozygous *emp5-4* and the wild type (WT). Bar = 5 cm.

(C) RT-PCR analysis of the transcripts in the *emp5-4* mutant and wild-type seedling leaves. Primer combinations are shown in the left. PCR amplification with Emp5-F4 and Emp5-R4 primers on genomic DNA was used as a control.

(D) Sequence chromatograms showing the C-to-U editing of *rpl16* and *nad9* transcripts in the *emp5-4* mutant and the wild type. The RNA editing site is described as the name of transcripts and the edited position from translation start codon. Arrows indicate the editing sites.

domains in the C terminus (Figure 8A). To address whether Os EMP5 has a conserved function similar to the Zm EMP5, we characterized EMP5 in rice. First, we determined the subcellular localization of rice EMP5 by expressing OsEMP5^{N372}:GFP fusion in tobacco (*Nicotiana tabacum*) leaf epidermal cells. Confocal laser scanning microscopy analysis revealed that the GFP signal of OsEMP5^{N372}:GFP was colocalized with MitoTracker Red in mitochondria (Figure 8B), confirming that EMP5 is targeted to the mitochondrion. To study the function of EMP5, Os *Emp5* RNAi transgenic rice was created. It is likely that severe knockdown transgenic lines would be lethal considering the essential role of EMP5 in maize, therefore only weak knockdown lines were likely generated. About 20 lines of transgenic rice were obtained and three representational lines were used for further molecular analysis. DNA gel blot analysis of the three Os *Emp5* RNAi transgenic lines (lines 19, 23, and 33), using the *hygromycin phosphotransferase* (*hpt*) gene as a probe, confirmed that they were independent transgenic lines, each carrying one copy of transgene (Figure 9A). RT-PCR analysis revealed the *Emp5* expression level was significantly decreased in lines 19 and 23 and slightly decreased in line 33 compared to the wild type (Figure 9B). The transgenic plants grew much slower than the wild type at seedling stage. But after the seedling stage, the plants gradually recovered and grew to normal adult plants (Figure 9C). The T1 progeny of all these three transgenic lines segregated defective seeds as different ratio (Figure 9C). The ratio of defective seeds in line 19 is 24.7% (199:807), line 23 is 22.3% (93:417), and line 33 is 9% (53:584). The severity of seed phenotype was roughly consistent with the suppressed *Emp5* expression level. This result indicated that similar to *Emp5* in maize, rice *Emp5* is essential to seed development in rice.

To reveal the effect on mitochondrial RNA editing in these Os *Emp5*-RNAi transgenic lines, mitochondrial *rpl16* transcripts were

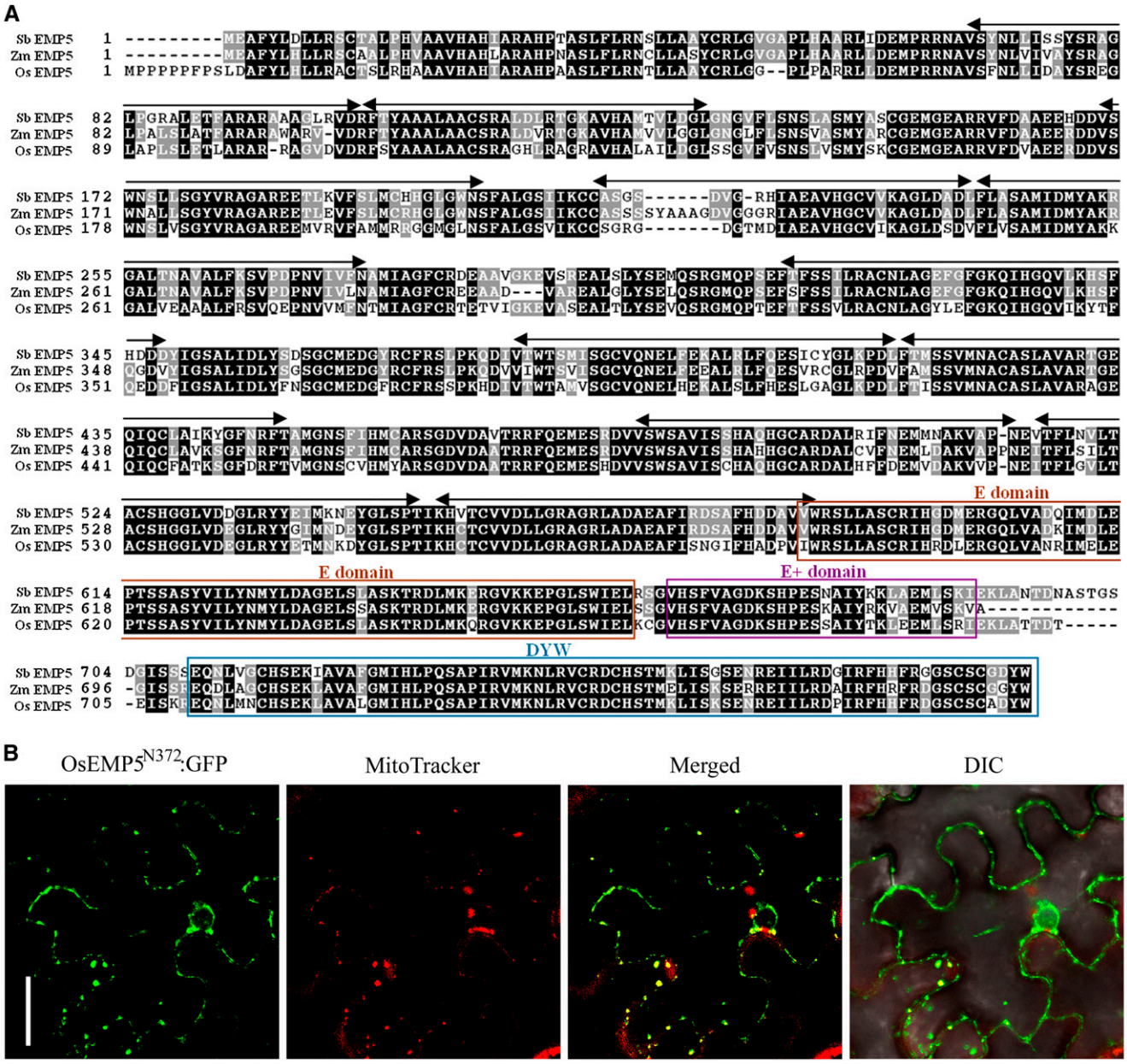

Figure 8. Os EMP5 Is a Mitochondrion-Localized PPR-DYW Protein.

(A) Alignment of maize Zm EMP5 protein with rice ortholog Os EMP5 and sorghum ortholog Sb EMP5 protein.
(B) OsEMP5^{N372}:GFP fusion protein was transient expressed in tobacco epidermal cells. Fluorescence signals from OsEMP5^{N372}:GFP (green) and MitoTracker stained mitochondria (red) were detected by confocal microscopy. DIC, differential interference contrast. Bar = 10 μm.

amplified and sequenced in the three Os *Emp5* RNAi transgenic lines and wild-type plants to compare the RNA editing difference. This analysis revealed that <50% *rpl16*-458 site in all three transgenic lines was edited, compared to 100% editing in the wild type (Figure 10). For *rpl16*-444 site, the editing level was increased in the three transgenic lines, which is similar to the maize *emp5-1* allele (Figure 10). This result confirmed that similar to maize EMP5, Os EMP5 is required for the editing of *rpl16*-458 in rice mitochondria.

DISCUSSION

Abortion of *emp5-1* Mutant Seed Development Is Caused by Defective Mitochondrial RNA Editing

The *emp* mutants are defined by a dramatic reduction in embryo and endosperm size, yet possess a normal pericarp (Sheridan and Neuffer, 1980). In maize, three mutants with a similar phenotype have been characterized. *Emp2* encodes a repressor of

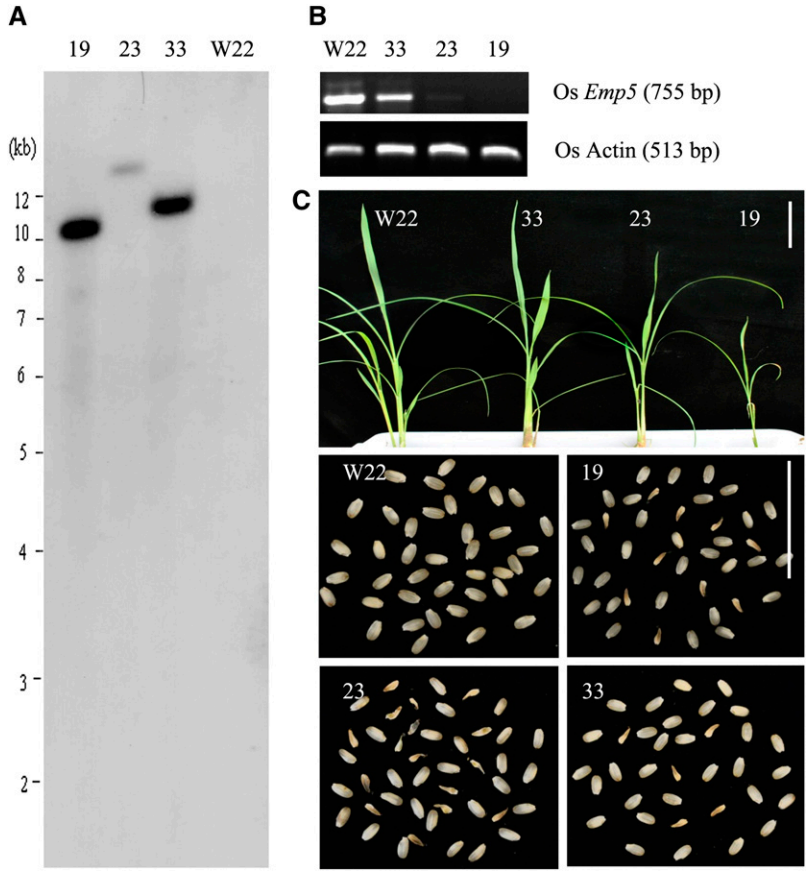

Figure 9. RNAi Knockdown Analysis of Os *Emp5* Expression in Transgenic Rice.

(A) DNA gel blot analysis of independent Os *Emp5* RNAi transgenic lines (lines 19, 23, and 33) using the *hpt* gene as a probe.
(B) RT-PCR analysis of endogenous *Emp5* expression in three transgenic lines. Os *Actin* (X15865.1) was used as control. Primers Osactin-F, Osactin-R, OsEmp5-F, and OsEmp5-R (see Supplemental Table 1 online) were used in RT-PCR.
(C) Phenotypes of Os *Emp5* RNAi transgenic plants at the seedling stage and in T1 progeny segregating defective seeds. Bars = 5 cm.

a heat shock response in seeds, mutation of which unleashes a heat shock response causing seed development abortion (Fu et al., 2002). *Emp4* encodes a PPR protein that is required for normal level expression of several mitochondrial genes (Gutiérrez-Marcos et al., 2007). Recently, MPPR6, a PPR gene whose mutation causes an empty pericarp phenotype, is required for maturation and translation initiation of *rps3* mRNA in mitochondria (Manavski et al., 2012). In this study, we show that *Emp5* encodes a DYW subclass PPR protein that functions in the editing of several maize mitochondrial gene transcripts. The cloning of *Emp5* is supported by multiple independent insertions in the *Emp5* gene that condition a typical *emp* phenotype and also by the further functional analysis in editing. Such function in mitochondrial editing is conserved in its ortholog *Emp5* in rice. The results indicate that the deficiency of editing in these mRNAs compromises the mitochondrial function, causing the embryo and endosperm development to be arrested at transition stage. Since the pericarp tissue is maternal, growth of the pericarp was not expected to be affected in heterozygous *Emp5*/*emp5* plants. Thus, the mutant kernels are appeared as *empty pericarp*. Considering the critical functions of mitochondria to both the embryo and the endosperm, the *emp* mutants should be enriched with genes that have key functions in mitochondria. In maize, the *emp* mutants form a distinct class that will be ideal genetic materials for dissecting the mitochondrial PPR functions.

In the severe allele of *emp5-1*, the editing of *rpl16*-458 was completely abolished, and the other nine editing sites in three different transcripts (*nad9*, *cox3*, and *rps12*) were also diminished. *rpl16* and *rps12* encode ribosomal proteins of the mitochondrial translation machinery. *nad9* and *cox3* are required for Complex I and Complex IV function in the electron transport chain, respectively. However, a significant portion of correctly edited transcripts of *nad9*, *cox3*, and *rps12* still exist. It is not clear of the impact on seed development as a result of reduced editing in these three transcripts. A fraction of the correctly edited mRNA may be sufficient for mitochondrial function to complete embryogenesis. This is indicated in other mitochondrial genes, such as *apocytochrome b* (*cob*). A complete loss of *cob*-908 editing severely reduces the growth in maize, whereas a residual level of *cob*-908 editing is sufficient to assure normal plant growth in *Arabidopsis* (Sosso et al., 2012). In addition, our editing analysis in maize mitochondrial transcripts showed that

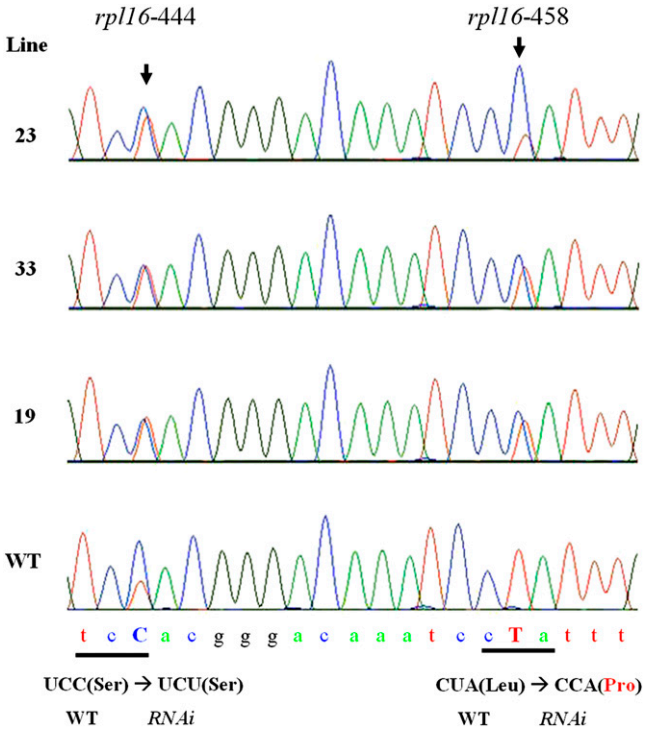

Figure 10. Os EMP5 Is Required for *rpl16* Editing in Rice Mitochondria.

Sequence chromatograms of the rice *rpl16* cDNA amplified by RT-PCR in wild-type (WT) and Os *Emp5* RNAi transgenic lines showed the editing sites. Arrowheads indicate the editing sites. The amino acid change is indicated at the bottom.

partial editing for a particular site is common in wild-type mitochondrial transcripts. This argues a possibility that the reduced editing in the nine sites of three transcripts (*nad9*, *cox3*, and *rps12*) may not be the major cause for the *emp* phenotype. Instead, the complete loss of a single *rpl16*-458 editing site may be attributable to the abortion of *emp5* mutant seed development.

The *rpl16* transcript lacking *rpl16*-458 editing translates to a protein with a Pro instead of Leu residue at position 153, which may severely compromise the RPL16 function in mitochondrial protein translation. Pro often acts as a structural disruptor in protein secondary structure, such as α-helices and β-sheets. Thus, this change may severely affect the structure and the function of the RPL16 protein. There are several cases where an unedited site coding for Pro causes a severe impact on plant growth and development. The maize mitochondrial *cob*-908 is normally C-to-U edited by PPR2263 to render a Pro-to-Leu change. The unedited *cob*-908 in *ppr2263* mutants caused defects in seed development and seedling growth (Sosso et al., 2012). In the *slg1* mutant of *Arabidopsis*, RNA editing in a single site *nad3*-250 in mitochondria is abolished, resulting in a codon for Pro instead of the edited for Ser. This single amino acid mutation caused slow growth and delayed development (Yuan and Liu, 2012). The mutations of OGR1, SLOW GROWTH1 (SLO1), and MITOCHONDRIAL EDITING FACTOR11(MEF11) also leaded to absence of RNA editing of mitochondrial transcripts, which all result in Leu-to-Pro changes in *nad4*-416, *nad4*-449, and *cox3*-422, respectively (Kim et al., 2009; Sung et al., 2010; Verbitskiy et al., 2010). All three mutants exhibited significant defects in plant growth and development. Conceivably, genetic screens will enrich the identification of changes critical to protein functions. These cases demonstrate that the editing for transition from Pro to other amino acids is critical to the function of multiple mitochondrial proteins.

The *rpl16* gene has been proved to be essential to mitochondrial gene expression as a key component in the translation machinery. Thus far, a complete deletion of the *rpl16* gene in mitochondria has not been isolated, presumably due to its lethality (Newton et al., 1996). In the *Arabidopsis maternal distorted leaf* mutant, rearrangement in two mitochondrial DNA fragments associated with the *rps3-rpl16* polycistron resulted in a deletion of part of the intron and exon b of *rps3* that is upstream of *rpl16* but without affecting the *rpl16* coding region. The mutant showed poor growth, distorted rough leaves, and aborted flowering organs (Sakamoto et al., 1996). A deletion of the 5′ untranslated region sequences, including the promoter for the transcription unit of the *rps3-rpl16* polycistron in maize *nonchromosomal stripe3* (*ncs3*) and *ncs4* mutants, caused severe stunted and striped leaves and male fertility, respectively (Hunt and Newton, 1991; Newton et al., 1996). The mRNA abundance corresponding to the *rps3/rpl16* coding region was specifically reduced in these mutants. Mitochondrial protein synthesis was dramatically reduced in severely affected mutant plants. These results reveal that the RPL16 protein is essential for protein synthesis in mitochondria, which consequently will be crucial for plant development. Therefore, the failed development in both embryo and endosperm in the *emp5* mutants is likely due to a loss of *rpl16*-458 editing.

Increased Editing in the *emp5* Mutant

The mechanism by which RNA editing is performed in plastids and mitochondria is not clear. PPR proteins are considered as the specificity determinants that recognize different transcripts (Shikanai, 2006; Chateigner-Boutin and Small, 2010). A single PPR protein, such as EMP5, that is responsible for multiple editing sites in multiple transcripts has been reported in several PPR-DYW proteins, such as MEF1, MEF11, and OGR1 in *Arabidopsis* and rice (Kim et al., 2009; Zehrmann et al., 2009; Verbitskiy et al., 2010). A considerate amount of RNA was found still correctly edited in the *emp5-1* mutant, raising a reasonable possibility that overlapping editing on the same site may be mediated by different PPR proteins. In addition, we found that five editing sites on four mitochondrial transcripts were increased in the *emp5-1* mutants, interspersing in *atp6*, *nad1*, *cob*, and *rpl16* transcripts (Figure 6B). This result was also confirmed in *Emp5* RNAi knockdown transgenic rice where the *rpl16*-444 editing level is increased in all three transgenic lines compared with the wild type. Intriguingly, the editing in these sites do not change the amino acid coding except in *atp6*-953 where editing converts Ser to Leu. This phenomenon has also been reported in PPR protein REME1, mutation of which reduces the editing of *nad2*-558 and *orfX*-552, but increases the editing extent in at least two sites, *matR*-1771 and *rpl5*-92

(Bentolila et al., 2010). It seems that PPR proteins may work as a positive and negative regulator of organelle RNA editing at the same time. One possibility is that overlapping functions by PPR proteins in editing a single site exist in plants such that the deficiency in one PPR leads to a compensational expression of another PPR protein that edits another set of sites, overlapping but not identical. In this case, some sites will be edited more than the wild type as a result of the compensational PPR proteins. Another possibility is that although a *Mu2* is inserted into the *Emp5* gene, it can still be transcribed and translated into a truncated protein that recognizes different RNA substrates. For the *emp5-1* allele, the *Mu2* insertion disrupted six of the 11 PPR motifs, allowing five PPR repeats remained in the mutant protein if it can be translated (Figure 4A).

Targeting Sequences of EMP5 Are Not Conserved

Two PPR proteins, CRR4 and RESTORER OF FERTILITY1 (RF1), were reported as possessing the RNA binding activity in vitro without other factors (Okuda et al., 2006; Kazama et al., 2008), leading to a hypothesis that PPR proteins can specifically recognize the *cis*-element of an editing site. Some RNA editing sites were shown to have conserved sequences upstream of the editing sites (Karcher et al., 2008; Kobayashi et al., 2008; Sosso et al., 2012). However, these conserved sequences were derived by comparing merely two editing sites. In cases where a PPR protein is involved in the editing of multiple sites, such as OGR1, MEF1, MEF11, and CRR22, no conserved sequences in the corresponding region can be identified (Kim et al., 2009; Okuda et al., 2009; Zehrmann et al., 2009; Verbitskiy et al., 2010). In CHLOROPLAST BIOGENESIS19 (CLB19), the sequences surrounding the two editing sites showed little sequence similarity (Chateigner-Boutin et al., 2008). In an attempt to identify conserved sequences for EMP5 edited sites, we aligned the adjacent sequences near the editing sites (region from −40 to +20). We did not find any conserved sequences except most of the −1 base is T (see Supplemental Figures 1 and 2 online). The likely possibility is that PPR proteins recognize a specific secondary structure of the transcripts, not the primary sequence. It is hypothesized that PPR editing factors can only distinguish pyrimidines from purines and, at some positions, must be able to recognize specific bases (Hammani et al., 2009). Recently, Barkan et al. (2012) used computational methods to infer a PPR-RNA recognition mechanism where a combination of position 6 amino acid residues in a PPR motif and the 1′ position of the next PPR motif recognizes one nucleotide in the RNA substrate. As such, the tandem PPR motifs are decoded to a nucleotide sequence that specifies the substrate RNA of the PPR protein. It was validated by recoding a PPR protein to bind novel RNA sequences in vitro. However, we failed to make the connection between EMP5 and *rpl16*.

The E+ and DYW Motifs of EMP5 Are Not Essential for PPR Protein Function

PPR proteins are classified into P, PPR-E, and PPR-DYW subclasses based on the presence of additional C-terminal motifs (Lurin et al., 2004). The PPR repeats are proposed to recognize the target RNA sequences (Shikanai, 2006). In MEF11, the second PPR repeat was shown to be crucial for the specific editing of *cox3*-422, *nad4*-124, and *ccb203*-344 (Verbitskiy et al., 2010). The DYW domain that showed significant similarity to cytidine deaminases was proposed to have catalytic editing activity (Salone et al., 2007). Indeed, its presence is correlated with presence of RNA editing in plant evolution (Fujii and Small, 2011). Although PPR proteins, such as CRR4, CLB19, MEF9, and SLO1, all lacking a DYW domain, still possess the function of C-to-U RNA editing (Kotera et al., 2005; Chateigner-Boutin et al., 2008; Sung et al., 2010; Takenaka, 2010), a recent report showed that the DYW domain can be supplied in trans to the CRR4 protein and it is essential for the editing (Boussardon et al., 2012). Therefore, the hypothesis that the DYW domain functions as the deaminase enzyme remains. Up to this work, all the PPR proteins involved in editing contain an E domain, but the E domain lacks any obvious catalytic characteristics, suggesting that the E domain is indispensable for editing with unknown function (Shikanai, 2006; Okuda et al., 2007). It is possible that the E domain mediates protein–protein interaction to recruit another protein with deaminase activity, which may include PPR-DYW proteins.

In the *emp5-4* allele, a *Mu1* element is inserted in the middle of E+ motif, disrupting the E+ motif and DYW motif. RT-PCR analysis using several sets of primers revealed the presence of two transcripts where one contained the 5′ region of the *Emp5* gene and ended inside the *Mu1*, and the other contained the 3′ region of the *Emp5* gene, but no transcript across the *Mu1* element existed. We analyzed the two transcripts for open reading frames that would possibly encode proteins with a scenario like the PPR-E protein with a *trans*-supplied DYW protein. As shown in Supplemental Figure 3 online, the translation of first transcript is predicted to terminate by a stop codon, TAA, only adding two amino acids encoded by the *Mu1* TIR sequence. This will produce a truncated EMP5 protein, similar to a PPR-E protein, and if translated, it should be able to target to mitochondria. The second transcript predicts only one open reading frame in frame with the DYW domain. The translation adds five amino acid residues (MAIIS) from the *Mu1* sequence to the N terminus of this hypothetical protein. However, the N terminus sequence does not predict a mitochondrion signal peptide by currently available algorisms. Therefore, it is highly unlikely that the protein would be targeted to the mitochondrion, even if it can be translated. Alternatively spliced transcripts were not detected, although we intentionally set the conditions favoring the amplification of potentially spliced *emp5-4* transcripts. This leads us to conclude that the insertion in the *emp5-4* allele causes a possible deletion of the C-terminal E+ domain and the entire DYW domain. However, a truncated version of the *EMP5-4* protein may be produced. Genetic analysis indicated that the *emp5-4* allele appeared normal in growth and development. And most of the editing events targeted by EMP5 showed similar editing level comparing to the wild type, except the editing of *rpl16*-458, which is considered critical showed partial editing. In addition, *rpl16*-444 and *nad9*-190 editing efficiency was also changed slightly. The presence of editing in *emp5-4* that lacks the E+ and DYW motif indicates that E+ and DYW domains are not essential for EMP5 editing function. This conclusion is consistent with the plastid-located CRR22, CRR28, and ORGANELLE TRANSCRIPT PROCESSING28 (OTP28) and the mitochondrial

factors MEF3 and MEF11 (Okuda et al., 2009, 2010; Verbitskiy et al., 2010, 2012; Zehrmann et al., 2011). However, the DYW domain of MEF1 cannot be destroyed without severe effects on its function in editing (Zehrmann et al., 2009). Therefore, it is possible that EMP5 associates with a DYW container partner, which can almost completely complement E+ and DYW motif truncation and carry out editing, although inefficiently. However, for MEF1, there is no such DYW container partner to associate, so the DYW domain cannot be deleted or mutated.

METHODS

Plant Materials

The *emp5-1* reference allele was isolated from the UniformMu population by introgressing *Mu* active lines into the inbred W22 genetic background (McCarty et al., 2005). The wild-type plants were either siblings of the mutant or W22. The *emp5-2*, *emp5-3*, and *emp5-4* alleles were isolated from the TUSC population (Pioneer Hi-Bred International) by PCR screening with *Emp5*-specific primers and Mu primers. The maize (*Zea mays*) plants were grown in the experimental field at the Chinese University of Hong Kong under natural conditions. Rice (*Oryza sativa* ssp *japonica*; cv Nipponbare) was used as the plant material for *Agrobacterium tumefaciens*–mediated rice transformation.

Light Microscopy of Cytological Sections

To enable a precise comparison, wild-type and *emp5-1* mutant kernels were harvested from the same ear of a self-pollinated heterozygous plant at 8 and 13 DAP. The kernel was cut along longitudinal axis, and the slice containing the embryo was fixed for 1 d at room temperature in 4% paraformaldehyde. The fixed material was dehydrated in an ethanol gradient series (50, 70, 85, 95, and 100% ethanol). After clearing with xylene and paraffin wax infiltration, the sample was embedded and sectioned at 6- to 10-μm thickness under a Leica 2035 Biocut. The sections were stained with Johansen's Safranin O and Fast Green and observed with a Nikon ECLIPSE 80i microscope.

Immunohistochemistry Analysis

The sections containing 13 DAP wild-type and *emp5-1* seeds were deparaffinized, then air-dried for 15 min, rinsed with PBS, and incubated in 0.2 to 0.3% Triton X-100 in PBS for 15 min. Nonspecific antibody binding was blocked with 3% BSA for 2 h. The tissues were then incubated with the primary antibody (a gift from Gregorio Hueros, Universidad de Alcalá, Spain; 1:400 diluted in 1% BSA and 0.05% Triton X-100 in PBS) at 4°C overnight. The slides were washed three times with PBS, 15 min each to completely remove the primary antibody. Then the slides were incubated with Alexa Fluor-568 anti-rabbit secondary antibodies (Invitrogen) for immunofluorescent detection (diluted 1:500 in PBS, room temperature, 1 h). After washing three times with PBS, the slides were viewed and imaged under an Olympus FluoView FV1000 confocal microscope. Control sections were incubated with PBS without the primary antibody and subsequently processed as described above.

DNA Gel Blot Analysis and Inverse PCR Cloning

For cosegregation analysis, maize genomic DNA was extracted from *emp5-1*/+ and wild-type seedlings using the urea extraction method as described previously (Tan et al., 2011) and digested by *Hin*dIII. DNA gel blot analysis was performed as previously described (Tan et al., 2011). The hybridization probe was the ~1-kb *Hin*fI fragment of the *Mu1* element internal sequence. Because *Mu1* and *Mu2* share a high degree of sequence identity, this probe hybridizes to both elements.

For inverse PCR cloning of the *Mu* flanking sequences in the 3.4-kb *Hin*dIII fragment, genomic DNA of heterozygous *emp5-1* plants was digested by *Hin*dIII and separated on 0.7% agarose gel. The 3.4-kb fragment was enriched by cutting a small gel slice around 3.4 kb and the DNA was purified. The DNA was self-ligated at 50 ng/μL concentration overnight at 4°C. Then, the ligated DNA was digested with *Not*I. *Mu1* and *Mu2* elements contain a *Not*I site in the internal sequences; hence, the digestion linearized the ligated circular DNA for efficient amplification. This DNA was used as the template in the inverse PCR amplification of the *Mu* flanking sequences. Annealing temperature for the first-round PCR is 60°C and *Mu*-specific degenerated TIR6 primer (5′-AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC-3′) was used. The second-round PCR was performed with a 56°C annealing temperature and Mu1-62 primer (5′-CCCTTCCCTCTTCGTCCATAAT-3′). The amplified fragment was cloned into pCR4-TOPO and sequenced.

RNA Extraction and RT-PCR

Approximately 100 mg of fresh tissue was quickly frozen in liquid nitrogen and ground to fine powder with a mortar and pestle. Total RNA was extracted with 1 mL Trizol reagent according to the manufacturer's instructions (Invitrogen). After isopropanol precipitation, the RNA was resuspended in 40 μL RNase-free water and treated with RNase-free DNase. RT-PCR analyses were performed using the SuperScript III One-Step RT-PCR system according to the manufacturer's instructions (Invitrogen). RT-PCR for the expression pattern analysis of *Emp5* in maize organs was performed with primers Emp5-F2 and Emp5-R2 and 57°C annealing temperature for 30 cycles. For analysis of *Emp5* expression in rice transgenic lines, RT-PCR was performed with primers OsEmp5-F and OsEmp5-R and 55°C annealing temperature for 28 cycles. The information of all primers is listed in Supplemental Table 1 online.

Subcellular Localization of EMP5

To generate a translational protein fusion between the EMP5 signal peptide and GFP, full-length *Emp5* and *Emp5*^{N469} fragment was amplified by PCR from maize inbred line W22 and cloned into pENTR/D-TOPO (Invitrogen), respectively. The fusion was introduced to binary vector *pGWB5* (a gift from Tsuyoshi Nakagawa, Shimane University) by Gateway site-specific recombination. The OsEMP5^{N372}:GFP fusion expression construct was constructed similarly. These fusion proteins were placed under the cauliflower mosaic virus 35S promoter for constitutive expression. Then, these constructs were transformed into *Agrobacterium* strain EHA105. The resulting strains harboring full-length EMP5:GFP and EMP5^{N469}:GFP expression plasmid were used to transform *Arabidopsis thaliana* Columbia ecotype by the flower dip method (Clough and Bent, 1998). The transgenic *Arabidopsis* was identified with PCR amplification of the *Hpt* gene in pGWB5 vector with primer Hpt-F3 and Hpt-R3. The protoplasts were isolated from the transgenic leaves by digesting with an enzyme solution (1.5% cellulose R10, 0.3% pectolyase Y23, 20 mM MES, pH 5.7, 0.4 M mannitol, 20 mM KCl, 10 mM $CaCl_2$, and 0.1% BSA). Using established protocols (van Herpen et al., 2010), the EHA105 strain harboring the OsEMP5^{N372}:GFP fusion construct was infiltrated into tobacco (*Nicotiana tabacum*) leaves to transiently express the fusion protein. The infiltrated tobacco leaves, transgenic *Arabidopsis* leaf samples, and protoplasts were used for GFP and MitoTracker red signals detection by an Olympus FluoView FV1000 confocal microscope. The working concentration of MitoTracker was 30 nM, and the samples were incubated at 37°C for 30 min.

In vitro chloroplast protein import assay was performed as described previously (Cline, 1986; Martin et al., 2009). *Emp5* cDNA was placed under the SP6 promoter in pGem-3Z vector. RNA transcripts of this construct were produced by in vitro transcription with SP6 polymerase (Promega).

The protein was translated with a homemade wheat germ translation system in the presence of ^{3}H-Leu (50 mCi, 3000 Ci/mol). Pea (*Pisum sativum* cv Laxton's Progress 9 Improved) used for chloroplast isolation were grown as described (Cline, 1986). The chloroplast import was performed as described (Martin et al., 2009).

Analysis of Mitochondrial RNA Editing

For RNA editing analysis in the wild type and the *emp5-1* allele, total RNAs were isolated from the immature embryos and endosperms by carefully removing the pericarp. For the *emp5-4* allele and rice RNAi transgenic lines, total RNAs were isolated from seedling leaves, all using the Trizol reagent according to the instructions of the manufacturer (Invitrogen). The RNA was treated with DNase I (New England Biolabs), and the complete removal of DNA was checked by PCR on genomic DNA. Then, the DNA-free RNAs were reverse transcribed with random hexamers and the high-fidelity reverse transcriptase SuperScript III (Invitrogen). Full sequences of total 35 protein-coded maize mitochondrial genes and rice mitochondrial *rpl16* gene were amplified by PCR. The RT-PCR products were sequenced directly. This analysis was done with three biological replicates using seeds of different days after pollination. These primers are listed in Supplemental Table 1 online.

Rice Transformation

For *OsEmp5* RNAi vector construction, a 518-bp *Emp5* (position: 1362 to 1879 bp from ATG) fragment was PCR amplified with primers OsEmp5-KpnI and OsEmp5-BamHI, and OsEmp5-SpeI and OsEmp5-SacI, respectively (see Supplemental Table 1 online). The PCR products were digested with *Bam*HI and *Kpn*I, *Spe*I, and *Sac*I, respectively, and ligated into the binary vector *pTCK303*. The resulting RNAi vector, *pTCK303-OsEmp5*, was introduced into the wild-type cultivar Nipponbare using *Agrobacterium*-mediated transformation. Transformation was performed as previously described (Hiei et al., 1994). DNA gel blot analysis of transgenic lines was performed as previously described (Tan et al., 2011), and the *hpt* gene amplified from vector pTCK303 with primer Hpt-F1 and Hpt-R1 (see Supplemental Table 1 online) was used as a probe.

Accession Numbers

Sequence data for *Emp5* genomic DNA, cDNA, alleles *emp5-1*, *emp5-2*, *emp5-3*, and *emp5-4* can be found in the GenBank/EMBL database under accession numbers JX308938, JX308939, JX308940, JX308941, JX308942, and JX308943, respectively.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Alignment of −40 to +20 Sequences of All 15 RNA Editing Sites in Seven Mitochondrial Gene Transcripts Changed in emp5-1.

Supplemental Figure 2. Alignment of −40 to +20 Sequences of 10 RNA Editing Sites in Four Mitochondrial Gene Transcripts Decreased and Increased in *emp5-1*.

Supplemental Figure 3. The Hypothetical Proteins as Predicted Based on the Two Transcripts Detected in the *emp5-4* Mutant Seeds.

Supplemental Table 1. Primers Used in This Study.

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AUTHOR CONTRIBUTIONS

Y.-J.L., Z.-H.X., and B.-C.T. designed and performed the research. R.M. isolated the alleles of *emp5-2* to *-4*. Y.-J.L., R.M., and B.-C.T. analyzed the data. Y.-J.L. and B.-C.T. wrote the article.

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