Editing of Mitochondrial Transcripts *nad3* and *cox2* by Dek10 Is Essential for Mitochondrial Function and Maize Plant Development

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ABSTRACT Respiration, the core of mitochondrial metabolism, depends on the function of five respiratory complexes. Many respiratory chain-related proteins are encoded by the mitochondrial genome and their RNAs undergo post-transcriptional modifications by nuclear genome-expressed factors, including pentatricopeptide repeat (PPR) proteins. Maize *defective kernel 10* (*dek10*) is a classic mutant with small kernels and delayed development. Through positional cloning, we found that *Dek10* encodes an E-subgroup PPR protein localized in mitochondria. Sequencing analysis indicated that Dek10 is responsible for the C-to-U editing at *nad3*-61, *nad3*-62, and *cox2*-550 sites, which are specific editing sites in monocots. The defects of these editing sites result in significant reduction of Nad3 and the loss of Cox2. Interestingly, the assembly of complex I was not reduced, but its NADH dehydrogenase activity was greatly decreased. The assembly of complex IV was significantly reduced. Transcriptome and transmission electron microscopy (TEM) analysis revealed that proper editing of *nad3* and *cox2* is critical for mitochondrial functions, biogenesis, and morphology. These results indicate that the E-subgroup PPR protein Dek10 is responsible for multiple editing sites in *nad3* and *cox2*, which are essential for mitochondrial functions and plant development in maize.

KEYWORDS *Zea mays*; *dek10*; pentatricopeptide repeat protein; mitochondrion; RNA editing; seed development

MITOCHONDRIA are the center of cellular energy homeostasis and redox regulation, and integrate numerous metabolic pathways (Sweetlove *et al.* 2007). Respiration is the core of mitochondrial metabolism for free energy release and ATP production. During respiration, electrons from NADPH and reduced flavin adenine dinucleotide (FADH2) are transferred to O_2 via the electron transport chain (ETC), generating ATP and oxidized NADP1 and FAD1 (Siedow and Day 2000). The ETC is composed of five respiratory complexes. Depending on the substrate, electrons are transported from complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) through ubiquinone and complex III [cytochrome c (Cyt-c) reductase] to Cyt-c and to complex IV [Cyt-c oxidase (Cox)], which produces water, while ATP is generated by complex V (ATP synthase) (Dudkina *et al.* 2006). In plants, respiratory metabolism can also be fulfilled by alternative glycolytic, phosphorylating, and electron transport pathways. When there is an electron transport defect in the Cyt-c pathway, alternative oxidases (AOXs) are activated to maintain the tricarboxylic acid cycle and electron transport, even in the absence of oxidative phosphorylation (Vanlerberghe and Ordog 2002).

Though the majority (~98%) of mitochondrial proteins are nuclear-encoded, the mitochondrial genome retains some genes encoding proteins involved in the respiratory chain. The primary mitochondrial genome-expressed pre-RNAs could be post-transcriptionally processed (Knoop 2013; Hammani and Giege 2014). These processes are mostly regulated by nuclear genome-expressed factors, including pentatricopeptide repeat (PPR) proteins, which are reported to play a critical role (Barkan and Small 2014). The PPR RNA-binding proteins are defined by the tandem repeats of a degenerate 35-aa motif. PPR proteins are classified into two major subgroups: the P-type PPR members only harbor tandem repeats of the canonical 35-aa PPR motif, while and the PLS-type PPR members are

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composed of sequential repeats of P, short (S), and long (L) PPR motifs and often carry an E or E–DYW C-terminal domain extension (Lurin *et al.* 2004). The PPR family comprises > 450 members in plants, and acts specifically in mitochondria or plastids for RNA editing, cleavage, splicing, and stability, as well as translational initiation and regulation (Schmitz-Linneweber and Small 2008; Fujii and Small 2011; S. Liu *et al.* 2013; Barkan and Small 2014). To date, the PPR proteins identified to be involved in mitochondrial or plastid RNA editing mostly belong to the E and DYW subgroups (Lurin *et al.* 2004). However, the P-subgroup protein PPR596 was also reported to be involved in editing efficiency (Doniwa *et al.* 2010; Takenaka 2010). A number of severe growth and developmental defects associated with loss-of-function PPR mutants have been previously described (Fujii and Small 2011; Sosso *et al.* 2012; Y. Liu *et al.* 2013; Colas des Francs-Small and Small 2014; Hammani and Giege 2014; Li *et al.* 2014; Sun *et al.* 2015; Haïli *et al.* 2016). However, the molecular roles and regulatory functions are still unknown for a great number of PPR proteins.

Maize (*Zea mays*) is highly suitable for genetics research, partly because of its numerous easily observable phenotypes (Neuffer and Sheridan 1980). Defective kernel (dek) mutants are a major class of maize kernel mutants that are good resource with which to investigate seed development (Neuffer and Sheridan 1980). *Dek1* encodes a calpain family protein affecting embryo and endosperm aleurone layer development (Becraft *et al.* 2002; Lid *et al.* 2002). *Dek** encodes ribosome biogenesis factor Rea1, and *dek** as a weak mutant allele partly represses the maturation and export of the 60S ribosomal subunit. Taking advantage of this mutant allele, comprehensive cellular responses to impaired 60S ribosomal subunit biogenesis were revealed (Qi *et al.* 2016a). dek mutants offer opportunities to investigate many basic biological processes during kernel development.

In this study, we characterized *dek10*, a dek mutant with small kernels and delayed development. We report the map-based cloning of *Dek10* and demonstrate that it encodes an E-subgroup PPR in maize. We present evidence that Dek10 is specifically involved in the C-to-U editing at *nad3*-61, *nad3*-62, and *cox2*-550. Defects in this editing reduces the functions of complex I and complex IV in the ETC. Consequently, it arrests mitochondrial oxidative phosphorylation, and embryo, endosperm, and seedling development.

Materials and Methods

Plant materials

The maize *dek10-N1176A* stock was obtained from the Maize Genetics Cooperation stock center. The mutant was crossed into a W22 genetic background to produce the F2 populations. Kernels of the F2 ears exhibited a 3:1 segregation of wild-type kernels (*dek10*/+ or +/+) and homozygous mutant kernels (*dek10/dek10*), which were used for analysis. The root, stem, third leaf, tassel, and ear tissues were collected from at least three W22 plants at the V12 stage. All the plants were cultivated in a field at the Shanghai University campus, in Shanghai, China.

Measurement of protein and starch

For the protein measurements, the endosperm of *dek10* and wild-type mature kernels was separated from the embryo and pericarp by dissection after soaking the kernels in water. The samples were dried to constant weights, pulverized with a mortar and pestle in liquid N_2, and then measured according to a previously described protocol (Wang *et al.* 2011). All the measurements were replicated at least three times.

For the starch measurements, five mature kernels of the wild type and *dek10* were ground in liquid N_2. The resulting powders were dried to a constant weight. Finally, the total starch was measured by using an amyloglucosidase/α-amylase starch assay kit (Megazyme). The protocol follows the method by Wang *et al.* (2014). All the measurements were replicated at least three times.

Scanning electron microscopy and transmission electron microscopy (TEM)

For scanning electron microscopy, *dek10* and wild-type kernels were prepared according to Lending and Larkins (1992): mature maize kernels were rifted with a razor at the peripheral region and placed in 2.5% glutaraldehyde. Samples were critically dried and spray-coated with gold. Gold-coated samples were then observed with a scanning electron microscope (S3400N; Hitachi).

For TEM, immature *dek10* and wild-type kernels were prepared according to Lending and Larkins (1992), with some modifications: 18 days after pollination (DAP) kernels of *dek10* and wild type were fixed in paraformaldehyde and postfixed in osmium tetraoxide. After being dehydrated in an ethanol gradient, samples were then transferred to a propylene oxide solution before being gradually embedded in acrylic resin (London Resin Company). Sections (70 nm) of samples were made with a diamond knife microtome (Reichert Ultracut E). Sample sections were stained with uranyl acetate and poststained with lead citrate. Sample sections were observed with a Hitachi H7600 transmission electron microscope.

Map-based cloning

A population of 947 homozygous mutant kernels from F2 ears was used for gene mapping. Molecular markers that are distributed throughout maize (*Z. mays*) chromosome 4 were used for preliminary mapping. Molecular markers for fine mapping (Supplemental Material, Table S2 in File S1) were developed to localize the *dek10* locus to a 225-kb region. The corresponding DNA fragments were amplified from *dek10* allele and wild-type plants using KOD Plus DNA polymerase (Toyobo) and sequenced using a MegaBACE 4500 DNA analysis system (Amersham, Piscataway, NJ).

The construction of transgene vectors and the transformation

For functional complementation, a 1416-bp coding sequence of *Dek10* was cloned into the pHB vector between *Bam*HI and *Sal*I restriction sites. A 2-kb upstream promoter sequence of *Dek10* was cloned into the pHB vector between *Eco*RI and *Bam*HI restriction sites replacing the CaMV 35S promoter. For clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene knockout transformation, designed transfer RNA–guide RNA (tRNA–gRNA) units for multiplex CRISPR/Cas9 editing were synthesized by Generey (Generey.com) and cloned into the *Psi*I and *Xba*I sites between maize the *U6* promoter and *U6* terminator (Qi *et al.* 2016b).

The construct was transferred into *Agrobacterium tumefaciens* (EHA105). *Agrobacterium*-mediated maize transformation was carried out according to known protocols (Frame *et al.* 2002). Seventeen independent transgenic lines were generated for functional complementation. Fourteen independent transgenic lines were generated for CRISPR/Cas9 gene knockout transformation. Target regions were amplified with specific primers (Table S2 in File S1) using KOD DNA polymerase (Toyobo). Selected PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI) for DNA sequencing.

RNA extraction and RT-PCR analysis

Total RNA was extracted with TRIzol reagent (Tiangen) and DNA was removed by treatment with RNase-free DNase I (Takara). Using ReverTra Ace reverse transcriptase (Toyobo), RNA was reverse transcribed to complementary DNA (cDNA). Quantitative real-time PCR (qRT-PCR) was performed with SYBR Green Real-Time PCR Master Mix (Toyobo) using a Mastercycler ep realplex 2 (Eppendorf) according to the standard protocol. Specific primers were designed (Table S2 in File S1) and the experiments were performed with two independent RNA sample sets, with ubiquitin as the reference gene. A final volume of 20 μl contained 1-μl reverse transcribed cDNA (1–100 ng), 10 μl 23 SYBR Green PCR buffer, and 1.8 μl 10 mM/L forward and reverse primers for each sample. Relative quantifiable differences in gene expression were analyzed as described previously (Livak and Schmittgen 2001).

Subcellular localization of Dek10

The full-length *Dek10* ORF without the stop codon was cloned into pB7YWG. The recombinant plasmid DNA was extracted (~1 μg total amount) and introduced into onion epidermal cells and tobacco leaf epidermal cells through transient transformation using Bio-Rad PDS-1000/HeTM biolistic particle delivery system (Bio-Rad, Hercules, CA). The fluorescence signals were detected using LSM710 (Occult International).

Isolation of mitochondria

The isolation of mitochondria was performed as described previously (de Longevialle *et al.* 2008). About 10 g immature seeds at 18 DAP were harvested and grounded with a mortar and pestle in liquid N_2, adding 20 ml extraction buffer (100 mM tricine, 300 mM sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA-K, 0.1% BSA, and 5 mM DTT, pH 7.4) and 60 μl plant protease inhibitor cocktail (Sigma [Sigma Chemical], St. Louis, MO). The samples were twice centrifuged at 2600 × *g* for 15 min, after filtration through a Miracloth membrane (Calbiochem, San Diego, CA; La Jolla, CA), retaining the supernatant, and then were centrifuged at 12,000 × *g* for 25 min to pellet crude mitochondria. The pellet was resuspended in wash buffer (100 mM tricine, 300 mM sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA-K, and 0.1% BSA, pH 7.4) and were loaded on sucrose density gradients of 1.5, 2.5, 2.5, 2, and 2 ml containing, respectively, 1.8, 1.45, 1.2, 0.9, and 0.6 M of sucrose diluted in wash buffer. After 90 min of centrifugation at 24,000 rpm at 4°, mitochondria were collected from the 1.2 M/1.45 M interface and diluted four times in wash buffer. The enriched mitochondria were collected after 20 min of centrifugation at 12,000 rpm at 4°.

Polyclonal antibodies

For anti-Dek10 antibody production, the full-length cDNA sequence of *Dek10* was inserted into pGEX-4T-1 (Amersham) at the *Eco*RI- and *Bam*HI-digested sites. The GST-tagged Dek10 fusion protein was purified and antibodies were produced in rabbits according to the standard protocols of Shanghai ImmunoGen Biological Technology.

For production of polyclonal antibody against NAD7, the first 21 aa were synthesized. Peptide synthesis, protein purification, and production of antibodies in rabbits were according to standard protocols of Shanghai ImmunoGen Biological Technology.

Immunoblot analysis

Proteins extracted from developing wild-type and *dek10* kernels were separated by SDS-PAGE. Separated protein samples were then transferred to a nitrocellulose membrane (0.45 mm; Millipore, Bedford, MA). The membrane with protein sample attached was incubated with primary and secondary antibodies. Using the Super Signal West Pico chemiluminescent substrate kit (Pierce Chemical, Rockford, IL), the signal was visualized according to the manufacturer's instructions. The antibody against Dek10 was used at 1:1000, the antibody against Nad3 (Agrisera) was used at 1:1000, the antibody against Cox2 (Agrisera) was used at 1:1000, the antibody against NAD7 was used at 1:500, the antibody against Cyt-c (Agrisera) was used at 1:5000, and the antibody against Tubulin (Sigma) was used at 1:5000.

Blue native (BN)-PAGE and complex I activity assay

The enriched mitochondria were resuspended in 50 μl B25G20 solution (25 mM Bis-Tris and 20% glycerin, pH 7.0), adding 20% n-dodecyl-β-D-maltoside (DDM) to the final concentration of 1% DDM, and were gently mixed on ice for 1 hr. After 15 min of centrifugation at 12,000 rpm at 4°, the supernatant was collected and added to the loading

buffer before BN-PAGE. The concentration of the separation gel was from 4 to 13%. Electrophoresis was first run at 50 V, adding 25 V every 20 min to a final 150 V until the loading dye migrated to the edge of the gel. The gel was stained by coomassie brilliant blue (Zhang *et al.* 2015). In-gel complex I activity assay was performed according to a previous report (Meyer *et al.* 2009).

RNA sequencing (RNA-seq) analysis

Total RNA (10 μg) was extracted from the endosperms of *dek10* and wild-type kernels harvested at 18 DAP, and three *dek10* or wild-type biological samples were pooled together. The poly(A)-selected RNA-seq library was prepared according to Illumina standard instruction (TruSeq Stranded RNA LT Guide). Library DNA was checked for concentration and size distribution in an Agilent2100 bioanalyzer before sequencing with an Illummina HiSequation 2500 system according to the manufacturer's instructions (HiSequation 2500 User Guide). Paired-end reads were aligned to the maize B73 genome build *Z. mays* AGPv2.15 using TopHat 2.0.6 (Langmead *et al.* 2009). Data were normalized as fragments per kilobase of exon per million fragments mapped (FPKM), since the sensitivity of RNA-seq depends on the transcript length. Significant differentially expressed genes (DEGs) were identified as those with a fold change and *P*-value of differential expression above the threshold (fold change > 2.0, $P < 0.05$).

Data availability

RNA-seq data are available from the National Center for Biotechnology Information Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) under the series entry GSE80091. Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *Dek10*, NM_001329449, NP_001316378; *nad3*, X14709, YP_740388; and *cox2*, AY973492, YP_740408.

Results

dek10 produces small kernels with delayed development

The *dek10-ref* (*dek10-N1176A*) mutant was obtained from the Maize Genetics Cooperation stock center. It was crossed to the W22 inbred line to produce a F2 population that displayed a 1:3 segregation of dek (*dek10/dek10*) and wild-type (+/+ and *dek10*/+) phenotypes (81:251, *P*-value < 0.05; Figure 1, A and B). Mature homozygous *dek10* kernels were small and shrunken. The 100-kernel weight of *dek10* was only ~27% of wild type (Figure 1C), but there was no significant difference in the total protein and zein content per endosperm weight (Figure S1, A and B in File S1). We also found no obvious difference in total starch content, amylase content per endosperm weight, and starch granule size in *dek10* and wild-type endosperms (Figure S1C and Figure S2 in File S1). We observed the seedlings of *dek10* and wild type at 10 and 30 days after germination (DAG). Fewer seedlings of *dek10* can be generated and the *dek10* seedlings can only grow into miniature plants (Figure 1, D and E). These results demonstrated that the growth and development of kernels and seedlings is affected in *dek10* mutants.

Wild-type and *dek10* kernels of 10, 12, 15, 18, 21, 24, and 27 DAP were analyzed by light microscopy to compare their development. Longitudinal sections of the whole kernel indicated a $>$ 10 days' developmental delay in *dek10* compared with wild type (Figure 2A). In particular, the development of the basal endosperm transfer layer (BETL) was dramatically arrested in *dek10* kernels (Figure 2B).

Positional cloning of Dek10

We performed a map-based approach to clone *Dek10*. After characterizing a population of 947 mutant kernels from the F2 population, the *dek10* gene was placed between the molecular markers InDel-AC204567.28 and InDel-GRMZM2G071304.4, which encompass a physical region of 225 kb. This region was covered by two BAC clones (AC204567 and AC186864). There are eight candidate genes within the interval (gene 1: *GRMZM2G339018*; gene 2: *GRMZM2G041310*; gene 3: *GRMZM2G041159*; gene 4: *GRMZM2G041060*; gene 5: *GRMZM2G580389*; gene 6: *GRMZM2G087226*; gene 7: *GRMZM2G088256*, and gene 8: *GRMZM2G168629*) (Figure 3A). Sequence comparison of the eight candidate genes between wild-type and mutant alleles revealed a 5-bp (CCTCC) insertion at 292 bp in gene 6 (*GRMZM2G087226*, Figure 3B). This insertion resulted in a frameshift and a premature stop codon at 370 bp in the mature transcript. There is no sequence difference in other candidate genes. Therefore, *GRMZM2G087226* appeared to be the candidate for the *Dek10*.

To confirm that *GRMZM2G087226* is the *Dek10* gene, functional complementation was performed by using a transformed wild-type candidate gene allele. Phenotyping and genotyping analysis indicated that the transgenic kernels carrying the transformed *GRMZM2G087226* sequence functionally complemented the lost function of *dek10* and rescued the mutant phenotype (Figure 3C). Targeted mutation of *GRMZM2G087226* was further created by using the multiplex CRISPR/Cas9 system (Qi *et al.* 2016b). *GRMZM2G087226* was targeted at two sites and the gRNA spacer sequences were at 327 and 553 bp of the ORF. The chromosomal fragment deletion between two gRNA spacers was detected in *GRMZM2G087226* Cas9 T0 lines (Figure 3D). The allelism test was done by crossing *dek10-ref* F1 (*dek10-ref*/+) and *dek10-Cas9* F1 (*dek10-Cas9*/+). The kernel phenotypes in the F2 ears displayed a 1:3 segregation of dek (*dek10-ref/dek10-Cas9*) and wild-type phenotype kernels (76:235, *P*-value < 0.05; Figure 3D), indicating that *dek10-Cas9* cannot complement *dek10-ref*. Therefore, *GRMZM2G087226* is indeed the *Dek10* gene.

Dek10 encodes a E-subgroup PPR protein

The genomic DNA sequence of *GRMZM2G087226* produces a transcript containing a 1422-bp coding sequence and encodes a ~52-kDa protein of 473 aa (Figure 3B). BLASTP

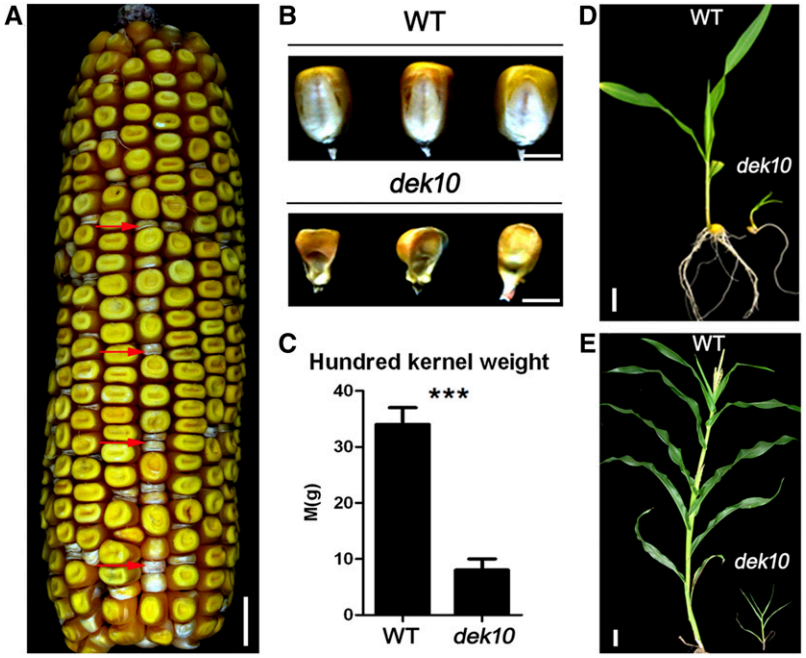

Figure 1 Phenotypic features of maize *dek10* mutant. (A) Mature F2 ear of *dek10* × W22 population, red arrow identifies the *dek10* kernel. Bar, 10 mm. (B) Randomly selected mature *dek10* and WT kernels from segregated F2 population. Bar, 5 mm. (C) Comparison of 100-grain weight of randomly selected mature *dek10* and WT kernels in segregated F2 population. Values are the mean values with SEs, $n = 3$ individuals (***$P < 0.001$, Student's *t*-test). (D and E) Phenotype of *dek10* and WT seedlings (10 DAG and 30 DAG). Bar, 5 cm. DAG, days after germination; WT, wild type.

searches of GenBank indicated that *GRMZM2G087226* encodes an E-subgroup PPR protein with the PLS motifs carrying an E-terminal domain extension (Lurin *et al.* 2004; Figure 3E and Figure S3 in File S1). The mutation in the *dek10-ref* allele results in a premature termination in the first P motif (Figure 3E). The mutant protein lost most functional domains.

To examine *Dek10* messenger RNA (mRNA) expression in *dek10*, we performed qRT-PCR with the total RNA extracted from 18 DAP to 21 DAP mutant and wild-type kernels. mRNA expression of *Dek10* was not downregulated in *dek10* mutants (Figure S4 in File S1). Using an antibody against Dek10, no band was found at the predicted size in 18 DAP *dek10* kernels by immunoblot analysis (Figure 3F), and what was detected was likely maternal contamination because of the experimental conditions of extraction.

The PPR proteins are prevalently expanded in plants (Fujii and Small 2011). We constructed a phylogenetic tree on the basis of the maize Dek10 full-length protein sequence and homologous protein sequences from other organisms (Figure 4A). The results suggest that Dek10 homologs are clearly diverged into two separated clades belonging to monocots and dicots, respectively, during the evolution. There is no paralog of Dek10 in maize, so a knockout in this single gene would result in a phenotype.

Dek10 is a ubiquitous mitochondrial protein

qRT-PCR analysis revealed that *Dek10* is expressed in a broad range of maize tissues, including tassel, ear, root, stem, leaf, and kernel (Figure 4B). Expression in the kernel is higher than in the root, stem, and leaf, but is similar to that in the ear and tassel.

PPR proteins are predominantly targeted to plastids or mitochondria (Colcombet *et al.* 2013). To determine its subcellular localization, full-length Dek10 was fused to yellow fluorescent protein (YFP) in a binary vector pB7YWG. The fusion was transiently expressed in onion (*Allium cepa*) epidermal cells by bombarding, and the fluorescent signal was detected by confocal laser microscopy. The YFP signals were detected in small dots that were identified as mitochondria by red fluorescence of Mito-Tracker pBIN20-MT-RK (Nelson *et al.* 2007; Figure 4C). The fusion was also transiently expressed in tobacco leaf epidermal cells by bombarding, and chloroplast chlorophyll autofluorescence was also detected. However, the Dek10 was not colocalized with chloroplasts (Figure 4D).

Cellular fractionation assay was then performed to confirm the Dek10 mitochondrial distribution in maize kernels. The total proteins extracted from 18 DAP wild-type kernels were separated into five sample layers in a sucrose density gradient after differential centrifugation (see *Materials and Methods*). The antibody against Nad7 was used as a mitochondrial indicator and the fourth sample layer was detected as the mitochondrial fraction. The immunoblot with Dek10-specific antibody showed that Dek10 signal was present in the mitochondrial fraction (Figure 4E). Thus, Dek10 is targeted to the mitochondria.

Dek10 is required for C-to-U RNA editing in nad3-61, nad3-62, and cox2-550 transcript in mitochondria

The E–PPR proteins were found to be involved in RNA editing (Barkan and Small 2014). RT-PCR was performed on RNAs isolated from *dek10* and wild-type kernels with the pericarp removed. Bulked sequencing and direct comparison of the mitochondrial transcripts of the *dek10* mutant were performed using 35 sets of primers (Y. Liu *et al.* 2013Y. Liu *et al.* 2013). Sequence analysis revealed three altered editing sites in the *dek10* mutant: at position 61 and 62 in the *nad3* transcript, and at position 550 in the *cox2* transcript.

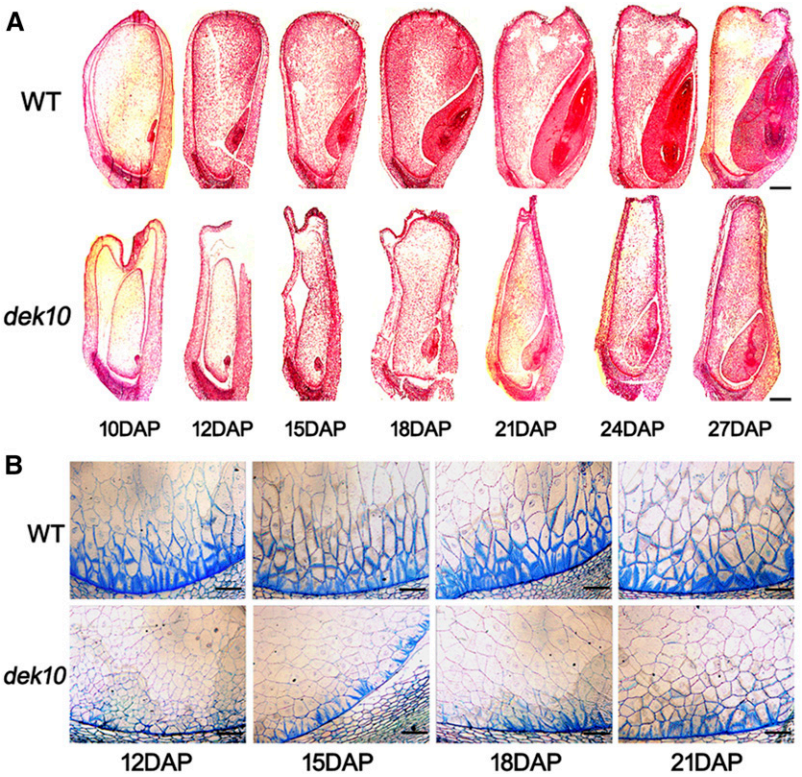

Figure 2 Developmental delay of *dek10* embryo and endosperm. (A) Paraffin sections of 10 DAP, 12 DAP, 15 DAP, 18 DAP, 21 DAP, 24 DAP, and 27 DAP *dek10* and WT kernels. Bar, 500 μm. (B) Microstructure of developing endosperm BETL of *dek10* and WT kernels (12 DAP, 15 DAP, 18 DAP, and 21 DAP). Bar, 100 μm. BETL, basal endosperm transfer layer; DAP, days after pollination; WT, wild type.

The editing defect results in a Leu-to-Pro mutation in the Nad3 protein and a Ser-to-Pro mutation in the Cox2 protein (Figure 5A). The editing deficiency of these two sites was confirmed in RNAs isolated from *dek10-Cas9* kernels (Figure 5A). The C-to-U editing at position *cox2*-550 is whole editing in wild-type kernels and was not detectable in *dek10*. However, for *nad3*-61 and *nad3*-62, a change of editing efficiency was observed. By sequencing 20 individual clones three times, we found that these two sites are edited to ~87% on average in wild-type kernels, while they are only edited to ~39% on average in *dek10* (P-value = 0.016). An alignment of the Dek10 target site flanking sequences in *nad3* and *cox2* revealed an identity of 43%, a value substantially higher than the 25% expected between unrelated sequences (Figure S5 in File S1), which was also reported by Sosso *et al.* (2012). *nad3* encodes subunit III of NADH dehydrogenase as part of complex I, and *cox2* encodes subunit II of Cox as part of complex IV, of the ETC. Editing deficiency in these sites affects highly conserved amino acids in *dek10* mutants, as leucine and serine are genomically encoded in Nad3 and Cox2 dicot orthologs (Figure 5B). Therefore, the editing of the CCA Pro codon to a UUA Leu codon and the CCC Pro codon to a UCC Ser codon is likely to be required in monocots for the maintenance of these strictly conserved amino acids.

dek10 affects Nad3 and Cox2 protein accumulation, NADH dehydrogenase activity, and Complex IV assembly

The defect in *nad3*-61, *nad3*-62, and *cox2*-550 editing might affect the level of the Nad3 and Cox2 proteins, which are essential for electron transport, as well as other components of the respiratory chain. The protein levels of Nad3 (complex I), Cox2 (complex IV), and Cyt-c were examined by immunoblot analysis of *dek10* and wild-type maize kernel proteins with specific antibodies. Nad3-specific antibody detected a protein of ~17 kDa in wild-type kernels that was significantly decreased in *dek10*. Cox2-specific antibody detected a protein of ~30 kDa in wild-type kernels, while this signal was absent in *dek10* (Figure 5C). In contrast, the level of Cyt-c was similar in the *dek10* mutant and wild-type maize kernels, indicating that other components of the ETC are not affected (Figure 5C).

To further investigate the assembly and quantity of respiratory complexes, mitochondrial proteins were isolated from *dek10* and wild-type endosperm, and were analyzed by blue native BN-PAGE. The bands of different complexes were recognized according to Zsigmond *et al.* (2008). The two profiles showed a decrease of complex IV, but no significant difference in the complex I band in the *dek10* mutant, which might be due to the small size of Nad3 and its partial decrease in *dek10*. There was a slight increase in the complex III band, which might occur to compensate for the functional defect of other complexes, as previously reported by Xiu *et al.* (2016) (Figure 5D). An in-gel NADH dehydrogenase activity test was carried out to inspect the activity of complex I. Significant reduction of complex I activity was observed in *dek10*. The activities of complex I and super-complex $I+III^2$ were much lower in the *dek10* mutant (Figure 5E). In summary, editing defects in *nad3* and *cox2* result in a reduction of Nad3 and the loss of Cox2 protein, which further causes the

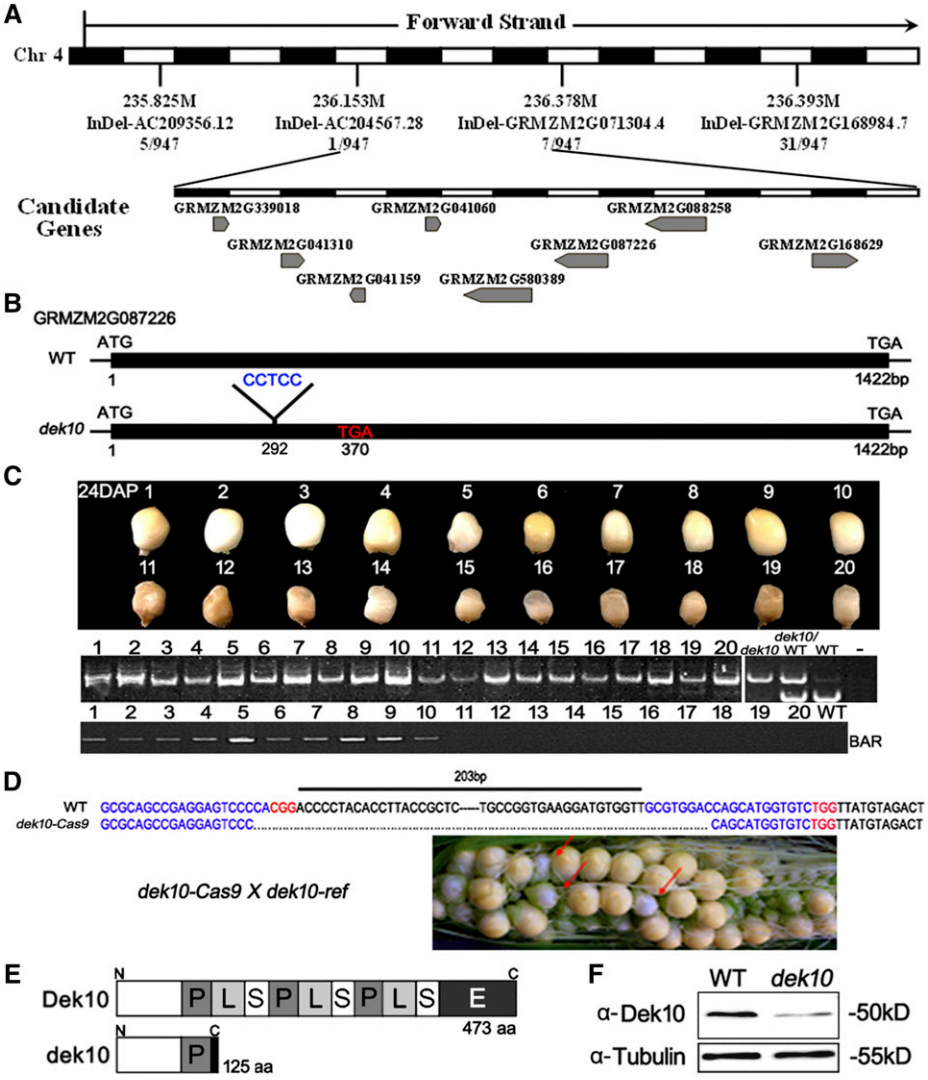

Figure 3 Map-based cloning and identification of *Dek10*. (A) The *Dek10* locus was mapped to a 225-kb region between molecular markers InDel-AC204567.28 and InDel-GRMZM2G071304.4 on chr 4, which contained eight candidate genes. See Table S2 in File S1 online for primer information. (B) Structure and mutation site of the *Dek10* gene. (C) The functional complementation transgenic lines were hybridized to the homozygous mutant (*dek10*/*dek10*) and self-pollinated (T2 lines). Using the molecular marker closely linked to the *Dek10* locus (primers AC204567.28 in Table S2 in File S1), 10 wild-type and 10 mutant kernels were sorted out from T2 ears both with mutant band patterns. The primers of the BAR gene were used to identify the transgenic positive and negative kernels. −, H_2O control. (D) The DNA sequence of the edited *Dek10* gene is provided. The 20-bp gRNA spacer sequence for the Cas9/gRNA complex is in blue, and the PAM site is in red. Deleted nucleotides are depicted as dots. Heterozygous *dek10-ref* and *dek10-Cas9* were used in an allelism test, red arrow identifies the mutant kernel. (E) Schematic diagram of the Dek10 and dek10 protein structures. (F) Western-blot analysis with antibody against Dek10 in *dek10* and WT kernel. Anti-Tubulin was used as sample loading control. aa, amino acid; BAR, Phosphinothricin (R); Chr, chromosome; DAP, days after pollination; gRNA, guideRNA; InDel, insertion/deletion; PAM, Protospacer Adjacent Motif; WT, wild type.

functional reduction of complex I and complex IV of the respiratory chain.

dek10 affects expression of genes related to mitochondrial functions

We compared the transcript profile of 18 DAP *dek10* and wild-type endosperm using RNA sequencing (RNA-seq). Among the 48,480 gene transcripts detected by RNA-seq, significantly DEGs were identified as those with a threshold fold change > 2 and P-value < 0.05. Based on this criterion, 2668 genes showed significant altered expression between *dek10* and the wild type. There were 2248 genes with increased transcription, while 420 genes showed decreased transcription. Within the DEGs, 736 genes could be functionally annotated [annotations were found using BLASTN and BLASTX analyses against the Genbank (http://www.ncbi.nlm.nih.gov/) database]. Gene Ontology (GO; http://bioinfo.cau.edu.cn/agriGO/) analysis indicated that DEGs were mostly related to seven GO terms (Figure 6A and Table S1 in File S1). Among them, three GO terms were closely related to mitochondrial function: GO: 0005740 (Mitochondrial envelope, P-value = 5.0E−18); GO: 0015078 (Hydrogen ion transmembrane transporter activity, P-value = 1.3E−13); and GO: 0015992 (Proton transport, P-value = 7.0E−06).

Forty-seven DEGs were classified to GO: 0005740 (Mitochondrial envelope), including *Alternative oxidase 2* (*Aox2*, GRMZM2G125669). The expression of *Aox2* was 399-fold upregulated in *dek10* (Table S1 in File S1), indicating that the alternative respiratory pathway was activated to compensate for the inefficient mitochondrial oxidative phosphorylation in *dek10*. Other DEGs related to mitochondrial function are Mitochondrial ATPases, ETC complex subunits, Cox subunits, and Porins. Transcription of all the genes related to mitochondrial function was increased in *dek10* endosperm (Table S1 in File S1). To validate the differences observed by RNA-seq, we performed qRT-PCR on the most significant DEGs and the results confirmed similar differences of mRNA accumulation (Figure 6B). To examine *nad3* and *cox2* mRNA expression in *dek10*, we also performed qRT-PCR with the total RNA extracted from 18 DAP to 21 DAP mutant and wild-type kernels. mRNA expression of *nad3* and *cox2* was not affected in the *dek10* mutant (Figure S6 in File S1).

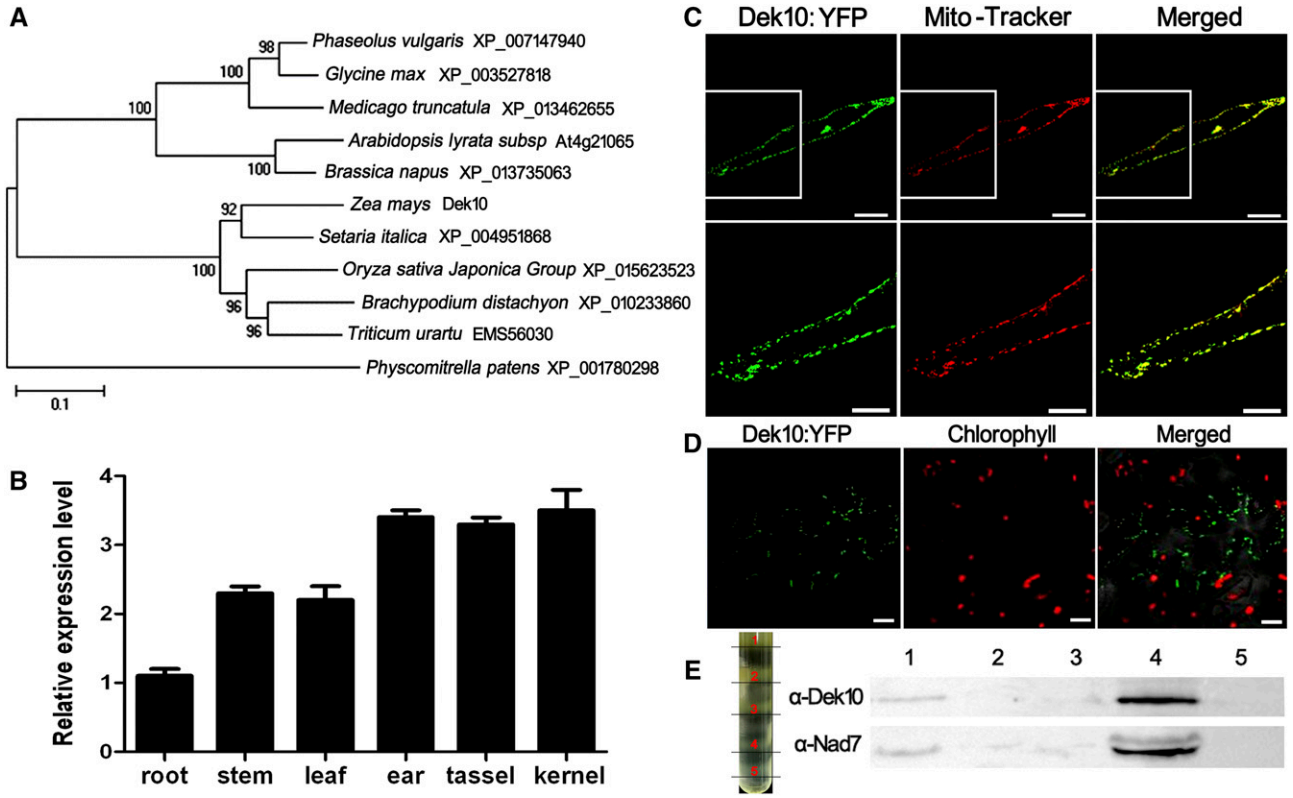

Figure 4 Phylogenetic analysis, expression pattern, and subcellular localization of Dek10. (A) Phylogenetic relationships of Dek10 and its homologs. Maize Dek10 and identified homologous proteins in *Setaria italica*, *Oryza sativa*, *Brachypodium distachyon*, *Triticum urartu*, *Arabidopsis*, *Brassica napus*, *Glycine-max*, *Phasedlus vulgaris*, *Medicago truncatula*, and *Physcomitrella patens* were aligned by MUSCLE method in the MEGA 5.2 software package. The phylogenetic tree was constructed using MEGA 5.2. The numbers at the nodes represent the percentage of 1000 bootstraps. (B) Expression profiles of *Dek10* in various tissues. Ubiquitin was used as an internal control. Representative results from two biological replicates are shown. For each RNA sample, three technical replicates were performed. Values are the mean values with SEs, $n = 6$ individuals. (C) Subcellular localization of Dek10. The Dek10 fusion protein with YFP at the C-terminus (green) and Mito-Tracker pBIN20-MT-RK (red) were transiently expressed in onion epidermal cells. Bar, 2 mm (top), Lower bar, 1 mm (bottom). (D) The Dek10 fusion protein with YFP at the C-terminus (green) was transiently expressed in tobacco leaf epidermal cells. Red spots were signals from chloroplast autofluorescence. Bar, 20 μm. (E) Cellular fractionation assay detecting localization of Dek10. Lanes 1–5 identify the five sample layers in a sucrose density gradient. Anti-Nad7 was used as mitochondrial indicator. YFP, yellow fluorescent protein.

dek10 affects mitochondria morphology

A mitochondrial function defect in the cytoplasm of 18 DAP *dek10* endosperm was also observed by TEM analysis. Normal activation of the ETC is required for the proper formation of the inner envelope cristae in mitochondria (Logan 2006). The mitochondria in the wild-type endosperm formed distinct inner envelope cristae surrounded by a dense matrix, while the internal structure of mitochondria in the *dek10* mutant lacked cristae and the mitochondrial matrix was extremely light. Furthermore, the whole structure of mitochondria in the *dek10* mutant were dilated and irregular (Figure 6C).

Discussion

Dek10 is a newly identified E-subgroup PPR protein member responsible for nad3-61, nad3-62, and cox2-550 editing

A number of maize dek mutants have been identified: *dek1* causes severe growth and development defects; *dek** causes only mild effects; and the mutants of PPRs, including *ppr2263*, *smk1*, *emp5*, *emp7*, and *dek10*, always have an obvious small kernel phenotype, with arrested development of the embryo, endosperm, and seedling (Figure 1 and Figure 2; Lid *et al.* 2002; Y. Liu *et al.* 2013; Li *et al.* 2014; Sun *et al.* 2015; Qi *et al.* 2016a). *dek10-ref* and *dek10-Cas9* are both severe mutant alleles with abolished function of Dek10, which is a newly identified E-subgroup PPR protein involved in mitochondrial RNA editing (Figure 3, Figure 4, and Figure 5).

To date, at least 46 RNA editing factors in plant mitochondria have been characterized (Hammani and Giege 2014; Li *et al.* 2014; Sun *et al.* 2015). These editing factors mostly belong to the E and DYW subgroups and function either at single or multiple editing sites. For DYW-subgroup PPR proteins, MEF1 is the first identified plant mitochondrial editing factor involved in multiple editing sites (Zehrmann *et al.* 2009). Rice OGR1 is involved in seven specific editing sites on five distinct mitochondrial transcripts (Kim *et al.* 2009). Moss (*Physcomitrella*) PPR_77 is involved in the editing

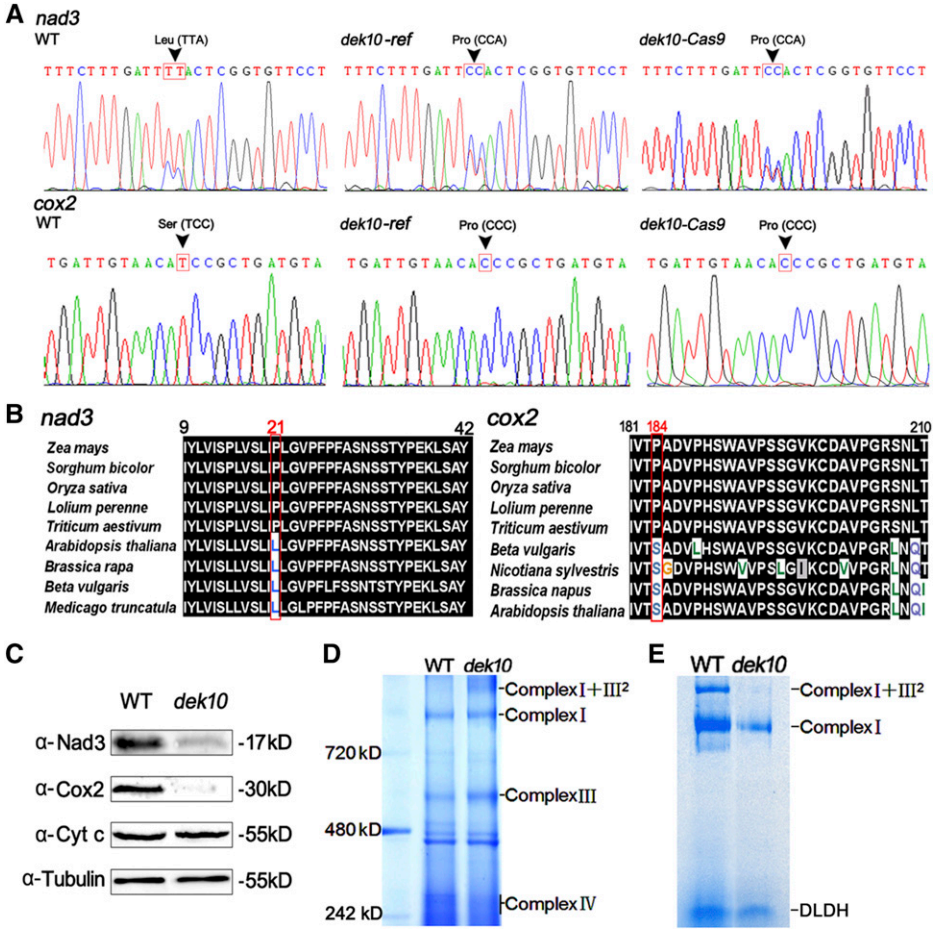

Figure 5 Dek10 is required for *nad3*-61, *nad3*-62, and *cox2*-550 editing in maize mitochondria. (A) Analysis of RNA editing at the *nad3*-61, *nad3*-62, and *cox2*-550 sites in the transcripts from developing kernels of the WT, *dek10-ref*, and *dek0-Cas9* mutants at 18 DAP. The arrow marks the editing site. (B) Alignment of the Nad3 sequences around amino acid 21 and alignment of the Cox2 sequences around amino acid 184. The protein sequences are derived from *Z. mays*, *Sorghum bicolor*, *O. sativa*, *Lolium perenne*, *T. aestivum*, *Beta vulgaris*, *Nicotiana sylvestris*, *B. napus*, *M. truncatula*, and *Arabidopsis thaliana*. Numbers indicate amino acid positions in the protein. The red box indicates the position affected by the *nad3*-61, *nad3*-62, and *cox2*-550 editing. (C) Western blot analysis with antibodies against Nad3, Cox2, and Cyt-c. Anti-Tub was used as sample loading control. (D) BN-PAGE of mitochondrial complexes. The positions of super complex I+III^2, complex I, complex III, and complex IV are indicated. (E) In-gel NADH dehydrogenase activity test analysis of complex I activity. The positions of super complex I+III^2 and complex I are indicated. The activity of the dehydrolipoamide dehydrogenase was used as a sample loading control. BN-PAGE, blue native polyacrylamide gel electrophoresis; Cox, Cyt-c oxidase; Cyt-c, cytochrome c; DAP, days after pollination; WT, wild type.

of *cox2-370* and *cox3-733* transcripts (Ohtani *et al.* 2010). Maize *PPR2263* also encodes a DYW-subgroup PPR protein responsible for multiple editing sites (Sosso *et al.* 2012). For E-subgroup PPR proteins, MEF9, MPR25, SLG1, AHG11, SMK1, and EMP7 are editing factors required for single editing sites in complex I (Takenaka 2010; Murayama *et al.* 2012; Toda *et al.* 2012; Yuan and Liu 2012; Li *et al.* 2014; Sun *et al.* 2015). *OTP87* encodes an E-subgroup PPR protein required for the editing of *nad7-24* and *atp1-1178* (Hammani *et al.* 2011). SLO2 affects several editing sites in *Arabidopsis* (Zhu *et al.* 2012). These previously reported editing defects often lead to a compromised or complete loss-of-function of the encoded protein, and affect plant growth and development (Sosso *et al.* 2012; Y. Liu *et al.* 2013; Li *et al.* 2014).

Dek10 is identified to be another E-subgroup PPR protein targeted to the mitochondria in maize. There is no paralog of Dek10 in maize (Figure 4). This newly characterized maize PPR is responsible for C-to-U RNA editing at *nad3*-61, *nad3*-62, and *cox2*-550 transcripts (Figure 5). Editing at *cox2*-550 is completely abolished while there is only editing efficiency reduction at *nad3*-61 and *nad3*-62, indicating that the editing at these two sites might also involve other proteins. Several PPR proteins have previously been reported to be mitochondrial RNA editing factors involved in multiple editing events (Kim *et al.* 2009; Zehrmann *et al.* 2009; Ohtani *et al.* 2010; Hammani *et al.* 2011; Sosso *et al.* 2012; Zhu *et al.* 2012). The corresponding mutants exhibit both whole editing abolishment and editing efficiency reduction (Sosso *et al.* 2012; Zhu *et al.* 2012). Among them, SLO2 is required for two contiguous editing sites, *mttB*-144 and *mttB*-145 (Zhu *et al.* 2012). Dek10 is a newly identified PPR protein responsible for the editing of two contiguous editing sites in the same transcript.

Dek10 editing sites are critical for mitochondrial function

Respiration, as the core process of mitochondrial metabolism, depends on the function of five complexes on the substrate (Dudkina *et al.* 2006). *nad3* encodes subunit III of NADH dehydrogenase as part of complex I. *cox2* encodes subunit II of Cox as part of complex IV. *nad3*-61, *nad3*-62, and *cox2*-550 editing are required for the maintenance of strictly conserved amino acids in Nad3 and Cox2 orthologs present in monocots. Comparison of the alignment of the amino acid encoded by unedited *nad3* and *cox2* revealed that Pro encoded by unedited *nad3*-61, *nad3*-62, and *cox2*-550 is clustered in all monocots investigated, indicating a need for RNA editing at these sites (Figure 5), which was also observed for *nad7-836* by Li *et al.* (2014). Dek10 is also

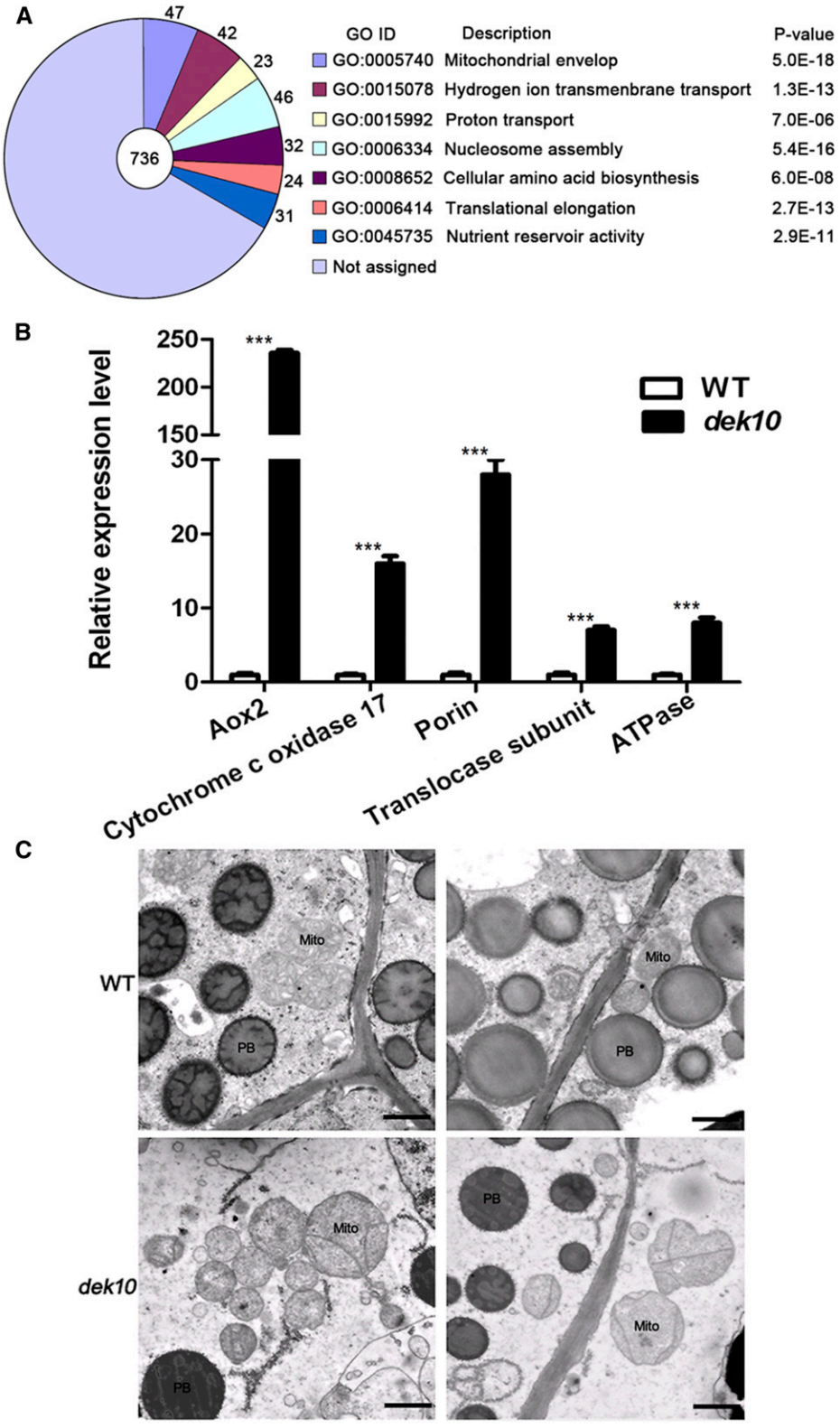

Figure 6 Disrupted mitochondrial function in *dek10* kernels. (A) The most significantly-related GO terms of the 736 functional annotated DEGs. The significance and number of genes classified within each GO term is shown. (B) qRT-PCR confirmation of DEGs associated with mitochondrial function, including GRMZM2G125669 (Aox2), GRMZM2G010933 (Cytochrome c oxidase 17), GRMZM2G115049 (Porin), GRMZM2G064600 (Mitochondrial inner membrane translocase subunit), and GRMZM2G069229 (ATPase). Ubiquitin was used as an internal control. Values are the mean values with SEs, $n = 6$ individuals (***$P < 0.001$, Student's *t*-test). (C) Ultrastructure of developing endosperms of WT and *dek10* (18 DAP) for mitochondria observation. Bar, 1 μm. DAP, days after pollination; DEGs, differentially expressed genes; GO, gene ontology; ID, identifier; Mito, mitochondrion; PB, protein body; qRT-PCR; quantitative real-time PCR WT, wild type.

highly conserved in monocots, implying a coordination of *Dek*10 with *nad3* and *cox*2 during evolution (Figure 4 and Figure 5). The selective pressure of Dek10 in monocots might be due to the need for RNA editing at *nad3*-61, *nad3*-62, and *cox2-550* in monocots.

Most of the characterized PPR mutants affect transcripts encoding proteins of complex I (Colas des Francs-Small and Small 2014; Hammani and Giege 2014). *Arabidopsis slg1* mutants with *nad3* transcript editing defects showed severe developmental delay and hypersensitivity to abiotic stresses

(Yuan and Liu 2012). Editing defects of subunits of complex III, complex IV, and Complex V have also been reported and the loss of these complexes is always lethal (Colas des Francs-Small and Small 2014). MEF32 was predicted to be required for multiple editing sites including *cox2*-27 (Takenaka *et al.* 2013). Abolished *cox2-370* and *cox3-733* transcript editing in a Moss *ppr_77* mutant resulted in severe developmental defects (Ohtani *et al.* 2010). *Arabidopsis cod1* mutants were defective in *cox2-253*, *cox2-698*, and *nad4-1129* editing leading to seed abortion (Dahan *et al.* 2014). Affecting *nad3*-61, *nad3*-62, and *cox2*-550 editing results in a significant decrease or complete loss of the encoded proteins in *dek10* (Figure 5). The discrepancy between transcription levels and protein accumulation levels of *nad3* and *cox2* in *dek10* (Figure 5 and Figure S6 in File S1) implies that the functional defect of these proteins affects their assembly into complexes and, thus, further affects protein stability. Respiratory metabolism was blocked in *dek10*, as *Aox2* and other mitochondrial function-related genes were dramatically upregulated to rescue the electron flux and functional tricarboxylic acid cycle (Figure 6 and Table S1 in File S1). AOXs can reduce the reactive oxygen species levels in situations when complexes III and IV are unable to function properly for the maintenance of electron flux. There is also rapid activation of AOXs in previously reported PPR mutants (Vanlerberghe and McIntosh 1994; Sun *et al.* 2015; Xiu *et al.* 2016).

ETC biogenesis was reported to be required for the proper morphology of the cristae in mitochondria (Logan 2006). Reduced complex I in the *Arabidopsis nmat1* mutant and reduced complex III in the maize *ppr2263* mutant caused compromised mitochondrial ultrastructure (Keren *et al.* 2012; Sosso *et al.* 2012). The cristae formed by the inner membrane are strongly reduced or completely missing in the maize *ppr2263* mutant (Sosso *et al.* 2012). Abnormal morphology of mitochondria was also observed in the *dek10* mutant. The loss of both Nad3 (complex I) and Cox2 (complex IV) function results in defects of ETC biogenesis, which not only affects the respiratory metabolism of mitochondria but also their proper morphology. Sosso *et al.* (2012) hypothesized that the structurally altered mitochondria were likely nonfunctional or at least less functional than mitochondria with a normal ultrastructure.

The mitochondrial function defect in the dek10 mutant affects kernel and seedling development

The development of the BETL was dramatically arrested in *dek10* endosperm (Figure 2). The BETL is the basal endosperm layer that develops extensive cell wall ingrowths supporting an enlarged plasma membrane surface, which promotes nutrient (primarily sucrose and amino acids) uptake by the endosperm (Pate and Gunning 1972; Thompson *et al.* 2001). BETL cells allow rapid solute transport at the interface between maternal vascular tissue and the endosperm (Offler *et al.* 2003), which requires high metabolic rates. Therefore, transfer cells typically have a dense cytoplasm that is rich in small, spherical mitochondria. The absence of a properly formed transfer cell layer is correlated with reduced rates of grain filling and seed abortion (Brink and Cooper 1947; Charlton *et al.* 1995). Mutation of the maize *EMP4* and *EMP16* genes, which encode PPR proteins, results in a defective transfer cell layer and endosperm (Gutierrez *et al.* 2007; Xiu *et al.* 2016).

Embryo lethality, reduced fertility, and dwarf phenotype are associated with several PPR mutants, highlighting the important functions of PPRs in plant growth and development (Lurin *et al.* 2004; Gutierrez *et al.* 2007). Because the *Dek10* gene is constitutively expressed in maize (Figure 4), the abolished growth of *dek10* plants can be attributed to the mitochondrial function defect in other tissues. The suppressed mitochondrial function in *dek10* also brings about changes in other important biological processes, including nucleosome assembly, cellular amino acid biosynthesis, translation elongation, and nutrient reservoir activity (Figure 6 and Table S1 in File S1). Nucleosome assembly is essential for a variety of biological processes, such as cell cycle progression, development, and senescence (Gal *et al.* 2015). Amino acid biosynthesis and translation elongation are both important for cellular protein expression and accumulation. Nutrient reservoir activity in the endosperm, the main storage tissue, largely determines the nutritional value of maize (Holding and Larkins 2006). All these biological processes consume energy produced by the mitochondria. Hence, maize *dek10* mutation affects kernel and seedling development due to mitochondrial dysfunction and other secondary biological defects.

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Author contributions: R.S. and W.Q. designed the experiment. W.Q., Z.T., L.L., Xiu.C., Xin.C., and W.Z. performed the experiments. W.Q., Z.T., L.L., and R.S. analyzed the data. W.Q. and R.S. wrote the article.

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Editing of Mitochondrial Transcripts *nad3* and *cox2* by Dek10 Is Essential for Mitochondrial Function and Maize Plant Development

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