Title: Editing of mitochondrial transcripts *nad3* and *cox2* by Dek10 is essential for mitochondrial function and maize plant development

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Editing of mitochondrial transcripts *nad3* and *cox2* by Dek10 is essential for mitochondrial function and maize plant development

**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, those 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*, that is essential for mitochondrial functions and plant development in maize.
Introduction

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 releasing and ATP production. During respiration, electrons from the NADPH and FADH2 are transferred to O2 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 reductase) to cytochrome c and to Complex IV (cytochrome 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 cytochrome 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 respiratory chain. The primary mitochondrial genome expressed pre-RNAs could be post-transcriptional 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-amino-acid motif. PPR proteins are classified into two major subgroups: the P-type PPR members only harbor tandem repeats of the canonical 35-amino-acid PPR motif, and the PLS-type PPR members are 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 more than 450 members in plants, and acts specifically in mitochondria or plastids for RNA editing, cleavage, splicing, stability, as well as translational initiation and regulation (Schmitz-Linneweber and Small, 2008; Fujii and Small, 2011; Liu et al., 2013a; 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 development defects associated with loss-of-function PPR mutants were described before (Fujii and Small, 2011; Sosso et al., 2012; Liu et al., 2013b; Colas des Francs-Small and Small, 2014; Hammani and Giege, 2014; Li et al., 2014; Sun et al., 2015; Haili et al., 2015). However, the molecular roles and regulatory functions are still unknown for a great number of PPR proteins.

Maize (Zea mays) is suitable material 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 is good resource to investigate seed development (Neuffer and Sheridan, 1980). Dek1 encodes a calpain family protein affecting embryo and
endosperm aleurone layer development (Lid et al., 2002; Becraft et al., 2002). *Dek* is a partial functional allele for *Dek10* and 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 was 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 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. Defect of these editing reduces the function of Complex I and Complex IV in 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 field at the Shanghai University.
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/\(\alpha\)-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

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 transmission electron microscopy, immature kernels of *dek10* and wild type 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 post-fixed in osmium tetroxide. After being dehydrated in an ethanol gradient, samples were then transferred to a propylene oxide solution along with 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 post-stained 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 (*Zea mays*) chromosome 4 were used for preliminary mapping. Molecular markers for fine mapping (Table S2) 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 Biosciences).

**The Construction of Transgene Vectors and the Transformation**

For functional complementation, 1416 bp coding sequence of *Dek10* was cloned into pHB vector between *Bam*HII and *Sal*I restriction sites. 2 kb upstream promoter sequence of *Dek10* was cloned into pHB vector between *Eco*RI and *Bam*HII restriction sites replacing the CaMV 35S promoter. For CRISPR/Cas9 gene knock-out transformation, designed tRNA-gRNA units for multiplex CRISPR/Cas9 editing were synthesized by Generey (Generey.com)
and cloned into the *Psil* and *Xbal* sites between maize *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 knock-out transformation. Target regions were amplified with specific primers (Table S2) using KOD DNA polymerase (Toyobo). Selected PCR products were cloned into pGEM-T Easy Vector (Promega) for DNA sequencing.

**RNA Extraction and RT-PCR Analysis**

Total RNA was extracted with TRIzol reagent (Tiangen) and DNA was removed by a treatment with RNase free DNase I (Takara). Using ReverTra Ace reverse transcriptase (Toyobo) RNA was reverse transcribed to cDNA. Quantitative real-time 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) and the experiments were performed with 2 independent RNA samples sets with ubiquitin as the reference gene. A final volume of 20 μL contained 1 μL reverse transcribed cDNA (1 to 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 was 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 (~1μg 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. The fluorescence signals were detected using LSM710 (Occult International Ltd.).

Isolation of mitochondria

The isolation of mitochondria was performed as described previously (De Longevialle et al., 2008). About 10g immature seeds at 18 DAP were harvested and were grinded with a mortar and pestle in liquid nitrogen, adding 20ml extraction buffer (100mM tricine, 300mM sucrose, 10mM KCl, 1mM MgCl2, 1mM EDTA-K, 0.1% BSA, 5mM DTT, pH7.4) and 60μL plant protease Inhibitor cocktail (Sigma-Aldrich). The samples were twice centrifuged at 2,600g for 15min, after filtration through a Miracloth membrane (Calbiochem), retaining the supernatant, and then were centrifuged at 12,000g for 25min to pellet crude mitochondria. The pellet was resuspended in wash buffer (100mM tricine, 300mM sucrose, 10mM KCl, 1mM MgCl2, 1mM EDTA-K, 0.1% BSA, pH7.4) and were loaded on sucrose density gradients of 1.5mL, 2.5mL, 2.5mL, 2mL and 2mL containing, respectively, 1.8M, 1.45M, 1.2M, 0.9M and 0.6M of sucrose diluted in wash buffer. After 90min of centrifugation at 24,000rpm at 4°C, mitochondria were collected from the 1.2M/1.45M interface and diluted 4 times in wash buffer. The enriched mitochondria were collected after 20min of centrifugation at 12,000 rpm at 4°C.
Polyclonal antibodies

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

For production of polyclonal antibody against NAD7, the first 21 amino acids were synthesized. Peptide synthesis, protein purification and producing 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 nitrocellulose membrane (0.45 mm; Millipore). The membrane with protein sample attached was incubated with primary and secondary antibodies. Using the Super Signal West Pico chemiluminescent substrate kit (Pierce), 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-Aldrich) was used at 1:5000.
**BN-PAGE and Complex I activity assay**

The enriched mitochondria were resuspended in 50μL B25G20 solution (25mM Bis-Tris, 20% glycerin, pH7.0), adding 20% n-dodecyl-β-D-maltoside (DDM) to the final concentration of 1% DDM, and were gently mixed on ice for 1h. After 15min of centrifugation at 12000rpm at 4°C, the supernatant was collected, and was added to the loading buffer before blue native polyacrylamide electrophoresis. The concentration of separation gel was from 4% to 13%. Electrophoresis was first run at 50V adding 25V every 20min to a final 150V till 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 basically according to a previous report (Meyer et al., 2009).

**RNA-seq Analysis**

Total RNA (10 μg) was extracted from endosperm 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 Illumina HiSeq 2500 system according to the manufacturer's instructions (HiSeq 2500 User Guide). Paired-end reads were aligned to the maize B73 genome build Zea 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 is available from the National Center for Biotechnology Information Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) under the series entry GSE80091.

Accession numbers
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; 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/dek10*) phenotypes (81:251, p-value<0.05, Figure 1A&B). Mature homozygous *dek10* kernels were small and shrunken. The 100-kernel weight of *dek10* was only about 27% of wild type (Figure 1C), but there was no significant difference in the total protein and zein content per endosperm weight (Figure S1A&B). We also found no obvious difference in total starch
content, amylase content per endosperm weight, and starch granule size in \textit{dek10} and wild type endosperms (Figure S1C&S2). We observed the seedlings of \textit{dek10} and wild type at 10 and 30 days after germination (DAG). Fewer seedlings of \textit{dek10} can be generated and the \textit{dek10} seedlings can only grow into miniature plants (Figure 1D&E). These results demonstrated that the growth and development of kernels and seedlings is affected in \textit{dek10} mutant.

\textbf{Figure 1.} Phenotypic features of maize \textit{dek10} mutant.

\begin{itemize}
  \item A. Mature F2 ear of \textit{dek10}×\textit{W22} population, red arrow identifies the \textit{dek10} kernel, Bar = 10 mm.
  \item B. Randomly selected mature \textit{dek10} and WT kernels from segregated F2 population, Bar = 5 mm.
  \item C. Comparison of 100-grain weight of randomly selected mature \textit{dek10} and WT kernels in segregated F2 population. Values are the mean values with standard errors, \( n = 3 \) individuals (***\( P < 0.001 \), Student’s t-test).
  \item D&E. Phenotype of \textit{dek10} and WT seedlings (10 DAG and 30 DAG). Bar = 5 cm.
\end{itemize}
Wild type and *dek10* kernels of 10 DAP, 12 DAP, 15 DAP, 18 DAP, 21 DAP, 24 DAP and 27 DAP were analyzed by light microscopy to compare their development. Longitudinal sections of the whole kernel indicated a more than ten days’ developmental delay in *dek10* compared to wild type (Figure 2A). Especially the development of basal endosperm transfer layer (BETL) was dramatically arrested in *dek10* kernels (Figure 2B).

**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. Bars = 500 μm.

B. Microstructure of developing endosperm BETL of *dek10* and WT kernels (12 DAP, 15 DAP, 18 DAP, and 21 DAP). Bars = 100 μm.

**Positional cloning of Dek10**
We performed 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 encompasses a physical region of 225 kb. This region was covered by 2 BAC clones (AC204567 and AC186864). There are eight candidate genes within the interval (Gene1: GRMZM2G339018, Gene2: GRMZM2G041310, Gene3: GRMZM2G041159, Gene4: GRMZM2G041060, Gene5: GRMZM2G580389, Gene6: GRMZM2G087226, Gene7: GRMZM2G088256 and Gene8: 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 Gene6 (GRMZM2G087226, Figure 3B). This insertion resulted in a frame-shift 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 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 were targeted at two sites and the gRNA spacer sequences were at the 327bp and 553bp of ORF. The chromosomal-fragment deletion between two gRNA spacers was detected in GRMZM2G087226 Cas9 T0 lines (Figure 3D). The
An 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* can’t complement *dek10-ref*. Therefore, *GRMZM2G087226* is indeed the Dek10 gene.

**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 chromosome 4, which contained eight candidate genes. See Supplemental Table 2 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 \( \text{dek10/dek10} \) and self-pollinated (T2 lines). Using the molecular marker closely linked to the \( \text{Dek10} \) locus (primers AC204567.28 in Supplemental table S2), ten wild type and ten mutant kernels were sorted out from T2 ears both with mutant band pattern. And the primers of BAR gene were used to identify the transgenic positive and negative kernels. -, \( \text{H}_2\text{O} \) control.

D. The DNA sequence of the edited \( \text{Dek10} \) 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. And heterozygous \( \text{dek10-ref} \) and \( \text{dek10-Cas9} \) were used in allelism test, red arrow identifies the mutant kernel.

E. Schematic diagram of the Dek10 and \( \text{dek10} \) protein structures. aa, amino acid.

F. Western-blot analysis with antibody against Dek10 in \( \text{dek10} \) and WT kernel. Anti-Tub was used as sample loading control.

**Dek10 encodes a E-subgroup PPR protein**

The genomic DNA sequence of \( \text{GRMZM2G087226} \) produces a transcript containing a 1,422 bp coding sequence and encodes a ~52 kD protein of 473 amino acids (Figure 3B). BLASTP searches of Genbank indicated that \( \text{GRMZM2G087226} \) encodes an E-subgroup PPR protein with the PLS (repeat P–L–S) motifs carrying an E–terminal domain extension (Lurin et al., 2004; Figure 3E & Figure S3). The mutation in the \( \text{dek10-ref} \) allele results in a premature termination in the first P motif (Figure 3E). The mutant protein lost most functional domains.

To examine \( \text{Dek10} \) mRNA expression in \( \text{dek10} \), we performed quantitative RT-PCR (qRT-PCR) with the total RNA extracted from 18 DAP and 21 DAP mutant and wild type kernels. mRNA expression of \( \text{Dek10} \) was not down-regulated in \( \text{dek10} \) mutant (Figure S4). Using antibody against Dek10,
no band was found at the predicted size in 18 DAP dek10 kernels by immunoblot analysis (Figure 3F) and what 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 homologue 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 that a knockout in this single gene would result in a phenotype.

Figure 4

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 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 standard errors, n= 6 individuals.

C. Subcellular localization of Dek10. The Dek10 fusion protein with YFP at 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 C-terminus (green) was transiently expressed in tobacco leaf epidermal cells. Red spots were signals from chloroplast autofluorescence. Bar=20μm.

E. Cellular fractionation assay detecting localization of Dek10. 1-5 identifies the five sample layers in sucrose density gradient. Anti-Nad7 was used as mitochondrial indicator.

**Dek10 is a ubiquitous mitochondrial protein**

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

PPR proteins are predominantly targeted to plastids or mitochondria (Colcombet et al., 2013). To determine the subcellular localization, full-length Dek10 was fused to 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. But the Dek10 was not co-localized with chloroplast (Figure 4D).

Cellular fractionation assay was then performed to confirm the Dek10 mitochondrial distribution in maize kernel. The total proteins extracted from 18 DAP wild type kernels were separated into five sample layers in sucrose density gradient after differential centrifugation (see Methods). The antibody against Nad7 was used as mitochondrial indicator and the forth sample layer was detected as 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 at 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 dek10 mutant were performed using 35 sets of primers (Liu et al., 2013b). 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.

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 and was not detectable in dek10. But for nad3-61 and nad3-62, it was change of editing efficiency. By sequencing of twenty individual clones three times, we found that these two sites are edited to about 87% in average in wild type, while they are only edited to about 39% in 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), 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 cytochrome c oxidase as part of Complex IV of 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 dicots 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 are likely to be required in monocots for the maintenance of these strictly conserved amino acids.
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 kernel of the WT and dek10-ref, 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 zea mays, Sorghum bicolor, Oryza sativa, Lolium perenne, Triticum aestivum, Beta vulgaris, Nicotiana sylvestris, Brassica napus, Medicago 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 and Complex I are indicated. The activity of the dehydrogenase was used as a sample loading control.

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 protein that 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 protein with specific antibodies. Nad3-specific antibody detected a protein of ~17 kD in wild type, which was significantly decreased in dek10. Cox2-specific antibody detected a protein of ~30 kD in wild type, 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 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 decrease of Complex IV region but no significant difference in Complex I band in dek10 mutant, which might due to the small size of Nad3 and its partial decrease in dek10. There was slight increase of Complex III band, which might occur to compensate the functional defect of other complexes, as previously reported by Xiu et al (2016) (Figure 5D). In-gel NADH dehydrogenase activity
test was further carried out to inspect the activity of Complex I. Significant reduction of Complex I activity was observed in *dek10*. The activity of Complex I and super-Complex I+III$^2$ were much lower in the *dek10* mutant (Figure 5E). In summary, editing defect in *nad3* and *cox2* results in reduction of Nad3 and loss of Cox2 protein, which further causes the 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 differentially expressed genes (DEGs) were identified as those with a threshold fold change $>$2 and p-value $<$0.05. Based on this criterion, 2,668 genes showed significant altered expression between *dek10* and the wild type. There were 2,248 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). Among them, three GO terms were closely related with 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 up-regulated in *dek10* (Table S1), indicating the alternative respiratory pathway was activated to compensate for the inefficient mitochondrial oxidative phosphorylation in *dek10*. Other DEGs related with mitochondrial function are Mitochondrial ATPases, ETC complex subunits, Cytochrome c oxidase subunits, and Porins. Transcription of all the genes related to mitochondrial function was increased in *dek10* endosperm (Table 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 and 21 DAP mutant and wild type kernels. mRNA expression of *nad3* and *cox2* was not affected in *dek10* mutant (Figure S6).
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 standard errors, n= 6 individuals (***P<0.001, Student’s t-test).

C. Ultrastructure of developing endosperms of WT and *dek10* (18 DAP) for mitochondria observation. Bars = 1 μm. PB, protein body; Mito, mitochondrion.
**dek10 affects mitochondria morphology**

A mitochondrial function defect in the cytoplasm of 18 DAP dek10 endosperm was also observed by Transmission Electron Microscopy (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 with dense matrix, while the internal structure of mitochondria in dek10 mutant lacked cristae and the mitochondria matrix was extremely light. Furthermore, the whole structure of mitochondria in dek10 mutant were dilated and irregular (Figure 6C).

**Discussion**

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

A number of maize dek mutants have been identified: \textit{dek1} causes severe growth and development defects; \textit{dek*} causes only mild effects; and the mutants of PPRs, including \textit{ppr2263}, \textit{smk1}, \textit{emp5}, \textit{emp7}, and \textit{dek10}, always have an obvious small kernel phenotype, with arrested development of embryo, endosperm and seedling (Figure 1&2; Lid et al., 2002; Liu et al., 2013b; Li et al., 2014; Sun et al., 2015; Qi et al., 2016a). \textit{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 3-5).
To date, at least 46 RNA editing factors in the 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 of cox2-370 and cox3-733 transcripts (Ohtani et al., 2010). Maize PPR2263 is also encoding 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; Toda et al., 2012; Yuan and Liu, 2012; Murayama et al., 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., 2011a). 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 affects plant growth and development (Sosso et al., 2012; Liu et al., 2013b; Li et al., 2014).

Dek10 is identified to be another E-subgroup PPR protein targeted to the mitochondrion 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 transcript (Figure 5). Editing at cox2-550 is completely abolished while there is only editing efficiency reduction at nad3-61 and nad3-62, indicating the editing at these two sites might also involve other
proteins. There were several reported PPR proteins as mitochondrial RNA editing factor involved in multiple editing events (Zehrmann et al., 2009; Kim et al., 2009; Ohtani et al., 2010; Hammani et al., 2011a; 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 cytochrome c oxidase 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 the unedited *nad3* and *cox2* revealed that Pro encoded by the 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 highly conserved in monocots, implying a coordination of Dek10 with *nad3* and *cox2* during evolution (Figure 4&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 mutant with nad3 transcript editing defect 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 were also somehow 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 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). Affected nad3-61, nad3-62, and cox2-550 editing results in significant decrease or complete loss of the encoded proteins in dek10 (Figures 5). The discrepancy between transcription levels and protein accumulation levels of nad3 and cox2 in dek10 (Figure 5 & Figure S6) implies that the functional defect of these proteins is affecting their assembling into complexes and further affecting protein stability. Respiratory metabolism was blocked in dek10 as Aox2 and other mitochondrial function related genes were dramatically up-regulated to rescue the electron flux and functional tricarboxylic acid cycle (Figure 6&Table S1). AOXs can reduce the reactive oxygen species (ROS) levels in situations when Complexes III and IV are unable to function properly for the maintenance of electron flux (Plaxton and Podesta’, 2006). 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 defect of ETC biogenesis, which is affecting not only 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 dek10 mutant affects kernel and seedling development

The development of BETL was dramatically arrested in dek10 endosperm (Figure 2). BETL is the basal endosperm layer that develops extensive cell wall ingrowths supporting an enlarged plasma membrane surface that promotes nutrient (primarily Suc 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 gene and EMP16 gene encoding PPR proteins results in a defective transfer cell layer and endosperm (Gutierrez-Marcos et al., 2007; Xiu et al., 2016).

Embryo lethality, reduced fertility, and dwarf phenotype are associated with several PPR mutants highlighting important functions of PPRs in plant growth and development (Lurin et al., 2004; Gutierrez-Marcos et al., 2007). Because Dek10 gene is constitutively expressed in maize (Figure 4), the abolished growth of dek10 plant 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&Table 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 endosperm, the main storage tissue, largely determines the nutritional value of maize (Holding and Larkins, 2006). All these biological processes are consuming energy produced by mitochondria. Hence, maize dek10 mutant affects kernel and seedling development due to mitochondrial dysfunction and other secondary biological defects.

Supplemental Data

Supplemental Figure S1. Biochemical analysis of WT and dek10 endosperm.
Supplemental Figure S2. Scanning electron microscopy analysis of the peripheral regions of mature WT and dek10 endosperm.

Supplemental Figure S3. Alignment of Dek10 full length protein sequence and homologue protein sequences from Setaria italica and Oryza sativa.

Supplemental Figure S4. qRT-PCR comparing expression level of Dek10 gene in the 18 DAP and 21 DAP dek10 and WT kernels.

Supplemental Figure S5. Alignment of the Dek10 target sequences in nad3 and cox2.

Supplemental Figure S6. qRT-PCR comparing expression level of nad3 and cox2 gene in the 18 DAP and 21 DAP dek10 and WT kernels.

Supplemental Table S1. Gene ontology classifications of DEGs with functional annotation.

Supplemental Table S2. Primers used in this work.

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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Conflict of interest

There is no conflict of interest.
References


Dudkina, N.V., Heinemeyer, J., Sunderhaus, S., Boekema, E.J., and Braun, H.P.


Figure legends

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 standard errors, *n* = 3 individuals (***P*<0.001, Student’s t-test).

D&E. Phenotype of *dek10* and WT seedlings (10 DAG and 30 DAG). Bar = 5 cm.

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. Bars = 500 μm.

B. Microstructure of developing endosperm BETL of *dek10* and WT kernels (12 DAP, 15 DAP, 18 DAP, and 21 DAP). Bars = 100 μm.

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 chromosome 4, which contained eight candidate genes. See Supplemental Table 2 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 Supplemental table S2), ten wild type and ten mutant kernels were sorted out from T2 ears both with mutant band pattern. And the primers of BAR gene were used to identify the transgenic positive and negative kernels. -, H₂O 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. And heterozygous dek10-ref and dek10-Cas9 were used in allelism test, red arrow identifies the mutant kernel.

E. Schematic diagram of the Dek10 and dek10 protein structures. aa, amino acid.

F. Western-blot analysis with antibody against Dek10 in dek10 and WT kernel. Anti-Tub was used as sample loading control.

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 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 standard errors, \( n = 6 \) individuals.

C. Subcellular localization of Dek10. The Dek10 fusion protein with YFP at 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 C-terminus (green) was transiently expressed in tobacco leaf epidermal cells. Red spots were signals from chloroplast autofluorescence. Bar=20μm.

E. Cellular fractionation assay detecting localization of Dek10. 1-5 identifies the five sample layers in sucrose density gradient. Anti-Nad7 was used as mitochondrial indicator.

Figure 5. Dek10 is required for \textit{nad3}-61, \textit{nad3}-62, and \textit{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 kernel of the WT and dek10-ref, 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 *zea mays*, *Sorghum bicolor*, *Oryza sativa*, *Lolium perenne*, *Triticum aestivum*, *Beta vulgaris*, *Nicotiana sylvestris*, *Brassica napus*, *Medicago 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+III², 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+III² and Complex I are indicated. The activity of the dehydrolipoamide dehydrogenase was used as a sample loading control.

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 standard errors, n= 6 individuals (**P<0.001, Student’s t-test).

C. Ultrastructure of developing endosperms of WT and dek10 (18 DAP) for mitochondria observation. Bars = 1 μm. PB, protein body; Mito, mitochondrion.
Supplemental Data

Figure S1. Biochemical analysis of WT and dek10 endosperm.
A. SDS–PAGE analysis of total, zein, and nonzein proteins from WT and dek10 mature endosperm.
B. Comparison of total, zein, and nonzein proteins from dek10 and WT mature endosperm. The measurements were done on per mg of dried endosperm. Values are the mean values with standard errors, n= 3 individuals.
C. Comparison of total starch and amyllose content in WT and dek10 mature endosperm. The measurements were done on per mg of dried endosperm. Values are the mean values with standard errors, n= 3 individuals.

Figure S2. Scanning electron microscopy analysis of the peripheral regions of mature WT and dek10 endosperm. Bars = 50 μm (top), Lower, Bars=20 μm (bottom).
Figure S3. Alignment of Dek10 full length protein sequence and homologue protein sequences from *Setaria italica* and *Oryza sativa*.

Figure S4. qRT-PCR comparing expression level of Dek10 gene in the 18 DAP and 21 DAP *dek10* and WT kernels. Ubiquitin was used as internal control. Values are the mean values with standard errors, *n* = 3 individuals.

Figure S5. Alignment of the Dek10 target sequences in *nad3* and *cox2*.

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**GO:0015078**
Hydrogen ion transmembrane transporter activity

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**GO:0006414**

**Translational elongation** 2.70E-13

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**GO:0045735**

**Nutrient reservoir activity** 2.90E-11

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