The novel E-subgroup pentatricopeptide repeat protein DEK55 is responsible for RNA editing at multiple sites and for the splicing of \textit{nad1} and \textit{nad4} in maize

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Abstract

Background: Pentatricopeptide repeat (PPR) proteins compose a large protein family whose members are involved in both RNA processing in organelles and plant growth. Previous reports have shown that E-subgroup PPR proteins are involved in RNA editing. However, the additional functions and roles of the E-subgroup PPR proteins are unknown.

Results: In this study, we developed and identified a new maize kernel mutant with arrested embryo and endosperm development, i.e., defective kernel (dek) 55 (dek55). Genetic and molecular evidence suggested that the defective kernels resulted from a mononucleotide alteration (C to T) at +449 bp within the open reading frame (ORF) of Zm00001d014471 (hereafter referred to as \textit{DEK55}). \textit{DEK55} encodes an E-subgroup PPR protein within the mitochondria. Molecular analyses showed that the editing percentage of 24 RNA editing sites decreased and that of seven RNA editing sites increased in dek55 kernels, the sites of which were distributed across 14 mitochondrial gene transcripts. Moreover, the splicing efficiency of \textit{nad1} introns 1 and 4 and \textit{nad4} intron 1 significantly decreased in dek55 compared with the wild type (WT). These results indicate that DEK55 plays a crucial role in RNA editing at multiple sites as well as in the splicing of \textit{nad1} and \textit{nad4} introns. Mutation in the \textit{DEK55} gene led to the dysfunction of mitochondrial complex I. Moreover, yeast two-hybrid assays showed that DEK55 interacts with two multiple organellar RNA-editing factors (MORFs), i.e., ZmMORF1 (Zm00001d049043) and ZmMORF8 (Zm00001d048291).

Conclusions: Our results demonstrated that a mutation in the \textit{DEK55} gene affects the mitochondrial function essential for maize kernel development. Our results also provide novel insight into the molecular functions of E-subgroup PPR proteins involved in plant organellar RNA processing.

Keywords: Defective kernel, Maize, Mitochondrion, Pentatricopeptide repeat proteins, RNA processing, Splicing

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Background

Pentatricopeptide repeat (PPR) proteins compose a large protein family found in most land plants, with more than 450 members identified in *Arabidopsis thaliana*, *Oryza sativa*, and *Zea mays* [1–5]. These proteins contain standard tandem degenerate repeat motifs, which form a helix-loop-helix structure of approximately 35 amino acids. PPR proteins are classified mainly into P- and PLS-type subfamilies according to their PPR repeat motifs [2, 6, 7]. P-subfamily PPR proteins contain only classic "P" motif repeats in tandem, while PLS-subfamily PPR proteins contain alternating repeats of three PPR motifs of different lengths. The latter are usually divided into PLS, E, E+, and DYW subgroups based on the presence of E, E+, or DYW domains at the carboxy-terminal end [2]. A new class of PPR proteins that contain small MutS-related domains at the carboxy-terminal end has also been identified, [8, 9].

P-type PPRs are considered to be involved in group II intron splicing, RNA stabilization, cleavage, translational activation, and transcript accumulation. In contrast, PLS-type PPRs play essential roles in the conversion of cytidine (C) to uridine (U) at specific sites of organelle transcripts [4, 10, 11]. Most plant PPR proteins are targeted to mitochondria, chloroplasts, or both and regulate the functions and development of those organelles [10]. In the mitochondrion, the oxidative phosphorylation system comprises five complexes (I-V) [12]. Normal assembly of these complexes is essential to maintain mitochondrial function, which requires the standard processing of mitochondrial pre-mRNAs, involving both RNA editing and intron splicing [13, 14]. Numerous PPRs are responsible for RNA posttranscriptional processes in mitochondria [15–21].

E-subgroup PPR proteins (e.g., slow growth 1 (SLO1), organelle transcript processing 87 (OTP87), mitochondrial editing factor 3 (MEF3), MEF9, MEF12, and mitochondrial PPR 25 (MPR25)) play vital roles in mitochondrial RNA editing and plant development [22–27]. In addition, several E-subgroup proteins in *Arabidopsis* and rice are involved in RNA splicing [28, 29]. In maize, five E-subgroup PPR proteins have been characterized, and all of them are involved in RNA editing. SMALL KERNEL (SMK) 1 (SMK1) is critical for *nad7–836* editing in mitochondrion and is conserved in maize and rice [14]. SMK4 is critical for RNA editing of *cytochrome c oxidase 1* (cox1) at position +1489 bp [30], *ccmF* is essential for cytochrome c maturation, and EMPTY PERICARP 7 (EMP7) is responsible for its editing at the +1553 bp position [31]. DEK39 is necessary for RNA editing of *nad3* transcripts in mitochondria [32], and Dek10 is responsible for RNA editing of three sites of *nad3* and *cox2* transcripts [33]. However, it is still unclear whether E-subgroup PPRs are involved in RNA editing and intron splicing in maize organelles.

Here, we identified maize mutant *dek55*, which has an embryo-lethal phenotype and arrested endosperm development, which is caused by a mutation of the mitochondrion-localized E-subgroup PPR protein DEK55. In the *dek55* mutant, the splicing efficiency of *nad1* introns 1 and 4 trans-splicing and *nad4* intron 1 cis-splicing decreased. Moreover, the editing percentages of 24 editing sites (*atp1*–1490, *atp8*–123, *ccmfC*–160, *ccmfC*–799, *ccmfC*–866, *ccmfC*–906, *ccmfC*–1144, *ccmfC*–1244, *ccmFn*–287, *ccmFn*–302, *cob*–564, *mat-r*–1877, *nad3*–146, *nad3*–190, *nad4*–77, *nad6*–25, *nad6*–138, *nad6*–146, *nad6*–159, *nad6*–61, *rps12*–ct–118, *rps12*–284, *ribosomal protein S13* (rps13)–56, and rps3–69) was also substantially reduced. Further, DEK55 could interact with ZmMORF1 and ZmMORF8 in yeast, which might be responsible for the activity of DEK55 on multiple editing sites. Taken together, our results suggest that the E-subgroup PPR protein DEK55 is involved in both RNA editing and group II intron splicing in maize mitochondria.

Results

Genetic and phenotypic analysis of the defective kernel 55–1 (*dek55–1*) mutant

A mutant with a defective kernel phenotype was isolated from an ethylmethanesulfonate-induced maize B73 background population and was subsequently named *dek55–1*. The *dek55–1* kernels segregated from self-pollinated progeny of *dek55–1/+* heterozygotes at a 1:3 mendelian ratio (Fig. 1a, Additional file 1: Table S1). These results suggested that *dek55–1*, which exhibits a recessive phenotype, is caused by a monogenic mutation, which was confirmed in other populations generated from *dek55–1/+* heterozygotes crossed with C733 or S162 inbred lines (Additional file 1: Table S1).

The *dek55–1* kernels were smaller and presented a whitish pericarp, and they could be distinguished from the wild-type (WT) kernels at 15 days after pollination (DAP) (Fig. 1a). At maturity, the *dek55–1* mutant kernels contained a smaller and shrivelled (Fig. 1b, c). To further investigate the developmental structure of *dek55–1* kernels, we examined the kernel tissue structure of the WT and *dek55–1* mutant at 12 and 18 DAP

To further investigate the developmental structure of *dek55–1* kernels, we examined the kernel tissue structure of the WT and *dek55–1* mutant at 12 and 18 DAP.
At 12 DAP, the dek55–1 embryo had only a small scutellum whose development was arrested at the coleoptile stage and a large interspace between the endosperm and the seed coat. In contrast, the WT embryo contained a visible coleoptile, a shoot apical meristem, a scutellum, and two leaf primordia, and the kernel was filled with endosperm cells (Fig. 1i, j). At 18 DAP, the WT embryo had developed complete structures, including four leaf primordia, a shoot apical meristem, and a clearly visible root apical meristem (Fig. 1k), while the dek55–1 embryos presented only a single leaf primordium (Fig. 1l). In addition, fewer starch grains accumulated in the dek55–1 endosperm cells than in the WT endosperm cells at this stage (Fig. 1k, l), and a cavity was observed in the dek55–1 endosperm (Fig. 1l). Taken together, these results indicate that developmental defects in the embryo and endosperm had occurred in the dek55–1 mutant.
Map-based cloning of DEK55

To identify the DEK55 gene, we applied the classic map-based cloning strategy to identify filial 2 (F2) mutant kernels, which segregated from a self-pollinated F1 hybrid ear. Four genomic DNA pools (10 mutant kernels per pool) and the DNA of both of the parents were used to identify the chromosomal location of the DEK55 gene. Six simple sequence repeat (SSR) markers on chromosome 5 were strongly correlated with defective kernel phenotypes, suggesting that the candidate gene may be on chromosome 5. Further analysis showed that the DEK55 gene is located between umc1705 and umc2302 on chromosome 5 (Fig. 2a). There are 25 putative protein-coding genes in this region (http://ensembl.gramene.org/Zea_mays/Info/Index). To identify the mutated genes, the genomic DNA of 25 candidate genes was amplified and sequenced. Sequence alignment revealed a single-nucleotide polymorphism in the E-subgroup PPR protein gene (Zm00001d014471). In the dek55–1 mutant, nucleotide C was replaced with nucleotide T at +449 bp, resulting in the substitution of the amino acid serine (Ser) with phenylalanine (Phe). However, no change in the mRNA expression level was observed (Fig. 2a-d). To validate our results, we obtained a new mutant, dek55–2, from the maize ethylmethanesulfonate-induced mutant database [34]. The dek55–2 mutant showed a single-nucleotide mutation (G to A) at +729 bp (Fig. 2b), which led to a truncated protein (Fig. 2d). The mutant dek55–2 also produced defective kernels with a small white pericarp (Fig. 2e). An allelic test between dek55–1 and dek55–2 heterozygotes revealed that normal and mutant kernels segregated at the expected 3:1 ratio (normal/mutant; 450/143; P = 0.62) in the F1 ear (Fig. 2e). For a control, all the kernels from the ear that were crossed between the dek55–2 heterozygote and WT were normal (Fig. 2e). Taken together, these results indicate that the mutation in the Zm00001d014471 PPR gene was responsible for the defective kernel phenotype, so the annotated gene was designated DEK55.

Fig. 2 Map-based cloning and identification of DEK55. a Fine mapping of the DEK55 locus. The DEK55 locus was mapped to a 1.29 Mb region between marker 3 (M3) and M4 on chromosome 5, in which there are 25 candidate genes. The physical location of polymorphic molecular markers and the number of recombinants are shown in the schematic diagram. b Schematic structure of the dek55 gene. The mutation sites of dek55–1 and dek55–2 are shown. c Relative expression level of DEK55 in WT and dek55–1 kernels. The values are the means of three biological replicates. The error bars represent the standard deviations. (Not significant (NS); P > 0.05, Student’s t-test). d Schematic diagram of the DEK55 protein, which contains a total of 13 PPR domains (P, L and S) and an E domain. The amino acid changes in dek55–1 and dek55–2 are indicated. e The self-pollinated dek55–2/+ (heterozygote) at 15 DAP, dek55–1/+ and dek55–2/+ were used in an allelism test of dek55. A cross between dek55–2/+ with B73 (WT) was used as a control. Several mutant kernels are indicated by the black arrowheads. Scale bars = 1 cm.
**DEK55 is a mitochondrial E-subgroup PPR protein**

Sequence alignment demonstrated that the **DEK55** gene is a 1893 bp long ORF with no introns. Moreover, **DEK55** encodes a 630 amino acid protein containing 13 PPR motifs and an E domain at the carboxy-terminal end (Fig. 2b-d and Additional file 1: Fig. S1). Mutations in **dek55–1** and **dek55–2** were located in the third and fifth PPR motifs, respectively (Fig. 2d). The mutation in **dek55–2** resulted in a truncated DEK55 protein missing the last eight PPR motifs and the E domain.

To examine the subcellular localization of DEK55, a p35S:DEK55-enhanced green fluorescent protein (EGFP) vector was constructed and transformed into maize protoplasts. The fluorescent signal of DEK55-EGFP overlapped with that of MitoTracker (a mitochondrion-specific dye) (Fig. 3a), suggesting that, in maize, **DEK55** is a mitochondrial PPR protein (Fig. 3a). In addition, expression analysis in various maize tissues demonstrated that **DEK55** is relatively highly expressed in the roots, anthers, and ears, with relatively low expression in the stems, leaves, silk, tassels, and kernels (Fig. 3b).

**DEK55 is involved in the C-to-U editing of 14 transcripts at multiple sites**

PPR proteins usually participate in modifying organelle transcripts [10]. It has been reported that E-subgroup PPRs are involved in the C-to-U editing of mitochondrial pre-mRNAs [14, 32, 33]. To explore whether **DEK55** is involved in this processing, the transcript levels of 35 maize mitochondrial genes that encode functional proteins were analysed in both WT and **dek55–1** kernels. RNA editing of these transcripts in the **dek55** (**dek55–1** and **dek55–2**) and WT (WT-1 and WT-2) kernels was detected via the strand- and transcript-specific RNA sequencing (ST-S-PCRseq) strategy [35]. The sequencing reads were mapped to the 35 mitochondrial gene transcripts, and 482 C-to-U RNA editing sites were examined in the WT and **dek55** kernels (Additional file 2: Table S1). The results revealed that, compared with that of these RNA editing sites between the WT and **dek55** kernels (Additional file 2: Table S2), the C-to-U editing percentage of 31 editing sites in 14 transcripts (**atp1**, **atp8**, **ccmFc**, **ccmFn**, **cob**, **mat-r**, **nad3**, **nad4**, **nad6**, **nad7**, **rps12-ct**, **rps12**, **rps13**, and **rps3**) was significantly altered in the **dek55** kernels (Fig. 4, Additional file 2: Tables S2-S4), whereas the editing percentage of 24 sites decreased (Fig. 4a) and that of seven sites increased in the **dek55** kernels compared with WT kernels (Fig. 4b, Additional file 2: Tables S2 and S4). The editing efficiency at the **atp1–1490**, **ccmFn-287**, **mat-r–1877**, and **rps13–56** sites dramatically decreased in the **dek55–1** and **dek55–2** kernels, and the editing percentage in the **dek55** mutants was more than 50% lower than that in the WT kernels (Fig. 4a, Additional file 2: Table S3). Direct sequencing of reverse transcription-polymerase chain reaction (RT-PCR) products of the **atp1–1490**, **ccmFn–287**, **mat-r–1877** and **rps13–56** sites also indicated that the editing efficiency was significantly reduced in the **dek55** kernels at these RNA editing sites (Fig. 4c). Deficient C-to-U RNA editing led to altered amino acid residues in **dek55** (Fig. 4c). Moreover, at the **atp8–123** site, the editing efficiency of only the **dek55–2** kernels (5%) was more than 50% lower than that of the WT kernels, and at **nad4–77** sites, the editing efficiency of only the **dek55–1** kernels (24.2%) was more than 50% lower than that of the WT kernels (Fig. 4a). Taken together, the above results indicated that **DEK55** is required for RNA editing at multiple sites, especially at the **atp1–1490**, **ccmFn–287**, **mat-r–1877**, and **rps13–56** sites.

**DEK55 is essential for the trans-splicing of **nad1** introns 1 and 4 and the cis-splicing of **nad4** intron 1**

The transcript levels of 35 maize mitochondrial genes were examined, and the results showed that the transcript levels of **nad1** and **nad4** were significantly down-regulated in the **dek55** mutants (Fig. 5a). The genomic DNA of **nad1** contains four group II introns, and with the exception of the 2nd intron, all are trans-splicing introns. (Fig. 5c). The genomic DNA of **nad4** has three cis-splicing introns (Fig. 5d) [13, 36]. The full maturation of **nad1** and **nad4** transcripts requires complete intron splicing. We therefore further analysed the intron splicing efficiency of **nad1**, **nad4**, and other genes in the **dek55–1** and WT kernels via quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Compared with that in the WT kernels, the splicing efficiency of the first and fourth introns of **nad1** and the first intron of **nad4** in the **dek55–1** mutant kernels decreased (Fig. 5b). Furthermore, we amplified each intron and full transcript of **nad1** and **nad4** via RT-PCR (Fig. 5c, d). The transcript abundance of **nad1** exons 1–2 and exons 4–5 and the full-length DNA fragments significantly decreased (Fig. 5c). RT-PCR could not amplify the intronic DNA fragments (1F + 2R, 3F + 4R, 4F + 5R) in the **dek55** and WT kernels because the 1st, 3rd, and 4th introns of **nad1** are trans-spliced. (Fig. 5c). The unspliced 2nd intronic fragments of **nad1** in the **dek55** mutant kernels were similar to those in the WT kernels (Fig. 5c). The abundance of **nad4** spliced exons 1–2 and full-length DNA fragments significantly decreased, and the abundance of **nad4** unspliced intron 1 transcripts significantly increased (Fig. 5d). Our findings suggest that the significant decrease in abundance of **nad4** and **nad1** transcript in the **dek55** mutant kernels was caused by the abnormal splicing of **nad4** intron 1, **nad1** intron 1, and intron 4 (Fig. 5a-d). Therefore, **DEK55** is necessary for the trans-splicing of the two **nad1** introns (1st
and 4th) and cis-splicing of the first nad4 intron in maize.

The dek55–1 mutant exhibits reduced complex I activity and increased alternative respiratory pathway activity

Four genes, i.e., nad1, nad4, nad3, and nad6, encode the subunits of complex I NAD1, NAD4, NAD3, and NAD6, respectively [36]. The rps13 gene encodes a ribosomal protein, and atp1 and atp8 encode the ATPase subunit 1 and subunit 8 subunits of ATP synthase F1, respectively [36]. Defects in the posttranscriptional processing of these genes may impair the biosynthesis of mitochondrial complexes [17, 37–39]. We performed blue native polyacrylamide gel electrophoresis (BN-PAGE) and an in-gel NADH dehydrogenase activity assay to investigate the accumulation level and activity of mitochondrial complexes in WT and dek55–1 endosperm. BN-PAGE showed that the abundance of complex I and supercomplex I+III₂ in the dek55–1 mutant significantly decreased (Fig. 6a). However, no significant differences for complex V were observed between the WT endosperm and dek55–1 endosperm (Fig. 6a). Furthermore, the activity of complexes I and I+III₂ was reduced in the dek55–1 mutant (Fig. 6b). Taken together, these results indicate that defects in mitochondrial transcript splicing and/or editing might affect the abundance and activity of mitochondrial complex I.

The mitochondrial respiratory chain in plants includes the cytochrome c and alternative oxidase (AOX) pathways [40]. When the main cytochrome c pathway is blocked, AOX activity can be increased to compensate for the respiratory pathway [41]. In dek55–1, the
Fig. 4 RNA C-to-U editing of 14 mitochondrial transcripts at multiple sites in maize mitochondria. a-b Heatmaps showing sites where the RNA editing efficiency in dek55 decreased (a) and increased (b) compared to that in the WT. The editing efficiency of each site in the WT and dek55 is indicated. The variation in editing efficiency of dek55 compared to the WT is denoted by dek55-WT. c Sequence chromatograms containing the editing sites are shown. The arrows mark the editing sites. The amino acid in the editing site is indicated at the bottom.
Fig. 5 (See legend on next page.)
functions of complex I were abolished (Fig. 6a, b). Thus, we performed qRT-PCR to detect the expression levels of AOX genes in WT and dek55–1 kernels, and the results showed a 512-fold increase in the expression level of the AOX2 gene in the dek55–1 mutant kernels compared to the WT kernels. (Fig. 6c). Collectively, our results indicate that the respiratory pathway is severely blocked in dek55–1 mitochondria.

DEK55 interacts with ZmMORF1 and ZmMORF8 in yeast
Previous studies have shown that MORFs directly interact with PPR proteins and play a role in RNA editing at numerous editing sites [42, 43]. In the present study, DEK55 was found to be responsible for 31 RNA editing events in maize, so we speculated that DEK55 might interact with MORFs to form an editing complex involved in RNA editing in maize. Thus, we used MORFs in Arabidopsis as bait to search for putative MORFs in maize; seven putative MORFs were identified in maize (Fig. 7a). A yeast two-hybrid assay was performed to screen for MORFs that interact with DEK55, and the results indicated that DEK55 can interact with ZmMORF1 and ZmMORF8 in yeast (Fig. 7b).

Discussion
DEK55 is required for maize kernel development

Previous reports have shown that PPR proteins play vital roles in maize kernel development, and the loss of function of some PPR proteins results in empty pericarp and small and defective kernel phenotypes in different genetic backgrounds [13, 14, 17–21, 30, 31, 39, 44, 45]. The ppr mutants exhibit developmentally arrested embryos and endosperm. The embryos usually reached the coleoptile stage or leaf stage 1 (L1), and the endosperm presented significantly reduced starch and protein levels [14, 33, 45]. The dek55 mutants produced small kernels with a shrivelled pericarp (Fig. 1a-c). Moreover, compared with the WT kernels, the mutant kernels had smaller embryos and a reduced proportion of hard endosperm (Fig. 1d-l). In particular, dek55–1 embryos were severely arrested and had only one leaf primordium. Thus, these mutant kernels would not be able to germinate in the field. Allelic tests indicated that the nonsense mutant dek55–2, an allelic mutant with dek55–1 and dek55–1/dek55–2 heterozygous kernels, exhibited a phenotype similar to that of dek55–1.
This suggests that *dek55* dysfunction is responsible for the defective kernel phenotype and that DEK55 is required for kernel development in maize.

The E-subgroup PPR proteins are characterized as having an E domain at their carboxy-terminal end, and this domain might be responsible for interactions between proteins [46, 47]. In the *dek55*–1 mutant, there was a single-nucleotide change (C to T) at +449 in the *dek55* gene, which resulted in Phe instead of Ser on the third PPR motif of DEK55 at position 150 in the polypeptide chain (Fig. 2b, d; Additional file 1: Fig. S1). This mutation from a hydrophilic amino acid to a hydrophobic amino acid would alter the affinity of the protein to water. Our evidence suggests that this amino acid change (Ser → Phe) is responsible for defective kernels in the *dek55* mutants. Therefore, the amino acid change at this site in DEK55 might cause conformational changes and loss of function.

In the *dek55*–2 mutant, the mutation resulted in a loss of the last eight PPR motifs and E domain at the carboxy-terminal end of the DEK55 protein (Fig. 2b, d), which might prevent it from forming complexes with other proteins and binding to its target sites.

**DEK55 is necessary for the C-to-U editing of multiple sites in the mitochondrial transcripts of maize**

PPR proteins, including DYW2, EMP21, NUWA, MEF8, and DEK53, are involved in C-to-U editing at multiple sites [48–52]. In this study, we demonstrated that DEK55 is involved in RNA editing at 31 sites; however, the editing percentage of 24 editing sites and that of seven editing sites decreased and increased, respectively, in *dek55*, suggesting that DEK55 is also necessary for RNA editing at multiple sites. Among them, DYW2 and MEF8 harbour only five PPR repeats and belong to an atypical DYW subgroup [48, 49]. NUWA belongs to the P-class of PPR proteins [48, 50], and EMP21 contains 11 PPR motifs in addition to the E and DYW domains and belongs to the PPR-DYW protein family [51]. Furthermore, DEK53 is an E-subgroup PPR protein with seven PPR repeats [52], and DEK55 is considered an E-subgroup PPR protein that contains the canonical E domain. Therefore, PPR proteins that target multiple sites for editing might have dissimilar structures.

MORFs can interact directly with PPR proteins to participate in RNA editing [42, 43, 51]. In *Arabidopsis*, MEF13 (an E-subgroup PPR protein) interacts with MORF3 and MORF8; this protein complex is responsible for RNA editing of the same sites among *morf3*, *morf8*, and *mef13* mutants [42]. EMP21 is necessary for the editing of ~17% of mitochondrial target Cs in maize [51]. Interestingly, 34 editing sites overlap in maize *emp21* mutants and *Arabidopsis* *morf8* mutants, and eight editing sites overlap in maize *emp5* mutants and *morf8* mutants. ZmMORF8 (GRMZM2G169384), an orthologue of MORF8 in maize, directly interacts with EMP21 and EMP5, suggesting that EMP21 and EMP5 participate in the editing of several sites by interacting with ZmMORF8 [51]. DEK53 is an E-subgroup PPR protein that interacts with ZmMORF1 to form an RNA-editing complex and is responsible for more than 60 RNA edits in the maize
mitochondrion [52]. In our study, DEK55 participated in the C-to-U editing of 14 mitochondrial transcripts at 31 editing sites, while the editing percentage of 24 sites decreased in dek55 (Fig. 4a, Additional file 2: Table S3) (Fig. 4). Moreover, comparative analyses of these mitochondrial transcript C-to-U editing events in both Arabidopsis and maize indicated that multiple sites, e.g., ccmFc-799, ccmFc-866, ccmFc-1144, ccmFc-1244, cob-564, mat-r-1877, nad3-190, nad4-77, and nad6-146, were not edited in Arabidopsis, as these sites are “Ts”. This suggests that editing of these sites is necessary in maize. In addition, editing was also substantially impaired in Arabidopsis morf8 mutants at the following four sites: atp1–1484 (atp1–1490 in the dek55 mutants), ccmFc-160, nad6–161, and rps12–284 [35]. DEK55 can interact with ZmMORF8 (orthologue of AtMORF8 in maize), as shown by yeast two-hybrid assays. Although a ZmMORF8 (orthologue of AtMORF8 in maize), as shown [161, and 1484 (Fig. 4)). Moreover, DEK55 can interact with ZmMORF8 (orthologue of AtMORF8 in maize), as shown by yeast two-hybrid assays. Although a ZmMORF8 (orthologue of AtMORF8 in maize), as shown by yeast two-hybrid assays. Although a ZmMORF1 [52, this study]. Therefore, DEK53 and DEK55 might be responsible for C-to-U RNA editing of these sites by interacting with ZmMORF1. Taken together, these results indicate that ZmMORF8 and ZmMORF1 might interact with DEK55 to form an editing complex for these multiple editing sites.

**DEK55 is involved in group II intron splicing in maize mitochondria**

E-subgroup PPR proteins are considered editing factors for RNA editing in organelles [10], but few of these proteins are considered to play a role in splicing [28, 29, 53]. SLO4 is necessary for RNA editing of nad4 and the efficient splicing of nad2 intron 1 in Arabidopsis mitochondria [29]. AEF1/MPR25 is involved in RNA editing of atpF and nad5 and modulates atpF splicing in both Arabidopsis and rice [28]. Furthermore, the plastid PPR protein OTP70 participates only in the intron splicing of the rpoC1 transcript [53]. In this study, DEK55 (an E-subgroup PPR protein) was shown to participate in both the RNA editing of 31 sites and group II intron splicing in maize mitochondrial transcripts (Figs. 4 and 5a–d). The RNA editing percentage of nad1 transcripts was not affected, and among the nad4 transcripts, only nad4–77 transcripts decreased. Moreover, it has been reported that intron splicing can be mediated by RNA editing events in which the key sites of introns are edited [54–56]. The editing efficiency of two RNA editing sites (147 and 409) on nad4 intron 1 increased in dek55 (Additional file 2: Table S1), which might have resulted from the reduction in the splicing efficiency of nad4 intron 1. Since splicing of nad1 introns 1 and 4 occurs in trans, the introns were unable to be amplified via RT-PCR with primer pairs (1F + 2R and 4F + 5R) (Fig. 5c). The editing efficiency of the RNA editing sites on nad1 introns 1 and 4 were not analysed. Thus, it could not be confirmed whether the decreased splicing efficiency of nad1 introns 1 and 4 in dek55 was caused by editing events of these introns.

Several proteins that participate in the splicing of nad1 and nad4 pre-mRNAs have been identified. Nuclear maturase 1 [57], DEK2 [45], and EMP11 [44] participate in the trans-splicing of nad1 intron 1, and EMP11, EMP8, and ZmSMK3 are required for nad1 intron 4 trans-splicing [44]. The proteins NMS1 [58], DEK35 [19], EMP8 [13], DEK43 [20], EMP602 [59], and ZmSMK3 [60] and are involved in cis-splicing of nad4 intron 1. The results of our study demonstrated that DEK55 is involved in both trans- and cis-splicing. It appears that splicing of one intron may require the involvement of multiple factors to constitute a putative spliceosome. This is supported by the finding that PPR-small MutS-related-1 can interact with Zm-mCSF1 to form a protein complex. This protein complex is subsequently involved in the intron splicing of multiple transcripts within the mitochondria [61]. Therefore, DEK55 might interact with P-type PPR proteins or other splicing factors involved in group II intron splicing.

**Conclusions**

In this study, we demonstrated that DEK55 is a mitochondrion-localized E-subgroup PPR protein. Mutation of DEK55 leads to embryo lethality and arrested endosperm development in maize. DEK55 is required for editing at 31 RNA editing sites, especially the atp1–1490, ccmFc-287, mat-r-1877, and rps13–56 sites (Fig. 4). Moreover, DEK55 can interact with ZmMORF1 and ZmMORF8 in yeast. In addition, DEK55 is responsible for the trans-splicing of two nad1 introns (intron 1 and intron 4) and the cis-splicing of nad4 intron 1 in the mitochondria. Our results suggest that the E-subgroup PPR protein DEK55 plays important roles in the RNA editing and splicing of introns of maize mitochondrial transcripts. These results provide a novel perspective for understanding the molecular function of E-subgroup PPR proteins in RNA processing in plant organelles.

**Methods**

**Plant materials**

The maize mutant dek55–1 identified from an ethyl-methanesulfonate population in the B73 background was kindly provided by Prof. Xiaoduo Lu of Qilu Normal University. The original name of the allele mutant dek55–2 was EMS4–073342, which was purchased from a maize ethylmethanesulfonate-induced mutant database (http://www.elabcaas.cn/memdd/) [34] and identified by searching for the gene ID (Zm00001d014471). To purify the genetic background, dek55–1 was back-crossed with
the B73 inbred line, and BC2F2 kernels were used in this study. A dek55–1 heterozygote (as the male parent) was crossed with our laboratory-grown inbred lines C733 and S162, after which the F1 progeny were self-pollinated to generate an F2 population that was used for gene mapping. Ru Chang Ren and Xu Wei Yan formally identified the plant materials. All the plant materials were sown at the experimental station of Shandong Agricultural University (Taian, Shandong Province).

**Histological analysis**

WT and defective kernels were obtained from self-pollinated heterozygous plants at 12 and 18 DAP, respectively. The middle part of the kernel along the longitudinal axis was selected and placed in formalin-acetic acid-alcohol solution for at least 12 h on ice, followed by treatment with 50, 70, 85, 95, and 100% ethanol. Afterwards, the seeds were placed in 100% xylene for 2–4 h. After dehydration, the materials were immersed in molten paraffin for 72 h at 60°C and then embedded in the paraffin. The paraffin-embedded samples were cut into 12 μm slices using a microtome (Leica RM2235, Germany). The sections were then stained based on the methods of Ren et al. [20]. The sections were ultimately imaged with a light microscope equipped with a camera (Olympus DP72, Olympus, Tokyo, Japan).

**Map-based cloning**

The DEK55 locus was identified using 1868 F2 defective kernels from the self-pollinated F1 population (C733 × dek55–1/+). For preliminary mapping, 73 polymorphic SSR markers selected from the entire genome were used to screen the DNA of the parents, individual F1 plants, and four groups of pooled F2 defective kernels. For fine mapping, new molecular markers were selected according to the parental DNA sequences. A website (http://ensembl.gramene.org/Zea_mays/Info/Index) was used to search for the genes annotated in the candidate regions of the Zea mays genome (B73_RefGen_v4) [62]. Phanta EVO Super-Fidelity DNA polymerase (catalogue number P503-d1, Vazyme Biotech Co., Nanjing, China) was used to clone all the candidate gene genomic DNA sequences and sequencing. The primers used were designed according to the candidate gene reference sequences. The primers used to clone the full-length DEK55 gene and used for map-based cloning are given in Additional file 1: Table S2.

**RNA extraction, RT-PCR, and qRT-PCR**

Total RNA from WT and dek55 mutant kernels without a pericarp and other tissues was extracted with an UltraPure RNA Kit (CWBIIO, China). Any residual DNA among the total RNA was removed by DNase. For RT-PCR, complementary DNA (cDNA) was obtained by reverse transcription and used as the template for polymerase chain reaction (PCR)-based amplification. KOD DNA polymerase (KOD FX Neo, code: KFX-201, Toyobo, Japan) was used for PCR. The PCR procedure was as follows: initial melting at 94°C for 2 min; 33 to 38 cycles of 15 s at 98°C and 30 s at the applicable annealing temperature (58°C to 61°C) for the various primer pairs; adequate extension (30 s/kb) at 68°C; and a final extension at 68°C for 7 min. RT-PCR was performed to amplify the mitochondrial transcripts, splicing efficiency of nad1 and nad4 introns. The DNA fragments obtained via RT-PCR were directly sequenced. The transcripts were amplified by the use of previously reported primers [63], which are listed in Additional file 1: Table S2. The primers used to amplify the introns of nad1 and nad4 are shown in Additional file 1: Table S2.

The qRT-PCR equipment and reaction system were the same as those of a previous report [20]. All the qRT-PCR assays were performed for three samples and with three technical repeats. The primers for group II intron splicing efficiency analysis in the mitochondria were designed according to previous reports [17, 18, 63]. The primers used to analyse DEK55 expression levels are shown in Additional file 1: Table S2.

**RNA editing efficiency analysis**

The RNA editing of 35 mitochondrial genes was analysed via the STS-PCRseq method described by Bentolila et al. [35], with slight modifications. The 35 mitochondrial gene transcripts and nad4 intron 1 were amplified through RT-PCR using four cDNA libraries as templates. These cDNA libraries were obtained from WT and dek55 mutant kernels (WT-1 and dek55–1; WT-2 and dek55–2) obtained from self-pollinated dek55–1/+ and dek55–2/+ heterozygous ears at 15 DAP. The PCR products were visualized on 1% agarose gels; the gel bands were excised, and the DNA fragments were purified using a Gel DNA Extraction Mini Kit (catalogue number DC301–01, Vazyme Biotech Co.,). Purified DNA samples amplified from the same cDNA library were mixed together in equimolar amounts and sonicated to generate 300–500 bp DNA fragments. The DNA library was constructed and sequenced on the Illumina platform by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). The sequenced data were filtered to remove any low-quality reads, adaptor reads, and unknown base reads. The clean reads were subsequently mapped to the 35 mitochondrial gene transcripts and nad4 intron 1 using Bowtie 2, and allele frequency counts were performed as described previously [52]. The editing efficiencies of 482 RNA editing sites were calculated, and the affected editing site in the dek55 mutants was defined as described previously [51]. Editing efficiency was considered to have decreased in the dek55 mutants when the T/(T + C)% in dek55–1/T/(T + C)% in
the WT was ≤ − 10%, while the editing efficiency was considered to have increased in the dek55 mutants when the T/(T + C)% in dek55-T/(T + C)% in the WT was ≥10%. Overlapping sites affected in dek55–1 vs. WT-1, dek55–1 vs. WT-2, dek55–2 vs. WT-1, and dek55–2 vs. WT-2 were considered affected editing sites [51].

Yeast two-hybrid assays
The full-length ORF of DEK55, excluding the signal peptide coding sequence (1–207 bp), was amplified using specific primers. The PCR products were subsequently cloned into a pGBK7 vector (Clontech, Kyoto, Japan) at the EcoRI and BamHI sites to generate a DEK55-BD bait vector. The coding sequences of seven ZmMORFs were then amplified using specific primers. The PCR products were subsequently cloned into the pGADT7 vectors (Clontech) to generate recombinant ZmMORF-AD prey vectors. The DEK55-BD and ZmMORF-AD recombinant vectors were cotransfected into Y2HGold competent cell (catalogue number YC1002, Weidi Biotechnology Co., Shanghai, China). Empty pGBK7 and pGADT7 vectors were used as negative controls. The transformed cells were incubated on synthetic dextrose (SD)/–leucine (Leu)-Trp dropout plates and SD/–Leu-Trp-His-Ade dropout plates supplemented with X-a-gal at 30 °C for 3 days. The primers used are listed in Additional file 1: Table S2.

Subcellular localization
The complete ORF (excluding the stop codon) of the DEK55 gene was incorporated into a pM999–EGFP vector, generating a DEK55–EGFP recombinant vector driven by the CaMV 35S promoter. Subcellular localization was performed as reported previously [64]. In brief, maize mesophyll cell protoplasts were obtained from etiolated leaves by enzymatic hydrolysis as described previously [21]. Recombinant vectors (20 µL, 15–20 µg) were added to a 200 µL maize protoplast solution, and 220 µL of 40% (w/v) PEG4000 solution was then added and mixed completely, after which the samples were incubated at 23 °C for 10–15 min. Afterwards, the protoplasts were washed using a W5 or WI solution and cultured for 12–16 h in the dark at 23 °C. Before being imaged, the protoplasts were stained with a mitochondrion-specific dye (MitoTracker Red CMXRos, Thermo Fisher Scientific, Waltham, MA, USA), with a mitochondrion-specific dye (MitoTracker Red CMXRos, Thermo Fisher Scientific, Waltham, MA, USA), and the samples were observed using a laser confocal microscope (LSM 880, Zeiss, Jena, Germany).

Isolation and analysis of mitochondrial complexes
A plant mitochondrial isolation kit (catalogue number P0045, Biohao, Wuhan, China) was used to isolate crude mitochondria from WT and dek55–1 seed tissue, excluding the pericarp (at 15 DAP), for BN-PAGE and complex I activity analysis. The collected mitochondrial precipitate was redissolved in 35 µL of solution buffer (50 mmol/L bis-Tris, 6 N HCl, 50 mmol/L NaCl, 10% w/v glycerol, 0.001% Ponceau S; pH 7.2) containing 20% n-dodecyl-b-D-maltoside (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 1% and then kept on ice for 30 min. The suspension was then centrifuged at 4 °C, after which the supernatant was collected and loaded on preprepared gradient gels (BN1002BOX, Thermo Fisher Scientific), and electrophoresis was performed according to the manufacturer’s instructions. Afterwards, the gels were placed in 100 mL of fixing solution (methanol:ddH2O:acetic acid, 4:5:1) for 30 min and then transferred to 0.02% Coomassie R-250 stain (Sigma-Aldrich, St. Louis, MO, USA) for mitochondrial complex abundance analysis. The gel strips were incubated in assay buffer (25 mg nitro blue tetrazolium and 100 µL of NADH (10 mg/mL) combined with 10 mL of 5 mmol/L Tris/HCl; pH 7.4) (Sigma-Aldrich) for 5 min, and the reaction was terminated with the fixing solution (40% methanol:10% acetic acid (v/v)) for analysis of complex I activity [44].

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12870-020-02765-x.

Additional file 1: Fig. S1. Amino acid alignment of maize DEK55 with homologous PPR proteins of other plant species. Fig. S2. Original gel images corresponding to Fig. 5a. Fig. S3. Original gel images corresponding to Fig. 6c-d. Fig. S4. Original gel images corresponding to Fig. 6a-b. Fig. S5. Original gel images corresponding to Fig. 7b. Table S1. Genetic analysis of the mutant kernels in the segregating ear. Table S2. Primers used in this study. Table S3. Number of reads at each editing site for each library. Table S2. Editing percentage at each editing site of each library. Table S3. The editing percentage of 24 RNA editing sites decreased in the dek55 mutant kernels. Table S4. The editing percentage of seven RNA editing sites increased in the dek55 mutant kernels.

Abbreviations
AOX: Alternative oxidase; atp1: ATP synthase subunit 1; BN-PAGE: Blue native polyacrylamide gel electrophoresis; C: Cytidine; cox: Cytochrome c oxidase; cDNA: Complementary DNA; DAP: Days after pollination; dek: Defective kernel; EGFP: Enhanced green fluorescent protein; EMP: Empty pericarp; Leu: Leucine; MEF: Mitochondrial editing factor; MORF: Multiple organellar RNA-editing factor; MPR2S: Mitochondrial PPR 2S; ORF: Open reading frame; OTP87: Organelle transcript processing 87; PCR: Polymerase chain reaction; Phe: Phenylalanine; PPR: Pentatricopeptide repeat; Pro: Proline; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; rps13: Ribosomal protein S13; RT-PCR: Reverse transcription-polymerase chain reaction; SD: Synthetic dextrose; Ser: Serine; SLO1: Slow growth 1; SMK: Small kernel; SSR: Simple sequence repeat; S2-PCRseq: Strand- and transcript-specific RNA sequencing; U: Uridine; WT: Wild type.

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Authors’ contributions
The experiments were conceived and supervised by XYZ. RCR, YXW, YJZ, YMW, JZ, JWW, and GMZ performed the experiments. XL isolated the dek55–
I mutant. The manuscript was drafted by XYZ and RCR and revised by XHD and XSZ. All the authors have read and approved the final manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information files. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. Sequencing data for RNA editing efficiency analysis has been deposited in National Center for Biotechnology Information Sequence Read Archive database (https://trace.ncbi.nlm.nih.gov/Traces/sra/), the BioProject accession number: PRJNA679100.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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