RESEARCH PAPER

Maize Dek33 encodes a pyrimidine reductase in riboflavin biosynthesis that is essential for oil-body formation and ABA biosynthesis during seed development

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Abstract

The maize (Zea mays) defective kernel 33 (dek33) mutant produces defective and occasionally viviparous kernel phenotypes. In this study, we cloned Dek33 by positional cloning and found that it encodes a pyrimidine reductase in riboflavin biosynthesis. In dek33, a single-base mutation (G to A) in the C-terminal COG3236 domain caused a premature stop codon (TGA), producing a weak mutant allele with only a truncated form of the DEK33 protein that occurred at much lower levels than the completed WT form, and with a reduced riboflavin content. The dek33 mutation significantly affected oil-body formation and suppressed endoreduplication. It also disrupted ABA biosynthesis, resulting in lower ABA content that might be responsible for the viviparous embryo. In addition, our results indicated that the COG3236 domain is important for the protein stability of DEK33. Yeast two-hybrid experiments identified several proteins that interacted with DEK33, including RGLG2 and SnRK1, suggesting possible post-translational regulation of DEK33 stability. The interaction between DEK33 and these proteins was further confirmed by luciferase complementation image assays. This study provides a weak mutant allele that can be utilized to explore cellular responses to impaired riboflavin biosynthesis during seed development. Our findings indicate that the COG3236 domain might be an essential regulatory structure for DEK33 stability in maize.

Keywords: ABA, COG3236, Dek33, oil body, riboflavin, Zea mays.

Introduction

Maize (Zea mays) is one of the world’s most important crops, providing food, feed, and biofuel (Godfray et al., 2010). It is also widely considered as a model plant for studies of diverse biological phenomena in monocots, such as heterosis, imprinting, transposons, and genetic diversity (Bennetzen and Hake, 2009). The seed is a key organ of maize and comprises three compartments: the embryo, endosperm, and seed coat (Reiser and Fischer, 1993; West and Harada, 1993). The study of maize seed development has been facilitated by the characterization of mutants. The normal product of fertilization is a mature dormant
seed with a well-developed embryo and starchy endosperm. Variations from this norm with significantly reduced embryo or endosperm development are considered as defective kernel (dek) types (Lowe and Nelson, 1946). Although a large number of classic dek mutants have been described (Neuffer et al., 1978; Neuffer and Sheridan, 1980; Sheridan and Neuffer, 1980), only dek1, dek2, and dek10 have been identified and characterized so far (Lid et al., 2002; Qi et al., 2017a, 2017b). In recent years, the number of known dek mutants has expanded, with more genes cloned from mutants isolated from different sources (Qi et al., 2016; Chen et al., 2017; García et al., 2017; Wang et al., 2017; Dai et al., 2018). A subclass of dek mutants is represented by the empty pericarp (emp) mutants (Scanlon et al., 1994), which display more severe endosperm and embryo defects (Gutiérrez-Marcos et al., 2007; Liu et al., 2013; Sun et al., 2015; Xu et al., 2016; Cai et al., 2017; Ren et al., 2017). Interestingly, the majority of these dek/emp mutants belong to the pentatricopeptide repeat (PPR) family proteins, except for dek1 (Lid et al., 2002), dek* (Qi et al., 2016), and dek38 (García et al., 2017). If we are to improve our knowledge of maize seed development, there is a need to identify and characterize more dek/emp mutants associated with other (i.e. non-PPR) mechanisms.

Riboflavin is the precursor of flavin mononucleotide and flavin adenine dinucleotide, which are essential co-factors involved in numerous critical cellular processes (Frenkel et al., 1979; Gastaldi et al., 1999). Riboflavin biosynthesis proceeds by the same reaction steps in higher plants as in eubacteria except for fungi, which use a different pathway (Roje, 2007; Fischer and Bacher, 2008). In Escherichia coli, the deamination and reduction steps are catalysed by a single bifunctional enzyme named RibD (Richter et al., 1997; Magalhães et al., 2008). In higher plants, there are two distinct RibD homologues, namely PyrD (Fischer et al., 2004) and PyrR (Hasnain et al., 2013). PyrR contains an extra C-terminal domain COG3236, although this has been characterized with experimental support in only one study (Frelin et al., 2015). The regulation mechanism of PyrR and the COG3236 domain remains elusive in higher plants.

In this study, we report the map–based cloning of the classic maize kernel mutant dek33, which encodes a pyrimidine reductase in riboflavin biosynthesis. Our results indicate that DEK33 is essential for oil-body formation, endoreduplication, and ABA biosynthesis during seed development. We also found that the COG3236 domain might be an essential regulatory structure for DEK33 stability. In addition, several proteins that interact with DEK33, including RGLG2 and SnRK1, were identified by yeast two-hybrid assays and confirmed by luciferase complementation image assays, suggesting possible post-translational regulation for DEK33 stability.

Materials and methods

Plant materials

The maize (Zea mays) dek33-N1299, dek33-N1145A, and UFMu05381 mutants were obtained from the Maize Genetics Cooperation Stock Center (http://maizecoop.cropsuiuc.edu/). Heterozygous dek33-N1299 and dek33-N1145A were crossed into the W22 and W64A genetic backgrounds to generate respective F1 populations. The F1 populations were then self-crossed to generate segregated F2 ears that displayed a 1:3 segregation of dek (dek33/dek33) and wild-type (WT) (+/+ and dek33/+) kernel phenotypes. Homozygous kernels from segregated F2 ears of dek33-N1299 (named as dek33-ref) were used as the mapping population. The heterozygous kernels from segregated F2 ears of dek33-N1145A and UFMu-05381 were used in allelism tests with dek33-N1299.

Kernels of dek33-N1299 and the WT were harvested and used for phenotypic observations, for preparation of paraffin and resin sections, TEM observations, RNA and protein extraction, analysis of riboflavin content, riboflavin feeding assays, isolation of oil bodies, flow cytometric analysis, analyses of carotenoid and ABA contents, and measurement of zeaxanthin epoxidase (ZEP) and aldehyde oxidase (AO) activities. For RNA-seq, three biological replicates were taken from three different segregated F2 ears of dek33-N1299 that were harvested at 15 d after pollination (DAP). One ear was harvested in the summer of 2015, and the other two ears were harvested in the summer of 2017. For analysis of Dek33 expression patterns, kernels at 3, 9, 12, 15, 18, 21, 24, 30, and 36 DAP were harvested for RNA and protein extraction from the same ear of a B73 inbred line. Other tissues were also harvested from the same plant, including roots, stem, leaves, tassels, silky, husks, and ears.

N. benthamiana plants were grown in a growth chamber at 22 °C and 70% relative humidity under a 16/8 h light/dark photoperiod for ~1–1.5 months before being used for infiltration. After infiltration, plants were kept under the same growth conditions.

Measurements of starch, proteins, and fatty acids

For starch and protein measurements, we followed the methods described previously by Qi et al. (2016). For fatty acid measurements, we followed the methods described by Feng et al. (2018). All the measurements were performed with at least three biological replicates.

Paraffin, resin, and TEM sections

For paraffin, resin, and TEM sections, samples were prepared as described by Dai et al. (2018). Bright-field images of paraffin and resin sections were taken using a Leica microscope at Shanghai University. Sample sections of TEM were observed using a Tecnai Spirit G2 BioTWIN (FEI Company).

Map-based cloning

For map–based cloning of Dek33, 1173 homozygous dek33-N1299 kernels from the segregated F2 ears were used as the mapping population. Molecular simple-sequence repeat (SSR) markers AC218-6, AC191-16, AC210-12, AC184-3, AC194-1, AC220-2, ACAC1-1, and the Indel (insertion-deletion) marker InD7 were developed to map the Dek33 locus on the maize chromosome. Fine–mapping of Dek33 indicated that the gene was localized to a 631 730-bp genomic interval between AC194-1 and AC211-5 on chromosome 5.

Polyclonal antibodies

For antibody production, the full-length DEK33 ORF sequences were cloned into the pGEX-4T -1 vector (Amersham Biosciences; primers are listed in Supplementary Table S4 at JXB online). Protein expression was followed using established procedures in E. coli strain BL21. Protein purification and production of antibodies in rabbits were performed according to standard protocols of ABClonal (China; https://abclonal.com/). The antibodies against GFP and GST were obtained from Clontech. The antibodies against FLAG and α-Tubulin were obtained from Sigma-Aldrich. Antibodies against the subunits of PSII proteins D1, D2, and CP43 were obtained from Agrisera.

Protein extraction and immunoblot analysis

Extraction of proteins from dek33, WT, and B73 tissues was performed according to the method described by Bernard et al. (1994). Extraction of
protein from *N. benthamiana* leaves was performed as described previously by Liu et al. (2010).

Separated protein samples were transferred to a nitrocellulose membrane (0.45-μm; Millipore). Membranes with attached protein samples were incubated with primary and secondary antibodies. Using a Super Signal West Pico chemiluminescent substrate kit (Pierce), the signal was visualized according to the manufacturer's instructions. Antibodies against DEK33 and CP43 were used at 1:2000, antibodies against FLAG, GFP, GST, D1, D2, and α-Tubulin were used at 1:5000.

**Subcellular localization of DEK33**

For subcellular localization of DEK33, the full-length DEK33 ORF sequence was cloned into the pSAT6-EYFP-N1 (yellow fluorescent protein) vector (Dai et al., 2018). The recombinant plasmid (~1 μg) was introduced into *N. benthamiana* leaf epidermal cells through transient transformation using the Bio-Rad PDS-1000/HeTM bioptic particle delivery system. The fluorescence signals were detected using LSM710 (Occult International).

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

Total RNA was extracted with TRIzol reagent (Tiangen). Using ReverTra Ace reverse transcriptase (Toyobo), RNA was reverse-transcribed to cDNA using the attached random primers. RT-PCR was performed for each individual fragment that was transiently expressed in *N. benthamiana* using Actin1 as an internal control. Quantitative RT-PCR was performed with SYBR Green Real-Time PCR Master Mix (Toyobo) using a Mastercycler ep realplex 2 (Eppendorf) according to the standard protocol (Livak and Schmittgen, 2001). All primers are listed in Supplementary Table S4.

**Bacterial strain DE3ΔribD and functional complementation experiments**

An *E. coli* ribD (DE3ΔribD) strain containing a chromosomal copy of the *erythrobacterium glutaminum* riboflavin transporter gene *ribM* was constructed from strain BL21 (DE3) using the CRISPR-Cas9 system (Mathes et al., 2009; Jiang et al., 2015). Correct replacement of the *ribD* gene was confirmed by sequencing (Supplementary Fig. S4). DE3ΔribD was maintained on LB medium containing 50 μM riboflavin.

For functional complementation experiments, maize DEK33 and PyrD were cloned into the modified pET-32a vector (Novagen) without the predicted N-terminal targeting peptides using a ClonExpress II One-Step Cloning Kit (Vazyme). *Escherichia coli* RibD was cloned into the same vector as a positive control. DEK33-i, DEK33-ii, and DEK33-iii were also cloned into the modified pE-F32a. The positions at which the various truncations were made are shown in Supplementary Fig. S11. All constructs were verified by sequencing. Functional complementation experiments were performed as described previously (Hasnain et al., 2013). All primers are listed in Supplementary Table S4.

**Preparation and quantification of riboflavin**

Riboflavin was extracted from *dek33* and WT mature kernels as described previously (Langer and Lodge, 2014). Quantification of riboflavin was determined using HPLC with standard protocols in the Shanghai Institute of Material Medica (Chinese Academy of Sciences).

**Riboflavin feeding assay**

Mature kernels of *dek33* and the WT were surface-sterilized for 30 min in 10% H2O2, followed by saturated CaSO4 for 3 h. Kernels were rinsed three times in sterile water and placed on a piece of sterile filter paper (Whatman Grade No. 3 MM) wetted with sterile water. After germination for 3 d, seedlings were transferred onto half-strength MS culture medium with or without riboflavin and placed in a growth chamber with a photoperiod of 16/8 h light/dark at 25 °C.

**PSII activity (Fv/Fm) analysis**

At 7 d after germination on basic growth medium, seedlings of *dek33* and the WT were detached and placed on a piece of wet paper. After dark adaption for 15 min, chlorophyll fluorescence was measured using an Imaging PAM 101 (Walz, Germany), and PSII activity (Fv/Fm) values were calculated according to the manufacturer's instructions.

**RNA-seq analysis**

For RNA-seq, three biological replicates were performed. For each replicate, kernels from the F2 ears were genotyped for *dek33* or WT using the molecular markers AC194-1 and AC211-5, and at least five individual genotyped endosperms were pooled for total RNA (20 μg) extraction. Library construction was performed according to Illumina standard protocols by Shanghai Biotechnology. Reads were aligned to the maize B73 genome using TopHat2 0.6. (Langmead et al., 2009). Data were normalized as fragments per kilobase of exon per million fragments mapped. Significant differentially expressed genes were identified as genes with a fold change ≥2 and q-value ≤0.05. Gene ontology (GO) enrichment was performed with the accession numbers of significant differentially expressed genes (DEGs) by agriGO Singular Enrichment (http://bioinfo.cau.edu.cn/agriGO/). RNA-seq raw data are available from the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under the series entry GSE80250.

**Isolation of oil bodies**

Ten kernels of *dek33* and of the WT were ground in liquid nitrogen and dried to a constant weight for subsequent analysis. Then, 150 mg of each ground sample was used for isolation of oil bodies according to an established protocol (Siloto et al., 2006). Oil body-associated proteins were analysed by SDS-PAGE using standard protocols (Sambrook et al., 1989) and stained with Coomassie Brilliant Blue R 250.

**Flow cytometry detection**

For extraction of nuclei, we followed the method described by (Wang et al., 2014). Each flow cytometric histogram was saved using PARTEC CA3 software and analysed using the WinMDI 2.8 software.

**Preparation and quantification of carotenoids**

Carotenoids were extracted from *dek33* and WT mature kernels as described previously (Kurilich and Juvik, 1999) and the contents were determined using HPLC at China Agricultural University according to the method described by (Shutu et al., 2012).

**Preparation and quantification of ABA, ZEP, and AO**

ABA was isolated from *dek33* and WT mature endosperms and embryos by the protocol described by Wang et al. (2012) and the content was determined using ELISA analysis according to Zhao et al., 2006. The ELISA kit was obtained from the Center of Crop Chemical Control, China Agricultural University, China.

Plant ZEP (zeaxanthin epoxidase, XY-00768) and AO (aldehyde oxidase, SY-G0070) ELISA kits were obtained from Shanghai Shuangying Biotechnology. The contents of ZEP and AO were determined following the manufacturer's protocols.

**Protein overexpression in *N. benthamiana***

For protein overexpression in *N. benthamiana*, full-length and truncated DEK33 were cloned into a pHB vector under the CaMV 35S promoter. DEK33, DEK33*, COG3236, and COG3236* were cloned into a modified pR101-AN vector (Takara). For modification of pR101-AN, a 6×FLAG fragment was cloned into the EcoRI and SacI sites. A GFP fragment was cloned into the NdeI and SalI sites, and EcoRI and SacI sites. A GST fragment was cloned into the NdeI and SalI sites.
sites. DEK33 and DEK33\* were cloned into pRJ101-AN with a FLAG tag. COG and COG\* were cloned into pRJ101-AN with a GFP tag. The positions at which the various truncations were made are shown in Supplementary Fig. S1. All primers are listed in Supplementary Table S4. Vectors were transformed into Agrobacterium tumefaciens strain GV3101. The agro-infiltration procedure was performed as previously described (Liu et al., 2010).

**Yeast two-hybrid assays**

Yeast two-hybrid (Y2H) assays were performed as follows. The ORFs of DEK33 and six selected DEK33-interacting proteins identified from Y2H screening were cloned into both JWW771 (NLUC) and JWW772 (CLUC) using a ClonExpress II One-Step Cloning Kit (Vazyme). All primers are listed in Supplementary Table S4. We then followed the method of Feng et al. (2018). The resulting luciferase signals were collected using a 5200 Chemiluminescent Imaging System (Tanon).

**Luciferase complementation image assays**

The luciferase complementation image (LCI) assays were performed as follows. The ORFs of DEK33 and six selected DEK33-interacting proteins identified from Y2H screening were cloned into both JWW771 (NLUC) and JWW772 (CLUC) using a ClonExpress II One-Step Cloning Kit (Vazyme). All primers are listed in Supplementary Table S4. We then followed the method of Feng et al. (2018). The resulting luciferase signals were collected using a 5200 Chemiluminescent Imaging System (Tanon).

**Results**

**The dek33 mutation produces defective kernels with reduced storage components and delayed development**

The dek33 mutant stock (dek33-N1299) was crossed into the W22 genetic background to generate the F1 population. F2 ears from self-crossed F1 plants displayed a 1:3 segregation of dek (dek33/dek33) and WT (+/+ or dek33/+). The heterozygous dek33 kernels could be distinguished from F2 ears as early as 12 DAP, as they exhibited smaller white endosperms compared with the WT. At maturity, dek33 kernels contained smaller and more opaque endosperms than the WT (Fig. 1A–C), and smaller and defective embryos that were occasionally viviparous (Fig. 1B, C). The 100-kernel weight of dek33 was 63.7% that of the WT (Fig. 1D).

To further determine the underlying biochemical basis for the dek phenotype of the mutant, the major storage components were examined in mature kernels. Total starch, total fatty acid, and protein (total protein, zein and non-zein) contents were determined. There was no significant difference in total starch content per unit weight between dek33 and the WT (Fig. 1E). However, the total fatty acid and total protein contents were generally reduced. GC analysis showed that total fatty acid content in dek33 was only 53.2% of that in the WT (Fig. 1F; Supplementary Fig. S1C, D) and SDS-PAGE analysis indicated a significant reduction of total protein in dek33 (Supplementary Fig. S1E). Quantitative analysis showed that the contents of total protein and zeins in dek33 were 95.6% and 86.2% of that in the WT, respectively (Fig. 1G); however, there was no significant change for the content of non-zein.

Seed development was observed through histological sections of kernels collected at different times after pollination. Longitudinal sections of the whole kernel showed affected seed development in dek33. At 12 DAP and 18 DAP, dek33 embryos were much smaller compared with the WT (Fig. 1H). Light microscopy clearly indicated that dek33 endosperm cells contained fewer starch granules than the WT at 18 DAP (Fig. 1I) and the aleurone layer cells were smaller in size had abnormal cellular morphology. In addition, TEM sections revealed that the number and size of protein bodies were drastically reduced in dek33 endosperms (Supplementary Fig. S1F). Fewer oil bodies were observed in both dek33 embryo (Fig. 1J) and aleurone layer cells (Fig. 1K). These results indicated that seed development was severely affected in dek33.

**Positional cloning of Dek33**

Genetic mapping of Dek33 was carried out with the F2 mapping population of dek33-N1299 (named as dek33-ref). After characterizing a population of 1173 homozygous dek33-ref individuals using molecular markers (Supplementary Table S4), Dek33 was placed between molecular markers AC194-1 and AC211-5, with an interval of 631 730 bp (Fig. 2A). Nucleotide sequence analysis within this interval combined with gene models identified nine predicted ORFs (Supplementary Table S1). Genomic sequence analysis revealed that only GRMZM2G090068 had a single-base mutation (G to A in exon 7 (Fig. 2B; Supplementary Fig. S2A), resulting in a premature stop codon TGA. Thus, GRMZM2G090068 was considered as the candidate gene for Dek33.

Further confirmation that GRMZM2G090068 was the Dek33 gene was provided by the fact that another dek mutant (dek33-N1145A) has been found to be allelic to dek33-N1299 (Neuffer and England, 1995; Dong et al., 2018), and this was also confirmed in our study (Supplementary Fig. S2B). Genomic sequence analysis of GRMZM2G090068 in dek33-N1145A indicated that it had the same mutation as dek33-N1299 (Supplementary Fig. S2A). In addition, a UniformMu mutant stock (UFMu05381) carrying a Mutator insertion in the first exon of GRMZM2G090068 (named as dek33-mu) was obtained from the Maize Genetics Stock Center (Fig. 2B), and was used for an allelism test. In dek33-mu, homozygous mutant kernels abort shortly after fertilization (Hasnain et al., 2013). An allelism test between dek33-ref and dek33-mu revealed a 1:3 segregation of dek and WT kernel phenotypes on the F2 ear, indicating that dek33-mu cannot complement dek33-ref (Supplementary Fig. S2C–E). Further genotyping analysis indicated that all dek kernels contained both the Mutator insertion and the single-base mutation, while WT kernels contained either of two alleles or nothing (Supplementary Fig. S2F; G). Thus, GRMZM2G090068 was indeed the Dek33 gene.

Dek33 encodes a pyrimidine reductase in riboflavin biosynthesis

Conserved domain analysis indicated that DEK33 encodes a pyrimidine reductase in riboflavin biosynthesis (Fig. 3A). This pyrimidine reductase is a functional homologue in plants to
Identification and characterization of dek33 in maize

To examine whether the dek33 mutation affected Dek33 transcriptional expression, quantitative RT-PCR was performed with total RNA extracted from dek33 and WT kernels at 15 DAP. The results showed no significant change of expression between dek33 and the WT (Fig. 3B), suggesting that the dek33 mutation did not affect the transcriptional level of Dek33.

To further investigate whether the dek33 mutation affected the DEK33 protein level, immunoblotting analysis was carried out for the total protein extracted from dek33 and WT kernels at 15 DAP. An expected band with molecular mass of ~68-kD was detected in the WT, whereas this band was not detected in dek33 (Fig. 3C; Supplementary Fig. S3A); however, a ~53-kD band corresponding to a truncated fragment of DEK33 was detected in dek33, and this had a much lower protein level compared with the complete DEK33 protein in the WT.
subsequently confirmed this result in *N. benthamiana* by transient expression of full-length DEK33 (WT) and truncated DEK33 (dek33) with the pHB vector under the CaMV 35S promoter. Western blot analysis using the same antibody detected the ~68-kD and the ~53-kD bands in samples expressed for the full-length DEK33 and the truncated DEK33, respectively (Supplementary Fig. S3B). These results indicated that the dek33 mutation caused truncated DEK33, which occurred at a low level.

The dek33 mutation does not affect the reductase activity of DEK33

A previous study had indicated that the COG3236 domain is not essential for DEK33 reductase activity in *E. coli* (Hasnain *et al.*, 2013). Because the dek33 mutation resulted in truncated DEK33, we used an *E. coli* strain, DE3ΔribD, to test whether this truncation affected the reductase activity (Supplementary Fig. S4). Three truncated proteins were tested for complementing activity: DEK33-i identified the truncated DEK33, DEK33-ii lacked the C-terminal COG3236 domain, and DEK33-iii lacked both the COG3236 and deaminase domains (Fig. 3D). The results showed that DEK33-i remained active (Fig. 3E; Supplementary Fig. S5), suggesting that the dek33 mutation did not affect the reductase activity of DEK33.

Riboflavin feeding partially rescues the phenotype of dek33 seedlings

To identify effects on its biosynthesis caused by the dek33 mutation, riboflavin content was examined in dek33 and WT mature kernels using HPLC analysis. The results showed that the content decreased by 48% in dek33 (Fig. 3F), indicating that the mutation greatly affected riboflavin biosynthesis.

DEK33 is expressed highly in leaves and kernels, and is localized in chloroplasts

A blastp sequence search using the DEK33 amino acid sequence identified homologous proteins from other species. Sequence alignment showed high similarities in the reductase
Fig. 3. Maize dek33 is a weak mutant allele. (A) Structure of maize DEK33. The arrow indicates the mutation site in the COG3236 domain. (B) Quantitative RT-PCR analysis of Dek33 expression in dek33 and wild-type (WT) kernels at 15 d after pollination (DAP). Ubiquitin was used as an internal control. Data are means (±SE), n=3 individuals (ns, not significant, Student’s t-test). (C) Immunoblots comparing the accumulation of DEK33 in total proteins extracted from dek33-ref and WT kernels at 15 DAP. α-Tubulin was used as the sample loading control. The arrow indicates the band detected in dek33 kernels. (D) DEK33, DEK33-i, DEK33-ii, and DEK33-iii were constructed for functional complementation analysis without N-terminal-targeting peptides. (E) Functional complementation of E. coli ribD deletant strain DE3ΔribD by genes encoding maize DEK33 and PyrD. For each construct, three independent isolates were cultured on LB medium containing 50 μg ml–1 ampicillin and 1 mM IPTG, with or without 50 μM riboflavin, for 60 h at 22°C. (F) HPLC analysis of riboflavin content in dek33 and WT mature kernels. For each sample, three independent biological replicates were performed. Data are means (±SE), n=3 individuals (***P<0.001, Student’s t-test). (G) Phenotypes of dek33 and WT seedlings cultivated in half-strength MS culture medium supplemented with or without riboflavin. Scale bar is 1 cm.
and COG3236 domains (Supplementary Fig. S7). A neighbor-joining phylogenetic tree was constructed (Supplementary Fig. S8A) and indicated that DEK33 homologs are highly conserved in angiosperms.

Quantitative RT-PCR revealed that Dek33 was constitutively expressed in all the tissues that we tested (Supplementary Fig. S8B). During seed development, expression of Dek33 occurred before 3 DAP and continued beyond 30 DAP. An immunoblot analysis with a DEK33-specific antibody showed that it was highly accumulated in kernels and leaves, being first detectable at 3 DAP (Supplementary Fig. S8C, D).

To determine its subcellular localization, full-length DEK33 was fused with yellow fluorescent protein (YFP) and transiently expressed in N. benthamiana leaf epidermal cells. Confocal laser scanning microscopy showed co-localization of the YFP signal with chloroplast autofluorescence (Supplementary Fig. S8E), indicating that DEK33 targets chloroplasts.

The dek33 mutation alters the transcriptome

To investigate the impact of the dek33 mutation on gene expression, RNA-seq analysis was carried out for endosperm from kernels at 15 DAP. Among the 33,284 transcripts detected by RNA-seq, significant (DEGs) were identified as those with a threshold fold-change ≥2 and a q-value ≤0.05. Based on these criteria, 855 genes showed significantly altered expression between dek33 and the WT.

Within the 855 DEGs, 645 genes could be functionally annotated. GO analysis indicated that these were mainly classified into eight terms (Fig. 4A; Supplementary Data Set 1). The GO classifications ‘cellular catabolic process’, ‘generation of precursor metabolites and energy’, ‘cellular amino acid metabolic process’, ‘organic acid metabolic process’, and ‘translation’ showed extensively up-regulated expression, indicating that these metabolic processes were greatly affected in dek33. Genes related to ‘nucleosome assembly’ also exhibited up-regulated expression, indicating that the function of endoreduplication was affected in dek33. In contrast, genes related to ‘monolayer-surrounded lipid storage body’ showed significant down-regulation, and this was related to the reduced total fatty acid content in dek33. In addition, genes related to ‘nutrient reservoir activity’ were also significantly down-regulated, which was related to the reduced zein content in dek33. The alterations in processes in dek33 suggested a critical role for riboflavin biosynthesis during seed development.

The dek33 mutation results in fewer oleosins

Transcriptome analysis indicated significant down-regulation in dek33 of genes related to lipid storage bodies surrounded by monolayers. To validate the differences observed by RNA-seq, quantitative RT-PCR was performed on the seven most significant genes related to lipid storage bodies from this GO category (Fig. 4B). The results confirmed that the expression level of these genes was reduced in dek33.

Oleosins are the major proteins associated with oil bodies and are usually present as two or more isoforms (Tzen et al., 1990). Because down-regulation of genes related to oil bodies was detected at the RNA level, we prepared oleosins from the total homogenates of mature dek33 and WT kernels. SDS-PAGE analysis of isolated oil body-related proteins indicated a significant reduction of oleosins in dek33 (Fig. 4C).

The dek33 mutation affects endoreduplication

Endoreduplication is a general feature of endosperm development in maize, involving replication of the nuclear genome without cell division and leading to elevated nucleic acid content (Qi et al., 2016). Endoreduplication is a variant of the mitotic cell cycle (G1-S-G2-M) with only the G1 and S phase. RNA-seq data showed up-regulation of genes related to nucleosome assembly (39 genes) and related to DNA replication (five genes) in dek33 (Supplementary Data Set 1). The expression level of 10 selected genes was further confirmed by quantitative RT-PCR (Fig. 4D; Supplementary Table S2).

Because obvious developmental delays were observed in dek33, we performed flow cytometric analysis to determine whether there was aberrant endoreduplication in the dek33 endosperm. The results indicated that in endosperm at 15 DAP endoreduplicated nuclei with C-values of 12 C or greater accounted for 29.11% of the DNA in dek33, but the corresponding value in the WT was 34.40% (Fig. 4E, Supplementary Fig. S9). The results therefore indicated that endoreduplication was suppressed in dek33 endosperm.

The dek33 mutation disrupts ABA biosynthesis

dek33 kernels exhibited a viviparous phenotype (Fig. 1B), similar to previously identified maize viviparous mutants (McCarty, 1995). It is known that carotenogenesis and ABA biosynthesis are affected at various stages in several maize viviparous mutants (Robertson, 1955; Robertson et al., 1978). To examine whether the dek33 mutation affected ABA biosynthesis, the content of carotenoids was determined by HPLC in mature kernels. The results showed significantly reduced contents of lutein, zeaxanthin, β-cryptoxanthin, β-carotene, and antheraxanthin in dek33 compared with the WT (Fig. 5A). ELISAs were used to examine the ABA contents in the embryo, endosperm, and seedlings, and showed that dek33 kernels and seedlings had reduced ABA compared with the WT (Fig. 5B, Supplementary Fig. S10).

Several enzymes have been identified as having key roles in ABA biosynthesis, including ZEP (Marin et al., 1996) and AO (Marin and Marion-Poll, 1997). ZEP and AO activities were therefore determined by ELISAs in kernels at 21 DAP, and significant decreases in activity were observed in dek33 compared with the WT (Fig. 5C). In addition, the expression of other genes involved in ABA biosynthesis (Vp5, Vp7, Vp10, and Vp14) (Hable et al., 1998; Burbidge et al., 1999; Singh et al., 2003; Porch et al., 2006) were also examined by quantitative RT-PCR in kernels at 21 DAP. The results showed up-regulation of Vp5, Vp7, and Vp10 in dek33, while Vp14 showed no significant difference in expression (Fig. 5D). These results suggested that the dek33 mutation significantly disrupted ABA biosynthesis.
The COG3236 domain might be important for DEK33 stability

Because the dek33 mutation resulted in levels of truncated DEK33 protein that were very low, we suspected that the COG3236 domain might have a stability function for DEK33. To explore this possibility, DEK33-FLAG and DEK33*-FLAG were transiently expressed in N. benthamiana leaves (Supplementary Fig. S11). RT-PCR was performed for each individual fragment that was transiently expressed in order to avoid experimental errors in parallel experiments. Western blot analysis using an
antibody against FLAG revealed a single ~60-kD band of DEK33*-FLAG with a lower protein level compared with the ~75-kD band of DEK33-FLAG (Fig. 6A, Supplementary Fig. S12A).

To further determine the stability function of the COG3236 domain, GFP-COG and GFP-COG* were transiently expressed in N. benthamiana leaves (Supplementary Fig. S11). As a single domain that fused to the C-terminus of GFP,

![Image](image_url)
GFP-COG was expressed normally with a clearly detected band, while the GFP-COG* band was detected with a lower protein level (Fig. 6B, Supplementary Fig. S12B). Because the conserved COG3236 domain is fused to both the C-terminus of DEK33 in plants and the N-terminus of RibA in bacteria such as *Vibrio vulnificus* (Frelin *et al.*, 2015), we subsequently detected the expression of the fused protein COG–GFP and COG*-GFP in *N. benthamiana* leaves. Interestingly, COG*-GFP was not detectable, while COG-GFP was expressed normally with a clearly detected band (Fig. 6C, Supplementary Fig. S12C). These results suggested that the COG3236 domain might be important for the protein stability of DEK33.

**DEK33 interacts with RGLG2 and SnRK1, which might provide possible post-translational regulation of DEK33 stability**

Y2H screening assays were performed to identify proteins that interacted with DEK33. After screening ~6×10^6 recombinant cDNA clones, 40 putative DEK33-interacting proteins were identified (Supplementary Table S3). Six proteins with independent positive clones from the Y2H screening, including RGLG2 and SnRK1, were then further analysed by Y2H and LCI assays (Fig. 7). The results indicated that DEK33 had protein–protein interactions with all these proteins in yeast and in *N. benthamiana*. Because the *dek33* mutation caused truncated DEK33 in maize, we tested the interactions between truncated DEK33 and five of the proteins (we did not test G6 because only weak interactions were observed in the Y2H and LCI assays). Interestingly, no interactions with any of these proteins were observed in yeast (Supplementary Fig. S13).

**Discussion**

In this study, we identified and characterized the classic maize kernel mutant *dek33*. *dek33* encodes a pyrimidine reductase in riboflavin biosynthesis. A premature stop codon in the COG3236 domain produced a weak mutant allele that resulted in a truncated DEK33 protein that occurred only at low levels and in reduced riboflavin content. This lead to abnormalities in oil-body formation, endoreduplication, and ABA biosynthesis during seed development (Fig. 8).

*dek33-ref* is a weak mutant allele that functions with reduced riboflavin content during seed development

Riboflavin is essential for basic metabolism and numerous critical cellular processes. Complete abolishment of the riboflavin pathway would produce pleiotropic phenotypes and affect the survival of plants (Xiao *et al.*, 2004). Although seven distinct enzymes are known to be involved in the riboflavin pathway in plants (Herz *et al.*, 2000; Schramek *et al.*, 2003; Fischer *et al.*, 2004; Sandoval *et al.*, 2008), only Arabidopsis *cos1* and *psh1* have been characterized (Xiao *et al.*, 2004; Ouyang *et al.*, 2010). To our knowledge, the characterization of *dek33* provides the first description of riboflavin deficiency during maize seed development. This will be useful for our further understanding of the essential role of riboflavin biosynthesis in maize.

![Fig. 7. Interactions between maize DEK33 and candidate proteins identified by yeast two-hybrid (Y2H) assays, as tested by Y2H and luciferase complementation image (LCI) assays. (A, B) Y2H assays between DEK33 and Y2H-identified proteins. The interaction between T-antigen and Human P53 was used as a positive control. AD, activating domain; BD, binding domain. (C) LCI assays between DEK33 and Y2H-identified proteins. The specific combinations used for each interaction are indicated. The fluorescence signal intensity represents their interaction activities. G1–G6 are the proteins encoded by GRMZM2G007647, GRMZM2G047607, GRMZM2G180704, GRMZM2G180704, GRMZM2G345544, and GRMZM2G119146, respectively (Supplementary Table S3).](image-url)
Compared with dek1, which causes severe effects in seed development (Becraft et al., 2002; Lid et al., 2002), dek33 is a weak mutant allele that is responsible for a dek phenotype with delayed seed development (Fig. 1). The homozygous dek33-mu allele displays a lethal phenotype (Hasnain et al., 2013), indicating that DEK33 is evidently required for seed development. In dek33, the G-to-A transition caused the presence of a premature stop codon that lead to truncation of the DEK33 protein and low levels of its production (Figs 2, 3). It is possible that the low protein level of truncated DEK33 in dek33 still retains its reductase activity and hence riboflavin can still be synthesized—albeit at reduced content—to maintain seed development (Fig. 8).

The embryonic development of dek33 was severely affected and embryonic cells had reduced cytoplasmic density with fewer oil bodies (Fig. 1). The maize embryo only represents 11% of the mass of the seed but accumulates 90% of the oil (Watson et al., 2003), and about 90% of the seed oil is accumulated in oil bodies (Murphy, 1993). Previous studies have shown that riboflavin deficiency produces changes in fatty acid composition (Taniguchi and Nakamura, 1976; Taniguchi et al., 1978), and probably affects lipid metabolism by interfering with β-oxidation of fatty acids (Olpin and Bates, 1982). In β-oxidation, acyl-CoA dehydrogenases (ACADs) are flavin adenine dinucleotide (FAD)-dependent enzymes that catalyse the alpha, beta-dehydrogenation of acyl-CoA esters (Thorpe and Kim, 1995; Arent et al., 2008; Swigonová et al., 2009). The expression of some ACAD genes was altered in dek33 (Supplementary Fig. S14); however, we considered that the mutation might not affect FAD-dependent enzymes directly. It is possible that reduced FAD content affects the FAD-dependent β-oxidation in dek33, leading to altered gene expression and a significant reduction in oil bodies, which might be one important reason for the defective embryo (Fig. 8).

Seed dormancy and germination are controlled by multiple phytohormones, with ABA and gibberellins acting in opposing roles (McCarty, 1995; Kucera et al., 2005). After maturation, dek33 kernels occasionally displayed s viviparous phenotype (Fig. 1). Previous studies have indicated that carotenogenesis and ABA biosynthesis are affected at various stages in several maize viviparous mutants (Robertson, 1955; Robertson et al., 1978; Neill et al., 1986; Durantini et al., 2008). In plants, carotenoids provide not only pigments, but also the precursors for ABA synthesis (Bartley and Scolnik, 1995). Carotenoids and ABA showed significant reductions in content in dek33 (Fig. 5). Therefore, it is reasonable to propose that the dek33 mutation affects carotenogenesis and ABA biosynthesis, resulting in reduced ABA content that might be responsible for viviparous embryos (Fig. 8). However, we observed that the expression of some FAD-dependent Vp genes was up-regulated in dek33 (Fig. 5). A previous study has reported a similar up-regulation of Arabidopsis AAO3, which encodes an enzyme that is active on abscisic aldehyde in ABA biosynthesis (Seo et al., 2000). Mutation of AAO3 results in complete loss of the AO enzyme and hence its activity, leading to reduced ABA content in rosettes; however, a more intense expression of AAO3 was observed in the mutants. We speculate that a feedback regulator mechanism may exist whereby an initial decrease in carotenoids under low-riboflavin stress may result in an immediate speed-up of transcriptional compensation.

Possible post-translational regulators of DEK33 protein stability

A previous study indicated that the COG3236 domain is not required for DEK33 reductase activity (Hasnain et al., 2013). However, the maize dek33 mutation in this domain resulted in defective kernels with delayed development (Fig. 1). In addition, the Arabidopsis pls1 mutation in this domain also causes severe photo-oxidative damage (Ouyang et al., 2010), suggesting essential roles for the COG3236 domain in higher plants.

If the COG3236 domain is not required for DEK33 reductase activity, it is possible that this domain maintains the stability of DEK33. This hypothesis comes from two observations: loss of part of the COG3236 domain resulted in low levels of truncated DEK33 in dek33 (Figs 2, 3) and less DEK33*-FLAG that was transiently expressed in N. benthamiana leaves.
A previous study had indicated that the enzymatic activity of this domain was as an N-glycosidase to prevent accumulation of toxic, reactive intermediates during riboflavin biosynthesis (Frelin et al., 2015). Thus, it is possible that the accumulation of reactive intermediates may be expected to cause protein turnover. However, the COG3236 family is diverse and probably encompasses functions unrelated to riboflavin (de Souza and Aravind, 2012). As a single protein fused to the C-terminus of GFP, the expression of COG* was not as stable as the full-length COG domain (Fig. 6). Interestingly, as a single protein fused to the N-terminal of GFP, the fused protein COG*-GFP was not detectable (Fig. 6). This evidence led us to speculate that the COG3236 domain is also important for DEK33 stability as well as its N-glycosidase activity.

A number of studies have indicated that ubiquitination and phosphorylation are involved in the regulation of protein stability (Piattoni et al., 2011; Tsai and Gazzarrini, 2012; Choi et al., 2016; Yang et al., 2018). In our study, Y2H and LCI assays demonstrated that DEK33 could interact with RGLG2 (Yin et al., 2007) and SnRK1 (Halford et al., 2003) (Fig. 7), suggesting possible post-translational regulation for DEK33 stability. Therefore, it is possible that the truncation of DEK33 resulted in the loss of its interaction with RGLG2/SnRK1 (Supplementary Fig. S13), which might have been responsible for the instability of the truncated DEK33 protein in dek33. It is also plausible that the denaturation of the C-terminus might directly result in the truncated DEK33 being unstable, as the COG3236 domain was indicated as a possible stability structure that is independent of the association with RGLG2/SnRK1. A recent study of maize o10 reported a similar observation (Yao et al., 2016) with the G-to-A transition causing a premature stop codon that lead to reduced protein levels in o10 kernels. Further experimental evidence will be needed to determine which mechanism regulates DEK33 protein stability during maize seed development.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Phenotypic features of the maize dek33 mutant.

Fig. S2. Cloning of Dek33.

Fig. S3. Full scan images of immunoblots for DEK33 in maize kernels and in N. benthamiana leaves expressing DEK33 fragments.

Fig. S4. Confirmation of E. coli strain DE3ΔAribD.

Fig. S5. Functional complementation assay of E. coli strain DE3ΔAribD.

Fig. S6. Seedling phenotypes of dek33 and wild-type maize.

Fig. S7. Amino acid sequences alignment of DEK33 and its homologues.

Fig. S8. Phylogenetic relationships, expression patterns, and subcellular localization of DEK33.

Fig. S9. Cell cycle diagrams of endosperms at 15 DAP analysed by flow cytometry.

Fig. S10. ELISA analysis of ABA content in dek33 and wild-type seedlings.

Fig. S11. Amino acid sequences of maize DEK33 and PyrD showing the points at which truncations were made to remove various regions.

Fig. S12. Full scan images of immunoblots for N. benthamiana leaves expressing DEK33-FLAG, DEK33-FLAG, GFP-COG, GFP-COG*, COG-GFP, and COG*-GFP.

Fig. S13. Results of the Y2H assays between DEK33* and Y2H-identified proteins.

Fig. S14. Quantitative RT-PCR analysis of ACAD genes in dek33 and wild-type kernels at 15 DAP.

Table S1. Summary of Dek33 candidate genes.

Table S2. Differentially expressed genes related to the cell cycle in dek33 and the wild-type.

Table S3. List of DEK33-interacting proteins, as identified by Y2H assays.

Table S4. Primers used in this study.

Data Set 1. Gene ontology classifications of DEGs, with functional annotations.

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**Author contributions**

RS and DD designed the experiments; DD, HT, LC, FP, and TZ performed the experiments; DD, TZ, WQ, and RS analysed the data; DD and RS wrote the manuscript.

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Identification and characterization of dek33 in maize | 5187

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