The mitochondrial pentatricopeptide repeat protein EMP12 is involved in the splicing of three nad2 introns and seed development in maize

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Abstract

Plant mitochondrial genes contain cis- and trans-group II introns that must be spliced before translation. The mechanism by which these introns are spliced is not well understood. Several families of proteins have been implicated in the intron splicing, of which the pentatricopeptide repeat (PPR) proteins are proposed to confer the substrate binding specificity. However, very few PPRs are characterized. Here, we report the function of a P-type PPR protein, EMP12, and its role in seed development. EMP12 is targeted to mitochondria. Loss-of-function mutation in Emp12 severely arrests embryo and endosperm development, causing embryo lethality. The trans-splicing of mitochondrial nad2 intron 2 and cis-splicing of nad2 intron 4 are abolished, whereas the cis-splicing of nad2 intron 1 is reduced in emp12 mutants. As a result, complex I assembly is disrupted, and its activity is strongly reduced in the mutants. The expression of the alternative oxidase and several components of other mitochondrial complexes is increased, possibly in response to the defective complex I. These results suggest that Emp12 is required for the trans-splicing of nad2 intron 2 and cis-splicing of nad2 introns 1 and 4, and is important to complex I biogenesis, and embryogenesis and endosperm development in maize.

Keywords: EMP12, maize, mitochondrion, PPR, RNA splicing, seed development.

Introduction

Plant mitochondrial genes have prokaryotic characteristics resulting from their origin from endosymbiosis of α-proteobacteria. Subsequently, they evolved novel features of RNA metabolism to adapt to the eukaryotic host cell environments. Mitochondria have lost most of the bacterial genes or transferred genes to the nucleus of the host cell during evolution (Gray et al., 1999; Hammani and Giegé, 2014). Therefore, the mitochondrial genome retains only a small percentage of genes, encoding proteins, tRNAs, and rRNAs that are essential for the oxidative phosphorylation system (OXPHOS) and the translation machinery (Richardson et al., 2013). The maize mitochondrial genome contains 58 genes, 18 of which encode subunits of complex I, III, IV, and V (atp1 double copies) in the OXPHOS system, 4 are involved in the cytochrome c
maturation process, 9 genes encode ribosomal proteins, and 21 genes encode tRNAs required for 14 amino acids. In addition, there are three rRNA genes (rrn5, rrn18, and rrn26), a mature rRNA (mat-r) gene residing within the fourth intron of the nad1 transcript (Clifton et al., 2004), and a transporter gene (mttB) in the genome (Clifton et al., 2004). Some genes are transcribed as long polycistronic RNA precursors. To form mature transcripts, these precursor RNAs undergo extensive post-transcriptional processing including RNA editing, which converts the cytidines to uridines (C-to-U) (Takenaka et al., 2013a); intron splicing that removes the cis- and trans-introns and joins the exons (Brown et al., 2014); RNA maturation that trims the 5' or 3' end of precursor mRNAs, and translation regulation that is facilitated by specific RNA-binding proteins (Colas des Francs–Small, et al., 2014; Haïli et al., 2016).

Some mitochondrial genes are interrupted by introns. Based on the structure and splicing mechanism, these introns are classified as group I or group II introns, the latter of which are prevalent in plant mitochondria (Bonen, 2011). Group II introns are large ribonucleoproteins consisting of a catalytic RNA (ribozyme) and an intron-encoded maturation protein with reverse transcriptase activity (Novikova and Belfort, 2017). Structurally, group II introns have six domains (D1–DVI), in which D1, DV, and DVI are essential for splicing (Novikova and Belfort, 2017). There are 19 group II introns in the genes of nad1, nad2, nad4, nad5, and nad7 (encoding components of complex I), and 3 introns in cox2 (cytochrome c oxidase 2 of complex IV), comFc (component of cytochrome c maturation), and rps3 (proteins and translation in the maize mitochondrial genome (Clifton et al., 2004; Brown et al., 2014). Most introns are in the cis configuration, but some are in trans which require trans-spooling. Trans–introns are believed to result from the DNA rearrangement, causing a break in DIV of the intron and splitting the transcript into two, such that the two exons with the flanking half-intron are transcribed independently in the genome (Malek and Knoop, 1998; Bonen, 2011).

In contrast to bacteria, plant mitochondrial group II introns have lost the activity of self-splicing because of degeneracy and loss of the cognate maturation, leaving only an immobile maturation gene (matR) encoded in nad1 intron 4 (de Longevialle et al., 2010; Sultan et al., 2016). To facilitate the splicing, nucleus-encoded RNA-binding cofactors are recruited, which are from different protein families. For instance, the plant organellar RNA recognition (PORR) protein, WTF9, is required for the splicing of rpl2 and comFc introns (Colas des Francs–Small, et al., 2012). Similarly, the REGULATOR OF CHROMOSOME CONDENSATION-like protein, RUG3, is associated with the splicing of nad2 intron 2 and intron 3 (Kühn et al., 2011). A member of the mitochondrial transcription termination factor (mTERF) protein family, mTERF15, is involved in the splicing of the nad2 cis-intron 3 (Hsu et al., 2014). Moreover, a chloroplast RNA splicing and ribosome maturation (CRM) protein, mCSF1 (Zmudjak et al., 2013), a putative DEAD-box RNA helicase PMH2 (Köhler et al., 2010), an RAD-52-like protein ODB1 (Samach et al., 2011), and two nucleus-encoded matrases (Keren et al., 2009, 2012) are required for the splicing of mitochondrial introns. In addition to these splicing factors, the prevalent RNA-binding proteins are from the large family of pentatricopeptide repeat (PPR) proteins (Barkan et al., 2014).

PPRs belong to the α-solenoid superfAMILY of helical repeat proteins, with a large number in nearly all eukaryotic lineages (Fujii and Small, 2011; Barkan and Small, 2014). The structure of PPRs is defined as tandem repeats of a degenerate 35 amino acid repeat motif and a right-handed superhelix that facilitates RNA binding (Ke et al., 2013; Yin et al., 2013; Barkan et al., 2014). PPR proteins are divided into the P- and PLS-subfamily based on the diversity of the C-terminal motif. The P-subfamily contains the canonical P-motif, whereas the PLS-subfamily additionally harbors longer (L) or shorter (S) variant PPR motifs and additional C-terminal domains (E, E+, and DYW) (Lurin et al., 2004). In land plants, >450 PPR proteins have been found (Fujii and Small, 2011) and they are mainly localized to plastids and mitochondria (Colcombet et al., 2013). Some mitochondrial PPR proteins have been functionally characterized in Arabidopsis, Physcomitrella, rice, and maize (Barkan and Small, 2014; Colas des Francs–Small and Small, 2014). The P-subfamily PPRs usually facilitate RNA intron splicing (Brown et al., 2014; Colas des Francs–Small, et al., 2014; Hsu et al., 2014; Hsieh et al., 2015; Xiu et al., 2016; Cai et al., 2017; Chen et al., 2017; Qi et al., 2017a; Ren et al., 2017; Dai et al., 2018; Sun et al., 2018), RNA stability (Colas des Francs–Small et al., 2014; Lee et al., 2017; Wang et al., 2017; Zhang et al., 2017) or RNA cleavage and translation (Colas des Francs–Small et al., 2014; Haïli et al., 2016), whereas the PLS-subfamily is predominantly involved in RNA editing (Takenaka et al., 2013a; Barkan and Small, 2014; Sun et al., 2015; Qi et al., 2017b; Wang et al., 2017; Yang et al., 2017; Li et al., 2018) and occasionally in RNA splicing (Chateigner-Boutin et al., 2011; Ichinose et al., 2012). However, very few PPRs involved in intron splicing have been functionally characterized in Arabidopsis, as disruption of their functions often causes embryo lethality (Colas des Francs–Small and Small, 2014).

The Nad2 protein is similar to the MRP family of Na+/H+ antipporters and is a likely site for proton transfer in complex I (Hirst, 2013). Previous genetic evidence has pointed to the involvement of RNA helix proteins from distinctive families for splicing of nad2 introns, including RUG3 (Kühn et al., 2011) and mTERF15 (Hsu et al., 2014) in Arabidopsis, loss of function of which results in reduced complex I activity and retarded growth. The maize kernel mutants are ideal materials to study embryo-lethal genes because of the large size and availability of homozygous endosperm and embryos. Currently, three PPR–mediated nad2 intron splicing events in maize have been described (Xiu et al., 2016; Cai et al., 2017; Dai et al., 2018). Male EMP16, a P-subfamily of PPR proteins, harboring 11 PPR motifs, is involved in the cis-spooling of nad2 intron 4, the lack of which precludes its normal intron splicing and complex I assembly, and, in turn, probably the defect in embryogenesis and endosperm development (Xiu et al., 2016). Mutation of another P-subfamily PPR protein, EMP10, causes loss of nad2 intron 1 splicing, which severely affects complex I activity, and the embryos in emp10 are blocked in the proembryo stage, producing non-viable maize kernels (Cai et al., 2017). Loss of DEK37 expression in the dele mutants leads
to reduced nad2 intron 1 splicing, such that the embryogenesis and endosperm development are relatively alleviated, displaying a small kernel phenotype (Dai et al., 2018).

In this study, we characterized a mitochondrial PPR protein designated EMP12 affecting the splicing of nad2 introns in maize. Disruption of Emp12 is lethal, giving rise to aborted embryogenesis and endosperm development. The splicing efficiency of nad2 cis-intron 1 and intron 4, and trans-intron 2 is reduced in emp12 mutants, leading to the disassembly of complex I and a reduced complex I activity. Our results imply that EMP12 plays an essential role in nad2 intron splicing, mitochondria functions, and embryo and endosperm development in maize.

Materials and methods

Plant materials

The maize kernel mutants, emp12-673 (UFMu-02085) and emp12-20 (UFMu-07644), in a W22 background were obtained from the Maize Genetics Cooperation Stock Center. The mutants were isolated from the UniformMu transposon tagging population and sequenced by high-throughput Mu-TAIL (thermal asymmetric interlaced) (Settles et al., 2004; McCarty et al., 2005). The Mu insertion was verified by genomic PCR amplification using EMP12-R: AACACACCACCTAATGTGTATCATATC and specific TIR8 primers (Tan et al., 2011). Subsequently the PCR products were recovered and subjected to sequencing to confirm the Mu insertion position. The Mu active line was introgressed into the W22 inbred background. Primers EMP12-F, CACCATGCTCTTCCTCGTCCGGCG; and EMP12-R2, GGAGCAGGTTGTTGCGTCTCTGTGC were used for detection of Emp12 expression in different tissues. EMP12-F; EMP12-F2, AAGACCCACACCTGCTCTCGTG; EMP12-F3, CACTTGCGATCCATCGTGTGGAATG; and EMP12-R were used for detection of Emp12 expression in emp12 mutants. Ubiquitin was used as an internal control that was amplified by primers UbiF and UbiR (for detection of unspliced RNA). The splicing efficiency is shown as a ratio of spliced to unspliced forms of each transcript in detection of spliced RNA, and the exon–intron flanking primers were used for detection of unspliced RNA. The splicing efficiency is shown as a ratio of spliced to unspliced forms of each transcript in

Sequence data for maize Emp12 was from the GenBank database under accession number GRMZM2G023071 and for alternative oxidases (AOXs) under accession numbers AY059646, AY059647, and AY059648.

Subcellular localization of EMP12

The full-length coding sequence of Emp12 was amplified from the maize cDNA of the W22 inbred line using primers EMP12-F and EMP12-R. The cDNA was cloned into pENTR/D-TOPO vector (ThermoFisher Scientific, http://www.thermofisher.com) and the binary pGWB5 vector. The fused Emp12-GFP was infiltrated into tobacco (Nicotiana tabacum) epidermal cells as described in Sun et al. (2015). The fluorescence signals of EMP12-green fluorescent protein (GFP) were detected at 28 h under the Olympus Fluoview FV1000 confocal microscope (Olympus, http://www.olympus-global.com). The leaf slices expressing EMP12-GFP signals were dipped in 30 nM MitoTracker solution (ThermoFisher Scientific) at room temperature for 30 min before confocal microscope detection. The excitation wavelengths of GFP and MitoTracker (containing chlorophyll) were 488 nm and 559 nm, respectively.

Light microscopy of cytological sections

The emp12-673 kernels were harvested from self-pollinated heterozygous maize plants at 12 days after pollination (DAP) and 16 DAP. Sectioned kernels were fixed, dehydrated, and stained with Jolanssen’s Safranin O, and observed under a microscope as described previously (Liu et al., 2013).

Mitochondrial RNA transcript analysis

Total RNAs of embryo and endosperm of kernels at 12 DAP were extracted by using the TRRiol reagent (ThermoFisher Scientific), and subsequently digested with DNase (NEB, USA) and purified using the Ambion PureLink Plant RNA Kit (ThermoFisher Scientific). The cDNA was transcribed using random hexamer primers. Analyses of mitochondrial gene expression and intron splicing were performed in emp12-673 and emp12-20 mutants by reverse transcription–PCR (RT–PCR) and quantitative real-time PCR (qRT–PCR) using the primers listed previously (Xiu et al., 2016; Yang et al., 2017). qRT–PCR analyses were performed using SYBR Green Master Mix (Roche) using a LightCycler (Roche). The flanking exon–exon primers were used for detection of spliced RNA, and the exon–intron flanking primers were used for detection of unspliced RNA. The splicing efficiency is shown as a ratio of spliced to unspliced forms of each transcript in emp12 mutants normalized to wild-type (WT) maize kernels (Colas des Francis-Small et al., 2014).

Mitochondrial protein and complexes analysis

Fresh embryo and endosperm of maize kernels between 12 and 14 DAP were ground in extraction buffer [0.3 M sucrose, 10 mM KH2PO4, pH 7.5, 5 mM tetrasodium pyrophosphate, 2 mM EDTA, 1% (w/v) BSA, 1% (w/v) polyvinylpyrrolidone 40, and 20 mM ascorbic acid] by using a porcelain mortar at 4 °C. The homogenate was filtered through two layers of Miracloth (Calbiochem Co., La Jolla, CA, USA) and centrifuged for 5 min at 30 000 g. Crude mitochondria were obtained by centrifugation of the clear supernatant at 20 000 g for 15 min. The mitochondrial membrane proteins were measured by the Bradford assay (Bio–Rad) and a total of 8 μg of denatured proteins were subjected to SDS–PAGE for western blotting analysis (Sun et al., 2015). A 100 μg aliquot of mitochondrial membrane protein solubilized in 1% N-dodecylmaltoside and separated by 3–12% blue native gel electrophoresis (BN-PAGE) (ThermoFisher Scientific) as described in Sun et al. (2015). The gel strips were stained by Coomassie Brilliant Blue R–250 (CBB) and in-gel nitroblue tetrazolium (NBT)–NADH as described in Meyer et al. (2009). The gel strips were incubated in 50 mM Tris–HCl, pH 6.8, 8 M urea, 1% (w/v) SDS, and 0.5% (w/v) β-mercaptoethanol for 30 min to denature the complexes and subjected to PVDF (polyvinylidene difluoride) membrane transfer and western blotting by incubating the antiserum against Nad9 (complex I/NADH dehydrogenase subunit 9) (Lamattina et al., 1993), maize cytochrome c1 (Cytc1), Arabidopsis Cox2 (Agrisera), ATPase α-subunit (ATP–A), and AOX for detection of complex I, III, VI, and V, and total AOXs, respectively (Xia et al., 2016).
Sun et al. performed to test the linkage of emp12-673 using Emp12-specific and Mu TIR8 primers (Tan et al., 2011). No recombination was detected from a segregating population from a self-progeny of an emp12-673/Emp12 plant, suggesting that the Mu insertion is tightly linked to the Emp12 mutation (see Supplementary Fig. S1 at JXB online). At 12 DAP, the emp12 kernels were much smaller than those of the WT, displaying obscure embryo structures and vitreous endosperm (Fig. 1C). Sectioned homozygous emp12-673 and WT kernels under microscopy indicated that the emp12-673 mutant kernel displays a remarkable developmental retardation of the embryo and endosperm at 12 DAP (Fig. 1F). In the WT, the embryo had already formed a scutellum and shoot apical meristem, and there were clearly visible endosperm cells (Fig. 1E). At 16 DAP, the WT kernels exhibited significant growth and the pericarp clung tightly to the endosperm (Fig. 1G), while the emp12-673 kernels grew more slowly, displaying a more crumpled empty pericarp. The emp12-673 endosperm accumulated less starch and the embryo development stagnated at the transitional stage, remaining as an undifferentiated embryo and suspensor (Fig. 1H). Taken together, the Emp12 mutation arrests embryo development at the transition stage and severely delays embryo and endosperm development, suggesting an essential role for Emp12 in embryogenesis and endosperm development.

To determine whether the mutation in GRMZM2G023071 accounts for the emp12 phenotype, another independent mutant of Emp12 from the UniformMu population was analyzed. This Mu element inserted at +20 bp downstream of the ATG in Emp12 (emp12-20) as indicated by the linkage and genomic PCR analysis (Fig. 1D). The selfed progeny of emp12-20 heterozygotes separated emp mutant kernels, confirming that each allele could not complement each other, hence GRMZM2G023071 is the causative gene for the emp12 phenotype. RT–PCR amplification of the Emp12 transcripts in these two alleles failed to detect WT Emp12 transcripts (Fig. 1I). However, we did detect the transcripts downstream of the Mu insertion, suggesting the expression of the Mu downstream region and/or the transcript containing the Mu insertion. In any case, the result indicates that the WT EMP12 cannot be produced in the two emp12 alleles. In summary, the disruption of Emp12 results in arrested embryogenesis and endosperm development.

Fig. 1. The maize Emp12 gene is involved in embryogenesis and endosperm development. (A) A self-pollinated ear segregating for emp12-673 mutant kernels at 15 days after pollination (DAP). Arrows show the emp maize kernels. Scale bar=0.5 cm. (B) The dried kernels of emp12-673 mutants and the wild type (WT). Scale bar=2 mm. (C) The embryo and endosperm of emp12-673 mutant and WT kernels at 12 DAP. Arrows indicate the embryo (Em). Scale bar=2 mm. (D) Schematic diagram of the Emp12 gene and its protein structure, showing the Mu insertion sites of emp12-673 and emp12-20. The expression of full-length and partial Emp12 (Emp12' and Emp12'') downstream of the insertion sites was detected by RT–PCR analysis, with the combinations of primers EMP12-F, EMP12-F2, EMP12-F3, and EMP12-R. PPR motifs (P) of EMP12 are predicted by TPRpred (https://toolkit.tuebingen.mpg.de/#/tools/tprpred). (E–H) Light microscopy of cytological sections of WT (E, G) and emp12-673 mutant kernels (F, H) are longitudinally sectioned early at 12 DAP (E, F) and late at 16 DAP (G, H). En, endosperm; Em, embryo; per, pericarp; sc, scutellum; su, suspensor; col, coleoptile; ep, embryo proper; sam, shoot apical meristem; ram, root apical meristem. Scale bar=1 mm. (I) RT–PCR analysis of full-length Emp12 and truncated Emp12' and Emp12'' expression indicated in (D) was performed in the emp12-673 and emp12-20 mutants and WT siblings at 12 DAP, with normalization by Ubiquitin primers.
EMP12 is vital to mitochondrial intron splicing

EMP12 is a P-subfamily PPR protein that localizes in mitochondria

Emp12 (GRMZM2G023071) is an intronless gene encoding a P-subfamily PPR protein. This PPR harbors 442 amino acids and is predicted to have 10 putative PPR motifs (Lurin et al., 2004; Cheng et al., 2016). EMP12 is closely related to Sb06g030430 from Sorghum bicolor (93%) and LOC_Os04g55090 from Oryza sativa (84%). However, no close homolog of EMP12 in Arabidopsis thaliana (Supplementary Fig. S2). The expression of Emp12 is ubiquitous in a range of vegetative and reproductive tissues, showing relatively higher levels in leaves, stems, roots, silk, and developing kernels (Supplementary Fig. S3).

EMP12 belongs to the P-subfamily PPR proteins that are involved in intron splicing, RNA stability, and RNA maturation (Barkan and Small, 2014). To gain insight into EMP12 function, the expression of mitochondrial transcripts was measured by RT–PCR and qRT-PCR analysis between the WT and emp12 mutant kernels (Fig. 3). The results indicated that the expression of the nad2 transcript which encodes the complex I/NADH dehydrogenase subunit2 (Nad2) was significantly reduced in the two emp12 mutant alleles. However, no distinguishable differences were found in the expression of other mitochondrial transcripts (Fig. 3), suggesting a defective RNA processing of the nad2 transcript in emp12 mutants.

The nad2 transcript in maize contains four introns; intron 2 is trans-spliced while the rest are cis-spliced (Fig. 4B). Since nad2 is greatly reduced in emp12 mutants, possible splicing defects in nad2 introns were monitored by using both exon–exon and exon–intron flanking primers (Xiu et al., 2016; Yang et al., 2017). The results show that in emp12-673 and emp12-20 alleles, the trans-splicing of intron 2 and cis-splicing of intron 4 of the nad2 transcript were lost, whereas the cis-splicing of intron 1 of nad2 was reduced (Fig. 4A), pointing to the requirement for Emp12 in nad2 intron splicing. Moreover, no differences were found in the introns of the other five mitochondrial transcripts (Fig. 4A). Prediction of the binding sites by the recognition code at positions 6 and 1′ indicated that these three introns might share similar binding sequences that are not present in other introns (Barkan et al., 2012; Takenaka et al., 2013b) (Supplementary Fig. S4). Therefore, the reduced splicing of nad2 introns 1, 2, and 4 is defective in emp12.

**Splicing of nad2 introns 1, 2, and 4 is defective in emp12**

EMP12:GFP

MitoTracker

*Merged*

*Merged + DIC*

**Fig. 2.** EMP12 localizes in the mitochondrion. Full-length Emp12 was fused to green fluorescent protein (GFP) and introduced into Nicotiana tabacum epidermal cells, and fluorescence signals were detected under a confocal microscope. EMP12:GFP co-localizes with the specific mitochondrial marker, MitoTracker, but not with the chlorophyll fluorescence. DIC, differential interference contrast. Scale bar=10 µm.

**Fig. 3.** The emp12 mutants only affect expression of nad2 in the mitochondria. Total RNA was extracted from fresh maize kernels at 12 DAP and reverse transcribed using hexamer primers. RT–PCR analysis was performed by using three biological replicates and was normalized to Ubiquitin. (A) Transcript analysis of nad genes in emp12 mutant alleles. (B) Expression levels of mitochondrial transcripts were quantified by qRT-PCR analysis. The transcript abundance was plotted as emp12/wild-type log2 ratios using Ubiquitin for normalization.
expression of *nad2* in *emp12* mutants is associated with the splicing defects of three *nad2* introns.

**Complex I biogenesis is reduced in emp12**

To gain insight into whether the splicing defect of *nad2* introns affects the function of the respiration chain in the *emp12* mutants, representative mitochondrion-encoded protein components of each of the mitochondrial complexes in the maize kernels were first monitored by western blot analysis. The results showed that the protein abundance of Nad9 (complex I/NADH dehydrogenase subunit 9) (Lamattina et al., 1993), which is a peripheral membrane subunit of complex I, was severely reduced in *emp12-673*. This scenario is also seen in other *nad2* intron splicing mutants such as *emp16* (Xiu et al., 2016), suggesting that the lack of *nad2* splicing results in the lost stability of peripheral proteins of complex I. In contrast, a core membrane subunit from complex III, Cytc1, was strongly increased. In addition, Cox2 (cytochrome oxidase subunit2) from complex IV, and mitochondrial ATP synthase α-subunit from complex V, were also increased in *emp12-673* mutants (Fig. 5). A possible explanation is that the mitochondrial respiratory chain contains 92 subunits, comprising both mitochondrion- and nucleus-encoded components (Jacoby et al., 2012; Subrahmanian et al., 2016). In *emp12* mutants, complex I is affected, so expression of proteins from other complexes or branched electron transport chains would be enhanced to adapt to the altered electron transfer state and NADH accumulation.

The assembly and amount of respiratory complexes of *emp12* were further determined by BN-PAGE using dissolved crude mitochondrial membrane proteins from embryo and endosperm. Complex I in *emp12* was strongly reduced, leaving little assembled complex I as indicated by the CBB and in-gel NBT-NADH activity staining. In addition, the supercomplex (I+III2) in *emp12-673* was strongly reduced, suggesting that the assembly of complex I in *emp12* mitochondria is severely impeded (Fig. 6A, B). In contrast, as shown by CBB staining and western blotting, complex III, IV, and V accumulated to levels greater than found in the WT (Fig. 6C–E). A similar shift in the relative amounts of these complexes was also noted with other complex I mutants such as *numat1*, *2, 4* mutants in Arabidopsis (Keren et al., 2009, 2012; Cohen et al., 2014) and *emp16* and *emp8* mutants in maize (Xiu et al., 2016; Sun et al., 2018).
Alternative oxidase is activated in emp12

AOX drains the electrons from the ubiquinone pool, by-passing the cytochrome c pathway for ATP synthesis (Moore and Siedow, 1991; Kühn et al., 2015). Both RT–PCR and qRT-PCR analyses indicated that among the three AOX genes, the AOX2 transcript in emp12 mutant alleles was strongly increased in comparison with the WT, whereas AOX1 and AOX3 showed an indistinguishable expression (Supplementary Fig. S5). Western blotting confirmed that the maize AOX proteins in the emp12 mutant were strongly increased (Fig. 6), which is consistent with the scenarios occurring in other complex I mutants (Li et al., 2014; Xiu et al., 2016; Chen et al., 2017; Cai et al., 2017; Qi et al., 2017a, b; Ren et al., 2017; Zhang et al., 2017; Dai et al., 2018; Sun et al., 2018). These results indicate that the alternative pathway is activated to reduce levels of reactive oxygen species (ROS) when electron flow is improperly maintained through the cytochrome c pathway in emp12 mutants (Wagner and Moore, 1997).

Splicing of one intron of nad2 involves the co-ordination of more than one splicing factor

EMP12 was found to act on nad2 intron 4 splicing, whereas EMP16 also specifically participates in the splicing of this intron, implying that more than one splicing factor is involved in the splicing of a specific intron (Supplementary Fig. S4B). EMP12 is also involved in the cis-splicing of nad2 intron 1, which has been described to require other PPR proteins, such as EMP10 (Cai et al., 2017), DEK37 (Dai et al., 2018), and EMP8 (Sun et al., 2018) in maize. In addition to maize, it has also been found that in Arabidopsis, splicing factors from various families, namely the PPR protein MTSF1 (Haili et al., 2013), together with the CRM protein mCSF1 (Zmudjak et al., 2013), the DEAD-box protein PMH2 (Köhler et al., 2010), a maturase nMAT1 (Keren et al., 2012), and a RAD-52-like protein ODB1 (Samach et al., 2011), are involved in the nad2 intron 1 splicing. Other nad2 introns, such as cis-intron 3, require the Arabidopsis PPR protein ABO5 (Liu et al., 2010), mCSF1 (Zmudjak et al., 2013), the TERF family protein mTERF15 (Hsu et al., 2014), and the RUG protein RUG3 (Kühn et al., 2011) for the splicing. These results suggest that splicing of a specific intron involves the co-ordination of specialized and general RNA-binding proteins. In addition, these splicing factors, particularly PPRs, showed distinct disparity (or at least lack of distinct sequence conservation) on one intron in monocots (i.e. maize) and dicots (i.e. Arabidopsis). It may reflect the evolutionary divergence and the complexity of splicing. The requirement for a splicing factor in organelles is probably a co-evolutionary result between the mutation in the intron and the corresponding recruitment of a nuclear-encoded protein such as the PPR proteins. In addition, it is also probable that the splicing of a specific intron requires multiple PPR proteins. If the mutation in the intron occurs after the divergence of monocots and eudicots, the splicing PPRs could be different in monocots and eudicots although they function on the same intron. If the mutation is prior to the divergence, the PPR proteins could be similar.
PPRs probably recognize the intron-binding sequences independently or, alternatively, they most probably form a highly dynamic complex similar to the nuclear spliceosome. Yeast two-hybrid analyses between EMP12 and EMP16 revealed no direct interaction (Supplementary Fig. S6). However, PPRs have been described to form a complex with other unknown proteins. PN1, a dual-targeted PPR protein, has been implicated in a 120 kDa complex (Hammani et al., 2011; Senkler et al., 2017). GRP23 (Ding et al., 2006) was recently identified in a 160 kDa complex (Senkler et al., 2017) and is in a complex including PMH2 and nMAT2 in mitochondria (Zmudjak et al., 2017). Two PPR proteins, DYW2 and NUWA (He et al., 2017), constitute the main components of the editosome in mitochondria. They interact with the mitochondrial PPR protein SLO2 and chloroplast PPR protein CLB19 for RNA editing. NUWA is thought to act as a general bridge for the editing of SLO2 and CLB19 at specific sites (Andres-Colas et al., 2017; Guillaumot et al., 2017). It is most likely that EMP12 constitutes a complex similar to the highly dynamic nuclear spliceosome for each intron, either transiently or stably, as it specifically acts on three introns of the nad2 transcript, to maintain the configuration in a ribozyme active state.

Supplementary data
Supplementary data are available at JXB online.

Fig. S1. The Mu insertion in Emp12 linked with the empty pericarp phenotype in the emp12-673 allele.

Fig. S2. The amino acid alignment of EMP12 homologs.

Fig. S3. qRT-PCR analysis of Emp12 expression in different tissues and kernels at different developing stages.

Fig. S4. Predicted binding sites of EMP12 and EMP16 in nad2 introns.

Fig. S5. AOX2 expression is increased in emp12 mutants.

Fig. S6. EMP12 did not interact with EMP16 as demonstrated by yeast two-hybrid assay.

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Author contributions
FS and BT conceived and designed the experiments: FS, ZX, RJ, YL, XZ, and YY performed the experiments; FS, ZX, WY, and BT analyzed the data; XL contributed reagents/materials/analysis tools; and FS and BT wrote the manuscript.

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