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RESEARCH PAPER

PPR-SMR1 is required for the splicing of multiple mitochondrial introns, interacts with Zm-mCSF1, and is essential for seed development in maize

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Abstract

Group II introns are ribozymes that can excise themselves from precursor-RNA transcripts, but plant organellar group II introns have structural deviations that inhibit ribozyme activity. Therefore, splicing of these introns requires the assistance of nuclear- and/or organellar-encoded splicing factors; however, how these splicing factors function remains unclear. In this study, we report the functions and interactions of two splicing factors, PPR-SMR1 and Zm-mCSF1, in intron splicing in maize mitochondria. PPR-SMR1 is a SMR domain-containing pentatricopeptide repeat (PPR) protein and Zm-mCSF1 is a CRM domain-containing protein, and both are targeted to mitochondria. Loss-of-function mutations in each of them severely arrests embryogenesis and endosperm development in maize. Functional analyses indicate that PPR-SMR1 and Zm-mCSF1 are required for the splicing of most mitochondrial group II introns. Among them, *nad2*-intron 2 and 3, and *nad5*-intron 1 are PPR-SMR1/Zm-mCSF1-dependent introns. Protein interaction assays suggest that PPR-SMR1 can interact with Zm-mCSF1 through its N-terminus, and that Zm-mCSF1 is self-interacting. Our findings suggest that PPR-SMR1, a novel splicing factor, acts in the splicing of multiple group II introns in maize mitochondria, and the protein–protein interaction between it and Zm-mCSF1 might allow the formation of large macromolecular splicing complexes.

Keywords: Group II introns, maize, mitochondria, organelle biogenesis, pentatricopeptide repeat (PPR) proteins, seed development.

Introduction

Group II introns are catalytic RNAs (ribozymes) that have six structurally conserved domains (I–VI) and are capable of self-splicing from precursor-RNAs. They are also mobile genetic elements via reverse splicing, and are hence considered to be the ancestors of nuclear introns and the spliceosome machinery in eukaryotes (Lambowitz and Zimmerly, 2011). Group II introns are numerous in eubacteria, and are also present in the chloroplast and mitochondrial genomes of plants, fungi, and some animals (Robart *et al.*, 2014). The chemical nature of group II intron splicing involves two transesterification reactions, in which the 5′-splice site is attacked by the 2′-OH of the bulged A-residue in domain VI to generate lariat RNA, followed by a nucleophilic attack by the 3′-OH of the 5′-exon at the 3′-splice site, forming an excised intron lariat and ligated exons (Toor *et al.*, 2008). Some bacterial introns can self-splice *in vitro* (Ferat and Michel, 1993); however, during the course of

evolution plant organellar group II introns have become highly degenerated and lost the capability of self-splicing.

Group II introns are prevalent in organellar genomes of land plants; however, no mitochondrial or chloroplast introns have been reported to have self-splicing activity *in vitro* (Vogel and Börner, 2002; Bonen, 2008), leading to the notion that these introns have lost this capability. In recent years, protein factors have been found to be required for the splicing of group II introns, and these come from different families and are encoded by both nuclear and organellar genomes. For example, maturase MatR encoded by the mitochondrial genome is required for the splicing of several group II introns (Sultan *et al.*, 2016). Four nucleus-encoded maturases (nMAT1–4) have been shown to reside in mitochondria, with nMAT4 having a possible dual-localization in chloroplasts as well (Keren *et al.*, 2009). Genetic studies in Arabidopsis mitochondria have suggested that nMAT1 is involved in the splicing of three introns, nMAT2 11 introns, and nMAT4 five introns (Keren *et al.*, 2009, 2012; Cohen *et al.*, 2014; Zmudjak *et al.*, 2017). In addition to MATs, different nucleus-encoded RNA-binding proteins have been demonstrated to be required for splicing of mitochondrial group II introns (Brown *et al.*, 2014; Hammani and Giegé, 2014). The CRM/CRS1-YhbY domain protein mCSF1 (a paralog of CAF1) is required for the splicing of more than half of the introns in Arabidopsis mitochondria (Zmudjak *et al.*, 2013). A member of the plant organellar RNA recognition (PORR) domain family WTF9 is essential for the splicing of the introns of *rpl2* and *ccmF*(*C*) in mitochondria (Francs-Small *et al.*, 2012). The mitochondrial transcription termination factor (mTERF) protein mTERF15 is required for splicing of mitochondrial *nad2* intron 3 (Hsu *et al.*, 2014). The DEAD-box RNA helicase PMH2 is required for optimal splicing of multiple mitochondrial group II introns (Köhler *et al.*, 2010; Zmudjak *et al.*, 2017), and the regulator of chromosome condensation (RCC) protein RUG3 is required for efficient splicing of the *nad2* introns 2 and 3 (Kühn *et al.*, 2011). Pentatricopeptide repeat (PPR) proteins are a large family of RNA-binding proteins that mediate organellar mRNA processing, including group II intron splicing (Barkan and Small, 2014; Hammani and Giegé, 2014). Many maize PPR proteins are found to be required for splicing of specific mitochondrial pre-mRNAs (Xiu *et al.*, 2016; Cai *et al.*, 2017; Chen *et al.*, 2017; Qi *et al.*, 2017; Ren *et al.*, 2017; Sun *et al.*, 2019).

PPR family proteins are divided into P-, PLS-, and PPR-SMR subfamilies according to their PPR motifs and carboxy-terminal domains (Barkan and Small, 2014). PPR-SMR is a small subfamily containing a small MutS-related (SMR) domain at the C-terminus (Liu *et al.*, 2013a). SMR domains are also found in proteins that serve as DNA or RNA endonucleases for nucleotide cleavage, or that structurally resemble the DNA and RNA binding domains for nucleic acid binding (Fukui and Kuramitsu, 2011). Recently, the SMR domain of one chloroplast-localized PPR-SMR protein, SOT1, was found to have DNA/RNA endonuclease activity *in vitro* and *in vivo* (Zhou *et al.*, 2017). In addition, several other chloroplast-localized PPR-SMR proteins have been found to play critical roles in retrograde signaling, chloroplast gene expression, and organelle biogenesis. In maize, two chloroplast-localized PPR-SMR proteins have been functionally characterized: the ATP4 protein promotes translation of the chloroplast *atpB/E* mRNA (Zoschke *et al.*, 2012), while the PPR53 protein protects mRNAs of *rrn23* and *ndhA* in chloroplasts (Zoschke *et al.*, 2016). In Arabidopsis, four chloroplast-localized PPR-SMR proteins have been functionally characterized: GUN1 physically interacts with plastid ribosomal protein S1 and some of the enzymes in the tetrapyrrole biosynthesis (TPB) pathway to play a role in plastid protein homeostasis (Tadini *et al.*, 2016); SOT1, the homolog of PPR53, binds to and protects the 5′-region of the 23S-4.5S rRNA dicistron (Wu *et al.*, 2016); SVR7, the homolog of ATP4, is associated with FtsH-mediated chloroplast biogenesis (Liu *et al.*, 2010); and pTAC2 is required for the activity of plastid-encoded RNA polymerase (Pfalz *et al.*, 2006). Nevertheless, the functions of several PPR-SMR proteins in mitochondria are still unknown.

In this study, we examined a new PPR-SMR protein, PPR-SMR1, and a CRM domain-containing protein, Zm-mCSF1, in maize. Our results showed that PPR-SMR1 is required for the splicing of nearly 75% of the mitochondrial group II introns, while Zm-mCSF1 is required for the splicing of six introns in mitochondria. We further provide evidence that PPR-SMR1 physically interacts with Zm-mCSF1. Our results imply that PPR-SMR1 and Zm-mCSF1 play important roles in the splicing of many of the group II introns, in mitochondrial functions, and in embryogenesis and endosperm development in maize.

Materials and methods

Plant material

ppr-smr1 and *Zm-mcsf1 Mutator*-insertion alleles were obtained from the UniformMu population in the maize (*Zea mays*) inbred W22 genetic background (McCarty *et al.*, 2005). The flanking sequences of *Mu* insertions in the mutant alleles were confirmed by PCR amplification using gene-specific primers and *Mu* primers (Tan *et al.*, 2011) and by direct sequencing. Wild-type material refers to normal siblings of the mutants. All the maize plants were grown in an experimental field under natural conditions.

DNA extraction and linkage analysis

Maize leaf genomic DNA was extracted using a urea-phenol-chloroform-based extraction method as described previously (Tan *et al.*, 2011). For linkage analysis, the leaf DNA was extracted from individual plants and the genotype was determined by PCR analysis as described above. Each individual plant was selfed to check the 3:1 segregation of *empty pericarp* (*emp*) mutants.

Light microscopy of cytological sections

Immature seeds of wild-types and mutants were dissected from the same ears of self-pollinated heterozygous plants at 9 d after pollination (DAP) and at 13 DAP. Slice samples were prepared as described previously (Liu *et al.*, 2013b). The embedded seeds were sectioned at 8 μm thickness using a Microm HM 315. The sections were stained with Johansen's Safranin O and observed using a Zeiss Scope A1 microscope.

Subcellular localization of PPR-SMR1 and Zm-mCSF1

The coding sequences of *PPR-SMR1* and *Zm-mCSF1* were cloned into the entry vector pENTR/D-TOPO (Invitrogen) and then transferred

into the pBI221 and pGWB5 vectors by Gateway site-specific recombination, generating the *PPR-SMR1::GFP* and *Zm-mCSF1::GFP* fusion genes driven by the Cauliflower mosaic virus 35S promoter (p35S). The constructs pBI221-*PPR-SMR1*, pBI221-*Zm-mCSF1*, and the mitochondria reporter gene p*35S*::*F1-ATPase-γ-RFP* fusion (Jin *et al.*, 2003) were purified using an EndoFree Maxi Plasmid Kit (TIANGEN). Following this, the plasmids pBI221-*PPR-SMR1* and pBI221-*Zm-mCSF1* were each transformed into mesophyll protoplasts of Arabidopsis together with the plasmid p*35S*::*F1-ATPase-γ-RFP*, as previously described (Yoo *et al.*, 2007). After incubation at 23 °C in the dark for 24–36 h, the GFP and RFP signals were recorded using a confocal laser-scanning microscope (LSM 880, Carl Zeiss). In addition, the pGWB5-*PPR-SMR1* and pGWB5-*Zm-mCSF1* constructs were each transformed into *Agrobacterium tumefaciens* strain EHA105. *Agrobacterium* cells containing each *GFP* fusion construct were infiltrated into tobacco (*Nicotiana tabacum*) leaves as described previously (van Herpen *et al.*, 2010). After incubation at 24 °C for 20–26 h, the GFP signal was recorded using a confocal laser-scanning microscope (LSM 700, Carl Zeiss) and mitochondria were stained with MitoTracker Red (Invitrogen) with a working concentration of 100 nM.

RNA extraction, RT-PCR, and qRT-PCR

Total RNA was isolated from ~100 mg of endosperm using an RNeasy® Plant Mini Kit (Qiagen). DNA contamination was removed from RNA samples using an RNase-free DNase I treatment (New England BioLabs, NEB). The removal of DNA contamination was confirmed by PCR amplification without reverse transcription. RNAs were reverse-transcribed into cDNAs using a *TransScript*® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, China). For analysis of *PPR-SMR1* expression in *ppr-smr1* mutant alleles and wild-type seeds, RT-PCR was performed with the primers 64mi-F1 and 63mi-R1 at an annealing temperature of 60 °C for 36 cycles. Similarly, RT-PCR analysis of *Zm-mCSF1* expression was performed with the primers CSFL-F1 and CSFL-R1 at an annealing temperature of 60 °C for 32 cycles. The expression of *ZmEF1α* (GRMZM2G153541) was used to normalize the cDNA levels between different samples. The primers are listed in Supplementary Table S2 at *JXB* online.

The expression levels of mitochondrial transcripts were analysed as described previously (Xiu *et al.*, 2016; Sun *et al.*, 2019). The intron splicing efficiency between the wild-type and *ppr-smr1*, and the wild-type and *Zm-mcsf1* was compared by RT-PCR and qRT-PCR. The qRT-PCR analysis was performed using FastStart Essential DNA Green Master and a LightCycler® 96 Instrument (Roche). The primers for the mitochondrial transcripts were from our previous study (Liu *et al.*, 2013b) and other primers are listed in Supplementary Table S2.

Western blotting assays

Western blotting was performed according to Sun *et al.* (2015). Total protein was extracted from 0.3 g of ground frozen endosperm with lysis buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, and 0.1% protease inhibitor. Loading samples were prepared from total protein for PPR-SMR1 and Zm-mCSF1. Primary antibodies were used against maize Cytc_1, ATPase α-subunit (MBL Beijing Biotech), AOX (Xiu *et al.*, 2016), wheat Nad9 (Lamattina *et al.*, 1993), and Arabidopsis Cox2 (Agrisera) .

Yeast two-hybrid assays

Coding sequences of *PPR-SMR1* and *Zm-mCSF1* were cloned into the bait (pGBKT7) and prey (pGADT7) vectors (Clontech). Different combinations of GAL4 DNA binding domain (BD) and GAL4 activation domain (AD) constructs were co-transformed into the yeast (*Saccharomyces cerevisiae*) strain Y2HGold (Clontech). The manipulations were performed according to the manufacturer's instructions.

Protein purification and pull-down analysis

The *Zm-mCSF1* coding sequence was cloned into the pMAL-c2x or pGEX4T-1 expression vectors to generate fusions construct with maltose-binding protein (MBP) or glutathione S-transferase (GST) tags. Coding sequences of *PPR-SMR1* (containing the 6xHis coding sequence at the C terminus) were cloned into the pMAL-c2x vector to generate fusion constructs with MBP and His tags. The primers are listed in Supplementary Table S2. These constructs were transformed into *Rosetta* strains of *Escherichia coli*. After protein expression in the bacterial cells, the MBP-Zm-mCSF1 protein was purified using amylose resin (NEB), GST-Zm-mCSF1 was purified using glutathione agarose beads (GE Healthcare), and MBP-PPR-SMR1-His was purified using Ni-NTA agarose (Qiagen) and amylose resin (NEB) according to the manufacturers' instructions.

For pull-down assays, equal amounts of the two interacting proteins as well as MBP protein and/or GST protein were mixed, and the mixture was incubated with amylose resin or glutathione agarose beads for 2–3 h at 4 °C. After incubation, the beads were collected by simple centrifugation (8000 *g*, 10 s) and then washed five times (>60 times bed volume) with a buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, and 0.5% Triton X-100. Finally, the bound proteins within 1×SDS loading buffer were released from the beads by heating at 95 °C and subjected to SDS-PAGE. Immunoblotting was used to detect the targeted recombinant proteins with anti-GST (GE Healthcare) and anti-MBP (NEB) antibodies. The primers used to make the constructs are listed in Supplementary Table S2.

Bimolecular fluorescence complementation assays

The coding sequences of *PPR-SMR1* and *Zm-mCSF1* without stop codons were cloned into pSPYNE-35S and pSPYCE-35S plasmids, respectively, as previously described (Walter *et al.*, 2004). YFP^N (aa 1–155) was fused to the C-terminus of PPR-SMR1, and YFP^C (aa 156–239) was fused to the C-terminus of Zm-mCSF1. Both resulting plasmids were mixed equally and transformed into mesophyll protoplasts of Arabidopsis as described previously (Yoo *et al.*, 2007). The YFP signal was imaged using a confocal laser-scanning microscope (LSM 880, Carl Zeiss). Mitochondria were labeled using ATPase:RFP marker (Jin *et al.*, 2003).

Phylogenetic analysis and multiple protein sequence alignment

The amino-acid sequences of PPR-SMR1, Zm-mCSF1, and their homologs were identified using BLAST searches in the UniProt database (http://www.uniprot.org). Multiple sequence alignment and construction of the phylogenetic tree (neighbor-joining algorithm with default parameters; bootstrap values were calculated from 1000 iterations) were performed using MEGA7 (Kumar *et al.*, 2016).

Results

PPR-SMR1 is essential for embryo and endosperm development in maize

We chose to study maize PPR-SMR1 (GRMZM2G345667; Supplementary Fig. S1) because of its unknown function and predicted mitochondrial localization (Emanuelsson *et al.*, 2000), which is different from previously studied PPR-SMR proteins (Zoschke *et al.*, 2012, 2016; Williams-Carrier *et al.*, 2014; Pfalz *et al.*, 2015). *PPR-SMR1* is intronless and encodes a protein with 12 PPR motifs, a C-terminal small MutS-related (SMR) domain, and an unknown N-terminus domain that is highly conserved among maize, rice, and sorghum (Fig. 1A; Supplementary Fig. S1C).

Two *Mutator* insertional mutants were identified from the UniformMu mutagenesis population (McCarty *et al.*, 2005), one with a *Mu* insertion at 166 bp and the other at 1084 bp downstream of the ATG, named *ppr-smr1-1* and *ppr-smr1-2*, respectively (Fig. 1A). Both alleles were probably null as the insertions were in the N-terminal coding region of *PPR-SMR1*

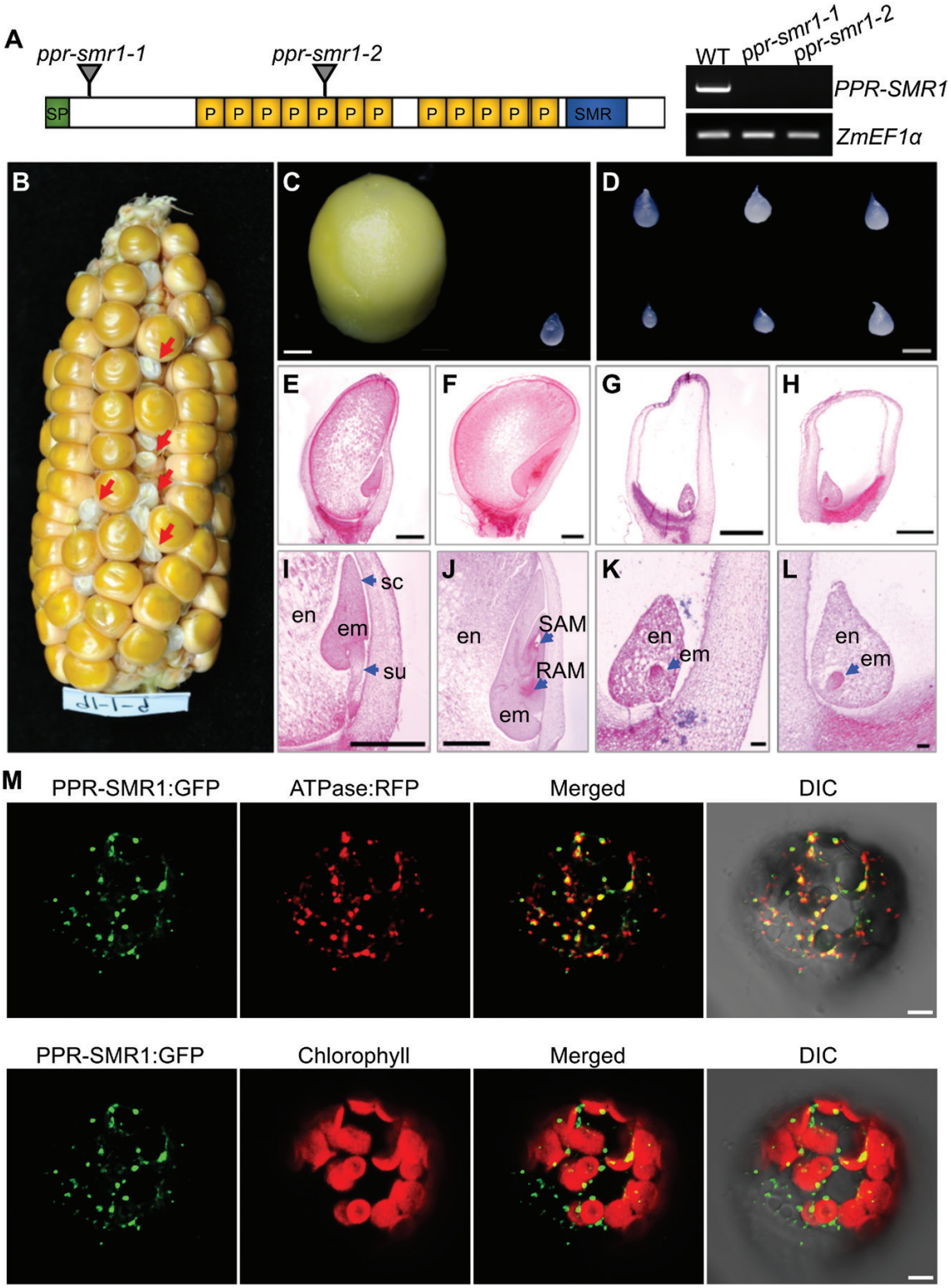

Fig. 1. Overview of maize *PPR-SMR1*. (A) Diagram of the PPR-SMR1 protein containing 12 PPR domains, with the positions of the *Mutator* insertions indicated. Expression of *PPR-SMR1* is absent from *ppr-smr1* mutants. SP, signaling peptide; P, PPR motif; SMR, small MutS-related. (B) A maize ear showing 3:1 segregation for wild-type (WT) and *ppr-smr1-1* mutant kernels (arrows). (C) Dissection of WT (left) and *ppr-smr1-1* (right) endosperm at 11 d after pollination (DAP). (D) Mutant endosperm dissected from reciprocal crosses of *ppr-smr1-1* and *ppr-smr1-2* kernels. (E–L) Comparisons of WT and *ppr-smr1-1* kernel development at 9 DAP and 13 DAP. WT kernels at 9 DAP (E, I) and 13 DAP (F, J); *ppr-smr1-1* kernels at 9 DAP (G, K) and 13 DAP (H, L). en, endosperm; em, embryo; su, suspensor; sc, scutellum; RAM, root apical meristem; SAM, shoot apical meristem. (M) Localization of the PPR-SMR1 protein. Mesophyll protoplasts from Arabidopsis were transformed with PPR-SMR1::GFP and ATPase::RFP constructs and imaged using confocal microscopy. In the upper images, mitochondria are labeled by fluorescence of ATPase::RFP, and in the lower images, chloroplasts are marked by autofluorescence. GFP, green fluorescent protein; DIC, differential interference contrast. Scale bars are 1 mm in (C–J), 0.1 mm in (K, L), and 5 μm in (M).

and wild-type transcripts were not detected. The selfed *ppr-smr1-1* (Fig. 1B) and *ppr-smr1-2* heterozygotes segregated *emp* kernels at a ratio of ~25% (63:260, $P<0.05$, and 75:270, $P<0.05$, respectively), indicating that the mutation was recessive. Both alleles were embryo-lethal, so the mutations were kept in heterozygotes. At 11 DAP, the endosperms dissected from mutant kernels were greatly reduced in size when compared with wild-type siblings (Fig. 1C). Linkage analyses indicated that the *emp* phenotype was tightly linked to the *Mu* insertions in PPR-SMR1 (Supplementary Fig. S2A, B). In addition, ears from reciprocal crosses between *ppr-smr1-1* and *ppr-smr1-2* heterozygotes segregated *emp* kernels, confirming that the *emp* phenotype was caused by mutations in the *PPR-SMR1* gene (Fig. 1D; Supplementary Fig. S2C).

The small and collapsed mature mutant kernels (Fig. 1B) and the extremely small endosperms (Fig. 1C) suggested early embryonic arrest in *ppr-smr1* seeds. To verify this, we performed histological examination of the mutant and wild-type kernels from segregating ears. Embryo development in maize is divided into transition, coleoptile, and late-embryogenesis stages (Vernoud *et al.*, 2005). Compared with the wild-type siblings, the embryos of which had long suspensors and scutellum (Fig. 1E, I) at 9 DAP and a developing shoot apical meristem (SAM) and root apical meristem (RAM) at 13 DAP (Fig. 1F, J), the *ppr-smr1* embryos were arrested at the transition stage and the endosperms showed extremely small size at 9 DAP (Fig. 1G, K); at 13 DAP, the embryos continued to stay at the transition stage and the endosperms grew slowly and remained small (Fig. 1H, L). These results indicated that both embryo and endosperm development were severely inhibited by the PPR-SMR1 mutation.

PPR-SMR1 is targeted to mitochondria and functions in the splicing of 16 introns

PPR-SMR1 was predicted to contain a mitochondrial-targeting peptide (Emanuelsson *et al.*, 2000). To test the protein localization, we fused PPR-SMR1 (at the N-terminus) with eGFP (at the C-terminus) and transiently expressed the fusion protein in mesophyll protoplasts prepared from Arabidopsis and also in the epidermal cells of tobacco leaves. Green fluorescence signals were detected in spots that merged with mitochondria labeled by the ATPase::RFP marker (Jin *et al.*, 2003) or stained with MitoTracker Red (Fig. 1M, Supplementary Fig. S2E), indicating that PPR-SMR1 was targeted to mitochondria. The non-targeted GFP protein in the negative control was detected in the cytoplasm (Supplementary Fig. S2D).

PPR proteins have been implicated in diverse functions of post-transcriptional RNA processing (Barkan and Small, 2014). To determine the molecular function of PPR-SMR1, we compared the transcript levels of 35 mitochondrial genes between the *ppr-smr1* mutant kernels and their wild-type siblings. The W22 inbred maize line contains the NB mitochondrial genome (Liu *et al.*, 2013b), and specific primers for the 35 mitochondrial genes were anchored to 5′- and 3′-proximal regions of each gene in order to ensure the analysis of nearly full transcripts. RNAs of *ppr-smr1* and their wild-type siblings were extracted from embryos and endosperms at 12 DAP from the same segregating ears. Residual genomic DNA was removed by RNase-free DNase I treatment before reverse-transcription into cDNA. The cDNAs were normalized against maize *elongation factor 1 alpha* (Lin *et al.*, 2014). The analyses showed that the transcript levels of *nad1*, *nad2*, *nad4*, *nad5*, and *nad7* were dramatically decreased in the *ppr-smr1* mutants (Supplementary Fig. S3A). To examine whether this was the result of defective intron splicing, we examined the splicing status of each intron by amplifying the transcript containing the intron and comparing between *ppr-smr1* and the wild-type. The maize NB mitochondrial genome encodes 22 group II introns, and primers were designed to anchor on two adjacent exons, as well as one end on exons and the other end on joint introns to detect unspliced introns. RT-PCR analysis revealed that splicing of *nad1*-introns 1 to 4, *nad2*-introns 1 to 4, *nad4*-introns 1 to 3, *nad5*-introns 1, 3, and 4, *nad7*-intron 2, and the *rps3* single intron was either abolished or significantly reduced in the *ppr-smr1* mutant in comparison with the wild-type (Fig. 2A). To quantify the intron splicing efficiency in *ppr-smr1*, we examined the splicing of 22 mitochondrial group II introns by qRT-PCR (Fig. 2B). Both the RT-PCR and qRT-PCR results indicated that the splicing of 16 group II introns was severely impaired in the *ppr-smr1-1* mutant (Supplementary Table S1); the remaining group II introns were spliced normally. These results suggested that PPR-SMR1 is a key splicing factor that is required for the splicing of 16 group II introns in maize mitochondria.

The Nad1, 2, 4, 5, and 7 proteins are components of mitochondrial complex I, which is the entry complex of the oxidative phosphorylation (OXPHOS) electron transfer chain that includes complexes I to V. Defects of intron-splicing in these genes would conceivably lead to deficiencies in the encoded proteins, which may affect the assembly of complexes. To assess the impact of PPR-SMR1 mutation on the expression of complex components, we used immunoblot analyses to examine the protein levels of representative components of each complex, namely NADH dehydrogenase subunit 9 (Nad9) for Complex I, cytochrome c_1 ($Cytc_1$) for Complex III, cytochrome oxidase subunit2 (Cox2) for Complex IV, and mitochondrial ATP synthase (ATPase) α subunit for Complex V. In the *ppr-smr1* mutants, the loss of PPR-SMR1 function resulted in dramatic reductions of Nad9, Cox2, and the ATPase α subunit, and also caused a moderate increase in the $Cytc_1$ subunit (Fig. 2C). Like the loss-of-function mutations in maize *Emp12* and *Emp16*, *nad2* RNA with unspliced introns in *emp12* and *emp16* mitochondria results in a reduction of Nad9 (Xiu *et al.*, 2016; Sun *et al.*, 2019), and the splicing defects in 15 introns of *nad* genes in *ppr-smr1* mitochondria clearly led to dramatic reductions of Nad9 (Fig. 2C). In addition, in line with the loss of RPS3 function in the maize *mppr6* mutant that results in much lower protein levels of Cox2 and Nad9 in mitochondria (Manavski *et al.*, 2012), the *rps3* RNA with a retained single intron conceivably encodes a defective RPS3 protein in *ppr-smr1* mitochondria, which can also lead to lower protein levels of Cox2 and Nad9 (Fig. 2C). Given the important function of RPS3 in mitochondrial translation, the lower level of organellar-encoded ATPase α subunit could have been caused by the defective RPS3 protein in *ppr-smr1* mitochondria. The high level of nuclear-encoded

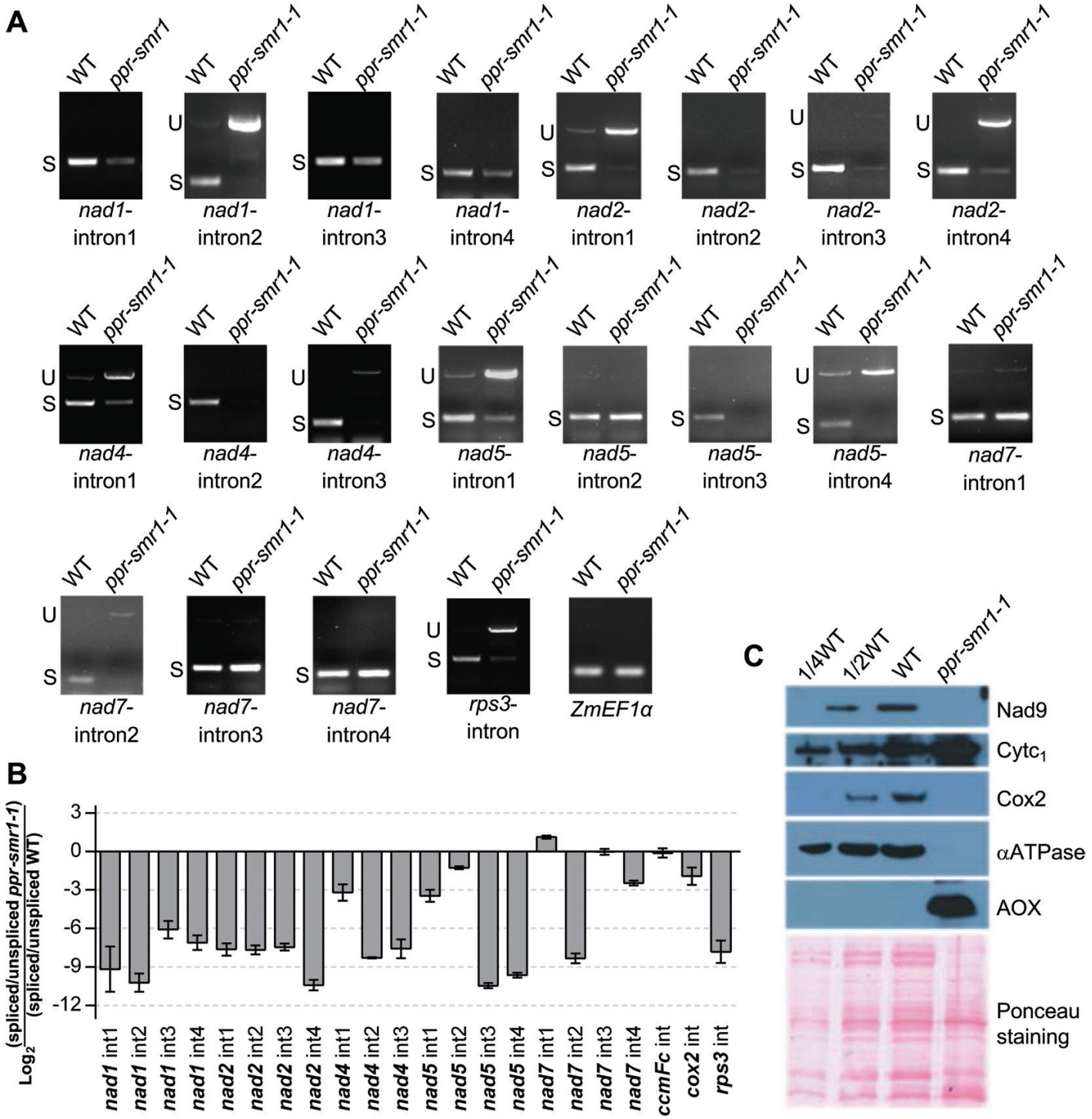

Fig. 2. PPR-SMR1 is involved in splicing of maize group II introns. (A) Inefficient splicing of group II introns of *nad1*, *nad2*, *nad4*, *nad5*, and *nad7* in the embryos and endosperms of *ppr-smr1-1* mutants as determined by reverse-transcription PCR (RT-PCR). S, introns are spliced; U, introns are retained. (B) Quantitative real-time RT-PCR (qRT-PCR) analysis of the 22 intron-containing mitochondrial transcripts in *ppr-smr1* mutants. Total RNA was extracted from embryos an endosperms of *ppr-smr1* mutants and their wild-type siblings at 12 d after pollination. *ZmEF1*α was used to normalize the quantifications. Data are means (±SD) of three biological replicates. (C) Immunoblot analysis of core subunits of mitochondrial complexes in *ppr-smr1-1* mutants. Immunoblots of embryo and endosperm extract (20 μg protein or the indicated dilutions) were probed with antibodies specific for subunits of Complex I (Nad9), Complex III (Cytochrome c_1, Cytc$_1$), Complex IV (Cox2), and ATP synthase (αATPase). AOX, alternative oxidase. Total protein input can be seen on the Ponceau S-stained blots below. (This figure is available in color at *JXB* online.)

Cytc$_1$ in *ppr-smr1* mitochondria might have been triggered by the accumulation of electrons and NADH in *ppr-smr1* mitochondria. As a result, the cytochrome pathway of the *ppr-smr1* mutant was disrupted, as revealed by the induction of AOX, the expression of which normally cannot be detected in wild type endosperm (Fig. 2C).

Zm-mCSF1 is required for the splicing of six group II introns in mitochondria

CRM domain-containing proteins are implicated in the splicing of group II introns in plant organelles (Asakura and Barkan, 2007; Barkan *et al.*, 2007). mCSF1, a

mitochondrion-targeted CRM domain-containing protein, is required for the splicing of more than half of the mitochondrial introns in Arabidopsis (Zmudjak *et al.*, 2013). Because null mutants of At-mCSF1 are embryo-lethal, which hinders analysis of its function, we sought to study the function of its ortholog in maize by examining its null mutants. The ortholog of At-mCSF1 is GRMZM2G087395, named Zm-mCSF1, which showed 67% similarity to At-mCSF1 (AT4G31010). Zm-mCSF1 contains a predicted mitochondrial targeting sequence, two CRM repeats, and a C-terminal domain with as yet unknown function (Fig. 3A, Supplementary Fig. S4A). To define the molecular functions of *Zm-mCSF1* in maize, we isolated two *Mutator*-insertional mutants, *Zm-mcsf1-1* and *Zm-mcsf1-2* (McCarty *et al.*, 2005). Both insertions are in the coding region of *Zm-mCSF1* (Fig. 3A). The selfed *Zm-mcsf1-1* and *Zm-mcsf1-2* heterozygotes segregated *emp* kernels at ratios of ~25% (169:643, $P<0.05$, and 233:949, $P<0.05$, respectively; Fig. 3B; Supplementary Fig. S4B), and reciprocal crosses between the two alleles yielded *emp* kernels as well (Supplementary Fig. S4C). At 15 DAP, the mutant kernels of both alleles were distinctly smaller than their wild-type siblings (Fig. 3B, C). Wild-type *Zm-mCSF1* transcripts were not detected in the mutant kernels (Fig. 3D). Taken together, these results indicated that *Zm-mCSF1* is the causal gene for the *emp* phenotype.

Similar to PPR-SMR1, Zm-mCSF1 protein is predicted to target to mitochondria. Subcellular localization using Zm-mCSF1::GFP fusion showed mitochondrial localization (Fig. 3E; Supplementary Fig. S3C). Hence, PPR-SMR1 and Zm-mCSF1 are nucleus-encoded proteins that are targeted to mitochondria in maize.

To determine the molecular functions of *Zm-mCSF1*, we used RT-PCR and qRT-PCR to examine transcripts of mitochondria carrying unspliced introns in *Zm-mcsf1* mutants and their wild-type siblings. Both sets of results indicated that splicing of six group II introns was severely impaired in *Zm-mcsf1-1*, namely *nad2*-introns 2 and 3, *nad5*-introns 1 and 2, *nad7*-intron 3, and the *ccmFc* intron (Fig. 4A, B, Supplementary Table S1). In contrast, the other mitochondrial group II introns were spliced normally in *Zm-mcsf1-1* (Fig. 4B, Supplementary Fig. S3B). In Arabidopsis, knockdown mutants of *At-mcsf1* have been reported to have splicing defects in more than half of the mitochondrial group II introns (13 out of 23) (Zmudjak *et al.*, 2013). We found that four of the group II introns were either incompletely spliced (*nad2*-introns 2 and 3, and *nad5*-intron 2) or completely unspliced (*nad5*-intron 1) in the *Zm-mcsf1* mutant, suggesting a conserved function of At-mCSF1 and Zm-mCSF1 in the splicing of these group II introns in mitochondria.

To assess the impact of the *Zm-mcsf1* mutation on the expression of mitochondrial complex components, we examined the protein levels of representative components of each complex by immunoblot analyses. In the *Zm-mcsf1* mutants, the loss-of-function mutation caused a decrease of Nad9 and Cytc_1 subunits, a slight increase of Cox2 and ATPase α subunit, and induction of AOX (Fig. 4C). Like the *emp12* (Sun *et al.*, 2019), *emp16* (Xiu *et al.*, 2016), and *ppr-smr1* (Fig. 2C) mutants, the lower protein level of Nad9 in *Zm-mcsf1* mitochondria was caused by defective Nad proteins. However, the decrease of Cytc_1 subunit can be attribute to defective CcmF$_C$ protein in *Zm-mcsf1* mitochondria, as the *ccmFc* transcripts contained an unspliced intron. The CcmF$_C$ protein together with the CcmF$_N$ protein forms a functional CcmF protein in plant mitochondria. In the maize *emp7* mutant, the abolishment of C-to-U editing at the *ccmF$_N$*-1553 editing site leads to loss-of-function in the CcmF$_N$ protein and a reduction in *c*-type cytochrome and, as a consequence, the protein level of the Cytc_1 subunit decreases (Sun *et al.*, 2015). Accordingly, the loss of function in the CcmF$_C$ protein in *Zm-mcsf1* mitochondria may also lead to reduction of the Cytc_1 subunit. In contrast, there was a slight increase in Cox2 and ATPase α subunit, which might be feedback regulation on the expression of components of complex IV and complex V.

PPR-SMR1 and Zm-mCSF1 physically interact

It has been suggested that several group II intron splicing factors, such as CRM, PPR, APO, and PORR proteins, are present in a high molecular weight complexes in chloroplasts (0.5–1 MDa; Asakura *et al.*, 2008; Hammani and Barkan, 2014; Reifschneider *et al.*, 2016). Our results indicated that splicing of *nad2*-introns 2 and 3 and *nad5*-intron 1 was impaired in both *ppr-smr1* and *Zm-mcsf1* (Figs 2A, B, 4A, B, Supplementary Table S1). Accordingly, it is possible that PPR-SMR1 interacts with Zm-mCSF1 to promote the splicing of these introns from pre-mRNAs in maize mitochondria.

We explored the interaction between PPR-SMR1 and Zm-mCSF1 using yeast two-hybrid (Y2H) assays. The predicted mature PPR-SMR1 (PPR-SMR1$_{49-787}$) interacted with the predicted mature Zm-mCSF1 (Zm-mCSF1$_{30-424}$) (Fig. 5A). To corroborate the Y2H analysis, we examined the interaction by *in vitro* pull-down assays and bimolecular fluorescence complementation (BiFC) analysis. PPR-SMR1 and Zm-mCSF1 were purified as fusion proteins with MBP, and the interaction between MBP-PPR-SMR1-His and MBP-Zm-mCSF1 was investigated using a His-mediated pull-down assay. Consistent with the observed interactions in the Y2H analysis, MBP-PPR-SMR1-His was able to bind to MBP-Zm-mCSF1 with weak interaction *in vitro* (Fig. 5B). The MBP protein used as a negative control that was added to the binding reaction between MBP-PPR-SMR1-His and MBP-Zm-mCSF1 failed to be retained in the resin after high-stringency washes (Fig. 5B). In the BiFC assays, YFP was split into N-terminus (YFP^N) and C-terminus (YFP^C) (Walter *et al.*, 2004), and YFP^N was fused to the C-terminus of PPR-SMR1, whilst YFP^C was fused to the C-terminus of Zm-mCSF1. The resulting constructs were mixed equally and then transformed into mesophyll protoplasts of Arabidopsis. In mitochondria of protoplasts labeled by ATPase::RFP, we were able to detect YFP fluorescence due to the reconstitution of intact YFP proteins by PPR-SMR1 interacting with Zm-mCSF1 (Fig. 5C). These results suggested that PPR-SMR1 physically interacts with Zm-mCSF1 *in vivo*. As negative controls, combinations of PPR-SMR1::YFP^N and YFP^C, YFP^N and Zm-mCSF1::YFP^C, YFP^N and YFP^C were transformed into mesophyll protoplasts, but no YFP signals could be detected (Fig. 5C).

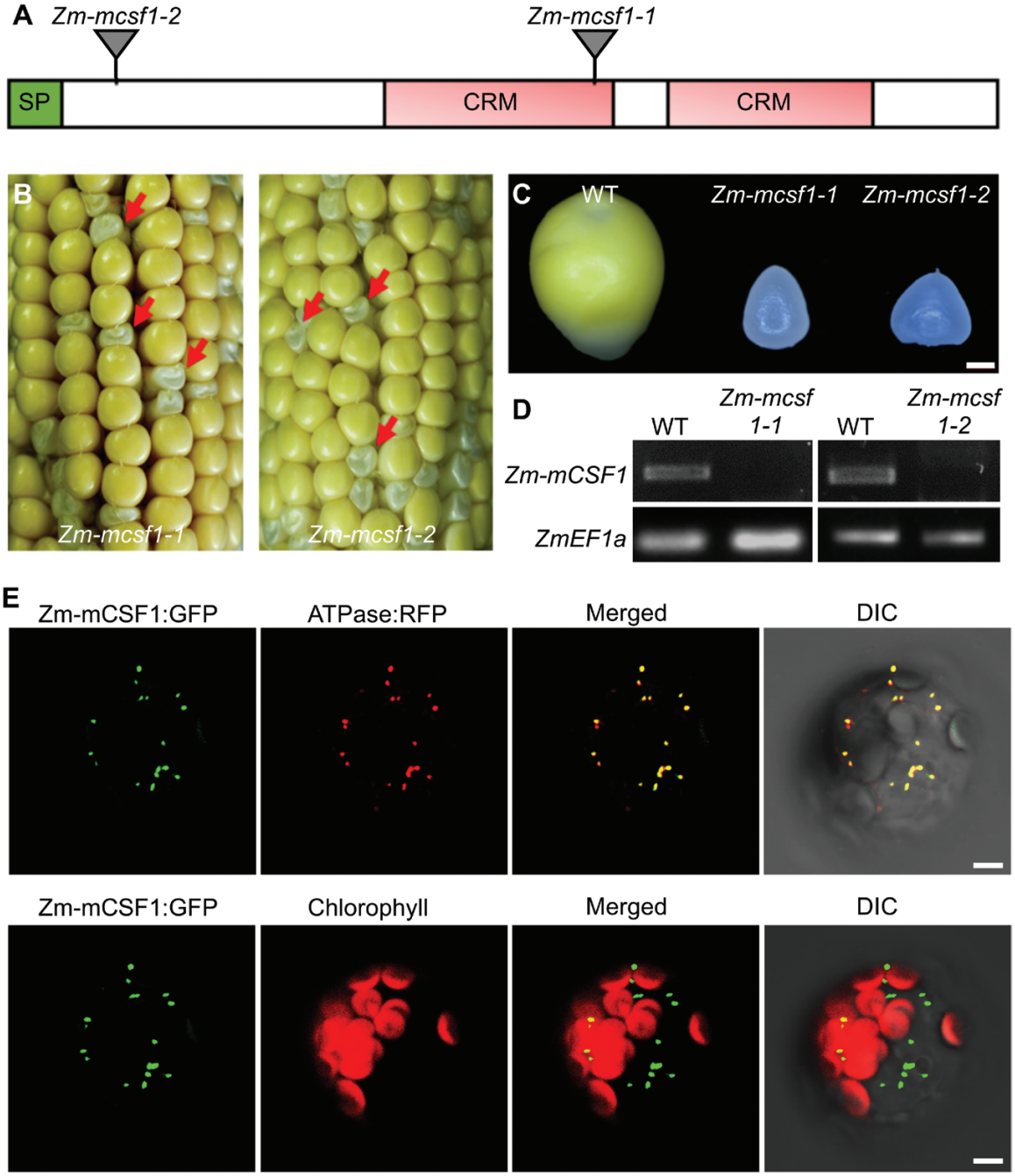

Fig. 3. Overview of *Zm-mCSF1*. (A) Diagram of the Zm-mCSF1 protein containing two CRS1-YhbY domains (CRM) with the positions of the *Mutator* insertions in alleles indicated. SP, signaling peptide. (B) Seed phenotype of *Zm-mcsf1* mutants. Ears of *Zm-mcsf1-1* and *Zm-mcsf1-2* were harvested at 15 d after pollination. The arrows indicate homozygous *Zm-mcsf1* seed mutants classified as *empty pericarp* (*emp*). (C) Comparison of endosperm morphologies of *Zm-mcsf1-1* and *Zm-mcsf1-2* with the wild-type (WT). (D) Reverse-transcription PCR demonstrating the absence of *Zm-mCSF1* mRNA in the *Zm-mcsf1* mutants. (E) Localization of the Zm-mCSF1 protein. Mesophyll protoplasts from Arabidopsis were transformed with Zm-mCSF1::GFP and ATPase::RFP constructs and imaged using confocal microscopy. In the upper images, mitochondria are labeled by fluorescence of ATPase::RFP, and in the lower images, chloroplasts are marked by autofluorescence. DIC, differential interference contrast. Scale bars are 1 mm in (C) and 5 μm in (E).

Self-interaction of Zm-mCSF1

The Y2H analysis showed Zm-mCSF1 to be a self-interacting protein (Fig. 5D). To confirm this, *in vitro* pull-down assays were performed. Zm-mCSF1 was purified as fusion proteins with MBP and GST, and the interaction was investigated via GST-mediated pull-down assays (Fig. 5E). MBP and GST proteins were used as negative controls, and could not be found in the elution after washes.

Identification of the interacting domains in the PPR-SMR1/Zm-mCSF1 proteins

To investigate the regions responsible for the interaction between PPR-SMR1 and Zm-mCSF1, we generated a series of proportional *PPR-SMR1* and *Zm-mCSF1* cDNAs and cloned them into the prey and bait constructs, respectively (Fig. 6A). The results showed that a construct containing the N terminus (NT, amino acids 49–193) of PPR-SMR1 was

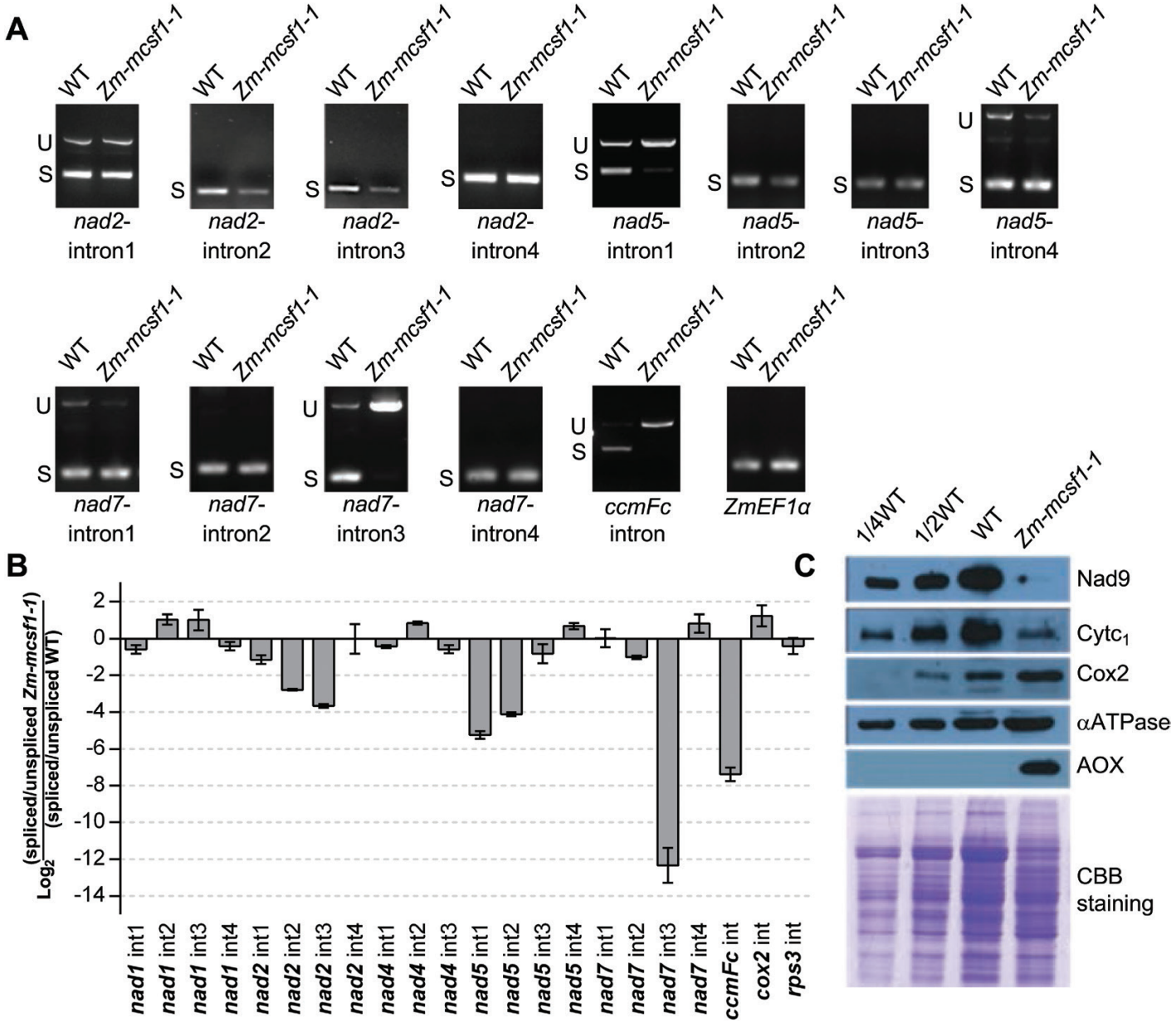

Fig. 4. Zm-mCSF1 is involved in splicing of maize group II introns. (A) Inefficient splicing of group II introns of *nad2*, *nad5*, *nad7*, and *ccmF*$_C$ in the endosperm of *Zm-mcsf1-1* mutants by reverse-transcription PCR (RT-PCR). S, introns are spliced; U, introns are retained. (B) qRT-PCR analysis of the 22 intron-containing mitochondrial transcripts in *Zm-mcsf1-1* mutants. RNA was extracted from the embryos and endosperms of *Zm-mcsf1-1* mutants and their wild-type siblings at 12 d after pollination. *ZmEF1*α was used to normalize the quantifications. Data are means (±SD) of three biological replicates. (C) Immunoblot analysis of core subunits of mitochondrial complexes in *Zm-mcsf1-1* mutants. Immunoblots of embryos and endosperms extract (20 μg protein or the indicated dilutions) were probed with antibodies specific for subunits of Complex I (Nad9), Complex III (Cytochrome c_1, Cytc$_1$), Complex IV (Cox2), and ATP synthase (αATPase). AOX, alternative oxidase. Total protein input can be seen in the Coomassie Brilliant Blue (CBB) staining below. (This figure is available in color at *JXB* online.)

sufficient for the interaction with mature Zm-mCSF1 (Fig. 6B), suggesting that the NT region of PPR-SMR1 with an unknown function could be involved in the interaction with Zm-mCSF1. In addition, constructs harboring the N terminus (NT, amino acids 30–161) or the CRM domain (amino acids 162–370) of Zm-mCSF1 were able to interact with the NT segment of PPR-SMR1 (Fig. 6D). However, neither the PPR domain (PPR-SMR1$_{194–652}$) nor the SMR segment (PPR-SMR1$_{653–787}$) could interact with the predicted mature Zm-mCSF1 or its split segments in the Y2H analysis (Fig. 6B, D). Zm-mCSF1 was able to interact with itself through the N-terminus (amino acids 30–161) and CRM domain (amino acids 162–370) (Fig. 6C).

Discussion

PPR-SMR1 and Zm-mCSF1 are involving in the splicing of multiple introns in mitochondria

Both the PPR-SMR1 and Zm-mCSF1 proteins were encoded by nuclear genes and targeted to mitochondria (Figs 1M, 3E), and contained mitochondrial signaling peptides as predicted

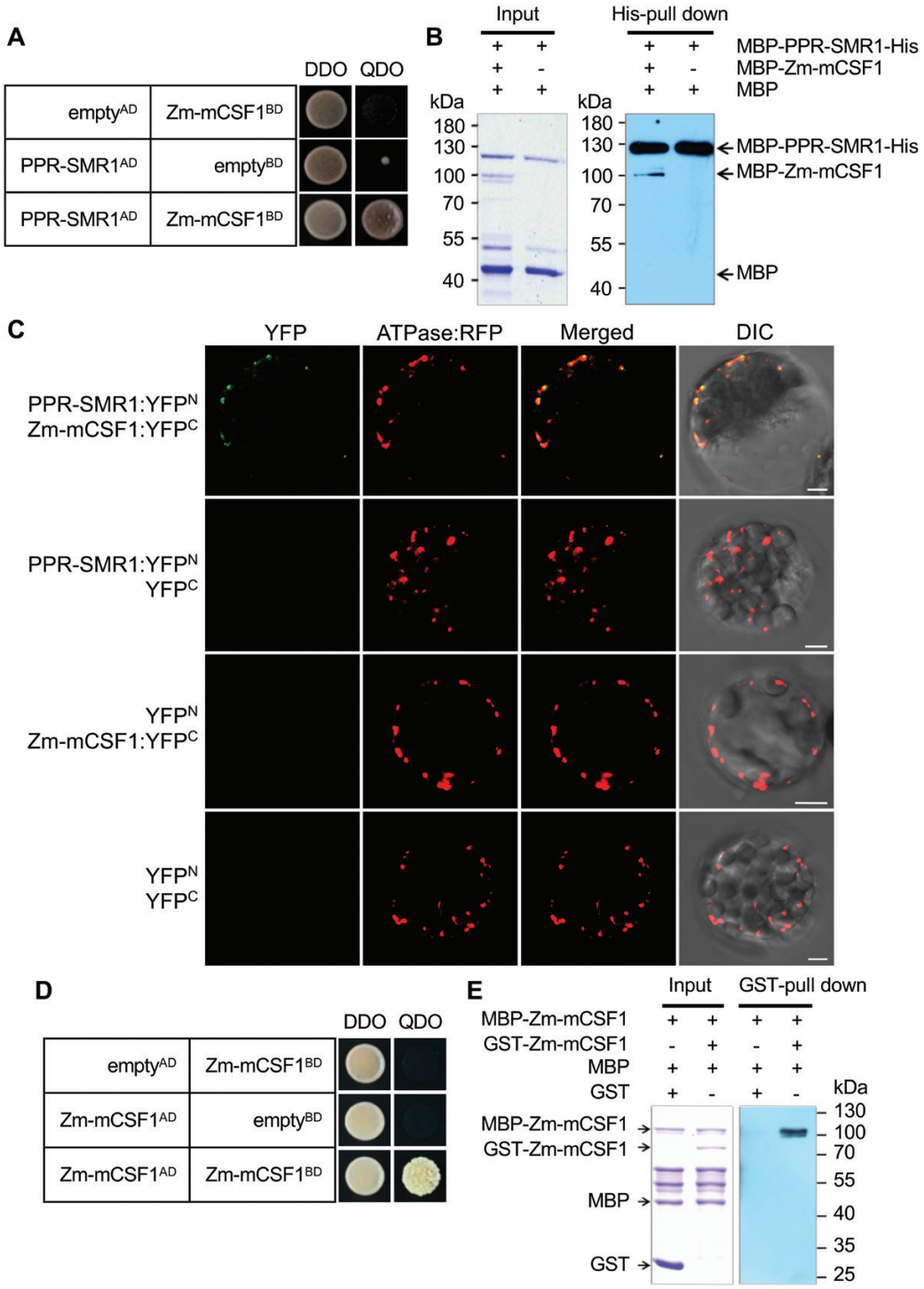

Fig. 5. PPR-SMR1 protein interacts with Zm-mCSF1. (A) Yeast two-hybrid (Y2H) analysis of PPR-SMR1 and Zm-mCSF1 interaction. The Y2HGold strain harboring the indicated bait and prey constructs were spotted on synthetic dropout (SD) medium without Leu and Trp (double-dropout, DDO) and SD without Ade, Leu, Trp, and His (quadruple-dropout, QDO). Yeast cultures on DDO control plates demonstrate the existence of both plasmids. Positive interactions were verified by growth on QDO plates. (B) Recombinant protein MBP-PPR-SMR1-His interacts with MBP-Zm-mCSF1 as determined using *in vitro* His pull-down assays. (C) Bimolecular fluorescence complementation (BiFC) analysis of the interaction of PPR-SMR1 and Zm-mCSF1 in mesophyll protoplasts of Arabidopsis. Yellow fluorescent protein (YFP) was split into N- and C-terminus, and PPR-SMR1 was fused with the N-terminus of YFP and Zm-mCSF1 was fused with C-terminus of YFP. Scale bars are 5 μm. (D) Zm-mCSF1^{AD} physically interacts with Zm-mCSF1^{BD} in Y2H assays. Yeast Y2HGold was transformed with paired constructs Zm-mCSF1^{AD} and Zm-mCSF1^{BD}, and AD (activating domain) and BD (binding domain) were used as negative controls. (E) Recombinant protein MBP-Zm-mCSF1 interacts with GST-Zm-mCSF1 as determined using *in vitro* glutathione S-transferase (GST) pull-down assays. '+' and '–' indicate the presence and absence of the corresponding proteins in the reactions, respectively.

by the TargetP and Predotar tools (Emanuelsson *et al.*, 2000). Using comparative analyses of the mutants and wild-type, we found evidence that loss-of-function mutations in each of these two genes caused defects in the splicing of multiple mitochondrial introns. The *Zm-mcsf1* mutants were defective in splicing of *nad2* introns 2 and 3, *nad5* introns 1 and 2, *nad7*

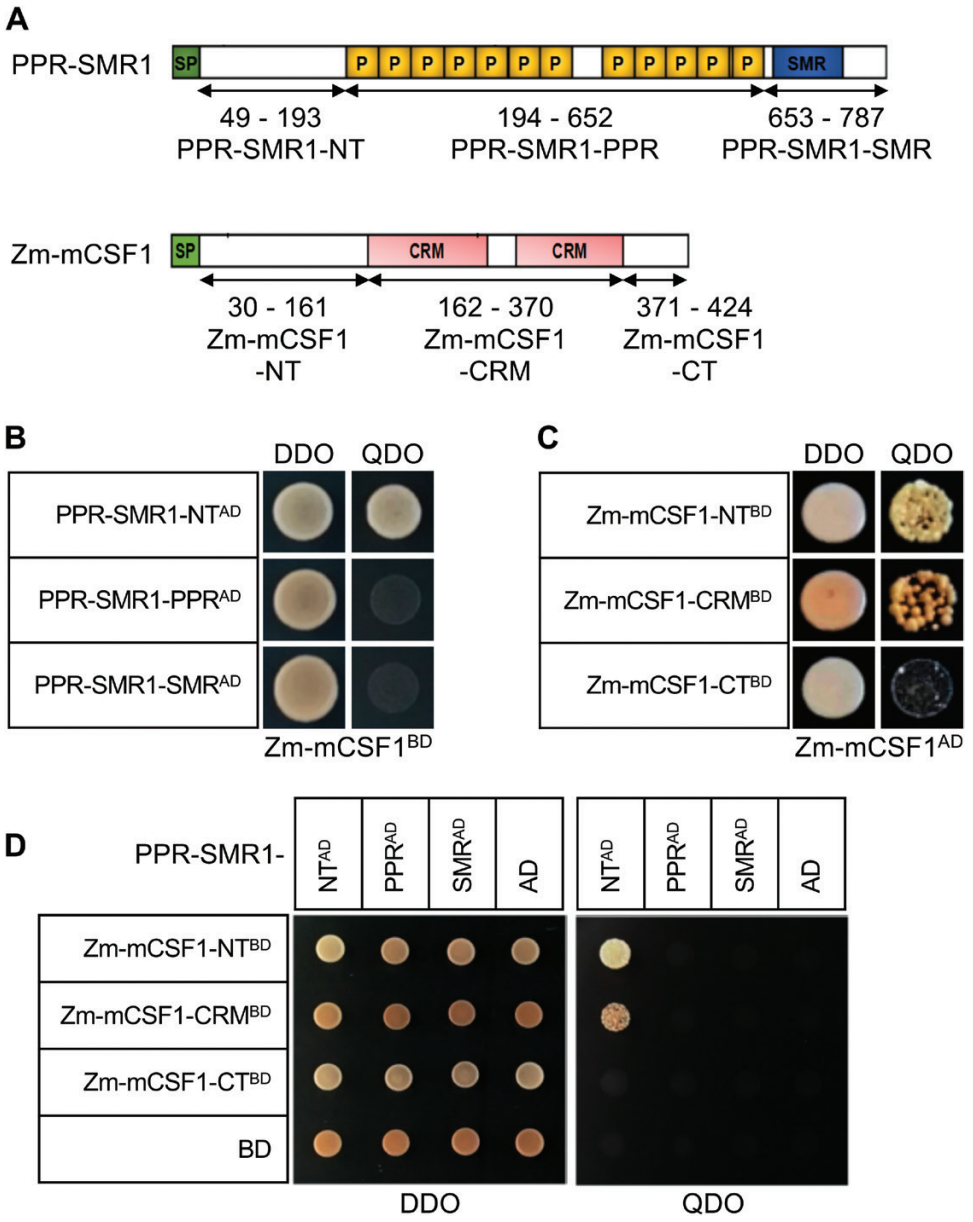

Fig. 6. Protein fragments required for interactions of maize PPR-SMR1 and Zm-mCSF1. (A) Schematic diagram of full-length and split fragment fusions of PPR-SMR1 and Zm-mCSF1 to the GAL4 activating domain (AD) or the GAL4 DNA binding domain (BD), respectively. The PPR-SMR1 protein is split into three fragments: PPR-SMR1-NT (N-terminus, 49–193), PPR-SMR1-PPR (194–652), and PPR-SMR1-SMR (653–787). The Zm-mCSF1 protein is split into three fragments: Zm-mCSF1-NT (N-terminus, 30–161), Zm-mCSF1-CRM (162–370), and Zm-mCSF1-CT (371–424). (B–D) Yeast two-hybrid (Y2H) assays demonstrating the physical interactions. The Y2HGold harboring the indicated bait and prey constructs were spotted on synthetic dropout (SD) medium without Leu and Trp (double-dropout, DDO) and SD without Ade, Leu, Trp, and His (quadruple-dropout, QDO). (B) PPR-SMR1-NT^{AD} physically interacts with Zm-mCSF1^{BD}. (C) Zm-mCSF1-NT^{BD} and Zm-mCSF1-CRM^{BD} interact with Zm-mCSF1^{AD}. (D) PPR-SMR1-NT^{AD} physically interacts with Zm-mCSF1-NT^{BD} and Zm-mCSF1-CRM^{BD}. (This figure is available in color at *JXB* online.)

intron 3, and the *ccmFc* intron (Fig. 4A, B). Four of their corresponding introns in Arabidopsis mitochondria required the involvement of mCSF1; however, the number of Zm-mCSF1-dependent introns was smaller than that of Arabidopsis mCSF1. Possible reasons for this may be that the highly degenerated sequences in these introns cannot be recognized by Zm-mCSF1, or that its homolog Zm-mCSF2 could take over and be required for the splicing of those introns. However, the functions of Zm-mCSF2 and Arabidopsis mCSF2 are still unknown due to the lack of loss-of-function mutations. Further efforts are needed to create knockout mutants for Zm-mCSF2 and mCSF2 and to characterize their molecular functions in maize and Arabidopsis, respectively. The *ppr-smr1* mutants were defective in splicing of a surprising 16 introns, namely *nad1* introns 1 to 4, *nad2* introns 1 to 4, *nad4* introns 1 to 3, *nad5* introns 1, 3, and 4, *nad7* intron 2, and the *rps3* intron (Fig. 2A, B). The failed splicing of these introns would undoubtedly cause a deficiency of these proteins. Because these Nad proteins are the essential subunits of mitochondrial complex I, which is the entry point of the oxidative phosphorylation (OXPHOS) machinery, the mitochondrial function will be severely impaired. This was manifested in several regards, including the impairment of mitochondrial complex assembly and the altered expression of OXPHOS component proteins (Figs 2C, 4C).

Failures in the cytochrome pathway of the OXPHOS machinery also caused up-regulation of AOX protein expression (Figs 2C, 4C), as has been documented previously (Xiu *et al.*, 2016; Sun *et al.*, 2019). Because mitochondria are the sites for the tricarboxylic acid cycle, NADH re-oxidization, reduction of oxygen, and ATP synthesis, they are essential to all cellular activities (Schertl and Braun, 2014). Thus, the dysfunction in mitochondria would be expected to impact seed development, resulting in severely inhibited embryogenesis and endosperm development in the mutants of *ppr-smr1* and *Zm-mcsf1*.

Unlike the P-type PPR proteins that are usually involved in the splicing of one or more introns (Xiu *et al.*, 2016; Cai *et al.*, 2017; Chen *et al.*, 2017; Qi *et al.*, 2017; Ren *et al.*, 2017), each of PPR-SMR1 and Zm-mCSF1 was required for the splicing of multiple introns in maize, particularly PPR-SMR1 that was required for nearly 75% of mitochondrial group II introns. Taken together, PPR-SMR1 and Zm-mCSF1 were required for the splicing of a subset of 19 mitochondrial introns, of which *nad2*-introns 2 and 3, and *nad5* intron 1 were dependent on both PPR-SMR1 and Zm-mCSF1. In addition, those known P-type PPR-dependent introns were also included in this subset of 19 mitochondrial introns. This raises questions as to whether PPR proteins and CRM domain-containing proteins are essential factors for the splicing of all introns in mitochondria, and whether PPR-SMR1 and Zm-mCSF1 are the major players of mitochondrial intron splicing. Since the group II introns in maize mitochondria are large in size (ranging from 792–3425 nt) and complex in structure with trans-introns (Supplementary Table S1), it appears that PPR-SMR1 and Zm-mCSF1 together with other P-type PPR proteins might be involved in the folding of catalytic RNAs (ribozymes) through directly binding to specific sequences within the corresponding introns to block random local RNA base-pairings, or through protein–protein interactions for long-range RNA domain interactions, thus increasing the splicing efficiencies of these introns.

PPR-SMR1 and Zm-mCSF1 may be involved in formation of large and dynamic splicing macromolecules

There is emerging evidence to suggest that large ribonucleoprotein (RNP) complexes related to intron splicing may be present in plant mitochondria (Zmudjak *et al.*, 2017). Moreover, an increasing number of splicing factors have been reported to be required for the splicing of distinct or overlapping mitochondrial introns, including the maturases nMAT1–4 (Keren *et al.*, 2009, 2012; Cohen *et al.*, 2014; Zmudjak *et al.*, 2017), PORR protein WTF9 (Kroeger *et al.*, 2009; Francs-Small *et al.*, 2012; Chettoor *et al.*, 2015), the CRM domain-containing protein mCSF1 (Zmudjak *et al.*, 2013), RUG3 (Kühn *et al.*, 2011), the RNA DEAD-box helicase PMH2 (Köhler *et al.*, 2010; Zmudjak *et al.*, 2017), and several P-type PPR proteins (Xiu *et al.*, 2016; Cai *et al.*, 2017; Chen *et al.*, 2017; Qi *et al.*, 2017; Ren *et al.*, 2017; Sun *et al.*, 2019). However, how these proteins participate in intron splicing is not known. Our results identified a novel central splicing factor PPR-SMR1 in maize and highlighted some of the conserved splicing functions of mCSF1 in plants. Notably, the CRM domain protein Zm-mCSF1 could form a homodimer and physically interact with PPR-SMR1 in Y2H, pull-down, and BiFC assays (Figs 5, 6). Given that many members of PPR proteins, CRM domain proteins, and other families of RNA-binding proteins are splicing factors, and the splicing of some particular introns required the involvement of PPR-SMR1, Zm-mCSF1, and some other P-type PPRs, it suggests that PPR-SMR1 and Zm-mCSF1 might be the core factors for forming large macromolecular complexes to mediate splicing of mitochondrial group II introns, and that these macromolecular complexes could also be able to interact through the interaction between PPR-SMR1 and Zm-mCSF1. However, further efforts need to be made to determine the binding properties for PPR-SMR1 and Zm-mCSF1, and PPR-SMR1 or Zm-mCSF1 should be used as targets to immunoprecipitate the large macromolecular complexes followed by protein mass spectrometry to reveal their members. Currently, we cannot exclude the possibility that these genes might have other activities.

In addition to mitochondria, similar protein complexes may exist to facilitate intron splicing in chloroplasts. Although the event of chloroplast endosymbiosis was 450 million years after that of mitochondria, the mechanism of intron splicing seems to be conserved in both organelles. In maize, chloroplasts have 15 group II introns in the genes *rps12*, *rps16*, *rpl2*, *rpl16*, *ndhA*, *ndhB*, *petB*, *petD*, *atpF*, and six *trn* genes (Maier *et al.*, 1995). Many PPR proteins are targeted to chloroplasts, and several of them are splicing factors that help to remove distinct introns (Barkan and Small, 2014). In addition, like CRM domain-containing proteins (Till *et al.*, 2001; Ostheimer *et al.*, 2003), the mTERF protein Zm-mTERF4 (Hammani and Barkan, 2014) and the PORR protein WTF1 have been reported to be required for splicing of introns in chloroplasts, and they have been found to be simultaneously present in a 0.55–1 MDa protein complex with potential physical interactions (Hammani and Barkan, 2014). Since the removal of plant organellar group II introns from pre-mRNAs requires the formation of ribozyme structures within introns, this protein complex could function in connecting those RNA domains (I–VI) to form the autocatalytic conformation to excise themselves from exons. In contrast to the nuclear intron splicing machinery that involves small nuclear RNAs (U1, U2, U4/U6, and U5 snRNPs) and ~170 distinct snRNA-associated proteins (Wahl *et al.*, 2009), the splicing of organellar group II introns probably preferentially involves the assistance of nuclear RNA-binding proteins, and evolution has favored nuclear control of organelle gene expression for an unknown reason of fitness.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. PPR-SMR1 and its homologs in Arabidopsis, rice, and sorghum.

Fig. S2. Co-segregation and allelism tests of *ppr-smr1* alleles and the *emp* phenotype.

Fig. S3. Altered expressions of mitochondrial transcripts in *ppr-smr1* and *Zm-mcsf1* endosperm.

Fig. S4. mCSF1 and mCSF2 of Arabidopsis and maize, and allelism test of *Zm-mcsf1* alleles.

Table S1. List of mitochondrial group II introns and their splicing efficiencies in *ppr-smr1* and *Zm-mcsf1* mutants.

Table S2. Primers used in this study.

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Author contributions

ZC and B-CT designed the research; ZC, H-CW, and JS conducted most of the experiments; ZC, FS, MW, CX, and B-CT analysed the data; ZC, H-CW, and B-CT wrote the manuscript.

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