

Two CRM protein subfamilies cooperate in the splicing of group IIB introns in chloroplasts

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ABSTRACT

Chloroplast genomes in angiosperms encode ~20 group II introns, approximately half of which are classified as subgroup IIB. The splicing of all but one of the subgroup IIB introns requires a heterodimer containing the peptidyl-tRNA hydrolase homolog CRS2 and one of two closely related proteins, CAF1 or CAF2, that harbor a recently recognized RNA binding domain called the CRM domain. Two CRS2/CAF-dependent introns require, in addition, a CRM domain protein called CFM2 that is only distantly related to CAF1 and CAF2. Here, we show that CFM3, a close relative of CFM2, associates in vivo with those CRS2/CAF-dependent introns that are not CFM2 ligands. Mutant phenotypes in rice and *Arabidopsis* support a role for CFM3 in the splicing of most of the introns with which it associates. These results show that either CAF1 or CAF2 and either CFM2 or CFM3 simultaneously bind most chloroplast subgroup IIB introns in vivo, and that the CAF and CFM subunits play nonredundant roles in splicing. These results suggest that the expansion of the CRM protein family in plants resulted in two subfamilies that play different roles in group II intron splicing, with further diversification within a subfamily to accommodate multiple intron ligands.

Keywords: group II intron; RIP-Chip; plastid; *Arabidopsis*; maize

INTRODUCTION

Group II introns are large ribozymes whose splicing involves the same chemical steps as spliceosome-catalyzed splicing in the nucleus (reviewed in Pyle and Lambowitz 2006). The mitochondrial and plastid genomes in land plants each encode ~20 group II introns; none of these have been reported to self-splice in vitro, and some lack intron motifs known to play important roles in splicing (for review, see Schmitz-Linneweber and Barkan 2007; Bonen 2008). Thus, most or all group II introns in plant organelles appear to have lost the capacity to self-splice even under the nonphysiological conditions that promote the self-splicing of several model group II introns. Indeed, the splicing of almost all of the introns in angiosperm chloroplasts has been shown to require the participation of nucleus-encoded proteins in vivo (reviewed in Schmitz-Linneweber and Barkan 2007).

The genetic identification of nuclear genes involved in chloroplast group II intron splicing led to the recognition

of a protein family defined by a novel RNA binding domain denoted the chloroplast RNA splicing and ribosome maturation (CRM) domain (also called the CRS1-YhbY domain/Pfam PF01985) (Till et al. 2001; Ostheimer et al. 2003; Barkan et al. 2007). This domain is derived from a prokaryotic pre-ribosome binding protein, and is found in a family comprising 14 orthologous groups in vascular plants (Barkan et al. 2007). Four CRM proteins have been shown to interact with group II introns in chloroplasts and to promote their splicing: CAF1, CAF2, CRS1, and CFM2. CAF1, and CAF2 are closely related paralogs, each with two CRM domains, that promote the splicing of different subsets of chloroplast subgroup IIB introns in heterodimeric complexes with the peptidyl-tRNA hydrolase homolog CRS2 (Ostheimer et al. 2003). CRS1 and CFM2 are closely related paralogs that belong to a different branch of the CRM protein family than CAF1/2 (see Fig. 1A): CRS1 is required for the splicing of the subgroup IIA *atpF* intron (Jenkins et al. 1997; Asakura and Barkan 2006), whereas CFM2 is required for the splicing of two subgroup IIB introns (*ndhA* and *ycf3-int1*) and for the splicing of the group I intron in *trnL-UAA* (Asakura and Barkan 2007). (See Fig. 11 below for summary of the intron specificities of each protein.) Coimmunoprecipitation assays showed that each of these proteins is found in stable ribonucleoprotein

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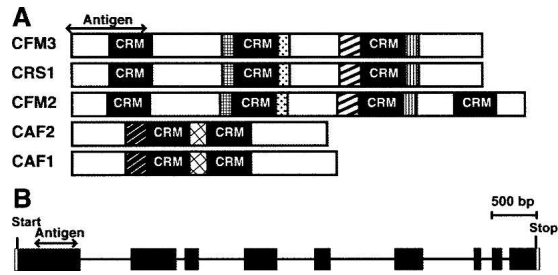


FIGURE 1. Graphical representations of CFM3. (A) Domain organization of CFM3 and previously characterized CRM-domain splicing factors. CFM3, CRS1, and CFM2 are in the “CRS1” subfamily, whereas CAF1 and CAF2 are in the “CAF” subfamily (Barkan et al. 2007). Regions of high sequence similarity are represented by like shading. There is no discernable sequence similarity between members of the CAF and CRS1 subfamilies outside of their CRM domains. The region used for production of CFM3 antisera is indicated. (B) The *ZmCfm3* gene. Exons are shown as rectangles and introns as lines.

complexes with those introns whose splicing it promotes (Ostheimer et al. 2003; Schmitz-Linneweber et al. 2005; Asakura and Barkan 2007).

To explore the functional repertoire of CRM domain proteins and to elucidate the protein machinery for plant organellar splicing, we are using reverse genetics and RNA coimmunoprecipitation assays to define the functions and RNA ligands of additional members of the CRM family. Here we present evidence that another member of the CRS1 subfamily, CFM3, acts in a nonredundant fashion with CRS2/CAF complexes to promote the splicing of a subset of group IIB introns in angiosperm chloroplasts. CFM2 and CFM3 interact with and promote the splicing of non-overlapping subsets of CRS2/CAF-dependent introns. Thus, most chloroplast subgroup IIB introns require either CAF1 or CAF2, together with either CFM2 or CFM3. The results suggest that CFM2/CFM3 paralogs and CAF1/CAF2 paralogs play distinct biochemical roles in splicing, and that diversification within each of these CRM protein subfamilies generated proteins that promote splicing in similar ways but that recognize distinct intron subsets.

RESULTS

CFM3 localizes to chloroplasts and mitochondria

Proteins in the angiosperm CRM domain family comprise 14 orthologous groups that can be grouped into four subfamilies according to their domain organization and sequence similarity (Barkan et al. 2007). Uncharacterized members of the family were assigned the names CRM Family Member (CFM) 1–11. CFM3 is in the “CRS1 subfamily,” which includes the group II intron splicing factors CRS1 and CFM2. CAF1 and CAF2 are members of a separate subfamily. The relationships between CAF1, CAF2, CRS1, CFM2, and CFM3 are diagrammed in Figure 1A. A phylogenetic tree of the CRS1 subfamily (Supplemental

Fig. 1) shows that CFM3 is represented by a single gene in rice (Os11g37990, denoted *OsCFM3*) and by two co-orthologs in *Arabidopsis* (At3g23070 and At4g14510); these genes are referred to here as *AtCFM3a* and *AtCFM3b*, respectively. Sequence for a putative maize ortholog was identified from public cDNA and genomic sequence data, and the sequence of a full-length cDNA was determined (GenBank Accession No. EU084957). Phylogenetic evidence for the orthology between the corresponding maize gene, *ZmCfm3*, and *Os/AtCFM3* is shown in Supplemental Figure 1. An alignment between the *Arabidopsis*, maize, and rice CFM3 proteins is shown as Supplemental Figure 2.

AtCFM3a, *AtCFM3b*, *ZmCFM3*, and *OsCFM3* are predicted to localize to chloroplasts and/or mitochondria by both the Target P (Emanuelsson and Heijne 2001) and Predotar (Small et al. 2004) algorithms. GFP fused to the N-terminal 113 amino acids of *ZmCFM3* localized to both chloroplasts and mitochondria when transiently expressed in onion epidermal cells (Fig. 2A). The analogous experiment with *OsCFM3* and *AtCFM3a* gave similar results, whereas the *AtCFM3b* transit peptide targeted GFP solely to chloroplasts (Fig. 2A).

An antibody was raised to a segment of *ZmCFM3* that does not show significant similarity to other plant proteins (amino acids 76–221). This antibody detected a protein of the size expected for mature CFM3 (~88 kDa) in chloroplast stroma and in mitochondria purified from green or etiolated maize leaf tissue, respectively (Fig. 2B). The GFP-fusion data together with the cell fractionation data demonstrate that *ZmCFM3* is dual-targeted to chloroplasts and mitochondria. Likewise, genetic data described below support the notion that *AtCFM3a* is dual targeted.

CFM3 and CFM2 associate in vivo with distinct subsets of CAF/CRS2-dependent group II introns

As an initial screen to identify the RNA ligands of *ZmCFM3*, we used a “RIP-Chip” assay in which RNAs that coimmunoprecipitate with *ZmCFM3* from chloroplast extract were identified by hybridization to a tiling microarray of the maize chloroplast genome (Schmitz-Linneweber et al. 2005). Immunoprecipitations were performed with antibodies from two different rabbits immunized with the CFM3 antigen. The results are summarized in Figure 3A as the log-transformed enrichment ratio for sequences corresponding to each array fragment, plotted according to chromosomal position. The previously reported data for CFM2 (Asakura and Barkan 2007) are plotted on the same graph for comparison (Fig. 3A, gray line). RNAs from six chloroplast loci showed strong enrichment in the CFM3 immunoprecipitations, in comparison with CFM2 and negative control immunoprecipitations (Fig. 3A; data not shown). The coimmunoprecipitation of these RNAs with CFM3 was validated by slot-blot hybridization of RNAs

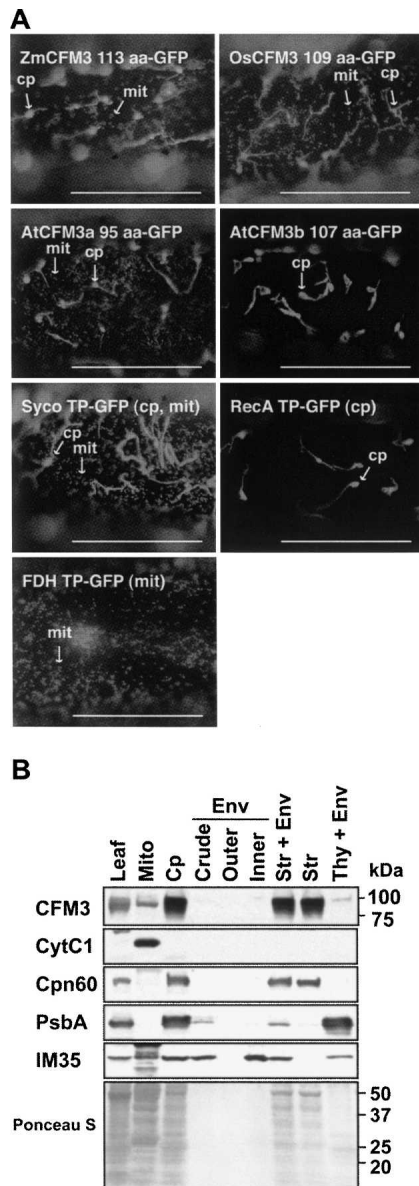


FIGURE 2. Subcellular localization of CFM3 orthologs. (A) Localization of GFP fused to the N terminus of maize (Zm), rice (Os), or *Arabidopsis* CFM3 orthologs when expressed transiently in onion epidermal cells. The number of CFM3 amino acids used in each construct is indicated. GFP fused to the transit peptide of chloroplast RecA, mitochondrial FDH, or Syco (cysteinyI-tRNA synthetase) (Peeters et al. 2000) were used as controls to visualize chloroplast, mitochondrial, or dual-targeted GFP. Bars=5 μ m. (B) Subcellular fractionation demonstrating the localization of ZmCFM3 to the chloroplast stroma and to mitochondria. Chloroplast (Cp) and chloroplast subfractions (Env, envelope membranes; Str, stroma; Thy, thylakoid membranes) were from a preparation described previously (Williams and Barkan 2003); each of these lanes contains material derived from the same amount of chloroplasts. Cytochrome C1 (Cyt C1), chaperonin 60 (Cpn60), the PsbA subunit of photosystem II (PsbA), and IM35 are markers for mitochondria, chloroplast stroma, thylakoid membranes, and chloroplast inner envelope membranes, respectively. The Ponceau S-stained filter is shown below.

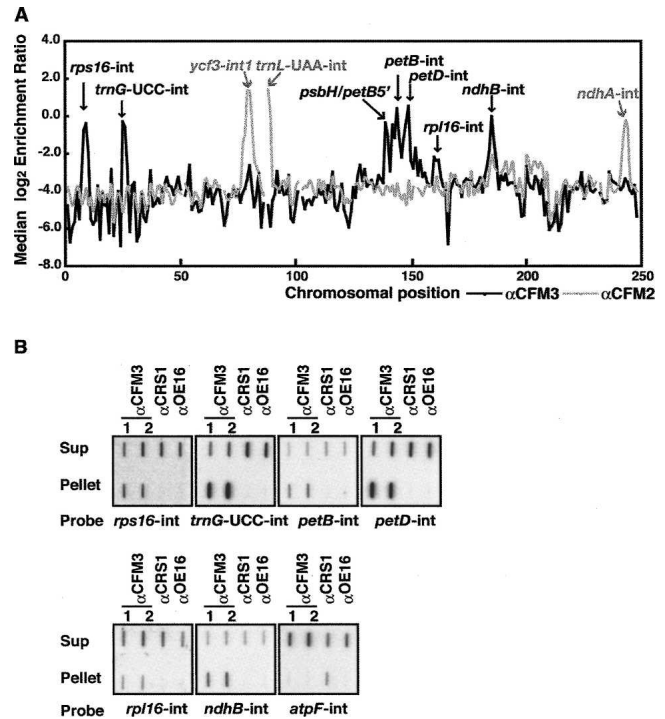


FIGURE 3. Identification of chloroplast RNA ligands of ZmCFM3 in coimmunoprecipitation assays. (A) Summary of RIP-Chip data demonstrating the different populations of chloroplast RNAs that associate with CFM3 and CFM2. The median log₂ transformed enrichment ratios (F635/F532, representing signal in the immunoprecipitation pellet/supernatant), are plotted according to chromosomal position for three replicate CFM3 RIP-Chip assays (black line). The RIP-Chip results reported previously for CFM2 (Asakura and Barkan 2007) are shown for comparison (gray line). Fragments for which fewer than two replicate spots per array yielded an F532 signal above background were excluded and appear as gaps in the curve. (B) Validation of RIP-Chip data by slot-blot hybridization. RNA purified from the pellets and supernatants of immunoprecipitations with antisera to the indicated proteins was applied to slot blots and hybridized to intron-specific probes. Antisera from different rabbits immunized with the CFM3 antigen were used in replicate assays (α CFM3 1 and 2). An immunoprecipitation with antibody to OE16, a protein in the thylakoid lumen that does not bind RNA, was used as a negative control. CRS1 is an *atpF*-specific chloroplast splicing factor and was analyzed as an additional control. One-third of the RNA recovered from each immunoprecipitation pellet and one-sixth of the RNA recovered from each supernatant was applied to each slot.

recovered from CFM3 immunoprecipitation pellets and supernatants (Fig. 3B).

These experiments showed that CFM3 is associated in vivo with RNAs from the *petB*, *petD*, *ndhB*, *rpl16*, *rps16*, and *trnG-UCC* loci. Each of these genes encodes a subgroup IIB intron that requires either CRS2/CAF1 or CRS2/CAF2 for its splicing. CFM2, by contrast, associates with RNAs from the *ycf3* and *ndhA* loci, which contain CRS2/CAF-dependent subgroup IIB introns, and the *trnL-UAA* locus, which contains a group I intron (Asakura and Barkan 2007). The single *trans*-spliced intron in land plant chloroplasts, *rps12-int1*, requires CRS2/CAF2 for its splicing

(Ostheimer et al. 2003) but is not represented among the ligands of CFM2 and CFM3. Thus, all of the *cis*-spliced introns in maize that require CRS2/CAF complexes for their splicing (*rps16*, *trnG-UCC*, *ycf3-int1*, *petB*, *petD*, *rpl16*, *ndhB*, *ndhA*) associate with either CFM2 or CFM3, but not both.

AtCFM3a and AtCFM3b act redundantly to promote an essential step in *Arabidopsis* seed development

To determine whether CFM3 promotes the splicing of the RNAs with which it associates, we sought *ZmCfm3* mutants in a reverse genetic screen of our collection of transposon-induced nonphotosynthetic maize mutants (Stern et al. 2004). Previous screens of this nature recovered mutations in many nuclear genes involved in chloroplast RNA splicing (*caf1*, *caf2*, *crs1*, *rnc1*, *ppr4*, and others), but we were unable to identify insertions in *ZmCfm3*. The mutant collection is near saturation for genes whose disruption results in chlorophyll-deficient seedlings (data not shown), so the failure to obtain *ZmCfm3* mutants suggests that disruption of this gene may not cause such a phenotype. A mitochondrial function for *ZmCFM3*, as suggested by its dual-targeting to chloroplasts and mitochondria, could result in an embryo-defective mutant phenotype, which would preclude representation of the mutant in our collection. Alternatively, a functionally redundant duplicate locus might account for the absence of *ZmCfm3* mutants in our collection, as partial sequence for a second *cfm3*-like gene can be detected in the available maize genome sequence data (data not shown).

Because genetic analysis of CFM3 in maize was problematic, we obtained *Arabidopsis* mutants with T-DNA insertions in *AtCFM3a* or *AtCFM3b* from the SALK and SAIL collections (Sessions et al. 2002; Alonso et al. 2003). Pale green seeds segregated among the progeny of plants heterozygous for each of two exonic insertions in *AtCFM3a* (*Atcfm3a-1* and *Atcfm3a-4*) (Fig. 4B). Approximately 40% of the pale seeds in each line failed to germinate; those that did germinate yielded very slow-growing seedlings (Fig. 4C) that are homozygous for the insertion (Supplemental Fig. 4A) and that lack *AtCFM3a* mRNA (Fig. 4D). The heteroallelic progeny of crosses between *Atcfm3a-1/+* and *Atcfm3a-4/+* plants exhibited these same phenotypes (Fig. 4B,C; Supplemental Fig. 4B), demonstrating that these phenotypes result from disruption of *AtCFM3a*. Thus, loss of *AtCFM3a* function results in an incompletely penetrant defective seed phenotype, with the “escapes” germinating to yield severely stunted plants.

Two T-DNA insertions in *AtCFM3b* likewise caused the loss of mRNA from the disrupted locus (Fig. 4D), but these did not cause a visible phenotype on their own. However, both insertions caused a synthetic phenotype when combined with a mutation in *AtCFM3a*: the progeny of self-pollinated

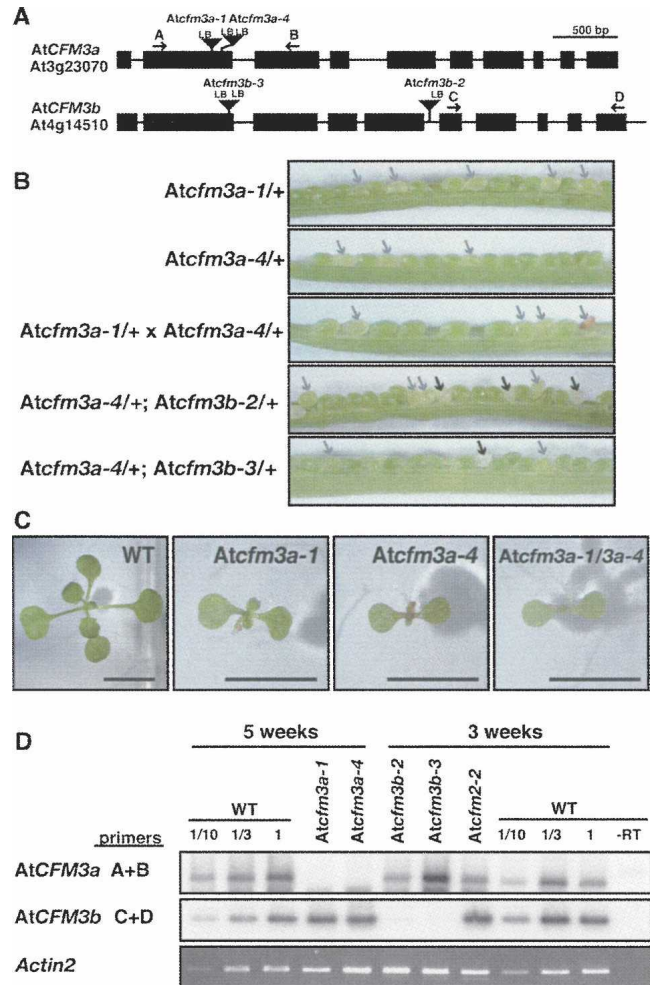


FIGURE 4. *AtCFM3a* and *CFM3b* insertion mutants. (A) T-DNA insertions in *AtCFM3a* (At3g23070) and *AtCFM3b* (At4g14510). Exons and introns are indicated by rectangles and lines, respectively. Arrows show primers used for RT-PCR. LB, T-DNA left border. The sequences flanking each insertion are shown in Supplemental Figure 3. (B) Seed phenotypes associated with insertions in *AtCFM3a* and *AtCFM3b*. The siliques shown result from crosses between plants of the indicated genotypes (self-pollination where just one genotype is shown). Pale green and white seeds are indicated with gray and black arrows, respectively. Approximately two-thirds of the pale green seeds germinated to yield slow-growing seedlings (see C), whereas approximately one-third did not germinate. The white seeds did not germinate. (C) Stunted seedling phenotype caused by insertions in *AtCFM3a*. Plants of the indicated genotype (homozygous unless otherwise indicated) were grown for 17 d on MS medium containing 2% sucrose. Plants homozygous for the insertions in *AtCFM3b* have no visible phenotype (data not shown). Bar=5 mm. (D) RT-PCR demonstrating a reduction in mRNA from the disrupted loci. Total leaf RNA (200 ng) was analyzed by RT-PCR using the primer pairs indicated to the left and diagrammed in A. The age of the plants used for RNA extraction is shown at the top; the very slow growth of *Atcfm3a* mutants required an extended period of growth to obtain sufficient material. The *Atcfm2-2* mutant was used as a control to show the mRNA levels in a different chloroplast splicing mutant. *Actin2* mRNA was used as an internal control. The dilution series of the wild-type sample demonstrates that the assay is roughly linear over the relevant concentration range. *CFM3* RT-PCR products were detected by DNA gel blot hybridization. The *actin2* RT-PCR product was detected with ethidium bromide.

plants that were heterozygous for either *AtCFM3b* mutant allele and for *Atcfm3a-4* segregated white seeds that failed to germinate (Fig. 4B). This phenotype was never observed among the progeny of plants harboring any of these mutant alleles on their own. Forty-seven germinating progeny of the double-heterozygotes were genotyped by PCR, and none of these were homozygous for both insertions (Supplemental Fig. 4C). The synthetic lethality resulting from the simultaneous disruption of both *AtCFM3* co-orthologs indicates that *AtCFM3a* and *AtCFM3b* act redundantly in a process that is necessary for seed development.

AtCFM3a is required for the splicing of the chloroplast *ndhB* intron

AtCFM3a homozygous mutants, although very slow growing, provided sufficient tissue for some analyses of organellar gene expression. Immunoblots were used to quantify components of the chloroplast multisubunit complexes that include plastid-encoded subunits (Fig. 5). The ribosomal protein Rpl2, the photosystem I subunit PsaD, the photosystem II subunit PsbA, the cytochrome *b₆f* subunit PetD, the ATP synthase subunit AtpA, and the Rubisco subunit RbcL accumulate to near normal levels in *AtCFM3a* mutants, indicating that chloroplast gene expression as a whole is not substantially compromised. The NdhH subunit of the chloroplast NADH dehydrogenase may be reduced slightly more than these other proteins.

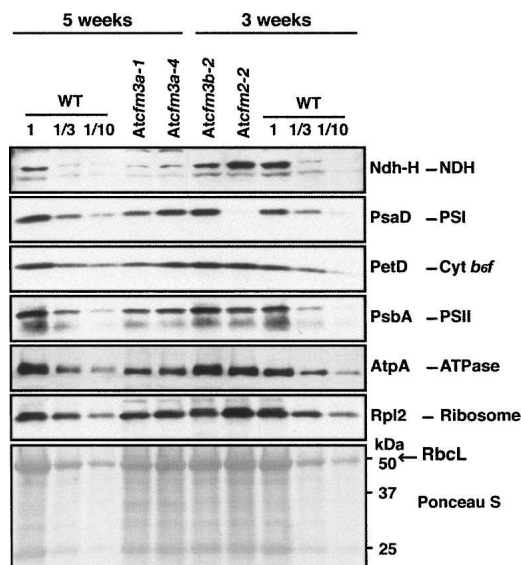


FIGURE 5. Immunoblot analyses of subunits of chloroplast protein complexes in *AtCFM3* mutants. Total leaf extract (20 μ g protein or dilutions as indicated) from seedlings of the ages indicated were analyzed on immunoblots by probing with antibodies for the proteins named to the right. The Ponceau S-stained membrane at the bottom illustrates sample loading and the abundance of the large subunit of Rubisco (RbcL).

These subtle protein defects are consistent with the fact that *AtCFM3a* mutants are not chlorophyll deficient, and contrast with the chlorosis and global loss of this same protein set in *AtCAF1* and *AtCAF2* mutants (Asakura and Barkan 2006).

The splicing of each chloroplast RNA whose ortholog coimmunoprecipitated with ZmCFM3 was analyzed in *Atcfm3* mutants. Poisoned-primer extension assays showed that the ratio of spliced to unspliced RNAs from the *ndhB* locus was dramatically reduced (Fig. 6A). RNA gel blot data showed that unspliced *ndhB* transcripts accumulate to increased levels, whereas the excised intron fails to accumulate in *AtCFM3a* mutants, confirming a defect in the splicing of the *ndhB* intron (Fig. 6B). The splicing of the five other introns orthologous to ZmCFM3's ligands, and of the three chloroplast introns that are found in *Arabidopsis* but not in maize (*clpP-int1*, *clpP-int2*, *rpoC1*), were unaffected in *AtCFM3a* mutants (Fig. 6A; Supplemental Fig. 5). A chloroplast defect specifically in the splicing of the *ndhB* intron in *Atcfm3a* mutants is consistent with their lack of chlorosis and their lack of gross plastid protein deficiencies, as an *Arabidopsis* mutant that fails to express *ndhB* due to a different RNA processing defect lacks a visible phenotype (Hashimoto et al. 2003). In contrast, the failure to splice any of the other chloroplast RNAs that associate with CFM3 would be expected to reduce the accumulation of one or more photosynthetic enzyme complex, compromise photosynthesis, and lead to chlorosis.

These results demonstrated that *AtCFM3a* is necessary for the splicing of the group II intron in the chloroplast *ndhB* pre-mRNA. Because *AtCFM3b* and *AtCFM3a* are, in part, functionally redundant, these results do not preclude a role for *AtCFM3a/b* in the splicing of additional introns. There is strong evidence that chloroplast translation is essential for embryogenesis in *Arabidopsis* (see, e.g., Stern et al. 2004; Duchene et al. 2005; Schmitz-Linneweber et al. 2006). Thus, a redundant role for *AtCFM3a* and *AtCFM3b* in the splicing of any of its ligands that function in translation (e.g., *trnG-UCC* or *rpl16*) could contribute to the defective seed phenotype of *Atcfm3a/Atcfm3b* double mutants.

OsCFM3 is required for the splicing of several CRS2/CAF-dependent introns

Mutants lacking plastid ribosomes in graminaceous plants (e.g., maize, barley, rice) have been reported frequently; these germinate to yield albino seedlings that die after several weeks, when seed reserves are exhausted (e.g., Walbot and Coe 1979; Hess et al. 1993; Jenkins et al. 1997; Williams and Barkan 2003; Gothandam et al. 2005; Schmitz-Linneweber et al. 2006). Therefore, we initiated a genetic analysis of the single *CFM3* ortholog in rice to bypass the difficulties introduced by the redundant

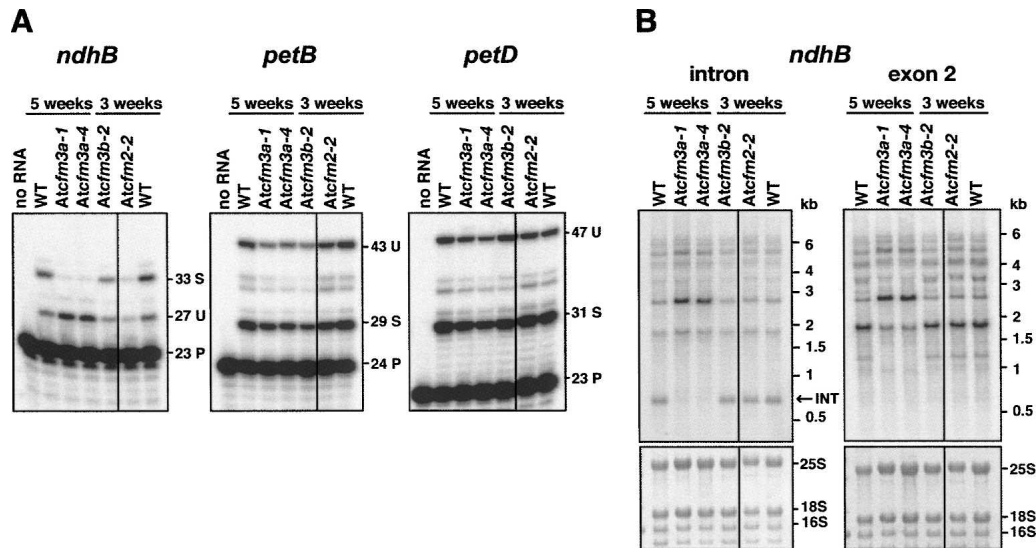


FIGURE 6. Chloroplast *ndhB* splicing defect in *AtCFM3a* mutants. (A) Poisoned-primer extension assays monitoring splicing of several introns. Ten micrograms of total leaf RNA from plants homozygous for the indicated alleles was analyzed. The length in nucleotides of each product is shown to the right. U, unspliced; S, spliced; P, primer. (B) RNA gel blots confirming an *ndhB* splicing defect in *AtCFM3a* mutants. Total leaf RNA (4 μ g) was probed with an intron-specific or exon 2-specific probe. The same blot stained with methylene blue is shown below, with rRNA bands marked. INT, excised intron.

embryo-essential functions of CFM3a and CFM3b in *Arabidopsis*.

We obtained a small number of seeds from the Postech insertion collection (Jeong et al. 2006) from a rice line with a T-DNA insertion in *OsCFM3*. Five of the seeds germinated, two of which were homozygous for the insertion in *OsCFM3* (Fig. 7B). The homozygous mutants had albino leaves (Fig. 7B) and contained little, if any, plastid rRNA (Fig. 7C). Albino leaves that lack plastid ribosomes would be expected to result from the failure to splice the CFM3 ligands *trnG-UCC*, *rps16*, and/or *rpl16*, each of which is essential for translation, and thus for synthesis of ribosomes themselves. A third rice seed gave rise to an albino plant that did not harbor the insertion in *OsCFM3* (Fig. 7B). This plant did, however, lack plastid rRNAs (Fig. 7C) and served as a control in subsequent experiments to reveal pleiotropic defects resulting from a plastid ribosome deficiency. This plant is referred to as the “ivory” control in experiments described below.

The number of experiments that could be performed with the *Oscfm3* mutant was limited by the very small amount of tissue available. Nonetheless, by using sensitive ribonuclease-protection assays, we were able to monitor the splicing of each RNA whose maize ortholog coimmunoprecipitated with ZmCFM3 (Fig. 8A). The results demonstrated that CFM3 is required for *ndhB* splicing in rice, as in *Arabidopsis*. The results also provided strong evidence that *OsCFM3* is necessary for the splicing of the *petD* and *rps16* RNAs: these spliced RNAs were virtually absent in the *Oscfm3* mutant, and were reduced less so in the ivory control mutant. The ribonuclease protection data further

suggested that *rpl16* splicing relies on *OsCFM3*, but noise in the data precluded an unambiguous interpretation. However, a poisoned-primer extension assay (Fig. 8B) confirmed the specific loss of spliced *rpl16* transcripts in the *Oscfm3* mutant. Ribonuclease protection and poisoned-primer extension assays demonstrated the absence of spliced *petB* RNA (Fig. 8A,B), and RNA gel blot hybridization demonstrated the absence of spliced *trnG-UCC* RNA (Fig. 8C) in the *Oscfm3* mutant, consistent with a requirement for CFM3 in their splicing. However, the control albino mutant also lacked these spliced RNAs so firm conclusions could not be made regarding a direct role for CFM3. The *yef3-int2* intron is spliced normally in the *Oscfm3* mutant (Fig. 8A), correlating with its lack of coimmunoprecipitation with ZmCFM3 (Fig. 3).

The genetic data from *Arabidopsis* and rice in conjunction with the RNA coimmunoprecipitation data from maize together provide strong evidence that CFM3 associates with and promotes the splicing of the chloroplast *ndhB*, *rpl16*, *rps16*, and *petD* introns. CFM3 also coimmunoprecipitates with the *trnG-UCC* and *petB* introns, and the genetic data are consistent with a requirement for CFM3 in their splicing.

CFM3 is associated in vivo with the group II intron splicing factors CAF1, CAF2, and RNC1

Among CFM3's RNA ligands are introns that also associate with CAF1, CAF2, and/or the RNAse III-domain splicing factor RNC1 (Ostheimer et al. 2003; Schmitz-Linneweber et al. 2005; Watkins et al. 2007). Therefore, it was of interest

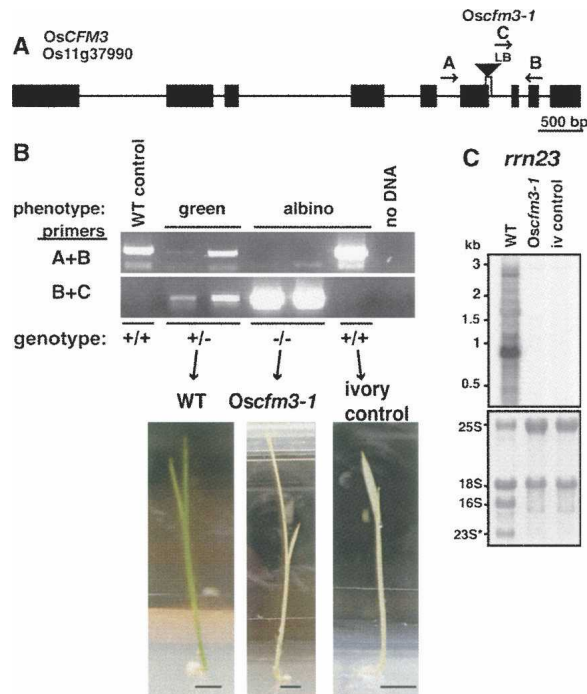


FIGURE 7. *OsCFM3* T-DNA insertion mutant. (A) T-DNA insertion in *OsCFM3*. Exons and introns are indicated by rectangles and lines, respectively. A deletion of a 3' splice site accompanied the T-DNA insertion, as shown by the vertical lines below the triangle. Arrows show the primers used for PCR genotyping. LB, T-DNA left border. (B) Phenotype and genotype of the five germinating plants from the Postech T-DNA line 3A-16097. Rice seedlings were grown for 3 wk on MS medium containing 2% sucrose. Bar=5 mm. The primer pairs used are indicated to the left and diagrammed in A. The inferred *OsCFM3* genotype is shown below. Two of the albino seedlings were homozygous for the insertion in *OsCFM3*. The third albino seedling lacked this insertion, and was used as a control for the RNA analyses (ivory control). (C) RNA gel blot showing loss of plastid 23S rRNA in *Oscfm3-1* mutant. The probe is from the 5' portion of the 23S rRNA gene and detects just one of the two 23S rRNA fragments that accumulates in vivo. Shown below is the same blot stained with methylene blue. 16S is the plastid 16S rRNA. 23S* marks the smaller of the two 23S rRNA fragments; the larger fragment comigrates with cytosolic 18S rRNA.

to determine whether CFM3 is found in the same ribonucleoprotein particles as these previously identified splicing factors. After sedimentation of stromal extract through a sucrose gradient, ZmCFM3 was found in a broad high molecular weight peak that sediments more rapidly than Rubisco (~550 kDa) (Fig. 9A) and that coincides with the peaks reported previously for RNC1, CAF1, and CAF2 (Watkins et al. 2007). Furthermore, antibodies to CFM3 coimmunoprecipitate each of these proteins from chloroplast extract and vice versa (Fig. 9B). Together, these cosedimentation and coimmunoprecipitation data provide strong evidence that CFM3, a CAF/CRS2 complex, and RNC1 associate simultaneously with their shared intron ligands.

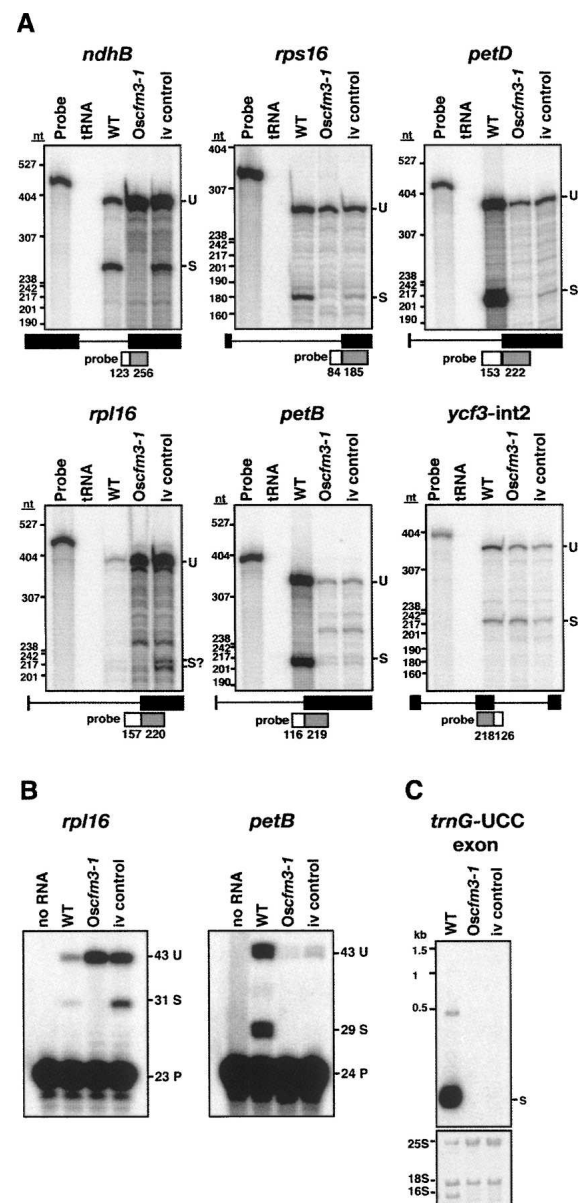


FIGURE 8. Chloroplast splicing defects in the *Oscfm3-1* mutant. RNA was analyzed from a normal seedling (WT), a *Oscfm3-1* homozygote, and from an *OsCFM3*⁺ albino mutant segregating in the same background (iv control). (A) Ribonuclease protection assays. Total leaf RNA (2 μg) was analyzed using probes spanning either the 3' or 5' splice junction, as diagrammed. The length in nucleotides of probe segments corresponding to intron and exon sequences are shown in the diagrams. The positions of DNA size standards are shown to the left. U, unspliced; S, spliced. (B) Poisoned-primer extension assays. Total leaf RNA (4 μg) was used in reverse-transcription reactions using primers mapping several nucleotides downstream of the indicated intron. A dideoxynucleotide present in each reaction terminates reverse-transcription after different distances on spliced and unspliced RNA templates. The length in nucleotides of each product is indicated. U, unspliced; S, spliced; P, primer. (C) RNA gel blot showing loss of *trnG-UCC* in *Oscfm3-1* mutant. Total leaf RNA (2 μg) was probed with a maize *trnG-UCC* exon probe. The same blot stained with methylene blue is shown below.

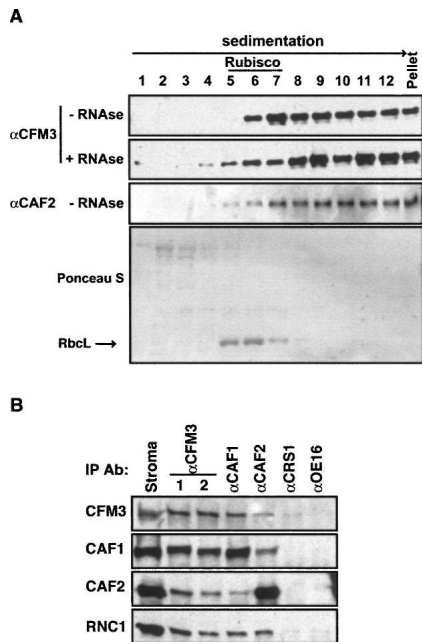


FIGURE 9. CFM3 is associated with CAF1 and CAF2 in large particles in the chloroplast stroma. (A) Untreated (–RNase) and ribonuclease-A-treated (+RNase) maize stromal extracts were sedimented through sucrose gradients under conditions in which particles greater than ~70S pellet. An equal portion of each fraction was analyzed on immunoblots by probing with CFM3 or CAF2 antiserum. The CFM3 blot stained with Ponceau S is shown below to illustrate the position of Rubisco in the gradient (RbcL, large subunit of Rubisco). The modest effect of RNase-treatment on the sedimentation of CFM3 particles is characteristic of group II intron ribonucleoprotein particles, which are resistant to RNase treatment (Till et al. 2001; Asakura and Barkan 2007; Watkins et al. 2007). CAF1 and RNC1 were shown previously to be recovered in analogous gradient fractions (Watkins et al. 2007). (B) CFM3 coimmunoprecipitates with CAF1 and CAF2. Chloroplast stroma was used for immunoprecipitations with the antibody indicated at the top. Antisera from two rabbits immunized with the CFM3 antigen were analyzed (lanes 1,2). Immunoblots of the immunoprecipitated proteins were probed with the antibodies indicated to the left.

Functions for CFM3 in mitochondria

GFP-fusion and cell fractionation data indicated that ZmCFM3 is dual targeted to chloroplasts and mitochondria. GFP-fusion data likewise suggested that AtCFM3a is dual-targeted. A mitochondrial function for AtCFM3a is supported by the slow-growth phenotype of AtCFM3a mutants because mutations in *Arabidopsis* that solely disrupt photosynthesis cause only a small reduction in growth rate when seeds are sown on medium containing sugars. For example, AtCAF1, AtCRS1, and AtCAF2 mutants are nearly albino, are severely deficient for numerous chloroplast-encoded proteins, yet grow much more rapidly than AtCFM3a mutants on MS medium (Asakura and Barkan 2006). The only molecular defect detected in the chloroplasts of AtCFM3a mutants was in *ndhB* splicing; this defect cannot account for their stunted growth, as *Arabidopsis* mutants lacking solely the chloroplast NDH

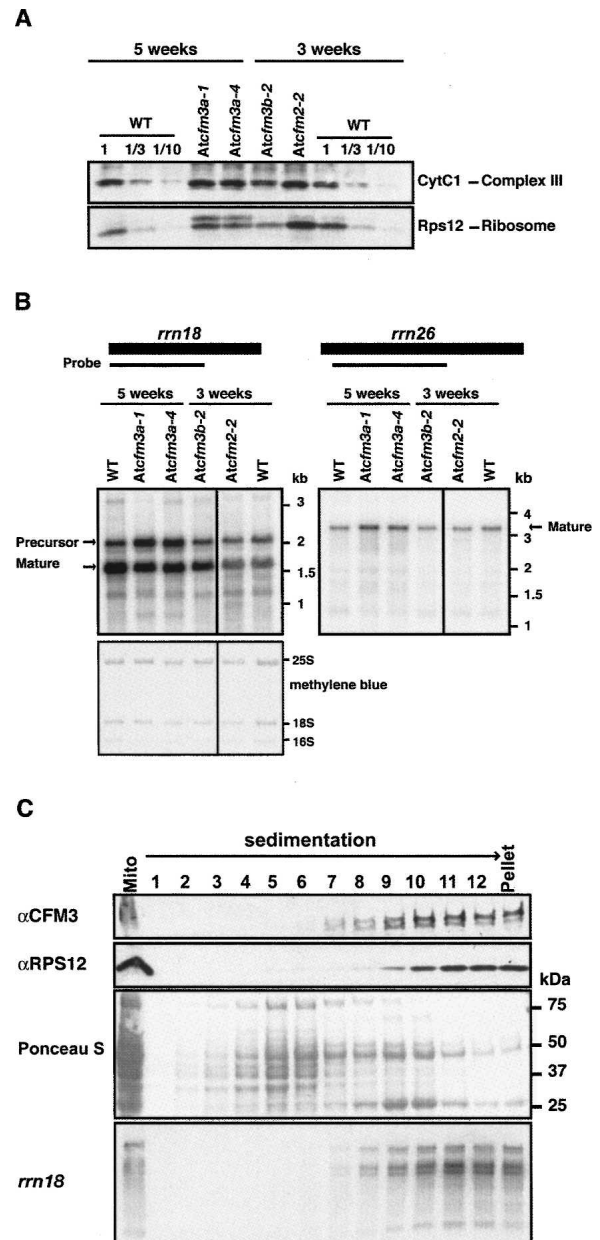


FIGURE 10. Analyses of CFM3 function in mitochondria. (A) Immunoblots of total leaf extract were probed with antibodies to cytochrome c1 or mitochondrial Rps12. The corresponding Ponceau S stained blot is shown in the bottom panel of Figure 5. (B) RNA gel blot analysis of total leaf RNA (0.5 μg), using probes from the mitochondrial *rrn18* and *rrn26* genes. The forms marked as “mature” are based on the annotated termini of these rRNAs. The methylene blue-stained blot is shown below. (C) Sucrose-gradient fractionation of maize mitochondrial extract. Maize mitochondria were solubilized with 1% NP-40 and sedimented through a sucrose gradient under conditions in which particles greater than ~70S pellet. An equal portion of each fraction was analyzed on immunoblots, by probing with CFM3 or RPS12 antiserum. RNA purified from the same fractions was analyzed by RNA gel blot hybridization, using a probe for *rrn18* (bottom panel).

complex have no visible phenotype (Hashimoto et al. 2003; Kotera et al. 2005).

To gain insight into the role of AtCFM3a in mitochondria, the abundance of several mitochondrial proteins was assayed in those *Atcfm3a* mutants that succeeded to germinate (Fig. 10A). Cytochrome c1, a nucleus-encoded subunit of the cytochrome *bc1* complex, accumulated to normal levels in these mutants. However, an antibody to the mitochondrially encoded ribosomal protein RPS12 detected an aberrant, slowly migrating isoform specifically in *Atcfm3a* mutants (Fig. 10A). The metabolism of mitochondrial 18S rRNA is also altered in these mutants: a large rRNA isoform (~2.1 kb), presumably a precursor, is overrepresented, whereas the presumed mature form (~1.7 kb) is underrepresented slightly (Fig. 10B). These results suggest that AtCFM3a influences the biogenesis of the mitochondrial small ribosomal subunit. Furthermore, ZmCFM3 cosediments with mitochondrial small ribosomal subunits, as indicated by its cosedimentation with 18S rRNA and RPS12 when mitochondrial extract was fractionated by sucrose gradient sedimentation (Fig. 10C). However, antibodies to ZmCFM3 did not unambiguously coimmunoprecipitate 18S rRNA from mitochondrial extract (data not shown). These results suggest that ZmCFM3 either associates with a low abundance intermediate in ribosome assembly, or that it influences ribosome metabolism indirectly and that its cosedimentation with ribosomes is coincidental. Clarification of the role of CFM3 in mitochondria will require further study.

DISCUSSION

The results presented here elucidate the nature of the chloroplast RNA splicing machinery and its relationship to the expansion and diversification of the CRM domain protein family in the plant lineage. The CRM domain family is derived from a single-domain prokaryotic protein whose extant representative in *Escherichia coli*, YhbY, functions in ribosome maturation (Barkan et al. 2007). The duplication of this ancestral gene to yield a family of single- and multidomain proteins appears to be confined to plants. All characterized members of the family in plants function in the splicing of chloroplast introns: 13 of the group II introns and the sole group I intron in angiosperm chloroplasts have been shown to require one or more CRM domain protein for their splicing (Fig. 11). Both the acquisition of group II introns in land plant chloroplasts and the expansion of the CRM domain family occurred after the divergence of land plants and chlorophytes (green algae) but prior to the divergence of vascular and non-vascular plants, suggesting that these two events may be evolutionarily linked.

The first three CRM domain splicing factors to be characterized, CRS1, CAF1, and CAF2, act on largely non-overlapping intron subsets, consistent with the possibility

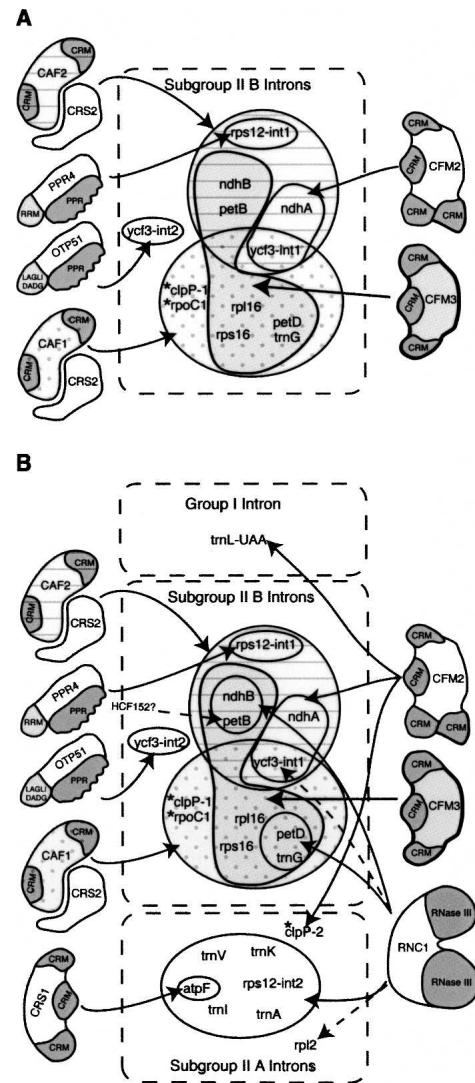


FIGURE 11. Chloroplast splicing factors in land plants. (A) Cooperation between CFM2/3 and CAF1/2 during the splicing of Subgroup IIB introns. The intron sets that require CAF1, CAF2, CFM2, or CFM3 are marked with distinct fill patterns. These assignments for CFM3 are based on a composite of the RNA coimmunoprecipitation data from maize, and the genetic data from rice and *Arabidopsis*. Rps12-int1 is the sole trans-spliced intron in land plant chloroplasts. Ycf3-int2 is the sole subgroup IIB intron whose splicing does not require a CAF/CRS2 complex. Introns found in *Arabidopsis* but not in maize and rice are marked with asterisks. Current data are consistent with the possibility that the AtCFM3 coorthologs play redundant roles in the splicing of the *clpP-1* and *rpoC1* introns, but such a role has not been demonstrated. (B) Summary of nucleus-encoded chloroplast splicing factors in land plants. The group II introns are divided into subgroup IIA and IIB according to Michel et al. (1989). Solid arrows mark interactions supported both by splicing defects in mutants and by RNA coimmunoprecipitation data. Dashed arrows mark interactions supported by either genetic or coimmunoprecipitation data, but not both. A role for the plastid-encoded protein MatK in subgroup IIA splicing has been proposed (reviewed in Bonen and Vogel 2001). HCF152 is required for the accumulation of spliced *petB* RNA but not for the accumulation of excised intron (Meierhoff et al. 2003). Results are summarized from this work and from Jenkins et al. (1997), Vogel et al. (1999), Ostheimer et al. (2003), Schmitz-Linneweber et al. (2005), Asakura and Barkan (2006), Schmitz-Linneweber et al. (2006), Asakura and Barkan 2007; Watkins et al. (2007), and de Longevialle et al. (2008).

that different CRM proteins interact with their intron substrates in similar locations and play similar roles in splicing. However, with the recent characterization of CFM2 and CFM3 that scenario is no longer plausible. CFM2 interacts with and promotes the splicing of two introns that also require CRS2/CAF complexes (*ycf3-int1* and *ndhA*) (Asakura and Barkan 2007). Data presented here show that CFM3 associates with all but one of the CRS2/CAF-dependent introns that do not associate with CFM2, and that there is no overlap among the intron sets that coimmunoprecipitate with CFM2 and CFM3 (see summary in Fig. 11A). Genetic data demonstrate that CFM3 promotes the splicing of four of the RNAs with which it associates (*ndhB*, *rpl16*, *rps16*, and *petD*), and are consistent with a role for CFM3 in the splicing of its remaining two ligands, the *trnG* and *petB* RNAs. However, the redundancy of the two CFM3 co-orthologs in *Arabidopsis* and the absence of these spliced RNAs in a different albino rice mutant preclude firm conclusions about *trnG* and *petB*. It seems likely, however, that CFM3 does promote the splicing of these introns, given the perfect correspondence between the set of introns that coimmunoprecipitate with each previously characterized CRM splicing factor (CRS1, CAF1, CAF2, CFM2) and the set that fails to splice in the corresponding mutants (Ostheimer et al. 2003; Schmitz-Linneweber et al. 2005; Asakura and Barkan 2007).

The division of labor among the CAF1/2 and CFM2/3 CRM proteins in the splicing of chloroplast subgroup IIB introns is summarized in Figure 11A. CRS2 functions in concert with either CAF1 or CAF2 to promote the splicing of all but one of the subgroup IIB introns. CRS2/CAF complexes, in turn, function in concert with either CFM2 or CFM3 to promote the splicing of all but one of the CRS2/CAF-dependent introns. The genetic disruption of either the CAF or CFM subunit of each ribonucleoprotein particle blocks splicing, indicating that these subunits play nonredundant roles. These results suggest that the closely related paralogs CAF1 and CAF2 play similar biochemical roles in splicing but bind different introns, and that the situation is analogous for the closely related paralogs CFM2 and CFM3. CRS1, which is in the same CRM subfamily as CFM2 and CFM3, has been shown to interact with domains 1 and 4 in its *atpF* intron substrate (a subgroup IIA intron), and to enhance intron folding (Ostersetzer et al. 2005); it will be interesting to determine whether CFM2 and CFM3 function analogously. CAF1 and CAF2, in contrast, recruit the splicing factor CRS2 to specific introns, and this may be their primary role. CRS2 is a peptidyl-tRNA hydrolase homolog that plays an unknown but essential role in the splicing of all but one of the subgroup IIB introns in the chloroplast.

The *rps12-int1* and *ycf3-int2* introns are the only plant chloroplast subgroup IIB introns that associate with neither CFM2 nor CFM3 (Fig. 11A). These introns are non-canonical in other ways as well. *rps12-int1* is the sole plant

chloroplast intron that is *trans*-spliced, and this intron requires a dedicated splicing factor called PPR4 (Schmitz-Linneweber et al. 2006). *ycf3-int2* is the only chloroplast subgroup IIB intron that splices independently of a CAF/CRS2 complex. This intron also relies on a dedicated splicing factor, called OTP51 (de Longevialle et al. 2008). Interestingly, both PPR4 and OTP51 consist of pentatricopeptide repeats (PPRs) followed by an accessory RNA binding domain: an RRM domain in the case of PPR4 and a LAGLIDADG domain in the case of OTP51. Although CRS1-like CRM splicing factors have not been identified for *rps12-int1*, *ycf3-int2*, or for most of the chloroplast subgroup IIA introns (Fig. 11B), two members of the CRS1 subfamily have not yet been analyzed.

Our results show that CFM3 is a dual-targeted protein that localizes not only to chloroplasts, but also to mitochondria. GFP fused to the N terminus of ZmCFM3, AtCFM3a, or OsCFM3 localized to both chloroplasts and mitochondria in transient expression assays. Furthermore, ZmCFM3 was detected in purified chloroplast and mitochondrial fractions by immunoblotting. Finally, the AtCFM3a mutant phenotype points to a mitochondrial function: *Atcfm3a* mutants grow very slowly even when supplied with exogenous sugars, unlike mutants whose defects are limited to photosynthesis. Furthermore, aberrant mitochondrial 18S rRNA metabolism and an aberrant mitochondrial RPS12 protein isoform were detected in these mutants. Elucidation of the precise role of CFM3 in mitochondria and whether this function is conserved among species will require further study.

To explore the functions of CFM3 we took advantage of three different organisms (maize, rice, and *Arabidopsis*). The information obtained from each was incomplete, but together the data provide a compelling picture for the role of CFM3 in chloroplast splicing. The consistency of the findings among the three species strongly suggests that CFM3's functions in the chloroplast are largely conserved among monocots and dicots, like those of CRS1, CAF1, and CAF2 (Asakura and Barkan 2006). Despite the powerful genetic resources available for *Arabidopsis*, its utility in this study was limited by the lineage-specific *CFM3* gene duplication and by the embryo-lethality resulting from the disruption of both *CFM3* co-orthologs. The embryo essentiality of chloroplast translation in *Arabidopsis* complicates the genetic analysis of nuclear genes that are necessary for the expression of any essential chloroplast ribosomal protein, tRNA or rRNA (Williams and Barkan 2003; Asakura and Barkan 2006; Schmitz-Linneweber et al. 2006; Asakura and Barkan 2007). Several such RNAs are included among CFM3's RNA ligands (*rpl16*, *rps16*, *trnG-UCC*); an essential role for AtCFM3 in the metabolism of any of these RNAs could account for the embryo-lethality associated with simultaneous disruption of both *AtCFM3a* and *AtCFM3b*. These introns fail to splice in the *Oscfm3* mutant, and in *crs2* and *caf1* mutants in maize (Jenkins

et al. 1997; Ostheimer et al. 2003); these and other mutants lacking plastid ribosomes in the grasses germinate to yield albino, seedling lethal mutants whose molecular defects can be analyzed. Thus, the use of a variety of model organisms will be necessary to achieve a comprehensive understanding of the mechanisms underlying chloroplast gene expression and its control in higher plants.

MATERIALS AND METHODS

Sequence analysis of maize *Cfm3* cDNAs

The nucleotide sequence of *OsCfm3* (Os11g37990) was used to identify closely related maize cDNAs at the POGs (<http://plantbrp.uoregon.edu/>) and Plant GDB (<http://www.plantgdb.org/>) databases. Corresponding maize cDNAs from the Arizona full-length cDNA collection (Accession numbers; DR823593.1 and EB158689.1) were obtained and sequenced. EB158689.1 was truncated at the 5' end, and DR823593.1 included an intron with an in-frame stop codon. To confirm the sequence of exon 1, a DNA fragment was amplified from our maize seedling leaf cDNA library (inbred line B73) by nested PCR using primers as follows: first PCR, YA654Cfm35' (5'-AGGGTCAAGGTCTCTGGCAC-3') and YA1191Cfm3' (5'-AGCAAAGTGAGGAGGCAGT-3'); nested PCR, YA680Cfm35' (5'-GCTCAATATGCGCCTCTTCC-3') and YA1153Cfm33' (5'-TTGGTAGTGTCCCTTCGGCT-3'). The PCR product was cloned into pGEM-T (Promega) and its sequence was determined. The *ZmCfm3* cDNA sequence deposited in GenBank (Accession No. EU084957) is the composite sequence derived from these three cDNAs.

Plant growth and extract preparation

Maize chloroplasts were purified from the leaves of seedlings (inbred line B73; Pioneer HiBred) grown in soil in a growth chamber at 28°C under a 16-h light (400 $\mu\text{E m}^{-2} \text{S}^{-1}$)/8-h dark cycle for several days until the coleoptiles emerged, transferred to the dark for ~6 d until two leaves have emerged, and then shifted to the light for ~12 h prior to harvest. Maize chloroplasts and stromal extract were prepared as described previously (Ostheimer et al. 2003). Maize chloroplast subfractions were from the same preparation described in Williams and Barkan (2003).

Maize mitochondria were purified from the leaves of seedlings grown in soil in a growth chamber under a 16-h light (28°C; 400 $\mu\text{E m}^{-2} \text{S}^{-1}$)/8-h dark cycle for 4 d, then incubated in the dark at 28°C for 6 to 7 d. Mitochondria were purified on sucrose step gradients as described in Newton (1994). Mitochondrial extract for sucrose gradient analysis was obtained by suspending the mitochondria in a minimal volume of 1% NP-40, 50 mM Tris-HCl at pH 8.0, 7 mM MgCl_2 , 150 mM KOAc, 2 mM DTT, 2 $\mu\text{g/mL}$ aprotinin, 2 $\mu\text{g/mL}$ leupeptin, 1 $\mu\text{g/mL}$ pepstein, 1 mM phenylmethylsulfonyl fluoride, 20 $\mu\text{L/mL}$ RNasin [Promega], and subjecting them to three freeze-thaw cycles. After addition of glycerol to 10% (v/v), the lysate was divided into small aliquots and stored at -80°C.

Arabidopsis T-DNA insertion lines were obtained from the Salk Institute Genomic Analysis Laboratory: SALK_125653 (*Atcfm3a-1*), SAIL_595_F03 (*Atcfm3a-4*), SALK_039803 (*Atcfm3b-2*), and SAIL_23_C02 (*Atcfm3b-3*). *Atcfm2-2* (SAIL_67_E05), which conditions pale green seedling-lethal mutants with several chloroplast

splicing defects (Asakura and Barkan 2007), was used as a mutant control. *Arabidopsis* plants used for RNA and protein analysis were 3-wk-old (*Atcfm3b-2* and *Atcfm2-2*) or 5-wk-old (*Atcfm3a-1* and *Atcfm3a-4*) seedlings grown on MS plates containing 2% sucrose, as described previously (Asakura and Barkan 2006). Total *Arabidopsis* leaf protein and RNA were extracted as described previously for maize (Barkan 1998).

The rice T-DNA insertion line 3A-16097 was obtained by Maureen Hanson (Cornell University) from POSTECH (Jeong et al. 2006). An albino mutant plant that segregated among the progeny but that lacked the T-DNA insertion in *OsCFM3* was used as a control for RNA analyses. The cultivar "Dongjing," which is the background from which this line was derived, was used as the source of wild-type RNA. The growth of the rice seedlings was performed at Cornell University by John Robbins. Rice seeds were surface sterilized and planted on Murashige and Skoog medium (0.5% [w/v] Caisson agar [Caisson Laboratories, North Logan, Utah], 1 \times Murashige and Skoog salts [Sigma-Aldrich], 1 \times Gamborg's B5 vitamin [Sigma-Aldrich], and 2% [w/v] sucrose at pH 5.8) until the third leaf stage under 12-h light (400 $\mu\text{E m}^{-2} \text{S}^{-1}$; 28°C)/12-h dark (21°C) cycle.

Production of anti-CFM3 antisera

A segment of *ZmCFM3* (amino acids 76–221) was expressed in *Escherichia coli* (BL21 Star (DE3), Invitrogen) as a 6xHis-tagged fusion protein from vector pET-28b(+) (Novagen). Protein expression was induced by the addition of 0.5 mM isopropyl-thio- β -D-galactopyranoside for 3 h at 37°C. The recombinant CFM3 fragment was recovered in inclusion bodies, which were solubilized with 8 M urea, 50 mM Tris-HCl at pH 7.5, and 300 mM NaCl. The protein was affinity purified with Ni-NTA agarose beads (Qiagen) according to the manufacturer's protocol. Ten milligrams of purified protein was dialyzed into PBS containing 2 M urea. Polyclonal antisera were generated in two rabbits at Alpha Diagnostic Inc. Antisera were affinity purified against the antigen coupled to HiTrap NHS-activated column (GE Healthcare Life Science).

Transient expression of GFP fusion proteins

Sequences encoding the N-terminal 113 amino acids of *ZmCFM3* were amplified by PCR from the *ZmCfm3* cDNA with the primers YA1Cfm35' (SpeI) (5'-CGTACTAGTATGGCCATGGCGTCTC-3') and YA339Cfm33' (KpnI) (5'-CGTGGTACCCGACGTCCTC GACCGACCCCC-3'). Sequences encoding the N-terminal 109 amino acids of *OsCFM3* were amplified by nested PCR from rice leaf DNA with the following primers: first PCR, YAOs257Cfm35' (5'-AAAATATCCTCACCCGCTAAACC-3') and YAOs1089Cfm33' (5'-AGCAATTCAGCATTTTCCCGCT-3'); nested PCR, YAOsCFM3-F (SpeI) (5'-CTTACTAGTATGGCCGCGCGCCATGGCC-3') and YAOsCFM3-R (KpnI) (5'-CTTGGTACCGAGCTCCGGGG GGGAGTAGGA-3'). Sequences encoding 95 and 107 amino acids of *AtCFM3a* and *AtCFM3b*, respectively, were amplified by nested PCR with the following primers: *AtCFM3a*, first PCR, *AtCfm3-7* 5' (5'-TTCTATGTGAATAAACAACAAT-3') and *AtCfm3-8* 3' (5'-TTAGTCTGATTCGGGAAGAGT-3'); nested PCR, *YAAtCfm3GFPF* (SpeI) (5'-CTTACTAGTATGGCGATGAAGCCAGTCTC-3') and *YAAtCfm3GFPR* (KpnI) (5'-CTTGGTACCTTTCTTTTCGA TAATTCACAAC-3'); *AtCFM3b*, first PCR, *AtCfm4-11* 5' (5'-GC GTAGCTGAAGGAATCAAAGGT-3') and *Atcfm4-6* (5'-GTTGC

TGCCACCTTTGTAATTG-3'); nested PCR, YAAAtCfm4GFPF (SpeI) (5'-CTTACTAGTATGGCGATTAATCAAGTC-3') and YAAAtCfm4GFPR (KpnI) (5'-CTTGGTACCTCTCACCACCTATA ACCCTAA-3'). PCR products were digested with SpeI and KpnI and cloned into pOL-LT, which was kindly supplied by Ian Small (University of Western Australia). The particle bombardment into onion epidermal tissues was performed as described previously (Barkan et al. 2007). Samples were observed 2–3 d after the bombardment using an Axoplan microscope (Zeiss) at 40× magnification with a FITC filter.

DNA isolation and PCR-genotyping

DNA was isolated from *Arabidopsis* as described previously (Asakura and Barkan 2006). T-DNA insertions were confirmed by PCR, using T-DNA left border primer LBa1 (5'-TGGTTCACG TAGTGGGCCATCG-3') for *Atcfm3a-1* and *Atcfm3b-2*, or LB(SAIL) (5'-TTCATAACCAATCTCGATACAC-3') for *Atcfm3a-4* and *Atcfm3b-3*. Gene specific primer pairs were as follows: *Atcfm3a-1* and *Atcfm3a-4*, *AtCfm3-5(787)* (5'-GGGAGATTTTCTGGGTCG GA-3') and *cfm32* (5'-CCGGCCAGTCCATGAACCTA-3'); *Atcfm3b-2*, *Atcfm4-3* (5'-GAAGTTGGAAAGGAACTTGCT-3'), and *Atcfm4-4* (5'-CGTTTTTGAAGCTCAAGAGAA-3'); *Atcfm3b-3*, *Atcfm4-5* (5'-ATTGGAATAGAACACAGAAGC-3'), and *Atcfm4-6* (5'-GTTGCTGCCACCTTTGTAATTG-3'). DNA was isolated from rice using Plant DNAzol reagent (Invitrogen) according to the manufacturer's instructions. The T-DNA insertion was confirmed by PCR using the T-DNA left border primer OsLB2(pGA2715_Postec) (5'-AAACGTCCGCAATGTGTTATT-3'), and the gene-specific primer pair OsYA4702Cfm35' (5'-CAGGCAGGAGAGGAGTTTT TGAT-3') and OsYA5411Cfm33' (5'-CACATCTTCTCGTCAC TGGAAAT-3').

Protein analyses

Antisera to the chloroplast proteins NDH-H, IM35, Cpn60, and RPL2 were kindly provided by Tsuyoshi Endo (Kyoto University), Danny Schnell (University of Massachusetts), Harry Roy (Rensselaer Polytechnic Institute), and A. Subramanian (University of Arizona), respectively. Antisera to maize mitochondrial RPS12, yeast cytochrome c1, and maize MDH were kindly provided by Michael Mulligan (University of California–Irvine), Masato Nakai (Osaka University), and Kathy Newton (University of Missouri), respectively. Antisera against maize CAF1, CRS1, CAF2, RNC1, PsbA, PsdD, PetD, and AtpA were generated by us and described previously (Roy and Barkan 1998; Till et al. 2001; Ostheimer et al. 2003; Watkins et al. 2007). Immunoblots and coimmunoprecipitations were performed as described in Ostheimer et al. (2003) except that immunoprecipitated proteins were detected on immunoblots using the One-Step Western Kit (GenScript Corporation).

RNA analyses

RNA-coimmunoprecipitation and the analyses of coimmunoprecipitated RNAs by RIP-Chip and slot-blot hybridization were carried out as described in Barkan (2008). Three replicate immunoprecipitations were performed for CFM3 RIP-Chip assays: two with antiserum from one rabbit, and one with antiserum from a second rabbit. RNA extraction, RNA gel blot hybridization and ribonuclease protection assays were performed as described previously (Jenkins et al. 1997). The probes for RNA-gel blot hybridization

of the *Arabidopsis* genes were amplified from *Arabidopsis* leaf DNA by PCR with the following primers: *ndhB* intron, YAAAtndhBF2 (5'-CGTTTGAGAAATTCCTACCTCTCT-3') and YAAAtndhBR3 (5'-AAAAGAGGATTCCTCACTTCTTTC-3'); *ndhB* exon 2, YAAAtndhB Ex F3 (5'-TCTCCCCTCCAGTCGTTGCT TT-3') and YAAAtndhB Ex R4 (5'-AAAAAGGGTATCCTGAGCAA TCG-3'); *rrn18*, YAAAt mit *rrn18F1* (5'-ATCATAGTCAAAAAGAAG AGT-3') and YAAAt mit *rrn18R1* (5'-TCAATTACTAGGTCTGGC AC-3'); *rrn26*, YAAAt mit *rrn26F1* (5'-TTAGTGTAGGCGCTTCC AA-3'), and YAAAt mit *rrn26R1* (5'-GTTTACCGGGGCTTCCA TTC-3'). Poisoned primer extension assays were performed as described previously (Asakura and Barkan 2006). RT-PCR analysis of cytosolic mRNA levels was performed using 200 ng of total leaf RNA, as described previously (Asakura and Barkan 2007). Primers for RT-PCR were as follows: *AtCFM3a*, same primer pair as for PCR genotyping; *AtCFM3b*, *AtCfm4-9* 5' (5'-ACCACACTGAGG CCTAAAAAC-3') and *AtCfm4-10* 3' (5'-AAGTTCCTCATTCTGC AGTTCTA-3'); *Actin2*, YAAAt actin2 F (5'-GAAGCTCTCCTTTGT TGCTGTT-3') and YAAAt actin2 R (5'-TTAGAAACATTTTCTGTG AACGA-3'). Probes used to detect RT-PCR products on DNA gel blots were amplified from *Arabidopsis* leaf DNA using the same primers as used for RT-PCR, and were radiolabeled by random hexamer priming in the presence of [α - 32 P]dCTP.

Rice RNA was extracted from the leaves of plants grown for 3 wk on MS medium containing 2% sucrose, using Trizol reagent. The probes for ribonuclease-protections were amplified from rice total DNA, and cloned into pBluescript SK+ (Stratagene). The primers used for PCR were as follows: *ndhB*, YAOs_ndhBF (SpeI) (5'-CTTACTAGTAATCAAAAAAGAAAGAAGACAA-3') and YAOs_ndhBR (HindIII) (5'-CTTAAGCTTTTGTAGTCTCCA ACAATTATTCC-3'); *petB*, YAOs_petBF2 (SmaI) (5'-CTTCCCG GGTGAACCTTTTTTCTAATATCC-3') and YAOs_petBR (HindIII) (5'-CTTAAGCTTCTCGTCAATTATGTATGAACCGA-3'); *petD*, YAOs_petDF (SpeI) (5'-CTTACTAGTCTTACTTAGAGCC ATTGAGCCA-3') and YAOs_petDR (HindIII) (5'-CTTAAGCTT GGTAATATTTCCAGAGGAGTTG-3'); *rps16*, YAOs_rps16F (SpeI) (5'-CTTACAGTGCAAGTCATATTCGAGCCGT-3') and YAOs_rps16R (HindIII) (5'-CTTAAGCTTTTCCGCCTTCCTTAA AATATCA-3'); *rpl16*, YAOs_rpl16F (SpeI) (5'-CTTACTAGTCTA TTAATGTGTATCTATCCGT-3') and YAOs_rpl16R (HindIII) (5'-CTTAAGCTTTTATTGTAACCGGTTTGTCGGG-3'); *ycf3* intron 2, YAOs_ycf3-2F (SpeI) (5'-CTTACTAGTCAATTCAGTTAAGA CAAGGA-3') and YAOs_ycf3-2R (XhoI) (5'-CTTCTCGAGGC GCTTCGTAATCTTCAACCAG-3'). Poisoned primer extension assays for rice used the following primers and dideoxynucleotide: *rpl16*, YAOs_rpl16-R5PPE, 5'-TGTTGTTTACGAAATCTGGTTC T-3', ddCTP; *petB*, YAOs_petB-R2PPE, 5'-ACGTTCTCAAACC AATCATATAC-3', ddCTP. The RNA gel blot probe for *trnG-UCC* was the maize cDNA probe described previously (Watkins et al. 2007). The *rrn18* probe was amplified from rice total DNA using primers; YAOs_rrn18-F (5'-AAAATCTGAGTTTGATCCT GGC-3') and (5'-AAAATCTGAGTTTGATCCTGGC-3').

Sucrose gradient fractionation of chloroplast and mitochondrial extract

Three hundred microliters of maize mitochondrial extract (~0.9 mg protein) was precleared by centrifugation at 21,000g for 30 min at 4°C. The supernatant was applied to a 10 to 30% sucrose gradient containing KEX buffer [30 mM HEPES-KOH at pH 8, 150

mM KOAc, 10 mM Mg(OAc)₂, and 5 mM DTT] and centrifuged in a Beckman SW55.1 rotor at 50,000 rpm for 4 h at 4°C. The sucrose-gradient fractionated maize chloroplast stroma was the same preparation described previously (Watkins et al. 2007).

Accession number

Sequence data for the maize *CFM3* cDNA can be found in the GenBank data library under Accession No. EU084957.

SUPPLEMENTAL DATA

Supplemental material can be found at <http://www.rnajournal.org>.

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NOTE ADDED IN PROOF

AtCFM3a mutants fail to splice the mitochondrial *nad1*-intron 2 group 2 intron, confirming that AtCFM3a plays a role in mitochondrial gene expression.

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