RNA PROCESSING FACTOR3 Is Crucial for the Accumulation of Mature *ccmC* Transcripts in Mitochondria of Arabidopsis Accession Columbia[1][W][OA]

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RNA PROCESSING FACTOR1 (RPF1) and RPF2 are pentatricopeptide repeat (PPR) proteins involved in 5′ processing of different mitochondrial mRNAs in Arabidopsis (*Arabidopsis thaliana*). Both factors are highly similar to RESTORERS OF FERTILITY (RF), which are part of cytoplasmic male sterility/restoration systems in various plant species. These findings suggest a predominant role of RF-like PPR proteins in posttranscriptional 5′ processing. To further explore the functions of this group of proteins, we examined a number of T-DNA lines carrying insertions in the corresponding PPR genes. This screening identified a nearly complete absence of mature *ccmC* transcripts in an At1g62930 T-DNA insertion line, a phenotype that could be restored by the introduction of the intact At1g62930 gene into the mutant. The insertion in this nuclear gene, which we now call RPF3, also leads to a severe reduction of the *CcmC* protein in mitochondria. The analysis of C24/*rpf3-1* F2 hybrids lacking functional RPF3 genes revealed that this gene has less influence on the generation of the mature *ccmC* 5′ transcript end derived from a distinct *ccmC* 5′ upstream configuration found in mitochondrial DNAs from C24 and other accessions. These data show that a particular function of an RF-like protein is required only in connection with a distinct mtDNA configuration. Our new results further substantiate the fundamental role of RF-like PPR proteins in the posttranscriptional generation of plant mitochondrial 5′ transcript termini.

Plant mitochondria contain a complex genetic framework to realize the genetic information encoded in their DNA (Kubo and Newton, 2008). These systems include many different proteins required for the posttranscriptional processing of the organellar transcripts. Among these factors, pentatricopeptide repeat (PPR) proteins play a crucial role (Delannoy et al., 2007; Schmitz-Linneweber and Small, 2008). In higher plants, PPR proteins form one of the largest protein families, comprising more than 400 members in Arabidopsis (*Arabidopsis thaliana*). PPR proteins, which are characterized by degenerate 35-amino acid repeats arranged in tandem, predominantly localize to chloroplasts or/and mitochondria and take part in virtually all processes affecting RNA (Small and Peeters, 2000; Lurin et al., 2004). This includes processes that link the formation of RNA extremities to RNA stability. In chloroplasts, this is achieved by specific binding of the PPR proteins to the target RNA (Loiselay et al., 2008; Pfalz et al., 2009; Johnson et al., 2010). The binding leads to the formation of so-called caps, which define the RNA ends by building barriers to 5′ or/and 3′ exonucleases. This relatively simple mechanism, which has been found for PPR10 from maize (*Zea mays*) and similarly for two other proteins from *Chlamydomonas*, might account for the formation and stabilization of the majority of chloroplast RNAs. But PPR proteins affect endonucleolytic processing reactions as well. For instance binding of PPR5 protects a distinct tRNA[Gly] precursor transcript from endonucleolytic cleavage in maize chloroplasts (Beick et al., 2008).

In mitochondria, nuclear-encoded PPR proteins also influence processing, accumulation, and stability of RNAs. Of the investigated proteins most function as RESTORERS OF FERTILITY (RF) in various cytoplasmic male sterility (CMS)/restoration systems described in different species. The majority of RF proteins reconstitutes male fertility by altering transcript levels and/or patterns of CMS-related mitochondrial genes, which reduces expression of the corresponding CMS-associated proteins (Budar and Pelletier, 2001; Bentolila et al., 2002; Brown et al., 2003; Desloire et al., 2003; Koizuka et al., 2003; Wang et al., 2006; Schmitz-Linneweber and Small, 2008). Up to now, the exact mechanisms influencing the steady-state levels of the CMS-specific transcripts are mostly unknown. The sole exception is the Boro II CMS/restoration system in rice (*Oryza sativa*). Here RF1A stimulates the cleavage of

the CMS-related *B-atp6/orf79* dicistronic transcript probably by directing an unknown endonuclease to a specific cleavage site (Wang et al., 2006; Kazama et al., 2008). Analogous functions have been suggested for RNA PROCESSING FACTOR1 (RPF1) and 2. These PPR proteins, which are highly similar to RFs, participate in 5′ processing of the major transcripts of the genes encoding subunits 4 and 9 of NADH dehydrogenase (*nad4* and *nad9*) and subunit 3 of cytochrome *c* oxidase (*cox3*) in mitochondria of the autogamous species Arabidopsis, where no CMS lines have been described (Jonietz et al., 2010; Hölzle et al., 2011).

Here, we report the identification of RPF3, which is required for formation of the mature mRNA of the gene encoding cytochrome *c* maturation protein C (*ccmC*) in mitochondria of the Arabidopsis accession Columbia (Col). The gene was identified by screening mutants with T-DNA insertions in *RF*-like PPR genes. The presence of the T-DNA in the *RPF3* gene provokes a nearly complete absence of the mature *ccmC* transcripts, accompanied by an increase of corresponding precursor RNAs. The recovery of *ccmC* transcript accumulation by the introduction of the intact *RPF3* gene into the mutant unambiguously demonstrates its function in 5′ processing of *ccmC* transcripts. In contrast to Col, RPF3 is less important for the formation of a mature *ccmC* 5′ transcript end, which is generated from a specific mtDNA configuration found in C24 and other accessions (Forner et al., 2008). In the *rpf3-1* mutant, the extremely reduced amount of the *CcmC* protein, a potential component of the cytochrome *c* biogenesis system, has no negative influence on the activity of the mitochondrial respiratory chain or on plant fitness.

RESULTS

At1g62930 Is Required for the Accumulation of *ccmC* Transcripts

We have recently identified two genes involved in the generation of 5′ ends of *nad4*, *nad9*, and *cox3* mRNAs in mitochondria of Arabidopsis (Jonietz et al., 2010; Hölzle et al., 2011). Both genes, *RPF1* (At1g12700) and *RPF2* (At1g62670) encode PPR proteins with high similarity to RF active in CMS-restoration systems in various plant species (Budar and Pelletier, 2001; Bentolila et al., 2002; Brown et al., 2003; Desloire et al., 2003; Koizuka et al., 2003; Wang et al., 2006). This suggests an important role of RF-like PPR proteins in 5′-end formation of mitochondrial mRNAs in the autogamous species Arabidopsis. Therefore, a circularized RNA (CR)-reverse transcription (RT)-PCR approach was used to analyze major mRNA extremities in various lines with T-DNA insertions in *RF*-like PPR genes (data not shown). In almost all mutants, no aberrant PCR product patterns were found when compared to wild type. This indicates that these PPR genes do not play an important role in the generation of main 5′ and 3′ ends of mitochondrial transcripts. However, in the SAIL line 18E04, we could

not amplify a CR-RT-PCR product that would be consistent with the previous results obtained for the *ccmC* mRNAs in accessions Col, Landsburg *erecta* (L*er*), and C24 (560 and 470 bp, respectively; Forner et al., 2008). Instead, comparatively weak products of about 700 and 760 bp were generated in the mutant (Fig. 1A). These results suggested the absence of the mature *ccmC* mRNA in this mutant.

To examine the *ccmC* transcripts by an alternative approach, a northern-blot analysis was performed with a probe covering the *ccmC* reading frame (from position 208 to 607 with respect to the ATG). In Col, L*er*, and C24 total RNA, *ccmC* transcripts of about 1,200 (Col and L*er*) and 1,100 (C24) nucleotides were detected, which is consistent with previous mapping results (Fig. 1B; Forner et al., 2007, 2008). In addition, weak signals corresponding to RNAs with sizes of about 1.4, 1.8, and 2.6 kb were seen in all RNA samples. In contrast, almost no mature *ccmC* mRNA was detected in the SAIL 18E04 mutant. Instead, the approximately 2.6-kb RNA was found to be increased. In addition, approximately 1.35- and 2.15-kb *ccmC* RNAs were detected, which were not seen in the wild-type RNAs of the different accessions (Fig. 1B). The elevated accumulation of the mutant-specific larger *ccmC* RNAs was confirmed by a probe corresponding to regions upstream of the *ccmC* reading frame (−513 to −734 with respect to the ATG). This hybridization experiment identifies the 1.35-, 2.15-, and 2.6-kb transcripts as *ccmC* precursor RNAs. To the contrary, no increase was observed for the signals corresponding to the 1.4- and 1.8-kb RNAs.

In addition, a primer extension analysis of total mitochondrial RNA (mtRNA) isolated from cell suspension cultures established from Col, C24, and the SAIL 18E04 line was performed (Fig. 1C). In Col wild type, strong extension products of about 390 and 370 nucleotides and a relatively weak 350-nucleotide cDNA, respectively, correspond to 5′ ends at −500, −484/482, and −465, all of which correlate with mature *ccmC* transcripts of approximately 1,200 nucleotides. In the mutant and C24, the 390- and 370-nucleotide extension products are barely detectable, which is consistent with the almost complete absence of the mature 1,200-nucleotide *ccmC* transcripts in these lines (Forner et al., 2008). Only the 350-nucleotide extension product is detectable in the mutant or even slightly increased in C24. In this accession, a very strong 270-nucleotide cDNA corresponds to the −391/390 5′ termini of the mature *ccmC* mRNA of about 1,100 nucleotides. These ends are also present in Col and the mutant, but only in trace amounts. Apart from that extension products of 440 and 650 nucleotides are present in similar amounts in all investigated lines or are overrepresented in the mutant (550, 1,300, and 1,900 nucleotides). The 5′ end indicated by the 1,900-nucleotide product very likely correlates with the 2.6-kb mRNA, which is also overrepresented in the SAIL 18E04 line.

Since the primer extension experiment indicated a complex 5′ end pattern for the mature *ccmC* transcript in Col, we reexamined this end by sequencing of 10
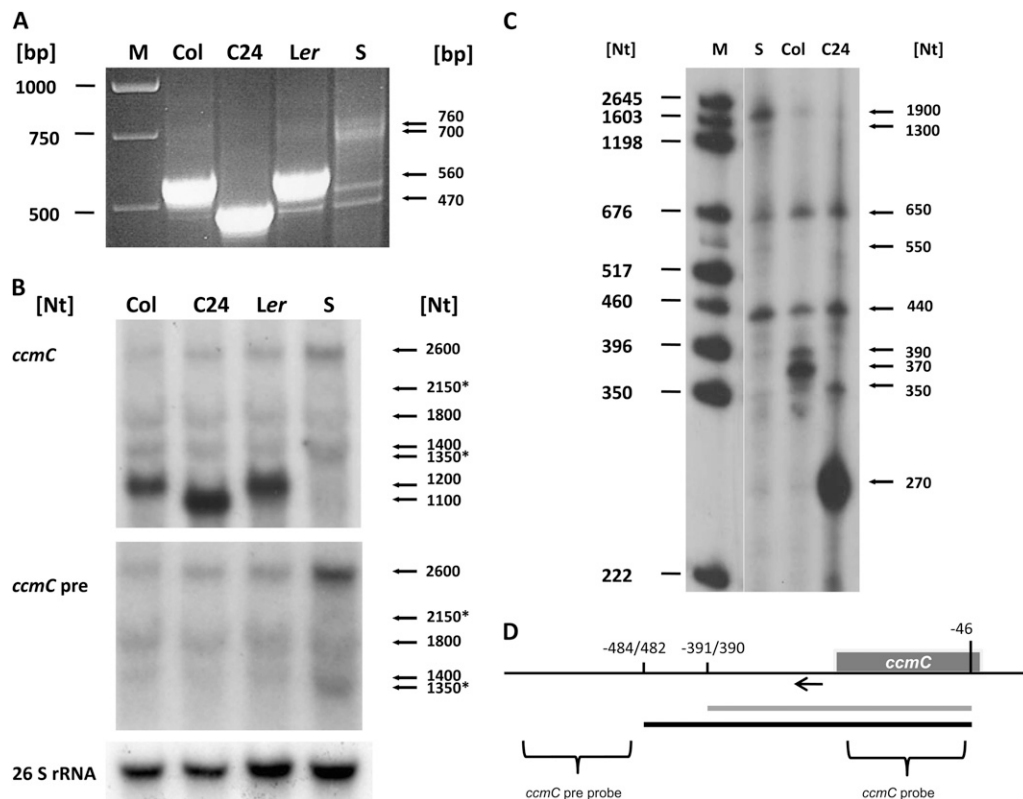
Figure 1. Analysis of *ccmC* transcripts. RNAs derived from the *ccmC* gene in accession Col, C24, and L*er* and the mutant line SAIL 18E04 (S) were analyzed by CR-RT-PCR (A), northern-blot hybridization (B), and primer extension analysis (C). Lengths of size markers are given on the left-hand sides of the images. Relevant products or signals are indicated by small arrows and corresponding sizes on the right-hand sides. Asterisks indicate mRNA species predominantly detected in the mutant (the 2.15-kb mRNA is difficult to see in the image, but can be seen on the x-ray film). D, Schema of the *ccmC* gene (dark-gray box) and the major transcripts (gray [C24] and black [Col] bold lines). Mature 5′ (relative to the translation start codon NATG, $n = -1$) and 3′ ends (relative to the translation stop codon, TAAN, $n = +1$) are indicated. Probes used for the northern analysis are depicted in the bottom part of the schema and indicated on the left-hand side of the images in B. The position of the oligonucleotide (Atccb3Mega5′.nah) used for the primer extension experiments is indicated by an arrow. Schema not drawn to scale.

clones obtained from the 560-bp CR-RT-PCR product. This revealed two clusters of ends (Fig. 2). One group of termini found in five clones surrounds the previously described ends at −484/−482, which had been determined by direct sequencing of a PCR product (Forner et al., 2007). The second cluster comprising four clones is found at positions −501 to −503. Both clusters correspond to the primer extension products of 370 and 390 bp, respectively, suggesting that the mature *ccmC* transcripts from Col have indeed two 5′ ends located about 20 nucleotides apart from each other. An additional 5′ end at position −476 was found represented only in single clone.

At1g62930 Rescues the Formation of Mature *ccmC* mRNAs in the SAIL 18E04 Mutant

The T-DNA in the SAIL 18E04 mutant is located 31 nucleotides downstream of the predicted translation start codon. An RT-PCR analysis showed that sequences downstream of the insertion are transcribed

(Supplemental Fig. S1). To test whether the absence of mature *ccmC* mRNAs is indeed linked to the T-DNA insertion in At1g62930, a 2.6-kb DNA fragment containing the At1g62930 gene and 0.2 kb of the 5′ and 0.5 kb of the 3′ flanking sequences from Col wild type was stably introduced into the SAIL 18E04 mutant. After selection of transformants, the presence of the transgene was confirmed by PCR with T-DNA-specific primers. The accumulation of the mature *ccmC* mRNAs was then examined by CR-RT-PCR and northern-blot analyses (Fig. 3).

In five independent transformants, a CR-RT-PCR analysis showed that the presence of At1g62930 rescues the formation of the mature *ccmC* mRNA. Using total cDNA as template, 560-bp PCR products were amplified in the transformed mutant as in Col and L*er* wild-type plants. No such product is seen in the untransformed mutant (Fig. 3A). Additionally, a northern-blot analysis of two complemented plants confirmed that mature *ccmC* mRNAs with sizes identical to wild type accumulate only in the presence of
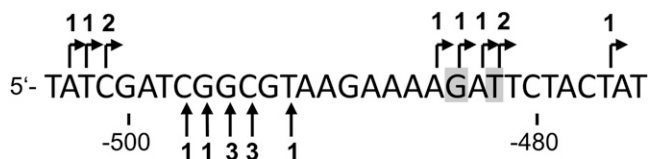
Figure 2. RNA extremities of *ccmC* RNAs. The 5′ termini found in 10 clones obtained after cloning of CR-RT-PCR products of Col *ccmC* transcripts (shown in Fig. 1A) are given as bent arrows above the sequence. Previously mapped 5′ ends are indicated by gray boxes (Forner et al., 2007). The 3′ ends of *ccmC* leader RNAs determined by sequence analysis of nine individual clones derived from the 3′-RACE analysis of the *ccmC* 5′ leader molecules (see also Fig. 4B) are indicated by vertical arrows below the sequence.

At1g62930, while no such transcript was seen in the untransformed mutant (1,200 nucleotides; Fig. 3B). These results unambiguously demonstrate that an intact At1g62930 gene is required for the formation of the mature *ccmC* mRNA in Arabidopsis accession Col. We designate this gene *RPF3* and the corresponding mutant *rpf3-1*.

RPF3 Is a Factor Involved in the Posttranscriptional Generation of *ccmC* Transcripts

The failure to generate mature *ccmC* mRNAs in the *rpf3-1* mutant can be attributed to a defect in transcription initiation or to a disturbance of posttranscriptional processing. To discriminate between both possibilities, we investigated whether the mature *ccmC* mRNAs carry 5′ triphosphate or 5′ monophosphate groups by primer extension analysis of terminator exonuclease-treated mtRNA. This exonuclease specifically degrades RNA containing 5′ monophosphate groups, which are generated by posttranscriptional cleavage. Primary RNAs having 5′ triphosphate groups, which derive from transcription initiation, are not degraded by this enzyme. As shown in Figure 4A, strong extension products of approximately 270 and 370/390 nucleotides were obtained from untreated mtRNA preparations. These indicate the mature −391/390 in C24 and −484/482 and −501 to −503 5′ termini in Col. Again further signals indicated 5′ termini of precursor molecules, consistent with the results shown in Figure 1. In contrast, no signals corresponding to the mature *ccmC* 5′ ends have been detected with terminator exonuclease-treated mtRNA from both accessions. Only the less-abundant extension products were seen after terminator exonuclease treatment. In both accessions, the strongest signals correspond to extension products of about 650 nucleotides. Additionally, relatively weak extension products of about 1 kb and larger cDNAs up to 1.9 kb are seen after exonuclease treatment. Although the exact sizes of these signals cannot be determined in our experiment, they might well correspond to different primary 5′ ends found around positions −1,800 and −1,600 in respect to the translation initiation codon (Kühn et al., 2009). Taken together,

these results strongly suggest that the −484/482 and −501 to −503 5′ termini in Col are generated posttranscriptionally, which makes a function of RPF3 in transcription initiation unlikely. Similarly, the mature 5′ ends of the *ccmC* transcripts in C24 are also formed posttranscriptionally.

To support the posttranscriptional origin of the major 5′ termini in Col and to obtain information whether these ends could be derived from endonucleolytic processing, total mtRNA was searched for the presence of *ccmC* 5′ leader RNAs that might result from a cleavage at positions −484/482 or −501 to −503. To this end, the mitochondrial 18S rRNA was used as anchor, which was linked to the 3′ end of such potential leader molecules in a bulk RNA ligation reaction. After cDNA synthesis initiated from an oligonucleotide annealing to the 18S rRNA the applied PCR strategy selected for *ccmC* 5′ leader-18S rRNA ligation products (Fig. 4B). After a single PCR, a product with a size of about 410 nucleotides was generated from Col mtRNA (Fig. 4C), while a much weaker product of the same size was amplified from *rpf3-1* mtRNA. A half-nested approach using an alternative primer annealing to 18S rRNA amplified a strong product of approximately 210 nucleotides. Almost no cDNA product of this size was seen in the mutant. The 210-bp product was inserted into pCR 2.1-
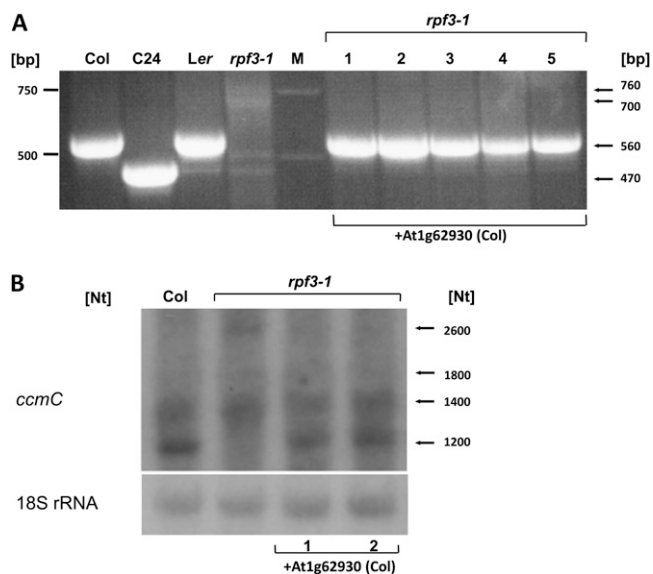

Figure 3. Complementation of *rpf3-1* with At1g62930 rescues the formation of mature *ccmC* mRNA. A, CR-RT-PCR analysis of five *rpf3-1* plants containing the wild-type allele of At1g62930. Five hundred and sixty base pair products were amplified from total RNA from all of these plants and from Col and L*er* wild-type plants, while no such product was amplified from the untransformed *rpf3-1* mutant. A 470-bp product was generated in C24. B, A northern analysis confirmed the generation of the 1,200-nucleotide *ccmC* in two complemented plants. The size of the *ccmC* transcripts is identical in the complemented plants and in Col wild type. No mature *ccmC* mRNA was detected in the untransformed mutant. Lengths of size markers are given in the left margin, relevant products are indicated in the right margin.
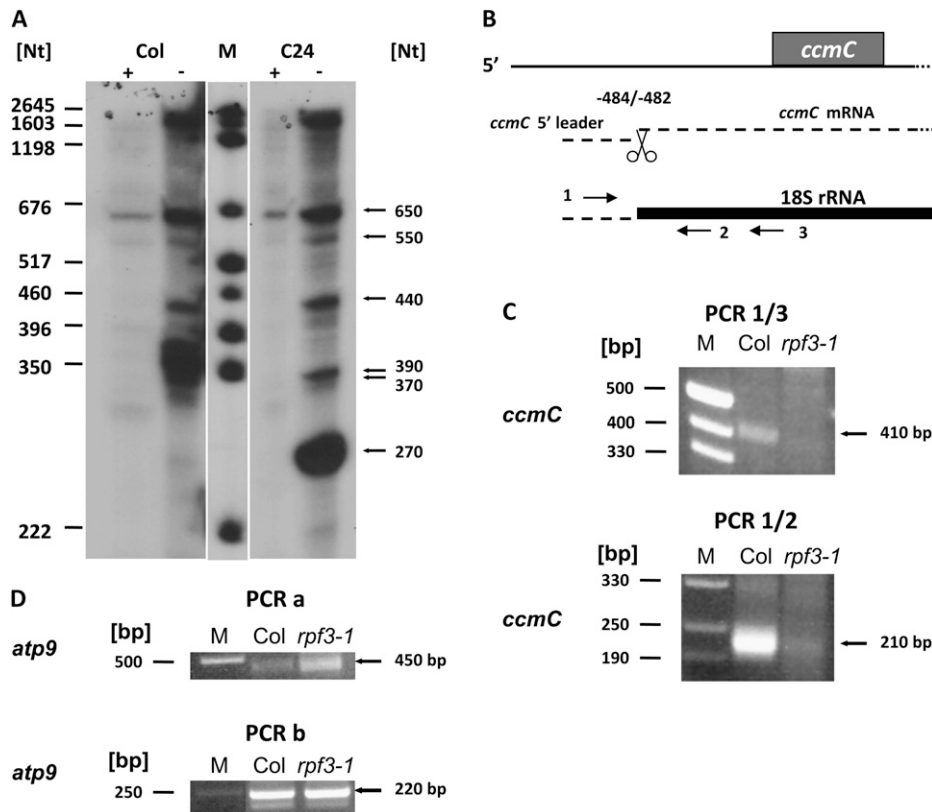
Figure 4. The mature *ccmC* 5′ ends are generated by posttranscriptional processing. A, Primer extension analysis of untreated (−) and terminator exonuclease-treated (+) mtRNA from Col and C24. B, Schema explaining the analysis of 3′ ends of potential *ccmC* 5′ leader molecules. The *ccmC* reading frame is indicated by a gray box, *ccmC* RNAs are given as dashed lines. The 18S rRNA, which was used as anchor molecule, is depicted as a black bold line. Primers (horizontal arrows) used for this analysis are Atccb3QPCR.3 (1), At18S-Adapter.R4 (2), and At18S-Adapter.R3 (3). C, PCR products amplified with primer pair 1/3 (top section) and 1/2 (bottom section) from Col wild type and from *rpf3-1* total mtRNA. Lengths of size markers are given on the left, relevant products are indicated on the right-hand side of the images (A and C). D, Control reactions performed with primer pairs Atatp9Mega-Endo.A/At18S-Adapter.R3 (PCR a) and Atatp9-11/At18S-Adapter.R4 (PCR b) amplified *atp9* leader RNA molecules as described previously (Forner et al., 2007). These reactions were performed with identical volumes of the same cDNA preparation used in the PCRs shown in C. The results of these amplifications demonstrate that in *rpf3-1* the reduced amount of PCR product derived of *ccmC* leader molecules is not due to the use of lower amounts or insufficient quality of the cDNA.

TOPO vectors and nine individual clones were sequenced. These sequences identified 5′ termini at positions −492 to −497 (Fig. 2). Taken together these results demonstrate that *ccmC* 5′ leader molecules exist in Arabidopsis mitochondria. The 3′ ends of these molecules are located 10 to 14 nucleotides upstream of the −484/482 and around eight nucleotides downstream of the −503 to −501 5′ ends of the mature *ccmC* mRNA. However, the preferential amplification of these leader molecules from wild-type mtRNA demonstrates that their accumulation depends on a functional *RPF3* gene. This correlation strongly suggests a function of RPF3 in endonucleolytic cleavage.

A *CcmC* Protein Is Detectable in the *rpf3-1* Mutant

The above-described northern-blot hybridization revealed (Fig. 1) that the mature *ccmC* transcript is almost completely absent in the mutant, which might be at least partially compensated by increased amounts of *ccmC* precursor molecules. To determine the abundance of transcripts containing the coding sequence (CDS) in the mutant, real-time quantitative RT-PCR was performed. Amplification of a product spanning the *ccmC* CDS from positions 273 to 368 relative to the ATG (A = +1) revealed no difference in the amount of *ccmC* RNAs containing the reading frame between *rpf3-1* and Col wild type. In contrast, an amplification reaction covering *ccmC* precursor molecules from −510 to −607 revealed about 6-times more *ccmC* precursor RNA species in the mutant, confirming a defect in *ccmC* 5′ processing in the *rpf3-1* mutant (Supplemental Fig. S2). These data suggested that a *CcmC* protein might still be translated in the mutant. To test this assumption, an immunodetection analysis was performed with total mitochondrial protein from *rpf3-1* and Col wild-type cell suspension cultures, respectively. Using an antiserum against the *CcmC* polypeptide, a strong signal

corresponding to a protein with the apparent molecular mass of about 34 kD detected the *CcmC* protein in Col wild type (Fig. 5). To the contrary, a much weaker signal shows a substantially reduced accumulation of the *CcmC* polypeptide in *rpf3-1* but confirmed that still minor amounts of this protein can be synthesized in this mutant. To test whether this severe reduction of the *CcmC* protein has any consequences on the activity of the respiratory chain, activities of complexes I, III, and IV were measured with mitochondrial protein isolated from the *rpf3-1* mutant and Col wild-type cell suspension cultures. However, no differences were observed between the activities of these complexes from the different lines (Supplemental Fig. S3).

RPF3 Is a Mitochondrial Protein

RPF3 is nuclear-encoded protein involved in a processing event that occurs in mitochondria. Accordingly this protein is expected to be imported into theses organelles. To test this assumption, a DNA fragment encoding 80 amino acids at the N terminus of RPF3 was fused in frame upstream of the gene encoding the GFP (Davis and Vierstra, 1998). After transient transformation of this construct into transgenic tobacco (*Nicotiana tabacum*) protoplasts, which express a isovaleryl-CoA dehydrogenase:red fluorescence fusion protein as mitochondrial marker (Forner and Binder, 2007), the subcellular sorting of the fusion proteins was inspected by fluorescence microscopy. Green fluorescence was exclusively observed in mitochondria, as demonstrated by the nearly identical pattern of the green and red fluorescence (Fig. 6). This result strongly suggests that RPF3 is also targeted to mitochondria in vivo.

RPF3 Has a Minor Influence on the Generation of the Mature *ccmC* Transcript in C24

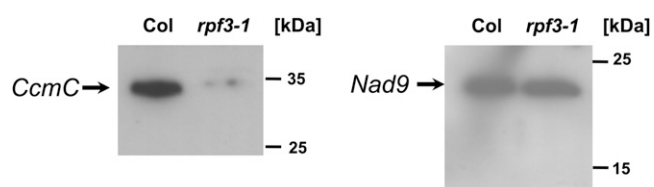In a previous analysis of mitochondrial transcript extremities in different Arabidopsis accessions we identified a *ccmC* 5′-end polymorphism between accessions Col and C24 (Forner et al., 2008). The analysis of reciprocal Col/C24 F1 hybrids clearly demonstrated that this polymorphism is linked to differences in the mitochondrial DNA sequences, most likely to a small segment located upstream of both accession-specific 5′ ends (Supplemental Fig. S4). Since RPF3 is crucial for the generation of the mature 5′ end of the *ccmC* transcript in Col, we wanted to know whether this protein might also be required for 5′ processing of the mature *ccmC* transcript derived from the C24 mtDNA configuration. Thus C24 (egg cell donor) was crossed with the *rpf3-1* mutant (pollen donor). Since in Arabidopsis mtDNA is maternally inherited, the offspring of this cross contains the C24 *ccmC* upstream configuration, which was confirmed by PCR. In the F2 generation, plants homozygous for the *rpf3-1* T-DNA allele were selected by PCR and investigated for their ability to generate mature *ccmC* transcripts. As shown in Figure 7, CR-RT-PCR products with sizes identical to the C24 wild-type control could be amplified from two F2 plants. This result was confirmed by a northern-blot hybridization, which confirmed identical sizes of mature *ccmC* mRNAs in these F2 and C24 wild-type plants. But the northern analysis also revealed a reduced level of the mature *ccmC* transcripts in the F2 hybrids. These results demonstrate that the function of the *RPF3* gene is per se not required for the generation of substantial amounts of the mature C24-type *ccmC* mRNA in these F2 hybrids and probably also in C24. These observations suggest that a distinct mtDNA configuration, i.e. in the *ccmC* upstream region, might be associated with a particular version of the *RPF3* reading frame. Thus we examined the *RPF3* coding region in C24. A PCR analysis of the CDS sequence generated products of identical sizes in Col and C24 (data not shown). Sequencing of the product from C24 identified 35 amino acid polymorphisms in comparison to Col (Supplemental Table S1). None of the nucleotide exchanges interrupts the coding region, suggesting that C24 retained a functional *RPF3* reading frame.

Taken together these results demonstrated that in both C24 and Col RPF3 is involved in *ccmC* transcript processing, but that the gene is of different importance for the generation of the mature 5′ ends of *ccmC* mRNAs in these accessions.

Figure 5. The steady-state level of the *CcmC* polypeptide is substantially reduced in the *rpf3-1* mutant. Immunodetection analyses of total mitochondrial protein isolated from cell suspension cultures from Col wild type (Col) and the *rpf3-1* mutant (*rpf3-1*). While in both lines a *Nad9* antiserum detects the target protein in similar amounts, an antiserum raised against an oligopeptide representing the core region of the *CcmC* protein (Kitazaki et al., 2009) revealed a substantially reduced level of *CcmC* polypeptide in the mutant. Sizes of marker proteins are indicated on the right-hand side of the images.

DISCUSSION

RPF3 Is Required for the Generation of Mature 5′ Ends of *ccmC* mRNAs in Arabidopsis Accession Col

In Arabidopsis mitochondria, RPF1 and RPF2, two PPR proteins with high similarity to RF, are involved in the generation of the mature 5′ ends of *nad9*, *cox3*, and *nad4* transcripts. This observation suggests that RF-like PPR proteins play a predominant role in 5′ processing. In accordance with this hypothesis, several lines of evidence now demonstrate an analogous function of the RF-like PPR protein RPF3 in the for-
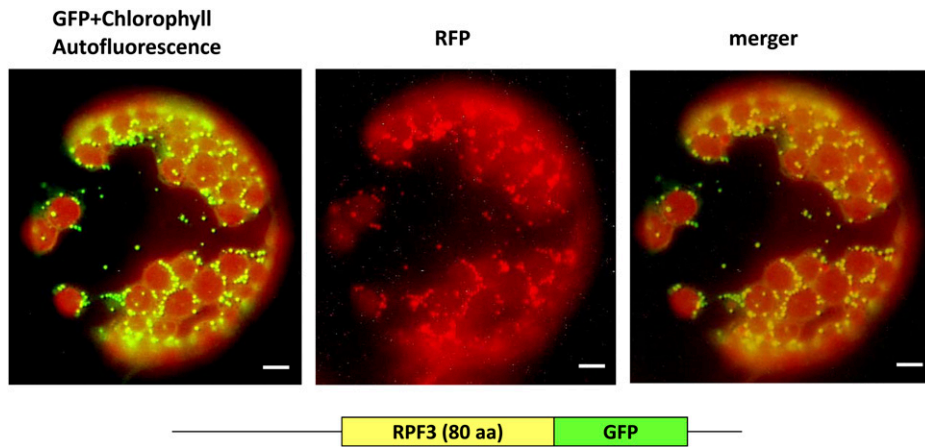
Figure 6. Subcellular targeting of RPF3. A hybrid protein consisting of the RPF3 mitochondrial targeting sequence (80 amino acids) and the GFP was transiently expressed in transgenic tobacco protoplasts. Fluorescence of GFP and chlorophyll and the red fluorescent protein from the sea anemone *Entacmaea quadricolor* (eqFP611) were visualized through different filter sets. eqFP611, which is C-terminally fused to the N-terminal part of the mitochondrial isovaleryl-CoA dehydrogenase had been established as mitochondrial marker in transgenic tobacco (Forner and Binder, 2007). A merger (GFP + eqFP611) of both images demonstrated the nearly perfect match of both patterns. Small differences were due to the rapid movement of mitochondria in the protoplast. Size bars correspond to 10 μm.

mation of mature 5′ end of *ccmC* mRNAs in Arabidopsis accession Col.

First, although the T-DNA insertion in the *rpf3-1* mutant does not interfere with the generation of *RPF3* transcripts corresponding to regions downstream of the insertion, it prevents the accumulation of the mature *ccmC* mRNA in the accession Col. Second, the generation of this mRNA could be restored by the introduction of the functional *RPF3* gene into *rpf3-1*. Third, as demonstrated by real-time quantitative RT-PCR, northern-blot hybridization, and primer extension analysis, the severely impaired generation of the mature *ccmC* mRNA is accompanied by an increase of different 5′-extended precursor molecules strongly

suggesting that RPF3 functions in posttranscriptional generation of the mature 5′ end. Fourth, this function is corroborated by the analysis of terminator exonuclease-treated RNA, which shows the investigated mature *ccmC* 5′ ends in Col but also the mature 5′ termini in C24 to be derived from processing rather than from transcription initiation, although the 5′-CRTA-3′ promoter motif is found at these transcript ends (Forner et al., 2007; Kühn et al., 2009). Fifth, the preferential detection of *ccmC* 5′ leader molecules in Col wild type indicates that the accumulation of these by products depends on an intact *RPF3* gene. This observation strongly suggests a function of RPF3 in endonucleolytic cleavage, although the identified 3′
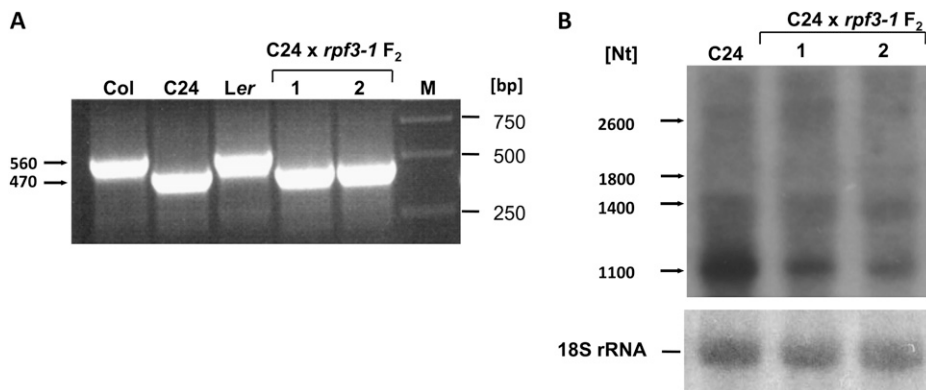

Figure 7. RPF3 is less important for the generation of the −391/390 5′ termini in C24. A, CR-RT-PCR analysis of total RNA from two F2 hybrids obtained from a cross of C24 ($) with the *rpf3-1* mutant (#) and from Col, C24, and L*er* wild type. Products of 470 bp were generated from total RNA of both F2 plants lacking an intact *RPF3* gene and from C24. Products of 560 bp were amplified from Col and L*er*. B, F2 hybrids were also analyzed by northern-blot hybridization. Relevant signals are indicted in the left. The northern probe against the CDS is shown in Figure 1D. A hybridization with an oligonucleotide complementary to the cytosolic 18S rRNA showed equal loading of the RNA gel (bottom part).

ends of these leader molecules slightly deviate from the detected 5′ ends. This pattern of ends indicates that after the endonucleolytic cuts, a few nucleotides are removed most likely by 3′ exonucleases. Alternatively, the few nucleotides might be lost by two endonucleolytic cuts in close vicinity to each other (e.g. −484/−482 mRNA 5′ ends and −492 to −497 3′ leader ends).

In summary, our data unambiguously demonstrate a function of RPF3 in the generation of the mature 5′ end of the mitochondrial ccmC transcript in the accession Col. Thus three RF-like PPR proteins are now shown to be required for the posttranscriptional generation of 5′ transcript termini corroborating the predominant role of this group of proteins for this process (Jonietz et al., 2010; Hölzle et al., 2011). In addition, RPF1 to RPF3 as well as RF1A from rice (Wang et al., 2006; Kazama et al., 2008) are involved in endonucleolytic cleavage while no evidence has been found that would support a function of these proteins as barriers to exonucleolytic degradation as it has been seen for some PPR proteins from chloroplasts (Loiselay et al., 2008; Pfalz et al., 2009; Johnson et al., 2010).

Function of RPF3 in Mitochondria of Arabidopsis Accession C24

In Col, the function of RPF3 is very important for the generation of the mature 5′ ends since almost no mature ccmC transcripts are detectable in the corresponding rpf3-1 mutant. In contrast, the analysis of C24 X rpf3-1 F2 plants without functional RPF3 indicated that this gene is per se not necessary for the generation of the −391/−390 5′ termini of ccmC RNAs derived from the mtDNA configuration present in C24 and other accessions (Fig. 7; Forner et al., 2008). In these plants still substantial amounts of mature ccmC mRNAs are detectable by northern-blot analysis. However, the level of these transcripts is reduced to about 50% (Fig. 7). This indicates that RPF3 has a function in RNA processing of ccmC transcripts originating from two different upstream configurations. While in Col the absence of an intact RPF3 almost completely prevents accumulation of mature ccmC mRNAs, in C24 RPF3 has only a moderate stimulatory effect on the processing event that results in the −391/−390 5′ termini.

The RPF3 protein from C24 shows 35 changes of amino acid identities in comparison to Col, most of them located in the N-terminal half. But all 15 repeats retain the characteristic degenerative motif, even repeat 6, where 12 amino acid exchanges were seen (Supplemental Table S1). These observations suggest that RPF3 might have a slightly different function in C24. However, we cannot exclude that the reduction of ccmC mRNA level in the F2 plants is due to other factors in the nuclear genome, which is combined and mixed up from C24 and Col. Thus further analyses are required to elucidate the exact function of the RPF3 gene in C24.

The Mature ccmC mRNA Is Almost Completely Absent in rpf3-1

In the previously analyzed rpf2-1 mutant still substantial amounts of mature nad9 and cox3 mRNA accumulate and accordingly no changes were seen in the steady-state levels of the corresponding proteins (Jonietz et al., 2010). Likewise, Ler plants lacking a functional RPF1 gene accumulate an approximately 170-nucleotide-longer precursor RNA to amounts comparable to the normal mature nad4 transcript found in Col with the intact RPF1 (Hölzle et al., 2011). In contrast to these observations, the northern analysis shows that the presence of the T-DNA insertion in RPF3 results in a nearly complete loss of the mature ccmC mRNA and accordingly only small amounts of this protein are detectable in the rpf3-1 mutant. On the one hand there might be still enough mature ccmC mRNA to be translated. On the other hand it is also possible that one or the other species of the precursor molecules can be translated. It is rather unlikely though that all of the ccmC pre-RNA species are used for protein biosynthesis. In this case more CcmC protein would be expected, since quantitative RT-PCR revealed no difference of transcripts containing the ccmC reading frame (Supplemental Fig. S2).

To generate functional c-type cytochromes, the prosthetic heme group has to be covalently attached to the corresponding apoproteins. For this biochemical process, three different systems have been described (Giegé et al., 2008; Hamel et al., 2009; Kranz et al., 2009; Sanders et al., 2010). The cytochrome c maturation (ccm) system (also called system I) is found in mitochondria of plants and red algae, in archaea, and in different subgroups of bacteria. In the latter, 12 proteins are involved in the heme delivery and attachment process, most of them being conserved in plants. In recent years, many of the plant mitochondrial Ccm proteins, either encoded in the mitochondrial or nuclear DNA, have been analyzed and partially characterized (Spielewoy et al., 2001; Meyer et al., 2005; Raczynska et al., 2006; Rayapuram et al., 2007, 2008). In Arabidopsis mitochondria, the CcmC polypeptide is translated from mRNAs that lack the predicted translation stop codon. As its counterpart in bacteria, the protein from Arabidopsis contains the WWD motif and an important His residue. In bacteria, the CcmC protein is an essential component of the cytochrome c biogenesis pathway where it is involved in the translocation of heme from the cytoplasm into the periplasm and for the ligation of heme to CcmE (Kranz et al., 2009). Likewise, plant ccmC might be involved in the delivery of heme from the mitochondrial matrix into the intermembrane space, however, experimental evidence for this potential function has not been obtained yet (Giegé et al., 2008). If mitochondrial CcmC also has a crucial function in plants, one might expect a reduction of the respiratory activity in the rpf3-1 mutant. However, the activities of complex I, III, and IV have been found to be identical in the mutant

and in wild-type mitochondria (Supplemental Fig. S3). In addition, no changes were seen in the macroscopic phenotype or in the development of the mutant under normal growth conditions (C. Jonietz and S. Binder, unpublished data). Thus mitochondria of *rpf3-1* seem to have retained normal activity, which can be explained by two scenarios. First, the residual amount of *CcmC* protein in the mutant still supports maturation of c-type cytochromes without negative consequences for the respiratory potential of mitochondria. It might be possible that levels of *c*-type cytochromes are reduced in the mutant (maybe not as much as the *CcmC* protein itself), but these lower amounts still support full function of the mitochondrial respiratory chain. Unfortunately no data are available whether plants would overcome reductions in *c*-type cytochromes as they do for instance with impaired complex I activity. Second, the plant mitochondrial *CcmC* protein has no essential function in cytochrome *c* biogenesis in plants. This scenario is consistent with the fact that the enzymes required for protoporphyrin IX biosynthesis localize to chloroplasts. Moreover, the mitochondrial localization of ferrochelatase, which catalyzes the insertion of iron into the protoporphyrin IX, has not yet been unambiguously demonstrated. Thus it is speculated that heme is delivered from the outside directly to the mitochondrial intermembrane space so that there is no need for a transport of heme across the inner mitochondrial membrane (Giegé et al., 2008). In this scenario, *CcmC* might fulfill an alternative function, which is not essential for cytochrome *c* biogenesis and for the function of the respiratory chain.

Presently there is no experimental evidence for these scenarios, but the *rpf3-1* mutant might be a valuable system for further studies of *CcmC* and its role in ccm in mitochondria of higher plants.

MATERIALS AND METHODS

Plants and Plant Cultivation

Arabidopsis (*Arabidopsis thaliana*) plants were grown and checked for correct genotypes as described (Forner et al., 2008). Cell suspension cultures were established and cultivated as outlined previously (Köhler et al., 2010). The Arabidopsis SAIL line 18E 04 was obtained from the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info/). Crosses to generate F1 and F2 hybrid plants were done following standard procedures (Weigel and Glazebrook, 2002).

Analysis of RNA

The 5′ ends of mitochondrial *ccmC* mRNA were determined by CR-RT-PCR analysis (Kuhn and Binder, 2002; Forner et al., 2007, 2008; Jonietz et al., 2010) using primers Atccb3Mega5′.nah and Atccb3Mega3′.nah. The 3′ termini of 5′ leader RNA molecules were determined by an established alternative 3′-RACE approach (Forner et al., 2007). First-strand cDNA synthesis was initiated from primer At18S-Adapter.R3 followed by the first PCR with primer pair Atccb3QPCR.3/At18S-Adapter.R3 and a second half-nested PCR with oligonucleotides Atccb3QPCR.3/At18S-Adapter.R4. PCR products were directly sequenced and cloned into pCR 2.1-TOPO (Invitrogen), respectively. *ccmC* 5′ ends were determined by sequence analysis of 10 clones. Total RNA and total mtRNA were isolated using RNeasy plant mini kits (Qiagen, http://www1.qiagen.com). Northern-blot hybridization, terminator exonuclease treatment, and primer extension analyses were done as described formerly (Jonietz et al., 2010).

Extension was initiated at oligonucleotide Atccb3Mega5′.nah, the probes for the northern analyses were amplified using primer pairs Atccb3-21/Atccb3-22 (CDS) and Atccb3-Mega5′-fern/Atccb3-12 (upstream probe). PCR products were labeled using a random-primed DNA labeling kit and [α32P]dCTP following the recommendations given by the manufacturer (GE Healthcare).

Real-time quantitative RT-PCR was done using transcriptor high-fidelity cDNA synthesis and light-cycler 480 SYBR green I master kits on a light-cycler 480 real-time PCR system according to instructions given by the manufacturer (Roche). Data were evaluated using the light-cycler 480 software (1.5). Relative transcript levels were measured in respect to the validated reference genes encoding ubiquitin conjugating enzyme 9 (UBC9, At4g27960) and protein phosphatase 2A subunit 3 (PP2A, At1g13320) with established primer pairs (Czechowski et al., 2005). Oligonucleotides for quantitative *ccmC* transcript analysis (Atccb3QPCR1–4) were selected using the primer design tool at the National Center for Biotechnology Information home page (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHomeAd).

Complementation Assays

A complementation construct containing the *RPF3* gene as well as 184- and 480-bp upstream and downstream regions (amplified with primers At1g62930-Kompl.H and At1g62930-Kompl.R) was established in pMDC123 using standard procedures (Sambrook and Russel, 2001; Curtis and Grossniklaus, 2003). To circumvent potential effects by PCR errors a mixture of 20 different clones was used for each construct. Plant transformation was done by floral dip (Clough and Bent, 1998). The presence of the transgene was tested with a PCR with primers KAN1 and KAN2.

Miscellaneous Methods

DNA sequencing was commercially performed (4baselab, http://www.4baselab.de/index_4.html). Immunodetection analysis of mitochondrial protein was performed as described (Jonietz et al., 2010). Basic methods of molecular biology followed standard protocols (Sambrook and Russel, 2001). Protoplast transformation and fluorescence microscopy were done following an established protocol (Matthes et al., 2007). In silico sequence analyses were done at the National Center for Biotechnology Information server applying various blast tools (McGinnis and Madden, 2004). Oligonucleotide sequences are listed in Supplemental Table S2.

Respiratory chain activity tests were performed at room temperature in 25 mM potassium phosphate buffer pH 7.4 using standard protocols for complex I (Singer, 1974) and complexes III and IV (Birch-Machin et al., 1994).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. RT-PCR analysis of *RPF3* transcripts in the SAIL 18E04 mutant.

Supplemental Figure S2. Real-time quantitative RT-PCR of *ccmC* transcripts in *rpf3-1*.

Supplemental Figure S3. Activities of respiratory chain complexes I, III, and IV in mitochondria from *rpf3-1* and Col wild type.

Supplemental Figure S4. Sequences of the *ccmC* upstream regions in the mitochondrial DNAs from Arabidopsis accessions Col and C24.

Supplemental Table S1. Amino acid exchanges between RPF3 proteins from Col and C24.

Supplemental Table S2. Oligonucleotide sequences.

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