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A DYW Domain–Containing Pentatricopeptide Repeat Protein Is Required for RNA Editing at Multiple Sites in Mitochondria of *Arabidopsis thaliana* [W]

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RNA editing in flowering plant mitochondria alters 400 to 500 nucleotides from C to U, changing the information content of most mRNAs and some tRNAs. So far, none of the specific or general factors responsible for RNA editing in plant mitochondria have been identified. Here, we characterize a nuclear-encoded gene that is involved in RNA editing of three specific sites in different mitochondrial mRNAs in *Arabidopsis thaliana*, editing sites *rps4*-956, *nad7*-963, and *nad2*-1160. The encoded protein MITOCHONDRIAL RNA EDITING FACTOR1 (MEF1) belongs to the DYW subfamily of pentatricopeptide repeat proteins. Amino acid identities altered in MEF1 from ecotype C24, in comparison to Columbia, lower the activity at these editing sites; single amino acid changes in mutant plants inactivate RNA editing. These variations most likely modify the affinity of the editing factor to the affected editing sites in C24 and in the mutant plants. Since lowered and even absent RNA editing is tolerated at these sites, the amino acid changes may be silent for the respective protein functions. Possibly more than these three identified editing sites are addressed by this first factor identified for RNA editing in plant mitochondria.

INTRODUCTION

In mitochondria of flowering plants ~450 selected cytosines are changed to uridines, most of them being observed in mRNAs (Giegé and Brennicke, 1999; Handa, 2003; Takenaka et al., 2008). The biochemical effect of this RNA editing is thus a site-specific deamination of C to U. So far the enzyme(s) involved in the biochemical reaction, as well as the mediators of the specific site recognition, are unknown. In the last few years in organello and in vitro analyses of mitochondrial RNA editing have delineated several *cis*-elements required for editing-site recognition in the affected RNA molecules but have not yet yielded any *trans*-factors (Farré et al., 2001; Neuwirt et al., 2005; van der Merwe et al., 2006).

In plastids, a similar type of RNA editing is found, its presence being correlated with the occurrence of editing in mitochondria. As in mitochondria, C nucleotides are altered to U in several plastid RNAs, but the total number is only 30 to 40 in plastids of most flowering plants (Sasaki et al., 2006; Shikanai, 2006). For RNA editing events in plastids, six specific nuclear factors have been identified, all of which are classified as pentatricopeptide repeat (PPR) proteins (Kotera et al., 2005; Okuda et al., 2007, 2009; Chateigner-Boutin et al., 2008; Zhou et al., 2008). In plants, the PPR protein coding genes form a large family with ~450 members, while animal genomes usually encode <10 such proteins (Small and Peeters, 2000; Schmitz-Linneweber and Small, 2008). Most of these PPR proteins are predicted to be targeted to plastids and/or mitochondria (Lurin et al., 2004). The PPR proteins in plants have been categorized according to the type and length of additional C-terminal extensions beyond the 6 to 12 repeats of 35 amino acids that gave these proteins their name (Small and Peeters, 2000). One of these extensions, the DYW domain, has been found to contain a signature characteristic of Zn-containing cytidine deaminases and has consequently been proposed to be potentially involved in the C-to-U RNA editing in the two plant organelles (Salone et al., 2007).

However, three of the six PPR proteins required for specific editing events in plastids are classified as containing E/E+ extensions but not the DYW domain. Accordingly, additional factors are being postulated for the enzymatic activity, and a model has been proposed in which a PPR protein recognizes a specific RNA sequence motif, binds there, and recruits through protein–protein interactions one or more additional proteins, including one with the enzymatic activity for the deamination step (Kotera et al., 2005; Okuda et al., 2007).

The RNA binding properties of these PPR proteins involved in plastid RNA editing have been experimentally verified for one of them (Okuda et al., 2006). This polypeptide specifically recognizes and attaches to an RNA region that covers its cognate editing site, the ACG to ATG alteration in the translation initiation codon of the *ndhD* gene. The region extends ~20 to 30 nucleotides upstream and 10 nucleotides downstream, similar to the specific *cis*-regions delineated by in vitro assays for several plastid, as well as mitochondrial, editing sites (Chaudhuri and Maliga, 1996; Hirose and Sugiura, 2001; Miyamoto et al., 2002; Sasaki et al., 2006; Takenaka et al., 2008).

Three of the six plastid RNA editing factors presently identified address single sites, while the other three PPR proteins seem to be required for editing of at least two sites in different plastid

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mRNAs (Kotera et al., 2005; Okuda et al., 2007, 2009; Chateigner-Boutin et al., 2008; Zhou et al., 2008). Interconnections between editing sites in plastids have been observed in transgenic and in vitro assays, in which introduction and overexpression of an additional editing site into the plastid system lowered RNA editing at other sites in *trans* (Chateigner-Boutin and Hanson, 2002; Heller et al., 2008; Kobayashi et al., 2007). The PPR proteins identified to be required for specific RNA editing events in plastids thus appear to be the specificity factors that recognize and bind to certain RNA motifs, which may be present at two or more different editing sites.

However, several of the PPR proteins encoded in plants have been implied to be active in other posttranscriptional processes in plastids. Some of the PPR proteins are required for specific intron splicing events, some are required for processing of multicistronic pre-mRNAs, for stabilization of pre-tRNAs, and yet others have been implicated in translation of specific genes in plastids (Beick et al., 2008; Williams-Carrier et al., 2008; for reviews, see Delannoy et al., 2007; Schmitz-Linneweber and Small, 2008).

In plant mitochondria, so far none of the RNA editing factors nor any of the cofactors of other processing events had been identified until recently. The first mitochondrial factor identified is a PPR protein that is involved in an intron splicing event (de Longevialle et al., 2007).

Considering these multiple functions of PPR proteins in various RNA maturation processes, it remains to be determined whether these PPR proteins are always involved in such *cis*-element guided reactions in the organelles and if so, how the 450 genes can accommodate addressing the >450 editing sites in mitochondria plus the >30 sites in plastids in addition to the many processing and splicing events required in both organelles.

The identification in this work of a *trans*-factor of RNA editing in plant mitochondria suggests as a solution to this problem that one *trans*-acting PPR protein can function as a specificity factor for several editing sites.

RESULTS

A Nuclear Gene Variation between Ecotypes Influences RNA Editing at a Site in the *rps4* mRNA

To identify site-specific *trans*-factors of RNA editing in plant mitochondria, we initiated a survey of *Arabidopsis thaliana* for ecotype-specific variations in RNA editing. Of the 379 editing sites analyzed in ecotypes Landsberg *erecta* (L*er*), Columbia (Col), and C24, seven show reduced RNA editing levels in one of the ecotypes (Zehrmann et al., 2008). One difference manifests as ~50% reduced RNA editing at nucleotide 956 in the *rps4* coding region in ecotype C24 (*rps4*-956; Figure 1A). The mitochondrial *rps4* gene codes for protein 4 of the small ribosomal subunit. Reciprocal crosses between ecotypes C24 and Col showed that the lowered editing is caused by a nuclear locus inherited as a recessive Mendelian trait (Zehrmann et al., 2008).

This nuclear locus was mapped by following the phenotype of reduced editing through a genetic screen of 199 individual F2 plants of a C24 × Col cross and its linkage to ecotype-specific sequence variations to an interval of 153 kb on chromosome 5. The region on chromosome 5 from nucleotides 21,320,166 to 21,472,719 contains 47 annotated genes between *At5g52510* and *At5g52900*, including two PPR genes. One of these two PPR genes, *At5g52850*, shows the same three polymorphisms (http://polymorph.weigelworld.org) in L*er* as it does in C24 in comparison to Col and can thus be excluded as a likely candidate gene. One annotated single nucleotide polymorphism (SNP) in the second PPR protein coding gene (*At5g52630*) is unique to C24 and shows a 100% correlation with the lowered editing phenotype, suggesting this gene as a candidate for being responsible for the lower RNA editing in C24. To analyze this possibility, C24 protoplasts were transfected with the wild-type Col version of this open reading frame under control of the 35S cauliflower mosaic virus promoter. The transfected protoplasts show RNA editing to be enhanced from ~20% editing in controls of untreated or green fluorescent protein (GFP) transfected C24 protoplasts to ~80% (Figure 1B). This result shows that the difference in RNA editing levels between ecotypes C24 and Col is indeed caused by this nuclear gene, now termed *MITOCHONDRIAL RNA EDITING FACTOR1* (*MEF1*).

Mutants in *MEF1* with No Detectable RNA Editing

To identify further mutations in *MEF1* and to investigate whether such mutants of MEF1 would be viable, we searched a collection of chemically mutagenized *Arabidopsis* Col plants for mutants deficient in editing the *rps4*-956 site. This editing site-targeted search was performed with the recently developed multiplexed single nucleotide extension protocol (Takenaka and Brennicke, 2008). The screen identified two mutant plants, *mef1-1* and *mef1-2*, which have lost detectable editing at this site (Figure 2; *rps4*-956 controls). Both plants show a normal growth phenotype under standard conditions. The unaffected growth habitus suggests that this editing event is not essential for ribosomal function although it changes an amino acid (S→L) in RPS4. The T-DNA insertion lines described for this region are annotated to contain insertions only upstream of the reading frame and were therefore not analyzed here.

For a cosegregation test of the connection between the mutations in *MEF1* and the RNA editing phenotype at the *rps4*-956 site, mutant line *mef1-1* was crossed with wild-type Col. Plants of the F2 generation were screened for individuals homozygous in the mutant *mef1-1* allele. The 11 homozygous mutant plants identified from 88 randomly selected plantlets were then analyzed for editing at the *rps4*-956 site. All 11 plants have lost RNA editing at this nucleotide, correlating the *MEF1* gene with this editing event.

The Wild-Type *MEF1* Gene Restores RNA Editing in the Mutants

The connection between the *MEF1* gene and RNA editing at the *rps4*-956 site was further assayed by complementation experiments with protoplasts from the mutants. The Col wild-type *MEF1* gene was transfected into *mef1-1* and *mef1-2* mutant protoplasts and was transiently expressed under the control of a strong 35S cauliflower mosaic virus promoter. When this

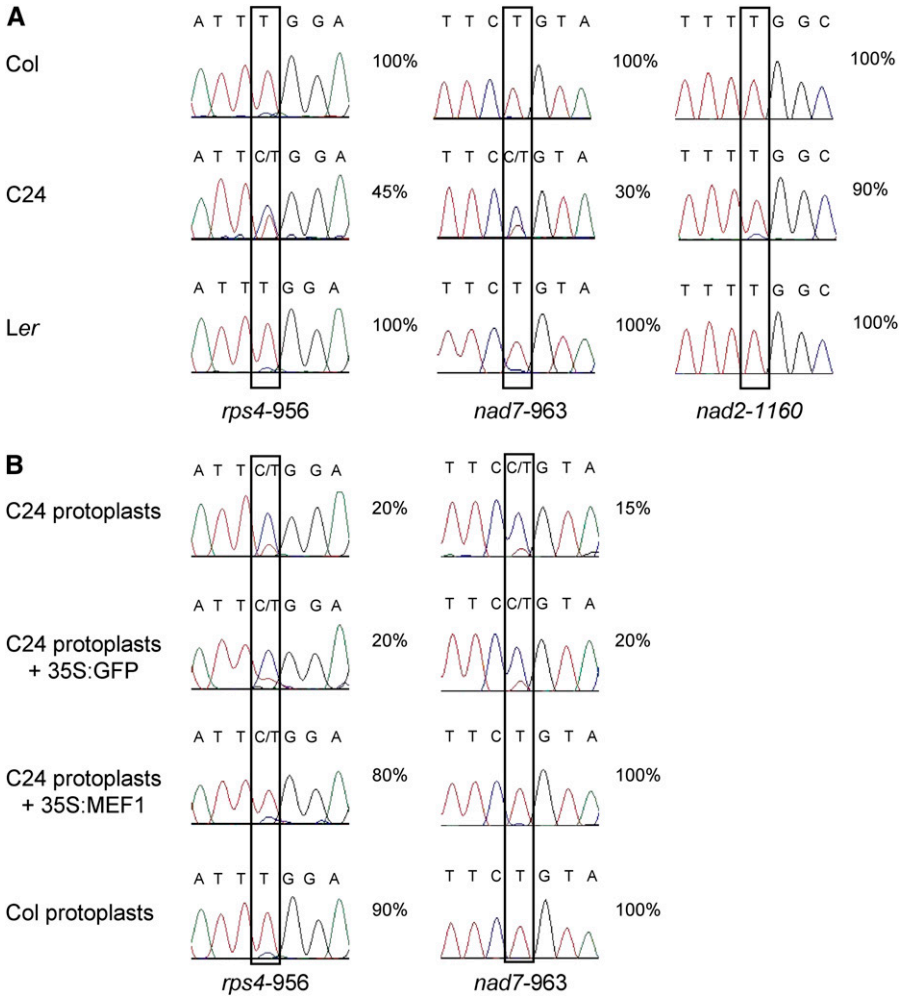

Figure 1. The *MEF1* Nuclear Gene Is Responsible for Ecotype-Specific RNA Editing Variations in *Arabidopsis* Mitochondria.

(A) Ecotype C24 shows partial RNA editing at the mitochondrial editing sites *rps4*-956 and *nad7*-963 where both L*er* and Col ecotypes are fully edited from C to U. At site *nad2*-1160, the RNA editing level in ecotype C24 is only slightly lower than the 100% in Col and L*er*.

(B) Introduction of the Col version of gene *MEF1* (35S:MEF1 sequence traces) into C24 protoplasts increases RNA editing at sites *rps4*-956 and *nad7*-963 to levels higher than those of untransfected C24 protoplasts (top traces). Sequence traces of wild-type ecotype Col (bottom sequence traces) and of C24 protoplasts transfected with GFP (35S:GFP) as a control are also shown. Other editing sites in the *rps4* mRNA are not affected.

construct is transfected into the mutant protoplasts, RNA editing at the *rps4*-956 site is restored (Figure 2; *rps4*-956). Although the full level of 100% editing as in Col-wt plants is not recovered, the ~50% observed editing versus the undetectable editing in control transfections (with only a GFP sequence in the vector) indicates that the Col-wt *MEF1* gene does recover RNA editing at this site. These results show that a functional *MEF1* gene is essential for RNA editing at the *rps4*-956 site.

Two Further Editing Sites in Different mRNAs Are Affected by the Nuclear *MEF1* Locus

Another RNA editing site differing between ecotypes Col and C24 (Zehrmann et al., 2008), the site at nucleotide 963 from the AUG in the *nad7* mRNA (*nad7*-963; Figure 1A), cosegregates with the *rps4*-956 lowered editing phenotype in the F2 generation of the cross between Col and C24. To further investigate this potential connection, the 11 plants identified to be homozygous in the mutant *mef1-1* allele and to be deficient in RNA editing at the *rps4*-956 site were probed for editing at the *nad7*-963 site. All 11 plants show no detectable RNA editing at this position, correlating the *MEF1* gene with the editing events at the *rps4*-956 site and the *nad7*-963 site. The *nad7* gene encodes subunit 7 of the NADH-dehydrogenase of the mitochondrial respiratory chain.

In the C24 protoplast transfection assays, the introduced wild-type Col *MEF1* gene boosts RNA editing at the *nad7*-963 site from 15 or 20% in the untransfected or GFP-transfected protoplasts to 100% edited mRNAs in the *MEF1*-transfected protoplasts (Figure 1). The wild-type Col *MEF1* gene introduced into the *mef1-1* and *mef1-2* protoplasts recovers detectable levels of RNA

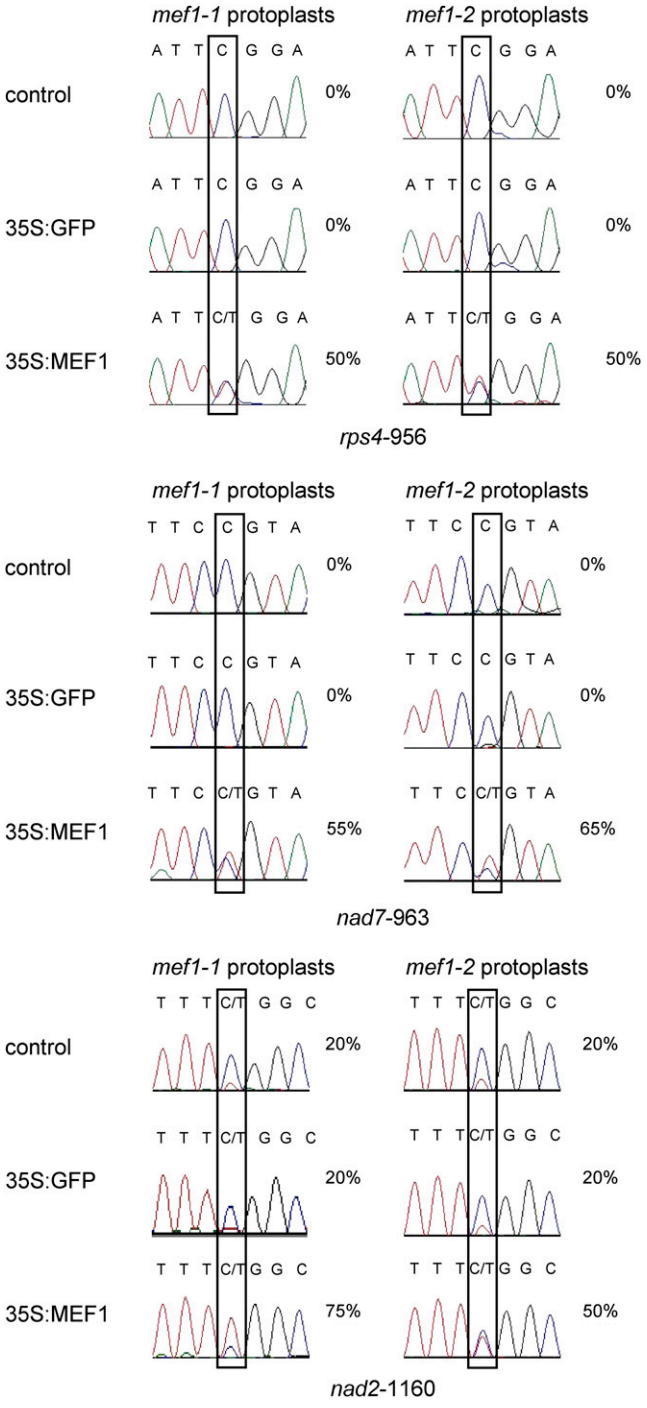

Figure 2. RNA Editing Analysis of Two Ecotype Col-Derived Plant Lines Mutated in the Nuclear Locus *MEF1*.

Two plant lines with independent mutations (*mef1-1* and *mef1-2*) in the *MEF1* gene show no editing at the *rps4*-956 site in leaves or in protoplasts (top panels, control). Transfection of protoplasts from mutants *mef1-1* and *mef1-2* with the wild-type Col *MEF1* gene restores the ability to edit this site in the *rps4* mRNAs. Introduction of the gene for GFP does not influence editing. The two mutants are also defective in editing at site *nad7*-963 (center panels, control). At the third site *nad2*-1160, RNA editing is lowered but still detectable (bottom panels, control). Transfection of protoplasts from the mutants *mef1-1* and *mef1-2* with the wild-type Col *MEF1* gene restores the ability to edit site *nad7*-963 and enhances editing at *nad2*-1160. The *MEF1* gene is thus required for, or at least involved in, RNA editing at these three sites in mitochondria of *Arabidopsis*.

editing at the *nad7*-963 site (Figure 2; *nad7*-963). The fact that RNA editing at the *nad7*-963 site is strongly enhanced by the introduced *MEF1* gene confirms that this gene is also involved in editing the *nad7* site and most likely is also required for this editing event in vivo (Figure 1B).

To determine whether the MEF1 protein targets editing at yet more sites, ~120 further mitochondrial editing sites were investigated in the two mutant plants *mef1-1* and *mef1-2*. Since the mutants do not show any gross growth deficits, we reasoned that most likely only nonessential editing sites should be affected, if any. Therefore, the sites investigated directly were primarily selected as possibly being dispensable (i.e., some changing a third codon position and being silent at the protein level and others at positions where no editing has been reported in other plant species, suggesting that both protein variants from edited and unedited mRNA might be functional). Several sites were included out of convenience such as being on fragments amplified for neighboring sites. In this screen, a third site was found to be affected, this one in the *nad2* transcript (nucleotide 1160 from the AUG, *nad2*-1160; Figure 2). The *nad2* gene encodes subunit 2 of the NADH-dehydrogenase, complex I of the mitochondrial respiratory chain. However, at this *nad2* site, RNA editing is still detectable in the mutants, although strongly reduced. The functional correlation between this *nad2*-1160 editing site and the *MEF1* gene was further investigated in mutant protoplasts complemented with the Col wild-type *MEF1* gene. Complementation increases RNA editing levels at this *nad2* site in both mutants (Figure 2), showing that the *MEF1* gene is indeed involved in RNA editing at this site also.

All three sites are completely edited in ecotypes Col and L*er*. In ecotype C24, sites *nad7*-963 and *rps4*-956 are edited in up to 50% of the steady state mRNAs, while *nad2*-1160 is edited to 90%. This suggests that the alteration of the *MEF1* gene in C24 affects editing at two of the three target sites more strongly than at the third site. The residual low level RNA editing at *nad2*-1160 site in mutant lines *mef1-1* and *mef1-2* (Figure 2; *nad2*-1160 control) confirms these site-specific effects of mutations in the *MEF1* gene. While editing at site *nad7*-963 alters a third codon position and is silent at the protein level, the C- to-U change at site *nad2*-1160 alters an amino acid (S→L) and is thus, like site *rps4*-956 (S→L), not silent. Both sites are well conserved in different plant species, the *rps4* site, for example, is also found in rice (*Oryza sativa*), magnolia (*Magnolia grandiflora*), and sunflower (*Helianthus annuus*), and site *nad2*-1160 is present also in wheat (*Triticum aestivum*), *Oenothera berteriana*, and sugar beet (*Beta vulgaris*), but encoded as a T in the genomic sequence of rice. This evolutionary stability of the encoded amino acids in RPS4 and NAD2 suggests that both are necessary for the respective functional proteins and thus mitochondrial competence, which is somewhat difficult to correlate with the normal growth phenotype

of the C24 and the two mutants (discussed further below). Beyond the three target sites identified here, the *MEF1* gene might affect other sites among the >280 editing sites that were not investigated in the mutants. Additionally targeted editing sites within or outside the 120 investigated sites may not be as strongly affected by these mutations. The single amino acid changes in MEF1 caused by the mutations in C24, *mef1-1*, and *mef1-2* differentially affect the three editing sites identified and may thus influence editing at other target sites very little if other regions of the MEF1 protein are involved in recognizing these additional target sites.

MEF1 Does Not Affect RNA Editing in Plastids

Most of the PPR proteins found in silico are predicted to be targeted to either or both, plastids and mitochondria (Lurin et al., 2004; Andrés et al., 2007). MEF1, a DYW-PPR protein, shows no clear prediction, while an experimental assay with an N-terminal peptide fused to a GFP reporter indicated a location in plastids (Lurin et al., 2004). To investigate a potential influence of MEF1 on RNA editing in plastids, we analyzed the status of all editing sites in plastids in the mutant *mef1-1*. All plastid sites are edited normally, confirming that MEF1 is involved in RNA editing in mitochondria, but not in chloroplasts.

Processing and RNA Stability Are Not Affected by the Mutations in *MEF1*

To investigate whether the observed RNA editing defects in C24 and in the mutants are caused by indirect effects, such as altered RNA processing or modified RNA stability, the transcript patterns of *rps4*, *nad7*, and *nad2* were analyzed (Figure 3). The *rps4* gene is located upstream of and cotranscribed with the first exon of the *trans*-spliced *nad2* gene, which leads to similarities in their transcript profiles (Lippok et al., 1996). The RNA gel blot hybridization reveals comparable amounts of the respective mature transcripts from all three genes in Col, C24, and the mutants *mef1-1* and *mef1-2*, suggesting that the mutations in *MEF1* do not alter the amount of these transcripts, which may for example, out-titrate a limited RNA editing factor for these sites. In turn, the RNA editing defects in the mutants have no detectable influence on processing and RNA stability. This result supports a direct and specific role of MEF1 in RNA editing at the three here identified sites.

Transcript Abundance of the *MEF1* Gene Is Not Affected in the Mutants

To investigate whether an altered transcription of the *MEF1* gene in *mef1-1* and *mef1-2* is responsible for the observed RNA editing defects, we compared the relative amounts of *MEF1* transcripts by limited-cycle RT-PCR (Figure 4). The absence of any EST in the databases and of signals in array analyses suggests that the transcription rate of *MEF1* is so low that RNA gel blot analysis is not feasible. The RT-PCR analysis performed with total cellular RNAs from Col wild-type plants and from the two mutants *mef1-1* and *mef1-2* shows that the transcript signals from wild type and altered plants are comparable. This observation suggests that

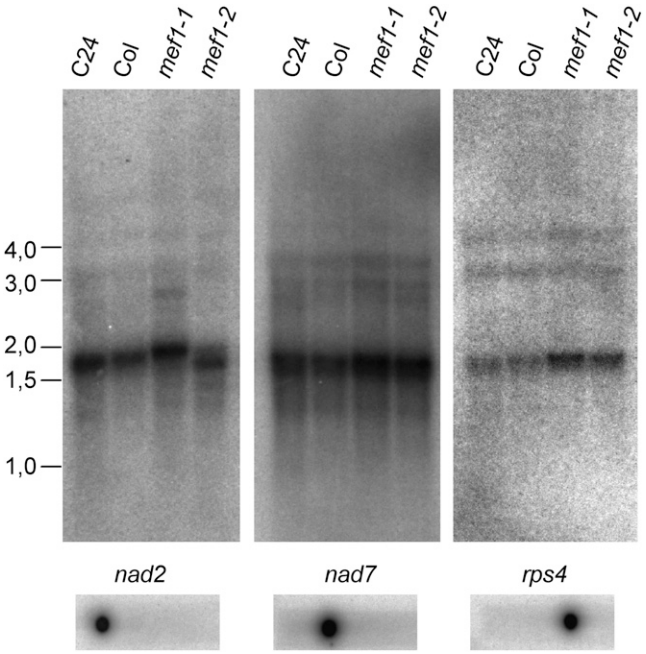

Figure 3. Transcript Patterns of the Mitochondrial Target RNAs in Col, C24, and the *MEF1* Mutants.

The transcript patterns of the mitochondrial *nad2*, *nad7*, and *rps4* genes are compared by RNA gel blot analysis. The hybridization signals of the gene-specific probes show comparable patterns and similar amounts of the respective precursors and mature transcripts from all three genes in Col, C24, and the *mef1-1* and *mef1-2* mutants. The specificity of the probes is demonstrated by dot blot hybridization to the three gene sequences shown underneath each panel, with the order of the dots being *nad2*, *nad7*, and *rps4* from left to right on each filter. The source of the respective total cellular RNA preparation is given above each lane. The positions of DNA size standards are indicated alongside in kilobase pairs.

altered transcript abundances of the *MEF1* gene are not responsible for the phenotype of diminished RNA editing.

The *MEF1* Gene Encodes a PPR Protein of the DYW Class

The genomic alterations responsible for the distinct editing phenotypes were analyzed in the different alleles of the *MEF1* gene in ecotypes Col, L*er*, and C24 and in the two mutant lines. The *MEF1* gene encodes, in a continuous open reading frame, a member of the DYW subgroup of the PPR protein family (Small and Peeters, 2000; Lurin et al., 2004; Figure 5). In ecotype C24, four nucleotide alterations change three amino acids in the encoded protein in comparison to the Col sequence, and four further SNPs are silent. A conserved Gly is altered to Ser in the E-domain, an Ala is changed to Thr in the first S-domain, and a Lys is substituted by Arg in the first P-domain. Between ecotypes Col and L*er*, two SNPs are silent and one SNP changes a Ser to Ala in L*er* without any apparent effect on editing. In the mutant line *mef1-1*, the single mutation alters a conserved Gly to Glu in the C-terminal L motif in one of the 35 amino acids repeats. In *mef1-2*, the sole amino acid change substitutes a Phe for a highly conserved Ser in the DYW domain.

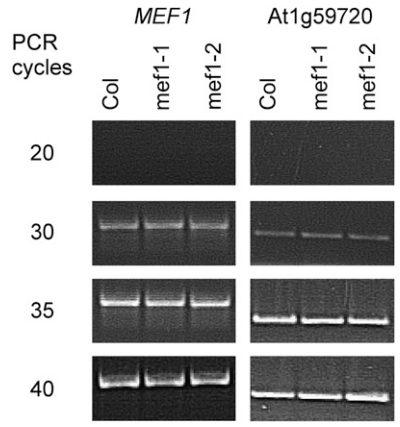

Figure 4. Analysis of the *MEF1* Transcript Abundance in Col Wild-Type and Mutant Plants.

The relative amounts of *MEF1* transcripts are analyzed by reverse transcription and subsequent cycle-limited PCR in Col and in the *mef1-1* and *mef1-2* mutants. The gel pictures show the transcript signals generated after the indicated number of cycles. Transcripts of another unrelated PPR gene (At1g59720) were amplified in parallel as a standard for the amount of RNA and as a PCR control.

DISCUSSION

A DYW Class PPR Protein Is Involved in RNA Editing in Mitochondria

Approximately 450 different PPR proteins are encoded in flowering plants, more than in any other organism. Based on these numbers, their coincidence with the presence of RNA editing in the plant kingdom, their being predicted to be mostly targeted to mitochondria and/or plastids, and their ability to bind RNA, the PPR proteins have been proposed to be involved in plastid and mitochondrial RNA editing (Small and Peeters, 2000; Lurin et al., 2004; Salone et al., 2007). Indeed, several PPR proteins have been found to be required for distinct RNA editing events in plastid mRNAs (Kotera et al., 2005; Okuda et al., 2006, 2007, 2009; Chateigner-Boutin et al., 2008; Zhou et al., 2008). The E/E+ adjacent DYW domain has been suggested from in silico analyses to harbor a deaminating activity for this type of RNA editing (Salone et al., 2007; Rüdinger et al., 2008), and the E/E+ domain without the adjacent DYW region has been proposed to interact with other proteins, including the editing enzyme (Kotera et al., 2005; Okuda et al., 2007). While the mitochondrial PPR protein identified in this work, MEF1, does display the entire E/E+/DYW extension similar to three PPR proteins recently identified for plastid editing (Zhou et al., 2008; Okuda et al., 2009), three PPRs required for specific editing sites in plastids contain only the E/E+ regions (Kotera et al., 2005; Okuda et al., 2007; Chateigner-Boutin et al., 2008). Another DYW domain-containing PPR protein for which a function has been identified is not involved in RNA editing but is required for an endonucleolytic RNA processing step in plastids (Hashimoto et al., 2003).

In several PPR proteins required for RNA editing at specific sites in the plastids, a novel 15–amino acid domain has been found to be conserved and has accordingly been proposed to play a role in the editing process (Okuda et al., 2007). This domain is not conserved in MEF1 and may be involved in a plastid-specific feature of the RNA editing process.

Requirement for RNA Editing at the Sites Affected by MEF1

The single nucleotide mutations in the *MEF1* gene identified here are not knockout mutations as the residual editing observed at the *nad2*-1160 site shows. A very low level of RNA editing below the limits of detection and thus escaping the analysis cannot be excluded at the *nad7*-963 and *rps4*-956 sites. This may provide sufficient edited mRNA for enough functional RPS4 protein to assemble the necessary set of ribosomes. Similarly, a low level of edited transcripts may supply enough competent NAD7 protein. In plastids of different tobacco (*Nicotiana* spp.) species, RNA editing at the *ndhD*-ACG codon varies in extent, and a level of ~10 to 15% appears to be sufficient to provide enough of the NDHD subunit for a functional NDH-complex (Okuda et al., 2008). By analogy, a low level of the edited *rps4* mRNA below the detection threshold of ~10% in the direct sequence analysis might be adequate to provide enough competent mitochondrial ribosomes for a normal growth habit. Alternatively, the RPS4 and NAD7 proteins synthesized from mRNAs unedited at these sites may be partially functional and may yield enough activity for growth under normal growth chamber conditions even though the amino acids encoded by the edited mRNA are conserved in the two proteins in many other plant species.

Nucleotides Conserved between the Affected RNA Editing Sites Are Not Sufficient for Specificity

Surprisingly, when comparing the three RNA editing sites addressed by the *MEF1* gene product, no extended similar sequence motif is obvious between their presumed specific recognition regions (Figure 6). In vitro and in organello investigations have delineated the *cis*-elements in the RNA context for several editing sites in mitochondria and plastids to ~20 to 25 nucleotides upstream (5′) and one to three nucleotides downstream (3′) of the edited C (Chaudhuri and Maliga, 1996; Farré et al., 2001; Miyamoto et al., 2002; Hegeman et al., 2005; Neuwirt et al., 2005; van der Merwe et al., 2006). Within this window, only five nucleotide identities are shared between all three sites in addition to the edited C, which is not enough to specify a unique site in the plant mitochondrial transcriptome. An in silico screen identified two further editing sites with these five nucleotides, sites *ccb203*-65 and *ccb382*-955 (Figure 6). These two sites are edited normally in C24 and in the mutant *mef1-2*, confirming that the five shared nucleotide positions are not sufficient to guide editing through MEF1 and that additional features defining the specificity of the interaction are required in the RNA targets. These may be further upstream and outside the window most commonly observed at editing sites. Influences of more distant elements have indeed been observed in in vitro assays of mitochondrial RNA editing and may be important in more instances than so far documented (van der Merwe et al., 2006).

The two editing sites in the plastid transcriptome targeted by the CLB19 PPR protein likewise show little sequence similarity in

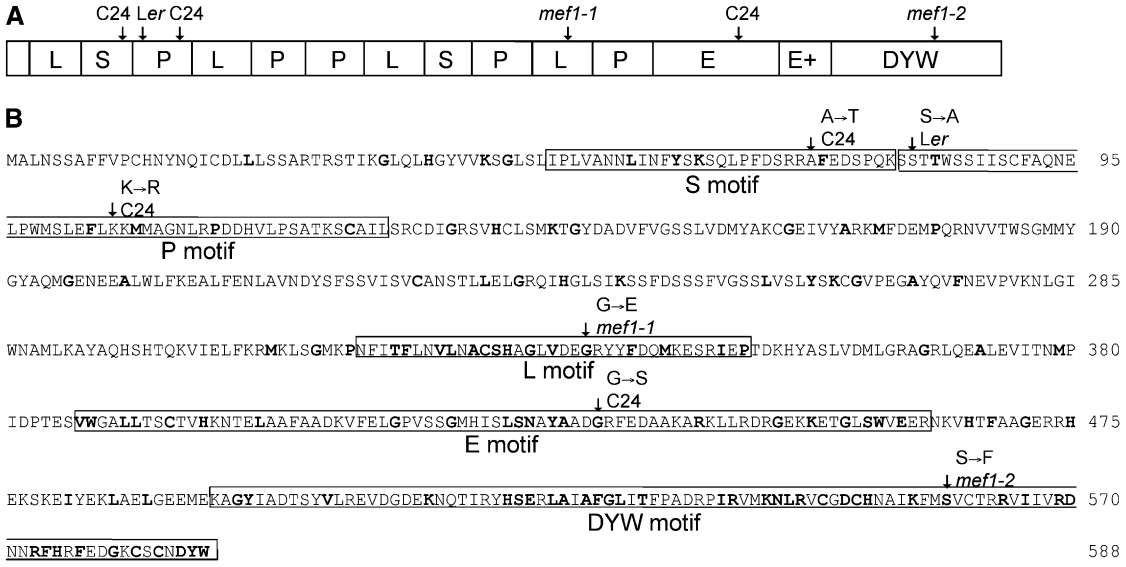

Figure 5. Structure of the DYW-PPR-Class MEF1 Protein and Locations of the Ecotype Variations and the *mef1-1* and *mef1-2* Mutations.

(A) The order of the PPR repeats and the E/E+/DYW domains are displayed in this protein schematic with the locations of the changes caused by the various SNPs (arrows). The labeling of the PPR repeats follows the nomenclature introduced by Lurin et al. (2004).
(B) The deduced amino acid sequence of MEF1 is shown. The three nonsilent variations between C24 and Col, the single amino acid change between Col and L*er*, and the single mutations in *mef1-1* and *mef1-2* are indicated. Residues conserved according to Lurin et al. (2004) are in bold. The motifs in which the changes are located are boxed and their classifications are given. The effected amino acid changes are indicated above the protein sequence.

their vicinity (Chateigner-Boutin et al., 2008). In addition, a thorough biochemical analysis of two RNA editing sites in tobacco suggests that one protein is involved in editing at both these sites, although they share very little conserved sequence (Kobayashi et al., 2007).

The Differential Site-Specific RNA Editing Phenotypes of the Mutants May Reflect Changes in the Properties of RNA Binding or of Connections to Specific RNA-Interacting Proteins

The effects of the amino acid alterations in C24 and in the two mutants *mef1-1* and *mef1-2* on RNA editing differ between the *nad7*-963 and *rps4*-956 sites and the *nad2*-1160 site. One possible explanation could be that the RNA binding properties of the MEF1 protein are altered by the various amino acid changes. Considering that the CRR4 protein required for editing at the *ndhD* initiation codon in plastids is a site-specific RNA binding protein and that RNA binding has been shown for several other PPR proteins (Okuda et al., 2006; Delannoy et al., 2007; Williams-Carrier et al., 2008), it would be reasonable to extrapolate that MEF1 can also bind to RNA directly. With this proviso, the amino acid changes in C24 versus Col and L*er* as well as in the two mutants would more strongly affect binding to the *nad7* and *rps4* sites than to the *nad2* site. Indeed, several nucleotides are shared between the *nad7* and *rps4* sequences but differ at the *nad2* site. Their relevance will have to be investigated experimentally by specific mutational analysis. The sequence

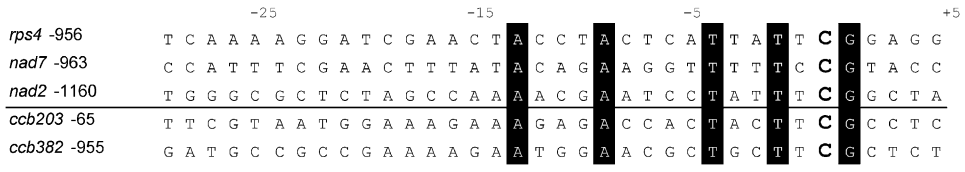

Figure 6. Alignment of the Putative *cis*-Specificity Regions around the RNA Editing Sites Targeted by *MEF1*.

Editing at the first two sites, *rps4*-956 and *nad7*-963, and to a lesser extent also at the third, *nad2*-1160, is influenced by the *mef1-1* and *mef1-2* mutations and the C24/Col variations. The edited C is in bold and a larger font. Five further nucleotide identities shared between the three sites are inversely shaded. Two other editing sites, *ccb203*-65 and *ccb382*-955, share the same five nucleotides, but their editing is not affected in C24 or in the mutant *mef1-2*. Editing sites *rps4*-956 and *nad2*-1160 alter amino acid codons, both changing S→L, while editing site *nad7*-963 is silent. The two unaffected editing sites, *ccb203*-65 and *ccb382*-955, both alter codons, the first also S→ L and the latter R→C.

comparison by itself does not allow any clear conclusion, since several other nucleotide positions are shared between *nad2* and either of the other two sites.

Alternatively, the MEF1 protein may not be binding to RNA selectively but could be an intermediate protein between one or more proteins binding specific RNA sequence motifs and the actual editing enzyme (and/or other intermediates) or may confer the actual editing activity for a select group of editing sites. The effects observed at specific editing sites may then be due to disturbances of specific protein–protein interactions. Another possible scenario could also be a combination of both, in which some nucleotide differences/mutations affect RNA binding properties, while others influence the connections to other proteins required for efficient editing.

The *mef1-2* mutation in the DYW domain alters a very conserved amino acid that should block whatever function this domain has, including the suggested deaminating enzymatic activity (Salone et al., 2007). In this scenario of the DYW domain as a cytidine deaminase, it would be somewhat difficult to explain the residual editing observed at the *nad2* site in the mutants in which no editing is detected at the *rps4* and *nad7* sites.

An alternative origin of the 10 to 20% residual editing at the *nad2* site may be that this site is targeted not only by MEF1, but also by a second protein that mediates this low-level editing. This second factor can explain that this residual editing is unaffected by the MEF1 deficiencies in the mutants as well as in ecotype C24 by having a lower, yet functioning, affinity to the *nad2* site. In the most likely scenario of the evolution of RNA editing sites in plant organelles, site recognition has to be able to adapt rather quickly to account for the numerous editing site differences between different flowering plant species. This evolutionary flexibility can be provided if the individual specificity factors are attracted by rather small and variable RNA sequence motifs, which allow these factors to attach to more than one specific region.

Indeed, the finding that MEF1 is involved in editing at least three sites supports such a scenario of rather loosely defined recognition sites for the editing factors. Additional binding sites to which the RNA editing activity will be guided by a shallow specificity of factors like MEF1 may not be bona fide observable editing sites if, for example, a U or another uneditable nucleotide is present in the appropriate position. Therefore, such RNA binding proteins with relaxed specificity may be tolerated in the organellar system as long as an essential C nucleotide is not inadvertently changed to a U.

Enough PPR Proteins for All RNA Processing Events in Organelles

Our finding that at least three distinct RNA editing sites are targeted by one PPR protein suggests that ~150 to 200 PPR proteins may be sufficient to address the ~450 RNA editing sites in a given flowering plant mitochondrial transcriptome and the ~35 editing sites in the plastid. Of the different PPR subclasses, the E/E+ only and the E/E+/DYW groups together number ~150 proteins (de Longevialle et al., 2007). The six editing factors in plastids and the mitochondrial protein identified in this work exclusively belong to these subgroups, which would be sufficient to supply all of the specific editing factors, provided that on average three sites are addressed by one protein. A considerable number of PPR proteins would then be left available for involvement in various other processes of RNA maturation in mitochondria (de Longevialle et al., 2007) and in plastids (Hashimoto et al., 2003; Schmitz-Linneweber et al., 2005, 2006; Beick et al., 2008; Williams-Carrier et al., 2008).

METHODS

Preparation of Nucleic Acids

Arabidopsis thaliana seeds for the three ecotypes Col, C24, and L*er* analyzed were kind gifts of J. Forner and S. Binder (Universität Ulm). Growth of the *Arabidopsis* plants and preparation of DNA or RNA from the leaves were as described (Takenaka and Brennicke, 2007; Zehrmann et al., 2008).

Analysis of RNA Editing Sites

Specific cDNA fragments were generated by RT-PCR amplification by established protocols (Takenaka and Brennicke, 2007). The cDNA sequences (4base lab; Macrogen) were compared for C-to-T differences resulting from RNA editing. For initial rapid screening of large numbers of samples, RT-PCR was initiated from primers (see Supplemental Table 1 online, primers 1 to 6) specific for either the edited or the unedited nucleotide.

Linkage-Based Cloning

The *MEF1* gene was identified by genomic mapping with InDel markers between Col and C24, a number of which were kindly made available by C. Jonietz and S. Binder (Universität Ulm). For additional markers, known SNPs between C24 and Col were used, and InDels between other ecotypes, such as L*er* and Col, available in the public databases (www.Arabidopsis.org/cereon/) were investigated for their relevance to the C24-Col comparison. An ethyl methanesulfonate mutant library of *Arabidopsis* ecotype Col obtained commercially (Lehle Seeds) was screened by multiplexed single base extension (Takenaka and Brennicke, 2008). The compromised RNA editing phenotype was verified by cDNA sequence analysis for the status of the respective investigated editing site (see Supplemental Table 1 online, primers 7 to 12). In the two mutants *mef1-1* and *mef1-2*, the deviant alleles of the *MEF1* gene were investigated by sequencing the relevant RT-PCR and PCR products (see Supplemental Table 1 online, primers 13 and 14).

Protoplast Complementation Assays

Protoplasts were prepared from 3- to 4-week-old plantlets and transfected by the method of Yoo et al. (2007). Transfected genes, including GFP as a control and the Col *MEF1* reading frame (see Supplemental Table 1 online, primers 13 and 14), were expressed from the 35S promoter in the *Sma*I cloning site of vector pSMGFP4 (Forner and Binder, 2007). Efficiency of the transfection was monitored by the signals from separately introduced or cotransfected GFP genes in the cytoplasm. Typically the GFP fluorescence was detected in >80% of the transfected protoplasts. Total RNA was isolated after 15 h of incubation at room temperature. Sequences of cDNAs were determined after RT-PCR with the respective specific primers (see Supplemental Table 1 online, primers 13 and 14). RNA editing levels were estimated by the relative heights of the respective nucleotide peaks in the sequence analyses. Comparison between values obtained from the peak areas with those obtained from the relative height yielded similar data, and for simplicity the latter was

employed. Data points were rounded to the nearest multiple of 5%; finer differences are not reproducible by such sequence analyses.

The lower levels of RNA editing recovered by transfection with the *MEF1* gene may be due to not all protoplasts being transfected successfully, to incomplete assembly of the introduced factor with the putative additional endogenous RNA editing components, or to the time frame in which the protoplasts are incubated. The incompletely recovered RNA editing may also be caused by inherent properties of the protoplasts in connection with the editing activity. In these protoplast preparations, the editing observed is generally lower than in total leaves as for example the 20% editing levels seen in untransfected C24 protoplasts in comparison to the 30 to 45% editing detected in C24 total leaves (Figure 1).

Assays with RT-PCR

Relative abundances of the *MEF1* transcripts in plants were estimated by comparing the amounts of RT-PCR products after increasing numbers of cycles (ranging from 20 to 40 cycles). Total cellular RNA (5.25 μg) was reverse transcribed into cDNA (M-MLV reverse transcriptase; Promega). For each sample analyzed on the gel, the cDNA obtained from 0.38 μg RNA was amplified between primers (see Supplemental Table 1 online, primers 15 and 16) to yield a product of 630 bp from the 3′ part of the open reading frame. Transcripts of the unrelated PPR gene *At1g59720* served as internal standard (see Supplemental Table 1 online, primers 17 and 18). The absence of contaminating DNA was controlled for by amplification without reverse transcriptase.

RNA Gel Blot Analysis

Total cellular RNAs (5.25 μg per lane) from leaves of 3-week-old plantlets were size fractionated in a formaldehyde-agarose gel. The RNAs from Col, C24, and the two mutants were probed with complementary strand DNA specific for the mitochondrial *rps4*, *nad7*, and *nad2* mRNA sequences, respectively. RT-PCR fragments (see Supplemental Table 1 online, primers 19 to 24) covering most of the respective reading frames were amplified from Col total cellular RNA. Probes were synthesized from these cDNAs by elongation of complementary strand primers (see Supplemental Table 1 online, primers 25 to 32) and were labeled with ^{32}P-α-dCTP by Klenow fragment (Fermentas) catalyzed DNA synthesis. Signals were visualized with a FLA-3000 phosphor imager (Fuji).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: MEF1 has the Arabidopsis Genome Initiative number At5g52630 and the gi number 18423413; the mitochondrial gene sequences for rps4, nad2, nad7, ccb203, and ccb382 are part of the mitochondrial genome under Y08501 and Y08502.

Supplemental Data

The following material is available in the online version of this article.

Supplemental Table 1. List of Primers.

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REFERENCES

Andrés, C., Lurin, C., and Small, I.D. (2007). The multifarious roles of PPR proteins in plant mitochondrial gene expression. Physiol. Plant. **129:** 14–22.

Beick, S., Schmitz-Linneweber, C., Williams-Carrier, R., Jensen, B., and Barkan, A. (2008). The pentatricopeptide repeat protein PPR5 stabilizes a specific tRNA precursor in maize chloroplasts. Mol. Cell. Biol. **28:** 5337–5347.

Chateigner-Boutin, A.L., and Hanson, M.R. (2002). Cross-competition in transgenic chloroplasts expressing single editing events reveals shared cis-elements. Mol. Cell. Biol. **22:** 8448–8456.

Chateigner-Boutin, A.L., Ramos-Vega, M., Guevara-García, A., Andrés, C., de la Luz Gutiérrez-Nava, M., Cantero, A., Delannoy, E., Jiménez, L.F., Lurin, C., Small, I., and León, P. (2008). CLB19, a pentatricopeptide repeat protein required for editing of rpoA and clpP chloroplast transcripts. Plant J. **56:** 590–602.

Chaudhuri, S., and Maliga, P. (1996). Sequences directing C to U editing of the plastid psbL mRNA are located within a 22 nucleotide segment spanning the editing site. EMBO J. **15:** 5958–5964.

Delannoy, E., Stanley, W.A., Bond, C.S., and Small, I.D. (2007). Pentatricopeptide repeat (PPR) proteins as sequence-specificity factors in post-transcriptional processes in organelles. Biochem. Soc. Trans. **35:** 1643–1647.

de Longevialle, A.F., Meyer, E.H., Andrés, C., Taylor, N.L., Lurin, C., Millar, A.H., and Small, I.D. (2007). The pentatricopeptide repeat gene OTP43 is required for trans-splicing of the mitochondrial nad1 intron 1 in *Arabidopsis thaliana*. Plant Cell **19:** 3256–3266.

Farré, J.-C., Leon, G., Jordana, X., and Araya, A. (2001). Cis recognition elements in plant mitochondrion RNA editing. Mol. Cell. Biol. **21:** 6731–6737.

Forner, J., and Binder, S. (2007). The red fluorescent protein eqFP611: Application in subcellular localization studies in higher plants. BMC Plant Biol. **7:** 28.

Giegé, P., and Brennicke, A. (1999). RNA editing in Arabidopsis mitochondria effects 441 C to U changes in ORFs. Proc. Natl. Acad. Sci. USA **96:** 15324–15329.

Handa, H. (2003). The complete nucleotide sequence and RNA editing content of the mitochondrial genome of rapeseed (*Brassica napus* L.): Comparative analysis of the mitochondrial genomes of rapeseed and *Arabidopsis thaliana.* Nucleic Acids Res. **31:** 5907–5916.

Hashimoto, M., Endo, T., Peltier, G., Tasaka, M., and Shikanai, T. (2003). A nucleus-encoded factor, CRR2, is essential for the expression of chloroplast ndhB in Arabidopsis. Plant J. **36:** 541–549.

Hegeman, C.E., Hayes, M.L., and Hanson, M.R. (2005). Substrate and cofactor requirements for RNA editing of chloroplast transcripts in Arabidopsis in vitro. Plant J. **42:** 124–132.

Heller, W.P., Hayes, M.L., and Hanson, M.R. (2008). Cross-competition in editing of chloroplast RNA transcripts in vitro implicates sharing of trans-factors between different C-targets. J. Biol. Chem. **283:** 7314–7319.

Hirose, T., and Sugiura, M. (2001). Involvement of a site-specific *trans*-acting factor and a common RNA-binding protein in the editing of

chloroplast RNA: Development of an in vitro RNA editing system. EMBO J. **20:** 1144–1152.

Kobayashi, Y., Matsuo, M., Sakamoto, K., Wakasugi, T., Yamada, K., and Obokata, J. (2007). Two RNA editing sites with cis-acting elements of moderate sequence identity are recognized by an identical site-recognition protein in tobacco chloroplasts. Nucleic Acids Res. **36:** 311–318.

Kotera, E., Tasaka, M., and Shikanai, T. (2005). A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. Nature **433:** 326–330.

Lippok, B., Brennicke, A., and Unseld, M. (1996). The rps4-gene is encoded upstream of the nad2 gene in Arabidopsis mitochondria. Biol. Chem. Hoppe Seyler **377:** 251–257.

Lurin, C., et al. (2004). Genome-wide analysis of Arabidopsis pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell **16:** 2089–2103.

Miyamoto, T., Obokata, J., and Sugiura, M. (2002). Recognition of RNA editing sites is directed by unique proteins in chloroplasts: Biochemical identification of cis-acting elements and trans-acting factors involved in RNA editing in tobacco and pea chloroplasts. Mol. Cell. Biol. **22:** 6726–6734.

Neuwirt, J., Takenaka, M., van der Merwe, J.A., and Brennicke, A. (2005). An in vitro RNA editing system from cauliflower mitochondria: Editing site recognition parameters can vary in different plant species. RNA **11:** 1563–1570.

Okuda, K., Chateigner-Boutin, A.-L., Nakamura, T., Delannoy, E., Sugita, M., Myouga, F., Motohashi, R., Shinozaki, K., Small, I., and Shikanai, T. (2009). Pentatricopeptide repeat proteins with the DYW motif have distinct molecular functions in RNA editing and RNA cleavage in Arabidopsis chloroplasts. Plant Cell **21:** 146–156.

Okuda, K., Habata, Y., Kobayashi, Y., and Shikanai, T. (2008). Amino acid sequence variations in Nicotiana CRR4 orthologs determine the species-specific efficiency of RNA editing in plastids. Nucleic Acids Res. **36:** 6155–6164.

Okuda, K., Myouga, R., Motohashi, K., Shinozaki, K., and Shikanai, T. (2007). Conserved domain structure of pentatricopeptide repeat proteins involved in chloroplast RNA editing. Proc. Natl. Acad. Sci. USA **104:** 8178–8183.

Okuda, K., Nakamura, T., Sugita, M., Shimizu, T., and Shikanai, T. (2006). A pentatricopeptide repeat protein is a site-recognition factor in chloroplast RNA editing. J. Biol. Chem. **281:** 37661–37667.

Rüdinger, M., Polsakiewicz, M., and Knoop, V. (2008). Organellar RNA editing and plant-specific extensions of pentatricopeptide repeat proteins in Jungermaniid but not in Marchantiid liverworts. Mol. Biol. Evol. **25:** 1405–1414.

Salone, V., Rüdinger, M., Polsakiewicz, M., Hoffmann, B., Groth-Malonek, M., Szurek, B., Small, I.D., Knoop, V., and Lurin, C. (2007). A hypothesis on the identification of the editing enzyme in plant organelles. FEBS Lett. **581:** 4132–4138.

Sasaki, T., Yukawa, Y., Wakasugi, T., Yamada, K., and Sugiura, M. (2006). A simple in vitro RNA editing assay for chloroplast transcripts using fluorescent dideoxynucleotides: Distinct types of sequence elements required for editing of ndh transcripts. Plant J. **47:** 802–810.

Schmitz-Linneweber, C., and Small, I. (2008). Pentatricopeptide repeat proteins: A socket set for organelle gene expression. Trends Plant Sci. **13:** 663–670.

Schmitz-Linneweber, C., Williams-Carrier, R.E., and Barkan, A. (2005). RNA immunoprecipitation and microarray analysis show a chloroplast pentatricopeptide repeat protein to be associated with the 5′ regions of mRNAs whose translation it activates. Plant Cell **17:** 2791–2804.

Schmitz-Linneweber, C., Williams-Carrier, R.E., Williams-Voelker, P.M., Kroeger, T.S., and Barkan, A. (2006). A pentatricopeptide repeat protein facilitates the trans-splicing of the maize chloroplast rps12 pre-mRNA. Plant Cell **18:** 2650–2663.

Shikanai, T. (2006). RNA editing in plant organelles: Machinery, physiological function and evolution. Cell. Mol. Life Sci. **63:** 689–708.

Small, I.D., and Peeters, N. (2000). The PPR motif – A TPR-related motif prevalent in plant organellar proteins. Trends Biochem. Sci. **25:** 46–47.

Takenaka, M., and Brennicke, A. (2007). RNA editing in plant mitochondria: Assays and biochemical approaches. Methods Enzymol. **424:** 439–458.

Takenaka, M., and Brennicke, A. (2008). Multiplex single base extension typing to identify nuclear genes required for RNA editing in plant organelles. Nucleic Acids Res. **37:** e13 10.1093/nar/gkn975.

Takenaka, M., Verbitskiy, D., van der Merwe, J.A., Zehrmann, A., and Brennicke, A. (2008). The process of RNA editing in plant mitochondria. Mitochondrion **8:** 35–46.

van der Merwe, J.A., Takenaka, M., Neuwirt, J., Verbitskiy, D., and Brennicke, A. (2006). RNA editing sites in plant mitochondria can share cis-elements. FEBS Lett. **580:** 268–272.

Williams-Carrier, R., Kroeger, T., and Barkan, A. (2008). Sequence-specific binding of a chloroplast pentatricopeptide repeat protein to its native group II intron ligand. RNA **14:** 1930–1941.

Yoo, S.-D., Cho, Y.-H., and Sheen, J. (2007). *Arabidopsis* mesophyll protoplasts: A versatile cell system for transient gene expression analysis. Nat. Protocols **2:** 1565–1572.

Zehrmann, A., van der Merwe, J.A., Verbitskiy, D., Brennicke, A., and Takenaka, M. (2008). Ecotype-specific variations in the extent of RNA editing in plant mitochondria. Mitochondrion **8:** 319–327.

Zhou, W., Cheng, Y., Yap, A., Chateigner-Boutin, A.-L., Delannoy, E., Hammani, K., Small, I., and Huang, J. (2008). The Arabidopsis gene *YS1* encoding a DYW protein is required for editing of *rpoB* transcripts and the rapid development of chloroplasts during early growth. Plant J., in press.