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Contiguous RNA editing sites in the mitochondrial *nad1* transcript of *Arabidopsis thaliana* are recognized by different proteins

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ABSTRACT

Pentatricopeptide repeat (PPR) proteins have been identified as site-specific factors for RNA editing in plant organelles. These proteins recognize *cis*-elements near the editing site. It is unclear how contiguous sites are addressed, and whether one or two factors are required. We here show the PPR MEF25 to be essential for RNA editing at the *nad1*-308 site in *Arabidopsis* mitochondria. Another editing site just one nucleotide upstream, *nad1*-307, is edited normally in *mef25* mutant lines. This finding shows that two independent factors recognizing similar *cis*-elements are involved at these contiguous sites without competing with each other in vivo.
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1. Introduction

RNA editing changes the nucleotide sequence of mature transcripts away from that of its DNA templates. In flowering plants, this change involves the deamination of more than 400 specific cytidines to uridines in organellar transcripts [1]. Since RNA editing was discovered in plant mitochondria, the two basic questions of the specificity determinants in the RNA substrate and the components of the "editosome" machinery have been answered only partially. Experiments with in organello [2] or in vitro [3] editing systems showed that a *cis*-region in the RNA between −20 and +6 nucleotides relative to the target site is generally necessary and sufficient for editing. However, until now the precise bases for specificity have not yet been established for any site. Recently, the MORF-protein family (Multiple Organellar Editing Factor) was found to be important for editing of many sites in mitochondria and all sites in plastids [4], but their precise role is still open. These MORFs can interact with selected *trans*-acting proteins necessary for editing single or few specific cytidines, the pentatricopeptide repeat (PPR) proteins [5–7]. Distinct functions have been assigned to several PPRs, all of them related to organellar RNA metabolism including splicing, endo- and exonucleolytic processing, translation initiation and editing [8]. PPR proteins are characterized by degenerate motifs of 35 amino acids arranged as tandem repeats. Some PPR proteins (PLS subfamily) contain repeats shorter (S) or longer (L) than the canonical (P) 35 amino acid repeat, and one, two or three additional domains in the C-terminal region: The E-domain, the E+-domain and the DYW-domain [5]. All of the organelle editing factors required for editing at specific sites contain the E, the E and E+ or the E, E+ and DYW domains. PPR proteins most likely bind specifically to RNA with the PPR motifs, and in the case of RNA editing recognize the *cis*-element at the target site (e.g. [9–11]).

Several RNA editing sites in plant organelles are located near or even contiguous to other sites. Two alternative scenarios are plausible for the recognition of contiguous nucleotides. In the first scenario one PPR protein binds to its cognate *cis*-element and facilitates RNA editing at both sites. In this case, the PPR protein must allow at least one nucleotide flexibility in the distance between the binding site and editing sites. In the second scenario, two PPR proteins independently mediate editing at the two sites. In this case, the *cis*-elements of the contiguous sites are expected to overlap for the two *trans*-factors and the mutually exclusive binding of the two PPR proteins must accommodate the potential competition. We here report the identification and analysis of the novel Mitochondrial RNA Editing Factor 25, MEF25, which very

Abbreviations: GFP, green fluorescent protein; GUS, β-glucuronidase; MEF, mitochondrial editing factor; MORF, multiple organellar editing factor; PPR, pentatricopeptide repeat

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specifically addresses only one of two contiguous RNA editing sites in the *nad1* transcript.

2. Materials and methods

2.1. Molecular and phenotypic analysis of mef25 mutant plants

The wild type *Arabidopsis thaliana* ecotype used was Columbia-0 (Col-0). The T-DNA insertion mutant lines SAIL 672 (*mef25-1*) and SALK_091381C (*mef25-2*) in the Col-0 background were obtained from the ABRC Stock Center. Seeds were sown on half-concentrated Murashige and Skoog medium supplemented with 1% (w/v) sucrose and solidified with 0.8% (w/v) agar. After 2 weeks in a 16/8-h day/night cycle at 22 °C, seedlings were transferred to soil and grown for 4–6 weeks under the same conditions. To isolate homozygous mutant plants, genotyping was performed by PCR as described [12], using the specific primers indicated in Supplementary Table 1 (see also Supplementary Fig. 2). Growth and phenotype of wild type and *mef25* mutant plants were analyzed at the seedling stage and in adult plants. Alexander staining of pollen was performed on anthers of recently opened flowers as described [12].

To characterize *nad1* transcripts in wild type and *mef25* mutant plants, total RNA was isolated from leaves of six-week-old plants or 15-days-old seedlings with the TRIzol reagent following the manufacturer's protocol (Invitrogen). Northern-blot analyses were performed as described, using a *nad1* ^{32}P-labeled probe [13].

2.2. Analysis of RNA editing sites

mef25-1 and *mef25-2* homozygous mutant plants were screened by multiplexed single base extension "SNapShot analysis" for altered RNA editing at specific sites [14]. To confirm the editing defect observed, specific cDNA fragments were generated by RT-PCR amplification [15] using primer *nad1ssampAC580R* for cDNA synthesis and primers *nad1ssamp-37F* and *nad1ssamp-CA376R* for PCR amplification (Supplementary Table 1). The status of the respective editing site was determined by sequence analysis (Macrogen, Seoul, Korea or 4base lab, Reutlingen, Germany). The cDNA sequences were compared for C to T differences resulting from RNA editing.

2.3. Constructs for MEF25 localization experiments and promoter analysis

MEF25 localization was investigated by GFP fusion to the MEF25 amino terminal 144 amino acids in vector pK7FWG2 [16]. The DNA encoding this amino terminal sequence was obtained by PCR with primers *amino-mef25F* and *amino-mef25R* (Supplementary Table 1). To construct GUS promoter fusions, two PCR fragments of 1000 and 500 bp from upstream of the MEF25 initiation codon were cloned into the pGEM-T easy plasmid (Promega), digested with *Sal*I and *Nco*I (restriction sites introduced by the primers, see Supplementary Fig. 1) and ligated to the GUS frame in pCAMBIA1381 (www.cambia.org). Primers were *Pro1mef25-SalIF* and *Promef25-NcoIR* for the 1.0 kb DNA fragment and *Pro2mef25-SalIF* and *Promef25-NcoIR* for the 0.5 kb fragment. Constructs were verified by DNA sequencing, introduced into *Agrobacterium tumefaciens* GV3101 by electroporation and *Arabidopsis* plants were transformed by floral dip [17]. Several independent transgenic lines were obtained for each construct. GFP and Mitotracker Orange (Invitrogen) fluorescence were analyzed with a confocal microscope (Nikon C2+) in seedling roots and protoplasts prepared as described [18]. Histochemical GUS staining was performed as described [19].

3. Results

3.1. At3g25060 encodes an E+-PPR protein targeted to mitochondria

To identify specific *trans*-factors involved in RNA editing of plant mitochondrial transcripts, we selected PPR candidate genes based on four criteria: (i) PPR genes with ESTs or cDNA sequences in public databases; (ii) mitochondrial destination predicted by different subcellular sorting algorithms (Predotar, TargetP, iPSORT, Mitoprot2); (iii) members of the PLS subfamily with E, E+ or DYW motifs; (iv) low sequence similarity with other *Arabidopsis* PPR proteins. One of the PPR genes thus selected is At3g25060, which encodes an E+-PPR protein.

To test the prediction that the protein translated from this gene, At3g25060, is targeted to mitochondria, a DNA fragment encoding the N-terminal 144 amino acids (up to the second PPR motif) was fused upstream of the GFP gene and stable transgenic *Arabidopsis* lines were generated. In leaf protoplasts and seedling roots from these transgenic lines GFP fluorescence showed the punctuate pattern characteristic of mitochondria (Fig. 1). Furthermore, GFP and Mitotracker-Orange fluorescence overlapped and were clearly distinct from chloroplast fluorescence, confirming that the AT3G25060 protein is targeted to mitochondria and not to chloroplasts.

To characterize *MEF25* gene expression, we fused either 1000 or 500 bp fragments from just upstream of the *MEF25* AUG codon to a GUS reporter gene. Transgenic lines for both constructs showed similar expression patterns, suggesting that the main promoter activity is located within 500 bp upstream of the *MEF25* AUG start codon (Supplementary Fig. 1). GUS activity was detected in cotyledons and hypocotyls of seedlings, in leaves, and in sepals, filaments and the pistil of flowers.

3.2. MEF25 is required for one mitochondrial RNA editing site

With the mitochondrial localization of AT3G25060 confirmed, we analyzed the RNA editing phenotypes in At3g25060 disrupted mutants. Two independent mutants were retrieved from the SALK and SAIL collections, homozygous plants were selected and the positions of both independent T-DNA insertions were confirmed by PCR and sequencing to be in the coding region (Fig. 2A, Supplementary Fig. 2).

For the identification of mitochondrial RNA-editing defects in the mutants, RNA from leaves was screened for deficiencies in RNA editing with the multiplexed SNaPshot approach [14,15]. With this assay, 367 annotated editing sites were analyzed, in addition, 45 further mitochondrial editing sites were examined by direct RT-PCR amplification and sequencing. In this analysis we found a defect in editing at one of the 25 editing events in the *nad1* transcript, site *nad1*-308 is not edited in both mutants (Fig. 2B and C). Surprisingly, the immediately adjacent site *nad1*-307 is completely edited in both wild type and mutant plants, suggesting that nucleotides 307 and 308 are recognized by different factors. When we analyzed the *nad1* RNA editing status by RT-PCR, we found that among 43 mutant cDNA clones, all were unedited at *nad1*-308 and edited at *nad1*-307 (Supplementary Fig. 3A). In contrast, among 82 wild type cDNA clones, all were edited at *nad1*-307 and 80 were also edited at *nad1*-308. When normally edited at positions 307 and 308, the CCG (pro) codon is changed to UUG (leu) codon. The leucine amino acid is highly conserved in plants. In both mutants the CCG codon is edited to CUG, encoding serine. The gene locus (TAIR ID: AT3G25060) was renamed *MEF25* and the mutant lines *mef25-1* and *mef25-2*, respectively.

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