FEBS Letters 587 (2013) 887–891

FEBS Letters

journal homepage: www.FEBSLetters.org

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

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ARTICLE INFO

Article history:
Received 22 November 2012
Revised 4 February 2013
Accepted 5 February 2013
Available online 14 February 2013

Edited by Michael Ibba

Keywords:
RNA editing factor
Plant mitochondria
Pentatricopeptide repeat protein
PPR protein
nad1
MEF25

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.

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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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2013.02.009

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.

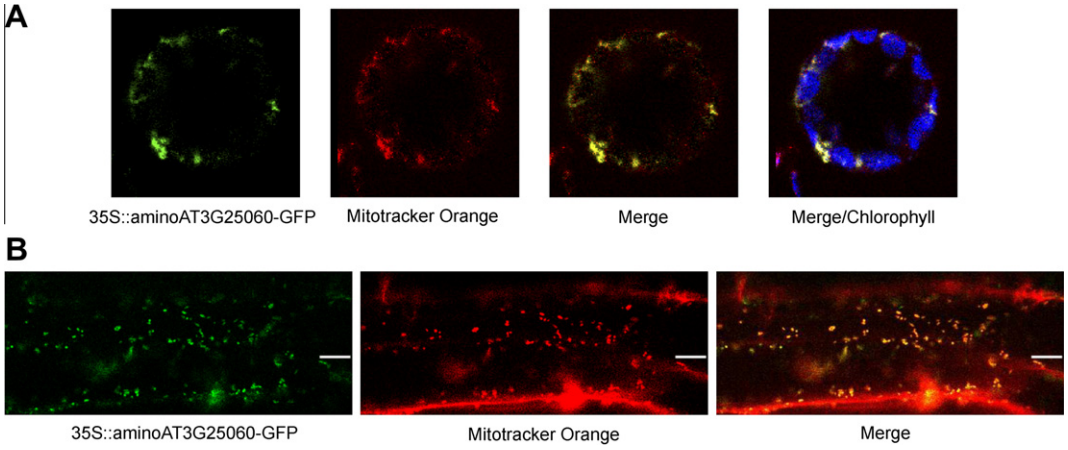

Fig. 1. The AT3G25060 (MEF25) amino terminus targets GFP to mitochondria. The aminoAT3G25060-GFP fusion protein was expressed in transgenic *Arabidopsis* plants under control of the CaMV35S promoter. (A) Protoplasts isolated from leaves and incubated with Mitotracker Orange, from left to right: AminoAT3G25060-GFP fluorescence; Mitotracker Orange signal; merged aminoAT3G25060-GFP and Mitotracker Orange signals (yellow indicates signal co-localization); overlay between the merged image of aminoAT3G25060-GFP and Mitotracker Orange signals, and chlorophyll autofluorescence. (B) Roots of a seedling, from left to right: aminoAT3G25060-GFP signal; Mitotracker Orange stain; merged GFP and Mitotracker Orange signals. Scale bar = 10 μm.

3.3. Phenotype and pattern of nad1 transcripts are not affected in mef25 mutants

When the mutants were compared to wild type plants, no changes were seen in the macroscopic phenotype or in the development of homozygous *mef25-1* and *mef25-2* plants under standard growth conditions (Supplementary Fig. 4). Neither was pollen viability affected. These results suggest that the lack of editing at site *nad1*-308 has little effect on normal mitochondrial function.

RNA processing of the *nad1* precursor to the mature transcript includes *cis*- and *trans*-splicing and 25 RNA editing events (Fig. 2B). To evaluate if the lack of RNA editing at the *nad1*-308 site alters other RNA processing steps in the *nad1* transcript, we assayed an RNA gel blot with a probe specific for the *nad1* transcript. No difference in the *nad1* transcript profiles was seen in leaves of wild type and *mef25-1* and *mef25-2* mutant plants, suggesting that stability, splicing and 5′ or 3′ processing are not affected (Supplementary Fig. 5). This result also precludes that the molecular phenotype of the loss of RNA editing at site *nad1*-308 is a secondary effect generated by defects in transcription or processing.

4. Discussion

Here we identify MEF25 as a mitochondrial PPR protein required for RNA editing specifically at site *nad1*-308. This assignment is supported by the lack of editing at this site in two independent *mef25* mutant lines. The unedited C changes a highly conserved leucine to a serine in the NAD1 protein. NAD1 is one of more than 40 subunits of respiratory Complex I (NADH dehydrogenase) in plants and one of nine coded in the mitochondrial genome [20,21]. Modular subunit arrangements proposed by Klodmann et al. [20] localize this subunit in the membrane arm, to the periphery at the interface of the two complex I arms.

The *mef25* mutant lines are phenotypically indistinguishable from wild type plants, at least under standard growth conditions (Supplementary Fig. 4), suggesting that the amino acid alteration caused by the absence of the *nad1*-308 editing event is tolerated in the NAD1 protein despite the conservation of the amino acid encoded by the edited codon in many plants (Fig. 3). Amino acid changes caused by other editing events in the *nad1* transcript may be more detrimental and it will be interesting to see if there is any cumulative effect detectable from the combination of several such amino acid alterations. Until now the only factors implicated in editing of other sites in the *nad1* mRNA are MORF factors acting at sites 500, 743 and 755 (Supplementary Fig. 6) [4].

There are several contiguous pairs of RNA editing events in *Arabidopsis* mitochondria, for example sites *mttB*-144 and 145 [22]. In the *slo2* mutants, RNA editing at both *mttB*-144 and 145 sites is completely lost, suggesting that binding of the SLO2 protein to its *cis*-element is essential for RNA editing at both sites. The action of MEF25 at contiguous sites *nad1*-307 and 308 is likely different from SLO2, since editing is lost only at site *nad1*-308 while the adjacent *nad1*-307 nucleotide is normally edited. More than 40 independent clones of the *nad1* cDNA from mutant plants confirm this specificity (Supplementary Fig. 3A). Therefore, these two adjacent editing sites must be recognized by (at least) two different editing factors. These two factors appear to solely act on their cognate sites, the factor specific for site *nad1*-307 cannot edit *nad1*-308 and site *nad1*-307 does not require MEF25. Furthermore, site *nad1*-307 does not require site *nad1*-308 to be edited and is recognized and processed independently from the identity of the downstream nucleotide at *nad1*-308. The contrasting behavior of SLO2 and MEF25 at respectively contiguous RNA editing events may be induced by different cofactors, however, it is still possible that SLO2 acts similar to MEF25. SLO2 may be responsible for editing at only one of the two *mttB* sites which may be a pre-requisite for binding of another *trans*-factor and editing at the second site.

Among the most closest situations in which mitochondrial editing at only one of two nearby sites is impaired in PPR mutants are sites *ccmF*-1 and *ccmF*-2 in *Physcomitrella patens*, separated by 18 nucleotides, and *cox3*-413 and *cox3*-422 in *Arabidopsis*, separated by 8 nucleotides [24,25]. In the *Physcomitrella* PpPPR_71 mutant editing at *ccmF*-2 was impaired while *ccmF*-1 was unaffected. In this case editing at *ccmF*-1 may be required for editing at *ccmF*-2 since cDNA sequence analysis suggested that *ccmF*-1 editing occurs before *ccmF*-2 editing, and the PpPPR_71 protein preferentially binds RNA edited at *ccmF*-1. In the *Arabidopsis mef11* mutant no detectable editing was observed at site *cox3*-422 while *cox3*-413 editing was unaffected, and editing at *cox3*-413 increases the similarity between the presumed *cox3*-422 recognition sequence and that of another editing site targeted by MEF11 (*nad4*-124). However, in contrast to the situation of *ccmF* sites, cDNA sequence analysis indicated that editing at the upstream *cox3*-413 is not required for editing at the *cox3*-422 site. It is clear from our results that editing at *nad1*-307 does not require previous editing at *nad1*-308.

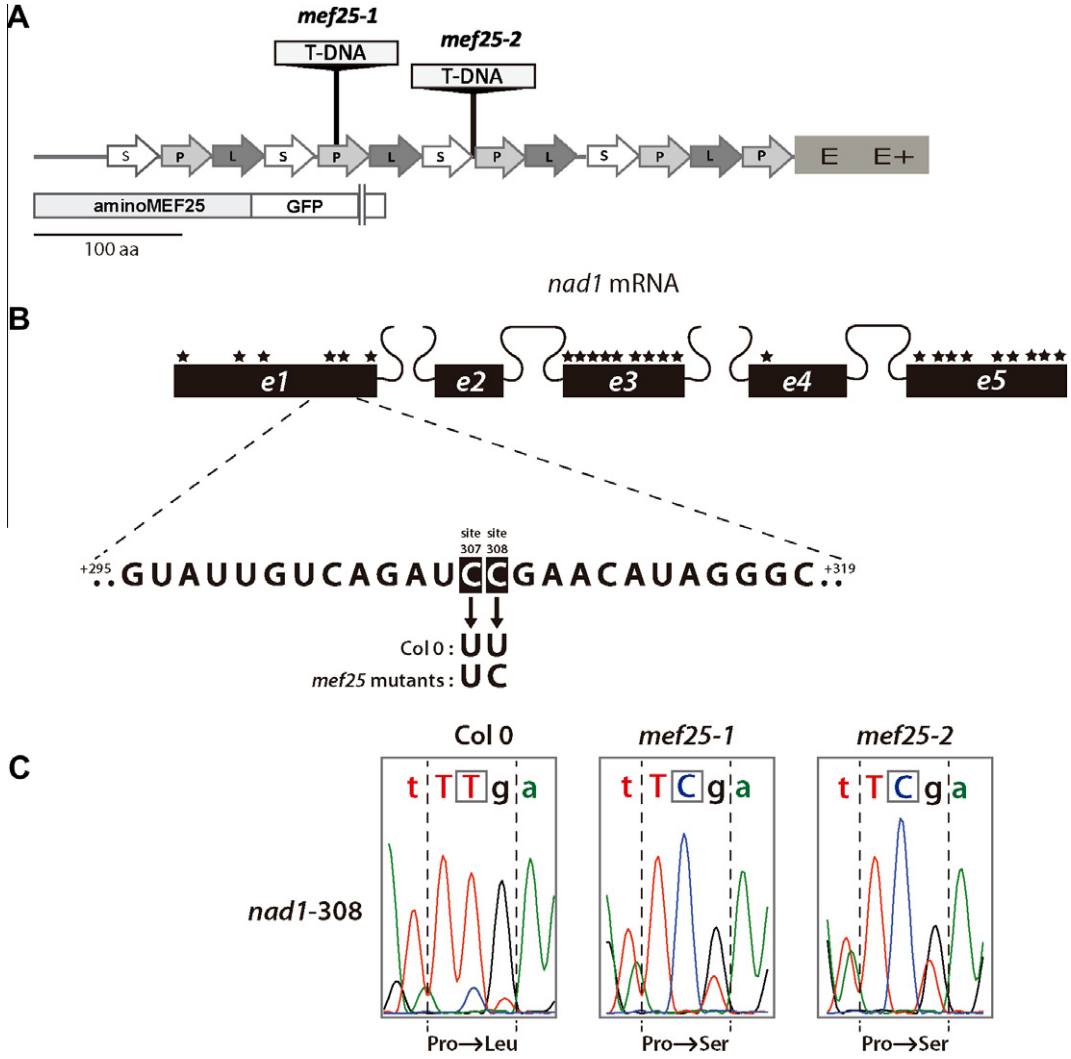

Fig. 2. AT3G25060 (MEF25) is necessary for one RNA editing event in the mitochondrial *nad1* transcript. (A) AT3G25060 (MEF25) structure and T-DNA insertion sites in the *mef25-1* and *mef25-2* mutants. MEF25 contains thirteen pentatricopeptide (PPR) motifs (arrows). P represents the classic PPR motif, L and S the variant "long" and "short" PPR motifs, and E and E+ the extension motifs at the C-terminus (box). The N-terminal sequence fused to GFP to analyze the subcellular localization is shown below the MEF25 structure. (B) Structure of the *nad1* mRNA. Five exons (black boxes), two *trans*- and two *cis*-splicing events (open and closed lines, respectively), and 25 editing sites (stars) are indicated. The zoomed sequence below gives the nucleotides from +295 to +319 nt in exon 1 (e1) with the *nad1*-308 site which is not edited in the *mef25* mutants. Nucleotides are counted from the AUG. (C) Sequence analyses of *nad1* cDNAs obtained by RT-PCR from wild type and *mef25* mutant plants are displayed. The codon containing both 307 and 308 editing sites is indicated by dashed lines, editing sites are in capitals and site *nad1*-308 is framed.

However, whether editing at *nad1*-308 requires *nad1*-307 to be edited or not is unresolved. cDNA sequence analysis showed that both sites are nearly fully edited in mature transcripts from wild type seedlings (82/82 clones edited at *nad1*-307 and 80/82 clones edited at *nad1*-308, Supplementary Fig. 3A). In an attempt to analyze partially edited transcripts, we sequenced 48 cDNAs containing exon 1 and part of intron 1 (Supplementary Fig. 3B). Forty-two cDNA clones (87.5%) were edited at both sites, four (8.3%) were unedited at both sites and two (4.2%) were edited at *nad1*-307 only. Although the number of cDNA clones edited at *nad1-307* only is low (two for mature transcripts and two for unspliced transcripts), the absence of cDNAs edited at *nad1*-308 only may suggest that *nad1*-307 editing occurs before and is required for editing at *nad1*-308. Alternatively, both sites may be edited independently with *nad1*-307 editing more efficient and faster. Unfortunately we did not obtain recombinant MEF25 protein to evaluate MEF25 in vitro binding to RNA edited or unedited at *nad1*-307.

Recently an amino acid code has been proposed for RNA recognition by PPR proteins [26]. This amino acid code does not assign any role to the −1 position of *nad1*-307 in *nad1*-308 editing. Based on the work of Lurin et al. [5] we carefully analyzed MEF25 amino acid sequence and manually predicted the structure of PPR, E and E+ motifs shown in Fig. 2. This structure is similar to that annotated in the UniProt database (http://www.uniprot.org) and allows us to identify in each motif the 6 and 1′ amino acids proposed to constitute the amino acid code [26]. However, application of this code to MEF25 does not show preferential targeting of *nad1*-308 over *nad1*-307, and does not allow to predict which PPR protein may target *nad1*-307. It will be interesting to identify the PPR protein factor for site *nad1*-307 which may have a similar set of PPR repeats recognizing similar nucleotides in the *cis*-element. Alternatively, the PPR repeats may be different from MEF25 and attach to a very different set of nucleotides in a shifted, possibly overlapping position. A Blast search using MEF25 as a query identified 80 PPR proteins with an overall identity of 30–35%, with AT3G12770 (MEF22) and AT2G29760 (OTP81) displaying the lowest e-values. A comparison between MEF25 and MEF22 or OTP81 is shown in Supplementary Fig. 7. MEF 22 is required for editing at *nad3*-149 and not at *nad1*-307 [27], and OTP81 is a plastid protein involved in *rps12* intron editing [28]. No significant similarities are observed between these and the *nad1*-307/308 target sites. Thus, the PPR protein targeting *nad1*-307 remains to be identified. In conclusion,

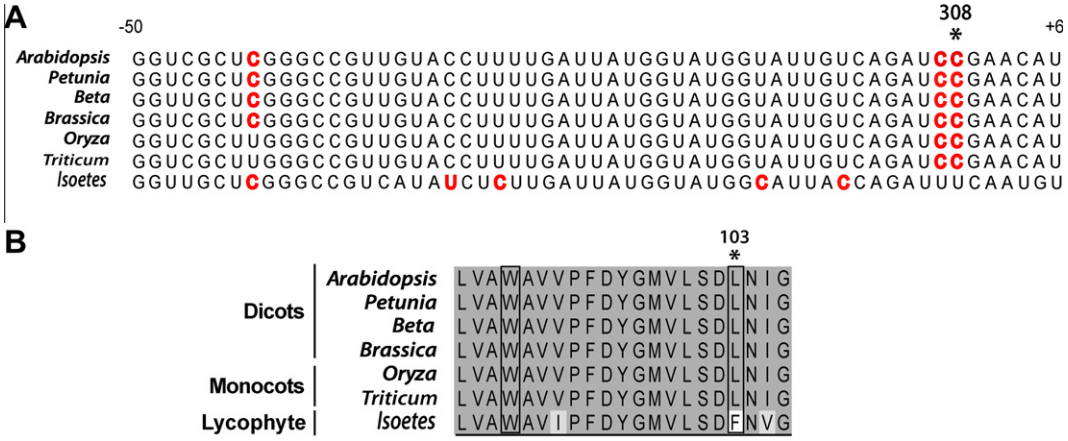

Fig. 3. Conservation of nucleotide and amino acid sequences around the *nad1*-308 editing site. (A) Alignment of nucleotide sequences around the editing site targeted by MEF25 in land plants. Nucleotide 308 (counted from the AUG) and other RNA editing sites are shown as unedited C or U in red. (B) Alignment of amino acid sequences around leucine 103 of the NAD1 protein. Amino acid sequences are from the UniProt database (http://www.uniprot.org), mRNA sequences from REDIdb (http://biologia.unical.it/py_script/REDIdb/) and lycophyte sequences from Grewe et al. [23]. Identical residues are in grey, similar amino acids are in light grey. Amino acids altered by RNA editing events in angiosperms are boxed.

our results thus suggest that two specificity factors can share a *cis*-region in the RNA without competition in vivo.

Acknowledgments

We are very grateful to Axel Brennicke for his critical reading of the manuscript, discussion of results and constant encouragement, and to Franko Restovic for his valuable help and suggestions. This work was supported by grants from CONICYT (AT24100161), FONDECYT (1100601), Millenium Nucleus in Plant Functional Genomics (P10-062-F, Millennium Scientific Initiative Program, Mideplan, Chile) and the Deutsche Forschungsgemeinschaft. Mizuli Takenaka is a Heisenberg fellow.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.02.009.

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