RNA editing competence of trans-factor MEF1 is modulated by ecotype-specific differences but requires the DYW domain

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ABSTRACT

RNA editing in plant mitochondria posttranscriptionally changes multiple cytidines to uridines. The RNA editing trans-factor MEF1 was identified via ecotype-specific editing polymorphisms in Arabidopsis thaliana. Complementation assays reveal that none of the three amino acid changes between Columbia (Col) and C24 individually alters RNA editing. Only one combination of these polymorphisms lowers editing at two of the three target sites, suggesting additive effects of the involved SNPs. Functional importance of the C-terminal DYW domain was analysed with DYW-truncated and extended constructs. These do not recover RNA editing in protoplasts and regain only low levels in stable transformants. In MEF1, the DYW domain is thus required for full competence in RNA editing and its C-terminus has to be accessible.

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1. Introduction

Since the detection of RNA editing in plant mitochondria more than 20 years ago, a lot of research has been done with the aim to identify the mechanism of the C to U-alteration and to determine the requirements of targeting the sites to be edited. Although the exact mode of action has not yet been revealed, it seems that the identity of the nucleotide is changed by a deamination reaction [1,2]. Concerning the cis-elements around the editing sites, a region between 20 nts upstream and only 3 nts downstream appears to be sufficient to identify a C-nucleotide target [3–5]. For the RNA editing in plastids several trans-factors have already been identified during the last few years [6–10], while the first trans-factors acting in mitochondria have been identified only recently [11–14].

All RNA editing trans-factors in plastids and mitochondria known so far belong to the class of pentatricopeptide repeat proteins (PPR proteins). Characteristic of these proteins is a repeated motif of about 35 amino acids [15]. The approximately 450 members of the nuclear-encoded protein family in flowering plants can be classified into different categories on the basis of the nature of the repeats and of various C-terminal extensions [16]. So far all factors involved in RNA editing exhibit at their C-terminus at least one extension, the so-called E-domain. Some possess in addition a region which is known as DYW domain. For two of the DYW-class PPR proteins involved in RNA editing in plastids, CRR22 and CRR28, it has been shown that their DYW domains are interchangeable and can be even completely removed without influence on the RNA editing efficiency of the respective target sites [8]. This observation suggests that the DYW domains are in vivo dispensable for correct function of these trans-factors. Removal of the E domain of the plastid editing factor CRR4 however resulted in significantly reduced editing efficiency of its target site in transgenic plants, indicating that the E domain is required for RNA editing. Exchanging the E domains of trans-factors CRR4 and CRR21 yielded functional chimeric proteins, suggesting that these E domains have a common function in RNA editing [7].

The first mitochondrial factor MEF1 was discovered via ecotype-specific editing polymorphisms in Arabidopsis thaliana. Two sites in mitochondrial transcripts, rps4-956 and nad7-963, show a lower editing efficiency of 40–50% in ecotype C24 when compared to 100% C to U alteration in ecotype Col [17]. In two independent EMS mutant lines no detectable editing is observed at these two sites and in addition RNA editing at a site in the nad2 transcript (nad2-1160) is strongly reduced. The nuclear-encoded editing factor was identified by linkage-based cloning and verified by complementation of C24 and mutant protoplasts. While in the mutant plants single amino acid changes in MEF1 inactivate RNA editing, the reduced editing of rps4-956 and nad7-963 in C24 is connected with three SNPs between the ecotypes Columbia (Col)
and C24 which alter the encoded amino acid sequence of MEF1 [11]. We here investigate the influence of each of these SNPs on the editing efficiency of the affected sites. Furthermore we examine the functional requirement for the DYW domain in this RNA editing trans-factor.

2. Materials and methods

2.1. Plant material

Seeds for the Arabidopsis thaliana ecotypes C24 and Col-0 were kind gifts of J. Forner and S. Binder (Universität Ulm). The two mutant lines mef1-1 and mef1-2 are derived from an EMS mutant population of Arabidopsis thaliana ecotype Col obtained from Lehle Seeds (http://www.arabidopsis.com). These had been identified by a multiplexed SNPshot approach [18]. All plants were grown as described previously [17].

2.2. Protoplast complementation assays

Preparation of protoplasts from 3- to 4-week-old individual plantlets and transfection was performed by the method of Yoo et al. [19]. Transfected genes were expressed from the 35S promoter in vector pSMGF4 [20]. The C24 ecotype-specific mutations were introduced into the Col MEF1 reading frame by site-directed mutagenesis [21]. Deletion of the region coding for the DYW domain was achieved by inverse PCR [22]. Efficiency of the transfections was monitored as the RNA editing levels obtained in control transfections with the intact Col MEF1 reading frame. Total RNA was prepared after 20–24 h incubation at room temperature with the illustra RNAspin Mini Kit (GE Healthcare). Specific cDNA fragments were generated by RT-PCR amplification by established protocols [23]. The cDNA sequences (4 base lab; Macrogen) were compared for differences in C to T ratios resulting from RNA editing. RNA editing levels were estimated by the relative height of the respective nucleotide peaks in the sequence analyses [11]. All assays were performed at least four times and interpreted according to the replicate results. These all agreed within the typical experimental variance of such biological assays. Seven assays were performed with different preparations of protoplasts from different plants. In three of these assays, one or the other data point (of a total of 16 parallel transfections and RNA preparations, and 48 RT-PCRs with sequence analyses in each assay series) had failed and was not interpretable. Thus four complete series of protoplast transfection assays could be used for the statistics in Fig. 2. The efficiency of parallel control transfections with the wt Col gene was taken as 100% in each separate set of assays to which each mutant was compared.

2.3. Plant transformation

To obtain transgenic plants, respective DNA sequences were cloned under control of the 35S promoter into the binary vector pMDC123 [24] and introduced into mef1-1 mutant plants via Agrobacterium tumefaciens GV2260 by the method of Clough and Bent [25].

3. Results

3.1. Influence of the SNPs between Col and C24 in MEF1 on RNA editing of the target sites

In the DYW-class PPR protein MEF1 three amino acids differ between Col and C24, caused by three SNPs between the two ecotypes (Fig. 1A). The polymorphism in the first S-domain (nucleotide position 214) changes an Ala in the Col sequence to Thr in C24, the SNP at nucleotide position 314 alters a Lys to an Arg residue. In the E domain a conserved Gly is altered to a Ser in C24 by the polymorphism at nucleotide position 1297. The ecotype C24-specific MEF1 variant reduces the editing efficiency at two of the mitochondrial target sites, rps4-956 and nad7-963, to 40–50% which are edited to 100% in Col. This effect can be caused by amino acid alterations from either of these SNPs individually or by a combination of them.

To address this question and to investigate the influence of each of these non-synonymous SNPs on the editing efficiency of the affected sites, we monitored the recovery of RNA editing in cells of the mutants mef1-1 and mef1-2, in which editing at the respective target sites rps4-956 and nad7-963 is absent. In the first series of experiments, we transfected mutant protoplasts with three different constructs of the Col-MEF1 gene mutated individually at each of the variant C24 nucleotide positions 214, 314 and 1297, respectively. Each of these variants recovered RNA editing at sites rps4-956 and nad7-963 in the transfection assays and increased the RNA editing efficiency at the third target site nad2-1160 (Fig. 1B). At this latter site, MEF1 does not seem to be required per se for editing, but enhances the reaction and is needed for complete C to U conversion in all steady state nad2 mRNA molecules. The editing levels achieved by each of the three SNP-constructs were similar to the recovery of editing by complementation with the Col wild-type MEF1. These results show that single mutations of the nucleotides in positions 214, 314 and 1297, respectively, of the Col MEF1 gene do not detract from the ability of the resulting MEF1 protein to complement editing deficient mutant protoplasts.

To analyse potential cumulative effects of the SNP-mutations, we next constructed derivatives of MEF1 with all possible combinations of the three non-silent SNPs between Col and C24 and transfected mef1-1 and mef1-2 EMS mutant protoplasts with each of these MEF1 gene variants. The constructs with combinations of two altered nucleotides at SNP positions 1+2 and 1+3 still restore the ability for RNA editing at rps4-956 and nad7-963 and enhance RNA editing at nad2-1160 to levels comparable to the control transfections with the Col version of the gene (Fig. 2A, +MEF1 C24-1+2, +MEF1 C24-1+3; Fig. 2B). While the achieved levels of editing at nad2-1160 for all constructs tested are comparable to those after transfection with the Col version of the gene, the editing extents at rps4-956 and nad7-963 are lower in the protoplasts transfected with MEF1 C24-2+3 than in protoplasts after introduction of the wild-type Col MEF1 gene (Fig. 2A, MEF1 C24-2+3; Fig. 2B).

Surprisingly, these lowered editing levels appear to slightly increase at rps4-956 and nad7-963, when mutant protoplasts are transfected with the C24 version of MEF1, almost up to the editing efficiency of protoplasts transfected with the Col version (+MEF1 C24-1+2+3). However, these differences in the relative quantifications are not statistically significant and thus only suggest a trend (Fig. 2B)

3.2. Requirement of the DYW domain for MEF1 function

To investigate the role of the DYW domain for the function of the RNA editing trans-factor MEF1 we pursued two lines of inquiries. The first was to test the requirement of the DYW domain for the editing activity, the second was to analyse the function of the highly conserved C-terminus of these proteins. For the first assays we deleted the DYW motif and tested the competence for the MEF1-DYW to recover editing in mutant mef1-1 protoplasts (MEF1-DYW; Fig. 3A). RNA editing is not recovered at any of the three target sites: editing at the rps4-956 site is detectable, but very low at around the background limit, C to U conversion at nad7-963 is not detectable at all and the residual editing of
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Fig. 1. Single mutations of the three non-silent nucleotide differences in the MEF1 gene between ecotypes Col and C24 do not change the ability of the resulting protein variants to complement mutant protoplasts. (A) MEF1 is a PPR protein of the DYW subclass. The positions of the SNPs (C24-1 to C24-3) between Col and C24 and of the point mutations in the EMS lines mef1-1 and mef1-2 are shown. (B) The editing efficiency at the three sites affected in mutant protoplasts is increased by transfection with the wild-type Col version of the gene MEF1 (+MEF1 Col traces). Introduction of MEF1 gene versions mutated in nucleotide positions 214, 314 or 1297, respectively, found in the C24 version of this gene still leads to recovered RNA editing levels comparable to the wild-type Col version of MEF1 (+MEF1 C24-1 traces, MEF1 C24-2 traces and MEF1 C24-3 traces).

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<tr>
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<td>50%</td>
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<tr>
<td>nad2-1160</td>
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<td>55%</td>
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In the second series of DYW functional investigations we tested how important the accessibility of the DYW domain of MEF1 is for its function in RNA editing. Six histidines were attached to the C-terminus of the protein and the resulting protein was stably introduced in mef1-1 plants. In contrast to editing efficiencies up to 100% attained in transgenic mef1-1 plants with the wild-type Col MEF1, the MEF1+His protein with its C-terminal extension of six histidines has only a weak positive effect on the extent of RNA editing at either site (Fig. 3C).

4. Discussion

4.1. Interactions of several SNPs between Col and C24 in MEF1 lead to ecotype-specific differences in RNA editing

The investigation of trans-factors involved in RNA editing in plant mitochondria has been considered to be challenging, as mutants defective in mitochondrial editing were suspected to cause severe phenotypes or to be even lethal. However, most of the mutants identified so far by screening a collection of chemically mutagenized Arabidopsis Col plants show completely abolished editing of the respective target sites without causing severe phenotypic
Fig. 2. Combinations of the ecotype C24-specific SNPs in MEF1 affect RNA editing levels differentially at the three target sites. (A) Transfection of mef1-1 (top part) and mef1-2 (bottom part) mutant protoplasts with versions of MEF1 after mutation of any of two or all three variant nucleotides still recovers editing and increases the editing efficiency at all three affected editing sites (+MEF1 C24-1+2, MEF1 C24-1+3, MEF1 C24-2+3 and MEF1 C24-1+2+3 traces). For site nad2-1160, editing recovery is with all constructs comparable to those achieved with the Col version of MEF1 (+MEF1 Col). In the experiment shown, the editing efficiencies at sites nad7-963 and rps4-956 reach the Col transfection levels in the protoplasts transfected with three of four versions of MEF1 mutated in the variant nucleotides (+MEF1 C24-1+2, MEF1 C24-1+3, and MEF1 C24-1+2+3 traces), while transfection with MEF1 C24-2+3 leads to slightly lower levels. (B) Editing of sites nad7-963 and rps4-956 in protoplasts transfected with MEF1 C24-2+3 is reduced in comparison to the transfection with the Col version of MEF1. Introduction of the C24 version (C24-1+2+3), however, appears to recover slightly higher editing levels. The error bars show that these interpretations are not statistically significant and thus only suggest possible trends. This graph summarizes the results of four independent experiments for each construct. RNA editing levels are shown relative to the levels recovered by transfection with the Col wt version of MEF1 (100%) in each series of assays to compensate for variations in protoplast quality and transfection. Individual transfections with other constructs may thus show better recovery of editing.
are altered to the C24 version, the mutated MEF1 protein is somewhat less active at the sites nad7-963 and rps4-956 than the Col wild-type version (Fig. 2A).

The reduced effectiveness in editing may originate from a lowered binding capacity to either of the target mRNAs or alternatively to other potential co-factors of the RNA editing reaction. Either scenario could be the result of a changed steric conformation caused by the altered amino acids. All three SNPs in C24 result in a decrease of the hydrophobicity of the encoded amino acid. This potentially leads to an aberrant solubility of parts of MEF1 and consequently may change folding of the C24 MEF1 protein and its editing activity.

Interesting is the differential effect of the ecotype-variations on the editing efficiency thus appear to be partially neutralized by the third C24 amino acid. Here it has to be taken into account that a presently unsolvable potential source of variation may reside in the Col nuclear background of the mef1-1 and mef1-2 mutants. The C24 plants may have accumulated potentially compensatory mutations in factors interacting with MEF1 arisen in C24 to adjust to the three C24-SNPs in MEF1. These mutations may not be present in the respective factors in Col.

These site-specific effects of combined amino acid changes in MEF1 may be caused by a disturbed binding to the RNA or by an altered interaction with other protein(s). The first alternative can now be specifically investigated and differential RNA binding studies with the various MEF1 variants will clarify this possibility and, if yes, may show which domains of the MEF-PPR proteins are crucial for interaction with target RNAs and which of the ecotype-variations will disturb the MEF1-RNA interaction through altered binding to the cis-motifs in the three RNA editing targets of MEF1.

4.2. The DYW domain is necessary for efficient function of MEF1

In the subclass of the E PPR proteins which contain an additional extension beyond the E-domain, most proteins terminate in the amino acids DYW, suggesting that this highly conserved

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**Fig. 3.** The DYW domain of MEF1 is required for full competence in RNA editing at all three target sites. (A) Schematic of the MEF1 structure after deletion of the DYW domain and addition of 6 histidines, respectively. (B) Transfection with the truncated gene MEF1-ΔDYW does not remarkably increase RNA editing in mutant protoplasts at rps4-956, nad7-963 and nad2-1160 (+MEF1-ΔDYW) compared to wild-type transfected mef1-1 protoplasts (+MEF1). (C) Introduction of the truncated gene into mef1-1 plants does increase RNA editing at all target sites, but only to low levels (+MEF1-ΔDYW). Similar low editing recovery can be observed in transgenic mef1-1 plants with an integrated MEF1+His gene (+MEF1+His), while the wild-type Col MEF1 gene reconstitutes editing at all sites to 100% (+MEF1).
trippeptide and its accessibility is very important for the function of these proteins. Contrary to this theoretical consideration, the two plastid RNA editing trans-factors CRR22 and CRR28 are still fully functional without their C-terminal DYW domain [8]. The DYW domain in MEF1 may be more important, since already the mutation of a single nucleotide in the EMS-line mef1-2 completely abolishes editing at two of the three target sites, rps4-956 and nad7-963, and results in a strong reduction at nad2-1160 [11]. Complete removal of the DYW motif from the MEF1 protein as assayed here could not recover or complement RNA editing at any of the three target sites in mutant protoplasts. Transgenic plants, however, in which the MEF1-ΔDYW gene is stably introduced, show during overexpression of the truncated protein recovery of or increased editing at these sites, although never reaching wild-type levels (Fig. 3C). This low level recovery in vivo may potentially be mediated by a recruitment of the mutant MEF1-1 protein, which is disabled in the region of the PPRs, but contains an intact DYW domain.

Similarly, masking of the DYW C-terminus by additional His-residues inhibits any increasing effect of the overexpressed MEF1+ His protein on RNA editing efficiency in transgenic plants (Fig. 3C). These observations suggest that the DYW domain in MEF1 can be functional without their C-terminal DYW domain[8]. The DYW domain of CRR22 and CRR28 is fully substituted by such additional, as yet unknown molecules, the activity of the MEF1-ΔDYW protein is not completely recovered in any of the assays, possibly due to a lower affinity or abundance of the co-factor of MEF1. The deleted DYW domain may be partially compensated for by either the high level of overexpression in the transgenic plants versus the protoplast assays, or alternatively the longer incubation time in the transgenic plants (versus the limited time frame in protoplast transfection assays) might be required to assemble the MEF1 editing complex with sufficient functionality. Other mutations in the DYW domain may destroy its binding activity to other proteins, but not the template RNA. Such mutations will in effect block the editing site (and concomitantly the editing reaction) and then cannot be compensated for or replaced by other co-factors.

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