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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.

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)

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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 SNaPshot 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 pSMGFP4 [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 (4base 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 promotor 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 two 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 derivates 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 persued 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-ΔDYW to recover editing in mutant *mef1-1* protoplasts (*MEF1*-ΔDYW; 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