THE PENTATRICOPEPTIDE REPEAT PROTEIN OTP87 IS ESSENTIAL FOR RNA EDITING OF nad7 AND atp1 TRANSCRIPTS IN ARABIDOPSIS MITOCHONDRIA

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Running head: PPR protein involved in editing of mitochondrial nad7 and atp1 transcripts
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In plant organelles, RNA editing is a post-transcriptional mechanism that converts specific cytidines to uridines in RNA of both mitochondria and plastids, altering the information encoded by the gene. The cytidine to be edited is determined by a cis-element surrounding the editing site that is specifically recognized and bound by a trans-acting factor. All the trans-acting editing factors identified so far in plant organelles are members of a large protein family, the pentatricopeptide repeat (PPR) proteins. We have identified the ORGANELLE TRANSCRIPT PROCESSING 87 (OTP87) gene, which is required for RNA editing of the nad7-C24 and atp1-C1178 sites in Arabidopsis mitochondria. OTP87 encodes an E-subclass PPR protein with an unusually short E-domain. The recombinant protein expressed in Escherichia coli specifically binds to RNAs comprising 30 nucleotides upstream and 10 nucleotides downstream of the nad7-C24 and atp1-C1178 editing sites. The loss-of-function of OTP87 results in small plants with growth and developmental delays. In the otp87 mutant, the amount of assembled respiratory complex V (ATP synthase) is highly reduced compared with the wild type suggesting that the amino acid alteration in ATP1 caused by loss of editing at the atp1-C1178 site affects complex V assembly in mitochondria.

In flowering plants, RNA editing comprises conversion of specific cytidine residues to uridine in both mitochondrial and plastid transcripts. RNA editing occurs most frequently in coding regions of mRNAs, with only few sites found in structural RNAs and introns. In Arabidopsis thaliana, more than 500 sites are edited in mitochondrial transcripts whereas 34 sites are affected in chloroplasts (1-4). The mechanism of plant RNA editing has been extensively studied since the late 1980s, but the identity of the enzyme catalyzing the editing reaction remains unknown. Genetic studies have identified nuclear encoded factors required for the editing of one or more specific cytidines in mitochondrial or chloroplast transcripts of Arabidopsis plants (5-21). All of these protein factors are pentatricopeptide repeat (PPR) proteins, a large family of over 450 RNA-binding proteins in Arabidopsis, the majority of which are thought to be targeted to mitochondria or chloroplasts (22,23). All of the organelle editing factors reported to be required for editing of specific sites are members of the E and DYW subclasses of the PPR family. An exception may be the P class protein PPR596 which when disabled increases editing at several mitochondrial sites (24). The distinctive plant-specific C-terminal DYW domain of most of these proteins correlates phylogenetically with plant organelle RNA editing (25,26). These PPR proteins are thought to play the role of trans-acting specificity factors in the current model of RNA editing in plant organelles. In this model, a protein trans-factor specifically binds a cis-element in the vicinity of the editing site, facilitating the access of a putative RNA editing enzyme (27,28). The proof that these genetically identified PPR factors directly target their editing sites comes from studies on the proteins CRR4 and PpPPR_71. CRR4 is required for editing of the initiation codon of the chloroplast ndhD transcript in Arabidopsis thaliana, and binds specifically within the region -25/+10 spanning the...
editing site (27). PpPPR_71 is required for editing of the ccmF-2 site of mitochondrial ccmFc transcript in Physcomitrella patens, and binds specifically within the region -40/+5 spanning the editing site (29). It is generally assumed that the other PPR proteins identified as editing factors function in a similar way (4).

Many editing sites in plant organelles affect the coding sequence of the target mRNA and therefore editing defects might be expected to lead to striking phenotypes due to mis-expression of proteins involved in photosynthesis or respiration. Indeed, forward genetic studies on Arabidopsis mutants based on photosynthetic phenotypes have identified many new Arabidopsis editing specificity factors in chloroplasts (5-9,12). Lately, reverse genetics approaches concentrating on genes encoding PPR proteins of the E and DYW sub-groups have identified many new Arabidopsis editing mutants (13,18). Despite the fact that these mutants lose editing at one or several sites in mitochondria or chloroplasts, the mutant plants show a normal growth phenotype. Among the Arabidopsis mutants characterized so far which are specifically impaired in the editing of one or several cytidine sites in mitochondria only the slo1 mutant shows a severe phenotype when grown on soil (11,14,17-19,21,30).

We report here the identification of the nuclear OTP87 gene, which encodes a PPR protein lacking a DYW domain and with only a partial E domain. Disruption of the OTP87 gene leads to a severe slow growth phenotype in Arabidopsis. The OTP87 protein is required for the editing of at least two sites, nad7-C24 and atp1-C1178 in mitochondria.

**EXPERIMENTAL PROCEDURES**

All primers used in this study are listed in Supplemental Table 1.

**Plant Material** - Arabidopsis thaliana ecotype Columbia (Col-0) was used in this study. The T-DNA insertion mutant line GABI_073C06 was obtained from the ABRC Stock Center.

**Phenotype and Genetic Analyses** - Seeds were germinated in soil or on half-strength Murashige and Skoog medium and grown in a growth chamber under a 16 hours light / 8 hours dark cycle.

Total cellular DNA was isolated as described (31). Homozygous otp87 plants (GABI_073C06) were genotyped by PCR and the insertion position (+710 with respect to the ATG) was confirmed by sequencing with a T-DNA left border primer.

**Analysis of targeting via GFP fusions** - The first 300 bp of the coding sequences of the PPR genes were amplified using Phusion DNA polymerase (Finnzymes) with primers listed in Supplemental Table 1 containing the attB sites for Gateway® cloning according to the manufacturer’s instructions (Invitrogen). This fragment was cloned in frame with the GFP coding sequence. The GFP vector used and the chloroplast targeting marker encoding red-fluorescent protein fused to the small subunit of Arabidopsis ribulose biphosphate carboxylase were kindly provided by Prof. James Whelan (The University of Western Australia) (32). Biolistic co-transformation of GFP and RFP fusion constructs were performed on Arabidopsis cell culture as described (33).

**Genetic complementation** - The 3099 bp fragment containing the coding sequence of OTP87 and the 5'-intergenic region was amplified by PCR on total cellular DNA. This construct was cloned into pGWB1 binary vectors and introduced into otp87 mutant via Agrobacterium tumefaciens GV3101. Transformants were obtained by selection on MS agar plates containing 25µg/mL hygromycin and confirmed by PCR.

**RNA gel blot and RT-PCR analysis** - Total RNA from leaves of 15-day-old plantlets was isolated using TRIzol reagent (Invitrogen) as recommended by the manufacturer’s instructions. Three micrograms of DNA-free RNA were reverse transcribed using Superscript III RT and random hexamers (Invitrogen) according to the manufacturers’ instructions. RT-PCR was performed with primers listed in Supplemental Table 1. In total, 45 and 25 cycles were used for OTP87 and ACTIN2 respectively. For northern blot analysis, ten micrograms of total RNA was fractionated on 1.2% (w/v) formaldehyde agarose gels and transferred onto Hybond N+ nylon membranes (GE Healthcare, Piscataway, NJ). RNA integrity, loading and transfer were checked by staining the membrane with methylene blue. Membranes were hybridized with nad7- and atp1-specific probes (Further information in the supplement) using DIG Easy Hyb kit (Roche) according to the manufacturer’s instructions.
Double stranded DNA probes were internally digoxigenin-labeled using PCR DIG Probe Synthesis kit (Roche) as recommended by the manufacturer.

**Analysis of RNA Editing**- Plastid RNA editing sites were screened by high-resolution melting analysis of amplicons as described (3) using the primers listed in (7). Mitochondrial RNA editing sites were screened by multiplexed single base extension as described (34). The editing defects were confirmed by sequencing specific RT-PCR products.

**Confocal microscopy**- Observation of chlorophyll fluorescence was performed using a Zeiss LSM510 confocal laser scanning microscope (Zeiss). Chloroplast images were taken in leaves of two-week-old wild-type and mutant plants. Chlorophyll fluorescence was excited with a 488/561 nm laser and collected through a long pass filter (>650 nm).

**Chlorophyll fluorescence analysis**- Chlorophyll fluorescence was measured on 4-week-old plants with a MINI-PAM portable chlorophyll fluorometer (Walz, Effeltrich, Germany) in ambient air at room temperature (25°C). Minimum fluorescence at open PSII centers in the dark-adapted state (Fo) was excited by a weak measuring light (wavelength 650 nm) at a PFD of 0.05-0.1 μmol m⁻² s⁻¹. A saturating pulse of white light (800 ms, 3,000 μmol photons m⁻² s⁻¹) was applied to determine the maximum fluorescence at closed PSII centers in the dark-adapted state (Fm) and during AL illumination (Fm'). The steady state fluorescence level (Fs) was recorded during AL illumination (15-1,000 μmol photons m⁻² s⁻¹). These photosynthetic parameters were determined 2 min after the change of AL intensity. NPQ was calculated as (Fm-Fm')/Fm'. The quantum yield of PSII (φPSII) was calculated as (Fm'-Fs)/Fm' (35). ETR was calculated as φPSIIxPFD. The transient increase in chlorophyll fluorescence after turning off actinic light was monitored as previously described (36).

**Chlorophyll content analysis**- Chlorophyll (chl) content was determined in intact leaves of 4-week-old plants using using a Chl meter (SPAD-502, Konica Minolta, Tokyo, Japan) and was calculated as 0.65 x SPAD value (microgram cm⁻²). For analysis of the chlorophyll a/b ratio, leaf tissue of 3-week-old plants was ground in liquid nitrogen and chlorophyll pigment was extracted into acetone. The ratio chlorophyll a/b was determined as described (37).

**Immunoblot analysis**- Chloroplasts were isolated from the leaves of 4-week-old plants as previously described (6) and samples were normalized by measuring chlorophyll concentration. Mitochondria were isolated from 3-week-old seedlings grown hydroponically in half-strength MS medium and samples were according to protein concentration. The protein samples were separated by SDS-PAGE. After electrophoresis, the proteins were transferred onto a Hybond-P membrane (GE Healthcare) and incubated with specific antibodies. Signals were detected using an ECL Plus Western Blotting Detection Kit (GE Healthcare) and visualized by a LAS1000 chemiluminescence analyzer (Fuji Film).

**BN PAGE**- Mitochondria were isolated from 3-week-old (Col-0) and 4-week-old (otp87) seedlings grown hydroponically in half-strength MS medium. Blue native polyacrylamide gel electrophoresis (BN PAGE) was performed as previously described (38). Proteins separated by BN PAGE were transferred onto a PVDF (BioRad) in cathode buffer for 15 h at 40 mA, using a BioRad Mini transblot cell. The membrane was then destained with ethanol and restained with Coomassie blue to visualise the complexes, and subsequently destained before probing the membrane with specific antibodies.

**Generation of recombinant OTP87**- Full-length mature OTP87 (i.e. lacking the transit peptide) fused to the N-terminal maltose binding protein (MBP) tag was generated by PCR amplification of its coding sequence from the gDNA, digestion of the product with BamHI and SalI, and cloning into pMAL-TEV. Subsequent steps in expressing and purifying recombinant MBP-OTP87 were as described previously for PPR10 (39) except that the MBP moiety was not cleaved off.

**Nucleic binding assays**- Gel mobility shift (GMS) assays were performed with the same procedures as described in (40). GMS assays used gel-purified synthetic RNA and DNA 31-mers derived from two regions of the 5'-untranslated region of the maize chloroplast petA mRNA (petA-1; TTCTAGTACAACATATGCAGTAGAAATC-39) and (petA-2; TTAGCTACCTATCATTTATTAGAATGACA A) and (petA-2; TTAGCTACCTATCATTTATTAGAATGACAC T-39) in both sense and antisense orientations. Binding reactions contained 40mM NaH2PO4 pH 7.5, 140mM NaCl, 5mM DTT, 40μg/ml BSA for the single and double-stranded nucleic acid
binding assays and 40mM NaH2PO4 pH 7.5, 250mM NaCl, 5mM DTT, 40µg/ml BSA, 2mg/ml heparin for specific RNA oligo (atp1a, atp1b, nad7) binding assays. Competition assays were carried out in the same conditions except that the unlabeled competitor RNA was pre-incubated with the protein for 10 min, and then the labeled RNA was added. The relative levels of shifted bands were quantified with a phosphorimag (Storm Molecular Imager).

Bioinformatic Analysis- Consensus for editing sites recognised by OTP87 was calculated by hand and searched against the Arabidopsis mitochondria or chloroplast genomes sequence using fuzznuc from the EMBOSS package (41).

RESULTS

Phenotypic analysis of Arabidopsis organelle transcript processing 87 mutant. To identify genes that are involved in the editing of organelar transcripts in Arabidopsis, we initiated a reverse genetic screen of T-DNA insertion lines in which genes encoding PPR proteins of the E and DYW subgroups are disturbed in Arabidopsis (Arabidopsis thaliana) ecotype Columbia (Col-0) plants. We isolated T-DNA homozygous mutant plants (GABI_076C04) for the At1g74600 gene (Figure 1A). This gene encodes a PPR protein of the E-subgroup and we named this gene OTP87 (for ORGANELLE TRANSCRIPT PROCESSING 87). We analyzed the T-DNA flanking genomic sequence by sequencing the PCR product amplified with a specific T-DNA left border primer and a gene specific right primer. The T-DNA insert (GABI_076C04) lies in the coding region of OTP87 (+710 from the ATG start codon) and disrupted the expression of OTP87, as RT-PCR analysis revealed that OTP87 transcripts were not detectable in the otp87 mutant (Figure 1B). Another line (SALK_112173C) is annotated to contain the T-DNA insertion in the promoter and does not show a phenotype (18).

The phenotype of otp87 mutant plants is severe. Mutant plants germinate late and continue to show slow growth and delayed development (Figure 1C-F). After 2 weeks growth, the size of otp87 plants is significantly smaller than wild-type plants (WT). In 4-week old wild-type Arabidopsis plants, the floral stalk starts to elongate whereas rosette leaves are still developing in otp87 plants. After 5 weeks growth, otp87 plants make the transition from vegetative growth to reproductive development and produce flowers and siliques, but the mutant plants remain smaller in size than the wild-type Arabidopsis plants.

The introduction of a wild-type copy of the OTP87 gene into the otp87 mutant (otp87_CP) restores the wild-type phenotype and the expression of OTP87 (Figure 1B, 1D-F). This result supports the otp87 phenotype as a direct consequence of the loss of OTP87 function in mutant Arabidopsis plants.

The OTP87 transit peptide targets GFP to both mitochondria and chloroplasts. Most of the PPR proteins are predicted to localize either in mitochondria or chloroplasts (22,23). Predotar (42) and TargetP (43) predict OTP87 to be localized in chloroplasts with high confidence. TargetP predicts a cleavage site predicted at the 71st amino acid residue. A search in the POGS database (44) for putative orthologs of OTP87 identified the Os09g24680 gene in Oryza sativa. This gene encodes the PPR protein OsPPR1, previously described in (45). In rice, OsPPR1 localizes to chloroplasts and the loss of function of OsPPR1 leads to chloroplast development phenotypes, including chlorophyll deficiency. However, the precise molecular function of OsPPR1 has not been elucidated. OTP87 only shares 36% identity and 60% similarity with OsPPR1 and their respective N-terminal target signal sequences differ suggesting that the two proteins may have a different localization and function (Supplemental Figure 1). The alignment of OTP87 with potential orthologs in various plant species clearly shows a difference in length of the putative targeting sequences between monocots and dicots (Supplemental Figure 2). In addition, for both the dicot and monocot protein sequences, some are predicted to be mitochondrial and some are predicted to be plastid (Supplemental Table 2).

To assess the sub-cellular localization of OTP87, the N-terminal 100 amino acids of the protein were expressed fused with green fluorescent protein (GFP) in wild-type Arabidopsis cells by bombardment. Analysis of GFP fluorescence in transformed cells revealed that the fluorescence co-localized with red fluorescent protein fused to a bona fide plastid protein as well as with a mitochondrial protein marker (Supplemental Figure 3). Thus, the first 100 amino
acids of OTP87 can dual-target GFP to mitochondria and chloroplasts and OTP87 may have a function in both organelles.

Chloroplast biogenesis is not affected in otp87. PPR proteins are involved in a wide range of post-transcriptional processes in plant organelles and we postulated that OTP87 is involved in one of these processes. OTP87 is a PPR protein of the E-subgroup. All the E-subgroup PPR proteins to which functions have been assigned so far are essential for RNA editing in Arabidopsis organelle transcripts (5,6,8,13,18,19) apart from OTP70, involved in splicing of rpoC1 (46). We examined the 34 Arabidopsis chloroplast editing sites in otp87 using a high-resolution melting screen (3). All of them were edited in the chloroplasts of otp87 demonstrating that OTP87 is not an essential editing factor in Arabidopsis chloroplasts (Supplemental Figure 4).

To test if other aspects of chloroplast biogenesis were affected in otp87 plants, we first examined autofluorescent mesophyll chloroplasts by confocal microscopy. We did not observe obvious differences in size, morphology, or numbers of chloroplasts between otp87 plants and wild type (Figure 2A). Levels of subunits in both photosystems and the cytochrome (Cyt) b6f complex were not substantially affected in otp87 (Figure 2B).

Consistent with these results, no alterations of the rate of electron transport through photosystem II or of non-photochemical quenching of chlorophyll fluorescence, which reflects ΔpH formation, were observed in otp87 (Figure 2C). However, otp87 showed a slightly higher NPQ at low light intensities (less than 400 µmol photons m⁻² s⁻¹). The chloroplast NAD(P)H dehydrogenase (NDH) complex is involved in photosystem I cyclic electron flow (47) and NDH activity can be monitored as a transient increase in chlorophyll fluorescence reflecting plastoquinone reduction after turning off actinic light (36). Figure 2D shows a typical chlorophyll fluorescence trace from wild-type Arabidopsis and in crr7, a mutant lacking NDH activity (48). The post-illumination increase of chlorophyll fluorescence in otp87 is not modified (Figure 2D), indicating that NDH activity is not affected in the Arabidopsis otp87 mutant.

The total chlorophyll content (in micrograms per square centimeter) as well as the ratio of chlorophyll a/b is unchanged in the mutant compare to wild type plants (Figure 2E).

Taken together, our results show that chloroplast biogenesis and function are not obviously affected in otp87. The slightly elevated NPQ may be secondarily caused by the growth phenotype of the mutant (Figure 2C). In contrast, the severe growth phenotype of otp87 cannot be explained by any particular defects in chloroplast function.

OTP87 is involved in RNA editing of mitochondrial transcripts. As the GFP fusion experiments showed that OTP87 is targeted to mitochondria as well, we screened otp87 mitochondrial editing defects using the recently developed multiplexed SNaPshot approach (34). With this approach, 335 Arabidopsis mitochondria editing sites were monitored. Our results revealed that editing at the two sites atp1-C1178 and nad7-C24 is specifically abolished in otp87 while the other sites are edited (Figure 3). Editing at these two sites is restored in the complemented otp87 plant (otp87_CP) demonstrating that OTP87 encodes an editing factor targeting the two sites nad7-C24 and atp1-C1178 in Arabidopsis mitochondria (Figure 3A).

To investigate whether the editing defects we observed in these mutants were secondarily caused by altered RNA processing or modified RNA stability, the transcripts affected were analyzed by RNA gel blots (Supplemental Figure 5). The hybridization patterns reveal no striking differences in the pattern and level of nad7 and atp1 transcripts whose editing is impaired in otp87.

The nad7-C24 site is found at the third position of codon 8 (encoding isoleucine) in NAD7, a subunit of the mitochondrial respiratory complex I (NADH dehydrogenase). This isoleucine is conserved in many plant species and may be important for the function of NAD7 (Figure 3B). Nevertheless, AUC and AUU both encode isoleucine and thus the loss of editing of this site is not expected to have a deleterious effect on the function of the protein. The atp1-C1178 is found at the second position of the codon encoding serine 393 in ATP1, a subunit of the mitochondrial respiratory complex V (ATP synthase). The conversion of cytidine to uridine at this site changes the encoded amino acid from serine to leucine. This editing event restores an amino acid conserved in many other plant species (Figure 3B).
An editing defect at this site could have a strong impact on the function of the encoded protein or act by destabilizing the protein or by affecting its ability to form complexes with other proteins. To assess whether ATP1 stably accumulates in otp87, mitochondrial protein blots were analyzed using antibodies against ATP1 and another ATP synthase subunit, ATP2 (Figure 4A). Levels of ATP1 and ATP2 were not affected in the mutant suggesting that the alteration of leucine 393 to serine in ATP1 does not impact the accumulation of the protein. Additional immunodetection shows that the Hsp70 chaperone is overexpressed in otp87 compared with the wild type. This overexpression may reflect a mitochondrial dysfunction in otp87 as previous studies reported the activation of hsp genes in response to mitochondrial impairment (49).

We further examined the formation of complex V in otp87 mitochondria by BN PAGE with subsequent immunodetection using antibodies against ATP1 (Figure 4B). Two bands of different size correspond to complex V due to the different electrophoretic mobilities between the F0F1-ATPase (F0F1) and the detached F1 moiety (F1) in BN PAGE. In otp87 mitochondria, these two bands are hardly detectable as compared with mitochondria from wild type. To assess if any complex V is assembled in the mutant, a similar BN PAGE gel was blotted, stained and probed with ATP1 antibodies (right panel). The immunodetection confirms that the formation of complex V in otp87 mitochondria is significantly reduced compared with the wild type. This result suggests that although the amino acid alteration in ATP1 does not affect ATP1 accumulation, it strongly affects the assembly of the ATP synthase in otp87 mitochondria.

Dysfunction of plant mitochondria is often associated with cytoplasmic male sterility leading to pollen abortion (50). Pollen formation is a highly energy-consuming process (51) and the energy is exclusively supplied by mitochondria in the developing pollen grains (52). In addition, it has been demonstrated that transgenic tobacco plants expressing an unedited version of the mitochondrial encoded wheat ATP synthase subunit 9 (ATP9) frequently exhibit either male-fertile phenotypes (53). To investigate whether the reduction of ATP synthase could affect pollen development and viability in otp87 plants, we compared the pollen grains in otp87 and wild-type plants after staining them with Alexander solution (54). We did not observe any difference in number (data not shown), shape or viability of pollen grains in the mutant compared to the wild type (Figure 5 and data not shown).

**Editing sites targeted by OTP87 share similar sequences** - In vitro and in organello investigations have delineated the cis-elements in the RNA for several editing sites in mitochondria and chloroplasts to lie within a region of ~30 nucleotides covering 20 to 25 nucleotides upstream and one to three nucleotides downstream of the edited C (55-60). A similar length for the cis elements of the nad7 and atp1 sites may be expected. Within this window between -25 and +3 relative to the edited C, 14 contiguous nucleotides are identical between the genomic nucleotide sequences around the two RNA editing sites targeted by the OTP87 gene product, nad7-C24 and atp1-1178 (Figure 6). We examined whether the two sites contain sufficient conserved nucleotides to be unambiguously defined as a binding site consensus. If an unambiguous consensus could be found, then this would be consistent with the OTP87 protein acting as a specific factor by binding to this consensus sequence. We searched in silico the entire mitochondrial genome of Arabidopsis for any match with the 14 nucleotide pattern shared between nad7-C24 and atp1-1178 and found that this sequence conservation is sufficient to define the specific recognition of the two editing sites by OTP87.

Only 76% of the 440 editing sites annotated in Arabidopsis mitochondria (1) were analyzed by the SNaP shot assay in the otp87 mutant. It is possible that other sites targeted by OTP87 escaped our analysis. To investigate whether other RNA editing sites could be targeted by OTP87, we performed an in silico search for annotated mitochondrial editing sites sharing nucleotide identities with the nad7-C24 and atp1-C1178 cis-elements. In the window -25 and +3 relative to the edited C, atp9-C53, cox2-C476 and nad5-C548 sites share some similarity with the two sites (Figure 6). We analyzed the status of these three sites in otp87 and found that they were correctly edited. A search of the entire Arabidopsis chloroplast genome did not reveal any match with the 14 nucleotides consensus pattern. However, we cannot rule out...
that other editing sites targeted by OTP87 may show less conserved but crucial shared cis-motifs within the window -25/+3 or may share cis-motifs outside of this window and thus would have escaped our analysis.

**OTP87 is a trans-acting factor required for RNA editing of nad7-C24 and atp1-C1178.** PPR proteins are predicted to be RNA binding proteins (61) and studies have already shown that two PPR editing trans-factors can indeed directly bind the RNA sequence surrounding their target editing site (27,29). In these two cases, the PPR trans-factor is involved in the editing of a single editing site and thus, the protein is only required to bind to a single RNA target. Other PPR trans-factors have been reported to be involved in the editing of multiple sites in distinct RNAs and thus are expected to bind more than one RNA target (13). Some confirmation of specific binding of a trans-factor to multiple target sites comes from UV cross-linking experiments in an *in vitro* tobacco (*Nicotiana tabacum*) editing system. A protein of 95 kDa was shown to specifically bind cis-acting elements of the two editing sites, ndhB-9 and ndhF-2 (62). This protein was later proposed to be the ortholog of OTP84, a PPR editing factor genetically identified in Arabidopsis (13). However, the biochemical evidence for direct binding of a PPR trans-factor to multiple target sites is lacking. To determine whether OTP87 preferentially binds to both nad7-C24 and atp1-C1178 cis-elements, recombinant OTP87 was generated by expression as a maltose-binding protein (MBP) fusion (Figure 7A). We tested if the recombinant protein showed nucleic acid binding capacity by gel mobility shift assays using a synthetic 31-mer oligonucleotide (either single-stranded or double-stranded RNA or DNA) (Figure 7B). The protein–nucleic acid complexes were detected as shifted bands that migrated more slowly than the free nucleic acid probes in the gel. The results showed that shifted bands were detected only when MBP-OTP87 was incubated with single-stranded RNA. To eliminate the possibility that MBP binds to the RNA probe, the RNA was incubated with recombinant MBP. Even a 1,500-fold amount of MBP (150 nM) added to the RNA (0.1 nM) did not induce any shifted bands, indicating that the RNA-binding activity depends on OTP87.

To further examine the binding of OTP87 to its target editing sites, we performed gel mobility shift assays using 41-nt RNA probes (nad7a, atp1a) that include 30 nt of the upstream sequence and 10 nt of the downstream sequence surrounding nad7-C24 and atp1-C1178 respectively (Figure 7C). Our assays showed that OTP87 binds to nad7a and atp1a in *in vitro*. To test if OTP87 shows preferential binding for nad7 and atp1a relative to other RNAs of similar size (atp1b), we performed gel mobility shift assays using cold RNA competitors (Figure 7D) and we compared their ability to reduce binding of labeled nad7 or atp1a probes to OTP87 (Figures 7E-F). The addition of a 2-fold excess of cold nad7 or atp1a probes resulted in the disappearance of the respective shifted band whereas the addition of a 4-fold excess of cold atp1b probe had no effect on the binding of OTP87 to nad7 and atp1a (Figure 7E). The addition of a 10-fold excess of cold nad7 or atp1a probes reduced the binding of OTP87 to nad7 and atp1a by more than 50%, whereas the addition of the same excess of cold atp1b inhibited the binding of OTP87 to nad7 and atp1a by only 20% (Figure 7F). These results indicate that OTP87 shows specificity towards the 40 nucleotides surrounding the nad7-C24 and atp1-C1178 editing sites.

**DISCUSSION**

**OTP87 is involved in editing two sites in mitochondrial RNAs.** OTP87 is a PPR protein of the E-subclass like many of the reported plant organelle editing factors and could play a role in RNA editing in chloroplasts and/or mitochondria. All 34 known editing sites in *Arabidopsis* chloroplasts are edited in the otp87 null mutant. Thus OTP87 is not essential for RNA editing in chloroplasts and we showed that the function of OTP87 is not required for major chloroplast functions. In mitochondria, OTP87 appears to act as a trans-factor involved in the recognition of the two editing sites nad7-C24 and atp1-C1178. Like other putative editing trans-factors, OTP87 is expected to bind specifically the RNA sequences surrounding the editing sites. Proof that PPR proteins can unambiguously bind to multiple targets with similar sequences has been provided for the maize proteins CRP1 (63) and PPR10 (39,64). In these two cases, the multiple target sites have almost identical sequences. Our *in vitro* binding assay using recombinant OTP87 revealed
that OTP87 preferentially binds to the putative nad7-C24 and atp1-C1178 cis-elements and provides biochemical evidence for the specific binding of a PPR trans-factor to multiple editing sites.

Fourteen nucleotides are identical between the putative cis-acting elements (-25 and +3 relative to the edited C) of nad7-C24 and atp1-C1178. Our analysis showed that these 14 nucleotides are sufficient to define specific recognition of these two sites by OTP87 in the Arabidopsis mitochondrial transcriptome. The recently identified LOI1 and SLO1 PPR proteins are involved in RNA editing at three and two sites respectively in Arabidopsis mitochondria (14,15,17). In these two cases, the multiple sites recognized by the same trans-factor also share high nucleotide identity in the window -25/+3 relative to the edited C. Together, these findings suggest that the high sequence identities of multiple cis-elements targeted by the same PPR trans-factor form the information required for substrate recognition by editing factors in Arabidopsis mitochondria. Similar conclusions were reached for most chloroplast editing factors (13).

The otp87 mutant shows similarity to other plant mitochondrial respiratory chain mutants—With the exception of slo1 (17), all the Arabidopsis mitochondrial editing mutants described so far do not show any obvious phenotype despite the fact that these editing defects introduce point mutations in proteins which are components of the respiratory chain. The germination delay, growth retardation and dwarf phenotype of otp87 are reminiscent of Arabidopsis mutants affected in the mitochondrial electron transport chain (38,65). We discovered two specific RNA editing defects in otp87 in two mitochondrial transcripts: nad7 and atp1. NAD7 is a subunit of Complex I. The amino acid residue at position 8 is not altered by the editing defect at nad7-C24 site and there is no reason to assume that the NAD7 protein is functionally affected in the otp87 mutant. In contrast, editing at atp1-C1178 induces an amino acid change (serine to leucine) in the a subunit of the ATP synthase complex (complex V) of the mitochondrial respiratory chain. An editing defect at this site may have a strong impact on the encoded protein as it exchanges a conserved hydrophobic amino acid for one with a very different character. Our biochemical data show that this amino-acid alteration in ATP1 does not affect its accumulation but does impair the assembly of the ATP synthase in otp87 mitochondria. The strong reduction of fully assembled ATP synthase in otp87 mitochondria could easily explain the severe phenotype of the mutant.

otp87 encodes a PPR protein with a partial E-domain—OTP87 is an E-subclass PPR protein. The E (extended) domain is a degenerate C-terminal motif with some similarities to PPR motifs identified in 194 PPR proteins in Arabidopsis thaliana (23). The E motif is followed by a more highly conserved DYW motif (named for its typical Asp-Tyr-Trp C-terminal tripeptide) in 87 PPR proteins. The DYW motif shows sequence similarity to the active site of cytidine deaminases and thus has been proposed to catalyse the editing reaction (25). However, the recombinant DYW motif of the chloroplast editing factor CRR22 does not show any editing activity in vitro and truncated proteins that lack the DYW domain can restore RNA editing in vivo (7,16). In contrast, the E motif has been shown to be essential for editing in all PPR editing factors where its role has been tested (6,7,19). It has been hypothesized that the E domain could recruit other essential components of the editing machinery such as the editing enzyme (5,7). The alignment of the OTP87 E domain with the E domains of the other editing factors identified so far shows that the E domain is not highly conserved between these proteins (Figure 8). OTP87 harbors the shortest E-domain observed so far amongst characterized editing factors; only the E-domain in OTP70 is shorter. However, OTP70 plays a role in splicing rather than in editing and its E-domain is not required for this processing event (46). Like several other mitochondrial editing factors, OTP87 is missing a conserved C-terminal 15 amino acid motif (5,6) that is present in the chloroplast editing factors. These observations could suggest a difference in the function and usage of the E-domain by the RNA editing machinery between the two organelles.
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FOOTNOTES

* This work is funded through the Australian Research Council Centres of Excellence scheme (CE0561495), an International Science Linkages grant (CG120098) from the Australian Government Department of Innovation, Industry, Science and Research, the Centre National de la Recherche Scientifique, the Deutsche Forschungsgeminschaft, and scholarships from the Region of Alsace and the University of Western Australia. IS is a West Australian State Premier’s Fellow. TS was supported by Grant-in-Aid (17GS0316 and 16085206) and by Genomics for Agricultural Innovation (GPN0008). We thank Philippe Giegé and Alice Barkan for useful discussions and Margarita Rojas for help with protein purification and GMS assays.

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The abbreviations used are: PPR: pentatricopeptide repeat; OTP: organelle transcript processing; NDH: NAD(P)H dehydrogenase; GFP: green fluorescent protein; RFP: red fluorescent protein; WT: wild type; GMS: gel mobility shift.

FIGURE LEGENDS

Fig. 1. Molecular characterization and phenotypic analysis of the Arabidopsis otp87 mutant. A. Schematic structure and domain composition of the OTP87 (At1g74600) gene. The position of the T-DNA insertion (GABI_073C06) is indicated by the black triangle. Arrows indicate the position of the primers used for RT-PCR in B. B. RT-PCR analysis of OTP87 and ACTIN2 in wild type WT (Columbia), otp87 and the complemented mutant otp87_CP plants. The OTP87 transcript was undetectable in otp87. Primers specific to ACTIN2 were used in a control RT-PCR. C. Four-day-old seedlings grown on half-strength Murashige and Skoog medium. D. Two-week-old plants grown on soil. E. Four-week-old plants. F. Five-week-old plants.

Fig. 2. Chloroplast biogenesis analyses in otp87. A. Confocal microscopy of mesophyll chloroplasts from rosette leaves of wild type Col-0 (WT) and otp87 mutant. Images are false-colored white to correspond to chlorophyll autofluorescence. B. Immunoblot analysis of photosynthetic proteins. Photosynthetic proteins were detected using specific antibodies against PsbA (PSII), PsaA (PSI), Cyt f (Cytochrome b6f complex). RbcL (the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase) was revealed by staining the membrane with Coomassie blue. The lanes were loaded with chloroplast membranes equivalent to 0.4 µg chlorophyll (100%) from the wild type and otp87, along with a series of dilutions of the wild-type sample. C. In vivo analysis of electron transport activity. Light-intensity dependence of ETR (electron transport rate) (upper graph). ETR is depicted relative to a value of ΦPSII × light intensity (µmol photons m⁻² sec⁻¹). Light-intensity dependence of NPQ (non-photochemical quenching) of chlorophyll fluorescence (lower graph). Symbols indicate the wild type (lozenge), and otp87 (square). Each point represents the mean ± SD (n = 5). D. Monitoring of NDH activity using chlorophyll fluorescence analysis for otp87. The curve shows a typical trace of chlorophyll fluorescence in the wild type and a mutant totally impaired in NDH activity (crr7) (48) compared with traces from otp87 mutant. Leaves were
exposed to actinic light (AL) (50 mmol of photons m\(^{-2}\) s\(^{-1}\)) for 5 min. AL was turned off, and the subsequent change in chlorophyll fluorescence level was monitored. The transient increase in chlorophyll fluorescence is due to the plastoquinone reduction based on NDH activity. Insets are magnified traces from the boxed area. The fluorescence levels were normalized by the maximum fluorescence at closed photosystem II centers in the dark (Fm) levels. ML, measuring light; SP, a saturating pulse of white light. E. Chlorophyll content in wild type and otp87. Each value represents the mean ± SD (n = 10 for chlorophyll and n=5 for chlorophyll \(a/b\)).

\(\text{Fig. 3.}\) OTP87 targets two editing sites in Arabidopsis mitochondria. A. Analysis of RNA editing at sites \(\text{atp1-C1178}\) and \(\text{nad7-C24}\) in the respective transcripts from wild type (WT), \(\text{otp87}\) and the complemented \(\text{otp87 (otp87\_CP)}\) plants. RT-PCR products containing the editing sites were directly sequenced. Arrowheads mark the editing sites and the amino acids resulting from in-frame translation of the transcripts are indicated below the nucleotide sequences. In the cDNA strand analyzed, the detected T nucleotide (red trace) corresponds to the edited U, the observed C (blue trace) is derived from an unedited C. The absence of RNA editing event \(\text{atp1-C1178}\) results in incorporation of the genomically encoded serine rather than the leucine specified by the edited codon. The retention of cytidine at position 24 in the \(\text{nad7}\) transcript does not change the identity of the amino acid in the predicted protein. B. Partial sequence alignments of ATP1 and NAD7 around the amino acids affected by RNA editing. \text{Arabidopsis} ATP1 and NAD7 were aligned with their homologs from other species. The alignment was performed using ClustalW (66). Identical amino acid residues are shaded in black and similar ones are shaded in grey. Numbers indicate amino acid positions in the protein. The arrows above the sequences indicate the positions of edited codons.

\(\text{Fig. 4.}\) Analysis of mitochondrial Complex V (ATP synthase) in \(\text{otp87}\). A. Immunoblot analysis of mitochondria respiratory complex V proteins. Mitochondrial proteins were detected using specific antibodies against HSP70 (heat shock protein 70) ATP1 (complex V), ATP2 (complex V) and PORIN. The lanes were loaded with 15 µg mitochondrial proteins (100%) from the wild type and \(\text{otp87}\), along with a series of dilutions of the wild-type sample. B. Mitochondrial membrane complexes (loading 150 µg) were resolved by BN PAGE, transferred onto a membrane which was subsequently probed with antibodies against ATP1. Bands corresponding to respiratory chain complexes I (CI), III (CIII), and to the \(\text{F}_0\text{F}_1\)-ATPase (CV) and its F1 subcomplex are indicated.

\(\text{Fig. 5.}\) Pollen structure of wild type (WT) and \(\text{otp87}\) plants. Pollen grains were stained according to (54). The pollen of \(\text{ otp87}\) was viable and stained comparable to the wild type.

\(\text{Fig. 6.}\) Comparison of nucleotide identities between the \textit{cis}-elements surrounding the two editing sites \(\text{nad7-C24}\) and \(\text{atp1-C1178}\) targeted by OTP87. In the window \(-25/+3\) relative to the editing site, 14 nucleotides are identical between \(\text{nad7-C24}\) and \(\text{atp1-C1178}\). \(\text{nad5-C548}\), \(\text{atp9-C53}\) and \(\text{cox2-C476}\) shares nucleotides similarities with the two editing sites but are nevertheless edited in \(\text{ otp87}\) mutant.

\(\text{Fig. 7.}\) Electrophoretic mobility shift assays using recombinant OTP87 protein. A. Purification of the recombinant OTP87 (MBP-OTP87) and MBP. The purified proteins were analyzed by SDS-PAGE and the gel was stained with Coomassie brilliant blue. B. Synthetic 31-mer oligonucleotide of RNA or DNA were radiolabeled, heated and either snap cooled (ssRNA, ssDNA) or cooled slowly in the presence of monovalent salts and a two-fold excess of its complement (dsRNA, dsDNA). The substrate (40 pM) was mixed with increasing concentrations of MBP-OTP87 represented by black triangles (0, 32.5, 75, 150nM) or 150nM of recombinant MBP. Protein binding is illustrated by the appearance of an upper band (B) and retention at the top of the gel, and by the disappearance of unbound substrate (U). C. Electrophoretic mobility shift assay with recombinant OTP87 (0, 15, 31, 62, 125, 250 nM) and target RNAs (15 pM) comprising 30 nucleotides upstream
and 10 downstream of unedited *nad7-C24 (nad7)* and *atp1-C1178 (atp1a)* respectively. All assays were in the presence of 2mg/mL heparin. D. Cold RNAs used in the electrophoretic mobility shift competition assays. 200 ng of each RNA was resolved on a 15% denaturing polyacrylamide gel and stained with ethidium bromide. E. Electrophoretic mobility shift assay using cold *nad7, atp1a* and a non-target competitor RNA of the same length, *atp1b*. Assays were carried out as in (C). The molar excess of competitor RNA relative to the labeled RNA (15 pM) is indicated above each lane. Unlabeled RNA was pre-incubated with recombinant OTP87 (150 nM) for 10 min before the labeled RNA was added. F. Relative levels of shifted bands at the indicated concentrations of cold competitor RNA. Each point represents the mean ± SD (n=3 for *nad7, atp1a* and n=6 for *atp1b*).

**Fig. 8** Comparison of the E-domain among the plastid and mitochondrial E-subclass PPR editing factors and the plastid splicing factor OTP70. The alignment was performed using ClustalW (66). Identical amino acid residues are shaded in black and similar ones are shaded in grey. The fifteen amino acid motif previously associated with editing (5,6) is indicated above the sequences.
Figure 1
Figure 2

A

B

C

D

E

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Figure 3

A

Col-0
cdp87
cdp87_CP

atp1-C1178

G T A G T A G T A A A A C T G
G S K L K

G G T A G T T C A A A A C T G
G S K L

G G T A G T T C A A A A C T G
G S K L

G G T A G T T C A A A A C T G
G S K L

G G T A G T T C A A A A C T G
G S K L

G G T A G T T C A A A A C T G
G S K L

G G T A G T T C A A A A C T G
G S K L

G G T A G T T C A A A A C T G
G S K L

nad7-C24

A G G C A A A T T A A A A A T
R Q I K N

A G G C A A A T T A A A A A T
R Q I K N

A G G C A A A T T A A A A A T
R Q I K N

A G G C A A A T T A A A A A T
R Q I K N

B

mRNA

atp1-C1178

nad7-C24

mRNA
Figure 4

A

| 50 | 100 | 200 | 100 |
|---|---|---|---|
| HSP70 | ATP1 | ATP2 | PORIN |

B

WT (Col)  dp-07  WT (Col)  dp-07  WT (Col)  dp-07

BN PAGE  membrane  ATP1

Cl → CV (F_1F_0) CV CI

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Figure 5

WT    otp87

Figure 6
Figure 7

A

B

C

D

E

F

relative level of shifted bands

Competitor RNA (molar excess)

nad7                     atp1a

rOTP87 (nM)

1 2 4 10 100

rOTP87 (nM)

1 2 4 10 100

nad7

atp1a

atp1b

rOTP87

rOTP87

nad7

atp1a

atp1b

rOTP87

rOTP87

nad7

atp1a

atp1b

rOTP87
The pentatricopeptide repeat protein OTP87 is essential for RNA editing of nad7 and atp1 transcripts in Arabidopsis mitochondria
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J. Biol. Chem. published online April 19, 2011

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