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The Pentatricopeptide Repeat Gene *OTP43* Is Required for *trans*-Splicing of the Mitochondrial *nad1* Intron 1 in *Arabidopsis thaliana* [W]

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The mitochondrial NADH:ubiquinone oxidoreductase complex (Complex I) is a large protein complex formed from both nuclearly and mitochondrially encoded subunits. Subunit ND1 is encoded by a mitochondrial gene comprising five exons, and the mature transcript requires four RNA splicing events, two of which involve *trans*-splicing independently transcribed RNAs. We have identified a nuclear gene (*OTP43*) absolutely required for *trans*-splicing of intron 1 (and only intron 1) of *Arabidopsis thaliana nad1* transcripts. This gene encodes a previously uncharacterized pentatricopeptide repeat protein. Mutant *Arabidopsis* plants with a disrupted *OTP43* gene do not present detectable mitochondrial Complex I activity and show severe defects in seed development, germination, and to a lesser extent in plant growth. The alternative respiratory pathway involving alternative oxidase is significantly induced in the mutant.

INTRODUCTION

NADH:ubiquinone oxidoreductase (Complex I) is the major entry point for the electron transport chain in plant and animal mitochondria (Brandt, 2006). Deficiency in Complex I function has been associated with various human diseases, including neurodegenerative diseases, and the aging process (Adam-Vizi and Chinopoulos, 2006; Janssen et al., 2006). In plants, this complex is still the major entry point under normal conditions, even though the electron transport chain is potentially more complicated due to the presence of alternative NAD(P)H dehydrogenases and alternative oxidase (reviewed in Noctor et al., 2007). Due to these alternative pathways, the plant mitochondrial respiratory chain has been a popular target for biochemical studies. Despite this widespread interest, very few studies have used genetic approaches to look at respiratory chain function. Mitochondrial Complex I is comprised of >40 subunits in *Arabidopsis thaliana* (Heazlewood et al., 2003). The majority of these are encoded by nuclear genes, and only nine genes have been retained in the land plant mitochondrial genome, namely, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, *nad6*, *nad7*, and *nad9* (Sugiyama et al., 2005). With such a plethora of genes involved, one might have expected many mutants with alterations in Complex I activity to have been investigated, but in fact, the literature is very sparse. Two mitochondrial mutants with similar deletions have been described from *Nicotiana sylvestris* (Pla et al., 1995) and another mitochondrial DNA deletion mutant from maize (*Zea mays*; Karpova and Newton, 1999). A nuclear mutant almost lacking Complex I was observed in *N. sylvestris* (Brangeon et al., 2000), while three nuclear mutants affected in Complex I have been identified in *Arabidopsis* (Lee et al., 2002; Perales et al., 2005; Nakagawa and Sakurai, 2006). Each of these mutants is defective in a different gene, and the phenotypes of these mutants are quite varied.

Five of the *nad* genes are fragmented into different exons that are flanked by sequences with group II intron features, and their transcripts need to be spliced. Although other mitochondrial transcripts (*rps3*, *cox2*, *ccmF*, and *rpl2*) contain introns, only *nad1*, *nad2*, and *nad5* transcripts require *trans*-splicing (Malek and Knoop, 1998). Very little is known about RNA splicing factors in plant mitochondria, but much more is known about the process in chloroplasts. In maize chloroplasts, four RNA splicing factors (CRS1, CRS2, CAF1, and CAF2) have been identified, and three chloroplast protein complexes have been proposed for the splicing of different intron subsets (Till et al., 2001; Ostheimer et al., 2003, 2005, 2006; Ostersetzer et al., 2005). Orthologs in *Arabidopsis* for these RNA splicing factors have been identified (Asakura and Barkan, 2006). In addition to these splicing factors, one highly specific factor has been identified that is required for the *trans*-splicing of *rps12* intron 1 in chloroplasts (Schmitz-Linneweber et al., 2006).

This *rps12* RNA splicing factor is a pentatricopeptide repeat (PPR) protein, one of a very large family in *Arabidopsis* of at least 450 proteins (Aubourg et al., 2000; Lurin et al., 2004; Rivals et al., 2006). They are characterized by tandemly repeated,

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degenerate, 35–amino acid motifs and have been proposed to be sequence-specific RNA binding proteins (Small and Peeters, 2000). Most of the few that have been characterized play a role in posttranscriptional processes in organelles (reviewed in Nakamura et al., 2004; Andres et al., 2007). PPR proteins have been implicated in RNA editing (Kotera et al., 2005; Okuda et al., 2007), RNA processing (Meierhoff et al., 2003; Hattori et al., 2007), RNA splicing (Schmitz-Linneweber et al., 2006), and translational activation (Schmitz-Linneweber et al., 2005). During a reverse genetics screen of PPR mutants, we identified one with a defect in splicing of *nad1* transcripts. Nothing is known of RNA *trans*-splicing factors in mitochondria; thus, this mutant is of considerable interest in understanding mitochondrial RNA splicing processes. This mutant is also specifically affected in *nad1* expression and appears to lack Complex I activity. It is thus equally interesting from the biochemical and physiological point of view and may be helpful in understanding the role of Complex I and the alternative pathways in plant mitochondria.

RESULTS

Macroscopic Phenotype

The gene studied in this work (At1g74900) was first flagged as worthy of interest from a high-throughput RNA interference screen of a large number of *Arabidopsis* genes, including many genes encoding PPR proteins (Hilson et al., 2004). Different independent lines of knockdown expression of At1g74900 showed delayed development and delayed flowering, so three T-DNA insertion lines putatively mutated in At1g74900 were obtained and analyzed. The positions of the three T-DNA inserts were confirmed by PCR and sequencing, but only one line, Sail867_F07, proved to contain the T-DNA insert within the coding region (130 bp upstream of the stop codon) (Figure 1A). Seeds from each of the three lines were sown on soil. For the two lines with insertions outside of the coding sequence, all seeds germinated and homozygous mutant plants exhibited no obvious phenotype. These lines were not considered further. For the line Sail867_F07, only three-quarters of the seeds germinated, and no homozygous mutant plants were obtained. Closer examination of seeds from Sail867_F07 plants heterozygous for the insertion revealed two kinds (Figure 1B); three-quarters of the seeds were wild-type, while the remaining quarter were smaller and darker. PCR amplification of DNA from both kinds of seeds confirmed that the smaller, darker seeds contained homozygous mutant embryos. A small fraction of mutant seeds could be germinated on half-strength Murashige and Skoog medium. The rare seedlings obtained were homozygous for the T-DNA insertion and could be subsequently transferred to soil. The mutant plants show obviously delayed development and delayed flowering and are smaller than the wild type, but the difficulty in reproducibly germinating significant numbers of seeds precluded any rigorous statistical analysis of growth rate. The leaves are often curled (Figure 1C), and the seeds produced from these plants are invariably malformed and impossible to germinate. Perhaps surprisingly, male and female gametogenesis appears to be relatively unaffected; heterozygous plants produce close to 20% phenotypically mutant seeds (38/207), and seed set in homozygous mutants plants is as good as could be expected given the poor growth of the plants. All the phenotypes observed were absent from mutant plants transformed with a genome fragment carrying the wild-type At1g74900 gene (Figure 1D).

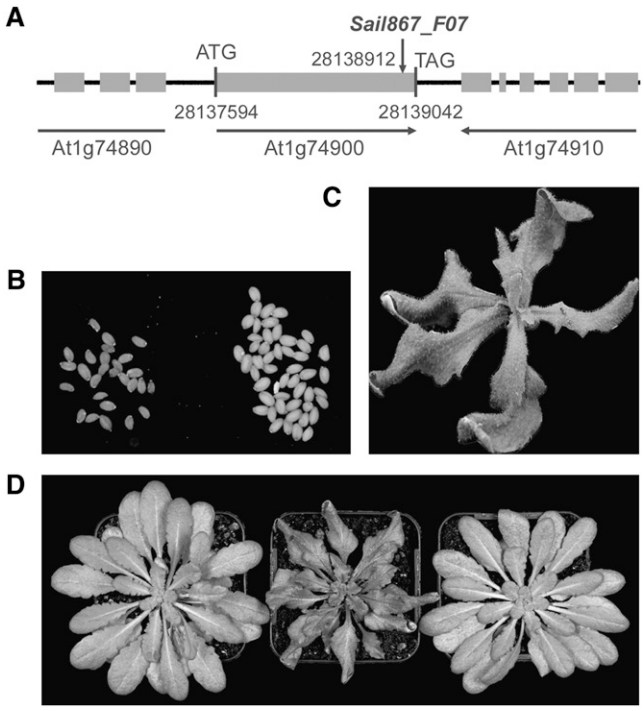

Figure 1. The *otp43* Mutant Line.

(A) T-DNA insertion map of the Sail_867F07 line.
(B) Mutant (left) and wild-type (right) seeds from a plant heterozygous for the Sail_867F07 insertion.
(C) Rosette from a homozygous *otp43* plant growing on soil in short-day conditions.
(D) Rosettes growing on soil in short-day conditions, from left to right: wild-type (Columbia [Col-0]), *otp43*, and complemented *otp43*. Rosettes from *otp43* are 4 weeks older than those of the wild type and the complemented *otp43* to approximately match the developmental stages of the plants.

Molecular Characterization

The protein encoded by At1g74900 is strongly predicted by Mitoprot (Claros, 1995) and Predotar (Small et al., 2004) to be mitochondrial, like the majority of PPR proteins. As virtually all of the PPR proteins characterized so far are involved in RNA processing within organelles, we employed a mitochondrial transcriptome mapping approach (C. Andrés, A. Falcon de Longevialle, A.L. Chateigner-Boutin, C. Lurin, and I.D. Small, unpublished data) to gain a general view of mitochondrial transcripts in the mutant plants and find any eventual RNA processing defects. The major defect found in the mutant was an absence of fully spliced *nad1* transcript (Figure 2). We thus named this mutant *otp43* (for *organelle transcript processing defect*) in line with other mutants identified by this screen. The *nad1* gene encoding

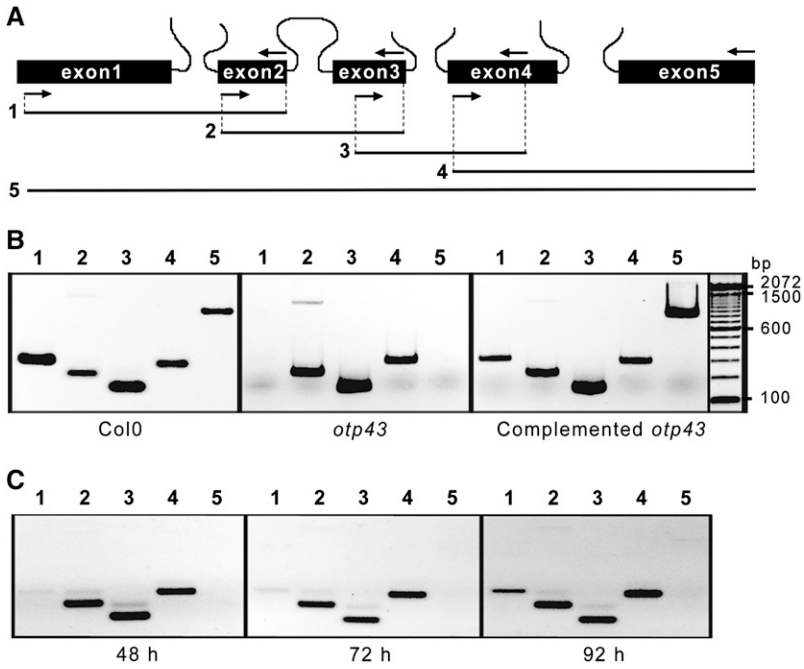

Figure 2. A Splicing Defect in *nad1* Intron 1 in *otp43*.

(A) Schematic representation of the localization of the primers used to detect the four splicing events by RT-PCR on the *nad1* pre-mRNA. 1, nad1exon1F/nad1exon2R; 2, nad1exon2F/nad1exon3R; 3, nad1exon3F/nad1exon4R; 4, nad1exon4F/nad1exon5R; 5, nad1exon1F/nad1exon5R.
(B) RT-PCR on Col-0 cDNA, on cDNA from *otp43*, and on cDNA from complemented *otp43* using primers designed to amplify across *nad1* splice junctions. No amplification is detected from *otp43* with primer pairs 1 and 5, showing that intron 1 is not spliced in the mutant. Molecular weight markers are shown on the right.
(C) PCR on cDNA from *otp43* leaves 48, 72, or 92 h after infiltration with *Agrobacterium* carrying a T-DNA containing the wild-type *OTP43* gene.

subunit 1 of Complex I is fragmented into five coding segments that are scattered over at least 175 kb and interspersed with other genes in the *Arabidopsis* mitochondrial genome. Two *trans*-splicing events and two *cis*-splicing events are necessary for the complete maturation of the *nad1* transcript. To verify if the absence of spliced *nad1* transcript is due to a defect in splicing of a specific intron, we designed primers to detect each splicing event by RT-PCR. In the mutant, *trans*-spliced mRNA joining exon 1 and exon 2 is not detected, whereas the three other introns of *nad1* are correctly and efficiently spliced (Figure 2). We tested all 22 other mitochondrial RNA splicing events by quantitative RT-PCR (see Supplemental Figure 1 and Supplemental Table 1 online) without observing any other qualitative differences between the mutant and the wild type.

Complementation of the RNA *trans*-Splicing Event by Wild-Type Copies of *OTP43*

To verify that the RNA splicing defect observed is due to a lack of functional *OTP43*, we directly transformed mutant plants with a wild-type copy of the gene (including the upstream sequences likely to contain the promoter). As homozygous mutant seeds almost never germinate, we reasoned that transformed seeds could be selected simply by their ability to grow, and this proved to be the case. The seedlings obtained were genotyped and shown to be carrying the *OTP43* transgene (see Supplemental Figure 2 online). Their growth and development were indistinguishable from wild-type plants (Figure 1D), and splicing of the *nad1* transcript was fully restored (Figure 2B). Incidentally, the easy recovery of complemented seedlings also demonstrates that the defective seed development and germination phenotype is a property of the mutant embryos, not a maternal effect due to the poor growth of the plants. To further investigate the restoration of RNA splicing, we infiltrated leaves of homozygous mutant plants with *Agrobacterium tumefaciens* carrying the T-DNA construct with the *OTP43* gene. By 48 h after inoculation, traces of spliced exon1-exon2 *nad1* mRNA could be detected by RT-PCR (Figure 2C), and by 92 h, the complete mature *nad1* mRNA could be amplified. We conclude that the defect observed in *nad1* transcript splicing in *otp43* plants is solely due to a lack of functional *OTP43*.

New RNA Editing Sites in *nad1* Intron 1 and Exon 4

As at least one PPR protein is known to be involved in RNA editing (Kotera et al., 2005), we sequenced cDNAs covering the five exons of *nad1* cDNA and both halves of intron 1 from the mutant in case an RNA editing defect was at the root of the RNA splicing defect. Twenty-four C-to-U editing sites have been described in exons 1, 3, and 5 of *nad1* mRNA from *Arabidopsis* (Giegé and Brennicke, 1999).

In wheat (*Triticum aestivum*), a C-to-U editing event in domain VI of the *nad1* intron 1 may have an impact on secondary structure (Li-Pook-Than and Bonen, 2006). In *Arabidopsis*, as previously described (Carrillo et al., 2001), this site is not edited. However, we found a new C-to-U editing site in domain V of the *nad1* intron 1 (Figure 3). This site is correctly edited in the mutant.

It has also been demonstrated in wheat that an editing site located very close to the intron/exon junction could be essential for the correct secondary structure of the group II intron and, thus, splicing (Li-Pook-Than et al., 2007). No differences between the mutant and the wild type were observed in any of the *nad1* exons, although we did find a new editing site in *nad1* exon 4 that was not described from *Arabidopsis* but has been observed in *Brassica napus* (Handa, 2003). Thus, the splicing defect is unlikely to be due to a deficiency in editing; furthermore, none of the editing events in *nad1* transcripts require prior splicing out of intron 1.

Loss of Mitochondrial Complex I

The NAD1 protein is a central anchor component of Complex I and would be expected to be essential for assembly of the complex in the membrane. As no mature *nad1* mRNA is detected, it is highly likely that no NAD1 protein is produced in the mutant plants. No effective antibodies against the highly hydrophobic NAD1 protein are available to verify this. However, separation of mitochondrial complexes by blue-native PAGE and NADH dehydrogenase activity staining (Figure 4A) fail to reveal any trace of Complex I activity. Two-dimensional blue-native/SDS-PAGE on the mutant confirms the specific lack of Complex I (Figure 4B); none of the other complexes appear to be affected. We cannot rule out that partial complexes formed from some of the other Complex I subunits are still present but not visible under the gel procedures used.

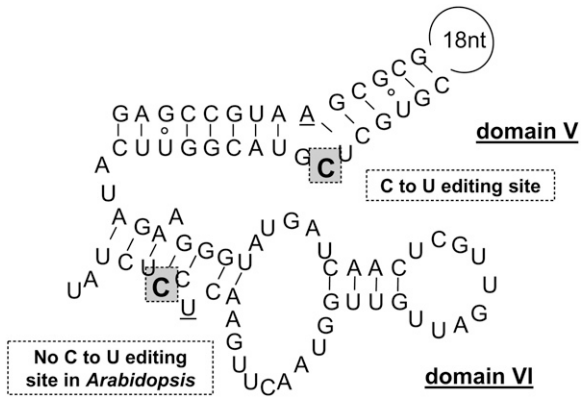

Figure 3. Secondary Structure Model of Domains V and VI of the *Arabidopsis nad1* Intron 1.

The C-to-U editing site found in *nad 1* intron 1 domain VI in wheat, highlighted in gray, is not edited in *Arabidopsis*. The second nucleotide highlighted in gray in domain V is a novel C-to-U editing site that was found to be edited in the wild type and in *otp43*. The underlined adenosine in domain V is a uracil in almost all other group II introns, while the underlined uracil in domain VI lies approximately where the bulged adenosine involved in lariat formation would normally be located.

Accumulation of Alternative Respiratory Pathway Transcripts and Proteins

The previously described *Nicotiana* and maize mutants lacking Complex I had elevated levels of alternative oxidase (AOX) (Karpova et al., 2002; Dutilleul et al., 2003b). To check whether this *Arabidopsis* mutant has the same phenotype, we performed quantitative RT-PCR assays for transcripts coding for alternative respiratory pathway proteins, namely, the alternative external (NDB1-4) and internal (NDA1-2 and NDC1) NAD(P)H dehydrogenases and the AOX isoforms AOX1a-c and AOX2 (Figure 5A). The levels of many of these transcripts are significantly altered in the *otp43* mutant, and some, such as *AOX1c*, *NDB2*, and *NDB4*, are highly induced. The transcript increases lead to protein accumulation, at least of the AOX, as revealed by a protein gel blot using an antibody against AOX. As shown in Figure 5B, the mutant plants contain considerably increased levels of AOX compared with wild-type plants.

DISCUSSION

Plant Organellar Splicing Factors

cis-spliced group II introns can self-splice in vitro, but both *cis*- and *trans*-spliced group II introns in the chloroplast and mitochondria require RNA splicing factors in vivo. Each organelle genome contains one group II intron that encodes a conserved maturase protein. In chloroplasts, the *matK* maturase is suspected to be involved in several different splicing events but is not required for splicing of all introns (Vogel et al., 1999). The other cofactors and RNA splicing factors are encoded by nuclear genes and are imported into organelles. Four of these (CRS1, CRS2, CAF1, and CAF2) that act as factors in splicing of one or more different introns have been identified from maize chloroplasts (Jenkins and Barkan, 2001; Till et al., 2001; Ostheimer et al., 2003, 2005, 2006; Ostersetzer et al., 2005), and a PPR protein has been characterized that is specifically needed for *trans*-splicing of the *rps12* transcript (Schmitz-Linneweber et al., 2006). RNA *trans*-splicing probably requires more proteins than *cis*-splicing, and many more may remain to be identified in land plants if we compare the currently known factors with the 14 nuclear genes that are required for the removal of the *trans*-spliced introns in the *psaA* gene in *Chlamydomonas reinhardtii* chloroplasts (Goldschmidt-Clermont et al., 1990; Merendino et al., 2006).

In plant mitochondria, much less is known about RNA splicing factors. Introns in fungal mitochondria usually require the activity of intron-encoded maturases, but various other RNA binding cofactors have been shown to be important for splicing of specific introns. In land plants, the *nad1* intron 4 encodes a maturase, but it is not known which introns, if any, require its activity for splicing. Recently, an *Arabidopsis* nuclear gene encoding a maturase-like protein was shown to be involved in splicing of the mitochondrial *nad4* transcript (Nakagawa and Sakurai, 2006), but some RNA splicing still occurs in the mutant described.

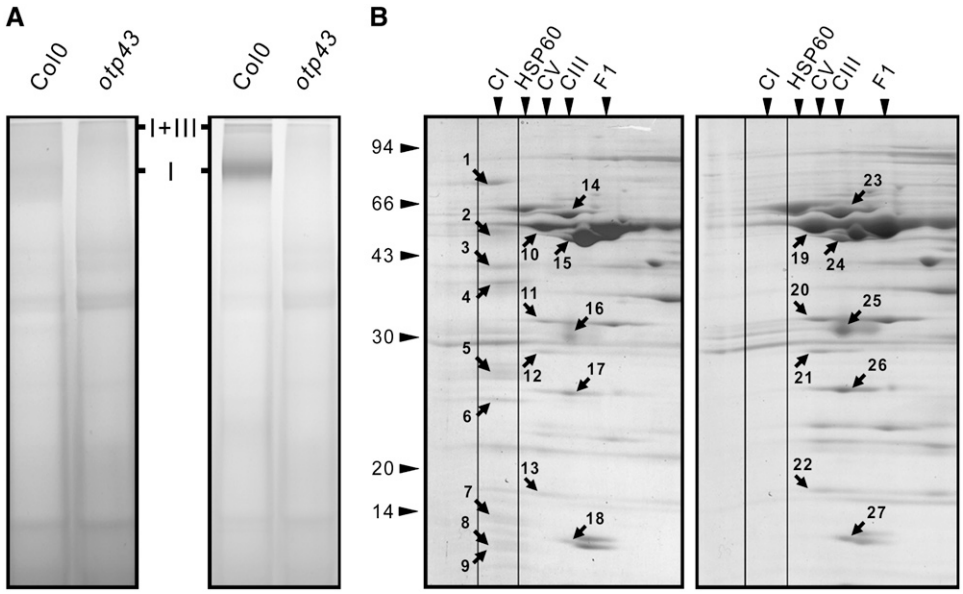

Figure 4. Complex I Is Lacking from *otp43* Mitochondria.

(A) Blue-native gel (left) and NADH dehydrogenase activity staining of the same gel (right) of isolated mitochondrial proteins from Col-0 rosettes and from *otp43* rosettes growing in short-day conditions. I and III+I indicate the positions of Complex I and the supercomplex of Complex I + Complex III. **(B)** Two-dimensional blue-native/SDS-PAGE of mitochondrial proteins from Col-0 wild-type plants (left) and *otp43* plants (right). The gels are stained with Coomassie blue. Apparent molecular mass of subunits is shown in kilodaltons. Arrows indicate spots that have been identified by mass spectrometry (see Supplemental Table 2 online): 1, 76-kD subunit (At5g37510); 2, 51-kD subunit (At5g08530), ND5 (NP_085478/AtMg01460); 3, ND7 (NP_085511/AtMg00510); 4, 39-kD subunit (At2g20360), ND2 (NP_085584/AtMg01450); 5, CAL1 (At5g63510), 23-kD subunit (At1g79010), ND1 (NP_085565/AtMg01275); 6, ND9 (NP_085479/AtMg0070); 7, 16-kD subunit (At2g27730), PDSW subunit (At3g18410, At1g49140); 8, B14 subunit (At3g12260), B18 subunit (At2g02050); 9, B18 subunit (At2g02050). These proteins are all subunits of Complex I and none are visible in the gel of *otp43* proteins. 10 and 19, ATP2 (At5g08670), ATP1 (NP_085571/AtMg01190); 11 and 20, ATP3 (At2g33040); 12 and 21, ATP FAD (At2g21870); 13 and 22, ATP8 (NP_085508/AtMg00480). These proteins, identified in both wild-type and *otp43* plants, are all subunits of Complex V. 14 and 23, MPP b (At3g02090); 15 and 24, MPP a (At1g51980); 16 and 25, Cyt c1-1 (At3g27240), Cyt c1-2 (At5g40810), COB (NP_085492/AtMg00220); 17 and 26: URC1 (At5g13430); 18 and 27, QCR7 (At4g32470). These proteins, identified in both wild-type and *otp43* plants, are all subunits of Complex III.

A Mitochondrial *trans*-Splicing Factor

Our results clearly show that *OTP43* is absolutely and specifically required for splicing of the first intron in the mitochondrial *nad1* transcript. In comparison, all other introns in the mutant mitochondria are correctly spliced to a large extent. A decrease in splicing of *nad2* intron 1 is also observed (see Supplemental Figure 1 online) and could be due to a minor role for OTP43 in splicing of this intron too or to pleiotropic effects of the loss of Complex I. The high degree of specificity of OTP43 resembles that shown for maize PPR4, but the two PPR proteins are not closely related. PPR4 and its probable *Arabidopsis* ortholog (At5g04810) contain an RNA recognition motif domain, and At5g04810 is the only one of the 450 *Arabidopsis* PPR proteins to do so. By contrast, At1g74900 is a more typical PPR protein, containing only canonical PPR motifs. There is a putative ortholog of At1g74900 in the rice (*Oryza sativa*) genome (Os02g45590 using the TIGR 3.0 annotation), as would be expected considering that the presence of *nad1* intron 1 is widely conserved across angiosperms.

Possible Mechanisms of Action

By immunoprecipitating PPR4-RNA complexes from maize chloroplast stromal extract, PPR4 was shown to bind specifically to *rps12* intron sequences and thus presumably helps the two intron halves to associate or fold into an active conformation (Schmitz-Linneweber et al., 2006). The extreme difficulty in obtaining sufficient *Arabidopsis* mitochondrial matrix extract (particularly from the rare mutant plants we have obtained that were needed to carry out the necessary controls) precludes an equivalent test of At1g74900 function. Nevertheless, it is likely that this PPR protein does interact in some way with the *nad1* intron 1. A second possibility is that it acts indirectly by being required for expression of another splicing factor (e.g., a small RNA or the mitochondrial *matR* maturase). A third possibility, which we have partially disproved, is that At1g74900 is required for accumulation and processing of *nad1* precursors such that they are in a correct state for RNA splicing. We observed no differences in RNA abundance, editing, or structure of the various *nad1* precursor transcripts in the mutant, and, indeed, all other *nad1* splicing events take place normally.

nad1 Intron 1 Has Some Unique Features

Based on extensive phylogenetic, biochemical, mutational, and biophysical data, a consensus sequence has been derived from 112 group IIA/IIB introns (Bonen and Vogel, 2001). The *nad1* intron 1 differs from this consensus at several points and has an

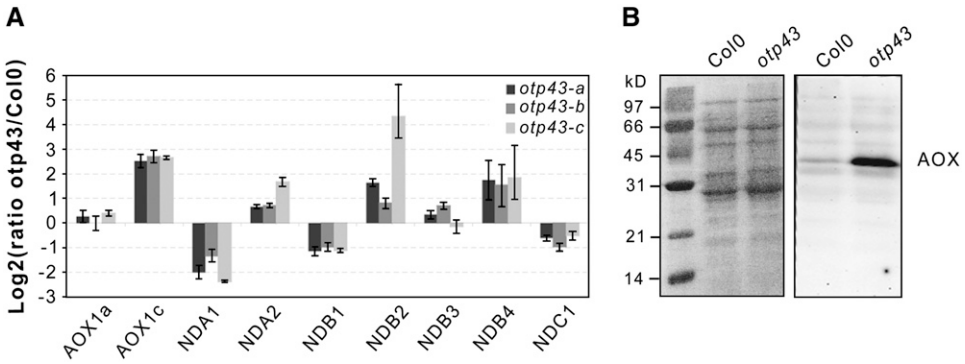

Figure 5. Alternative Electron Transport Pathway Transcripts and Proteins Are Induced in *otp43*.

(A) Quantitative RT-PCR on cDNA from three independent *otp43* plants (*otp43-a*, *otp43-b*, and *otp43-c*).
(B) Immunoblot analysis of AOX proteins. Coomassie blue–stained SDS-PAGE gel (left) of mitochondrial proteins extracted from wild-type and *otp43* plants. This gel was used for a protein gel blot (right) using anti-AOX antibodies. Bound antibodies were revealed by chemiluminescence.

unconventional domain V sequence with a stem loop of 18 nucleotides instead of the four nucleotides found in the conventional domain V. A second major difference is the presence of an adenosine nine nucleotides downstream from the 5′ end of domain V (Figure 3). Among 112 domain V sequences from group II introns, only angiosperm *nad1* intron 1 and the *Marchantia polymorpha petB* intron contain an adenosine at what is usually a highly conserved uracil. The base at this position is thought to be paired with the conserved guanine at position 26 (Costa et al., 1998). The cytidine at position 25 and the guanosine in position 26 are the site of important 2′-OH and phosphoryl groups involved in intron structure. Domain VI is also unusual in this intron, and, notably, the bulged adenosine that typically acts as the attacking nucleophile at the 5′ splice site in other group II introns is missing (Figure 3). It has been proposed that *nad1* intron 1 splicing may involve hydrolysis rather than trans-esterification (Carrillo et al., 2001). It is possible that one or more of these unique features explain the need for a specific splicing factor.

Plant Complex I Mutants

In *Arabidopsis*, three nuclear mutants of Complex I have been described so far. The *fro1* mutant was identified as being cold sensitive (Lee et al., 2002) and has a mutation affecting the expression of the *FRO1*, which codes for an 18-kD subunit of Complex I. The phenotype of the *fro1* mutant is similar to the phenotype of the *otp43* mutant described here: slow growth, retarded development, and delayed germination. However, no problems with seed formation were observed, and the *fro1* germination phenotype is less severe. Another mutant contained a T-DNA insertion in a nuclear gene (At1g47260) encoding a subunit of Complex I with some similarity to carbonic anhydrases (Perales et al., 2005). These mutants were not visually distinguishable from wild-type plants, although Complex I activity was decreased by 80%. The third mutant, *css1*, was identified by its sensitivity to an inhibitor of cellulose biosynthesis (Nakagawa and Sakurai, 2006). The nuclear gene affected in this mutant has a high similarity to group II intron maturases, and the affected plants show impaired splicing of the mitochondrial *nad4* transcript. The molecular phenotype of this mutant resembles that of the NMS1 mutant from *N. sylvestris*, also specifically defective in splicing of *nad4* transcripts (Brangeon et al., 2000). Complex I activity was not measured in the *css1* mutant, but like the other previously described *Arabidopsis* Complex I mutants, the overall phenotype appears to be milder than the *otp43* mutant described here.

Two apparently total knockouts of Complex I activity have been described from other species, and in both cases, they are mitochondrial mutants. The best-characterized mutant to date is the CMSII mutant in *N. sylvestris*. This mutant carries a mitochondrial deletion in the *nad7* gene encoding a 40-kD subunit of Complex I (Pla et al., 1995). The mutant plants develop slowly, exhibit conditional male sterility, and have no detectable Complex I, increased amounts and activity of AOX, and multiple perturbations to primary metabolism (Gutierres et al., 1997, 1999; Lelandais et al., 1998; Sabar et al., 2000; Dutilleul et al., 2003a, 2003b, 2005; Pineau et al., 2005; Priault et al., 2006a, 2006b; Vidal et al., 2007). A second Complex I mutant has been described from maize (Karpova and Newton, 1999). The NCS2 mutant carries a deletion of the 3′ end of the *nad4* gene. This deletion causes lethality during kernel development, and only mutant plants containing at least some normal mitochondria can propagate, giving rise to striped plants containing sectors with varying ratios of mutant to wild-type mitochondria. The mutant sectors have no Complex I activity but a partially assembled Complex I (Karpova and Newton, 1999). As observed in the CMSII mutant, the amount of AOX is increased in NCS2 sectors (Karpova et al., 2002). Both of these mutants resemble *otp43* in that they lack a mitochondrially encoded subunit of Complex I, leading to a partial or complete loss of the complex and a complete loss of activity. However, the physiological effect on the plants is quite distinct; in *N. sylvestris*, loss of Complex I leads to conditional male sterility but relatively minor effects on growth and seed development. In maize and *Arabidopsis*, on the contrary, male fertility is near normal, but seed development is severely compromised and plant growth drastically affected (to the point in maize where it is impossible to recover completely mutant plants). Further research is needed to discover whether

these differences are due to differences in the relative importance of Complex I to the physiology of these different species or whether the differences arise because different Complex I subunits are missing in each mutant.

The Effects of a Loss of Complex I Activity

Inhibition of Complex I by inhibitors, such as rotenone, or by loss of subunits as in the mutants described above leads to problems with primary metabolism in the affected plants (reviewed in Noctor et al., 2007). The primary entry point for electrons into the electron transport chain is missing, leading to a loss in ATP synthesis and an initial buildup of unoxidized NADH. It has been previously shown in other plants that under these conditions the alternative respiratory pathway is engaged, comprising the induction of alternative NAD(P)H dehydrogenases and AOXs (Gutierres et al., 1997; Sabar et al., 2000; Karpova et al., 2002; Dutilleul et al., 2003b), bypassing the classical cytochrome electron transport chain. Our data show that this response is significantly induced in this mutant, with transcriptional induction of both alternative NADH dehydogenases and AOX and a significant increase in total AOX protein revealed by immunodetection. The availability of an *Arabidopsis* mutant lacking expression of a major Complex I subunit will be valuable for genetically dissecting the retrograde signaling pathways involved in this response.

METHODS

Plant Material and Culture Conditions

The *Arabidopsis thaliana* Sail867_F07 mutant from the SAIL collection (Sessions et al., 2002) and Col-0 wild type were germinated on half-strength Murashige and Skoog medium and 1% sucrose at 22°C with a 16-h photoperiod. Two-week-old rosettes were transferred to soil, and plants were grown at 22°C with a 8-h photoperiod for isolation of mitochondria and RNA and also for the transient complementation experiments. For the other experiments described, rosettes were transferred to soil and grown at 22°C with a 16-h photoperiod.

DNA and RNA Isolation and RT-PCR Analysis

Leaf DNA was isolated using the DNA plant extraction protocol as described by Edwards et al. (1991), and 10 ng from the extraction was used for PCR with primers sail_867_F07_LP (5′-TGGTTACAGGAGACTCGGTTG-3′), sail_867_F07_RP (5′-GGTGAGGAGAGGTTATGAGCC-3′), and LB1 (5′-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC-3′). Leaf RNA was isolated using the plant RNeasy extraction kit (Qiagen) according to the manufacturer's protocols. The leaf RNA was treated with DNase (Ambion) according to the manufacturer's protocols, and 3 μg was reverse transcribed with Superscript III (Invitrogen) according to the manufacturer's directions using random hexamers (Invitrogen). Primers designed to detect splice junctions were as follows: nad1exon1F (5′-GCAACGTAGAAAGGGTCCTG-3′), nad1exon2R (5′-TGAGCTGCAGATCGTAATGC-3′), nad1exon2F (5′-TCGAAATATGCCTTTCTAGGAG-3′), nad1exon3R (5′-ATTCAGCTTCCGCTTCTGG-3′), nad1exon3F (5′-GTCATGGCGCAAAAGCAGATATGG-3′), nad1exon4R (5′-AGAGCAGACCCCATTGAAGA-3′), nad1exon4F (5′-TCTTCAATGGGGTCTGCTCT-3′), and nad1exon5R (5′-AGGAAGCCATTGAAAGGTGA-3′). One microliter of a 33-fold dilution of the reverse transcription reaction was used for PCR with *Taq* DNA polymerase (New England Biolabs).

Quantitative RT-PCR Analysis

Quantitative RT-PCR was performed using LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics) and a LightCycler 480 Roche real-time PCR system with the following thermal cycling program: 95°C for 10 min, followed by 18 touchdown cycles of denaturation at 94°C for 10 s, 85°C (−2°C per cycle) for 10 s, and extension at 72°C for 20 s, followed by 40 cycles of 95°C for 10 s, 50°C for 10 s, and 72°C for 20 s. Primer sequences used were as follows: AOX1a (At3g22370) forward (F), 5′-GACGGTCCGTACGGTTTCG-3′, and reverse (R), 5′-CTTCTGATTCGCGTCCTCCTCCT-3′; AOX1c (At3g27620) F, 5′-GCATCAAAGCAAGCGACATCC-3′, and R, 5′-TGGCTTCACGCCCCAATAAC-3′; NDA1 (At1g07180) F, 5′-GTCCGTGAGAGCAAGGAAGG-3′, and R, 5′-GGCGAAGTGGAGGGGATATG-3′; NDA2 (At2g29990) F, 5′-CGAGAGCAAGGACGCAAAAG-3′, and R, 5′-CAGTAGGCCGAGATTGAGAC-3′; NDB1 (At4g28220) F, 5′-CTGTGACTGATAAAGATATC-3′, and R, 5′-GCCAACTGCATATACATTC-3′; NDB2 (At4g05020) F, 5′-CTGACTCTCTCAAAGAGTTCC-3′, and R, 5′-CGATTTGAACTCTTCGATC-3′; NDB3 (At4g21490) F, 5′-GCAAATCCATCATATAGCAAC-3′, and R, 5′-CAAGGGAGTGAAAGCAAAG-3′; NDB4 (At2g20800) F, 5′-TCAAGTCTTAAGGGCACAACA-3′, and R, 5′-CGGAAGAGAGAGGCTCTCTCG-3′; NDC1 (At5g08740) F, 5′-TCCATAAAGAGAGCAAGCAAC-3′, and R, 5′-CGAATCACCTAATGCAAATATC-3′; Actin2.8, Actin2 (At3g18780), and Actin8 (At1g49240) F, 5′-GGTAACATTGTGCTCAGTGGTGG-3′, and R, 5′-AACGACCTTAATCTTCATGCTGC-3′. Actin2.8 was used as an internal reference amplicon. Data were analyzed using LightCycler 480 software version 1.2 (Roche Diagnostics).

Complementation

Genomic DNA fragments were amplified using *Pfx* polymerase (Invitrogen) and the primers 5′-AAGGTACCAGGGACCCAATTTTCTTTGA-3′ and 5′-AAGGTACCGGGAGCAGCCACACTATCTC-3′. The products were subcloned into pGEM-T Easy (Promega) and sequenced. The resulting plasmid was cut by *Kpn*I and transferred into pCAMBIA-1301 (www.cambia.org). The resulting vector was transformed into *Agrobacterium tumefaciens* GV3101pMP90 and used for infiltration of leaves and floral dip transformation. *Agrobacterium*-mediated transient expression by leaf infiltration assays was performed with this strain as described by Marois et al. (2002) and floral dip transformation of homozygous plants as described by Clough and Bent (1998). Resulting seeds were sown on soil without selection because only transformed seeds can germinate and grow.

Mitochondrial Isolation

Ten grams of plant tissue were ground in extraction buffer (0.45 M sucrose, 1.5 mM EDTA, 0.2% BSA, 0.6% PVP40, and 15 mM MOPS/KOH, pH 7.4) using a mortar and pestle. The resulting extract was filtered through two layers of Miracloth and spun 5 min at 2500*g*. The pellet was discarded, and the supernatant was spun 10 min at 15,000g at 4°C. The pellet was resuspended in 1 mL of wash buffer (0.3 M sucrose and 10 mM MOPS/KOH, pH 7.2), loaded on a linear 0 to 4% (w/v) PVP-40 gradient in wash buffer containing 28% Percoll, and spun 45 min at 40,000*g* at 4°C. The mitochondrial band located in the bottom of the gradient was recovered, diluted in wash buffer, and loaded on top of a 35% Percoll gradient and spun 45 min at 40,000*g* at 4°C. Mitochondria form a band in the top part of the gradient. This band was collected and washed twice in wash buffer.

Blue-Native and Two-Dimensional SDS-PAGE and NADH Dehydrogenase Activity Stain

Four hundred micrograms of mitochondrial membrane proteins were solubilized in 1% *N*-dodecylmaltoside in ACA750 buffer as described

by Schagger and von Jagow (1991). Blue-native strips were excised, incubated in 1% (w/v) SDS and 1% (w/v) β-mercaptoethanol for 30 min to denature the complexes, and placed horizontally on top of a 12% SDS-PAGE. The resulting gels were stained with colloidal Coomassie Brilliant Blue G 250 as described previously (Millar et al., 2001).

The NADH dehydrogenase activity of Complex I was revealed by incubating the Blue-Native gel in 1 mM nitroblue tetrazolium and 0.14 mM NADH in 0.1 M Tris, pH 7.4. The reaction was stopped by soaking the gel in fixing solution (30% [v/v] methanol and 10% [v/v] acetic acid).

Immunoblot Analysis

Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane (Amersham Pharmacia Biotech). Protein gel blots with AOX antibodies were performed at a dilution of 1/5000. Goat anti-mouse antibodies conjugated to horseradish peroxidase (GE Healthcare) were used as secondary antibodies and revealed with ECL reagents (GE Healthcare).

Identification of Gel Spots by Mass Spectrometry

The protein samples to be analyzed were cut from the gels, destained in 10 mM NH_4CO_3 and 50% (v/v) acetonitrile, dehydrated at 50°C, rehydrated with 15 μL of digestion solution (10 mM NH_4CO_3 and 12.5 μg/mL of trypsin in 0.01% trifluoroacetic acid), and incubated overnight at 37°C. Peptides were extracted according to Taylor et al. (2005). Samples were loaded onto self-packed Microsorb C18 (5 μm, 100 Å; Varian) reverse phase columns (0.5 × 50 mm) using an Agilent Technologies 1100 series capillary liquid chromatography system and eluted into a XCT Ultra IonTrap mass spectrometer (Agilent Technologies) with an electrospray ionization source equipped with a low flow nebulizer in positive mode and controlled by Chemstation (rev B.01.03 [204]; Agilent Technologies) and MSD Trap Control version 6.0 software (build 38.15; Bruker Daltonik). Peptides were eluted from the C18 reverse phase column at 10 μL/min using a 9-min acetonitrile gradient (5 to 60%) in 0.1% formic acid at a regulated temperature of 50°C. Ions were selected for tandem mass spectrometry (MS/MS) after reaching an intensity of 25,000 counts per second, and two precursor ions were selected from the initial MS scan. The resulting MS/MS spectra were exported, and the extracted results were queried against the *Arabidopsis* protein set (TAIR7) using the Mascot search engine v2.1.01 (Matrix Science) using error tolerances of ± 1.2 D for MS and ± 0.6 D for MS/MS, maximum missed cleavages set to 1, and peptide charge set at 2+ and 3+. Results were filtered using standard scoring, maximum number of hits set to 20, significance threshold at $P < 0.05$, and ion score cutoff at 0.

Accession Number

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under accession number At1g74900 (*OTP43*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Intron Splicing in *otp43* Mutant Plants.

Supplemental Figure 2. Genotyping of Complemented *otp43* Plants.

Supplemental Table 1. Primers Used for RT-PCR Assays.

Supplemental Table 2. Mass Spectrometry Results from the Analysis of Two-Dimensional BN-PAGE Gels.

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