ORFB is a subunit of F_1F_0 -ATP synthase: insight into the basis of cytoplasmic male sterility in sunflower

Mohammed Sabar, Dominique Gagliardi*, Janneke Balk† & Christopher J. Leaver[‡]

Department of Plant Sciences, University of Oxford, Oxford, UK

ORFB is the product of a gene that is conserved in plant mitochondrial genomes, and which, on the basis of sequence motif and structural similarity, is predicted to be the homologue of yeast and mammalian ATP8, part of the F_0 component of the F_1F_0 -ATP synthase. We have shown that, in sunflower, *orfB* transcripts are edited, increasing the similarity of the predicted protein to ATP8 proteins from non-plant species. Blue-native polyacrylamide gel electrophoresis and peptide sequencing confirm that ORFB localizes to the ATP synthase complex. The predicted amino-terminal 19 amino acids of ORFB are identical to those in the chimeric mitochondrial ORF522 protein, which is associated with cytoplasmic male sterility (CMS) in sunflower. Assays comparing respiratory complexes from a male-sterile line expressing ORF522 with those from a male-fertile line show a specific decrease in ATP hydrolysis by the ATP synthase. These observations allow us to propose a mechanism underlying CMS that is associated with the expression of chimeric open reading frames containing part of the *orfB* gene.

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INTRODUCTION

Plant mitochondrial genomes contain approximately 60 open reading frames (ORFs), most of which have been identified (Unseld *et al.*, 1997). Only a few, such as *orfB*, *orf25* and *orfX*, which are conserved across all plant mitochondrial genomes sequenced so far, have no known function. Gray *et al.* (1998), on the basis of a comparison of the genome sequences of a range of protists (for example, *Reclinomonas americana*), predicted that the ORFB protein in plants is the homologue of the yeast and mammalian ATP8. The *atp8* gene encodes a core proteolipid subunit of the F_0 component of the F_1F_0 -ATP synthase (complex V), which is thought to be involved in the assembly of F_0 (Hadikusumo *et al.*, 1988; Mueller, 2000). However, the sequence conservation between ORFB and ATP8 is low and ORFB has not been shown to be a component of the plant F_1F_0 -ATP synthase.

We, and others, have shown that chimeric mitochondrial genes that contain part, or all, of the *orfB* gene are involved in cytoplasmic male sterility (CMS) in several plant species (Laver *et al.*, 1991; Schnable & Wise, 1998; Horn & Friedt, 1999; Nakajima *et al.*, 2001). CMS is a maternally inherited trait that has been described in more than 150 plant species, and is characterized by the inability to produce functional pollen, although vegetative development is unaffected. The CMS phenotype, in conjunction with the nuclear *restorer of fertility* genes (*Rf* genes), is used in the production of F_1 hybrid crops. In the best-characterized systems, CMS is associated with aberrant recombination events in the mitochondrial genome that result in the generation of chimeric ORFs, which are expressed as novel polypeptides (Schnable & Wise, 1998). In sunflower, the PET1–CMS protein is associated with the expression of a novel mitochondrial gene, *orf522*, which is located downstream of the *atp1* gene. *orf522* is co-transcribed with the *atp1* gene, and is expressed as a mitochondrial polypeptide of ~15 kDa in all tissues (Horn *et al.*, 1991; Köhler *et al.*, 1991; Laver *et al.*, 1991). The male-fertile phenotype can be restored by the introduction, in a cross, of nuclear *Rf* genes, which lead to a specific reduction in the levels of the *atp1*–*orf522* co-transcript and of the ORF522 protein in the male florets (Monéger *et al.*, 1994; Smart *et al.*, 1994). In addition, we have shown that increases in the level of polyadenylation of the *atp1*–*orf522* transcript are correlated with the tissue-specific instability of the transcript in the male florets of the hybrid plants with restored fertility (Gagliardi & Leaver, 1999).

In this study, we show that, in sunflower, ORFB is localized to the F_1F_0 -ATP synthase complex, and that editing of the *orfB* transcript creates a leucine in the amino terminus that increases the level of sequence similarity with the N-terminal amino acids of ATP8 from non-plant species. This provides further evidence that ORFB is the plant equivalent of ATP8. In-gel assays of the enzyme activities associated with the main enzyme complexes of the mitochondrial inner membrane revealed a specific reduction in the ATPase activity of the F_1F_0 complex in mitochondria from seedlings of the cytoplasmic male-sterile line compared with that in fertile lines. In contrast, the activities associated with other respiratory complexes were unaffected. These observations allow us to propose a mechanism underlying CMS in higher plants in which part of the *orfB* gene contributes to the generation of novel chimeric genes.

Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK

*Present address: IBMP-CNRS, 12 Rue du Général Zimmer, 67084 Strasbourg, France

†Present address: Institut für Zytobiologie und Zytopathologie, Philipps-Universität Marburg, Robert-Koch-Strasse 5, D-35032 Marburg, Germany

‡Corresponding author. Tel: +44 1865 275 143; Fax: +44 1865 275 144;

E-mail: chris.leaver@plants.ox.ac.uk

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RESULTS

ORFB localizes to the F₁F₀-ATP synthase complex

To determine whether ORFB is a subunit of the mitochondrial F₁F₀-ATP synthase (complex V), highly purified mitochondria were isolated from four-day-old, dark-grown sunflower seedlings or florets, and the mitochondrial respiratory complexes were separated in their native forms by blue-native polyacrylamide gel electrophoresis (BN-PAGE).

Fig. 1 | Localization of ORFB in F₁F₀-ATP synthase (complex V). (A) Blue-native polyacrylamide gel electrophoresis of 200 µg of solubilized floret mitochondrial membranes (first dimension) followed by tricine-SDS-polyacrylamide gel electrophoresis in the second dimension. The arrowhead indicates the protein analysed in (C). (B) Western blot analysis of membrane proteins separated as in (A) and labelled with an antibody against spinach F₁-ATP synthase. (C) Sequence alignment of amino acids 46–69 of several plant ORFB proteins and two trypsin-digested fragments of the protein indicated in (A). The arrowheads indicate trypsin cleavage sites. CI, CV and CIII, respiratory complexes I, V and III. *A. thaliana*, *Arabidopsis thaliana*; *B. napus*, *Brassica napus*; *B. vulgaris*, *Beta vulgaris*; *H. annuus*/*H. a.*, *Helianthus annuus*.

The component subunits in each complex were then resolved using SDS-PAGE in the second dimension (Fig. 1A). The complexes were identified by their order of separation in the gel on the basis of their known molecular weight, the patterns of subunit composition resolved in the second dimension (Jänsch *et al.*, 1996) and by the use of histochemical staining to detect the characteristic enzyme activities associated with each complex (see below). The identity of the F₁F₀-ATP synthase (550–600 kDa) was confirmed further by peptide mass fingerprinting of the F₁ α-subunit (data not shown), and by immunological staining of the α- and β-subunits (Fig. 1B) with an antibody against the whole F₁ complex from spinach mitochondria (Hamasur *et al.*, 1990). In sunflower mitochondria, this antibody recognizes the α- and β-subunits and, to a lesser extent, the γ-subunit. Combining with a 3:3:1 stoichiometry of α:β:γ only the α- and β-subunits are usually detected. In addition, these two subunits are similar in terms of molecular weight (approximately 54 kDa each), which prevents good resolution of them, especially when tricine is used instead of glycine in the SDS-PAGE. This results in the detection of only one spot on immunoblots. A band from the F₁F₀-ATP synthase complex containing a polypeptide of approximately 15 kDa (indicated by an arrowhead in Fig. 1A) was excised from the gel. In-gel trypsin digestion was then carried out on the excised band, and peptide mass fingerprinting was performed using matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry. The spectrum obtained contained peptides matching those predicted for the mitochondrially encoded ORFB from sunflower (Monéger *et al.*, 1994) and *Arabidopsis* (GenBank accession number NP_085508). Mass spectrometry was carried out on two peptides that exactly matched the predicted molecular mass for ORFB in order to determine their amino-acid sequences. The sequences of these peptides were identical to sequences found in the sunflower ORFB protein, as predicted from the genomic sequence (Fig. 1C; Quagliariello *et al.*, 1990). These results provide the first biochemical evidence that ORFB is a subunit of the F₁F₀-ATP synthase.

The sunflower *orfB* transcript is edited

Editing of mitochondrial transcripts is a common phenomenon in plants, often leading to codon changes (Maier *et al.*, 1996). To obtain a more accurate prediction of the amino-acid sequence of sunflower ORFB, seven complementary DNAs corresponding to the *orfB* transcript were sequenced in both the forward and reverse directions, and were compared with the *orfB* genomic sequence. Three C to T conversions were found, in all of the cDNAs sequenced, at nucleotide positions 47, 58 and 452 of the coding sequence. Each of these results in the change of an amino-acid residue as compared with the sequence predicted from the genomic DNA as follows: amino-acid residues S16 to L, L21 to F and P151 to L (Fig. 2). Residues L16 and F21 are conserved in ORFB proteins from higher plants, and L16 is also conserved in ATP8 of the protist *Reclinomonas americana* and in the mammalian ATP8 orthologue. The editing site at nucleotide 47 (amino acid S16 to L) is also present in the related chimeric *orf522* gene of sunflower (Monéger *et al.*, 1994), confirming that the predicted N-terminal 19 amino acids of ORFB and ORF522 are identical.

ATPase activity in mitochondria expressing ORF522

To determine whether there is a correlation between the presence of ORF522 and any change in the activity of the F₁F₀-ATP synthase that might be linked to pollen abortion in the sterile PET1-CMS line, we analysed the activity of the native ATP synthase in mitochondria

Fig. 2 | Protein sequence alignment of ORF522, ORFB and ATP8. The alignment was generated using the ClustalW programme. The 19 amino acids of ORF522 that are identical to ORFB are shown by black letters; the rest of the ORF522 sequence is shown by grey letters. Boxes indicate identical (grey shading) and similar (blue shading) residues. Amino acids of edited codons are in bold, and are indicated by numbers above the sequence. h, hydrophobic amino acid; +, positively charged amino acid. Ha, *Helianthus annuus*; Hs, *Homo sapiens*; Ra, *Reclinomonas americana*; Sc, *Saccharomyces cerevisiae*.

isolated from seedlings of fertile, sterile and hybrid sunflower lines. Mitochondrial membranes were solubilized using N-dodecyl β -D-maltoside, and the electron transport complexes and F_1F_0 -ATP synthase were separated by BN-PAGE. The ATPase activity of the F_1F_0 -ATP synthase complex was visualized by histochemical staining on the basis of the precipitation of lead nitrate by the inorganic phosphate produced during ATP hydrolysis (Yoshida *et al.*, 1975). The in-gel ATPase activity of the isolated complex was reproducibly and significantly lower in mitochondria from the sterile compared with those from the fertile line, but was higher in the hybrid line (Fig. 3A, B). To investigate whether this pattern of activity was unique to the ATP synthase complex, the enzyme activities associated with complex I (NADH dehydrogenase), complex II (succinate dehydrogenase) and complex IV (cytochrome c oxidase) were visualized in parallel gels (Fig. 3A). Although slight differences were observed, they were not statistically significant (Fig. 3B), nor did they correlate with the consistently reproducible lower level of ATPase activity observed in the sterile line.

To investigate whether the decrease in ATPase activity was linked to a decreased protein level on the gel in the sterile line, the bands containing the F_1F_0 -ATP synthase complex in the gel stained with Coomassie blue were scanned and quantified using Multi-Analyst software (Biorad) (Fig. 3C). The variations in the intensities of total areas of the bands in all three lines were not significantly different, except for complex V. The apparent slight decrease in band intensity in complex I of the sterile line is not correlated with any significant change in the activity of the complex (Fig. 3B).

DISCUSSION

The existence of *orfB* in plant mitochondrial genomes was first reported in *Oenothera* by Hiesel *et al.* (1987), identified in sunflower by Quagliariello *et al.* (1990) and subsequently shown to be conserved in the mitochondrial genome of diverse plant species. Gualberto *et al.* (1991) reported that the ORFB protein is expressed extensively in wheat, suggesting that it has biological significance. However, it

was not until the genome sequences of a range of protists became available that it was suggested, on the basis of structural and sequence motif similarities, that ORFB is the homologue of yeast and mammalian ATP8, a subunit of the F_0 component of F_1F_0 -ATP synthase (Gray *et al.*, 1998). However, the overall sequence similarity is weak, and the predicted molecular weight of ORFB (18.240 kDa) is much higher than that predicted for yeast ATP8 (5.822 kDa) and the human homologue (7.992 kDa). Our demonstration that ORFB is a subunit of the F_1F_0 -ATP synthase complex (Fig. 1), together with our demonstration that editing of the *orfB* transcript increases the similarity of the N-terminal 19 amino acids to ATP8 from non-plant species, provides further support for the above suggestion (Fig. 2).

Numerous studies have shown that cytoplasmic male sterility in plants is associated with aberrant recombination events in the mitochondrial genome, resulting in the generation of chimeric ORFs that are expressed as novel polypeptides (Schnable & Wise, 1998). The sequences that contribute to the generation of these chimeric ORFs are usually derived from coding and non-coding regions of existing genes, but are occasionally from unknown origins. ORF522, the chimeric protein responsible for the CMS phenotype in sunflower, shares similarities with ORFB in its N-terminal amino-acid sequence, polypeptide domain structure and molecular weight (Fig. 2). The sequence identity of the N-terminal 19 amino acids of ORFB and ORF522, coupled to a novel carboxy-terminal domain, could result in competition between the two proteins, leading to impaired biogenesis and uncoupling or decreased phosphorylation activity of the F_1F_0 -ATP synthase complex (Balk & Leaver, 2001). In support of this, we have shown, by comparing the in-gel enzyme activities associated with several mitochondrial respiratory complexes between fertile and sterile lines, that the ATPase activity of the F_1F_0 -ATP synthase is specifically and significantly reduced in the sterile line as compared with the fertile line. The reduction in ATPase activity in the male-sterile line correlates with the expression of ORF522, and is restored in the presence of nuclear *restorer of fertility* genes in the fertile hybrid line (Fig. 3).

Fig. 3 | (A) In-gel enzyme activities of mitochondrial respiratory complexes. Solubilized mitochondrial membranes (200 µg) from seedlings of the fertile (F), sterile (S) and hybrid (H) sunflower lines were fractionated by blue-native polyacrylamide gel electrophoresis. Replicate gels were stained with Coomassie blue to visualize the protein complexes, or incubated with appropriate reagents to visualize the activities of NADH dehydrogenases (complex I and alternative dehydrogenases: DHase), succinate dehydrogenase, cytochrome (cyt) *c* oxidase and ATPase. Enzyme activity is visualized as purple (NADH and succinate dehydrogenases) or green–brown (cytochrome *c* oxidase) precipitates against a background of partially destained Coomassie-blue-stained protein complexes. The lead-phosphate precipitate, indicating ATPase activity, was imaged using a standard flatbed scanner with a black background. (B) Quantification of histochemical reactions associated with mitochondrial respiratory complex activities. Gels were scanned using a Fluor-S MultiImager (Biorad). Band intensities were estimated as the volume of optical density (OD) per millimetre squared of band area using Multi-Analyst software (Biorad). Each bar represents data from three independent experiments for complex I, complex II and complex IV, and at least five for complex V. (C) Quantification of protein content of respiratory complexes I, III and V after Coomassie blue staining. Band-area intensity was estimated by scanning as in (B).

Segments of *orfB* and *atp9* have been shown previously to be involved in the generation of the chimeric proteins associated with CMS in a variety of plant species (Schnable & Wise, 1998). Some of these *orfB*-related CMS genes include either part of the sequence of the *orfB* gene, for example, the CMS-associated *orf224* in *Brassica*, *orf222* and *orf138* in radish (Schnable & Wise, 1998), *orf324* in sugar beet (Kubo & Mikami 1996), or the entire sequence of the gene, as in carrot (Nakajima *et al.*, 2001). The derivation of these chimeric sequences from progenitor genes that encode subunits of the F_0 portion of the ATP synthase, suggests that the molecular mechanisms underlying all of these CMS phenotypes may be similar to that for PET1–CMS in sunflower, which is proposed in this work.

It is interesting to speculate as to why mutations in the mitochondrial genome in plants only seem to affect cellular development during male flower development (male gamete production), whereas vegetative development and female flower development are unaffected. We have demonstrated previously that high levels of mitochondrial gene expression, associated with a marked increase in mitochondrial biogenesis (Smart *et al.*, 1994), occur in the meiocyte cells, which give rise to four independent haploid microspores (pollen grains) during anther development in sunflower. The data presented here support the hypothesis that the increased demand for respiratory function and cellular energy in the form of ATP generated by oxidative phosphorylation during anther development may be compromised by the expression of ORF522. The inability to meet the increased energy demand required to sustain anther development may therefore not be met in the sterile line, leading to pollen abortion. Our data also support an alternative hypothesis that we proposed recently, which suggests that impairment of the capacity for mitochondrial oxidative phosphorylation in sterile sunflower lines leads to premature programmed cell death during anther development (Balk & Leaver, 2001). It is of interest to note that the F_1F_0 -ATP synthase is thought to have a role in apoptosis in mammals, and possibly yeast, although the precise mechanism remains to be determined (reviewed in Matsuyama & Reed, 2000).

Unexpectedly, we observed that the ATPase activity in hybrid sunflower seedlings was increased as compared with that of fertile and sterile seedlings. It is tempting to speculate that an increased activity or efficiency of the F_1F_0 -ATP synthase in the F_1 hybrid, by an as yet unknown mechanism, could contribute to the phenomenon known as 'hybrid vigour', one of the important goals of plant breeding.

METHODS

Plant material. Sunflower (*Helianthus annuus* L.) lines and growth conditions were as described by Smart *et al.* (1994).

Analysis of editing sites. Genomic DNA and *orfB* cDNAs were amplified, cloned and sequenced using standard molecular biological techniques. Sequences of the primers used in this analysis are available on request to the authors.

Isolation of mitochondria. Mitochondria from sunflower florets and four-day-old etiolated seedlings were isolated by differential centrifugation and gradient purification as described previously (Leaver *et al.*, 1983).

Blue-native (first dimension) and tricine-SDS (second dimension) polyacrylamide gel electrophoresis. Sample preparation, electrophoresis conditions, gel fixation and Coomassie blue staining were adapted from Jansch *et al.* (1996) and Schägger & von Jagow (1991). A final ratio of 1 g of detergent (N-dodecyl β -D-maltoside) to

1 g of protein gave the best solubilization of respiratory complexes from sunflower mitochondria. Western blot analysis was performed as described in Balk & Leaver (2001).

MALDI-TOF and mass spectrometry for peptide fingerprint analysis. The in-gel digestion and mass spectrometry was performed as a service by GARNET (<http://www.york.ac.uk/res/garnet/garnet.htm>). The results from mass spectrometry data were analysed using the MASCOT search program, available at the website <http://www.matrixscience.com/>.

In-gel activity assays of respiratory complexes. After electrophoresis, gels were rinsed briefly with MilliQ water, equilibrated in reaction buffer without reagents for 10 min and incubated in fresh buffer with reagents (Zerbetto *et al.*, 1997; Jung *et al.*, 2000). Gels were then fixed and destained in 45% (v/v) methanol and 10% (v/v) acetic acid.

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