

# A mitochondrial rRNA dimethyladenosine methyltransferase in Arabidopsis

Uwe Richter<sup>1,†</sup>, Kristina Kühn<sup>1,2,†</sup>, Sachiko Okada<sup>3</sup>, Axel Brennicke<sup>3</sup>, Andreas Weihe<sup>1</sup> and Thomas Börner<sup>1,\*</sup>

<sup>1</sup>Institut für Biologie/Genetik, Humboldt-Universität, Chausseestr. 117, 10115 Berlin, Germany,

<sup>2</sup>Australian Research Council Centre of Excellence in Plant Energy Biology, University of Western Australia, Crawley 6009, WA, Australia, and

<sup>3</sup>Molekulare Botanik, Universität Ulm, 89069 Ulm, Germany

Received 4 September 2009; revised 19 October 2009; accepted 3 November 2009; published online 17 December 2009.

\*For correspondence (fax +49 302 093 8141; e-mail thomas.boerner@rz.hu-berlin.de).

†These authors contributed equally to this work.

## SUMMARY

**S-adenosyl-L-methionine-dependent rRNA dimethylases mediate the methylation of two conserved adenosines near the 3' end of the rRNA in the small ribosomal subunits of bacteria, archaea and eukaryotes. Proteins related to this family of dimethylases play an essential role as transcription factors (mtTFBs) in fungal and animal mitochondria. Human mitochondrial rRNA is methylated and human mitochondria contain two related mtTFBs, one proposed to act as rRNA dimethylase, the other as transcription factor. The nuclear genome of *Arabidopsis thaliana* encodes three dimethylase/mtTFB-like proteins, one of which, Dim1B, is shown here to be imported into mitochondria. Transcription initiation by mitochondrial RNA polymerases appears not to be stimulated by Dim1B *in vitro*. In line with this finding, phylogenetic analyses revealed Dim1B to be more closely related to a group of eukaryotic non-mitochondrial rRNA dimethylases (Dim1s) than to fungal and animal mtTFBs. We found that Dim1B was capable of substituting the *E. coli* rRNA dimethylase activity of KsgA. Moreover, we observed methylation of the conserved adenines in the 18S rRNA of Arabidopsis mitochondria; this modification was not detectable in a mutant lacking Dim1B. These data provide evidence: (i) for rRNA methylation in Arabidopsis mitochondria; and (ii) that Dim1B is the enzyme catalyzing this process.**

**Keywords:** rRNA dimethyltransferases, mitochondria, Arabidopsis, mitochondrial transcription, molecular phylogeny.

## INTRODUCTION

Mitochondria and plastids are descendants of bacterial endosymbionts (Margulis, 1970, 1981) and both possess their own vestigial genomes. Analyses of the mitochondrial DNA (mtDNA) trace the evolutionary predecessor of mitochondria to a single ancestor whose closest contemporary relatives are found within the  $\alpha$  division of the proteobacteria (Yang *et al.*, 1985). The origin of chloroplasts traces back to a primary endosymbiotic event between a eukaryotic host and a relative of extant cyanobacteria representing the root of the plant kingdom (Rodríguez-Ezpeleta *et al.*, 2005). The majority of the original set of mitochondrial and plastid genes was either relocated to the nuclear genome or lost from the cell relatively early in the process of both endo-

symbiotic events (Gray *et al.*, 1999). As a consequence of the unidirectional functional gene transfer, components participating in the diverse mitochondrial and plastid metabolic pathways and genetic processes are largely encoded in the nucleus and, following synthesis in the cytosol, are imported into the organelles (Peeters and Small, 2001; Herrmann, 2003).

Surprisingly, several mitochondrial DNA replication genes were acquired probably from a T-odd phage early in the evolution of the eukaryotic cell, at the time of the mitochondrial endosymbiosis (Shutt and Gray, 2006a). Similarly, in mitochondria of the budding yeast *Saccharomyces cerevisiae* (Greenleaf *et al.*, 1986; Masters *et al.*, 1987), mammals (Tiranti *et al.*, 1997; Gaspari *et al.*, 2004), plants (Weihe *et al.*, 1997) and other eukaryotes (Cermakian *et al.*, 1996), a nucleus-encoded T-odd phage-type RNA polymerase (RNAP) has replaced the ancestral bacterial-type RNAP.

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Unlike the single-subunit RNA polymerases of bacteriophages, mitochondrial phage-type RNA polymerases require auxiliary factors to initiate transcription at promoter sequences. To date, two types of nucleus-encoded mitochondrial transcription factors, designated here as mtTFA and mtTFB, have been characterized in *S. cerevisiae* (Winkley *et al.*, 1985; Schinkel *et al.*, 1987a; Matsunaga and Jaehning, 2004), *X. laevis* (Bogenhagen and Insdorf, 1988; Bogenhagen, 1996), *Drosophila melanogaster* (Matsushima *et al.*, 2004, 2005), humans (Fisher and Clayton, 1988; Falkenberg *et al.*, 2002; McCulloch *et al.*, 2002) and mouse (Parisi and Clayton, 1991; Gaspari *et al.*, 2004). The HMG-box protein mtTFA is an essential transcription factor in human (Gaspari *et al.*, 2004), but not in yeast mitochondria where it is an abundant DNA-binding protein that enhances transcriptional activity (Parisi *et al.*, 1993; Kanki *et al.*, 2004).

In contrast, mtTFBs belonging to the family of *S*-adenosyl-L-methionine (SAM)-dependent rRNA adenine dimethyltransferases were found to be essential for transcription in yeast and mammalian mitochondria (Jang and Jaehning, 1991; Asin-Cayuela and Gustafsson, 2007). Homologues of this rRNA adenine dimethylase protein family are found to function in all domains of life. SAM-dependent rRNA dimethylases, such as KsgA from *E. coli* or the nucleolar/cytoplasmic Dim1 of *S. cerevisiae*, usually mediate the name-giving methylation activity and modify two specific adenosines in a highly conserved stem-loop near the 3' end of small subunit ribosomal RNAs. While the significance of this methylation activity is poorly understood, it seems that KsgA/Dim1 orthologues may have the general potential to play diverse additional roles within different compartments of the cell (Lafontaine *et al.*, 1995, 1998; Tokuhisa *et al.*, 1998; Metodiev *et al.*, 2009). For the yeast protein ScDim1 it was shown that the enzymatic cytoplasmic function of Dim1 in dimethylation can be separated from an involvement in pre-rRNA processing (Lafontaine *et al.*, 1998).

Mitochondria of all metazoan species investigated thus far possess two mtTFB proteins. In contrast, mitochondria of *S. cerevisiae* and other fungal species possess only one mtTFB. Yeast sc-mtTFB lacks rRNA dimethyltransferase activity correlated with the lack of the corresponding modification in the mitochondrial rRNA of budding yeast (Cotney and Shadel, 2006). Recent observations led to the conclusion that despite the similarity of both proteins to rRNA dimethyltransferases and their ability to act as transcription factors *in vitro*, the methyltransferase and cofactor roles are distributed *in vivo* between TFB1M (methyltransferase) and TFB2M (transcription factor) in animal mitochondria (Falkenberg *et al.*, 2002; McCulloch and Shadel, 2003; Matsushima *et al.*, 2004, 2005; Cotney *et al.*, 2007; Metodiev *et al.*, 2009).

In dicotyledonous plants such as *Arabidopsis thaliana*, three phage-type RNAPs are involved in the transcription of organellar genes: a mitochondrial RNAP (RpoTm), a plastid

RNAP (RpoTp) and an RNAP dual-targeted to both mitochondria and plastids (RpoTmp; Hedtke *et al.*, 1997, 2000). Previous studies suggested that factors supporting mitochondrial RNAPs in promoter recognition and/or transcription initiation should exist also in higher plants (Newton *et al.*, 1995; Young and Lonsdale, 1997; Binder and Brennicke, 2003). More recently we found that *Arabidopsis* RpoTm and RpoTp are able to correctly start transcription *in vitro* from several mitochondrial and at least one plastid promoter without the assistance of further protein factors. Selective promoter recognition by RpoTm and RpoTp and the apparent inability of RpoTmp to correctly initiate transcription at mitochondrial and plastidial NEP promoters imply, however, that auxiliary factors are required for efficient initiation of transcription *in vivo* (Kühn *et al.*, 2007).

Only limited information is available about mitochondrial rRNA methylation in the green branch of life (Schnare and Gray, 1982) and so far mtTFA- and mtTFB-related transcription factor proteins have not been found in plants. We therefore initiated this study to ascertain if the *Arabidopsis* mitochondrial rRNA shows the conserved type of adenine dimethylation and if plant mitochondria have one or more dimethylases belonging to the KsgA/Dim1 family. Moreover, we were interested in the potential function of such a methyltransferase as a mitochondrial transcription factor.

We report here on KsgA/Dim1-like *Arabidopsis* proteins initially identified by *in-silico* screening of databases. One of these proteins is the Pfc1 previously demonstrated to function as a plastidial 16S rRNA dimethylase (Tokuhisa *et al.*, 1998). The two others are shown here to be localized to the nucleus and to mitochondria, respectively. We found no evidence for an mtTFB-like role of the mitochondrial protein, designated as Dim1B (adenosine dimethyl transferase 1B), in mitochondrial transcription. Our data demonstrate, however, that Dim1B is an rRNA methyltransferase and is required for the dimethylation of two conserved adenines in the mitochondrial 18S rRNA of *Arabidopsis*.

## RESULTS

### mtTFB/rRNA dimethyltransferase-like proteins in *Arabidopsis*

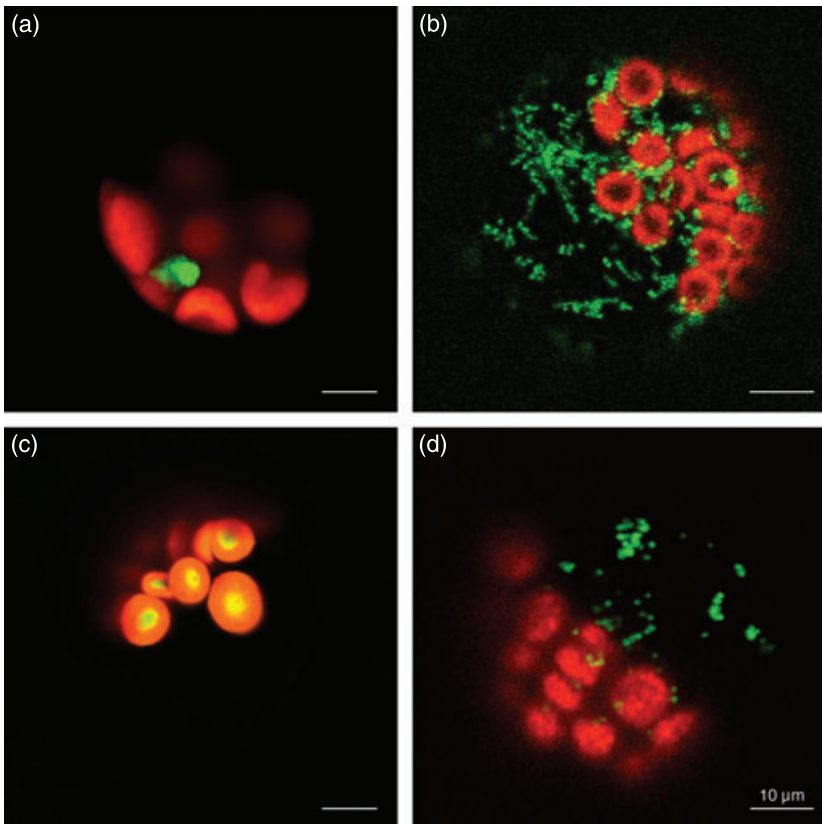
To screen the *Arabidopsis* genome for sequences encoding mtTFB-like proteins we queried the genome and EST databases with the putative *Schizosaccharomyces pombe* mtTFB amino acid sequence (McCulloch *et al.*, 2002) and the human mtTFB1 and mtTFB2 sequences (Falkenberg *et al.*, 2002) using the BLASTP and TBLASTN algorithms available at the National Centre for Biotechnology Information (NCBI). The search predicted three mtTFB/rRNA dimethyltransferase-like proteins to be encoded by the loci At5g66360, At2g47420 and At1g01860 in *Arabidopsis*. At1g01860 corresponds to the previously characterized *Pfc1* gene coding for a plastidial 16S rRNA dimethylase (Tokuhisa *et al.*, 1998).

The putative rRNA dimethyltransferase genes at loci At2g47420 and At5g66360 were tentatively designated *Dim1A* and *Dim1B*, respectively. *Dim1A* is predicted to encode a 353-amino acid polypeptide. Available EST data support alternative splicing of the hypothetical mRNA deriving from *Dim1B*, which would give rise to two different polypeptides of 352 and 380 amino acids (Accession numbers NP\_201437 and NP\_975003). However, PCR amplification of the *Dim1B* coding sequence from cDNA yielded only the splice product coding for the longer of the two predicted products. For *Dim1A*, a cDNA sequence of the expected length was amplified (data not shown). Protein sequence comparisons revealed that the annotated optional *Dim1B* intron codes for amino acid sequence motifs that are conserved among rRNA dimethylase-like proteins (amino acids 214–241 of the derived *Dim1B* polypeptide). Hence, further sequence analyses were based on the longer deduced *Dim1B* polypeptide. Both *Dim1A* and *Dim1B* display ~27% and ~30% amino acid sequence similarity to mtTFB from *S. pombe* and *S. cerevisiae*, respectively; ~12% and ~15% of the predicted amino acids are identical when sequences exclusive of the predicted transit peptides are compared. These levels of similarity approximately correspond to those between human and fungal mtTFB sequences. Similarities of *Dim1A* and *Dim1B* with each human TFB1M and TFB2M are ~37%; amino acids are for

both *Dim1A* and *Dim1B* ~20% identical with the human sequences.

#### Subcellular localization of the mtTFB-like proteins *Dim1A* and *Dim1B*

Several computer algorithms consistently predict *Dim1B* to possess an N-terminal transit peptide mediating the import of the protein into mitochondria while *Dim1A* might be a nuclear protein (Table S1). To experimentally investigate the subcellular localization of *Dim1A* and *Dim1B*, nucleotide sequences encoding the full length and 64 N-terminal amino acids of *Dim1A* and *Dim1B*, respectively, were fused in-frame to the green fluorescent protein (GFP) coding sequence. Arabidopsis protoplasts were transformed with the *Dim1A*- and *Dim1B*-GFP fusion constructs and transient expression of the fusion proteins was monitored using confocal laser fluorescence microscopy. Two plasmids encoding a mitochondrial Cox4-GFP or a plastidial RecA-GFP fusion protein were used for reference transformations of Arabidopsis protoplasts. Protoplasts expressing *Dim1B*-GFP displayed green fluorescence of small structures resembling the fluorescent mitochondria of protoplasts synthesizing Cox4-GFP (Figure 1), substantiating a mitochondrial localization of *Dim1B*. *Dim1A*-GFP fluorescence, on the other hand, was found to localize to the nucleus and for the most part enveloped a small globular structure



**Figure 1.** Transient expression of GFP fusion proteins in *Arabidopsis thaliana* protoplasts. The *Dim1A* and *Dim1B* gene fragments encoding putative transit peptides were inserted into plasmid pOL-GFPS65C to generate vectors driving the expression of *Dim1A*-GFP (a) and *Dim1B*-GFP (b) showing nuclear (a) and mitochondrial (b) GFP localisation. Control constructs code for plastidial RecA-GFP (c) and mitochondrial Cox-IV-GFP (d), respectively. Images were taken by confocal fluorescence microscopy.

therein, which likely corresponds to the nucleolus. These data indicate that Dim1A does not possess a mitochondrial or plastidial transit peptide.

### Phylogenetic analysis of plant, fungal and animal rRNA dimethylase-like proteins

In order to assess the phylogenetic relationships of Arabidopsis Dim1A, Dim1B and of the plastidial methyltransferase Pfc1 to established mitochondrial transcription factors such as yeast and animal mtTFBs and to other rRNA dimethylases such as *E. coli* KsgA, the Dim1A, Dim1B and Pfc1 sequences were compared with available mtTFB sequences and to a set of sequences of characterized and predicted rRNA dimethylases. Blast analyses with *Arabidopsis thaliana* Dim1A and Dim1B of all plant genomes available at the Joint Genome Initiative database (<http://genome.jgi-psf.org/>) including *Cyanidioschyzon merolae*, *Chlorella vulgaris*, *Ostreococcus tauri*, *Ostreococcus lucimarinus*, *Volvox carteri*, *Chlamydomonas reinhardtii*, *Physcomitrella patens* and *Selaginella moellendorffii* combined with searches against the NCBI Plant EST database revealed that Dim1B-like proteins might be restricted to flowering plants. For identified plant Dim proteins, subcellular targeting was predicted employing the *TargetP*, *Predotar*, *iPSort* and *MultiLoc* algorithms (Table 1). All sequences with high similarity to Arabidopsis Dim1B,

which are therefore designated Dim1B in the phylogenetic tree, were predicted to contain putative mitochondrial transit peptides. Proteins most similar to Arabidopsis Dim1A were predicted to be neither plastidial nor mitochondrial (Table 1) and are referred to as Dim1A. Notably, the *Physcomitrella* genome was found to lack a Dim1B homologue but encodes two potential dimethyltransferases which best align with plant Dim1A proteins, PpDim1A-1 being predicted to be neither mitochondrial nor plastidial and PpDim1A-2 containing a putative mitochondrial transit peptide (Table 1). Amino acid sequences were compared using the Multalin algorithm (Corpet, 1988) and the alignment was refined according to a structure-based alignment generated for fungal mtTFBs and the *Bacillus subtilis* rRNA dimethylase ErmC' (Schubot *et al.*, 2001; see Figure S1). Based on the selected sequence blocks indicated in the alignment, a Bayesian phylogenetic tree was derived (Figures 2 and S2). Essentially the same tree topology was obtained employing neighbour joining (NJ) or maximum likelihood (ML) analysis.

Arabidopsis Dim1A and its plant orthologues appear to be most closely related to a group of rRNA dimethylases containing the yeast nucleolar 18S rRNA dimethylase Dim1. Therefore, they most probably represent nuclear/cytoplasmic enzymes, a suggestion which would be consistent with computational predictions of the subcellular localization of these proteins and with our GFP import experiments conducted with Arabidopsis Dim1A. A sister group to plant, fungal and animal nuclear Dim1-like dimethylases is formed by the predicted mitochondrial rRNA dimethylases of plants including Arabidopsis Dim1B. Remarkably, amino acid sequence similarities of Arabidopsis Dim1A and Dim1B to yeast Dim1 are 68% and 54%, respectively and considerably exceed similarities to yeast or animal mtTFBs. This is contrasted by similarities of yeast mtTFB, human TFB1M and TFB2M to yeast Dim1 with only 36%, 35% and 28%. The phylogram shows that the presumed plant mitochondrial rRNA dimethylases clustering with either Dim1A or Dim1B are decidedly more closely related to nuclear/cytoplasmic enzymes of this type – notably with the archaeal Dim1-like proteins at the root of this clade – than to fungal and animal mtTFBs.

On the other hand, the mtTFB sequences form a common branch with alpha-proteobacterial dimethyltransferases as previously described (Shutt and Gray, 2006b). Arabidopsis Pfc1 and its plant orthologues compose a distinct group apart from the Dim1A/Dim1B cluster and from three other well separated groups formed by animal TFB1Ms, animal TFB2Ms and the highly diverse fungal mtTFBs. In agreement with a recent study (Park *et al.*, 2009), the Pfc1-like sequences, which likely code for plastidial dimethyltransferases, unexpectedly form a common clade with chlamydial sequences but not with cyanobacterial or mitochondrial dimethyltransferases (Figures 2 and S2).

**Table 1** Prediction of subcellular localisations for Dim1A, Dim1B and Pfc-like proteins

	Predotar <sup>a</sup>	iPSORT <sup>b</sup>	TargetP <sup>c</sup>	MultiLoc <sup>d</sup>
CrPfc	Other	mt	mt/pt	pt
VcPfc	mt	mt	mt/pt	pt
OtPfc	mt/pt	mt	mt/pt	pt
PpPfc	pt	pt	pt	pt
SmPfc	mt	pt	pt	pt
PgPfc	pt	pt	pt	pt
Magnoliophyta Pfc	pt	pt	pt	pt
Magnoliophyta Dim1A	NOR (mt,pt)	NOR (mt,pt)	NOR (mt,pt)	nc or cyt
Magnoliophyta Dim1B	mt	mt	mt	mt
PpDim1A-2	mt	mt	mt	mt

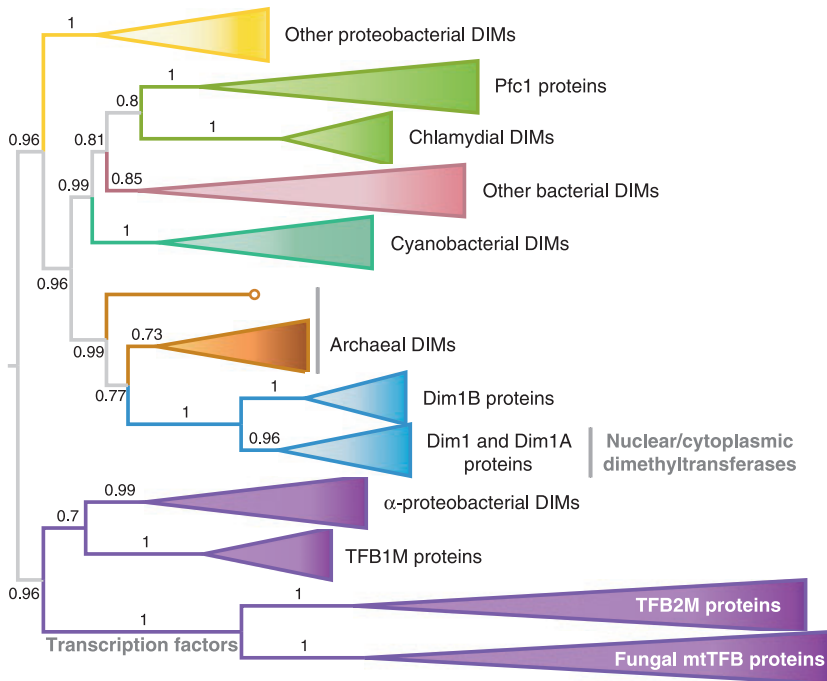
Predictions of targeting to the mitochondrion (mt), nucleus (nc), cytoplasm (cyt) or plastid (pt) by the respective computer algorithms are documented. Proteins are designated as follows: CrPfc, *Chlamydomonas reinhardtii* Pfc; VcPfc, *Volvox carteri* Pfc; OtPfc, *Ostreococcus tauri* Pfc; PpPfc, *Physcomitrella patens* Pfc; SmPfc, *Selaginella moellendorffii* Pfc; PgPfc, *Pinus glauca* Pfc; PpDim1A-2, *Physcomitrella patens* Dim1A-2. Magnoliophyta Dim1A and Dim1B include predictions for proteins from *Arabidopsis thaliana*, *Vitis vinifera*, *Medicago truncatula*, *Lycopersicon esculentum*, *Populus trichocarpa*, *Zea mays* and *Oryza sativa*.

<sup>a</sup><http://urgi.versailles.inra.fr/predotar/predotar.html>.

<sup>b</sup><http://hc.ims.u-tokyo.ac.jp/iPSORT/>.

<sup>c</sup><http://www.cbs.dtu.dk/services/TargetP/>.

<sup>d</sup><http://www-bs.informatik.uni-tuebingen.de/Services/MultiLoc>.



**Figure 2.** Phylogeny of mitochondrial transcription factors and small-subunit rRNA dimethylases. Bayesian phylogenetic analysis from conserved amino acid sequence sections indicated in the amino acid sequence alignment (see Figure S1). Collapsed branches of orthologous and paralogous proteins from the detailed phylogenetic tree (see Figure S2) are displayed as triangles. Total branch length to the closest and the farthest leaf are used to calculate the length of the triangles.

Given the presence of apparently only one mtTFB/rRNA dimethylase-like protein in plant mitochondria, our further experiments were designed to answer the question of whether *Arabidopsis* Dim1B could play a dual role as a mitochondrial rRNA dimethyltransferase and as a mitochondrial transcription factor.

#### Promoter-specific transcription initiation is not stimulated by Dim1B

Recombinant *Arabidopsis* Dim1B was prepared in order to assay the protein *in vitro* for a possible function as cofactor in mitochondrial transcription. Dim1B fused to an N-terminal hexahistidine tag was expressed in *E. coli* and soluble recombinant protein was enriched from bacterial extracts; Dim1A was similarly prepared so as to have a non-mitochondrial rRNA dimethylase-like protein available for control experiments (Figure S3). In order to confirm the correct folding and native conformation of the recombinant proteins, electrophoretic mobility shift assays of Dim1A and Dim1B were carried out with mitochondrial DNA probes following the protocol described for testing DNA binding by human TFB1M (McCulloch *et al.*, 2002). Both *Arabidopsis* proteins were found to bind to DNA without showing sequence specificity (Figure S3).

It has been demonstrated that the yeast mitochondrial phage-type RNA polymerase RPO41, which on its own transcribes double-stranded linear DNA only non-specifically, recognizes mitochondrial promoters on a linear DNA template when complemented with sc-mtTFB in *in vitro* transcription experiments (Schinkel *et al.*, 1987b). We there-

fore used our previously established *in vitro* transcription system (Kühn *et al.*, 2007) to test if recombinant Dim1B enabled the *Arabidopsis* mitochondrial RNA polymerases RPOTm or RPOTmp to specifically transcribe from mitochondrial promoters located on linear DNA templates. We also examined if, in the presence of Dim1B, the two RNA polymerases recognized promoters on both linear and supercoiled DNA that in our earlier study (Kühn *et al.*, 2007) were not specifically utilized by any of the two enzymes.

Recombinant RPOTm and RPOTmp were assayed for transcription initiation at several mitochondrial promoters (promoter sequences are listed in Table S2) in the presence or absence of equimolar amounts of Dim1B; in order to discern non-specific effects of Dim1B addition, control reactions were set up with Dim1A (Figure S4). Figure S4 shows the PAGE analysis of transcripts synthesized *in vitro* from linear or supercoiled DNA templates containing the mitochondrial promoters *Patp6-1-156* and *Patp6-1-200*, *Patp8-157* and *Patp8-228/226*, or *Prrn18-69* and *Prrn18-156*. RPOTm did not specifically transcribe from any mitochondrial promoter on a linear DNA template in the presence or absence of Dim1B; neither did the enzyme show increased mitochondrial promoter specificity due to Dim1B addition when supercoiled templates were provided. None of the promoters not utilized by RPOTm in the absence of Dim1B, like *Patp6-1-156* or *Prrn18-69*, was recognized when Dim1B was added. The transcriptional activity and initiation specificity of RPOTmp were similarly unaltered by the addition of Dim1B; no significant promoter specificity of this enzyme

was seen in the presence or absence of Dim1B with any mitochondrial promoter tested (only experiments with supercoiled templates are shown for RPOTmp). In addition to the three DNA templates analysed as shown in Figure S4, several other DNA templates with different promoters were tested. Promoter recognition assays are summarized in Table 2. Altogether, the addition of Dim1B appears to have no effect on RPOTm- or RPOTmp-driven transcription.

### Dim1B is an rRNA dimethyltransferase

To determine if the Dim1B protein is a functional rRNA dimethyltransferase, the complementation of an *E. coli* mutant lacking the N6-adenine dimethyltransferase encoded by the *KsgA* gene (*KsgA*<sup>-</sup> *E. coli*) was investigated. The bacterial strain was assayed for complementation by the introduced *Dim1B* gene from Arabidopsis. This strategy essentially follows the complementation assays as described by Seidel-Rogol *et al.* (2003) for complementing *KsgA*-deficient *E. coli* with human TFB1M. N6-adenine methyltransferase activity in *KsgA*<sup>-</sup> *E. coli* was recovered when the Arabidopsis *Dim1B* gene was introduced (Figure 3), indicating that Dim1B indeed is a functional and site-specific rRNA dimethylase. In both expression systems used, with the *Dim1B* gene activated either from the LacZ promoter (JM101) or from the T7 promoter (BL21(DE3)), N6-adenine methyltransferase activity was observed. In the LacZ system, the methylating enzyme activity seems to be weaker than in the T7 system, which may be caused by the weaker promoter and the resulting lower level of protein

expression. In the T7 system, an extra primer extension product terminating at G<sub>1517</sub> is observed. In the bacterial small ribosomal rRNAs such an m<sup>2</sup>G modification at position G<sub>1516</sub> has been reported. However, the correspondence of this extension product with this modification needs to be confirmed by further analysis.

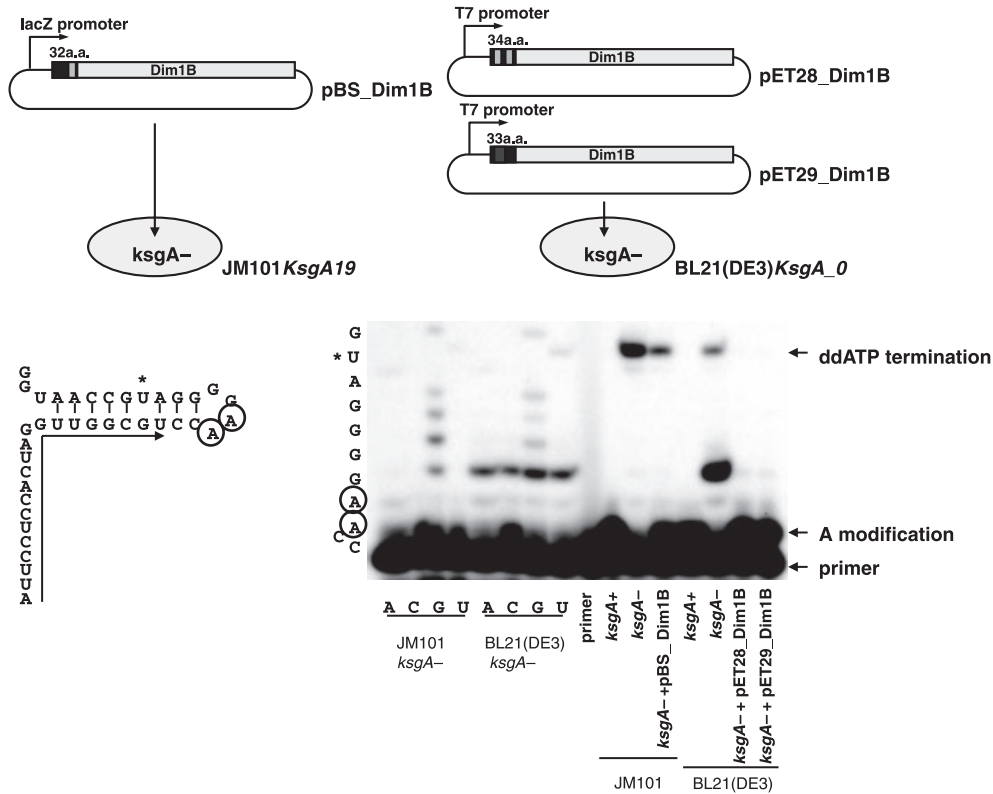
These complementation assays show that the Dim1B protein encoded by the *Dim1B* gene is an active N6-adenine methyltransferase which modifies the AA dinucleotide at the bacterial 16S rRNA nucleotides A1518 and A1519, the positions equivalent to the plant mitochondrial 18S rRNA nucleotides A1914 and A1915. To analyse the functional importance of this modification, the requirement of the *Dim1B* gene for plant growth was investigated.

### A *dim1B* mutant lacks adenosine modification of the mitochondrial 18S rRNA

A mutant line of Arabidopsis (GABI-Kat 071F10) that contains a T-DNA fragment inserted in the second exon of the *Dim1B* gene (see Experimental Procedures) and designated as *dim1B* was employed to analyse the role of Dim1B in mitochondrial RNA metabolism. The mutation had no apparent effect on plant growth or development under standard growth conditions (data not shown). As reverse transcriptase is unable to extend a DNA oligonucleotide primer if it encounters an adenosine dimethylated at the N-6 position of the base (Hagenbüchle *et al.*, 1978; Sigmund *et al.*, 1988), we used primer extension to distinguish between the modification states of wild-type and *dim1B*

**Table 2** Promoter recognition by RPOTm *in vitro*

<i>In vitro</i> transcripti on template		Promoter recognition RPOTm				Promoter recognition RPOTmp			
		ccc template		Linear template		ccc template		Linear template	
Plasmid	Promoters	-Dim1B	+Dim1B	-Dim1B	+Dim1B	-Dim1B	+Dim1B	-Dim1B	+Dim1B
pKL23- <i>atp6-1</i>	<i>Patp6-1-156</i>	-	-	-	-	-	-	-	-
	<i>Patp6-1-200</i>	+	+	-	-	-	-	-	-
pKL23- <i>atp6-1-B</i>	<i>Patp6-1-916/913</i>	+	+	na	na	-	-	na	na
pKL23- <i>atp6-2</i>	<i>Patp6-2-436</i>	+	+	na	na	-	-	na	na
	<i>Patp6-2-507</i>	+	+	na	na	-	-	na	na
pKL23- <i>atp8</i>	<i>Patp8-157</i>	-	-	-	-	-	-	na	na
	<i>Patp8-228/226</i>	+	+	-	-	-	-	na	na
pKL23- <i>atp9-B</i>	<i>Patp9-239</i>	-	-	-	-	-	-	na	na
	<i>Patp9-295</i>	+	+	-	-	-	-	na	na
pKL23- <i>atp9</i>	<i>Patp9-487</i>	+	+	na	na	-	-	na	na
	<i>Patp9-652</i>	+	+	na	na	-	-	na	na
pKL23- <i>cox2</i>	<i>Pcox2-210</i>	+	+	-	-	-	-	na	na
	<i>Pcox2-481</i>	+	+	-	-	-	-	na	na
pKL23- <i>rps3</i>	<i>Prps3-1053</i>	-	-	-	-	-	-	-	-
	<i>Prps3-1133</i>	-	-	-	-	-	-	-	-
pKL23- <i>rrn18</i>	<i>Prrn18-69</i>	-	-	-	-	-	-	na	na
	<i>Prrn18-156</i>	+	+	-	-	-	-	na	na
pKL23- <i>rrn26</i>	<i>Prrn26-893</i>	+	+	-	-	-	-	na	na
pKL23- <i>trnM</i>	<i>PtrnM-98</i>	+	+	-	-	-	-	na	na
pKL23- <i>trnM-B</i>	<i>PtrnM-574/573</i>	-	-	na	na	-	-	na	na



**Figure 3.** The *Dim1B* gene from *Arabidopsis thaliana* can complement site specific methylation defects in *E. coli*. The coding sequence of the *Dim1B* gene from *Arabidopsis thaliana* was cloned into vectors driving transcription in *E. coli* from either a lacZ or a T7 promoter as indicated in the scheme and introduced into the *E. coli* strains JM101 or BL21(DE3) lacking the *ksgA* gene. The specific adenosine dimethylation in the 3' terminal stem-loop structure of the bacterial 16S rRNA is depicted on the left. Primer extension in the presence of ddATP confirms the absence of this methylation in the *ksgA*- mutant bacteria and yields no signal at the adenosine doublet but terminates primer extension at the next uridine in the 16S rRNA sequence. Introduction of a functional *ksgA* gene or either of the expression constructs of the *Dim1B* gene from *Arabidopsis* restores the methylation signal at the adenosine dinucleotide. The left part of the gel image shows the corresponding RNA sequence reactions for orientation of the ddATP termination signals.

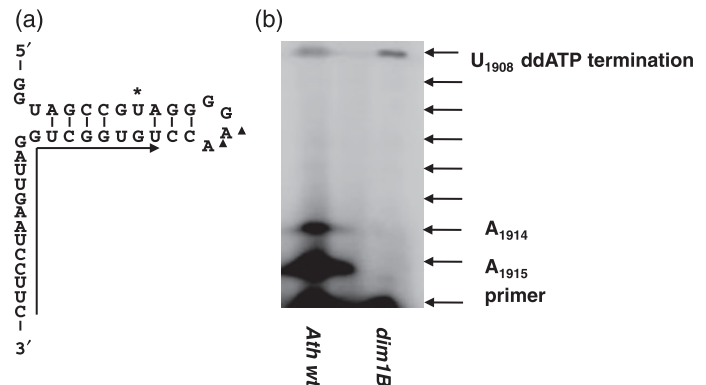
rRNAs. As shown in Figure 4, primer extension was inhibited at positions A1914/A1915 suggesting modification of the mitochondrial 18S rRNA at these nucleotides in the wild type. This signal was absent in RNA from the homozygous insertion line *dim1B*, where reverse transcription did continue uninhibited to position U1908. These results indicate that the conserved modification of two adenosines at the 3' end of the *ssrRNA* is present in the 18S rRNA of *Arabidopsis*

wild-type mitochondria and that *dim1B* plants lack this methylation, confirming that *Dim1B* is essential for methylation of the adenosine dinucleotide.

**DISCUSSION**

To investigate their potential function in mitochondrial transcription initiation and/or as bona fide dimethyltransferases, we screened the *Arabidopsis* genome and plant EST

**Figure 4.** *Arabidopsis dim1B* plants lack adenosine modification of the mitochondrial 18S rRNA. (a) Sequence and predicted structure of the 3' end of the mitochondrial small subunit 18S rRNA, the arrow indicating the reverse complementary primer used in the assay. (b) Poisoned primer extension products using wild-type (wt) and *Arabidopsis thaliana dim1B* plants ( $\Delta Atdim1B$ ) in which the *Dim1B* gene is disrupted. In wt plants ddATP incorporation into the cDNA strand at the methylated adenosine doublet results in a strong termination signal at these nucleotides and only a weak read-through signal at the next uridine. In the *dim1B* mutant no termination is detected at the adenosines and the read-through signal is increased.



databases for genes related to members of a gene family encoding rRNA dimethyltransferases, some of which act as transcription factors in fungal and animal mitochondria. These screens revealed two uncharacterized genes encoding dimethylase-like proteins, *Dim1A* (At2g47420) and *Dim1B* (At5g66360), in addition to *Pfc1* (At1g01860) previously characterized as the gene coding for a plastidial 16S rRNA adenosine dimethylase (Tokuhisa *et al.*, 1998). The results of the present study strongly suggest that *Dim1A* encodes a nuclear and *Dim1B* encodes a mitochondrial rRNA dimethyltransferase. The protein structure and the observed non-specific DNA binding activities of Dim1A and Dim1B are compatible with a functional role of both proteins as rRNA dimethyltransferases, but had also been observed with mtTFBs (Riemen and Michaelis, 1993; McCulloch *et al.*, 2002).

The expression of Dim1A fused to GFP in Arabidopsis protoplasts led to the appearance of GFP fluorescence within the nucleus where it localized to structures probably representing nucleoli. Such a localization of Dim1A is in agreement with predictions based on properties of the N-terminal domain of this protein. Moreover, it would also fit a role of Dim1A as an enzyme that interacts with pre-90S during early nucleolar processing events and methylates 18S rRNA after shuttling of pre-40S with bound Dim1 in the cytosol as shown for the yeast rRNA dimethylase Dim1 (Lafontaine *et al.*, 1994, 1995, 1998; Zemp and Kutay, 2007). The suggested function of Arabidopsis Dim1A as a nucleolar/cytoplasmic rRNA dimethylase is supported by its position on the phylogenetic tree where it appears to be most closely related to other eukaryotic rRNA dimethylases like ScDim1. Our search detected Dim1 homologues in all completely sequenced plant genomes from algae to flowering plants suggesting that the 18S rRNA of cytoplasmic ribosomes might generally be a target for methylation in plants.

Dim1B was found to possess a mitochondrial transit peptide. Further results strongly support the suggestion that Dim1B is a mitochondrial 18S rRNA adenosine dimethyltransferase. Firstly, we found that recombinant Arabidopsis Dim1B is capable of correctly methylating *E. coli* 16S rRNA at the conserved sites in *ksgA* mutant strains lacking the 16S rRNA methyltransferase activity of KsgA. Secondly, while we observed adenosine modification of mitochondrial 18S rRNA at the conserved site in wild-type Arabidopsis, we found no indication of mitochondrial 18S rRNA methylation in an Arabidopsis mutant line bearing a T-DNA insertion in the *Dim1B* gene, suggesting that the lack of mitochondrial 18S rRNA methylation is caused by the missing Dim1B activity.

The methylation of bacterial 16S rRNA is commonly discussed as contributing to the fidelity of translation. Recent reports, however, suggest a role in ribosome assembly. KsgA interacts with small ribosomal subunits near the binding sites of initiation factor 3 and the 50S

subunit (Xu *et al.*, 2008). Unassembled ribosomal subunits accumulated in an *E. coli* mutant lacking KsgA. Interestingly, this defect was exacerbated by growth at low temperature, i.e. the *ksgA* mutant exhibited a cold-sensitive phenotype (Connolly *et al.*, 2008). While the absence of methylation of chloroplast 16S rRNA has no influence on growth of the Arabidopsis *Pfc1* mutant under normal temperature, growth of the *pcf1* mutant at low temperature led to impaired chloroplast development (Tokuhisa *et al.*, 1998). In mouse mitochondria, dramatic, even lethal effects were reported from the loss of adenine dimethylation of 12S rRNA. This defect was shown to impair the stability of the small ribosomal subunit, ribosome assembly and translation (Metodieiev *et al.*, 2009). In striking contrast, lack of adenine dimethylation of 18S rRNA in Arabidopsis mitochondria has no obvious effect on growth and development of the investigated T-DNA insertion line, i.e. this conserved rRNA methylation seems not to be essential for Arabidopsis under the conditions of growth in our experiments. This conclusion is also supported by an unaltered phenotype of the *dim1B* mutant under cold stress (data not shown).

Several observations suggest that mitochondrial transcription in Arabidopsis requires auxiliary factors: (i) RpoTmp, the dual-targeted phage-type RNA polymerase, shows virtually no specific *in vitro* transcription initiation regardless of the promoter offered, (ii) RpoTp and RpoTm initiate transcription correctly *in vitro* only with some of the tested promoters; and (iii) correct *in vitro* transcription initiation by the RNA polymerases RpoTp or RpoTm is only observed if the DNA template is in a supercoiled conformation (Kühn *et al.*, 2007). Similar observations have previously been reported for the mitochondrial RNA polymerase Rpo41 from yeast which recognizes mitochondrial promoters on supercoiled, but not linear DNA templates *in vitro* (Matsunaga and Jaehning, 2004). Complementation with sc-mtTFB, however, did enable promoter-specific initiation of transcription on linear templates (Matsunaga and Jaehning, 2004). The results of the *in vitro* experiments performed in this study indicate that Dim1B does not have a function in transcription analogous to sc-mtTFB. Addition of Dim1B to the transcription assays did not lead to correct initiation of transcription by RPOTmp with supercoiled or linear template DNAs whatever Arabidopsis mitochondrial promoter was offered. Moreover, Dim1B assisted RPOTm neither in using additional promoters on supercoiled templates nor in recognizing any promoter on linear templates.

mtTFBs are essential transcription factors in fungal and animal mitochondria (Falkenberg *et al.*, 2007). A similar role of Dim1B as a transcription factor in Arabidopsis mitochondria would not be compatible with our finding that a mutant line lacking functional Dim1B grows and develops normally. Microarray analyses revealed no significant down- or up-regulation of the expression of mitochondrial genes in the *dim1B* mutant in comparison with the wild type (data not



shown). Moreover, the positioning of Dim1B in phylogenetic trees supports the conclusion that Dim1B acts as an rRNA dimethyltransferase but not as a mitochondrial transcription factor. The group of predicted mitochondrial rRNA dimethylases, which is only found in flowering plants and includes Arabidopsis Dim1B, does not branch together with mtTFB sequences but appears as a sister group of all eukaryotic Dim1-like proteins.

It is very likely that the evolutionary processes leading to the recruitment of an rRNA dimethylase as auxiliary factor of the mitochondrial transcription machinery in fungi and animals took place after the separation of the common ancestor of these groups from the green algae and plant lineage (Figures 2 and S2; for eukaryotic phylogeny, see (Burger *et al.*, 2003). The position of Arabidopsis Pfc1 and other plant Pfc1 homologues in the phylogram (Figures 2 and S2) is similarly inconclusive with respect to a possible additional role of these proteins as cofactors of phage-type RNA polymerases in plastids. Pfc1 appears to be separated from the Dim1 and mtTFB groups and to be most closely related to chlamydial KsgA homologues. This is in congruence with data from a recent publication (Park *et al.*, 2009) where implications of this positioning are discussed.

No Dim1B homologues have so far been detected in non-flowering plants, raising the question of whether mitochondrial small-subunit rRNAs in algae and in deep branching land plants such as mosses and lycopods lack methylation like the yeast 15S rRNA. In plants without a Dim1B homologue, Dim1A or Pfc1 may be recruited additionally to mitochondria by dual targeting of these proteins. Predictions of the subcellular localization of algal Pfc1-like proteins yield equally high scores for plastidial and mitochondrial localization (Table 1). On the other hand, two Dim1A-like proteins are found in the fully sequenced genome of *P. patens*, with PpDim1A-2 being predicted to have an N-terminal mitochondrial transit peptide (Table 1), suggesting that this protein might replace Dim1B in its function as a mitochondrial rRNA dimethylase.

In conclusion, we found no evidence for a role of Dim1B in mitochondrial transcription in Arabidopsis. Instead, our data provide conclusive evidence of Dim1B functioning as a bona fide rRNA dimethyltransferase methylating the mitochondrial 18S rRNA. The observation of genes orthologous to *Dim1B* in the genome of other angiosperms suggests that adenine dimethylation of mitochondrial 18S rRNA is a common phenomenon among higher plants, the functional importance of which remains to be investigated.

## EXPERIMENTAL PROCEDURES

### Plant material and growth condition

*Arabidopsis thaliana* (ecotype Columbia) wild type and T-DNA insertion line 071F10 were grown under short-day conditions (8 h of light/16 h of dark) for 14 days and then transferred to long-day conditions (16 h of light/8 h of dark) in a growth chamber.

### Analysis of an Arabidopsis *dim1B* mutant

The T-DNA insertion line 071F10 was obtained from the GABI-Kat collection. Plants homozygous for the mutation were identified by PCR analysis of a segregating population. Segregation analysis showed the mutant trait to be inherited in a monogenic fashion (3:1 segregation). Sequencing of T-DNA borders identified the insertion site within exon 2 of the genomic *Dim1B* sequence on chromosome V (At5g66360), 947 nucleotides downstream of the ATG initiation start codon in the CDS (see Figure S5). From the selfed line, we selected a homozygous mutant by PCR analysis and named it *dim1B*. The presence of a single T-DNA insertion in *dim1B* was shown by Southern blot analysis of the mutant using the T-DNA probe (data not shown).

### Isolation of RNA from Arabidopsis

Total cellular RNA was extracted from leaves and flowers of Arabidopsis plants using TRIZOL (Invitrogen, <http://www.invitrogen.com/>) according to the protocol provided by the manufacturer. Total cDNA was synthesized from 1 µg mRNA-enriched Arabidopsis RNA using Poly(A)Purist™ kit (Ambion, <http://www.ambion.com>) and Omniscript RT kit (QIAGEN, <http://www.qiagen.com/>) according to the manufacturer's instructions. Protein-coding sequences were amplified from the Arabidopsis cDNA with *Pfu* DNA polymerase (Promega, <http://www.promega.com/>). Sanger dideoxy sequencing was performed on an ABI3130xl sequencer (Applied Biosystems, <http://www.appliedbiosystems.com/>).

### Generation of targeting constructs, transient expression and confocal microscopy

To generate the pDim1B-GFP and pDim1A-GFP constructs driving the expression of fusion proteins Dim1B-GFP and Dim1A-GFP, sequences encoding the 64 N-terminal amino acids of Dim1B and the complete coding sequence of Dim1A were amplified from reverse-transcribed mRNA-enriched Arabidopsis RNA using primer pairs gfp-Dim1B-P/gfp-Dim1B-M (5'-cagctctagaATGATTCTCGATTGAAAGACCA-3'/5'-cagcgtcgacTGCACAGAAACAATCCATCG-3') and gfp-Dim1A-P/gfp-Dim1A-4M (5'-cagctctagaATGGCGGGAGGCAAGATC-3'/5'-cagcgtcgacTGAAGTGAATACCAGACTTGTACA-3'), respectively. PCR products were *Xba*I/*Sal*I-digested and inserted into the *Spe*I/*Sal*I-cleaved vector pOL-GFP S65C (Peeters *et al.*, 2000). Control constructs encoding mitochondrial Cox4-GFP and plastidial RecA-GFP were kindly provided by I. Small (Peeters *et al.*, 2000).

GFP-constructs were used to transform isolated Arabidopsis protoplasts as described in Yin *et al.* (2009). Protoplasts were examined 2 days after transformation by confocal laser scanning microscopy with a Leica TCS SP2 using 488 nm excitation and two-channel measurement of emission from 510 to 580 nm (green/GFP) and >590 nm (red/chlorophyll).

### Sequence data and multiple alignments

Genomic sequences, EST sequences and amino acid sequences were retrieved from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>) employing the BLASTP and TBLASTN algorithms and from the Joint Genome Institute (<http://genome.jgi-psf.org/>). *E. coli* KsgA, human Dim1, AtPfc1 and h-TFB1M sequences were used as query sequences. For translation and alignment, sequences were subsequently imported into GENEIOUS v4.5 (Drummond *et al.*, 2008). A multiple protein sequence alignment was generated using CLUSTALW (Thompson *et al.*, 1994) implemented in the GENEIOUS package using GONNET cost matrix with gap open cost set to 10 and gap extend cost set to

0.1. The alignment was refined manually with respect to the known secondary structural homology and poorly aligned regions were excluded from following analyses.

For a phylogenetic reconstruction based on Bayesian statistics, the MRBAYES program version 3.1 (Ronquist and Huelsenbeck, 2003) was used. In addition, maximum likelihood (phyML; Guindon and Gascuel, 2003) and neighbour joining analysis (Saitou and Nei, 1987) implemented in the GENEIOUS v4.5 package were conducted.

### Plasmids for the expression of recombinant proteins

Sequences encoding amino acids 18–353 of Dim1A (AGI At2g47420) and amino acids 26–380 of Dim1B (AGI At5g66360) were amplified from reverse-transcribed mRNA-enriched *Arabidopsis* RNA using oligonucleotide primer pairs Dim1A-PROP/Dim1A-PROM (cagcgtcgacTCGAACCATACCAAGGAGGAATAT/cagcgcggccgcACACCACAAAACGATTATGTGAAGTG) and Dim1B-PROP/Dim1B-PROM (cagcgtcgacCGAGATTCTCACTCGCAGGC/cagcgcggccgcTTATTCGTGTAGATCCATTGTGAATGATG), respectively. PCR products were ligated into pPROTet.E121 (Clontech, <http://www.clontech.com/>) to generate plasmids pPRO-Dim1A and pPRO-Dim1B.

### Poisoned primer extension

Primer extension reactions were carried out according to standard protocols (Sambrook *et al.*, 1989) using 10 µg of total *Arabidopsis* RNA isolated from wild-type plants and the homozygous dim1B insertion line each. The DNA oligonucleotide primer PPE *Atmt18S* (5'-GAAGGATTCATCCAGCCACA-3') was end-labelled with [ $\gamma$ -<sup>32</sup>P]ATP and T4-poly-nucleotide kinase (Fermentas, <http://www.fermentas.com>). Primer extensions were performed using Superscript-III MMLV reverse transcriptase (Invitrogen) at 50°C, with nucleotides ddATP, dCTP, dGTP, dTTP at 0.02 mM and with 2 pmol end-labelled primer. The end-labelled oligonucleotide 5'-TAAGGAGGTGATCCAACCGCA-3' was used for reverse transcription for the analysis of the AA dinucleotide at the bacterial rRNA nucleotides A1518 and A1519, the positions equivalent to the plant mitochondrial rRNA nucleotides A1914 and A1915. RNA sequencing reactions were performed as described previously (Lowe and Eddy, 1999) using total RNA of strains JM101*KsgA19* and BL21(DE2)*KsgA\_0*, respectively. Resulting products were analysed on a 10% sequencing gel. Visualization was done with a Phosphorimaging system and the complementary software (Bio-Rad, <http://www.bio-rad.com/>).

### Complementation of bacterial *KsgA* knock-out mutants

For the bacterial complementation tests, *E. coli* strains JM101*KsgA19* and BL21(DE3)*KsgA\_0* lacking the *KsgA* gene and JM101 were kindly provided by Dr A. Vila-Sanjurjo. The *Dim1B* gene lacking the N-terminal targeting signal was ligated into pBluescriptII KS (pBS\_Dim1B) and into the *Bam*HI and *Xho*I sites of pET28a (pET28\_Dim1B) and pET29a (pET29\_Dim1B) (Novagen, <http://www.novagen.com>), respectively. Bacterial strains Strain BL21 (DE3)pLys (Stratagene, <http://www.stratagene.com/>), as well as JM101, were used as positive controls. Strain JM101*KsgA19* was transformed with pBS\_Dim1B. Strain BL21(DE2)*KsgA\_0* was transformed with pET28\_Dim1B and pET29\_Dim1B, respectively. Each bacterial strain was grown up to  $A_{600nm} \sim 0.6$  and total RNA was isolated using a previously described method (Khodursky *et al.*, 2003).

### Protein expression and purification

Recombinant Dim1A and Dim1B were overexpressed in *Escherichia coli* BL21 Codon Plus RIL (Stratagene), purified over Ni<sup>2+</sup>-NTA-agarose (QIAGEN) and stored in 20 mM Tris/HCl, pH 7.8, 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 50% (v/v) glycerol at -20°C for 2–6 weeks. Expression and purification of recombinant

RPOTm and RPOTmp was performed as described (Kühn *et al.*, 2007).

### *In vitro* transcription assay

To generate DNA templates for *in vitro* transcription assays, mitochondrial DNA fragments were ligated into pKL23 (Liere and Maliga, 1999) upstream of terminator sequences; all plasmids used in this study have been described earlier (Kühn *et al.*, 2007). Linearized templates were generated by *Xho*I or *Eco*RI digests and purified via QIAquick spin columns (QIAGEN).

*In vitro* transcription assays were carried out for 45 min at 30°C essentially as described in (Kühn *et al.*, 2007). Reactions contained 6.7 mM Tris/HCl (pH 7.9), 6.7 mM KCl, 6.7 mM MgCl<sub>2</sub>, 0.67 mM DTT, 0.067% (w/v) BSA, 267 µM each ATP, CTP and GTP, 13 µM unlabelled UTP and 10 µCi of [ $\gamma$ -<sup>32</sup>P]-UTP (3000 Ci/mmol), 24 U RNase inhibitor and 200 ng of template DNA in a final volume of 15 µl. Reactions were started by adding 400 fmol of recombinant RPOTm or RPOTmp and 400 fmol of recombinant Dim1A or Dim1B where indicated and stopped with 115 µl RNA extraction buffer [6 M urea, 360 mM NaCl, 20 mM EDTA, 10 mM Tris/HCl pH 8.0, 1% (w/v) SDS] and 20 µl 2.25 M sodium acetate (pH 5.2). Nucleic acids were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol precipitated. Transcripts were then dissolved in formamide buffer [95% (v/v) formamide; 0.02% (w/v) bromphenol blue; 0.02% (w/v) xylene cyanol] and resolved using a Protean II xi electrophoresis unit (Bio-Rad) on 7 M urea, 5% polyacrylamide gels using 0.6× TBE as electrophoresis buffer. A radiolabeled RNA length standard was generated using the RNA Century Marker Template Plus (Ambion) and MAXscript kit (Ambion) according to the manufacturer's instructions and separated alongside RNA samples. Following electrophoresis, gels were dried and subjected to autoradiography employing a phosphorimager (Molecular Imager FX; Bio-Rad).

### ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft, Bonn (SFB 429) to T.B. and A.W. S.O. was supported by a fellowship of the Alexander von Humboldt-Foundation, Bonn. We thank Chang Yin for help with transient expression of Dim1A-GFP constructs in *Arabidopsis* protoplasts and confocal microscopy, C. Stock for excellent technical assistance, K. Liere (all Humboldt University, Berlin) for fruitful advice and discussion, A. Vila-Sanjurjo (UC, Berkeley) for kindly providing *E. coli* strains for complementation studies.

### SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Alignment of bacterial and eukaryotic rRNA methyltransferases.

**Figure S2.** Phylogeny of mitochondrial transcription factors and small-subunit rRNA dimethylases.

**Figure S3.** Expression of recombinant Dim1A and Dim1B and analysis of their DNA binding capacity by electrophoretic mobility shift assays (EMSAs).

**Figure S4.** Run-off transcription from the mitochondrial *atp6-1*, *atp8* and *rrn18* up-stream regions by RPOTm and RPOTmp.

**Figure S5.** Schematic representation of T-DNA insertion in *dim1B* plants.

**Table S1.** Physicochemical properties and targeting prediction results for *Arabidopsis* Dim1A (At2g474420) and Dim1B (At5g666360).

**Table S2.** Promoter sequences analysed in an *in vitro* transcription assay.

## References S1

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