Arabidopsis MDA1, a Nuclear-Encoded Protein, Functions in Chloroplast Development and Abiotic Stress Responses

Pedro Robles, José Luis Micol, Víctor Quesada*  
Instituto de Bioingeniería, Universidad Miguel Hernández, Campus de Elche, Elche, Spain

Abstract

Most chloroplast and mitochondrial proteins are encoded by nuclear genes, whose functions remain largely unknown because mutant alleles are lacking. A reverse genetics screen for mutations affecting the mitochondrial transcription termination factor (mTERF) family in Arabidopsis thaliana allowed us to identify 75 lines carrying T-DNA insertions. Two of them were homozygous for insertions in the At4g14605 gene, which we dubbed MDA1 (MTERF DEFECTIVE IN Arabidopsis1). The mda1 mutants exhibited altered chloroplast morphology and plant growth, and reduced pigmentation of cotyledons, leaves, stems and sepals. The mda1 mutations enhanced salt and osmotic stress tolerance and altered sugar responses during seedling establishment, possibly as a result of reduced ABA sensitivity. Loss of MDA1 function caused up-regulation of the RpoTp/SCA3 nuclear gene encoding a plastid RNA polymerase and modified the steady-state levels of chloroplast gene transcripts. Double mutant analyses indicated that MDA1 and the previously described mTERF genes SOLDAT10 and RUG2 act in different pathways. Our findings reveal a new role for mTERF proteins in the response to abiotic stress, probably through perturbed ABA retrograde signalling resulting from a disruption in chloroplast homeostasis.

Introduction

Chloroplast and mitochondrial genomes are assumed to derive from ancestral prokaryotes that established an endosymbiotic relationship with a primitive eukaryotic cell. Since many genes of those ancestral endosymbionts have been transferred to the nucleus, contemporary plant organelle genomes harbour only 100–200 genes, which encode components of photosynthetic, transcriptional and translational apparatuses. Recent estimations indicate, however, that 3,000 and 2,000 proteins localise in plant chloroplasts and mitochondria, respectively [1]. Most of these proteins are encoded by nuclear genes, synthesised in the cytoplasm and subsequently transported to their target organelle [1]. Therefore, the expression of the nuclear and organelar genomes has to be very precisely coordinated in the plant cell.

The mitochondrial transcription termination factor (mTERF) family was first identified and characterised in humans and other metazoans. Members of this family have been found in monocotyledons and dicotyledonous plants, and also in the moss Physcomitrella patens, but not in fungi and prokaryotes [2]. Metazoan mTERFs are required for the termination and initiation of transcription in mitochondria. Four vertebrate MTERF subfamilies have been described [2,3], which suggests that mitochondrial transcription regulation is more complex than initially anticipated. Human MTERF1 simultaneously binds to the mitochondrial transcription initiation and termination sites. This creates a DNA loop that promotes the direct delivery of mitochondrial RNA polymerase from the termination to the initiation site, thus accounting for the high mitochondrial rRNA synthesis rate in this organelle [4]. MTERF1 might also modulate mitochondrial DNA replication since the replication pause was sensitive to MTERF1 over-expression in human cultured cells [5]. Inactivation of the mouse MTERF2 gene leads to myopathies and memory deficits, which are associated with decreased levels of mitochondrial transcripts and proteins of respiratory chain complexes, thus impairing the respiratory function [6]. In addition, loss of the mouse MTERF3 or the MTERF4 function very early in development is lethal [7,8]. MTERF3 functions in vivo as a repressor of mitochondrial transcription [7], while MTERF4 regulates mitochondrial translation by targeting the methyltransferase NSUN4 to ribosomes [9].

Information on the function of mTERF genes in plants is still scarce [9]. An Arabidopsis mTERF protein (PTAC15) has been described as a member of the TAC (transcriptionally active chromosome) multi-protein complex involved in the transcription of chloroplast genes [10]. A proteomic study of plastid-enriched nucleoid fractions of maize leaves identified 10 mTERFs among 750 nucleoid-associated proteins [11], indicating a function for mTERFs in the expression of plastid genomes. Three mTERF nuclear genes from photosynthetic organisms have been cloned and functionally characterised: MOC1 (mtorf-like gene of Chlamydomonas) in the unicellular alga Chlamydomonas reinhardtii [12], and SOLDAT10 (SINGLET OXYGEN-LINKED DEATH ACTIVATOR10; [13] and BELAYA SMERT (BSM)/RUGOSA2 (RUG2)
[14,15] in Arabidopsis. Perturbation of the MOC1 mitochondrial protein alters the expression profiles of those genes encoding components of mitochondrial respiratory complexes. SOLDAT10 is a chloroplast-localized protein. The soldat10 mutation alters chloroplast function and suppresses the cell death caused by singlet oxygen in the Arabidopsis flu (fluorescent) mutant [13]. Of these proteins, only BSM/RUG2 is dually targeted to chloroplasts and mitochondria. The bsm and rug2-1 mutations alter the expression of chloroplast genes at the RNA and protein levels, causing stunted plant growth and paleness [14,15]. The bsm mutant also exhibits arrested embryo development. Besides, impaired RUG2 function results in decreased levels of mitochondrial transcripts, including those encoding subunits of the respiratory chain [15].

We identified T-DNA alleles of Arabidopsis mTERF genes and characterised a member of this family, which we named MDA1 (for MTERF DEFECTIVE IN Arabidopsis). MDA1 loss of function alters plant development, leading to defective chloroplast function and gene expression, early flowering, reduced plant growth and paleness [14,15]. The bsm mutant also exhibits impaired embryo development. Besides, impaired RUG2 function results in decreased levels of mitochondrial transcripts, including those encoding subunits of the respiratory chain [15].

We followed a reverse genetics approach in Arabidopsis to isolate mutations in the mTERF genes with a morphological phenotype. With this purpose in mind, we screened 75 T-DNA insertion lines from different publicly available collections [Table S3; SIGnalAL [19]; SAIL (Syngenta Arabidopsis Insertion Library; [20]) and WiscDsLox (https://mywebspace.wisc.edu/groups/Krysan/Web/2010/default.html)] by presumably tagging 29 of the 35 mTERF genes of Arabidopsis. Regardless of their phenotype, eight T3 plants from each line were transferred to soil and genotyped by PCR for the presence of the annotated T-DNA insertion (Table S4), and the T4 plants displaying a mutant phenotype were selected for further studies, thus confirming the mutant phenotype in the T3, T4 and T5 generations.

The N597243 and N819625 lines, putatively carrying a T-DNA insertion in the At4g14605 gene, exhibited a mutant phenotype that was inherited with complete penetrance and constant expressivity. They were backcrossed to the Col-0 wild type and their F2 progenies showed a 3:1 wild-type:mutant segregation ratio ($\chi^2 = 0.81$, $P = 0.37$ and $\chi^2 = 1.93$, $P = 0.16$, for N597243 and N819625, respectively), indicating that the mutant phenotypes were monogenic and recessive. The presence of insertions in the At4g14605 gene was confirmed by PCR (see Materials and Methods), affecting the fifth exon of the gene (Figure S1A) as annotated on the SIGnalAL website (http://signal.salk.edu). For each line, all the F2 mutant plants and their F3 progenies were homozygous for the T-DNA insertions. The mutant phenotype of the F1 plants from an N597243 × N819625 cross further confirmed their allelism. We named these mutants mda1-1 (mTERF defective in Arabidopsis1; N597243) and mda1-2 (N819625).

Bioinformatics Analysis of the MDA1 Protein

The MDA1 gene encodes a predicted protein of 493 amino acids with a molecular mass of 55.9 kDa (http://www.arabidopsis.org/index.jsp). The number of mTERF motifs predicted by SMART was eight (Figure 1A). Database searches allowed us to identify proteins similar to MDA1 in metazoans and plants, but not in archea, eubacteria or fungi. An alignment of the amino acid sequences of the mTERF motifs in MDA1 with those of other plant and metazoan mTERFs [see below] revealed the conservation of a proline residue at position 8 (Figure 1B). The iPSORT, ProteinProwler and TargetP programs yielded a high probability for chloroplast localisation for MDA1 (Table S1), as also reported in the SubCellular Proteomic Database (http://saba.plantenergy.uwa.edu.au/flatfile.php?id). These results are consistent with those obtained by Babiychuk et al. [14] using GFP fusions.

The closest identity with MDA1 was found for the ARALY-DRAFT_915404 protein from Arabidopsis lyrata. 95.6% amino acid identity and 97.8% similarity (Figure 1C). The rice Os02g39040.1 gene product displayed 54.5% amino acid identity and 82.3% amino acid similarity. The amino acid sequence of MDA1 exhibited 31.3% identity and 70.4% similarity to SOLDAT10 [13], and 25.5% identity and 57.8% similarity to BSM/RUG2 [14,15] (Figure 1C). Other Arabidopsis mTERFs closely related to MDA1 were the products of the At4g38160 (27.8% identity and 65.0% similarity) and At2g44020 (24.6% identity and 59.6% similarity) genes. Furthermore, MDA1 displayed 25.7%, 25.5%, 23.9% and 22.9% overall sequence identity with the human MTERF3, MTERF4, MTERF1 and MTERF2 proteins, respectively, showing a closer similarity to the MTERF3 subfamily members from rat, mouse, sea urchin (Paracentrotus lividus) and Drosophila (from 26.6% to 21.7% amino acid identity) than to the remaining subfamily members (Figure 1C). Our results indicate that MDA1 shows closer identities to mTERFs from plants than...
from metazoans, and hint at a functional conservation of this protein in Arabidopsis and rice.

**MDA1 Expression Analyses**

We examined the expression of the MDA1 gene in *mda1* mutants. For this purpose, total RNA was extracted from mutant plants on 14 das (days after stratification), reverse-transcribed and PCR-amplified using different primer combinations (Table S4 and Figure S1A). A single band of the expected size was obtained from Col-0 cDNA, but not from *mda1-1* or *mda1-2* cDNAs, by using as primers the oligonucleotides RP and R2a flanking the insertions (Figure S1B). In addition, chimeric transcripts were detected in *mda1* plants when using a primer (F1) which hybridises upstream the T-DNA insertions that disrupt At4g14605 and an LB-specific primer (LBb1.3 or LB1 for *mda1-1* or *mda1-2*, respectively; Figure S1C). The translation of these chimeric transcripts is predicted to

---

**Figure 1. mTERF motifs in members of the mTERF family.** (A) Modular architecture of the *Arabidopsis thaliana* (At) mTERF proteins MDA1, BSM/RUG2 and SOLDAT10, human (Hs) MTERF3, *Drosophila melanogaster* (Dm) D-MTERF3 and *Paracentrotus lividus* (Pl) mtDBP. The diagram was drawn using SMART. mTERF motifs are shown as ellipses. The number of amino acids of each protein is indicated. The sequence logo was derived using WebLogo (http://weblogo.berkeley.edu/). (C) Multiple alignment of the amino acid sequence of part of the proteins encoded by the At MDA1, SOLDAT10 and BSM/RUG2, *Arabidopsis lyrata* A1915404, *Oryza sativa* Os02g39040.1, mouse [*Mus musculus* (Mm)] Mterf3, rat [*Rattus norvegicus* (Rn) Mterf3], Hs MTERF3, Dm D-MTERF3 and Pl mtDBP genes. Residues conserved across five or more sequences are shaded in black, and similar residues are shaded in grey. Numbers indicate amino acid positions. The alignment was obtained using ClustalX v1.5b. A continuous line indicates an mTERF motif in MDA1.

doi:10.1371/journal.pone.0042924.g001
yield truncated proteins lacking 110 and 238 C-terminal amino acids in the mda1-1 and mda1-2 mutants (deleting 2 and 3 mTERF motifs), respectively. In addition, primers annealing upstream the T-DNA insertions revealed by quantitative RT-PCR (qRT-PCR) down-regulation of MDA1 expression in mda1-1 and mda1-2 compared with Col-0 (2.0- and 1.75-fold, respectively; Figure S2A).

We followed in silico and experimental approaches to study the spatial expression of the MDA1 gene in Arabidopsis. In the first case, we examined the results from different publicly available microarray databases [Genevestigator (http://www.genevestigator.com/gv/), and the BIO-array resource (BAR; http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi)]. We found MDA1 to be ubiquitously expressed, reaching its highest level of expression in the aerial parts of seedlings, and in young and senescent leaves, and its lowest level in roots and old flowers, according to the tiling array data available at BAR [21,22]. Similar results were obtained when using Genevestigator. We confirmed the ubiquitous expression of MDA1 by qRT-PCR. MDA1 transcripts were detected in all organs analyzed (Figure S2B), with the lowest expression found in roots and the highest in stems and vegetative leaves (2.9- and 2.5-fold higher than in roots, respectively).

External Morphology and Histology of mda1 Mutants

The most noticeable phenotype of mda1 plants is the pale pigmentation of their rosette and cauline leaves, stems, sepals and siliques, which was visible early in development. The cotyledons of the mda1 seedlings exhibited less green pigmentation than the wild type (Figure 2A–C). Consistently, we noted a significant reduction in chlorophyll a, b and carotenoid levels in mda1 mutants compared with Col-0 (Figure 2E; mda1 leaves were rounded, their margins lacked indentations and the lamina was uniformly pale (Figure 2D–F); mda1 plants were small in size if compared with Col-0 plants, as confirmed by measuring several body parameters, which revealed a significant decrease in mda1 fresh and dry weights, root, hypocotyl and main stem lengths (Figure 2G, H; Table 1). Furthermore, we found that the mda1 mutations did not affect flower development, although they significantly accelerated flowering, while the mda1-1 mutation diminished fertility (Table 1).

The internal anatomy of mda1 leaves was studied by confocal microscopy using chlorophyll autofluorescence of mesophyll cells. We did not find any noticeable differences in the mda1-1 and mda1-2 mesophyll cells when we compared them with Col-0, although a slight decrease in chloroplast autofluorescence was observed (Figure 3A, D and G). We examined the leaf chloroplast ultrastructure in both mda1 and wild-type plants by transmission electron microscopy, which allowed us to identify defects in the mda1 chloroplast structure (Figure 3B, C, E, F, H and I). Accordingly, chloroplasts in mda1 mesophyll cells had a low starch grain number, suggesting low photosynthetic activity, as well as enlarged thylakoid lamellas, probably due to a breakdown of thylakoid membranes. Consistently, mda1 chloroplasts revealed an accumulation of plastoglobuli, storage sites for membrane degradation material (Figure 3C, F and I). Vacuoles were occasionally found in mda1-2 (Figure 3C and I). Our microscopy studies did not reveal any significant differences in either the number of chloroplasts or the morphology of the mitochondria between mda1 mutants and Col-0.

Abiotic Stress Responses of the mda1 Mutants

Dozens of genes associated with abiotic stress are up-regulated in the Arabidopsis soldat10 mutant, which displays a very similar morphological phenotype to that of the mda1 mutants [13]. By employing the BIO-array website resources [21,22], our expression analysis revealed changes in the MDA1 transcript levels in those Col-0 plants grown in vitro in response to different stress stimuli over time, the most significant being a substantial down-regulation after abscisic acid (ABA) or salt treatments. Besides, we analysed all the Arabidopsis mTERF genes using the Arabidopsis whole-genome tiling array express (At-TAX) data (http://www.weigelworld.org/resources/microarray/at-tax) [23] to get a wider perspective on the role of mTERF family members in abiotic stress responses. Most mTERF genes (including RUG2 and SOLDAT10) were largely down-regulated in response to ABA, salt or mannitol (Table S5), being these differences usually higher when the exposure of the seedlings to the stress was more prolonged (12 h vs. 1 h; Table S5).

These results prompted us to study the response of the mda1 mutants to different agents causing abiotic stress. We first examined the ability of our mutants to form fully expanded green cotyledons at different NaCl concentrations (seedling establishment; Figure 4A, B). We found that mda1 mutants showed this trait at 200 mM NaCl, a concentration that almost abolished it in Col-0 (53%, 58% and 6% of the mda1-1, mda1-2 and Col-0 seeds, respectively; Figure 4C). Interestingly, we found that 52% of soldat10 (in a Ler genetic background) and 13% of Ler seeds developed expanded cotyledons at 150 mM NaCl, indicating that an impaired SOLDAT10 function also causes reduced sensitivity to this salt (neither Ler nor soldat10 seeds expanded cotyledons at 200 mM NaCl). To determine whether mda1 mutants were less sensitive to specific ions or osmotic stress than Col-0, we grew them on media supplemented with KCl or mannitol. mda1 seeds yielded higher rates of seedling establishment than Col-0 on media containing high KCl or mannitol concentrations (Figure 4D, E), indicating that mda1 mutants are less sensitive than the wild type to K+ and Cl− ions and to osmotic stress produced by the osmoticum mannitol.

It is well-known that plant responses to environmental stresses are regulated by ABA, a hormone that can inhibit seed germination in response to high ionic and/or osmotic stress produced by salt, cold or drought, and that the mutations affecting ABA synthesis or signalling enhance germination under these stress conditions [24–26]. Hence, we investigated the behavior of mda1 and Col-0 seeds on media containing different ABA concentrations. As depicted in Figure 5A, mda1 seeds clearly showed higher levels of seedling establishment than Col-0 on medium supplemented with either 2 μM ABA (97%, 98% and 65% of the mda1-1, mda1-2 and Col-0 seeds yielded green expanded cotyledons, respectively) or 3 μM ABA (51%, 38% and 6% of the mda1-1, mda1-2 and Col-0 seeds lead to seedlings with green expanded cotyledons, respectively). Nevertheless, ABA sensitivity was greater in mda1 than in the null ABA insensitive san5/2 (also named abi4-2) mutant [24] used as a control (Figure 5A). Taken together, these data indicate that the increased tolerance to salt and osmotic stress shown by mda1 mutants might be explained by their reduced sensitivity to ABA.

To characterise the response of mda1 mutants to salt and ABA later in development, Col-0 and mutant plants were transferred 9 das from a non-supplemented agar medium to media containing 100 mM NaCl or 4 μM ABA. Their root length and fresh weight were determined after a 12-day growth period and were referred to those of the Col-0 or mda1 plants transferred at the same time to non-supplemented media. Compared with Col-0, mda1-1 and mda1-2 plants subjected to salt stress had a significantly reduced fresh weight (20%, 34% and 41% respectively; Figure 5B) and mda1-2 also displayed shortened roots (31% and 43% length reduction for Col-0 and mda1-2, respectively; Figure 5C). When grown on NaCl, the soldat10 plants exhibited significantly less fresh
weight when compared with Ler (63.6% and 53.9%, respectively; P = 0.015). On the ABA-supplemented media, mda1-1 and mda1-2 exhibited considerably shortened roots compared with Col-0 (28%, 20% and 9%, respectively; Figure 5C), while mda1-1 individuals displayed significantly lower fresh weight values than Col-0 (63% and 80% of non-stressed plants, respectively; Figure 5B).

We evaluated the effect of temperature stress on mda1 mutants because we previously reported that the phenotype of the mTERF-defective rug2-1 mutant was temperature-dependent [15]. Growth of mda1 mutants and Col-0 was similarly affected by culture at 25°C (we normally grow our plants at 20°C; Figure S3D-I). Compared with 20°C, at 15°C (Figure S3A-F) the mutant plants showed reductions in fresh weight significantly higher than Col-0 (65.5%) for mda1-1 (73.8%), but not significant ones for mda1-2 (71.9%; Figure S3J). Taken together, our results indicate that mda1 mutations diminish salt, mild cold and ABA tolerance during vegetative growth.
Sugar Sensitivity of the mda1 Mutants

It is known that ABA-deficient or insensitive mutants display altered responses to sugars upon germination (reviewed in [27]). Besides, the Arabidopsis mutant shi1-1 (salt hypersensitive1-1) shows altered sensitivity to NaCl, ABA and sugars, together with inhibition of chlorophyll synthesis, very early in development [28]. Therefore, given the paleness of mda1 mutants and their reduced sensitivity to salts and ABA during early seedling development, we decided to examine their responses to sugars. Seeds were sown on media containing different concentrations of sucrose (0, 30, 90, 175 and 290 mM) or glucose (330 and 390 mM). We normally grow our mutants on 30 mM sucrose. The mda1-1 mutant was clearly less sensitive to sugars: on the media containing high concentrations of glucose (330 or 390 mM) or sucrose (290 mM), mutant seedlings developed fully expanded leaves (observed 14 das). Later in development, paleness of the green cotyledons at higher rates than Col-0 (Table 2). Consistent with the above observation, it was confirmed by analysing the expression of nuclear genes through retrograde signalling [30]. Consequently, the transcript levels of RpoTp/SCA3 in the Col-0 and Ler genetic backgrounds, respectively. In all cases, we confirmed the phenotypes of the double mutants identified in F2 by studying the F3 progenies derived from selfed F2 plants displaying single or double mutant phenotypes, whose genotypes were verified by PCR (see Materials and Methods).

| Body parameters | Genotype | mda1-1 | mda1-2 |
|-----------------|----------|--------|--------|
| Fresh weight    | Col-0    | 26.1±7.1 | 9.0±3.0* | 9.8±2.9* |
| Dry weight      | Col-0    | 2.7±0.8  | 0.8±0.5* | 0.6±0.4* |
| Root length     | Col-0    | 75.0±0.3 | 59.2±6.2* | 49.3±4.6* |
| Hypocotyl length| Col-0    | 15.3±1.6 | 13.0±1.2* | 12.8±1.3* |
| Primary stem length | Col-0 | 77.0±3.2 | 36.6±3.8* | 27.2±3.9* |
| Siliquae length | Col-0    | 13.6±0.9 | 13.1±1.2 | N.D. |
| Number of seeds per siliquae | Col-0 | 50.7±7.3 | 40.9±7.7* | N.D. |
| Number of vegetative leaves at bolting | Col-0 | 13.4±2.2 | 12.0±1.1* | 9.1±1.1* |
| Number of days for bolting | Col-0 | 27.4±2.5 | N.D. | 24.2±4.3* |

Values shown are the mean of at least 15 measurements ± standard deviation (SD). Lengths are indicated in mm and weights in mg. Measurements were obtained from plant material collected at 14, 16, 19, 60, 84 and 105 days after stratification (das). Seedlings grown in the dark. Values were significantly different (Student’s t-test, P < 0.01) from those of the wild type (Col-0); N.D. = not determined.

doi:10.1371/journal.pone.0042924.t001

Effects of mda1-1 Mutation on the Expression of Plastid and Nuclear Genes

Considering that MDA1 is an mTERF-plastid protein which is putatively involved in transcriptional control and that the mutants in the previously characterized mTERF genes SOLDAT10 and BSM/RUG2 modify the levels of chloroplast transcripts, we decided to study the expression of several plastid genes by qRT-PCR using the RNA extracted from seedlings 14 das. We selected genes whose expression is known to be affected in the soldat10, rug2-1 and/or bsm mutants. Besides, we included representatives of the three classes of genes that are transcribed by different RNA polymerases: only the plastid-encoded polymerase (PEP; class I), PEP and the nuclear-encoded polymerase (NEP; class II), and only NEP (class III) [29]. According to these criteria, the expression of the psbA [class I encoding a core subunit of the photosystem II], matk [class II encoding a matrerase], clpP [class II encoding the proteolytic subunit of the Clp ATP-dependent protease], rps18, rps18 and acsD [class III encoding the core β subunit of PEP, a ribosomal protein and a subunit of the acetyl-Coa carboxylase for lipid biosynthesis, respectively] genes was studied. In comparison to Col-0, in mda1-1, we found significant differences in the expression of psbA, accD, rps18 [2.2-fold (2.2±0.6; P = 0.01), 1.7-fold (1.7±0.6; P = 0.04) and 1.6-fold (1.6±0.3; P = 0.01) up-regulated, respectively] and of clpP [1.7-fold down-regulated (0.6±0.2; P = 0.008)], whereas the transcript levels of matk [1.3-fold (1.3±0.5; P = 0.2) and rug2B (0.9±0.1; P = 0.3) were only slightly affected.

Defective chloroplast development may modify the expression of nuclear genes through retrograde signalling [30]. Consequently, the transcript levels of the RpoTp/SCA3 nuclear gene, encoding a plastid RNA polymerase [31], change in Arabidopsis mutants which are impaired in plastid development, such as rug2-1 [15]. For this reason, we investigated whether the expression of RpoTp/SCA3 was affected in mda1 mutants: RpoTp/SCA3 was found significantly up-regulated in mda1-1 (1.7±0.1; P = 0.01) and mda1-2 (1.6±0.3; P = 0.008) when compared with Col-0.

Genetic Interactions among mTERF-defective Mutants

To identify the genetic interactions among the mTERF genes whose perturbation led to a mutant phenotype, we crossed mda1 mutants with rug2-2 [15] and soldat10 [13] in the Col-0 and Ler genetic backgrounds, respectively. In all cases, we confirmed the phenotypes of the double mutants identified in F2 by studying the F3 progenies derived from selfed F2 plants displaying single or double mutant phenotypes, whose genotypes were verified by PCR (see Materials and Methods).

The rug2-2 × mda1-1 and rug2-2 × mda1-2 crosses allowed us to identify additive double mutant phenotypes in their F2 progenies since they exhibited a combination of phenotypic traits from their corresponding parents (Figure 6A). Since MDA1 and RUG2 are linked, the four phenotypic classes found did not fit the expected 9:3:3:1 ratio. As regards the mda1-1 × soldat10 and mda1-2 × soldat10 crosses, we identified double mutants with a clearly additive phenotype in all the F2 progenies studied: they had much smaller leaves and rosettes than mda1 or soldat10 (Figure 6A). Only three phenotypic classes were found in the F2 progenies (wild-type, single or double mutant), probably because of the similarity between the phenotypes of soldat10 and mda1. Additivity of the phenotypes caused by soldat10 and mda1 was confirmed by analysing the F3 progenies. We also crossed rug2-1 with soldat10, both in the Ler genetic background, and the F2 progeny showed a 9:3:3:1 segregation ratio (x² = 0.97; P = 0.82), and the F2 and F3 double mutants were smaller and paler than their single mutant siblings (Figure 6B), a phenotype which we interpreted as additive.

We evaluated the response of the double mutants to ABA by measuring root growth and seedling establishment. Root growth assays revealed that the rug2-1 and soldat10 mutations increase ABA sensitivity whereas no differences were found for rug2-2 compared with Col-0 (Table 3). On ABA, root length of the mda1-2 rug2-2 plants was similar to that of mda1-2 whereas it was shortened in rug2-1 soldat10 and mda1-1 soldat10 over that of the single mutants, which suggests additivity (Table 3). Regarding seedling establishment on ABA, rug2-1, soldat10 and the rug2-1 soldat10 double mutant were as sensitive as Ler. On the contrary, the rug2-2, mda1-2 rug2-2 and mda1-2 soldat10 seedlings were more tolerant than the corresponding wild type (Table 3). The rate of
seedling establishment on ABA for mda1-2 rug2-2 was similar to that of the single mutants, whereas mda1-2 soldat10 exhibited higher and lower rates than soldat10 and mda1-2, respectively, likely due to its mixed genetic backgrounds (Table 3).

We studied by qRT-PCR the expression of the nuclear RpoTp/SCA3 and plastid psbA genes, in the mda1-1 rug2-2 and mda1-1 soldat10 double mutants. In mda1-1 rug2-2 compared with Col-0, RpoTp/SCA3 (2.5 ± 0.2; P = 0.008) and psbA (2.8 ± 0.6; P = 0.05) were up-regulated to levels slightly higher than those of mda1-1 (see above). psbA was up-regulated in mda1-1 soldat10 compared with Col-0 (2.5 ± 0.8; P = 0.008) or Ler (2.5 ± 0.8; P = 0.01) at a similar extent than mda1-1 (see above). RpoTp/SCA3 expression was clearly dependent on the genetic background; it was up-regulated in Col-0 (2.8 ± 0.2; P = 0.006) compared with Ler. This would explain the differences found in RpoTp/SCA3 expression between mda1-1 soldat10 and either Ler (1.9-fold up-regulated) or Col-0 (1.3-fold down-regulated).

**Discussion**

Although chloroplasts and mitochondria are essential for life, and despite experimental and *in silico* studies estimating the number of proteins which are located in these organelles to run into thousands, knowledge of their role in plant biology is limited. To help elucidate the function of the nuclear-encoded proteins localised to chloroplasts and/or mitochondria, we studied the Arabidopsis mTERF family of transcriptional regulators. To this end, we first searched for members of this gene family in the genomes of the dicotyledonous Arabidopsis thaliana and the monocotyledonous Oryza sativa. Our bioinformatics analyses revealed that the numbers of annotated mTERF genes in the Arabidopsis and rice genomes are similar and substantially higher than in metazoan genomes (four in vertebrates, three in Drosophila melanogaster or one in Caenorhabditis elegans [2,14]). We found experimental evidence for the expression of these genes, thus validating the *in silico* identification. Furthermore, our results

---

**Figure 3. Chloroplast structure in the mda1 mutants.** (A, D, G) Confocal micrographs showing chlorophyll autofluorescence in mesophyll cells of Col-0 (A), mda1-1 (D) and mda1-2 (G) third-node leaves. (B, C, E, F, H and I). Transmission electron micrographs of chloroplasts of a Col-0 (B), mda1-1 (E) and mda1-2 (H) mesophyll cell. Close-up views of Col-0 (C), mda1-1 (F) and mda1-2 (I) chloroplasts. Photographs were taken 21 days after stratification (das). Bars = 20 μm (A, D, G), 5 μm (B, E, H) and 2 μm (C, F, I).

doi:10.1371/journal.pone.0042924.g003
suggest that Arabidopsis and rice mTERFs are targeted to chloroplasts or mitochondria, most of which are potentially located in mitochondria. This has been experimentally confirmed in Arabidopsis by GFP fusions [14]. In animals, mTERF proteins are mitochondrial, and their molecular characterisation has revealed their participation in mitochondrial transcription initiation, termination, translation and mtDNA replication. We speculate that the large number of mTERF genes in plants might be explained by them requiring the accurate expression of not only mitochondrial, but also plastid genes. Besides, mTERFs might differentially contribute to regulate the organelle gene expression in distinct developmental stages and tissues, or in response to environmental demands or stress.

Our reverse genetics approach underpins the importance of the mTERF gene family in Arabidopsis, as previously shown throughout the characterisation of the mTERF-related genes SOLDAT10 [13] and BSM/RUG2 [14,15]. mda1 insertional mutants curtail MDA1 expression. Besides, they would encode truncated proteins lacking 110 (mda1-1) or 238 (mda1-2) residues from the C-terminus, which would likely include divergent amino acids translated from T-DNA because we detected chimeric transcripts. This suggests that mda1 mutants are not null. Despite all this, the mda1-1 and mda1-2 phenotypes are indistinguishable, which might be explained by the existence of a redundant function supplying MDA1 deficiency regardless of the extent of mutational damage.

Our morphological, physiological and molecular analyses of the mda1 soldat10 and mda1 rug2-2 double mutants suggest additivity rather than synergy which would rule out RUG2 or SOLDAT10 as that redundant function. This indicates that the mTERF genes so far characterised in Arabidopsis participate in different pathways. Alternatively, the mTERF domains remaining in mda1-2 may be sufficient to accomplish a level of activity similar to that of mda1-1.

Consistent with the chloroplastic targeting of the MDA1 protein, loss-of-function mda1 alleles alter chloroplast morphology and cause disorganised thylakoid membranes and a reduction in starch grains, indicating diminished photosynthetic activity. Impaired chloroplast activity results in reduced chlorophyll levels in mda1 plants, leading to pale green organs and general stunted growth, as shown by the lower weight and reduced height of mda1 plants. In line with the pleiotropic effects of mda1, the MBM1 gene was broadly expressed. Leaf morphology was almost normal in mda1 mutants, except for their roundness. Stunted plant growth, chlorophyll levels and green pigmentation are phenotypic traits shared by other previously characterised mTERF mutants, such as rug2 [15] and soldat10 [13]. Like mda1, rug2-1 also showed abnormal chloroplasts. Interestingly, the short hypocotyls of mda1 plants suggest that MDA1 is required to complete the cotyledon developmental program in the dark and also suggests a role for this gene in etioplasts. The most severe phenotype described so far for an mTERF-defective mutant was displayed by bsm, which is likely a null allele of the BSM/RUG2 gene, exhibiting albino cells and severe alterations in organogenesis. In addition, bsm cells require hormone supplementation to proliferate as well as to grow in vitro [14]. Like bsm, rug2-1 and soldat10, mda1-1 plants exhibit an altered plastidic gene expression. The fact that the mda1-1 mutation modifies the transcript levels of those genes transcribed by NEP, PEP or both, suggests that MDA1 might be directly or indirectly required by different plastid transcriptional machineries for appropriate gene expression.

Figure 4. Effects of NaCl, KCl and mannitol on seedling establishment in the mda1 mutants. (A, B) Col-0 (A) and mda1-1 (B) seedlings germinating on growth medium supplemented with 200 mM NaCl. The inset images correspond to magnifications of the seedlings indicated by arrows. The mutants display green expanded cotyledons, a phenotypic trait barely observed in Col-0. Scale bars: 1 mm. (C–E) Seedling establishment of the mda1 mutants and Col-0 in various NaCl (C), KCl (D) and mannitol (E) concentrations. We considered only those seedlings displaying green expanded cotyledons. Error bars represent SD. Each value corresponds to the average of two independent experiments with two to four replicates of 50–100 seeds each. Germination was scored at 10 das.

doi:10.1371/journal.pone.0042924.g004
The proper activity of the different plant cell genomes entails tight coordination. Thus, retrograde pathways transmit the developmental, metabolic or physiological chloroplast status to the nucleus by modifying the expression of those nuclear genes whose products act on chloroplasts, such as the RpoTp/SCA3 gene, which encodes a plastid RNA polymerase [31]. Accordingly, we found that RpoTp/SCA3 up-regulates in mda1 plants as in Arabidopsis rug2-1 [15] and rpoT2 (affected in the chloroplast and mitochondria-targeted RNA polymerase; [32]) mutants and the plastid-ribosome deficient albostrans mutant from barley [33]. This up-regulation may attempt to compensate for the defective plastid function caused by the altered plastome expression in mutants.

Our findings reveal that disrupted MDA1 activity causes altered responses to abiotic stresses, according to the in silico microarray-based results, and they support previous observations suggesting a role for mTERFs in plant stress. Thus, soldat10 is constitutively adapted to light stress [13], whereas a Brassica napus mTERF gene and its Arabidopsis orthologue are up-regulated under abiotic stress conditions [34]. Consistent with this, our analysis using the At-TAX tiling array data showed differences in the expression of most Arabidopsis mTERF genes after salt, mannitol or ABA treatments, suggesting a role for mTERFs in abiotic stress responses. mda1 mutants exhibit reduced seed sensitivity to the inhibition of seedling establishment caused by high NaCl, KCl or mannitol concentrations, yielding higher rates than those of Col-0 under stress conditions. As ABA plays a central role in plant adaptive responses to environmental stresses, and since the perturbation of ABA signalling in Arabidopsis abi4-2 (ABA insensitive) mutants results in increased salt tolerance [24,35], we evaluated the mda1 response to ABA. We found mda1 early seedling development to be less sensitive to this hormone, indicating that the MDA1 function is required for a proper ABA response in this stage. The experimental results support that there is an interaction between sugar and ABA signalling during early seedling development in Arabidopsis, to the extent that ABA biosynthesis and signalling pathways are at least partially modulated by glucose (for reviews, see [36–38]). Consequently, the mutants identified in screens for an altered sugar response were actually allelic to aba2 (ABA deficient2) or abi4 mutants [27]. Therefore, we decided to investigate if mda1 mutants, which are partially insensitive to ABA, also show an altered sugar response. We found this to be the case because, in the presence of high glucose or sucrose concentrations, mda1-1 seeds yielded higher seedling establishment rates than Col-0.

Altered sugar, salt and ABA responses during early seedling development were also observed in the Arabidopsis shs1-1 mutant [28]. SHS1 belongs to the mitochondrial carrier family of proteins involved in the energy transfer being located in plants in plastids [39] or the endoplasmic reticulum [28]. We considered reduced sensitivity to ABA to be a likely explanation for the altered responses to salts, sugar and osmotic stress exhibited by mda1 mutants because, as previously mentioned, any perturbation in the plastid-ribosome deficient albostrans mutant from barley [33]. The plastid function caused by the altered plastome expression in mda1 mutants because, as previously mentioned, any perturbation in any plastid-ribosome deficient albostrans mutant from barley [33]. The plastid function caused by the altered plastome expression in mda1 mutants because, as previously mentioned, any perturbation in

---

**Figure 5. Tolerance to ABA and NaCl of the mda1 mutants.** (A) Effects of different ABA concentrations on seedling establishment in the mda1 mutants. Data are means of two independent experiments with three replicates of 50–100 seeds each scored 10 das. Error bars represent SD. The xan5 (abi4-2) ABA insensitive and salt-tolerant mutant was used as a positive control [24]. (B, C) Sensitivity to ABA and NaCl of mda1 plants. The individuals were transplanted 9 das from non-supplemented growth media to media supplemented with 0, 100 mM NaCl or 4 µM ABA. 12 days after the transfer, tolerance was estimated by determining the fresh weight and root length of the plants transferred to NaCl or ABA supplemented media and referring them to those of the same genotypes transferred to non-supplemented media. These values are represented as percentages of (B) fresh weight and (C) root length of plants transferred to non-supplemented media. Each value corresponds to the mean ± SD of the fresh weight or root length of 15 plants of each genotype. One and two asterisks indicate that the value is significantly different from the wild-type at P<0.05 or P<0.01, respectively, using Student’s t-test. doi:10.1371/journal.pone.0042924.g005
Apart from germination and seedling establishment, flowering time, the other fundamental developmental transition, is also influenced by mTERF genes. Accordingly, mda1, soldat10 (data not shown) and rug2 [15] mutants are early flowering, which is in agreement with the finding that germination and flowering share genetic controls [41]. mTERFs might affect flowering by their

Table 2. Effect of sugars on the germination of the mda1 mutants.

| Genotype | Seedlings displaying green expanded cotyledons (%) | Non supplemented | Sucrose (mM) | Glucose (mM) |
|----------|--------------------------------------------------|-----------------|--------------|--------------|
|          |                                                  |                 | 30⁰          | 90⁰          | 175⁰         | 290⁰         | 330⁰         | 390⁰         |
| Col-0    |                                                  | 98.6±0.4        | 89.4±3.3     | 76.7±2.0     | 83.3±3.4     | 11.7±2.0     | 29.8±4.7     | 2.6±3.2      |
| mda1-1   |                                                  | 95.4±2.4        | 93.7±5.5     | 65.0±0.0     | 76.9±16.2    | 31.8±0.7     | 68.3±12.3    | 11.2±1.4     |
| mda1-2   |                                                  | 96.0±0.2        | 73.0±11.6    | 71.4±24.0    | 71.7±16.5    | N.D.         | 31.5±15.8    | 9.8±4.3      |

Values shown are the mean ± SD of the percentages of green expanded cotyledons seedlings referred to germinated seeds and obtained in two different experiments with at least 100 seeds per genotype in each experiment. Measurements were performed 4 and 10 das. N.D.: not determined.

doi:10.1371/journal.pone.0042924.t002

Figure 6. Genetic interactions between mTERF mutants. (A) Additive phenotypes of mda1 rug2-2 and mda1 soldat10 double mutants. Rosettes from a wild-type (Col-0) and the single mutants rug2-2, mda1-1 and mda1-2 (in a Col-0 genetic background), soldat10 (in a Ler genetic background) and the mda1-1 rug2-2, mda1-1 soldat10, mda1-2 rug2-2 and mda1-2 soldat10 double mutants. (B) Genetic interaction between rug2-1 and soldat10. Rosettes from the rug2-1 and soldat10 single mutants, both in a Ler genetic background, and the rug2-1 soldat10 double mutant. Pictures were taken 21 das. Scale bars: 1 mm.

doi:10.1371/journal.pone.0042924.g006
Table 3. Tolerance to ABA of the double mutants.

| Genotype | Remaining root length (%) a (4 μM ABA) | Seeding establishment (%) b (3 μM ABA) |
|----------|----------------------------------------|---------------------------------------|
| Col-0    | 91.4 ± 9.8                             | 63 ± 4.4                              |
| mda1-1   | 72.7 ± 11.2**                          | 53.4 ± 9.5                            |
| mda1-2   | 80.2 ± 12.5**                          | 38.4 ± 10.9                           |
| rug2-2   | 93.9 ± 15.7                            | 39.6 ± 10.9                           |
| mda1-1 rug2-2  | 80.7 ± 17.3*                          | 32.8 ± 5.2                            |
| Ler      | 77.3 ± 19.0                            | 1.9 ± 2.7                             |
| rug2-1   | 58.8 ± 17.1*                           | 0                                     |
| soldat10 | 67.0 ± 14.6                            | 1.1 ± 1.3                             |
| rug2-1 soldat10  | 49.3 ± 10.1**                          | 0                                     |
| mda1-1 soldat10 | 53.3 ± 23.4**f                        | N.D.                                  |
| mda1-2 soldat10 | 69.5 ± 24.7**e                        | 21.1 ± 12.5                           |

a These values are represented as percentages of root length of plants transferred to non-supplemented media as described in Methods. Each value corresponds to the mean ± SD of the root length of 15 plants of each genotype.
b Values shown are the mean ± SD of the percentages of green expanded cotyledons of seedlings of two replicates of 50 seeds each scored 10 das. N.D.: not determined.

*Mutant genotypes in a Col-0, *Ler or *Ler/Col-0 mixed genetic background, respectively.
**Differences were significant from Col-0 and Ler or *only from Col-0, respectively. Values were significantly different from the corresponding wild type at *P<0.05 or **P<0.01 using Student’s t-test. doi:10.1371/journal.pone.0042924.t003

...abiotic stress responses, probably through ABA signalling, in connecting chloroplast gene expression, ABA activity and plant adaptation to stress.

Materials and Methods

Plant Material and Growth Conditions

 Cultures and crosses were performed as described by Ponce et al. [52] and Bernár et al. [53], respectively. The seeds of the Arabidopsis thaliana (L.) Heynh. wild-type accessions Landsberg erecta (Ler) and Columbia-0 (Col-0) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The seeds of the T-DNA insertion lines (Table S3) were provided by the NASC and are described on the SIGnAL website ([19]; http://signal.salk.edu). The rug2-1 mutant was isolated in a Ler background after ethyl methanesulphonate (EMS) mutagenesis [53] and was backcrossed twice to the wild-type Ler [15]. The rug2-2 mutant in a Col-0 genetic background was characterised in a previous work [15]. The soldat10 seeds in a Ler genetic background were kindly provided by Klaus Apel (the Boyce Thompson Institute for Plant Research, Ithaca, NY). Pigment extraction and quantification were carried out as previously described [31].

Bioinformatics Analyses

Amino acid sequence comparisons and similarity searches were performed by using FASTA ([http://fasta.bioch.virginia.edu/fasta www2/fasta www.cgi, [54]]) and BLAST ([http://blast.ncbi.nlm.nih.gov/Blast.cgi, [55]])

Morphological and Ultrastructural Analyses

Dry weight was measured in plants that were oven-dried overnight at 55°C. Root, silique, stem and hypocotyl measurements were obtained with the ImageJ program ([http://rsb.info.nih.gov/ij/docs/menus/file.html] from pictures taken by a Leica MZ6 stereomicroscope equipped with a Nikon DXM1200 digital camera. A Student’s t-test was applied to the data obtained, with

| Genotype | Remaining root length (%) a (4 μM ABA) | Seeding establishment (%) b (3 μM ABA) |
|----------|----------------------------------------|---------------------------------------|
| Col-0    | 91.4 ± 9.8                             | 63 ± 4.4                              |
| mda1-1   | 72.7 ± 11.2**                          | 53.4 ± 9.5                            |
| mda1-2   | 80.2 ± 12.5**                          | 38.4 ± 10.9                           |
| rug2-2   | 93.9 ± 15.7                            | 39.6 ± 10.9                           |
| mda1-1 rug2-2  | 80.7 ± 17.3*                          | 32.8 ± 5.2                            |
| Ler      | 77.3 ± 19.0                            | 1.9 ± 2.7                             |
| rug2-1   | 58.8 ± 17.1*                           | 0                                     |
| soldat10 | 67.0 ± 14.6                            | 1.1 ± 1.3                             |
| rug2-1 soldat10  | 49.3 ± 10.1**                          | 0                                     |
| mda1-1 soldat10 | 53.3 ± 23.4**f                        | N.D.                                  |
| mda1-2 soldat10 | 69.5 ± 24.7**e                        | 21.1 ± 12.5                           |
a significance level of 0.01. Confocal imaging was performed as described by Hricová et al. [31]. For transmission electron microscopy, mutant and wild-type plant material was harvested at the same time of the day and prepared as described by Hricová et al. [31]. Samples were visualised under a Zeiss EM10C transmission electron microscope.

Identification of the T-DNA Insertions in Mutant Lines

To genotype mutants, we extracted DNA from the T₃, T₄ and T₅ mutant plants, from the F₂ segregating plants derived from the backcrosses, and from the F₁ plants derived from the complementation analyses. The DNA was PCR-amplified using the primers (RP and LP) designed by the T-DNA Primer Design (http://signal.salk.edu/tdnaprimer2.2html) tool, which were hybridised with the genomic sequences flanking the insertions in combination with the T-DNA specific primers LB1 or LBb1.3 (Table S4).

RNA Extraction and Semi-quantitative RT-PCR

Total RNA was extracted from 50–70 mg of 14-day-old Col-0, mda1-1 and mda1-2 14 das seedlings using TRIzol (Bioline) and treated with DNase I following the manufacturer’s instructions. RNA was ethanol-precipitated and resuspended in 40 µl of RNase-free water. Two to four micrograms of each sample were reverse-transcribed using random hexamers, and PCR amplification of first strand cDNA was performed as described by Quesada et al. [56]. Next, 1 µl of the resulting cDNA solution was used for the qRT-PCR amplifications. To detect the MDA1 transcripts in the Col-0 and mda1 mutants, different primers combinations were used as described in Table S4 and Figure S1A.

Quantitative RT-PCR

Total RNA was extracted from 50–70 mg of 14-day-old Col-0, Lrr, mda1-1, mda1-2, mda1-1 nrg2-2 and mda1-1 soldat10 seedlings, 3-week-old roots and vegetative leaves, and 45-day-old stems, cauline leaves and flowers and it was treated with DNase I following the manufacturer’s instructions. RNA was ethanol-precipitated and resuspended in 40 µl of RNase-free water. Two micrograms of RNA from each sample were reverse-transcribed using random hexamers, as described by Quesada et al. [56]. cDNAs were diluted three times with water and 1 µl of the resulting solution was used for qRT-PCR amplifications, which were carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Oligonucleotides (Table S4) were designed as described in Quesada et al. [15]. Each 25-µl reaction mix contained 12.5 µl of the SYBR-Green/ROX qPCR Master Kit (Fermentas), 0.4 µM of primers and 1 µl of the cDNA solution. Relative quantification of the gene expression data was performed by following the 2^{ΔΔCT} method [57]. Each reaction was done in two or three replicates and three different biological replicates were used. The expression levels were normalised to the Ct value obtained for the housekeeping OTC gene [56].

Seedling Establishment and Growth Sensitivity Assays

For the seedling establishment assays, sowings were conducted by plating seeds in a water suspension using a Pasteur pipette at a density of 100 regularly spaced seeds per Petri dish of GM agar medium (Murashige and Skoog medium containing 1% sucrose) supplemented with NaCl (0–200 mM), KCl (0–150 mM), mannitol (0–500 mM), ABA (1.5–6 µM), sucrose (30–290 mM) or glucose (330 and 390 mM). Seed germination was scored at 4, 10 or 14 das on Petri dishes kept at 20±1°C under 72 µmol m⁻² s⁻¹ of continuous light, considering that only those seedlings exhibiting green and fully expanded cotyledons displayed seedling establishment.

To evaluate the salt and ABA responses during later stages of plant growth, seeds were sown on Petri dishes containing GM agar medium. The seedlings were transferred 9 das to new plates containing agar medium supplemented with 100 mM NaCl or 4 mM ABA. Tolerance was estimated by determining plant fresh weight and root length after 12 days of stress treatment and by referring the values to those of plants transferred to non-supplemented media. Temperature-sensitivity assays were performed as previously described [31].

Supporting Information

Figure S1 Detection of MDA1 transcripts in Col-0 and mda1 mutants. (A) Structure of the At4g14605 (MDA1) gene indicating the positions of the T-DNA insertions in mda1-1 and mda1-2 by triangles. Boxes and lines indicate exons and introns, respectively. White boxes correspond to the 5’ and 3’ untranslated regions. Oligonucleotides used to study MDA1 expression are represented by horizontal arrows (not drawn to scale; Table S4). (B–C) PCR amplifications were performed using genomic DNA (gDNA) or complementary DNA (cDNA) from 2-week-old plants and primers hybridizing with (B) genomic sequences flanking the insertions in the mda1 mutants or (C) the LB of the T-DNAS and the upstream genomic region. The OTC gene was used as an internal control [56]. (PPT)

Figure S2 Quantitative RT-PCR analysis of the expression of the MDA1 gene. (A) Level of expression of the MDA1 gene in the wild type (Col-0) and the mda1 mutants and (B) in different organs of Col-0 plants after normalisation with those of the OTC gene (see Material and methods). Bars indicate relative levels of expression, determined as 2^{ΔΔCT}. A value of 1 is assigned to MDA1 expression in Col-0 (A) and roots (B). Error bars indicate the range of variation of the 2^{ΔΔCT} values, obtained using three different biological replicates and triplicate reactions. (PPT)

Figure S3 Effect of temperature on the growth of the mda1 mutants. Representative plants are shown for (A, D, G) Col-0, (B, E, H) mda1-1 and (C, F, I) mda1-2, which were grown at 15 (A-C), 20 (D-F) or 25°C (G-I). (J) Fresh weight of Col-0 and mda1 individuals grown 21 days at 15°C. Each value represents the percentage of fresh weight of unstressed plants (grown at 20°C). Error bars indicate the mean ± standard deviation (SD) of the fresh weight of 15 plants of each genotype. An asterisk indicates that the value is significantly different from the wild type at P<0.05 using Student’s t-test. Pictures were taken 21 das. Scale bars indicate 1 mm. (PPT)

Figure S4 Sugar sensitivity of the mda1-1 mutant. Pictures are shown for (A, B) Col-0 and (C, D) mda1-1 plants grown (A, C) in the presence of 30 mM or (B, D) 175 mM of sucrose. (E) Concentration (µg/g of fresh weight) of chlorophyll a (Ca) and b (Cb) in Col-0 and mda1-1 plants grown on media supplemented with 30 or 175 mM of sucrose. Data represent mean of 10 samples of 15-day-old plants per genotype ± SD. One and two asterisks indicate that the values are significantly different at P<0.05 or P<0.01, respectively, using Student’s t-test. Pictures were taken 21 das. Scale bars indicate 1 mm. (PPT)

Table S1 Arabidopsis mTERF proteins.
Table S2  Rice mTERF proteins.

Table S3  T-DNA lines for mTERF genes used in this work.

Table S4  Primers used in this work.

Table S5  Tiling array expression for Arabidopsis mTERF genes.

References

1. Binder S, Brennicke A (2003) Gene expression in plant mitochondria: transcriptional and post-transcriptional control. Philos Trans R Soc Lond B Biol Sci 358: 181–189.
2. Linder T, Park CB, Asín-Cayuela J, Pellegrini M, Larsson NG, et al. (2005) A family of putative transcription termination factors shared among metazoans and plants. Curr Genet 46: 265–269.
3. Roberti M, Polosa PL, Bruni F, Manzari C, Deceglie S, et al. (2009) The mTERF family of proteins: mitochondrial transcription regulates and beyond. Biochim Biophys Acta 1787: 303–311.
4. Martín M, Cho J, Cesare AJ, Griffith JD, Attardi G (2005) Formation-mediated DNA loop between termination and initiation sites drives mitochondrial RNA synthesis. Cell 123: 1227–1240.
5. Hyvarinen AK, Pohjoismäki JL, Reyes A, Wamposoq, Y. Sasakawa T, et al. (2007) The mitochondrial transcription termination factor mTERF modulates replication pausing in human mitochondrial DNA. Nucleic Acids Res 35: 6536–6547.
6. Wenz T, Laca G, Terraço A, Moraes CT (2009) mTERF regulates oxidative phosphorylation by modulating mtDNA transcription. Cell Metab 9: 499–511.
7. Park CB, Asín-Cayuela J, Cámara Y, Shi Y, Pellegrini M, et al. (2007) MTERF is a negative regulator of mammalian mtDNA transcription. Cell 130: 273–285.
8. Cámara Y, Asín-Cayuela J, Park CB, Metodiev MD, Shi Y, et al. (2011) MTERF4 regulates translation by targeting the mitophagy factor NSUN4 to the mammalian mitochondrial ribosome. Cell Metab 13: 527–539.
9. Köhler P, Micol JL, Quesada V (2012) Unveiling plant mTERF functions. Mol Plant 5: 294–296.
10. Pfalz J, Lièvre K, Kandelbinder A, Dietz JK, Oelman R (2006) PTA2C-6, and-12 are components of the transcriptionally active plastid chromosome that are required for plastid gene expression. Plant Cell 18: 176–197.
11. Majerus W, Frito G, Askarova Y, Xu X, Huang M, et al. (2011) Nucleoid-enriched proteomes in developing plastids and chloroplasts from maize leaves: a new conceptual framework for nuclearuld functions. Plant Physiol 158: 156–189.
12. Schondel C, Wobbe L, Borgetzad R, Kienast A, Nixon PJ, et al. (2004) The mtDNA-encoded mitochondrial light-actin gene is essential for mitochondrial light function in Chlamydomonas reinhardtii. J Biol Chem 279: 50366–50374.
13. Meskauskienë R, Wurth M, Lalo C, Vidi S, Coll N, et al. (2009) A mutation in the Arabidopsis mTERF-related plastid protein SOLDAT10 activates retrograde signaling and suppresses Vrn-induced cell death. Plant J 60: 399–410.
14. Babitsch K, Vandevoorde K, Wissing J, Garcia-Diaz M, De Rycke R, et al. (2011) Plastid gene expression and plant development require a plastidic protein of the mitochondrial transcription termination factor family. Proc Natl Acad Sci U S A 108: 6674–6679.
15. Quesada V, Sarmiento-Manús R, González-Bayoń R, Hricová A, Pérez-Marcos E, et al. (2011) Arabidopsis RUGG4Encodes an mTERF family member required for mitochondrial, chloroplast and leaf development. Plant J 68: 738–753.
16. Emmanuelson O, Nielsen H, Brunnak S, von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol 300: 1005–1016.
17. Small I, Feeters N, Legge F, Larin C (2004) Predstar: A tool for rapidly screening proteomes for N-terminal targeting sequences. Proteomics 4: 298–306.
18. Bannai H, Tamada Y, Maruyama O, Nakai K, Miyano S (2002) Extensive feature detection of N-terminal protein sorting signals. Bioinformatics 18: 298–309.
19. Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, et al. (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science 301: 653–657.
20. Sessions A, Burke E, Presting G, Aux G, McElver J, et al. (2002) A high-throughput Arabidopsis reverse genetics system. Plant Cell 14: 2985–2994.
21. Winter D, Vinegar B, Nahal H, Ammar M, Wilson GV, et al. (2007) An “Electronic Fluorescent Pictograph” browser for exploring and analyzing large-scale biological data sets. PLoS One 2: e178.
22. Kilian J, Whitehead D, He J, Wanke D, Weinl S, et al. (2007) The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. Plant J 50: 347–363.

Acknowledgments

We thank H. Candela for his useful comments on the manuscript, the NASC for providing seeds and J.M. Serrano, S. Moya-Raez, E. Castellano-Martínez, R. Cano-García, F. Gea-Caselles and A.M. Hernández-Miranzo for their excellent technical assistance.

Author Contributions

Conceived and designed the experiments: VQ. Performed the experiments: PR. VQ. Analyzed the data: PR. JLM. VQ. Wrote the paper: VQ.
47. Chan KX, Crisp PA, Estavillo GM, Popson JJ (2010) Chloroplast-to-nucleus communication: current knowledge, experimental strategies and relationship to drought stress signaling. Plant Signal Behav 5: 1575–1582.
48. Koussevitzky S, Nott A, Mockler TC, Hong F, Sachetto-Martins G, et al. (2007) Signals from chloroplasts converge to regulate nuclear gene expression. Science 316: 715–719.
49. Giraud E, Van Aken O, Ho LH, Whelan J (2009) The transcription factor ABF4 is a regulator of mitochondrial retrograde expression of ALTERNATIVE OXIDASE1a. Plant Physiol 150: 1286–1296.
50. Kim C, Lee KP, Baruah A, Nater M, Gobel C, et al. (2009) 1O2-mediated retrograde signaling during late embryogenesis predetermines plastid differentiation in seedlings by recruiting abscisic acid. Proc Natl Acad Sci U S A 106: 9920–9924.
51. Zhao Z, Zhang W, Stanley BA, Assmann SM (2008) Functional proteomics of Arabidopsis thaliana guard cells uncovers new stomatal signaling pathways. Plant Cell 20: 3210–3226.
52. Ponce MR, Quesada V, Micol JL (1998) Rapid discrimination of sequences flanking and within T-DNA insertions in the Arabidopsis genome. Plant J 14: 497–501.
53. Berna G, Robles P, Micol JL (1999) A mutational analysis of leaf morphogenesis in Arabidopsis thaliana. Genetics 152: 729–742.
54. Lipman DJ, Pearson WR (1985) Rapid and sensitive protein similarity searches. Science 227: 1435–1441.55.
55. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410.
56. Quesada V, Ponce MR, Micol JL (1999) OTF and AUL1, two convergent and overlapping genes in the nuclear genome of Arabidopsis thaliana. FEBS Lett 461: 101–106.
57. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta CT). Methods 25: 402–408.