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Arabidopsis mTERF6 is required for leaf patterning

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ABSTRACT

To enhance our understanding of the roles of mitochondrial transcription termination factors (mTERFs) in plants, we have taken a reverse genetic approach in *Arabidopsis thaliana*. One of the mutants isolated carried a novel allele of the *mTERF6* gene, which we named *mterf6-5*. mTERF6 is a chloroplast and mitochondrial localised protein required for the maturation of chloroplast isoleucine tRNA. The *mterf6-5* plants are pale and exhibit markedly reduced growth, and altered leaf and chloroplast development. Our qRT-PCR analyses revealed misexpression of several plastid, mitochondrial and nuclear genes in *mterf6-5* plants. Synergistic phenotypes were observed in double mutant combinations of *mterf6-5* with alleles of other *mTERF* genes as well as with *scabra3-2*, affected in the plastid RpoTp RNA polymerase; these observations suggest a functional relationship between *mTERF6*, other *mTERFs* and *SCA3*. The *mterf6-5* mutation also enhanced the leaf dorsoventral polarity defects of the *asymmetric leaves1-1* (*as1-1*) mutant, which resulted in radial leaves. This interaction seemed specific of the impaired *mTERF6* function because mutations in the *mTERF* genes *MDA1* or *TWR-1/mTERF9* did not result in radialised leaves. Furthermore, the *mterf6-5* mutation dramatically increased the leaf phenotype of *as2-1* and caused lethality early in vegetative development. Our results uncover a new role for *mTERF6* in leaf patterning and highlight the importance of mTERFs in plant development.

1. Introduction

Chloroplasts are organelles with a double membrane that possess their own genome since they evolved from a cyanobacteria-like prokaryote that established an endosymbiotic relationship with an ancestral eukaryotic cell. During the course of evolution, the endosymbiont genome underwent an intense process of erosion, with the vast majority of its genes being relocated to the genome of the host. Consequently, the chloroplast genomes of higher plants range from 120 to 160 kb in size and contain about 100 genes that encode proteins for plastid gene expression (PGE) and photosynthesis [1]. The genes for rRNA, tRNA, and numerous non-coding RNAs have also been identified [2,3]. Experimental and bioinformatic estimates indicate that the chloroplast proteome includes around 3000 proteins [4], most of which are encoded by nuclear genes. These proteins are synthesised in the cytoplasm and later transported to the organelle. Hence, chloroplast multiprotein complexes are mosaics of nuclear- and plastid-genome encoded products.

Given the small size and number of genes of the chloroplast genomes, their transcriptional regulation seems unexpectedly complex. This complexity can be explained only partially with the transcriptional components known to date. Transcription of chloroplast genes is carried out by two types of RNA polymerases; a plastid-encoded polymerase (PEP), which derives from the cyanobacteria ancestor, and a single-subunit-bacteriophage type, which is encoded by the nucleus (NEP: nuclear encoded polymerase) [5]. Monocotyledonous plants present a specific NEP of mitochondria and another one of chloroplasts, whereas three NEPs, RpoTm, RpoTp and RpoTmp, which are located in mitochondria, chloroplasts and in both organelles respectively, have been identified in dicotyledonous plants (reviewed in [6]). For a proper transcriptional regulation in mitochondria and chloroplasts, especially in the latter, regulatory proteins encoded by nuclear genes are also required. In fact, PEP is a complex composed of multiple subunits, which requires sigma factors encoded by the nuclear genome to recognise promoters, similarly to what happens in prokaryotes. Transcription of the genome of mature chloroplasts occurs within large DNA and protein complexes called nucleoids or pTACs (plastid transcriptionally active chromosomes), which include PEP-core subunits and more than 50 additional nuclear-encoded proteins [7]. The proteins identified as pTAC components that fulfil RNA/DNA-associated

Abbreviations: DAS, days after stratification; Fv/Fm, variable fluorescence/maximum fluorescence; mTERFs, mitochondrial transcription termination factors; NEP, nuclear encoded polymerase; PEP, plastid-encoded polymerase; PSII, photosystem II; pTAC, plastid transcriptionally active chromosome; WT, wild-type

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functions, and which potentially act during post-transcriptional processes, include PPRs (pentatricopeptide repeat proteins) and mTERFs (mitochondrial transcription termination factors) [8–10]. mTERFs and PPRs are modular helical-repeat proteins, and some are able to bind to RNA and/or DNA, and to participate in different organelle gene expression-related aspects (OGE). Interestingly, plant genomes contain many more PPR and mTERF proteins than animals, but only a few have been characterised, as described below [11–13].

Despite there being more mTERFs in plants than in mammals [11], information on the function of plant mTERF genes is still rather limited. Indeed, whereas four mammal mTERFs have been characterized in detail, only a mutant phenotype has been reported for a few of the 35 Arabidopsis and 31 maize mTERFs already identified [12], and a molecular function can be assigned to an even smaller number. Paleness (due to low chlorophyll levels), low growth rates and altered transcript levels of chloroplast and/or mitochondrial genes have been associated to loss-of-function alleles of the *Zea mays Zm-mTERF4* [14] and Arabidopsis *SOLDAT10* (*SINGLET OXYGEN-LINKED DEATH ACTIVATOR10*) [15], *BSM/RUG2* (*BELAYA SMERT/RUGOSA2*) [16,17], *MDA1* (*mTERF DEFECTIVE IN Arabidopsis1*) [18], *mTERF6* [19,20], *TWR-1/mTERF9* (*TWIRT1/mTERF9*) [21,22], *mTERF15* [23] and *SHOT1* (*SUPPRESSOR OF HOT1-4*) [24] genes. Some *mterf* mutants, such as *rug2*, *mda1* and *mterf9,* also show impaired chloroplast biogenesis and aberrant internal leaf structure [17,18,22]. Furthermore, *soldat10*, *shot1*, *mda1* and *mterf9* display an altered response to stress, which supports an emerging role for plant mTERFs in response to abiotic stress [13]. Consistent with this, an involvement for Arabidopsis mTERF10 and mTERF11 in the response to salt stress has been recently proposed [25]. All characterised plant mTERFs are chloroplast proteins, except for mitochondrial SHOT1 and mTERF15, and chloroplast and mitochondrial BSM/RUG2 and mTERF6. Additional evidence of the importance of plant mTERFs is provided by alleles of *mTERF2/EMB2219* (*mTERF2/EMBRYO DEFECTIVE 2219*), *BSM/RUG2* and *mTERF16* that arrest embryonic development [12].

In the last few years, a molecular function has been proposed for some *mTERF* genes characterised in photosynthetic organisms. The mTERF-like MOC1 protein from the green alga *Chlamydomonas reinhardtii* acts as a terminator of mitochondrial antisense transcription [26]. BSM/RUG2 and its orthologue in maize, *Zm-mTERF4,* are required for the splicing of the second group IIa intron of the plastid *clpP* gene [16] and the mitochondrial group II introns [14], respectively. mTERF15 is also involved in intron splicing, specifically in *nad2* intron 3 splicing in mitochondria [23]. Recently, Arabidopsis mTERF6, a protein dually targeted to chloroplasts and mitochondria, was found to bind to the transcript of the chloroplast isoleucine transfer RNA (*trnI.2*) gene and to promote its maturation [19]. Also recently, a prominent role in the aminoacylation of tRNA for isoleucine has been demonstrated for mTERF6 [20]. Taken together, these results suggest additional molecular functions for plant mTERFs.

Here, we describe the characterisation of a new loss-of-function mutant allele of the *mTERF6* gene, which we named *mterf6-5* according to the work by Romani et al. [19]. *mterf6-5* plants exhibited markedly reduced growth, paleness and abnormal leaf development and chloroplast biogenesis. Altered expression of several organellar and nuclear genes in this mutant suggests that defective chloroplast and/or mitochondrial function was signalled to the nucleus. *mterf6-5* interacted in double mutant combinations with other mutations impairing chloroplast biogenesis, and unexpectedly enhanced the polarity defects of the leaf patterning mutant *asymmetric leaves1-1* (*as1-1*). In addition, *mterf6-5 as2-1* double mutants resulted lethal shortly after germination. In conclusion, our analysis of the *mterf6-5* mutant revealed an unexpected role for *mTERFs* in abaxial-adaxial leaf patterning and highlighted the importance of this family of proteins in plant development.

2. Material and methods

2.1. Plant material and growth conditions

Plant cultures and crosses were performed as previously described [18]. The seeds of the *Arabidopsis thaliana* (L.) Heynh. wild-type (WT) accession Columbia-0 (Col-0) were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC). Seeds of transferred DNA (T-DNA) insertion lines SAIL_360_H09 (*mterf6-2*), SALK_116335 (*mterf6-5*), SALK_010822 (*rpl24-1*) and SALK_095863 (*rps5*) were provided by the NASC and are described on the SIGnAL website (http://signal.salk.edu). The *rug2-2, mda1-1*, *mterf9*, *sca3-2*, *as1-1* and *as2-1* mutants, in a Col-0 genetic background, have been previously described [17,18,22,27,28]. *soldat10* seeds [in a Landsberg *erecta* (L*er*) genetic background] were kindly provided by Klaus Apel (Boyce Thompson Institute for Plant Research, Ithaca, NY, USA).

2.2. Pigment determination and photosynthesis analysis

Pigment extraction and quantification were performed as already reported [27]. The photosynthetic maximum quantum yield was measured 20 days after stratification (DAS) by a DUAL-PAM/F fluorometer and a DUAL-BA leaf-positioning device (WALZ), as described in [28].

2.3. In silico analyses

Comparisons of amino acid sequences and similarity searches were made using FASTA [29] and BLAST [30]. Co-expression data for the *mTERF6* gene were retrieved from Genevestigator [31]. The GO annotation tool from TAIR [32] was used for the functional categorisation of the co-expressed genes and the annotation of the Gene Ontology (GO) terms. The PLAZA database for comparative plant genomics [33,34] was used to identify *mTERF6* orthologues in photosynthetic species.

2.4. Morphological and ultrastructural analyses

For light microscopy, plant material was fixed with FAA/Triton (1.85% formaldehyde, 45% ethanol, 5% acetic acid, and 1% Triton X-100). Transverse sections of leaves were prepared as described in [17], and were observed under a Leica DMRB microscope equipped with a Nikon DXM1200 digital camera under bright-field illumination. Confocal imaging was performed as reported in [18]. For transmission electron microscopy, plant material was collected at the same time of day and prepared as described by Hricová et al. [27]. A Zeiss EM10C transmission electron microscope (Zeiss, http://www.zeis.com) was used to visualise samples. Flowering time was scored as the number of vegetative leaves and the number of days upon bolting of plants grown under continuous light.

2.5. Identification of T-DNA in insertional lines

DNA was extracted from T_3, T_4 or T_5 *mterf6-5* and *mterf6-2* mutant plants, from F_2 and F_3 double mutant segregating plants, and from F_1 plants derived from the *mterf6-5* x *mterf6-2* cross. The DNA of *rpl24-1, rps5* and *mterf6-5* DNA was PCR-amplified using the primers (RP and LP) designed by the T-DNA Primer Design Tool (http://signal.salk.edu/tdnaprimers.2.html), which hybridised with the genomic sequences that flanked the insertions, in combination with the T-DNA-specific primer LBa1 (Table S1). *mterf6-2* DNA was PCR-amplified using primers At4g38160-R1, At4g38160-F2 and LB1 (Table S1).

2.6. RNA extraction and semi-quantitative RT-PCR (sqRT-PCR)

Total RNA was extracted using TRIsure (Bioline) and treated with DNase I following the manufacturer's instructions, from 80 mg of Col-0 and *mterf6-5* of 13 days after stratification (DAS) seedlings. RNA was