the plant journal

SEB Society for Experimental Biology

The Plant Journal (2011) **68**, 738–753 doi: 10.1111/j.1365-313X.2011.04726.x

Arabidopsis *RUGOSA2* encodes an mTERF family member required for mitochondrion, chloroplast and leaf development

Víctor Quesada[†], Raquel Sarmiento-Mañús[†], Rebeca González-Bayón, Andrea Hricová[‡], Rubén Pérez-Marcos, Eva Graciá-Martínez, Laura Medina-Ruiz, Eduardo Leyva-Díaz, María Rosa Ponce and José Luis Micol[*]

Instituto de Bioingeniería, Universidad Miguel Hernández, Campus de Elche, 03202 Elche, Spain

Received 23 June 2011; revised 20 July 2011; accepted 22 July 2011; published online 13 September 2011.
[*]For correspondence (fax+ 34 96 665 85 11; e-mail jlmicol@umh.es).
[†]These authors contributed equally to this work.
[‡]Present address: Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Akademická 2, 95007 Nitra, Slovak Republic.

SUMMARY

Little is known about the mechanisms that control transcription of the mitochondrial and chloroplastic genomes, and their interplay within plant cells. Here, we describe the positional cloning of the Arabidopsis *RUG2* gene, which encodes a protein that is dual-targeted to mitochondria and chloroplasts, and is homologous with the metazoan mitochondrial transcription termination factors (mTERFs). In the loss-of-function *rug2* mutants, most organs were pale and showed reduced growth, and the leaves exhibited both green and pale sectors, with the latter containing sparsely packed mesophyll cells. Chloroplast and mitochondrion development were strongly perturbed in the *rug2-1* mutant, particularly in pale leaf sectors, in which chloroplasts were abnormally shaped and reduced in number, thereby impairing photoautotrophic growth. As expected from the pleiotropic phenotypes caused by its loss-of-function alleles, the *RUG2* gene was ubiquitously expressed. In a microarray analysis of the mitochondrial and chloroplastic genomes, 56 genes were differentially expressed between *rug2-1* and the wild type: most mitochondrial genes were downregulated, whereas the majority of the chloroplastic genes were upregulated. Quantitative RT-PCR analyses showed that the *rug2-1* mutation specifically increases expression of the *RpoTp* nuclear gene, which encodes chloroplastic RNA polymerase. Therefore, the *RUG2* nuclear gene seems to be crucial for the maintenance of the correct levels of transcripts in the mitochondria and chloroplasts, which is essential for optimized functions of these organelles and proper plant development. Our results highlight the complexity of the functional interaction between these two organelles and the nucleus.

Keywords: leaf development, Arabidopsis, chloroplast and mitochondrion biogenesis, mTERF, variegation, *rugosa*.

INTRODUCTION

The molecular mechanisms that regulate leaf morphogenesis are far from being fully understood. One of the most successful approaches to the dissection of this process has been the isolation and characterization of mutants with altered leaf morphology, hundreds of which have been identified thus far in different plant species (Berná *et al.*, 1999; Micol and Hake, 2003; Fleming, 2005; Tsukaya *et al.*, 2007; Micol, 2009). Some leaf mutants show a severely altered internal leaf structure, with mesophyll cells containing defective plastids. A category of these mutants exhibits patches of green and white or yellowish sectors in normally green tissues or organs, a phenotype known as variegation. Typically, chloroplasts are morphologically normal in the green tissues of variegated mutants, whereas those in the pale sectors are defective (Rodermel, 2001).

Leaf variegation has been described in a number of plant species as a consequence of mutations in mitochondrial genes (Newton and Coe, 1986; Roussell *et al.*, 1991; Gu *et al.*, 1993) as well as in nuclear genes, the products of which are targeted to mitochondria (Abdelnoor *et al.*, 2003) or chloroplasts (Chatterjee *et al.*, 1996; Keddie *et al.*, 1996; Berthold *et al.*, 2000; Chen *et al.*, 2000; Takechi *et al.*, 2000; Sakamoto *et al.*, 2002; Naested *et al.*, 2004). Other cases of variegation result from the instability of transposable

© 2011 The Authors
The Plant Journal © 2011 Blackwell Publishing Ltd

element insertions in nuclear genes of maize (Rhoades, 1943), *Oenothera* (Epp, 1973) and barley (Ahokas, 1976).

Hundreds of variegated mutants have been isolated in *Arabidopsis thaliana* (Yu *et al.*, 2007). Recessive alleles of some nuclear genes that affect chloroplast development and cause leaf variegation have made it possible to ascertain their functions. Examples are *chloroplast mutator* (*chm*) and *immutans* (*im*) (Rédei and Plurad, 1973), *yellow variegated 1* (*var1*), *var2* (Martínez-Zapater, 1993), *var3* (Sundaresan *et al.*, 1995) and *thylakoid formation 1* (*thf1*; Wang *et al.*, 2004; Keren *et al.*, 2005; Huang *et al.*, 2006). The *IM* gene encodes a chloroplast protein similar to the alternative oxidases (AOXs) of the mitochondrial inner membrane that act as terminal oxidases in the alternative pathway of mitochondrial respiration (Berthold *et al.*, 2000). Variegation in *im* mutants is likely to result from photo-oxidation in developing plastids. VAR1 and VAR2 are chloroplast-localized homologs of the *Escherichia coli* FtsH protein, an ATP-dependent zinc metalloprotease (Chen *et al.*, 2000). Variegation in the *var1* and *var2* mutants has been proposed to be the result of photosystem II (PSII) photo-inhibition caused by the accumulation of photodamaged D1 protein, a subunit of the PSII reaction center core (Lindahl *et al.*, 2000; Sakamoto *et al.*, 2002; Bailey *et al.*, 2002). Although *VAR3,* which is unrelated to *VAR1* and *VAR2*, encodes a protein containing two zinc-finger domains, the *var3* variegation phenotype resembles those of *var1* and *var2* (Naested *et al.*, 2004). VAR3 might participate in carotenoid biosynthesis, because it interacts with the NCED4 carotenoid dioxygenase in yeast two-hybrid experiments (Naested *et al.*, 2004). The loss of function of the *THF1* gene, which encodes a chloroplast-targeted protein, causes variegation (Wang *et al.*, 2004) and impairs PSII function (Keren *et al.*, 2005). Like Psb29, THF1 is a PSII component (Keren *et al.*, 2005), and a role for THF1 in sugar signaling through its interaction with the plasma membrane-delimited G protein GPA1 has recently been demonstrated (Huang *et al.*, 2006). Variegation is also exhibited by antisense lines in which the Arabidopsis *ClpP4* and *ClpP6* nuclear genes, encoding proteolytic subunits of the plastid-localized ATP-dependent Clp protease, are knocked down (Zheng *et al.*, 2006; Sjögren *et al.*, 2006).

The aforementioned *IM*, *VAR1*, *VAR2*, *VAR3* and *THF1* genes encode chloroplast-localized proteins, and their loss of function causes variegation by perturbing chloroplast function. A different cause of variegation is the impairment of mitochondria, which, in turn, affects chloroplast development as a consequence of the metabolic interconnection between these organelles. Mitochondria provide ATP via the electron transfer chain, but their activity is also essential for dissipation of excess redox equivalents in the chloroplast stroma, for photosynthesis optimization and for protection of chloroplasts against photoinhibition (Raghavendra and Padmasree, 2003). Mutations affecting mitochondrial development are known to severely disrupt chloroplast morphology, function and gene expression, leading to variegated phenotypes, as observed in non-chromosomal stripe (NCS) mutants of maize and the Arabidopsis *chm* mutant. Several mitochondrial functions are impaired in maize NCS mutants as a consequence of specific deletions in essential mitochondrial genes, such as *cox2* (which encodes cytochrome oxidase subunit 2) in NCS6 (Newton *et al.*, 1990; Gu *et al.*, 1993; Jiao *et al.*, 2005), *rps3* and *rpL16* (encoding ribosomal proteins; Hunt and Newton, 1991) in NCS3, and *nad4* and *nad7* (encoding subunits of the electron transfer chain complex I; Marienfeld and Newton, 1994) in NCS2. The Arabidopsis mitochondrial CHM protein is similar to the yeast Msh1p and *E. coli* MutS proteins, which are both required for DNA mismatch repair and recombination (Abdelnoor *et al.*, 2003). A function for *CHM* has been proposed in the control of mitochondrial genome substoichometric shifting (differential copy number control) to repress aberrant recombination and/or the replication of abnormal genomes.

Here we report the genetic and molecular characterization of two hypomorphic alleles of the *RUGOSA2* (*RUG2*; Berná *et al.*, 1999) gene, which was found to encode a protein targeted to mitochondria and chloroplasts, with similarity to metazoan mitochondrial transcription termination factors (mTERFs). Our results indicate that RUG2 activity is required for leaf development in Arabidopsis, and its loss-of-function leads to a pleiotropic phenotype that includes leaf variegation, reduced growth, and perturbed mitochondrial and chloroplastic gene expression and development.

RESULTS

Positional cloning of the *RUG2* gene

In a large-scale screen for EMS-induced mutations affecting leaf morphology, we identified two Arabidopsis mutants with small vegetative leaves and rounded and protruding laminae (Figure 1), which were assigned to a phenotypic class that we named Rugosa (Rug), and were found to fall into two complementation groups (*RUG1* and *RUG2*; Berná *et al.*, 1999). We have already low-resolution mapped the *rug1* and *rug2* recessive mutations (Robles and Micol, 2001).

A Col-0 × *rug2-1* (in a L*er* background) cross was performed, and the DNA of F_2 plants exhibiting a leaf phenotype similar to that of the *rug2-1* parental (Figure 1 and above) was extracted and used to carry out linkage analysis (Table S1). A 72-kb candidate interval was defined in this way on chromosome 4, encompassing 17 annotated genes (Figure 2a). Sequencing of the candidate genes in the *rug2-1* mutant and its wild-type L*er* identified a C → T transition mutation at position 1259 (relative to the predicted translation initiation codon; Figure 2b) of the *At4g02990* gene, which substitutes an extremely conserved proline residue by leucine at position 420 of the RUG2 protein (Figure 2c).

© 2011 The Authors

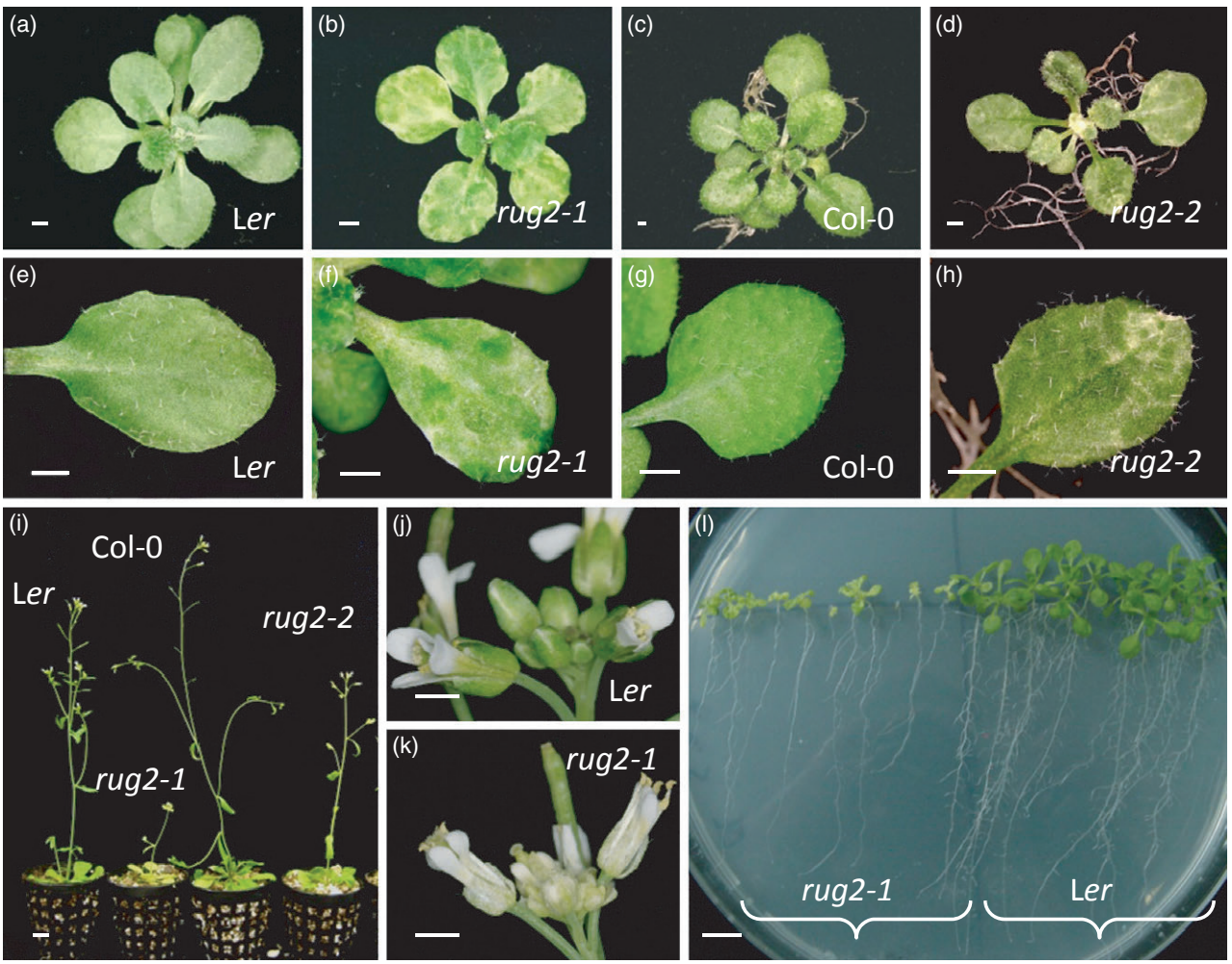

Figure 1. Some morphological traits of the *rug2* mutants. (a–d) Representative rosettes and (e–h) third vegetative leaves of the *rug2-1* (in a L*er* genetic background) and *rug2-2* (in a Col-0 genetic background) mutants, and their corresponding wild types. (i) Plants grown in soil (45-days old). (j, k) Details of *rug2-1* and L*er* inflorescences. (l) Plants grown on vertically orientated agar plates (15-days old). All plants were homozygous for the mutations shown. Scale bars: 1 mm (a, b, d–h, j, k); 2 mm (c); 1 cm (i, l).

A second mutant allele of *RUG2* was found in the Salk collection of T-DNA lines. The N533963 line, as annotated on the SIGnAL website (http://signal.salk.edu/), bears a T-DNA insertion at nucleotide 2269 (Figure 2b; Table S1) in the 3′ untranslated region (3′-UTR) of the *At4g02990* transcription unit, 143 nucleotides downstream of the predicted stop codon (TAA; Figure 2b). The F_1 progeny of a cross between *rug2-1* and N533963 displayed a mutant phenotype, confirming that mutation at *At4g02990* results in the phenotypes of *rug2-1* and N533963, which we named *rug2-2.* The mutant phenotype of *rug2-1* was complemented by a transgene carrying the wild-type allele of *RUG2* fused to the *35S* promoter (Figure S1; see Experimental procedures).

RUG2 is an mTERF family member

The predicted product of the *At4g02990* (*RUG2*) gene is a 61.5-kDa protein of 541 amino acids in length that shares similarity to mTERFs, a large family present in metazoans and plants (Linder *et al.*, 2005). The IPSORT, MITOPROT II and TARGETP programs predicted RUG2 to be mitochondrial, as indicated in the Subcellular Proteomic Database (SUBA, http://suba.plantenergy.uwa.edu.au/index.php), although for the latter program, similar scores for mitochondria (0.264) or chloroplasts (0.191) were obtained. To experimentally determine the subcellular localization of the RUG2 protein, stably transgenic Arabidopsis plants expressing a construct encoding the first 103 amino acid residues of RUG2 fused to GFP (RUG2-N-terminal:GFP) were obtained, and GFP signals were detected in large, oval-shaped subcellular structures completely overlapping with chlorophyll autofluorescence (Figure 3a–c, g–i). Palisade cells from Col-0 and a transgenic line carrying a mitochondria-targeted GFP construct were used as controls (Logan and Leaver, 2000; Figure S2; see Experimental proceudres). Leaf epidermal and mesophyll cells also displayed punctate green fluorescence, the size, shape and

© 2011 The Authors

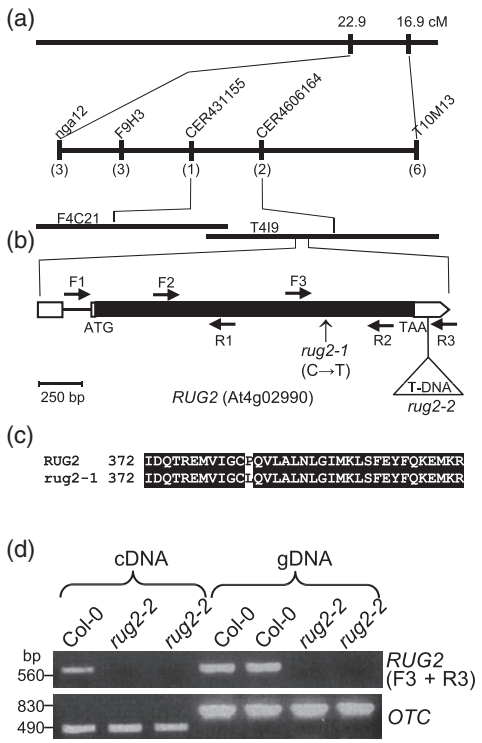

Figure 2. Identification and structural analysis of the *RUG2* gene.
(a) Map-based cloning strategy used to identify the *RUG2* gene. We identified 15 recombination events (in brackets) in a 6-cM region on chromosome 4 flanked by the nga12 and T10M13 markers. A 72-kb candidate interval partly encompassed the F4C21 and T4I9 BAC clones, and included 17 annotated genes (*At4g02960*–*At4g03115*).
(b) Structure of the *RUG2* gene indicating the nature and positions of the *rug2* mutations. Boxes and lines indicate exons and introns, respectively. White boxes correspond to the 5′- and 3′-UTRs. The triangle indicates a T-DNA insertion. Oligonucleotides used to study *RUG2* are represented by horizontal arrows (not drawn to scale; see Table S1).
(c) Partial alignment of the amino acid sequences of the RUG2 and rug2-1 protein products.
(d) Detection of *RUG2* transcripts in *rug2-2* and Col-0. PCR amplifications were performed using genomic DNA (gDNA) or complementary DNA (cDNA) from 3-week-old plants and the primers shown (see Table S1). The *OTC* gene was used as an internal control.

subcellular distribution of which was consistent with mitochondria (Figure 3b,e,f). In order to confirm the identity of the organelle giving the punctate staining pattern, protoplasts were isolated according to Yoo *et al.* (2007) from RUG2-N-terminal:GFP stably transgenic plants, and were stained with MitoTracker Red CMX-ROS, a specific mitochondria fluorescence dye (see Experimental procedures). The green fluorescence signals in the punctate structures co-localized with the MitoTracker Red signals (Figure 3j,l), suggesting that RUG2 accumulates within the mitochondria and, hence, supporting the dual localization of this protein in chloroplasts and mitochondria.

Analysis of RUG2 with SMART (http://smart.embl-heidelberg.de) revealed a modular architecture with 10 mTERF motif repeats, each of which is about 31 amino acids in length (Figure 4). Interestingly, a proline residue is highly conserved at position 8 of every mTERF motif, and this residue is replaced by leucine at motif 9 in the *rug2-1* allele (Figure 4). Human MTERF1 contains leucine zipper domains (Fernández-Silva *et al.*, 1997), but we found no clear evidence for these in RUG2, although some leucines and similar hydrophobic amino acids were highly conserved among different mTERF family members (Figure 4).

We identify 34 putative *RUG2* paralogs in the Arabidopsis genome (http://www.ncbi.nlm.nih.gov/BLAST; Figure S3). RUG2 displayed 55.0, 44.7, 41.6, 25.8, 25.2, 25.1, 24.1 and 23.3% overall sequence identity with mTERF proteins from rice, *Medicago truncatula*, Arabidopsis, *Drosophila melanogaster*, *Paracentrotus lividus*, *Chlamydomonas reinhardtii*, human and mouse, respectively (Figure 4). Hence, the amino acid sequence similarity was higher among terrestrial plants than in unicellular algae or animals.

External morphology of the *rug2* mutants

The most conspicuous trait of the *rug2* mutants was the presence of green and yellowish leaf sectors in their vegetative leaves (Figure 1a–h), a phenotype reminiscent of some variegated mutants (Sakamoto, 2003). The paleness of the *rug2* mutants also affects the cotyledons, stems, cauline leaves and sepals (Figure 1i–k). Consistent with this, chlorophyll content was reduced in *rug2* mutants compared with the corresponding wild types (Table S2).

The *rug2* mutants showed a pleiotropic phenotype (all traits were more extreme in *rug2-1*), retarded growth and a general reduction in size, clearly visible in their hypocotyls, rosettes, stems, fruits and roots (Figure 1; Table S3). Accordingly, we found a decrease in the fresh weight of *rug2-1* and *rug2-2*, and in the dry weight of *rug2-1*. Leaves exhibited reduced expansion along the proximodistal axis, affecting the final length of both the lamina and the petiole in the mutants (Table S3). No obvious alterations in floral development were detected in the *rug2* mutants (Figure 1j,k), although fertility was reduced in *rug2-1* (Table S3).

RUG2 expression analyses

To examine the effect of *rug2* mutations on the expression of the *RUG2* gene, total RNA was extracted from 3-week-old Col-0, L*er*, *rug2-1* and *rug2-2* plants, reverse transcribed and PCR amplified. Primers flanking the *rug2-2* T-DNA insertion (F3 and R3 in Figure 2b) yielded amplification products only from Col-0, but not from *rug2-2* cDNA or genomic DNA (Figure 2d). Primers hybridizing upstream of the T-DNA insertion in *rug2-2* (F3 and R2; Figure 2b) yielded a single band of the expected size (455 bp) for the mutant and for Col-0. Similar *RUG2* transcript abundance was detected by quantitative RT (qRT)-PCR in the *rug2* mutants and their respective wild types.

We studied the spatial and temporal expression patterns of *RUG2* in Col-0 by qRT-PCR, and detected *RUG2* transcripts

© 2011 The Authors

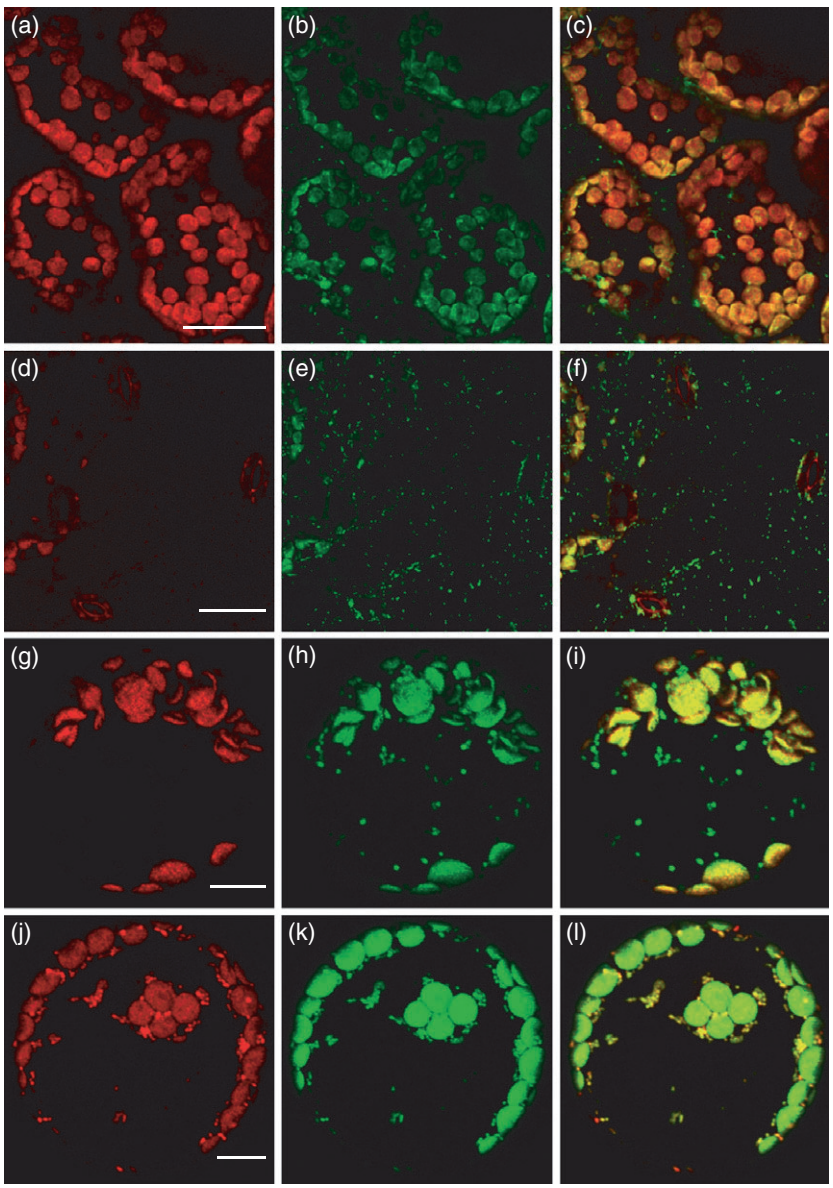

Figure 3. Subcellular localization of RUG2 in Arabidopsis transgenic plants and protoplasts.
(a–f) Third leaf (a–c) mesophyll and (d–f) epidermal cells from RUG2-N-terminal:GFP transgenic plants showing (a, d) chlorophyll autofluorescence of the chloroplasts, (b, e) GFP fluorescence, and (c, f) an overlay of the GFP and chlorophyll signals, showing that RUG2 co-localizes with the chloroplasts, and that it is also detected in the mitochondria.
(g–l) Arabidopsis protoplasts from RUG2-N-terminal:GFP transgenic plants showing (g) chlorophyll autofluorescence, (j) chlorophyll autofluorescence and the mitochondrial marker MitoTracker signal, (h, k) GFP fluorescence, overlay of GFP and (i) chlorophyll and (l) chlorophyll and MitoTracker signals, confirming the localization of RUG2 in mitochondria and chloroplasts. Scale bars: 31 μm (a–f); 7.75 μm (g–l).

in all organs and stages analyzed (Figure S4). The lowest expression was found in roots and the highest in cauline leaves and flowers (7.2- and 4.3-fold higher than in roots, respectively). Equivalent levels were detected in vegetative leaves and stems (2.9- and 3.3-fold higher than in roots, respectively). Temporally, the highest *RUG2* expression was observed in 4-day-old seedlings (1.7- and 2.6-fold higher than in 12- and 21-day-old plants, respectively).

RUG2 expression in response to different conditions and in different genotypes that might affect chloroplast function was studied using GENEVESTIGATOR (http://www.genevestigator.com/gv). No clear-cut differences were found, the most significant being a twofold *RUG2* upregulation in Col-0 seedlings exposed to cold (4°C, 10 days) or to high light conditions (1600–1800 μmol m^{-2} s^{-1}).

Effects of environmental factors on the phenotypes of *rug2* mutants

The *rug2-1* mutant was sensitive to temperature stress. When grown at 26°C (our normal growth temperature is

© 2011 The Authors

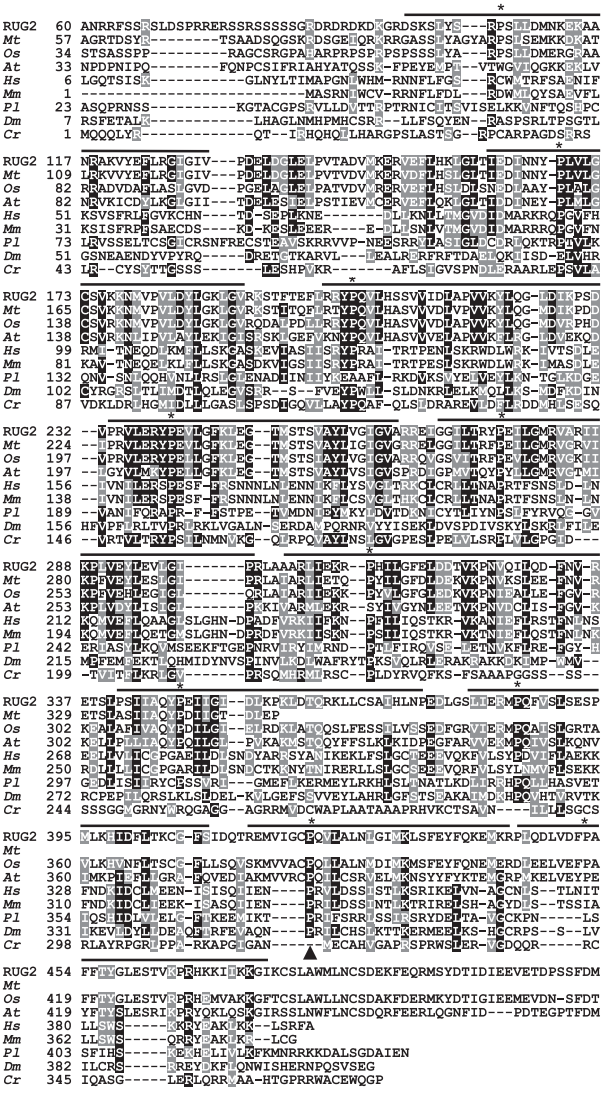

Figure 4. Multiple alignment of the amino acid sequence of RUG2 and homologous proteins.
RUG2 (NM_116533) is aligned with mTERF proteins from *Arabidopsis thaliana* (*At*; AY079380), *Oryza sativa* (*Os*; NP_001055500), *Medicago truncatula* (*Mt*; ABE94205), *Paracentrotus lividus* (*Pl*; CAB40796), *Drosophila melanogaster* (*Dm*; AY196479), *Mus musculus* (*Mm*; AY726770), *Homo sapiens* (*Hs*; Q99551) and *Chlamydomonas reinhardtii* (*Cr*; AAM96690). Residues conserved across five or more sequences are shaded in black, and similar residues conserved across five or more sequences are shaded in gray. Numbers indicate amino acid positions. The alignment was obtained using CLUSTALX 1.5b (Chenna *et al.*, 2003). Continuous lines indicate the mTERF motifs. Asterisks and an arrowhead indicate the highly conserved proline residues and the amino acid change in the *rug2-1* mutant allele, respectively.

20°C), *rug2-1* development was severely impaired and its growth was completely arrested (Figure S5e,f,i,j), whereas at 16°C its mutant phenotype was fully suppressed (Figure S5a,b,e,f). Growth of the *rug2-2* mutant and Col-0 was affected similarly by culture at 26 or 16°C (Figure S5c, d,g,h,k,l). Almost no differences were found in *RUG2* transcript abundance in *rug2-1* compared with L*er* in qRT-PCR amplifications performed on RNA from 3-week-old L*er* and *rug2-1* plants grown at 16, 20 or 26°C, indicating that the temperature sensitivity of *rug2-1* was not the result of changes in *RUG2* mRNA levels.

Consistent with the pale pigmentation and the reduction in size of *rug2-1*, we found that *RUG2* is important for photoautotrophic growth, because the growth of *rug2-1* and *rug2-2* was impaired *in vitro* in the absence of sucrose, a condition that did not affect wild-type development (Figure S6). Accordingly, *rug2-1* failed to grow when sown on soil.

Leaf ultrastructure of the *rug2* mutants

Internal leaf anatomy was studied in the *rug2* mutants by confocal microscopy of intact leaves and cross sections (Figure 5). Plastid chlorophyll autofluorescence was almost undetectable in the pale sectors of *rug2-1*. In the green sectors, mesophyll cell density was similar to the wild type, although a decrease in chlorophyll autofluorescence per chloroplast was observed (Figure 5a,b,e,f). In the yellowish sectors of *rug2-2* a decrease in chlorophyll autofluorescence and palisade cell number was found (Figure 5c,d,g,h), and this observation was confirmed in cross sections (Figure 5k,l).

Chloroplast and mitochondrion morphology was studied by transmission electron microscopy of leaf palisade cells. Chloroplasts in the green sectors of *rug2-1* were similar in size to those of L*er* but displayed fewer thylakoid membranes, and starch grains were considerably reduced in abundance (Figure 6a,b). Mesophyll cells in the yellowish sectors of *rug2-1* exhibited a large reduction in the number of chloroplasts, which were extremely vacuolated and lacked starch grains and organized thylakoid membranes (Figure 6a,c). We did not observe mesophyll cells containing both normal and underdeveloped plastids in *rug2-1*, indicating that these cells were not heteroplastidic. Chloroplasts in *rug2-2* were indistinguishable from those of Col-0, although their number was reduced in the pale sectors (Figure 6a,d).

Mitochondria in the mesophyll cells of the *rug2-2* mutant did not differ appreciably from Col-0 with respect to their relative size and abundance of cristae (Figure 6e,i,h,l). In contrast, *rug2-1* mitochondria showed abnormal morphology in palisade cells of both green and pale sectors, with markedly reduced cristae (Figure 6e–g, i–k). Mitochondria in the pale regions were similar to L*er* in size and number, but displayed less intermembrane spaces (Figure 6e,i,g,k). Accordingly, no clear differences were found in mitochondria size or distribution within cells in *rug2-1* compared with L*er*, as demonstrated by the introduction (by crossing) of a mitochondria-targeted GFP construct (Logan and Leaver, 2000) in *rug2-1* (Figure S7).

Maintaining the correct membrane potential across the mitochondrial inner membrane is crucial for mitochondrial

© 2011 The Authors

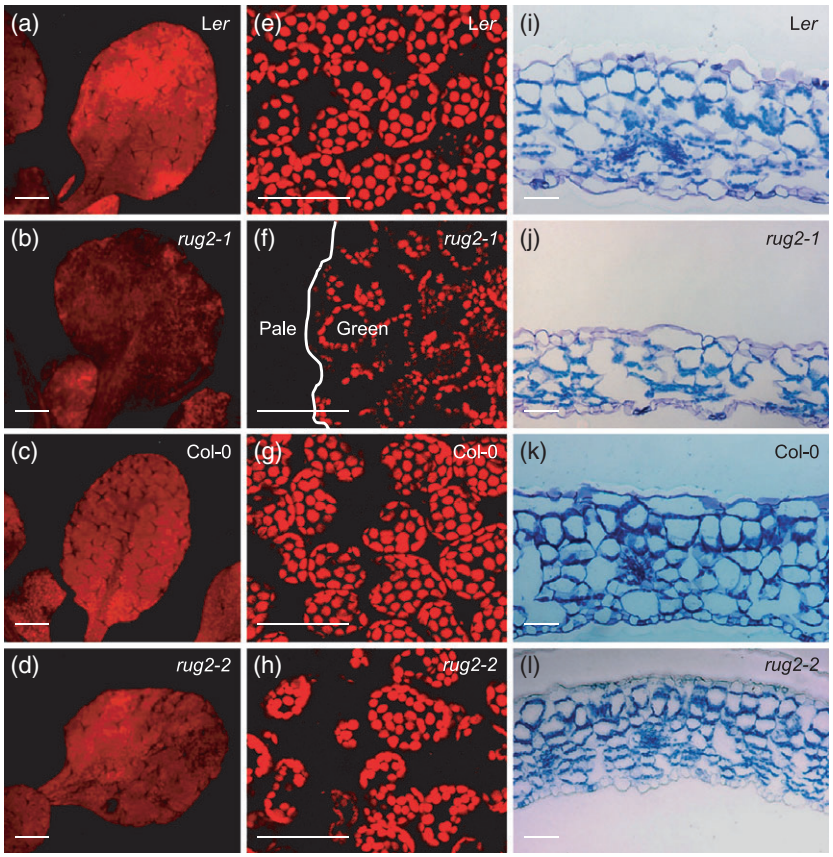

Figure 5. Internal leaf structure in the *rug2* mutants.
(a–h) Confocal micrographs showing chlorophyll autofluorescence in mesophyll cells of whole third leaves (a–d) and details of the mesophyll (e–h), depicting a border between green (right) and pale (left) sectors in *rug2-1* (f).
(i–l) Transverse sections of third leaves. Pictures were taken 21 days after sowing. All plants were homozygous for the mutations shown. Scale bars: 1 mm (a–d); 50 μm (e–h); 40 μm (i–l).

function and integrity. Aiming at visualizing the functional state of *rug2-1* mitochondria, protoplasts isolated from leaves of this mutant and L*er* were stained with tetramethylrhodamine methyl ester (TMRM; see Experimental procedures), a lipophilic cationic dye that accumulates proportionally to the mitochondrial membrane potential (Zhang *et al.*, 2001; Kim *et al.*, 2006). The lower the mitochondrial membrane potential the less fluorescence can be detected because the capability of the mitochondria to retain the probe is diminished. A threefold reduction in the fluorescence of protoplasts from *rug2-1* compared with L*er* was found, indicating that the mitochondrial membrane potential and hence mitochondrial function is perturbed in the mutant (Figure 7).

Double mutant analyses

We crossed *rug2-1* with *sca3-1* (*scabra3-1*), a mutant defective in chloroplast and leaf development. SCA3 is the nuclear-encoded RNA polymerase RpoTp targeted to chloroplasts (Hricová *et al.*, 2006). Four phenotypic classes were found in the F_2 progeny of the *rug2-1* × *sca3-1* cross, conforming to a 9:3:3:1 ratio (χ^2 = 4.94; P = 0.17). The genotypes of the double mutants were confirmed by sequencing of the *RUG2* and *SCA3* genes (see Experimental procedures). We considered the phenotype of the *rug2-1 sca3-1* double mutants to be synergistic because they displayed very small and narrow pale green leaves, and plant size was substantially reduced compared with that of the parental mutants (Figure 8).

Expression analysis of organelle genes in the *rug2-1* mutant

The similarity of the RUG2 protein to mTERF factors involved in transcriptional control, and its dual localization in chloroplasts and mitochondria, prompted us to investigate the expression of both organelle genomes in the severe *rug2-1* mutant. For this purpose, we constructed a DNA microarray containing probes representing 121 genes of the Arabidopsis mitochondrial genome (ftp://ftp.arabidopsis.org/home/tair/home/tair/Sequences/chloroplast) and 128 genes of the chloroplastic genome (ftp://ftp.arabidopsis.org/home/tair/home/tair/Sequences/mitochondrial) (Table S4). We detected 56 genes (22.5%) that were deregulated in

© 2011 The Authors

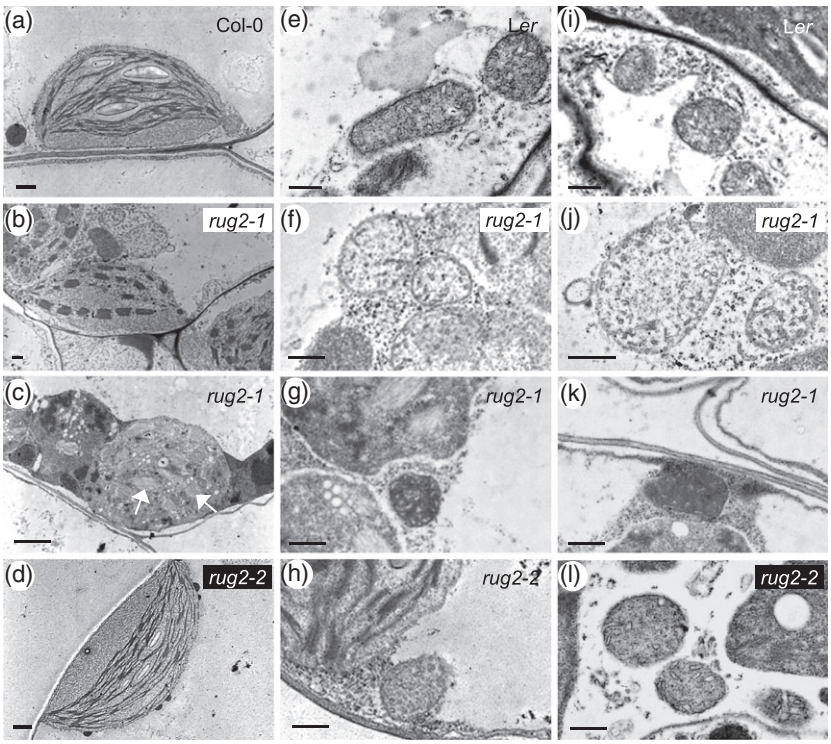

Figure 6. Transmission electron micrographs of third-node leaf mesophyll cell chloroplasts and mitochondria of the *rug2* mutants.
(a–d) Representative chloroplasts and
(e–l) mitochondria of Col-0, L*er* and the *rug2* mutants. Chloroplasts and mitochondria of L*er* and Col-0 were indistinguishable. (b) Abnormal chloroplasts and (f, j) mitochondria in mesophyll cells of a green sector of the *rug2-1* mutant showing their defectively organized internal membranes. (c) An extremely abnormal chloroplast showing enlarged thylakoids (arrows) and (g, k) mitochondria in cells of a pale sector of the *rug2-1* mutant. (d) Chloroplasts and (h, l) mitochondria of mesophyll cells in the *rug2-2* mutant. All plants were homozygous for the mutations shown. Leaves were collected 21 days after sowing. Scale bars: 2 μm (a–d); 1 μm (e–l).

rug2-1: 26 mitochondrial (21.5% of all mitochondrial genes) and 30 plastidic (23.4% of all chloroplast genes); 31 (55.3%) genes were downregulated and 25 (44.7%) genes were upregulated. Interestingly, 23 of the downregulated genes (74.2% of all downregulated genes) were mitochondrial, including most of those encoding subunits of the major respiratory complexes of the inner mitochondrial membrane (complexes I, III, IV and V; Table S5). Besides, we found that mitochondrial genes encoding hypothetical proteins (AtMg00500, AtMg00520 and AtMg00590), open reading frames without assigned function (*orf25*, *orf101b*, *orf113*, *orf149* and *orf294*), the *rpS3* and *rpL5* genes (encoding the S3 and L5 ribosomal proteins, respectively) and the *ccb206* gene (encoding a protein involved in cytochrome *c* maturation) were downregulated (Table S5). Only three mitochondrial genes of unknown function (*orf116*, *orf109b* and *orf121a*) were upregulated in *rug2-1*. In the chloroplastic genome, *trnc* and *trns.2* (encoding the Cys and Ser tRNAs), *rrn4.5*, *rrn16* and *rrn23* (encoding *16S*, *23S* and *4.5S* rRNAs), *psbA* and *psbN* (encoding subunits of the PSII core complex) were all downregulated (Table S5). Nevertheless, the transcripts of the majority of plastid genes detected in our microarray (23; 92% of all upregulated genes) accumulated at higher levels in *rug2-1* compared with L*er*. These included *rpoB* and *C2* (encoding the RNA polymerase subunits β and β″), *trna.1*, *trni.2*, *trnl.1*, *trnk*, *trnv.3* and *trnt.2* (encoding Ala, Ile, Leu, Thr, Lys and Val tRNAs, respectively), *rpS2*, *rpS3*, *rpS4*, *rpS15*, *rpL2*, *rpL16* and *rpL20* (encoding S2, S3, S4, S15, L2, L16 and L20 ribosomal proteins, respectively), *ndhE* and *ndhB.2* (encoding the NADPH dehydrogenase subunits), *psbL* and *psbF* (the products of which are subunits of the PSII core complex), *matK* (encoding a maturase), *ccsA* (the product of which is a cytochrome *c* biogenesis protein), *atpH* (encoding an ATPase III subunit) and *ycf1.2* (encoding a hypothetical protein).

We validated our microarray results by qRT-PCR and confirmed that in *rug2-1* the *matK*, *rpoB*, *rpoC2* and *rpsL20* genes were upregulated both by qRT-PCR (12.1-, 9.2-, 7.5- and 3.9-fold, respectively) and microarray analyses (6.0-, 2.2-, 2.4- and 2.0-fold, respectively), whereas the *cox1* (encoding cytochrome oxidase subunit 1) and *psbA* genes were downregulated both by qRT-PCR (1.2- and 1.3-fold, respectively) and microarray analyses (1.9- and 2.4-fold, respectively).

The levels of the plastid-encoded RbcL (large subunit of RUBISCO), atpB (ATP synthase subunit β) and psbA (PSII

© 2011 The Authors

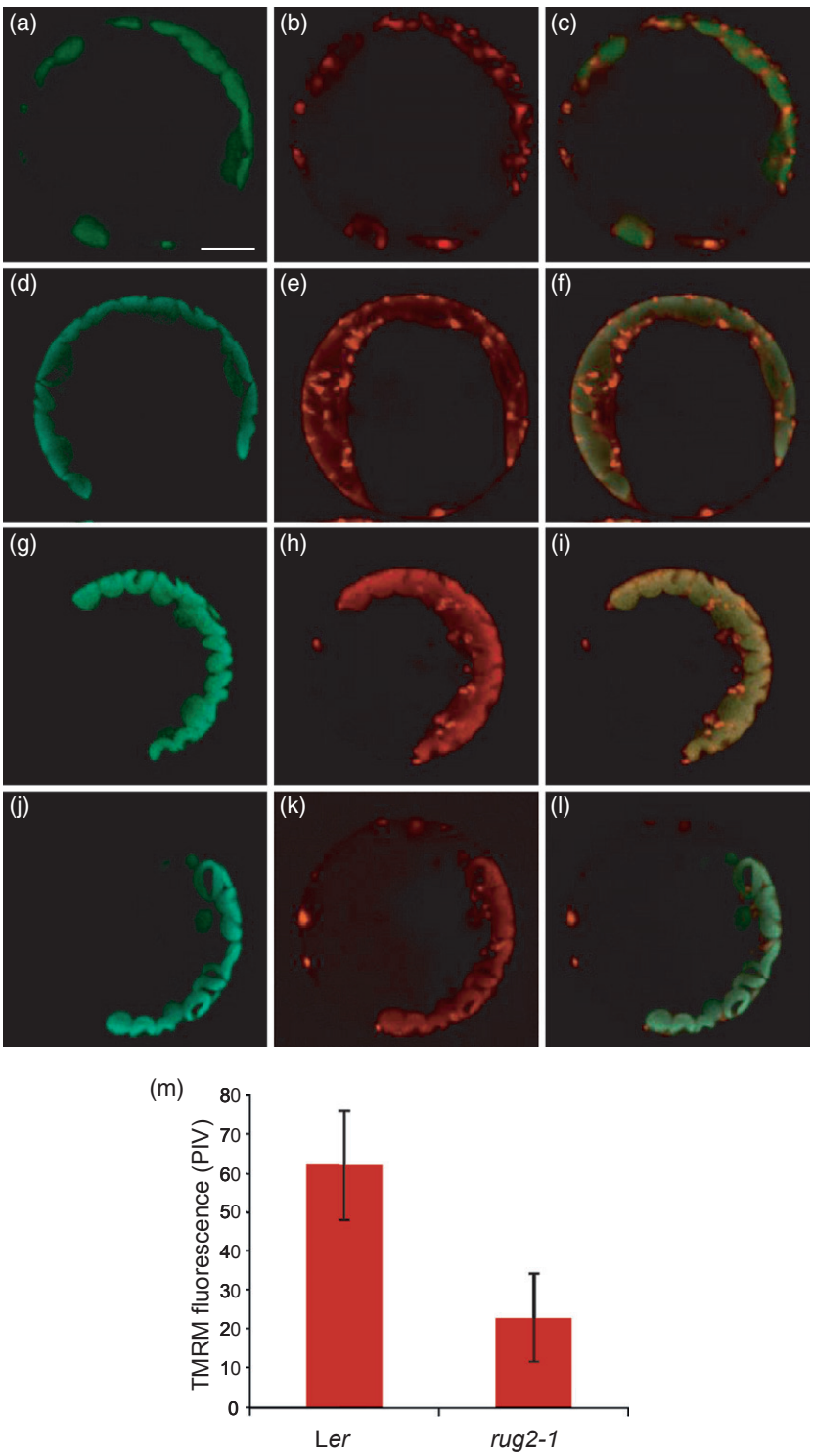

Figure 7. Mitochondrial membrane integrity.
Confocal laser scanning images of Arabidopsis protoplasts from (a–c, g–i) L*er* and (d–f, j–l) *rug2-1*, showing (a, d, g, j) chlorophyll autofluorescence of the chloroplasts, (b, e) MitoTracker and (h, k) tetramethylrhodamine methyl ester (TMRM) signals. *rug2-1* showed lower TMRM fluorescence than L*er*, suggesting a reduction of the mitochondrial inner membrane potential in *rug2-1*. The overlay of chlorophyll autofluorescence with (c, f) MitoTracker and with (i, l) TMRM is also shown. Scale bar: 7.75 μm.
(m) Quantification of the TMRM average fluorescence, confirming the reduction in the TMRM fluorescence in *rug2-1*. Data points represent means ± SDs of 15 individual protoplasts. PIV: pixel intensity values.

core complex D1) proteins and the nuclear-encoded and mitochondrial-localized FtsH10 protein were investigated by western blot analysis. All the chloroplastic proteins were accumulated in *rug2-1* and *soldat10*, an Arabidopsis mutant defective in an mTERF factor exclusively localized in the chloroplasts (Meskauskiene *et al.*, 2009), to lower levels than in L*er* (Figure S9). Interestingly, compared with the wild type, much less FtsH10 protein was detected in *rug2-1* but not in *soldat10,* consistent with the dual targeting of the RUG2 protein to chloroplast and mitochondria (Figure 9).

© 2011 The Authors

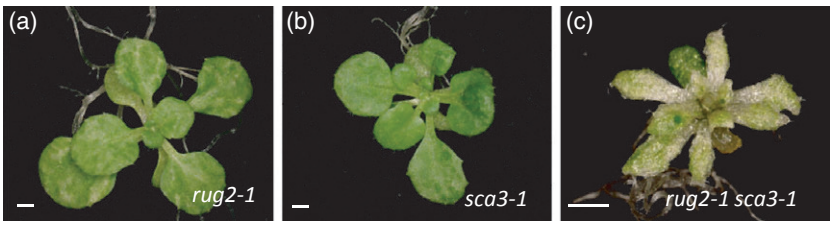

Figure 8. Synergistic phenotype of the *rug2-1 sca3-1* double mutant.
(a–c) The (a) *rug2-1* and (b) *sca3-1* single mutants, both in a L*er* genetic background, and (c) the *rug2-1 sca3-1* double mutant. Pictures were taken 21 days after sowing. Scale bars: 1 mm.

RpoT transcript accumulation in the *rug2-1* mutant

The *RpoT* nuclear genes encode T7 phage-type RNA polymerases [nuclear encoded polymerases (NEPs); Hess and Börner, 1999; Shiina *et al.*, 2005], which transcribe mitochondrial and chloroplastic genes. Experimental results indicate the existence of crosstalk between the regulatory pathways of the NEPs through organelle-to-nucleus communication (retrograde signaling; Emanuel *et al.*, 2004; Baba *et al.*, 2004; Hricová *et al.*, 2006). This evidence and the severe perturbation of mitochondrial and plastidic gene expression in *rug2-1* (Table S5) prompted us to investigate whether expression of the Arabidopsis *RpoT* nuclear genes (*RpoTm*, *RpoTp/SCA3* and *RpoTmp*, the products of which are targeted to the mitochondria, chloroplast and both organelles, respectively) was affected in *rug2-1*. The *RpoTp/SCA3* gene was upregulated 1.8-fold compared with L*er* (1.8 ± 0.3; $P = 0.002$), whereas *RpoTmp* and *RpoTm* were only slightly affected [down- and upregulated 1.1-fold (1.1 ± 0.0; $P = 0.001$) and 1.2-fold (1.2 ± 0.1; $P = 0.08$), respectively]. Consistent with the accumulation of *RpoTp/SCA3* transcripts in *rug2-1*, the NEP-transcribed plastid genes *rpoB* and *rpoC2* were upregulated in this mutant (Table S5).

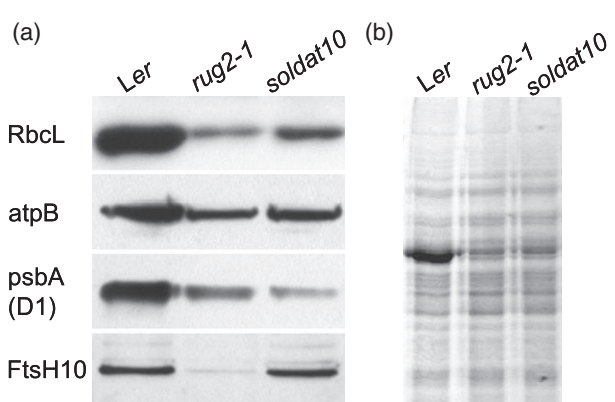

Figure 9. Western blot analysis of plastid and mitochondrial proteins.
(a) Western blot analysis of total protein extracts from 6-day-old L*er*, *rug2-1* and *soldat10* seedlings with the indicated antibodies. (b) BioSafe Coomassie blue was used as a loading control. Each lane contains 20 μg of protein.

DISCUSSION

The *RUG2* gene encodes an mTERF-related protein

This work aimed to characterize the Arabidopsis *rug2* mutants at the genetic and molecular levels. We positionally cloned the *RUG2* gene and found it to encode a protein dual targeted to leaf chloroplasts and mitochondria, and displaying similarity to mTERFs, a wide family of proteins identified in metazoans and plants (Linder *et al.*, 2005). Different mTERF proteins have been characterized in animals: human MTERF1 (Fernández-Silva *et al.*, 1997; Asín-Cayuela *et al.*, 2004) and mTERFL (mTERF-like; Chen *et al.*, 2005), murine mTERF (Li *et al.*, 2005), MTERF3 (Park *et al.*, 2007), MTERF2 (Wenz *et al.*, 2009) and MTERF4 (Cámara *et al.*, 2011), rat mTERF (Prieto-Martín *et al.*, 2004), sea urchin mtDBP (Loguercio-Polosa *et al.*, 1999, 2002) and DmTTF in *D. melanogaster* (Roberti *et al.*, 2006). The founder member of this protein family, the human MTERF1, is a multizipper protein that regulates mitochondrial gene transcription through binding as a monomer (it is inactive as a homotrimer) to a 28-nucleotide sequence located at the 3′ end of the *16S* rRNA gene, promoting termination of transcripts from the first transcription initiation site (H1). After processing, this polycistronic transcript yields *12S* and *16S* rRNAs, and $tRNA^{Phe}$ and $tRNA^{Val}$. MTERF1 was also found to stimulate transcription initiation through binding to H1 (Prieto-Martín *et al.*, 2004; Martin *et al.*, 2005). Park *et al.* (2007) have shown that the mouse *Mterf3* gene is a negative regulator of mitochondrial transcription initiation, and its function is essential for embryo development. Wenz *et al.* (2009) reported that the inactivation of the mouse mitochondrial transcriptional modulator MTERF2 reduces mitochondrial transcripts, resulting in myopathy and memory deficits, and very recently, Cámara *et al.* (2011) found that mouse MTERF4 controls mitochondrial ribosomal biogenesis and translation. Additional evidence supports a role for MTERF1, 2 and 3 in mitochondrial DNA replication (Hyvärinen *et al.*, 2007, 2010). In humans, an A → G substitution in the recognition site of MTERF1 results in the severe impairment of *16S* rRNA transcription termination, which is associated with mitochondrial encephalomyopathy lactic

© 2011 The Authors

acidosis and stroke-like episodes (MELAS) syndrome (Hess *et al.*, 1991).

In stark contrast to mammals, the control of mitochondrial gene expression in plants is poorly understood. Although mitochondrial metabolic functions are basically the same in all organisms, plant mitochondrial genomes display distinctive features from those of animals and fungi: larger size, wide rearrangements and transcription units usually separated by long intergenic sequences of unknown function (Forner *et al.*, 2007). In contrast to plastid genes, plant mitochondrial genes are transcribed exclusively by nuclear-encoded proteins. Two Arabidopsis genes encoding mitochondrial RNA polymerases have been identified, and the functional analysis of one of them, *RpoTmp*, has been reported (Baba *et al.*, 2004). Despite this, very little is known about other factors controlling gene expression in plant mitochondria (Binder and Brennicke, 2003). When this work was submitted, only two genes encoding mTERF-related factors had been characterized based on the analysis of a mutant phenotype in photosynthetic organisms: the *MOC1* gene in the green unicellular alga *C. reinhardtii* (Schonfeld *et al.*, 2004) and *SOLDAT10* in Arabidopsis (Meskauskiene *et al.*, 2009). Perturbation of *MOC1* function causes a light-sensitive phenotype and alters the expression of mitochondrial genes encoding proteins of the respiratory complexes. A mutation in the mTERF-related gene *SOLDAT10* (the product of which is plastid localized), abrogates $^1O_2$-mediated cell death in the Arabidopsis *flu* mutant, which usually accumulates $^1O_2$ during a shift from dark to light. Very recently, Babiychuk *et al.* (2011) reported the characterization of the Arabidopsis mutant *belaya smert* (*bsm*) affected in the *At4g02990* (*RUG2*) gene. The authors detected the BSM protein exclusively in the chloroplasts, and growth of the *bsm* mutant was seriously compromised, leading to malformed albino seedlings that required hormone supplementation for cell proliferation and organogenesis.

Organelle and mesophyll cell development are impaired in *rug2* mutants

The *rug2-1* mutant and, to a lesser extent, *rug2-2*, displayed a variegated phenotype, with green and yellowish sectors in their leaves and notable paleness in the remaining organs. Consistent with their molecular lesions, the mutant phenotype of *rug2-1* was stronger than that of *rug2-2*, as *rug2-1* carries a missense mutation that affects a highly conserved residue in the RUG2 protein, whereas *rug2-2* bears a T-DNA insertion in the 3′-UTR of *RUG2*. As expected from the pleiotropic phenotype found in the *rug2* mutants, *RUG2* is ubiquitously expressed.

Our findings suggest that Arabidopsis RUG2 protein is dually targeted to mitochondria and chloroplasts. Consistent with this, a proteomic study identified RUG2 in the stroma of chloroplasts (Olinares *et al.*, 2010) and, moreover, it was predicted to be in the mitochondrial proteome based on a computational framework developed by Cui *et al.* (2011) aimed at identifying Arabidopsis mitochondrial proteins according to a probabilistic model (called the Naive Bayesian Network), which integrates different genomic data generated from bioinformatics tools, multiple orthologous mappings, protein domain properties and co-expression patterns using microarray profiles.

According to the dual subcellular localization of RUG2, chloroplasts and mitochondria showed abnormal morphologies in *rug2-1*. Thus, chloroplasts were not properly developed in mesophyll cells of the green areas of *rug2-1* leaves, and were extremely abnormal in the pale ones, lacking organized internal membrane structures and containing undeveloped lamellae leading to diminished chlorophyll levels, paleness and defective photoautotrophic growth. However, as opposed to other well-characterized variegated mutants, such as *im* or *var2* (Sakamoto, 2003), chloroplasts were not completely normal in the green sectors of *rug2-1* mutant leaves. Therefore, the loss of function of the *RUG2* gene seems to disrupt a process required for proper chloroplast development, which, in turn, might perturb internal leaf anatomy. This would support the hypothesis of a putative plastid-to-nucleus developmental signal controlling mesophyll cell differentiation and proliferation (Rodermel, 2001; Hricová *et al.*, 2006). Alternatively, *RUG2* might be directly necessary for both plastid development and mesophyll cell morphogenesis.

The mitochondria of palisade cells were similar in size in *rug2-1* and L*er*, but displayed abnormal morphologies in both the green and the pale leaf sectors of the mutant, indicating that *RUG2* activity is required for chloroplastic and mitochondrial function, as expected from its subcellular localization. Consistent with a mitochondrial activity for RUG2, our TMRM staining results clearly revealed a disruption in the mitochondrial potential membrane in *rug2-1*.

RUG2 is involved in chloroplast and mitochondrion function, and gene expression

Consistent with its similarity to metazoan mTERFs and dual targeting to mitochondria and chloroplasts our microarray analyses revealed an important role for *RUG2* in plant organelle genome expression. In Arabidopsis, proteins dually targeted to both mitochondria and plastids function in DNA and RNA maintenance, translation and cellular defense responses (Mackenzie, 2005).

Interestingly, nearly all of the altered mitochondrial genes in *rug2-1* were downregulated, including most of those encoding subunits of the I, II, IV and V respiratory omplexes, as was the *ccb206* gene, involved in cytochrome *c* biogenesis. Likewise, a decrease in the steady-state levels of RNAs and proteins of the different oxidative phosphorylation systems has recently been reported in mouse *MTERF2* knock-out mutants (Wenz *et al.*, 2009). As described for the *stm6* mutant of Chlamydomonas, defective in the

© 2011 The Authors

mTERF-related *MOC1* gene (Schonfeld *et al.*, 2004), the *cox1* gene encoding a key subunit of the cytochrome oxidase complex is also repressed in *rug2-1*. Furthermore, we found in *rug2-1* but not in *soldat10*, a significant reduction in the level of nuclear-encoded and mitochondria-localized FtsH10 protease. This protein assembles with prohibitins in a high-molecular weight complex that putatively acts to stabilize respiratory chain subunits, and/or acts as protein and lipid scaffolding, controlling inner membrane organization and integrity (Piechota *et al.*, 2010). This result would point towards a direct role for RUG2 in mitochondrial function. Our data suggest that in *rug2-1* the mitochondrial respiratory chain would have a reduced capacity to oxidize chloroplast reductants, resulting in the over-reduction of the stroma of chloroplasts. The redox state of plastoquinone (PQ), a thylakoid membrane electron carrier, governs the transcription of plastid genes encoding reaction-center apoproteins of PSI and PSII, such as *psbA* (encoding the D1 reaction center protein of PSII; Pfannschmidt *et al.*, 1999), the expression of which can be induced or repressed when PQ is oxidized or reduced, respectively. Interestingly, we found at the mRNA and protein levels the *psbA* gene to be downregulated in *rug2-1* plants, supporting our hypothesis of an over-reduced stroma. Less *psbA* transcripts have also been detected in the yellow sectors of the variegated mutants NCS6 of maize (Jiao *et al.*, 2005) and *im* of Arabidopsis (Wetzel *et al.*, 1994).

In contrast to mitochondria, most of the altered chloroplast genes were upregulated in the *rug2-1* mutant, indicating that RUG2 would act principally as a negative regulator of chloroplast gene expression. Several of these genes encode transcription and translation components. Intriguingly, genes encoding the *16S*, *23S* and *4.5S* rRNAs were downregulated in *rug2-1*, as in the Arabidopsis *soldat10* and *bsm* mutants. This would reduce protein synthesis in the chloroplasts mostly affecting those with higher turnover rates, such as the D1 protein (Meskauskiene *et al.*, 2009). We confirmed this by western analysis: less D1 protein accumulated in *rug2-1* compared with L*er*. Because the D1 protein is a component of the reaction center of PSII, and participates in its photoprotection and in photosynthetic electron transport, we consider it likely that photosynthetic activity is perturbed in *rug2-1* as in *soldat10*. Accordingly, much less RbcL protein was found in *rug2-1* and *soldat10*, and a decrease in RbcL transcript levels has also been reported in the *bsm* mutant (Babiychuk *et al.*, 2011). Contrary to *SOLDAT10* that seems to more selectively affect chloroplast gene expression, the *RUG2* lesion would have a broader effect in this organelle. Nevertheless, we cannot rule out an alternative scenario: *RUG2* might have a more specific effect on plastid gene activity, being principally required for the expression of the rRNA genes. *RUG2* deficiency would stimulate a compensatory activity (e.g. a functionally redundant factor) accounting for the accumulation of various plastid transcripts in *rug2-1*. The upregulation in *rug2-1* of nuclear genes encoding proteins involved in plastid or mitochondria transcription would support this hypothesis (see below).

The alteration caused by the *rug2-1* mutation in mitochondrion and chloroplast transcript profiles reinforces the idea of the existence of functional interaction between both organelles. As *RUG2* is a nuclear gene, this suggests a function in the coordination of the expression of chloroplast and mitochondrial genomes mediated by the nucleus. Although there is currently no evidence for direct signaling between these organelles, different signaling molecules (e.g. ascorbate, active oxygen species and nitric oxide) have been proposed as candidates (Raghavendra and Padmasree, 2003). Further results suggest that mitochondrion–chloroplast functional interaction could be even more complex, involving a retrograde signaling mechanism (from one of the organelles to the nucleus) in order to modulate anterograde (from the nucleus to an organelle) control of gene expression in both organelles. Thus, in the leaves of the *albostrians* mutant of barley containing ribosome-deficient undifferentiated plastids, insufficient chloroplast development is signaled to the nucleus, giving rise to upregulation of plastid *RpoTp/SCA3* and mitochondrial *RpoTm* NEP genes, leading to increased chloroplast and mitochondrion genome gene expression (Emanuel *et al.*, 2004). Accordingly, we found the accumulation of *RpoTp/SCA3* and, to a lesser extent, *RpoTm* transcripts in *rug2-1* plants. Enhanced *RpoTp/SCA3* activity would contribute to the accumulation of NEP-transcribed genes such as *rpoB*, *rpoC2* or *rpsL2*. The synergistic phenotype of the *rug2-1 sca3-1* double mutant supports the functional relationship of *RUG2* and *SCA3* genes. Nevertheless, as RUG2 is plastid localized, we cannot rule out that these changes in transcript levels could be caused at least in part, directly by *RUG2* perturbation.

Mechanism of *rug2* variegation

In *im* and *var2* mutants, a threshold model of activity for the IM and VAR2 proteins, has been proposed to explain variegation (Wu *et al.*, 1999; Yu *et al.*, 2004). Likewise, based on our results, we put forward the hypothesis that a threshold level of *RUG2* activity is required for normal mitochondrion and chloroplast development in Arabidopsis cells. Thus, defects in mesophyll cell morphogenesis, and hence in leaf anatomy, would be a secondary effect of the failure in organelle function caused by *RUG2* loss-of-function mutations. When the threshold is not attained in the *rug2* mutants, another of the Arabidopsis *RUG2* mTERF paralogs would compensate for *RUG2* reduced activity, leading to green or yellowish sectors. Nevertheless, chloroplasts and mitochondria are not completely normal, even in *rug2-1* green areas, indicating that this compensation would not be fully accomplished. Our results are in agreement with a cell-autonomous mechanism of variegation, which has been invoked to explain the phenotype of reticulate mutants (Yu

© 2011 The Authors

et al., 2007), which exhibit a dark-green vasculature on a pale leaf lamina, probably as a consequence of the existence of cell-specific functional redundancy. Our results highlight the complexity of mechanisms governing concerted gene expression among the different genomes coexisting within plant cells. Further investigations will be required to elucidate the molecular mechanism by which *RUG2* influences gene expression in chloroplasts and mitochondria.

EXPERIMENTAL PROCEDURES

Plant material, growth conditions and growth assays

Cultures and crosses were performed as described by Ponce *et al.* (1998) and Berná *et al.* (1999), respectively. Seeds of the *A. thaliana* (L.) Heynh. wild-type accessions L*er* and Col-0 were obtained from the Nottingham Arabidopsis Stock Centre (NASC, http://arabidopsis.info). The *rug2-1* and *sca3-1* mutants were isolated in a L*er* background after ethyl methanesulfonate (EMS) mutagenesis, and were backcrossed twice to L*er* (Berná *et al.*, 1999). Seeds of the N533963 (*rug2-2*) T-DNA insertion line were provided by the NASC, and are described at the SIGnAL website (http://signal.salk.edu; Alonso *et al.*, 2003). The 43C5 transgenic line, containing the mitochondrial signal sequence of the β-ATPase subunit from *Nicotiana plumbaginifolia* fused to GFP, was kindly provided by David Logan (University of St Andrews, Scotland, UK). Temperature sensitivity and autotrophic growth analyses, and root studies, were performed as previously described (Hricová *et al.*, 2006).

Morphological and ultrastructural analyses

Dry weight was measured in plants that were oven dried overnight at 55°C. Whole rosette, third leaf, silique, stem and inflorescence measurements were performed using IMAGEJ (http://rsb.info.nih.gov/ij/docs/menus/file.html) from pictures taken using a Leica MZ6 stereomicroscope (Leica, http://www.leica.com) equipped with a Nikon DXM1200 digital camera (Nikon, http://www.nikon.com). A Student's *t*-test was applied to the data obtained, with a significance level of 0.01. Confocal imaging was performed as described by Pérez-Pérez *et al.* (2002).

For light microscopy, plant material was fixed with FAA/Triton (1.85% formaldehyde, 45% ethanol, 5% acetic acid and 1% Triton X-100), as described by Serrano-Cartagena *et al.* (2000). Transverse sections of leaves (0.5-μm thick) were cut on a microtome (Microm International HM350S; http://www.microm-online.com), stained with 0.1% toluidine blue and observed using a Leica DMRB microscope equipped with a Nikon DXM1200 digital camera under bright-field illumination.

For transmission electron microscopy, mutant and wild-type plant material was harvested at the same time of day and prepared as described by Hricová *et al.* (2006). Visualization of the samples was performed using a Zeiss EM10C transmission electron microscope (Zeiss, http://www.zeiss.com).

For the measurement of mitochondrial membrane potential, TMRM (T668; Invitrogen, http://www.invitrogen.com) was added to a final concentration of 200 nM to *rug2-1* and L*er* protoplasts, which were immediately transferred to microscope slide wells and observed with a confocal microscope. Fluorescence intensity was quantified using IMAGEJ.

Complementation of the *rug2-1* mutation

The coding region of *At4g02990* was amplified by PCR using the primers presented in Table S1 and a proofreading polymerase (Pfu Ultra; Stratagene, now Agilent, http://www.genomics.agilent.com). The product was cloned directionally into the GATEWAY entry vector pENTR-TOPO (Invitrogen), and then subcloned into the pMDC32 vector by site-directed recombination according to the manufacturers' instructions (Invitrogen). Competent *E. coli* DH5α cells were transformed and the isolated transformants were tested by PCR for the presence of the construct. Plasmid DNA was obtained from positive clones and used to transform competent *Agrobacterium tumefaciens* LBA4404 cells, and the positives clones were used for the *in planta* transformation of *rug2-1* plants (Clough and Bent, 1998). For isolation of transformant plants, T_2 seeds were sown in agar plates supplemented with 40 mg ml^{-1} hygromycin. The presence of the transgene in the putative transformants was verified by PCR.

Subcellular localization of the RUG2 protein

The first 311 nucleotides of the RUG2 coding region were amplified from Col-0 DNA using a proofreading polymerase (Pfu Ultra; Stratagene) and the primers presented in Table S1, which contain the attB1 and attB2 sequences, for recombination and directional cloning of the PCR product in the pGEMteasy221 vector, using the BP clonase enzyme (Invitrogen). The PCR product insert in pGEMteasy221 was then transferred to a pMDC85 Gateway vector (which contains the GFP-coding sequence), by site-directed recombination using LR clonase (Invitrogen), and competent *Agrobacterium tumefaciens* LBA4404 cells were transformed. Positive transformants were inoculated and used for *in planta* transformation of Col-0 plants (Clough and Bent, 1998). Protoplasts were prepared from 36-day-old transgenic plants stably expressing the RUG2-N-terminal:GFP construct under the control of two CaMV35S promoters, as described by Yoo *et al.* (2007), except that Viscozyme L (Sigma V2010, Bagsvaerd, Denmark) was used instead of macerozyme. Protoplasts were incubated for 15 min in culture medium supplemented with 50 nM MitoTracker Red CMX ROS (Molecular Probes, http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes.html), and washed three times before confocal analysis. Sample visualization was carried out in a Nikon Eclipse C1 confocal microscope. The chlorophyll, GFP and MitoTracker signals were taken from the same leaf or protoplast preparation by selecting different excitation and emission wavelengths (excitation – chlorophyll and MitoTracker, 540/525 nm; GFP, 460/500 nm; emission – chlorophyll and MitoTracker, 605/55 nm; GFP, 510/560 nm).

Quantitative RT-PCR

Total RNA was extracted from 50–70 mg of 4- and 12-day-old seedlings, 3-week-old rosettes, roots and vegetative leaves, and 45-day-old stems, cauline leaves and flowers (L*er*, *rug2-1*, Col-0 and *rug2-2*), and DNase-I treated using the Qiagen RNeasy Plant Mini kit, following the manufacturer's instructions. RNA was ethanol precipitated and resuspended in 40 μl of RNase-free water. A 5-mg portion of each sample was reverse transcribed using random hexamer primers, as described by Quesada *et al.* (1999). A 1-μl portion of the resulting cDNA solution was used for qRT-PCR amplifications, which were carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, http://www.appliedbiosystems.com), as described by Pérez-Pérez *et al.* (2004). As *RUG2* lacks introns, reverse-transcription reactions without reverse transcriptase were performed in parallel, and 1 μl of the reaction mixture used for qRT-PCR amplification confirms the absence of contaminating genomic DNA in the cDNA sample. Oligonucleotides were designed to yield amplification products of approximately 100 bp (Table S1). For all other genes containing

© 2011 The Authors

introns, one primer from each primer pair was designed to span the ends of two adjacent exons so that genomic DNA would not be amplified. Each 25-μl reaction mix contained 12.5 μl of the SYBR-Green PCR Master kit (Applied Biosystems), 0.4 μM of primers and 1 μl of cDNA solution. Relative quantification of the gene expression data was performed using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Each reaction was performed using three biological replicates and the expression levels were normalized to the C_t values obtained for the housekeeping *OTC* gene (Quesada *et al.*, 1999).

Protein analysis

Proteins from 6-day-old seedlings of the wild type (L*er*), *rug2-1* and *soldat10* were isolated as described in Quesada *et al.* (2003). A portion of total protein (20 μg) from each genotype was separated on 10% SDS-PAGE, blotted on nitrocellulose membranes (Amersham Hybond ECL, RPN203D; GE Healthcare, http://www.gelifesciences.com) and subjected to immunoblot analysis with specific antibodies (AS03 037-10, AS03 030-10, AS01 016-10 and AS07 251 (AgriSera AB, http://www.agrisera.com). BioSafe Coomassie blue (161-0786, Bio-Rad) was used as a loading control.

ACKNOWLEDGEMENTS

We thank H. Candela, J.M. Pérez-Pérez and P. Robles for their useful comments on the manuscript, the NASC for providing seeds, R. Solano for performing the microarray analyses and J.M. Serrano, T. Trujillo and V. García-Sempere for their excellent technical assistance. This work was supported by the Ministerio de Ciencia e Innovación of Spain (research grants BFU2005-01031 and BIO2008-04075), the Generalitat Valenciana (PROMETEO/2009/112) and the European Research Training Network HPRN-CT-2002-00267 (DAGOLIGN). RS-M is indebted to the Ministerio de Ciencia e Innovación for her PhD scholarship.

SUPPORTING INFORMATION

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

Figure S1. Complementation of the mutant phenotype of *rug2-1*.

Figure S2. Absence of GFP signal in chloroplasts from control plants.

Figure S3. An unrooted phylogenetic tree of mTERF proteins.

Figure S4. Quantitative RT-PCR analysis of *RUG2* expression.

Figure S5. Temperature sensitivity of the *rug2* mutants.

Figure S6. Effects of sucrose on the growth of the *rug2* mutants.

Figure S7. Distribution of mitochondria in *rug2-1.*

Table S1. Primers used in this work.

Table S2. Chlorophyll content in the *rug2* mutants.

Table S3. Morphometric analysis of the *rug2* mutants.

Table S4. Chloroplast and mitochondria custom array.

Table S5. Changes in plastidic and mitochondrial transcript abundance in the *rug2-1* mutant.

Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

REFERENCES

Abdelnoor, R.V., Yule, R., Elo, A., Christensen, A.C., Meyer-Gauen, G. and Mackenzie, S.A. (2003) Substoichiometric shifting in the plant mitochondrial genome is influenced by a gene homologous to MutS. *Proc. Natl. Acad. Sci. USA*, **100**, 5968–5973.

Ahokas, H. (1976) Two segregating cytoplasmic mutants of barley. *Hereditas*, **82**, 187–192.

Alonso, J.M., Stepanova, A.N., Leisse, T.J. *et al.* (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**, 653–657.

Asin-Cayuela, J., Helm, M. and Attardi, G. (2004) A monomer-to-trimer transition of the human mitochondrial transcription termination factor (mTERF) is associated with a loss of *in vitro* activity. *J. Biol. Chem.* **279**, 15670–15677.

Baba, K., Schmidt, J., Espinosa-Ruiz, A., Villarejo, A., Shiina, T., Gardestrom, P., Sane, A.P. and Bhalerao, R.P. (2004) Organellar gene transcription and early seedling development are affected in the *rpoT;2* mutant of Arabidopsis. *Plant J.* **38**, 38–48.

Babiychuk, E., Vandepoele, K., Wissing, J. *et al.* (2011) Plastid gene expression and plant development require a plastidic protein of the mitochondrial transcription termination factor family. *Proc. Natl. Acad. Sci. USA*, **108**, 6674–6679.

Bailey, S., Thompson, E., Nixon, P.J., Horton, P., Mullineaux, C.W., Robinson, C. and Mann, N.H. (2002) A critical role for the Var2 FtsH homologue of *Arabidopsis thaliana* in the photosystem II repair cycle *in vivo*. *J. Biol. Chem.* **277**, 2006–2011.

Berná, G., Robles, P. and Micol, J.L. (1999) A mutational analysis of leaf morphogenesis in *Arabidopsis thaliana*. *Genetics*, **152**, 729–742.

Berthold, D.A., Andersson, M.E. and Nordlund, P. (2000) New insight into the structure and function of the alternative oxidase. *Biochim. Biophys. Acta*, **1460**, 241–254.

Binder, S. and Brennicke, A. (2003) Gene expression in plant mitochondria: transcriptional and post-transcriptional control. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **358**, 181–188.

Cámara, Y., Asin-Cayuela, J., Park, C.B. *et al.* (2011) MTERF4 regulates translation by targeting the methyltransferase NSUN4 to the mammalian mitochondrial ribosome. *Cell Metab.* **13**, 527–539.

Chatterjee, M., Sparvoli, S., Edmunds, C., Garosi, P., Findlay, K. and Martin, C. (1996) *DAG*, a gene required for chloroplast differentiation and palisade development in *Antirrhinum majus*. *EMBO J.* **15**, 4194–4207.

Chen, M., Choi, Y.D., Voytas, D.F. and Rodermel, S. (2000) Mutations in the Arabidopsis *VAR2* locus cause leaf variegation due to the loss of a chloroplast FtsH protease. *Plant J.* **22**, 303–313.

Chen, Y., Zhou, G.J., Yu, M., He, Y.G., Tang, W., Lai, J.H., He, J., Liu, W.G. and Tan., D.Y. (2005) Cloning and functional analysis of human *mTERFL* encoding a novel mitochondrial transcription termination factor-like protein. *Biochem. Biophys. Res. Commun.* **337**, 1112–1118.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.G. and Thompson, J.D. (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res.* **31**, 3497–3500.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

Cui, J., Liu, J., Li, Y. and Shi, T. (2011) Integrative identification of Arabidopsis mitochondrial proteome and its function exploitation through protein interaction network. *PLoS One* **6**, e16022.

Emanuel, C., Weihe, A., Graner, A., Hess, W.R. and Borner, T. (2004) Chloroplast development affects expression of phage-type RNA polymerases in barley leaves. *Plant J.* **38**, 460–472.

Epp, M.D. (1973) Nuclear gene-induced plastome mutations in *Oenothera-hookeri* .1. genetic-analysis. *Genetics*, **75**, 465–483.

Fernández-Silva, P., Martínez-Azorín, F., Micol, V. and Attardi, G. (1997) The human mitochondrial transcription termination factor (mTERF) is a multizipper protein but binds to DNA as a monomer, with evidence pointing to intramolecular leucine zipper interactions. *EMBO J.* **16**, 1066–1079.

Fleming, A.J. (2005) The control of leaf development. *New Phytol.* **166**, 9–20.

Forner, J., Weber, B., Thuss, S., Wildum, S. and Binder, S. (2007) Mapping of mitochondrial mRNA termini in *Arabidopsis thaliana*: t-elements contribute to 5 'and 3 'end formation. *Nucleic Acids Res.* **35**, 3676–3692.

Gu, J.Y., Miles, D. and Newton, K.J. (1993) Analysis of leaf sectors in the NCS6 mitochondrial mutant of maize. *Plant Cell*, **5**, 963–971.

Hess, W.R. and Börner, T. (1999) Organellar RNA polymerases of higher plants. *Int. Rev. Cytol.* **190**, 1–59.

Hess, J.F., Parisi, M.A., Bennett, J.L. and Clayton, D.A. (1991) Impairment of mitochondrial transcription termination by a point mutation associated with the MELAS subgroup of mitochondrial encephalomyopathies. *Nature*, **351**, 236–239.

© 2011 The Authors
The Plant Journal © 2011 Blackwell Publishing Ltd,

Hricová, A., Quesada, V. and Micol, J.L. (2006) The *SCABRA3* nuclear gene encodes the plastid RpoTp RNA polymerase, which is required for chloroplast biogenesis and mesophyll cell proliferation in Arabidopsis. *Plant Physiol.* **141**, 942–956.

Huang, J., Taylor, J.P., Chen, J.G., Uhrig, J.F., Schnell, D.J., Nakagawa, T., Korth, K.L. and Jones, A.M. (2006) The plastid protein THYLAKOID FORMATION1 and the plasma membrane G-protein GPA1 interact in a novel sugar-signaling mechanism in Arabidopsis. *Plant Cell*, **18**, 1226–1238.

Hunt, M.D. and Newton, K.J. (1991) The NCS3 mutation: genetic-evidence for the expression of ribosomal protein genes in *Zea mays* mitochondria. *EMBO J.* **10**, 1045–1052.

Hyvärinen, A.K., Pohjoismäki, J.L., Reyes, A., Wanrooij, S., Yasukawa, T., Karhunen, P.J., Spelbrink, J.N., Holt, I.J. and Jacobs, H.T. (2007) The mitochondrial transcription termination factor mTERF modulates replication pausing in human mitochondrial DNA. *Nucleic Acids Res.* **35**, 6458–6474.

Hyvärinen, A.K., Pohjoismäki, J.L., Holt, I.J. and Jacobs, H.T. (2010) Overexpression of MTERFD1 or MTERFD3 impairs the completion of mitochondrial DNA replication. *Mol. Biol. Rep.* **38**, 1321–1328.

Jiao, S.X., Thornsberry, J.M., Elthon, T.E. and Newton, K.J. (2005) Biochemical and molecular characterization of photosystem I deficiency in the NCS6 mitochondrial mutant of maize. *Plant Mol. Biol.* **57**, 303–313.

Keddie, J.S., Carroll, B., Jones, J.D. and Gruissem, W. (1996) The *DCL* gene of tomato is required for chloroplast development and palisade cell morphogenesis in leaves. *EMBO J.* **15**, 4208–4217.

Keren, N., Ohkawa, H., Welsh, E.A., Liberton, M. and Pakrasi, H.B. (2005) Psb29, a conserved 22-kD protein, functions in the biogenesis of photosystem II complexes in *Synechocystis* and Arabidopsis. *Plant Cell*, **17**, 2768–2781.

Kim, M., Lim, J.H., Ahn, C.S., Park, K., Kim, G.T., Kim, W.T. and Pai, H.S. (2006) Mitochondria-associated hexokinases play a role in the control of programmed cell death in *Nicotiana benthamiana*. *Plant Cell*, **18**, 2341–2355.

Li, X.M., Zhang, L.S. and Guan, M.X. (2005) Cloning and characterization of mouse mTERF encoding a mitochondrial transcriptional termination factor. *Biochem. Biophys. Res. Commun.* **326**, 505–510.

Lindahl, M., Spetea, C., Hundal, T., Oppenheim, A.B., Adam, Z. and Andersson, B. (2000) The thylakoid FtsH protease plays a role in the light-induced turnover of the photosystem II D1 protein. *Plant Cell*, **12**, 419–431.

Linder, T., Park, C.B., Asin-Cayuela, J., Pellegrini, M., Larsson, N.G., Falkenberg, M., Samuelsson, T. and Gustafsson, C.M. (2005) A family of putative transcription termination factors shared amongst metazoans and plants. *Curr. Genet.* **48**, 265–269.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_{T}$ method. *Methods*, **25**, 402–408.

Logan, D.C. and Leaver, C.J. (2000) Mitochondria-targeted GFP highlights the heterogeneity of mitochondrial shape, size and movement within living plant cells. *J. Exp. Bot.* **51**, 865–871.

Mackenzie, S.A. (2005) Plant organellar protein targeting: a traffic plan still under construction. *Trends Cell Biol.* **15**, 548–554.

Marienfeld, J.R. and Newton, K.J. (1994) The maize NCS2 abnormal growth mutant has a chimeric *nad4-nad7* mitochondrial gene and is associated with reduced complex I function. *Genetics*, **138**, 855–863.

Martin, M., Cho, J.Y., Cesare, A.J., Griffith, J.D. and Attardi, G. (2005) Termination factor-mediated DNA loop between termination and initiation sites drives mitochondrial rRNA synthesis. *Cell*, **123**, 1227–1240.

Martínez-Zapater, J.M. (1993) Genetic-analysis of variegated mutants in Arabidopsis. *J. Hered.* **84**, 138–140.

Meskauskiene, R., Würsch, M., Laloi, C., Vidi, S., Coll, N., Kessler, F., Baruah, A., Kim, C. and Apel, K. (2009) A mutation in the Arabidopsis mTERF-related plastid protein SOLDAT10 activates retrograde signaling and suppresses $^{1}O_{2}$-induced cell death. *Plant J.* **60**, 399–410.

Micol, J.L. (2009) Leaf development: time to turn over a new leaf? *Curr. Opin. Plant Biol.* **12**, 9–16.

Micol, J.L. and Hake, S. (2003) The development of plant leaves. *Plant Physiol.* **131**, 389–394.

Naested, H., Holm, A., Jenkins, T. *et al.* (2004) Arabidopsis *VARIEGATED3* encodes a chloroplast-targeted, zinc-finger protein required for chloroplast and palisade cell development. *J. Cell Sci.* **117**, 4807–4818.

Newton, K.J. and Coe, E.H. (1986) Mitochondrial-DNA changes in abnormal growth (nonchromosomal stripe) mutants of maize. *Proc. Natl. Acad. Sci. USA,* **83**, 7363–7366.

Newton, K.J., Knudsen, C., Gabaylaughnan, S. and Laughnan, J.R. (1990) An abnormal growth mutant in maize has a defective mitochondrial cytochrome-oxidase gene. *Plant Cell*, **2**, 107–113.

Olinares, P.D., Ponnala, L. and van Wijk, K.J. (2010) Megadalton complexes in the chloroplast stroma of *Arabidopsis thaliana* characterized by size exclusion chromatography, mass spectrometry, and hierarchical clustering. *Mol. Cell Proteomics* **9**, 1594–1615.

Park, C.B., Asin-Cayuela, J., Cámara, Y. *et al.* (2007) MTERF3 Is a Negative Regulator of Mammalian mtDNA Transcription. *Cell*, **130**, 273–285.

Pérez-Pérez, J.M., Ponce, M.R. and Micol, J.L. (2002) The *UCU1* Arabidopsis gene encodes a SHAGGY/GSK3-like kinase required for cell expansion along the proximodistal axis. *Dev. Biol.* **242**, 161–173.

Pérez-Pérez, J.M., Ponce, M.R. and Micol, J.L. (2004) The *ULTRACURVATA2* gene of Arabidopsis encodes an FK506-binding protein involved in auxin and brassinosteroid signaling. *Plant Physiol.* **134**, 101–117.

Pfannschmidt, T., Nilsson, A. and Allen, J.F. (1999) Photosynthetic control of chloroplast gene expression. *Nature*, **397**, 625–628.

Piechota, J., Kolodziejczak, M., Juszczak, I., Sakamoto, W. and Janska, H. (2010) Identification and characterization of high molecular weight complexes formed by matrix AAA proteases and prohibitins in mitochondria of *Arabidopsis thaliana*. *J. Biol. Chem.* **285**, 12512–12521.

Polosa, L.P., Roberti, M., Musicco, C., Gadaleta, M.N., Quagliariello, E. and Cantatore, P. (1999) Cloning and characterisation of mtDBP, a DNA-binding protein which binds two distinct regions of sea urchin mitochondrial DNA. *Nucl. Acids Res.* **27**, 1890–1899.

Polosa, P.L., Megli, F., Di Ponzio., B., Gadaleta, M.N., Cantatore, P. and Roberti, M. (2002) Cloning of two sea urchin DNA-binding proteins involved in mitochondrial DNA replication and transcription. *Gene*, **286**, 113–120.

Ponce, M.R., Quesada, V. and Micol, J.L. (1998) Rapid discrimination of sequences flanking and within T-DNA insertions in the Arabidopsis genome. *Plant J.* **14**, 497–501.

Prieto-Martín, A., Montoya, J. and Martinez-Azorín, F. (2004) Phosphorylation of rat mitochondrial transcription termination factor (mTERF) is required for transcription termination but not for binding to DNA. *Nucleic Acids Res.* **32**, 2059–2068.

Quesada, V., Ponce, M.R. and Micol, J.L. (1999) *OTC* and *AUL1*, two convergent and overlapping genes in the nuclear genome of *Arabidopsis thaliana*. *FEBS Lett.* **461**, 101–106.

Quesada, V., Macknight, R., Dean, C. and Simpson, G.G. (2003) Autoregulation of FCA pre-mRNA processing controls Arabidopsis flowering time. *EMBO J.* **22**, 3142–3152.

Raghavendra, A.S. and Padmasree, K. (2003) Beneficial interactions of mitochondrial metabolism with photosynthetic carbon assimilation. *Trends Plant Sci.* **8**, 546–553.

Rédei, G.P. and Plurad, S.B. (1973) Hereditary structural alterations of plastids induced by a nuclear mutator gene in Arabidopsis. *Protoplasma*, **77**, 361–380.

Rhoades, M.M. (1943) Genic induction of an inherited cytoplasmic difference. *Proc. Natl. Acad. Sci. USA*, **29**, 327–329.

Roberti, M., Bruni, F., Polosa, P.L., Gadaleta, M.N. and Cantatore, P. (2006) The *Drosophila* termination factor DmTTF regulates *in vivo* mitochondrial transcription. *Nucleic Acids Res.* **34**, 2109–2116.

Robles, P. and Micol, J.L. (2001) Genome-wide linkage analysis of Arabidopsis genes required for leaf development. *Mol. Genet. Genomics* **266**, 12–19.

Rodermel, S. (2001) Pathways of plastid-to-nucleus signaling. *Trends Plant Sci.* **6**, 471–478.

Roussell, D.L., Thompson, D.L., Pallardy, S.G., Miles, D. and Newton, K.J. (1991) Chloroplast structure and function is altered in the NCS2 maize mitochondrial mutant. *Plant Physiol.* **96**, 232–238.

Sakamoto, W. (2003) Leaf-variegated mutations and their responsible genes in *Arabidopsis thaliana*. *Genes Genet. Syst.* **78**, 1–9.

Sakamoto, W., Tamura, T., Hanba-Tomita, Y. and Murata, M. (2002) The *VAR1* locus of Arabidopsis encodes a chloroplastic FtsH and is responsible for leaf variegation in the mutant alleles. *Genes Cells*, **7**, 769–780.

Schonfeld, C., Wobbe, L., Borgstadt, R., Kienast, A., Nixon, P.J. and Kruse, O. (2004) The nucleus-encoded protein MOC1 is essential for mitochondrial light acclimation in *Chlamydomonas reinhardtii*. *J. Biol. Chem.* **279**, 50366–50374.

Serrano-Cartagena, J., Candela, H., Robles, P., Ponce, M.R., Pérez-Pérez, J.M., Piqueras, P. and Micol, J.L. (2000) Genetic analysis of *incurvata* mutants

© 2011 The Authors

reveals three independent genetic operations at work in Arabidopsis leaf morphogenesis. *Genetics*, **156**, 1363–1377.

Shiina, T., Tsunoyama, Y., Nakahira, Y., Khan, M.S. and Kwang, W.J. (2005) Plastid RNA polymerases, promoters, and transcription regulators in higher plants. *Int. Rev. Cytol.* **24**, 1–68.

Sjögren, L.L.E., Stanne, T.M., Zheng, B., Sutinen, S. and Clarke, A.K. (2006) Structural and functional insights into the chloroplast ATP-dependent Clp protease in Arabidopsis. *Plant Cell*, **18**, 2635–2649.

Sundaresan, V., Springer, P., Volpe, T., Haward, S., Jones, J.D.G., Dean, C., Ma, H. and Martienssen, R. (1995) Patterns of gene-action in plant development revealed by enhancer trap and gene trap transposable elements. *Genes Dev.* **9**, 1797–1810.

Takechi, K., Murata, M., Motoyoshi, F. and Sakamoto, W. (2000) The *YELLOW VARIEGATED* (*VAR2*) locus encodes a homologue of FtsH, an ATP-dependent protease in Arabidopsis. *Plant Cell Physiol.* **41**, 1334–1346.

Tsukaya, H., Tsujino, R., Ikeuchi, M., Isshiki, Y., Kono, M., Takeuchi, T. and Araki, T. (2007) Morphological variation in leaf shape in *Ainsliaea apiculata* with special reference to the endemic characters of populations on Yakushima Island, Japan. *J. Plant. Res.* **120**, 351–358.

Wang, Q., Sullivan, R.W., Kight, A., Henry, R.L., Huang, J.R., Jones, A.M. and Korth, K.L. (2004) Deletion of the chloroplast-localized *Thylakoid formation1* gene product in Arabidopsis leads to deficient thylakoid formation and variegated leaves. *Plant Physiol.* **136**, 3594–3604.

Wenz, T., Luca, C., Torraco, A. and Moraes, C.T. (2009) mTERF2 regulates oxidative phosphorylation by modulating mtDNA transcription. *Cell Metab.* **9**, 499–511.

Wetzel, C.M., Jiang, C.Z., Meehan, L.J., Voytas, D.F. and Rodermel, S.R. (1994) Nuclear organelle interactions: the *immutans* variegation mutant of Arabidopsis is plastid autonomous and impaired in carotenoid biosynthesis. *Plant J.* **6**, 161–175.

Wu, D., Wright, D.A., Wetzel, C., Voytas, D.F. and Rodermel, S. (1999) The *IMMUTANS* variegation locus of Arabidopsis defines a mitochondrial alternative oxidase homolog that functions during early chloroplast biogenesis. *Plant Cell*, **11**, 43–56.

Yoo, S.-D., Cho, Y.-H. and Sheen, J. (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Protocols* **2**, 1565–1572.

Yu, F., Park, S. and Rodermel, S.R. (2004) The Arabidopsis FtsH metalloprotease gene family: interchangeability of subunits in chloroplast oligomeric complexes. *Plant J.* **37**, 864–876.

Yu, F., Fu, A.G., Aluru, M., Park, S., Xu, Y., Liu, H.Y., Liu, X.Y., Foudree, A., Nambogga, M. and Rodermel, S. (2007) Variegation mutants and mechanisms of chloroplast biogenesis. *Plant Cell Environ.* **30**, 350–365.

Zhang, H., Huang, H.M., Carson, R.C., Mahmood, J., Thomas, H.M. and Gibson, G.E. (2001) Assessment of membrane potentials of mitochondrial populations in living cells. *Anal. Biochem.* **298**, 170–180.

Zheng, B., MacDonald, T.M., Sutinen, S., Hurry, V. and Clarke, A.K. (2006) A nuclear-encoded ClpP subunit of the chloroplast ATP-dependent Clp protease is essential for early development in *Arabidopsis thaliana*. *Planta*, **224**, 1103–1115.

© 2011 The Authors
The Plant Journal © 2011 Blackwell Publishing Ltd,