LETM Proteins Play a Role in the Accumulation of Mitochondrially Encoded Proteins in Arabidopsis thaliana and AtLETM2 Displays Parent of Origin Effects*5

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Botao Zhang‡, Chris Carrie‡, Aneta Ivanova‡, Reena Narasai‡§, Monika W. Murcha‡, Owen Duncan‡, Yan Wang‡, Simon R. Law‡, Verónica Albrecht‡, Barry Pogson‡, Estelle Giraud‡, Olivier Van Aken‡1, and James Whelan‡1,‡

From the ‡Australian Research Council Centre of Excellence in Plant Energy Biology and ‡Centre for Computational Systems Biology, Bayliss Building M316 University of Western Australia, 35 Stirling Highway, Crawley 6009, Western Australia, Australia and the ‡Australian Research Council Centre of Excellence in Plant Energy Biology, Research School of Biology, Australian National University, Acton 2601, Australian Capital Territory, Australia

Background: Plants contain homologs of the conserved mitochondrial protein LETM1.

Results: AtLETM1 and AtLETM2 are important proteins for accumulation of ATP synthase proteins in Arabidopsis thaliana, and double mutants are not viable. AtLETM2 expression displays parent of origin effects during seed development.

Conclusion: Accumulation of mitochondrially encoded proteins is under maternal control during early development.

Significance: This work reveals that mitochondrial gene expression is under maternal control early in development.

The Arabidopsis thaliana genome contains two genes with homology to the mitochondrial protein LETM1 (leucine zipper-EF-hand-containing transmembrane protein). Inactivation of both genes, Atletm1 and Atletm2, together is lethal. Plants that are hemizygous for AtLETM2 and homozygous for Atletm1 (letm1(−/−) LETM2(+/-)) displayed a mild retarded growth phenotype during early seedling growth. It was shown that accumulation of mitochondrial proteins was reduced in hemizygous (letm1(−/−) LETM2(+/-)) plants. Examination of respiratory chain proteins by Western blotting, blue native PAGE, and enzymatic activity assays revealed that the steady state level of ATP synthase was reduced in abundance, whereas the steady state levels of other respiratory chain proteins remained unchanged. The absence of a functional maternal AtLETM2 allele in an Atletm1 mutant background resulted in early seed abortion. Reciprocal crosses revealed that maternally, but not paternally, derived AtLETM2 was absolutely required for seed development. This requirement for a functional maternal allele of AtLETM2 was confirmed using direct sequencing of reciprocal crosses of Col-0 and Ler accessions. Furthermore, AtLETM2 promoter β-glucuronidase constructs displayed exclusive maternal expression patterns.

The endosymbiotic event that led to the formation of mitochondria occurred over one billion years ago (1), yet the mitochondrial genome from a wide variety of phylogenetic lineages still contains a small number of genes that encode from three to over 50 proteins (2–5). These proteins are each found in multisubunit protein complexes and must be assembled along with nucleus-encoded proteins to form functional protein complexes (6). Although the coding capacity of mitochondria is ≈5% of the total number of proteins that are located in mitochondria, which is estimated to be >1000 (7), the proteins encoded in the mitochondrial genome are essential for normal growth and development. In many cases, mutations or disruption of mitochondrial genes results in a lethal or severely retarded growth phenotype (8). The majority of the proteins required for the transcription, translation, and replication of mitochondrial DNA are encoded in the nucleus and must be translated in the cytosol before being imported into the mitochondrion (7). Literally hundreds of nucleus-located genes are required for the expression of mitochondrial genes, from the proteins required for DNA replication and transcription to processing of transcripts. Translation requires discrete translation machinery, ranging from ribosomes to aminoacyl-tRNA synthetases and various other factors that make up the translational machinery. In higher plants, hundreds of genes that encode pentatricopeptide proteins are required for editing and processing of mitochondrial RNA (9), and many aspects of organelle transcription and RNA processing have been studied in detail. In contrast, little is known about factors that may affect or regulate the translation of organelle-encoded transcripts in plant mitochondria (10).

Yeast are distinct from other eukaryotes in that mitochondrial inheritance is biparental. In multicellular eukaryotes, inheritance of mitochondria is uniparental (11), with some minor exceptions, such as the parental “leakage” observed in Silene vulgaris (12). The precise mechanism(s) defining maternal inheritance of mitochondria are not clear, but the identification and characterization of specific endonucleases expressed in pollen cells in Arabidopsis thaliana (Arabidopsis) provides a mechanism that selectively degrades paternal mitochondrial DNA (13). Although the nuclear genome is clearly inherited in a Mendelian manner, studies have shown that sub-

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1 Supported by Australian Research Council APD Discovery Fellowship DP110102868.
2 To whom correspondence should be addressed. Tel.: 61-8-6488-1749; Fax: 61-8-6488-4401; E-mail: jim.whelan@uwa.edu.au.
sets of genes are preferentially expressed based on the parent of origin and are defined as imprinted genes (14). Imprinting primarily occurs in the endosperm in *Arabidopsis* and is proposed to occur because of the parental conflict hypothesis (15). This hypothesis states that in mammals and flowering plants with multiple embryos, where different parental fertilization of embryos can occur and where the embryo receives nutrition from the maternal tissue via the placenta or endosperm, there is a conflict because the male parent would favor investment of more resources in the embryo fertilized by its gametes, whereas the female would favor equal investments in all embryos. This hypothesis is proposed as a driving force for exclusive or biased maternal expression of genes during embryo development.

Here, we detail the characterization of two nucleus-encoded, mitochondrial proteins, AtLETM1 and AtLETM2 (lecine zipper-EF-hand-containing transmembrane protein). AtLETM1 and AtLETM2 show similarity to the mammalian protein LETM1 and yeast Mdm38 (16, 17). Human LETM1 is associated with Wolf-Hirschhorn syndrome. Yeast mdm38 mutants display abnormal mitochondrial morphology and potassium homeostasis (18, 19). The demonstration that LETM1 in mitochondria from *Drosophila* mediates Ca\(^{2+}\)/H\(^+\) supports a role in maintaining ionic balance (20). In addition, a role for Mdm38 in mitochondrial translation has been shown in yeast. In yeast, at least three proteins bind to ribosomes to affect translation: Oxa1, Mba1, and Mdm38 (16). Specifically, Mdm38 functions as a membrane-bound ribosome receptor that has overlapping functions with Mba1 and is required for the translation of COX1 and CytB mRNA in yeast; a double mutant of mdm38 and mba1 lacks complex III and IV (16). This role in translation is distinct from that in regulating ionic balance because the latter can be rescued by nigericin, whereas the former cannot (16). Additionally, it should be noted that a variety of translational activators have been identified in yeast that are specific for each transcript (21), and substituting the COX1 UTRs for that of COX2 results in the recovery of translation of COX1 (16). We show that AtLETM1 and AtLETM2 are required for efficient translation in mitochondria in *Arabidopsis*. We demonstrate that maternal expression of LETM2 is required for successful seed development and, furthermore, that the amount of LETM2 protein in mitochondria is correlated with gene dosage for LETM2.

**EXPERIMENTAL PROCEDURES**

**T-DNA Insertion Lines**—The following T-DNA insertion lines were obtained from the ABRC stock center: *AtLETM1* (At3g59820): SALK_067558C (letm1-1), SALK_058471 (letm1-2) and *AtLETM2* (At1g65540): SALK_068877 (letm2-1), WIScDsoX15S16g10B (letm2-2). Plants were genotyped by PCR, and the insert positions were confirmed by sequencing with T-DNA left border primers. Primer sequences are listed in supplemental Table 1. Phenotype analysis of all mutants was carried out as described by Boyes et al. (22).

**Construction of GFP Fusion Vectors**—Coding sequences for *LETM1* and *LETM2* were amplified from *Arabidopsis* cDNA using standard protocols with the Roche Expand High Fidelity PCR system (Roche Applied Science), using gene-specific primers flanked by Gateway recombination cassettes (Invitrogen) (supplemental Table 1). The first 300 bp as well as the full-length coding sequences were cloned into pDONR201 according to the manufacturer’s instructions. Cloning into the final GFP vectors was as described by Carrie et al. (23). As a mitochondrial marker, the alternative oxidase (AOX)\(^{3}\) targeting signal of 42 amino acids fused to GFP was used (23).

**Stable Transformation, Biolistic Transformation, and Microscopy**—To monitor mitochondrial morphology in the hemizygous AtLETM2 (letm1(−/−) LETM2(+/−)), a cross was carried out between the aforementioned hemizygous mutant and a line of *Arabidopsis* stably transformed with a mitochondrial targeted GFP and backcrossed to obtain the letm1(−/−) LETM2(+/−) hemizygous genotype. The resulting seeds were then grown on Murashige and Skoog (MS) medium. Biolistic co-transformation of the GFP and RFP fusion vectors was performed on *Arabidopsis* cell culture and onion epidermal cells as previously reported (23). In brief, GFP and RFP plasmids (5 μg each) were co-precipitated onto gold particles and transformed using a PDS-1000/He biolistic transformation system (Bio-Rad). Two ml of *Arabidopsis* cell suspension (on a paper filter) or freshly peeled onion epidermal cells were placed on osmotically medium and bombarded. Cells were then incubated for 24–48 h at 22°C in the dark. GFP and RFP expression in both the permanent and transient transformants were visualized using a BX61 Olympus microscope (Olympus, Melbourne, Australia) using excitation wavelengths of 460/480 nm (GFP) and 535/555 nm (RFP) and emission wavelengths of 495–540 nm (GFP) and 570–625 nm (RFP). Subsequent images were captured using Cell\(^{3}\) imaging software as described previously (24).

**Mitochondrial Purification**—To obtain mitochondria for biochemical analysis, wild-type (Col-0) and hemizygous AtLETM2 (letm1(−/−) LETM2(+/−)) seeds were first vertically grown on MS agar plates for 3 days under continuous light conditions before being transferred to liquid culture medium to allow visual selection of hemizygous AtLETM2 seedlings. Seedlings were grown for another 7 days in MS medium under long day conditions (16 h light, 8 h darkness) before harvesting for mitochondrial preparations. Mitochondria were harvested from 10-day-old seedlings as described previously (25). Typically 2–3 mg of mitochondrial protein were obtained from ~20 g of starting tissue. Mitochondrial protein concentrations were determined using the Coomassie protein assay reagent (Thermo Scientific, Rockford, IL) in triplicate.

**In Organello Protein Synthesis**—In organello protein synthesis assays were carried out as described previously (10), using freshly prepared mitochondria. The reactions were resolved by SDS-PAGE (26), and the incorporation of \[^{35}\text{S}\]\text{Met} into protein bands was determined by exposing a phosphorimaging plate to a dried gel for 48 h. The exposed plate was visualized using the Personal Molecular Imager (Bio-Rad) at high resolution. Radio-labeled bands corresponding to the correct apparent molecular weights for ATPα, CYTB, COXII, NAD9, NAD6, and ATP9 where quantitated at 10, 30, and 60 min time points using

\(^{3}\)The abbreviations used are: AOX, alternative oxidase; MS, Murashige and Skoog; GUS, β-glucuronidase; BN-PAGE, blue native PAGE; PIC, mitochondrial phosphate carrier; Ler, Landsberg erecta.
Quantity One software (Bio-Rad). Radiolabeled bands where uniformly boxed, and the pixel density was determined for each band minus the background. The pixel densities were expressed relative to Col-0 at 60 min, which was adjusted to 1. Three biological replicates were performed and quantitated, and the average and S.E. relative to Col-0 were determined. The incorporation of \(^{35}S\) Met was compared for each band across the four genotypes to give the relative efficiency of translation of each protein in each genotype (Fig. 4A), and also the incorporation of \(^{35}S\) Met into each band was expressed as a percentage of the total incorporation for the six identified bands in each genotype, to give a measure of the total incorporation into each band in each genotype (supplemental Fig. 4).

**In Vitro Import Studies**—\(^{35}S\) Met-labeled precursor proteins for AOX (X68702) (27) and mitochondrial phosphate carrier (Pic) (AB016064) (28, 29) were synthesized using rabbit reticulocyte TNT in vitro transcription/translation lysis (Pro-mega, Melbourne, Australia) as described previously (27). Time course analysis of precursor protein import into intact mitochondria isolated from wild-type (Col-0) or mutant plants was performed as described previously, and radiolabeled imported proteins were quantified at each time point and normalized to the highest time point for Col-0, which was set to 100% using Quantity One software (Bio-Rad). Three biological replicates were carried out for each mutant and precursor protein, and the average and S.E. of import amount relative to Col-0 were determined (25).

**Immunodetection of Proteins**—Isolated mitochondria (15 \(\mu\)g) were resolved by SDS-PAGE, transferred to Hybond-C extra nitrocellulose membrane, and immunodetected as outlined previously (23). To generate antibodies to AtLETM1, recombinant proteins containing amino acids 387–525 (LETM1 AB1) and amino acids 540–667 (LETM1 AB2) of AtLETM1 fused to an N-terminal His\(_6\) affinity purification tag were expressed in Escherichia coli strain BL21 (DE3) pLys (Stratagene, La Jolla, CA) using the pDEST17 expression vector (Invitrogen). The recombinant protein was purified by denaturing immobilized metal affinity chromatography, using the Profinia protein purification system (Bio-Rad). The resultant eluate was separated by SDS-PAGE, and the recombinant protein was extracted using a Bio-Rad model 422 Electro-Eluter. Buffer exchange was performed using an Amicon Ultracel 5K centrifugal filter device (Millipore) such that the antigen was resuspended in PBS solution, recovering a total of 3 mg (LETM1 AB1) and 4 mg (LETM1 AB2) of proteins for inoculation. Four separate doses were administered to a rabbit at regular intervals over a 3-month period using standard protocols and Freund’s complete adjuvant solution (30). Other antibodies used have been described previously: RISP (31), NAD9 (32), uncoupling protein (33), TOM40 (24), and TIM17-2 (34). ATP\(_\beta\) and COXII were obtained from Agrisera (Vannas, Sweden). ATP\(_\alpha\), porin, E1\(_x\), and AOX were obtained from Dr. Tom Elthon (University of Nebraska, Lincoln, NE). Detection of cross-reacting products was carried out using chemiluminescence, and the signal was captured using ImageQuant RT ECL (GE Healthcare) with the signal intensity (pixel intensity) adjusted relative to wild type (Col-0) and set to 100. The abundance of each protein was normalized to wild type (Col-0), which was set to 100%. Three biological replicates were carried out, and the average and S.E. relative to wild type (Col-0) were determined.

**Promoter Reporter Analysis**—1 kb region upstream of the start codons of LETM1 and LETM2 was cloned into pBGWSF7 (35) in fusion with GUS, by Gateway recombination (Invitrogen). The homozygous promoter-GUS fusion transgenic plants from five independent lines were stained with 5-bromo-4-chloro-3-indolyl-\(\beta\)-glucuronic acid (GoldBio) as described (36).

**Analysis of Gametophytes and Seed Development and Sequencing of LETM2 Transcript from Embryo and Endosperm**—Unfertilized ovules and seeds at different developmental stages of Col-0 and AtLETM1 mutant lines were cleared using chloral hydrate and mounted on microscope carriers. Microscopy was then performed on a Zeiss Axioplan differential interference contrast microscope. Endosperm nuclei were counted using images displayed on a video monitor attached to the microscope, as reported before (37). Viability staining and counting of pollen were performed as described previously (38). Statistical tests for viable/aborted ratios and progeny genotyping of the reciprocal crosses were performed using a Pearson’s \(\chi^2\) test. Flower buds of Col-0 and Landsberg erecta (Ler) plants were emasculated and reciprocally pollinated 36 h later. Embryo and endosperm were dissected under a dissecting microscope 6 days after pollination, which corresponded to the late torpedo stages of embryogenesis. Embryos were washed to remove contaminating endosperm and seed coats using RLT buffer (RNAeasy kit, Qiagen, Melbourne, Australia). Approximately 10 siliques were dissected, and total RNA was extracted using the RNAeasy kit plus DNase digestion. Three independent sets of total RNA were obtained from each direction of the crosses. Total RNA was converted to double-stranded cDNA using the Invitrogen cDNA synthesis kit (Melbourne, Australia). Fragments of the LETM2 gene, containing SNP between Col-0 and Ler ecotypes, were amplified with primers 5’-GGTACGCGACGATGTTTCGG-3’ and 5’-CTGAAAAGATGAATT-TAAGTC-3’ and sequenced by the Sanger method.

**Quantitative RT-PCR**—RNA isolation, cDNA generation, and quantitative RT-PCR on the mitochondrial transcriptome were performed as described (39) using the LightCycler 480 SYBR Green I Master Mix (Roche Applied Science) and the primer pairs listed in supplemental Table 1 on a LightCycler 480 real-time PCR system (Roche Applied Science). The nuclear 18S rRNA gene was used for data normalization. Transcripts were measured in technical triplicate from three independent RNA isolations in separate experiments.

**Global Transcript Analysis**—Analysis of the changes in transcript abundance between Col-0, and double mutants was performed using Affymetrix GeneChip\textsuperscript{TM} Arabidopsis ATH1 Genome Arrays (Affymetrix, Santa Clara, CA), along with preliminary data quality assessment, as described previously (31). Once processed, GC Robust Multiarray Average normalized gene expression values were analyzed to identify differentially expressed genes by a regularized \(t\) test based on a Bayesian statistical framework using the software program Cyber-T (40) as in Ref. 39. Changes were considered significant at an false discovery rate correction level of PPDE (\(>P\)) >0.95 and a \(-fold\) change greater than 1.5. Hierarchical clusters were generated in
Partek Genomics Suite software, version 6.3 (Partek, St. Louis, MO) using Euclidean distance and average linkage measures. Overrepresented gene ontology full categories were identified using a hypergeometric test with a significance threshold of 0.05 after a Benjamini and Hochberg false discovery rate correction (41).

**Measurement of ATP Concentration**—Total ATP levels were measured using the ATP bioluminescent assay kit (Sigma). Leaves from Col-0 and hemizygous AtLETM2 plants were frozen in liquid nitrogen and ground using a ball mixer. Plant extracts were resuspended in 500 μl of 2.3% (v/v) TCA. After centrifugation for 15 min at 20,000 × g, the supernatant was recovered and neutralized using a few drops of 2.5 M K2CO3. Total ATP levels were measured using the ATP bioluminescent assay kit (Sigma).

**Mitochondrial Respiration and Complex Activity Assays**—Isolated mitochondria of *in vitro* grown seedlings were used for respiration measurements and succinate dehydrogenase activity assays as described previously (42).

**Starch Staining**—For the staining of plants for starch, leaves were boiled in 80% (v/v) ethanol in order to remove chlorophyll. After washing three times in water, leaves were placed in 50% (v/v) Lugol solution (Sigma) for 5 min. Leaves were destained in water for 60 min before being imaged.

**Identification of Protein Complex Subunits**—Isolated mitochondria were separated by blue native PAGE as described previously (43). Complexes of interest were excised and were recovered and neutralized using a few drops of 2.5 M K2CO3. The supernatant was then dialyzed for 30 min against 2.5 M K2CO3 to remove KCl. Mitochondria were separated by blue native PAGE as described previously (44). After digestion and analysis by LC-MS as described previously (44).

**Accession Numbers**—The genes described in this article correspond to the following Arabidopsis Genome Initiative codes: At3g59820 (AtLETM1) and At1g65540 (AtLETM2). A complete list of oligonucleotides used in this study is available in supplemental Table 1. All raw and processed microarray data files can be found at ArrayExpress under the accession number E-MEXP-3328 (www.ebi.ac.uk).

**RESULTS**

**AtLETM1 and AtLETM2 Are Conserved Mitochondrial Proteins**—Previous studies have defined a number of nucleus-located genes encoding mitochondrial proteins that respond to a wide variety of stresses in *Arabidopsis* (45). Several of these genes have no defined function, and many have additional alleles that display high levels of sequence identity and constitutive expression, suggesting basic housekeeping functions. Thus, we undertook studies to determine the function of these genes. One gene, encoded at locus At3g59820, was annotated as a putative calcium-binding protein, but no other functional data have been reported. A close homolog of this gene, At1g65540, was identified in the *Arabidopsis* genome, with the predicted protein displaying 58% amino acid identity and 72% amino acid similarity. A BLAST search identified these genes as homologous to the mitochondrial proteins, LETM1, in humans (46) and Mdm38 in yeast (47). These two orthologs display 48 and 43% amino acid identity, respectively, in the conserved LETM1 domain of AtLETM1. Therefore, the *Arabidopsis* genes were labeled *AtLETM1* (At3g59820) and *AtLETM2* (At1g65540). Structural analysis revealed that all LETM1-like proteins contain a central conserved domain, termed the LETM1-like domain, which features a highly conserved 22-amino acid transmembrane domain (Fig. 1A). Furthermore, the plant and animal lineages have acquired a C-terminal Ca2+-binding EF-hand domain, which is absent from the yeast lineage. The leucine zipper domain seems to be only present in the animal LETM1-like proteins and has diverged in plants and yeast.

**Simultaneous Mutations in AtLETM1 and AtLETM2 Affect Plant Viability**—To characterize the function of *Arabidopsis* AtLETM proteins *in planta*, two independent knock-out lines were obtained for each gene, and the site of the T-DNA insertion was confirmed by DNA sequencing (Fig. 1B). Homozygous knock-out plants for each gene were obtained, and for single homozygous knock-out plants, shoot growth, leaf development, and flowering appeared normal (Fig. 1C and supplemental Fig. 1). Analysis of root growth on vertical MS plates revealed a noticeable reduction in root length of ~15% in *letm1* plants (supplemental Fig. 1A). Attempts to obtain double homozygous knock-out plants were not successful, and only plants that were homozygous knock-out for one of the *LETM* genes and hemizygous for the other could be obtained (Fig. 1A). It was noticed that there was a phenotypic difference in the hemizygous plants, which differed between which of the two *LETM* genes were present in hemizygous form in the genome. *LETM1*/+/*letm2*/(−/−) displayed a normal phenotype, similar to any single homozygous knock-out (Fig. 1A). However, *letm1*/(−/−) *LETM2*/(+/+) plants consistently displayed retarded growth in the first few weeks after germination, with true leaves 1–6 showing irregular deformations (Fig. 1, C and D). Early root growth was severely impaired to only 40% of that in wild-type (Col-0) plants but improved to normal rates and length with age (supplemental Fig. 1A). Germination itself did not appear to be affected in any of the mutant lines. Leaves 1 and 2 (growth stage 1.02) emerged around day 10 in wild-type and single mutant plants, whereas true leaves in *LETM1*/(+/−) *letm2*/(−/−) plants emerged 2 days later (supplemental Fig. 1, B and E). Emergence of true leaves was delayed until leaf 6 (stage 1.06), after which no significant difference in emergence time could be observed with wild-type (Col-0) plants (supplemental Fig. 1, B and F). Similarly, morphological aberrations were observed in leaves 1–6, whereas leaves 7 and onward developed normally, similar to wild-type plants. The maximum rosette radius was 10–15% smaller in *LETM1*/(+/−) *letm2*/(−/−) plants until 30 days after sowing, upon which mutant plants caught up with wild-type (Col-0) plants (supplemental Fig. 1C). Thus, after 4 or more weeks, the plants appeared essentially normal. The appearance of the first open flower (stage 6.00) was caught up with wild-type (Col-0) plants (supplemental Fig. 1).

It was also noticeable in the hemizygous *letm1*/(−/−) *LETM2*/(+/+) plants that the lesions at the leaf edges corresponded to where *AtLETM1* was expressed, as determined by *AtLETM1* promoter:GUS fusion (Fig. 1E).
FIGURE 1. Identification of AtLETM1 and AtLETM2 mutants. A, diagram of the domain structure of LETM1-like proteins. TM, transmembrane region; cc, coiled-coil; LZ, leucine zipper; EF, EF-hand domain.

B, diagram of insertion positions of T-DNAs in AtLETM1 and AtLETM2. Black boxes, open boxes, and black lines indicate exons, untranslated regions, and introns, respectively. Insertion sites were verified by sequencing of PCR products.

C, 18-day-old wild type (Col-0) and AtLETM1 and AtLETM2 single and double mutants grown under long day regime (16-h photoperiod). The arrows indicate the leaves harvested for genotyping.

D, arrangement of cotyledons and leaves 1–12 of wild-type and hemizygous letm1-1(-/-) LETM2-1(+/+) plants. The arrows indicate deformations at the leaf edge of young leaves. E, comparison of 12-day-old mutant letm1-1(-/-) LETM2-1(+/+) plant with 6–8-day-old AtLETM1 promoter-GUS reporter plants. The arrows indicate malformed leaf edges, which correspond with GUS expression pattern.
AtLETM1 and AtLETM2 Are Mitochondrial Inner Membrane Proteins—Although we have previously demonstrated that AtLETM1 is a mitochondrial protein (45), analysis of the targeting specificity for AtLETM2 was also carried out to confirm a mitochondrial location. Transient transformation of full-length AtLETM2 or the first 100-amino acid sequence of AtLETM2 coupled to GFP via the N terminus into Arabidopsis suspension cells or onion epidermal cells resulted in an exclusively mitochondrial pattern (Fig. 2A). In order to determine the intraorganellar location of AtLETM1 and AtLETM2, in vitro import assays were carried out (Fig. 2B). For this, radiolabeled full-length proteins were incubated with mitochondria under conditions that support protein uptake into mitochondria. The uptake of the AOX from Arabidopsis and PIC from maize was used as a control for mitochondrial import. Mit, mitochondria; Mit-OM, mitochondria with the outer membrane ruptured; PK, proteinase K; Val, valinomycin; P, precursor protein band; M, inner membrane protected protein band; I, intermediate protein; I’, inner membrane protected mature band protein when the outer membrane was ruptured prior to protease treatment. C, Western blotting of isolated mitochondria of wild-type (Col-0) and mitochondrial soluble and membrane fractions extracted by sodium carbonate. Two antibodies raised against ATLETM1 (ATLETM1 AB1 and ATLETM1 AB2) and antibodies against TIM17-2 and pyruvate dehydrogenase E1α were used as controls for membrane and soluble proteins, respectively. The apparent molecular masses are indicated in kDa as calculated using an LMW calibration kit (GE Healthcare).

AtLETM1 and AtLETM2 Are Mitochondrial Inner Membrane Proteins—Although we have previously demonstrated that AtLETM1 is a mitochondrial protein (45), analysis of the targeting specificity for AtLETM2 was also carried out to confirm a mitochondrial location. Transient transformation of full-length AtLETM2 or the first 100-amino acid sequence of AtLETM2 coupled to GFP via the N terminus into Arabidopsis suspension cells or onion epidermal cells resulted in an exclusively mitochondrial pattern (Fig. 2A). In order to determine the intraorganellar location of AtLETM1 and AtLETM2, in vitro import assays were carried out (Fig. 2B). For this, radiolabeled full-length proteins were incubated with freshly isolated mitochondria. In vitro import of both AtLETM1 and AtLETM2 was observed, as evidenced by the appearance of a processed peptide designated the mature protein, using both intact mitochondria and mitochondria with the outer membrane removed. The processed peptide was protected against proteinase K degradation and required a membrane potential to cross the inner mitochondrial membrane, as shown by the lack of import in the presence of a transmembrane potential inhibitor, valinomycin. These results indicate that AtLETM1 and AtLETM2 proteins are imported into or across the inner mitochondrial membrane (Fig. 2B).

Antibodies were raised against two regions of AtLETM1 protein sequence (supplemental Fig. 2). Using full-length in vitro expressed AtLETM1 and AtLETM2 proteins, it was demonstrated that the AtLETM1 antibody 1 (AtLETM1 AB1) cross-reacted with both AtLETM1 and AtLETM2 proteins, whereas AtLETM1 antibody 2 (AtLETM1 AB2) only cross-reacted with AtLETM1 (supplemental Fig. 2C). Probing intact mitochondria along with membrane and soluble mitochondrial fractions, obtained using carbonate extractions from wild-type Col-0 plants, indicated that both AtLETM1 and AtLETM2 were uniquely located in the membrane fraction (Fig. 2C). These results taken together with the in vitro import assays demonstrate that AtLETM1 and AtLETM2 are mitochondrial inner membrane proteins, in agreement with the presence of a transmembrane domain (Fig. 1B). It is also worth noting that the absence of AtLETM1 could be clearly demonstrated in mitochondria from the letm1-1 (−/−) LETM2-1 (+/+ ) line because
probing mitochondria isolated from this line with both antibodies detected a specific product when probed with AtLETM1 A1B1, but no product was detected when probed with AtLETM1 A2 (supplemental Fig. 2D).

AtLETM1 or AtLETM2 Is Required for Efficient Translation in Mitochondria—To determine the reason for the early growth defect observed with letm1(−/−) LETM2(+/+) plants, a variety of assays characterizing mitochondrial function were carried out. Transmission electron microscopy analysis of mitochondria and crossing letm1-1(−/−) LETM2-1(+/+) plants stably transformed with GFP fused to a mitochondrial targeting signal (48) did not reveal any differences in morphology, number, or abundance of mitochondria (supplemental Fig. 3). However, it was apparent that chloroplast structure was altered in leaves in that there was a large accumulation of starch in the chloroplasts, and plastoglobules were noticeably larger (supplemental Fig. 3C). The increased accumulation of starch in letm1(−/−) LETM2(+/+) lines was confirmed with starch leaf stains carried out at the end of the light period and at the end of the dark period (supplemental Fig. 3C). Significantly more starch was apparent in the letm1(−/−) LETM2(+/+) plants compared with wild-type tissue. Notably, the accumulation and breakdown of starch in the cotyledons was unaffected and was similar to wild type (supplemental Fig. 3C).

Because hemizygous (letm1-1(−/−) LETM2-1(+/−)) plants still contain a functional LETM gene, the reason for the aberrant growth phenotype was investigated. Using antibodies raised against the LETM protein, Western blot analysis was carried out on mitochondria isolated from wild-type (Col-0) plants and each mutant allele, letm1(−/−) LETM2(+/-), LETM1(+/+) letm2(−/−), and hemizygous (letm1-1(−/−) LETM2-1(+/−)) (Fig. 3). Strikingly, mitochondria from hemizygous plants (letm1-1(−/−) LETM2-1(+/−)) contained half the amount of LETM protein compared with wild-type (Col-0) or when two alleles were present, letm1(−/−) LETM2(+/+) or LETM1(+/+) letm2(−/−) (Fig. 3A). Noticeably, letm1(−/−) LETM2(+/+) plants that displayed a mild root growth phenotype and had a slight reduction in the amount of LETM2 protein (supplemental Fig. 1A). The linearity of detection with the LETM antibody was demonstrated with dilution of mitochondrial protein (Fig. 3A). To investigate if the abundance of additional mitochondrial proteins was being modulated, Western blot analyses were carried out. It was revealed that the abundance of proteins in Complex I (NAD9), Complex III (RISP), and Complex IV (COX II) was not significantly changed compared with wild-type (Col-0). However, the α-subunit of ATP synthase (ATPα) was consistently reduced in abundance by almost 50% in hemizygous plants (Fig. 3B). To further investigate if any other mitochondrial processes were affected, Western blot analysis was carried out using antibodies to AOX, a stress-induced bypass component of the plant respiratory chain (49). AOX is induced by a variety of stresses, in particular perturbation of Complex I (50) and IV (51) of the cytochrome respiratory chain. There was no increase in abundance of AOX. Also, there was no increase in the amount of the mitochondrial uncoupling protein (Fig. 3B). Porin and TOM40 antibodies were used to verify equal protein loading (Fig. 3B).

Because yeast Mdm38 had been previously demonstrated to be required for the translation of COXII and CYTB (16), the rate of translation of proteins in mitochondria was investigated. Mitochondria were isolated from Arabidopsis seedlings, and in organello protein synthesis revealed that the translation of multiple proteins was reduced in mitochondria isolated from letm1-1(−/−) LETM2-1(+/+) mutant plants (Fig. 4A). Because over 50 proteins are encoded in the Arabidopsis mitochondrial genome (53), it is not possible to identify all protein bands based on apparent molecular mass; however, based on previous studies, six protein bands (namely ATPα, ATPβ, NAD6 and -9, CYTB, and COXII) can be identified based on apparent molecular mass (10, 26). There was some decrease or increase observed in the single mutants, with translation in the single mutant letm1(−/−) LETM2(+/+) consistently displaying an approximately 10–20% reduction and translation in LETM1(+/+) letm2(−/−) displaying a 10–20% increase, with the exception of subunit 9 of ATP synthase, which was translated at the highest levels in wild type (Fig. 4A). Translation of subunit 9 in all mutants was reduced, but it was reduced by the greatest amount in the hemizygous mutant, where it reached 35% of wild-type levels (Fig. 4A). It was notable that the difference in incorporation of [35S]Met into protein was greatest after 60 min, with significant differences (p < 0.01, Student’s t test) between incorporation from mitochondria from Col-0 and letm1-1(−/−) LETM2-1(+/−) observed for all proteins. However, after 10 min, little or no incorporation was detected, and after 30 min, the difference in incorporation of [35S]Met for ATPα and CYTB was not significant (Fig. 3A). Thus, the differences were most apparent after 60 min.

Analysis of the incorporation of [35S]methionine into ATP9, compared with incorporation into the other protein bands that could be identified revealed that it accounted for >50% of incorporation of [35S]Met in mitochondria from wild-type plants (supplemental Fig. 4). Note that although the incorporation of [35S]Met was still 42% into ATP9 in mitochondria from hemizygous letm1-1(−/−) LETM2-1(+/+) plants, incorporation into ATP9 was only one-third compared with wild type. Thus, Western blot analysis showed that the steady state abundance of the α-subunit of ATP synthase decreases in mitochondria from letm1-1(−/−) LETM2-1(+/+) plants compared with the other genotypes; in organelle translation indicated that the translation of all mitochondrial products detected by SDS-PAGE was affected in the letm1-1(−/−) LETM2-1(+/+) hemizygous background and that the greatest reduction was observed with ATP9, but the translation of ATPα was also reduced by almost 50% in mitochondria from letm1-1(−/−) LETM2-1(+/+) plants.

To investigate this further, mitochondria from Col-0, letm1(−/−) LETM2(+/+), LETM1(+/+) letm2(−/−), and hemizygous (letm1-1(−/−) LETM2-1(+/+)) plants were analyzed by BN-PAGE to determine any differences in pattern and abundance (Fig. 4B). It was apparent that upon examination of the Coomassie-stained gel, the mitochondria from Col-0, letm1(−/−) LETM2(+/+), and LETM1(+/+) letm2(−/−) displayed the same pattern of protein complexes, but mitochondria from hemizygous letm1-1(−/−) LETM2-1(+/+) plants had a relative decrease in the amount of Complex V and
an increase in a distinct band with an apparent molecular mass of ~800 kDa (Fig. 4B). To verify the identity of these bands, mass spectrometry was utilized, confirming that the band that decreased in intensity was Complex V, with the \( \beta \)-subunit and subunit 8 of ATP synthase identified from an excised band (Fig. 4B and supplemental Table 2). The band that increased in abundance in mitochondria from hemizygous \( \text{letm1}^{-/-} \) \( \text{LETM2}^{-/-} \) \( \text{LETM1}^{+/+} \) \( \text{letm2}^{-/-} \) \( \text{LETM2}^{+/+} \) \( \text{letm1}^{-/-} \) \( \text{LETM2}^{-/-} \) with antibodies raised against LETM1. Equal amounts of mitochondrial protein were loaded in each lane. Dilutions of mitochondrial proteins from 20 to 5 \( \mu \)g were separated by SDS-PAGE, blotted to supported nitrocellulose, and probed with the LETM1 antibody. Detection of cross-reacting products was carried out using chemiluminescence, and the signal was captured using ImageQuant RT ECL (GE Healthcare) with the signal intensity (pixel intensity) adjusted relative to wild type (Col-0) and set to 100. Numbers in gray represent intensity with S.E. from \( n = 3 \). B, mitochondria from 10-day-old water culture-grown plants were isolated and separated by SDS-PAGE and probed with antibodies to a variety of mitochondrial proteins. The change in abundance of protein in the \( \text{letm1}^{-/-} \) \( \text{LETM2}^{-/-} \) \( \text{LETM1}^{+/+} \) \( \text{letm2}^{-/-} \) \( \text{LETM2}^{+/+} \) \( \text{letm1}^{-/-} \) \( \text{LETM2}^{-/-} \) line compared with wild type (Col-0) is indicated beside each blot with S.E. values. UCP, uncoupling protein; RISP, Rieske FeS protein.

**FIGURE 3. Analysis of mitochondrial protein abundance.** A, Western blot analysis of mitochondria isolated from 10-day-old plants of wild-type (Col-0), \( \text{letm1}^{-/-} \) \( \text{LETM2}^{+/+} \), \( \text{LETM1}^{+/+} \) \( \text{letm2}^{-/-} \) \( \text{LETM2}^{-/-} \), and \( \text{letm1}^{-/-} \) \( \text{LETM2}^{+/+} \) with antibodies raised against LETM1. Equal amounts of mitochondrial protein were loaded in each lane. Dilutions of mitochondrial proteins from 20 to 5 \( \mu \)g were separated by SDS-PAGE, blotted to supported nitrocellulose, and probed with the LETM1 antibody. Detection of cross-reacting products was carried out using chemiluminescence, and the signal was captured using ImageQuant RT ECL (GE Healthcare) with the signal intensity (pixel intensity) adjusted relative to wild type (Col-0) and set to 100. Numbers in gray represent intensity with S.E. from \( n = 3 \). B, mitochondria from 10-day-old water culture-grown plants were isolated and separated by SDS-PAGE and probed with antibodies to a variety of mitochondrial proteins. The change in abundance of protein in the \( \text{letm1}^{-/-} \) \( \text{LETM2}^{-/-} \) \( \text{LETM1}^{+/+} \) \( \text{letm2}^{-/-} \) \( \text{LETM2}^{+/+} \) \( \text{letm1}^{-/-} \) \( \text{LETM2}^{-/-} \) line compared with wild type (Col-0) is indicated beside each blot with S.E. values. UCP, uncoupling protein; RISP, Rieske FeS protein.

**Translation in Mitochondria**
AOX and PiC revealed no significant differences in import rate over time. For AOX, which is imported via the general import pathway, import increased over time, as judged by protection from externally added protease (Fig. 5A). In the case of PiC, which is imported by the carrier import pathway, import was observed to increase over time, where initial protease protection of the precursor was evident, representing stage III of the carrier import pathway (55), and this was converted to the mature protein over time (Fig. 5A, M). A weak intermediate band was also observed (Fig. 5A, I) because this carrier protein
contains a cleavable presequence that is processed in two steps (29). Analysis of transcript abundance for mitochondrially encoded transcripts revealed no significant change. Notably, no changes were detected in the transcript abundance of ATP/H9251.

Finally, the transcript abundance of nucleus-encoded genes was measured using microarray analysis. Analysis of transcript levels for 1202 nuclear genes encoding mitochondrial proteins (48) revealed that only 10% (124) of these genes were altered in abundance by more than 1.5-fold in either of the letm1–1 LETM2–1 lines compared with Col-0 (supplemental Table 3). Furthermore, only six and eight mitochondrial components were down- or up-regulated, respectively, by 2-fold or more in both letm1–1 LETM2–1 and letm1–2 LETM2–1 lines based on global transcript abundances, indicating very minor reprogramming in regard to mitochondrial metabolism (supplemental Table 3 and Fig. 5 C). Functional categorization analysis of both the up- and down-regulated transcripts encoding mitochondrial proteins revealed an overrepresentation of import and RNA binding-related functions in up-regulated transcripts, whereas transcripts encoding...
proteins with transport and metabolism functions were over-represented in the down-regulated transcripts (Fig. 5C). This is consistent with a general role for the involvement of AtLETM in translation, with a reduction in AtLETM protein abundance compensated for by the induction of other RNA-binding proteins as a possible mechanism to stabilize transcripts from degradation. The lack of induction of a variety of transcripts encoding mitochondrial proteins is consistent with the lack of significant changes of protein abundances in Complex I, III, IV or the respiratory chain, as revealed by Western blotting.

letm2 Exhibits Maternal Effect Seed Abortion—Analysis of developing seeds within the siliques of hemizygous letm1(−/−) LETM2(+/-) self-fertilized mutants revealed that half of the developing seeds were aborted (Fig. 6A and supplemental Table 4). To examine whether the LETM2 gene is required for the development of the female gametophyte, we inspected unpollinated ovules. In Arabidopsis, the mature embryo sac consists of an egg cell, a central cell, two synergid cells, and three degenerated antipodal cells (57–59). Morphologically, embryo sac development in ovules of letm1(−/−) LETM2(+/-) plants was indistinguishable from wild type (Fig. 6B, 1 and 5). Holow-mount preparations of ovules 12–60 h after pollination revealed that more than 90% of the embryo sacs had initiated embryo and endosperm development in the heterozygous mutants letm1(−/-) LETM2(+/-), and during early embryo-genesis (12–16 h after pollination) there was no difference between the developing seeds within the hemizygous siliques (Fig. 6B, 2 and 6). However, embryo and endosperm were aborted at later stages of development in 50% of the seeds (Fig. 6A, 4 and 5, and supplemental Table 4). Among the aborted seeds, an apparently random proportion (supplemental Table 4) were arrested at quadrant/octant embryo stage with an endo-sperm arrested even earlier (Fig. 6B, 7). At quadrant/octant embryo stage, the non-aborted seeds contained 36–48 ± 12 endosperm nuclei (n = 10), whereas aborted seeds had only 10–16 ± 5 nuclei (n = 10). Another proportion of the aborted seeds (supplemental Table 4) were arrested at early heart embryo stage (Fig. 3B, 8) with an endosperm of 78–90 ± 11 nuclei (n = 10) as opposed to non-aborted seeds, which had 188–194 ± 8 endosperm nuclei (n = 10) at that stage.

Because 50% seed abortion is a strong indication for a defect that is under gametophytic control, we performed reciprocal crosses between letm1(−/-) LETM2(+/−) and letm1(−/-) LETM2(+/-) plants. When letm1(−/-) LETM2(+/-) plants were used as female, the letm1(−/-) LETM2(+/-) seed phenotype could not be complemented by fertilization with a wild-type LETM2 allele derived from the pollen in ~50% of the F1 seeds (Fig. 6A). Genotyping revealed that all viable seeds from this cross (239 of 239; Table 1) gave rise to letm1(−/-) LETM2(+/-) plants. In contrast, using maternal letm1(−/-) LETM2(+/-) in a cross with letm1(−/-) LETM2(+/+) did not impair seed development (Fig. 6A and supplemental Table 4), resulting in 100% viable seeds. F1 plants from this cross displayed ~1:7:1 segregation of the letm1(−/-) LETM2(+/+) and letm1(−/-) LETM2(+/+) genotypes (271 and 155 individuals, respectively; Table 1). This segregation ratio is significantly different from the expected 1:1 ratio (p < 0.005), indicating that pollen with the genotype letm1(−) letm2(−) is viable yet slightly less fertile than letm1(−) LETM2(+) or wild-type pollen. When crossing letm1(−/-) LETM2(+/+) with Col-0, 100% seed viability was observed in either direction. However, genotyping of the F1 progeny indicated that using paternal Col-0 showed 1:1 (124:116 individuals; Table 1) LETM1(+/−) LETM2(+/+) versus LETM1(−/-) LETM2(+/-) segregation, whereas using paternal letm1(−/-) LETM2(+/+) resulted in ~1.6:1 (146:94 individuals) LETM1(+/-) LETM2(+/-) versus LETM1(−/-) LETM2(+/-) segregation. This segregation ratio is also significantly different from the expected 1:1 ratio (p < 0.005). Pollen viability and number were assessed using the Alexander staining method (Fig. 6C) (38). A slight increase in defective pollen was observed in pollen of letm1(−/-) LETM2(+/+) plants compared with Col-0. letm1(−/-) LETM2(+/+) flowers produced ~60% less pollen than Col-0. Again, these observations suggest that pollen with genotype letm1(−) and letm2(−) is viable yet slightly less fertile than letm1(−) LETM2(+) or wild-type pollen. Similar results were obtained using independent alleles for all mutations.

The parent of origin effect on seed viability observed with the letm2 mutant could be due to a requirement for LETM2 protein during female gametophyte development or due to the maternal allele being expressed during seed development. To investigate the maternal expression of LETM2, we performed reciprocal crosses of the LETM2::GUS promoter reporter lines with Col-0 plants (Fig. 7, A and B). Although there is some loss of cellular resolution due to the staining procedure, a phenomenon that is routinely observed (37, 60, 61), GUS expression under the control of the AtLETM2 promoter is observed in the female gametophyte and in developing seed (Fig. 7A). When using the LETM2::GUS promoter reporter line as a female in a cross with Col-0, GUS expression was observed (Fig. 7B). However, when using the LETM2::GUS promoter reporter line as male, no GUS activity was observed (Fig. 7B). Notably, in a similar experiment using the AtLETM1 promoter, no GUS expression was observed in endosperm or embryo, consistent with previous findings that AtLETM1 is stress-induced (45).

Furthermore, we dissected 6-day-old seeds of reciprocal crosses between Col-0 and Ler plants and isolated embryo and endosperm tissues for transcript sequencing. The T′ C polymorphism between Col-0 and Ler in the mRNA sequence of LETM2 allowed differentiation of transcripts originating from the Col-0 or Ler allele. As a control, a 1:1 mix of Col-0 and Ler cDNA was used, and as expected, this cDNA showed equal amounts of each polymorphism in the chromatograms (Fig. 7C). Analysis of the Col-0 × Ler crosses revealed a clear abundance of the maternal transcript in both embryo and endosperm (Fig. 7C). Thus, seed viability depends only upon the presence of a wild-type maternal LETM2 allele.

DISCUSSION

This study has defined the overlapping functions performed by AtLETM1 and AtLETM2 as essential for mitochondrial function and viability. Mechanistically, LETM function was defined as being important for accumulation of mitochondrial...
proteins. Although the translation of potentially all mitochondrial proteins was reduced in the hemizygous letm1(−/−) LETM2(+/-) plants, only the steady state level of ATP synthase was reduced, as determined by both Western blot analysis and BN-PAGE. Also the amount of ATP was reduced on a tissue basis, consistent with the decrease in the amount of ATP synthase. Note that although Western blot analysis revealed a reduction in the amount of ATPα, other subunits of this complex are also likely to be reduced in abundance, but there are no specific antibodies available for other subunits, apart from the β-subunit, which was not decreased in abundance. No evidence was found for a reduction in abundance of the other respiratory chain complexes, despite the fact that Complex I, III, and IV all contain mitochondrially encoded subunits, where the rate of translation was also reduced. It is proposed that this is due to the fact that mitochondrial biogenesis takes place during early development in plants; a directed study revealed that mitochondrial transcription and translation were the earliest mitochondrial events that could be detected after imbibition during germination (48). Second, the abundance of the ATP synthase complex can be seen to be greater than the other respiratory complexes that contain mitochondrially encoded subunits (Fig. 4B), and in general, the abundance of the ATP synthase complex is the highest of the respiratory chain complexes in isolated plant mitochondria, as evidenced by staining with Coomassie Brilliant Blue (62, 63). Furthermore, the incorporation of [35S]Met into ATP6 represented >50% of the incorporated methionine in mitochondria from the six bands identified from Col-0 plants. ATP9 is present at a stoichiometry of 10 copies in yeast and mammalian mitochondria and thus contains 10 times more subunit 9 than subunit 6 or 8 (64). Consistent with our observation, the incorporation of radiolabeled methionine into ATP9 in yeast is also severalfold greater than other ATP synthase subunits (64). Five subunits of the ATP synthase are encoded in the mitochondrial genome of Arabidopsis: four subunits of the F8 (namely orfB, orf25, subunit 6, and subunit 9) (54) and ATP6 in F1 (53). Thus, it is proposed that during the rapid burst of mitochondrial biogenesis that occurs early in germination, the rate of synthesis of the ATP synthase subunits is rate-limiting for assembly and thus is reduced in abundance compared with mitochondria from wild-type plants. This is especially relevant for the F6 module, where subunit 9 is proposed as the initiating event for assembly of the F6 module (64).

Although the reduction in the accumulation of the mitochondrial ATP synthase does not lead to any detectable changes in mitochondrial morphology, notably there was an increased in starch and plastoglobule accumulation in chloroplasts. This is proposed to be due to the fact that a reduced mitochondrial ATP synthase results in an inability to respire all of the carbon that is fixed in photosynthesis, and it accumulates in storage products.

However, for the other respiratory chain complexes that contain mitochondrial encoded subunits, the rate of synthesis is not limiting, and thus no differences are observed in steady state abundance. This is also consistent with the fact that the phenotype is only observed early in development, where mitochondrial biogenesis peaks, and later even the reduced rate of synthesis is sufficient for these plants to achieve a normal phenotype. Additionally, the fact that the alternative respiratory chain is not induced, because the reduction in the ATP synthase leads to a reduction in the amount of ATP and thus induction of a non-phosphorylating bypass pathway, would not alleviate this biochemical lesion. Recently, an RNAi approach to reduce the abundance of the nucleus-encoded δ-subunit of ATP synthase also observed a defect in pollen formation, consistent with the finding in this study that the ATP synthase is critical for pollen development (65). Finally, although the abundance of Complex I, II, III, and IV was not reduced, despite the decrease in the rate of translation, it may also indicate that the rate of degradation of these complexes or subunits in these complexes can be adjusted, so that steady state abundance is unaffected. However for the ATP synthase, if the rate of degradation was adjusted, it cannot compensate for the reduced rate of synthesis. Because the difference in incorporation of [35S]Met into protein was observed to be greatest after 60 min and much less at 30 min, it is possible

| TABLE 1 |
| --- |
| PCR genotyping of LETM crosses in the F1 progeny |
| Reciprocal crosses were performed between different combinations of Col-0 and LETM mutant genotypes. The genotype of the F1 progeny was determined using PCR-based genotyping. |
| Parents | F1 progeny | Ratio |
| --- | --- | --- |
| letm1-1/- x letm1-1/- | letm1-1/- x LETM2-1+/+; letm1-1/- x LETM2-1 (+/-) | 149:81 |
| letm1-1/- x LETM2-1+/+ x letm1-1/- | letm1-1/- x LETM2-1 (+/-); letm1-1/- x LETM2-1 (+/-) | 239:0 |
| letm1-1/- x LETM2-1+/+ x Col-0 | LETM1-1 (+/-) x LETM2-1 (+/-); LETM1-1 (+/-) x LETM2-1 (+/-) | 59:61 |
| Col-0 x letm1-1/- x LETM2-1+/+ | LETM1-1 (+/-) x LETM2-1 (+/-); LETM1-1 (+/-) x LETM2-1 (+/-) | 75:47 |
| letm1-2/- x LETM2-2+/+ x letm1-2/- | letm1-2/- x LETM2-2 (+/-); letm1-2/- x LETM2-2 (+/-) | 122:74 |
| letm1-2/- x LETM2-2+/+ x Col-0 | LETM1-2 (+/-) x LETM2-2 (+/-); LETM1-2 (+/-) x LETM2-2 (+/-) | 208:0 |
| Col-0 x letm1-2/- x LETM2-2+/+ | LETM1-2 (+/-) x LETM2-2 (+/-); LETM1-2 (+/-) x LETM2-2 (+/-) | 65:55 |
| Col-0 x letm1-2/- x LETM2-2+/+ | LETM1-2 (+/-) x LETM2-2 (+/-); LETM1-2 (+/-) x LETM2-2 (+/-) | 73:47 |

FIGURE 6. Seed sets of crossing of AtLETM1 and AtLETM2 single and double mutants. A, silique from the self-crossing of double mutant letm1-1(-/-) LETM2-1(+/-) and the crossing of double mutant letm1-1(-/-) LETM2-1(+/-) with single mutant letm1-1(-/-) LETM2-1(+/-) and with wild-type (Col-0), +, aborted embryos. Values indicate the average number of viable and aborted progeny per silique ± S.D. (n = 10). Scale bar, 0.5 cm. The crosses of both alleles are shown, with the top five panels representing one allele of AtLETM1 and AtLETM2 and the bottom five panels representing similar crosses with the second allele for both AtLETM1 and AtLETM2. +, statistically similar to theoretical 1:1 ratio (p > 0.50); *, statistically similar to theoretical 1:0 ratio (p > 0.90). B, microscopic analysis of different stages of development for embryos in letm1-1(-/-) LETM2-1(+/-) mutant plants. Gametophyte, early embryo, and seeds 3 and 7 days after pollination (DAP) are shown. Some embryos of letm1-1(-/-) LETM2-1(+/-) plants are aborted in the 2–4 cell stage (7), whereas other embryos are aborted at the globular stage (8). cc, central cell; ec, egg cell; s, synergid; es, endosperm; z, zygote. C, number of pollen grains and percentage of defective pollen as determined by Alexander staining. Arrow, defective pollen grain. * statistically significant difference compared with wild-type (Col-0) (p < 0.01). Scale bars, 20 μm. Error bars, S.E.
that the stability or insertion of organelle-encoded subunits of the ATP synthase is affected. The detection of a protein complex in BN-PAGE with several subunits of ATP synthase with HSP60 is consistent with this (Fig. 4B). Thus, it is possible that LETM proteins are required for the correct orientation of ribosomes for efficient co-translational insertion of subunit 9 into the membrane and that limiting the abundance of LETM proteins results in degradation of subunit 9 that is not correctly inserted into the inner membrane, akin to the role played by Mba1 in yeast in assembly of cytochrome oxidase (66). Although this possibility cannot be excluded, it is notable that the accumulation of soluble subunit ATPα is also reduced, but not the nucleus-encoded β-subunit of γ, suggesting that the rate of synthesis is affected. The smaller differences in incorporation of [35S]Met at 30 min may simply reflect that all of the amino acids required for in organelle translation need to be transported across the inner mitochondrial membrane, and thus the difference in capacity for translation may only become apparent after some time, when all added amino acids are not limiting in the mitochondrial matrix.

It was determined that maternal \( \text{LETM2} \) was absolutely required for seed development; otherwise, the seed aborted at an early stage. Three independent methods support the biased parent of origin effect for \( \text{LETM2} \). (i) The ratio of viable and aborted embryos in reciprocal crosses is indicative of a parent of origin effect but does not rule out a gametophyte effect. However, analysis of unfertilized ovules and pollen revealed normal development prior to fertilization. (ii) Reciprocal crosses of \( \text{LETM2} \)::GUS promoter reporter lines with Col-0 clearly demonstrated that the paternal \( \text{LETM2} \) promoter was silenced during early embryogenesis, and the maternal \( \text{LETM2} \) promoter...
was active in female gametophyte before pollination and during early seed development. (iii) By analyzing maternal and paternal transcripts by dissecting endosperm and embryo tissues, we found that LETM2 transcripts are of maternal origin for both embryo and endosperm as late as 6 days after pollination. Furthermore, the silencing of the LETM2 promoter GUS construct hints at the mechanism that may underlie the parent of origin effect observed. The presence of an LTR/Gypsy transposon (AT1TE80065) in the promoter region of AtLETM2 may act as a target site for methylation and the silencing observed via RNA-directed DNA methylation (56). Analysis of pollen and female gametophyte viability indicated that although the amount of pollen produced is reduced, the viability of the gametes is not affected. Thus, the failure of embryo development is due to a lack of expression in the early stages of seed development. Finally, analysis of the spatial expression pattern of AtLETM1 using AtLETM1 promoter GUS plants revealed that it was not detected in early endosperm or embryo (Fig. 7C), revealing that in planta, under normal situations, only the maternal AtLETM2 controls translation of mRNA of mitochondrionally encoded genes.

Although a number of studies have defined a variety of genes that display a biased parent of origin effect during early seed development, these genes encode proteins that are located in the nucleus or involved with regulation of nuclear genes and hormonal signaling pathways (52). Because biased parent of origin expression is proposed to occur due to the parent conflict hypothesis (15), it is consistent that mitochondrial gene expression is also under maternal control during early development (48). Early embryo development following fertilization and the earliest events in germination are crucial for plant development and success. The production of multiple offspring with potentially many different parental inputs would select for vigor in early embryo development and germination in parental gametes because they could out-compete other siblings for resources. However the maternal parent can counteract any such parental vigor by ensuring equal resource allocation during embryo development and early seedling development, after which environmental resources dominate growth and development. Given the essential role of mitochondrial function in embryo development and early germination, maternal control of mitochondrial gene expression ensures equal allocation to all offspring. Furthermore, maternal control of translation of mitochondrial genes could act to coordinate organelle and nuclear genome expression at the earliest stages of development because several nuclear genes have been characterized to display a biased maternal expression pattern that affects nuclear gene expression. Finally, given that the AtLETM protein contains an EF-hand domain and that AtLETM1 is stress-inducible, it is suggested that mitochondrial gene expression at the level of translation may play a role in stress responses.

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