EMB2473/MIRO1, an Arabidopsis Miro GTPase, Is Required for Embryogenesis and Influences Mitochondrial Morphology in Pollen

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The regulation of mitochondrial biogenesis, subcellular distribution, morphology, and metabolism are essential for all aspects of plant growth and development. However, the molecular mechanisms involved are still unclear. Here, we describe an analysis of the three Arabidopsis thaliana orthologs of the evolutionarily conserved Miro GTPases. Two of the genes, MIRO1 and MIRO2, are transcribed ubiquitously throughout the plant tissues, and their gene products localize to mitochondria via their C-terminal transmembrane domains. While insertional mutations in the MIRO2 gene do not have any visible impact on plant development, an insertional mutation in the MIRO1 gene is lethal during embryogenesis at the zygote to four-terminal-cell embryo stage. It also substantially impairs pollen germination and tube growth. Laser confocal and transmission electron microscopy revealed that the miro1 mutant pollen exhibits abnormally enlarged or tube-like mitochondrial morphology, leading to the disruption of continuous streaming of mitochondria in the growing pollen tube. Our findings suggest that mitochondrial morphology is influenced by MIRO1 and plays a vital role during embryogenesis and pollen tube growth.

INTRODUCTION

Mitochondria are the main sites for aerobic energy production and supply various intermediates necessary for cellular metabolism. They are also involved in cellular processes, including the defense response against oxidative stress, calcium signaling, and the regulation and execution of programmed cell death (reviewed in Logan, 2006a; McBride et al., 2006). Thus, the coordination and regulation of mitochondrial biogenesis and function are vital during plant growth and development from zygote to mature plant. In addition, molecular mechanisms also exist to ensure that mitochondria are equally segregated between daughter cells during cell division and are properly distributed in cells undergoing rapid morphological differentiation and metabolic change. Such mechanisms are particularly important in plants, in which mitochondrial function and morphology must adapt in response not only to intracellular signals regulating plant development and differentiation but also to constantly changing external environmental conditions and biotic stresses.

In other eukaryotes, it has been shown that mitochondrial fusion and fission rely on evolutionarily conserved dynamin-like GTPases and their binding partners (reviewed in Yaffe, 1999; Okamoto and Shaw, 2005; Chan, 2006b), while mitochondrial motility is dependent on the cytoskeletal network (reviewed in Hollenbeck, 1996; Yaffe, 1999; Hollenbeck and Saxton, 2005; Boldogh and Pon, 2006, 2007; Frederick and Shaw, 2007). Dysfunction of these dynamic processes often causes developmental defects and diseases in mammals and insects (reviewed in Chan, 2006a, 2006b). Loss of the dynamin-like GTPases required for mitochondrial fusion causes embryonic lethality in mice (Chen et al., 2003) and neurodegenerative diseases in humans (Alexander et al., 2000; Delettre et al., 2000; Züchner et al., 2004). In Drosophila, severe loss of mitochondria in synaptic termini and impaired neurotransmission are caused by disruption of the axonal transport machinery of mitochondria (Stowers et al., 2002; Guo et al., 2005).

Our understanding of the molecular machinery that regulates mitochondrial morphology and motility in plants is still at an early stage (reviewed in Logan, 2006b). It has been shown that mitochondrial fission requires the dynamin-like GTPases (Arimura and Tsutsumi, 2002; Arimura et al., 2004a; Fujimoto et al., 2004; Logan et al., 2004). Although frequent fusion events have been demonstrated using fluorescent reporter proteins (Arimura et al., 2004b; Sheahan et al., 2005), the genes mediating mitochondrial fusion have not been identified. The motility of mitochondria is dependent mainly on actin filaments (Van Gestel et al., 2002) and is vital for proper mitochondrial inheritance (Barr et al., 2005) and during cell division (Sheahan et al., 2004). Changes in mitochondrial morphology and motility are associated with cell growth (Sheahan et al., 2004, 2005), senescence (Zottini et al., 2006), cell death induced by reactive oxygen species (Yoshinaga et al., 2005), and various physiological responses (Stickens and...
Verbelen, 1996; Armstrong et al., 2006), implying an important role in developmental processes and physiological function in plants.

A novel class of GTPases, the Miro GTPase family, was recently identified in humans (Fransson et al., 2003, 2006), mice (Shan et al., 2004), yeast (Frederick et al., 2004), and Drosophila (Guo et al., 2005). They contain two GTPase domains and two calcium binding EF-hand motifs and are associated with the outer mitochondrial membrane via their C-terminal transmembrane domains (TMs) (Figure 1A). The overexpression of human GTPases containing wild-type and mutated functional domains leads to aggregated or thread-like mitochondrial morphology (Fransson et al., 2003, 2006). In the budding yeast Saccharomyces cerevisiae, loss of the Miro GTPase induces pleiotropic effects on mitochondrial morphology, including swollen and tubule-like structures (Frederick et al., 2004). Loss of the Drosophila Miro GTPase causes a dysfunction of the axonal mitochondrial transport, leading to abnormal subcellular distribution of mitochondria in neurons and muscles, with the result that mutant larvae do not accumulate mitochondria at the synaptic termini and thus exhibit abnormal locomotion and premature lethality (Guo et al., 2005).

In this study, we have shown that the Arabidopsis thaliana genome contains three genes encoding putative Miro-related GTPases, that two of the genes, MIRO1 and MIRO2, are transcribed throughout the plant tissues, and that their gene products localize to mitochondria. While mutations in the MIRO2 gene do not have any obvious impact on plant development, a mutation in the MIRO1 gene leads to an arrest of embryogenesis at an early stage and substantially impairs pollen germination and tube growth. Microscopic analysis and live-cell imaging revealed that the miro1 mutation causes abnormal mitochondrial morphology, leading to the disruption of continuous streaming of mitochondria in growing pollen tubes. Our data suggest that proper mitochondrial morphology influenced by MIRO1 is essential for embryogenesis and pollen tube growth in Arabidopsis.

Figure 1. Alignment of the Predicted Amino Acid Sequences of Miro GTPases.

(A) Schematic diagram of the predicted protein structure of the Miro GTPases. EF, EF hand motif.

(B) Alignment of the predicted amino acid sequences of Arabidopsis (MIRO1, -2, and -3), human (hMiro-1 and -2), Drosophila (dMiro), and yeast (Gem1p) Miro GTPases. Black boxes indicate identical amino acid residues, gray boxes indicate conserved amino acid substitutions, solid underlines indicate the GTPase domains, dotted underlines indicate the EF-hand motifs, and the black open box indicates the TM. Sequences were aligned using the default settings in ClustalW version 1.81 (Thompson et al., 1994) and shaded using BOXHARE 3.21 (http://ch.embnet.org/software/BOX_form.html).
RESULTS

The Arabidopsis Genome Contains Three Miro-Related GTPase Genes

Three orthologous genes of the Miro GTPase, MIRO1 (At5g27540; also known as EMB2473) (Tzafrir et al., 2004), MIRO2 (At3g63150), and MIRO3 (At3g05310), were identified in a basic BLAST search (Altschul et al., 1997) of the Arabidopsis cDNA database (www.Arabidopsis.org/Blast; Arabidopsis Genome Initiative, 2000) using the human Miro-1 (Fransson et al., 2003) as a query sequence. They shared 36, 38, and 33% amino acid identity with human Miro-1, respectively, and each contained a putative GTPase domain in the N-terminal region followed by a pair of EF-hand motifs and a second distinct GTPase domain. Single TMs were also predicted at their C termini (Figures 1A and 1B). In previous studies, the C-terminal TM was shown to be responsible for anchoring Miro GTPases onto the outer mitochondrial membrane in yeast (Frederick et al., 2004) and humans (Fransson et al., 2006).

RT-PCR analysis of all three Arabidopsis Miro-related genes revealed that MIRO1 and MIRO2 are expressed in seedlings, flowers, siliques, stems, mature and senescent rosette leaves, and roots, whereas MIRO3 transcripts were not amplified in any of these tissues (see Supplemental Figure 1 online). Further assessment was done by reference to the Arabidopsis microarray gene expression database GENEVESTIGATOR (https://www.genevestigator.ethz.ch/; Zimmermann et al., 2004), which revealed that MIRO1 and MIRO2 are ubiquitously expressed in all plant tissues, and therefore presumably have a vital role, whereas the expression of MIRO3 is almost undetectable (Figure 2).

The C-Terminal TM Is Required for Mitochondrial Localization of MIRO1 and MIRO2

Transient expression of cDNA constructs (GFP-MIRO1TM and GFP-MIRO2TM) containing the putative C-terminal TMs of MIRO1 (amino acid residues 602 to 648) and MIRO2 (amino acid residues 599 to 643) fused to the C terminus of an enhanced green fluorescent protein (GFP) in tobacco (Nicotiana tabacum) leaf epidermal cells showed colocalization with mitochondria labeled with the mitochondria-specific probe MitoTracker Orange (n = 150) (Figure 3). By contrast, C-terminal TM-deleted mutant proteins of MIRO1 (GFP-MIRO1ΔTM) and MIRO2 (GFP-MIRO2ΔTM) were excluded from mitochondria and dispersed throughout the cytosol (n = 150) (Figure 3). These results suggest that the C-terminal TM is required for mitochondrial localization of MIRO1 and MIRO2.

Embryogenesis Is Arrested at an Early Stage in the miro1 Mutant

To investigate the role of Arabidopsis Miro-related GTPase genes during plant development, we identified Arabidopsis mutant lines from the public mutant collections. The miro1 mutant, previously designated emb2473, was obtained from the Seed Genes Project collection (www.seedgenes.org; Tzafrir et al., 2004). The miro1 mutant was generated by the introduction of pCSA110 T-DNA, which carries a BASTA resistance gene and a β-glucuronidase (GUS) reporter gene under the control of the pollen-specific LAT52 promoter, into Arabidopsis plants harboring the homozygous quartet1 (qrt1) mutation, which causes mature fertile pollen grains to remain attached to each other (Preuss et al., 1994; McElver et al., 2001). Sequence analysis revealed that the T-DNA was inserted at the border of the 12th intron and 13th exon of the MIRO1 gene with a 16-bp deletion (Figure 4A). DNA gel blot analysis demonstrated that the T-DNA insertion was present as a single copy in the miro1 mutant genome (see Supplemental Figure 2 online). Segregation analysis of the self-fertilized miro1 progeny demonstrated that 54% of the progeny were resistant to BASTA, instead of the expected value of 75% (Table 1), implying embryonic and/or gametophytic lethality of the miro1 mutant. PCR analysis revealed that a similar percentage (51%; n = 112) of the randomly selected progeny and all of the BASTA-resistant mutant progeny tested (n = 50) carried the T-DNA insertion, suggesting that the BASTA resistance and T-DNA insertion of the miro1 mutant are tightly linked. No homozygous mutant plants were found, suggesting that the homozygous miro1 mutant causes seed lethality.

We obtained two mutant lines, miro2-1 and miro2-2, harboring T-DNA insertions in the 14th and 12th exon of the MIRO2 gene, respectively, from the SALK collection (Alonso et al., 2003). Segregation analysis (see Supplemental Table 1 online) of the heterozygous mutant progeny suggested single T-DNA insertions in both the miro2-1 and miro2-2 mutants. Neither of the homozygous mutant progeny accumulated the full-length MIRO2 transcripts (see Supplemental Figure 3 online). The phenotypes (see Supplemental Figure 4 online) and root cell mitochondrial morphology of both were indistinguishable from those of wild-type plants (see Supplemental Figure 5 online). These findings...
suggest that MIRO2 does not have a major role in plant development and does not share redundant functions with MIRO1; therefore, we concentrated our further analysis on the miro1 mutant.

To investigate the developmental basis of the lethal phenotype displayed by the miro1 mutant, maturing siliques from wild-type and heterozygous miro1 mutant plants were dissected. The siliques from homozygous qrt1 plants used as the wild-type control contained a well-ordered array of maturing green seeds (Figure 5A). By contrast, the siliques from heterozygous miro1 mutant plants contained a proportion of aborted white seeds together with wild-type green seeds (Figure 5B). The white aborted seeds then turned brown and did not develop further, while the wild-type seeds developed to maturity (data not shown).

More than 500 BASTA-resistant progeny of the miro1 mutant displayed the aborted seed phenotype, suggesting that this mutant phenotype is tightly linked to BASTA resistance and the T-DNA insertion. Together, these findings suggest that the homozygous miro1 mutation causes embryo lethality. However, the percentage of aborted seeds in the mutant siliques was significantly lower (13%; n = 3154) than the expected value of 25%, implying impaired pollen tube growth in the miro1 mutant (Meinke, 1982).

To identify the developmental stage at which embryo development was arrested in the miro1 mutant, immature seeds from heterozygous miro1 mutant siliques were examined by whole-mount clearing and differential interference contrast (DIC) microscopy. A comparison of embryo development in normal seeds (Figures 5C and 5E to 5G) and aborted seeds (Figures 5D and 5H to 5L) revealed that embryogenesis was arrested at the early developmental stages between the zygote and the four-terminal-cell stage in the miro1 mutant. Of the 80 mutant embryos examined, 8 embryos (10%) did not develop beyond the zygote (Figure 5I), 61 embryos (76%) aborted at the two-cell stage (Figures 5H and 5J), 7 embryos (9%) aborted at the three-cell stage (Figure 5K), and 4 embryos (5%) aborted at the four-terminal-cell stage (Figure 5L). These observations indicate that the homozygous miro1 mutation causes abortion of the embryo at an early stage of development.

**Pollen Germination and Tube Growth Are Impaired in the miro1 Mutant**

The abnormal segregation of selfed miro1 mutant progeny (Table 1) and the lower than expected number of aborted seeds in the mutant siliques suggested that, in addition to the embryo...
lethality, genetic transmission through the gametophytes also was impaired. Reciprocal crosses with wild-type plants as the female parent demonstrated severely impaired male genetic transmission in the heterozygous miro1 mutant (Table 1), suggesting that the miro1 mutation strongly affects pollen development and/or germination and tube growth. If this is the case, the apical half region of the mutant silique should display a significantly higher percentage of aborted seeds than the expected 50% (Meinke, 1982). Indeed, 84% of the aborted seeds were located in the apical half regions of the miro1 mutant siliques (n = 408). Additionally, the female genetic transmission of the miro1 mutant was reduced slightly (Table 1), suggesting that the miro1 mutation has a weak impact on female gametogenesis. This finding was supported by the fact that the frequency of unfertilized ovules was slightly higher in miro1 mutant siliques (10%; n = 1254) than in wild-type siliques (4%; n = 1288).

The homozygous qrt1 background causes mature fertile pollen grains to remain attached to each other and thus enables tetrad analysis to be performed (Preuss et al., 1994). Accordingly, the pollen tetrads from a heterozygous miro1 mutant with the genotype qrt1/qrt1;+/miro1 should contain two mutant and two wild-type pollen grains. However, the four grains were indistinguishable from each other in terms of their size and shape (Figure 6A). 4′,6-Diamidino-2-phenylindole (DAPI) and fluorescein diacetate (FDA) staining demonstrated that all four of the grains showed correctly differentiated one vegetative and two sperm nuclei (Figure 6B) and equal viability (Figure 6C). These findings suggest that miro1 mutant pollen development is normal and thus does not explain the impaired male genetic transmission.

The LAT52 promoter:GUS pollen-specific reporter gene contained in the pCSA110 T-DNA (McElver et al., 2001) makes it possible to distinguish between the miro1 mutant and wild-type pollen by staining for GUS activity. To assess pollen viability in the miro1 mutant, pollen from wild-type and heterozygous miro1 mutant plants were germinated and stained in 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) solution, and the

Table 1. Segregation and Genetic Transmission of the miro1 Mutation

<table>
<thead>
<tr>
<th>Cross (Female × Male)</th>
<th>BASTAR²</th>
<th>BASTAS²</th>
<th>BASTAS/Total</th>
<th>TE (Female)</th>
<th>TE (Male)</th>
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<tr>
<td>miro1/×miro1/×</td>
<td>1480</td>
<td>1259</td>
<td>54.0%</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>miro1/××+/×</td>
<td>215</td>
<td>286</td>
<td>42.9%</td>
<td>75.2%</td>
<td>NA</td>
</tr>
<tr>
<td>×+/×miro1/+</td>
<td>75</td>
<td>588</td>
<td>11.3%</td>
<td>NA</td>
<td>12.8%</td>
</tr>
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Transmission efficiency (TE) was calculated according to Howden et al. (1998). BASTAR, phosphinothricin-resistant; BASTAS, phosphinothricin-sensitive; NA, not applicable; TE, BASTAR/BASTAS × 100.

Figure 5. The Homozygous miro1 Mutant Is Embryo-Lethal.

(A) and (B) Siliques from wild-type (A) and heterozygous miro1 (B) plants. Arrowheads indicate the aborted mutant seeds. Bars = 1 mm.
(C) to (L) DIC images of seeds from heterozygous miro1 mutant siliques. (C) Wild-type seed containing a transition-stage embryo. Bar = 25 μm.
(D) and (H) miro1 mutant seed containing a two-cell-stage embryo (arrow) (D). A higher magnification of the mutant embryo exhibiting differentiated cell nuclei (arrowheads) is shown in (H). Bars = 25 μm in (D) and 10 μm in (H).
(E) to (G) Wild-type embryos: globular stage (E), transition stage (F), and heart stage (G). Bars = 25 μm.
(I) to (L) miro1 mutant embryos: zygote (I), two-cell stage (J), three-cell stage (K), and four-terminal-cell stage (L). Arrowheads indicate the mutant embryo nuclei. Bars = 10 μm.
germination and tube growth efficiencies were compared (Figures 7A and 7B). The GUS-negative wild-type pollen showed normal tube growth, while the GUS-positive mutant pollen showed impaired germination and tube growth. Approximately 70% of the wild-type pollen as well as the GUS-negative wild-type pollen from the heterozygous miro1 mutant germinated, and the average tube length reached was ~150 μm (Figure 7D). By contrast, the germination rate of the GUS-positive mutant pollen was decreased to ~40%, and tube growth was reduced as well (Figure 7D). These findings suggest that the miro1 mutation severely impairs pollen germination and tube growth.

A Mutation in MIRO1 Causes Abnormal Mitochondrial Morphology in Pollen

In order to investigate the role of MIRO1 in regulating mitochondrial morphology and intracellular distribution, mitochondria were visualized in pollen by creating transgenic plants containing
GFP fused to a mitochondrial targeting signal sequence (mtGFP) (Logan and Leaver, 2000) under the control of the pollen-specific LAT52 promoter (Twell et al., 1990; Yang et al., 2001). Homozygous qrt1 plants as the wild-type control were compared with heterozygous miro1 mutant plants. Laser confocal microscopy revealed that all four pollen grains in the tetrads from control qrt1 plants (n = 50) contained normal spherical or rod-like mitochondria (Figure 8A). By contrast, tetrads from heterozygous miro1 mutant plants (n = 50) contained two pollen grains with normal mitochondrial morphology and two with abnormally enlarged mitochondria (Figure 8B). Following germination, using time-lapse confocal microscopy, we showed that in wild-type pollen (n = 34) mitochondria exhibited uniform rod-like morphology (Figure 8C) and high motility, leading to continuous streaming of mitochondria throughout the cytoplasm (Figure 8E; see Supplemental Movie 1 online). This motility is dependent on actin filaments, because disruption of actin filaments in pollen by latrunculin B or cytochalasin D (see Supplemental Figure 6A online) arrested mitochondrial motility (see Supplemental Movies 3 and 4 online), whereas disruption of microtubules by oryzalin or nocodazole (see Supplemental Figure 6B online) did not have any impact on the streaming of mitochondria (see Supplemental Movies 5 and 6 online). Mitochondria in the miro1 mutant pollen (n = 29) exhibited an abnormal morphology and intracellular distribution (Figure 8D). In addition to the abnormal size (Figure 8F; time = 146 s), a large number of mitochondria also showed a tube-like morphology (Figure 8F; time = 49 s). These abnormal mitochondria were motile (see Supplemental Movie 2 online), often leading to transient absence from regions of the pollen cytoplasm (Figure 8F; time = 177 s). This motility was also actin-dependent (see Supplemental Movies 7 to 10 online), suggesting that the miro1 mutation does not affect actin-dependent mitochondrial motility. Together, these observations suggest that the miro1 mutation causes abnormally enlarged or tubulated mitochondrial morphology, leading to disruption of the continuous streaming of mitochondria in the growing pollen tube.

To investigate the detailed size and structures of their mitochondria, wild-type and miro1 mutant pollen were germinated

Figure 8. A Mutation in MIRO1 Causes Abnormal Mitochondrial Morphology in Pollen.

(A) and (B) Merged confocal images of mitochondrial morphology and distribution in pollen tetrads labeled with mtGFP from wild-type (qrt1/qrt1; +/+ ) (A) and heterozygous miro1 (qrt1/qrt1; miro1/+ ) (B) plants. Note that the two miro1 mutant pollen grains (arrowheads in [B]) exhibit abnormally enlarged mitochondrial morphology. Bars = 5 μm.

(C) and (D) Merged confocal images of mitochondrial morphology and intracellular distribution in germinating wild-type (C) and miro1 mutant (D) pollen. Top, GFP fluorescence; bottom, bright-field images. Bars = 5 μm.

(E) and (F) Time-lapse single confocal images of mitochondrial morphology and intracellular distribution in wild-type (E) and miro1 mutant (F) pollen. t indicates the time point of the image in seconds from Supplemental Movies 1 and 2 online ([E] and [F], respectively), in which mitochondrial movement can be seen. In (F), arrows indicate abnormal mitochondria showing tube-like morphology, the arrowhead indicates abnormally enlarged mitochondria, and the dotted area indicates the absence of mitochondria in pollen cytoplasm. Bars = 5 μm.

(G) and (H) Transmission electron micrographs of mitochondria in wild-type (G) and miro1 mutant (H) pollen. Open arrowheads indicate the outer mitochondrial membrane. Bars = 0.2 μm.
and visualized by transmission electron microscopy. The wild-type pollen contained rod-like mitochondria with an electron-dense matrix and well-developed inner membrane cristae (Figure 8G). The miro1 mutant pollen contained much larger globular mitochondria (Figure 8H). These findings further support our suggestion that the abnormal mitochondrial morphology seen in miro1 mutant pollen is due to the enlargement but not the aggregation of mitochondria.

**MIRO1 cDNA Complements the miro1 Mutant Phenotype**

To confirm that the embryo lethality observed in the homozygous miro1 mutant is caused by the insertional mutation in MIRO1, heterozygous miro1 plants were transformed with a vector containing a MIRO1 cDNA fused to the 1.7-kb genomic sequence 5’ upstream of the predicted translational start codon of the MIRO1 gene (MIRO1 promoter:cDNA) and the kanamycin-resistant marker gene. Three pairs of primers were designed to amplify the wild-type locus, the miro1 mutant locus containing the inserted T-DNA, and the transgene (Figure 4A). Control PCR amplifications showed that the primer pairs were able to amplify the wild-type and mutant loci but not the transgene in the heterozygous miro1 plants (Figure 4B). PCR screening of the kanamycin-resistant T1 plants with these primer pairs allowed us to identify four independent lines that were homozygous for the miro1 mutant locus and harbored the transgene (Figure 4B). This result demonstrated that the embryo lethality observed in the homozygous miro1 mutant was complemented by the MIRO1 promoter:cDNA transgene.

Two of the homozygous miro1 mutant lines (cDNA1 and cDNA4) harboring the transgene were assessed for pollen viability. The T3 plants from these lines produced pollen tetrads in which all four grains were GUS-positive (Figure 7C), germinated, and exhibited normal tube growth (Figure 7D). The mitochondrial morphology in these pollen tetrads was indistinguishable from that of wild-type plants (see Supplemental Figure 7 online). These results confirm that the MIRO1 transgene reversed the impaired pollen viability and aberrant mitochondrial morphology seen in the miro1 mutant. Together, these data confirm that expression of MIRO1 cDNA under the control of the endogenous promoter complements the miro1 mutant phenotype, namely embryo lethality and impaired pollen germination and tube growth.

**DISCUSSION**

**Identification of Arabidopsis Miro-Related GTPases**

Mitochondria are dynamic organelles that frequently change their morphology and intracellular distribution by various processes, including fission, fusion, and motility. Recent studies have revealed that plants are likely to share similar machinery for the regulation of mitochondrial morphology and motility with other eukaryotic organisms (reviewed in Logan, 2006b). In various organisms, the Mito GTPases were identified recently as novel mitochondria-localized GTPases that are involved in the regulation of mitochondrial morphology and intracellular distribution (Fransson et al., 2003, 2006; Frederick et al., 2004; Guo et al., 2005). Using the predicted human protein sequence, we identified three orthologs of the Mito GTPase encoded in the Arabidopsis genome. The predicted protein structures of these orthologs are highly conserved among the species (Figure 1), and we have shown that two of the genes, MIRO1 and MIRO2, encode proteins that localize to mitochondria via their C-terminal TMs (Figure 3), suggesting that they share similar functions with the previously characterized Mito GTPases.

Gene expression analysis revealed that MIRO1 and MIRO2 are expressed in all tissues examined (Figure 2), suggesting that they are functional in Arabidopsis. However, while a mutation in MIRO1 has a severe impact on mitochondrial morphology (Figure 8) and causes embryo lethality (Figure 5) and impaired pollen germination and tube growth (Figure 7), insertional mutations in MIRO2 do not have any impact on mitochondrial morphology (see Supplemental Figure 5 online) and show no obvious effects on plant development (see Supplemental Figure 4 online). These findings suggest that MIRO1 and MIRO2 are not functionally redundant but, rather, that MIRO1 has a major role in the regulation of mitochondrial morphology during the reproductive process and subsequent embryo development. The role of MIRO1 in later developmental stages was not examined in this study because of the homozygous lethality of the insertional mutation. It could be investigated in the future using another experimental system, for example, by creating transgenic plants that enable the inducible control of MIRO1 expression.

**MIRO1 Is Required for Embryogenesis and Pollen Tube Growth**

Mutations in the genes involved in the regulation of morphology and intracellular distribution of mitochondria often cause developmental defects and human genetic disease, indicating that mitochondrial morphology and distribution are indispensable for animal development (reviewed in Chan, 2006b). In plants, however, the identities of the genes influencing mitochondrial morphology and distribution are not known, and neither are their effects on development. In this study, we have demonstrated that MIRO1 is required for embryogenesis and pollen viability and function in Arabidopsis.

Analysis of an insertional mutant of the MIRO1 gene revealed that it was not possible to recover homozygous mutant plants. The siliques from the heterozygous mutant plants contained a significant proportion of aborted seeds (Figure 5B), suggesting that the homozygous miro1 mutation causes embryo lethality. Most of the aborted seeds contained two-cell-stage embryos (Figures 5D, 5H, and 5J), indicating that the homozygous miro1 mutation severely inhibits embryonic cell division. The Arabidopsis zygote undergoes polarized cell elongation after fertilization and the subsequent asymmetric division to produce the apical and basal cells. The elongating zygote accumulates mitochondria in its apical region, whereas the cytoplasmic density of mitochondria remains uniform in the subsequent apical cell division (Mansfield and Briarty, 1990), implying that precise regulation of mitochondrial distribution is required for the progress of embryogenesis. Mito GTPases are known to be involved in regulating mitochondrial morphology and distribution in various organisms (Fransson et al., 2003, 2006; Frederick et al., 2004; Guo et al., 2005), and in this study, we demonstrated that
MIRO1 has a role in influencing mitochondrial morphology in pollen (Figure 8). Therefore, we propose that the intracellular distribution of mitochondria is regulated by MIRO1 and is essential for the normal progress of embryo development.

We also observed that mutation in MIRO1 severely reduced male genetic transmission (Table 1). Heterozygous miro1 mutant plants produced siliques in which the apical half contains a significantly higher number of aborted seeds than expected, suggesting that pollen development or function is impaired in the miro1 mutant (Meinke, 1982). Microscopic analysis and in vitro germination assays revealed that a mutation in MIRO1 does not affect pollen development (Figure 6) but impairs pollen germination and tube growth (Figure 7). Approximately 60% of miro1 mutant pollen grains did not germinate, although the remains were capable of initiating tube growth. However, subsequent tube growth was impaired significantly compared with the wild-type pollen, and <4% were capable of growing to >150 μm in length (Figure 7D). These findings suggest that MIRO1 is required for normal pollen germination and tube growth.

It has been reported that during pollen tube growth mitochondria exhibit continuous streaming in the cytoplasm (Parrot et al., 2003; Lovy-Wheeler et al., 2007). Miro GTPases have been shown to be involved in the regulation of mitochondrial morphology and distribution in various organisms; therefore, we postulated that MIRO1 may be required for proper mitochondrial streaming during pollen germination and tube growth. In order to investigate this, we visualized mitochondria by confocal microscopy of the wild-type and miro1 mutant pollen from transgenic plants containing mtGFP under the control of the pollen-specific LAT52 promoter. While uniform morphology (Figures 8A and 8C) and continuous streaming of mitochondria (Figure 8E; see Supplemental Movie 1 online) were observed in wild-type pollen, miro1 mutant pollen exhibited abnormal morphology (Figures 8B, 8D, 8F) and disrupted streaming of mitochondria (Figure 8F; see Supplemental Movie 2 online), including transient absences from regions of the cytoplasm (Figure 8F; time = 177 s). Pollen germination and tube growth involve a significant number of energy- and metabolite-dependent cellular processes, including cell wall synthesis and vesicular trafficking, specifically in the apical region of the tube (reviewed in Krichevsky et al., 2007). The disruption of streaming and the abnormal morphology of mitochondria in miro1 mutant pollen may result in reduced energy and metabolite supply to those cytoplasmic regions where such cellular processes are vital for pollen germination and tube growth.

**MIRO1 Is Involved in the Regulation of Plant Mitochondrial Morphology**

Pollen-specific expression of mtGFP revealed that a miro1 mutation leads to abnormally enlarged or tube-like mitochondria (Figure 8). This finding suggests that MIRO1 plays a role in the regulation of mitochondrial morphology and that the mutation in MIRO1 may cause increased fusion and/or decreased fission of mitochondria. Similar observations have been reported in the analysis of GEM1, a single-copy gene encoding a Miro GTPase in the budding yeast. Loss of GEM1 leads to a pleiotropic defect in mitochondrial morphology, including swollen and tube-like structures (Frederick et al., 2004). These data suggest that Miro GTPases may play a role in regulating mitochondrial fusion/fission events in both budding yeast and plants. However, Gem1p is not associated directly with the mitochondrial fusion/fission machineries reported previously, suggesting that Miro GTPases may be involved in a novel pathway for regulating mitochondrial morphology (Frederick et al., 2004).

Recent studies in *Drosophila* suggested the involvement of a Miro GTPase in mitochondrial transport in the neural axon (Guo et al., 2005). The *Drosophila* Miro GTPase associated with a 120-kD protein, designated Milton, to form a protein complex that bound to the kinesin heavy chain, leading to the recruitment of mitochondria to microtubules (Glatier et al., 2006). The human Miro GTPases also are associated with two Milton orthologs, GRIF-1 and OIP106, which interact with kinesin and mitochondria (Brickley et al., 2005; Fransson et al., 2006). These studies suggest that the Miro GTPase may be a component of the microtubule-dependent transport machinery of mitochondria (Glatier et al., 2006; Rice and Gelfand, 2006; Frederick and Shaw, 2007). Our study demonstrated that mitochondria motility in pollen is actin-dependent and not likely to be affected by the miro1 mutation (see Supplemental Movies 3 to 12 online). We also failed to identify any obvious ortholog of *Drosophila* Milton in *Arabidopsis* by a basic BLAST search of the cDNA database (data not shown). These observations suggest that, similar to yeast Gem1p (Frederick et al., 2004; Rice and Gelfand, 2006; Boldogh and Pon, 2007; Frederick and Shaw, 2007), MIRO1 plays a major role in influencing mitochondrial morphology and might interact with partner proteins that differ from human and *Drosophila* Miro GTPases. Future studies on the proteins interacting with MIRO1 may give further insight into the mechanism by which Miro GTPases regulate mitochondrial morphology in plants.

**METHODS**

**Expression Analysis**

Gene expression data were collected from the GENEVESTIGATOR website (www.genevestigator.ethz.ch; Zimmermann et al., 2004) with the default settings and processed using Microsoft Excel.

**Subcellular Localization Assay**

The partial cDNA sequences of MIRO1 and MIRO2 were amplified using *Arabidopsis thaliana* leaf cDNA (for preparation, see Supplemental Methods online) as template and primer pairs as follows: for MIRO1TM, 5′-TTAAGCGGCCGCTCGAGAAGAGTCAG-3′ and 5′-AATTGGTACCA-GGAAGACGAGGCAA-3′; for MIRO1ATM, 5′-AATTGGTACCA- TGGGAGAGTAGTACGTCGC-3′ and 5′-AATTGGCCGCTATGGAATGCTC- AATTAGGGG-3′; for MIRO2TM, 5′-AATTGGTACCA-GGACAGACAGAGCTCAG-GGAAGAAAG-3′ and 5′-AATTGGCAGGCCTGATGAGATCCTAGATTTTCTGCC-3′; and for MIRO2TM, 5′-ATGGGTACCATCCGATGATGGACTCGTGGTGAAG-3′ and 5′-AATTGGCAGGCCTGATGAGATCCTAGATTTTCTGCC-3′. Ten cycles of PCR were performed at 98°C for 10 s, 55°C for 30 s, and 72°C for 30 s or 2 min, followed by 20 cycles at 98°C for 10 s and 68°C for 30 s or 2 min. The PCR fragments were digested with *KpnI* and *NotI* and ligated into the pENTRα vector (Invitrogen). DNA inserts were transferred into pK7WG2 (Karimi et al., 2005) by Gateway recombination (Invitrogen). *Agrobacterium tumefaciens* containing the resultant vectors was infiltrated into the...
leaf epidermal cells of tobacco (Nicotiana tabacum cv SR1) seedlings grown at 21°C on soil under continuous light for 4 to 5 weeks (Bartkó et al., 2000). Fluorescence was visualized at 30 to 35 h later. GFP fluorescence of the fusion proteins was excited at 488 nm with a 25-mW argon laser, and the emitted light was collected through a 40× C-Apochromat water-immersion lens (numerical aperture = 1.2).

Mitochondria were stained by infiltration of 200 nM MitoTracker Orange CMTMRos (Invitrogen) into the apoplastic space of tobacco leaves. The fluorescence of MitoTracker was excited at 543 nm with a HeNe laser, and the emitted light was collected through a 585- to 615-nm filter. Images were acquired as z-series with 3-μm intervals, merged, and processed using Zeiss LSM Examiner and Adobe Photoshop 7.0.

**Mutant Lines and Growth Conditions**

A mutant line in the SeedGenes Project collection, emb2473, and two Salk lines, SALK_060075 and SALK_151790 (background Columbia), were obtained from the Nottingham Arabidopsis Stock Centre and designated miro1, miro2-1, and miro2-2, respectively. These genotypes were confirmed by PCR amplification using gene-specific primers for miro1, 5′-TCTGCTGTTGTTGGTG-3′ and 5′-CTGTTG-GAATTTGTGAGA-3′; for miro2-1, 5′-ATCAGCTACGTTGAA-TAG-3′ and 5′-AGGAAAACGATCAAGTTTACTAAGT-3′; for miro2-2, 5′-GCCAGGGAATACGTGATGA-3′ and 5′-TTACAGGTTGGCAG-GATATT-3′ and T-DNA-specific primers for miro1, 5′-GCTTCTTCA-GAAATGATAAAATAGCTTTGCTTC-3′; for miro2-1 and miro2-2, 5′-TTGTCACATGAGCAGTAC-3′. The template DNA was prepared by the method of Neff et al. (1998). PCR was performed at 96°C for 1 min followed by 35 cycles at 96°C for 30 s, 57°C for 30 s, and 72°C for 1 min. Seeds were surface-sterilized and plated onto 0.8% (w/v) agar medium containing half-strength Murashige and Skoog salts (pH 5.8; Duchefa Biochemie), which were supplemented with 7.5 mg/L phosphinothricin or 30 mg/L kanamycin as required. The seeds were vernalized and germinated, and the 10-d-old seedlings were transferred onto soil and grown at 21°C under a 16-h-light/8-h-dark regime.

**Histological Analysis**

Characterization of the seed phenotype in siliques from homozygous qrt and heterozygous miro1 plants was performed according to the protocol of Yadegari et al. (1994). Seeds were fixed and cleared in chloral hydrate solution, and the embryos were observed using a Zeiss Axiosplan 2 microscope with DIC optics.

**Cytological Analysis and in Vitro Germination Assay of Pollen**

Cytological analysis of mature pollen grains was performed by incubating pollen in 1 μg/mL DAPI and 20 μM FDA solutions and visualizing using a fluorescence microscope (Olympus BX50). The in vitro germination assay was performed by dipping mature flowers into 1% (w/v) agar medium containing germination buffer (50 mM MES, pH 5.8, 1 mM KCl, 10 mM CaCl₂, 0.8 mM MgSO₄, 0.01% [w/v] H₂O₂, and 18% [w/v] sucrose) (Fan et al., 2001), after which the plates were sealed and incubated at 20°C for 8 to 9 h. Pollen grains were stained at 37°C overnight in 1 mM X-Gluc solution containing 50 mM NaPO₄, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, 0.01% [w/v] Triton X-100, 10 mM EDTA, and 18% (w/v) sucrose and then observed and scored with a Leica MZFLIII microscope. The pollen tube lengths were measured in the captured images using NIH Image version 1.63 (http://rsb.info.nih.gov/nih-image/).

**Visualization of Mitochondria**

Plant mitochondria were visualized in plants transformed with a vector containing LAT52 promoter–tobacco etch virus translational enhancer (TEVL) sequence (Yang et al., 2001), tobacco mitochondrial β-ATPase signal sequence (Logan and Leaver, 2000), and the GFP sequence, which were amplified using pLAT52-TEVL GFP, pGEM-β-ATPase signal sequence:mGFP5, and pK7WG2F2 as templates, respectively. Sequences of the primer pairs were as follows: for LAT52 promoter–TEVL, 5′-GGG-GACCATTGTTATAGAAAATGTTGTCGATCAGTACGGAGTA-3′ and 5′-GGGGACTCTTTGTGATGAGCAGGCGGCTC-3′; for tobacco mitochondrial β-ATPase signal, 5′-GGGGACAAGTGTGACACAAAAGGCGGCTCAGCAGGCGGCTC-3′ and 5′-GGGGACCATTGTTACAAAGAAGCTGGTTACAGGGCGGCTC-3′; and for GFP, 5′-GGGGAGACCTCTTTGTGATGAGCAGGCGGCTC-3′ and 5′-GGGGACCATTGTTACAAAGAAGCTGGTTACAGGGCGGCTC-3′. Thirty-five cycles of PCR were performed at 98°C for 10 s, 55°C for 5 s, and 72°C for 1 min. The amplified sequences were transferred into the pk7m34GW vector (Karimi et al., 2005) using the MultiSite Gateway system (Invitrogen).

The construct was introduced into the homozygous qrt1 and heterozygous miro1 mutant plants according to the method of Clough and Bent (1998), and three independent T2 plants were used for the microscopic analysis. Pollen grains from these transgenic plants were germinated on 1% (w/v) agar medium containing germination buffer poured on the slide glass and incubated at 20°C for 4 to 6 h. GFP fluorescence of pollen mitochondria was acquired using the confocal microscope as described above. Time-lapse imaging was performed with a series of single confocal images collected by scanning at 2- to 6-s intervals. Mitochondria were also visualized by staining plant materials with 200 nM MitoTracker Orange (Invitrogen). All images were processed using Zeiss LSM Examiner and Adobe Photoshop 7.0.

**Transmission Electron Microscopy**

Pollen grains from homozygous qrt1 and heterozygous miro1 plants were germinated on 1% (w/v) agar medium containing germination buffer for 2 h. Phosphate buffer (50 mM; pH 7.0) containing 4% (w/v) paraformaldehyde, 3% (w/v) glutaraldehyde, and 18% (w/v) sucrose was then poured onto the agar medium, and the plates were incubated at room temperature for 2 h. The surfaces of the agar plates were rinsed twice with 50 mM phosphate buffer containing 18% (w/v) sucrose and incubated for a further 2 h in 1% (w/v) osmium tetroxide. After rinsing with water, agar cubes carrying the germinated pollen grains were excised, dehydrated in a series of acetone solutions (25, 50, 75, and 100% [v/v]) followed by a resin/acetone dilution series (25, 50, and 75% [v/v]), and embedded in epoxy resin. Serial cross sections were examined with a JEOL JEM1010 transmission electron microscope and a Kodak Megaplus model 1.4 digital camera. Images were processed using Adobe Photoshop 7.0.

**Cytoskeletal Inhibitor Experiments**

Stock solutions of 1 mM latrunculin B, 10 mM cytochalasin D, 10 mM oryzalin, and 10 mM nocodazole were prepared in DMSO and diluted in germination buffer. Pollen grains were germinated on 1% (w/v) agar medium containing germination buffer for 4 to 6 h and excised as small agar blocks. They were incubated in germination buffer containing 1 μM latrunculin B, 10 μM cytochalasin D, 10 μM oryzalin, and 10 μM nocodazole for 30 min, then mitochondria were visualized as described above. Treatment with 0.1% (w/v) DMSO did not have any effect on mitochondrial motility in pollen (see Supplemental Movies 11 and 12 online).

**Gene Complementation**

The coding sequence of Miro1 cDNA and the 1.7-kb upstream sequence were amplified using Arabidopsis leaf cDNA and pBeloBAC clone F15A18 (Arabidopsis Genome Initiative, 2000) as templates, respectively.
Sequences for the primer pairs are as follows: for the coding sequence, 5’-AATTGTTACAGTAGGCGGAGATCGTG-3’ and 5’-TAAAGCCGCGCCCTGACGAGCTCTT-3’; for the upstream sequence, 5’-AGGTGATCCCCTTAAGTGTTGGTGGG-3’ and 5’-GTACGCTTCTTGAATCTGAAACACATACTCA-3’. Thirty-five cycles of PCR were performed at 98°C for 10 s, 55°C for 5 s, and 72°C for 2 min. The PCR fragments were digested with KpnI/NotI and BamHI/KpnI for Miro1 cDNA and the upstream sequence, respectively, and ligated into the BamHI–NotI sites of pENTR1a vector (Invitrogen). The cauliflower mosaic virus 3SS terminator sequence was amplified by 25 cycles of PCR at 98°C for 10 s, 55°C for 5 s, and 72°C for 30 s using the primers 5’-CGCAGCTATGATGCTAGTAGTGCCGCAAAAAAT-3’ and 5’-GCGCTTAAGCTTGGCTAGTTTTGTTT-3’ and pK7WG2 as template. The PCR fragment was digested and inserted into the SpeI–HindIII sites of pKGW (Karimi et al., 2005), and these two vectors were used to perform Gateway recombination (Invitrogen). The resultant construct (Miro1 promoter: cDNA) was confirmed by sequencing and introduced into heterozygous miro1 plants according to the method of Clough and Bent (1998). The T1 plants were selected on 30 mg/L kanamycin, and plants homozygous for the miro1 mutant locus were identified by PCR amplification. Sequences of the primers used were as follows: 5’, 5’-AATCGCCCTTGGAGGAGGCTCCCTTAC-3’, 7F, 5’-TCTCTCCCAGTTC-3’, 3R, 5’-CTCTCCTTTGTTAC-3’, and LB1, 5’-GCTCTTTTCTCAGAAAATGTAATAGCCTTGCTCC-3’. The positions of these primers are indicated in Figure 4. The primers specific for Fim1 (At4g27600) (5’-TGACAAGGTCCTCTCCAGTTC-3’ and 5’-GACACGCCGAGTCTCTTCTTGC-3’) were used for the positive control. The templates were prepared by the method of Neff et al. (1998), and PCR was performed at 98°C for 1 min followed by 35 cycles at 96°C for 30 s, 57°C for 30 s, and 72°C for 1 min. The T2 plants homozygous for both the miro1 mutant locus and the transgene were identified by segregation analysis for kanamycin resistance, and the T3 plants were used for further analysis.

Accession Numbers

Arabidopsis Genome Initiative numbers for the genes mentioned in this study are as follows: Miro1, At5g27540; Miro2, At3g63150; Miro3, At3g05310; UBO10, At4g05320; and Fim1, At4g28760.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. RT-PCR Analysis of the Miro Genes.

Supplemental Figure 2. DNA Gel Blot Analysis of the miro1 Mutant.

Supplemental Figure 3. Identification of miro2 Mutants.

Supplemental Figure 4. Phenotypes of Homozygous miro2 Mutants Are Indistinguishable from Those of Wild-Type Plants.

Supplemental Figure 5. Mitochondrial Morphology Is Normal in the Root Cells of miro2 Mutants.

Supplemental Figure 6. Disruption of the Cytoskeleton in Pollen by Cytoskeletal Inhibitors.

Supplemental Figure 7. Miro1 cDNA under the Control of Its Endogenous Promoter Restores Normal Mitochondrial Morphology in miro1 Mutant Pollen.

Supplemental Table 1. Segregation Analysis of the Heterozygous miro2 Mutants.

Supplemental Movie 1. Mitochondrial Movement in Wild-Type Pollen.


