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Phosphorylation and ubiquitination of Dynamin related proteins (AtDRP3A/3B) synergically regulate mitochondrial proliferation during mitosis

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Running title: Posttranslational modifications of DRP3A/3B in cell cycle

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ABBREVIATIONS:

AtDRP3A/3B, dynamin related protein 3A/3B; BY-2, *Nicotiana tabacum* L. cv. Bright Yellow 2 suspension cell; DIC, differential interference contrast microscopy; dimethyl sulfoxide (DMSO); IP, immunoprecipitation; LP, leaf primordium; LSCM, laser scanning confocal microscopy; MS, mass spectrometry; mtDNA, mitochondrial DNA; PAFP, photo-activatable fluorescent protein; SAM, shoot apical meristem; TEM, transmission electron microscopy;

SUMMARY

The balance between mitochondrial fission and fusion is disrupted during mitosis, but the mechanism governing this phenomenon in plant cells remains enigmatic. Here, we used mitochondrial matrix-localized Kaede (mt-Kaede) to analyze the dynamics of mitochondrial fission in BY-2 suspension cells. Analysis of the photoactivatable fluorescence of mt-Kaede suggested that the fission process is dominant during mitosis. This finding was confirmed by an electron microscopic analysis of the size distribution of mitochondria in BY-2 suspension cells at different stages. Cellular proteins interacting with Myc-tagged dynamin related protein 3A/3B (AtDRP3A and AtDRP3B) were immunoprecipitated with anti-Myc antibody-conjugated beads and subsequently identified by microcapillary liquid chromatography–quadrupole time-of-flight mass spectrometry (CapLC Q-TOF) MS/MS. The identified proteins functions were broadly associated with cytoskeletal (microtubular), phosphorylation, or ubiquitination functions. Mitotic phosphorylation of AtDRP3A/AtDRP3B and mitochondrial fission at metaphase were inhibited by treatment of the cells with a CdkB/cyclin B inhibitor or a serine/threonine protein kinase inhibitor. The fate of AtDRP3A/3B during the cell cycle was followed by time-lapse imaging of the fluorescence of Dendra2-tagged AtDRP3A/3B after green-to-red photoconversion; this experiment showed that AtDRP3A/3B is partially degraded during interphase. Additionally, we found that microtubules are involved in mitochondrial fission during mitosis and that the actin cytoskeleton defines mitochondrial inheritance as early as

metaphase. Taken together, these findings suggest that mitotic phosphorylation of AtDRP3A/3B promotes mitochondrial fission during plant cell mitosis and that AtDRP3A/3B is partially degraded at interphase, providing mechanistic insight into the mitochondrial morphologic changes associated with cell cycle transitions in BY-2 suspension cells.

Keywords: Mitochondrial fission; Mitosis; Photoactivatable fluorescent protein; Phosphorylation; Ubiquitination

INTRODUCTION

Mitochondria are dynamic entities that undergo frequent division and fusion and move three-dimensionally throughout the cell (Arimura *et al.* 2004, Berman *et al.* 2008, Logan 2010, Zheng *et al.* 2009a). Their morphology varies in diverse organisms and differs with the developmental stage and physiological conditions of the cell (Braschi and McBride 2010, Okamoto and Shaw 2005, Scott *et al.* 2008). The mechanism of mitochondrial dynamics during mitosis, especially of yeast and mammalian cells, has received considerable attention (Gorsich and Shaw 2004, Horn *et al.* 2011, Taguchi *et al.* 2007, Tanaka *et al.* 1985, Zunino *et al.* 2009). Three-dimensional electron microscopic (EM) analysis of the yeast mitochondrial network has demonstrated that it fragments during mitosis and resumes a single giant-form before cytokinesis (Gorsich and Shaw 2004, Tanaka *et al.* 1985). Similarly, the long, tubular mitochondria of HeLa cells fragment early in mitosis, and the filamentous network structures subsequently reform in the daughter cells (Horn *et al.* 2011, Taguchi *et al.* 2007, Zunino *et al.* 2009). In contrast, in the only published study on the mitochondrial morphology in *Arabidopsis* shoot apical meristem (SAM) and leaf primordium (LP) meristematic cells, punctate mitochondria fused to form a cage-like structure during mitosis; this structure divided into two independent tentacular mitochondria during cytokinesis and continued to divide into very small

particles (Seguí-Simarro *et al.* 2008). However, these conclusions are undermined because ascertaining the specific stage of SAM and LP meristematic cells is difficult. An understanding of how the various pleomorphic mitochondria function in the conserved process of mitosis requires further investigation, particularly in the area of regulation of plant mitochondrial morphology.

Mitochondrial morphology is regulated by the dynamic equilibrium between fission and fusion. In mammalian cells, mitochondria fusion is mediated by FZO1, OPA1, and Mitofusin1/2 (Chan 2006, Okamoto and Shaw 2005), but no obvious homologs of these proteins have been identified in plants (Arimura *et al*. 2004, Logan 2006). Studies on mitochondrial fission in plants have been more fruitful, resulting in the identification of two *Arabidopsis* homologs of the mitochondrial fission protein hFis1, AtBIGYIN1 (AtFIS1A), and AtBIGYIN2 (AtFIS1B) (Scott *et al.* 2006, Zhang and Hu 2009) and two *Arabidopsis* homologs of dynamin-related protein (DRP) 1, AtDRP3A, and AtDRP3B (hereafter, "AtDRP3A/3B"). These latter two proteins shuttle between the cytosol and the outer surface of mitochondrial fission sites (Arimura *et al.* 2004, Arimura and Tsutsumi 2002, Fujimoto *et al.* 2009, Hong *et al.* 2003, Logan *et al.* 2004). Another protein, ELONGATED MITOCHONDRIA 1 (ELM1), localizes on the outer mitochondrial membrane and is required for the relocalization of DRP3A (and possibly also DRP3B) from the cytosol to mitochondrial fission sites (Arimura *et al.* 2008).

As an evolutionarily conserved large dynamin-like GTPase, DRP appears to be the major orchestrator protein for mitochondrial outer membrane fission and for response to cellular signals, particularly in mammalian cells (Chang and Blackstone 2007, Cho *et al.* 2009, Cribbs and Strack 2007, Han *et al.* 2008, Karbowski *et al.* 2007, Zunino *et al*. 2009). However, no evidence exists to suggest that DRP3A/3B is involved in the regulation of mitochondrial fission in plants.

Recent studies on mammalian cells have demonstrated that posttranslational modifications, such as phosphorylation, ubiquitination, nitrosylation, and sumoylation (the attachment of small ubiquitin-like modifier (SUMO) proteins), are involved in

the control of mitochondrial fission in DRP-dependent ways (Chang and Blackstone 2010, Santel and Frank 2008). In mammalian cells, hDrp1 was phosphorylated by Cdk1/cyclin B and Aurora A, thereby mediating mitochondrial fission during mitosis (Taguchi *et al.* 2007, Kashatus *et al.* 2011, Yamano and Youle 2011). Ubiquitination regulates protein degradation or quality control. As cells exit mitosis, the APC/CCdh1 E3 ubiquitin ligase complex partially ubiquitinates Drp1, thereby driving reassembly of the mitochondrial network (Horn *et al.* 2011). Sumoylation usually alters the subcellular localization of substrates or protects them from ubiquitin-triggered destruction. Zunino et al. (2009) reported that the SUMO-specific protease SenP5 relocalizes from the nucleoli to the mitochondria at the G_2/M phase transition, driving mitochondrial fragmentation by desumoylating Drp1. Whether similar posttranslational modifications also regulate plant mitochondrial morphology during mitosis remains to be determined.

In the present study, we used mitochondrial matrix-localized Kaede (mt-Kaede), a photoconvertible green-to-red fluorescent protein, to qualitatively examine the dynamic imbalance between fission and fusion during mitosis of BY-2 *Nicotiana tabacum* cells in suspension. This imbalance was further investigated using transmission electron microscopy (TEM). Heterologously expressed AtDRP3A/3B was used to investigate the regulatory mechanisms underlying mitochondrial morphologic changes, and proteins putatively interacting with AtDRP3A/3B were identified by immunoprecipitation (IP) and mass spectrometry (MS). Finally, the phosphorylation and ubiquitination of AtDRP3A/3B during mitosis were analyzed. Our findings support the hypothesis that posttranslational modifications of DRP3A/3B in BY-2 suspension cells regulate the morphologic dynamics of mitochondria during mitosis.

RESULTS

Laser scanning–confocal microscopic (LSCM) analysis of mitochondrial morphology in BY-2 suspension cells at various cell stages

LCSM analysis of BY-2 tobacco cells in different phases of the cell cycle showed that mitochondrial morphology varied greatly with cell phase. In most interphase cells, the nucleus was located to one side, near the plasma membrane, and large, punctate mitochondria with strong mt-Kaede fluorescence signals were distributed in the cortex region. Smaller, punctate mitochondria with a moderate mt-Kaede fluorescence signal were distributed randomly throughout the cell at midplane (Figure 1a, b). At prometaphase, the chromosomes moved toward the equatorial plate, and the mitotic apparatus was surrounded by small, round mitochondrial particles (Figure 1c, d). In metaphase cells, the chromosomes were aligned into a straight line, with smaller, punctate mitochondria at the cortex region and weak mitochondrial mt-Kaede fluorescence observed at midplane (Figure 1e, f). At anaphase, two straight lines of chromosomes moved toward opposite poles, with small particles at the cortex region and weak mitochondrial fluorescence observed at midplane (Figure 1g, h). In early cytokinesis, prominent phragmoplasts formed between the daughter nuclei, and the mitochondrial fluorescence signal increased (Figure 1i, j). In late cytokinesis, a complete cell plate formed and two daughter nucleoli emerged. Punctate mitochondria were distributed throughout the region of the phragmoplast (Figure 1k, l).

Dynamic analysis of mitochondrial fission and fusion revealed by photoconversion at different stages

To examine the balance between mitochondrial fission and fusion during mitosis, we followed the changes in partially photoconverted mt-Kaede protein in BY-2 cells in interphase (G_1/S), prophase, and metaphase (G_2/M). The green and red mt-Kaede fluorescence signals were monitored for at least 2 h after exposure to ultraviolet light (UV) to determine the period required for full colocalization. The data were analyzed using Manders' colocalization coefficients.

We first compared the colocalization periods of mitochondria at different stages. At interphase, granular mitochondria were distributed throughout the cytoplasm. After photoconversion, the green and red signals were completely colocalized within 2 h, so that almost all of the mitochondria were uniformly yellow (Figure 2a). When prophase cells were exposed to UV, the cells entered metaphase after 3 h, with weak and diffused green and red fluorescence intermingled with each other, only a few yellow signals appeared (Figure 2b). When metaphase cells were exposed to UV, the cell went through anaphase, cytokinesis and reentered into interphase, uniform mitochondrial fluorescence did not occur until 3 h after photoconversion (Figure 2c).

Next, we examined the fate of mt-Kaede protein synthesized in the mitochondrial matrix during interphase and prophase (Figure 2a). At interphase, small green particles corresponding to newly synthesized mt-Kaede were attached to the yellow mitochondria (Figure S1, magnified version of Figure 2a, row 5, column 4), indicating that the newly synthesized protein was incorporated into existing mitochondria. When cells were photoconverted at prophase, the intensity of the green signal declined after 1 h but significantly increased after 2 or 3 h (Figure 2b, row 3, column 3). The nascent green signal was evenly distributed and intermingled with the preexisting red signal, without colocalization (Figure 2b, rows 5 and 6, column 4).

Finally, we longitudinally converted BY-2 cells in metaphase (or early cytokinesis), when the condensed chromosomes were arranged in straight lines (or in a newly formed cell plate). No merging between the red and green signals was observed, even after late cytokinesis (Figure 2d), suggesting that mitochondrial inheritance by daughter cells is determined before the cell wall can serve as a physical barrier.

Cell stage-dependent analysis of mitochondrial size distribution in BY-2 suspension cells

Mitochondrial morphology in BY-2 cells at various stages was also examined using TEM to analyze the size and distribution of mitochondria in the cells. Wild-type cells stained with 4′,6-diamidino-2-phenylindole (DAPI) were sorted into different cell stages under an epifluorescence microscope and relocated before sectioning. At interphase, nucleoli with complete nuclear envelop displaced near one side of the plasma membrane and mitochondria were distributed throughout cytoplasm (Figure 3a). At metaphase, chromosomes distributed in the nucleoplasm and mitochondria presented as small, round particles (Figure 3b). At anaphase, tubular vesicle network joined together at the equatorial plate (Figure 3c*i*, *ii*), and most of the observed mitochondria were small, both in the vicinity of the daughter nucleolus and in the vicinity of the newly forming cell plate (Figure 3c, d).

The size distributions of randomly selected mitochondria in cells at different stages are shown in the histogram in Figure 3d. Small mitochondria (area, <0.3 μm^2) comprised 34.3% of the mitochondria in metaphase cells and 64.8% of those in anaphase cells, but only 28.2% of those in interphase cells. In contrast, 32.4% of the mitochondria in interphase cells were large (area, >0.5 μm^2), whereas only 7.2% of those in anaphase cells were large. Statistical analysis of the EM data revealed that the fraction of mitochondria with areas less than 0.3 μm^2 increased significantly during

mitosis.

Heterologously expressed AtDRP3A/3B functions in mitochondrial fission in BY-2 suspension cells

No homologs of known mitochondrial fusion proteins have yet been identified in plants. A BLAST search of the National Centers for Biotechnology database with the AtDRP3A/3B sequence yielded two contigs (BP136295 and DV160432) from *N. tabacum* expressed sequence tags. These sequences were identified as belonging to a single NtDRP3 candidate (Hamada *et al.* 2006). Pairwise alignment using the EMBOSS program (http://www.ebi.ac.uk/emboss/align/index.html) showed that NtDRP3 is quite similar to sequence to AtDRP3A (59.9% identity, 69.1% similarity) and AtDRP3B (59.8% identity, 70.8% similarity) (Figure S2).

When AtDRP3A/3B constructs with Dendra2 fused to their C-termini were expressed in BY-2 suspension sells, counterstaining of the mitochondria showed that the constructs promoted mitochondrial fission (Figure S3). Consistent with a previous report that the overexpression of ΔDRP3A/3B containing a defective GTPase domain in BY-2 suspension cells induces the formation of tubular mitochondria (Arimura *et al.* 2004), our finding indicates that heterogeneously expressed AtDRP3A and AtDRP3B are functional in mitochondrial fission in BY-2 suspension cells.

Identification of proteins putatively interacting with Myc-AtDRP3A/3B by IP and MS

To explore the molecular mechanisms that govern mitochondrial fission in plant cells, we transformed BY-2 suspension cells with a vector expressing Myc-tagged AtDRP3A, Myc-AtDRP3B, or Myc. After 3 days of growth, the total cellular protein was extracted from each culture and mixed with anti-Myc antibody (Ab)-conjugated agarose beads. Electrophoretic separation and staining of the resulting

immunoprecipitated protein showed three main bands. These bands were excised from the gel and analyzed by microcapillary liquid chromatography–quadrupole time-of-flight (CapLC Q-TOF) MS/MS. Both bands at 130 and 100 kDa were identified as Myc-AtDRP3A or Myc-AtDRP3B, respectively. Comparisons of data from wild-type and Myc-expressing cells allowed the exclusion of some proteins interacting nonspecifically with Myc-DRP3A and Myc-DRP3B. The remaining proteins were assigned to one of three categories (Table 1). Since most components of the cytoskeleton have been reported to interact with dynamin *in vitro* (Praefcke and McMahon 2004, Shpetner and Vallee 1989), the high-confidence identification of tubulin as protein interacting with AtDRP3A/3B suggested that our IP/CapLC Q-TOF MS/MS procedure was effective for identifying AtDRP3A/3B-interacting proteins.

Among the other proteins identified by this method were protein phosphatases 2A and 2C; these members of the mitogen-activated protein kinase kinase family were classified as candidates involved in (de)phosphorylation. Also identified were E3 ubiquitin protein ligase, the 26S proteasome AAA-ATPase and regulatory subunits; these proteins were classified as candidates involved in ubiquitination. However, the confidence scores for the identified phosphorylation- and ubiquitination-related proteins were low, probably because these proteins, being involved in signal transduction, are expressed at very low levels. In a Western blot analysis using a polyclonal Ab against BIGYIN, the *Arabidopsis* ortholog of yeast Fis1p, NtBIGYIN, appeared in the column input and flow-through fractions, but not in the anti-Myc Ab IP fraction (Figure S4a), serving as negative control for indirect interaction with DRP3A/3B, which is also confirmed by our co-immunoprecipitation experiment (Figure S4b).

Oryzalin treatment of BY-2 suspension cells enhances mitochondrial fission

To investigate the role of microtubules in mitochondrial fission, we examined the effects of two different microtubule inhibitors, oryzalin and taxol, on interphase BY-2 suspension cells expressing mt-Kaede (Figure 4). The mean mitochondrial area

decreased by approximately one-half (51%) after 10 min of exposure to 10 μM oryzalin, whereas it increased by approximately one-third (38.0%) after 10 min of exposure to 5 μM taxol, and the phenotype induced by oryzalin was reversed by taxol when both inhibitors were simultaneously applied (Figure 4b, c).

Using photoconversion, we also examined the effects of these inhibitors on the balance between mitochondrial fission and fusion on interphase BY-2 suspension cell. After a lengthy incubation with oryzalin, smaller mitochondria with weak mt-Kaede fluorescence were observed (similar to the pattern seen at metaphase), and almost no colocalization of the green and red signals was detected (Figure 4c). In contrast, incubation with taxol or double treatment (oryzalin + taxol) did not enhance mitochondrial fission (Figure 4c).

Mitotic phosphorylation of Myc-AtDRP3A/3B is important in mitochondrial fission during mitosis

We next investigated whether Myc-DRP3A/3B is phosphorylated during mitosis using G_2/M-synchronized Myc-AtDRP3A- and Myc-AtDRP3B-expressing BY-2 suspension cells. After the fraction of mitotic cells was enriched by a sequential aphidicolin and propyzamide treatment, flow cytometric analyses showed that approximately 30-50% of the cells were mitotic. Non-synchronized cells were used as interphase (G_1/S) cells. After synchronization, the total cellular protein was extracted, subjected to IP with anti-Myc Ab-conjugated agarose beads, and subjected to electrophoresis. Phosphorylation was visualized using Pro-Q Diamond phosphoprotein gel staining, and the amount of protein was monitored using Coomassie brilliant blue staining. The specificity of Pro-Q Diamond staining was validated by calf intestinal phosphatase (CIP) treatment (Figure S5). The ratio of the phosphorylated Myc-DRP3A/3B signals (Pro-Q Diamond staining of 130 kDa) to their respective Myc-DRP3A/3B signals (protein amount of 130 kDa) at G_1/S or G_2/M stages suggests that Myc-DRP3A/3B is phosphorylated during mitosis (Figure 5a, b).

Protein phosphorylation during mitosis is predominantly driven by cyclin-dependent kinases. To determine whether CdkB/cyclin B is involved in the mitotic phosphorylation of DRP3A/3B, we investigated the effects of kinase inhibitors both on phosphorylation level of DRP3A/3B and mitochondrial morphology. When synchronized Myc-DRP3A/3B-expressing BY-2 suspension cells was treated with olomoucine, a potent, selective, ATP-competitive inhibitor of CdkB/cyclin B and related kinases, the phosphorylation level of Myc-DRP3A/3B (~130 kDa) at G_2/M stages was inhibited. A similar phenomenon was observed when treated with staurosporine, a serine/threonine protein-kinase inhibitor (Figure 5a, c). When Mt-Kaede-expressing BY-2 suspension cells were treated with olomoucine, mitochondrial fission at metaphase was impaired, with granular mitochondria appeared instead (Figure 5d, e). When the cells were treated with staurosporine, punctate mitochondria appeared in both interphase and metaphase cells, but almost no cells undergoing cytokinesis were detected (Figure S6).

DRP3A/3B is partially degraded at interphase

To investigate DRP3A/3B protein degradation during the cell cycle, we fused the photoconvertible fluorescent protein Dendra2 to the C-terminus of AtDRP3A/3B and expressed the fusion proteins in BY-2 suspension cells. By exposing the cells to UV, we were able to activate the preexisting AtDRP3A/3B-Dendra2 protein, marking it with red fluorescence, and exclude any newly synthesized (green) AtDRP3A/3B-Dendra2 protein from analysis. We followed the decay of the red fluorescence intensity as a direct marker of the degradation of AtDRP3A/3B-Dendra2 (Gerbin and Landgraf 2010, Zhang *et al.* 2007).

Using this method, we were able to monitor protein degradation at the single-cell level and we used 3 days culture to monitor both interphase and mitotic trajectories. As shown in Figure 6a and 6b, an analysis of red DRP3A-Dendra2 fluorescence showed that during the first 2 h after UV exposure, the cell went through metaphase

and entered into cytokinesis, with a concomitant 20% decrease in red fluorescence. During the next 4 h, the cell finished cytokinesis and reentered G_1/S stages, with a concomitant 30% decrease in red fluorescence. In the meanwhile, the red DRP3A-Dendra2 fluorescence of interphase cell declined 35% during the first 2 h, and 55% during the whole 6 h (Figure 6a, c).

BY-2 cells transformed with DRP3B-Dendra2 or Dendra2 at metaphase or interphase were also UV exposed and analyzed (Figure 6d, g). By 6 h after UV exposure, the red DRP3B-Dendra2 fluorescence signals declined 40% and 52%, respectively, from their metaphase and interphase values (Figure 6d, e, respectively). Nevertheless, the decrease of the red Dendra2 fluorescence ranged from 14% to 20%, respectively, from their metaphase and interphase values (Figure 6f, g, respectively). The red fluorescence of DRP3A/3B-Dendra2 cells decreased dramatically during G_1/S stages, and had a relatively slower declined tendency when the cell was UV exposed at metaphase.

To investigate the ubiquitination of Myc-AtDRP3A/3B, we used BY-2 cells grown in suspension for 7 days as an interphase sample. Because of the relatively low synchronization efficiency, mitotic samples intermingled with interphase cells can interfere with the protein expression tendency, therefore samples at G_2/M stages were not analyzed. Two main protein bands of approximately 130 and 100 kDa were detected in the interphase cells, and the intensity of both bands increased when the stationary cells transfer to fresh medium were used (without any treatments) (Figure 7a). When the cells were treated with dimethyl sulfoxide (DMSO), similar tendency to independent turnover of Myc-DRP3A/3B was observed (Figure 7b, c, and Figure S7). When the Myc-DRP3A/3B cells were treated only with the protein synthesis inhibitor cycloheximide (CHX), the intensity of the lower band (~100 kDa, both Myc-DRP3A and Myc-DRP3B) increased, whereas that of the upper band (~130 kDa) of Myc-DRP3A remained almost at similar level, with the intensity of the upper band (~130 kDa) of Myc-DRP3B was constantly decreased (Figure 7b, c and Figure S7) over time. When Myc-DRP3A/3B cells were treated with both MG132, an inhibitor of

the chymotrypsin-like activity of the proteasome, and CHX, the intensity of both upper band and lower band remained nearly constant, comparing with samples treated with DMSO.

DISCUSSION

Networked or tubular mitochondria in yeast or mammalian cells fragment early in mitosis (Taguchi *et al.* 2007, Zunino *et al.* 2009). However, the punctate nature of plant mitochondria hinders the direct quantification of the mitochondrial fission and fusion processes (Arimura *et al.* 2004, Berman *et al.* 2008), and the morphologic dynamics of mitochondria during mitosis in dedifferentiated plant cells in suspension or in differentiated plant cells have not yet been reported. In the present study, we used mt-Kaede to evaluate mitochondrial fission and fusion during mitosis.

In our dynamics analysis, we found that mitochondria at the cortex or midplane regions of BY-2 suspension cells decrease in size at metaphase and anaphase. Photoconversion–colocalization experiments also demonstrated that the period required for complete mitochondrial fusion is significantly longer during G_2/M stages than in G_1/S stages (3 h vs. 2 h, respectively). Moreover, high-resolution EM observations showed that the fraction of small mitochondria (<0.3 μm^2) increases significantly at anaphase. In addition, we noted an increase in green fluorescence corresponding to non-photoconverted mt-Kaede during time-lapse imaging in interphase and prophase, indicating that new protein is synthesized and incorporated into preexisting mitochondria during these stages. Based on our findings, we conclude that mitochondrial matrix proteins are continuously synthesized and transported into preexisting mitochondria during G_1/S stages and that mitochondrial fission dominates at G_2/M stages, ensuring mitochondrial proliferation during mitosis.

Our findings are in agreement with previous reports that networked tubular mitochondria in fission yeast and HeLa cells fragment during mitosis (Horn *et al.* 2011, Taguchi *et al.* 2007, Tanaka *et al*. 1985, Zunino *et al*. 2009). A recent study, however, reported that small mitochondria in *Arabidopsis* SAM and LP meristematic

cells fuse with a single large mitochondrion, and that this structure eventually reorganizes into a cage-like structure encompassing the mitotic spindle (Seguí-Simarro *et al.* 2008). This apparent discrepancy may have occurred because only static observations were used in previous report. However, we cannot rule out the possibility that the difference is a result of using different tissues.

Actin cytoskeletons play a vital role in cellular organization, organelle movement and inheritance, cytokinesis, and signal transduction. Actin bundles have been reported to provide the main track for mitochondrial movement in fission yeast and plant cells (Schauss and McBride 2007, Zheng *et al.* 2009b), and furthermore, the actin-depleted zone observed at metaphase has been reported to correspond exactly to the site of cell plate formation during cytokinesis (Sano *et al.* 2005, Sheahan *et al.* 2004). In our photoconversion experiments, however, we found that longitudinal photoconversion of metaphase cells (UV exposure of half the cell, with the equatorial plate as the border) resulted in no detectable fusion between red and green mitochondria, similar to our observations of cytokinesis after longitudinal photoconversion with the newly formed cell plate as the border. We speculate that this discrepancy in findings arose, at least in part, because the actin-depleted zone at the metaphase plate limits mitochondrial movement between the daughter cells. Our finding that mitochondrial inheritance by daughter cells is determined as early as metaphase provides strong support for the hypothesis that mitochondrial movement is limited by the distribution of the actin cytoskeleton and that the cell plate serves as a physical barrier for mitochondrial inheritance in BY-2 suspension cells.

Microtubules have also been reported to play important roles in mitochondrial positioning and fission (Jourdain *et al.* 2009, Yaffe *et al.* 2003). Using the *dnm1*Δ fission yeast strain, Jourdain et al. showed that Dnm1p (dynamin-related protein 1) fragmented mitochondria in a microtubule-dependent manner (Jourdain, *et al.* 2009). In our IP experiments, we identified microtubular proteins as principal candidates for BY-2 proteins interacting with DRP. Furthermore, oryzalin treatment caused the BY-2 mitochondria to dramatically decrease in size and gradually but significantly reduce their mt-Kaede fluorescence. These findings are consistent with the previously reported conclusion that dynamin associates with microtubules *in vitro* (Obar *et al.* 1990, Shpetner and Vallee 1989) and provide evidence that mitochondrial morphology *in vivo* is affected by microtubule inhibitors. Given the link between

microtubule dynamics (Vos *et al.* 2004) and mitochondrial morphologic changes during mitosis, we propose that microtubule depolymerization is involved in mitochondrial fission during the cell cycle.

AtDRP3A/3B is phosphorylated and recruited to mediate mitochondrial and peroxisomal fission. In mammalian cells, hDrp1 is phosphorylated by several different kinases; it is phosphorylated by Cdk1/cyclin B at Ser585 during mitosis, by cAMP-dependent protein kinase at Ser656, and by Ca^{2+}/calmodulin-dependent protein kinase Iα at Ser600 (Cribbs and Strack 2007, Han *et al*. 2008, Taguchi *et al*. 2007). However, the only data concerning the putative posttranslational modification of AtDRP3A/3B in plant cells are in the PhosPhate database of The Arabidopsis Information Resource (TAIR), which shows that Ser575 (SRS#FLGR) of DRP3A and Ser560 (TRS#FLGR) of DRP3B are confirmed phosphorylation sites. Our experiments using phosphoprotein staining showed that mitotic phosphorylation of AtDRP3A/3B also occurs in BY-2 suspension cells, in agreement with a previous report stating that mitotic phosphorylation of hDrp1 is important in mitochondrial fission in HeLa cells (Taguchi *et al*. 2007). We also detected a low level of basal phosphorylation. Treatment of BY-2 cells with olomoucine, an inhibitor of CdkB/cyclin B and related kinases, impaired both mitotic phosphorylation of Myc-AtDRP3A/3B and mitochondrial fission at metaphase. Treatment of the cells with staurosporine, a Ser/Thr protein kinase inhibitor, had a similar effect on mitotic phosphorylation of Myc-AtDRP3A/3B and mitochondrial fission at metaphase, as well as severe physiological effects. These results are consistent with those of recent studies showing that the Cdk1/cyclin B complex and the mitotic Ser/Thr kinase Aurora A cooperate with the small Ras-like GTPase RalA and its effector, RalA-binding protein 1, to promote DRP1 phosphorylation and mitochondrial fission during mitosis (Kashatus *et al.* 2011, Yamano and Youle 2011). From these results, we infer that AtDRP3A/3B has more than one phosphorylation site and that CdkB/cyclin B might be an upstream regulator of AtDRP3A/3B mitotic phosphorylation.

The Dendra2-based fluorescence decay assay is a powerful and effective technique that not only allows spectroscopic monitoring of protein degradation at the single-cell level (Gerbin and Landgraf 2010, Zhang *et al.* 2007), but also is also useful for correlating protein degradation with cell cycle stages. The technique circumvents the low synchronization efficiency associated with the CHX chase technique.

Although Drp1 ubiquitination has been studied extensively in mammalian cells (Horn *et al.* 2011, Karbowski *et al.* 2007, Wang *et al.* 2011), the posttranslational modifications of AtDRP3A/3B in plant cells remain to be further investigated. Our IP and MS analyses identified a series of ubiquitin pathway-related proteins, including the AAA-ATPase and regulatory subunits of the 26S proteasome and ubiquitin protein ligase. In live cells, red Dendra2 fluorescence from AtDRP3A/3B-Dendra2 dramatically decreased at interphase. This finding was confirmed by the effects of time-dependent treatment with CHX and MG132. Thus, we speculate that the smaller Myc-AtDRP3A/3B electrophoretic band was the product of protein degradation by the 26S proteasome. These findings are in accord with a more recent report that partial ubiquitination of Drp1 drives the reformation of tubular mitochondria in mammalian cells as the cells exit mitosis (Horn *et al.*, 2011). Therefore, the partial degradation of DRP3A/3B-Dendra2 seen at interphase suggests that AtDRP3A/3B is partially degraded at interphase to ensure that it remains at a relatively low level. However, our results about protein degradation investigation with CHX chase or fluorescence decay with Dendra2 does not match pretty well. This discrepancy may have occurred because of different interphase sample were used (7 days culture for CHX chase vs. 3 days culture for fluorescence decay assay). Another possible reason is that fluorescence bleaching and time lag of fluorophore maturation during fluorescence decay assay (Gerbin and Landgraf 2010, Zhang *et al.* 2007).

Our investigations into DRP3A/3B regulation at different stages of the plant cell cycle have provided new perspectives concerning the morphologic dynamics of mitochondria during mitosis (Figure 8). Our three principal findings are that the dominance of mitochondrial fission at metaphase and of mitochondrial growth at G1/S stages provides a foundation for mitochondrial proliferation during the cell cycle, AtDRP3A/3B undergoes mitotic phosphorylation and partial degradation at interphase, and microtubules depolymerization and the distribution of actin cytoskeleton are involved in mitochondrial fission and inheritance during mitosis. Taken together, our findings help to expand our insights into the molecular mechanism underlying the morphologic control of mitochondria during mitosis in BY-2 suspension cells.

EXPERIMENTAL PROCEDUES

Plasmid construction

The coding region of transient expression vector mito-kaede (Arimura *et al*. 2004), was amplified by PCR with primerSTAR^{TM} (Takara, Dalian, China), subcloned with SmaI and XbaI into stable transformation vector as pCM2300-Mt-kaede (kana^{r}, both in bacteria and plants) for mitochondrial morphology visualization.

For immuno-precipitation and western blotting experiment, the cDNAs of Arabidopsis *AtDRP3A* and *AtDRP3B* (mitochondrial fission complex member) were cloned into stable transformation vector *pCM1307-6×Myc* with SpeI/SalI or SpeI/KpnI as *pCM1307-Myc-AtDRP3A* and *pCM1307-Myc-AtDRP3B*, respectively. For protein degradation analysis, *Dendra2* (kindly provided by Dr. Landgraf, University of Miami), with/without stop codon, inserted into the plasmid of *pCM1307-6×Myc* with SalI and KpnI as *pCM1307-Myc-Dendra2* (Dendra2), then *AtDRP3A* and *AtDRP3B* was constructed at the N-terminal of *Dendra2*, as *pCM1307-Myc-AtDrp3A/3B-Dendra2* (DRP3A-Dendra2/DRP3B-Dendra2).

Plant materials, maintenance, and transformation

Maintenance and transformation of BY-2 cells were performed as previously described (Nebenfuhr *et al.* 2000, Nagata *et al.* 2006) (http://mrg.psc.riken.go.jp/strc/index.htm), maintained both in liquid culture and solid plate via sub-culturing (once per week for suspension cultures and twice per month for calli on agar plates).

By Agrobacterium mediated transformation, the pCM2300-*Mt-Kaede*, pCM1307-*Myc-DRP3A/3B*, pCM1307-*Myc-Drp3A/3B-Dendra2* and corresponding empty vector pCM1307-*Myc* and pCM1307-*Myc-Dendra2* (with stop codon) were introduced into BY-2 suspension cells. The transgenic BY-2 cell lines were screened by kanamycin (pCM2300-mt-kaede) or hgromycin (pCM1307 related vectors) and carbenicillin resistance and incubated at room temperature for 3 to 4 weeks until transformed colonies were visible. Resistant cell colonies (50 to 100 colonies for each construct) were subjected to preliminary screening by PCR identification or fluorescence detection. Selected transgenic cell lines (5 to 10 per construct) were

further transferred into MS liquid media containing antibiotics to initiate suspension culture and used for subsequent analysis.

Confocal imaging, photoconversion of kaede and Dendra2

Unsynchronized BY-2 suspension cells of 3 days after transferred to fresh medium were used for mitotic process observation. The samples were transferred into glass bottom dishes (MatTek Corp., Ashland, MA), observed with Leica SP5 (Leica, Germany) inverted laser scanning confocal microscope. Firstly, DIC was used to find the cell undergoing mitosis, then mitochondrial morphology visualized with mt-kaede was excited with 488 nm (green kaede), emission spectra were collected between 500 and 550 nm for green-kaede. For kaede photo-conversion, wavelength of 405 nm (30% intensity of full power, only illuminated the selected ROIs, three pulse with imaging speed-1024×1024 pixel at 100 Hz) were used, then imaging at 488 nm for green-kaede and 543 nm for red-kaede (Arimura *et al.* 2004). For Dendra2 photo-conversion, wavelength of 488 nm (strong blue light-100% intensity of full power, illuminated the whole cell, 3 pulse with imaging speed-1024×1024 at 10 Hz) was used. Photo-converted cells were incubated for 6 h and imaged every 2 h. The fluorescence decay of the red-Dendra2 was recorded for protein degradation analysis. All images were captured with a 63 × 1.40 numerical aperture oil objective lens (Leica). Image analysis was performed with Adobe Photoshop CS2 (Adobe Systems) and Image J 1.42e (Wayne Rasband, National Institutes of Health).

Correlated light and Electron microscope

Synchronized wild type BY-2 suspension cells were collected and fixed with 2.5% glutaraldehyde and 4% paraformaldehyde, washed with PBS for twice, after staining with DAPI, the cell pellet was mixed with 2% low melting-point agarose. After mounting the cell slush on slides, the cells were gently pressed with a cover glass in order to make the agarose layer as thin as possible and cut into small pieces. Then small BY-2 agarose pieces were observed under the epifluorescence microscope with DAPI channel, cells at different stages were chosen, washed with PBS for two more times, and fixed with 1% osmium tetraxide, and followed by routine EM procedures. The agarose pieces were embedded on slides and thermal cure at 60 □ for 24 h. BY-2 cells at specific stages were relocated with semi-ultra section. Ultrathin sections were prepared in a thickness of 70 nm and post-stained with uranylacetate

and lead citrate, and examined at 80 KV under a Hitachi H-600 transmission electron microscope.

Protein extraction and immuno-precipitation

For immuno-precipitation experiments, suspension cells of Myc, Myc-DRP3A and Myc-DRP3B were collected and grinded with liquid N_2. The crude extract was suspended in the homogenization buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol; 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM NaF, 10 mM sodium orthovanadates, protease inhibitor and Phosstop cocktail tablets (Roche Diagnostics, Beijing, China)). After ultracentrifugation at 15, 000 × g for 20 min, at 4 °C, the supernatant was normalized for protein content and incubated with protein A- agarose beads to reduce the non-specific binding. Then the supernatant was incubated with anti-Myc agarose beads (Sigma Aldrich, Beijing, China) for 2 h with end-over-end rotation at 4℃. Precipitates were recovered by centrifugation (2 min at 2, 000g) and washed five times in the homogenization buffer, and the beads were transferred into a fresh tube after the fifth wash to minimize carry-over of proteins from the lysate. Proteins were eluted from the beads by boiling in SDS sample buffer and resolved on 8% polyacrylamide gels. The gel was cut out for identification with CapLC Q-Tof MS/MS, or stained with Pro Q diamond for phosphorylation detection.

MS Identification and Data Analysis

To identify the putative Myc-DRP3A and Myc-DRP3B interacting protein, after electrophoresis and stained with Coomassie brilliant blue, the immunoprecipitation sample in gel slices were excised. In-gel digestion and CapLC Q-Tof MS/MS were performed according to previous report (Chen *et al*. 2006).

To qualify as positive identification, both NCBInr and ESTobacco database were used, the NCBInr was searched with MASCORT with following parameters: taxonomy, Viridiplantae (green plants); one missed cleavage was allowed; peptide tolerance, 0.6; MS/MS tolerance, 0.5; enzyme, trypsin; fixed modifications (carbamidomethyl) and variable modifications (oxidation, Phospho (S and T) and Phospho(Y)) were used. For more accurate information, the ORFs of ESTobacco database (http://www.estobacco.info/annotations.html) was used as local protein database and searched with pFind (http://pfind.jdl.ac.cn/index.htm) and same

parameters.

Pharmaceutical treatments

For microtubule cytoskeleton analysis, stock concentrations of 1 mM taxol were made up in DMSO, the final concentration was used at 10 μM oryzalin were prepared as 20 mM stocks in 100% ethanol and used as 5 μM for work solution (Zheng *et al.* 2009b). Both taxol and oryzalin were used to examine the microtubule disorder effect on mitochondrial morphology.

For phosphorylation analysis, the transgenic BY-2 cell lines of Myc-DRP3A/DRP3B or Mt-kaede were synchronized according to previous report (Samuels *et al.* 1998). For cell stages phosphorylation analysis, synchronized Myc-DRP3A/DRP3B cell cultures at G_1/S or G_2/M were used for immuno-precipitation and pro-Q diamond staining. To determine the putative protein kinase of Myc-AtDRP3A/3B, 10 μM Staurosporine (Ser/Thr protein kinase inhibitor) or 10 μM Olomoucine (CDKB/Cyclin B inhibitor) were added to Myc-AtDRP3A/3B or mt-Kaede cell cultures at G_2/M stage after released from propyzamide. After treated for 2 h, samples were used to investigate the phosphorylation level or mitochondrial morphology changes upon treatment with these kinase inhibitors.

For protein degradation analysis, 20 μM CHX was used to inhibit the protein synthesis (Gerbin and Landgraf 2010), and 50 μM MG132 was used to detect the proteasome interactions. After incubation for different periods, the samples were harvested for western blotting detection. After transferring proteins to nitrocellulose membranes, blots were detected with anti-Myc mAbs (eluted at 1:5000) and developed with horseradish peroxidase–linked secondary antibodies and the ECL chemiluminescence detection system (Applygen, Beijing, China)

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SUPPLEMENTAL FIGURE LEGENDS

Table S1 Sequences of primers used in this study**.**

Figure S1. Magnified version of Figure 2a (2 h, row 5, column 4).

Figure S2. Sequence alignments of the NtDRP3 candidate protein and AtDRP3A/3B.

Figure S3. Dynamic transitions of AtDRP3A-Dendra2 and AtDRP3B-Dendra2 during mitochondrial fission in BY-2 suspension cells.

Figure S4. BIGYIN1/2 and DRP3A/3B do not appear to physically interact in BY-2 cells.

Figure S5. Specificity confirmation of Pro-Q diamond staining.

Figure S6. Mitochondrial morphology in BY-2 cells at interphase and metaphase after treatment with staurosporine.

Figure S7. Quantification of data shown in Figure 7b and 7c.

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Table 1 Identification of putative Myc-AtDRP3A/3B-interacting proteins in BY-2 suspension cells by CapLC Q-TOF MS/MS. Peptides, number of distinct peptides identified for the protein; No. of peptides, number of peptides identified in the MS analysis; Score, the score retrieved by the MASCOT program. With the exception of AtDRP3A and AtDRP3B, the identified proteins are listed according to score. A score of 25 or greater indicates an identification event of at least 95% confidence.

Figure legends

Figure 1. Mitochondrial distribution at different stages of the BY-2 cell cycle (bar = 10 μm); Chr, chromosome; CP, cell plate; Nu, nucleolus). BY-2 suspension cells expressing mitochondrial localized N-δ-ATPase-Kaede (mt-Kaede) were imaged at the cortex (a, c, e, g, i, k) and midplane (b, d, f, h, j, l) regions by fluorescence microscopy (488 nm) and differential interference contrast (DIC) microscopy; the fluorescence and bright field images were merged. Green dots correspond to punctate mitochondria.

(a, b) In the interphase cell, the nucleus is displaced to one side, almost touching the plasma membrane.

(c, d) In prometaphase, the chromosomes are condensed and moving toward the equatorial plate.

(e, f) In metaphase, the chromosomes are aligned at the equatorial plate.

(g, h) In anaphase, the chromosomes are moving toward opposite spindle poles.

(i, j) In early cytokinesis, a prominent phragmoplast is present between the daughter nuclei.

(k, l) In late cytokinesis, formation of the cell plate is complete and two daughter nuclei are present.

Figure 2. Mitochondrial fission/fusion dynamics at different stages of the cell cycle in BY-2 suspension cells. Bar = 20 μm. In each panel, column 1 (reading left to right) shows mitochondrial fluorescence due to mt-Kaede at the G_1/S or G_2/M stages, column 2 shows the corresponding time-lapse DIC images, column 3 shows the colocalization analysis with Manders' coefficients, and column 4 is an enlargement of the boxed area in column 1.

(a) Longitudinal photoconversion of mt-Kaede fluorescence at interphase ($n = 4$).

(b) Transverse photoconversion at prometaphase ($n = 3$). The increase in green fluorescence over time is indicated by an asterisk.

(c) Transverse photoconversion at metaphase ($n = 4$).

(d) After longitudinal photoconversion at metaphase or early cytokinesis with the equatorial plate as the border, no fusion is detected between red and green mt-Kaede signals ($n = 3$).

Figure 3. EM and statistical analysis of mitochondrial morphology in BY-2 suspension cells at different stages (Chr, chromosome; M, mitochondria; Nu, nucleolus; TVN, tubulovesicular network).

(a) The cell is in interphase, as shown by the complete nuclear membrane and nucleus in (*i*) (bar = 5 μm). Images (*ii*) and (*iii*) are enlargements of the boxed areas in (*i*) (bars = 0.5 μm).

(b) The cell is in metaphase, as determined by chromosome condensation and DAPI staining in (*i*). Image (*ii*) is an enlargement of the boxed area in (*i*) (bar = 5 μm). Images (*iii–v*) are enlargements of the boxed areas in (*ii*) (bars = 0.2 μm).

(c) The cell is in anaphase, as determined by DAPI staining of the aligned, condensed chromosomes and the developing cell plate in (*i*). Image (*ii*) is an enlargement of the boxed area in (*i*) (bar = 2 μm). Images (*iii–v*) are enlargements of the boxed areas in (*ii*) (bars = 0.2 μm).

(d) Size distribution of mitochondrial area in cells at different stages. The areas of at least 100 randomly selected mitochondria were measured in each cell, and three or four cells were analyzed for each stage.

Figure 4. Changes in mitochondrial morphology induced by oryzalin and/or taxol treatment of interphase BY-2 suspension cells.

(a) Mitochondrial morphology in cells treated with DMSO (control), 10 μM oryzalin, 5 μM Taxol, or 10 μM oryzalin + 5 μM Taxol for 10 min. mt-Kaede fluorescence merged with DIC bright-field images are shown (bars = 20 μm). Images shown are representative of results obtained for cells treated with DMSO ($n = 4$), oryzalin ($n = 6$), taxol ($n = 5$) and oryzalin/taxol (n=5).

(b) Quantitative analysis of mitochondrial size (in μm^2) in cells treated as in (a). Differences in the mitochondrial mean area treated with DMSO vs. oryzalin, DMSO

vs. taxol, or oryzalin vs. taxol are statistically significant by Student's t test (*p <0.01). Nevertheless, difference in mitochondrial mean area treated with taxol vs. oryzalin + taxol is not significant by Student's t test (p>0.05).

(c) Images showing progress of mitochondrial morphology changes over time after treatment with oryzalin and/or taxol as in (a) (bars = 20 μm).

Figure 5. Mitotic phosphorylation of DRP3A/3B is involved in mitochondrial fission during mitosis.

(a) Phosphoprotein (Pro-Q staining) and protein amount (Coomassie brilliant blue, CBB staining) immunoprecipitated from Myc-DRP3A/3B-expressing BY-2 cells with anti-Myc Ab-conjugated agarose beads at the G_1/S or G_2/M stages, and G_2/M samples treated with olomoucine (olo) or staurosporine (sta).

(b) Signal ratio plot showing the relative phosphorylation levels of DRP3A/3B (~130 kDa) at the G_1/S and G_2/M stages, respectively.

(c) Signal ratio plot showing the relative phosphorylation levels of DRP3A/3B (~130 kDa) at G_2/M stages (untreated) or treated with olomoucine (olo) or staurosporine (sta), respectively.

(d) Mitochondrial morphology at different stages with olomoucine treatment (bar = 20 μm). BY-2 suspension cells expressing mitochondrial localized N-δ-ATPase-Kaede were imaged at the cortex (i, iii, v, vii, ix, xi) and midplane (ii, iv, vi, viii, x, xii) regions by fluorescence microscopy (488 nm) and differential interference contrast (DIC) microscopy; the fluorescence and bright field images were merged. (i–vi) Cells were treated with DMSO and imaged at the indicated stages. (vii–xii) Cells were treated with olomoucine and imaged at the indicated stages.

(e) Quantitative analysis of mitochondrial size (in μm^2) in cells treated with DMSO or olomoucine. Differences in the mitochondrial mean area at interphase and at metaphase treated with DMSO vs. olomoucine are statistically significant by Student's t test (*p <0.01). Nevertheless, difference in mitochondrial mean area at cytokinesis treated with DMSO vs. olomoucine is not significant by Student's t test (p>0.05).

Figure 6. Fluorescence decay analysis of DRP3A/3B-Dendra2 degradation in BY-2 suspension cells during mitosis.

(a) DIC and fluorescence images of cells expressing DRP3A-Dendra2. Images were recorded before and after photoconversion both for cell at G_1/S stage and cell went through metaphase (bar = 50 μm).
(b-c) Normalized red and green fluorescence intensities of (a), DRP3A-Dendra2 in cells photoconverted at metaphase or at interphase, respectively.
(d-e) Normalized red and green fluorescence intensities due to DRP3B-Dendra2 in cells photoconverted at metaphase or at interphase, respectively.
(f-g) Normalized red and green fluorescence intensities due to Dendra2 (control) in cells photoconverted at metaphase or at interphase, respectively.

Figure 7. Western blot analysis of Myc-DRP3A/3B degradation in BY-2 suspension cells at interphase. Tubulin is shown as a loading control.
(a) Interphase Myc-DRP3A/3B-expressing cells after transfer to fresh medium were collected at the indicated times and analyzed for the amount of Myc-DRP3A/3B over time.
(b) Cells were treated with DMSO (control), CHX, or CHX/MG132 at interphase and analyzed for the amount of Myc-DRP3A over time.
(c) Cells were treated with DMSO (control), CHX, or CHX/MG132 at interphase and analyzed for the amount of Myc-DRP3B over time.

Figure 8. Schematic model for phosphorylation and ubiquitination of DRP3A/3B during mitosis and the subsequent induction of mitochondrial morphologic changes during the cell cycle. During the G_2/M stages, mitotic phosphorylation of AtDRP3A/3B ensures high activity. Subsequently, GTP hydrolysis and AtDRP3A/3B dephosphorylation induce mitochondrial fission. During the G_1/S stages, ubiquitination and partial protein degradation maintain AtDRP3A/3B activity at a relatively low level, allowing a dynamic balance between the fission and fusion processes.

Table 1

Putative interacting protein of Myc-DRP3A

Function	Protein Name	Accession No.	Peptides	Score
Structural constituent of cytoskeleton	α-tubulin	gi|11967906; [*Nicotiana tabacum*]	3	180
	TUBA2/4		3	94
	TUBB1		2	57
Ubiquitination	E3 SINA-like 2	At1g66620	1	30
	E3 ubiquitin-protein ligase	gi|7715601; gi|42567657 gi|10176735	1	29
	26S protease regulatory subunit homolog A/B	GN=RPT5A/RPT5B	2/2	36
	AAA-type ATPase family protein	AT5G16930.1	1	27
Protein kinase	protein kinase family protein	gi|76869647|gb|DV160639.1|DV160639	2	31
	MAP kinase kinase	gi|32873530|gb|BP130645.1|BP1306 [*Nicotiana tabacum*]	1	28

Putative interacting protein of Myc-DRP3B

Function	Protein Name	Accession No.	Peptides	Score
Structural constituent of cytoskeleton	α-tubulin	CONTIG5175; CONTIG3008 [*Nicotiana tabacum*]	2	49
	TUBB8		7	273
	TUBB1/5/6		2	116
	TUB7;	AT2G29550.1; AT5G62690.1	4	137
	TUA4;	AT1G04820.1; AT1G50010.1	2	84
	ACT8	AT1G49240.1; AT3G18780.1/2	2	71
	TUA3	AT5G19770.1; AT5G19780.1	1	80
Ubiquitination	Probable E3, ARI3	ARI3	2	25
	AAA-ATPase subunit RPT2a	CONTIG2316	2	105
	26S proteasome subunit RPN6a	CONTIG5120	2	80

	26S proteasome regulatory subunit-like	CONTIG4095	2	66
Phosphatase/ Protein kinase	PP2A	gi|1568511; [*Nicotiana tabacum*]	2	73
	MAP kinase kinase	gi|32873530|gb|BP130645.1|BP130645; [*Nicotiana tabacum*]	2	39
	Serine/threonine-protein phosphatase 2A	PP2AA2 gi|543715	3	45
	protein kinase family protein	AT1G67580.1; AT1G67580.2	1	31
	Ribose-phosphate pyrophosphokinase 3	CONTIG394	2	83

Table 1 Identification of putative Myc-AtDRP3A/3B-interacting proteins in BY-2 suspension cells by capLC-Q-TOF MS. Peptides, number of distinct peptides identified for the protein; No. of peptides, number of peptides identified in the MS analysis; Score, the score retrieved by the MASCOT program. With the exception of AtDRP3A and AtDRP3B, the identified proteins are listed according to score. A score of 25 or greater indicates an identification event of at least 95% confidence.