Investigations on Mitochondrial Pleomorphy and Interactions with the Endoplasmic Reticulum and Peroxisomes

by

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Guelph, Ontario, Canada

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ABSTRACT

INVESTIGATIONS ON MITOCHONDRIA PLEOMORPHY AND INTERACTIONS WITH THE ENDOPLASMIC RETICULUM AND PEROXISOMES

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University of Guelph, 2015

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Mitochondria are pleomorphic organelles capable of constant fusion and fission. Live fluorescent microscopy was employed to investigate mitochondrial pleomorphy during fluctuations in light, sugar and O₂. Light and sugar are shown to induce fission whereas a green-to-red photo-convertible mEos fluorescent protein targeted to mitochondria (mitoEos) reveals that hypoxia induces fusion, leading to giant mitochondria. The endoplasmic reticulum (ER) is shown to be a mediator of mitochondrial fission and acts as a mould for morphological transitions displayed by mitochondria. Simultaneous live-imaging also reveals sustained mitochondria-peroxisome interactions, which are apparent in anisotropy1, a cell wall mutant shown here to be light sensitive. Triple transgenic lines were created to differentially label mitochondria, peroxisomes and the ER and show that the ER cages the other organelles including chloroplasts during these sustained interactions. Together, simultaneous live-imaging of mitochondria, peroxisomes and the ER using double and triple Arabidopsis thaliana transgenics further illuminate organelle pleomorphy and interactivity.
ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ADP         adenosine diphosphate
AIM         Agrobacterium infiltration media
any1        anisotropy1
AOX         alternative oxidase
AsA-GSH     ascorbate-glutathione cycle
ATP         adenosine triphosphate
C           cytochrome c complex
Caf4p       CCR4p-associated factor 4
[Ca^{2+}]_m  matrix calcium
CAT         catalase
cDNA        complementary DNA
CesA        cellulose synthase
CLSM        confocal laser scanning microscopy
Complex I    NADH dehydrogenase
Complex II   succinate dehydrogenase
Complex III  cytochrome c reductase
Complex IV   cytochrome c oxidase
DAB         3,3-diaminobenzidine
DAL1/DAL2    Drosophila inhibitor of apoptosis-like protein 1/2
DLP         Dynamin-Like Protein
Dnm         Dynamin
DRP         Dynamin Related Protein
ELM1        Elongated Mitochondria1
ER           endoplasmic reticulum
ETC         electron transport chain
FAD         oxidized flavin adenine dinucleotide
FADH_{2}    reduced flavin adenine dinucleotide
FIS1        Fission1
FP          fluorescent protein
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>Fzo1</td>
<td>fuzzy onions homolog 1</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic DNA</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GOX</td>
<td>glycollate oxidase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine-5’-triphosphate</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HL</td>
<td>high light</td>
</tr>
<tr>
<td>HO’</td>
<td>hydroxyl radicals</td>
</tr>
<tr>
<td>IMM</td>
<td>inner mitochondrial membrane</td>
</tr>
<tr>
<td>IMS</td>
<td>intermembrane space</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>MAL</td>
<td>maltose</td>
</tr>
<tr>
<td>MAPL RING</td>
<td>MAPL-Really Interesting New Gene finger domain</td>
</tr>
<tr>
<td>MAPL</td>
<td>Mitochondria-Anchored Protein Ligase</td>
</tr>
<tr>
<td>MCS</td>
<td>membrane contact site</td>
</tr>
<tr>
<td>Mdv1</td>
<td>Mitochondrial Division Protein 1</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>Mfn1/Mfn2</td>
<td>Mitofusin1/2</td>
</tr>
<tr>
<td>Mgm1</td>
<td>Mitochondrial Genome Maintenance</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog medium</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>oxidized nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NOS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>$^1$O$_2$</td>
<td>singlet oxygen</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>superoxide anion radicals</td>
</tr>
<tr>
<td>OAA</td>
<td>oxaloacetate</td>
</tr>
<tr>
<td>OMM</td>
<td>outer mitochondrial membrane</td>
</tr>
<tr>
<td>Opa1</td>
<td>Optic Atrophy1</td>
</tr>
<tr>
<td>p35S</td>
<td>Cauliflower Mosaic Virus 35S promoter</td>
</tr>
<tr>
<td>PYR</td>
<td>pyruvate</td>
</tr>
<tr>
<td>PSI</td>
<td>photosystem I</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PSII</td>
<td>photosystem II</td>
</tr>
<tr>
<td>Q_{10}</td>
<td>coenzyme Q_{10}</td>
</tr>
<tr>
<td>r</td>
<td>radius of curvature</td>
</tr>
<tr>
<td>RFP</td>
<td>Red Fluorescent Protein</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>Rubisco</td>
<td>ribulose-1,5 bisphosphate carboxylase-oxygenase</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TPR</td>
<td>tetratricopeptide repeat</td>
</tr>
<tr>
<td>tr</td>
<td>trichomes</td>
</tr>
<tr>
<td>Ugo1</td>
<td>Ugo (Japanese for fusion)</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>YEB</td>
<td>Yeast Extract Broth</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
</tr>
<tr>
<td>ΔΨ_{m}</td>
<td>membrane potential</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 MITOCHONDRIAL PLEOMORPHY AND FUNCTION

*Sui generis*: in a class of its own; unique. This phrase was used to describe mitochondria just over a century ago by Cavers (1914) because of the plethora of appearances they can display. Mitochondria are typically long and filamentous in fibroblast cells, whereas hepatocyte cells have a predominance of spherical or ovoid mitochondria (Youle and van der Bliek 2012). In yeast cells, they usually form a network consisting of 1-10 mitochondria (Hoffman and Avers 1973; Stevens 1981; Nunnari et al. 1997). In most plant cells however, they are typically small, spherical or rod shaped (Logan 2010), although the first published image of mitochondria in plants showed them to be elongated ‘fila’ (Meves 1904). This organelle can also exhibit pleomorphy within one particular cell under observation; the same mitochondrion could be spherical and stretch into a small rod one moment or become elongated the next (Cavers 1914; Logan and Leaver 2000). Flemming (1882) first called this truly dynamic organelle ‘fila’ as they appeared thread-like, but Altman (1890) described them as granules that resembled bacteria, calling them ‘plastosomes’. Since then, they have been called bioblasts, chondriomiten, chondriosomes, chromidia, plastokonts etc., all describing a particular form of the same
organelle. For this reason, Benda (1897) justly assigned the name ‘mitochondria’ (Gr. mitos = thread, chondrion = granule), in acceptance that this organelle was capable of a wide range of appearances. Although many factors, stresses (Table 1.1) and cell types, functions, and requirements seem to dictate the appearance this organelle assumes, there is a clear link between the energy and metabolic status of a cell and the pleomorphy that mitochondria exhibit. This is especially apparent at the ultrastructural level.

**Table 1.1 Factors influencing mitochondrial morphology**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Influence on Shape</th>
<th>References</th>
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<tbody>
<tr>
<td>Glutamate</td>
<td>Increase roundness</td>
<td>Rintoul et al. 2003</td>
</tr>
<tr>
<td>Calcium</td>
<td>Increase roundness</td>
<td>Boustany et al. 2002; Stolz and Bereiter-Hahn 1987</td>
</tr>
<tr>
<td>Glucose</td>
<td>Fragmentation, Increase roundness</td>
<td>Hackenbrock 1966</td>
</tr>
<tr>
<td>$\text{H}_2\text{O}_2$</td>
<td>Increase roundness</td>
<td>Yoshinaga et al. 2005</td>
</tr>
<tr>
<td>NO</td>
<td>Fragmentation</td>
<td>Barsoum et al. 2006</td>
</tr>
<tr>
<td>$\text{CO}_2$</td>
<td>Fragmentation</td>
<td>Lewis and Lewis 1914</td>
</tr>
<tr>
<td>Riboflavin deficiency</td>
<td>Enlargement</td>
<td>Tandler et al. 1969; Bereiter-Hahn and Voth 1994</td>
</tr>
<tr>
<td>ATP</td>
<td>Orthodox ultrastructure</td>
<td>Novikoff and Holtzman 1976; Bereiter-Hahn and Voth 1994</td>
</tr>
<tr>
<td>ADP</td>
<td>Condensed ultrastructure</td>
<td>Novikoff and Holtzman 1976; Bereiter-Hahn and Voth 1994</td>
</tr>
</tbody>
</table>

1.1.1 Mitochondrial ultrastructure: Orthodox and condensed states

Mitochondria can exist in two general conformational states: condensed and orthodox (Fig. 1.1B). The balance between these states is influenced by changing environmental conditions such as fluctuations in oxygen, carbon and metabolite availability (Hackenbrock 1968; Hackenbrock 1971; Novikoff and Holtzman 1976; Bereiter-Hahn and Vöth 1994; Logan and Leaver 2000). In the condensed state, the intracristal spaces are dilated which condenses the matrix. This state is seen when adenosine diphosphate (ADP) levels are high and the respiratory pathways are active (Fig. 1.2). But when adenosine triphosphate (ATP) levels increase, respiration and ADP phosphorylation are signalled to stop. In this resting state, the intracristal
spaces become narrow again, allowing the matrix to expand (Novikoff and Holtzman 1976; Bereiter-Hahn and Vöth 1994). This is the orthodox state. In cells with high energy functions, such as muscle cells, cristae are closely packed and more abundant (Novikoff and Holtzman 1976), most likely because of the greater requirement for active respiration (Fig. 1.2). The rearrangement of the inner mitochondrial membrane (IMM) is seen in response to fluctuating ATP/ADP levels because this is where the electron transport chain (ETC) is located and where oxidative phosphorylation takes place in mitochondria.

1.1.2 The electron transport chain and ATP synthesis

Cellular respiration consists of several pathways whereby nutrients are metabolized to produce ATP. During glycolysis, glucose is catabolized to produce pyruvate, reduced nicotinamide adenine dinucleotide (NADH) and ATP. Pyruvate is further metabolized by the tricarboxylic acid (TCA) cycle. This cycle also oxidizes NAD\(^+\) and FADH to NADH and FADH\(_2\), respectively. Available NADH and FADH\(_2\) enter the oxidative phosphorylation pathway where they are reduced by the ETC (Fig. 1.1C). The electrons released are transported down a chain of complexes in the IMM until they are finally accepted by O\(_2\). As the electrons are being transported, the complexes also pump H\(^+\) from the matrix into the intermembrane space (IMS). This creates an electrochemical gradient where the membrane potential (\(\Delta \Psi_m\)) is negative on the matrix side but positive on the IMS side (Hoefnagel et al. 1998; Brookes et al. 2004). This gradient is used by ATP synthase to phosphorylate ADP to ATP. The more energy utilized by the cells, the more rapidly ATP is converted back to ADP. This increase in ADP level subsequently permits a cycle whereby the rate of electron transport, oxygen consumption and ATP production increases again (Novikoff and Holtzman 1976). However, as this oxidative phosphorylation process becomes more frequent, higher levels of reactive oxygen species (ROS) are produced by the mitochondrial complexes. This leads to damage of lipids, proteins, and mtDNA.
Figure 1.1 Mitochondria structure and inner membrane composition. (A) Mitochondria are semi-autonomous organelles with two membranes. The IMM encapsulates the matrix. It is enclosed by the OMM, which creates a space between them called the IMS. The folds created by the IMM are cristae. (B) When the intracristal spaces become dilated, thereby condensing the matrix, a mitochondrion is said to be in the condensed state. If the intracristal spaces are narrow, the matrix expands. This is the orthodox state. (C) The IMM is home to the ETC. NADH and FADH$_2$ are reduced by complex I and II, respectively. The $2e^-$ released from each reaction are transported from complex to complex. In the process, an electrochemical gradient is created as H$^+$ are pumped out of the matrix by complexes I, III and IV. This gradient is used to drive the production of ATP by ATP synthase. Here, ADP is phosphorylated to ATP. The condensed state is electron dense and is seen when ADP levels are high. Here, respiration is taking place to phosphorylate ADP to ATP. The matrix is electron dense as the gradient is being created to drive ATP synthesis. When ADP levels are low however, ATP levels are high, respiration and phosphorylation cease and the orthodox state is assumed. Hence, the condensed state can be associated with active respiration and the orthodox state with inactive respiration and high ATP levels. OMM = outer mitochondrial membrane; IMM = inner mitochondrial membrane; IMS = intermembrane space; ETC = electron transport chain; complex I = NADH dehydrogenase; complex II = succinate dehydrogenase; complex III = cytochrome c reductase; complex IV = cytochrome c oxidase; Q$_{10}$ = coenzyme Q$_{10}$; C = cytochrome c complex; dotted arrows = path of $2e^-$ released from the reduction of each NADH and FADH$_2$ before being accepted by O$_2$; dashed arrows = transport of H$^+$. Compiled from Hackenbrock 1968; Hackenbrock et al. 1971 (source of TEMs); Bereiter-Hahn and Vöth 1994; Hoefnagel et al. 1998; Brookes et al. 2004.
1.2 TRIGGERS AND CONSEQUENCES OF MITOCHONDRIAL FUSION AND FISSION

The pleomorphic nature of mitochondria is permitted by constant membrane fusion and fission. Fusion and fission serve different purposes but the balance between both events ultimately leads to cellular homeostasis. Fusion of the OMM and IMM of two or more mitochondria allows complementation of damage caused by oxidative stress (Fig. 1.3). By forming a continuous network of membranes, contents of the IMS and of the matrix of each
mitochondrion become mixed. As a result, solutes, metabolites, proteins and electrochemical
gradients are shared (Nakada et al. 2001; Skulachev 2001; Arimura et al. 2004; Chen et al. 2005;
Twig et al. 2006; Benard et al. 2009). This is an important process because it allows the effects
of damaged constituents to be diluted, thereby permitting recovery and continued functionality
(Chan 2006; Twig et al. 2008). Subsequent fission allows each mitochondrion to disperse within
the cell where the process can occur again if necessary. Thus, it comes as no surprise that
fragmented ‘plastosomes’ are found in cells of high energy demand such as muscle and near
flagella (Bereiter-Hahn and Vöth 1994). This is because the damage caused by higher rates of
respiration is more severe and this process of complementation must occur more rapidly.

1.3 ROS PRODUCTION IN PLANTS

A key signal responsible for stimulating oxidative phosphorylation in mitochondria is
calcium (Ca$^{2+}$). If the concentration of matrix Ca$^{2+}$ ([Ca$^{2+}$]$_{m}$) becomes elevated, oxidative
phosphorylation and ATP output are also upregulated (Brookes et al. 2004). [Ca$^{2+}$]$_{m}$ is regulated
by several mechanisms such as the Ca$^{2+}$ uniporter (UP), ryanodine receptor (RyR), the
permeability transition pore (PTP) and $\Delta$$\Psi$$_{m}$. Under normal conditions, Ca$^{2+}$ is pumped into the
mitochondria through the PTP and signals the TCA cycle. This feeds electrons into the ETC
(Fig. 1.4). Under certain stress conditions that lead to increased [Ca$^{2+}$]$_{m}$, oxidative
phosphorylation occurs more rapidly causing an even greater increase in ATP synthesis. Such a
scenario is seen during a cell death response. This is because ROS, a signal for cell death and
apoptosis, is overproduced (Foyer and Noctor 2003; Apel and Hirt 2004; Brookes et al. 2004;

ROS are reactive derivatives of oxygen due to their partially reduced or activated states. It
includes singlet oxygen ($^{1}$O$_{2}$), superoxide anion (O$_{2}^{-}$), hydroxyl radical (OH’) and hydrogen
peroxide (H$_{2}$O$_{2}$) (Klotz 2002; Apel and Hirt 2004). ROS are involved in signaling and activating
transcription factors, but can be detrimental at high levels. Due to the highly reactive nature of
ROS, they can cause severe oxidative damage to proteins, lipids and DNA (Apel and Hirt 2004;
Bhattacharjee 2011), which is why the complexity and sheer abundance of the mechanisms
involved in ROS regulation (Table 1.2) is not surprising; the detoxification of ROS is crucial for maintaining cell survival.

A fundamental difference between animals and plants is a plant’s ability to carry out photosynthesis through chloroplasts. Photosystem I (PSI) and PSII of thylakoid membranes within chloroplasts are considered the primary sites of ROS production. Light drives the photosynthetic process in which O$_2$ is continuously produced by photosynthetic electron transport in chloroplasts (Fig. 1.4). The photo-reduction of O$_2$ by PSI then yields O$_2^{•−}$ (the Mehler reaction; Mehler 1951; Asada 1999; 2006). This is because PSI electron carriers have

![Diagram of mitochondrial fusion and fission](image)

**Figure 1.3 Triggers and consequences of mitochondrial fusion and fission.** Fusion and fission activity constantly fluctuates as a cell attempts to achieve homeostasis. Under low energy, such as during metabolic starvation, ATP levels are depleted and $\Delta\Psi_m$ becomes depolarized. Mitochondria undergo fusion to help repolarize the $\Delta\Psi_m$ and maximize oxidative phosphorylation. The mitochondria then undergo fission and disperse. This dispersal permits the transfer of energy throughout the cell. When mitochondrial constituents such as proteins, lipids and mtDNA become damaged (i.e. by oxidative damage via the ETC), mitochondria also fuse. This allows the mixing of wild type and damaged constituents and complementation. The mitochondrion then undergoes fission and the resulting mitochondria disperse. However, when the damage is too much, the mitochondrion enters the autophagic pathway. Such a case is seen during ROS-induced apoptosis. Red = damaged mitochondrion; green = normal, wild type mitochondrion; yellow = fusion between damaged and normal mitochondria; $\Delta\Psi_m$ = membrane potential. Adapted from and based on Amchenkova et al. 1988; Bereiter-Hahn and Vöth 1994; Legros et al. 2002; Yu et al. 2006; Twig et al. 2008; Youle and van der Bliek 2012.
negative electrochemical potentials and therefore the electrons are leaked to \( O_2 \) (Bhattacharjee 2011). \( O_2^- \) can be further photoreduced to \( H_2O_2 \). When there is excess photochemical or light energy, the generation of \( O_2^- \) and \( H_2O_2 \) is heightened. \( H_2O_2 \) then actually diffuses out of the chloroplast membranes into the cytosol. It can also travel short distances into peroxisomes (Table 1.2; Foyer and Noctor 2003; Bhattacharjee 2011).

**Table 1.2 Common ROS and their removal**

<table>
<thead>
<tr>
<th>ROS</th>
<th>Removal Mechanism (Product)</th>
<th>Removal Mechanisms found in</th>
<th>Diffusion Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide (O(_2^-))</td>
<td>Superoxide dismutase (H(_2)O(_2))</td>
<td>C, Cyt, M, P</td>
<td>30nm</td>
</tr>
<tr>
<td>Hydrogen peroxide (H(_2)O(_2))</td>
<td>Catalase (H(_2)O) Peroxidase (H(_2)O) Ascorbate/glutathione cycle (H(_2)O) Glutathione peroxidase (H(_2)O)</td>
<td>M, P C, Cyt?, M, P C, Cyt, ER, M</td>
<td>1( \mu )m</td>
</tr>
<tr>
<td>Singlet oxygen (('O_2))</td>
<td>Carotenes and tocopherol (O(_2))</td>
<td>C</td>
<td>30nm</td>
</tr>
</tbody>
</table>

C = chloroplasts; Cyt = cytosol; M = mitochondria; P = peroxisomes; ER = endoplasmic reticulum. Adapted from Bhattacharjee 2011.

Peroxisomes, initially termed microbodies (Rhodin 1954), are organelles that play crucial roles in eukaryotic cells. They are involved in fatty acid \( \beta \)-oxidation and photorespiration (Beevers 1979). Through its resident antioxidant enzymes such as peroxidase, superoxide dismutase (SOD) and catalase, this organelle plays an important role in detoxifying aerobic cells of free radicals by aiding in the scavenging of ROS and reactive nitrogen species (NOS) (de Duve and Baudhuin 1966; Foyer and Noctor 2003; del Río et al. 2006; Bhattacharjee 2011). When \( H_2O_2 \) enters peroxisomes, it is reduced by catalase to \( H_2O \) (Foyer and Noctor 2003; Asada 2006). Another pathway in which chloroplasts and peroxisomes are linked is by the Calvin cycle. During the Calvin cycle in chloroplasts, glycolate is produced through an oxygenation reaction (Fig. 1.4). Glycolate is translocated from chloroplasts to peroxisomes where it is converted to glyoxylate by glycolate oxidase (GOX). In the process, \( H_2O_2 \) is generated again but is also reduced by catalase (Fig. 1.4; Foyer and Noctor 2003).

Mitochondria are also producers of ROS due to the ETC (Halliwell and Gutteridge 1999; Chen et al. 2003; Foyer and Noctor 2003; Apel and Hirt 2004; del Río et al. 2006; Bhattacharjee 2011). For example, \( O_2^- \) is produced during the very first steps of the ETC when NADH
dehydrogenase (complex I) and succinate dehydrogenase (complex II) reduce NADH and FADH$_2$, respectively. O$_2^-$ then goes to the mitochondrial matrix-antioxidant enzyme system, such as alternative oxidase (AOX) and catalase where it is inactivated (Chen et al. 2003; Foyer and Noctor 2003; Bhattacharjee 2011). Cytochrome c reductase (complex III) is another major producer of O$_2^-$ in mitochondria, however. Here, O$_2^-$ is released to the IMS rather than to the matrix. SOD is found in the IMS and converts O$_2^-$ to H$_2$O$_2$ (Foyer and Noctor 2003). At high concentrations, H$_2$O$_2$ can diffuse through the OMM into the cytosol as well (Chen et al. 2003). The H$_2$O$_2$ released can diffuse through the peroxisomal membrane where it can also be reduced to H$_2$O. These metabolic and biochemical pathways are not the only way peroxisomes and mitochondria are linked.

1.4 MITOCHONDRIA AND PEROXISOME PLEOMORPHY IN RESPONSE TO ROS

Imaging of peroxisomes in living cells has revealed them to be pleomorphic as well, with spherical, tubular and beaded forms being observed (Fig. 1.5A). It has also provided an appreciation of their dynamic nature and altered motility during ROS stress. Sinclair et al. (2009) used a copper chloride/ascorbate mixture to produce hydroxyl radicals. After 1-2min of exposure, peroxisomes, which are spherical under normal physiological conditions in plant cells, became immotile and elongated after 1min. Elongated peroxisomes then became beaded and resumed motility around 2h after the copper chloride/ascorbate mixture was washed off. The beaded state was followed by fission and then dispersal of the new peroxisome counterparts. The authors also demonstrated that UV irradiation, which induces ROS production, of single Arabidopsis thaliana (Arabidopsis) hypocotyl cells caused all peroxisomes to elongate after 90s. Neighbouring cells however, which received lower, peripheral exposure to UV, displayed spherical peroxisomes with thin projections known as peroxules.
Named for their morphological resemblance to plastid stromules, peroxules are thin, short-lived tubular projections from a main spherical to ovate peroxisome body (Scott et al. 2007; Sinclair et al. 2009; Delille et al. 2010; Barton et al. 2014). In contrast to completely elongated...
peroxisomes, it has never been shown that peroxules undergo fission. These elastic peroxules may therefore be a mere "stress threshold sensing mechanism" (Sinclair et al. 2009; Mathur et al. 2012). They may extend and retract in response to low fluctuations of ROS, but once levels exceed a certain threshold, peroxisomal elongation and fission may be signalled in order to increase peroxisomal numbers and dispersal throughout the stressed cell (Schrader et al. 1999; Scott et al. 2007; Sinclair et al. 2009; Delille et al. 2010; Mathur et al. 2012; Barton et al. 2014).

This morphological progression leading to fission is also exhibited by mitochondria (Fig. 1.5B), which become extensively fragmented in response to high ROS levels during mitophagy, apoptosis and cell death (Ingerman et al. 2005; Yoshinaga et al. 2005; Jendrach et al. 2008; Yu et al. 2006; 2008; Frank et al. 2012). Mitochondria have also been reported to have thin projections called terminal matrixules. Additionally, when a tubular mitochondrion divides, it becomes constricted causing it to appear as beads-on-a-string; medial matrixules were described as the string portion (Logan et al. 2004; Logan 2006). Although the exact roles of peroxules and matrixules are not fully understood, key proteins involved in the fission process of elongated peroxisomes and mitochondria are known. Furthermore, it has become very evident that apart from the biochemical and metabolic links and commonalities in morphological processions during fission, they also share common fission machineries.
Figure 1.5 Sequential events leading to peroxisome and mitochondria fission reveals morphological similarities in the division pathway. (A) Under low ROS levels, peroxules extend and retract rapidly from the main peroxisome body. Upon high levels of ROS, peroxisomes elongate. These elongated peroxisomes become beaded before finally undergoing fission. (B) ROS also leads to mitochondria fission, whereby the same division factors, DRP3 and FIS1 constrict the membranes of the elongated form. Peroxisome division complied from compiled from the reports of Tabak et al., 2006; Titorenko and Mullen, 2006; Scott et al., 2007; Sinclair et al., 2009; Delille et al., 2010; Mathur et al., 2012; Barton et al., 2014. Mitochondria division complied from Ingerman et al., 2005; Yoshinaga et al., 2005; Jendrach et al., 2008; Yu et al., 2006, 2008; Frank et al., 2012.
1.5 MECHANISMS OF MITOCHONDRIAL FUSION AND FISSION

Several components of the fusion and fission machinery of mitochondria in yeast and mammals have been described (Table 1.3). No plant orthologues for fusion have been identified to date, but three have for fission: FISSION1 (FIS1), DYNAMIN RELATED PROTEIN3 (DRP3) and ELONGATED MITOCHONDRIA1 (ELM1). The former two are also involved in peroxisomal fission and the latter is thought to be a functional homologue of the yeast Mdv1/Caf4 fission proteins.

Table 1.3 Proteins involved in mitochondrial fusion and fission

<table>
<thead>
<tr>
<th>Fusion/Fission</th>
<th>Component (Yeast/Mammals/Plants)</th>
<th>Location</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fission</td>
<td>DNM1/ DRP1 or DLP1/ DRP3A &amp; DRP3B</td>
<td>Cytosol &amp; OMM</td>
<td>Fission of OMM</td>
</tr>
<tr>
<td></td>
<td>FIS1p/ hFIS1/ FIS1</td>
<td>OMM</td>
<td>Receptor for DNM1/ DRP1 or DLP1/ DRP3A &amp; DRP3B, respectively</td>
</tr>
<tr>
<td></td>
<td>MDV1/ –/ ELM1</td>
<td>Cytosol &amp; OMM</td>
<td>Mediator between the two above fission proteins</td>
</tr>
<tr>
<td>Fusion</td>
<td>CAF4p/ –/ –</td>
<td>Cytosol &amp; OMM</td>
<td>MDV1 homolog; redundant function</td>
</tr>
<tr>
<td></td>
<td>FZO1/ MFN1 &amp; MFN2/ –</td>
<td>OMM</td>
<td>Fusion of OMM</td>
</tr>
<tr>
<td></td>
<td>UGO1/ –/ –</td>
<td>OMM</td>
<td>Coordinates OMM and IMM fusion</td>
</tr>
<tr>
<td></td>
<td>MGM1/ OPA1/ –</td>
<td>IMM &amp; IMS</td>
<td>IMM fusion</td>
</tr>
</tbody>
</table>

DRP3A and DRP3B are also referred to as ADL1 and ADL2, respectively.
FIS1 is also referred to as BIGYIN.
All three orthologues are also involved in peroxisomal fission.
No sequence similarity, but proposed function of EML1 is similar to MDV1 and CAF4p.
OMM = outer mitochondrial membrane; IMM = inner mitochondrial membrane; IMS = intermembrane space. Adapted and compiled from Arimura et al. 2008; Westermann 2008.

Arabidopsis has two DRP3 homologs, DRP3A and DRP3B, which have partially redundant functions and are involved in both mitochondria and peroxisome fission (Arimura and Tsutsumi 2002; Arimura et al. 2004; Logan et al. 2004; Mano et al. 2004; Zhang and Hu 2009). DRPs are GTPases that are recruited to mitochondria and peroxisome membranes by FIS1, form spirals around membranes thereby tubulating and constricting them, and mechanochemically...
mediate the fission and fusion of those membranes (Yoon et al. 2001; 2003; Kobayashi et al. 2007). Mitochondria and peroxisomes in the *drp3a/apml* mutant have an aberrant morphology where they appear greatly elongated (Mano et al. 2004), which suggests that the actual fission of the organelles, not the tubulation, is affected.

FIS1 has two homologues in plants as well: FIS1A and FIS1B (Scott et al. 2006; Zhang and Hu 2008). Both have a C-terminal transmembrane domain that anchors them to the OMM and peroxisome membrane and a C-terminal tail that extends into the matrix (Yoon et al. 2003; Scott et al. 2006). The N-terminal region is exposed to the cytoplasm and has a tetratricopeptide repeat (TPR)-like binding domain that can interact with Dlp1 (animal DRP3 homologue) in the case of hFis1 and supposedly DRP3 in the case of plant FIS1 (Yu et al. 2005). In *fis1p/hfis1* mutants (yeast and mammalian homologs, respectively), mitochondria form a net-like sheet, but mitochondria in the plant *fis1* mutant decrease in number and increase in area (Scott et al. 2006). Overexpression of both FIS1A and FIS1B on the other hand, results in an increase in the number of mitochondria and peroxisomes (Zhang and Hu 2008). These phenotypes can be explained by linking FIS1 to a role in fission; knockouts of FIS1 mean DRP3 cannot bind to it to constrict and facilitate the scission of mitochondria and peroxisomes, but overexpression makes it more available, leading to increased fission.

The elongated mitochondrial phenotype of *drp3a/apml* is also seen in *elm1* mutants. ELM1 is thought to be a functional homolog of the mitochondrial fission proteins Mdv1 and Caf4p in yeast. The mutants not only have elongated mitochondria, but they form extensive branched networks, resembling a net-like sheet similar in appearance to the polygon network of the endoplasmic reticulum (ER; Arimura et al. 2004; Friedman and Voeltz 2011). For these reasons, ELM1 is proposed to be a mediator protein between FIS1 and DRP3 for mitochondrial fission, but not peroxisomal fission as the peroxisome morphology in *elm1* is unaffected (Arimura et al. 2008).

It is interesting that mitochondria in *elm1* form networks that resemble the ER network because Friedman et al. (2011) showed that in yeast and mammalian COS-7 cells, the ER is involved in the fission of mitochondria as well. The authors used three-dimensional reconstruction to show that ER tubules constrict mitochondria at potential fission sites. The
helices formed by Dnm1 and Drp1 are smaller in diameter than that of a non-constricted mitochondrion. The authors proposed that because of this, the ER wraps around mitochondria to constrict them enough for Dnm1/Drp1 to fit around the tubular structure. This has not been shown in plants to date, but given that the functions of FIS1 are DRP3 conserved, this interaction between the ER and mitochondria may occur in plant cells as well and should be investigated.

1.6 LIVE IMAGING OF ORGANELLES

Small organic dyes can be used for antibody targeting of specific intracellular proteins, however utilization of these requires fixation, deeming live-imaging impossible (Giepmans et al. 2006). There are dyes that can penetrate the living cell to visualize particular components, such as MitoTracker dyes that stain for mitochondria (Invitrogen/Molecular Probes; Chazotte 2011). However, these dyes are often toxic, therefore there is only a short period of time to conduct live-imaging observations (Mathur 2007). But since the cloning of the Green Fluorescent Protein (GFP) from the jellyfish Aequorea victoria (Prasher et al. 1992), this is no longer a major barrier, at least not for studying Arabidopsis. This is because the fluorescent protein (FP) can be fused to a specific nucleotide sequence and incorporated into the Arabidopsis genome for stable transgene expression (Giepmans et al. 2006; Haseloff and Siemering 2006; Mathur 2007; 2010). For example, GFP can be fused to an ER membrane peptide sequence, calnexin (CX; Fig. 1.6). The cells produce this GFP-CX fusion normally and the result is a fluorescently labelled ER still capable of movement and trafficking. There is now a wide spectrum of FP available and because a FP can be fused to a specific nucleotide sequence and therefore targeted to a particular organelle, more than one FP-fusion can be expressed simultaneously. As a result, a key attribute to cell dynamics, organelle interactions, can be investigated using live fluorescent microscopy.
1.7 OBJECTIVES

A living cell is quite a dynamic system. This is attributed to the constant movement of organelles and trafficking of constituents between the organelles such as metabolites and signalling compounds like ROS. Because of this, even though organelles are membrane bound entities, the processes occurring within them are by no means isolated from the rest of the cell – organelles are truly interactive. While moving throughout the cell and interacting with one another, they display morphological rearrangements. Some of these morphological transitions have been associated with cell stress and fluctuating inter- and intracellular environmental conditions.

The main objectives of this thesis are to:

1) identify conditions to reproducibly induce morphological transitions of mitochondria and peroxisomes to better understand how pleomorphy can be an indicator of cell stress;
2) better understand the mechanisms behind their pleomorphy; and
3) better understand organelle interactivity during light stress.

Double and triple Arabidopsis transgenics will be created and used to simultaneously visualize fluorescently labelled mitochondria, peroxisomes and the ER using live imaging. The photosynthetic impacts of light stress such as fluctuations in cytosolic sugar content, \( \text{O}_2 \) depletion and ROS production, are of key interest.

1.8 HYPOTHESES

The main hypotheses that are investigated in this thesis are:

1) Mitochondria in plant cells will exhibit fragmentation in response to high cytosolic sugar content, just as in mammalian cells;
2) Mitochondria in dark grown plants will be elongated, morphologically resembling those of animal cells. These elongated mitochondria will fragment in response to light;
3) The endoplasmic reticulum will mediate mitochondrial fragmentation and pleomorphy in plant cells;
4) The interactivity between fragmented mitochondria and peroxisomes will increase in response to high light and ROS stress; and
5) Peroxules will facilitate mitochondria-peroxisome interactions.
CHAPTER 2

MATERIALS AND METHODS

2.1 CLONING OF FLUORESCENT PROTEIN-FUSION CONSTRUCTS

RNA was extracted from Col-0 leaf tissue or whole seedlings with the EZ-10 Spin Column Plant RNA Mini-Prep Kit (BioBasic Inc.) and used to prepare complementary DNA (cDNA) using the Thermo Scientific Revertaid H Minus First Strand cDNA Synthesis Kit (Fermentas). This was then used to PCR amplify the coding sequences (CDS) for each gene of interest. Promoters were PCR amplified from Col-0 genomic DNA (gDNA). Primers flanked the sequence upstream of the CDS of the gene of interest, including the 5'-UTR, up to 1kb without including the sequence of another gene. Primer sequences and restriction enzyme sites
introduced for subsequent clonings are available in Appendix III (Table III-1).

Once purified [EZ-10 Spin Column Plasmid DNA Kit (BioBasic Inc.)], all amplified PCR products were ligated into a base vector, pGEM®-T Easy vector (Promega), and transformed into *Escherichia coli* DH5α competent cells. Blue-white colony screening for successfully ligated products was carried out by plating transformants on Luria-Bertani (LB) medium (Bertani 1951) with ampicillin and X-galactosidase (Promega). White colonies which contained the recombinant DNA were selected and used to inoculate overnight LB + ampicillin cultures. DNA was purified and screened by digestion using the restriction enzymes introduced by the forward and reverse primers during PCR amplification. The CDS and promoters were then digested from the pGEM®-T Easy vectors and placed into a pCAMBIA 1300 binary vector already containing a fluorescent protein (FP) of interest (Fig. 2.1). The cloning strategies for most FP-fusion constructs were similar, but an example is described here.

**Figure 2.1 Core vectors used for clonings.** The CDS (blue arrows) were replaced with the CDS of a gene of interest using the restriction enzymes flanking the C- and N-terminus. The p35S (CaMV35S) was replaced with the native promoter sequence (~1kb upstream of the respective CDS including the 5'UTR). Vector 943 was created by inserting the RFP sequence (Ching et al. 2012) into the previously available vector 620 in place of the mEosFP sequence in the BamHI/SacI position. The antibiotic resistance for bacterial/plant selection are indicated. Vector 648 contained a pS0022 backbone which lacks a plant selection marker required for subsequent transgenic plant screening and was therefore used only as an intermediate vector. Vectors 620 and 943 have a pCAMBIA 1300 backbone which has a hygromycin resistant gene marker. This was therefore used as a binary vector.
pfis1a-YFP-FIS1A (Ruberti 2014) was used to investigate the localization of FIS1A to peroxisomes, mitochondria and chloroplasts. Also created was a mEosFP targeted FIS1A probe driven by the constitutive Cauliflower Mosaic Virus 35S (CaMV35S) promoter (referred to as p35S from here on). This was generated because the green-to-red photo-convertibility of mEosFP may give further insight into the distribution and possible translocation of FIS1A between the membranes of the three organelles.

The CDS of FIS1A (AT3G57090.1) was PCR amplified to introduce 5’-NaeI and 3’-SpeI restriction enzyme sites using the following primers: forward 5’-ATATGCCGGCATGGATGCTAAGATCGGACA; and reverse 3’-GCGC ACTAGTTCAATTCTTGCGAGACATCG. This was then ligated into a pGEM®-T Easy vector and screened as described above. The fis1a gene was then ligated into an existing pS0022 vector in which the green-to-red photo-convertible mEosFP was present in the XhoI and NaeI position. The mEosFP-FIS1A fusion was excised as XbaI (upstream of the XhoI site in pS0022) and SpeI and then ligated into an existing pCAMBIA 1300 binary vector. In the binary vector, p35S was placed into the HindIII and XbaI position of pCAMBIA 1300 and a nos terminator (Tnos) in the SacI and EcoRI position. The p35S-mEosFP-FIS1A construct was used to transform electro-competent Agrobacterium (GV301) cells according to Weigel and Glazebrook (2006), which were plated on Yeast Extract Medium (YEB) medium with rifampicin, gentamycin and kanamycin. Plates were grown to confluence for 2 days at 28°C. mEosFP-FIS1A driven by the native promoter was also created by replacing the p35S with pfis1a.

All binary constructs were tested for FP expression using a Nicotiana bentamiana (tobacco) co-infiltration method. Small streaks the size of a pipette tip from the Agrobacterium culture plates were resuspended in Agrobacterium infiltration media (AIM) [50mL of ddH2O, 7.5μL of acetosyringone, 0.5mL of 0.5M MgCl and 0.5mL of 2-(N-morpholino)ethanesulfonic acid (MES)] for 2h at room temperature. The suspension was then diluted if necessary with AIM to an optical density of 0.8. This was done for the construct of interest as well as a control construct for which the localization was known, preferably one labeling an organelle in which the protein of interest was thought to localize [ex. p35S-mEosFP-FIS1A was co-infiltrated with mito-GFP [GFP-fused to the N-terminal pre-sequence of the mitochondrial β-ATPase subunit (Logan and Leaver 2000)]. Using a 2mL syringe, equal amounts of each suspension were
injected through the abaxial side of ~1 month old tobacco leaves. Leaves were checked for fluorescence 2-3 days later using a Leica epi-fluorescent microscope or confocal laser scanning microscopy (CLSM).

2.2 CREATING TRANSGENIC ARABIDOPSIS LINES

*A. tumefaciens* GV3101 was used to introduce the FP-fusion constructs into Arabidopsis (Columbia). This strain has a rifampicin resistance gene in its genome and a tumor inducing (Ti) plasmid that carries a gentamycin resistance gene and virulence (*vir*) genes that are flanked by a T-DNA border that incorporates into the plant genome during infection. One reason the pCAMBIA 1300 backbone was used is that it also has this T-DNA border. 5mL YEB cultures of the *Agrobacterium* containing a binary FP-fusion construct incubated at 28°C overnight. The YEB contained rifampicin, gentamycin and kanamycin for selection. This was then used to create a 500mL overnight culture. The culture was centrifuged at 3,000rpm for 20min at 4°C and the pellets were re-suspended in a solution of 5% sucrose and 0.5% Silwet (~100mL). This was used to transform Arabidopsis plants that were just beginning to flower using the *A. tumefaciens* floral dip method (Clough and Bent 1998).

Arabidopsis ecotype Col-0 was transformed with all constructs created (Appendix II and III). Double and triple transgenics were created in the same manner. For example, mito-GFP (Logan and Leaver 2000) plants were transformed with pfis1a-mEosFP-FIS1A (double transgenic) and mito-GFP Yperoxi (YFP-PST1; Mathur et al. 2002) was transformed with RFP-ER (RER; triple transgenic; Sinclair et al. 2009).

2.2.1 SELECTION OF STABLE LINES

All lines created using the pCAMBIA 1300 backbone were selected by growing sterilized seeds on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) with hygromycin (12.5μg/mL). All plants that developed proper root systems that extended well into the medium and were able to produce their first leaves were screened for fluorescence using an epi- or confocal microscope. If positive for fluorescence, plants were planted in soil and grown for second generation seeds. In the case of double and triple transgenics which already had a transgene(s) and therefore already had the hygromycin resistance gene, seeds were plated on MS
without antibiotics. Lines were selected by screening all seedlings for all fluorescent proteins visually, rather than through genotyping. Doubly or triply positive plants were harvested for seeds. The second generation seeds were always plated on MS without antibiotic and selected for fluorescence again. 10-16 plants were harvested for seeds. These were considered stable and were used for subsequent experimentation. Double and triple transgenics were also created by crossing and were visually selected during the second and third generations.

2.3 GROWTH CONDITIONS

Sterilized seeds were plated on MS with Gamborg vitamins and 3% sucrose, Parafilm-sealed, stratified for 2-3 days, and grown for 7 days, unless stated otherwise. When light and dark comparisons were being made, plants were either grown in light (16h/8h light/dark cycle) or plates were wrapped with tinfoil immediately after vernalization for growth in the dark. Plates for both conditions were always kept near one another to exclude temperature differences. When sugar comparisons were being made, plants were either plated on MS with 3% or with no added sucrose. The MS medium in all cases contained 3g/L of Phytagel (Sigma-Aldrich).

2.4 COLORIMETRIC SUGAR QUANTIFICATION

The phenol-sulfuric acid colorimetric method for quantifying the total soluble sugar of plant tissue described by Buysse and Merckx (1993) was implemented to determine the relative levels of sugar of plants grown in the light and dark. Plants were grown in the light (155μmol m$^{-2}$s$^{-1}$) or dark for 7 days on MS medium with or without 3% sucrose. 50 plants were quickly and carefully harvested from each treatment either in light 1.5h into the light cycle or in a room in near darkness. The plants were immediately placed into pre-weighed 2mL microfuge tubes during harvest. The tissue was immediately frozen in liquid nitrogen and the tubes were weighed after. The tissue fresh-weight was calculated by subtracting the post-harvest weight from the pre-weighed weight of the tube.
2.4.1 Sample preparation

The tissue was ground using magnetic beads and a bead beater (Retsch MM301) for 2 min at a frequency of 30.0 1/s and placed on ice immediately. After removing the magnetic beads, 500μl of 80% ethanol was added to the tissue and vortexed to homogenize the tissue in solution. The tubes were centrifuged at 14,000rpm for 5 min at 4°C. The supernatant was transferred to a new tube on ice. This elution in ethanol was repeated two more times. After the final elution, tubes were centrifuged one more time to remove residual tissue that could hamper quantification.

2.4.2 Quantification

A stock of 100μg/mL of glucose in 80% ethanol was diluted to 10 to 90μg/mL. These were used to create a standard line. 500μL of the standard or sugar elution was added to a cuvette. To each cuvette, 500μL of 28% phenol (w/w) was added. 2.5mL of concentrated sulphuric acid was added directly to the liquid surface in a steady stream which helped with mixing. After 15 min, the absorbances were read at 490nm. Note that the blank was made the same way, except 80% ethanol was used as the sample.

2.5 ASSESSING LIGHT-INDUCED STRESS

2.5.1 Induction of High Light Stress

Plants were all grown under intermediate light (50-164μmolm$^{-2}$s$^{-1}$). Observations were made on the seventh day of growth, at least 1.5 h into the light cycle for the day of experimentation. High light (HL) (850 ± 50μmolm$^{-2}$s$^{-1}$) was only used for shorter treatments (1-5 min) to observe the more rapid responses to light stress. Observations were taken immediately after the light treatments. This was possible because the microscope used had a bright field light which allowed plants to be irradiated directly on the microscope stage so that imaging could commence immediately after irradiation. Time-lapses were at least 2 min long. The time taken to scan the entire field of view for each frame of the time-lapse was 3.93 s.
2.5.2 3,3-diaminobenzidine Staining

To compare light-induced production of H$_2$O$_2$ in Col-0 and the *anisotrophy1* (*any1*) mutant (Fujita et al. 2013), plants were grown in the light (164μmolm$^{-2}$s$^{-1}$) for 8 days, transferred to the dark for 24h and then exposed to light (164μmolm$^{-2}$s$^{-1}$) for 30min, 1h or 2h. To look at the effects of light intensity, seedlings were grown in the dark for 6 days, transferred to low light (30μmolm$^{-2}$s$^{-1}$) for 2 days and then subjected to 0, 100, 200 or 300μmolm$^{-2}$s$^{-1}$ of light for 24h. Cotyledon and hypocotyl tissue were submerged in a solution of 3,3-diaminobenzidine (DAB) (SIGMAFAST™ DAB with Metal Enhancer, Sigma-Aldrich) or distilled water and left under vacuum (-50KPa) for 4h. The tissue was cleared with ethanol by washing the samples with 100% ethanol, incubating them in 85% ethanol + 15% methanol overnight, and rinsing them in 70% ethanol and then distilled water. The samples were mounted in 50% glycerol and sealed with nail polish. All images were acquired at the same light intensity and microscope settings to permit direct comparisons between treatments. The DAB stain intensity was measured as the average inverse grey value using ImageJ (http://imagej.nih.gov/ij/) which was subtracted from the background (the average inverse grey values of distilled water treated seedlings). The staining intensity was representative of the amount of H$_2$O$_2$ produced during the relative light intensity treatments.

2.5.3 H$_2$O$_2$ Responsive Fluorescent Probe

Arabidopsis transgenics expressing cytosolic HyPer-GFP$^{11}$, hydrogen peroxide responsive probe (http://www.evrogen.com/products/HyPer/HyPer.shtml, Evrogen, Russia; Belousov et al. 2006; Costa et al. 2010) were used to look at the real-time production and dissemination of H$_2$O$_2$ in response to a short light stimulus. Seedlings were given 1 to 5min of HL and the change in GFP fluorescence was imaged right away. The ImageJ RGB Profile Plot plugin was used to determine the changes in fluorescence intensity before and after HL treatment.

2.6 ASSESSING anisotrophy1 MUTANT SUSCEPTIBILITY TO STRESS

Scanning electron microscopy (SEM) and toluidine blue (TBO) staining was used to
assess the alteration of the cell wall in the any1 mutant. Leaf cross sections of 12 day old seedlings grown in low light (70µmolm⁻²s⁻¹) were stained with TBO to analyse the alterations in cell isotropy and cell-to-cell connectivity. SEM of whole leaf samples allowed the intercellular spaces to be visualized with a higher resolution or clarity.

The any1 mutant was used to investigate the effects of altered light penetration, given its reduced cell wall crystallinity (Fujita et al. 2013) and increased inter-cellular spaces. any1 Yperoxi mito-GFP double transgenic lines were also created to evaluate the mutant’s susceptibility to HL stress at the subcellular level relative to wild type plants to aid in understanding organelle interactions. This was done by elucidating if any1 was more susceptible to HL stress. The production of H₂O₂ and the duration of interactions between the organelles were compared in any1 and wild type plants.

2.6.1 Criteria for estimating interactions

mito-GFP Yperoxi and any1 Yperoxi mito-GFP double transgenics were used to visualize peroxisomes and mitochondria simultaneously. An encounter between the mitochondria and peroxisomes was considered an interaction if it lasted for ≥4 frames (15.72s) during a given time-lapse sequence. An encounter was considered coincidental if the organelles moved apart in

![Figure 2.2 Criteria for classifying a sustained interaction.](image)
less than 4 frames. The diffusion distance of \( \text{H}_2\text{O}_2 \) and \( \text{O}^- \) is \( \sim 1\mu\text{m} \) and 30nm, respectively (Table 1.2), so an encounter was declared if the fluorescence of the organelles were overlapping (Figure 2.2). A cut off of 4 frames was used because it was important to distinguish between coincidence (i.e. the organelles were merely moving in the same direction) and an actual sustained interaction (the organelles remained in contact long enough for the transfer of ions, metabolites, membrane constituents etc.).

2.7 MICROSCOPIC VISUALIZATION

2.7.1 Epi-fluorescent microscopy

The Nikon Eclipse 80i epi-fluorescent microscope was used for selecting transgenic lines. The filters available were Endow EGFP Longpass filter (41018; exciter HQ470/40X, dichroic Q495LP, emitter HQ500LP), TRITC (41002c; exciter HQ545/30x, dichroic Q570LP, emitter HQ620/60m) and DAPI (31000v2; exciter AT350/50x, dichroic 400LP, emitter 460/50m). There were two major limitations with this microscope: it could not be used to visualize organelle behaviour and punctate localization of FIS1 with high enough resolution; and only one filter could be used at a time, making simultaneous visualization of organelles near impossible. CLSM was therefore used for experimental observations.

2.7.2 Confocal Laser Scanning Microscopy

Fluorescent CLSM was used to conduct simultaneous, live imaging of organelle behaviour and interactions. A Leica DM6000 microscope with a Leica TCS-SP5 scanning head was used. A 488nm argon and 543nm helium-neon laser was used to excite GFP and RFP based probes, respectively. Settings used for visualizing multiple FPs at once are shown in Table 2.1. Time-lapse and 3D imaging was conducted using the Leica microscope software, Confocal LAS AF. The bright field of this microscope was use for HL treatments. The 40X water immersion lens (numerical aperture 0.80) was used for all imaging. Further zoom was achieved with the manual zoom of the Leica software. The format used was 1024 x 512 pixels meaning that the field of view using the 40X immersion lens with a zoom of 1.00 was 387.5 x 193.56\( \mu\text{m} \). The line average was set to 3, which meant that it took 3.945s to scan the entire field of view; each frame
in a time-lapse series was therefore separated by 3.945s.

Table 2.1 Emissions used to simultaneously visualize multiple FP

<table>
<thead>
<tr>
<th>FPs Visualized</th>
<th>Green</th>
<th>Red</th>
<th>Chlorophyll</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP, RFP, Chlorophyll</td>
<td>503-524nm</td>
<td>571-643nm</td>
<td>664-750nm</td>
</tr>
<tr>
<td>GFP, YFP, Chlorophyll</td>
<td>503-528nm</td>
<td>550-643nm</td>
<td>643-750nm</td>
</tr>
<tr>
<td>EosFP, Chlorophyll</td>
<td>500-536nm</td>
<td>580-653nm</td>
<td>653-754nm</td>
</tr>
<tr>
<td>GFP, MitoTracker Orange</td>
<td>500-535nm</td>
<td>555-622nm</td>
<td>634-781nm</td>
</tr>
</tbody>
</table>

2.8 STATISTICAL ANALYSIS

Unless stated otherwise, data was compiled from 4 cells per plant, 5 plants per treatment. For mitochondrial length analysis, 10 mitochondria per cell were randomly measured. For mitochondria-peroxisome morphological comparisons, a minimum of 6 of each organelle was analyzed, and all organelles in the field of view were measured for each image. All experiments consisted of at least 4 biological and 2 technical replications. When there was an unequal sample size per treatment, a two-tailed t-test was used to determine the significance of results. For equal sample sizes, a Tukey’s test of significance was used (Tukey 1949). Significance was predetermined as having a p-value < 0.05 (95% confidence interval).
CHAPTER 3

RESULTS

3.1 IDENTIFYING CONDITIONS TO INDUCE TRANSITIONS BETWEEN MITOCHONDRIAL MORPHOLOGIES

3.1.1 Mitochondria are predominantly small when grown with exogenous sugar

In hyperglycemic animal cells, elongated mitochondria fragment into small, spherical or ovate forms (Yu et al. 2006; 2008; Jhun et al. 2013). As mitochondria in plant cells are typically described as small, spherical to ovate in shape but can also be tubular (Meves 1904; Cavers 1914; Frey-Wyssling and Mühlethaler 1965; Logan and Leaver 2000), it was investigated whether higher levels of cytosolic sugar can lead to the fragmentation of mitochondria in plant cells as well. To do this, comparisons of mitochondria length in plants grown on MS medium with and without sucrose were made.

First however, to be certain that the differences in exogenous sugar between the treatments were reflected in the cytosolic sugar levels, the total soluble sugar levels were estimated using a phenol-sulphuric acid colorimetric method (Buysse and Merckx 1993). Indeed, plants grown on medium with sucrose had a higher level of total soluble sugar than plants grown without sucrose (Fig. 3.1H). Confocal imaging showed that plants grown with sucrose had a
greater proportion of mitochondria ≤0.85μm in length than plants grown without exogenous sugar (73% and 42%, respectively; Fig. 3.1A-D, G). Mitochondria were indeed small in response to exogenous sugar uptake. As the production of carbohydrates increases during the process of photosynthesis (Azcón-Bieto et al. 1983; Azcón-Bieto and Osmond 1983), the length of mitochondria of plants grown in the light and dark without exogenous sucrose was compared next.
3.1.2 Light induces mitochondrial fission

When plants were grown in the dark, a greater proportion of mitochondria were elongated and tubular (Fig. 3.1D and G), but mitochondria were primarily small and fragmented when plants were grown in the light (Fig. 3.1C and G). This trend held true when plants were grown with sucrose, however it was more apparent in plants grown without. To determine if light could induce the fragmentation of elongated mitochondria, dark grown plants grown without exogenous sugar were exposed to light for 1 and 12h. Plants grown in the dark without sucrose were used here because this treatment yielded the highest proportion of elongated and tubular mitochondria. During the light treatment, mitochondria started to undergo fission (Fig. 3.1E). The elongated forms first started to exhibit a beads-on-a-string appearance. As fission occurred, the mitochondria were aligned at first but later dispersed throughout the cell. This supported that light induces the fission of mitochondria.

3.1.3 Mitochondria elongate and become giant under O₂ limited conditions

While studying mitochondrial pleomorphy by live-imaging, it became apparent that the longer a plant remained mounted in water between a slide and coverslip for observations, the more elongated and tubular the mitochondria became (Movie 3.1). After 30min, round mitochondria began to elongate and by 45min-1h they formed expanded ring-shaped structures (Fig. 3.2). After 2-3h, extensive giant mitochondria were formed. Using mitoEos transgenics, it was also observed that the giant mitochondria could fuse with one another, permitting a mixing of green and green-to-red photo-converted fluorescent protein in the matrix of two of mitochondria (Fig. 3.3 and Movie 3.2). These mitochondria were similar in appearance to giant mitochondria formed under low oxygen pressure (Van Gestel and Verbelen 2002). The prospect that O₂ depletion during observations was inducing fusion was therefore investigated next.

Janus green B is used as a supravital stain to differentiate mitochondria from other organelles (Brenner 1953). The stain is reduced to a colourless leuco-form under anaerobic conditions. This discolouration takes place over time when observing mitochondria under a coverslip using Janus green B (Frey-Wyssling and Mühlethaler 1965). To further validate that giant mitochondria were forming due to oxygen depletion, a mineral oil overlay of plants was employed.
Figure 3.2 Morphological transition of small to giant mitochondria in response to oxygen depletion during lengthy imaging. (A) At the beginning of imaging, mitochondria are round and/or ovate in shape. (B-D) After about 45min-1h, they begin to elongate or form ring shaped structures. At this point, giant mitochondria also start forming as the mitochondria keep fusing (D). (E-G) During the early stages of O$_2$ depletion, the giant mitochondria are relatively stationary, but exhibit some degree of rearrangement, taking on various irregular shapes. (H) Eventually, after ~2-3h of submergence in water, they form extensive giant and very elongated mitochondria. Scale = 2μm.
An overlay of mineral oil over medium is commonly used to culture anaerobic bacteria. A layer of oil 1cm thick is usually sufficient to limit the diffusion of O\textsubscript{2} into and out of the underlying media. This results in the death of aerobes as O\textsubscript{2} becomes depleted (Rahn and Richardson 1940; Jacobson et al. 2004). Edwards et al. (1947), showed that after 1h of immersion in mineral oil the average oxygen consumption rate of a fungal culture decreased. As mineral oil minimizes the diffusion of oxygen and decreases respiration, plants were mounted in mineral oil to investigate if the elongation of mitochondria in plants mounted in water was due to the depletion of oxygen over time. Mitochondria were elongated after 1h under water. After 2h, elongated and giant mitochondria were seen for both treatments (Fig. 3.4). Although it took longer for small mitochondria to elongate under mineral oil than water, the elongated to giant mitochondria transition was most rapid under the mineral oil. Thus, O\textsubscript{2} limiting conditions induce fusion, elongation and the subsequent formation of giant mitochondria.
3.2 THE ENDOPLASMIC RETICULUM MEDIATES MITOCHONDRIAL PLEOMORPHY

The ER has been shown to aid in mitochondrial fission in yeast and animal cells (Friedman et al. 2011). This has not been investigated in plant cells. The notion that the ER could be a mediator of the various morphological transitions reported here in response to light, sugar
and hypoxia was therefore investigated next.

3.2.1 The endoplasmic reticulum is compact in light grown plants

The ER is a meshwork of tubular membrane that forms polygons (Fig. 3.5A and B). It can also flatten out into a sheet of membrane, forming what is termed cisternae (Fig. 3.5A (*); Friedman and Voeltz 2011). As mitochondria were predominantly elongated or tubular in dark grown plants but small and round under light grown conditions, the effect of light on ER polygon size or morphology in general was investigated first. The average area and perimeter of ER polygons were significantly greater in dark grown plants (area = 55.07 ± 39.44; perimeter = 30.06 ± 11.06) than light grown plants (area = 3.27 ± 2.05; perimeter = 7.07 ± 2.26; p-value < 0.01, n = 240; Fig. 3.5). It was also noted in dark grown plants that although the ER polygons were large on average, there were smaller ones within the meshwork (Fig. 3.5B). There was a greater occurrence of aggregated ER tubules or cisternae in light grown plants than those grown in the dark (Fig. 3.5A).

![Figure 3.5](image)

**Figure 3.5 Average planar ER polygon size comparison between light and dark growth conditions.** 8 day old plants of mito-GFP RER Arabidopsis transgenics were grown in the light (164μmolm⁻²s⁻¹, 16h/8h light/dark) (A) and complete darkness (B) were used to determine the average ER polygon area (C) and perimeter (D) to compare ER arrangement between the two treatments. Area and perimeter were significantly different between light and dark grown plants (p-value < 0.01, n = 240). ER cisternae (*) and smaller depressions or polygons within the larger polygon meshwork of dark grown plants (arrows) are indicated. Standard error bars are shown. Scale bars = 10μm.
3.2.2 The endoplasmic reticulum acts as a mould for mitochondrial shape

Live imaging of mito-GFP RER double transgenics revealed that the various shapes of mitochondria discussed above were reflected in the arrangement of the surrounding ER. When mitochondria were round and spherical, as seen in light grown plants, they were found within the small depressions in the ER polygon meshwork (Fig. 3.6A). When mitochondria were elongated and tubular, they were outlined by ER tubules (Fig. 3.6C and D). As these tubular forms became twisted or contorted, they remained aligned with the ER (Movie 3.3 and 3.4). Furthermore, as the ER flattened out into sheets of cisternae, the mitochondria were also flattened out (Fig. 3.6D; Movie 3.3). This was especially evident under hypoxic conditions.

Giant mitochondria displayed the most pleomorphy, taking on a wide range of appearances. Visualization of these shapes made it apparent that as the ER rearranged, mitochondria followed suit. The mitochondria appeared to flow into gaps in the ER (Movie 3.3). This phenomenon resembled a fluid (mitochondria) being poured into a mould (the ER meshwork).

Figure 3.6 Mitochondria are embedded in the ER. (A) When mitochondria are small and spherical, they are embedded within small ER polygons. (C) When mitochondria are elongated and tubular they are closely aligned by the ER. ER tubules also constrict the mitochondria at potential sites of fission (arrow). (D) As the ER flattens out into sheets of cisternae, mitochondria expand and also flatten. As the ER rearranges, the mitochondria follow suit, acting in a fluid-like motion and flowing into gaps created by the ER. RFP=ER; GFP=mitochondria. Scale bars = 2μm.
3.2.3 The endoplasmic reticulum aids in mitochondrial fragmentation

The ER has been shown in yeast and animal cells to aid in the fission of mitochondria by wrapping around the mitochondrial fission sites (Friedman et al. 2011). This has not been shown in plant cells. This aspect of the ER-mitochondrial relationship was investigated next. Mitochondrial elongation was induced by either growing plants in the dark or by inducing hypoxia by submerging the plants in water for ≥1h. Plants were then exposed to HL to induce mitochondrial fission. The elongated mitochondria were closely surrounded by ER tubules which often wrapped around the mitochondria, constricting them at sites of fission (Fig. 3.7; Movie 3.4). As the ER was rearranged, the mitochondria also appeared to be tugged accordingly, suggesting that the ER also applies a pulling force that allows mitochondria to be pulled apart during fission. This separation also aids in mitochondrial dispersal as the fragmented portions are pulled in different directions.

3.2.4 Mitochondrial beading and the endoplasmic reticulum

Mutants where mitochondrial fission is impaired were used to further validate the ER involvement in this process. The drp3a/apm1-13 mutant displays an aberrant mitochondrial phenotype (Fig. 3.7B, C; Mano et al. 2004). Plants of this mutant were grown in the dark for 7 days and stained with MitoTracker Orange for 10min prior to live imaging. When treated with HL for 2min, the elongated, tubular mitochondria typical of this mutant displayed a beads-on-a-string appearance as they were stretched throughout the cell (Fig. 3.7D). This beading phenomenon was also seen in plants of elm1-1. As the beading is also seen in wild type plants when tubular mitochondria are undergoing fission, it was investigated whether the ER constriction was actually the cause of the beading.

elm1-1 Mt-GFP (mitochondrial targeted GFP; Feng et al. 2004; Arimura et al. 2008) were transformed with RER to gain insight into the stretching and beading phenomenon. Elongated mitochondria appeared to thread through the ER polygons. When the ER was rearranged, the mitochondria often became constricted at points where the ER became constricted. This resulted in the beading appearance. Furthermore, as tubular mitochondria moved through a ‘tunnel’ or between ER tubules, they remained thin. Once it reached an area of this ER tunnel that was
**Figure 3.7 ER-mediated fission and beading of elongated mitochondria.** (A) Sequence of mitochondrial fission from Movie 3.4. The solid arrows indicate sites where the ER constricts the mitochondrion. Dotted arrows indicate the movement of the ER and subsequent movement of the fragmented mitochondrion. The white occurred before the yellow which occurred before the blue. The sequences show that the ER constricts the mitochondrion and as the ER is rearranged, the mitochondrion fragments at this site. The fragmented portion of the mitochondrion disperses in the direction of the ER rearrangement. (B-D) Plants of *drp3a/apm1-13* stably expressing GFP-labelled peroxisomes were stained with MitoTracker Orange (Molecular Probes). Both organelles mostly displayed an aberrant phenotype whereby they were very elongated. As the organelles stretched, they beaded (C, arrows). (D) When given a 2min HL treatment, the mitochondria rapidly stretched and exhibited beading. (E-I) Mutants of *elm1-1* stably expressing GFP-labelled mitochondria were transformed with RER. (E) Mitochondria were typically elongated, but were capable of beading as well in this mutant (F-I). Where the tubular mitochondria threaded through the ER, beading was often seen (solid arrows). This was especially the case when the mitochondrion encountered aggregated ER (dotted arrows). H is a magnification of the boxed-in region of G. *apm1-13* and *elm1-1* seeds were kindly provided by Shoji Mano and Shin-ichi Arimura, respectively.
dilated, the mitochondria followed suit and also dilated (Fig. 3.7F-I). A similar beading phenomenon was also witnessed with elongated peroxisomes (Fig. 3.7B; Sinclair et al. 2009; Delille et al. 2010) and stromules (Pkye and Howells 2002; Gunning 2005; Hanson and Sattarzadeh 2008; Schattat et al. 2011).

3.3 THE ER CAGES MITOCHONDRIA, PLASTIDS AND PEROXISOMES

The ER clearly plays a major role in the pleomorphic nature of mitochondria, peroxisomes and plastids. To gain a better understanding of how all four organelles interact in a live system, triple Arabidopsis transgenics, mito-GFP Yperoxi RER, were created. This was done by crossing mito-GFP (Logan and Leaver 2000) plants with Yperoxi (Mathur et al. 2002) and transforming the stable transgenic plants with RER (Sinclair et al. 2009). The chlorophyll auto-fluorescence could be used to visualize chloroplasts as well, therefore simultaneous visualization of all four organelles was possible.

It was previously shown that the ER forms a cage that can enclose plastids (Schattat et al. 2011). As the cage moved, the plastids moved with it. Live-imaging of the triple transgenics here showed that mitochondria and peroxisomes also become stuck in this cage (Fig. 3.8; Movie 3.5). Mitochondria and peroxisomes were also seen moving along ER bundles in the cytoplasmic stream, but where this may be co- incidental, the ER cage which has minimal movement in comparison to cytoplasmic stream may serve a higher purpose. Containment of mitochondria, peroxisomes and chloroplasts within the ER network may consequently permit sustained interactions to increase the efficiency of metabolite or ROS transfers between all four organelles.

Figure 3.8 The ER cages mitochondria, peroxisomes and chloroplasts. A cluster of organelles maintaining a sustained interaction is shown. GFP=mitochondria; YFP=peroxisome; RFP=ER; auto-fluorescence false coloured blue=chloroplasts.
Since the ER plays a role in regulating peroxisome (Sinclair et al. 2009; Barton et al. 2014) and mitochondrial pleomorphy, and both organelles were found to exist in close association with one another in ER cages, the mitochondria-peroxisome interactions were investigated in more depth next. The interactive behaviours in response to light, sugar and ROS stress were of particular interest because these are all factors that link the two organelles and suggest a cause for their close interactions.

3.4 INVESTIGATION OF MITOCHONDRIA AND PEROXISOME INTERACTIONS

3.4.1 Mitochondria and peroxisome morphology in response to H$_2$O$_2$

mito-GFP Yperoxi plants were grown in the dark for 7 days and then placed in water, 0.3% or 3% H$_2$O$_2$ for 30s. The plants were quickly rinsed with and mounted in water. Plants grown continuously in the dark without sucrose were used because mitochondria in these plants were most elongated. Since ROS has been shown to fragment mitochondria, responses may be most apparent in these plants. Mitochondria were significantly longer when plants were treated with 0.3% and 3% H$_2$O$_2$ (Fig. 3.9; p-value < 0.01, n = 204). There was no significant difference in length between the two 0.3% and 3% treatments, however. Peroxisomes were elongated in all three treatments, but H$_2$O$_2$ did not result in a significant increase in the average length (p-value > 0.05, n = 201). Peroxules did form occasionally, and some beading of elongated mitochondria and peroxisomes were seen (Fig. 3.9). No clear interaction between mitochondria and peroxisomes were seen.

3.4.2 Peroxisome morphology in response to light, sugar and low O$_2$ conditions

mito-GFP Yperoxi double transgenics were grown either in the light (16h/8h light/dark cycle) or continuously in the dark on MS medium with or without sucrose. Mitochondria were predominantly longer (>1.3μm in length) when plants were grown in the dark without sucrose but were predominantly shorter (≤1.3μm in length) when grown in the light with sucrose (p-value < 0.05; Fig. 3.10A and C-F). This was consistent with the above results. The trends reflected in peroxisomal morphology were nearly opposite. There was a significantly greater proportion of large (>1.3μm in length) peroxisomes than small (≤1.3μm in length) ones when
plants were grown in the light with and without sugar (p-value < 0.05; Fig. 3.10B, C-F). There was also a significant decrease in the proportion of large or elongated peroxisomes when plants were grown in the dark without sucrose (p-value < 0.05; Fig. 3.10B-F).

Peroxules and actively dividing peroxisomes were rarely seen even in the light grown plants. Since the plants were grown under the respective treatments for 7 days prior to analysis, they may have adapted and achieved homeostasis. It has been suggested that peroxisomes are constantly sensing or monitoring cellular ROS levels given their role in ROS scavenging (Sinclair et al. 2009; Mathur et al. 2012). As such, the effects of light on peroxisomal pleomorphy may be more rapid and apparent under shorter treatments where the cell is quickly trying to attain homeostasis.

Arabidopsis transgenics expressing HyPer-GFP were given a short HL treatment to look at the rapidity of H$_2$O$_2$ production in response to light. Prior to HL treatment, the intensity of the

**Figure 3.9 Effects of H$_2$O$_2$ on mitochondria and peroxisome morphology.** mito-GFP Yperoxi plants grown in the dark without sucrose for 7 days were placed in a solution of 0.3% or 3% H$_2$O$_2$, or in water (0%) for 30s. Mitochondria were significantly longer when treated with H$_2$O$_2$. Although peroxisomes were elongated, there was no significant increase in length. Mitochondria and peroxisomes did exhibit some beading during fission (arrows). GFP=mitochondria; YFP=peroxisomes. Scale bars = 2μm.
basal GFP fluorescence was low (Fig. 3.11A and C). After only 5min of HL, the intensity increased (Fig. 3.11B and D). This showed that the H₂O₂ levels can indeed increase very rapidly in response to HL. HL irradiation of dark grown plants for short intervals – 30s, 1, 2 or 4min – were therefore used to investigate rapid morphological responses of mitochondria and peroxisomes in response to light, sugar and hypoxia. The average lengths of mitochondria and peroxisomes were measured using 7 day old mito-GFP Yperoxi plants grown in the light or dark, with or without sucrose in the medium. An organelle was considered large if the length was >1.3μm and small if it was ≤1.3μm. (A) Mitochondria were more elongated or larger when plants were grown in the dark (D, F), and shorter when grown in the light (C, E) (p-value < 0.05). (B) Peroxisomes were predominantly larger when plants were grown in the light, with and without sucrose than dark grown plants without sucrose (p-value < 0.05). Peroxules and elongated peroxisomes were rarely seen in all treatments. Standard deviation bars are shown. * = significant difference in the proportion of long and short organelles within each treatment; a = significant difference in the proportions between treatments; NS = no significant difference; α=0.05. (G-H) During hypoxia, one or more spherical peroxisomes were seen often clustered to ring-shaped or giant mitochondria. Ends of peroxules were never witnessed interacting with mitochondria exhibiting hypoxic morphologies. Scale bars = 2μm.
peroxisomes in response to HL-induced ROS stress. But again, no clear morphological responses of peroxisomes to the shorter HL treatments were observed. What was witnessed however was an interactive behaviour of peroxisomes around mitochondria.

When hypoxia was induced by submerging mito-GFP Yperoxi plants in water for 45min onwards, ring-shaped and giant mitochondria were formed reproducibly as before. These mitochondria often had one or more spherical peroxisomes clustered around them (Fig. 3.10G and H). The interactivity of these two organelles was investigated in more depth.

### 3.4.3 Mitochondria and peroxisomes have sustained interactions

Mitochondria and peroxisomes are linked metabolically and biochemically, but their physical interactions have not been fully investigated in plants. This was looked at next. Simultaneous visualization of mitochondria and peroxisomes using mito-GFP Yperoxi double transgenics showed that although the two organelles would often move along similar paths, for example along the cytoplasmic stream (Movie 3.6) and sometimes crossed paths, there was no
impression of an interaction. However, often mitochondria would cluster around peroxisome(s) for long periods of time (Fig. 3.12; Movie 3.6, 3.7). The number of these interactions between the two organelles peaked after 1min of HL irradiation, but no clear trend was observed (Fig. 3.12E). However, the duration of the interactions did increase between 0-2min of HL, but decreased after 4min. Furthermore, the motility of these clusters was minimal in comparison to the rapid movement of the surrounding organelles. The clusters often consisted of small, spherical mitochondria around spherical peroxisomes with and without peroxules (Fig. 3.12A and B). Since the fluctuation of light is a major contributor to ROS production, a mutant where the effects of light were implicated was of interest.

3.5 **THE anisotropy1 MUTANT RESPONSE TO LIGHT**

Plant epidermal cells act as lenses (Haberlandt 1914; Vogelmann 1993; Vogelmann et al. 1996). When collimated light strikes these cells, it is refracted and passes through the epidermis to the underlying mesophyll layer where it is absorbed by plastids. The way the light is refracted and how it is focused in the cells below the epidermal cell wall is determined by such characteristics as the cell surface area, cell wall composition, waxes, and the degree and uniformity of curvature (Vogelmann et al. 1996). If the radius of curvature (r) is small (i.e. the cell is very round), the light is focused with minimal scattering immediately below the epidermis at the top of the mesophyll layer. Conversely, if r is large (i.e. the cell is flattened), the light is focussed farther below the epidermis. But the deeper the focal point of light, the greater the scattering and hindrance of absorption there is (Vogelmann et al. 1996). It would therefore be expected that the chloroplasts of the mesophyll layer would absorb the light better if the cells in the outermost epidermal layer are round – the light would be better focused. This would also mean that the more light absorbed by the chloroplasts, the higher the rate of photosynthesis and therefore the higher the levels of ROS. The expectation would be that the interactivity between the mitochondria and peroxisomes in response to light-induced ROS would be heightened in a mutant exhibiting such a phenomenon.
3.5.1 Production of light-induced H$_2$O$_2$ is more rapid in *any1* than wild type

One of the mutants available was *anisotropy1 (any1)*. *any1* has a mutation in a cellulose synthase (CesA) gene that causes a reduction in cell wall crystallinity (Fujita et al. 2013). As the
When cell wall crystallinity was affected, the refraction of light would be expected to be altered as well. Comparisons of wild type and any1 revealed that the epidermal cells in any1 are nacreous and often swollen or bulged (Fig. 3.13). The reduction in growth anisotropy of the cells also created larger intercellular spaces. As the arrangement and the curvature of the cells were altered, this mutant was a good contender for investigating the effects of light-induced ROS production.

Columbia and any1 seedlings were grown in the dark for 6 days and then transferred to low light (30μmolm⁻²s⁻¹) for 2 days prior to subjecting them to 0, 100, 200 or 300μmolm⁻²s⁻¹ of light for 24h. At this stage the seedlings did not have their first true leaves fully emerged yet. The plants were submerged in a solution of DAB with a metal enhancer (Sigma-Aldrich) under vacuum (-50KPa) for 4h. The tissue was cleared with ethanol and mounted in 50% glycerol. Images were acquired at the same light intensity and microscope settings for direct comparisons. The staining was representative of the amount of H₂O₂ produced during the relative light intensity treatments. DAB staining illustrated that any1 produces more H₂O₂ in response to light than wild type (Fig. 3.14B). When seedlings were transferred to the dark for 24h and then exposed to light (164μmolm⁻²s⁻¹) for 30min, 1h or 2h, H₂O₂ levels were also greater in any1 seedlings in each case (Fig. 3.14A). This also showed that response to light occurs well before 24h, as staining intensified over the span of 2h.
Figure 3.14 Comparisons of wild type (WT) and any1 responses to light. (A) Seedlings of any1 and WT were transferred to the dark for 24h prior to their exposure to light (164μmolm⁻²s⁻¹) for 0, 0.5, 1 or 2h prior to DAB staining. (B) Dark grown seedlings were also transferred to low light (30μmolm⁻²s⁻¹) for 2 days and then transferred to 0, 100, 200 or 300μmolm⁻²s⁻¹ of light for 24h prior to DAB staining. The average inverse gray values of DAB staining for H₂O₂ were greater for any1 in the cotyledon and hypocotyl. (D) A boxplot (R, http://www.r-project.org/) showing that mitochondria were smaller on average in any1. (C) The interactions between mitochondria and peroxisomes and peroxules were more apparent and frequent in the mutant. Scale bars = 0.5mm and 2μm, A and C, respectively.
3.5.2 Mitochondria-peroxisome interactions in any1

Using mitochondrial size as an indicator for light stress, 7 day old any1 mutants were grown in the light or dark, with or without sucrose. The average length of mitochondria was smaller in any1 than wild type for all treatments (Fig. 3.14D). The mitochondria-peroxisome interaction was more apparent in these plants than wild type as well (Fig. 3.14C; Movie 3.8). Peroxules were also observed more frequently. These extensions were seen interacting with small, fragmented mitochondria and within clusters of chloroplasts. These observations were very apparent in cells that exhibited the most isotropy and were bulbous or nacreous. Overall, the morphological transitions and interactive behaviour of mitochondria and peroxisomes in response to light stress was most apparent in any1 plants than wild type.

3.6 INVESTIGATION OF MITOCHONDRIA FISSION MACHINERY

Investigations of mitochondria and peroxisome morphology brought to light overlaps in the appearances of each organelle, in particular during their fission (Fig. 1.5). When undergoing fission, both organelles elongate. They then become constricted sometimes exhibiting a beads-on-a-string appearance. Fission then takes place at the site(s) of constriction and the new entities disperse throughout the cell. As mitochondria and peroxisomes share this morphological procession during division, the mechanism behind fission was looked at closer. The possibility that FIS1A, a tail-anchored membrane protein, in particular could be exchanged between mitochondria and peroxisomes through mitochondrial derived vesicles (MDVs) was explored in particular.

3.6.1 DAL as possible mechanism of hierarchical fission between mitochondria and peroxisomes

Neuspiel et al. (2008) described a novel interaction between mitochondria and peroxisomes in mammalian cells by demonstrating that mitochondria derived vesicles (MDVs) carrying an outer membrane Mitochondria-Anchored Protein Ligase (MAPL) were transported from mitochondria to peroxisomes. They demonstrated that MAPL becomes enriched along tubular mitochondria and that MAPL over-expression resulted in fragmentation of mitochondria,
a phenomenon that was deemed dependent on the MAPL-RING domain and Drp1.

Since 1) FIS1 is anchored to the peroxisomal membrane and the OMM, 2) MAPL is localized to the OMM and 3) MDVs bud off of the OMM, it was questioned if the MDVs translocate MAPL and FIS1 to peroxisomes, which could be the result of close interactions seen with small mitochondria and peroxisomes and/or peroxules. This could result in the subsequent fragmentation of peroxisomes as well.

FIS1 is C-terminally anchored to the peroxisome membrane and to the OMM, thus mEosFP was N-terminally fused to FIS1A and to FIS1B. The expressions of both fusions were driven by the p35S. A construct in which Eos-FIS1A was driven by the native promoter (pfis1a-Eos-FIS1A) was also created. When *N. benthamiana* was co-infiltrated with Eos-FIS1B, mito-GFP and Yperoxi, FIS1B was localized to mitochondria, peroxisomes, plastids and stromules (Fig. 3.15; Ruberti 2014). This was also the case for Eos-FIS1A under the p35S and pfis1a. As this could have been an artifact of transient expression, stable lines of YFP-FIS1A (Ruberti 2014) crossed with mito-GFP and with FNR-GFP were created. Again, FIS1A was localized to the entire OMM, plastid bodies and stromules (Fig. 3.15). Dal1-Eos and Dal2-Eos lines were also created, where DAL1 and 2 are the plant homologues of MAPL. A major issue in testing the hypothesis that FIS1A or FIS1B and DAL1 and/or DAL2 were translocated from fragmented mitochondria to peroxisomes during their interaction for subsequent peroxisome fission was the experimental approach. mEosFP was used in particular with the hopes of photo-converting a small region or subset of FIS1/DAL1/DAL2 on mitochondria from green to red. The red would then be tracked to see if they are indeed translocated to peroxisomes during fission. Given the motility and dispersal of these organelles during fission, it was not possible to do that. The lines are available however, if approaches are available in the future.
Figure 3.15 Localization of FIS1A and FIS1B to mitochondria, peroxisomes and plastids. (A-E) mito-GFP plants crossed with YFP-FIS1A. FIS1A was localized to mitochondria, as shown by the green-red overlay making yellow. It was also localized to peroxisomes and peroxules, which are indicated (p and px, respectively) by just the yellow in B and C. It formed rings around chloroplasts and also localized to stromules (str). (F) YFP-FIS1A was crossed to FNRGFP (GFP labelling plastid stroma) and further validated that FIS1A localized to the plastids and stromules. (H-L) The Eos-FIS1A and Eos-FIS1B constructs co-infiltrated with mito-GFP and Yperoxi showed that FIS1A and FIS1B (seen in red in all images) were localized to plastids as well. Note that it was difficult to photo-convert a single or very small portion of the mitochondria and peroxisome population.
CHAPTER 4

DISCUSSION

4.1 MITOCHONDRIA MORPHOLOGY IN RESPONSE TO LIGHT, SUGAR AND O₂ LIMITED CONDITIONS

4.1.1 Morphological differences between mitochondria in light and dark grown plants

Fluctuating cytosolic sugar levels is a phenomenon plant cells must constantly overcome. This is because of fluctuations in light and photosynthetic activity (Azcón-Bieto et al. 1983; Azcón-Bieto and Osmond 1983). Because of their photosynthetic capabilities, plants are able to synthesize carbohydrates when they are exposed to light. Any excess sugar produced is converted to and stored as starch which is broken down when cytosolic sugar levels are too low, such as at night (Fig. 4.1). The carbohydrates produced are metabolized by glycolysis and the TCA cycle during cellular respiration to produce ATP, NADH and FADH₂. The latter two are reduced by complexes I and II of the ETC in mitochondria to supply electrons. This is necessary to create an electrochemical gradient that is needed to drive ATP synthesis (Fig. 1.1).

One of the major causes of damaged mitochondrial constituents is an overwhelmed ETC, where there is an excessive amount of electrons that are not reduced. These electrons then react with O₂, producing ROS. Such a situation is seen in animal cells when hyperglycemic conditions
provide an overwhelming metabolic input that causes the ETC to overwork. There is a consequential rise in ROS that leads to cell death (Yu et al. 2006; 2008). Overcoming detrimental levels of ROS is a challenge photosynthetic plants are constantly faced with because excessive amounts of light can be quite damaging by producing too much ROS (Bhattacharjee 2011). Mitochondria of animal cells fragment under hyperglycemic conditions (Yu et al. 2006; 2008; Jhun et al. 2013), but mitochondrial pleomorphy in response to fluctuations in cytosolic sugar in plant cells had not been investigated. As mitochondria are essential for cellular respiration in eukaryotes, and mitochondrial fission and fusion play an important role in regulating this activity, an investigation on the pleomorphy of mitochondria in response to light and sugar was carried out.

When plants are grown without exogenous sugar, mitochondria are predominantly elongated. When plants are grown with sucrose however, mitochondria are primarily small and round to ovate in shape. Thus, sugar is an inducer of mitochondrial fragmentation in plant cells as well. Most striking however, is the effect light has on the appearance of mitochondria. When plants are grown in the light, the mitochondrial population consists of fragmented, small and spherical entities but more tubular and elongated mitochondria are seen in cells of dark grown plants. This trend holds true whether plants are grown with or without exogenous sugar. When dark grown plants are exposed to light, the mitochondria are quick to undergo fission, rapidly fragmenting from a tubular, elongated population to a small and spherical one. Light therefore has a greater influence on what the more dominant shape and size of the mitochondria is than exogenous sugar levels.

Dark grown plants are unable to photosynthesize and they quickly deplete the lipids, starch and carbohydrates stored in their cotyledons (Fig. 4.2). As the cell is not respiring, NADH and FADH$_2$ are low; electrons are not provided to the ETC to create the electrochemical gradient required to produce ATP. Mitochondria undergo fusion when the $\Delta \Psi_m$ is depolarized which explains why mitochondria are predominantly longer in plants grown in the dark without sucrose; fusing of the membranes allows the repolarization of the $\Delta \Psi_m$ so that oxidative respiration can still occur to synthesize ATP.
Figure 4.1 Influence of light, sugar and energy status on mitochondrial shape. When there is light, plants are able to photosynthesize and produce carbohydrates. Excess sugar is converted to starch which is broken down when cytosolic sugar levels are too low, such as at night. Under controlled conditions discussed in this thesis, plants are also able to uptake exogenous sugar supplied in the growth medium (see Section 3.1.1). Glycolysis breaks down glucose, yielding ATP, NADH and pyruvate. The TCA cycle further metabolizes pyruvate, producing GTP, NADH and FADH₂. NADH is reduced by NADH dehydrogenase (complex I) and FADH₂ by succinate dehydrogenase (complex II) of the ETC during oxidative phosphorylation. This occurs on the IMM. The electrons released are transported down the ETC pathway as H⁺ are pumped out of the matrix into the IMS creating an electrochemical gradient. The electrons are accepted by O₂ (Fig. 1.1). The gradient is used by ATP synthase to produce ATP. The mitochondria would then be in the orthodox state, which occurs when ATP levels are high. Mitochondria then undergo fission, increasing their numbers. The cell is in a high energy state now, and the mitochondria are constantly dispersing to other areas of the cell. They will interact with one another, transferring the energy to each other and parts of the cell where energy is required. ATP levels are decreasing at this point, and ADP levels are increasing. As the membrane is therefore depolarizing, mitochondria must keep keep respiring to replenish ATP.
Figure 4.2 The effects of dark, hypoxia and starvation on respiration and mitochondria morphology. Plants were grown in the dark for seven days post germination. As no light was available, photosynthesis was not occurring and carbohydrates were not being produced. Seedlings were then entirely dependent on lipids, carbohydrates and other nutrients stored in the cotyledons and also on the nutrients other than sugar in MS medium. Once the starch, lipid and carbohydrate stores were depleted, ATP levels became low. With no NADH and FADH$_2$ feeding the ETC due to the lack of glycolysis, β-oxidation and TCA cycling, oxidative phosphorylation is also not taking place. The IMM becomes depolarized. Mitochondria attempt to fuse, which helps to increase the ΔΨ$_m$, however any ATP produced becomes depleted as well. Mitochondria also have minimal motility and very little energy is being dispersed. The cell continues to starve. A similar situation occurs when a plant is submerged in nutrient-free water, except O$_2$ is also being depleted and eventually becomes unavailable for oxidative phosphorylation.
After the plants are given a HL treatment, photosynthesis can occur, thereby increasing cytosolic sugar levels (Fig. 4.3). Once carbohydrates become available, respiration may commence. At this point, the IMM becomes progressively hyperpolarized and ATP levels rises. The mitochondria then undergo fission to disperse the energy throughout the cell (Amchenkova et al. 1988; Bereiter-Hahn and Vöth 1994). High energy mitochondria might also fuse with those of lower ΔΨₘ to achieve an intermediate state of polarization. This fission and fusion activity continues until a proper balance is achieved. Since plants grown in the light have higher levels of total soluble sugar than those grown in the dark, it makes sense that the mitochondria are fragmented in the light grown plants; there is a greater metabolic supply for respiration in the presence of light which essentially leads to more hyperpolarized, energy-filled mitochondria that must constantly disperse the energy throughout the cell. A similar situation occurs when a plant is submerged in water during microscopic observations for too long.

### 4.1.2 Mitochondria morphology under O₂ deplete conditions

Whole plants are mounted in water on a slide and coverslip during observations. When they are left submerged for long periods of time, the carbohydrates, starch, lipid stores etc. get depleted. As cells respire, O₂ is also consumed (Frey-Wyssling and Mühlethaler 1965; Hackenbrock et al. 1971) and eventually becomes unavailable for oxidative phosphorylation (Fig. 4.2). If O₂ is too low, electrons start to accumulate. This accumulation results in an increase in ROS which triggers mitochondrial fragmentation and mitophagy (Yu et al. 2008). However, after being submerged in water ≥1h, nutrients (carbohydrate, starch and lipid stores etc.) become depleted as well. Just as in the dark grown plants, very little electrons are therefore fed into the ETC regardless. This again causes a depolarization of the ΔΨₘ. Mitochondria are forced to fuse in an attempt to repolarize the IMM. As both the metabolic input and O₂ availability for oxidative phosphorylation are low, the fusion has to be more extensive. Giant mitochondria form.
1. **Before HL**: The mitochondrion is elongated. ATP levels are low or basal, but there is no metabolic input; the cell is not respiring. Intracristal spaces are narrow; the orthodox state is assumed.

2. **After HL**: Photosynthesis yields an increase in cytosolic sugar levels. Respiration commences. The intracristal spaces have expanded; the condensed state is acquired. The mitochondrion is still elongated.

3. The ATP levels are high and the IMM is hyperpolarized in this condensed state. The mitochondrion undergoes fission and disperses throughout the cell, usually to an area of high energy demand.

4. A high energy mitochondrion fuses with one of lower ATP and lower $\Delta \Psi_m$ which may be in the orthodox state. The longer the HL treatment, the greater the energy input and the higher the rate of fission and motility. The population may become predominantly small and spherical.

5. After fusion, the mitochondrion is in an intermediate state of $\Delta \Psi_m$ and ATP. If the membrane is still too hyperpolarized, steps 3 and 4 may be repeated until homeostasis is achieved or the cell has sufficient energy to function.

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**Figure 4.3 Proposed model for orthodox to condensed state transition to explain fusion-fission activity in response to HL.** When plants with a predominantly elongated population receive a HL treatment, the mitochondria may undergo an ultrastructural and then energetic state transition. (1) An elongated mitochondrion in the orthodox state (i.e. the intracristal spaces are narrow, the matrix is expanded and ATP levels are low) transitions to (2) the condensed state upon HL irradiation. With the HL stimulus, the plant is actively respiring and the mitochondria undergo oxidative phosphorylation. (3) The IMM becomes hyperpolarized and the mitochondrion must undergo fission to disperse the energy it now contains. (4) After they disperse throughout the cell, the hyperpolarized, energy-filled mitochondria may fuse to a mitochondrion of lower $\Delta \Psi_m$ or lower energy. This transferral of electrochemical potential and energy results in a mitochondrion of intermediate $\Delta \Psi_m$ and an ultrastructure somewhere in between the orthodox and condensed state. Red = orthodox state, low ATP, high ADP; green = condensed state, high ATP, low ADP; yellow = intermediate orthodox-condensed state and ATP/ADP levels; the darker shaded regions are the intracristal spaces and the light regions are the matrix to better distinguish between the orthodox (more lighter regions/expanded matrix) and condensed state (more darker regions/expanded intracristal spaces).
4.2 MITOCHONDRIA AND ENDOPLASMIC RETICULUM INTERACTIONS

The ER has been shown to be a key player in peroxisome (Sinclair et al. 2009; Barton et al. 2014) and plastid morphology (Pyke and Howells 2002; Gunning 2005; Hanson and Sattarzadeh 2008; Schattat et al. 2011). The extent of the ER-mitochondria interactions in plants cells had not been elucidated yet. Mitochondria exhibit quite an array of morphological appearances (Appendix I). Not only can they exist as ‘fila’ or ‘plastosomes’, but when they form giant mitochondria, they display very irregular shapes, which were shown here to be mediated by the ER. As the ER rearranges, the mitochondria follow suit: flattened ER result in flattened mitochondria; small mitochondria are embedded within small ER polygons; elongated, beaded mitochondria are constricted by ER tubules; and ER rearrangement pulls or tugs constricted mitochondria, and as they undergo fission, they disperse with the ER.

Membrane contact sites (MCS) have been suggested to exist between mitochondria and the ER. These MCS are thought to have several purposes, such as tethering the ER and mitochondria, lipid transfer, Ca\(^{2+}\) transport and apoptosis signalling (Rizzuto et al. 1998; Achleitner et al. 1999; De Brito and Scorrano 2008; Osman et al. 2011; Prinz 2014; Vance 2014). Interestingly, one of the proposed tethers between these two organelles is Fis1 in the OMM which is thought to tether to Bap31 in the ER membrane (Prinz 2014). This may explain why the mitochondria can be tugged by the ER during fission and why DRP3, which binds to Fis1, is found at sites of ER-constricted mitochondria (Fig. 4.4). If Fis1 tethers the ER around a tubular mitochondrion, it would allow the ER to constrict the mitochondrion tight enough and long enough for DRP3 to bind to FIS1 and form helices around the constricted site. DRP3 could then mechanochemically cause fission and refuse the ends of the IMM and the OMM. If the newly divided mitochondria are still tethered to the ER, either through FIS1 or other tethering proteins (i.e. VDAC for Ca\(^{2+}\) transport), when the ER rearranges, it would pull the mitochondria along with it. The mitochondria would disperse throughout the cell (Fig. 4.4E). Simultaneous visualization of mito-GFP RER double transgenics here supports this.

It was also shown here that when the ER is flattened out (i.e. sheets of cisternae), mitochondria often are as well. As the ER is stretched out, a meshwork of distinct tubules is created. As this occurs, mitochondria too, are stretched out, and become tubular. It is proposed
that this stretching out and tubulation of these two organelles are correlated and may be due to membrane contact sites (MCS). If mitochondria are attached to the ER through MCS, as the ER membrane is stretched, the MCS are pulled along with it causing the mitochondrial membrane to stretch as well (Fig. 4.4D). In some situations, it was noticed that a piece of the mitochondrion that fragmented from a tubular mitochondrion appeared small and round. This may be because there are now less MCS with the ER to allow the mitochondria membranes to be stretched out or elongate; the mitochondria by default would ball up. This may explain why mitochondria are described as spherical structures in mitochondria isolations; there is no ER to stretch the outer membrane out.

It is interesting that FIS1 is suggested to be involved in tethering mitochondria. The ER has also been shown to play a role in peroxisome and plastid morphology and FIS1 was shown here and by Ruberti (2014) to localize to mitochondria, peroxisomes and plastids. It was also shown here through simultaneous visualization of triple transgenics that the ER cages these three organelles. Other tethering proteins have been suggested between the ER and the other organelles, so perhaps MCS are involved in bringing these organelles together during inter-organelle transfers. The metabolic and biochemical basis for close ER-plastid, ER-peroxisome and mitochondria-plastid interactions have been discussed previously, but analysis of mitochondria-peroxisome physical interactions in plant cells was lacking. The clustering of mitochondria and peroxisomes was therefore investigated further.

### 4.3 MITOCHONDRIA AND PEROXISOME INTERACTIONS

Both mitochondria and peroxisomes can exist in tubular and spherical forms. During fission, both organelles constrict and divide to increase their population and dispersal during various stresses. Since mitochondria fragment in response to light and increased cytosolic sugar, the effects these factors may have on peroxisome morphology was investigated.
Figure 4.4 Proposed models to explain ER-mediated mitochondrial pleomorphy. (A, B) FIS1 acts as a tether for mitochondria to the ER during mitochondrial fission. Tethered to the mitochondrion, the ER constricts it. DRP3 may bind to FIS1, but as the mitochondrion is constricted, DRP3 can form a helix around the mitochondria and mechanochemically cause fission. (C) VDAC can also act as an ER-mitochondrial tether during Ca^{2+} transport. (D) Mitochondria are often embedded or surrounded by the ER. During mitochondrial fission, the ER constricts a mitochondrion. Narrow enough now, DRP3 wraps around this site. Fission occurs as the ER also helps to apply a pulling force. (E) as the ER rearranges, the mitochondria, tethered to the ER by MCS(s), moves along with the ER. Based on observations reported here and adapted from models proposed by Friedman and Voeltz 2011; Westerman 2011; Prinz 2014 in the yeast and animal systems.
Mitochondria and peroxisomes are both essential organelles in eukaryotic cells. In plant cells, peroxisomes are the only site of β-oxidation, products of which enter the mitochondria matrix for use in ATP synthesis (Schrader and Yoon 2007). During ATP synthesis, electron leakage from the ETC often occurs. When these electrons spontaneously react with O₂, ROS are produced. Although mitochondria house enzymes to reduce these highly reactive species, ROS such as H₂O₂ can diffuse out of the mitochondrial membranes into the cytosol. Being notorious for causing lipid peroxidation, this increase in ROS can be damaging to all membranes in the cell. Plants are quite vulnerable to lipid peroxidation because, where mitochondria are the major producers of ROS in animal and yeast cells (Foyer and Noctor 2003; Apel and Hirt 2004; Ježek and Plecitá-Hlavatá 2009), chloroplasts also produce them in plants during photorespiration. Just as with mitochondria, ROS produced by chloroplasts may also diffuse through the chloroplast membranes into the cytosol. Peroxisomes, which are also producers of ROS themselves, are most famous for their ROS scavenging capabilities. The ROS released into the cytosol can enter the peroxisomal matrix. Having enzymes such as catalase and peroxidase, the ROS can then be reduced to more stable forms, i.e. catalase reduction of H₂O₂ to H₂O (Foyer and Noctor 2003; Apel and Hirt 2004; Schrader and Yoon 2007; Bhattacharjee 2011).

It is quite clear that these organelles are linked metabolically and biochemically in the cell. What has also become apparent, but not investigated in depth, is their physical interactivity. The half-life of O₂⁻ is seconds and that of \(^1\)O₂ is a matter of microseconds. This means that they have short diffusion distances (Halliwell and Gutteridge 1999). For this reason, it would be expected that the scavenger, peroxisomes, would be found within very short distances of the ROS producers, chloroplasts and mitochondria. Fredrick and Newcomb (1969) published a series of TEM images which supported this. They showed peroxisomes in close, physical proximity with mitochondria and chloroplasts. When transgenics expressing a cytosolic hydrogen peroxide responsive probe, HyPer-GFP (Costa et al. 2010), were given a short 5min HL irradiation, the GFP fluorescence in the cytosol increased. This demonstrates the sheer rapidity of H₂O₂ production and release into the cytosol during HL irradiation. Simultaneous visualization of mitochondria and peroxisomes revealed that the length of interaction between these two organelles in dark grown plants increased within 30s to 2min of HL. This further supports that ROS production-scavenging in response to light stress is a contributor to mitochondria-
peroxisome interactions, which may be one of the necessary efficient mechanisms for maintaining ROS homeostasis.

The duration of interactions between the two organelles did however decrease after 4min of HL irradiation. Dark grown plants have etioplasts which contain protochlorophyllide. When etioplasts are photoactivated, they become chloroplasts because protochlorophyllide is converted to chlorophyll. Protochlorophyllide accumulates in the dark and when the dark-to-light shift occurs, it becomes chlorophyll. But $^{1}\text{O}_2$ is produced in the process. If the photoperiod and light intensity are too high, $^{1}\text{O}_2$ levels become cytotoxic and cell death occurs (Kim et al. 2008; Przybyla et al. 2008; Gechev et al. 2010). The decrease in interactivity of mitochondria and peroxisomes may have been because the HL stimulus caused a cytotoxic level of $^{1}\text{O}_2$ to be produced between 2-4min of irradiation, causing the cell to prepare for death or the cell was in disarray, attempting a last ditch effort to save itself.

FIS1 and DRP3 are involved in the fission of both peroxisomes and mitochondria (Mano et al. 2004; Arimura et al. 2008; Zhang and Hu 2008; 2009; Pan and Hu 2011; Logan et al. 2004; Scott et al. 2006; Schrader 2006; Delille et al. 2009). Neuspiel et al. (2008) showed that MDVs carrying MAPL was translocated from the OMM to the peroxisome membrane. MDVs have been suggested to be one way in which intramembranous material and even lipids can be delivered from mitochondria to peroxisomes (Schumann and Subramani 2008). Another means of transfer between these two organelles that has been suggested is through the increase of surface area of one of the organelles to increase the physical contact available for efficient transfer (Schumann and Subramani 2008). This was of particular interest.

Both peroxisome and mitochondrial extensions called peroxules and matrixules, respectively, have been reported (peroxules: Scott et al. 2007; Sinclair et al. 2009; Delille et al. 2010; Barton et al. 2014; matrixules: Logan et al. 2004; Logan 2006). Their functions however, have yet to be elucidated. Extensions are thought to permit increased surface area for inter-organelle transport. Sustained interactions between peroxules and small, fragmented mitochondria were reported here. This interaction is more apparent in $\text{any}1$, a mutant that was shown to be more vulnerable to light stress, than wild type. Given that 1) this mutant is more susceptible to light stress, 2) peroxules have been suggested to be stress sensors (Mathur et al.
that peroxules are produced during ROS stress, the interaction may be a means of transporting ROS and Ca$^{2+}$, both of which are signals involved in regulating stress responses (Brookes et al. 2004).

**CONCLUSIONS**

Mitochondria are one of the most pleomorphic organelles found in eukaryotic cells because of their constant fusion and fission. Light and sugar induce the fission of mitochondria into small, spherical forms typically seen in plant cells. O$_2$ limited conditions however, induce mitochondrial fusion which results in elongation and eventually expanded, giant mitochondria.
The ER is a mediator of these transitions because it constricts tubular mitochondria and applies a pulling force to separate the mitochondria after fission. Also, as ER tubules rearrange or flatten out into cisternae, the mitochondria rearrange accordingly. Mitochondria also interact with peroxisomes. During HL stress, which results in increased ROS levels, peroxisomes extend thin membrane projections called peroxules. Peroxules often extend around chloroplasts and have small mitochondria clustered around them. Simultaneous, live-imaging further reveals that ER tubules form a cage around mitochondria, peroxisomes and chloroplasts and that all four organelles are truly interactive.

**FUTURE DIRECTIONS**

The effects of light, sugar and O\textsubscript{2} depletion on mitochondria morphology in plant cells was of key interest here. These factors were shown to induce morphological transitions that may ultimately be linked to the metabolic status of the cell, which could be explored further. Future studies could also apply these conditions to better understand the mechanism behind fusion and fission. For example, the ER is now known to constrict mitochondria during fission in yeast, mammalian and plant cells. Since dynamin related proteins have a smaller diameter than that of a tubular mitochondrion, it has been suggested that the ER must constrict the mitochondrion enough to allow the dynamin to wrap around the membrane to facilitate fission (Friedman et al. 2011). However, why it is the ER that does this and how the site of fission is decided remains unknown. Investigations into MCS may give insight into this. One MCS that may be of interest is the voltage dependent anion channel (VDAC), for which primers and base vectors are available (Appendix III). This may give insight into one of the major reasons for ER-mitochondria interactions: the transport of Ca\textsuperscript{2+}, a key stress signal that has also been shown to regulate mitochondrial fission (Brookes et al. 2004).

Simultaneous live imaging of the ER, mitochondria, peroxisomes and chloroplasts was also carried out. This gave a greater appreciation for how dynamic and interactive the living cell is. These organelles are often found in close, sustained clusters which may permit inter-organelle transfer of lipids, metabolites, and signalling molecules such as ROS and Ca\textsuperscript{2+}. One means of
enabling efficient transfers between organelles is by increasing membrane surface area such as through extensions. Peroxules were shown here to interact with small, fragmented mitochondria. ROS is released from mitochondria during their fragmentation. Peroxules may be one way to effectively transport ROS or other metabolites. MDVs carrying MAPL have been shown previously to translocate from mitochondria to peroxisomes. Transgenic lines of DAL1-Eos and DAL2-Eos, Arabidopsis homologues of MAPL have been created and may be of interest to further investigate the transport aspect of explaining the sustained mitochondria-peroxisome interactions reported here. How peroxules are extended during these interactions could also be investigated. Peroxin11 (PEX11) is known to be a peroxisome elongation factor (Koch et al. 2010) and may be of interest in such an investigation.

*Sui generis*: in a class of its own; unique. This phrase was initially used to describe the very pleomorphic organelle, mitochondria (Cavers 1914). However, investigations of their morphological behaviours and interactions with other organelles during stress contradict this. No organelle is truly in a class of its own; they are all interactive entities that work together to permit life.
REFERENCES


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and ultrastructural transformation in mitochondria in the intact ascites tumor cell. *J Cell Bio* 51:123-137


69


Rahn O, Richardson GL (1940) Oxygen demand and supply. J Bact 41:225-249


Shockey JM, Gidda SK, Chapital DC, Kuan JC, Dhanoa PK, Bland JM, Rothstein SJ, Mullen RT, Dyer JM (2006) Tung tree DGAT1 and DGAT2 have nonredundant functions in triacylglycerol biosynthesis and are localized to different subdomains of the endoplasmic reticulum. *Plant Cell* **18**:2294-2313


Zhang X, Hu J (2009) Two small protein families, DYNAMIN-RELATED PROTEIN3 and FISSION1, are required for peroxisome fission in Arabidopsis. *Plant J* **57**:146-159


APPENDIX I
SUMMARY OF MITOCHONDRIAL MORPHOLOGY DISCUSSED

Figure I-1 Effects of light, dark and sugar on mitochondrial morphology
Table I-1 Cytosolic sugar, light and oxygen effects on mitochondrial morphology

<table>
<thead>
<tr>
<th>Condition</th>
<th>Effect on Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosolic Sugar Increase</td>
<td>fragmented</td>
</tr>
<tr>
<td>No Exogenous Sugar</td>
<td>enlarged, tubular</td>
</tr>
<tr>
<td>Light Exposure</td>
<td>fragmented</td>
</tr>
<tr>
<td>Dark Grown</td>
<td>enlarged to tubular</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>tubular, ring shaped, giant</td>
</tr>
</tbody>
</table>

**Figure I-2** Morphological effects of O₂ depletion on mitochondria
### APPENDIX II

**MUTANTS AND TRANSGENIC LINES**

Table II-1 Arabidopsis transgenics and mutants used and discussed in this thesis

<table>
<thead>
<tr>
<th>Name</th>
<th>FP-fusion(s) Expressed</th>
<th>Utilization for work in this thesis and potential follow-up work</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>any1</em> mito-GFP RER</td>
<td>GFP-β-ATPase RFP-HDEL</td>
<td>Investigation of ER role in mitochondrial pleomorphy and fission in response to HL stress</td>
</tr>
<tr>
<td><em>any1</em> mito-GFP Yperoxi</td>
<td>GFP-β-ATPase YFP-PTS1 RFP-HDEL</td>
<td>Investigation of mitochondria-peroxisome interactions and morphological correlations in response to HL stress</td>
</tr>
<tr>
<td><em>any1</em> mito-GFP Yperoxi RER</td>
<td>GFP-β-ATPase YFP-PTS1 RFP-HDEL</td>
<td>Investigation of mitochondria, peroxisome, chloroplast and ER interactions during HL stress</td>
</tr>
<tr>
<td><em>apm1-13</em> x YFIS1A</td>
<td>GFP-PTS1 pfis1a-YFP-FIS1A</td>
<td>Investigation of FIS1 localization and role in fission</td>
</tr>
<tr>
<td><em>BIGYN p35S-Eos-FIS1A</em></td>
<td>mEosFP-FIS1A under 35S promoter</td>
<td>Investigation of mitochondria and peroxisome interactions and fission</td>
</tr>
<tr>
<td><em>BIGYN pfis1a-Eos-FIS1A</em></td>
<td>mEosFP-FIS1A under native promoter</td>
<td>Investigation of mitochondria and peroxisome interactions and fission</td>
</tr>
<tr>
<td>CX-GFP</td>
<td>Calnexin signal peptide fused to GFP</td>
<td>To create a base vector whereby GFP could be C-terminally fused to a nucleotide sequence; to create another expression probe for visualizing the ER</td>
</tr>
<tr>
<td>CX-YFP</td>
<td>Calnexin signal peptide fused to YFP</td>
<td>To create a base vector whereby YFP could be C-terminally fused to a nucleotide sequence; to create another expression probe for visualizing the ER</td>
</tr>
<tr>
<td>CX-RFP</td>
<td>Calnexin signal peptide fused to YFP</td>
<td>To create a base vector whereby RFP could be C-terminally fused to a nucleotide sequence; to create another expression probe for visualizing the ER</td>
</tr>
<tr>
<td>DAL1-Eos</td>
<td>DAL1-mEosFP</td>
<td>Investigation of fission machinery translocated from mitochondria to peroxisomes for hierarchical fission events</td>
</tr>
<tr>
<td>DAL2-Eos</td>
<td>DAL2-mEosFP</td>
<td>Investigation of fission machinery translocated from mitochondria to peroxisomes for hierarchical fission events</td>
</tr>
<tr>
<td><em>drp3a</em> mito-GFP</td>
<td>GFP-β-ATPase</td>
<td>Investigation of mitochondrial pleomorphy during impaired fission</td>
</tr>
<tr>
<td>Name</td>
<td>FP-fusion(s) Expressed</td>
<td>Utilization for work in this thesis and potential follow-up work</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>drp3a RER</td>
<td>RFP-HDEL</td>
<td>Investigation of ER pleomorphy during impaired mitochondrial fission</td>
</tr>
<tr>
<td>drp3a Yperoxi</td>
<td>YFP-PTS1</td>
<td>Investigation of peroxisomal pleomorphy during impaired fission</td>
</tr>
<tr>
<td>elm1-1 RER</td>
<td>GFP-β-ATPase, RFP-HDEL</td>
<td>Investigation of ER-mitochondria interactions during impair mitochondrial fission</td>
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<tr>
<td>elm1-1 Yperoxi</td>
<td>GFP-β-ATPase, YFP-PTS1</td>
<td>Investigation of mitochondria-peroxisome interactions and pleomorphy</td>
</tr>
<tr>
<td>FIS1B-RFP</td>
<td>p35S-FIS1B-RFP</td>
<td>Investigation of mitochondria and peroxisome interactions and fission</td>
</tr>
<tr>
<td>mito-GFP RER</td>
<td>GFP-β-ATPase, RFP-HDEL</td>
<td>Investigation of ER role in mitochondrial pleomorphy and fission</td>
</tr>
<tr>
<td>mito-GFP Yperoxi</td>
<td>GFP-β-ATPase, YFP-PTS1</td>
<td>Investigation of mitochondria-peroxisome interactions and morphological correlations</td>
</tr>
<tr>
<td>mito-GFP Yperoxi RER</td>
<td>GFP-β-ATPase, YFP-PTS1, RFP-HDEL</td>
<td>Investigation of mitochondria, peroxisome, chloroplast and ER interactions</td>
</tr>
<tr>
<td>p35S-mEos-FIS1A</td>
<td>mEosFP-FIS1A under 35S promoter</td>
<td>Investigation of mitochondria and peroxisome interactions and fission</td>
</tr>
<tr>
<td>pah1 pha2 mito-GFP</td>
<td>GFP-β-ATPase</td>
<td>Investigation of effects of ER disruption on mitochondrial pleomorphy</td>
</tr>
<tr>
<td>pahl pha2 EosHDEL mito-GFP</td>
<td>GFP-β-ATPase, mEosFP-HDEL</td>
<td>Investigation of effects of ER disruption on mitochondrial pleomorphy</td>
</tr>
<tr>
<td>pah1 pha2 RER mito-GFP</td>
<td>RFP-HDEL, GFP-β-ATPase</td>
<td>Investigation of effects of ER disruption on mitochondrial pleomorphy</td>
</tr>
<tr>
<td>pfis1a-mEos-FIS1A</td>
<td>mEosFP-FIS1A under native promoter</td>
<td>Investigation of mitochondria and peroxisome interactions and fission</td>
</tr>
<tr>
<td>RFP-FIS1B</td>
<td>p35S-RFP-FIS1B</td>
<td>Investigation of mitochondria and peroxisome interactions and fission</td>
</tr>
<tr>
<td>VPS26A-Eos</td>
<td>VPS26A-mEosFP</td>
<td>Investigation of ER-mitochondrial MCS and interactions</td>
</tr>
<tr>
<td>VPS26A-RFP</td>
<td>VPS26A-RFP</td>
<td>Investigation of ER-mitochondrial MCS and interactions</td>
</tr>
<tr>
<td>Name</td>
<td>FP-fusion(s) Expressed</td>
<td>Utilization for work in this thesis and potential follow-up work</td>
</tr>
<tr>
<td>--------------------</td>
<td>------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>VDAC-RFP</td>
<td>VDAC-RFP</td>
<td>Investigation of ER-mitochondrial MCS and interactions (ex. tethering)</td>
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<tr>
<td>YFIS1A x FNRGFP</td>
<td>pfis1a-YFP-FIS1A FNRGP</td>
<td>Investigation of FIS1 localization</td>
</tr>
<tr>
<td>YFIS1A x mito-GFP</td>
<td>pfis1a-YFP-FIS1A GFP-β-ATPase</td>
<td>Investigation of FIS1 localization and role in mitochondrial fission</td>
</tr>
<tr>
<td>YFIS1A x mCherryPTS1</td>
<td>pfis1a-YFP-FIS1A mCherry-PTS1</td>
<td>Investigation of FIS1 localization and role in peroxisome fission</td>
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</tbody>
</table>

**Table II-2 References for mutants and probes used**

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<thead>
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<tr>
<td>any1</td>
<td>Fujita et al., 2013</td>
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<tr>
<td>apm1-13</td>
<td>Mano et al., 2004</td>
</tr>
<tr>
<td>BIGYIN</td>
<td>Scott et al., 2006</td>
</tr>
<tr>
<td>elm1-1</td>
<td>Arimura et al., 2008</td>
</tr>
<tr>
<td>FIS1B</td>
<td>Lingard et al., 2008</td>
</tr>
<tr>
<td>mCherryPTS1</td>
<td>Ching et al., 2012</td>
</tr>
<tr>
<td>mEosFP</td>
<td>Mathur et al., 2010</td>
</tr>
<tr>
<td>mito-GFP</td>
<td>Logan and Leaver, 2000</td>
</tr>
<tr>
<td>pah1 pah2</td>
<td>Eastmond et al., 2010</td>
</tr>
<tr>
<td>pfis1a-YFP-FIS1A</td>
<td>Ruberti, 2014</td>
</tr>
<tr>
<td>RER</td>
<td>Sinclair et al., 2009</td>
</tr>
<tr>
<td>RFP</td>
<td>Shockey et al., 2006</td>
</tr>
<tr>
<td>Yperoxi</td>
<td>Mathur et al., 2002</td>
</tr>
</tbody>
</table>
APPENDIX III
PRIMER INFORMATION

The following is a list of primers and plasmids created and discussed in thesis. Unless stated otherwise, all vectors have a pCAMBIA 1300 backbone, expressed under the pCaMV35S and terminated by the nos terminator.

Table III-1 Primer sequences

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Primer ID</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>dal1-mEosFP</td>
<td>Forward Spel JM311</td>
<td>GGACTAGTATGATTTGGGATCGGTTGAGTT</td>
</tr>
<tr>
<td></td>
<td>Reverse BamHI JM312</td>
<td>GGATCCTGACGATGATGTCTTTAGGAGCGCA</td>
</tr>
<tr>
<td>dal2-mEosFP</td>
<td>Forward Xhol JM329</td>
<td>CTCGAGATGATACATTGCCTGGGTGATTAC</td>
</tr>
<tr>
<td></td>
<td>Reverse BamHI JM330</td>
<td>CCGGATCCATGGCGGTAATTTCTAAAAAC</td>
</tr>
<tr>
<td>RFP (Ching et al., 2012) in 620</td>
<td>Forward BamHI JM345</td>
<td>GGATCCATGGCGGTCCTGGCGAGCTCAT</td>
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<tr>
<td></td>
<td>Reverse SacI JM346</td>
<td>TATGAGCTCTTAGGGCGCCGAGGACTGGC</td>
</tr>
<tr>
<td>pfis1a</td>
<td>Forward HindIII JM456</td>
<td>AAGCTTCACTACAGGCTACATCACATAC</td>
</tr>
<tr>
<td></td>
<td>Reverse Xbal JM457</td>
<td>TCTAGATAGGGCGGATTTTTAGGTTT</td>
</tr>
<tr>
<td>mEosFP-fis1a</td>
<td>Forward Nael JM458</td>
<td>ATATGCGCGATGGATGCTAAGACCTGACATCGA</td>
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<td></td>
<td>Reverse Spel</td>
<td>GCGCAGCTAGTTCACCTTCTGGAGCATGGAC</td>
</tr>
<tr>
<td>fis1b-RFP</td>
<td>Forward Sall JM339</td>
<td>GTCGACATGGACGCCGCGATAGGGGAAG</td>
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<td></td>
<td>Reverse BamHI JM340</td>
<td>GGATCCCGAGCTGATATGCGTCG</td>
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<td>mEosFP-fis1b</td>
<td>Forward Nael JM425</td>
<td>TTGCGCCGCATGGCGCGGCGGCGATAGGGGAAG</td>
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<td>Reverse Spel JM426</td>
<td>CGGAGACTAGTTAGCGGTGAAATGCTG</td>
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<tr>
<td>vps26A-RFP</td>
<td>Forward Xbal JM409</td>
<td>GCTTGAAGATGATGCTAAGGTCGACTG</td>
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<tr>
<td></td>
<td>Reverse BamHI JM410</td>
<td>GGATCCAGATGCTCTTTCCTGGAGCCTG</td>
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<tr>
<td>pdrp3a</td>
<td>Forward HindIII JM518</td>
<td>GAAGCTTACCTCAATTTCCGAGCTAT</td>
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<td>Reverse Xbal JM546</td>
<td>GTCTAGACGTTGATCGGATTTTCGAAT</td>
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<td>drp3a-GFP</td>
<td>Forward Nael JM520</td>
<td>CGCCGCGATGACTTGGAGAGGTCCAGGTCCTTCG</td>
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<tr>
<td></td>
<td>Reverse Spel JM547</td>
<td>GGACTAGTACGATTTGGAGAGGTCTTG</td>
</tr>
<tr>
<td>vdac3-RFP</td>
<td>Forward Xbal JM548</td>
<td>CTTCTGAAGATGATGCTAAGGTCGACTG</td>
</tr>
<tr>
<td></td>
<td>Reverse BamHI JM549</td>
<td>GGATCCGCGGATTTGAGAGAGGAGCTAAT</td>
</tr>
</tbody>
</table>
APPENDIX IV
DESCRIPTIONS OF SUPPLEMENTAL MOVIES

The following is a list and description of the movies used in this thesis. All movies were acquired with a scan time of 3.945s per frame and shown as 5 frames per second.

Movie 3.1 Mitochondrial elongation during water submergence

7 day old elm1-1 Mt-GFP RER plants grown in the light on MS medium with 3% sucrose were submerged in water under a coverslip. Over the span of the video, just over 15min, small spherical mitochondria continuously fused and became elongated, tubular structures.

Movie 3.2 Giant mitochondrial fusion

Plants expressing green-to-red photo-convertible mitoEos (mEosFP N-terminally fused to the β-ATPase subunit) were submerged in water under a coverslip for over 1h until giant mitochondria formed. One giant mitochondrion was UV irradiated for 30s, which photo-converted it from green to red. The other two were not photo-converted, and therefore remained green. The red and green giant mitochondria moved together and fused. This is indicated by a mixing of the green and red fluorophores within the matrix.

Movie 3.3 ER moulding a giant mitochondrion

A cell expressing Gmito and RER is shown. The ER around the mitochondria is flattened in sheets or cisternae. The mitochondria are also expanded. As the ER rearranges, the mitochondria on the left also shift into the space created by the surrounding ER. As the ER narrows, a mitochondrion forms a tubule. When the ER dilates, the mitochondrion does as well, which creates a beaded appearance.

Movie 3.4 ER-mediated mitochondrial fission

Elongated mitochondria are constricted by the ER. The ER is very dynamic, and constantly rearranging. The mitochondria move along with the ER. The ER-constricted area creates a point of weakness and as the mitochondria are pulled, fission occurs. The fragments are pulled apart and disperse throughout the cell. GFP=mitochondria; RFP=ER.
Movie 3.5 ER caging chloroplasts, mitochondria and peroxisomes

A triple transgenic plant expressing Gmito Yperoxi RER and chloroplast auto-fluorescence is shown. Some mitochondria and peroxisomes are seen moving quickly towards the bottom left of the frame. However, mitochondria and peroxisomes found within ER cages with chloroplasts are relatively immotile in comparison. The ER appears to contain the other three organelles. Consequently, all four organelles maintain sustained interactions. GFP=mitochondria; YFP=peroxisomes; RFP=ER; red chlorophyll auto-fluorescence false coloured blue=chloroplasts.

Movie 3.6 Sustained mitochondria-peroxisome interactions

A cluster of mitochondria, peroxisomes and a chloroplast moves together while maintaining an interaction with each other. Mitochondria and peroxisomes outside of this cluster move quickly in the direction of the cytoplasmic streaming without interacting. GFP=mitochondria; YFP=peroxisomes; blue=chloroplast.

Movie 3.7 Fragmented mitochondria clustering a peroxule

The peroxisomes have extended peroxules. The peroxisome body on the bottom right maintains contact with the chloroplasts. Small, spherical mitochondria are clustered around the peroxule. As the peroxule draws in towards the peroxisome body, the mitochondria cluster moves with it. Note that mitochondria and peroxisomes to the left are moving rapidly without interacting. GFP=mitochondria; YFP=peroxisomes; blue=chloroplasts.

Movie 3.8 Abundant peroxules in any1

This any1 cotyledon cell has abundant chloroplasts, small, spherical mitochondria and peroxisomes with peroxules. The organelles are quite clustered and the peroxules are very long. The peroxules give the impression that they are extending and retracting to grab hold of something within the cytosol. GFP=mitochondria; YFP=peroxisomes; blue=chloroplasts.