FRIENDLY REGULATES MITOCHONDRIAL DISTRIBUTION, FUSION, AND QUALITY CONTROL IN ARABIDOPSIS

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In Partial Fulfillment of the Requirements
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University of Saskatchewan
Saskatoon
Saskatchewan, CANADA

By

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Mitochondria are vital semi-autonomous organelles, containing their own genome (mtDNA) and protein synthesizing machinery, that provide energy for life and a trigger for death. Known as the powerhouse of the cell due to their roles in energy transduction, plant mitochondria are highly dynamic and their distribution inside a cell can be strikingly heterogeneous as a result of their differentially controlled motility on the cytoskeleton. Plant mitochondrial dysfunction can cause male sterility, defects in carbon metabolism and intracellular signaling. In humans, loss of mitochondrial function is implicated in numerous diseases such as cancer, Alzheimer’s, diabetes, blindness, Parkinson’s and many additional neurological and neuromuscular diseases. Mitochondria have key roles in energy conversion and integrated signaling pathways such as regulation of Ca\(^{2+}\) signals and programmed cell death (PCD) in plants and animals. Despite the highly dynamic nature of plant mitochondria, there is little specific scientific evidence linking mitochondrial morphology, distribution and movement to organelle and cell function. Plant mitochondria are normally distributed throughout the cytosol as single, discrete, spherical, or tubular-shaped organelles. However, the friendly mitochondria (fmt, friendly) mutant of Arabidopsis thaliana (Arabidopsis) contains clusters of tens of mitochondria in contrast to the wild type distribution. The results presented in this thesis show that the mutant has a short-root phenotype, non-viable cells in the roots, increased size and number of acidic compartments (autolysosomes) and displays an increased frequency of transient depolarization events, termed “pulses”, that are indicative of cellular stress and a failure of mitochondrial quality control. Furthermore, transmission electron microscopy (TEM) of ultra-thin sections shows that clustered mitochondria are discrete organelles. Fluorescence recovery after photobleaching (FRAP) studies confirmed the TEM results by showing no obvious connectivity of the matrices within a cluster. A recent study that identified the Drosophila homologue of the FRIENDLY, clueless, demonstrated that it genetically interacts with parkin, the Drosophila orthologue of a human gene responsible for many familial cases of Parkinson’s disease. Despite this genetic evidence linking clueless to a putative mitochondrial quality control pathway, the function of FRIENDLY protein and its orthologues and, therefore, the mechanism(s) underpinning the mutant clustered-mitochondria phenotype, remain unknown. Furthermore, we show that the C.elegans CLU-1 protein is required for normal mitochondrial
morphology and *clu-1* mutants display an increased number and size of autophagosomes compared to wild type. Moreover, interrelationships between clustered mitochondria, actin, and microtubules were studied, demonstrating a clear dynamic association between clustered mitochondria and actin, with no observed association between mitochondria and microtubules. We hypothesized that *friendly* mitochondrial clusters develop due to a fusion defect. In wild type cells, mitochondria that meet due to either rearrangement of, or movement on, actin filaments first interact through a “handshake process” involving FRIENDLY. However, in the absence of FRIENDLY, the handshake is prolonged such that clusters of mitochondria develop between slow hand-shaking organelles. Overall, it is proposed that FRIENDLY is the first protein to be identified that is involved in plant mitochondrial fusion and that fusion is necessary to allow mitochondria to minimize damage arising from oxidative stress. Lastly, this research suggests that disruption of mitochondrial association, motility, and chondriome structure in *friendly* affects mitochondrial quality control, and leads to mitochondrial stress, photosynthetic dysfunction, and cell death.
ACKNOWLEDGEMENTS

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In the end, I would like to thank my friends in Egypt and the faculty of Science for their support and help.
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<tr>
<td>ABP</td>
<td>Actin binding protein</td>
</tr>
<tr>
<td>AOX</td>
<td>Alternative oxidase</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>Clu</td>
<td>CLUstered mitochondria</td>
</tr>
<tr>
<td>Col-0</td>
<td>Columbia ecotype of <em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Drp1</td>
<td>Dynamin-related protein 1</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
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<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>$F_1$</td>
<td>First generation</td>
</tr>
<tr>
<td>$T_1$</td>
<td>First generation after transformation</td>
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<tr>
<td>Fis1</td>
<td>Fission 1 protein</td>
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<tr>
<td>$fmt$</td>
<td><em>friendly</em> (<em>friendly mitochondria</em>)</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LatB</td>
<td>Latrunculin B</td>
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<tr>
<td>L</td>
<td>Liter</td>
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<tr>
<td>LB</td>
<td>Luria Broth</td>
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<td>$\mu$L</td>
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<td>$\mu$m</td>
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<td>MT</td>
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<td>MAP4</td>
<td>Microtubule associated protein 4</td>
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<tr>
<td>mL</td>
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<td>mM</td>
<td>Millimolar</td>
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<td>min</td>
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<tr>
<td>mDNA</td>
<td>Mitochondrial DNA</td>
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<tr>
<td>Mfn2</td>
<td>Mitofusin 2</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PCD</td>
<td>Programmed cell death</td>
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<td>35S</td>
<td>Promoter of the Cauliflower Mosaic Virus 35S protein</td>
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<td>RFP</td>
<td>Red fluorescent protein</td>
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<tr>
<td>s</td>
<td>Second</td>
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<tr>
<td>F₂</td>
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<tr>
<td>TPR</td>
<td>Tetratricopeptide repeat</td>
</tr>
<tr>
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<td>Tricarboxylic acid</td>
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<td>UV</td>
<td>Ultraviolet</td>
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<td>wt</td>
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<td>in vivo</td>
<td>Within the living</td>
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CHAPTER 1. GENERAL INTRODUCTION

1.1. What are mitochondria?

Since 1857, when Albert von Kolliker, a Swiss physiologist and anatomist, identified small granule-like structures in muscle cells (0.5 to 10 $\mu$m in diameter), which he named bioblasts, mitochondrial researchers have focused on elucidating the roles and functions of the mitochondria in living cells. In 1898, Carl Benda renamed bioblasts as mitochondria or ‘thread granules’ from the Greek ($mitos$ meaning thread and $chondros$ meaning granule) (Ingman and Gyllensten, 2003; Williamson, 2002). The endosymbiotic theory is used to explain the origin of mitochondria. This theory posits that an ancestor of extant alpha-proteobacteria formed a permanent partnership with “protoeukaryotes” (Emelyanov, 2003; Gray et al., 1999). Supporting evidence for this endosymbiotic theory indicates the facts that mitochondrial DNA (mtDNA) is organized as single and circular chromosomes like that found in prokaryotes (Karlberg and Andersson, 2003), is packed in nucleoids, and uses a variable genetic code like that of proteobacteria (Knoop et al., 2011). In addition, ribosomes located in plant mitochondria closely resemble those from bacteria in size and structure (Bonen and Calixte, 2006); mitochondria contain smaller ribosomes that found in the cytoplasm of eukaryotes. Mitochondria also contain the bacterial 70S ribosome, and not the 80S cytoplasmic ribosome found in eukaryotes (O’Brien, 2003). Mitochondria are said to be semi-autonomous: the nuclear genome encodes the majority of mitochondrial polypeptides but others are encoded by the mitochondrial genome. These mitochondrial polypeptides are translated in the cytosol and post-transcriptionally imported into mitochondria (Unseld et al., 1997; Duby and Boutry, 2002). Thus mitochondria are dependent on the cell for replication and growth, but also on their own genome (Westermann, 2010). Mitochondria cannot be synthesized by the cell, instead they replicate by binary fission, much like in bacteria. Thus, there is overwhelming evidence supporting the endosymbiotic origin of mitochondria.

As mentioned, despite their reliance on the nucleus, mitochondria contain their own genome and protein synthesizing machinery (Unseld et al., 1997; Gray et al., 1999). While their chief role is the production of cellular energy, they are involved in many metabolic processes such as the biosynthesis of amino acids, vitamin cofactors, iron-sulfur clusters and fatty acids.
(Mackenzie and McIntosh, 1999; Bowsher and Tobin, 2001). In plant cells, mitochondria are also required to maintain optimal rates of photosynthesis, especially in changing light conditions. Mitochondria have also been associated with cell signaling (especially calcium based processes) and linked to the initiation of programmed cell death (PCD) (Douce and Neuburger, 1999; Jones, 2000; Logan and Knight, 2003; Youle and Karpowski, 2005).

To perform these cellular functions, mitochondria are highly dynamic, pleomorphic and have a complex, dynamic internal structure (Fig. 1.1a, b & c). They are composed of at least six compartments: an outer membrane that encloses the entire organelle; an inner boundary membrane that encloses almost all of the mitochondrial proteins; an inter-membrane space that separates the inner and outer membrane; a cristal membrane or a folded inner membrane, an inter-cristal space, and the matrix which is enclosed by the inner membrane (Fig. 1.1a & b) (Karlberg and Andersson, 2003; Logan, 2006b).

Mitochondria in plants, like in most animals, are inherited maternally, however, plants, unlike animals, have a highly variable-sized mitochondrial genome (reviewed in Rose and Sheahan, 2012). The simple circular mtDNA of *Marchantia polymorpha* (liverwort) was the first complete sequenced mtDNA of a land plant, and its size (186 kbp) was significantly larger than that of the human mitochondrial genome (16.6 kbp) (Oda et al., 1992).

Interestingly, plant mitochondrial genomes are composed of DNA molecules found in small and large circular, or circular permuted forms (Lonsdale et al., 1988; Bendich, 1996; Oldenburg and Bendich, 1996) rather than the linear chromosomes. These sub-chromosomes may autonomously replicate, once produced (Lonsdale et al., 1988; Janska et al., 1998; Abdelnoor et al., 2003). This is known to lead to the existence of discrete mitochondria within plant cells that lack a complete genome (Preuten et al., 2010). Thus, not all mitochondria in plant cells are identical. A series of repeat regions in plant mitochondrial genomes allow intra- and intermolecular recombination to occur with high frequency, therefore the genomes of mitochondria exist as a subgenomic series (Bendich, 1996). Due to the organization of plant mitochondria as hundreds of physically discrete organelles per cell, and the recombination driven organization of the mitochondrial genome into subgenomic units, plant mitochondria undergo frequent inter-mitochondrial fusion. Fusion, followed by division, solves the problem of variable gene content by enabling mixing of the mtDNA and thus plant mitochondria can be viewed as forming a ‘discontinuous whole’, at least genetically (Sheahan et al., 2005; Logan, 2006b).
Figure 1.1. The schematic shows the structure of mitochondria. (a & b) Show the structure of mitochondria (Lodish et al., 2000; Logan, 2006b) (Permission is obtained from the author, (Logan, 2006)).(c) A transmission electron microscopy image of mitochondria in Arabidopsis leaf after fixation by high pressure freezing.
Despite the great differences in the sizes of plant mitochondrial genomes, partly due to the presence of the repeat regions (Wolstensholme and Fauron, 1995), the mitochondrial genomes of all species sequenced to date encode a similar set of proteins. There are 58 open reading frames in the Arabidopsis mitochondrial genome, which encode subunits of the electron transport chain (ETC) complexes and some components of the translational apparatus (reviewed in Rose and Sheahan, 2012).

As stated previously, most mitochondrial proteins must be imported because they are nuclear encoded. The import of mitochondrial proteins involves a chaperone that unfolds the protein. The translocase of the outer membrane (TOM) is the complex that then threads the protein through the mitochondrial membrane and acts as a selective importer of mitochondrial proteins from the cytosolic protein pool (reviewed in Rose and Sheahan, 2012). Three different pathways mediate protein transport through the intermembrane space. The first two pathways involve either the translocase of the intermembrane (TIM) complex pathways (TIM17:23 or TIM22) and the third pathway is through the mitochondrial intermembrane space import and assembly (MIA) pathway. TIM17:23 complex is composed of eight proteins in Arabidopsis while the TIM22 pathway has only one member (Tim22) which is responsible for transporting proteins containing internal targeting signals into the inner membrane of mitochondria (Perry et al., 2008; Carrie et al., 2010). This complex protein import process is part of the trade off of the endosymbiotic partnership: reduction of the size of the mitochondrial genome due to transfer of the genes to the nucleus meant that a process was required to get the protein back in to the mitochondrial compartment.

The transport of mitochondrial metabolites and small molecules is also facilitated by pores formed by voltage-dependent anion channels (VDACs; Mannella, 1992)). Duncan et al. identified four different VDACs in Arabidopsis (Duncan et al., 2011). Of course there are also many specialized membrane transporters such as the pyruvate transporter, which brings the end product of glycolysis to the mitochondria where it is decarboxylated and passed on to the Citric Acid Cycle or tricarboxylic acid cycle (TCA). In summary, mitochondria are complex, semi-autonomous structures found in eukaryotic cells. Many of their complexities are adaptations necessary to adapt from the ancestral free-living life to their role as vital components of the eukaryotic cell.
1.1.1. Mitochondrial metabolism generates ATP for the cell

To perform its function as the major supplier of ATP in aerobic cells, the TCA cycle enzymes are found within the mitochondrial matrix, which produces NADH and FADH2, the reduced electron carriers, from pyruvate. Pyruvate is transported from the cytosol directly into the mitochondria but can also be produced from the conversion of malate, by malic enzyme, into pyruvate within the mitochondrial matrix (Tronconi et al., 2008). The pyruvate dehydrogenase complex oxidizes pyruvate into acetyl-CoA, which subsequently enters the TCA cycle. It oxidizes acetyl-CoA to carbon dioxide and produces reduced NADH and FADH2.

Plant mitochondria, like other eukaryotes, have an ETC embedded in the inner and cristal membranes where reduced electron carriers from the TCA cycle are oxidized. This enables protons to be pumped into the intermembrane and intercristal spaces. The resulting $H^+$ and electrochemical gradient drives ATP synthesis using an ATP synthase that is confined to the cristal membrane. To power the rest of the cell, the ATP produced in the matrix is exported through an ADP/ATP antiporter.

The ETC in plant mitochondria, where reduced electron carriers are oxidized, allows protons to move across into the intercristal and intermembrane spaces, which is used to drive the process of ATP production via $H^+$-ATP synthase. Ubiquinone and cytochrome $c$ act as mobile electron carriers that are used by the four ETC protein complexes to communicate with each other. The largest complex contains 49 subunits, called Complex I or NADH-ubiquinone oxidoreductase, which oxidizes NADH to reduce ubiquinone. Succinate-ubiquinone oxidoreductase or Complex II has 4 core subunits and is the smallest of the complexes. It reduces ubiquinone by the oxidation of succinate to fumarate. Complex III is called ubiquinone cytochrome $c$ oxidoreductase and contains 10 subunits. It oxidizes ubiquinone to reduce cytochrome $c$. Finally, Complex IV or cytochrome $c$ oxidase, has 4 subunits and reduces oxygen to water from the oxidization of cytochrome $c$. A pH and electrical gradient is created due to the proton translocation at complexes I, III, and the reduction of $O_2$ to $H_2O$ by complex IV. This gradient generates ATP energy as protons are allowed back into the matrix via $H^+$-ATP synthase. This complex has 5 subunits making up a ‘knob’ that sticks into the matrix, which is connected to a 14-subunit ‘stalk’ found along the inner membrane. Interestingly, the ATP synthase may
also be responsible for the inner mitochondrial membrane folding into cristae (Dudkina et al., 2006).

1.1.2. Special features of electron transport in plants

An alternative nonphosphorylating ETC is also present in plants and some fungi in addition to the classic ETC. The inner membrane of the mitochondria contains a cyanide-insensitive alternative oxidase (AOX), and on both sides of the inner membrane contains rotenone-insensitive alternative NAD(P)H dehydrogenases (NDs) (Moller, 2001; Elhafez et al., 2006). The alternative ETC catalyzes the movement of electrons transferred from ubiquinone to oxygen (AOX) or from NAD(P)H to ubiquinone, which occurs without concurrently translocating protons. This alternative ETC serves to uncouple electron transport from the production of ATP. Plant mitochondria also contain in the inner membranes uncoupling mitochondrial proteins (PUMPs; (Zhu et al., 2011)), which are homologues of mitochondrial uncoupling proteins seen in mammals. PUMPs transport protons from the mitochondrial intermembrane space into the matrix, which diminishes the electrochemical gradient formed by the ETC without forming ATP.

Many studies of alternative electron transport in plant mitochondria have focused on the role of these processes in plant thermogenesis - the process whereby plants increase their temperature above ambient as a result of the uncoupling of respiration from oxidative phosphorylation. This phenomenon occurs from the dissipation of energy from the proton gradient generated by PUMPs and instead of using the energy from electron flow to produce ATP, it is diverted into heat production. The exact mechanisms are not clear but it could involve the mitochondrial pyruvate concentration and the redox state of the pool of ubiquinone (Zhu et al., 2011).

In addition to the role of alternative electron transport in thermogenesis, much research is being conducted into the role of the process with regard to plant responses to stress. The plant alternative oxidase (AOX) typically responds to stress conditions (Polidoros et al., 2009). While substantially more ATP is generated by aerobic metabolism as opposed to anaerobic metabolism, the disadvantage of aerobic metabolism is the production of the toxic byproducts, reactive oxygen species (ROS). ROS are produced through mitochondrial respiration. Production of ROS...
in excess leads to loss of cell function, and ultimately damage to the cell (DNA, proteins and lipids). ROS are therefore considered an important stress-signaling molecule (Navrot et al., 2007). ROS production, however, can be controlled by plant mitochondria by diverting electron flow to the alternative electron pathway, thereby minimizing mitochondrial ROS production to sustain ROS homoeostasis (Polidoros et al., 2009).

Several studies indicate that mitochondria play a key role in regulating this ROS homeostasis and even direct appropriate cellular responses to stress. Plants have several defense systems to prevent deleterious ROS production including an antioxidative defense complex (Minibayeva et al., 2009). This mitochondrial mechanism detoxifies ROS by utilizing antioxidants such as manganese superoxide dismutase from the ascorbate-glutathione cycle as well as from the peroxiredoxin/thioredoxin system (Navrot et al., 2007). Interestingly enough, plant mitochondria are also able to directly modify the production of ROS by diverting the electron flow from classic ETC to alternative ETC. Nevertheless, antioxidant-recycling can fail under continual stress, leading to the accumulation of ROS (Schafer and Buettner, 2001). Of concern for the cell, the role of mitochondria as containers for their own DNA is incompatible with the bioenergetics role of mitochondria since the transport of electrons generates ROS that can induce mtDNA lesions. While the mtDNA encodes only a small number of genes, the ones it contains are critical for cell survival. Thus, a mechanism must exist to protect and repair the mtDNA. This is one reason that has been suggested for the transfer of proteins encoding genes to the nucleus over evolutionary time.

1.1.3. Mitochondrial pulsing is a relief valve that protects the organelle from an over reduced ETC

In healthy, unstressed mitochondria, a balance between proton (H+) pumping electron transport and utilization of the H+ gradient to produce ATP is maintained. The proton pumping components of the electron transport chain, complexes I, III and IV, pump H+ from the matrix into the intermembrane space. The ATP-synthase uses the potential energy associated with the electrochemical gradient to convert ADP + Pi into ATP. Under stress conditions, such as high or low temperature or salt exposure, the balance between electron transport and ATP synthesis can be disrupted. As a result, if the ETC becomes highly reduced it can pass e− to O2 to form ROS.
Figure 1.2. Pulsing. (A) Images showing mitochondria stained with TMRM in Arabidopsis root epidermal cells. (B) Images showing a single mitochondrion (white arrow) undergoing a pulse. (C) Fluorescence intensity traces of mitochondria targeted GFP (green) and TMRM (red). (Permission was obtained from the author, (Schwarzlander et al., 2012)).
Figure 1.3. Frequency of mitochondrial pulses in response to abiotic stress treatments (heat, oxidative, salt, osmotic, and heavy metal) in Arabidopsis epidermal root cells expressing mito-GFP (permission was obtained from the author, (Schwarzlander et al., 2012)).
Similarly, if the gradient is too large, the ETC complexes will be unable to pump H\(^+\) ions across the inner membrane. As the ETC complexes remain reduced, the chance of them transferring electrons to oxygen increases.

Recently, Schwarzlander et al. discovered a protective mechanism called “pulsing” whereby the mitochondrial inner membrane becomes partially and transiently depolarized (Schwarzlander et al., 2012). This transient depolarization results in the dissipation of the H\(^+\) gradient, allowing the ETC complexes to once again transport H\(^+\) ions across the membrane. This protects the mitochondrion and thus the cell from the damaging production of ROS caused by over reduction of the electron transport chain. The change in the H\(^+\) gradient, more accurately described as an electrical potential, can be detected using TMRM (tetramethyl rhodamine methyl ester) fluorescent dye. When the electrochemical gradient is high and hence the ETC is highly reduced, the fluorescent dye accumulates in the mitochondrial matrix in proportion to the membrane potential (Brand and Nicholls, 2011). Thus, pulsing can be visualized and quantified in single mitochondria in living Arabidopsis root cells using fluorescent microscopy, (Fig. 1.2). The frequency of pulses increases with environmental stresses such as salt, heat, osmosis, oxidative and heavy metal stress treatments (Fig. 1.3) (Schwarzlander et al., 2012).

1.2. Mitochondrial dynamics

Mitochondrial dynamics is the study of mitochondrial behaviour, distribution, shape, size and number. This also includes the mechanisms, genes and proteins controlling their movement in vivo (Scott and Logan, 2011). Mitochondrial dynamics vary according to cell type and organism. The higher plant chondriome (the collective term for all mitochondria in a cell) differs from that of animal cells and yeast (Fig. 1.4) (Logan, 2010a). In yeast, mitochondria are organized as reticulo-tubular-shaped, interconnected organelles (Prokisch et al., 2000). In animals, mitochondria are generally elongated and tubular-shaped, while in higher plants, mitochondria are normally oval-shaped to sausage-shaped organelles (Fig.1.4) (Logan and Leaver, 2000; Frank et al., 2001; Logan, 2006a).

Mitochondria are highly dynamic in plant cells, which contain several hundred physically discrete mitochondria. For instance, Arabidopsis mesophyll cells typically contain 200-300 mitochondria (Logan, 2010b). The production of stable Arabidopsis lines expressing
Figure 1.4. Mitochondrial morphology in yeast, animal and plant cells. (a) Mitochondrial phenotype in yeast; (b) human Hela cell; (c) in Arabidopsis mesophyll protoplast. Scale bar = 5 µm (Permission was obtained from the author (Scott and Logan, 2011)).
mitochondria-targeted fluorescent proteins has enabled researchers to visualize mitochondria to study their dynamics in plants (Logan and Leaver, 2000).

Regulation of mitochondrial movement and position within the cytosol has been demonstrated to be essential for correct mitochondrial and cellular function in yeast (Frederick and Shaw, 2007; Boldogh and Pon, 2007; Logan, 2010a; Shutt and Mcbride, 2013). Indeed, mitochondria display altered motility and distribution under stress conditions or when functionality is impaired, in a variety of cell types across diverse eukaryotic lineages (Nunnari and Suomalainen, 2012; Chen and Chan, 2009). For example, in Arabidopsis, clustering and arrest of mitochondrial movement has been identified as a response to biotic and abiotic stress: in response to UV exposure (Gao et al., 2008); to the presence of methyljasmonate (Zhang and Xing, 2008); following treatment with the oxylipin 9-HOT (Vellosillo et al., 2013); and in response to heat shock or application of ROS (Scott and Logan, 2008).

In plant cells grown in culture, during the various growth stages, mitochondrial morphology and dynamics change. Cells that are healthy and growing have a reticular arrangement of mitochondria. Once cells begin to enter senescence, the network of mitochondria disintegrates (reviewed in (Welchen et al., 2014)). Other studies have shown that mitochondria change shape from tubular to spherical in Arabidopsis during the onset of PCD (Yoshinaga et al., 2005). This evidence supports the notion that mitochondria are dynamic, changing shape, position, and number inside living cells. These changes occur in response to a number of factors, including the phase of the cell cycle, the developmental stage, tissue type, external stimuli, cell demand of energy, and PCD, showing the central role mitochondria play with their dynamic behavior in many important cellular activities (reviewed in (Welchen et al., 2014)). Mitochondrial dynamics is therefore not only key to the cell functioning but also to the cellular response to stress.

Evidence showing that mitochondrial dynamics and their function are interrelated was provided by studies investigating the various stages of the cell cycle in plant mitochondria. Serial thin-sections of Arabidopsis observed by electron micrograph were prepared at various growth stages from the apical meristem, showing mitochondria in a large sheet-like manner, undergoing distinctive structural differences throughout the cell cycle (Seguí-Simarro and Staehelin, 2009). These researchers proposed that large reticulate mitochondria deliver ATP in an efficient way for cytokinesis and the cell cycle, allowing for effective mitochondrial DNA recombination and
mixing (mitochondrial DNA; Segui-Simarro et al., 2008).

Recent research by Islam and Takagi (2010) indicated changes in the location of mitochondria that were associated with movement of chloroplasts under various light regimes. In the palisade mesophyll cells, mitochondria were distributed randomly when plants were in the dark regime. But when different light intensities were used, mitochondria moved in a coordinated fashion along with chloroplasts, suggesting that they are either physically associated with the chloroplasts by the cytoskeleton or they are following a specific signal. Even though there is no specific evidence to prove this, it is thought that metabolites are exchanged through this close association which is required in order for efficient photosynthesis to be maintained (Islam and Takagi, 2010).

Despite these findings, little is known about the mechanisms of regulating mitochondrial dynamics in higher plants. However, disruption of the actin cytoskeleton has resulted in an aberrant distribution of mitochondria (Sheahan et al., 2004). Therefore, it is important to understand the relation between the cytoskeleton and mitochondria. Combined use of fluorescent markers for mitochondria and cytoskeleton elements has facilitated the understanding of mitochondrial dynamics. Thus an understanding of the plant cytoskeleton is intimately related to the study of mitochondrial dynamics.

1.2.1. Cytoskeleton elements

The plant cytoskeleton consists of: microtubules and actin filaments. These two distinct networks are highly dynamic and perform many functions with the assistance of motor proteins. Unlike animal cells, plants do not have intermediate filaments.

1.2.1.1. Microtubules

Microtubules (MTs) are crucial components of the cytoskeleton. MTs are cylindrical tubes formed from parallel polymers known as protofilaments in which the α- and β- tubulin heterodimers are assembled in a head and tail polar manner. Each tubulin subunit is a heterodimer that consists of two polypeptides, α- and β- tubulin. Guanosine triphosphate (GTP) binds to the β-tubulin subunit of the heterodimeric tubulin molecule, causing the α- and β-
tubulin subunit to hook onto another α-β-tubulin at the plus-end of a microtubule, forming a protofilament. The protofilament then lines up with another protofilament, forming flat sheets which eventually roll up into a hollow cylinder forming a MT (Mitchison and Kirschner, 1984; Erickson and O’Brien, 1992). MTs are composed of 13 protofilaments running in a parallel fashion (Hirokawa, 1998; Vale, 2003). Because of the polar nature of their assembly, MTs have two ends, the plus-end and the minus-end. When MT ends are stable, microtubules grow by the addition of more subunits. However, when one of its ends disassembles, pressure is put on the microtubules causing the protofilaments to splay apart like the peeling of a banana (Desai and Mitchison, 1997). GTP hydrolysis destabilizes MTs favoring their disassembly (catastrophe).

Dynamic instability, required for cytoskeletal behavior in the cell cycle, occurs when a microtubule rapidly grows by the addition of tubulin subunits to the polymer plus-end faster than the hydrolysis of GTP at the minus-end (Erickson and O’Brien, 1992; Ludueña, 1993; Sirajuddin et al., 2014). The dynamics of microtubules (growing and shrinking) occurs by binding the ends of microtubules to cellular structures such as chromosomes, mitotic spindles, the plasma membrane and other organelles (Howard and Hyman, 2003). It was reported that GTP-tubulin forms a cap at the tip of the fiber that stabilizes the plus-end. The breaking of this cap enhances the catastrophe phase or disassembly (Blume et al., 2008). It has been proposed that catastrophes are induced by kinesin-8 family depolymerizing motors, a form of microtubule-associated protein (MAP), whereby removal of the terminal tubulin subunit occurs by depolymerizing motors moving down to the plus-end of the microtubule in yeast (Varga et al., 2006, 2009). The direct mechanism of MAPs altering the properties of microtubule dynamics is not well understood.

MT polymerization and depolymerization processes are very important for the construction of microtubule arrays. Studying polymerization of tubulin molecules in vitro has enhanced the understanding of MT dynamics, showing that elongation of MTs is rapid, while the nucleation of new MTs is slow. Many MT arrays are highly dynamic and sensitive to specific antimitotic drugs. Some antimitotic drugs inhibit MT polymerization by binding to the tubulin subunit. Taxol has the opposite effect. It stabilizes MT polymerization by binding to the β-tubulin subunit, preventing hydrolysis from occurring. Using these drugs, it was demonstrated that MTs polymerize and depolymerize during mitosis. Many biomolecules targeting tubulin have been widely used to study microtubule structure and dynamics. Several tubulin binding
drugs, such as Colchicine, Nocodazole and Benomyl inhibit the assembly of MTs. Other tubulin binding agents such as Vinblastine, Oryzalin, and Trifluralin, bind between heterodimers and depolymerize MT. These molecules are varied in structure and have many effects on MTs by interacting with tubulin proteins or by shifting the balance between free dimers and MT composition (Amos, 2011). These types of drugs have also allowed for the examination of MT based trafficking.

In higher plants, MT nucleation is distributed throughout the cytoplasm, while in animals and yeast, microtubule organizing centers (MTOCs) such as centrosomes and spindle pole bodies exist (Hamada, 2007). In animal cells, the interphase cortical array of MTs has the minus-ends of microtubules anchored to the MTOCs. Because higher plants do not have discrete MTOCs, it is difficult to identify where cortical microtubules originate (Goddard et al., 1994; Wade and Hyman, 1997). During the cell cycle, microtubule assembly and disassembly forms different MT arrays (Amos and Klug, 1974).

Many proteins are associated with microtubules. Motor proteins and vesicle transport proteins are important. Two classes of MT-dependent motor proteins were identified in animals: cytoplasmic dyneins and kinesins (Skoufias and Scholey, 1993). Cytoplasmic dyneins are involved in mitosis and vesicle transport. Kinesins are involved in organelle transport, in mitosis and meiosis, and in the transport of synaptic vesicles (Skoufias and Scholey, 1993).

In plants, only kinesin has been discovered as a microtubule motor protein (Mitsui et al., 1993). Kinesin super-family proteins are crucial microtubule-based motor proteins with a kinesin motor domain found in all eukaryotes. Kinesins function in cytokinesis, signal transduction, morphogenesis and transporting unidirectional vesicles and organelles (Reddy and Day, 2001; Verhey et al., 2001; Hirokawa et al., 2009). Kinesin motor proteins are composed of two heavy chains and several light chains. Every heavy chain contains conserved ATP binding heads and a tail composed of rod-like domains (Skoufias and Scholey, 1993). The two head domains are ATPase motors that bind to MTs; while the tail binds to specific cell components specifying the type of cargo the protein transports (Skoufias and Scholey, 1993). It was reported that the motor protein kinesin is unidirectional on MTs, meaning it moves, either toward the plus-end or toward the minus-end. As such, kinesins are responsible for the unidirectional transport of cargo such as membranous organelles, mRNAs, and protein complexes via MT filaments, or cell highways (Miki et al., 2001).
1.2.1.2. Actin filaments

Like microtubules, actin filaments are polar structures with a fast growing plus (or barbed) end and a slow growing minus (or pointed) end. The polymerization rate between the two ends is different and the polymerization of actin in vitro requires ATP (Forscher and Smith, 1988). The actin cytoskeleton supports many cellular processes such as cell expansion, cell division, organelle motility, vesicle trafficking and cell elongation (Bannigan and Baskin, 2005; Hussey et al., 2006). It was recently shown that the actin cytoskeleton plays a crucial role in plant cell growth and morphogenesis (Mathur, 2004; Hussey et al., 2006). The role of actin filaments in mediating mitochondrial dynamics in plant cells has been known for many years (Van Gestel et al., 2002).

A large superfamily of ATPases activated by actin, known as myosins, function as motors, propelling cargo along actin filaments or propelling filaments of actin along stationary cell structures. Myosins are involved in transporting organelles such as peroxisomes, Golgi and mitochondria on filaments of actin (Jedd and Chua, 2002; Sparkes et al., 2008). Seventeen myosin genes have been identified in the Arabidopsis genome (Reddy and Day, 2001). Using colocalization with Arabidopsis proteins that are transiently expressed in tobacco, the myosin XI-J protein was found to colocalize with mitochondria (Resin and Hanson 2007). However, a pharmaceutical approach found evidence that there are other mechanisms for moving mitochondria on the actin cytoskeleton using proteins other than myosins (Zheng et al., 2009).

Thus, mitochondria appear to use myosin-dependent and independent motor mechanisms to move (Zheng et al., 2009). Zheng et al. (2009) concluded that mitochondrial speed, positioning, and directional movement were controlled and coordinated by the rate of actin turnover and myosin activity. Complicating the story further, Zheng et al. (2009) found that the arrangement of actin filaments was also influenced by microtubule dynamics. It was also reported by Wada and Suetsugu (2004), that actin filaments are involved in the transport of organelles, such as chloroplasts, endoplasmic reticulum, peroxisomes and Golgi stacks (Wada and Suetsugu, 2004).

Several fluorescent fusion proteins have contributed substantially in visualizing actin organization in living plant cells: one of them is GFP-mTalin (an in frame fusion of GFP to the C-terminal 197 amino acids of mouse talin which forms the actin binding domain (ABD)), and
fusion protein is GFP-ABD2-FIMBRIN1 (Kost et al., 1998; Sheahan et al., 2004).

Drugs such as latrunculin B and jasplakinolide can be used to disrupt or stabilize actin, respectively. As a result, the structure of the actin cytoskeleton changes immediately (Morton et al., 2000). A recent study by Wang et al., indicated that actin filament disruption caused by latrunculin B or jasplakinolide resulted in mitochondrial calcium release into the cytoplasm suggesting that actin polymerization and depolymerization are important for mitochondrial calcium storage in Arabidopsis root hairs (Wang et al., 2010).

Fluorescent fusion proteins and microscopic systems has facilitated the study of MT and actin dynamics and interactions with their motor proteins and mitochondria.

1.2.1.3. Cytoskeleton and mitochondria in plants

In plants, the organization of decentralized microtubule arrays is largely dependent on the ability of microtubules to self-organize. This organization is based on the activity of kinesin. It has been shown with multinucleate endosperm tissue that the direction of MT array extension and retraction is controlled by the linking of microtubule plus-ends to the MT convergence center (Smirnova and Bajer, 1994, 1998).

As mentioned above, the cytoskeleton plays a crucial role in regulating mitochondrial shape and function. Movement of mitochondria allows them to be transported into specialized cellular sites (Anesti and Scorrano, 2006). Animal and yeast mitochondria (with the notable exception of Saccharomyces cerevisiae) move on MTs, whereas in plants, mitochondria move on actin. Unlike in animals and yeast, plant mitochondria dynamics do not directly depend on MT arrangements (Scott and Logan, 2010). The difference in the cytoskeletal track used for movement may in part explain the difference in gross chondriome structure. Compelling evidence has emerged indicating that mitochondria associate with energy-consuming structures such as sperm axoneme, endoplasmic reticulum, and flagella (Hales and Fuller, 1997; Bereiter-Hahn and Vöth, 1994; Scott and Logan, 2010).

Mitochondria move on actin filaments in plant cells (Olyslaegers and Verbelen, 1998; Van Gestel and Verbelen, 2002) while microtubules (MTs) have been suggested to play a role in positioning and/or tethering mitochondria in the cortical cytoplasm of elongated tobacco cells (Van Gestel and Verbelen, 2002). This mitochondrial positioning might influence mitochondrial
activity (Logan, 2010b). Myosin and kinesin are actin and microtubule-specific motor proteins. Kinesin is known to bind mitochondria via the tetratricopeptide repeat (TPR) domains of the kinesin light chains, suggesting that kinesin light chains control binding of kinesin to its cargo (Khodjakov et al., 1998).

1.3. The role of fluorescent proteins in studying mitochondrial dynamics

Fluorescence protein (FP) technology is an extremely powerful tool for use in cell biology allowing researchers to monitor the protein location within a cell. Green fluorescent protein (GFP) is an in vivo marker that can be used to color living organelles (Shimomura et al., 1962; Tsien, 1998). GFP is a stable protein that emits green light at a peak emission of 509-511 nm, with a shoulder at 540 nm when absorbing blue light (Morin and Hastings, 1971; Morise et al., 1974; Prasher et al., 1992). It can be fused to a protein of interest and the functional integrity of both FP and the protein of interest are usually retained. Because FP fusions can be targeted to discrete subcellular compartments, FP technology can also be used to monitor the morphology of organelles in real-time (Prasher et al., 1992; Tsien, 1998). Logan and Leaver (2000) generated the first stable transgenic Arabidopsis lines expressing mitochondrial targeted GFP. These Arabidopsis lines have allowed plant mitochondrial researchers to study the dynamics of mitochondria, and the heterogeneity of mitochondrial dynamics in living cells.

The discovery of other fluorescing proteins has expanded the versatility of FP technology. For instance the stony coral, *Trachyphyllia geoffroyi*, expresses proteins that emit green, red or yellow light. The fluorescent protein responsible, Kaede, was found to be a tetrameric protein that converts irreversibly from green to red forms when exposed to near UV light (Ando et al., 2002). Wiedenmann et al., (2004), suggested that FP Kaede could be a useful and valuable tool for localized subcellular marking.

EosFP is a photoconvertible protein discovered from the Scleractinian coral, *Lobophyllia hemiprichii*. Its emission switches from green fluorescence (at 516 nm) to red fluorescence (at 581 nm) following photo-induced modification using near UV light as well (Wiedenmann et al., 2004). EosFP (226 amino acids) showed a high percentage of identity (84%) on the basis of amino acid sequence with FP Kaede (Ando et al., 2002; Wiedenmann et al., 2004). EosFP, therefore, represents a potential fusion marker protein that can be used for
tracking mitochondrial dynamics in the living cell (Wiedenmann et al., 2004). An engineered monomeric EosFP (mEosFP) form has been produced without any loss in properties (photoconvertibility or fluorescence). In plant cells, the mEosFP produces green fluorescence, which is converted into a red color a few seconds after exposure to UV light in plants (Nienhaus et al., 2005; Mathur et al., 2010; Wiedenmann et al., 2004). According to Mathur et al., 2010, mEosFP protein can be localized to several organelles in living cells such as: mitochondria, Golgi bodies, peroxisomes, endoplasmic reticulum or cytoskeletal elements. This protein does not show any interference with other fluorochromes (cyan FP and yellow FP), and thus can be used in tandem with other fluorescent proteins to monitor membrane and cytoskeletal dynamics (Mathur et al., 2010). Overall, using FP markers to examine mitochondria in live cells has tremendous potential to increase the understanding of mitochondrial dynamics, morphology and function.

1.4. Mitochondrial fission and fusion

As mitochondria cannot be generated de novo, they proliferate through growth and fission of pre-existing organelles (Westermann, 2010). The formation of two or more daughter organelles by scission of a single mitochondrion is known as mitochondrial division (Scott and Logan, 2011). Defects in division can lead to cellular dysfunction and even cell death, in both plant and mammalian cells (Logan, 2006b; Chen and Chan, 2009). Mitochondrial fission enables the number of mitochondria per cell to be maintained during cell division (mitosis and meiosis) (Hales, 2004).

The process of mitochondrial division is similar to that seen in bacteria. Scott and Logan (2011) summarized mitochondrial division machinery in a simple cartoon (Fig. 1.5). Dynamin-related proteins (DRPs) are key regulators of mitochondrial division. During fission events, they are recruited from the cytosol to the outer mitochondrial membrane at scission sites by a group of adapter proteins. DRPs belong to the dynamin superfamily of GTP-hydrolyzing proteins (Arimura and Tsutsumi, 2002; Arimura et al., 2004a; Logan et al., 2004; Mozdy et al., 2000; Otsuga et al., 1998; Smirnova et al., 1998, 2001; Labrousse et al., 1999).

In Arabidopsis, DRP3A and DRP3B are involved in mitochondrial division (Logan et al., 2004; Arimura and Tsutsumi, 2002). They localize to both constriction sites of dividing
mitochondria and to the tips of mitochondria (Arimura et al., 2004b, 2004a; Logan et al., 2004; Mano et al., 2004; Logan unpublished).
Figure 1.5. A simple cartoon representing mitochondrial division proteins. This figure represents mitochondrial division machineries of plants (DRP3A & DRP3B), yeast (Dnm1p) and mammals (Drp1). In plants, the two DRP proteins may interact with BIGYIN1 and BIGYIN2 using the plant specific adaptor protein NMT. In yeast, Dnm1p interacts with Fis1p, while Mdv1 and Caf4p mediate the interaction. In animals, Drp1 may interact directly with Fis1 or Mff proteins (Permission was obtained from the author, (Scott and Logan, 2011)).
Disruption of either DRP3A or DRP3B has been shown to increase the number of large and elongated mitochondria (Logan et al., 2004; Arimura et al., 2004a; Arimura and Tsutsumi, 2002; Arimura et al., 2004b). Scott et al., identified two Arabidopsis orthologues of the yeast outer mitochondrial membrane protein Fis1p, which interacts with the yeast dynamin, Dnm1p (Mozdy et al., 2000), called BIGYIN1 and BIGYIN2 (Scott et al., 2006). It is unknown if there are interaction between DRP3A/B and the BIGYINs in Arabidopsis.

NETWORK (NMT) was identified as a plant specific adaptor protein that is involved in mitochondrial division and the maintenance of normal mitochondrial structure in the cell (Logan et al., 2003; Arimura et al., 2008). NMT is the first plant-specific protein identified in mitochondrial division since there are no obvious homologues in non-plant eukaryotes (Logan, 2010a). It was demonstrated that Arabidopsis NMT recruits DRP3A and DRP3B to the outer mitochondrial membrane and is required for mitochondrial division (Arimura et al., 2008).

Mitochondrial fusion occurs when two or more individual organelles join to create a single, large mitochondrion. Fusion allows genetic complementation between two mitochondrial genomes, ensuring all required components for functional mitochondria are present (Hales, 2004).

Hydrolysis of GTP provides the energy required for fusion between membranes (Meeusen et al., 2004; Scott and Logan, 2011). The first gene to be identified to be involved in mitochondrial fusion was the \textit{Drosophila melanogaster} fuzzy onions (Fzo) gene. In the \textit{fzo} mutant, the mitochondrial defect affects spermatogenesis and results in male sterility (Hales and Fuller, 1997; Scott and Logan, 2011). Mammalian homologues of Fzo are known as Mitofusins (Mfn1 and Mfn2) and in yeast, Fzo1p (Hermann et al., 1998). Chen et al., (2003) generated mice that lack functional mitofusins and found that mitochondrial fusion is essential not only for cell survival but also the movement of mitochondria. Chen et al., discovered that mice deficient in Mfn1 or Mfn2 have embryonic developmental abnormalities (Chen et al., 2003).

To date, no genes functioning in higher plant mitochondrial fusion have been identified. However, it is known that mitochondria do undergo fusion in higher plants (Sheahan et al., 2004; Arimura et al., 2004b) and therefore it is apparent that plants utilize different proteins for the fusion process. In yeast and animal cells, the conserved Fzo/Mfn protein is important for mitochondrial fusion. While Arabidopsis has a gene encoding a protein similar to Fzo/Mfn, it influences chloroplast but not mitochondrial morphology (Gao et al., 2006). Scott and Logan
(2011) summarized mitochondrial fusion machinery in mammals, yeast and plants as shown in Fig.1.6.

Several studies have confirmed that mitochondrial fusion occurs in planta. One of these studies used the photoconvertible fluorescent protein Kaede (mitochondrial ATPase subunit was subcloned into the Kaede plasmid) (Arimura et al., 2004b). In that study, mitochondrial fusion was investigated in onion epidermal cells expressing mitochondrial targeted Kaede (emission color can be irreversibly converted upon exposure to light of 350-400nm). Arimura et al., (2004b) detected the occurrence of fusion by the presence of yellow mitochondria through fusion of green and red mitochondria. They concluded that fusion is a relatively frequent event that occurs between plant mitochondria under normal conditions (Arimura et al., 2004b).

A study by Sheahan et al., (2004) using Nicotiana tobacum mesophyll protoplasts, illustrated that mitochondrial fusion following protoplast fusion causes extensive mitochondrial elongation whereas fission resulted in increased numbers of small mitochondria in daughter cells. Plant mitochondria also undergo a process called Massive Mitochondrial Fusion (MMF) prior to cell division. The MMF is followed by extensive mitochondrial fission to enable proper redistribution of the mitochondria in daughter cells. Sheahan et al., (2005) demonstrated fusion events between two populations of plant protoplasts and confirmed that mitochondrial elongation is a result of massive mitochondrial fusion. Despite the genetic and cell biological evidence for mitochondrial fusion in plants the molecular components and mechanism remain unknown.

1.5. The role of mitochondria in autophagy

Autophagy is a conserved process amongst eukaryotes and is defined as the degradation of cellular contents as a means to recycle nutrients or degrade toxic and damaged components (Fig.1.7). It is a natural biological process but it becomes elevated with higher levels of stress. As a result of autophagy, cytoplasmic components are transported to the vacuole (yeast and plants) or lysosome (animals) during stress and starvation conditions (Klionsky, 2007; Mizushima, 2007). Many studies support the theory that one form of autophagy, termed mitophagy, is a mechanism controlling mitochondrial turnover during physiological distress or under pathological conditions (Tolkovsky, 2009).
Figure 1.6. Mitochondrial fusion proteins in mammals, yeast and plants. In yeast, the outer membrane protein Fzo1p and Mgm1 on the inner membrane, interact via an adaptor known as Ugo1p while no human protein homologous to Ugo1p has been identified (Sesaki and Jensen, 2001). In Arabidopsis, there are no homologues of Fzo1p or Ugo1p while the closest plant protein to Mgm1 is DRP3A that is involved in mitochondrial division (Permission was obtained from the author, (Scott and Logan, 2011)).
Recent advances have identified several components of the autophagic machinery which can be visualized using fluorescent fusion protein technology and have also led to the identification and validation of specific dyes to label components of the autophagy apparatus providing means to rapidly and easily detect autophagosomes or autolysosomes in plant cells. LysoTracker has been used for assessing autophagy in plants, and whilst not specific for autophagosomes, it does label acid vacuoles required for the process (Yoshimoto, 2012). For example, LysoTracker Red was used to label autolysosomes in tobacco cells and it was demonstrated that the dye was concentrated in autolysosomes (Moriyasu et al., 2003).

In addition, the autophagosome marker ATG8 (AuTophaGy)-GFP has been used to visualize autophagy in Arabidopsis cells (Contento et al., 2005; Yoshimoto, 2012; Yoshimoto et al., 2004). Yoshimoto et al., (2004) used atg4a4b-1 plants (mutants with a T-DNA insert in ATG4) to determine that plant autophagy is involved in root development under nutrient limiting conditions. Use of these different markers has enabled researchers to demonstrate that the formation of autophagosomes in mammals, yeast and plants involves the same molecular mechanisms (Hayward et al., 2009; Minibayeva et al., 2012). They also allow researchers to identify autophagosomes and increase the ease of studying them.

Mitophagy involves the selective removal of dysfunctional mitochondria (Lemasters, 2005; Minibayeva et al., 2012). Ashford and Porter (1962) first observed autophagy of mitochondria, where they found mitochondria present in the autophagosomes of the human liver. In mammalian cells, oxidative stress generates dysfunctional and damaged mitochondria that have the potential for futile ATP hydrolysis leading to increasing ROS production and release of pro-apoptotic proteins (Kim et al., 2007; Minibayeva et al., 2012). Thus, one can imagine why it is important for the cell to disassemble and remove such potentially damaging organelles. Compared to yeast and mammalian cells, oxidative stress-induced mitophagy has not been studied extensively in plants. A recent study demonstrated that oxidative stress in wheat root cells induces mitophagy and causes mitochondria to be encapsulated within autophagosomes with subsequent delivery and degradation in the central vacuole (Minibayeva et al., 2012). Therefore, investigating and understanding mitophagy enables researchers to study the pathway of dysfunctional mitochondria.
Figure 1.7. Plant macroautophagy pathway. Autophagy surrounds a portion of the cytoplasm (cargo), which is then transported to the vacuole via fusion of the outer autophagosomes membrane with the vacuole membrane. The autophagic body is then broken down by vacuolar hydrolases with the products exported for recycling (Permission was given by the author (Liu and Bassham, 2012)).
1.6. The role of mitochondria in programmed cell death

As one can imagine, the mitochondria are such an important part of the cell that mitochondrial dysfunction has detrimental effects on biological functions that, in turn, lead to several degenerative diseases in humans (Chen and Chan, 2009). Mitochondria are linked to many developmental and aging processes. Defects in mitochondrial dynamics lead to many diseases such as; Alzheimer’s (Selfridge et al., 2013), diabetes (Shenouda et al., 2011), Parkinson’s and many additional neurological and neuromuscular diseases. A recent study showed the relationship between mitochondrial dynamics and cellular metabolism, autophagy or apoptosis in cancer cells (Grandemange et al., 2009). We now know that mitochondrial dynamics are crucial for the health and development of all organisms that contain the organelle. As mentioned above, it has been well established that any direct or indirect changes in mitochondrial movement can be linked to neurodegenerative diseases that affects humans (Chen and Chan, 2009). PINK1 and PARK2 are genes found to be a determining factor in Parkinson’s disease in humans. Parkinson’s is a neurodegenerative disease characterized by movement disorders. PINK1 is a serine/threonine kinase found on the cristal membranes of the mitochondria while Parkin, is an E3 ubiquitin-protein ligase found in the cytoplasm (Cookson et al., 2003). Parkin and PINK1 respectively, are proposed to be involved in mitochondrial malfunctions and dynamics in humans (Narendra et al., 2008; Yang et al., 2008).

In addition, mitochondria act as regulators of PCD (Westermann, 2010). PCD represents a form of cellular suicide and is involved in all plant life cycle phases from germination until senescence. PCD in plants shares many characteristics seen in animals such as DNA fragmentation, cytoplasmic condensation, nuclear condensation (Gunawardena et al., 2004; Lord and Gunawardena, 2011), increased formation of vesicles (Lord and Gunawardena, 2011; Reisen et al., 2005) and DNA laddering (Balk et al., 2003; Lord and Gunawardena, 2012). Recently, plant PCD was divided into two categories: vacuolar cell death, and necrosis (van Doorn et al., 2011). Removal of cell contents via a combination of autophagy-like processes and the release of hydrolases from collapsed vacuoles are characteristics of vacuolar cell death. Necrotic cell death, however, is characterized by the rupture of the plasma membrane (van Doorn et al., 2011).

Changes in mitochondrial dynamics may be signaled by changes in the mitochondrial inner membrane permeability (Scott and Logan, 2011). Oxidative stress-induced mitochondrial
swelling leads to the disruption of the outer mitochondrial membrane and not the inner membrane. As a result, cytochrome c (in the intermembrane space) release is a consequence of swelling of the mitochondria (Arpagaus et al., 2002; Virolainen et al., 2002; Scott and Logan, 2011). It is not clear if cytochrome c plays a specific role in the execution of a PCD pathway in plants, although the release of cytochrome c has a role in apoptosis in animals via the formation of the caspase-activating apoptosome (Scott and Logan, 2011). It was believed until recently that apoptosis and PCD have no effect on mitochondrial dynamics, however, using fluorescent microscopy and fluorescent proteins to visualize mitochondria allow for the observation of changes in mitochondrial morphology in plants under stress conditions (Scott and Logan, 2008).

1.7. The first plant mitochondrial dynamics mutants

Due to the fact that the field of mitochondrial dynamics in higher plants is relatively new, our understanding and knowledge of the underlying processes is limited. In the early 2000s, with the aim of identifying plant genes controlling mitochondrial dynamics, eight mutants with altered mitochondrial morphology or distribution were identified in Arabidopsis (Logan et al., 2003). These were the first plant mitochondrial dynamics mutants. One of these was called the friendly mitochondrial mutant (fmt, friendly) (Logan et al., 2003; Fig. 1.8). These mitochondrial dynamics mutants were identified by microscopy-based screen of EMS (ethyl methansulfonate)-mutagenized plants expressing mito-GFP (mitochondrial–targeted GFP; Fig. 1.9; Logan et al., 2003; Logan and Leaver, 2000).

The phenotype of the fmt chondriome displays discrete clusters of tens of mitochondria in all cell types, while in wild type (wt), mitochondria are normally equally distributed throughout the cytosol as single, discrete, spherical, or tubular-shaped organelles, as shown in Fig. 1.8 (Logan et al., 2003). FMT (on chromosome III, (Logan et al., 2003)) encodes a TPR of 150-kDa that is homologous to the Clu1 protein of Saccharomyces cerevisiae, CluA of Dictyostelium discoideum and Clueless of Drosophila (Zhu et al., 1997; Logan et al., 2003; Cox and Spradling, 2009).

Similarities with the cluA- phenotype in Dictyostelium (the mutation resulted in mitochondria being clustered near the cell center rather than being distributed throughout the cytoplasm, (Zhu et al., 1997)) helped in identifying the FMT gene (Logan et al., 2003). When the
Figure 1.8. Arabidopsis chondriome organization in wt and in the fmt mutant. Arrows indicate clusters of mitochondria in the cell (Logan et al., 2003). Scale bar = 10 μm
Figure 1.9. Identification of EMS mutants. Eight mitochondrial dynamic mutants were identified by EMS mutagenesis using wide-field epifluorescence microscopy, one of which is fmt (Logan et al., 2003).
Arabidopsis CluA orthologue, At3g52140, was sequenced in fmt, a single G to A point mutation was found at the first base of the second intron, thus destroying the intron-exon consensus motif (Logan et al., 2003). Consistent with this, Northern blot analysis showed that the At3g52140 transcript was undetectable in fmt (Logan et al., 2003).

A recent study identified a series of Arabidopsis mutants (known as non-responding to oxylipins or noxy) that are insensitive to oxylipins, the products of 9-lipoxygenases (9-LOX). 9-LOX activates plant defenses against pathogenic bacteria by the formation of oxylipins (Vellosillo et al., 2013). Oxylipins are formed from the oxidation of fatty acids by lipoxygenase enzymes (Blée, 2002; Andreou et al., 2009), although they can also be produced non-enzymatically by free radical-mediated oxygenation (Durand et al., 2009; Vellosillo et al., 2013). The production of 9-LOX oxylipins is important in activating local defenses against Pseudomonas syringae in plants (Hwang and Hwang, 2010; López et al., 2011; Vicente et al., 2012).

The noxy mutants are more susceptible to P. syringae DC3000 and also show reduced activation of gene expression in response to salicylic acid (Vellosillo et al., 2013). The noxy38 mutation was mapped to the FMT locus. Microscopic analysis of mitochondrial morphology in noxy38 mutants using MitoTracker showed that noxy38 plants display a mitochondrial phenotype identical to that of fmt (strong mitochondrial aggregation, Vellosillo et al., (2013)). The noxy38 mutant has an impaired response to the 9-LOX oxylipins product 9-hydroxy-10, 12, 15 - octadecatrienoic acid (9-HOT) (Vellosillo et al., 2013). Thus, the noxy38 mutation lowered the defense potential of plants and stronger disease symptoms were observed in noxy38 plants compared to wt plants when incubated for three days with P. syringae DC3000 (Vellosillo et al., 2013). Vellosillo et al., (2013), concluded that the NOXY38/FMT protein plays a role in plant defense and that mitochondria participate in the activation of plant innate immunity signaling to protect plant tissues against pathogen infection (Vellosillo et al., 2013).

clueless in Drosophila melanogaster is another functional orthologue of FMT. It has been shown that functional loss of PINK1 or Parkin results in cellular deterioration and mitochondrial aggregation leading to motor impairment and decreased fertility in Drosophila (Yang et al., 2006; Park et al., 2006; Clark et al., 2006). Interestingly, several studies have shown that PINK1 and Parkin mutant phenotypes were partly rescued by increasing the activity of one of the mitochondrial division machinery proteins known as Drp1 in Drosophila. Amazingly, a
A reduction in the activity of members of the mitochondrial fusion machinery, Mfn or OPA1, has the same effect (Deng et al., 2008; Poole et al., 2008; Yang et al., 2008; Imai and Lu, 2011). Thus, one could suggest that increased numbers of mitochondria could help reduce the impact of PINK1 and Parkin defects. The phenotype caused by PINK1 deficiency was rescued by wt Parkin overexpression (Yang et al., 2006; Park et al., 2006; Clark et al., 2006). They also demonstrated that parkin and clueless genetically interact (Cox and Spradling, 2009). Drosophila clu-1 mutants displayed phenotypes that strongly resemble those in parkin mutants (Cox and Spradling, 2009). Cox and Spradling (2009) concluded that Drosophila Clu and Parkin have a functionally related pathway, and that any defects in the human Clu orthologues, might be involved in Parkinson’s disease.

In the absence of clueless, Cox and Spradling (2009) suggest that the mitochondrial quality control system is compromised and the number of dysfunctional mitochondria increases. The dysfunctional mitochondria form clusters when they are tethered to the plus-ends of microtubules, where they will undergo mitophagy. It was also hypothesized that Parkin is recruited to depolarized mitochondria, as has already been demonstrated in human cells (Narendra et al., 2008), and that Parkin has a role in promoting mitophagy. Under this hypothesis, failure to eliminate dysfunctional mitochondria due to the mutation of Parkin would result in the onset of Parkinson’s disease. This hypothesis is consistent with a role for Parkin in mitophagy of damaged mitochondria by autophagosomes (Narendra et al., 2008).

1.8. Aim, hypotheses and objectives

The aim of the research described in this thesis is to improve our understanding of the roles of mitochondrial dynamics in plants and to discover key mechanisms underpinning the organization of plant chondriome structure.

In pursuit of this aim, three hypotheses will be tested:

1- Mitochondria function as sentinels of cell health and disturbance of the ‘discontinuous whole’ (Logan, 2006b) homeostasis leads to mitochondrial and cell stress and thereby defective whole plant physiology.
2- The clustered mitochondrial phenotype in the friendly mitochondria mutant results from tethering of mitochondria to microtubules.

3- If hypothesis 2 is unsupported by experimental evidence, it is hypothesized that mitochondrial clusters in friendly are indicative of a failure of mitochondrial fusion such that the mitochondria are trying but failing to initiate or complete fusion.

The research takes a molecular cell biological approach using mitochondrial dynamics mutants identified in a forwards genetics screen. As mentioned previously (section 1.7), Logan et al. (2003) identified a suite of plant mitochondrial dynamics mutants. One of these, the friendly mitochondrial mutant (fmt), forms the core experimental material for the research but due to the conserved nature of mitochondrial function, it has also been possible to use an alternative model system, Caenorhabditis elegans, to further elucidate the cellular function of FRIENDLY and its orthologues.

The research objectives to test the above hypotheses are:

- To examine the phenotypic characteristics of the friendly mutant and determine whether or not friendly plants display indicators of higher levels of stress than wt plants.
- To determine whether or not mitochondria in clusters are discrete organelles or are connected by their outer membranes and have contiguous matrices.
- Establish any associations between friendly mitochondrial clusters and the F-actin and microtubule cytoskeleton.
- Determine the subcellular location of FRIENDLY and the role of lysine acetylation in FRIENDLY function.
- Identify FRIENDLY paralogues and determine whether or not the Caenorhabditis elegans clu-I mutant shows aberrant mitochondrial morphology.
CHAPTER 2. METHODOLOGY

2.1. Materials and Methods

2.1.1. Plant material and growth conditions

Seeds were surface sterilized by soaking them in 80% (v/v) ethanol for five minutes with continual inversion, followed by discarding the supernatant. Seeds were then soaked in 30% (v/v) household bleach for 7 minutes. Finally, seeds were rinsed 3 times with sterile, distilled water. Seeds were germinated on Petri dishes containing Murashige and Skoog (MS) (1962) medium, supplemented with 8% (w/v) agar (Type M, Sigma Chemical Co., St Louis, USA), 1% (w/v) sucrose and 0.05% (w/v) MES, (pH 5.8). Seeds were stratified for three days by incubation in the refrigerator at 4º C then transferred into a controlled environmental growth chamber at 23ºC under a 16 hour (h) light and 8 h dark photoperiod using cool-white light at 100 μmol m⁻² s⁻¹. The EMS generated mutant is named fmt1-1 and T-DNA lines are named fmt1-2 and fmt1-3 (see section 3.3.1. for more details)

2.1.2. fmt T-DNA insertion lines

Seeds of the fmt1-2 (SALK_046271) homozygous mutant plants with a T-DNA insertion in the 18th intron of the FMT gene (At3g52140), were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/) (ABRC). The gene specific LP (5'-ATACCTGCAGCAGTTTGCAAC-3') and RP (5'CTAGCGCCAACAGCTCTACTG-3') primers were used for genotyping SALK_046271 plants in conjunction with the left border T-DNA primer (LBb1) 5' GCGTGGACCGCTTGCTGCAACT-3'. Homozygous mutants of SALK_046271 were crossed with mito-GFP (Logan and Leaver, 2000) to produce fmt1-2 mito-GFP plants. The fmt1-3 (SAIL _ 513) (Table 3.1) was obtained also from ABRC and crossed with mito-GFP.
2.1.3. Microscope systems

For live imaging, plant tissues and seedlings were visualized using three different microscope systems unless otherwise specified:

**Microscope system 1** consists of an Olympus BX61 with 100x U-PLAN S-APO oil immersion objective (NA=1.4), Semrock Brightline cubes for GFP (Ex: 473/31 nm, Dichroic: 495 nm, EM: 520/35 nm) and mRFP/mCherry (Ex: 543/22 nm, Dichroic: 563 nm, EM: 593/40 nm) and X-Cite exacte illumination. Images were captured with a QImaging Rolera-MGi Plus EMCCD camera attached to a computer workstation running Metamorph software.

**Microscope system 2** is comprised of an inverted Zeiss META 510 confocal laser-scanning microscope (CLSM) (Jena, Germany) with a water immersion objectives 25x and 63x, 1.2 N.A). CLSM equipped with a 488 nm argon laser and a 543 nm helium-neon laser. GFP signal was detected using the Argon laser tuned to 488 nm and emission ranged between 505-530 nm while the red fluorescent signals were detected by using excitation at 543 nm lasers. LSM510/ConfoCor2 software, Version 3.2 SP2 was used. Fluorescence and transmitted light images were collected simultaneously.

**Microscope system 3** is the Transmission Electron Microscopy (TEM) and it was used specifically for observing the ultrastructure of mitochondria in wt and fmt. Ultra-thin sections (60nm) were generated from High Pressure Frozen (HPF) (prepared by The High Pressure Freezing Facility at Oxford Brookes University) Arabidopsis roots and leaves using Microstar diamond knife (Huntsville, US) on a Reichert-Jung microtome (Reichert microscopic service; Depew, US). These thin sections were captured onto single slot copper grids (Ted Pella; Redding, US) coated with 0.6% Formvar in chloroform. Uranyl acetate (2%) and Reynolds lead citrate solution (Reynolds, 1963) drops were used to stain the grids. After the stain had dried, samples were visualized using Philip CM10 transmission electron microscope (Netherlands).

2.1.4. Mitochondrial cluster size and number in wt and fmt mutants (fmt1-1 and fmt1-2)

Seven day-old Arabidopsis plants expressing mito-GFP (wt and fmt1-1 or fmt1-2) were used for analysis. Fluorescence microscopy system 1 was used to visualize mitochondria of the epidermal cells in leaves and roots. Images were obtained from 40 independent seedlings of each
Data were quantified using the ‘Analyze particles’ command in ImageJ software (Abràmoff et al., 2004). In ImageJ, brightness or contrasts were adjusted and then the threshold was adjusted to the exact size of particles, and then particles area size was measured. Clusters of mitochondria in fmt mutants were minimally defined as discrete “particles” consisting of mitochondria covering an area of at least 15 pixels² (equal to 0.384 µm²). Groups of wt mitochondrial were defined as discrete “particles” covering an area of at least 3 pixels² (0.077 µm²).

2.1.5. Fluorescence recovery after photobleaching

Fluorescence Recovery after Photobleaching (FRAP) was used to examine whether or not clustered mitochondria were connected via their matrices. Regions of interest in fields of view in Arabidopsis plants expressing mito-GFP in the fmt1-1 mutant background and the network mitochondria mutant (nmt, Logan et al., 2003)) background (as a control) were photobleached. Zeiss LSM 410 inverted CLSM (Jena, Germany) driven by a PC running LSM410/Zeiss LSM software was used. The microscope was equipped with a 25x and a 63x water immersion objective (NA=1.2). GFP was excited with the 488 nm argon laser (HFT 488/594 dichroic) and emission collected between 505-530 nm. GFP was photobleached using the 488 nm laser in a section of a randomly chosen mitochondrial cluster in fmt1-1 or nmt interconnected tubules. Ten images were captured of the whole cluster or elongated mitochondria prior to bleaching. A section of mitochondrial cluster or elongated mitochondria was bleached using 80% laser power and then immediately laser power was reduced to 10% and 300 images (one second a part) were captured sequentially to observe any GFP recovery. The experiment was repeated three times with similar results.

2.1.6. Root growth of wt and fmt mutant lines

To measure the rate of plant root growth for wt and fmt mutants, seeds were planted on agar plates (MS petri dishes) vertically in order to allow roots to grow along the agar surface. MS plates were held vertically in the growth chamber. At each time point (7, 14, 21 and 28-days old) the root length of 20 seedlings were measured and the experiment was repeated a further two
times (n=3). Wild type Arabidopsis lines mito-GFP (43C5) or Col-0 were used as controls for fmt1-1 and fmt1-2 respectively. Seedlings used for the measurements at one point were not used for any other time point. Light and temperature conditions were the same for all plant lines.

2.1.7. Etiolated hypocotyl length during dark germination

To determine the etiolated hypocotyl length, seeds were plated vertically on MS Petri dishes and covered with aluminum foil. Hypocotyl length was measured and recorded for 7-day old seedlings. Data were collected from three independent replicates and each replica comprised 20 seedlings.

2.1.8. Biomass measurements

Wild type and fmt mutant seeds were sown on MS plates. Fifteen seedlings of each line (wt, fmt1-1 and fmt1-2) were sampled at each time point (7, 14, 21 and 28-days). Free surface moisture was removed by gently blotting with soft paper towel and then seedlings were weighed immediately using an electronic balance scale and measurements were recorded as fresh weight. After wrapping the seedlings in filter paper and incubating at 40º C for two hours, the dry weight of groups of seedlings was measured. Seedlings were weighed and incubated again for three hours and four hours until seedlings reached a final dry weight and then the weight was recorded.

2.1.9. Three-dimensional reconstructions

AutoDeblur software (AutoQuant X3, Media Cybernetics) was used to deconvolute time-lapse images and z-stack images. IMARIS 7.4.2 software (Bitplane, Zurich, Switzerland) was used for image analysis and 3D reconstructions.

2.1.10. Root cell viability measurements

SYTOX dye is widely used to visualize cell death in Arabidopsis tissues. SYTOX stain nucleic acid of compromised or non-viable cells and the stained nucleus fluoresces brightly.
when the dye is bound to DNA. However, it cannot permeate the plasma membrane of living plant cells (Truernit and Haseloff, 2008).

SYTOX-Orange (Invitrogen) was used to detect dead cells in Arabidopsis roots. SYTOX-Orange was chosen since its excitation and emission wavelengths do not overlap with those of GFP. SYTOX dye was supplied as a 5 mM stock solution in DMSO and a final concentration of 250 nM SYTOX in ultra-pure water was used. Roots from 6 day-old plants of wt or fmt1-1 were incubated with SYTOX-Orange for 20 minutes at room temperature in the dark followed by a quick washing step in distilled water prior to visualization. Z-stack images were captured using microscope system 2 using a 514 nm laser and emission wavelengths selected with 560-615 nm band-pass filter. The main dichroic was HFT 488/514 and the secondary dichroic was NFT 545. SYTOX orange fluorescence was detected in the root cap, division, elongation, and differentiation zones. Cells with a stained nucleus were counted. Observation time did not exceed 30 minutes.

2.1.11. Root cellular structure using propidium iodide

Propidium iodide (PI) (Molecular Probes, Eugene, USA) outlines cells in living plant tissue and stains nuclei of dead cells. Roots were counterstained with PI to visualize the root anatomy of fmt1-1. PI was used at a working concentration of 3 µg /mL from a stock of 3 mg/mL in distilled water. Whole seedlings (6 day-old) were stained in PI solution for 2 min followed by a quick wash in distilled water. Root cells were visualized using microscope system 2 equipped with 25x and 63x objectives, a 488 nm argon laser at 12 % power, and emission was captured using a 610-660 nm band pass emission filter. Fluorescence and transmitted light images were collected simultaneously. Over 20 seedlings of wt and fmt1-1 roots were visualized and seedlings remained healthy during visualization time. All plant materials were imaged in water.

2.1.12. Autolysosome observation in Arabidopsis

Wild type and fmt1-1 seedlings (6 day-old) were stained with 1 µM LysoTracker Red DND-99 solution (Molecular Probes, Invitrogen, Cat no. L7528) in 0.5% MS for 5 minutes at room temperature to visualize LysoTracker-stained acidic compartments. After staining, the
seedlings were washed with distilled water to remove excess stain. The root cells were visualized using the Zeiss META 510 confocal microscope with a 63x, 1.2 N.A. water immersion objective, using the 543 nm helium-neon laser at 20% power, and a long pass 650 nm emission filter. The GFP signal was detected using the 488 nm laser and 505-530 nm emission band-pass filter. The main dichroic is HFT 405/488/543 and the secondary dichroic is NFT 545. Given that this stain does not overlap with mito-GFP, it was possible to simultaneously visualize GFP and LysoTracker red fluorescence in the same plant. Z-stacks were generated and LysoTracker-stained puncta were quantified using ImageJ.

2.1.13. Mitochondrial membrane potential measurement

Seedlings were grown on vertical plates containing 0.5x MS media + 0.8% phytagel. The membrane potential of mitochondria in living tissue was determined by CLSM using the red fluorescent potentiometric dye TMRM that accumulates reversibly in mitochondria in response to the inner membrane potential (Brand and Nicholls, 2011). After 4 days seedlings were incubated in freshly prepared 50 nM TMRM for >15 min before confocal imaging of roots bathed in fresh probe. Confocal microscopy, performed using a Zeiss LSM 780 confocal microscope (Carl Zeiss MicroImaging), and data analysis were performed as previously described (Schwarzlander et al., 2012).

2.1.14. Trichome visualization

Fourteen day-old seedlings were visualized using Nikon SMZ1500 zoom stereomicroscope bright field (Nikon, Melville, NY) equipped with a Nikon 1.0 x WD 54 objective lens camera.

2.1.15. Root zone measurements

Images of seven-day-old seedlings of wt and fmt1-1 were captured using a simple compound light microscope equiped with Dino-Eye Digital Eyepiece Camera AM423X with “Dino Capture” software. ImageJ software was used to measure the length and diameter of the
root zones (root cap, meristematic zone and elongation zone). Six independent root samples were used.

2.1.16. Cytoskeleton and mitochondrial visualization

In order to visualize actin filaments and mitochondria, a double transgenic line was generated by crossing mito-GFP with a second line (mCherry-mTalin) expressing an in frame fusion of mCherry to the C-terminal 197 amino acids of mouse talin which forms the actin binding domain (ABD). The mCherry-mTalin line was generated by transformation of Arabidopsis Col-0 with pDCLmcherry-mTalin. The backbone of pDCLmcherry-mTalin, the pDCLmcherry-X destination vector, was a modified pMDC43 in which mGFP6 was replaced by mCherry via pMDC7, and pMDC24. A cDNA fragment encoding the mTalin ABD was PCR-amplified from the pZP202-GFP-mTalin vector (see (Kost et al., 1998)) using attB Gateway primers and recombined into pDONRzeocin. Recombination between pDONRz-mTalin and pDCLmcherry-X created pDCLmcherry-mTalin. Actin and mitochondria were imaged using microscopy system 1.

To visualize MTs, microtubule-associated protein 4 (MAP4) (Olson et al., 1995) was amplified by PCR using Gateway attB primers, from a GFP-MAP4 construct that was a kind of gift from Jaideep Mathur, University of Guelph (Mathur and Chua, 2000). The MAP4 cDNA was recombined with pDONRzeocin, which was in turn recombined with the pDCLmcherry-X destination vector. A stable Arabidopsis line expressing mCherry-MAP4 was crossed with mito-GFP or with the fmt1-1 mutant expressing GFP and the crossed lines were analyzed using fluorescent microscopy. To visualize the plus-end of MTs, the EB1b coding sequence from GFP-EB1b (Mathur et al., 2003) was amplified by PCR using attB1 primers, and this was cloned and recombined with pDCLmcherry-X as above. A stable Arabidopsis line expressing mCherry-EB1b was crossed to wt and fmt1-1 as above.

2.1.17. Measurement of mitochondrial association time

Movies were generated from 250 images captured at 1 s intervals using microscopy system 1 of the control mito-GFP line or of fmt1-1. Five movies were captured of different
regions of the epidermis of the true leaves of three, 7-day old seedlings to give 15 movies (technical replicates). Results presented are means of associations measured in three independent experimental repeats each comprised of 15 movies (n=3). Association time was calculated as the number of frames (seconds) counted from the time a single mitochondrion was within one mitochondrial-width of either a second mitochondrion (in the case of wt mito-GFP) or a cluster of mitochondria (in the case of fmt1-1), until the same mitochondrion moved at least one apparent mitochondrial-width from its partner(s). When a cluster of mitochondria joined a second cluster, frames were counted until a group of mitochondria (at least 10 organelles) became separated from the large cluster.

2.1.18. Pharmaceutical treatments

Stocks concentrations of 2 mM and 30 mM lat-B and Oryzalin respectively were dissolved using ethanol (100%) while Nocodazole was prepared using DMSO. Ultra-pure water was used to dilute each stock solution to the working concentration as well as ethanol was prepared with the same dilution factor of the stock.

2.1.19. RNA isolation and cDNA generation

Total RNA was extracted from 14 day-old Arabidopsis seedlings using the RNeasy plant mini kit (Qiagen, Crawley, UK), using the manufacturers instructions. dNTPs (10 mM, 2 µL), oligo dT (1 µL), RNA (10 µg/mL, 1 µL) were combined with distilled water (8 µL) to a volume of 12 µL. These components were mixed and heated to 65°C for 5 min then placed on ice for one minute. cDNA synthase buffer (4 µL), 0.1M DTT (1 µL), RNease out (1 µL), and 1 µL Thermoscript RT (Invitrogen) were added prior to incubation at 55°C for 1h then 85°C for 5 min.

2.1.20. FMT gene amplification

The FMT coding sequence was amplified from cDNA using Phusion DNA polymerase (Invitrogen) using the primers (forward, 5’-ATGGCTGGGAAGTCGAACAAATCGAAGGCCAAG-3’ and reverse 5’-
TTATTTTTTGCTTTTTGCTTCTTCTTATCCAAAG-3'). After an initial denaturation at 94°C for 2 min, PCR was performed using the following program: 94°C for 1 min; 60°C for 1 min; 72°C for 1 min for 30 cycles followed by a final extension at 72°C for 5 min. The amplified FMT fragment (4300 bp) was excised from the agarose gel and purified.

2.1.21. FMT cloning

The extracted blunt-end PCR product was cloned using the Zero Blunt Topo PCR Cloning Kit for sequencing (Invitrogen) according to the manufacturers instructions. Two microliters of the mixed components were used to transform electro-competent cells, E. coli (DH5α). Transformed cells were plated on LB agar plates containing 50 μg/mL ampicillin and incubated overnight at 37°C. Several colonies were inoculated independently in Falcon tubes containing LB broth and ampicillin and incubated overnight at 37°C. The overnight cultures were used for plasmid mini-prep using Plasmid Mini Kit I (OMEGA, USA). The resulting plasmids were subjected to restriction analysis using HindIII. According to NEB Cutter website, the correctly amplified product would yield three bands (6437, 925 and 806 bp). Cultures which contained positively identified plasmids were sent for sequencing at NRC-Saskatoon using sequencing primers flanking the insertion site within the plasmid and internal primers as displayed in Table 9.1 (Appendix A). An error-free clone was used for all subsequent cloning.

2.1.22. Gateway BP and LR recombination reactions

Gateway technology was used, according to the manufacturers instructions, to generate an entry clone containing the FMT gene by performing a BP recombination reaction between the vector pDONR/Zeo (Gateway Donor vector from Invitrogen) and the purified PCR product (Table 9.2, Appendix A for more detail about the gateway primers). The recombined insert-plasmid was used to transform competent E.coli, which were selected on low salt LB agar plates containing Zeocin (50 μg/mL). Overnight incubation resulted in many colonies and several of these colonies were grown overnight individually in Falcon tubes containing low salt LB broth and Zeocin before plasmid isolation and restriction digest analysis as described above. Putative positive plasmids were subjected to sequencing as described above.
The LR reaction was performed using LR Clonase II, according to the manufacturers instructions, between the entry clone (pDONR/Zeo-FMT) and the Gateway destination vectors pMDC83 and pMDC45 to generate N- or C-terminal translation fusions to mGFP6, with pMDC32 for FMT overexpression and FMT complementation experiments, or with pDCL-X-mrfp or pDCL-mrfp-X which are modified versions of pMDC45 and pMDC83 respectively where the mgfp6 coding sequence was replaced with that for mrfp1 (re-engineered by DC Logan). The pMDC vectors were a kind gift from Mark Curtis’s Gateway (http://botserv1.uzh.ch/home/grossnik/curtisvector/index_2.html).

Two microliters from the LR reactions was used to transform competent *E. coli* cells and grown on LB Kanamycin plates overnight at 37°C. Liquid cultures, plasmid purification and analytical restriction digests were performed as described above.

Plasmids were sequenced and used to transform *Agrobacterium tumefaciens* (strain GV3101). The electroporated Agrobacterium cells were grown at 28°C in 10 mL YEP medium (10 g/L yeast extract, 10 g/L Bacto peptone, 5 g/L NaCl) with appropriate antibiotics (50 μg/mL kanamycin plus 25 μg/mL gentamycin). Four colonies from each plate were grown in YEP broth containing kanamycin and gentamycin for 18-24 h. Sequenced clones were selected for transient expression in *Nicotiana benthamiana* (tobacco) and stable transformation in Arabidopsis.

### 2.1.23. Fast transformation technique using tobacco plants

FMT constructs were used to agro-infiltrate tobacco leaves as described by Sparkes et al., (2006). Small segments of infiltrated tobacco leaves were visualized after 2-3 days using fluorescent microscopy system 1. A variety of concentrations of culture (OD$_{600}$ 0.1 to 0.5) were used for infiltration to determine the best OD as overexpression could result in cell death (Sparkes et al., 2006).

### 2.1.24. Stable transgenic lines

*Agrobacterium* containing the constructs pMDC83-FMT (FMT-GFP), pMDC45-FMT (GFP-FMT), pDCL-FMT-mRFP (FMT-mRFP), pDCLmRFP-FMT (mRFP-FMT) and pMDC32-FMT were used to transform Arabidopsis plants (*fml1-1*, wt) using the floral dip method (Clough
and Bent, 1998). 250 mL cultures of each Agrobacterium strain were incubated and grown for 18-24 h at 28°C with appropriate antibiotics. Cultures were centrifuged at 6,000 g at 4°C for 20 minutes. Supernatants were discarded and the pelleted cells were resuspended in 500 mL of infiltration medium containing 0.05 % (v/v) Silwet L-77. Arabidopsis plants at the correct developmental stage (flower buds present within the rosette) were dipped in the infiltration medium for approximately 15 s before covering in Saran wrap overnight followed by transfer back to the growth chamber (for more information see (Zhang et al., 2006)). For the selection of T1 transformations seeds were sown on Murashige and Skoog (1962) media as mentioned previously containing 100 µg/mL Timentin and 25 µg/mL hygromycin. After two weeks of growth putative transformants were transferred to fresh selection plates and screened by fluorescent microscopy system 1 for expression of the transgene if appropriate.

2.1.25. Site-directed mutagenesis

Unique GeneTailor Site-Directed Mutagenesis kit (Invitrogen) was used to generate base substitutions (mutations) in FMT (see primers in Table 9.3 in the Appendix A). DNA oligos were designed to encode the desired amino acids. The methylation reaction was performed as follows: 10x Methylation Buffer (1.6 µl), 10X fresh SAM (S-adenosine methionine) (200X stock) (1.6 µl), pDONR/Zeo-FMT (1 µl), DNA Methylase enzyme (1 µl) and sterile water (10.8 µl). The reaction was incubated at 37°C for 1h and the methylated DNA was kept at -20°C until use. PCR was carried out as follows: 10X HiFi buffer (5 µl), 10mM dNTPs (1.5 µl), 50mM MgSO4, HiFi Taq (0.2 µl), methylated DNA (2 µl) and primers displayed in Table 9.3 (Appendix A). The PCR program used was: 94°C for 2min, followed by 94°C for 30 s; 55°C for 30 s; 68°C for 8 min for 20 cycles with a final extension at 68°C for 10min.

The mutagenized vectors were used to transform electro-competent E.coli cells and the resultant mutants were confirmed by restriction analysis and sequencing. Ultimately, the plasmids were cloned into plasmid pMDC32 for plant transformation. Arabidopsis floral dip transformation performed as described previously. Transformed plant seedlings expressing mito-GFP were observed using fluorescence microscopy system 1.
2.1.26. 4', 6-Diamidino-2-phenylindole (DAPI) staining

The nucleic acid marker (DAPI) was used to visualize the nucleus. Prior to visualization, small regions of tobacco leaves infiltrated with FMT construct were immersed in DAPI solution (1 µg/mL) prepared from a stock solution (10 mg/µl) in an Eppendorf tube and vacuum infiltrated for 1 h. Tubes were covered with aluminum foil and kept at room temperature for 2 h.

2.1.27. Arabidopsis oligonucleotide microarray

Seeds of Arabidopsis (fmt1-1 and mito-GFP) were germinated and grown as described in 2.1.1. Both lines were grown under the same conditions and 0.1g of leaves were harvested from each of four plates (each representing a biological replicate) then samples were frozen in liquid nitrogen immediately before RNA isolation using RNeasy Plant Mini Kit according to manufacturer’s protocol. The RNA samples representing four biological replicates of each line (fmt1-1 and mito-GFP) were labelled (two cy3 and two cy5) and hybridized to slides prepared by the University of Arizona following the protocol described at http://ag.arizona.edu/microarray. Scanning, image analysis and quantification was as described previously (Xiang et al., 2011). Raw data was saved as a.gpr-file and converted into a.mev-file using the Express Converter software (version 2.1, Dana Faber Cancer Institute, Boston, MA, USA). Data was normalized using the lowess (locfit) algorithm and block normalization in the MIDAS software (version 2.22, Dana Faber Cancer Institute, Boston, MA, USA). Duplicates in the dataset were removed by averaging intensity values using the FiRe macro (Garcion et al., 2006). Significantly regulated transcripts using Cybr-T Bayesian probabilistic framework analysis ((Baldi and Long, 2001); Bayes p < 0.05). Functional class scoring was implemented using MapMan software (Thimm et al., 2004) applying the Benjamani-Hochberg correction. The datasets were deposited at the EBI ArrayExpress database (accessions) according to the MIAME guidelines.

2.1.28. Analysis of photosynthesis

Plants were grown as described in 2.1.1. Fully developed rosette leaves were placed in the dark for 15 min prior to determining dark-adapted F_o and F_m. All measurements were
performed using a XE-PAM system (Heinz Walz GmbH, Germany) and the data were collected via the PAM-Data Acquisition System (PDA-100) interfaced with the WinControl Software V2.08 (following the manufacturer's instructions). To induce minimal fluorescence the weak modulated light source was turned on, and data collected for 30s. Once the signal had stabilized an 800 ms pulse of 4000 µmol photons m⁻² s⁻¹ was applied to the leaf to close the PSII reaction centers and generate $F_M$. The leaf was then exposed to an actinic light source until a steady-state level of fluorescence was reached ($F_S$). To estimate the fraction of closed PSII reaction centers at steady-state, a saturating flash was applied and $F_{M'}$ determined. Following the saturating flash, the actinic light was removed and a weak far-red light (102-FR, Heinz Walz GmbH) was applied to oxidize the electron transport chain. When a steady-state was reached, $F_{O'}$ was determined. The actinic light intensity was then increased and the same steps were repeated. The dark-adapted optimal quantum yield of photosynthesis was calculated as $F_v/F_M$ according to the equation $F_v/F_M = (F_M-F_O)/F_{M'}$ (Krause and Weis, 1991); van Kooten and Snel, (1990). The relative redox state of PSII at steady-state was estimated at each light level using the term $1-q_L$, where $q_L = [(F_M-F_S)/(F_M-F_O)](F_O/F_S)$ (Kramer et al., 2004). Similarly, the amount of nonphotochemical quenching was estimated at each light level using the term NPQ, where NPQ = $(F_M-F_{M'})/F_M$ (Genty et al., 1990)

To estimate the functional activity of photosystem I in fmt1-1, fully developed rosette leaves from the same plants used for PSII analyses were examined using a PAM-101 control unit equipped with the ED-P700DW Dual-Wavelength-P700 emitter/detector (Heinz Walz GmbH). Data were collected using the PDA-100 interfaced as described V2.08 (Ivanov et al, 1998; Schreiber et al, 1988). Far-red light was applied to the leaf using the 102-FR light source. The deflection in the signal ($\Delta A_{820}/A_{820}$) was used to estimate the amount of oxidizable P700 (Schreiber et al., 1988). The functional pool size of intersystem electrons was determined by applying single (ST) and multiple (MT) turnover flashes (Asada et al., 1992; Ivanov et al., 1998).

### 2.1.29. DNA extraction from *C. elegans*

Worms were grown on 60 mm plates for few days (until pre-starved) following which the plates were washed with M9 buffer (3 g KH₂PO₄, 6 g Na₂HPO₄, 5 g NaCl, 1 mL 1 M MgSO₄, H₂O to 1 L) and worms, collected in 15 mL falcon tubes, washed twice in M9 and
pelleted on ice. Worms were then collected in two 1.5 mL Eppendorf tubes and 400 μL of freshly made lysis buffer (100 mM Tris pH 8.0, 100 mM NaCl, 50mM EDTA, 1% SDS, 1% β-mercaptoethanol, 100 μg/mL proteinase K solution) was added to both the Eppendorf tubes. The two Eppendorf tubes were then placed at -80°C for 30 min and then incubated at 60°C for 4 hr. Five microliters of 20 mg/mL proteinase K solution was added every 30 min and the tube contents were mixed by inversion. Debris was removed by centrifuging at 13,500 rpm for 30 s. The supernatant was transferred to a new tube along with 400 μL lysis buffer. 400 μL of phenol: chloroform: isoamyl-alcohol was added and tubes contents were mixed by inversion. Centrifugation was performed at 13,500 rpm and the supernatant was collected in a separate tube. 350 μL of phenol: chloroform: isoamyl-alcohol was added and after gentle mixing and a 2 min centrifugation, supernatant was collected into new tubes. 350 μL of chloroform: isoamyl-alcohol was added for the last extraction cycle. The supernatant was adjusted to 400 μL with sterile water. Two volumes of pre-chilled 95% ethanol (800 μL) was added to the tubes. DNA was spooled with a sterile glass rod and washed with 300 μL of pre-chilled 70% ethanol. An air-drying step was performed for a minute and the glass rod was placed in an Eppendorf tube with 200 μL of TE. Tubes were sealed with Parafilm and DNA eluted overnight at 4°C.

2.1.30. *C. elegans* clu-1 mutant

The *clu-1(ok2)* deletion mutant was ordered from the *Caenorhabditis* Genetics Center (CGC) (http://www.cgc.cbs.umn.edu/). In order to verify the *clu-1* deletion, forward primer 5’-ATGATGCTCAATCATATTTG-3’ and reverse primer 5’-CTTGCGGAGAGTTGATCAGC-3’ were designed. Touchdown PCR was performed and the thermal profile used as the following: initial denaturation (94 °C for 1 min) followed by denaturation (94°C for 15 s), annealing (60°C for 15 s, 1°C decrease/cycle) and extension (72°C for 1 min) for 9 cycles (from 60 to 50); denaturation (94°C for 15 s), annealing (50°C for 15 s) and extension (72°C for 5 min) for 29 cycles. The expected PCR sizes were 800 bp in the mutant due to fragment deletion and the 2023 bp in case of the wt.
2.1.31. *clu-1; LGG-1::mCherry* lines

To determine signs of upregulation of autophagy apparatus in *clu-1* mutants, the intestinal-driven promoter *mCherry::LGG-1* transgene (*nhx-2p::mCherry::LGG-1*) was used. LGG-1 is the worm homolog of ATG8 (Gosai et al., 2010) and marks autophagosomes. An *unc-119* mutation (*ed3*) was used as a trans marker for the *clu-1* locus on chromosome III to facilitate the identification of *clu-1/clu-1* homozygous. *clu-1* males generated by heat shock were crossed to *unc-119; nhx-2p::mCherry::LGG-1* hermaphrodites and homozygous *clu-1; nhx-2p::mCherry::LGG-1* progeny identified by loss of the Unc worms in selfing F$_2$ populations.
CHAPTER 3. CHARACTERIZATION OF PLANT MITOCHONDRIAL DYNAMIC MUTANT “friendly”

3.1. Introduction

As mentioned in Chapter 1, mitochondria in wt plants are traditionally distributed throughout the cytosol and depicted as single, discrete, spherical, or sausage-shaped organelles (Fig. 1.8). In contrast, the phenotype of friendly chondriome contains discrete clusters of tens of mitochondria (Fig. 1.8).

FMT is a functional orthologue of the CluA gene of Dictyostelium discoideum. CluA is required for correct mitochondrial distribution and morphology in the cell (Zhu et al., 1997; Logan et al., 2003) (Fig. 3.1). Furthermore, the fmt mitochondrial phenotype is similar to that observed in Clu1 deletion mutants of yeast (Fields et al., 1998). Additionally, clueless in Drosophila is another functional orthologue of FMT (Fig. 3.1). FMT, like its orthologues, has no significant homology to other proteins of known function. The only identified protein domains are a CLU domain (conserved in all eukaryotic CLU-type proteins) towards the N-terminus and TPR domains towards the C-terminus. TPR domains are thought to function in protein-protein interactions and it has been shown that mitochondria can associate with the microtubule-specific motor protein, kinesin, that binds cargo at the TPR domains in the kinesin light chains (Khodjakov et al., 1998).
Figure 3.1. *FMT* orthologues in *Dictyostelium* and *Drosophila*. (a) wt (mito-GFP), (b) *fmt1-1* (mito-GFP) in Arabidopsis epidermal pavement cells (Logan et al., 2003). (c) wt (d) *cluA* cells in Dictyostelium (Zhu et al., 1997). (e) Mitochondria in control and (f) *clueless* in *Drosophila* (using antibody)(Cox and Spradling, 2009). Scale bars = 10 μm in all panels. (Image is adapted from, Logan, 2010).
3.2. Objectives

It is known from Logan et al., (2003) that the *friendly* plants exhibit clusters of mitochondria. However, it is not known how the FRIENDLY protein plays a role in maintaining a “normal” mitochondrial population. It is also not known what effects *friendly* has on cellular and whole plant physiology. I predict that the defective mitochondrial positioning observed in *friendly* and plants will hinder plant growth and development by negatively affecting energy distribution within the cells. To investigate this, I will:

- Characterize the *friendly* mitochondrial phenotype at the cellular and whole plant level
- Measure the effects of *friendly* on shoot and root development
- Examine mitochondrial health using markers for mitophagy and membrane depolarization
- Determine whether photosynthetic electron transport is affected by mitochondrial positioning. cDNA microarray data (will be mentioned later) suggested that expression of genes related to photosynthetic system are affected in the *fmt1-1* mutant therefore it was decided to investigate whether *fmt1-1* has a defective photosynthetic system.
3.3. Results

3.3.1. Identification and characterization of FMT alleles

As mentioned previously (Chapter 1, section 1.7), Logan et al., (2003) EMS mutagenized Arabidopsis lines expressing mito-GFP with the aim of identifying mitochondrial dynamics mutants. The presence of GFP in fmt1-1 (EMS- fmt) caused one issue for the work described in this thesis since GFP is incompatible with green fluorescence from other dyes and stains, such as mito-mEosFP (mitochondria tagged with monomeric EosFP, see Chapter 1). Therefore, it was important to identify other alleles of fmt1-1 using T-DNA insertion lines. Logan et al., (2003) had identified T-DNA lines that displayed leaky clustered phenotypes and therefore it was decided to identify additional T-DNA alleles with a stronger mutant phenotype. The Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/) search engine was used to identify different independent Arabidopsis T-DNA insertions lines containing inserts in the FMT gene (Table 3.1).

PCR was carried out using sets of primers (Table 3.1) to identify homozygous lines for the T-DNA insertion. For example, as seen in Fig. 3.2, PCR of genomic DNA from T-DNA individuals (5 & 11, circled) did not produce the expected PCR product using a set of FMT specific oligonucleotides. However, both # 5 & # 11 did produce a PCR product, which correspond to the wt gene and the border of the T-DNA insert. Thus, it can be concluded that they are homozygous for the insert allele. This confirms the T-DNA insert is present in the FMT gene locus and that lines 5 and 11 are homozygous for the disrupted fmt1-2 allele.

An initial screen using the mitochondria dye TMRM on the homozygous mutants was used as a quick step to observe whether these lines had aberrant mitochondrial phenotypes. T-DNA lines 5 and 11 (Fig. 3.2) were identified as homozygous mutants with strong phenotypes and were crossed with plants expressing mito-GFP or mito-mEosFP mitochondrial fluorescent markers.
Figure 3.2. An example showing Arabidopsis plants homozygous for a T-DNA insertion in the At3g52140 (*FMT*) gene. This representative gel shows results of a PCR-based screen of Arabidopsis line Salk-046271 expected to contain a T-DNA insertion in At3g52140. The larger band (~1100bp) represents a PCR product generated from a wild type *FMT* allele. The smaller band (500-600bp) a PCR product generated from oligos annealing to the *FMT* gene and the border of the T-DNA insert. If only the larger band is present, the plant would be homozygous for the wild type *FMT* allele. While if only the lower band were present the plant would be homozygous for the *FMT* T-DNA insert. If both bands are present the plant would be hemizygous (white arrows). Lines 5 and 11 are homozygous *fmt* T-DNA mutants (showing only smaller band), as shown by yellow arrows. While line 14 is the wt (control) displaying only the larger band (red arrows).
Table 3.1. Independent alleles at the FMT locus.

<table>
<thead>
<tr>
<th>T-DNA lines</th>
<th>Insertion position</th>
<th>Primers</th>
<th>Product size</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>SALK_046271</td>
<td>Intron</td>
<td>LP (ATACCTGCAGCAGTTTGGCAAC) RP (CTACGCCCAACAGCTCTACTG)</td>
<td>502-802</td>
<td>Strong fmt phenotype</td>
</tr>
<tr>
<td>fmt1-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SALK_094731</td>
<td>Exon</td>
<td>LP (CTTGGAAATGGTTGCAGGTAG) RP (TACGTCTGTGCAAGATGGATG)</td>
<td>439-739</td>
<td>No homozygous mutants found</td>
</tr>
<tr>
<td>SAIL_513</td>
<td>Promoter</td>
<td>LP (AGGACGACCAAATGTGACAAC) RP (TTCATTTGACGGAAACAGAGG)</td>
<td>430-730</td>
<td>Strong fmt phenotype</td>
</tr>
<tr>
<td>fmt1-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAIL_294</td>
<td>Promoter</td>
<td>LP (GAGCAGTACAAGAAGCGGATG) RP (TTTTAAGCGGAAAGATGC)</td>
<td>610-910</td>
<td>No homozygous mutants found</td>
</tr>
</tbody>
</table>

As depicted in Table 3.1, homozygous mutants of lines SALK_046271 and SAIL_513 exhibited the fmt1-1 phenotype (clustered mitochondria) and contained T-DNA insertions in the intron and promoter of the FMT gene, respectively. The EMS generated fmt mutant (Logan et al., 2003) was named fmt1-1 while SALK_046271 was named fmt1-2 and SAIL_513 was named fmt1-3.

3.3.2. Mitochondria in fmt are organized into large clusters

To compare the mitochondrial clustered phenotype between fmt1-1 and fmt1-2, cluster size and number were quantified in wt and mutant seedlings using fluorescent microscope system 1. A typical Arabidopsis mitochondrial population is composed of pleomorphic, physically discrete organelles (0.5 to 1 µm in diameter) which are highly dynamic (Logan, 2006). Mitochondria in wt plants were found to be distributed evenly throughout the cytoplasm in both leaves and roots, whilst in fmt1-1, many mitochondria were arranged in large clusters of tens of organelles in addition to singletons which had a distribution resembling wt (Fig. 3.3A). In order to quantify cluster number and size, clusters were defined as discrete associations in an area of at least 0.38 µm² (equal to 15 pixels², see 2.1.4.), a phenotype that was not observed in healthy wt plants. While no clusters were observed in wt, some mitochondria were observed to be in loose groups with an average area of 0.105 µm² in leaf epidermal pavement cells and 0.202
Figure 3.3. The *fmt1-1* mutant is characterized by the presence of large clusters of mitochondria.

(A) Epifluorescent micrographs of root and leaf cells of 7 day-old Arabidopsis seedlings of the *fmt1-1* mutant and its wt (mito-GFP). Arrowheads indicate groups of mitochondria in wt, arrows indicate clusters. Scale bar = 10 μm. (B) Group size and number in wt. (C) Cluster size and number in *fmt1-1*. Values in (B) and (C) are averages calculated from 40 pairs of images of leaf (solid bars) or root (open bars) epidermal cells, each of the 40 pairs of images was of a separate plant. Error bars = SEM (standard error of the mean), n=40. (D) & (E) Frequency distribution of group sizes in wt or cluster size in *fmt1-1* respectively. Solid bars represent values from leaves, open bars from roots.
μm² in root epidermal cells (Fig. 3.3B). The average mitochondrial cluster size in fmt1-1 leaf epidermal cells was 2.491 μm², while significantly larger clusters covering an average area of 4.11 μm² were observed in roots (Fig. 3.3C). There was no significant difference in the number of clusters in leaves or roots in fmt1-1 (Fig. 3.3C). The majority of groups in wt covered an area less than 0.20 μm² in both leaves and roots while the larger size classes were more frequently observed in root cells reflecting the greater mean group size in this cell type (Fig. 3.3D & E). In fmt1-1 the majority of clusters fell within the 0.38–2 μm² range while, as in wt, clusters covering larger areas were more frequently present in roots than in leaves (Fig. 3.3E). A similar phenotype was observed in the fmt1-2 T-DNA mutant (Fig. 3.4).

3.3.3. Ultrastructure of mitochondria in fmt1-1 mutants

To determine whether clusters of mitochondria in fmt1-1 were connected via their outer membranes, leaves of wt and fmt1-1 were prepared for TEM by HPF (high pressure freezing) in order to better preserve membrane structure relative to chemical fixation (see 2.1.3, system 3). Clusters in the fmt1-1 mutant and groups of mitochondria in wt were observed using TEM (Fig. 3.5) (microscopy system 2). Ultra-thin sections were analyzed from at least 3 of fmt1-1 plants but there was no evidence of outer membrane connectivity between adjacent mitochondria in leaf cells (Fig. 3.5), rather each mitochondrion was physically discrete as in wt.

3.3.4. FRAP analysis of protein transfer in fmt1-1 mitochondrial clusters

On the basis of analysis of the TEM micrographs, it was concluded that mitochondrial clusters are composed of physically discrete organelles. However, since connectivity may be less obvious in 2D TEM images, it was decided to evaluate possible connectivity of the mitochondrial matrices in fmt1-1 cells using FRAP of the matrix-localized mito-GFP. A section of a mitochondrial cluster was photobleached (Fig. 3.6A) and fluorescence in the bleached area was examined for 300 s (Fig. 3.6B) to monitor any recovery. As shown in the images of Fig. 3.6, no recovery of mito-GFP fluorescence was visible in adjacent mitochondria within a cluster in fmt1-1. Mitochondria in wt are physically discrete and so a wt control for this FRAP experiment is not possible.
Figure 3.4. Analysis of cluster size and number in the fmt1-2 T-DNA. Data are averages taken from 20 independent images, each from a separate plant. Error bars = SEM, n=20.
Figure 3.5. TEM micrographs of mitochondrial clusters in *fmt1-1* and wt leaves. M = Mitochondria and C = Chloroplast. Scale bar = 0.5 μm
Figure 3.6. FRAP analysis of a mitochondrial cluster in *fmt*1*-1*. (A) Epifluorescent images of a cluster before photobleaching and after photobleaching (1 s after photobleaching). (B) Series of images were captured one second apart after photobleaching of the bleached clustered mitochondria using CLSM (see Materials and Methods, 2.1.5).
However, to ensure that photobleaching recovery could occur in connected mitochondria in Arabidopsis, another mitochondrial dynamics mutant known as nmt (the network mutant, see Chapter 1) was used. In the nmt mutant, mitochondria form an interconnected network of tubules extending throughout the cytosol as observed in Fig. 3.7 (Logan et al., 2003).

A region of the interconnected mitochondria in the nmt was photobleached as shown in Fig. 3.8. Ten images of elongated mitochondria were captured before photobleaching and then series of 300 images were taken one second apart after photobleaching. GFP fluorescence recovery was observed within 1.5 s after photobleaching in the nmt mutant (Fig. 3.8).

As can be observed in Fig. 3.8, recovery of GFP fluorescence in nmt (as a control) is rapid due to the movement of GFP molecules within the matrix of the long tubule. Additional confirmation of the motility of GFP if matrices were connected, is the observation that 30 s were sufficient for mitochondria to recover as shown in giant GFP-labeled mitochondria in tobacco cells (Van Gestel and Verbelen, 2002). Since no recovery was observed in FRAP analyses of clusters in fmt1-1, it was concluded that there is no connectivity of the matrices within a cluster.

### 3.3.5. Root length in wt and fmt1-1 or fmt1-2 seedlings

It was decided next to determine whether or not the mutation of FMT resulted in a whole plant phenotype. As shown in the images in Fig. 3.9 and quantitative data shown in Fig. 3.10, it was observed that fmt1-1 mutant plants have significantly shorter roots compared to wt in 7, 14, 21 and 28 day-old seedlings.

Significantly shorter roots were also observed for fmt1-2 compared to its wt (Col-0) (Fig. 3.11A) at all time points (Fig. 3.11B). Overall, primary root length is significantly shorter in fmt1-1 and fmt1-2 seedlings compared to wt at 7, 14, 21 and 28 days old (Figs. 3.10 & 3.11B) such that by 28 days the root of the mutant is only half the length of the wt.

### 3.3.6. Etiolated hypocotyl length

As can be seen from the images in Fig. 3.12A and quantitative data in Fig. 3.12B, fmt1-1 has a shorter etiolated hypocotyl compared to its wt (mito-GFP) and fmt1-2 has a shorter hypocotyl compared to its wt (Col-0) after seven days of growth in the dark.
Figure 3.7. Epifluorescent image shows elongated mitochondria in nmt expressing mito-GFP. Image show interconnected mitochondria in nmt of an epidermal cell of Arabidopsis leaf captured by fluorescent microscopy system 1. Scale bar =10 µm.

Figure 3.8. Series of images show the quick recovery after photobleaching in nmt. The left top panel shows the long mitochondrial tubule before bleach at zero s and right top panel show the long tubule after bleach (5 s) while other panels show the same view at 40 s and 100 s post-bleach. After 5 s of post-bleach, the GFP partial recovery can be observed (arrow). Scale bar =10 µm.
Figure 3.9. Wild type and *fmt1-1* gross phenotype. Scale bar = 5 mm.
Figure 3.10. Average root length of wt and fmt1-1 seedlings. Root length of wt and fmt1-1 seedlings (n = 20) at 7, 14, 21 and 28 days growth. Error bars = 1 SD. (*** = p<0.001).
Figure 3.11. *fmt1-2* has shorter roots and smaller leaves. (A) Root length of 7-day wt and *fmt1-2* seedlings. Scale bar = 5 mm. (B) Average root length for wt (Col-0) and *fmt1-2*. Error bars = 1 SD. (p<0.001)
Figure 3.12. Etiolated hypocotyl length of 7 day-old seedlings of wt and fmt mutants. (A) Seven day-old seedlings grown in the dark. Wild type is mito-GFP or Col-0 plants as controls for fmt1-1 or fmt1-2 respectively. Scale bar = 5 mm. (B) Quantification of hypocotyl length of dark grown etiolated seedlings 7 days after onset of germination. The top panel is showing representative images for the hypocotyl length of wt and mutant genotype (fmt1-1 & fmt1-2) seedlings, (n=20). Error bars = 1 SD. (p<0.001).
3.3.7. Effects of the mutation of *FMT* on above-ground biomass

Given the short root and etiolated hypocotyl phenotype, it was decided to measure biomass (Fig. 3.13) of the *fmt* mutants. Fresh weight and dry weight measurements were made for seedlings of each line at 7, 14, 21 and 28 days old. As demonstrated in the data shown in Fig. 3.14 & 3.15 the biomass in *fmt1-1* is reduced significantly at 7 and 21 days old and similar results was obtained with *fmt1-2*.

3.3.8. *fmt1-1* leaves have fewer trichomes

To further characterize the *fmt1-1* phenotype, the leaf surfaces of 14 day-old seedlings were visualized using a stereomicroscope (see, 2.1.14), and trichome numbers were counted using captured images for wt and *fmt1-1*. Figure 3.16 show the observation of reduced trichome numbers of *fmt1-1* leaves compared to wt.

3.3.9. Root zone measurements in wt and *fmt1-1*

As shown previously, the primary root length in the *fmt1-1* mutant is significantly shorter at 7, 14, 21 and 28 days (Figs. 3.9 and 3.10). To determine whether the reduction in whole root length in *fmt1-1* mutant is due to a reduction in length of specific root zones, the width and length of the root zones in wt and *fmt1-1* seedlings were measured using ImageJ.

The root cap, meristematic and division zones of wt and *fmt1-1* seedlings were visualized using a compound microscope. As can be seen from the images in Figs. 3.17 & 3.18, the reduction in total root length is due to a significant reduction in the length of the cap/meristematic region and a slightly greater reduction in the elongation zone of *fmt1-1* compared to the wt.

Six independent biological replicates of each line were examined and as shown by the representative images in Fig. 3.17 and the graph shown in Fig. 3.18, the average combined root cap and meristematic zone length of the *fmt1-1* was 0.18 mm and the division zone length was 0.375 mm, relative to a root cap length of 0.324 mm and meristematic zone length of 0.715 mm in the wt.
Figure 3.13. Comparison of phenotype of aerial components of wt and fmt mutant plants. Above-ground biomass for wt and fmt mutants. Images are showing 28 days (left panel) and 40 day-old plants (right panel) for wt and fmt (fmt1-1 and fmt1-2) mutants.
Figure 3.14. Dry weight of wt and fmt1-1 mutant genotype seedlings. Average dry weight measurements of wt and fmt1-1 at four different time points. Three independent replicates were used each containing 15 plants. Error bars = 1 SD. (**) = p<0.01.)
Figure 3.15. Dry weight of wt and fmt1-2 seedlings. Average dry weight of wt and fmt1-2 at four different time points. Means are of biological replicates each composed of 15 plants. Error bars = 1 SD. (* = p<0.1) for 7 and 28 days while p<0.001 for 14 days.
Figure 3.16. Rosette leaf trichome phenotype of wt and fmt1-1 plants. Fourteen-day old true leaves were visualized using brightfield on Nikon SMZ 1500 zoom stereomicroscope. Scale bar =1mm.
**Figure 3.17.** Measurements of wt and fmt1-1 root zones. A representative image shows the length of root zones of 7 day-old seedlings of wt and fmt1-1. Seedlings of wt or fmt1-1 were screened using compound microscope (10x objective lens) equipped with “Dino capture” software.
Figure 3.18. Length and width of cap/meristematic and elongation zones. Bars represent the mean measurements from 6 plants; error bars = SEM, n=6
Overall, the *fmt1-1* root elongation region is reduced and narrower in diameter when compared to wt roots (Fig. 3.18). Because roots were shorter in the *fmt* mutants, it was decided next to examine root cell anatomy.

### 3.3.10. Root cell anatomy

#### 3.3.10.1. Roots of *fmt1-1* contain more dead cells than in wt using propidium iodide (PI)

Roots were stained with propidium iodide (PI) to visualize root cell architecture in order to determine whether it was a reduction in cell number and/or cell size that was responsible for the reduction in root length. As can be seen in the images shown in Fig. 3.19 the roots of *fmt1-1* are composed of smaller cells and therefore there are a greater number of cells in a defined length of root tissue. Quantification of cell size showed a significant reduction in cell area of *fmt1-1* compared to wt (Fig. 3.20). However, PI staining also revealed that roots of *fmt1-1* contained many dead cells, which are permeable to PI, while few dead cells were observed in wt roots (Fig. 3.21).

#### 3.3.10.2. Roots of *fmt1-1* contain more dead cells than in wt using SYTOX-Orange

To further investigate cell death in the roots of *fmt1-1*, it was decided to use the more specific cell death probe SYTOX-Orange at 250 nM (Fig. 3.22). As illustrated by the images in Fig. 3.22, and quantification shown in Fig. 3.23, *fmt1-1* contained more dead cells, relative to wt, throughout the root.

These results showed that *fmt1-1* primary roots have more dead cells than wt and that the elongation and differentiation regions of *fmt1-1* are most affected (Figs. 3.22 & 3.23). SYTOX-Orange confirmed the PI staining observation, that the presence of dead cells likely contributes to the root growth defects in *fmt1-1* plants.
Figure 3.19. CLSM optical sections through a wt or fmt1-1 root. Over 20 six day-old seedlings of wt and fmt1-1 roots were imaged and used for quantification. Arrows indicate representative cells in the same cell type and developmental zone. This was a single optical section. Scale bar = 20 µm.
Figure 3.20. Analysis of the cell area and number of wt and *fmt1-1* roots. Cell area and number of root epidermal cells per fixed length in the elongation zone of 6 day-old seedlings. Images were captured using microscopy system 2. Bars represent mean measurements from 4 cells from each of 3 plants. Error bars = SEM.
Figure 3.21. CLSM z-projections of PI stained root of wt or fmt1-1. Six day-old wt and fmt1-1 seedlings were used. Arrows indicate dead cells identified by the entry of PI into the cells of different zones in wt and fmt1-1 root. Scale bar = 50 µm.
Figure 3.22. SYTOX-Orange staining of dead root cells in wt and fmt1-1. Six day-old wt and fmt1-1 roots were stained with SYTOX-Orange to identify dead cells in wt and fmt1-1 roots. Top panels show confocal z-projections of the red, SYTOX-Orange channel, middle panels show the brightfield images while the bottom panels show merged images. Arrows indicate dead cells in the elongation zone. Scale bar = 50 µm.
Figure 3.23. Average number of dead cells in *wt* and *fmt1-1* root zones. Dead cells were identified by the presence of a SYTOX-Orange stained nucleus. Average number of non-viable cells in *wt* and *fmt1-1* primary root was counted in each zone (root cap, dividing, elongation and differentiation). Five independent biological samples were used for the quantification analysis. There was significant difference in dead cells numbers of elongation and differentiation zones in *fmt1-1* compared to *wt*. Error bars = SEM. (**) = *p* < 0.01.
3.3.10.3. Identification of acidic compartments in fmt1-1

A conserved mitochondrial quality control pathway rids the cell of dysfunctional mitochondria. This pathway functions by firstly, isolating defective organelles from the otherwise healthy mitochondrial population, and secondly, by destroying these dysfunctional organelles. It was hypothesized that FMT is a key conserved component of this pathway. Therefore, it was decided to investigate mitochondrial quality control by measuring the stability of mitochondrial membrane potential using TMRM, and the possible induction of mitophagy using markers of autophagosomes.

The significant increase in root cell death led us to investigate a potential breakdown in the mitochondrial quality control process in fmt1-1. LysoTracker has been used for assessing autophagy in plants, and whilst not specific for autophagosomes, does label acid vacuoles required for the process (Yoshimoto, 2012). In Arabidopsis, autophagosomes fuse with the tonoplast and deliver damaged components directly to the lumen of the vacuole (Inoue et al., 2006; Mitou et al., 2009). Six day-old wt and fmt1-1 seedlings were stained using 1 µM LysoTracker Red DND-99 for five minutes followed by capture of z-stacks by CLSM of the root elongation zone (see, 2.1.12). As depicted in the images shown in Fig. 3.24A, Increased size and number of LysoTracker-stained acidic compartments in fmt1-1 were observed compared to wt. Quantification of LysoTracker stained compartments demonstrated that there was a significant increase in the number (54 ± 8.6 in fmt1-1 compared to 19 ± 3.6 in wt) and area (9.2 ± 1.2 µm² in fmt1-1 compared to 3.7 ± 0.8 µm² in wt) of LysoTracker-stained compartments in roots of fmt1-1 compared to wt (Fig. 3.24B). The increase in size and number of acid compartments in fmt1-1 is consistent with a role for FMT in the maintenance of cell health.

3.3.10.4. Mitochondrial membrane potential pulsing in fmt

Since fmt roots have dead cells and an increased number and size of acid compartments (as stained with LysoTracker Red), it was suspected that the fmt mutants were stressed. As outlined in Chapter 1, it was recently demonstrated that mitochondrial stress and dysfunction results in an increase in the frequency of a phenomenon termed “pulsing” whereby individual mitochondria experience a transient partial depolarization (Schwarzlander et al., 2012) that can be measured
Figure 3.24. The size and number of Lysotracker-stained acidic compartments are increased in fmt1-1. (A) Confocal microscopic images show representative maximum projections of 16 x 1µm optical sections through the epidermal cells of the root elongation zone in 6 day-old seedlings. Scale bar = 10 µm. (B) Quantification analysis of area and number of Lysotracker stained compartments in roots imaged as in (A) using the “Analyse particles” option in ImageJ. Error bars = SEM and the p<0.01, n=5.
using the fluorescent cationic probe, TMRM, as a reporter of the mitochondrial membrane potential (Bernardi et al., 1999). Several trials were attempted and our preliminary data suggested that the \textit{fmt1-1} mutant displayed an increase in membrane potential pulsing (Fig. 3.25).

As seen in the images of Fig. 3.25, mitochondrial inner membranes were depolarized more frequently in \textit{fmt1-1} than in \textit{wt}. However, because the required confocal laser in the Department of Biology was broken for a prolonged period, we collaborated with Dr. Markus Schwärzlander (Bonn University, Germany) to perform the pulsing experiment and subsequent quantification (Fig 3.26).

Since pulses are rare events under controlled conditions, it was immediately evident from CLSM imaging that the number of mitochondria exhibiting pulses and the frequency of pulses was greater in the mutant than in \textit{wt}. Mitochondria undergoing a pulse in the images of hypocotyl cells stably expressing mito-GFP shown in Fig. 3.26 are identified by their green color, due to mito-GFP fluorescence in the absence of the matrix-localized red TMRM, while mitochondria with a greater membrane potential are yellow-orange due to the colocalization of TMRM and mito-GFP in their matrices. There was no obvious difference in the pulsing behaviour of the more physically discrete mitochondria compared to those arranged into clusters in the mutant. Quantification of the number of pulses per hundred mitochondria per minute showed that the frequency of pulses was 0.12 per 100 mitochondria per minute in \textit{wt} while in \textit{fmt1-1} it was increased to 11.14 per 100 mitochondria per minute, an increase of approximately 100x (Fig. 3.26).

\subsection*{3.3.11. \textit{fmt} shows global transcriptome reprogramming indicating mitochondrial dysfunction, cellular stress, and repression of photosynthesis}

To understand the overall impact of the \textit{fmt} mutation and of compromised fusion control on whole cell homeostasis, a set of microarray-based transcriptome studies on whole seedlings was performed (Collaboration with Dr. Raju Datla, Plant Biotechnology Institute, National Research Council of Canada, Saskatoon). Although, in general, only small fold changes of abundance in \textit{fmt1-1} relative to \textit{wt} were observed, these changes were determined to be significant using Cybr-T (Baldi and Long, 2001) for 4121 nonredundant transcripts (
Figure 3.25. Mitochondrial membrane potential in fmt1-1. Epifluorescent images show a representative ‘snapshot’ of a root epidermal cell from fmt1-2 with merged channels of TMRM (red) and mito-GFP (green). Mitochondria with a relatively higher membrane potential appears as orange (red arrows) while mitochondria with relatively lower membrane potential (perhaps undergoing a pulse) appears as green (white arrows). Scale bar = 10 μm
Figure 3.26. The frequency of mitochondrial membrane potential pulsing is increased in fmt1-1. (A) Representative images of mitochondria in control (wt, mito-GFP) or fmt1-1 hypocotyl cells stained with 50 nM TMRM. The images are merged composites of green (GFP) and red (TMRM) channels. Arrows indicate mitochondria undergoing a transiently depolarizing pulse. Scale bar = 5 µm. (B) Quantification of the number of pulses per 100 mitochondria in root epidermal cells per minute. 219 mitochondria were scored on average for each biological replicate. Error bars = 95% confidence limits, p< 0.01, n=7 (wt) or 6 (fmt1-1).
El Zawily et al., 2014), supplementary Table 1, Appendix B). The significantly regulated transcripts were allocated to functional-groups using MapMan (Usadel et al., 2005) resulting in the identification of 54 significantly regulated functional groups of genes (Analysis was performed by Dr. Finkemeier, Max Planck Institute, Cologne, Germany, El Zawily et al., 2014, see Appendix B). The functional groups included ‘protein synthesis’ and ‘protein degradation’, consistent with the observed induction of autophagy. Together with ‘photosynthesis’ and ‘photosynthetic light reactions’, which were also significantly regulated, this functional group set was previously identified by meta-analysis of multiple transcriptomic data sets (Schwarzländer et al., 2012a) as being a general indicator for mitochondrial dysfunction.

3.3.12. Photosynthetic performance is reduced in fmt due to a defect in external electron flow to Photosystem I

To test the results from the transcriptome analysis suggesting photosynthetic defects in fmt1-1, it was next decided to investigate PSI and PSII activity. Collaboration for these experiments with Dr. Ken Wilson (University of Saskatchewan) was carried out. The results showed that there was no difference in steady-state PSII redox state between wt and fmt1-1, as estimated by the chlorophyll fluorescence parameter 1-qL (fraction of open PSII centers) (Fig. 3.27A). This observation was true both at the growth irradiance of 100 μmol photons m⁻² s⁻¹, and when the leaf being sampled was exposed to an actinic light of 910 μmol photons m⁻² s⁻¹ (Fig. 3.27A). Similarly, there was no difference in capacity for nonphotochemical dissipation of excess light (NPQ) between the fmt1-1 and wt (Fig. 3.27B).

PSI activity measured as the change in absorbance at 820 nm was used to probe possible effects of the fmt mutation on PSI function and its supply of electrons. The wt plants exhibited a greater level of PSI activity (Table 3.2) and an increased ΔA₈₂₀/ΔA₈₂₀ value. PSI was also more slowly reduced in fmt1-1: this measure of how rapidly PSI is re-reduced following the removal of far-red illumination indicates that PSI is receiving a diminished supply of electrons from plastocyanin and the photosynthetic electron transport chain. To probe whether the number of electrons available to PSI from the electron transport chain was diminished in fmt1-1, the areas under the curve of the single and multiple turnover flashes was used. The results confirm the PSI
Figure 3.27. The activity of Photosystem II is unaffected in *fmt1-1*. (A) Steady-state PSII redox state (as estimated by the chlorophyll fluorescence parameter 1-Q1) in wt and *fmt1-1*. (B) Capacity for nonphotochemical dissipation of excess light (NPQ) in *fmt1-1* and wt.
re-reduction observations, with \textit{fmt1-1} having a lower number of electrons available from sources outside of the linear electron transport chain (El Zawily et al., 2014, Appendix B).

**Table 3.2. PSI activity is reduced in \textit{fmt1-1}.** Values shown are means ± SEM (n=10 independent biological replicates). For experimental details see Materials and Methods.

<table>
<thead>
<tr>
<th>Plant Line</th>
<th>ΔA_{820}/A_{820} (relative units)</th>
<th>T_{1/2} PSI re-reduction (ms)</th>
<th>Intersystem e− Pool e−/P700 (MT_{Area}/ST_{Area})</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>570 ± 17</td>
<td>73 ± 4.0</td>
<td>20.9 ± 2.5</td>
</tr>
<tr>
<td>\textit{fmt1-1}</td>
<td>509 ± 25</td>
<td>86 ± 6.7</td>
<td>15.1 ± 1.3</td>
</tr>
</tbody>
</table>

1. The ΔA820/A820 ratios are significantly different (p = 0.03).
2. The T1/2 values are significantly different (p = 0.04).

### 3.4. Summary and conclusion

\textit{FMT} is conserved amongst eukaryotes but despite encoding a protein with a molecular mass of approximately 150 kDa, there are few clues to the protein function in its sequence. The \textit{fmt1-1} mutant was generated using EMS mutagenesis of an Arabidopsis line expressing mito-GFP (Logan et al., 2003). Mitochondria in \textit{fmt1-1} mutants form large clusters of tens of organelles, although some mitochondria retain a more randomly distributed wt distribution (Logan et al., 2003). The aim of the work described in this chapter was to better quantify the mitochondrial phenotype of \textit{fmt1-1}, to quantify aspects of the whole plant phenotype of the mutant and to assess general indicators of mitochondrial dysfunction.

Quantification of cluster size and number showed that clusters, as defined, were rarely seen in wt plants and that the roots of \textit{fnt} mutants contained larger clusters than leaf epidermal cells while there was no difference in the numbers of clusters in roots versus leaves. It was next investigated whether or not clusters in \textit{fmt1-1} were composed of discrete organelles by examining the ultrastructure of mitochondria in thin sections by TEM. Analyses clearly showed that the \textit{fmt1-1} clusters are composed of physically separate organelles (Figs. 3.5). In addition, FRAP analysis of a region within a cluster demonstrated that there was no visible GFP
fluorescence recovery in the mitochondrial matrix of bleached mitochondria indicating that mitochondria in clusters do not have connected matrices (Fig. 3.6). In contrast, photobleaching of a region within a long mitochondrial tubule in the nmt mutant (as control) showed almost immediate recovery of the fluorescent label (Figs. 3.8), demonstrating that GFP can move rapidly within mitochondria when their matrices are connected. It can be concluded that mitochondria within a cluster in fmt1-1 are composed of physically discrete organelles.

Whole plant phenotyping experiments were performed to determine the impact of fmt on primary root length, hypocotyl length and biomass. A diminished growth phenotype was observed in fmt mutant plants. The data revealed a significant difference in the root length of fmt mutant seedlings at 7, 14, 21 and 28 days old compared to wt (Figs. 3.10 & 3.11).

It was next decided to investigate the effect of the fmt mutation on the etiolated growth of the hypocotyl in order to determine if growth defects could also be observed in aerial parts of the plant in the absence of photosynthetic activity. fmt seedlings showed reduction in hypocotyl growth compared to wt (Fig. 3.12). In addition, fmt has reduced biomass compared to wt (Fig. 3.14 & 3.15), which is consistent with the short root and hypocotyl results.

Taken together, all phenotypic experiments suggest that fmt plants are stressed and that they might have structural defects. The use of PI and SYTOX-Orange demonstrated that fmt1-1 seedlings contain significantly more dead cells, specifically in the elongation and differentiation root zones of fmt1-1 than in wt as illustrated in Figs. 3.21, 3.22 & 3.23. This observation is consistent with the fact that fmt1-1 roots have a shorter elongation zone than wt.

As mentioned in Chapter 1, pulsing is a transient depolarization of the inner mitochondrial membrane. It was determined that pulsing is a mechanism that regulates the bioenergetic balance in individual mitochondria and it was proposed that pulsing is a mechanism to counteract mitochondrial dysfunction (Schwarzlander et al., 2012).

The increased frequency of membrane potential pulsing in fmt1-1, which is indicative of mitochondrial stress, has been postulated to be a component of a mitochondrial quality control pathway (Schwarzlander et al., 2012). This result, together with the whole plant phenotypes, especially the significant increase in root cell death, led us to seek additional evidence for a breakdown in the mitochondrial quality control process in fmt1-1 (Fig. 3.26).

Mitochondria are one of the main sources of cellular ROS and increased production of ROS causes oxidative damage to mitochondrial DNA, lipids and proteins leading to a vicious
cycle of ROS induced ROS production. Thus, removal of damaged and therefore ROS-overproducing mitochondria is a strategy the cell utilizes to survive oxidative stress (Minibayeva et al., 2012) and is crucial to protect cells from deleterious effects of disordered mitochondrial metabolism and to maintain cellular homeostasis. The results presented in this chapter showed that Lysotracker-stained acidic compartments in fmt1-1 are larger and increased in number compared to wt, suggesting that damaged mitochondria, identified by their pulsing behaviour, are assigned for recycling or removal (Fig. 3.24).

In summary, disrupted whole plant growth and development, increased cell death, increased frequency of mitochondrial membrane potential pulsing, and the increased size and number of autolysosomes in fmt suggest defects to mitochondrial quality control.
CHAPTER 4. FRIENDLY IS THE FIRST PLANT MITOCHONDRIAL PROTEIN IDENTIFIED TO BE INVOLVED IN MITOCHONDRIAL FUSION

4.1. Introduction

Key aspects of mitochondrial dynamics are the fusion and fission processes. Mitochondrial fusion and fission research has attracted a great deal of attention in recent years (see Chapter 1) due to their vital roles in cell death, ageing and the general maintenance of mitochondrial function in non-plant eukaryotes (Detmer and Chan, 2007; Westermann, 2010). In all eukaryotes studied to date, mitochondrial fusion and fission play a fundamental role in controlling the number, size, morphology and distribution of mitochondria (Logan, 2010a; Shaw and Nunnari, 2002).

Based on the fact that FMT contains TPR domains at the C-terminus, Logan et al., (2003) hypothesized that FMT/Clu is involved in regulating mitochondrial association with the cytoskeleton. It was proposed that FMT acts as an adaptor that binds to mitochondria at sites involved in the tethering of mitochondria to MTs. According to this hypothesis, the absence of FMT results in mitochondrial binding to MTs, which prevents them from moving freely on the actin cytoskeleton. It was also suggested that mitochondrial division may be affected by the lack of free movement on actin, which leads to clustered mitochondria (Logan et al., 2003).

Based on this hypothesis, Cox and Spradling (2009) suggested that clustered mitochondria in the Drosophila clueless mutant are tethered to the plus-ends of MTs. They speculated that Clu and Parkin (located in the cytoplasm) interfere with the dynein-regulated movement of mitochondria towards the microtubule minus-end and that mitochondria move to different sites of the cell according to their physiological states. They provided evidence that the absence of Clu resulted in impairment of correct mitochondrial movement and this was an integral part of a mitochondrial quality control pathway involving Clu and Parkin (Cox and Spradling, 2009; Logan et al., 2003).
4.2. Objectives

The *friendly* phenotype suggests that mitochondrial movement may be impaired. However, this has not been demonstrated *in vivo* in plants. Based on Logan et al., 2003 and Drosophila work (Cox and Spradling, 2009), I predict that by specifically disrupting cytoskeleton elements, clustered mitochondria tether to microtubule plus-ends. The objectives of this chapter are as follows:

- Determine the interrelationship between *fmt* mitochondrial clusters and actin filaments.
- Establish any association between *fmt* mitochondrial clusters, and MTs.
- Determine whether *friendly* clustered mitochondria are tethered to microtubule plus-ends.
- Determine how mitochondrial clusters move in the cell and how they interact with the cytoskeleton.
- Investigate how *fmt* clusters are formed.
4.3. Results

4.3.1. The interrelationship between clustered mitochondria and actin filaments

In order to understand the interaction between fmt clusters and F-actin, it was first decided to examine the effect of latrunculin-B (lat-B) (which depolymerizes actin) on mitochondrial dynamics in wt and fmt1-1. The results showed that depolymerization of the actin cytoskeleton in wt using lat-B resulted in a similar mitochondrial clustered phenotype as in fmt1-1 (Fig. 4.1), while in fmt1-1, lat-B resulted in more clusters but no quantification analysis was done (Fig. 4.2). It was also observed that depolymerization of F-actin with lat-B arrests the movement of mitochondria in wt and fmt1-1.

4.3.2. Mitochondria in fmt move on the actin cytoskeleton and clusters can be disrupted by the dynamic actin cytoskeleton

A double transgenic line expressing mCherry-mTalin in the fmt1-1 background was used to visualize mitochondria and actin. Results illustrated that mitochondrial clusters in fmt1-1 move along actin filaments (Fig. 4.3A), the same as in wt. Single mitochondria in fmt1-1 also move on actin filaments (Fig. 4.3A) as in wt. However, larger clusters are less able to move through the cytosol intact and are disrupted by the forces of cytoplasmic streaming. Large clusters assemble due to the aggregation of smaller clusters that are brought together through movement on actin and clusters can be disrupted by sudden changes in the organization of the actin cytoskeleton (Fig. 4.3B). Clusters of mitochondria in fmt1-1 are therefore highly dynamic transient structures composed of discrete organelles (Chapter 3) that are able to interact with the actin cytoskeleton in a wt manner.
Figure 4.1. Effects of lat-B on mitochondrial morphology in wt. Seven day-old mito-GFP (wt) seedlings were treated with lat-B (2 μM) or 0.1% (v/v) ethanol (EtOH). Arrows show clustered mitochondria. Scale bar =10 μm.
Figure 4.2. Effects of lat-B on mitochondrial dynamics in fmt1-1. Seven day-old fmt1-1 seedlings were treated with lat-B (2 μM) or 0.1% (v/v) mock treatments with ethanol (EtOH). Arrows show enlarged clusters. Scale bar =10 μm.
Figure 4.3. Mitochondria in fmt1-1 move on the actin cytoskeleton and clusters are disrupted by the dynamic actin cytoskeleton. Mitochondria were visualized by mito-GFP fluorescence and actin by mCherry-mTalin. (A) Single images from a time-lapse movie showing a small cluster of mitochondria (arrows) moving on actin. (B) Single images from a time-lapse movie showing a cluster developing (white arrows) up to 60 s when it is disrupted by rearrangement of the actin cytoskeleton (yellow arrows). Scale bars = 5 µm.
4.3.3. Visualization of \textit{fmt} mitochondrial clusters, microtubule plus-ends and the microtubule cytoskeleton

Logan et al., (2003) hypothesized that clustered mitochondria in \textit{fmt1-1} are tethered to MTs. To examine this hypothesis, an Arabidopsis line expressing End-Binding 1 protein (EB1b, to visualize the plus-ends of microtubules) was crossed with wt and \textit{fmt1-1} expressing mito-GFP. The double transgenic lines (F2 generation) were screened and analyzed using the fluorescent microscopy system 1. The results showed that MT plus-ends and mitochondria are in different focal planes and that clusters and plus-ends moved independently of each other (Figs. 4.4 & 4.5). Thus it was concluded that there was no obvious association between clustered mitochondria in the \textit{fmt1-1} and EB1b (Figs. 4.4 & 4.5).

Next, the interaction between clustered mitochondria and EB1b in \textit{fmt1-1} was also investigated using drugs inducing MTs and actin depolymerization. Double transgenic lines expressing mito-GFP/ mCherry-EB1b were treated with oryzalin (inhibits microtubule polymerization) and lat-B. Time laps images were captured for mito-GFP/ mCherry-EB1b from 10 different cells of each plant for a total of 3 plants for each line. No interaction between clustered mitochondria and EB1b in \textit{fmt1-1} was observed when lines were treated with 30 \( \mu \text{M} \) oryzalin for 3 hr (Fig. 4.6). It was also observed that the clustered mitochondria appeared to move on a different focal plane similar to the untreated samples (Fig. 4.4 & 4.5). The results demonstrated that depolymerization of microtubules with oryzalin failed to disrupt the clusters or affect their movement.

No specific association between clustered mitochondria and EB1b was apparent when \textit{fmt1-1} expressing mCherry-EB1b was treated with 1 \( \mu \text{M} \) lat-B (Fig. 4.7A & B), although the widespread distribution of both mitochondria and plus-ends does result in some spatial colocalization.

To determine if there is any association between clustered mitochondria and MTs, images and movies were captured and analyzed of Arabidopsis lines (mito-GFP and \textit{fmt1-1}) expressing mCherry-MAP4 (Fig. 4.8). According to the images shown in Fig. 4.8 there was no obvious association observed between \textit{fmt1-1} clustered mitochondria and microtubules (arrows). In addition, clustered mitochondria and MTs are in two different focal planes.
Figure 4.5. Microtubule plus-ends and clustered mitochondria in fnt1-1. Mitochondria visualized by mito-GFP (green) and MT plus-ends by mCherry-EB1b (red). Single images from a time-lapse movie show no association between clustered mitochondria and MT plus-ends (arrows). Scale bar = 5 µm.

Figure 4.4. No association between microtubule plus-ends and clustered mitochondria in fnt1-1. Epifluorescent images of a leaf epidermal cell in fnt1-1 expressing mCherry-EB1b (red) and mito-GFP (green) captured using fluorescent microscopy system 1. Arrows show mitochondrial clusters in different locations from the plus-ends. Scale bar = 10 µm.
Figure 4.6. Effects of oryzalin on clustered mitochondria and MTs plus-ends in *fmt1-1*.
Mitochondria and MTs plus-ends were visualized by mito-GFP (green) fluorescence and mCherry-EB1b (red), respectively, using microscopy system 1 after 3 hr of oryzalin (30 μM) treatment. The arrows show EB1b puncta. Scale bar = 10 μM.
Figure 4.7. Effects of lat-B treatments on clustered mitochondria and MTs plus-ends in fmt1-1. Mitochondria and MTs plus-ends (7 day-old of fmt1-1 seedlings) were visualized using mito-GFP (green) fluorescence and mCherry-EB1b (red), respectively, in fmt1-1 using microscopic system 1. (A) Control lines treated with 0.1% (v/v) EtOH (B) Seedlings treated with 1μM lat-B after 12 hr and 24 hr. Scale bar = 10 μm.
Figure 4.8. Mitochondria (green) in either mito-GFP wt, or fmt1-1 are not associated with microtubules (mCherry-MAP4, red). Arrows indicate mitochondria or clusters of mitochondria that are clear of MTs. Scale bar = 5 µm.
MT depolymerization in wt and in fmt1-1 was observed after treatment with 30 μM oryzalin (Figs. 4.9 & 4.10). Fragmented MTs were observed when plant lines were treated with 20 μM oryzalin (Fig. 4.10, middle panel), while no changes to mitochondria or MTs were observed in the mock treatment of 0.1% (v/v) ethanol (Fig. 4.10, top panels). No obvious changes in mitochondrial cluster morphology or dynamics were observed in cells treated with oryzalin (Fig. 4.11).

Given the effect of lat-B on the formation of clusters of mitochondria, it was decided to next investigate the effect of lat-B treatment on MTs. Wild type and fmt1-1 seedlings (7 day-old) expressing mCherry-MAP4 were treated with lat-B which led to the formation of clusters in wt or larger clusters in fmt1-1 as previously demonstrated (Figs. 4.1 & 4.2). However, no effect on the organization of MTs was observed (Figs. 4.12 & 4.13).

4.3.4. Clusters in fmt1-1 form due to an increase in the time of association between mitochondria that meet through movement on actin

Following the discoveries that mitochondrial clusters in fmt1-1 move on actin, and the role of actin in bringing mitochondria together in the formation and disruption of clusters, it was decided to focus on identifying a mechanism to explain how clusters are generated. Mitochondria in wt cells that meet one another through movement on actin, often display a short period of association (Fig. 4.14A) during which time fusion and subsequent fission can occur: a phenomenon also seen in some mammalian cells and termed “kiss and run” (Twig et al., 2008). The panels in Fig. 4.14A display individual frames from a movie which show two association/disassociation events in wt: the panels show one of these associations that occurs within 20 s (at 19 s in the movie) and the subsequent disassociation that occurs within a further 10 s (at 28 s in the movie). In contrast, the panels in Fig. 4.14B show an association/disassociation event between a single mitochondrion and the exterior of a cluster in fmt1-1: the single mitochondrion associates with the cluster within 20 s (at 18 s in the movie) and disassociates 62 s later. By analyzing 45 movies each of wt and fmt1-1, it was possible to quantify the average length of inter-mitochondrial association: in wt the average is 15.0 ± 0.7 s (Fig. 4.14C), however, in the fmt1-1 mutant the average time of association is significantly greater at 61.5 ± 1.4 s (Fig. 4.14C). This extended association time is sufficient to lead to the
Figure 4.9. Effects of oryzalin on mitochondria (mito-GFP, green) and microtubules (mCherry-MAP4, red) in wt. Seven day-old seedlings were treated with 30 μM oryzalin for 3 hr or 0.1% (v/v) EtOH as control. Arrows show depolymerized MTs. Scale bar = 10 μm.
Figure 4.10. Effects of oryzalin treatments on clustered mitochondria (mito-GFP) and MTs (mCherry-MAP4) in fmt1-1. (A) fmt1-1/mCherry-MAP4 seedlings (7 day-old) treated with 0.1% (v/v) EtOH. (B) fmt1-1/mCherry-MAP4 seedlings treated with 20 μM oryzalin for 3 hr. (C) Seedlings treated with 30 μM oryzalin for 3 hr. Arrows show clustered mitochondria. Scale bar = 10 μm.
Figure 4.11. No associations between clustered mitochondria and MTs in fmt1-1. Mitochondria were visualized by mito-GFP fluorescence and MTs by mCherry-MAP4. Single images from a time-lapse movie showing a cluster of mitochondria (arrows) do not interact with fragmented MTs and no changes in clustered mitochondria phenotype. Arabidopsis lines were treated with 20 μM oryzalin for 3 hr. Scale bars = 10 μm.
Figure 4.12. Effects of lat-B treatments on mitochondria and MTs in wt seedlings. Double transgenic lines expressing mito-GFP (green color) and mCherry-MAP4 (red color) of wt were treated with 2 μM lat-B for 2 hr or with 0.1% EtOH as a control. Images were captured using microscopy system 1. Arrows show clustered mitochondria. Scale bar = 10 μm.
Figure 4.13. Effects of lat-B treatment on clustered mitochondria (arrow) and MTs in fmt1-1. fmt1-1 seedlings (7 day-old) expressing mito-GFP (green color) and mCherry- MAP4 (red color) were treated with 2 μM lat-B for 2 hr or with 0.1% EtOH (top panel). Images were captured using microscopy system 1. The focal point was the cortical MT array and therefore the mitochondrial cluster is out of focus since the two are in different focal planes. Scale bar = 10 μm.
build up of a cluster as more mitochondria temporarily associate with the growing cluster before eventually being able to leave the cluster if sufficiently close to a filament of actin (Fig. 4.3 B).

It was also observed, in fmtl-1, that sometimes a cluster of mitochondria joined a second cluster. For example, as can be seen from the images in Fig. 4.15, a cluster associated with another at 5 s and a group of at least 10 organelles disassociated from the large cluster at 60 s, while another group of mitochondria dissociated until the large cluster separated into a few groups at 150 s.
Figure 4.14. Association time between mitochondria in wt and the fmt1-1 mutant. (A) Panels show single images from a movie of mitochondria in a wt leaf epidermal cell in which two mitochondria meet by 20 s (19 s in the movie) and separate by 30 s (28 s in the movie). (B) Panels show single images of mitochondria in leaf epidermal cell from the fmt1-1 mutant in which a mitochondrion joins a cluster at 18 s and then separates from the cluster at 1 min 20 s. Scale bars = 10 µm. (C) Quantification of the duration, in seconds, of association of single mitochondria in wt (solid bar) or a mitochondrion and a cluster in fmt1-1 (open bar). Bars represent the mean duration calculated from three independent experiments (n=3) of individual mitochondrial associations calculated from movies of five cells in each of three plants per experiment (15 movies per experiment). $p = 0.0002$
Figure 4.15. Association of two clusters in fmt1-1. Series of epifluorescent images from a time-lapse are showing a cluster (yellow arrow) associating with another cluster (red arrow) in fmt1-1. This large cluster later breaks apart by first losing a few mitochondria (60s, white arrows) before further dissociation into smaller clusters (150 s, white arrows) until the large cluster disassociates into small clusters. The different colors show the association/dissociation between clusters. Scale bar =10 µm.
4.4. Summary and conclusion

Homeostasis of chondriome structure in Arabidopsis depends on frequent “kiss and run” fusion and movement along the actin cytoskeleton. By these mechanisms, mitochondria are correctly positioned within the cell to provide metabolic support and fulfill their signaling functions (Jayashankar and Rafelski, 2014).

Logan et al. (2003) hypothesized that clusters of mitochondria in fmt1-1 arose via the association of mitochondria with microtubules and this hypothesis underpinned the work described in this chapter. The aim was to provide useful functional information about the role of FMT in maintaining chondriome structure. Firstly, it was decided to investigate the cytoskeleton structure in fmt1-1: the results presented show that the cytoskeleton structure appeared normal in fmt1-1. Next, it was decided to test the hypothesis, suggested by Logan et al. (2003) and extended by Cox and Spradling (2009) that clusters arose due to the tethering of mitochondria to MTs, and specifically the plus-ends of MTs. The results indicated that there was no obvious association between clustered mitochondria and MTs, or the plus-ends of MTs. Furthermore, the use of cytoskeleton-disrupting drugs confirmed the conclusion that there was no association between clustered mitochondria and MTs or their plus-ends. There was, however, a clear dynamic interaction between clustered mitochondria in fmt1-1 and the actin cytoskeleton as in wt. It is concluded that microtubules have little if any effect on fmt1-1 cluster development and dynamics beyond the role of microtubules in shaping F-actin networks and therefore mitochondrial dynamics (Zheng et al., 2009).

Fields et al. (2002) cited the similarity of the mitochondrial organization in yeast clu1Δ cells to that in cells lacking the mitochondrial division protein Dnm1p as further evidence of a role for CluA in mitochondrial division in Dictyostelium. However, applying the same argument to Arabidopsis leads to the opposite conclusion. Arabidopsis mutants of proteins involved in mitochondrial division, such as the dynamin-like proteins DRP3A (Logan et al., 2004) and DRP3B (Arimura and Tsutsumi, 2002), the Fis1 orthologue BIGYIN1 (Scott et al., 2006), or the plant-specific protein NMT (Logan et al., 2003; Arimura et al., 2008) contain greatly enlarged, elongated or reticular mitochondria, which are completely different from the clustered phenotype of fmt mutants. NMT is involved in mitochondrial division and defects caused elongated mitochondria (Logan et al., 2004; Scott and Logan, 2011; Arimura et al., 2008; Logan et al.,
Defects in division or motility can lead to mitochondrial and cellular dysfunction and cell death in both plant and mammalian cells (Logan, 2006b; Chen and Chan, 2009).

The plant chondriome, which has been termed a discontinuous whole, is typically composed of physically discrete organelles that interact genetically via fusion, while, the animal chondriome is typically more reticular in organization, although this varies greatly depending on cell type (see Chapter 1). In mammals and yeast, a predominance of fusion over fission, caused by genetic deletion of components of the fission apparatus, leads to a more reticular network chondriome, whilst genetic deletion of components of the fusion apparatus in yeast and animal cells leads to a fragmentation of the chondriome (Karbowski and Youle, 2003; Okamoto and Shaw, 2005; Shaw and Nunnari, 2002; Hoppins and Nunnari, 2009). In plants, disruption of mitochondrial fission leads to the production of enlarged spherical or elongated and occasionally reticular mitochondria (Scott et al., 2006; Logan, 2010b; Logan et al., 2003; Arimura and Tsutsumi, 2002). However, we know nothing of the plant fusion apparatus.

The length of association time between mitochondria was measured and indicated a significant difference between wt and fmt1-1 in the duration of association of mitochondria. It was hypothesized that after mitochondria meet due to either rearrangement of, or movement on, actin filaments they interact first via a brief association step termed a “handshake” involving FMT and that FMT aids in the timing of this handshake. In the absence of FMT, however, the handshake is prolonged such that clusters of mitochondria develop between slow hand-shaking organelles.

In wt, a single mitochondrion joined another mitochondrion or a small group of mitochondria and separated quickly therefore: this is termed a ‘fast-handshake’ (Fig 4.14A). While in fmt1-1, I observed mitochondrial disassociation after a mitochondrion joined a cluster took much longer and therefore the association was termed a ‘slow-handshake’ (Fig. 4.14B).

This extended association time, or ‘handshake’ is sufficient to lead to the build up of a cluster as more mitochondria temporarily associate with the growing cluster before eventually being able to leave the cluster if sufficiently close to a filament of actin.

This slow-fast handshake phenomenon provides a mechanism to explain how a cluster is formed in fmt1-1. Since mitochondrial association is a pre-requisite to fusion, it follows that FMT is the first protein to be identified as involved directly in plant mitochondrial fusion.
CHAPTER 5. MOLECULAR AND PHYSIOLOGICAL ANALYSES OF FRIENDLY FUNCTION

5.1. Introduction

The aim of the work described in the previous chapters aims to understand how disruption of FMT function leads to the clustered mitochondrial phenotype and the effects of this aberrant mitochondrial morphology on cell and whole plant physiology. As mentioned previously, fmt mutants display an increased frequency of mitochondrial membrane potential pulsing (Chapter 3), which is indicative of cellular stress (Schwarzlander et al., 2012) and this stress likely contributes to death of root cells. Furthermore, there is an increase in the number and size of acidic compartments (putatively identified as autolysosomes) in fmt1-1 compared to wt, suggesting defective mitochondria are being removed by auto/mitophagy (Chapter 3).

Next, it was decided to investigate the interrelationship between clustered mitochondria and the cytoskeleton. It was initially hypothesized that mitochondrial clusters in fmt resulted from tethering of mitochondria to microtubules (Logan et al., 2003) with this hypothesis being refined by Cox and Spradling (2009). They suggested that clusters in a Drosophila clu mutant formed due to a failure of mitochondrial quality control, whereby clusters are composed of dysfunctional mitochondria transported to the plus-ends of microtubules, thereby distancing them from the nucleus. Mitochondrial clusters in the Drosophila clu mutant are in the cortical cytoplasm along with the plus-ends of microtubules (Cox and Spradling, 2009), and mitochondria move on microtubules in Drosophila, but the experiments with Arabidopsis (Chapter 4) provide no evidence of tethering of clusters to microtubules. The results show that clusters in fmt1-1 move on the actin cytoskeleton, as shown for individual mitochondria in wt tobacco, onion, Arabidopsis and Picea wilsonii cells (Olyslaegers and Verbelen, 1998; Van Gestel et al., 2002; Zheng et al., 2010, 2009).

In addition, there was no evidence for the association of clusters with microtubules, or with their plus-ends and the results show that depolymerization of microtubules with oryzalin fails to disrupt the clusters or affect their movement (Chapter 4). It was concluded that in Arabidopsis, microtubules have little if any effect on fmt1-1 cluster development and dynamics beyond the role of microtubules in shaping F-actin networks (Zheng et al., 2010, 2009).
association time between adjacent mitochondria in a cluster in \textit{fmt1-1} is prolonged compared to wt (Chapter 4). Since a necessary prelude to mitochondrial fusion is physical association of two physically discrete organelles, and this is affected in \textit{fmt1-1}, it can be concluded that FMT is part of the Arabidopsis mitochondrial fusion apparatus with a role regulating the length of physical interaction between fusing mitochondria (Chapter 4). Collectively, previous chapters showed that the disruption of normal mitochondrial association and chondriome structure not only has negative effects on mitochondrial quality control (pulsing, autolysosomes) and increases cell death, but it also leads to changes in whole organism physiology.

5.2. Objectives

My objectives for this chapter are:

- Determine the subcellular location of \textit{FMT} (through transient and stable transformation) in order to form hypotheses about the function of the protein. The subcellular location of \textit{cluA} and \textit{CLU1} suggests that the FMT protein may be located in the cytosol, but the role of this protein in mitochondrial dynamics (Zhu et al., 1997; Fields et al., 1998, 2002) suggests it should be located in or near the mitochondria.

- Identify the importance of acetylated lysines at positions 1022 and 1029 in FMT. This will aid with understanding of the role of the acetylated region in FMT that was identified by Dr. Finkemeier (Max Planck Insititute, Cologne, Germany, personal communication).
5.3. Results

5.3.1. Subcellular localization of FMT

To gain insight into the subcellular localization of FMT in plants, both homologous stable expression in Arabidopsis and heterologous transient expression in tobacco of N- and C-terminal translational fusions of FMT to GFP using pMDC83, pMDC45 or to mRFP1 using pDCL-X-mrfp1 or pDCL-mrfp1-X (http://botserv1.uzh.ch/home/grossnik/curtisvector/index_2.html) and Logan (unpublished), were used (Fig. 5.1) (see Materials and Methods, 2.1.19 – 2.1.24).

5.3.1.1. Transient expression in tobacco

To determine the subcellular location of FMT in tobacco, a variety of concentrations of cultures of Agrobacterium (0.2 to 0.5 at OD₆₀₀) containing FMT (FMT fused to the C- and N-terminus of GFP or mRFP1) were injected into the abaxial side of tobacco leaves (see section 2.1.23). Leaf sections were visualized after a 2-3 day incubation using fluorescent microscopy system 1. The results illustrated that transient expression of GFP-FMT (FMT used to the C-terminus of GFP, pMDC45) in tobacco, displayed a pattern typical of GFP alone (cytoplasm and nuclear lumen) suggesting that a truncated fusion protein was being expressed (Figs. 5.2 & 5.3). For confirmation, DAPI staining was used to visualize the nucleus. The results indicated that the large object (white arrows) in each cell emitting GFP fluorescence was the nucleus (blue arrows) (Fig. 5.3).

In contrast, transient expression of FMT-GFP (FMT fused to N-terminus of GFP, pMDC83) in tobacco displayed two cytosolic localization patterns. As shown in Fig. 5.4, FMT is a cytosolic protein as can be seen from its location in transvacuolar cytoplasmic strands and shows no localization to the nucleus (Fig. 5.5). It was also observed that FMT-GFP localized to mobile cytosolic puncta (white arrows) (Fig. 5.4 bottom panel). Expression of FMT-GFP enabled visualization of two cytosolic location patterns, likely due to the stability of the fusion protein in this configuration compared to GFP-FMT. Similar localization patterns were observed using transient expression in tobacco. Co-transformation of tobacco with FMT-GFP and mito-
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<th>35S Promoter</th>
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Figure 5.1. Different fluorescent fusion constructs of FMT. N- and C-terminal translational fusions of GFP/mRFP1 to the full length FMT coding sequence were generated to visualize FMT location.

Figure 5.2. FMT localization in tobacco. Blue and red arrows point to areas of GFP-FMT fluorescence. Scale bar =10 µm.

Figure 5.3. GFP-FMT is found in the nucleus. Tobacco epidermal cells of tobacco expressing GFP-FMT were stained with DAPI (nucleus marker). Scale bar =10 µm.
Figure 5.4. FMT-GFP displays a diffuse cytosolic localization but sometimes also organizes into punctate structures. Representative images of a transiently transformed leaf epidermal cell of *Nicotiana benthamiana* expressing FMT, fused to GFP (FMT-GFP). Red arrows signify cytoplasmic strands; blue arrow represents the nucleus while the white arrows indicate puncta. Scale bar =10 µm
Figure 5.5. **FMT-GFP localizes to the cytosol and not the nucleus.** Image shows the subcellular location of FMT-GFP, green, in the cytoplasm and not the nucleus (blue arrow) in tobacco. Transvacuolar strands of cytoplasm are clearly visible (red arrow). The red puncta show mitochondria using (mito-mCherry). The inset, focusing on the area around the nucleus, has been subjected to changes to brightness and contrast and has the un-sharp mask filter applied to aid visualization. Scale bar =10 µm.

Figure 5.6. **Visualization of FMT puncta and mitochondria.** FMT-GFP (magenta) together with mito-mCherry (cyan) were visualized using fluorescence microscopy system 1. White arrows indicate FMT-GFP puncta while the yellow arrows indicate mitochondria. The image has been subjected to deconvolution using Auto-Deblur. Scale bar = 3 µm.
mCherry demonstrated that the FMT-GFP puncta were frequently associated with mitochondria (Fig. 5.6).

To determine whether, or not, the reticular location pattern was due to FMT-GFP in the cortical cytosol (Figs 5.4 and 5.5) and not endoplasmic reticulum (ER), co-infiltration using agrobacterium with FMT-GFP and ER-rb (a mCherry-HDEL construct, ABRC stock CD3-960, (Nelson et al., 2007)) was expressed transiently in tobacco leaves. Results showed that FMT is not an ER protein but is cytosolic (Fig. 5.7).

In an attempt to determine the origin of the discrete mobile puncta observed with the FMT-GFP construct (Fig. 5.4, bottom panel, white arrows), FMT-GFP was co-infiltrated with Golgi using ST-mRFP1 (Teh and Moore, 2007). Results showed no co-localization between mobile cytosolic FMT hotspots and Golgi (Fig. 5.8). Similarly, a peroxisome marker (pRCS2/mCherry-PTS1, a kind gift from Dr. Robert Mullen, University of Guelph), was co-infiltrated with FMT-GFP. The results and analyses indicated that there was no co-localization between mobile cytosolic FMT puncta and peroxisomes (Fig. 5.9).

5.3.1.2. Arabidopsis stable transgenic lines expressing FMT-GFP

Arabidopsis stable transgenic lines were generated by transforming Arabidopsis plants with FMT-GFP. Leaves of two week-old T₁ seedlings were examined using microscopy system 1 and seedlings were grown in soil to complete their life cycle. T₂ seeds were germinated on selected media and only positive seedlings for Kanamycin selection were selected. Results from the stably transformed Arabidopsis lines showed cytosolic puncta (Fig. 5.10A) and transvacuolar cytoplasmic strands (Fig. 5.10B). These results are consistent with the results obtained from transient transformation. However, in some cells of stably transformed Arabidopsis lines, a punctate cytoplasmic distribution was observed, whilst in other cells this pattern was not evident, and instead GFP fluorescence was detected in the cortical cytoplasm and transvacuolar cytoplasmic strands.
**Figure 5.7.** FMT is not an ER protein. A transiently transformed leaf epidermal cell of tobacco expressing FMT fused to GFP, together with an ER marker (red). Scale bar = 10 µm.

**Figure 5.8.** Co-localization between FMT puncta and Golgi apparatus. (A) FRIENDLY-GFP with Golgi (ST-mRFP1, red). Arrows indicate FMT cytosolic puncta. Scale bar = 10 µm.
Figure 5.9. **Co-localization of FMT puncta and peroxisomes.** FMT-GFP and pRCS2-mCherry-PTS1(red) were co-expressed in tobacco leaves. Fluorescent microscopy system 1 was used to visualize the FMT puncta (arrows) and peroxisomes (red). Scale bar =10 µm.

Figure 5.10. **FMT localization in Arabidopsis.** FMT subcellular location in cytoplasmic strands and mobile cytoplasmic puncta. Some cells of stably transformed Arabidopsis lines showed punctate cytoplasmic distribution (A) while in other cells puncta were not observed, but GFP fluorescence was clearly seen in the cortical cytoplasm and transvacuolar strands (B). Scale bar =10 µm.
5.3.2. FMT is lysine-acetylated at two sites in the C-terminal region which alters the ability of FMT to mediate mitochondrial association

Recently, it has been discovered that lysine acetylation is not only limited to histone proteins but is also a common posttranslational modification for a large number of proteins with a variety of functions including DNA-protein interaction, subcellular localization, enzymatic activity and protein stability (Kim et al., 2006; Glozak et al., 2005). The p53 protein was the first non-histone protein identified to have acetylated lysines (Gu and Roeder, 1997). Since then, tens of lysine-acetylated proteins have been shown to have roles in leukemia and other human diseases (Kouzarides, 2000; Yang, 2004). A proteomic study identified 388 acetylation sites in 195 proteins from HeLa cells and mouse liver mitochondria (Kim et al., 2006). Most of the proteins found to be lysine acetylated are involved in mitochondrial metabolism (Kim et al., 2006).

Little is known about non-histone lysine acetylation in plants, however, Finkemeier et al. (2011), reported that lysine acetylation also occurs on cytosolic proteins. They also suggested that non-histone lysine acetylation might be crucial to the chloroplast. In addition, they determined several enzymes were acetylated and suggested the importance of lysine acetylation in the regulation of metabolic enzymes in Arabidopsis (Finkemeier et al., 2011).

A proteomic analysis of lysine acetylation in Arabidopsis (Finkemeier et al., 2011) identified two acetylated lysine residues (K1022 and K1029) in the TPR domain of FMT. To validate the biological meaning of both sites and rule out artifacts, it was decided to study the effect of lysine acetylation of K1022 and K1029 on the function of FMT by site-directed mutagenesis (see Materials and Methods section 2.1.25). The respective lysine residues were mutated to either arginine (to abolish lysine-acetylation) or glutamine (to mimic lysine-acetylation) and the mutagenized cDNA coding sequences as well as the wt coding sequences were used to stably transform either fmt1-1 or its wt, followed by analysis of mitochondrial clustering relative to overexpression or complementation (Fig. 5.11). The fact that the mitochondrial phenotype of wt plants is altered by overexpression of FMT, suggests that FMT function is dependent on its expression level (Fig. 5.11B). Both K1022R and K1029R mutants were able to complement fmt1-1 to levels (44% and 38% for K1022R and K1029R, respectively) similar to expression of the wt coding sequence (46%, Fig. 5.11A & B). In contrast, mutation of

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Figure 5.11. Lysine acetylation alters the ability of FMT to mediate mitochondrial association. (A) Quantification of the percentage of wt or fmt1-1 plants displaying a wt phenotype when stably transformed with transgenes encoding for mutant FMT proteins incorporating K to Q or K to R mutations at residues 1022 or 1029. Numbers within the bar are the number of T1 individuals identified within each transgene/background combination. (B) Percentage of T1 plants (22 individuals were analyzed) displaying a wt phenotype after transformation with 35S-FMT of either fmt1-1 (complementation) or wt (overexpression).
either K to Q decreased complementation (29% and 6% for K1022Q and K1029Q respectively) relative to the K to R mutants (Fig. 5.11A) or the wt coding sequence (Fig. 5.11B). The reverse results were obtained when wt plants were transformed with the site-directed mutants indicating that only the K to Q mutations can counter the clustered phenotype caused by overexpression in wt plants or transformation of wt with either K to R mutants (Fig. 5.11A) or the wt construct (Fig. 5.11B). The low level of complementation by the K to Q mutants and the relative lack of effect of transformation with either of the K to Q mutants in wt supports the hypothesis that FMT activity can be regulated by lysine-acetylation.

5.4. Summary and conclusion

Heterologous or homologous expression was used to determine the subcellular location of FMT. Microscopic analyses of the expression patterns of the FMT fusion proteins demonstrated that FMT displays a diffuse cytosolic location but sometimes also organizes into cytosolic mobile puncta. These mobile cytosolic puncta were found to associate frequently with mitochondria.

The results showed that FMT is a cytosolic protein in agreement with a recent proteomic study (Ito et al., 2011). Another two studies based on proteomic analysis concluded that FMT was localized to the chloroplast (Kleffmann et al., 2004) or the plasma membrane (Benschop et al., 2007). However, yeast FMT orthologue CLU1, has been localized to the cytosol (Kumar et al., 2002) and the Drosophila orthologue, Clu, has been shown to be a cytosolic protein (Cox and Spradling, 2009; Sen et al., 2013). Consistently with our results with FMT-GFP in Arabidopsis and tobacco leaf cells, Clu protein particles are associated peripherally with mitochondria in Drosophila follicle nurse cells (Cox and Spradling, 2009).

A lysine-acetylated peptide of the FMT protein containing two acetylation sites, K1022 and K1029, were identified in the analysis performed by Finkemeier et al., (2011). Hence, each of the two nucleotide triplets encoding the acetylated lysine were mutagenized to encode either arginine (R) or glutamine (Q) using site-directed mutagenesis in wt and fmt1-1 plants. It was speculated that changing lysine into arginine abolishes acetylation and changing lysine into glutamine mimics acetylation (fewer mitochondrial clusters). The discovery that FMT can be lysine acetylated and that preventing acetylation by mutation of either of the two-acetylation
sites to R, increases molecular complementation relative to the effect of expressing the wt coding sequence, suggesting that one mechanism to regulate inter-mitochondrial aggregation “stickiness” may be changing the acetylation state of FMT. These results collectively suggest that lysine acetylation at positions (1022 and 1029) in FMT is an important posttranslational modification in Arabidopsis and that acetylation alters the ability of FMT to mediate mitochondrial association.
CHAPTER 6. FRIENDLY PARALOGUES AND THE IDENTIFICATION OF ABERRANT MITOCHONDRIAL PHENOTYPE IN THE *Caenorhabditis elegans clu-1* MUTANT

6.1. Introduction

*C. elegans* are self-fertilizing hermaphrodites that can generate large homogenous populations making them an excellent genetic model (Samara and Tavernarakis, 2008). *C. elegans* is a small nematode worm ~ 1.3 mm long and ~ 100 μm in diameter. They can be cultured in the lab on Petri dishes or in liquid cultures with *E. coli* as food. Their reproductive cycle takes ~ 2.5 days at 25°C and can live up to 2-3 weeks (Samara and Tavernarakis, 2008). The fact that they are colorless, and thus visualization of fluorescent protein markers is unhindered, has led to their extensive use in cell biology.

6.2. Objectives

Interestingly, BLAST search showed similarities between *FMT* and a protein annotated as CLU-1 in *C. elegans* but there have been no published reports about an aberrant mitochondrial phenotype in *C. elegans clu-1* mutants. Therefore, it was decided to investigate whether or not *C. elegans clu-1* mutants have aberrant mitochondrial dynamics. Objectives for this chapter are:

- Identification of *FRIENDLY* paralogues in Arabidopsis.
- Determine whether the *C. elegans clu-1* mutant has an aberrant mitochondrial phenotype.
- Identify the breakdown in the mitochondrial quality control process in *C. elegans clu-1*. It was proposed that the aberrant mitochondria in *C. elegans clu-1* are damaged and degraded by mitophagy.
6.3. Results

6.3.1. *FRIENDLY* paralogues

BLAST search identified *FMT* paralogues in Arabidopsis and six T-DNA lines were ordered from TAIR (Table 6.1). Seeds were grown on MS plates and DNA extraction (CTAB extraction) was performed. PCR was performed to determine the homozygous T-DNA mutant lines as explained in Materials and Methods in Chapter 2. The identified homozygous T-DNA mutant lines were crossed with Arabidopsis plants expressing mito-GFP. Mitochondrial phenotypes in the F₂ progeny of stable transgenic lines were visualized using fluorescent microscopy system 1. As shown in Table 6.1, homozygous mutant plants were identified by PCR screening for SAIL _633, SAIL_1164, SALK_013537 and SALK_033576 Arabidopsis lines; however, homozygous lines could not be identified for SALK_131234 and SALK_123933 lines. The results based on fluorescent microscopy analysis revealed no obvious aberrant mitochondrial dynamic phenotypes in any of these T-DNA mutant lines (Table. 6.1).
Table 6.1. T-DNA mutant lines and their aberrant mitochondrial phenotype.

<table>
<thead>
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<th>Insertion position</th>
<th>Primers</th>
<th>PCR product size</th>
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<td>AT1G15290</td>
<td>Exon</td>
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<td>476-776</td>
<td>No fmt phenotype observed</td>
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<tr>
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<td>AT1G01320</td>
<td>Exon</td>
<td>LP (ACTTCGGAGTCTTCTTCAGCC) RP (ATACTGTGGTTGATGAAGCCG)</td>
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<td>467-767</td>
<td>PCR did not show any homozygous mutant genotype</td>
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</table>
6.3.2. Mutations in CLU-type genes cause mitochondrial clustering

Alignment between Arabidopsis (FMT), D. discoideum (CluA), S. cerevisiae (Clu1p), D. melanogaster (Clueless) and C. elegans (Clu1) proteins was performed. FMT is 26% identical and 41% similar to the CluA protein, 20% identical and 34% similar to Clu1p, 32% identical and 43% similar to Clueless, while C. elegans Clu-1 showed 31% identity and 44% similarity to FMT. At the start of this work the D. melanogaster study had not been published, therefore it was unknown whether metazoans with defective CLU-type proteins also displayed a clustered mitochondrial phenotype. For this reason it was decided to investigate the effects of the mutation of the C. elegans Clu-1 homologue.

C. elegans clu-1 (ok2) mutants were obtained from CGC and PCR was carried out to confirm the genotype (see Materials and Methods, sections 2.1.28 & 2.1.29). The ok2 allele is an in-frame 1189 bp deletion (amino acids 314 to 693 are removed and glutamic acid is added at the deletion site). Gel electrophoresis analysis confirmed the expected 2023 bp band in wt and 800 bp band in the clu-1 deletion mutants (see, 2.1.29).

In order to visualize mitochondria, a pyc-1p::pyc-1(1st exon)::GFP construct was injected into clu-1 hermaphrodites. This transgene was ubiquitously expressed and generated a GFP tagged with the N-terminal mitochondria internalization signal from the worm pyruvate carboxylate (PYC-1). This enabled real-time assessment of mitochondrial location and morphology. Microscopic analysis system 2 was carried out and the results indicated that the C. elegans clu1Δ mutant has an aberrant mitochondrial phenotype in the cells examined (Fig. 6.1).

In wt muscle cells, mitochondria are in parallel lines to each other or in tracks, while in C. elegans clu-1 these mitochondria appear to be hairy and forming clumps. The same aberrant mitochondrial phenotype was also observed in the intestinal cells of C. elegans clu-1 cells (Fig. 6.1A & B).

6.3.3. Visualization of autophagosomes in C. elegans clu-1 mutant

To investigate whether clu-1 mutant worms show upregulation of autophagocytotic machinery, it was decided to visualize autophagosome formation. A total of 49 independent
Figure 6.1. *C. elegans* clu1Δ mutant has an aberrant mitochondrial phenotype in muscle and intestine cells. (A) The top image shows wt of *C. elegans* with mitochondria arranged in tracks or parallel lines (red arrows) in muscle cells, while the bottom image is showing clu-1 and mitochondria in a hairy-like structure (white arrows) causing mitochondrial clumps in muscle cells. ‘N’ refers to the nucleus. (B) Abnormal mitochondrial phenotype in intestine cells, images captured by Markus Schwarzlander. Scale bar = 5 µm.
worms were used to compare autophagosomes of the intestinal region between wt (nhx-2p::LGG-1::mCherry) and clu-1;nhx-2p::LGG-1::mCherry mutant lines (see section 2.1.30).

As shown in Fig. 6.2, an increase in the number and size of autophagosome puncta in *C. elegans clu-1* compared to wt was observed. Quantification demonstrated an approximate 4-fold increase in autophagosome size and a more than 2.5-fold increase in their number (Fig. 6.3A & B), within intestinal cells compared to wt.

### 6.3.4. Juxtaposition of mitochondria with autophagosome puncta

It was decided next to investigate the localization of mitochondria and autophagosome puncta. WT strain (*pyc-1::GFP; lgg-1::mCherry*) and *clu-1* strain (*pyc-1::GFP; lgg-1::mCherry; clu-1*) were generated (see Materials and Methods). These lines were visualized using microscopic system 2. As can be seen in Figs. 6.4 & 6.5, there was no obvious overlapping between autophagosomes and mitochondria in wt or *clu-1* cells.
Figure 6.2. Autophagosome visualization in *C. elegans* intestinal cells. *lgg-1::mCherry* (ATG8), marker of autophagosomes, was used to visualize autophagosome puncta in the intestines of *C. elegans* wt and *clu-1* cells. (A) Top panel represents the intestine region in wt while the bottom panel shows more numerous and larger autophagosomes in *clu-1*. (B) Higher magnifications (63x) clearly show that autophagosomes in the intestine of *clu-1* mutants are increased in size and number compared to wt cells. LGG-1 puncta are interpreted as autophagosomes while the more dispersed signal indicated cytosolic (non vesicle-bound) LGG-1. Scale bar = 20 µm.
Figure 6.3. Average size and number of autophagosome puncta in the intestine region of WT and clu-1 mutant. (A) The average size of autophagosomes in wildtype is 2.7 µm² while in clu-1 is 11.7 µm² (n=49). (B) wt average autophagosome number is 15.14, while the clu-1 mutant is 40.61(n=49). Error bars = 1SE (** = p<0.001).
**Figure 6.4. Autophagosome and mitochondria in wt.** Each image is a single optical slice from a Z-stack captured by CSLM. (A) Images (under 25x objective lens) are showing mito-GFP (green), autophagosome puncta (red, white arrows), brightfield (grey) in wt cells. (B) Higher magnifications (63x). Scale bar = 20 µm.
Figure 6.5. Autophagosomes and mitochondria in *C. elegans clu-1*. Each image represents a single optical slice from the original Z-stack captured by CSLM. Images are showing mito-GFP (green), autophagosome puncta (red, white arrows), Brightfield (grey) in *clu-1* cells. (A) Images captured using 25x objective lens. (B) Images captured using 63x objective lens. Scale bar = 20 µm.
6.4. Summary and conclusion

The results in this chapter show that none of the Arabidopsis genes identified as homologous to *FMT* are functional paralogues. Mutation of *FMT* orthologues in diverse eukaryotes has been shown to result in aberrant mitochondrial morphology and distribution (Fields et al., 1998; Zhu et al., 1997; Cox and Spradling, 2009): the typical cortical mitochondrial network in *S. cerevisiae* is replaced in *clu1Δ* cells with a collapsed aggregation of mitochondria to one side of the cell (Fields et al., 1998) and a clustered mitochondrial phenotype is observed in the *D. discoideum cluA* mutant (Zhu et al., 1997) and in the *D. melanogaster clueless* mutant (Cox and Spradling, 2009) (Fig. 3.1). My research demonstrates that an aberrant mitochondrial phenotype is also found in *C. elegans* since the *clu-1* deletion mutant shows an aberrant mitochondrial phenotype as illustrated in Fig. 6.1.

As mentioned previously (Chapter 3), autophagy is important in maintaining cell health by the removal of compromised or superfluous organelles and proteins (Edinger and Thompson, 2004; Elmore, 2007; Kroemer et al., 2007). Autophagosomes fuse with acidic lysosomes resulting in acidic autolysosomes (Bassham, 2007). This process occurs to recycle nutrients essential for cell survival under stressful conditions (Kim et al., 2007). Oxidative stress in wheat root cells induced mitophagy where mitochondria were damaged in the central vacuole (Minibayeva et al., 2012). Proper elimination of malfunctioning and damaged mitochondria is crucial to protect cells from the deleterious effects of disordered mitochondrial metabolism and to maintain optimal cellular homeostasis. There are many identified genes encoding proteins involved in autophagy in mammals, yeast and *C. elegans* (Klionsky et al., 2003; Klionsky, 2007; Samara and Tavernarakis, 2008). These proteins are involved in the induction, formation, expansion and maturation of autophagosomes (Klionsky, 2005). A study by Ogura et al., (1994) showed that some *C. elegans* proteins play additional roles unique from their role in autophagy. For instance, *C. elegans unc-51* gene (encodes serine/threonine kinase) is expressed in *C. elegans* neurons and is required for axonal elongation throughout development (Ogura et al., 1994).

Autophagosome puncta were increased in number and size in the *C. elegans clu-1* mutant compared to wt in the intestine region (this work, Figs. 6.2 & 6.3). Mitochondrial dysfunction can be one of the consequences of disease processes. Thus, retaining mitochondrial health is
important to the well-being of organisms (Kim et al., 2007). In this research it was hypothesized that mitochondria are degraded and removed by autophagosomes (Figs. 6.4 & 6.5). However, no obvious co-localization between mitochondria and autophagosomes was observed. This result does not prove that mitochondria are not being removed as a result of the upregulation of the autophagy machinery since there are several reasons or combinations of reasons why mitochondria were not observed to be within the LGG-1 marked structures. Firstly, it is possible that the LGG-1 labeled structures were autophagolysosomes (i.e. the fusion product of autophagosomes and the lysosome), since the turnover of autophagosomes is quite rapid. As such, the GFP fluorescence would be quenched by the acidic environment within the autophagolysosomes. It is also possible that the frequency of mitophagy is too low to see the co-localization of mitochondria with lgg-1::mCherry puncta, or indeed that the resolution of the microscope was not sufficient to clearly visualize mito-GFP within the lgg-1::mCherry puncta. It would be possible to test these explanations by blocking autophagolysosomal fusion in the worm by treating worms with chloroquine, an autophagomal/lysosomal fusion inhibitor, by generating GFP fusion to LGG-1 and using this with mito-RFP (red FPs are not sensitive to acid pH), or by using a microscope with greater resolution. Finally, another possible interpretation is that the hypothesis is not correct and that while clu-1 affects mitochondrial dynamics and there is an upregulation of autophagy machinery, there is no specific upregulation of mitophagy.
CHAPTER 7. DISCUSSION AND FUTURE WORK

7.1. Thesis discussion

FMT, like its orthologues, has no significant homology to proteins of known function. The CLU-family of proteins is grouped due to the presence of a CLU domain (conserved in all eukaryotic clu-type proteins) towards the N-terminus and TPR domains towards the C-terminus. Despite encoding a protein with a molecular mass of ~ 150 kDa, there are no evidences to protein function in its sequence. The research described in this thesis was performed to try to understand how disruption of FMT function leads to the clustered mitochondrial phenotype and the effects of this aberrant chondriome on cell and whole plant physiology.

TEM analysis of clusters in the Dictyostelium cluA mutant by Field et al., (2002) showed that the outer mitochondrial membranes of adjacent mitochondria were connected and there was sometimes penetration of the inner membrane between such adjacent mitochondria. Thus, it was concluded that CluA was involved in mitochondrial division due to the connected mitochondria. In contrast, the TEM results presented in this thesis showed that the outer membranes of the adjacent mitochondrial organelles in fmt1-1 were not connected (Fig. 3.5 & 3.6). FRAP studies (this work) showed matrix connectivity in elongated mitochondria (nmt) as a control (Fig. 3.9), while fmt1-1 clusters showed no mito-GFP fluorescence recovery confirming the lack of matrices connectivity between adjacent mitochondria in the cluster (Fig. 3.7). In spite of the lack of evidence for a membranous connection between mitochondria in a cluster, the fact that clustered mitochondria move as a unit through the cytosol suggests that individual organelles in a cluster are physically associated in some way. In Chapter 3, Fig. 3.5, arrows indicate electron dense patches between some adjacent mitochondria in the fmt1-1 cluster visualized by TEM.

Various lines of evidence suggest that a clustered mitochondrial phenotype leads to stress and that mitochondria in the fmt mutants may be dysfunctional. As described in Chapter 3, proper elimination of malfunctioning and damaged mitochondria is crucial to protect cells from the deleterious effect of disordered mitochondrial metabolism and to maintain optimal cellular homeostasis. The fmt1-1 mutant displays numerous indicators of stress and mitochondrial dysfunction such as up-regulation of autophagy machinery, an increased number of dead root cells, and the increased frequency of mitochondrial membrane potential pulses. Further evidence
for fmt1-I being affected by stress are displayed on a transcriptomic level (El Zawily et al., 2014, see Appendix B) including significant regulation of functional gene groups previously identified as targets of mitochondrial dysfunctions and oxidative stress (Schwarzlander et al., 2012). Similar stress phenotypes result from disruption of FMT orthologues in other model eukaryotes. For example, lack of the Drosophila FMT orthologue in the clu mutant leads to mitochondrial oxidative stress as measured by strongly decreased aconitase activity (Sen et al., 2013). In addition, the results obtained using C. elegans presented in this thesis (Chapter 6) show that disruption of the FMT orthologue, Clu-1, leads to up-regulation of autophagy machinery (Figs. 6.2 & 6.3).

Due to their sessile lifestyle, plants cannot escape environment-induced stressors such as excessive heat, drought, or irradiance. Instead, flexible biochemical mechanisms have evolved to allow survival by acclimation. Plant mitochondrial biochemistry integrates with the chloroplastic, peroxisomal and cytosolic compartments to provide cellular energy and redox balance and there are major differences relative to animals (Braun et al., 2014; Mackenzie and McIntosh, 1999). For example, plant mitochondria have alternative electron transport pathways consisting of non-proton pumping NAD(P)H dehydrogenases and alternative oxidase (AOX), that provide a sink for electrons from NAD(P)H, not coupled to membrane potential or ATP production (Chapter 1). While this system comes with an energetic cost, it provides biochemical flexibility in redox maintenance, regulation of ROS production and interorganellar metabolic balance, meeting the particular needs of autotrophic and sessile photosynthetic organisms. In addition to these biochemical adaptations of mitochondrial function in plants, I propose that the spatial organization of the plant chondriome as a dynamic ‘discontinuous whole’ is a further adaptation that minimizes, on a cell biological level, the negative effects of cellular stress caused by the sessile growth habit of plants.

Animal and yeast (with the notable exception of S. cerevisiae) mitochondria move on microtubules whereas in Arabidopsis, mitochondria move on actin. Mitochondria are highly dynamic and their movements on actin ensures that mitochondria physically come into contact with one another allowing transient fusion followed by subsequent fission (Logan, 2010a). The difference in the cytoskeletal track used for movement may in part explain the difference in gross chondriome structure (see Chapter 1, Fig. 1.4). The work presented in this thesis on interrelationships between clustered mitochondria, actin, and microtubules demonstrate a clear
dynamic association between clustered mitochondria and actin, but no observed association between mitochondria and microtubules (Chapter 4).

Some components of the plant mitochondrial division machinery have been identified but no components of the plant mitochondrial fusion apparatus have been discovered despite genetic and cell biological evidence that fusion occurs in plants (see Chapter 4). One of the first pieces of genetic evidence for fusion was the fact that mtDNA in cybrids (following protoplast fusion) was a unique combination of the two distinct mitochondrial genomes (Pelletier et al., 1985) and it is hypothesized that mitochondrial fusion in plants is driven by the distribution and organization of plant mtDNA (Logan, 2010a). In addition to the size and complexity of plant mitochondrial genomes relative to non-plant eukaryotes, it has been shown that mtDNA is unevenly distributed amongst the population of several hundred physically discrete organelles with some mitochondria containing less than a full genome (Lonsdale et al., 1988; Arimura et al., 2004b)(see Chapter 1). This heterogeneity led to the plant chondriome being termed a discontinuous whole whereby physically discrete mitochondria move through the cell on the actin skeleton enabling mitochondria to meet and exchange mtDNA and perhaps other components (Logan, 2006a, 2010a) to describe the functional interdependence of these physically discrete organelles. This interdependence has been suggested to drive a “need-to-meet” such that there is a requirement for mitochondria to be mobile so that they can associate with and then fuse with one another to enable transfer of mtDNA (Logan, 2010a). Fusion enables mtDNA transfer between physically discrete organelles and this transfer supports the high degree of recombination activity in mitochondrial genomes (Lonsdale et al., 1988). Studies using yeast have also demonstrated the tight links between mitochondrial dynamics and maintenance of mtDNA. For instance, fragmentation of the mitochondrial network due to decreased mitochondrial fusion led to a reduction of the mtDNA abundance or even total loss of mtDNA (Chen et al., 2010; Hermann et al., 1998).

The results presented in this thesis show that disruption of the ability of mitochondria to fuse freely with one another through mutation of FMT is accompanied by changes to plant growth (see Chapter 3). Total biomass and root size of fmt plants are decreased, likely due to the reduction in photosynthetic capacity (Chapter 3, see 3.3.12) and increased activity of AOX (collaboration between the Logan group and Dr. David Macherel, El Zawily et al., 2014, see Appendix B) and concomitant disruption of energy homeostasis. Similar effects on
photosynthesis due to mutations primarily affecting mitochondrial function have been reported previously (Dutilleul et al., 2003; Fiorani et al., 2005). For instance, Zhou et al. (2011) identified and characterized RETARDED ROOT GROWTH gene that encodes a mitochondrial-localized protein in Arabidopsis and required for root meristem cell division (Zhou et al., 2011). Additionally, the nonchromosomal stripe mutation in maize, NCS6, caused by a partial deletion of the mitochondrial Cox2 gene, was shown to lead to a PSI defect and while this was related to reductions in the expression of certain PSI genes, it is not known which are key determinants for the effects on PSI function nor if mitochondrial-plastid signaling or mitochondrial-nucleus retrograde signaling pathways are involved (Jiao et al., 2005). In the case of fmt1-1, it is postulated that it is a combination of imbalance in energy and redox metabolism between both organelles and disruption to the physical juxtaposition of mitochondria and chloroplasts directly arising from the clustered phenotype that causes PSI dysfunction (Chapter 3, Table 3.2).

Chondriome structure in eukaryotes is maintained by a balance of fusion, between physically discrete parts of the chondriome, and subsequent fission such that a predominance of fusion over fission, caused by genetic deletion of components of the fission apparatus, leads to a more reticular network chondriome whilst genetic deletion of components of the fusion apparatus in yeast and animal cells leads to a fragmentation of the chondriome (Karbowsk and Youle, 2003; Okamoto and Shaw, 2005; Shaw and Nunnari, 2002; Hoppins and Nunnari, 2009). In contrast, in Drosophila, disruption of mitochondrial fusion leads to mitochondrial aggregation (Hales and Fuller, 1997).

It is clear that disruption of mitochondrial fission in plants leads to the production of long, reticular mitochondria (Logan et al., 2004; Scott et al., 2006; Logan et al., 2003; Arimura and Tsutsumi, 2002) but, perhaps due to the chondriome organization in Arabidopsis, no genetic mutations, environmental or chemical treatments have been shown to lead to mitochondrial fragmentation as seen in other eukaryotes when fusion is disrupted. As a result, and similar to the Drosophila work (Hales and Fuller, 1997) it is hypothesized that mitochondrial fusion defects in Arabidopsis lead to mitochondrial aggregation.

I have demonstrated that the duration of mitochondrial association is greatly increased in fmt1-1 and assume that this defective association/disassociation leads to the formation of clusters (Chapter 4). Indeed, a change in association time has been demonstrated to be sufficient to induce clustering by mathematical modeling (collaboration between the Logan group and Drs.
The ability of mitochondria in the fmt mutant to meet in order to fuse is disrupted by at least three mechanisms. Firstly, the size of the clusters hampers their movement through the densely packed cytosol; secondly, many mitochondria within the inner reaches of a cluster can only associate with their equally trapped neighbours until clusters are broken apart by shear forces of F-actin remodelling, or by transport through physical bottlenecks in the cytosol; thirdly, the increased association/handshake time in fmt1-1 necessarily decreases the number of new associations and thus new, rather than repeated, fusion events that can take place in a given time-period. To investigate fusion in fmt1-1, an in vivo assay was used (collaboration between the Logan group and Dr. Markus Schwarzlander, El Zawily et al., 2014, see Appendix B) to determine whether or not the increased association time affected mitochondrial fusion between the interacting organelles. This assay involved the use of mitochondrial-targeted photoconvertible protein (mito-mEos) allowing conversion of green-emitting to red-emitting highly motile mitochondria in a region of the leaf epidermis (mito-mEos plants produced by Amr El Zawily, experiment performed by Dr. DC Logan and quantification by Dr. M Schwarzlander, see Appendix B). The analysis of fusion showed that there was a significant increase in the exchange of matrix localized photoconverted mEOS (thus a greater reduction in the average number of green mitochondria) in the fmt chondriome relative to wt. I conclude that although mitochondria in different regions of the cell are less likely to meet in fmt, when they do meet they stay associated for longer (increased handshake duration) forming clusters of potential fusing partners, which increases the number of fusion events even though these events are unlikely to generate as even a mixing of mitochondrial matrix within the chondriome as occurs in wt. In addition to the fusion assay (El Zawily et al., 2014, see Appendix B) and handshake data, the results of the analysis of mitochondrial membrane potential pulsing suggest that mitochondrial fusion is disrupted in fmt (Fig. 3.27A). Santo-Domingo et al. (2013) presented evidence that transient depolarisation and matrix alkalinization (pulsing) represented the formation of a fusion initiation pore within the mitochondrial inner membranes, which is not necessarily followed by an exchange of matrix constituents. The absence of the pro-fusion protein OPA1 (Alexander et al., 2000; Delettre et al., 2000) completely abolished pulsing (Santo-Domingo et al., 2013). The one hundred-fold increase in pulsing rate in fmt1-1 may therefore be indicative of either actively stimulated fusion initiation or loss of control over that process (Fig.
3.27B). That putative fusion pore opening is so much more frequent in fmt1-1 and may be due to the increased duration that fusion-defective mitochondria are associated (handshake) with one another. In the absence of known pro-fusion factors in plants (including a homologue of OPA1) fmt is thus a powerful genetic tool to dissect the precise physiological role of pulsing and the role of FMT in plant mitochondrial fusion in general.

A recent study provides additional evidence regarding the importance of chondriome dynamics, and FMT in particular, in the response to biotic stress. A mutant allele of FMT (noxy38) was identified in a screen for insensitivity to the oxylipin pathogen defence mediator 9-HOT in Arabidopsis (Chapter 1, section 1.7) (Vicente et al., 2001; Vellosillo et al., 2013). The noxy38 mutant showed an up-regulation of AOX expression and a decreased capacity to fight bacterial infection (Vellosillo et al., 2013). Interestingly, a mutant of the mitochondrial division protein DRP3A, with a chondriome comprising elongated and interconnected mitochondrial tubules, was identified in the same screen suggesting that chondriome structure is important for plant defence responses to pathogens. Taken together, the disruption of mitochondrial chondriome structure in response to biotic and abiotic stress (Gao et al., 2008; Zhang and Xing, 2008; Scott and Logan, 2008), the reduced ability of plants with altered chondriome structure to fight pathogen attack (Vellosillo et al., 2013) and the stress phenotypes in the fmt mutant (this work) suggests that in plants, as in animals (Liu et al., 2014; Runkel et al., 2013), mitochondria perform the role of sentinels whereby disruption of mitochondrial function is interpreted as an attack on the cell and that chondriome structure is important for the defence response.

It is proposed that FMT is an important component of the mitochondrial “kiss and run” fusion apparatus and functions by regulating inter-mitochondrial association. No other gene involved in mediating fusion of plant mitochondria is currently known. Further, preliminary evidence is presented to show that FMT function may be regulated by lysine acetylation (Chapter 5, 5.3.2) thereby controlling the association of FMT with mitochondria.

In summary, I have provided several independent lines of strong evidence that FMT functions in mitochondrial homeostasis by maintaining normal mitochondrial distribution, motility and fusion via regulation of inter-mitochondrial association time. Disruption of these key elements of mitochondrial dynamics leads to mitochondrial dysfunction and whole organism stress (this work) and increased susceptibility to pathogens (Vellosillo et al., 2013).
**Whole plant**

- Defect on whole plant physiology (see Ch.3)
- Greater number of dead cells in roots
- Increased susceptibility to pathogens (Vellosillo et al., 2013)

**Cell**

- Defect in Photosystem I
- Reduction in the activity of AOX pathway (El Zawily et al., 2014)
- FRIENDLY is a cytosolic protein with frequent association with mitochondria
  - Enlarged acidic compartments (autophagosomes)
  - FRIENDLY and cytoskeleton (Ch.4)
  - *C. elegans clu-1* has aberrant mitochondrial phenotype (Ch.6)

**Mitochondria**

- Clusters composed of discrete organelles
- Increased mitochondrial membrane potential pulsing (Ch.3)
- Increased matrix mixing (mitoEOS) (El Zawily et al., 2014)
- FRIENDLY regulates association time between mitochondria (handshake hypothesis) (Ch.4, El Zawily et al., 2014)

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Figure 7.1. Summary of the effects that the *friendly* mutation, and resultant clustered mitochondria, have on whole plant, cell and mitochondrial physiology.
7.2. Future directions

This work provides preliminary evidence that FMT function may be regulated by lysine acetylation (see Chapter 5, 5.3.2) thereby controlling the association of FMT with mitochondria. Future experimentation refining the role of FMT lysine acetylation may thus lead to a greater mechanistic understanding of the dynamic regulation of chondriome structure and its affects on cell physiology. Future experiments could be performed to identify proteins interacting with FMT that may, therefore, be involved in the inter-mitochondrial “stickiness” that causes them to aggregate if movement on the actin cytoskeleton is disrupted (e.g. by actin depolymerization). These same proteins may also be involved in the change of mitochondrial distribution and motility that results from biotic and abiotic stress. It is suggested that future work should focus on the molecular mechanism explaining how FMT regulates mitochondrial association. One step should be to identify proteins interacting with FMT. This approach could use protein pull down assays either using antibodies to FMT, if these can be generated, or anti-GFP antibodies and the FMT-GFP lines. The same approaches could be used in the lysine acetylation mutants to determine if interactions depended on the acetylation of those residues. A further powerful approach could be to screen for and identify second site modifiers of FMT function. This would be a quick and easy screen, possibly even automated, and genetic identification of the modifying locus could be quickly achieved with deep sequencing technology, avoiding costly and time consuming positional cloning which was necessary at the time when FMT was identified.
REFERENCES


Abràmoff, M.D., Magalhães, P.J., and Ram, S.J. (2004). Image processing with ImageJ. Biophotonics Int. 11: 36–42.


APPENDICES

APPENDIX A.

Table A.1. Primers used for sequencing FMT.

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<td>F3 5’-ATGCACAGAAGGAACCTG-3’</td>
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<td>F4 5’-ACTCAGTGCAACACG-3’</td>
</tr>
<tr>
<td>F5 5’-ATGGGGCTTCAAATTCTG-3’</td>
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<tr>
<td>R1 5’-AACGATGAGAACACAAATG-3’</td>
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<td>R2 5’-CAGGAAACAGCTATGAC-3’</td>
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Table A.2. Gateway primers used to amplify FMT.

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Table A.3. Primers for site-directed mutagenesis of FMT.

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APPENDIX B.

Attached FRIEnDLY manuscript (movies are in the attached DVD).
David C Logan
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Email: david.logan@univ-angers.fr

Research area: Cell Biology
FRIENDLY and mitochondrial fusion

FRIENDLY regulates mitochondrial distribution, fusion, and quality control in Arabidopsis

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Summary
Disruption of inter-mitochondrial association affects mitochondrial quality control leading to mitochondrial stress, cell death and strong growth defects.
ABSTRACT

Mitochondria are defining components of most eukaryotes. However, higher plant mitochondria differ biochemically, morphologically and dynamically from those in other eukaryotes. FRIENDLY, a member of the clu-superfamily, is conserved amongst eukaryotes and is required for correct distribution of mitochondria within the cell. We sought to understand how disruption of FRIENDLY function leads to mitochondrial clustering and the effects of this aberrant chondriome on cell and whole plant physiology. We present evidence for a role of FRIENDLY in mediating inter-mitochondrial association, which is a necessary prelude to mitochondrial fusion. We demonstrate that disruption of mitochondrial association, motility, and chondriome structure in friendly affects mitochondrial quality control, and leads to mitochondrial stress, cell death and strong growth phenotypes.
INTRODUCTION

Mitochondria are a defining component of eukaryotes: even the few groups of amitochondriate protists are believed to have secondarily lost the organelle (Clark and Roger, 1995; Bui et al., 1996; Gray et al., 1999). The long shared history of eukaryotes has led to considerable conservation of mitochondrial structure and function, although some divergence of both has occurred between plants, fungi and animals since they split from their most recent common ancestor approximately 1,500 million years ago (Feng et al., 1997; Douzery et al., 2004; Hedges et al., 2004).

The higher plant chondriome (the collective term for all mitochondria in a cell, Logan (2006)), differs from the chondriome in animal cells and in yeast in terms of motility and structural organisation (Logan, 2010a). In general, animal and yeast mitochondria move on microtubules whereas in higher plants mitochondria move on actin. The difference in cytoskeletal track used for movement may in part explain the difference in gross chondriome structure. The plant chondriome typically exists as a population of several hundred small, physically discrete organelles that interact genetically via fusion (i.e. as a “discontinuous whole”, (Logan, 2006)), while the animal chondriome, although varying according to cell type, is typically more of a reticular structure as in yeast (Bereiter-Hahn and Vöth, 1994; Rafelski, 2013).

Chondriome structure in eukaryotes is maintained by a balance of fusion, between physically discrete parts of the chondriome, and subsequent fission. A predominance of fusion over fission, caused by genetic deletion of components of the fission apparatus, leads to a more reticular network chondriome, whilst genetic deletion of components of the fusion apparatus in yeast and animal cells leads to a fragmentation of the chondriome (Shaw and Nunnari, 2002; Karbowski and Youle, 2003; Okamoto and Shaw, 2005; Hoppins and Nunnari, 2009). It is clear that disruption of mitochondrial fission in plants leads to the production of long, reticular mitochondria (Arimura and Tsutsumi, 2002; Logan et al., 2003; Logan et al., 2004; Scott et al., 2006). However, we know nothing of the plant fusion apparatus.

The fundamental differences in the structure of plant and non-plant chondriomes and the lack of conservation of genes, identified to date, as involved in mitochondrial
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fusion (Logan, 2003; Logan, 2010b) led one of us to perform a microscopy-based screen to identify Arabidopsis mutants with disrupted chrondriome structure with the goal of discovering genes involved in plant mitochondrial fusion and plant-specific genes involved in plant mitochondrial fission (Logan et al., 2003). One of the mutants, called friendly (fmt – friendly mitochondria) was identified due to the presence of large clusters of mitochondria rather than the homogeneous distribution of mitochondria in wild type (Logan et al., 2003). FRIENDLY, At3g52140, was identified by a combination of map-based cloning and candidate gene sequencing and the mutant null-allele contains a single G to A nucleotide mutation destroying the second exon-intron consensus motif (Logan et al., 2003). FRIENDLY is a member of the CLUstered mitochondria (CLU)-superfamily of conserved eukaryotic proteins (NCBI CDD accession cl16180) but despite encoding a protein with a molecular mass of approximately 150 kDa, there are no clues to protein function in its sequence. Here we set out to understand how disruption of FRIENDLY function leads to the clustered mitochondrial phenotype and the effects of this aberrant chondriome on cell and whole plant physiology.

We present empirical cell biological and theoretical mathematical evidence that the mechanism responsible for the friendly mutant phenotype is an extended duration of inter-mitochondrial association, which is a necessary prelude to inter-mitochondrial fusion. We demonstrate that disruption of normal mitochondrial association and chondriome structure has negative effects on cell and whole organism health including mitochondrial stress, cell death and modifications to whole organism growth.

RESULTS

Mitochondria in friendly are organised into large clusters
The friendly mutant was originally identified (Logan et al., 2003), due to the clustered mitochondrial phenotype, however, no analysis was made regarding the size distribution of clusters relative to wild type. Here, mitochondria in wild-type plants were found to be distributed evenly throughout the cytoplasm in both leaves and roots, whilst in friendly many mitochondria were arranged in large clusters of tens of organelles in addition to
singletons that had a distribution resembling wild type (Figure 1A, supplementary movie 1). In order to quantify cluster number and size, clusters were defined as discrete associations in an area of at least to 0.384 \( \mu \text{m}^2 \) (equal to 15 pixels\(^2 \), see Methods), a phenotype that was not observed in healthy wild-type plants. Whilst no clusters were observed in wild type, some mitochondria were observed to be in loose groups with an average area of 0.105 \( \mu \text{m}^2 \) in leaf epidermal pavement cells and 0.202 \( \mu \text{m}^2 \) in root epidermal cells (Figure 1B). The average mitochondrial cluster size in friendly leaf epidermal pavements cells was 2.491 \( \mu \text{m}^2 \), while significantly larger clusters covering an average of 4.11 \( \mu \text{m}^2 \) were observed in roots (Figure 1C). There was no significant difference in the number of clusters in leaves or roots in friendly (Figure 1C). The majority of groups in wild type covered an area less than 0.2 \( \mu \text{m}^2 \) in both leaves and roots while the larger size classes were more frequently observed in root cells reflecting the greater mean group size in this cell type (Figure 1D cf. 1E). In friendly, the majority of clusters fell within the 0.384 – 2 \( \mu \text{m}^2 \) range while, as in wild type, clusters covering larger areas were more frequently present in roots than in leaves (Figure 1E). A similar phenotype was observed in the friendly-3 T-DNA mutant (supplementary figure 1).

Clusters in the friendly mutant are comprised of discrete organelles.

Mitochondrial clusters in leaves prepared for transmission electron microscopy (TEM) by chemical fixation showed no obvious outer membrane connectivity (Logan et al., 2003). However, given the importance any inter-mitochondrial connection in the mutant to the role of FRIENDLY we decided to investigate this further by two complementary methods. In order to determine whether, or not, mitochondria in clusters in friendly were connected to each other by a contiguous outer membrane, leaf material prepared by High Pressure Freezing was viewed by TEM. Membranous structures were well preserved within the cytoplasm of both wild type and mutant (Figure 2A) leaves allowing clear identification of fine membranous organelles such as Golgi and endoplasmic reticulum and thylakoids within chloroplasts. No organelles other than mitochondria appeared to be affected, morphologically, by the friendly mutation. Over 40 ultra thin sections were observed from at least three plants and there was no evidence of continuity of the outer membrane.
between mitochondria in a cluster. However, electron dense regions were observed between individual mitochondria within a cluster (Figure 2A & B, arrows).

An independent approach was used to investigate connectivity of the mitochondrial matrices of adjacent mitochondria in a cluster using FRAP of the mito-GFP fluorescence. A strip through a cluster was photobleached using the 488 nm laser and no recovery was observed up to 300 seconds after the bleach (Figure 2C and supplemental movie 2). Due to the physically dispersed nature of the wild-type Arabidopsis chondriome, a meaningful photobleaching control could not be performed on wild type. Instead, in order to observe how quickly GFP could diffuse within a mitochondrion (as a proxy for contiguous matrices within a cluster) we performed FRAP on mitochondrial tubules of the network mutant first identified by Logan et al (2003). mito-GFP was able to diffuse into bleached areas of the matrix within 1.5 seconds (Figure 2D, supplementary movie 2).

Mitochondria in friendly move on F-actin and clusters can be disrupted by restructuring of the F-actin cytoskeleton

We next focused on the dynamism of clusters in friendly and their interaction with the cytoskeleton. Single mitochondria in friendly move on actin filaments as in wild type (supplementary movie 3). Similarly, small clusters of mitochondria in friendly are also able to move on actin filaments (Figure 3A and supplementary movie 3) and depolymerisation of F-actin with latrunculin-B arrests this movement (supplementary movie 3). Larger clusters assemble due to the aggregation of smaller clusters that are brought together through movement on actin and these large clusters are less able to move through the cytosol intact and are disrupted by the forces of cytoplasmic streaming, for example re-structuring of the actin cytoskeleton and physical bottlenecks in the cytoplasm (Figure 3B and supplementary movies 4 & 5). Clusters of mitochondria in friendly are therefore highly dynamic transient structures composed of discrete organelles that are able to interact with the actin cytoskeleton in a wild-type manner. Furthermore, while we had previously hypothesised that clusters in friendly may be tethered to microtubules we found no evidence of any association with either
microtubules themselves (supplementary movie 6), or more specifically the plus-ends of microtubules (supplementary movie 6); and partial depolymerisation of microtubules did not affect the movement of clusters (supplementary movie 6). In contrast, depolymerisation of the actin cytoskeleton in wild type using latrunculin-B was able to phenocopy the friendly phenotype (supplementary figure 2A). This apparent “stickiness” of mitochondria in the absence of their actin scaffold is not observed with other organelles that move on actin. For example, whilst peroxisomes and Golgi bodies move on actin, and latrunculin-B arrests this movement, neither organelle forms large clusters in response to actin depolymerisation (Nebenfuhr et al., 1999; Brandizzi et al., 2002; Mano et al., 2002; Mathur et al., 2002; Sparkes et al., 2008). Furthermore, the clustering of mitochondria in friendly is not due to a general cytoskeletal or motor defect since the distribution and movement of peroxisomes are not affected in the mutant (Supplementary figure 2B) and no altered distribution of other organelles was observed in TEM images (Figure 2A & B).

These observations support the hypothesis that mitochondria are attracted to one another, and that movement on actin filaments counters this attraction by providing the scaffold and driving force to keep mitochondria separate except during their regulated association and transient fusion.

**FRIENDLY is a cytosolic protein with either a diffuse expression pattern or a speckled pattern associated with mitochondria.**

A C-terminal translational fusion of GFP to the full length FRIENDLY cDNA coding sequence was generated to visualise FRIENDLY location. Expression of FRIENDLY-GFP in wild-type Arabidopsis showed a cytosolic location, often with a punctate distribution (Figure 4A & B). Similar localisation patterns were observed using transient expression in tobacco (Figure 4C-E). To demonstrate that this expression pattern was unlikely to be due to overexpression we performed transient expression in friendly seedlings: both C- and N-terminal fusions to mRFP1 showed a punctate distribution in close association with mitochondria (Supplementary figure 3). Co-transformation of tobacco with FRIENDLY-GFP and mito-mCherry demonstrated that the FRIENDLY-GFP puncta were frequently associated with mitochondria (Figure 4C). That this was a
dynamic association is evident from the supplemental time-lapse movie (supplementary movie 7). As with stable transformation in Arabidopsis, a punctate pattern was not always observed and instead a more diffuse cytoplasmic distribution was apparent (Figure 4D). In the cortical cytoplasm this appeared as a reticulum as the fusion protein was excluded from areas occupied by other organelles (Figure 4E). In order to exclude the possibility that FRIENDLY-GFP was localising to organelles exhibiting a similar punctate pattern, co-expression in tobacco was performed with fluorescent markers of peroxisomes (mCherry-PTS1, Figure 4F) and Golgi (ST-mRFP1, Figure 4G): no co-localisation was apparent. Similarly, the cortical reticular pattern of FRIENDLY-GFP was distinct from that of the ER visualised by co-expression of the ER marker er-RB (Figure 4H). Our data demonstrating a cytosolic localisation is in agreement with a recent cytosol proteomic study (Ito et al., 2011). The yeast FRIENDLY orthologue, CLU1, has also been localised by proteomics to the cytosol (Kumar et al., 2002) and the Drosophila orthologue, Clu, has been shown to be a cytosolic protein that associates peripherally with mitochondria in Drosophila follicle nurse cells (Cox and Spradling, 2009).

Clusters in friendly form due to an increase in the time of association between mitochondria that meet through movement on actin

Our observations of the movement of mitochondrial clusters on actin, the role of actin in bringing mitochondria together in the formation of clusters, and the dynamism of clusters led us to consider how clusters form. Mitochondria in wild-type Arabidopsis that meet one another through movement on actin often display a short period of association (Figure 5A) during which time fusion and subsequent fission can occur: a phenomenon also seen in some mammalian cells and termed “kiss and run” (Twig et al., 2008). The panels in Figure 5A display individual frames from supplementary movie 8 which shows two association/disassociation events in wild type: the panels reference one of these associations that occurs within 20 s (at 19 s in the movie) and the subsequent disassociation that occurs within a further 10 s (at 28 s in the movie). In contrast, the panels in Figure 5B reference an association/disassociation event between a single
mitochondrion and the exterior of a cluster in *friendly* (see supplementary movie 8): the single mitochondrion associates with the cluster within 20 s (at 18 s in the movie) and disassociates 62 s later. By analysing 45 movies each of wild type and *friendly* we were able to quantify the average length of inter-mitochondrial association: in wild type the average is $15.0 \pm 0.7$ s (Figure 5C), however, in the *friendly* mutant the average time of association is significantly greater at $61.5 \pm 1.4$ s (Figure 5C). This extended association time is sufficient to lead to the build up of a cluster as more mitochondria temporarily associate with the growing cluster before eventually being able to leave the cluster if sufficiently close to a filament of actin (see Figure 3B and supplementary movie 3).

To demonstrate that longer association times lead to more mitochondrial clustering, we constructed and simulated a simple stochastic model of mitochondrial dynamics (see Methods). In this model, mitochondria, represented as individual agents, undergo random motion on a 3D lattice, with the rate of mitochondrial motion depending on whether a mitochondrion is clustered (adjacent to other mitochondria) or free. As mean association time increases, the proportion of mitochondria existing in clusters in the steady state increases in concert (Figure 5D, supplementary movie 8). This provides independent theoretical evidence that alteration of mitochondrial association alone could be responsible for the clustering of mitochondria in *friendly*. In the supplementary information (Supplementary doc 1), we also provide a simple analytic argument that qualitatively recapitulates this behaviour.

The frequency of mitochondrial membrane potential pulsing is increased in *friendly*.

It has been suggested recently that mitochondrial fusion involves the formation of a pore in the IMM thereby causing transient changes in the membrane potential of the fusion partners (Santo-Domingo et al., 2013). We decided, therefore, to investigate membrane potential dynamics in *friendly*. Transient changes in membrane potential, that we term pulses (Schwarzlander et al., 2012b), are rare events in wild type and thus it was immediately evident from CSLM imaging that the number of mitochondria exhibiting pulses and the frequency of pulses was greater in *friendly* than wild type (Figure 6A).
Mitochondria undergoing a pulse in the reference images of hypocotyl cells expressing mito-GFP (Figure 6A, supplemental movie 9) are identified by their green colour, due to mito-GFP fluorescence in the absence of the matrix-localised red TMRM, while mitochondria with a greater membrane potential are yellow-orange due to the colocalisation of TMRM and mito-GFP in their matrices. In *friendly*, the extent of pulsing was highly variable between cells and there was considerable heterogeneity of membrane potential within the chondriome, although in general mitochondria in clusters showed the same baseline membrane potential as those not in clusters (Figure 6A). Quantification of the number of pulses showed that the average frequency of pulses was 0.12 per 100 mitochondria per minute in wild type, whilst in *friendly* it was increased to 11.14 per 100 mitochondria per minute, an increase of approximately 100x (Figure 6B). There was no significant difference in the frequency of pulses for mitochondria in a cluster (mean = 9.82, 95% CI [4.08, 18.03]) compared to those not in clusters (mean = 12.29, 95% CI [5.65, 21.48]). Our interpretation of these results is that fusion initiation events, via formation of a fusion pore, occur with higher frequency in *friendly* relative to wild type.

**An in vivo fusion assay demonstrates that matrix mixing is increased in *friendly***

To test whether or not the increased rate of pulsing correlated with an increase in the extent of matrix mixing, and therefore inter-mitochondrial fusion, we devised a quantitative in vivo fusion assay using a mitochondrial-targeted photoconvertible protein mito-mEos (Mathur et al., 2010). Matrix-localised mEos was photoconverted within highly motile mitochondria in a region of leaf epidermis (Figure 6C, supplemental movie 10). The percentage reduction in numbers of green mitochondria, as a result of the exchange of matrix contents during repeated transient fusion events with mitochondria containing photoconverted mEOS, was significantly greater at the end of the assay time period in *friendly* (30.4%) relative to that in wild type (14.2%) (Figure 6D). The fact that exchange of matrix contents was not seen in the FRAP experiments can likely be accounted for simply because we analysed, by necessity, a single relatively immobile cluster by photobleaching and therefore interaction with other mitochondria outside of
the cluster could not occur. Similarly, the fusion assay, by necessity, required mitochondria to be motile since there would be no fusion between dispersed mitochondria unless they were able to meet. In addition, observation of hundreds of individual association events between a single red mitochondrion and a single green mitochondrion failed to demonstrate extensive mixing of the two mEos species, suggesting that there is limited matrix exchange per fusion event, as reported for mammalian cells (Huang et al., 2013; Santo-Domingo et al., 2013).

The result of this fusion assay demonstrates that the increased association time is not simply due to mitochondria trying but failing to fuse with one another, but that it increases the chance that transient fusion events can occur, facilitating exchange of matrix content.

The mitochondrial phenotype in friendly is associated with defective whole plant phenotypes including lower biomass, shorter roots and shorter etiolated hypocotyls

Whilst friendly was identified solely on the basis of its mitochondrial phenotype, there is a series of whole plant defective phenotypes as a consequence. Plants of the same age are smaller and have shorter, more rounded leaves (Figure 7A). Biomass is reduced in friendly, significantly so at 7-21-days-old (Figure 7B), however, the most obvious phenotypic difference between mutant and wild type is the length of the primary root (Figure 7C & D). Primary root length is significantly shorter in friendly seedlings at 7, 14, 21 and 28 days (Figure 7D) such that by 28 days the root of the mutant is almost half the length of the wild type (4.72 ± 0.16 versus 9.00 ± 0.11 cm). Similarly, when seedlings are grown in darkness the hypocotyl length is significantly shorter in friendly than in wild type (Figure 7F & G). The reduction in total root length (Figure 7C & D) is reflected in a significant reduction in the length of the cap/meristematic region (in friendly this zone is 69% the length of the zone in wild type, Figure 7E) and a slightly greater reduction in the elongation zone (in friendly this zone is 67% of the length of the zone in wild type; Figure 7E). Root width was also reduced significantly in friendly compared to its wild type.
(Figure 7E). Similar whole plant phenotypes were apparent in the friendly-3 T-DNA mutant (supplementary figure 4).

The roots of friendly contain significantly greater numbers of dead cells
Propidium iodide (PI) staining of roots was performed to visualise root cell architecture in order to determine whether it was a reduction in cell number and/or cell size that was responsible for the reduction in root length. As can be seen from the data in Figure 7H, the roots of friendly are composed of smaller cells and therefore there are a greater number of cells in a defined length of root tissue. However, PI staining also revealed that roots of friendly contained many dead cells, which are permeable to PI, while few dead cells were observed in wild-type roots (Figure 8A). For independent confirmation using a more specific and robust probe, SYTOX Orange was used as a probe that readily and selectively enters cells with a damaged plasma membrane and stains DNA. A greater number of dead cells were observed in friendly than in wild type in all developmental zones (e.g. elongation zone, Figure 8B), with significant increases measured in the elongation and differentiation zones (Figure 8C).

The size and number of Lysotracker-stained acidic compartments are increased in friendly
The significant increase in root cell death led us to investigate a potential breakdown in the mitochondrial quality control process in friendly. Lysotracker has been used for assessing autophagy in plants, and whilst not specific for autophagosomes, it does label acid vacuoles required for the process (Yoshimoto, 2012). We stained six day-old wild-type and friendly seedlings using 1 µM Lysotracker Red DND-99 for five minutes followed by capture of z-stacks by CSLM of the root elongation zone. We observed both an increase in the size and number of Lysotracker-stained compartments in friendly compared to wild type (Figure 8D). Quantification of Lysotracker stained compartments demonstrated that there was a significant increase in the number (54 ± 8.6 in friendly compared to 19 ± 3.6 in wild type) and area (9.2 ± 1.2 µm² in friendly compared to 3.7 ±
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0.8 µm² in wild type) of Lysotracker-stained compartments in roots of friendly compared to wild type (Figure 8E). The increased size and number of acid compartments in friendly are consistent with a role for FRIENDLY in the maintenance of cell health. To discover what other processes and functions are altered in friendly that may contribute to the loss of cell health and the whole plant phenotypes we decided to analyse the friendly transcriptome.

**friendly shows global transcriptome re-programming indicating mitochondrial dysfunction, cellular stress and repression of photosynthesis**

To understand the molecular implications and overall impact of the friendly mutation and of compromised fusion control on whole cell homeostasis we performed a set of microarray-based transcriptome studies on whole seedlings. Although, in general, only small fold changes of abundance in friendly relative to wild type were observed, these changes were determined to be significant using Cybr-T (Baldi and Long, 2001) for 4121 non-redundant transcripts (supplementary table 1). The significantly regulated transcripts were allocated to functional-groups using MapMan (Usadel et al., 2005) resulting in the identification of 54 significantly regulated functional groups of genes (Table 1). The functional groups included ‘protein synthesis’ and ‘protein degradation’, consistent with the observed induction of autophagy. Together with ‘photosynthesis’ and ‘photosynthetic light reactions’, which were also significantly regulated, this functional group set was previously identified by meta-analysis of multiple transcriptomic data sets (Schwarzlander et al., 2012a) as being a general indicator for mitochondrial dysfunction. In addition to these classes we also observed regulation of ‘abiotic stress’, ‘redox’, and ‘TCA cycle’ (Table 1) that can clearly be linked to mitochondrial dysfunction. Interestingly, significant regulation was also determined for ‘transcription’ including by the NAC domain superfamily of transcription factors, members of which have been implicated in mitochondrial retrograde signalling (Tran et al., 2004; Chen et al., 2008; Nakashima et al., 2012; De Clercq et al., 2013; Ng et al., 2013).
The activity of the Alternative Oxidase pathway is increased in *friendly*

To determine the impact of the *friendly* mutation on mitochondrial respiration, and specifically the alternative pathway, we measured \( O_2 \) uptake by whole seedlings in the aqueous phase using a Clark-type \( O_2 \) electrode. There was no significant difference in average \( O_2 \) uptake between wild type (25.5 ± 3.0 µmoles \( O_2 \) h\(^{-1}\) µg chl\(^{-1}\)) and *friendly* (29.9 ± 2.8 µmoles \( O_2 \) h\(^{-1}\) µg chl\(^{-1}\)) under non-inhibited baseline conditions (Figure 9A). However, in the presence of 0.5 mM KCN, an inhibitor of the cytochrome pathway, average \( O_2 \) uptake by wild-type seedlings was reduced to 12.5 ± 3.4 µmoles \( O_2 \) h\(^{-1}\) µg chl\(^{-1}\) (Figure 9A), a percentage inhibition of 51.2 ± 10.8 % (Figure 9B), whilst average \( O_2 \) uptake by *friendly* seedlings was only reduced to 25.3 ± 4.3 µmoles \( O_2 \) h\(^{-1}\) µg chl\(^{-1}\) (Figure 9A) which was not a statistically significant change relative to uptake in the absence of KCN, and equated to an inhibition of only 16 ± 8.5 % (Figure 9B). This suggested that much of the \( O_2 \) uptake of *friendly* seedlings was due to the alternative pathway. To test this, we next measured the effect of 0.1 mM propylgallate, an inhibitor of the alternative oxidase, on the KCN-insensitive \( O_2 \) uptake. Propylgallate in combination with KCN reduced \( O_2 \) uptake by wild type or *friendly* seedlings to approximately the same rate (3.4 ± 1.4 and 4.0 ± 1.0 µmoles \( O_2 \) h\(^{-1}\) µg chl\(^{-1}\) respectively, \( p=0.498 \)) equating to a combined inhibition of KCN and propylgallate of baseline uninhibited \( O_2 \) uptake of approximately 87% confirming that AOX induction is responsible for the KCN-insensitive \( O_2 \) consumption in *friendly* (Figure 9B).

Photosynthetic performance is reduced in *friendly* due to a defect in external electron flow to photosystem I

To test the result from the transcriptome analysis suggesting photosynthetic defects in *friendly* we next investigated PSI and PSII activity. There was no difference in steady-state PSII redox state between wild type and *friendly*, as estimated by the chlorophyll fluorescence parameter 1-qL (fraction of open PSII centres) (Figure 9C). This observation was true both at the growth irradiance of 100 µmol photons m\(^{-2}\) s\(^{-1}\), and when the leaf being sampled was exposed to an actinic light of 910 µmol photons m\(^{-2}\) s\(^{-1}\).
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(Figure 9C). Similarly, there was no difference in capacity for nonphotochemical dissipation of excess light (NPQ) between the friendly mutant and wild type (Figure 9D). PSI activity measured as the change in absorbance at 820 nm was used to probe possible effects of the friendly mutation on PSI function and its supply of electrons. The wild-type plants exhibited a greater level of PSI activity (Table 2) and an increased ΔA820/A820 value. PSI was also more slowly reduced in friendly: this measure of how rapidly PSI is re-reduced following the removal of far-red illumination indicates that PSI is receiving a diminished supply of electrons from plastocyanin and the photosynthetic electron transport chain. To probe whether the number of electrons available to PSI from the electron transport chain was diminished in friendly we used the areas under the curve of the single and multiple turnover flashes. The results confirm the PSI re-reduction observations, with friendly having a lower number of electrons available from sources outside of the linear electron transport chain (Table 2).

FRIENDLY is lysine-acetylated at two sites in the C-terminal region which alters the ability of FRIENDLY to mediate mitochondrial association

A lysine-acetylated peptide of the FRIENDLY protein containing the two acetylation sites, K1022 and K1029 was identified in the analysis performed by Finkemeier et al, (2011) (supplementary figure 5). To validate the biological meaning of both sites and rule out artefacts, we studied the effect of lysine-acetylation of K1022 and K1029 on the function of FRIENDLY by site-directed mutagenesis. The respective lysine residues were mutated to either arginine (to abolish lys-acetylation) or glutamine (to mimic lys-acetylation) and the mutagenised cDNA coding sequences as well as the wild-type coding sequences were used to stably transform either friendly or its wild type followed by analysis of mitochondrial clustering relative to overexpression or complementation (Figure 10). The fact that the mitochondrial phenotype of wild-type plants is altered by overexpression of FRIENDLY suggests that FRIENDLY function is dependent on its expression level (Figure 10B). Both K1022R and K1029R mutants were able to complement friendly to levels (44% and 38% for K1022R and K1029R respectively) similar to expression of the wild type coding sequence (46%, Figure 10A and B).
contrast, mutation of either K to Q decreased complementation (29% and 6% for K1022Q and K1029Q respectively) relative to the K to R mutants (Figure 10A) or the wild type coding sequence (Figure 10B). The reverse results were obtained when wild-type plants were transformed with the site-directed mutants indicating that only the K to Q mutations can counter the clustered phenotype caused by overexpression in wild-type plants or transformation of wild type with either K to R mutants (Figure 10A) or the wild type construct (Figure 10B). The low level of complementation by the K to Q mutants and the relative lack of effect of transformation with either of the K to Q mutants in wild type supports the hypothesis that FRIENDLY activity can be regulated by lysine acetylation.

**DISCUSSION**

Regulation of mitochondrial movement and position within the cytosol has been demonstrated to be essential for correct mitochondrial and cellular function (Frederick & Shaw, 2007; Boldogh & Pon, 2007; Logan, 2010b; Shutt & McBride, 2013). Moreover, mitochondria display altered motility and distribution under stress conditions, or when their function is impaired, in a variety of cell types across diverse eukaryote lineages (Chen and Chan, 2009; Nunnari and Suomalainen, 2012). For example, in Arabidopsis clustering and arrest of mitochondrial movement has been identified as a response to biotic and abiotic stress: in response to UV exposure (Gao et al., 2008); to the presence of methyljasmonate (Zhang and Xing, 2008); following treatment with the oxylipin 9-HOT (Vellosillo et al., 2013); and in response to heat shock or application of ROS (Scott and Logan, 2008). Mitochondrial dynamics is therefore not only key to cell function but also to the cellular response to stress. Here we provide new insights into the effects of mitochondrial clustering induced by mutation of the Arabidopsis CLU-type **FRIENDLY** gene, and evidence for the role of this gene in regulating mitochondrial association and fusion.

Work on *Dictyostelium* first identified the involvement of **FRIENDLY**-like (clu-type) genes in the maintenance of mitochondrial distribution (Zhu et al., 1997). As a result of TEM analysis of clusters in the *Dictyostelium cluA* mutant showing continuity of the
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outer mitochondrial membrane of adjacent mitochondria, and sometimes penetration of the inner membrane between such connected mitochondria, it was concluded that CluA was involved in mitochondrial division (Fields et al., 2002). While we sometimes see an electron dense patch between adjacent mitochondria by TEM of leaf tissue fixed by HPF, we do not see any obvious outer membrane connectivity between mitochondria in HPF fixed material (this work), nor did we with chemically fixed leaves (Logan et al., 2003). In addition, the lack of mito-GFP fluorescence recovery in our photobleaching experiments confirms a lack of matrix continuity between adjacent mitochondria in a cluster. Fields et al (2002) cited the similarity of the mitochondrial organisation in yeast clu1Δ cells to that in cells lacking the mitochondrial division protein Dnm1p as further evidence of a role for CluA in mitochondrial division in Dictyostelium. However, applying the same argument to Arabidopsis leads to the opposite conclusion. Arabidopsis mutants of proteins involved in mitochondrial division, such as the dynamin-like proteins DRP3A (Logan et al., 2004) and DRP3B (Arimura and Tsutsumi, 2002), the Fis1 orthologue BIGYIN1 (Scott et al., 2006), or the plant-specific protein NMT1 (Logan et al., 2003; Arimura et al., 2008) contain greatly enlarged, elongated or reticular mitochondria, completely different from the clustered mitochondrial phenotype of friendly mutants. Notwithstanding the lack of evidence for a membranous connection between mitochondria in a cluster, the fact that clusters can move through the cytosol as units demonstrates that individual mitochondria in a cluster are physically associated, as suggested by our observation of electron dense patches between mitochondria visualised by TEM. While the specific identity of the proteins involved in this physical inter-mitochondrial association, that we term a “handshake”, are currently unknown, it is clear that FRIENDLY is required to regulate handshake duration and therefore the speed that mitochondria can undergo complete “kiss and run” fusion and division events that shape the plant chondriome. Future experiments will seek to identify proteins interacting with FRIENDLY that may, therefore, be involved in the inter-mitochondrial “stickiness” that causes them to aggregate if movement on the actin cytoskeleton is disrupted (e.g. by actin depolymerisation). These same proteins may also be involved in the change of mitochondrial distribution and motility that results from biotic and abiotic stress. Our discovery that FRIENDLY can be lysine acetylated and that preventing acetylation by
mutation of either of the two acetylation sites to Q significantly decreases (R increases) the molecular complementation, relative to the effect of expressing the wild type coding sequence, suggests that one mechanism to regulate inter-mitochondrial “stickiness” may be changing the acetylation state of FRIENDLY.

No components of the plant mitochondrial fusion apparatus have been identified despite genetic and cell biological evidence that fusion occurs in plants. For example, fusion enables mtDNA transfer between physically discrete organelles and this transfer supports the high degree of recombination activity in mitochondrial genomes (Lonsdale et al., 1988). That such fusion-driven recombination takes place in plants has been shown by the identification of novel non-parental arrangements of mtDNA in cybrids resulting from protoplast fusion (Belliard et al., 1979; Aviv and Galun, 1987). Indeed, it has recently been argued that the massively expanded mitochondrial genomes of higher plants such as *Amborella trichopoda*, that contains the entire mitochondrial genomes of other plant species (Rice et al., 2013), is a result of mtDNA recombination facilitated by mitochondrial fusion. In addition to the size and complexity of plant mitochondrial genomes relative to non-plant eukaryotes, it has been shown that mtDNA is unevenly distributed amongst the population of several hundred physically discrete organelles with some mitochondria containing less than a full genome (Lonsdale et al., 1988; Arimura et al., 2004). This heterogeneity led to the plant chondriome being termed a dynamic syncytium (Lonsdale et al., 1988) or as a discontinuous whole (Logan, 2006) to describe the functional interdependence of the physically discrete organelles. This interdependence has been suggested to drive a “need-to-meet” such that there is a requirement for mitochondria to be mobile so that they can associate with and then fuse with one another to enable transfer of mtDNA (Logan, 2010b).

The ability of mitochondria in the *friendly* mutant to meet in order to fuse is disrupted by at least three mechanisms. Firstly, the size of the clusters hampers their movement through the densely packed cytosol; secondly, many mitochondria within the inner reaches of a cluster can only associate with their equally trapped neighbours until clusters are broken apart by shear forces of F-actin remodelling, or by transport through physical bottlenecks in the cytosol; thirdly, the increased association/handshake time in *friendly* necessarily decreases the number of new associations and thus new, rather
than repeated, fusion events that can take place in a given time-period. Our analysis of fusion shows that there is a significant increase in the exchange of matrix localised photoconverted mEOS (thus a greater reduction in the average number of green mitochondria) in the friendly chondriome relative to wild type. Our interpretation of these data is that although mitochondria in different regions of the cell are less likely to meet in friendly, when they do meet they stay associated for longer (increased handshake duration) forming clusters of potential fusing partners, which increases the number of fusion events even though these events are unlikely to generate as even a mixing of mitochondrial matrix within the chondriome as occurs in wild type. In addition to the fusion assay and handshake data, the results of our analysis of mitochondrial membrane potential pulsing suggest that mitochondrial fusion is disrupted in friendly. Santo-Domingo et al (2013) presented evidence that transient depolarisation and matrix alkalinisation (pulsing) represented the formation of a fusion initiation pore within the mitochondrial inner membranes, which is not necessarily followed by an exchange of matrix constituents. The absence of the pro-fusion protein OPA1 (Alexander et al., 2000; Delettre et al., 2000) completely abolished pulsing (Santo-Domingo et al., 2013). The one hundred-fold increase in pulsing rate in friendly may therefore be indicative of either actively stimulated fusion initiation or loss of control over that process. That putative fusion pore opening is so much more frequent in friendly may be due to the increased duration that fusion-defective mitochondria are associated (handshake) with one another. Given the absence in plants of homologues of pro-fusion factors present in other eukaryotes (including OPA1) friendly is thus a powerful genetic tool to dissect the precise physiological role of pulsing and the role of FRIENDLY in plant mitochondrial fusion in general.

friendly displays numerous indicators of stress such as an increased number of dead root cells, increased number and size of acidic compartments, tentatively identified as autolysosomes, and the increased frequency of mitochondrial membrane potential pulses. Further evidence for friendly being affected by stress are displayed on a transcriptomic level including significant regulation of functional gene groups previously identified as targets of mitochondrial dysfunction and oxidative stress (Schwarzlander et al., 2012a). Lack of the Drosophila FRIENDLY orthologue in the clu mutant also leads to
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mitochondrial oxidative stress as measured by strongly decreased aconitase activity (Sen et al., 2013).

A recent study provides additional evidence regarding the importance of chondriome dynamics, and FRIENDLY in particular, this time in the response to biotic stress. A mutant allele of FRIENDLY (noxy38) was identified in a screen for insensitivity to the oxylipin pathogen defence mediator 9-HOT in Arabidopsis (Vicente et al., 2001; Vellosillo et al., 2013). The noxy38 mutant showed an up-regulation of AOX expression and a decreased capacity to fight bacterial infection (Vellosillo et al., 2013). Interestingly, a mutant of the mitochondrial division protein DRP3A, with a chondriome comprising elongated and interconnected mitochondrial tubules, was identified in the same screen suggesting that chondriome structure per se is important for plant defence responses to pathogens. Taken together, the disruption of mitochondrial chondriome structure in response to biotic and abiotic stress (Gao et al., 2008; Scott and Logan, 2008; Zhang and Xing, 2008), the reduced ability of plants with altered chondriome structure to fight pathogen attack (Vellosillo et al., 2013) and the stress phenotypes in the friendly mutant (this work) suggests that in plants, as in animals (Runkel et al., 2013; Liu et al., 2014), mitochondria perform the role of sentinels whereby disruption of mitochondrial function is interpreted as an attack on the cell and that chondriome structure is important for the defence response.

CONCLUSION

We show here that the clustered mitochondrial phenotype in the friendly mutant arises by increased duration of inter-mitochondrial handshake events. We propose that FRIENDLY is an important component of the mitochondrial “kiss and run” fusion apparatus and functions by regulating inter-mitochondrial association. No other gene involved in mediating fusion of plant mitochondria is currently known. Further, we provide preliminary evidence that FRIENDLY function may be regulated by lysine acetylation thereby controlling the association of FRIENDLY with mitochondria. Future experimentation refining the role of FRIENDLY lysine acetylation may thus lead to a greater mechanistic understanding of the dynamic regulation of chondriome structure.
and its affects on cell physiology. In summary, we have provided several independent lines of strong evidence that FRIENDLY functions in mitochondrial homeostasis by maintaining normal mitochondrial distribution, motility and fusion via regulation of inter-mitochondrial association time.

MATERIALS AND METHODS

Plant materials and growth conditions
All experiments using *Arabidopsis thaliana* were conducted using the Columbia ecotype (Col-0). Except when used for transformation, Arabidopsis seeds were first germinated on agar under aseptic conditions as follows: seeds were surface sterilized by washing in 80% (v/v) ethanol for five minutes with continual inversion, this wash was replaced with 30% (v/v) household bleach before three rinses in sterile type III water. Seeds were spread on plates and stratified as described in Logan et al (2003) before moving to a growth chamber at 23 ºC under a 16 h light/ 8 h dark photoperiod using cool-white light at 100 μmol m−2 s−1. For transformation, Arabidopsis seeds were stratified before direct sowing on a 1:1 compost (Sunshine LG3, www.sungro.com): vermiculite mixture. Plants to be crossed were first grown on agar as above before transplanting after two weeks to the compost:vermiculite mixture. Tobacco (*Nicotiana tabacum*) seed were also sown directly on this compost:vermiculite mixture and transferred to a growth chamber at 23 ºC under a 16 h light/ 8 h dark photoperiod using cool-white light at 150 μmol m−2 s−1.

**friendly** EMS and T-DNA insertion lines
*friendly* (fmt) was identified in a microscope-based screen of a mito-GFP line mutated with EMS (Logan and Leaver, 2000; Logan et al., 2003). The mutant allele was identified by map-based cloning, and DNA sequencing, as At3g52140 bearing a single G to A mutation in the first intron-exon boundary (Logan et al., 2003). The *friendly* mutant was backcrossed to its wild type (mito-GFP) and the homozygous recessive mutant re-identified 5 times to clean-up the genetic background (Feldmann et al., 1994). T-DNA Express (http://signal.salk.edu/cgi-bin/tdnaexpress) was used to identify the T-DNA
insertion line SALK_046271 bearing an insertion in the 18th intron of the *FRIENDLY* gene (At3g52140) and seed was obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/). Homozygous mutant plants (named friendly-3) were identified using the gene-specific LP (5'- ATACCTGCAGCAGTTTGCAAC-3') and RP (5'-CTAGCGCCAACAGCTCTACTG-3') together with the left border T-DNA primer (LBb1) 5' GCGTGGACCGCTTGCAACT-3'. Homozygous friendly-3 mutants were crossed with lines expressing mito-GFP or mito-mEosFP (using the same targeting signal as mito-GFP but fused to mEOS; Mathur *et al*, 2010). Crossing was performed as described by Meyerowitz & Somervile (1994).

**FRIENDLY cloning**

Total RNA was extracted from 14-day-old Arabidopsis Col-0 seedlings using the RNeasy plant mini kit (Qiagen, Crawley, UK). RNA was reverse-transcribed using Thermoscript RT according to the manufacturer's instructions (LifeTechnologies). *FRIENDLY* was amplified from cDNA using Phusion DNA polymerase (NEB) and primers: forward, 5'-ATGGCTGGGAAGTCGAACAAATCGAAGGCCAAG-3', reverse 5'-TTATTTTTTGCTTTTTGTCTTCTTCTTATCCAAAG-3'. A PCR product of the expected size was amplified, gel purified and cloned using the Zero Blunt Topo PCR Cloning Kit (Life Technologies). Putative positive clones were cultured overnight before plasmid extraction and restriction digest analysis using HindIII. DNA sequencing followed to identify an error-free clone, named pCR-FRIENDLY which was 100% identical to the coding sequence of gene model At3g52140.1 in the TAIR database (http://www.arabidopsis.org/servlets/TairObject?id=37255&type=locus).

**Amplification of FRIENDLY cDNA for Gateway fluorescent fusion constructs and site-directed mutagenesis**

pCR-FRIENDLY was used as template for re-cloning with Gateway attB primers. The PCR product was gel-purified recombined into pDONRzeocin (Life Technologies) to give pDONRz-FRIENDLY and transformed into *E. coli*. Positive clones were identified as above and subjected to DNA sequencing to confirm error free amplification. pDONRz-
FRIENDLY was recombined with the Gateway destination binary vectors pMDC83, pDCLmrfp1-X and pDCLX-mrfp1 (modified versions of pMDC43 and pMDC83 respectively, Curtis & Grossniklaus, 2003; http://botserv1.uzh.ch/home/grossnik/curtisvector/index_2.html) to generate a C-terminal translational fusions to mGFP6, and N- and C-terminal fusions to mRFP1, and with pMDC32 for FRIENDLY overexpression and complementation experiments. Positive clones were identified and verified as described above.

Site-directed mutagenesis
Site directed mutagenesis of FRIENDLY (in pDONRz-FRIENDLY) to convert K1022 or K1029 to Q or R was performed using the GeneTailor Site-Directed Mutagenesis kit (Life Technologies) following the manufacturers instructions and using the oligonucleotide primers listed in supplementary table 1. The mutated cDNAs were cloned using Gateway technology (Life Technologies) into pMDC32 which was used to stably transform friendly and its wild type (mito-GFP line 43C5; expressing a translational fusion of 93 N-terminal amino-acids of the beta-ATPase subunit of Nicotiana plumbaginifolia to mGFP5, Logan & Leaver, 2000) as described below.

Live cell imaging
Live imaging (except FRAP, pulsing analysis and the in vivo fusion assay) used one of two microscope systems:

System 1 consisted of an Olympus BX61 with 100x U-PLAN S-APO oil immersion objective (NA=1.4), Semrock Brightline cubes for GFP (Ex: 473/31 nm, Dichroic: 495 nm, EM:520/35 nm) and mRFP/mCherry (Ex: 543/22 nm, Dichroic: 563 nm, EM: 593/40 nm) and X-Cite exacte illumination. Images were captured with a QImaging Rolera-MGi Plus EMCCD camera attached to a computer workstation running Metamorph.

System 2 consisted of a Zeiss META 510 confocal laser-scanning microscope (CLSM) (Carl Zeiss, MicroImaging) driven by a PC running LSM510/ConfoCor2 software, Version 3.2. The microscope was equipped with a 25x and a 63x water immersion
objective (NA=1.2). GFP was excited with the 488 nm argon laser (HFT 405/488/543 dichroic) and emission collected between 505-530 nm. Propidium iodide stained tissue was excited using the 488 nm argon laser (HFT 405/488/543 dichroic) and emission collected between 610-660 nm. LysoTracker stained tissues was excited using the 543 nm helium-neon laser (HFT 405/488/543 dichroic) and emission collected between 585-650 nm. SYTOX stained tissues was excited with the 514 nm laser (488/514 dichroic) and emission collected between 560-615 nm. IMARIS 7.4.2 software (Bitplane, Zurich, Switzerland) was used for image analysis and 3D reconstructions. Images were deconvolved using AutoQuant X2 (MediaCybernetics) as appropriate.

**Transformation of Arabidopsis**

Binary vectors were used to transform electro-competent *Agrobacterium tumefaciens* (strain GV3101) and positive clones were identified by restriction digest as above following a modified plasmid extraction protocol. Wild type (Col-0), mito-GFP (Logan and Leaver, 2000) or friendly EMS mutant (which is in mito-GFP background, Logan *et al*, 2003) were transformed using the floral-dip method (Clough and Bent, 1998). Transformed plants were selected on appropriate antibiotics and then screened by fluorescence microscopy, if appropriate, using microscopy system 1.

**Transient transformation of tobacco leaf cells**

Fluorescent protein fusion constructs were transformed into *Nicotiana tabacum* (tobacco) leaves using agro-infiltration using a variety of *A. tumefaciens* cell densities as using the method described by (Sparkes et al., 2006). Microscopy using system 1 was performed 3-4 days after infiltration. Peroxisomes were visualised using the construct pRCS2/mCherry-PTS1 (a kind gift from Dr Robert Mullen, University of Guelph), ER using ER-rb (a mCherry-HDEL construct, ABRC stock CD3-960, (Nelson *et al.*, 2007)) and Golgi using ST-mRFP1 (Teh and Moore, 2007).
Transient transformation of Arabidopsis seedlings
Seedlings of friendly were transformed essentially as described in Candat et al. (2014), except that 0.01% (v/v) Silwet L-77 was used instead of Tween 20. Microscopy of transformed seedlings was performed using a Nikon A1 CLSM equipped with a 40X water-immersion objective NA=0.9, and using 488 nm and 561 nm excitation for GFP and mRFP1 respectively.

Transmission Electron Microscopy
Sections of the true leaves of 14 day-old Arabidopsis seedlings were high-pressure frozen at the BBSRC Oxford Brookes High Pressure Freezing Facility using a Baltec HPF010 and freeze-substituted in the presence of 2% osmium using an AFS apparatus (Leica) as described by Tse et al., (2006). After freeze substitution, sections were embedded, sectioned, and observed by transmission electron microscope according to Osterrieder et al., (2010).

Clusters size and number in wild type and friendly
Epidermal cells of 7-day old friendly plants, and their wild type were imaged using microscopy system 1. Images were obtained from 40 randomly chosen independent seedlings of each line. Mitochondrial clusters or groups were quantified using the ‘Analyze particles’ command in Fiji (ImageJ) software (Schindelin et al., 2012). Clusters of mitochondria in friendly were minimally defined as discrete “particles” consisting of mitochondria covering an area of at least 15 pixels² (equal to 0.384 µm²). Groups of WT mitochondrial were defined as discrete “particles” covering an area of at least 3 pixels² (0.077 µm²).

Fluorescence recovery after photobleaching (FRAP)
For FRAP we used a Zeiss LSM 410 inverted CSLM (Carl Zeiss, MicroImaging) equipped with a 25x and a 63x water immersion objective (NA=1.2) and driven by a PC running LSM410/Zeiss LSM software. The microscope was GFP was excited with the
488 nm argon laser (HFT 488/594 dichroic) and emission collected between 505-530 nm. Leaf epidermal pavement cells of 7 day-old seedlings were used. A portion of a randomly chosen mitochondrial cluster of *friendly*, or of a tubule in the *network* mutant, was bleached using 80% argon laser power followed immediately by a reduction of the argon laser power to 10% and capture of 300 images one second apart to observe any GFP recovery.

**Cytoskeleton and mitochondria visualization**

In order to visualize actin filaments and mitochondria, a double transgenic line was generated by crossing mito-GFP with a second line (mCherry-mTn) expressing an in-frame fusion of mCherry (Shaner et al., 2004) to the C-terminal 197 amino acids of mouse talin which forms the actin binding domain (ABD). The mCherry-mTn line was generated by transformation of Arabidopsis Col-0 with pDCLmcherry-mTn. The backbone of pDCLmcherry-mTn, the pDCLmcherry-X destination vector, was a modified pMDC43 (Curtis and Grossniklaus, 2003) in which mGFP6 was replaced by mCherry via pMDC7 and pMDC24. A cDNA fragment encoding the mTalin ABD was PCR-amplified from the pZP202-GFP-mTn vector (see Kost et al, 1998) using attB Gateway primers and recombined into pDONRz. Recombination between pDONRz-mTn and pDCLmCherry-X created pDCLmcherry-mTn. Actin and mitochondria were imaged using microscopy system 1.

To visualize microtubules we PCR amplified, using Gateway attB primers, microtubule-associated protein 4 (Olson et al., 1995) from a GFP-MAP4 construct that was a kind gift from Jaideep Mathur, University of Toronto (Mathur and Chua, 2000). The MAP4 cDNA was recombined with pDONRz, which was in turn recombined with the pDCLmcherry-X destination vector. A stable Arabidopsis line expressing mCherry-MAP4 was crossed with mito-GFP or with the *friendly* mutant expressing GFP and the crossed lines were analyzed using fluorescent microscopy. To visualize the plus end of microtubules, we PCR amplified, using attB1 primers, the EB1b coding sequence from GFP-EB1b (Mathur et al., 2003) and this was cloned and recombined with pDCLmcherry-X as above. A stable Arabidopsis line expressing mCherry-EB1b was crossed to wild type and *friendly*
as above.

**Measurement of mitochondrial association time**

Movies were generated from 250 images of the control mito-GFP line or of *friendly* captured at 1 s intervals using microscopy system 1. Five movies were captured of different regions of the epidermis of the true leaves of 3, 7-day old seedlings to give 15 movies (technical replicates). Results presented are means of associations measured in three independent experimental repeats each comprised of 15 movies (n=3). Association time was calculated as the number of frames (seconds), counted from the time a single mitochondrion was within one mitochondrial-width of either a second mitochondrion in the case of the wild-type mito-GFP line, or of a mitochondrion at the exterior surface of a cluster of mitochondria in the case of *friendly*, until the same mitochondrion moved at least one mitochondrial-width from its partner(s).

**Dynamic model of plant mitochondrial dynamics**

The dynamic model consists of a set of *n* model mitochondria positioned on an *l*×*l*×*l* cubic lattice with periodic boundary conditions. Two or more mitochondria cannot occupy the same lattice site. A mitochondrion is labelled as 'free' if none of its 6 nearest-neighbour sites are occupied by another mitochondria; otherwise it is labelled 'clustered'. The model is initialised by placing the *n* mitochondria randomly on the lattice. We use the Gillespie stochastic simulation algorithm (Gillespie, 1977) to simulate the subsequent dynamics of the model, with each mitochondrion able to move to any of its empty nearest-neighbour sites. The rate with which a free mitochondrion moves to a given neighbour is *λ*₁ and the rate with which a clustered mitochondrion moves to a given neighbour is *λ*₂. If mitochondria are very sticky then *λ*₂ / *λ*₁ will be much less than 1. The algorithm is run until the number of clustered mitochondria converges to a steady state *n*C. We also define the association time *τ* as the time interval between an initially free mitochondrion first becoming clustered and thereafter becoming free. We record the average association time over all mitochondria and over simulation time. We use *l* = 20,
n = 400, $\lambda_1 = 1$ for these simulations; the results are qualitatively robust across the full range of other reasonable parameterisations (data not shown). We vary $\lambda_2$, running 20 Gillespie simulations for each value, and record mean values of cluster proportion $c = n_c / n$ and $\tau$.

**Pulsing measurement and analysis**

Seedlings were grown on vertical plates containing 0.5x MS media + 0.8% phytagel. The membrane potential of mitochondria in living tissue was determined by CSLM using the red fluorescent potentiometric dye TMRM that accumulates reversibly in mitochondria in response to the inner membrane potential (Brand and Nicholls, 2011). After 4 days seedlings were incubated in freshly prepared 50 nM TMRM for >15 min before confocal imaging of roots bathed in fresh probe. Confocal microscopy, performed using a Zeiss LSM 780 confocal microscope (Carl Zeiss MicroImaging), and data analysis were performed as previously described (Schwarzlander et al., 2012b).

**In vivo mitochondrial fusion assay**

Exchange of matrix-localised mito-mEosFP (Mathur et al., 2010) was analysed between mitochondria in the leaves of 14 day-old Arabidopsis seedlings. Pieces of detached leaf were mounted in water between slide and cover slip on a chambered slide made from two parallel strips of ultra-thin double-sided adhesive tape (Ekanayake et al., in press). Imaging was performed using a Nikon A1 CSLM fitted with a 40x NA=1.25 water immersion objective and using the photoconversion dialogue within the Elements software package. Two frames (1024x1024 pixels) were captured pre-conversion at $\frac{1}{4}$ fps (pixel dwell time of 2.4 $\mu$s). Photoconversion was performed at $\frac{1}{4}$ fps (pixel dwell time of 2.2 $\mu$s) within a rectangular ROI using the 405 nm laser at 40% power output and for a total of 6 s. After photoconversion, image capture continued for 72 loops equating to 4.39 mins. Green and red channels were captured simultaneously pre- and post-conversion using a 488 nm laser at 1% and a 561 nm laser at 1.5% respectively. CSLM
time-courses were saved as .avi files and analysed using a custom designed Matlab program (Schwarzlander et al., 2012b). Green:red ratios were calculated for an identical number of pixels covered by each identified mitochondrion in each of the 74 movie frames of each biological replicate. The green:red pixel ratio was calculated for each identified mitochondrion at each time point. Calculation of the numbers of “green” mitochondria used a threshold of average + 1SD of the green:red pixel ratio pre-conversion. The initial and final percentages of “green” mitochondria post-conversion were calculated using the average of the first three (i.e. frames 4, 5 & 6) or last three time-points (i.e. frames 72, 73 & 74), respectively.

**Whole plant phenotyping**
To measure the rate of plant root growth, seeds were germinated and grown on aseptic agar plates as described above, with the plates maintained in a vertical orientation so that the roots grew along the surface of the agar. Measurements were made of 20 seedlings (technical replicate) for each time point per experiment (biological replicate) and results presented are means of the three biological replicates. Wild type mito-GFP or Col-0 were used as controls for *friendly* or *friendly-3* respectively. To measure etiolated hypocotyl length, seeds were germinated and grown on MS agar plates in the dark (plates covered with aluminium foil). Hypocotyl length was measured after 7 days and technical and biological replication was as described for root length. To measure biomass, seeds were germinated and grown on MS agar plates as above for 7, 14, 21 or 28 days. At each time point free surface moisture of 15 seedlings was removed gently using soft paper towel before their total fresh weight was recorded. The seedlings were then completely dried and re-weighed. The experiment was repeated three times and the results presented represent the mean (n=3).

**Root cellular structure and cells viability measurements**
Whole seedlings (6-day-old) were stained in PI solution (3 µg/ml) for two minutes followed by a quick wash in distilled water. Stained roots were mounted in water
between slide and coverslip and observed by CLSM. To visualize dead cells using SYTOX, roots of 6-day-old seedlings were stained in 250 nM SYTOX Orange (Life Technologies) for 20 minutes followed with a quick washing step in distilled water. Z-stacks were captured by CSLM using imaging parameters as described above and the number of dead cells, as visualized by a SYTOX Orange-stained nucleus, was counted in the root cap, division zone, elongation zone, and differentiating zone.

**Staining of acidic compartments using LysoTracker**

Arabidopsis mito-GFP and friendly were grown on MS agar for six days. Seedlings were stained with 1 µM LysoTracker Red DND-99 (Life Technologies) in 0.5x MS media for five minutes at room temperature to visualize acidic compartments. After staining, the seedlings were washed with distilled water to remove excess stain. The root cells (elongation zone) were visualized by CSLM. LysoTracker-stained structures were analysed using the “analyse particles” command in Fiji (ImageJ).

**Arabidopsis oligonucleotide microarray**

Seeds of Arabidopsis (friendly and mito-GFP) were germinated and grown as described above. Both lines were grown under the same conditions and 0.1g of leaves were harvested from each of four plates (each representing a biological replicate) then frozen in liquid nitrogen immediately before RNA isolation using RNeasy plant mini kit according to manufacturer’s protocol. The RNA samples representing four biological replicates of each line (friendly and mito-GFP) were labelled (two cy3 and two cy5) and hybridized to slides prepared by the University of Arizona following the protocol described at http://ag.arizona.edu/microarray. Scanning, image analysis and quantification was as described previously (Xiang et al., 2011). Raw data was saved as a .gpr-file and converted into a .mev-file using the Express Converter software (version 2.1, Dana Faber Cancer Institute, Boston, MA, USA). Data was normalized using the lowess (locfit) algorithm and block normalization in the MIDAS software (version 2.22,
Dana Faber Cancer Institute, Boston, MA, USA). Duplicates in the dataset were removed by averaging intensity values using the FiRe macro (Garcion et al., 2006). Significantly regulated transcripts were detected using Cybr-T Bayesian probabilistic framework analysis ((Baldi and Long, 2001); Bayes p < 0.05). Functional class scoring was implemented using MapMan software (Thimm et al., 2004) applying the Benjamani-Hochberg correction. The datasets were deposited at the EBI ArrayExpress database (accessions) according to the MIAME guidelines.

Respiration
Seed of wild type or friendly were germinated and grown hydroponically for seven days as described by Benamar et al (2013). Oxygen uptake was measured in the same growth medium using a Clark-type O2 electrode (Hansatech). Cyanide sensitive O2 uptake was measured in the presence of 0.5 mM KCN before addition of propylgallate to 0.1 mM. Total chlorophyll content (µg/ml = 7.04*A664 + 20.27*A647), was measured using the DMF method of Moran (1982). Measurements were made using four biological replicates (n=4) and data are presented as means ± standard deviation.

Analysis of photosynthesis
Plants were grown as described above. Fully developed rosette leaves were placed in the dark for 15 min prior to determining dark-adapted F0 and Fm. All measurements were performed using a XE-PAM system (Heinz Walz GmbH, Germany) and the data were collected via the PAM-Data Acquisition System (PDA-100) interfaced with the WinControl Software V2.08 (following the manufacturer's instructions). To induce minimal fluorescence the weak modulated light source was turned on, and data collected for 30s. Once the signal had stabilized an 800 ms pulse of 4000 µmol photons m⁻² s⁻¹ was applied to the leaf to close the PSII reaction centres and generate Fm. The leaf was then exposed to an actinic light source until a steady-state level of fluorescence was reached (Fₛ). To estimate the fraction of closed PSII reaction centres at steady-
state, a saturating flash was applied and \( F_M' \) determined. Following the saturating flash, the actinic light was removed and a weak far-red light (102-FR, Heinz Walz GmbH) was applied to oxidize the electron transport chain. When a steady-state was reached, \( F_O' \) was determined. The actinic light intensity was then increased and the same steps were repeated. The dark-adapted optimal quantum yield of photosynthesis was calculated as \( F_V/F_M \) according to the equation \( F_V/F_M = (F_M-F_O)/F_M \); Krause & Weis, (1991); van Kooten & Snel, (1990). The relative redox state of PSII at steady-state was estimated at each light level using the term \( 1-q_L \), where \[ q_L = \left[ \frac{(F_M-F_S)}{(F_M-F_O)} \times \frac{(F_O/F_S)} \right] \] (Kramer et al., 2004). Similarly, the amount of nonphotochemical quenching was estimated at each light level using the term \( NPQ \), where \( NPQ = (F_M-F_M')/F_M \) (Genty et al., 1990).

To estimate the functional activity of photosystem I in friendly, fully developed rosette leaves from the same plants used for PSII analyses were examined using a PAM-101 control unit equipped with the ED-P700DW Dual-Wavelength-P700 emitter/detector (Heinz Walz GmbH). Data were collected using the PDA-100 interfaced as described V2.08 (Ivanov et al, 1998; Schreiber et al, 1988). Far-red light was applied to the leaf using the 102-FR light source. The deflection in the signal (\( \Delta A_{820}/A_{820} \)) was used to estimate the amount of oxidizable P700 (Schreiber et al 1988). The functional pool size of intersystem electrons was determined by applying single (ST) and multiple (MT) turnover flashes (Asada et al., 1992; Ivanov et al., 1998). The ST flash had a ½-peak width of 10 µs produced by the XE-Pump Flash Control Unit - XE-STC (Heinz Walz GmbH). The MT flash had a duration of 50 ms. Both flashes were produced using the XMT-103 power/control unit (Heinz Walz GmbH). The number of intersystem electrons per P700 was calculated using the equation \( e^-/P700 = MT_{Area}/ST_{Area} \). The areas under the curve were calculated using the Origin 9.0 software package (OriginLab, Northamptonton, MA). The relative rate of P700 reduction following removal of the far-red light was calculated by finding the time required to reach ½ of the dark reduction level and is thus presented as a \( T_{1/2} \) value (Berry et al., 2011).
Lysine acetylation
Experimental details for the extraction of proteins from Arabidopsis, digestion, enrichment and MS analysis of Lys-acetylated peptides is given in (Finkemeier et al, 2011).

Statistical analysis
Unless stated otherwise, results are expressed as means ± s.e.m. of data obtained from independent experiments representing true biological replicates. Significant differences between mean values were calculated using t-tests.

Preparation of figures
Fluorescence micrographs including time-lapse movies and z-stacks had within-image annotations (arrows, scale bars, time stamps) added using Fiji (Schindelin et al., 2012). Occasional additional annotation was performed in Photoshop. All images were exported as TIFFs and composite figures were constructed in Adobe Illustrator before exporting as TIFFs.

ACKNOWLEDGEMENTS
Thanks to Barry Martin, Dr Sarah Irons, and Professor Chris Hawes, Oxford Brookes University, for HPF and microscopy expertise under UK BBSRC Grant no. BB/D001080/1. We thank Dr Mark D. Fricker from the University of Oxford for providing the software used for the quantitative mEOS analysis. We also thank the microscopy platform within the QUASAV SFR, IMAC, for support given to the functioning of the Nikon A1 confocal. Thank you to Harry Hodge, for excellent technical assistance at the University of St Andrews. The majority of this work was funded by a NSERC (Canada) Discovery Grant to DCL using equipment purchased using grants to DCL from the Canada Foundation for Innovation, NSERC, and the University of Saskatchewan. Parts of this work were performed under BBSRC (UK) Grant no. BB/C000129/1, and others
FRIENDLY and mitochondrial fusion

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AUTHOR CONTRIBUTIONS
AME, MS, IF, IGJ, AB, YC, CG, KW, DM & DCL designed and performed experiments and analysed data. MS, IF, IGJ, AJM, KW, RD, DM, NSJ and DCL interpreted data and contributed to the editing of the manuscript. DCL conceived the project, prepared the figures and wrote the manuscript.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

REFERENCES


**Asada K, Heber U, Schreiber U** (1992) Pool Size of Electrons That Can Be Donated to P700+ As Determined in Intact Leaves: Donation to P700+ from Stromal Components Via the Intersystem Chain. Plant Cell Physiol **33**: 927–932


Figure legends

Figure 1. The **friendly** mutant is characterized by the presence of large clusters of mitochondria. (A) Epifluorescent micrographs of root and leaf cells of 7 day-old Arabidopsis seedlings of the **friendly** mutant and its wild type (mito-GFP). Arrowheads
indicate groups of mitochondria in wild type, arrows indicate clusters. Scale bar = 10 µm.

(B) Group size and number in wild type. (C) Cluster size and number in friendly. Values in (B) and (C) are averages calculated from 40 pairs of images of leaf (solid bars) or root (open bars) epidermal cells, each of the 40 pairs of images was of a separate plant. Error bars = SEM, n=40. (C) & (D) Frequency distribution of group sizes in wild type or cluster size in friendly respectively. Solid bars represent values from leaves, open bars from roots.

Figure 2. Clusters in the friendly mutant are discrete organelles. Mesophyll cells in High Pressure Frozen sections of Arabidopsis leaf of wild type (A), or friendly (B) at the same magnification, m=mitochondrion; c=chloroplast; rer = rough ER; G = Golgi apparatus. (C) Higher magnification TEM of the cluster in (B). Arrows in (B) and (C) indicate electron dense regions between mitochondria. (D) FRAP of a cluster in friendly, left panel shows the cluster pre-bleach while the right panel shows the cluster 100 seconds post-bleach (see supplementary movie 2). (E) FRAP of a long mitochondrial tubule in the network mutant (nmt), the top left panel shows the tubule pre-bleach and the other panels show the same view 1.5, 15 or 30 s post-bleach (see supplementary movie 2). Scale bar in (C) = 200 nm and in (D) and (E) = 10 µm.

Figure 3. Mitochondria in friendly move on the actin cytoskeleton and clusters are disrupted by the dynamic actin cytoskeleton. Mitochondria are visualised by mito-GFP fluorescence and actin by mCherry-mTalin. (A) Single images from a time-lapse movie showing a small cluster of mitochondria (arrows) moving on actin (see supplementary movie 3). (B) Single images from a time-lapse movie showing a cluster developing (white arrows) up to 60 s when it is disrupted by rearrangement of the actin cytoskeleton (yellow arrows, see supplementary movie 5). Scale bars in (A) & (B) = 5 µm.
Figure 4. FRIENDLY displays a diffuse cytosolic location but sometimes also organises into puncta. (A) Localisation of FRIENDLY-GFP to discrete puncta (indicated by arrows) in epidermal pavement cells of stable transformed wild-type Arabidopsis. Scale bar = 10 µm. (B) Diffuse localisation of FRIENDLY-GFP in the cytosol of stable transformed wild-type Arabidopsis. Arrows indicate transvacuolar cytoplasmic strands. Scale as in (A). (C) Localisation of FRIENDLY-GFP (magenta) and mito-mCherry (cyan) by transient expression in tobacco leaf epidermal pavement cells, arrows indicate FRIENDLY-GFP puncta. The image has been subjected to deconvolution using Auto-Deblur and the original processed image is shown in the inset (see supplementary movie 7). Scale bar = 5 µm. (D) & (E) Localisation of FRIENDLY-GFP by transient expression in tobacco leaf epidermal pavement cells. Arrows in (D) indicate transvacuolar cytoplasmic strands. The cortical layer of a cell is imaged in (E) and arrows indicate negatively stained organelles. (F – H) Co-transformation of FRIENDLY-GFP and various organelle markers by transient transformation in tobacco as for (C) to (E): (F) peroxisomes (mCherry-PTS1, arrows, yellow) and FRIENDLY-GFP (arrowheads = puncta, magenta); (G) Golgi ST-RFP (arrows, cyan) and FRIENDLY-GFP (arrowheads = puncta, magenta). (H) ER marker ER-rb (cyan) and FRIENDLY-GFP (red), arrows indicate regions where the two colours are clearly not coincident. (I) Control tobacco leaf epidermis infiltrated with buffer only. Scale bars in (D) – (I) = 10 µm.

Figure 5. Association time between mitochondria in wild type and the friendly mutant. (A) Panels show single images from a movie (supplementary movie 8) of mitochondria in a wild type leaf epidermal pavement cell in which two mitochondria meet by 20 s (19 s in the movie) and separate by 30 s (28 s in the movie). (B) Panels show single images of mitochondria in leaf epidermal pavement cell from the friendly mutant in which a mitochondrion joins a cluster at 18 s and then separates from the cluster at 1 min 20 s (see supplementary movie 8). Scale bars = 10 µm. (C) Quantification of the duration, in seconds, of association of single mitochondria in wild type (solid bar) or a mitochondrion and a cluster in friendly (open bar). Bars represent the mean duration calculated from three independent experiments (n=3) of individual mitochondrial
associations calculated from movies of five cells in each of three plants per experiment (15 movies per experiment). Error bars = SEM, $p=0.0002$. (D) Stochastic modelling demonstrates that increased association time between mitochondria leads to increased cluster formation. The proportion of model mitochondria in a stochastic simulation (see Methods) existing as part of a cluster, as the mean association time between adjacent mitochondria varies. To obtain this relationship, $\lambda_1$, the diffusion rate of free mitochondria, is fixed to define unit time, and $\lambda_2$, the diffusion rate of clustered mitochondria, is varied: inset plot shows how clustered proportion and mean association time vary with $\lambda_2$. If mitochondria are very sticky then $\lambda_2 / \lambda_1$ will be much less than 1. Inset images show example snapshots (with periodic boundary conditions) of the stochastic model at the given association times; clustered mitochondria are shown in dark red.

**Figure 6. The frequency of mitochondrial membrane potential pulsing and matrix mixing are increased in friendly.** (A) Representative images of mitochondria in control (wild type, mito-GFP) or friendly hypocotyl cells stained with 50 nM TMRM. The images are merged composites of green (GFP) and red (TMRM) channels (see supplemental movie 9). Arrows indicate mitochondria undergoing a transiently depolarising pulse. Scale bar = 5µm.

(B) Quantification of the number of pulses per 100 mitochondria in root epidermal cells per minute. 219 mitochondria were scored on average for each biological replicate. Means and 95% confidence limits are shown as error bars after back-transforming from a square root transformation, $p<0.01$, n=7 (wild type) or 6 (friendly). (C) In vivo mitochondrial fusion assay in Arabidopsis leaf epidermal pavement cells using mitochondrial targeted mEos (mito-mEos) photoconvertable protein. Images created by merging the red and green channels of single optical slices of leaves of wild type or the friendly-3 mutant stably transformed with mito-mEos. The left panels show the pre-converted state at zero time and the outline boxes indicate the region subjected to photoconversion. Images were captured at continuously at $\frac{1}{4}$ fps until 5 minutes from
the start of the experiment. The right hand panels show the last single slice images captured at the end of the experiment (see supplementary movie 10). Scale bar = 10 µm. (D) Quantification of the percentage reduction in the average number of green mitochondria per field of view (the whole frames as shown in (C)). Error bars = SEM, n=8, p<0.01.

**Figure 7. Whole plant phenotype of wild type and the friendly mutant.** (A) Rosettes in mutant and wild type. (B) Dry weight of wild type and mutant 7, 14, 21 or 28 days after the onset of germination. Bars represent mean of 3 independent experiments each using 15 plants; error bars = SEM, n=3. (C) Root length of 7 day-old wild type or mutant plants. Scale bar = 0.5 cm. (D) Quantification of root length at 7, 14, 21 or 28 days after the onset of germination. For (C) and (D) bars represent the mean of 3 independent experiments each using 20 plants; error bars = SEM, n=3. (E) Length and widths of cap/meristematic or elongation developmental zones. Bars represent the mean measurements from 6 plants; error bars = SEM, n=6. (F) Dark grown, etiolated seedlings after 7 days growth in the dark. (G) Quantification of hypocotyl length of dark grown etiolated seedlings 7 days after onset of germination. Bars represent the mean of 3 independent experiments each using 20 plants; error bars = SEM, n=3. (H) Cell area and number of root epidermal cells per fixed length in the elongation zone of 6 day-old seedlings. Bars represent mean measurements from 4 cells from each of 3 plants; error bars = SEM, n=3.

**Figure 8. There are more dead cells and the size and number of Lysotracker-stained acidic compartments are increased in the roots of friendly.** (A) Confocal z-projections of roots of wild type or friendly mutant after staining with propidium iodide. Arrows indicate dead cells identified by entry of propidium iodide into the cell. Scale bar = 100 µm. (B) Identification of dead cells in wild type or friendly roots using SYTOX Orange. Top panels show confocal z-projections of the red, SYTOX Orange channel, middle panels bright field, and bottom panels the merged SYTOX Orange and bright-
field channels. Scale bar = 50 µm. (C) Quantification of number of cells in each zone of the roots of wild type (solid bars) or friendly (open bars). Dead cells were identified by the presence of a SYTOX Orange stained nucleus. Bars represent the mean number of dead cells in 5 plants; error bars = SEM, n=5. (D) Representative maximum projections of 16 x 1 µm optical sections through the epidermal cells of the root elongation zone: bright field, Lysotracker stained compartments, or mitochondria. Scale bar = 10 µm. (E) Quantification of area and number of Lysotracker stained compartments in roots imaged as in (A) using the “Analyze particles” option in Image J. Error bars = SEM, p<0.01, n=5.

Figure 9. The activity of the Alternative Oxidase pathway is increased in friendly but the activity of Photosystem II is unaffected. (A) Oxygen uptake in wild type versus the friendly mutant under baseline conditions or in the presence of 0.5 mM KCN or 0.5 mM KCN and 0.1 mM propylgallate (Pgal). The rate of oxygen uptake in the presence of KCN was significantly different between wild type and friendly (p<0.01). Error bars = SD, n=4. (B) Percentage inhibition of respiration in wild type or friendly relative to the baselines values, calculated using the data depicted in (A). (C) Steady-state PSII redox state (as estimated by the chlorophyll fluorescence parameter 1-qL) in wild type and friendly. (D) Capacity for nonphotochemical dissipation of excess light (NPQ) in friendly and wild type.

Figure 10. Lysine acetylation alters the ability of FRIENDLY to mediate mitochondrial association. (A) Quantification of the percentage of wild type or friendly plants displaying a wild type phenotype when stably transformed with transgenes encoding for mutant FRIENDLY proteins incorporating K->Q or K->R mutations at residues 1022 or 1029. Numbers within the bar are the number of T1 individuals identified within each transgene/background combination. (B) Percentage of T1 plants (22 individuals were analysed) displaying a wild-type phenotype after transformation with 35S-FRIENDLY of either friendly (complementation) or wild type (overexpression).
Table 1. Overview of significantly affected functional class gene bins.

Mean log2 fold ratios of transcripts were analysed by functional class scoring for each experiment using MapMan software and applying the Hochberg correction. Numbers of transcript elements within each bin are indicated along with the p-value.

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Table 2. PSI activity is reduced in friendly. Values shown are means ± SEM (n=10 independent biological replicates). For experimental details see Materials and Methods.

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¹ The ΔA₈₂₀/A₈₂₀ ratios are significantly different (p=0.03). ² The T₁/₂ values are significantly different (p=0.04).

Supplementary information

FIGURE LEGENDS

Supplementary figure 1. Analysis of cluster size and number in the friendly-3 T-DNA mutant. Data are averages taken from 20 independent images, each from a separate plant. Error bars = SEM, n=20.
Supplementary figure 2. (A) Disruption of the actin cytoskeleton can phenocopy for *FRIENDLY* mutation. Mitochondria in a leaf epidermal cell of a wild type mito-GFP seedling treated with either solvent (0.1% ethanol) or with 2 µM latrunculin-B. Scale bar = 10 µm. (B) Peroxisome distribution is unaffected in *friendly*. Mitochondria (mito-GFP, red) and peroxisomes (YFP-SKL, green) in the *friendly* mutant.

Supplementary figure 3. Transient expression of *FRIENDLY* N- and C-terminal mRFP1 fusions in *friendly* mutant seedlings. (A) *FRIENDLY*-mRFP1, or (B) mRFP1-*FRIENDLY* shows a punctate distribution in the cytosol. Arrows indicate mitochondrial clusters (mito-GFP, green) and arrowheads indicate *FRIENDLY*-mRFP1 or mRFP1-*FRIENDLY* puncta (magenta). Scale bars = 5 µm.

Supplementary figure 4. Whole plant phenotype of wild type (Col-0) and the *friendly*-3 T-DNA mutant. (A) Dry weight of wild type and mutant 7, 14, 21 or 28 days after the onset of germination. Bars represent mean of three independent experiments each using 15 plants; error bars = SEM, n=3. (B) Quantification of root length at 7, 14, 21 or 28 days after the onset of germination. Bars represent the mean of three independent experiments each using 20 plants; error bars = SEM, n=3. (C) Quantification of hypocotyl length of dark grown etiolated seedlings 7 days after onset of germination. Bars represent the mean of three independent experiments each using 20 plants; error bars = SEM, n=3.

Supplementary figure 5. Fragmentation spectrum of a lysine-acetylated peptide unique to *FRIENDLY* (At3g52140).
SUPPLEMENTARY MOVIES

**Movie 1.** The first part of the movie shows a z-stack through the epidermis and palisade mesophyll of a leaf of the wild type mito-GFP line while the second part shows a z-stack through a leaf of the *friendly* mutant. Yellow – mito-GFP, blue – chlorophyll autofluorescence.

**Movie 2.** The first part of the movie shows FRAP of a ROI within a mitochondrial cluster in *friendly*. The second part of the movie shows FRAP of a ROI within the elongated mitochondrial of the *network* mutant.

**Movie 3.** The first part of the movie shows mitochondria (mito-GFP) and actin (mCherry-mTalin) in a double stable transgenic but otherwise wild-type Arabidopsis line. The second part shows mitochondria (mito-GFP) and actin (mCherry-mTalin) in the *friendly* mutant. Arrows indicate clusters moving on an actin bundle, while arrowheads indicate a single moving mitochondrion. The third part of the movie shows mitochondria (mito-GFP) and actin (mCherry-mTalin) in the *friendly* mutant treated with 2 µM latrunculin-B for 2 hr. All captured at 1 frame per 3 s with playback at 2fps. Scale bars = 10 µm.

**Movie 4.** Mitochondrial clusters in the *friendly* mutant are modified by movement through the cytoplasm when they likely meet physical bottlenecks due to the presence of other organelles which limit the cytosolic space through which clusters can move.

**Movie 5.** Mitochondria and actin in the *friendly* mutant showing a cluster developing (arrow) up to 60 s when it is disrupted by rearrangement of the actin cytoskeleton. Full movie containing the snap-shots shown in Figure 3. Scale bar = 5 µm.

**Movie 6.** The first part of the movie shows mitochondrial clusters (mito-GFP) and
microtubules (mCherry-MAP4) in the friendly mutant. The second part shows mitochondrial clusters and the microtubule plus-end marker mCherry-EB1b in the friendly mutant. The third part of the movie shows mitochondrial clusters and partially depolymerised microtubules (mCherry-MAP4) in the friendly mutant treated with 20 µM oryzalin for 3 h. Arrows indicate clusters moving in regions devoid of visible microtubules. It is inevitable that some clusters appear to track along microtubules since F-actin and microtubules are known to co-align (see Sampathkumar et al. (2011) and references therein. Scale bars = 10 µm.

**Movie 7.** Dual transient expression of FRIENDLY-GFP (magenta) and mito-mCherry (cyan) in a tobacco epidermal cell displaying a punctate FRIENDLY-GFP pattern. Deconvolved using AutoQuant X2. Captured at 1fps with playback at 2fps. Scale bar = 5 µm.

**Movie 8.** The first part of the movie shows mitochondrial association/disassociation events in wild type as depicted in the static images in Figure 5A. One association occurs at 19 s and the subsequent disassociation that occurs within at 28 s. Another association occurs at 20 s with the dissociation at 54 s. The second part of the movie shows mitochondrial association/disassociation events in friendly as depicted in the static images in Figure 5B. A single mitochondrion associates with a cluster at 20 s and disassociates 60 s later. Scale bars = 5 µm. The third part shows behaviour of a stochastic model for mitochondrial dynamics at low association time. Video demonstrates the steady state of the model (low proportion of clustered mitochondria); evolution from a random initial state (clustered proportion decreases with time); and behaviour of a small-scale cluster (clustering is quickly lost). While the fourth part shows Behaviour of a stochastic model for mitochondrial dynamics at high association time. Video demonstrates the steady state of the model (high proportion of clustered mitochondria); evolution from a random initial state (clustered proportion increases with time); and behaviour of a small-scale cluster (clustering is generally retained).
Movie 9. The first part of the movie shows a region of one of the movies used for quantification of mitochondrial membrane potential pulsing in a hypocotyl cell of wild type (mito-GFP) incubated in TMRM. The second part of the movie shows a region of one of the movies used for quantification of mitochondrial membrane potential pulsing in a hypocotyl cell of the *friendly* mutant incubated in TMRM. Scale bars = 10 µm.

Movie 10. The first part of the movie shows a time-course of mito-mEosFP photoconversion in wild type and the second part of the film shows a time-course in *friendly*-3 as used for the data shown in Figure 6. Field-of-view captured at a scan rate of ¼ fps therefore each frame is 4 s apart. Movie runs at 2fps.
Figure 1. The friendly mutant is characterized by the presence of large clusters of mitochondria. (A) Epifluorescent micrographs of root and leaf cells of 7 day-old Arabidopsis seedlings of the friendly mutant and its wild type (mito-GFP). Arrowheads indicate groups of mitochondria in wild type, arrows indicate clusters. Scale bar = 10 µm. (B) Group size and number in wild type. (C) Cluster size and number in friendly. Values in (B) and (C) are averages calculated from 40 pairs of images of leaf (solid bars) or root (open bars) epidermal cells, each of the 40 pairs of images was of a separate plant. Error bars = SEM, n=40. (D) & (E) Frequency distribution of group sizes in wild type or cluster size in friendly respectively. Solid bars represent values from leaves, open bars from roots.
Figure 2. Clusters in the friendly mutant are discrete organelles. Mesophyll cells in High Pressure Frozen sections of Arabidopsis leaf of wild type (A), or friendly (B) at the same magnification. m = mitochondrion; cp = chloroplast; rer = rough ER; G = Golgi apparatus. (C) Higher magnification TEM of the cluster in (B). Arrows in (B) and (C) indicate electron dense regions between mitochondria. (D) FRAP of a cluster in friendly, left panel shows the cluster pre-bleach while the right panel shows the cluster 100 seconds post-bleach (see supplementary movie 2). (E) FRAP of a long mitochondrial tubule in the network mutant (nnf), the top left panel shows the tubule pre-bleach and the other panels show the same view 1.5, 15 or 30 s post-bleach (see supplementary movie 2). Scale bar in (C) = 200 nm and in (D) and (E) = 10 μm.
Figure 3. Mitochondria in a friendly move on the actin cytoskeleton and clusters are disrupted by the dynamic actin cytoskeleton. Mitochondria are visualised by mito-GFP fluorescence and actin by mCherry-mTalin. (A) Single images from a time-lapse movie showing a small cluster of mitochondria (arrows) moving on actin (see supplementary movie 3). (B) Single images from a time-lapse movie showing a cluster developing (white arrows) up to 60 s when it is disrupted by rearrangement of the actin cytoskeleton (yellow arrows, see supplementary movie 5). Scale bars in (A) & (B) = 5 μm.
Figure 4. FRIENDLY displays a diffuse cytosolic location but sometimes also organises into puncta. (A) Localisation of FRIENDLY-GFP to discrete puncta (indicated by arrows) in epidermal pavement cells of stable transformed wild-type Arabidopsis. Scale bar = 10 μm. (B) Diffuse localisation of FRIENDLY-GFP in the cytosol of stable transformed wild-type Arabidopsis. Arrows indicate transvacuolar cytoplasmic strands. Scale as in (A). (C) Localisation of FRIENDLY-GFP (magenta) and mito-mCherry (cyan) by transient expression in tobacco leaf epidermal pavement cells, arrows indicate FRIENDLY-GFP puncta. The image has been subjected to deconvolution using Auto-Deblur and the original processed image is shown in the inset (see supplementary movie 7). Scale bar = 5 μm. (D) & (E) Localisation of FRIENDLY-GFP by transient expression in tobacco leaf epidermal pavement cells. Arrows in (D) indicate transvacuolar cytoplasmic strands. The cortical layer of a cell is imaged in (E) and arrows indicate negatively stained organelles. (F – H) Co-transformation of FRIENDLY-GFP and various organelle markers by transient transformation in tobacco as for (C) to (E): (F) peroxisomes (mCherry-PBT1, arrows, yellow) and FRIENDLY-GFP (arrowheads = puncta, magenta); (G) Golgi ST-RFP (arrows, cyan) and FRIENDLY-GFP (arrowheads = puncta, magenta); (H) ER marker ER-rb (cyan) and FRIENDLY-GFP (red), arrows indicate regions where the two colours are clearly not coincident. (I) Control tobacco leaf epidermis infiltrated with buffer only. Scale bars in (D) – (I) = 10 μm.
Figure 5. Association time between mitochondria in wild type and the friendly mutant. (A) Panels show single images from a movie (supplementary movie B) of mitochondria in a wild type leaf epidermal pavement cell in which two mitochondria meet by 20 s (19 s in the movie) and separate by 30 s (28 s in the movie). (B) Panels show single images of mitochondria in leaf epidermal pavement cell from the friendly mutant in which a mitochondrion joins a cluster at 18 s and then separates from the cluster at 11 s (19 s in supplementary movie B). Scale bars = 10 μm. (C) Quantification of the duration, in seconds, of association of single mitochondria in wild type (solid bar) or a mitochondrion and a cluster in friendly (open bar). Bars represent the mean duration calculated from three independent experiments (n=3) of individual mitochondrial associations calculated from movies of five cells in each of three plants per experiment (15 movies per experiment). Error bars = SEM, p<0.0002. (D) Stochastic modelling demonstrates that increased association time between mitochondria leads to increased cluster formation. The proportion of model mitochondria in a stochastic simulation (see Methods) existing as part of a cluster, as the mean association time between adjacent mitochondria varies. To obtain this relationship, λ, the diffusion rate of free mitochondria, is fixed to define unit time, and $\lambda_0$, the diffusion rate of clustered mitochondria, is varied: inset plot shows how clustered proportion and mean association time vary with $\lambda_0$. If mitochondria are very sticky then $\lambda_0/\lambda_1$ will be much less than 1. Inset images show example snapshots (with periodic boundary conditions) of the stochastic model at the given association times; clustered mitochondria are shown in dark red.
Figure 6. The frequency of mitochondrial membrane potential pulsing and matrix mixing are increased in friendly. (A) Representative images of mitochondria in control (wild type, mito-GFP) or friendly hypocotyl cells stained with 50 nM TMRM. The images are merged composites of green (GFP) and red (TMRM) channels (see supplemental movie 9). Arrows indicate mitochondria undergoing a transiently depolarising pulse. Scale bar = 5μm. (B) Quantification of the number of pulses per 100 mitochondria in root epidermal cells per minute. 219 mitochondria were scored on average for each biological replicate. Means and 95% confidence limits are shown as error bars after back-transforming from a square root transformation, p=0.01, n=7 (wild type) or 5 (friendly). (C) In vivo mitochondrial fusion assay in Arabidopsis leaf epidermal pavement cells using mitochondrial targeted mEos (mito-mEos) photoconvertible protein. Images created by merging the red and green channels of single optical slices of leaves of wild type or the friendly-3 mutant stably transformed with mito-mEos. The left panels show the pre-converted state at zero time and the outline boxes indicate the region subjected to photococonversion. Images were captured at continuously at 1/6 fps until 5 minutes from the start of the experiment. The right hand panels show the last single slice images captured at the end of the experiment (see supplementary movie 10). Scale bar = 10 μm. (D) Quantification of the percentage reduction in the average number of green mitochondria per field of view (the whole frames as shown in (C)). Error bars = SEM, n=6, p<0.01.
Figure 7. Whole plant phenotype of wild type and the friendly mutant. (A) Rosettes in mutant and wild type. (B) Dry weight of wild type and mutant 7, 14, 21 or 28 days after the onset of germination. Bars represent mean of 3 independent experiments each using 15 plants; error bars = SEM, n=3. (C) Root length of 7 day-old wild type or mutant plants. Scale bar = 0.5 cm. (D) Quantification of root length at 7, 14, 21 or 28 days after the onset of germination. For (C) and (D) bars represent the mean of 3 independent experiments each using 20 plants; error bars = SEM, n=3. (E) Length and widths of cap/meristematic or elongation developmental zones. Bars represent the mean measurements from 6 plants; error bars = SEM, n=6 (F) Dark grown, etiolated seedlings after 7 days growth in the dark. (G) Quantification of hypocotyl length of dark grown etiolated seedlings 7 days after onset of germination. Bars represent the mean of 3 independent experiments each using 20 plants; error bars = SEM, n=3. (H) Cell area and number of root epidermal cells per fixed length in the elongation zone of 6 day-old seedlings. Bars represent mean measurements from 4 cells from each of 3 plants; error bars = SEM, n=3.
Figure 8. There are more dead cells and the size and number of Lysotracker-stained acidic compartments are increased in the roots of friendly. (A) Confocal z-projections of roots of wild type or friendly mutant after staining with propidium iodide. Arrows indicate dead cells identified by entry of propidium iodide into the cell. Scale bar = 100 μm. (B) Identification of dead cells in wild type or friendly roots using SYTOX Orange. Top panels show confocal z-projections of the red, SYTOX Orange channel, middle panels bright field, and bottom panels the merged SYTOX Orange and bright-field channels. Scale bar = 50 μm. (C) Quantification of number of cells in each zone of the roots of wild type (solid bars) or friendly (open bars). Dead cells were identified by the presence of a SYTOX Orange stained nucleus. Bars represent the mean number of dead cells in 5 plants; error bars = SEM, n=5. (D) Representative maximum projections of 16 x 1 μm optical sections through the epidermal cells of the root elongation zone: bright field, Lysotracker stained compartments, or mitochondria. Scale bar = 10 μm. (E) Quantification of area and number of Lysotracker stained compartments in roots imaged as in (A) using the “Analyze particles” option in Image J. Error bars = SEM, p<0.01, n=5.
Figure 9. The activity of the Alternative Oxidase pathway is increased in friendly but the activity of Photosystem II is unaffected. (A) Oxygen uptake in wild type versus the friendly mutant under baseline conditions or in the presence of 0.5 mM KCN or 0.5 mM KCN and 0.1 mM propyl gallate (Pgal). The rate of oxygen uptake in the presence of KCN was significantly different between wild type and friendly (p<0.01). Error bars = SD, n=4. (B) Percentage inhibition of respiration in wild type or friendly relative to the baselines values, calculated using the data depicted in (A). (C) Steady-state PSI redox state (as estimated by the chlorophyll fluorescence parameter \(1-q_L\)) in wild type and friendly. (D) Capacity for nonphotochemical dissipation of excess light (NPQ) in friendly and wild type.
Figure 10. Lysine acetylation alters the ability of FRIENDLY to mediate mitochondrial association. (A) Quantification of the percentage of wild type or friendly plants displaying a wild type phenotype when stably transformed with transgenes encoding for mutant FRIENDLY proteins incorporating K→Q or K→R mutations at residues 1022 or 1029. Numbers within the bar are the number of T1 individuals identified within each transgene/background combination. (B) Percentage of T1 plants (22 individuals were analysed) displaying a wild-type phenotype after transformation with 35S-FRIENDLY of either friendly (complementation) or wild type (overexpression).
Supplementary figure 1. Analysis of cluster size and number in the friendly-3 T-DNA mutant. Data are averages taken from 20 independent images, each from a separate plant. Error bars = SEM, n=20.
Supplementary figure 2. (A) Disruption of the actin cytoskeleton can phenocopy for FRIENDLY mutation. Mitochondria in a leaf epidermal cell of a wild type mito-GFP seedling treated with either solvent (0.1% ethanol) or with 2 μM latrunculin-B. Scale bar = 10 μm. (B) Peroxisome distribution is unaffected in friendly. Mitochondria (mito-GFP, red) and peroxisomes (YFP-SKL, green) in the friendly mutant.
Supplementary figure 3. Transient expression of FRIENDLY N- and C-terminal mRFP1 fusions in friendly mutant seedlings. (A) FRIENDLY-mRFP1, or (B) mRFP1-FRIENDLY shows a punctate distribution in the cytosol. Arrows indicate mitochondrial clusters (mito-GFP, green) and arrowheads indicate FRIENDLY-mRFP1 or mRFP1-FRIENDLY puncta (magenta). Scale bars = 5 μm.
Supplementary figure 4. Whole plant phenotype of wild type (Col-0) and the friendly-3 T-DNA mutant. (A) Dry weight of wild type and mutant 7, 14, 21 or 28 days after the onset of germination. Bars represent mean of three independent experiments each using 15 plants; error bars = SEM, n=3. (B) Quantification of root length at 7, 14, 21 or 28 days after the onset of germination. Bars represent the mean of three independent experiments each using 20 plants; error bars = SEM, n=3. (C) Quantification of hypocotyl length of dark grown etiolated seedlings 7 days after onset of germination. Bars represent the mean of three independent experiments each using 20 plants; error bars = SEM, n=3.
Supplementary figure 5. Fragmentation spectrum of a lysine-acetylated peptide unique to FRIENDLY (At3g52140).