INVESTIGATING DYNAMIC SPATIAL INTERACTIONS 
BETWEEN MITOCHONDRIA AND ENDOPLASMIC 
RETICULUM IN LIVING PLANT CELLS AND THEIR 
POSSIBLE ROLE IN CONTROLLING 
MITOCHONDRIAL CALCIUM FLUX

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By

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ABSTRACT

Mitochondria are dynamic organelles known primarily for their roles in oxidative metabolism and programmed cell death. Both of these processes are regulated by the mitochondrial matrix calcium concentration. Little is known about how mitochondrial calcium is regulated: no plant mitochondrial Ca\(^{2+}\)-ATPase pumps or no mitochondrial Ca\(^{2+}\) channels have been identified to date. In addition, little is known concerning any physical interactions between mitochondria and endoplasmic reticulum (ER), an important cellular calcium store, and how these modulate cellular calcium fluxes. In this work stable transgenic Arabidopsis lines expressing fluorescent marker proteins were generated to allow visualisation of mitochondria and the ER in the same cells, and to measure mitochondrial calcium fluxes using aequorin. According to my results, there is a physical association between mitochondria and ER and this association cannot be disrupted by chemical treatments (latrunculin B, methyl viologen and antimycin A). As part of this work I identified an Arabidopsis gene, *Mitochondrial Calcium Uptake 1 (MCU1)*, which encodes a protein with features that suggest a role in mitochondrial calcium dynamics. Fluorescent protein fusions of this protein demonstrated that it localizes to mitochondria. An Arabidopsis T-DNA line was identified with an insertion in *MCU1*. However, little effect of the insertion on transcript abundance of *MCU1* was observed.
TABLE OF CONTENTS

ABSTRACT .................................................................................................................................ii
TABLE OF CONTENTS ..................................................................................................................iii
LIST OF FIGURES .......................................................................................................................vi
LIST OF TABLES ........................................................................................................................vii
LIST OF ABBREVIATIONS .........................................................................................................viii

CHAPTER 1. LITERATURE REVIEW .........................................................................................1
  1.1 Mitochondria in eukaryotic cells .........................................................................................1
  1.2 Mitochondria in plants .......................................................................................................1
    1.2.1 The structure of plant mitochondria ...........................................................................1
    1.2.2 Plant mitochondrial dynamics and morphology .......................................................2
  1.3 Endoplasmic reticulum (ER) in plants ..............................................................................4
    1.3.1 Dynamics and morphology of ER .............................................................................4
  1.4 The role of calcium in plant cell function .........................................................................5
  1.5 Interaction between mitochondria, ER and calcium dynamics .......................................7
  1.6 The effects of latrunculin B (Lat-B), methyl viologen (MV) and antimycin A (AA) 
    treatments on plant mitochondria .....................................................................................8
  1.7 The aequorin protein .......................................................................................................9
    1.7.1 Measuring intracellular calcium in living cells ........................................................9
  1.8 Mitochondrial Calcium Uptake 1 (MICU1) ..................................................................10
  1.9 Programmed cell death (PCD) ........................................................................................10
  1.10 Aims of this study ........................................................................................................12

CHAPTER 2. MATERIALS AND METHODS .........................................................................13
  2.1 Plant material ................................................................................................................13
  2.2 Seed sterilization and sowing ........................................................................................13
  2.3 Crossing Arabidopsis plants ..........................................................................................14
  2.4 Development of Arabidopsis line to visualize ER and mitochondria .........................14
  2.5 Identification of MCU1 in Arabidopsis ........................................................................14
  2.6 Sub-cellular localization of MCU1 ................................................................................15
2.6.1 Polymerase chain reaction (PCR) amplification of \textit{MCU1} \hspace{1cm} 15
2.6.2 Preparation of electroporation-competent \textit{Escherichia coli} \hspace{1cm} 15
2.6.3 Transformation of \textit{E. coli} \hspace{1cm} 16
2.6.4 Purification of plasmid DNA \hspace{1cm} 16
2.6.5 Generation of reporter constructs \hspace{1cm} 16
2.6.6 Preparation of electroporation competent \textit{Agrobacterium tumefaciens} \hspace{1cm} 17
2.6.7 Transformation of \textit{A. tumefaciens} \hspace{1cm} 17
2.6.8 Transformation of Arabidopsis plants \hspace{1cm} 18
2.7 \textit{mcu1} T-DNA lines \hspace{1cm} 18
   2.7.1 Plant genomic DNA extraction \hspace{1cm} 18
   2.7.2 Genotyping \hspace{1cm} 19
2.8 Semi-quantitative reverse transcription-PCR (sqRT-PCR) \hspace{1cm} 19
2.9 Tetramethylrhodamine staining \hspace{1cm} 20
2.10 Isolation of protoplasts from Arabidopsis seedlings \hspace{1cm} 20
   2.10.1 Protoplast isolation (Sandwich method) \hspace{1cm} 20
   2.10.2 Protoplast isolation (Chopped method) \hspace{1cm} 22
   2.10.3 Luminescence measurements of mitochondrial Ca$^{2+}$ \hspace{1cm} 22
2.11 Fluorescence microscopy \hspace{1cm} 24
2.12 Chemical treatments \hspace{1cm} 24
2.13 Mitochondrial and ER morphology and dynamics \hspace{1cm} 24
2.14 Visualization of mitochondrial or ER movement \hspace{1cm} 25
2.15 Data analysis \hspace{1cm} 25

CHAPTER 3. RESULTS \hspace{1cm} 26
3.1 Identification of MCU1 in Arabidopsis plants \hspace{1cm} 26
   3.1.1 Does MICU1 exist in plants? \hspace{1cm} 26
   3.1.2 Sub-cellular localization of MCU1 \hspace{1cm} 26
   3.1.3 \textit{mcu1} T-DNA line \hspace{1cm} 31
   3.1.4 \textit{MCU1} transcript levels in T-DNA insertion mutants \hspace{1cm} 31
   3.1.5 Phenotype of \textit{mcu1} \hspace{1cm} 31
      3.1.5.1 Mitochondrial and ER morphology and phenotype in \textit{mcu1} \hspace{1cm} 31
3.1.5.2  mcu1 plant morphology ................................................................. 37
3.1.6  Measurement of mitochondrial calcium dynamics .......................... 37
3.2  The interactions between mitochondria and ER ............................... 37
   3.2.1  Production of a transgenic line expressing both mito-CFP and ER-YFP-HDEL..37
   3.2.2  The effect of chemical treatments on ER-mitochondrial dynamics ........... 41
       3.2.2.1  Effects of Lat-B on the morphology and motility of mitochondria and
               ER in Arabidopsis .................................................................. 41
       3.2.2.2  Effects of MV on the morphology and motility of mitochondria and
               ER in Arabidopsis .................................................................. 41
       3.2.2.3  Effects of AA on the morphology and motility of mitochondria and
               ER in Arabidopsis .................................................................. 47

CHAPTER 4. DISCUSSION AND CONCLUSIONS ........................................... 55
   4.1  Identification of mitochondrial Ca\(^{2+}\) ions channels and Ca\(^{2+}\)-ATPases ...... 55
       4.1.1  Sub-cellular localization of MCU1 ................................................. 56
   4.2  Measuring calcium fluxes in Arabidopsis ........................................... 56
   4.3  Mitochondria and ER juxtaposition ................................................... 57
   4.4  ER and mitochondrial dynamics ....................................................... 58
   4.5  Conclusions and future work .......................................................... 60

CHAPTER 5. REFERENCES ......................................................................... 62
LIST OF FIGURES

Figure 2-1: Sandwich method for Arabidopsis protoplast isolation ..........................21
Figure 2-2: Chopped method for Arabidopsis protoplast isolation .........................23
Figure 3-1: Amino acid alignment of human MICU1 and Arabidopsis MCU1 ..........27
Figure 3-2: Amplification of MCU1 ........................................................................28
Figure 3-3: N- and C-terminus GFP fusion protein constructs with MCU1 ...........29
Figure 3-4: Localization of MCU1 to Arabidopsis mitochondria in root and leaf tissues......30
Figure 3-5: Structure of mature RNA for MCU1 .......................................................32
Figure 3-6: Identification of homozygous mutants in the SAIL_359 line ...................33
Figure 3-7: Expression of MCU in plant lines used for this study ..............................34
Figure 3-8: Images of wild type (A) and mcu1 expressing mito-GFP-aequorin (B) ........35
Figure 3-9: Images of wild type (A) and mcu1 expressing ER-YFP-HDEL (B) ..........36
Figure 3-10: Plant morphology of mito-GFP-aequorin and mcu1 lines .....................38
Figure 3-11: Mitochondrial calcium signatures .........................................................39
Figure 3-12: Association of mitochondria and ER ....................................................40
Figure 3-13: Mitochondrial and ER dynamics upon treatment with Lat-B .........42
Figure 3-14: Effect of Lat-B treatment on mitochondria motility ..........................43
Figure 3-15: Effect of Lat-B treatment on ER morphology and movement ...........44
Figure 3-16: The effects of Lat-B on mitochondria and ER ..................................45
Figure 3-17: Mitochondrial and ER dynamics upon treatment with MV ..........46
Figure 3-18: Effect of MV treatment on mitochondria motility ..............................48
Figure 3-19: Effect of MV treatment on ER morphology and movement ...........49
Figure 3-20: The effects of MV on mitochondria and ER ..................................50
Figure 3-21: Mitochondrial and ER dynamics upon treatment with AA ..........51
Figure 3-22: Effect of AA treatment on mitochondria motility ..............................52
Figure 3-23: Effect of AA treatment on ER morphology and movement ...........53
Figure 3-24: The effects of AA on mitochondria and ER ..................................54
LIST OF TABLES

Table 2-1: Gateway primers used for cloning of Arabidopsis *MCUI* ...........................................18
Table 2-2: Primers used for the genotyping of *mcu1* ........................................................................19
Table 2-3: Primers for sqRT-PCR of *MCUI* ......................................................................................20
LIST OF ABBREVIATIONS

AA    antimycin A
ABRC  Arabidopsis Biological Resource Center
ANOVA Analysis of Variance
bp    base pair
CFP   cyan fluorescent protein
ER    endoplasmic reticulum
EtOH  ethanol
GFP   green fluorescent protein
g     gravity
Lat-B  Latrunculin B
LB    Luria-Bertani
LSD   Least Significant Difference
MAM   mitochondria-associated ER membrane
MCU1  Arabidopsis ortholog of $MICU1$
mcu1  Arabidopsis mutant of $MCU1$
$MICU1$ Mitochondrial Calcium Uptake 1 from Homo sapiens
MS    Murashige and Skoog
MV    methyl viologen
NCBI  National Center for Biotechnology Information
PCD   programmed cell death
PCR   polymerase chain reaction
RER   rough endoplasmic reticulum
RFP   red fluorescent protein
ROS   reactive oxygen species
SER   smooth endoplasmic reticulum
sqRT-PCR semi-quantitative reverse transcription-PCR
TMRM  tetramethyl rhodamine methyl ester
YFP   yellow fluorescent protein
CHAPTER 1. LITERATURE REVIEW

1.1 Mitochondria in eukaryotic cells

Eukaryotic cells have specialized structures called organelles to perform specific functions within the cell. Most of these organelles are compartments, surrounded with bilipid membranes that separate them from the rest of the cell in order to facilitate their function. One example of an organelle is the mitochondrion, known as the “powerhouse of the cell.” Mitochondria possess their own genome (mtDNA) and are semiautonomous. Moreover, they are believed to have evolved from a free α-proteobacterium-like organism (Takasugi et al., 2010). The word “mitochondrion” is itself derived from two Greek words, “mitos” (thread) and “chondros” (grain) which provide an apt description of the mitochondrial morphology as observed by early cytologists (referenced in Scott and Logan, 2007). Mitochondria play critical roles in cells because they convert carbon compounds into ATP, the vital form of energy required by the cell. The structure of mitochondria remained a mystery until the 1950s when the advent of the electron microscope permitted elucidation of cellular structure. Mitochondria in eukaryotes are not only the sites of cellular respiration, but are also involved in oxidative phosphorylation and programmed cell death (Liesa et al., 2009).

1.2 Mitochondria in plants

1.2.1 The structure of plant mitochondria

Over the last half century, electron microscopy has revealed that the mitochondrion consists of a matrix entirely surrounded by an inner membrane and an outer membrane. The inner and outer membranes, are separated by an inter-membrane space (Lodish et al., 1995; Frey and Mannella, 2000). Based on 3D tomogram analysis, it has been shown that mitochondria are made up of six compartments: matrix, cristae membrane, inter-cristae space, inner membrane, inter-membrane space, and outer membrane (Pical et al., 1993). Advances in electron microscopy and 3D analysis have subsequently led to the discovery of cristae that are pleomorphic in shape, with highly tubular structures interconnected both to one another and to the inner membrane (Logan, 2006). The electron transport chain is located in the cristae membrane, while enzymes of the TCA cycle, ribosomes, and mtDNA are situated in the mitochondrial matrix. The majority of mitochondrial proteins are encoded in the nuclear genome.
and synthesized in the cytosol before import into the mitochondria (Mackenzie and McIntosh, 1999).

1.2.2 Plant mitochondrial dynamics and morphology

Mitochondrial dynamics refers to the process of maintaining the shape, size, number and distribution of mitochondria within the cell (Logan et al., 2003). Mitochondria undergo changes in shape, size, and cellular distribution, depending on their prevailing conditions. For instance, in yeast and animal cells, mitochondria can form network-like structures after division and fusion (Chen et al., 2003; Meeusen et al., 2004). In plants, mitochondria movement or joining allows normal function. Mitochondrial dynamics in plants are determined by the metabolism and the architecture of the cell, along with mechanoproteins and regulatory proteins, which participate in the fusion and fission of organelles (Logan, 2010).

Forces due to cytoskeletal and intrinsic factors are responsible for various changes and displacements of mitochondria (Bereiter-Hahn and Vöth, 1994). In yeast, it has been observed that the microtubular cytoskeleton is crucial for mitochondrial distribution (Glick, 1996; Yaffe et al., 1996; Yaffe et al., 2003; Weir and Yaffe, 2004; Wiley et al., 2008). Translocation of mitochondria depends on the intermediate filaments and the actin filaments (Van Gestel et al., 2002). Mitochondria labeled with fluorochrome exhibit stop-go movements in BY-2 cells of tobacco (Mitsuhashi et al., 2000).

The dynamic nature of plant mitochondria is partly attributed to the need to exchange or complement mtDNA, a process facilitated by the physical meeting of discrete mitochondria (Logan et al., 2003). Mitochondria can move at speeds of up to 10 µm/s in the cytosol of root hairs of Arabidopsis; the speed varying depending on the position of the mitochondria within such root hairs. The speed at which mitochondria move varies, with a single mitochondrion moving at speeds between 4.19 µm/sec to 9.96 µm/s, depending on location within the cell (Zheng et al., 2009). The mechanisms underlying mitochondrial movement can also vary according to the organisms. In most yeast and animal cells, the movement of the mitochondria is microtubule-based (Boldogh and Pon, 2007). However, this movement in fungi *Aspergillus oryzae*, *Saccharomyces cerevisiae*, and plants depends on the actin cytoskeleton (Sheahan et al., 2004).
Despite the motility of mitochondria allowing for close association with other organelles, it remains unknown whether this association has an impact on function. In one study, the movement of chloroplasts under differing lighting conditions also resulted in changes in the positioning of the mitochondria (Islam et al., 2009). However, it remained inconclusive as to whether the movement of mitochondria was independent of chloroplast movement; it was not clear whether mitochondrial movement resulted from association with chloroplasts via indirect or direct interactions with the cytoskeleton (Islam et al., 2009). Overall, there is little conclusive information regarding exactly which mechanisms determine the cellular distribution of mitochondria in higher plants.

Mitochondrial dynamics in plants also includes fusion and fission of the organelles themselves, which are important aspects in the distribution and complementation of mtDNA. The mitochondrial division machinery in plants is made up of several proteins. For example, about 16 dynamin homologues are found in the Arabidopsis genome, and each homologue appears to act in the division of mitochondria (Scott and Logan, 2008). Two of these proteins, DRP3A and DRP3B, have a 37 to 41% similarity to the proteins involved in mitochondrial division in yeast and animal cells (Logan et al., 2004). Interruption of DRP3A and DRP3B function as a result of gene knockouts increases the quantity of large and elongated mitochondria, therefore indicating that these proteins play a role in the division of mitochondria (Logan, 2006).

In addition, different types of adapters direct these proteins to sites of scission. In plants, DRP3A and DRP3B interact with two adapters: Fis-1-type proteins, known in plants as BIGYIN1 and BIGYIN2 (Scott et al., 2006). The two proteins may also interact directly with the mitochondrial outer membrane as a result of interaction with the plant specific adapter NETWORK1. After appropriate localization by adapters, the dynamin homologues around the organelle constrict and ultimately split the mitochondrion into two (Logan et al., 2003; Arimura et al., 2008). Fusion in plant mitochondria has yet to be explained, although evidence thus far indicates that it is a rapid process (Arimura and Tsutsumi, 2002).

The higher plant chondriome is typically composed of several hundred physically discrete organelles, and its structure is known to undergo changes at different phases of the cell cycle (Logan, 2006). At G1 to S phase, a mitochondrion envelopes one end of the nucleus, in addition to displaying a tentaculate morphology (Segui-Simarro et al., 2008). During G2 phase, a large mitochondrion forms a cage around the nucleus, while both large and small mitochondria
increase in number within the cell. During cytokinesis, the division of the cage-like mitochondria occurs, giving rise to two distinct mitochondria which undergo further divisions within the two new daughter cells (Segui-Simarro et al., 2008).

### 1.3 Endoplasmic reticulum (ER) in plants

The ER in cells occurs as a system of sheets and membrane tubules distributed throughout the cytoplasm (Giorgi et al., 2009), and also continuous with the nucleus (Chevet et al., 2001). Interestingly, three-dimensional reconstructions of the ER indicate that it resembles a basket entirely enclosing the cellular space. The ER occupies a cortical position just inside the plasma membrane (Runions, 2007). Two types of ER are revealed by transmission electron microscopy: cisternal ER, coated with ribosomes (also referred to as rough endoplasmic reticulum (RER)) and smooth endoplasmic reticulum (SER) (Bootman et al., 2002). Generally, RER appears more common in cells that secrete proteins, while SER is more common in cells that actively secrete lipids and synthesize membranes (Borgese et al., 2006).

SER is found in abundance only in specific types of cells, particularly in animal cells such as hepatocytes, neurons, and muscle cells (Voeltz et al., 2002). Other functions, performed by the ER, include storage of calcium ions in the lumen of the ER and the subsequent calcium release into the cytosol, translocation of proteins across the membrane of the ER, modification and folding of proteins, and the synthesis of both phospholipids and steroids. The ER is a major component of the secretory pathway, given that it is comprised of a series of membranes from the site of synthesis of proteins. Proteins associated with the membrane and those destined for purposes such as storage vesicles are synthesized through the translation of mRNA at the ribosome (Cooper, 2000). They are synthesized and inserted concurrently into the ER via the translocon where they undergo modification. Protein insertion is a process that begins with an amino acid signal or a transit peptide sequence within the protein that directs the proteins into the ER lumen (Runions, 2007).

Under light microscopy of living cells, and after staining with fluorescent dyes, interphase ER appears to be made up of two components: the peripheral and the nuclear ER (Lee and Chen, 1988). The nuclear ER, or envelope, is comprised of two sheets of membranes with a lumen that encompasses the nucleus, and inner and outer membranes connecting only at nuclear pores (Staehelin, 1997). The peripheral ER is comprised of a series of interconnected tubules that
extend throughout the cell (Sparkes et al., 2009). Obvious morphological differences are apparent between SER and RER; SER is more convoluted than RER (Prinz et al., 2000).

### 1.3.1 Dynamics and morphology of ER

The ER is a highly dynamic organelle, the dynamism of which has been conserved by evolution, but the exact relationship between the dynamism of the ER and its function has yet to be fully understood (Berridge, 2002). Some dynamic modifications exhibited by the ER include budding and incorporation of vesicles into the ER membrane, emergence and retraction of tubules, transition of sheets into tubules and vice versa, as well as fusion and fission of tubules (English et al., 2009). The shape of the membrane changes during cell division, metabolic state, and growth. The intrinsic curvature of the membranes is attributed to the presence of peripheral proteins contorting the membrane. Most notably, integral membrane proteins have specialized hydrophobic domains that are responsible for generating the curved shape, since they wedge themselves into the outer layer of the membranes (Shibata et al., 2009).

Another critical component of ER dynamics is membrane fission and fusion (Du et al., 2004). Moreover fusion, crucial to the preservation of the ER structure in both plants and animals, has been demonstrated to be dependent on GTP and atlastins (Anderson and Hetzer, 2007). Atlastins are integral membrane proteins that have an N-terminal GTPase and two transmembrane domains, the insertion of which into the lipid bilayer results in the N- and C-termini protruding into the cytoplasm (Puhka et al., 2007). The exact mechanism of fission in ER and the proteins that are involved in the process are not known. Researchers have speculated that ER membranes undergo fission, exactly as the mitochondria undergo fission in order to strike a balance. Membrane fission of the ER can be seen during mitosis and it is speculated that the process of fission aids in shape maintenance of the ER (Voeltz et al., 2002).

### 1.4 The role of calcium in plant cell function

Calcium is known to play a key role in the normal growth of plants. The importance of calcium for whole plant function is, quite logically, reflected at the cell level. Ca$^{2+}$ is not normally limiting under field conditions, but a number of outcomes can be linked to low calcium levels, including poor development of roots, fruit cracking, and leaf curling and necrosis (White and Broadley, 2003). The significant cell targets are the cell wall in which Ca$^{2+}$ plays a major
function in crosslinking residues of acidic pectin, and the cell membrane system where a low calcium ion level increases the plasma membrane permeability (Hepler, 2005).

In the cell wall, calcium forms calcium pectates, compounds that provide cell wall stability and cements cells together (Holdaway-Clarke and Hepler, 2003). During formation of the cell wall, residues of acidic pectin are released as methyl esters that are later deesterified by pectin methylesterase exposing carboxyl groups that bind Ca$^{2+}$. It follows that low calcium ion levels will make the cell wall more easily ruptured and pliable, while high concentrations will rigidify the cell wall making it less plastic. Ca$^{2+}$ is also important in controlling the structure, as well as the function of cell membranes (Hepler, 2005). Through binding to phospholipids, Ca$^{2+}$ stabilizes the lipid bilayers and therefore offers cellular membranes some structural integrity.

Ca$^{2+}$ is a significant component of both structure and function of plant cells. Ca$^{2+}$ can act as a secondary messenger in processes to mediate biotic and abiotic stresses (Rudd and Franklin-Tong, 2001). The structural fundamentals for Ca$^{2+}$ plant signaling, the generation of Ca$^{2+}$ signatures as well as the nature of Ca$^{2+}$ sensors, are viewed in association with the functioning of plant cryptochromes, photoreceptors, and phytochromes, as well as phototropins.

The integration of intracellular signalling is basically achieved by the ER that extends throughout the cytosol of a typical eukaryotic cell. The ER membrane encloses an internally uninterrupted ER lumen, forming a specialized pathway for communication among different compartments of a cell (Verkhratsky, 2005). The integrative role of the ER is aided by several molecular cascades that allow it to decipher incoming information and to give output signals causing effects on cellular function in different spatial as well as temporal domains (Berridge, 2002).

Overall, Ca$^{2+}$ acts as a crucial signalling messenger involved in the regulation of many vital functions and the ER plays a key role in rapid physiological signalling because it can function as a dynamic storehouse for Ca$^{2+}$. The ER can accumulate Ca$^{2+}$ to levels 1000s of times greater than that in the cytosol. The Ca$^{2+}$ store in the ER is involved in the production of rapid intracellular signals of Ca$^{2+}$ after plasma membrane electrical excitation or following chemical activation of plasmalemmal receptors (Burdakov et al., 2005). In addition, the ER serves as a powerful intracellular Ca$^{2+}$ buffer, crucial in removing excess Ca$^{2+}$ that accumulates during physiological stimulation. Ca$^{2+}$ concentration inside the ER lumen serves as a primary determinant of ER homeostasis, controlling the activation of ER channels for release of Ca$^{2+}$, the
mechanism of endomembrane Ca$^{2+}$ uptake and of several enzymatic cascades (Burdakov et al., 2005).

Mitochondria play an important role in the provision of a Ca$^{2+}$ source in suspension cells of maize reacting to anoxia (Subbaiah et al., 1994), or by reacting to rising levels of cytosolic Ca$^{2+}$ through increasing their internal Ca$^{2+}$ concentration in *Fucus* rhizoid cells (Coleho et al., 2002).

### 1.5 Interaction between mitochondria, ER and calcium dynamics

In general, Ca$^{2+}$ signalling is necessary for regulating mitochondrial responses to both extra and intra-cellular stimuli. Satrústegui et al., (2007) theorized that more energy may be made available for mitochondrial function when NADH/NAD ratios are (Satrústegui et al., 2007).

Recently, Wang et al. (2010) investigated the role of microfilaments in the plant cell, focusing on both their positioning in the cell and the properties of plant mitochondria. This is important because actin filaments regulate mitochondrial movement (Wang et al., 2010).

The physical association between mitochondria and the ER is known as the mitochondria-associated ER membrane (MAM). MAM was first described in 1959 (Copeland and Dalton, 1959) and later, various groups have observed the close apposition of the ER (or sarcoplasmic reticulum, SR) and outer mitochondrial membrane (OMM) in rat liver and brain, mouse liver, Chinese hamster ovary cells, *Saccharomyces cerevisiae*, and fungal hyphae (Bracker and Grove, 1971; Vance, 1990; Ardail et al., 1993; Camici and Corazzi, 1995; Gaigg et al., 1995; Shiao et al., 1995). However, the physical contact of the ER-mitochondria tether in Arabidopsis has not yet been identified.

In order to perform the complex tasks like Ca$^{2+}$ signalling and programmed cell death (PCD), mitochondria arrange themselves in a dynamically interconnected network of organelles, especially with endoplasmic reticulum, the mechanism by which are mostly unknown (de Brito and Scorrano, 2008). A few studies have revealed that a dynamin like protein, DLP1, is required for normal distribution of ER and mitochondria in mammalian cells (Pitts et al., 1999). But not much is known about their interaction pattern in higher plants.
1.6 The effects of latrunculin B (Lat-B), methyl viologen (MV) and antimycin A (AA) treatments on plant mitochondria

Latrunculin B (Lat-B), a member of the latrunculins which inhibits actin polymerization, affects plant mitochondria motility through the inhibition of F-actin assembly and sequestering of G-actin (Van Gestel et al., 2002). In Arabidopsis leaf epidermal cells and tobacco cells, treatment with Lat-B showed a clustered mitochondrial behavior and inhibited the motility of mitochondria (Van Gestel et al., 2002).

In contrast, antimycin A (AA) is a chemical compound produced by *Streptomyces kitazawensis* bacteria (Nakayama et al., 1956) that binds to the *Q*₁ site of cytochrome *c* reductase in the mitochondrial complex III to restrain the oxidation of ubiquinol in the electron transport chain, blocking mitochondrial electron transfer between cytochrome *b* and *c* (Alexandre and Lehninger, 1984; Campo et al., 1992; Maguire et al., 1992; Pham et al., 2000; Xia et al., 1997; Dairaku et al., 2004; Han et., 2008). The inhibition of this reaction disrupts the formation of the proton gradient across the mitochondrial inner membrane, leading to the loss of the mitochondrial membrane potential (ΔΨₘ) (Campo et al., 1992; Pham et al., 2000). The significance of inhibiting complex III is an increase in the production of reactive oxygen species (ROS) (Park et al., 2007).

Methyl viologen (MV), also known as Paraquat, is a synthetic compound, extensively used as a herbicide because of its devastating effect on plant chlorophyll (Palatnik et al., 1997). This compound functions as a herbicide via its photochemical functions, inhibiting photosynthesis by producing destructive reactive oxygen species in the ferredoxin quinone reductase pathway, where it accepts electrons from photosystem I in turn channelling them to molecular oxygen (Allen, 1978). Through this process, reactive oxygen species are generated creating an oxidized form of MV that shuts down electron transport from photosystem I; thus inhibiting the plant cell cyclic electron flow. Accumulation of oxidized MV results in a production of free radicals, such as hydrogen peroxide, and causes superoxide production in the chloroplasts (Qingping et al., 1996). This compound affects mitochondria by catalysing the establishment of ROS in the cristae, where the compound is reduced by NADPH and in turn oxidized by dioxygen-producing superoxides (Ikuma and Bonner, 1967). According to a previous study, treatment of Arabidopsis leaves with MV for 4 hr caused mitochondria to cluster (Scott and Logan, 2008).
1.7 The aequorin protein

Aequorin is a photoprotein obtained from the jellyfish *Aequorea victoria*, that is also a calcium-sensitive fluorescent probe (Allen et al., 1977; Baubet et al., 2000; Mithöfer and Mazars, 2002). The photoprotein consists of coelenterazine as a luciferin, apoaequorin as the apoprotein with bound oxygen molecules, with an overall molecular weight of 22 kDa. The photoprotein has a unique high affinity for free calcium ions, a trait that makes it useful in measuring cellular calcium (Nelson et al., 2004).

In addition, aequorin has unique properties that make it effective as a cellular calcium reporter. It is non-cytotoxic, has a high selectivity for free calcium, a lack of calcium ion concentration buffering, a wide dynamic range, and an ability to be retained within targeted cell organelles such as the mitochondrion (Takahashi et al., 1999). Using appropriate signal sequences and promoters, the photoprotein can be expressed in mitochondria (Chiesa et al., 2001). Aequorin emits light in proportion to concentration of calcium ions. The active aequorin is formed by a complex between apoaequorin, oxygen and coelenterazine. In addition, on binding calcium, the complex is broken down emitting light and rendering the free apoaequorin and coeleteramide (Toma et al., 2005). A successful use of aequorin in plants involved targeting it to plant mitochondria in which the resting concentration of free Ca\(^{2+}\) was found to differ by 100 nM between the cytosol (100 nM) and the mitochondria (200 nM) (Logan and Knight, 2003).

1.7.1 Measuring intracellular calcium in living cells

Several methods for measuring intracellular calcium or calcium in living cells, including calcium-sensitive photoproteins, calcium-sensitive microelectrodes, and calcium-sensitive fluorescence have been established (Nelson et al., 2004). In order to measure calcium in plant mitochondria, the photoprotein must be transferred to the mitochondria to enable the detection of calcium.

In the case of the Arabidopsis plant, the chimeric construct should consist of the β-ATPase-m GFP5 cDNA and the aequorin cDNA required to express a GFP-aequorin fusion protein within the mitochondria (Logan and Knight, 2003). Expression of the GFP-aequorin fusion protein within the mitochondria allows detection and measurement of the calcium
specifically within the matrix (Logan and Knight, 2003). Since fused aequorin protein is expressed in the plant mitochondria, the concentration of the calcium ions can be detected through luminometry. Because mitochondria are dispersed in the cytoplasm, the concentration of calcium in the mitochondria can be determined through evaluation of the calcium attached to the fused aequorin protein expressed only within the mitochondria using a luminometer.

1.8 Mitochondrial Calcium Uptake 1 (MICU1)

Mitochondrial calcium uptake 1 (MICU1) is attached to the inner membrane, where it can sense Ca^{2+} in the cell. Studies have shown that MICU1 has an ability to let calcium across the inner membrane. The availability of calcium in a mitochondrion is therefore highly dependent on MICU1 (Balaban, 2009). The presence of MICUI may also have effects on various physiological factors, ranging from cell secretion to differentiation (Gunter and Gunter, 1994). Retention of large quantities of Ca^{2+} in mitochondria may cause bursting of the mitochondria.

The movement of cytosolic calcium into mitochondria is not shutdown even when MICU1 is silenced (Hajnóczky et al., 1995). However, intake is slowed, thus, most of the physiological functions of mitochondria are also slowed in the cell (Sparagna et al., 1995). Studies carried out on animal cell mitochondria by Rizzuto et al., (2012) identified MCU (mitochondrial calcium uniporter) and this work greatly added in the understanding of mitochondrial calcium transport mechanisms and function in animal cells. Two regulators of MCU, MCUR1 and MICU1, have since been identified (Perocchi et al., 2010; De Stefani et al., 2011; Mallilankaraman et al., 2012).

1.9 Programmed cell death (PCD)

Every living multicellular organism undergoes death at the end of the life cycle. Cell death can occur either as the result of intense damage during a process called necrosis, or through programmed cell death (PCD) which involves intricate cellular processes, machinery, and programs that lead to biochemical and morphological changes and result in self-induced, controlled death (Muñoz-Pinedo, 2012; Peng et al., 2013). PCD is governed by genetic instructions, and has been found both in animal and plant cells, though with marked differences.

By taking account morphological changes, animal PCD can be classified as first, apoptosis or type I PCD, characterized by chromatin condensation and fragmentation,
chromosomal DNA and nuclear fragmentation, cell shrinkage, disintegration, and formation of apoptotic bodies; second, type II PCD or autophagy involves catabolic processes, the emergence of autophagosomes and structures surrounding macromolecules and organelles in order to be recycled; and last, necrosis or type III PCD which involves genetically-controlled cell swelling, organelle dysfunction, and cell lysis (Reape et al., 2008; Peng et al., 2013).

In contrast, PCD in plants has not been yet successfully classified, although plant PCD can be broadly classified into several groups using various criteria. According to Lord and Gunawardena (2011), PCD in plants can be classified depending on the source of the induction as developmentally regulated and environmentally induced. The former is induced by internal factors and occurs at a predictable time and location, whereas the latter is initiated due to external abiotic or biotic signals (Lord and Gunawardena, 2011).

Another classification proposed by van Doorn et al., (2011) involves focusing on morphological changes during the process. By using the rupture of the tonoplast followed by a quick clearance of the cytoplasm and most of the cell walls as a criterion, PCD could be classified as autolytic and non-autolytic (van Doorn, 2011). In the autolytic PCD exists a rapid clearance of the cytoplasm, plus chromatin condensation, increase in vacuolar volume (e.g. for this type of PCD are developmental PCD, heat, oxygen and drought stress) and decrease of cytoplasmic volume, whereas in non-autolytic PCD although swelling of organelles (e.g. hypersensitive response) exists, there is no rapid clearance of cytoplasm or increase in vacuolar volume (van Doorn, 2011). However, overall, there is an overlap that exists between each of these modes in the plant PCD spectrum (Reape et al., 2008).

At an infection scenario, the hypersensitive response is a non-autolytics form of PCD at the site of attempted pathogen invasion (Mur et al., 2008). Reactive oxygen species (ROS) participate in the signaling pathways involved in hypersensitive responses (Coll et al., 2011). In animal cells, the most described form of PCD is apoptosis, which is mediated by ROS and proteins called caspases (or homologous proteins). Despite the fact that plants lack the necessary genes to encode caspases, caspase-like protease activity has been described in plants (Korthout et al., 2000; Lam and del Pozo, 2000; Chichkova et al., 2010; Watanabe and Lam, 2011). They do exhibit, however, metacaspases which are proteins related to caspases with emerging suggestions that they are playing a role in oxidative stress generating physical (heat) and chemical (H$_2$O$_2$, methyl viologen) treatment induced PCD (Vercammen et al., 2007; He et al., 2008; Coll et al.,
The mechanisms that involve ROS are related to organelles such as ER. It has been argued that the ER could suffer stress from the accumulation of unfolded proteins in its lumen, thus increasing the levels of intracellular Ca\(^{2+}\) (Mur et al., 2008) and activating cell death responses (Ron and Walter, 2007; Liu et al., 2012). In animal cells, the role of Bcl-2, a proto-oncogene that protects against apoptosis, is involved in pathways that regulate ER ionic homeostasis, decreasing intracellular Ca\(^{2+}\) concentrations, and preventing cell death (Foyouzi-Youssef et al., 2000). However, similar mechanisms are yet to be explored in plants.

In plant cells, the major site of ROS production is mitochondria and chloroplasts, and there is evidence that ROS produced in mitochondria are critical to PCD induction (Gechev et al., 2006). Mitochondria are highly dynamic organelles, but there is little information about mitochondrial dynamics during plant PCD. In a previous study, Scott and Logan (2008) suggested the mitochondrial morphology transition (swelling) is an early indicator for plant PCD events. In that study, cell death was induced by treating with a heat shock or ROS-inducing chemicals (methyl viologen, hydrogen peroxide or s-triazine) in Arabidopsis leaves and protoplasts (Scott and Logan, 2008).

### 1.10 Aims of this study

This thesis aims to contribute to our knowledge on MICU1 in mitochondrial calcium dynamics in the model plant species *Arabidopsis thaliana*, and to investigate interactions between ER and mitochondria. Such interactions are important for the maintenance of cell health while the induction of programmed cell death causes changes in the mitochondrial-ER dynamics central to the PCD process (Giorgi et al., 2009). Regulation of mitochondrial calcium activity underpins mitochondrial function and is closely associated with the regulation of ER-calcium dynamics (Giorgi et al., 2009). In addition, it is hypothesized that genetic perturbation of mitochondrial calcium dynamics will influence mitochondrial association with the ER.

The current work has two main objectives which offer new insights into the role of mitochondrial calcium dynamics, along with the interrelationship between mitochondria and ER. The first objective is to determine the role of MICU1 in mitochondrial calcium dynamics.

The second objective is to investigate the association between mitochondria and the ER in wild type and in micu1 mutants, as well as to discover if these interactions are affected by chemical treatments such as Lat-B, AA and MV.
CHAPTER 2. MATERIALS AND METHODS

The molecular and cellular biology techniques used in this study were carried out following Sambrook and Russell (2001), or by modification of manufacturer’s instructions. Protocols and techniques that were significantly changed from previously published works are described below.

2.1 Plant material

Seeds of wild type *Arabidopsis thaliana* (ecotype Col-0) and the T-DNA insertion line SAIL_359 were obtained from the Arabidopsis Biological Resource Center (ABRC). The transgenic Arabidopsis mitochondrial-GFP-aequorin (mito-GFP-aequorin) and mitochondrial-cfp pBIN-CFP (mito-CFP) lines were obtained from Dr. David Logan (University of Saskatchewan, Saskatoon, Canada; Logan and Knight, 2003; Logan, unpublished). The seed for the Arabidopsis ER-YFP-HDEL line was obtained from Dr. Ian Moore (University of Oxford, Oxford, UK). This line contains a construct comprising the retention signal for ER, represented by the four amino acids, H-histidine, D-aspartic acid, E-glutamic acid and L-leucine (Teh and Moore, 2007).

2.2 Seed sterilization and sowing

Up to a 50 µL equivalent of Arabidopsis seeds were surface sterilized in a 1.5 mL microfuge tube by immersion in 1 mL of 80% (v/v) EtOH, mixed by inversion for 5 min, decanted and followed by 1 mL of 30% (v/v) household bleach mixed by inversion for 7 min then decanted. Finally, the seeds were rinsed three times with 1 mL of sterile distilled water. Seeds were spread onto Murashige and Skoog (MS) agar plates (0.43% (w/v) 1X MS salts, 0.8% (w/v) type M agar, 1% (w/v) sucrose and 0.05% (w/v) MES, pH 5.8). Spread plates were allowed to dry for 10 min before sealing with 3M Micropore tape (1.25 cm x 9.1 m). Plates were stored at 4°C for 3 days to synchronize germination and were then placed in a growth chamber (SANYO, Osaka, Japan) set with a 16/8 hr light/dark cycle at 23°C. Most experiments described were performed on 14-day-old seedlings unless otherwise noted.
2.3 Crossing Arabidopsis plants

To cross Arabidopsis plants all secondary bolts, as well as all flowers and siliques were removed, from the female recipient plant. On the main bolt, the three largest closed buds were used, and all others removed. Forceps and scissors were sterilized with 70% EtOH (v/v). Under a low power dissecting microscope (10X magnification) flowers were emasculated through the removal of sepals, petals and stamens, leaving only the carpels intact.

From the male donor plant, flowers that were visibly shedding pollen were detached, and the convex surface of the anthers brushed against the stigma of the female plant to transfer the pollen. After crossing, any newly developed inflorescences were removed from the female plant. Mature siliques were collected in a 1.5 mL microfuge tube, and stored at -4°C for further use.

2.4 Development of Arabidopsis lines to visualize the ER and mitochondria

T3 seeds of the mito-CFP and ER-YFP-HDEL Arabidopsis lines were germinated on MS plates and screened using epifluorescent microscopy. Highly fluorescent lines were crossed, resulting in Arabidopsis transgenic lines that co-expressed a mitochondria-targeted cyan fluorescence protein reporter (mito-CFP) and a yellow fluorescent protein tagged ER marker (ER-YFP-HDEL). The best-expressing lines were grown to seed for the production of future generations. Results presented are from either T3 generations.

2.5 Identification of MCU1 in Arabidopsis

In order to identify the Arabidopsis ortholog of the human mitochondrial calcium uptake 1 (MICU1) protein, a homology search of the non-redundant protein sequence database at NCBI (www.ncbi.nlm.gov) using a protein-protein BLAST (BLASTP; http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al., 1997) was performed. The MICU1 (mitochondrial isoform 1 from Homo sapiens; Accession No. NP_006068.2) protein sequence was used as a query. This returned an EF-hand, calcium binding motif-containing protein from Arabidopsis (Accession No. NP_001078476.1) and was renamed MCU1. Protein sequences were aligned using CLUSTALW2.1 at the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/msa/clustalw2/; Larkin et al., 2007). The gene encoding this protein in Arabidopsis was identified as At4g32060 and also renamed MCU1. The nucleotide sequence
(Accession No. NM_001085007.1) was obtained from GenBank at NCBI and used for primer design.

2.6 Sub-cellular localization of MCU1

2.6.1 Polymerase chain reaction (PCR) amplification of *MCU1*

Total RNA was extracted from wild type Arabidopsis seedlings genomic clones using the RNeasy Plant Mini (Qiagen Inc., Valencia, CA), followed by cDNA synthesis using a QuantiTect reverse transcription kit (Qiagen) as described by the manufacturer. PCR was carried out using a Mastercycler (Eppendorf AG, Hamburg, Germany) and BIOTAQ Red DNA polymerase (Bioline, Taunton, MA), with cycling conditions as follows: after an initial denaturation at 94°C for 2 min, PCR was performed for 30 cycles using 94°C for 1 min, 60°C for 1 min, 68°C for 1 min; followed by a final extension at 72°C for 5 min. Gene-specific primers for *MCU1* with adaptors to facilitate Gateway cloning are shown in Table 2-1. The reaction mixture (20 µL) was prepared according to the manufacturer’s recommendations with 1 µL (20 µM) of each forward and reverse primer and 1 µL of cDNA template. Following PCR, the DNA products were separated by gel electrophoresis in a 1% (w/v) agarose gel run in 1X TBE buffer (45 mM Tris-borate, 1 mM EDTA pH 8.3) containing (1 µg/mL) ethidium bromide to visualize products. Fragments were extracted from the gel using an E.Z.N.A Gel Extraction Kit (Omega Bio-Tek, Norcross, GA) as described by the supplier and identity confirmed as *MCU1* by sequencing (National Research Council, Saskatoon).

2.6.2 Preparation of electroporation-competent *Escherichia coli*

An *E. coli* starter culture was produced by inoculating 10 mL Luria-Bertani (LB) medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract) with 10 µL of a previously made aliquot of competent cells. The culture was grown overnight at 37°C and 500 µL of this overnight culture was used to inoculate 500 mL of LB medium that was incubated at 37°C with shaking until optimal growth (OD$_{600}$ = 0.5) was attained. The cultures were centrifuged at 4000 g for 15 min (4°C) using a bucket-rotor and refrigerated centrifuge (Avanti J-25, Beckman Coulter, Mississauga, ON). Cell pellets were washed in 500 mL of ice cold sterile water. The wash step was repeated twice and the final pellet was re-suspended in 10 mL 10% (v/v) glycerol in a 50 mL sterile conical centrifuge. This was centrifuged at 4,000 g for 15 min (4°C) and the resulting
pellet was re-suspended in 1.5 mL ice cold 10% (v/v) glycerol and distributed into 50 µL aliquots in 1.5 mL microfuge tubes, frozen in liquid nitrogen, and kept at -70°C.

2.6.3 Transformation of E. coli

Electroporation-competent E. coli cells were thawed at room temperature and directly placed on ice; 40 µL of competent cells were transferred into a pre-chilled 2 mm gap cuvette on ice. Approximately 2 µL plasmid DNA (0.1 ng/µL) in 0.1X TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer was added to the competent cells and transformation was carried out at 1.44 KV (130-200 W, 5 ms) using a MicroPulser electroporator (Bio-Rad Laboratories, Hercules, CA). Cells were immediately suspended in 1 mL SOC medium (20 g/L tryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) and incubated with shaking for 1 hr at 37°C. Cultures were spread on LB agar plates containing the selective antibiotic and incubated at 37°C overnight.

2.6.4 Purification of plasmid DNA

A single colony from each plate was used to inoculate 10 mL LB plus selective antibiotic and incubated overnight at 37°C. Plasmid DNA was isolated using the E.Z.N.A Plasmid Mini Kit I (Omega Bio-Tek Inc.), according to the manufacturer’s guidelines. To confirm the transformation, restriction enzyme digestion was performed, as described in the protocol accompanying the restriction enzymes Bgl II (New England Biolabs, Beverly, MA), with resulting digest visualized by 1% (w/v) agarose gel electrophoresis (section 2.6.1). The concentration and quality of DNA was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

2.6.5 Generation of reporter constructs

Gateway technology (Invitrogen/Life Technologies, Carlsbad, CA) was used to produce an entry clone containing MCU1. The purified attB PCR product (section 2.6.1) was combined with the pDONR™/Zeo donor vector according to the manufacturer’s recommendations and used to transform E. coli. Following plasmid purification, restriction digest and gel analysis (sections 2.6.1 and 2.6.4), positive plasmids were confirmed by sequencing (National Research Council, Saskatoon). This entry clone was combined with the destination vectors pMDC45 and
pMDC83 (gifts from Dr. Mark D. Curtis, University of Zürich, Zürich, Switzerland; Curtis and Grossniklaus, 2003), as described by the manufacture, to generate C- and N-terminal fusions to GFP respectively. The ligation products were either used immediately in bacterial transformations by electroporation, or stored at -20°C for future use.

2.6.6 Preparation of electroporation competent Agrobacterium tumefaciens

In order to prepare electroporation competent A. tumefaciens (strain GV3101), a 10 µL culture was used to inoculate 500 mL of YEP liquid medium (10 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl) containing kanamycin and gentamycin (50 mg/mL and 25 mg/mL respectively) and incubated at 28°C to an OD₆₀₀ ~ 0.5 (early to mid-log phase). Cultures were centrifuged at 4000 g for 15 min (4°C). Cell pellets were washed three times in 500 mL of 1 mM HEPES (4-(2-hydroxyethyl)-1-piperazineëthanesulfonic acid, pH 7.4), and the final pellet was re-suspended in 10 mL of 1 mM HEPES. The suspension was dispensed into a 50 mL Falcon tube and centrifuged at 4,000 g for 15 min (4°C). The pellet was re-suspended in 1.5 mL ice-cold glycerol (10% v/v) and dispensed into 50 µL aliquots, frozen under liquid nitrogen, and stored at -70°C.

2.6.7 Transformation of A. tumefaciens

Electroporation was performed using a MicroPulser (Bio-Rad) with 100 ng of plasmid DNA and 40 µL of electroporation-competent A. tumefaciens as described in Section 2.6.3. Following electroporation 1 mL SOC medium was added to the cells and the whole volume was transferred to a 15 mL Falcon tube and incubated for one hr at 28°C before plating 100 µL on YEP-agar plates containing kanamycin and gentamycin (50 mg/mL and 25 mg/mL respectively) and grown over 24 hr at 28°C. A single colony from each plate was used to inoculate 10 mL of YEP medium containing the selective antibiotic and incubated over 24 hr at 28°C with shaking. Plasmid DNA was extracted, quantified and subjected to confirmation by restriction digestion using Bgl II as described in section 2.6.4. Glycerol stocks were prepared (300 µL culture + 300 µL glycerol), stored at -80°C, and used for the transformation of Arabidopsis to determine the sub-cellular localization of MCU1.
2.6.8 Transformation of Arabidopsis plants

Arabidopsis Colombia ecotype (Col-0) plants were transformed using an *A. tumefaciens* floral dip method as described by Clough and Bent (1998). The T1 plants were grown and advanced to the next generation. T2 seed was collected and screened for segregation.

Table 2-1: Gateway primers used for cloning of Arabidopsis *MCU1*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession #</th>
<th>Name</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At4g32060</td>
<td>NM_001085007.1</td>
<td>MCU1</td>
<td>F:GGGGACAAGTTTTGTACAAAAAAGCAGGCT TCGAAGGACATACATTATTATGCCCCGCTT R:GGGACCACCTTTGTACGGAAGCTGGAAGGTTA GACGATGAGGAACAGTTCTTAGAAC</td>
<td>1462</td>
</tr>
</tbody>
</table>

F, forward; R, reverse

2.7 *mcu1* T-DNA lines

A T3 homozygous T-DNA insertion line (SAIL_359, hereafter referred to as mcu1) of Arabidopsis *MCU1* was obtained from ABRC and independently crossed to the mito-GFP-aequorin and ER-YFP-HDEL lines to assess the effect of this knockout on mitochondrial and ER morphology, as well as calcium dynamics. The best-expressing lines were screened using epifluorescent microscopy and grown to seed for the production of future generations. Results presented are from T3 generations.

2.7.1 Plant genomic DNA extraction

Plant leaves (0.5 g) from 14-day-old plants were frozen in liquid nitrogen and ground in a 1.5 mL tube prior to the addition of 300 μL 2% (w/v) CTAB (hexadecyl trimethyl-ammonium bromide; Sigma-Aldrich, St. Louis, MO) buffer followed by incubation at 65°C for 10 min. After cooling to room temperature, 300 μL of chloroform was added, mixed by vortexing and centrifuged briefly at 15,000 g to separate the phases. The upper, aqueous phase was transferred to a fresh tube and 300 μL of 2-propanol was added and mixed. Tubes were centrifuged again in a microfuge for 5 min at maximum speed to pellet the DNA. The supernatant was removed, and the pellet was washed with 500 μL 70% (v/v) EtOH and centrifuged for 1 min at maximum speed. The EtOH layer was discarded and the DNA was air dried. The DNA pellet was dissolved in 50 μL of 0.1X TE buffer and used for PCR.
2.7.2 Genotyping

Polymerase chain reaction (PCR) was used to confirm homozygous mcu1 mutant T-DNA lines as described on the SALK institute website (http://signal.salk.edu/). A combination of gene specific primers (LP and RP), as well as a T-DNA specific primer (left border, LB1) must be used in order to detect the presence of the T-DNA insertion. The PCR reactions are set up using LP+RP and LB1+RP; the presence of a PCR product using only LP+RP indicates wild type, while a product only with LB1+RP is indicative of homozygous plants. PCR products with both primer sets indicates hemizygosity. The primers used for the genotyping are listed in Table 2-2. All PCR reactions were prepared as described in section 2.6.1 with 1 µL of DNA template. A touchdown PCR program between 60 and 50°C was used with the annealing temperature dropping 1°C every cycle for 10 cycles, followed by 20 cycles at 50°C. Following an initial denaturation for 1 min at 98°C, cycling conditions were 15 sec at 98°C, 15 sec of annealing, 1 min at 72°C and one final elongation step of 5 min at 72°C. After all amplifications products were separated and visualized as described in section 2.6.1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAIL_359 (LP)</td>
<td>F: AACTGTTGCTTACCGTTGCAG</td>
</tr>
<tr>
<td>SAIL_359 (RP)</td>
<td>R: GAAGAAGATCACCGAACCTCC</td>
</tr>
<tr>
<td>T-DNA (LB1)</td>
<td>F: GCCCTTTTCAGAAATGGATAATAGCCTTTCC</td>
</tr>
</tbody>
</table>

F, forward; R, reverse

2.8 Semi-quantitative reverse transcription-PCR (sqRT-PCR)

To determine the expression level of MCU1, a sqRT-PCR protocol using primers shown in Table 2-3 was employed. The RNeasy Plant Mini Kit (Qiagen) was used to extract total RNA from Arabidopsis plants following the manufacturer’s instructions. After RNA extraction, one-step sqRT-PCR was carried out using Super Script III/RT/Platinum Taq Mix (Invitrogen) according to the manufacturer’s recommendations. PCR was carried out using a Mastercycler (Eppendorf AG) with cycling conditions as follows: the reverse transcription step was for 30 min at 55°C, and then 2 min at 94°C. The cDNA amplification cycle was 1 min at 94°C, 30 sec at 60°C, 90 sec at 68°C for 30 cycles. The final elongation step was for 5 min at 72°C. After all amplifications products were separated and visualized as described in section 2.6.1.
Table 2-3: Primers for sqRT-PCR of MCU1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession #</th>
<th>Name</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At4g32060</td>
<td>NM_001085007.1</td>
<td>MCU1</td>
<td>F: ATGGATCACCGGATTTTCAGC R: TCATTGTAAGTAAACCATTAGCTTTGCC</td>
<td>1082</td>
</tr>
<tr>
<td>At5g09810</td>
<td>NM_121018.3</td>
<td>Act7</td>
<td>F:GATATTCCAGCCACTTGTCTGTGAC R:CATGTTCGATTTGGATCTTCAGAG</td>
<td>211</td>
</tr>
</tbody>
</table>

F, forward; R, reverse

2.9 Tetramethylrhodamine staining

To confirm the location of MCU1, mitochondria were stained briefly with 20 nM TMRM (tetramethylrhodamine methyl ester). TMRM (Sigma-Aldrich) is a sensor for membrane potential that stains mitochondria based on their membrane potential and accumulates in the mitochondrial matrix (Bernardi et al., 1999). Whole seedlings were equilibrated in 20 nM TMRM in 0.5X MS medium for 5 min in the darkness before fluorescence imaging of tissues.

2.10 Isolation of protoplasts from Arabidopsis seedlings

2.10.1 Protoplast isolation (Sandwich method)

Leaves were collected from 14-day-old Arabidopsis plants grown under 16 hr light, 8 hr dark cycle at 23°C. The epidermal cells of the leaves were peeled using the Arabidopsis Sandwich method (Wu et al., 2009). Leaves were peeled (about 1 to 2 g, up to 5 g), and peeled epidermal layers were transferred to a Petri dish containing 10 mL of the enzyme solution (1 M mannitol, 1 M KCl, 0.25 M MES, 1 M glucose, 1% (w/v) cellulase, 0.25% (w/v) pectinase). The leaves were incubated at room temperature for one hr with gentle shaking (40 rpm) on a platform shaker (Fig. 2-1). The solution containing protoplasts was centrifuged at 100 g for 5 min in a microfuge using an Eppendorf A-4-44 rotor (Hamburg, Germany), and the pellet washed once with 10 mL of protoplast buffer (1 M mannitol, 1 M KCl, 0.25 M MES, 1 M glucose).
Figure 2-1: Sandwich method for Arabidopsis protoplast isolation. (A) 14-day-old Arabidopsis seedlings. (B) the upper epidermis was stuck onto Time tape (the blue colour) and the lower epidermis was stuck to clear 3M Scotch Magic tape. (C) the leaves with Time tape still adhering to the top surface (with blue backing) were incubated in enzyme solution.
2.10.2 Protoplast isolation (Chopped method)

A second protocol was also used to isolate protoplasts, for mitochondrial calcium dynamics measurements: 14-day-old leaves were chopped into about 0.5 mm pieces with a fresh razor blade in Petri dishes containing 1 mL of enzyme solution (see section 2.10.1). The tissue slices were transferred to 15 mL of enzyme solution in Petri dishes and incubated for 30 min at room temperature, with continuous gentle mixing (~ 40-50 rpm) (Fig. 2-2). The digested material was filtered through a 35-75 μm nylon mesh and transferred into a 15 mL Falcon tube for collection. Protoplasts were pelleted by centrifugation for 5 min at 100 g using an A-4-44 rotor (Eppendorf). The protoplast pellet was washed in 15 mL of enzyme solution, and centrifuged for 5 min at 60 g. The supernatant was removed and the pellet washed again with 15 mL of the protoplast solution (see section 2.10.1). Evaluating protoplast yield was done using a hemocytometer (Zhai et al., 2009).

2.10.3 Luminescence measurements of mitochondrial Ca\(^{2+}\)

Luminescence was used to measure intracellular Ca\(^{2+}\) in mitochondrial protoplasts using a Varioskan Flash Instrument and SkanIt Software (Thermo Fisher Scientific). The instrument was operated as described in the user’s guide provided with the instrument. Protoplasts from the mcu1 x mito-GFP-aequorin line were isolated as described in sections 2.10.1 and 2.10.2. Coelenterazine (Biosynth, Naperville, IL) was dissolved in a small amount of 100% EtOH and then distilled water to a final concentration of 10 µM. Protoplasts were incubated in a freshly prepared coelenterazine solution for 2 or 24 hr in the darkness to reconstitute aequorin. A 50 µL aliquot of the reconstituted protoplasts was pipetted into a 1-well plate and fluorescence emission collected at 469 nm every sec for 100 sec to establish a background level. A cold shock was applied by introducing 50 µL of ice cold protoplast solution (described in section 2.10.1) which generated a Ca\(^{2+}\) spike. Following a 2 min resting period, back ground fluorescence was collected for 50 sec at which time 100 µL of 1 M CaCl\(_2\) and 10% EtOH (v/v) was provided to determine maximal aequorin-Ca\(^{2+}\) luminescence allowing for correction of variation in aequorin in the protoplasts.
Figure 2-2: Chopped method for Arabidopsis protoplast isolation: (A) 14-day-old Arabidopsis plants. (B) leaves chopped into 0.5 mm pieces in sterile disposable Petri dishes. (C) 20 to 60 min of gentle shaking allows the isolation of protoplasts.
2.11 Fluorescence Microscopy

An epifluorescence microscope (Olympus BX61) system was employed for reporter protein visualization, with 100X lens and 1.40 numerical aperture plan-apochromat oil-immersion objective. Fluorescent light is separated from the excitation light using an amalgamation of a dichroic mirror (FF562-Di02-25x36) and a band-pass filter FF01-543/22-25 to visualize red fluorescent signals or Bright-line GFP-3035B to discern green fluorescent signals. Cyan fluorescent signals were visualized using a CFP430/24 nm and CFP470/24 nm excitation/emission filter set while yellow fluorescent signals used a YFP500/20 nm and YFP535/30 nm filter set. All the above filter cubes were by Semrock (Rochester, NY, USA). Images were captured with MetaMorph Basic system (Advanced Scientific, Meraux, LA).

2.12 Chemical treatments

Stock concentrations of 25 mM antimycin A (Sigma-Aldrich; A0274) and 2 mM Latrunculin B (Sigma-Aldrich; L5288) were prepared using 100% EtOH as the solvent, and 1 mM methyl viologen dichloride hydrate (Sigma-Aldrich; 856177) was prepared using ultra-pure H2O. Each stock solution was diluted with ultra-pure H2O to the appropriate test concentration just before use. Control solutions consisted of the same dilution of the stock solvent (EtOH). Leaves were floated in Petri dishes containing 1 mL of these chemical or control solution for 3 or 24 hr and the effects of these compounds on mitochondrial and ER morphology, dynamics, and movement was assessed. Three replicates for each time-point and treatment were averaged.

2.13 Mitochondrial and ER morphology and dynamics

Leaf epidermal cells from transgenics harbouring mito-CFP and ER-YFP-HDEL were examined by epifluorescence microscopy to examine organelle morphology and dynamics. Clustered mitochondria (5 to 15 mitochondria) were counted and if there were at least two mitochondria clusters per field of view mitochondria were considered affected. However, if there was only one mitochondrial cluster or no apparent effect on mitochondrial morphology at all, we considered them non-affected. ER morphology changes (for example, enlargement of ER cisternae or broken) were also examined. A change in ER morphology was measured as affected;
no change (ER looked normal) or was not affected. Five plants were examined, and three views were measured.

2.14 Visualization of mitochondrial or ER movement

In order to visualize movement of mitochondria or ER, two images of the same field of view were taken 10 sec apart with identical settings. Those two images were false-colored as red or green, and were merged to produce a combination image using MetaMorph Basic system (Advanced Scientific). If mitochondria or ER moved within the 10 sec time period, they were seen as separate colors (red or green), but if a given mitochondrion or ER did not move it was identified as yellow (merged). If two or more mitochondria moved (green and red colors) per view, it was considered as movement. If a single mitochondrion moved or no mitochondria moved per view (yellow color), it was measured as non-affected. To measure ER movement, if two tubes or more of the tubular ER network moved as detached colors (red and green), it was considered as movement. Conversely, if there were less than two tubes of the tubular ER network moving as separate colors or not moving at all as combined color (yellow colour), these cases were counted as non-movement.

2.15 Data analysis

Three replicates for each-point and treatment were averaged. Prior to data analysis, raw data were attested for normality and homogeneity of variance using the Shapiro-Wilk and Levene’s tests respectively. This was followed by a one-way analysis of variance (ANOVA) to analyze treatment effects on response variables. Means were separated using Fisher’s Least Significant Difference (LSD) test and declared significant at a probability of 0.05. Statistical analyses were performed using Excel (Microsoft, Redmond, WA) software.
CHAPTER 3. RESULTS

3.1 Identification of MCU1 in Arabidopsis plants

3.1.1 Does MICU1 exist in plants?

A protein-protein BLAST search of the non-redundant protein sequence database at NCBI was performed using human MICU1 as the query sequence. An orthologous protein was identified in Arabidopsis as an EF-hand, calcium binding motif-containing protein (Accession No. NP_001078476.1) and was renamed MCU1. Alignment results indicated that there is 23.57% identity and 36.78% similarity between MICU1 and MCU1 at the amino acid level (Fig. 3-1).

3.1.2 Sub-cellular localization of MCU1

Primers were designed to amplify the open reading frame for the gene encoding this protein (At4g32060). PCR amplification successfully amplified a fragment of the expected size (Fig. 3-2) which was confirmed by sequencing to encode MCU1. The PCR product was used to generate N- and C-terminal fusions between MCU1 and GFP were generated to determine the sub-cellular localization of MCU1.

The DNA fragment from MCU1 was ligated in both pMDC45 and pMDC83 vectors to produce the fusion cassettes shown in Fig. 3-3. In pMDC45 the GFP coding region was fused to the N-terminus of MCU1; in pMDC83, GFP was fused to the C-terminus of MCU1 (Fig. 3-3). This resulted in the generation of MCU1-GFP and GFP-MCU1 respectively. These Gateway constructs were used to transform Agrobacterium and subsequently Arabidopsis plants.

Arabidopsis stable lines expressing the MCU1-GFP construct (C-terminal GFP fusion) showed that MCU1 is targeted to discrete punctate structures (Fig. 3-4 A and D). In contrast, GFP-MCU1 (N-terminal fusion) did not reveal a discrete location (data not shown). To confirm the location of MCU1 to mitochondria, the red fluorescent cationic probe TMRM was used. TMRM is a sensor for membrane potential that stains mitochondria based on their membrane potential (Bernardi et al., 1999). The results show that there is a co-localization between TMRM (red) and MCU1 (green) [merge-yellow] confirming that MCU1 localizes to mitochondria (Fig. 3-4 B).
Figure 3-1: Amino acid alignment of human MICU1 and Arabidopsis MCU1. The accession numbers for MICU1 and MCU1 are NP_006068.2 and NP_001078476.1 respectively. Black shading, identical; grey shading, conserved substitutions.
Figure 3-2: Amplification of *MCUI*. Agarose gel showing amplified 1462 bp fragment of *MCUI* from 7-day-old Arabidopsis seedling cDNA. A Hyperladder DNA marker is shown in lane 1.
Figure 3-3: N- and C-terminus GFP fusion protein constructs with MCU1.
Figure 3-4: Localization of MCU1 to Arabidopsis mitochondria in root and leaf tissues. MCU1 was visualized by MCU1-GFP (green) and mitochondria by TMRM (red). (A) Expression of the MCU1-GFP construct in roots. (B) Staining of mitochondrial matrix marker (TMRM) in roots. (C) Merge of (A) and (B) in root tissue. (D) Expression of MCU1-GFP in leaf tissue. Images are representative of three independent transformed lines. Scale bars = 10 µm.
3.1.3 mcu1 T-DNA line

In order to determine the role of MCU1 in Arabidopsis, a T-DNA insertion mutant line (SAIL_359) was identified in the gene MCU1 (At4g32060). The genomic structure of MCU1 (At4g32060) in Arabidopsis is shown in Fig. 3-5. The T-DNA insertion in SAIL_359 is located in exon 2 (Fig. 3-5). Homozygosity of the T-DNA line was confirmed by PCR using genomic DNA extracted from 18 seedlings of the SAIL_359 line (Fig. 3-6). Only homozygous plants were pursued further and renamed mcu1.

3.1.4 MCU1 transcript levels in T-DNA insertion mutants

To determine whether the mutants were either a knockdown or knockout, the expression level of MCU1 in wild type and mcu1 Arabidopsis seedlings was determined using sqRT-PCR (Fig. 3-7). The primers used were expected to amplify a fragment of 1082 bp and results demonstrated that the mutant line was a knockdown for MCU1, as transcript levels were somewhat lower for mcu1 in comparison to wild type (Fig. 3-7). There was also an unexpected band present in all samples analyzed at approximately 1400 bp whose expression mirrored that of MCU1 (Fig. 3-7).

3.1.5 Phenotype of mcu1

3.1.5.1 Mitochondrial and ER morphology and phenotype in mcu1

In order to visualize mitochondria in the mcu1 mutants and to determine if there is any aberrant mitochondrial phenotype, mcu1 mutants were crossed with Arabidopsis lines expressing mitochondrial targeted GFP and analyzed using fluorescence microscopy. Mitochondrial visualization of the mcu1 x mito-GFP-aequorin cross using fluorescence microscopy displayed no aberrant phenotypes (Fig. 3-8).

In order to visualize ER in mcu1 mutants and to determine whether there is any aberrant ER phenotype, these mutants were crossed with an Arabidopsis line expressing ER-targeted YFP followed by visualization by fluorescent microscopy. The results indicated that the ER phenotype in the mcu1 x ER-YFP-HDEL cross was not obviously aberrant compared to the wild type (Fig. 3-9).
Figure 3-5: Structure of mature RNA for *MCUI*. The mature RNA comprises 1712 bp over 6 exons and the open reading frame is located between positions 57 and 1421. Horizontal arrows indicate the positions of forward and reverse primers used for sqRT-PCR resulting in a 1082 bp fragment. The T-DNA insertion in the *MCUI* gene of the mutant line SAIL_359 is in exon 1 (vertical arrow).
Figure 3-6: Identification of homozygous mutants in the SAIL_359 line. PCR products using primer sets LP+RP (Wt) and LB1+RP (M) were run in adjacent lanes for each plant. Plants #1, 2, 3, 4, 7 and 13 are wild type while #8, 14, and 16 represent homozygous mutants and plants #5, 6, 9 and 10 are heterozygous. For plants #11, 12 and 15 no DNA was amplified.
**Figure 3-7:** Expression of *MCUI* in plant lines used for this study. Lane 1 (wild type, Col-0), lane 3 (mito-CFP x ER-YFP-HDEL), lane 9 (mito-GFP-aequorin), lane 11 (ER-YFP-HDEL), lane 5 (*mcu1* x mito-GFP-aequorin). Lanes 2, 4, 6, 8, and 10 show actin (211 bp) used as an internal control. Image is representative of three independent replicates.
**Figure 3-8:** Images of wild type (A) and *mcu1* expressing mito-GFP-aequorin (B). No abnormal mitochondrial phenotype was observed in *mcu1* cross. Images are representative of three independent replicates. Scale bars = 10 µm.
Figure 3-9: Images of wild type (A) and mcu1 expressing ER-YFP-HDEL (B). No abnormal ER phenotype was observed in the mcu1 cross. Images are representative of three independent replicates. Scale bars = 5 μm.
3.1.5.2 *mcu1* plant morphology

To determine whether *mcu1* plants showed any growth or developmental phenotypes, seedlings were grown on MS media on vertical plates for 14 days. No variation was observed between the *mcu1* mutant and wild type in growth and development (Fig. 3-10).

3.1.6 Measurement of mitochondrial calcium dynamics

Little is known about how mitochondrial calcium is regulated as no plant calcium mitochondrial Ca$^{2+}$-ATPase pumps and no mitochondrial calcium channels have been identified to date. In order to identify the role of *MCU1* in mitochondrial calcium dynamics, the *mcu1* x mito-GFP-aequorin line was utilized. In addition to GFP, these plants express a mitochondrial-targeted version of the calcium binding protein aequorin (Logan and Knight, 2003). Aequorin emits blue light when it binds to calcium (as described in section 1.7).

Initial testing illustrated that mitochondrial calcium could be measured in the protoplasts of Arabidopsis and showed a characteristic response to cold shock. A trace from a typical experiment is illustrated in Fig. 3-11. As shown in Fig. 3-11, there was an obvious response of mitochondrial aequorin in protoplasts to cold shock and CaCl$_2$ treatment. The experiment was repeated several times, but was unsuccessful even with the different methods of protoplast isolation (data not shown). One method used to try and overcome this problem was to reconstitute the aequorin overnight before measuring the mitochondrial calcium in the protoplasts, but it also did not work (data not shown). Overall, I was unable to establish whether *MCU1* has a role in mitochondrial calcium dynamics or not.

3.2 The interactions between mitochondria and ER

3.2.1 Production of a transgenic line expressing both mito-CFP and ER-YFP-HDEL

In order to investigate the interactions between ER and mitochondria, crosses were made between Arabidopsis plants individually expressing mito-CFP and ER-YFP-HDEL to obtain a transgenic line expressing CFP targeted to mitochondria and YFP targeted to ER (Fig. 3-12). A number of compounds known to affect cellular constituents were then employed to explore the relationship between mitochondria and the ER.
Figure 3-10: Plant morphology of mito-GFP-aequorin and *mcu1* lines. Two seedlings (14-day-old Arabidopsis plants) of each genotype are shown. Seedlings were grown in vertical plates on MS media for 14 days. Image is representative of three independent replicates. Scale bar = 2 cm.
**Figure 3-11**: Mitochondrial calcium signatures. Mitochondria in isolated mesophyll cell protoplasts responded to a cold shock by transiently accumulating calcium (red trace). Calcium+ethanol were given after a 2 min rest period (indicated by XXX) to same sample to determine maximal luminescence (blue trace).
**Figure 3-12:** Association of mitochondria and ER. Mitochondria and ER were visualized using mito-CFP and ER-YFP-HDEL respectively. Close association of mitochondria (blue, mito-CFP) and ER (ER-YFP-HDEL) in homozygous stable transgenic Arabidopsis lines. Image is representative of three independent stable transgenic lines. Scale bar = 10 μm.
3.2.2 The effect of chemical treatments on ER-mitochondrial dynamics

3.2.2.1 Effects of Lat-B on the morphology and motility of mitochondria and ER in Arabidopsis

In mock controls (0.1% v/v EtOH), mitochondria and ER association remains unaffected (Fig. 3-13 C). In contrast, mitochondria were clearly clustered after treatment with Lat-B for 3 hr (Fig. 3-13 D and F) or 24 hr (Fig. 3-13 G and I). ER morphology also changed with enlargement of ER-termed cisternae (Fig. 3-13 E and H). Alterations in the relationship between ER and mitochondrial dynamics were observed after 3 and 24 hr of treatment with 2 µM Lat-B in leaf epidermal cells.

To determine mitochondrial mobility two images of the same field of view were taken 10 seconds apart with identical settings, false-coloured red and green, and overlaid. The picture taken at 0 sec was false-coloured green and the 10 sec picture false-coloured red, and then it was merged to get a composite image using Metamorph software. Mitochondria stopped moving after treatment with Lat-B for 3 and 24 hr periods (Fig. 3-14 F and I) compared to control (Fig. 3-14 C).

Using a similar approach I observed that ER was seen to also stop moving after treatment with Lat-B for 3 and 24 hr (Fig. 3-15 F and I) compared to the control (Fig. 3-15 C).

Additionally, there was a significant difference in mitochondrial clustering and movement between Lat-B treated and controls. Also, significant differences in ER morphology and movement were observed in Lat-B treated tissues when compared to the EtOH controls. However, there was no difference in mitochondrial clustering and movements or ER morphology and movement when comparing the 3 and 24 hr Lat-B treatments (Fig. 3-16 A and B).

3.2.2.2 Effects of MV on the morphology and motility of mitochondria and ER in Arabidopsis

Treatments of transgenic Arabidopsis lines expressing ER-YFP-HDEL and mito-CFP with 100 µM MV for 3 and 24 hr, produced clustering of mitochondria as well as an effect on ER morphology in MV treated samples compared to EtOH controls at 3 and 24 hr (Fig. 3-17). Arrows show clustered swollen mitochondria and enlarged ER cisternae following treatment (Fig. 3-17 F and I).
**Figure 3-13:** Mitochondrial and ER dynamics upon treatment with Lat-B. Tissues of transgenic Arabidopsis expressing ER-YFP-HDEL and mito-CFP were treated with 2 μM Lat-B for 3 and 24 hr, respectively. A, D and G; images for mito-CFP, B, E and H: images for ER-YFP-HDEL, C, F and I: merged images. Control treated with 0.1% EtOH (A-C), treatment for 3 hr with 2 μM Lat-B (D-F), treatment for 24 hr with 2 μM Lat-B (G-I). Mitochondria are false-coloured green and ER false-coloured red. Arrows show the clustering of mitochondria and the enlargement of ER cisternae. Scale bars = 10 μm.

<table>
<thead>
<tr>
<th>Control</th>
<th>Lat-B for 3 hr</th>
<th>Lat-B for 24 hr</th>
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<tbody>
<tr>
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<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
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<tr>
<td>ER-YFP-HDEL</td>
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<td><img src="image5" alt="Image" /></td>
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<tr>
<td>Merged</td>
<td><img src="image7" alt="Image" /></td>
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**Figure 3-14:** Effect of Lat-B treatment on mitochondria motility. The mito-CFP line was treated with 2 μM Lat-B for 3 and 24 hr, respectively. A, D and G; Images were taken at 0 sec and false-coloured green, B, E and H: images were taken 10 sec later and false-coloured red, C, F and I: merged images to show mitochondrial movements. Control treated with 0.1% EtOH (A-C), treatment for 3 hr with 2 μM Lat-B (D-F), treatment for 24 hr with 2 μM Lat-B (G-I). Arrows show where mitochondria are moving. Image is representative of results from three independent experiments. Scale bars = 10 μm.
**Figure 3-15**: Effect of Lat-B on ER morphology and movement. The ER-YFP-HDEL line was treated with 2 µM Lat-B for 3 and 24 hr, respectively. A, D and G; Images were taken at 0 sec and false-coloured green, B, E and H: images were taken 10 sec later and false-coloured red, C, F and I: merged images to show ER movement. Control treated with 0.1% EtOH (A-C), treatment for 3 hr with 2 µM Lat-B (D-F), treatment for 24 hr with 2 µM Lat-B (G-I). Arrows show where ER is moving. Image is representative of results from three independent experiments. Scale bars = 10 µm.
Figure 3-16: The effects of Lat-B on mitochondria and ER. (A) mitochondrial clustering and movements and (B) ER morphology and motility. Transgenic Arabidopsis lines expressing ER-YFP-HDEL and mito-CFP were treated with 2 µM Lat-B or 0.1% EtOH control for 3 and 24 hr, respectively. Graphs are means of three independent experiments; error bars represent the standard errors of the means (n = 3). Different letters above the bars indicate significant differences (P ≤ 0.05) between treatments as indicated by a Fisher’s LSD test.
Figure 3-17: Mitochondrial and ER dynamics upon treatment with MV. Tissues of transgenic Arabidopsis expressing ER-YFP-HDEL and mito-CFP were treated with 100 µM MV for 3 and 24 hr, respectively. A, D and G: images for mito-CFP, B, E and H: images for ER-YFP-HDEL, C, F and I: merged images. A-C: Control treated with 0.1% EtOH. D-F: tissues treated with MV for 3 hr. G-I: tissues treated with MV for 24 hr. Mitochondria are false-coloured green and ER false-coloured red. Arrows show clustered swollen mitochondria and enlarged ER cisternae following treatment. Scale bars = 10 µm.
Mitochondria stopped moving following treatments with 100 µMV for 3 and 24 hr (Fig. 3-18 F and L: yellow color) compared to the EtOH control (Fig. 3-18 C: red and green). In addition, ER also stopped moving after treatment with 100 µM MV for 3 and 24 hr (Fig. 3-19).

There was a significant difference in both mitochondrial moving and clustering between MV treated and EtOH control samples (Fig. 3-20 A). At two time points 3 and 24 hr post MV treatments, no significant difference was detected in mitochondrial movement, but there was a significant difference in mitochondrial clustering (Fig. 3-20 A). MV treatment resulted in changes in both ER morphology and movement between MV treated and EtOH control samples. Also, significant differences in ER morphology and movement were observed in MV treated samples between 3 and 24 hr treatments (Fig. 3-20 B).

3.2.2.3 Effects of AA on the morphology and motility of mitochondria and ER in Arabidopsis

When transgenic Arabidopsis lines expressing ER-YFP-HDEL and mito-CFP were treated with 25 µM AA for 3 or 24 hr, there was a clear clustering of mitochondria in AA treated (Fig. 3-21 D and G) versus EtOH control (Fig. 3-21 A), as well as an effect on ER morphology (Fig. 3-21 E and H) compared to control (Fig. 3-21 B). Mitochondria became clustered, and ER appeared to be larger in membrane sheets of ER network compared to the control sample. Arrows show clustered swollen mitochondria and enlarged ER cisternae following treatment (Fig. 3-21 F and I).

AA treatments also resulted in mitochondria not moving after 3 or 24 hr (Fig. 3-22 F and I) compared to the EtOH control (Fig. 3-22 C). Arrows show where mitochondria were moving (green and red color) (Fig. 3-22). ER also stopped moving after treatment with 25 µM AA for 3 or 24 hr (Fig. 3-23, arrows indicate ER movement).

There was a significant difference in percentage of mitochondrial clustering resulting from AA treatment compared to EtOH control. However, no significant difference was detected between mitochondrial clustering or movement when the treatment with AA was extended to 24 hr (Fig. 3-24 A). There was a significant difference in ER morphology at 3 and 24 hr AA treatment compared to the EtOH control. There was also a significant difference in ER morphology and movement between 3 and 24 hr treatments (Fig. 3-24 B).
Figure 3-18: Effect of MV treatment on mitochondria motility. The mito-CFP line was treated with 100 µM MV for 3 and 24 hr, respectively. A, D and G; Images were taken at 0 sec and false-coloured green, B, E and H: images were taken 10 sec later and false-coloured red, C, F and I: merged images to show mitochondrial movements. Control treated with 0.1% EtOH (A-C), treatment for 3 hr with 100 µM MV (D-F), treatment for 24 hr with 100 µM MV (G-I). Arrows show where mitochondria are moving. Image is representative of results from three independent experiments. Scale bars = 10 µm.
**Figure 3-19**: Effect of MV treatment on ER morphology and movement. The ER-YFP-HDEL line was treated with 100 µM MV for 3 and 24 hr, respectively. A, D and G; Images were taken at 0 sec and false-coloured green, B, E and H: images were taken 10 sec later and false-coloured red, C, F and I: merged images to show ER movement. Control treated with 0.1% EtOH (A-C), treatment for 3 hr with 100 µM MV (D-F), treatment for 24 hr with 100 µM MV (G-I). Arrows show movement of ER. Image is representative of results from three independent experiments. Scale bars = 10 µm.
**Figure 3-20:** The effects of MV on mitochondria and ER. (A) mitochondrial clustering and movements and (B) ER morphology and motility. Transgenic Arabidopsis lines expressing ER-YFP-HDEL and mito-CFP were treated with 100 µM MV or 0.1% EtOH control for 3 and 24 hr, respectively. Graphs are means of three independent experiments; error bars represent the standard errors of the means (n = 3). Different letters above the bars indicate significant differences (P ≤ 0.05) between treatments as indicated by a Fisher’s LSD test.
**Figure 3-21:** Mitochondrial and ER dynamics upon treatment with AA. Tissues of transgenic Arabidopsis expressing ER-YFP-HDEL and mito-CFP were treated with 25 µM AA for 3 and 24 hr, respectively. A, D and G: images for mito-CFP, B, E and H: images for ER-YFP-HDEL, C, F and I: merged images. A-C: Control treated with 0.1% EtOH. D-F: tissues treated with 25 µM AA for 3 hr. G-I: tissues treated with 25 µM AA for 24 hr. Mitochondria are false-coloured green and ER false-coloured red. Arrows show clustered swollen mitochondria and disruption of ER network following treatment. Scale bars = 10 µm.
Figure 3-22: Effect of AA treatment on mitochondria motility. The mito-CFP line was treated with 25 µM AA for 3 or 24 hr, respectively. A, D and G; Images were taken at 0 sec and false-coloured green, B, E and H: images were taken 10 sec later and false-coloured red, C, F and I: merged images to show mitochondrial movements. Control treated with 0.1% EtOH (A-C), treatment for 3 hr with 25 µM AA (D-F), treatment for 24 hr with 25 µM AA (G-I). Arrows show where mitochondria are moving. Image is representative of results from three independent experiments. Scale bars = 10 µm.
Figure 3-23: Effect of AA treatment on ER morphology and movement. The ER-YFP-HDEL line was treated with 25 µM AA for 3 and 24 hr, respectively. A, D and G; Images were taken 0 sec and false-coloured green, B, E and H: images were taken 10 sec later and false-coloured red, C, F and I: merged images to show ER movement. Control treated with 0.1% EtOH (A-C), treatment for 3 hr with 25 µM AA (D-F), treatment for 24 hr with 25 µM AA (G-I). Arrows show where ER is moving. Image is representative of results from three independent experiments. Scale bars = 10 µm.
Figure 3-24: The effects of AA on mitochondria and ER. (A) mitochondrial clustering and movements and (B) ER morphology and motility. Transgenic Arabidopsis lines expressing ER-YFP-HDEL and mito-CFP were treated with 25 µM AA or 0.1% EtOH control for 3 and 24 hr, respectively. Graphs are means of three independent experiments; error bars represent the standard errors of the means (n = 3). Different letters above the bars indicate significant differences (P ≤ 0.05) between treatments as indicated by a Fisher’s LSD test.
CHAPTER 4. DISCUSSION AND CONCLUSIONS

4.1 Identification of mitochondrial Ca\(^{2+}\) ions channels and Ca\(^{2+}\)-ATPases

It has been well established that a variety of ion channels exist in plant cells of all species (Sanders et al., 1999). For example, mechanically gated ion channels in plant cells prevent lysis that arises during hypo-osmotic stress and also frees the cell of osmolytes (Hwang et al., 2000). Calcium- anion- and potassium channels in the vacuole and plasma membranes of plant cells have been categorized and identified (Skerrett and Tyerman, 1993), however the protein components of these channels have yet to be identified (Roberts and Tester, 1997). Some plant ion channels, such as, SLAC (S-type anion channels) in guard cell plasma membranes and ALMT aluminium activated malate transporter) channels of the vacuole, are unique (Hille, 2001), while others are encoded by genes with orthologs in animals. Examples of such families are: cyclic nucleotide-gated channels, ion-tropic glutamate receptor homologs, and both depolarization- and hyperpolarization-triggered Shaker-type potassium channels. The plant-specific ion channels clearly provide unique opportunities for evaluation of the structural mechanisms and functions of ion channels with regard to the special physiological requirements of plants (Hua et al., 2003).

Studies carried out on animal cell mitochondria by Rizzuto et al., (2012) identified MCU (mitochondrial calcium uniporter) and this work greatly added to the understanding of mitochondrial calcium transport mechanisms and function in animal cells. Two regulators of MCU called MCUR1 (mitochondrial calcium uniporter regulator 1) and MICU1 (mitochondrial calcium uptake 1) have since been identified (Rizzuto et al., 2012). Interestingly, how mitochondrial calcium is regulated in plants, is not well known since no plant mitochondrial Ca\(^{2+}\)-ATPase pump and no mitochondrial calcium channel proteins have been identified. This work successfully identified a MICU1 ortholog in Arabidopsis (At4g32060). The identification was achieved through a database search of the Arabidopsis genome using BLASTP and human MICU1 as the query. The Arabidopsis gene, \textit{MCU1}, encodes an open reading frame of 1324 bp. A T-DNA Arabidopsis line \textit{mcu1} (SAIL_359) was identified and used in this study. This line was crossed with Arabidopsis lines tagged with mitochondrial and ER marker proteins and no aberrant phenotype was observed in the resulting homozygous \textit{mcu1} line. However, when sqRT-PCR was performed with the T-DNA line, only a slight reduction in transcript level was observed (Fig. 3-7).
4.1.1 Sub-cellular localization of MCU1

A red fluorescent cationic probe, TMRM, was used as a mitochondrial specific stain to aid in my investigation of the intracellular location of MCU1 in plant cells. I generated stable transgenic Arabidopsis plants expressing a MCU1-GFP fusion and by comparing TMRM fluorescence with that of GFP, I found that MCU1 localized to mitochondria (Fig. 3-4). In contrast, the N-terminal cassette did not reveal a discrete location for MCU1 because N-terminal modification makes the MCU1 protein non-functional. However, without appropriate markers determination of the exact sub-compartment is difficult. It is likely that MCU1 is localized in a different sub-compartment of the mitochondria, but without higher resolution microscopy, I am unable to conclude that the C-terminal region of MCU1 is in the inter-membrane space. However, this location is likely based on the fact that human MICU1 is an inner membrane protein and the C-terminus, which is predicted to be more critical for function, is located in the inter-membrane space (Buchan et al., 2010). Recent studies question the localization of MICU1 and the function of the MICU1 at high [Ca$^{2+}$] (Mallilankaraman et al., 2012; Csordas et al., 2013; Hoffman et al., 2013; Sancak et al., 2013). Only one study has concluded that MICU1 localized in the mitochondrial matrix and senses matrix [Ca$^{2+}$] (Mallilankaraman et al., 2012).

4.2 Measuring calcium fluxes in Arabidopsis

Calcium is an essential regulator of various signaling pathways in plants since it serves as a secondary messenger (Tuteja and Mahajan, 2007). Thus, it is important to determine the signaling pathways involved in calcium response to various stimuli such as osmotic shocks and elicitors (Tuteja and Mahajan, 2007). Various calcium sensors including aequorin can be used to measure calcium fluxes in various compartments of a plant cell, including the nucleus, mitochondria, tonoplast, chloroplast and cytosol (Baubet et al., 2000). Aequorin can be directed to various organelles, such as the mitochondria, to measure changes in calcium ions in the response to environmental or physiological stimuli (Logan and Knight, 2003).

The experiments I performed to measure calcium in the mcu1 x mito-GFP-aequorin line proved challenging. Despite significant troubleshooting including many changes to the methods, such as modifying the coelenterazine reconstitution and protoplast isolation procedures, I was
unable to repeatedly reproduce the first successful trial. As a result of the lack of results it is unknown whether MCU1 has a role in mitochondria calcium dynamics.

4.3 Mitochondrial and ER juxtaposition

Juxtaposition between ER and mitochondria is known to provide a platform for communication during Ca$^{2+}$ signaling and apoptosis in non-plant models (Annunziata and d’Azzo, 2013). However, the molecular mechanisms that are responsible for controlling this interaction are uncertain. In mammals, mitofusin-2 is a mitochondrial dynamin-related protein localized at the ER that facilitates its interaction with mitochondrion, thus linking the ER to the mitochondrion (de Brito and Scorrano, 2008; Cali et al., 2013). A role for ER and ER-mitochondrion tethering has been suggested during pathogenesis (Cali et al., 2013). Little is known about the importance of mitochondrial-ER association in plants, for example, there are no mitofusin homologues in sequenced plant genomes, although it is clear from this work that there is a very close positional relationship of the two organelles (Fig. 3-12). Little is known about what keeps mitochondria associated with the tubular ER network. However, my results indicate that mitochondria and ER are connected to each other, and mitochondria are sitting in the tubular ER network on lacuna (Fig. 3-12 merged).

There are two main functional interrelationships between mitochondria and ER that are likely drivers for the close physical association between them. The first function is the facilitation of lipid exchange between the two organelles (Rath and Haller, 2012). Lipid exchange is the central point in the aminoglycerophospholipid biothynthesis pathway, which begins in the ER with the synthesis of phosphatidylserine. The second function is the exchange of Ca$^{2+}$ between ER and mitochondria (Annunziata and d’Azzo, 2013). The physical interaction between mitochondria and the ER is known as the mitochondria-associated ER membrane (MAM). MAM has not been confirmed in planta to date. A study indicated that there is a partial co-localization among ER, mitochondria, and microfilaments (Wang et al., 2000). Additionally, previous research has enabled easy identification of the MAM membranes from various tissues and organs in different cell lines in yeast (Achleitner et al., 1999). MAMs have since been observed in various organisms including animals and other fungi but not yet in plants (Vance, 1990; Ardail et al., 1993; Camici and Corazzi, 1995; Gaigg et al., 1995; Shiao et al., 1995).
Previous work using human cells has identified a functional $Ca^{2+}$ link between the ER and the mitochondria (Patergnani et al., 2011). Mitochondria and ER networks are vital for the maintenance of calcium homeostasis in human cells (de Brito and Scorrano, 2010). $Ca^{2+}$ plays a role in three main functions of the MAM. These include the regulation of cellular bioenergizers (Cali et al., 2013), cytoprotection through buffering inositol 1,4,5-triphosphate (IP3) $Ca^{2+}$ efflux and the regulation of cytotoxic processes (Rath and Haller, 2012). In addition, $Ca^{2+}$ plays a significant role in the mitochondria that is necessary for the activation of the tricarboxylic acid cycle (Krebs cycle) (Annunziata and d’Azzo, 2013). The tricarboxylic cycle is a series of chemical reactions that fuels ATP generation and thus $Ca^{2+}$ can then be shared between the two organelles through the MAM (Rath and Haller, 2012). From the above discussion it is clear that ER and mitochondria are dependent on each other and that the interdependent processes are regulated by $Ca^{2+}$. I found there is a MAM in plants, and there is a physical interaction between mitochondria and ER (Fig. 3-13, 3-17 and 3-21). However, from my experiments, I cannot identify if there is a functional $Ca^{2+}$ link between the ER and the mitochondria.

4.4 ER and mitochondrial dynamics

Plant ER forms an interconnected membrane network that extends from the outermost nuclear region to the periphery of the cell wall (Evert, 2006). Mitochondria are known for their roles in energy conservation and cell death programmes. They are highly dynamic organelles that move on the actin cytoskeleton to enable continuous fusion and fission of the cellular mitochondrial population (the chondriome) that in turn controls the size and number of mitochondria in the cell (Scott and Logan, 2007).

Methyl viologen (commonly called paraquat) is a colorless compound that is often used as a herbicide (Beligni and Lamattina, 2009). It works by causing an overproduction of ROS within chloroplasts. This type of reaction causes a severe oxidative stress in plants that may be lethal. Cell ion leakage to intercellular compartments occurs as an early step during oxidative stress and may be a first step in PCD (Beligni and Lamattina, 2009). When I treated transgenic Arabidopsis lines expressing ER-YFP-HDEL and mito-CFP with 100 µM MV for 3 and 24 hr, there was a clear clustering of swollen mitochondria in MV treated examples (Fig. 3-17 D and G) compared with controls at 3 and 24 hr (Fig. 3-17 A). I also saw enlarged ER cisternae, illustrated by comparing Fig. 3-17 E and H with the control in Fig. 3-17 B. Similarly,
Arabidopsis leaves treated with 100 µm MV showed an aggregated distribution in mitochondria, with tens of mitochondria arranged into tight clusters (Scott and Logan, 2007). In MV treated samples, mitochondria were found to be associated with the ER network.

When I treated the transgenic Arabidopsis lines expressing ER-YFP-HDEL and mito-CFP with 25 µM AA for 3 or 24 hr, there was a clear clustering of mitochondria in AA treated (Fig. 3-21 D and G) versus EtOH control plants (Fig. 3-21 A), as well as an effect on ER morphology (Fig. 3-21 E and H) compared to control (Fig. 3-21 B). Mitochondria became clustered, and ER appeared to be larger in membrane sheets of ER compared to the control sample. Similar studies in Hela cells, mitochondria clustered after treatment with 10 µM AA (Park et al., 2007; Han et al., 2008). The morphological changes of mitochondria were noticed under fluorescence time-lapse microscope after treatment with different concentrations of AA (10 to 100 µM) (Park et al., 2007; Han et al., 2008).

The cytoskeleton is a network of protein elements that extends throughout the cytoplasm in eukaryotic cells and is composed of three types of protein polymers: microtubules, actin filaments and intermediate filaments (Ingber, 2003). Lat-B alters the state of actin polymerization or the organization of actin filaments and microfilaments (Wakatsuki et al., 2001). Actin filaments in animal cells are responsible for “transmitting traction and contraction forces generated within a cell to the extracellular matrix during growth and development, wound healing, and cell motility, and to maintain tissue structure and more” (Chen et al., 2013). In plant cells remodeling of actin filaments and the movement of organelles on actin filaments causes cytoplasmic streaming.

The results showed that the treatment with Lat-B (actin-disrupting drug) closely resembles the clustering morphology observed with MV or AA (Fig. 3-13, 3-17 and 3-21), although the clusters found with Lat-B treatment were generally much bigger than that found with MV or AA (Fig. 3-13, 3-17 and 3-21). From observation, it was clear that both mitochondria and ER were affected in their motility and morphology after Lat-B treatment (Fig. 3-13) compared to the control. Following Lat-B treatment, the association of mitochondrial clusters with ER was obvious after 3 and 24 hr (Fig. 3-13). Large mitochondrial clusters appeared to be completely connected to the ER network. The concentration of Lat-B (2 µM) used for this experiment was significantly lower than the concentrations (10 µM for 1 hr) used by other authors (Doniwa et al., 2007), but it was used for up to 3 hr. In the Lat-B treated
samples, mitochondria were found to be associated with the ER network (see arrows in Fig 3-13 F and C).

Actin depolarization, caused by treatment with Lat-B, increased mitochondrial clustering through the reduction of mitochondrial movement. Within one hour of the addition of 2 μM Lat-B major disruption of the movement of mitochondrion and ER was evident as mentioned above (Doniwa et al., 2007). In Arabidopsis, mitochondria move on actin (Van Gestel et al., 2002) as does the ER network and therefore the effect of Lat-B on ER and mitochondria is as expected and replicates previous studies (Wang et al., 2010). However, what is original about this study is that I visualized the two organelles simultaneously in the same tissue. Since mitochondria and ER both move on the same filamentous actin network, they move together during the highly dynamic motility of cell contents that is a feature of plant cells cytoplasmic streaming. Cytoplasmic streaming is the visual consequence of both the effects of the changing dynamics of the actin cytoskeleton itself on the cytosol and the force affected on the cytosol and other cytoplasmic organelles by the movement of organelles such as the mitochondria and ER on actin. Cytoplasmic streaming is most clearly seen in the cortical cytoplasm of cells with a large central vacuole but can also be seen within the transvacuolar strands that connect regions of cortical cytoplasm otherwise separated by the vacuole.

I show here in Arabidopsis, that mitochondria and ER are closely associated, with mitochondria sitting within the ER lacunae such that they are enrobed in ER. My observations of mitochondria and ER following treatment of seedlings with drugs such as Lat-B, MV, and AA allows me to conclude that there is a physical interaction between the ER and mitochondria since although these drugs have different modes of action yet affect mitochondrial dynamics and function, they do not alter the association of mitochondria and the ER. I suggest that there is a physical interaction between mitochondria and ER that explains how the two organelles show tightly associated dynamics that cannot be disrupted by otherwise powerful drug treatments. This structure is conserved therefore in plant cells as in mammals, and fungi.

4.5 Conclusions and future work

The results presented in this thesis show that there is a tight physical association between mitochondria and the ER. The drugs used to disrupt either cytoskeleton or mitochondrial morphology and function failed to cause disruption to the mitochondrial and ER association. My
results also show that Arabidopsis MCU1 is a mitochondrial protein and future work using high-resolution microscopy or sub-fractionation of mitochondria and antibodies to GFP will provide definitive evidence of the precise sub-mitochondrial location of MCU1.

Despite my efforts in generating transgenic lines of *mcu1* mutants expressing mitochondrial targeted aequorin, technical difficulties with the measurement of aequorin luminescence undermined the chance to provide evidence for the function of *MCU1* in plant mitochondrial Ca$^{2+}$ dynamics. Although no differences in mitochondrial dynamics between wild type and *mcu1* plants were apparent, future work could focus on cross-talk, via Ca$^{2+}$ flux, between mitochondria and other cell compartments. This may be of more importance, for example, when plants are under biotic or abiotic stress. The experimental material generated in my research could be extremely useful for further investigation on this topic either using aequorin measured with a more robust and refined protocol, or by using newly generated mitochondrial ratiometric calcium sensors (Loro et al., 2012).

The chemical treatments used in the research reported in this thesis appeared to cause no gross changes to the interaction between ER and mitochondria, future studies could use different, more refined techniques to dissect the structure and physiological importance of MAMs in plants. For example, putative interacting proteins on the ER and mitochondrial outer membrane could be identified by homology searching and these used in fluorescence interaction studies such as FRET (fluorescence resonance energy transfer). Alternatively, a mutant screen could be performed as for the first mitochondrial dynamics mutants (Logan et al., 2003) but instead of visualizing mito-CFP alone, the researcher could visualize mito-CFP and ER-YFP-HDEL as a marker for the ER. Additional future research could also try to identify the roles, if any, of the plant cytoskeleton in the generation and maintenance of mitochondria and ER association. While my initial results suggest actin is not involved, this study was performed using high concentrations of Lat-B; future studies could take a more gentle approach by using low concentration over longer periods of time. In addition experiments could investigate the role, if any, of microtubules in the mitochondrial-ER association.
CHAPTER 5. REFERENCES


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