ABSTRACT

INVESTIGATION OF A PUTATIVE MITOCHONDRIAL TWIN ARGinine TRANSLOCATION PATHWAY IN ARABIDOPSIS THALIANA

by Tasmeen Shiny Weerakoon

The unique Twin Arginine Translocation (Tat) pathway which is composed of TatA, TatB and TatC, transports fully folded proteins without the use of ATP energy. It was thought to be lost from the mitochondrion, the powerhouse of the cell during post-endosymbiotic evolution. This study investigates the stress induced expression of a minimal, mitochondrial Tat pathway in Arabidopsis thaliana. We treated plants with salicylic acid (SA) stress which mimics the hypersensitive response and studied the putative mitoTat components over a 24-hour time period. qRT-PCR analysis indicated the upregulation of the mitochondrial gene mtTATC. Furthermore, immunoblot detection confirmed that the mtTATC protein is expressed at high SA concentrations. However, a minimal Tat system capable of translocation requires TatC as well as TatA (Tha4). Confocal microscopy data established the dual localization of nuclear encoded, chloroplast Tha4-GFP to the mitochondria. Together, our data illustrate the existence of a putative minimal mitoTat pathway at 4 hours post-treatment with high SA concentrations. Finally, we discuss the physiological significance of a SA-induced minimal mitoTat pathway in plant pathology.
INVESTIGATION OF A PUTATIVE MITOCHONDRIAL TWIN ARGinine
TRANSLOCATION PATHWAY IN ARABIDOPSIS THALIANA

A Thesis

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Dedication

This thesis is dedicated to my loving Grandmother, Acha. Thank you for laying the foundation for a Lifetime of Learning.
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Chapter 1: Introduction
1.1 Protein transport and trafficking in cells

The cell is the smallest functional unit of life. Life sustaining biochemical reactions occur at this level. However, they display incredible diversity across the phylogenetic Tree of Life (Hug et al., 2016). For instance, most single-celled bacteria and archaea have a single plasma membrane where the majority of membrane biochemistry takes place. Eukaryotes are more complex multicellular organisms with many different intracellular compartments or organelles for spatially and temporally separated biochemical reactions. Each compartment is separated from its external environment by a phospholipid membrane.

As the name suggests, phospholipid membranes are bilayer structures composed of lipids and proteins interspersed in a fluid-mosaic model structure. Membrane proteins make up nearly one-third of the cells protein content (Pielak and Tian, 2012). They are either i) integral proteins embedded in the membrane as transmembrane, monolayer associated or lipid linked proteins, or ii) peripheral proteins not directly associated with the membrane but attached through another protein or lipid. Membrane proteins function as receptors, enzymes, anchors and transporters.

In eukaryotic cells, protein synthesis occurs in three distinct intracellular compartments each with its own internal genome: nucleus, mitochondria and chloroplast. However, the latter two organelles lost the majority of their genome during its evolutionary development. Hence, most of their proteins are now nuclear-encoded and synthesized in the cytoplasm as precursor proteins. The eukaryotic cell therefore has to transport and target important proteins from the site of synthesis to the site of function (Sommer and Schleiff, 2014) (Figure 1.1).

Cells achieve protein transport in one of two main ways. One is vesicular trafficking via receptor proteins, where proteins are transported by exocytosis or endocytosis though vesicular fusion and membrane invaginations (Gagescu et al., 2000; Goldstein et al., 1979; Südhof and Rothman, 2009). Alternatively, proteins can be translocated across the membrane via protein transport pathways. In fact, nearly 50% of all cytosol synthesized proteins are eventually inserted in or transported across a membrane (Schatz and Dobberstein, 1996). Some well characterized protein translocases are found in the prokaryotic plasma membrane (Rollauer et al., 2012b; Van den
Berg et al., 2004) and in eukaryotes, the endoplasmic reticulum (ER) membrane (Simon and Blobel, 1991; Wonderlin, 2009), mitochondrial membranes (Hartl et al., 1986; Kiebler et al., 1990; Murcha et al., 2014a) and chloroplast membranes (Li and Chiu, 2010; Skalitzky et al., 2011; Vojta et al., 2007).

Figure 1.1: Protein targeting from site of synthesis to function. The nucleus, chloroplast and mitochondria have their own protein synthetics machinery (DNA and ribosomes). However, over 90% of proteins are nuclear encoded and cytosolically synthesized as precursor polypeptides. Protein translocation machinery is required for these proteins to be targeted to the mitochondria or chloroplast and be transported across their double membranes (Dabney-Smith, unpublished).

1.1.1. Protein Translocases: Common Principles

Protein transporter systems are found in a wide range of cell types. Prokaryotic cells have only protein export systems while eukaryotic cells have both import and export systems. Export systems facilitate transport out of the cytosol or cytosol like compartments into extracytosolic compartments (cytosol to mitochondrial inner membrane). Import systems allow transport into the cytosol or compartments similar to the cytosol (into the mitochondrial matrix or chloroplast
stroma) (Gupta, 2008). However, most major membrane protein transport systems share some common features and mechanisms (Schatz and Dobberstein, 1996).

The substrate or precursor protein is targeted to its designated location via a N-terminal signal peptide which has a tripartite structure (1) A hydrophilic, n-region usually 16-30 amino acid residues in length, (2) a central hydrophobic h-region and (3) a c-region which houses the post-translational cleavage site (Figure 1.2). A signal peptidase catalyzes cleavage at this site to generate the mature protein on the trans side of the membrane (Martoglio, 2003). Usually, export signals are more hydrophobic while import signals are more hydrophilic. However, in general protein signals lack a consensus sequence, highlighting the degeneracy of the protein primary sequence and ability to form similarly organized secondary structures (Schatz and Dobberstein, 1996).

**Figure 1.2: The tripartite structure of a signal peptide.** Most signal peptides are 30-70 amino acid residues and have a positive n-region, hydrophobic h-region and a neutral or polar c-region. The signal is cleaved at the cleavage site to produce the mature protein. Image adapted from (Palmer and Berks, 2012).

The translocase itself is composed of a membrane receptor which interacts with the signal sequence of the precursor protein, and a transmembrane protein channel which forms a membrane pore for the polypeptide to go through. The protein channel is usually a hydrophilic, hetero-oligomeric, transmembrane structure composed of integral proteins (Agarraberes and Dice, 2001). There are two types of translocons. One is signal-gated translocons which remain the same size and can accommodate many substrates as they are transported as unfolded polypeptide chains. The other
is signal-assembled translocons which are thought to assemble and adjust around the size of the fully folded substrate being transported (Blobel and Dobberstein, 1975; Cline and Mori, 2001). Chaperone proteins on both the cis and trans side of the membrane assist in protein translocation from helping with docking the precursor protein to the membrane receptor, to ‘pushing’ the mature protein through to more internal organelle membranes (Jackson-Constan et al., 2001).

This complex process exerts high energy demands on the cell. Most protein translocation systems utilize ATP/ GTP hydrolysis or the proton motif force (PMF) via a transmembrane proton electrochemical gradient to generate energy. Some systems such as the Sec system in the bacterial plasma membrane and the thylakoid membrane, use both forms of energy (Mori and Ito, 2001; Santini et al., 1998). But very rarely does the PMF act as the sole energy generator for protein translocation; the Tat (Twin Arginine Transport) system is the only such system characterized so far (Alder and Theg, 2003).

1.2 Protein translocation in the chloroplast

The eukaryotic plant cell has nearly 30 different compartments that are structurally and functionally distinct. These compartments are maintained by efficient targeting of cytosol synthesized proteins which occur along two main intracellular paths; integrated into cellular organelles such as the mitochondria, chloroplast, nucleus and microbodies or secreted outside via the ER secretory pathway (Matsuoka and Bednarek, 1998; Sharova et al., 2002). A newly synthesized proteins N-terminal signal sequence determines the pathway and destination in the plant cell.

1.2.1 Chloroplast Protein Targeting

The chloroplast is a specialized, double membrane bound organelle containing the structures and pigments required to carry out photosynthesis. This complex biochemical process converts solar energy to chemical energy necessary for plant development. Chloroplasts evolved from an endosymbiotic cyanobacteria which lost the majority of its 3000 genes to the nucleus due to energy implications. The present day chloroplast genome has only about 100 protein encoding genes and
the rest are nuclear encoded (Martin et al., 2002). Therefore the cell developed novel ways to target and transport nuclear encoded proteins to the inner chloroplast, the main site of photosynthesis.

Protein targeting in the compartmentalized chloroplast is an elaborate process. It contains three compartments: i) the inter membrane space (IMS) enclosed by the outer and inner membrane, ii) stromal space in between the inner membrane and thylakoid membrane and iii) a thylakoid lumen separated by a thylakoid membrane. The current plant cell has a conservative sorting mechanism which combines previous prokaryotic with new eukaryotic transport systems (Figure 1.3) (Celedon and Cline, 2013).

**Figure 1.3: Chloroplast protein translocation systems.** The TOC/TIC pathways on the chloroplast membrane are novel protein systems found in the eukaryotic plant cell. In contrast, the Sec, SRP, Spontaneous and TAT systems on the thylakoid membrane are evolutionarily conserved transport pathways derived from ancestral cyanobacteria. OM; outer membrane, IM; inner membrane. The above image is adapted from (Jarvis and Robinson, 2004).

The general import apparatus or main pathway used by chloroplast targeted housekeeping and photosynthetic proteins is the Toc/Tic system. Toc (Translocon at the outer chloroplast envelope) and Tic (Translocon at the inner chloroplast envelope) are novel protein transport systems which developed in the evolving eukaryotic cell. Although they are distinct systems, they work together
at sites of membrane contact to export unfolded proteins from the cytosol into the chloroplast stroma (Cline and Dabney-Smith, 2008). However, proteins may solely be translocated across the Toc system, and be targeted to the intermembrane space or integrated in the inner membrane via a “stop transfer” mechanism (Brink et al., 1995; Steiner et al., 2005).

Energy is not expended when the precursor protein forms contacts with the Toc complex. However when proteins move across Toc and form contacts with the inner membrane Tic machinery, a low ATP and GTP concentration is expended. Finally, high levels of ATP are required to move the protein moves across the inner membrane Tic machinery (Jarvis, 2008).

1.2.2 Thylakoid Protein Targeting

Thylakoid protein targeting is vital for the plant cell. Half of ~100 thylakoid proteins and all ~150 luminal proteins are nuclear encoded must be targeted to the inner chloroplast for photosynthesis reactions (Leister, 2003). The thylakoid lumen includes proteins which support or regulate photosynthesis (Dekker and Boekema, 2005). The assembly of these dynamic, flexible thylakoid membrane systems is one of the most complex eukaryotic biological processes (Iwai et al., 2014). Thylakoid protein targeting is determined by the precursor protein signal sequences. All proteins destined to the chloroplast have a chloroplast signal sequence, which translocates them through the Toc/Tic system. Following translocation, the chloroplast signal sequence is cleaved in the stroma. Proteins destined to either be integrated in the thylakoid membrane or transported across the thylakoid membrane into the lumen, have an additional thylakoid signal sequence which is then exposed. Following thylakoid translocation, this sequence too is cleaved to give the mature protein (Cline and Dabney-Smith, 2008).

Four diverse thylakoid targeting pathways have been studied to date, all of which existed in ancient cyanobacteria as well; the Spontaneous Insertion Pathway and the signal recognition particle (SRP) pathway pathways are responsible for membrane protein integration while the secretory 1 (Sec1) pathway and the chloroplast Twin arginine translocation (cpTat) pathways are able to translocate proteins across the thylakoid membrane (Dabney-Smith and Storm, 2014).
1.2.2.1 Spontaneous Insertion Pathway
Some thylakoid targeted proteins do not require either transporter proteins or energy to be transported. Instead, they are spontaneously inserted into the membrane by interacting directly with the lipid membrane. This pathway also helps build the cpTat system by spontaneously inserting the single transmembrane cpTha4 and Hcf106 components into the thylakoid membrane (Fincher et al., 2003). This possibly explains why substrate binding in the presence of a proton motive force can easily trigger cpTha4 assembly on the thylakoid membrane (Cline and Mori, 2001).

1.2.2.2 Signal Recognition Particle (SRP) Pathway
Every domain of life has a Signal Recognition Particle (SRP) transport pathway. However, the chloroplast SRP pathway, despite evolving from the prokaryotic SRP pathway remains unique to facilitate the complex thylakoid protein sorting process. Although most SRP pathways transport precursor proteins co-translationally, the chloroplast SRP pathway does so post-translationally.

1.2.2.3 Chloroplast Sec 1 Pathway
All Sec pathways transport unfolded precursor protein through a channel of fixed size. The Sec pathway is an evolutionarily conserved protein translocation pathway found in a wide variety of membranes; the archaeal plasma membrane (Robinson and Bolhuis, 2004), bacterial plasma membrane, eukaryotic endoplasmic reticulum (Park and Rapoport, 2012) and the thylakoid of plant and algal chloroplasts. The cpSec system contains cp-homologs for each of the three main components in the bacterial Sec system; cpSecA, cpSecE and cpSecY (Dabney-Smith and Storm, 2014). Hence, although most Sec pathway studies have been done on bacteria, it is applicable to the chloroplast Sec pathway as well (Figure 1.2).
Figure 1.4: \textbf{cpSec translocation: unfolded preproteins.} \textit{cpSecA} functions as an ATPase to power translocation of the unfolded preprotein through the \textit{cpSecYE} channel. On the luminal end processing peptidase cleaves the signal peptide to generate the mature folded protein. Image adapted from (Agarraberes and Dice, 2001).

\textbf{1.2.2.4 cpTat Pathway}

\textit{i) Overview}

The twin-arginine translocation (Tat) pathway is a unique protein export system found in a variety of biological membranes; the cellular membrane of $\sim$80\% prokaryotes and many archaea (Berks, 2015), the thylakoid membrane of plants and algae (Celedon and Cline, 2013) and the mitochondrial membrane of jakobid protists (Burger et al., 2013). Although the Tat pathway is a fairly recent discovery, since the 1990’s it has been extensively characterized in plant thylakoids and prokaryotes, the model organisms used in subsequent discussions.

It is a unique translocation pathway for two main reasons. Firstly it can translocate fully folded proteins of varying sizes. Only two other biological pathways can achieve this feat; protein import pathway into peroxisome matrix and the ESX secretion system in \textit{Bacillus subtilis} (Hasan et al., 2013; Sysoeva et al., 2014). In fact, discovery of a Tat system stemmed from investigations into the transport of fully folded hydrogenases in \textit{Desulfovibrio vulgaris} (van Dongen et al., 1988).
Secondly, the Tat pathway solely uses a proton-motive force (PMF) or electrochemical energy, instead of chemical energy forms such as ATP or GTP to translocate proteins. This also earned it the name “ΔpH pathway” (Henry et al., 1997).

ii) Function and substrates
The prokaryotic Tat system transports fully folded protein substrates required for energy metabolism, nutrient acquisition, virulence and survival (Joshi et al., 2010). The thylakoid Tat system targets nearly 50% of all protein subunits required for photosynthesis, from the chloroplast stroma to the thylakoid lumen (Celedon and Cline, 2013). The ability to transport fully folded proteins is especially useful when transporting substrates which assemble cofactors prior to translocation. Common co-factor containing substrates are oxidoreductases important in prokaryotic redox pathways, and Rieske [2Fe-2S] protein subunit of the mitochondrial or prokaryotic cytochrome b.1 complex (Bachmann et al., 2006; Weiner et al., 1998).

All Tat substrates have an N-terminal signal sequence structurally similar to the Sec signal sequence: N-terminal basic region or N-domain, a hydrophobic central core or H-domain and a polar C-terminal region or C-domain (Figure 1.2). Another unique feature of the Tat pathway is Tat substrate specificity; they contain a highly conserved twin-arginine (RR) motif at the interface of the N- and H-domains of the signal peptide. In bacteria, this motif has been characterized as Z-R-R-Φ-Φ, where Z is any polar residue and Φ is any hydrophobic residue (Natale et al., 2008). With slight deviations such as RK and KR substrate translocation is significantly limited, and completely abolished in the case of KK (Blaudeck et al., 2001; Ize et al., 2002). Tat signal peptides are also characterized by acidic residues closer to the signal peptide cleavage site and lower hydrophobicity compared to Sec signal peptides (Cristobal et al., 1999).

iii) Structure and protein translocation
Bacterial Tat pathway studies have mostly focused on the gram-negative bacteria E.Coli. All three Tat components, TatA (9.6 kDa), TatB (18.4 kDa) and TatC (28.9 kDa) are encoded by the tatABC operon (Robinson and Bolhuis, 2004). A functional chloroplast Tat pathway requires the dynamic assembly of three membrane components: cpTha4, Hcf106 and cpTatC, which are homologous to the bacterial components TatA, TatB and TatC (Table 1.1) (Figure 1.3). Hcf106-cpTatC forms a
“receptor complex” in a 1:1 molar ratio to recognize and binds the precursor protein. Studies have shown that 8 subunits of each make a receptor complex and hence a fully saturated precursor can bind 8 precursor proteins. cpTha4 forms a homo-oligomeric “pore complex” for protein translocation (Cline and Mori, 2001).

However, recent evidence suggests increased recruitment of Tha4 to the membrane when there is a PMF and precursor protein, to form a dynamic Tha4-cpTatC receptor complex interaction (Aldridge et al., 2014). This suggests that Tha4 has pore- and receptor-like functional properties in the chloroplast Tat system. Such dynamic recruitment, specifically ~26 Tha4 molecules for maximal transport efficiency, complements the recent “membrane weakening” mechanism of Tat protein translocation. A length mismatch between the short Tha4 transmembrane helix and bilayer width ruptures the membrane to make it substrate permeable (Brüser and Sanders, 2003). It also helps explain how an assembled protein pore can accommodate fully folded substrates of varying sizes; Tat substrates diameters range from 25 to 70 Å while peptide strand diameters are much smaller at ~12 Å (Gohlke et al., 2005). All these lend credit to the unique functionality of the Tat translocation pathway.

Table 1.1. Summary of cpTat pathway components. This table highlights the physical (size, structure) and biochemical (function and protein-protein interactions) of the three components of the cpTat pathway. The figures are adapted from (Berks, 2015). NMR structure of the E.coli TatA (2MN7) and TatB (2MI2) and X-ray structure of Aquifex aeolius TatC (4B4A) are illustrated as cartoons. The N-terminal (N), C-terminal (C), transmembrane helix (TMH), amphipathic helix (APH) and the 6 TatC TMH are shown. These solved structures of Tat components in bacteria serve as a useful guide for studying the cpTat system. Images from (Ma, 2016).

<table>
<thead>
<tr>
<th>Component</th>
<th>TatA; cpTha4</th>
<th>TatB; Hcf106</th>
<th>TatC; cpTatC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>Smallest component (9.6 kDa)</td>
<td>Multiple Hcf106 (TatB) sized at 18.4 kDa and cpTatC (TatC) components form a large 700 kDa complex in equimolar amounts(Cline and Mori, 2001).</td>
<td>Largest component (28.9 kDa) (Robinson and Bolhuis, 2004).</td>
</tr>
<tr>
<td></td>
<td>Most Tha4 proteins form a homo-oligomeric complex of about 400 kDa (Dabney-Smith et al., 2006).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Structure**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TatA</strong></td>
<td>L shaped. Consists of an N-terminal TMH, a short APH, and a C-terminal domain (Hu et al., 2010; Walther et al., 2010). TatA TMH is only 14 amino acids in length and very short. The N-terminal APH is tilted and buried in the membrane (Muller et al., 2007).</td>
</tr>
<tr>
<td><strong>TatB</strong></td>
<td>Similar core structure to Tha4 (TatA), but has a longer APH and C-terminal domain (Zhang et al., 2014). <em>HCF106 (TATB)</em> may have arisen by an early gene duplication of <em>THA4 (TATA)</em> but has evolved into a functionally distinct protein TatB (Yen et al., 2002).</td>
</tr>
<tr>
<td><strong>TatC</strong></td>
<td>TatC has six TMHs and adopts a glove-shaped structure with a lipid-exposed pocket which binds the signal sequence (Behrendt et al., 2004; Ramasamy et al., 2013). The N-terminus, the first stromal loop) and the two luminal loops are most important for signal peptide recognition and transport activity (Holzapfel et al., 2007; Kneuper et al., 2012; Ma, 2016).</td>
</tr>
</tbody>
</table>

**Function & Protein Interactions**

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forms the pore and present in the receptor complex, occupying a similar position as Hcf106.</strong></td>
<td></td>
</tr>
<tr>
<td><strong>In a stimulated state with bound precursor protein and a PMF, additional Tha4 are recruited to the receptor complex forming the active Tha4-Hcf106-TatC translocase system.</strong> (Aldridge et al., 2014).</td>
<td></td>
</tr>
<tr>
<td><strong>Hcf106 forms direct contacts with the signal peptide and precursor mature domains</strong> (Ma and Cline, 2013).</td>
<td></td>
</tr>
<tr>
<td><strong>Hcf106 (TatB) binds to cpTatC (TatC) to form the receptor complex which recognizes and binds peptides</strong> (Cline and Mori, 2001). TatC is a substrate specificity determinant (Jongbloed et al., 2000).</td>
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1.3 Protein translocation in mitochondria

1.3.1 Mitochondria overview

The mitochondrion is the powerhouse of the eukaryotic cell. The endosymbiotic hypothesis states that the mitochondrion arose 2 billion years ago through a fusion between a eukaryotic host cell and an α-Proteobacteria (Alphaproteobacteria) (Margulis, 1970). However, the hydrogen hypothesis states that an anaerobic archaebacterial (host) associated with a eubacterium that generated hydrogen as a byproduct of anaerobic respiration. The host could now use hydrogen to produce ATP energy to facilitate an energetically favorable expansion of the nuclear genome (Martin and Müller, 1998). This theory implies that the origin of the mitochondrion helped create the first eukaryotic cell too (Lane and Martin, 2010; Martin, 2003).

Given their eubacterial/prokaryotic origin, the organelle is double membrane bound with a porous outer membrane (OM) and a more selective inner membrane (IM) which is highly folded into cristae structures. There are two aqueous spaces, the proton rich intermembrane space (IMS) and matrix which houses most of the mitochondrial proteins (Figure 1.5). The mitochondria structure is dynamically integrated into a network through fusion and fission and is sometimes tethered to the Endoplasmic Reticulum (ER) (Lackner, 2014; van der Laan et al., 2012).

![Figure 1.5: Mitochondrion structure](image)

**Figure 1.5: Mitochondrion structure.** Double membrane bound organelle enclosing the IMS (Inter Membrane Space). IM = Inner membrane, OM = outer membrane. The IM is highly folded into cristae and houses most of the protein translocation pathways. It encloses the central matrix space rich in mitochondrial functional proteins, ribosomes and the mitochondrial genome mtDNA.
Mitochondria are cellular powerhouses which produce ATP via the electron transport chain (ETC) and oxidative phosphorylation. The tricarboxylic acid cycle (TCA) in the matrix produces the electron carriers NADH and FADH2 which donate electrons to the ETC. Multiple redox electron transfers occur between the protein complexes (I-IV) on the IM, until they reach molecular oxygen, oxygen, the final electron acceptor in the ETC. Each redox reaction releases free energy used to pump H+ from the matrix to the intermembrane space. Proton gradient energy is harvested through an ATPase catalyzed oxidative phosphorylation reaction which produces viable energy in the form of ATP (Hüttemann et al., 2007).

Mitochondria have several important functions which extend beyond its role as the cellular powerhouse (McBride et al., 2006; Nunnari and Suomalainen, 2012). Proteomic analysis of ≈90% mitochondrial proteins in *Saccharomyces cerevisiae* yeast showed that a quarter of the proteins were involved maintaining the mitochondrial genome, 14% constituted bioenergetics systems such as oxidative phosphorylation machinery, the tricarboxylic acid cycle (TCA) and pyruvate dehydrogenase, 14% acted as transporters for amino acid, lipid and iron metabolism and an additional 8% were involved in protein translocation, while approximately 6% functioned in cellular apoptosis and signaling and 3% were involved in mitochondrial fission and fusion (Sickmann et al., 2003). In plants, mitochondria additionally play a major role in redox homeostasis such as processing of reactive oxygen species (ROS), cellular signaling and in innate immunity (Chandel, 2015; Noctor et al., 2007; West et al., 2011).

1.3.2 Mitochondrial protein targeting

Mitochondria are semi-autonomous organelles having their own internal mtDNA genome complete with ribosomes in the matrix (Figure 1.5). However, due to energy implications most of the post-symbiotic mitochondrial genome was lost or transferred to the eukaryotic nucleus (Herrmann, 2003). 10- 15% of the eukaryotic nucleus encodes solely mitochondrial proteins (Marcotte et al., 2000). In *Arabidopsis thaliana*, only 33 of the 1000 mitochondrial proteins are encoded in the mitochondrial genome and the rest are nuclear encoded and cytosolically synthesized (Millar et al., 2008; Unseld et al., 1997). Given the numerous important functions of
the mitochondria, efficient protein import across the outer and inner membrane is vital for cell viability, even under conditions where aerobic respiration is not likely (Schatz, 1996).

Cytosolically synthesized precursor proteins are conservatively sorted to the mitochondrial membrane and compartments via signal sequences (Schmidt et al., 2010). They can be grouped into two classes based on the type of signal sequence i) Precursor proteins with an internal targeting signal to form transmembrane domains ii) Precursor proteins with cleavable N-terminal presequences which are targeted to mitochondrial spaces. A 20-60 amino acid residue long N-terminal sequence with an amphipathic helix that has a hydrophobic and positively charged face, directs the precursor to the matrix (von Heijne, 1986; Vögtle et al., 2009). In Arabidopsis thaliana the majority of N-terminal presequences are between 20-70 amino acids in length and average at 50 residues. This is longer than the average yeast or mammalian presequence. Additionally, plant presequences have higher levels of serine and positively charged arginine, serine and alanine, leucine account for about 50% of all amino acids (Huang et al., 2009).

1.3.3 Plant mitochondrial protein translocation machinery

Plant mitochondrial protein translocation systems transport cytosolically synthesized precursor proteins into mitochondrial compartments. Most import is done post-translationally, although some proteins are transported co-translationally, prior to being associated with co-factors in the matrix and then exported or integrated into the inner membrane (Pfanner and Geissler, 2001).

Studies on plant mitochondrial protein translocases were mostly performed on unicellular Saccharomyces cerevisiae and Neurospora crassa. General mitochondrial protein translocation systems consist of three main components: i) membrane translocase which recognizes and transports the protein, ii) chaperones such as Hsp70 and Hsp90 to maintain the unfolded mitochondria in a stable conformation and iii) a mitochondrial processing peptidase (MPP) or processing enhancing protein (PEP) to remove the signal sequence after targeting (Dudek et al., 2013; Hawlitschek et al., 1988; Vögtle et al., 2009). Although the general features are similar, these observations are not fully applicable to complex, multicellular plants such as Arabidopsis thaliana (Chaumont et al., 1990; Lister et al., 2003).
However, four main pathways have been characterized in mitochondria of multicellular plants. Translocase of the outer membrane (TOM) complex and sorting and assembly machinery (SAM) on the outer membrane, mitochondrial intermembrane space assembly pathway in the intermembrane space and translocase of the inner membrane (TIM) complex on the inner membrane (Figure 1.6). The general import pathway describes translocation of proteins through the TOM and TIM. The carrier import pathway describes translocation and integration of inner-transmembrane proteins which have an internal targeting sequence (Murcha et al., 2014a).

Figure 1.6: Plant mitochondrial protein translocation pathways. IM = Inner membrane, OM = Outer Membrane, IMS = Inter Membrane Space. TOM is the entry gate for all mitochondrial proteins. SAM embeds proteins in the OM. Matrix proteins go through the TIM23 complex and PAM. IM integral proteins are inserted by TIM23 and TIM22. MIA localizes IMS proteins. OXA embeds mitochondria encoded proteins into the IM. Image adapted from (Stojanovski et al., 2012).

1.3.3.1  TOM pathway

The TOM pathway imports all proteins that contain a mitochondria specific N-terminal presequence. This pathway is the entry gate in plant mitochondrial import and recent studies speculate its role in tRNA import and in binding glycolytic enzymes to the mitochondrial surface (Graham et al., 2007; Sieber et al., 2011). This 250 kDa system is composed of several subunits (Figure 1.7) (Jänisch et al., 1996). The Tom20 and Tom 22 (or plant Tom9) cytosolic facing receptors binds the amphiphilic α-helix of the mitochondrial presequence to a binding site as small as 8 residues (Saitoh et al., 2007). Inactivation of three of the four Tom20 genes in Arabidopsis thaliana reduced protein import by 70%, suggesting that other receptors exists such as Tom22
Another novel receptor protein in plant TOM is outer-membrane protein 64 (OM64), a paralogue of Toc64 a protein located on the outer envelope of chloroplasts (Chew et al., 2004). The TOM40 subunit, a dimerized β-barrel protein forms the pore-like translocation channel and is associated with small Tom5, Tom6, and Tom7 subunits which are conserved across all plant mitochondria (Künkele et al., 1998; Lister et al., 2003).

**Figure 1.7. TOM plant mitochondrial import pathway.** Translocase of the Outer Membrane is the entry path for all mitochondria targeted proteins. Tom20/Tom70 recognize the preproteins and direct them to the Tom40 channel for protein translocation. OM; outer membrane, IMS; intermembrane space. Image adapted from (Duncan et al., 2013).

### 1.3.3.2 SAM pathway

The SAM pathway on the outer membrane transfers TOM translocated β-barrel proteins such as porin, Tom40 and Sam50 to the outer mitochondrial membrane. The yeast SAM pathway is composed Sam50, Sam35 and Sam37 (Wenz et al., 2014). Sam50 functions as the translocation channel and its orthologue is present in plants. Sam35 and Sam37 associate with Sam50 to bind substrates and import substrates (Kozjak-Pavlovic et al., 2007).

### 1.3.3.3 MIA pathway

The MIA pathway transports cysteine-rich proteins such as Tim8, Tim9, Tim10, and Tim13 which assemble the TIM and SAM complex through the IMS (Chacinska et al., 2009). It also transports proteins involved in ROS metabolism and ETC complex IV assembly by reducing cytochrome C (Murcha et al., 2014a). The two main plant MIA pathway components are Mia40 and Erv1. Only
Erv1, a sulfhydryl oxidase which catalyzes thiol oxidation to form a protein structure stabilizing disulfide bond is essential for higher order plants (Basu et al., 2013; Stojanovski et al., 2012).

1.3.3.4 TIM pathway

![TIM pathway](image)

**Figure 1.8: TIM plant mitochondrial protein import pathway.** Translocase of the Inner Membrane translocates matrix destined preproteins threaded across the TOM, through either the Tim22 or Tim23/Tim17 complex and mediates protein integration into the IM. This process is aided by chaperones (Hsp60/10) and mitochondrial processing peptidases (MPP) in the mitochondrial matrix. IM; inner membrane, IMS; inter membrane space. Image adapted from (Duncan et al., 2013).

Proteins destined to the matrix and inner membrane are translocated by the IM protein translocator complexes TIM 17:23 via the general pathway or TIM 22 via the carrier import pathway (Figure 1.8). The dynamic plant TIM 17:23 consists of four integral proteins: the protein channel Tim23 associated with Tim17 and the membrane-potential dependent translocation regulators Tim50 and Tim21. The Tim17 C-terminal extends to and associates with the OM to form a TOM-TIM supercomplex which threads the precursor protein through both mitochondrial membranes. Tim23 also cooperates with the IM presequence translocase-associated motor (PAM) complex, to transport precursors into the matrix in an ATP-dependent manner. Tim23 and PAM cochaperones such as Tim44 and Pam16 respectively, regulate the PAM ATPase component mtHsp70, and provide a binding site for the precursors close to the Tim23 channel (Chacinska et al., 2010).
Plant TIM 22 complex translocates precursors without a cleavable sequence that get embedded as IM carrier proteins. Tim22 operates independently as the translocation channel. However, recent studies indicate that Tim22 associates with a large 500kDa component representing either the large respiratory complexes III or other unidentified proteins (Klodmann and Braun, 2011; Murcha et al., 2012). Tim21 also interacts with complexes I and III (Murcha et al., 2014b). More interestingly, Tim23 and another subunit B14.7 have been characterized in both the TIM 17:23 translocase and in respiratory complex I and III, indicating dual functions for these importer proteins (Murcha et al., 2012).

Another unique translocase on the mitochondrial inner membrane is OXA translocase, a 170-180 kDa transmembrane insertase which integrates mitochondrial encoded subunits co-translationally into the IM (Braun and Schmitz, 1999; Nargang et al., 2002). Accordingly, the Oxal mitochondrial protein is evolutionary conserved in bacteria (YidC) and chloroplasts (Alb3) as well (Preuss et al., 2005). In yeast and plant mitochondria, OXA is important for ETC complex I and IV assembly through export of matrix synthesized proteins into the inner membrane (He and Fox, 1997; Nargang et al., 2002). Furthermore the yeast OXA homolog was capable of inserting a large, nuclear-encoded multi-spanning ABC transporter protein which originally localized in the matrix, into the inner membrane. It achieved this through cooperative conservative sorting and a stop-transfer mechanism from both sides of the membrane (Bohnert et al., 2010).

1.3.4 Plant mitochondria: Stress sensor

Plant growth and reproduction are compromised by both abiotic and biotic stresses. Some abiotic plant stress factors are heat, cold and drought and biotic plant stress factors are pathogens. However unlike animals, plants are nonlocomotive and lack an immune system to evade these stresses. Instead, stress factors induce an initial hypersensitive response (HR) and eventually localized programmed cell death (PCD) where proximal tissue from the site of stress is killed to protect the neighboring healthy tissues (Morel and Dangl, 1997). These appear as dark tissue lesions on the plant (Govrin and Levine, 2000). Increasing evidence suggests the role of mitochondria as plant stress sensors, by orchestrating the HR, release of apoptotic factors for PCD and longer term immunity against a future pathogenic attack (Bartoli et al., 2004; Giraud et al., 2008).
The early hypersensitive response is characterized by a burst of Reactive Oxygen Species (ROS) and superoxide hydrogen peroxide (H$_2$O$_2$) which are byproducts of the mitochondrial ETC and photosynthesis (Lamb and Dixon, 1997). ROS induced oxidative stress damages the mitochondrial genome and increases mtDNA copy number and respiratory genes, especially in mammals (Lee and Wei, 2005; Wei and Lee, 2002). In higher plants, mitochondrial genomic redundancy, unequal genome distribution and circular permuted structure through recombination have helped minimize damage to mitochondria (Logan, 2010; Rhoads et al., 2006). However, excessive cellular mitochondrial ROS (mtROS) production causes significant mitochondrial morphological changes and eventually mitophagy, an early sign of cellular death (Huang et al., 2016).

The stress signal H$_2$O$_2$ diffuses to proximal tissues around the site of infection, acts as a threshold signal for the PCD process and increases endogenous levels of the plant hormone salicylic acid (SA, 2-hydroxy benzoic acid) (Levine et al., 1994) (Leon et al., 1995) (Leon et al., 1995; Levine et al., 1994). Research suggests that the stress induced SA affects plant mitochondrial respiration functions which then inhibit ATP formation and O$_2$ uptake to cause PCD (Tiwari et al., 2002; Xie and Chen, 1999; Xie and Chen, 2000). Additionally, endogenous SA induces pathogen-resistant (PR) genes and the complex plant systemic acquired resistance (SAR) response which provides the plant immunity against future pathogen attacks (Métraux et al., 1990; Vlot et al., 2009). Exogenous SA can also induce SAR (White, 1979). The mitochondrial complex II, which contributes to mtROS production may play a role in expression of SA-responsive PR genes (Gleason et al., 2011).

Since the mitochondria has lost ~ 99% of its internal genome the PR-genes for mitochondrial proteins such as antioxidant defense enzymes and mitochondrial carrier proteins are nuclear encoded (De Gara et al., 2010; Scott and Logan, 2008; Yao et al., 2002). A few are alternative oxidase (AOX) and alternative NAD(P)H dehydrogenases (NDs), Mn superoxide dismutase (MnSOD), mitochondrial ascorbate peroxidase (APX) and glutathione peroxidase (Chew et al., 2003; Van Aken et al., 2009a; Van Aken et al., 2009b). However given the interconnectedness of complex signaling pathways, the exact mechanism of plant SAR is unknown.
1.3.5 Mitochondrial Tat system during plant stress

Although the chloroplast and mitochondria lost most of their genome after endosymbiosis, the chloroplast retained a functional Tat pathway while the mitochondria did not. But one component of the mitochondrial Tat pathway, mtTATC is expressed in prokaryotes, plastids and one animal, the Oscarellidae family of homoscleromorph sponges although not in higher order plants (Bogsch et al., 1998; Pett and Lavrov, 2013; Yen et al., 2002).

However, a study in *Nicotiana tabacum* investigated a mitochondrial gene responsive to salicylic acid stress. They found that the *N. tabacum* mitochondrial genome contained an open reading frame (orfX) with 98.5% conserved domain homology to the *E.coli* TatC. When treated with exogenous SA this orfX was expressed and upregulated, and they named this stress responsive transcript, *mtTATC* (van der Merwe and Dubery, 2007). This is significant because during stress conditions, the mitochondrion expressed a gene which was previously thought to be lost during its evolutionary process. Furthermore it suggests that *mtTATC* may play a role in the plant stress response.

But so far only Oscarellidae have a functional mtTATC component; the single gene encoded pathway is capable of inserting proteins into the IM (Pett and Lavrov, 2013). However, the exact function and substrates for such a pathway are unknown, although a few have been suggested. Like higher order plants, Oscarellidae lack the mitochondrial Bcs1 transporter which exports and assembles Rieske Fe/S protein (Rip1) into mitochondrial complex III (Nobrega et al., 1992; Wagener and Neupert, 2012). Conversely, organisms which encode Bcs1 lack *mtTATC* (Carrie et al., 2016). This suggests that mtTATC maybe functionally redundant to Bcs1 in Oscarellidae. Accordingly, in higher order plants which also lack Bcs1, a functional mtTATC may perform the role of Bcs1 in exporting Rip1. This would be especially beneficial during the plant stress response, since Rip1 coordinates the release of apoptotic factors from the mitochondria during stress conditions (Minibayeva et al., 2012). Therefore it would be interesting to study the expression of a mtTATC component in plants during stress conditions.

However, it is unlikely that in higher order plants the mtTATC component alone could translocate a fully folded protein such as Rip1. Studies in gram-positive bacterium *Bacillus subtilis*, show that
a minimal Tat translocase system capable of transporting proteins is composed of both TatC, and the TatA component which functions as the pore (Jongbloed et al., 2004). Later studies also confirmed the presence of mtTATC and a TATB homolog in the absence of stress in Arabidopsis thaliana but not TatA (Carrie et al., 2016). Therefore we are yet to find a functional mtTATC component and a minimal, functional mitochondrial Tat pathway in plants.

1.4  Protein dual targeting in eukaryotic cells

1.4.1  Overview

Eukaryotic cells are subdivided into compartments with functionally specialized sets of proteins. However, sometimes an identical protein encoded by the same gene is targeted to more than one compartment (dual targeting). Such proteins arise in two ways: i) alternative transcriptional initiation or ii) an ambiguous N-terminal signal peptide sequence allowing recognition by protein import machineries in both compartments (Carrie et al., 2009b; Pujol et al., 2007).

1.4.2  Dual targeting in plant cells

Dual targeting was first observed between the mitochondria and chloroplast of tobacco plants (Creissen et al., 1995). Later studies found several proteins targeted to both the chloroplast and nucleus, chloroplast and endoplasmic reticulum, or nucleus and mitochondria (Carrie et al., 2009a; Mireau et al., 1996).

More significantly, over 100 proteins are dual targeted to both the chloroplast and mitochondria (Carrie et al., 2009a). This is not surprising given that they both have prokaryotic origin and their presequences are enriched with positive residues and have an amphipathic alpha-helix (Brink et al., 1997). Most dual targeted presequences are ambiguous; their arginine and serine content values range in between that of mitochondrial and chloroplast presequences. This sequence ambiguity also helps explain how non-homologous protein import machineries can import the same protein (Peeters and Small, 2001).
Dual targeted proteins may carry out different functions in either location, allowing the cell to perform additional cellular functions with the same number of genes (Carrie et al., 2009a). Alternatively, the protein may carry out similar functions in both the chloroplast and mitochondria, complementing their shared cellular functions such as energy metabolism and oxidative stress mediation (Silva-Filho, 2003).

1.4.3 cpTha4 dual localization during stress conditions

The mitochondrial genome of higher plants such as Beta vulgaris and Arabidopsis thaliana contain and open reading frame (OrfX) with high sequence similarity to bacterial tatC (Unseld et al., 1997; van der Merwe and Dubery, 2007). However, the minimal functional Tat pathway found in gram-positive bacteria is composed of tatC as well as the TatA orthologue, Tha4 (Celedon and Cline, 2013; Jongbloed et al., 2004). Additionally the mitochondrial tatC sequence studies of plants and green algae indicate an unstable negative charge in its hydrophobic region, which requires stabilization by TatA (Tha4) (Pett and Lavrov, 2013; Rollauer et al., 2012a).

But, plants are not known to have a mitochondrial OrfX coding for a mitochondrial TatA orthologue, mtTha4. Presequence analysis of nuclear encoded chloroplast Tha4 (cpTha4) with Ambiguous Targeting Prediction (ATP) software indicated that it can be dual targeted to both the chloroplast and mitochondria (Pal, 2014). Therefore, cpTha4 dual targeted to the mitochondria could provide this stability (Pal, 2014; Pett and Lavrov, 2013).

Furthermore, cells are able to respond to environmental stresses by altering the distribution of a dual targeted protein to either compartment (Carrie and Small, 2013; Stengel et al., 2010). It would be interesting to see whether the distribution pattern of cpTha4 between the chloroplast and mitochondria could also be altered during stress.
1.5 Thesis Goals and Specific Aims

The novel twin-arginine translocation (Tat) pathway has gained scientific interest since its discovery in the 1990’s and is mainly characterized in prokaryotes and plastids. This unique pathway translocates fully folded proteins of varying sizes using only a protein-motive force. However, it is unknown whether higher order plants express a minimally functional mitochondrial Tat pathway during oxidative stress. Such a novel pathway would allow the mitochondria to transport fully-folded precursors necessary for elevated mitochondrial biogenesis of redox components in the ETC or the stress induced plant hypersensitive response and apoptosis. Furthermore, evaluating the presence of a mitochondrial Tat pathway will shed light on a unique protein sorting and transport mechanism in plant mitochondria, a facility previously thought to be lost during the mitochondrial evolutionary process. Therefore, it is of great significance to (1) investigate expression of a minimal mitoTat pathway in higher order plants and (2) analyze the role of the mitochondrial Tat pathway during oxidative stress.

The main goal of this research was to investigate a minimal, putative mitochondrial twin-arginine translocation (mitoTat) pathway in Arabidopsis thaliana during salicylic acid (SA) treatment. Overall, we hoped to gain a better understanding of the stress-mediated expression of mitochondrial genes and the proteins it encodes. This thesis centers on SA induced expression of the mitochondrial gene mtTATC, the putative mtTATC component and the dual localization of the nuclear encoded cpTat component to form a minimal mitoTat pathway. We then evaluate the physiological implications of such a pathway in higher order plants, especially its role as a mitochondrial stress sensor in plant pathology.

Our first goal investigated mtTATC transcript expression in Arabidopsis thaliana during SA treatment and shows that it is both expressed and responsive to oxidative stress. Recent evidence showed expression of a putative mitochondrial TatC transcript during SA stress conditions in Nicotiana tabacum, the first instance in higher order plants. This suggested the presence of genes previously thought to be lost from the mitochondria during post-symbiotic evolution. However, this has not been investigated in the model plant Arabidopsis thaliana with SA stress. We designed
specific primers against \textit{mtTATC} and aimed to quantify transcript levels during SA treatment with qRT-PCR technique.

These results set the stage for our second goal, where we investigated the expression of the mtTATC protein in \textit{Arabidopsis thaliana} during oxidative stress. We designed antibodies and peptides against the putative mtTATC protein and performed immunoblot precipitation analysis. Previous studies have found a functional mtTATC in Oscarellidae. However, its expression and role in the mitochondria during oxidative stress is not yet characterized. To help understand the SA mediated mitochondrial stress response over time, we aimed to perform a time-course study of mtTATC protein levels at different SA concentrations.

For our third goal we investigated the dual localization of the chloroplast-Tha4 component to the mitochondria in \textit{A. thaliana} during stress. A minimal Tat pathway in bacteria is composed of both TatC and the TatA (Tha4) (Celedon and Cline, 2013; Jongbloed et al., 2004). Research indicates significant cross-talk between the chloroplast and mitochondria and over 100 dual targeted proteins (Carrie and Small, 2013; Ma, 2016; Ulrich et al., 2012). Furthermore, high cpTha4 presequence ambiguity makes cpTha4 a viable dual targeting protein (Pal, 2014). Hence, we aimed to identify whether cpTha4 is localized to the mitochondria during stress conditions to form a minimal mtTat pathway. We generated cpTha4-GFP transformed \textit{A. thaliana} plants to conduct a qualitative and quantitative confocal microscopy study.

Research shows that the membranes of plant organelles experience significant oxidative stress related morphological damages such as leaky membranes or decreased surface area. For our fourth goal we aimed to characterize SA stress mediated changes to the membranes of chloroplast and mitochondria that have a Tat pathway, in the higher order plant \textit{Pisum sativum}. Furthermore, since oxidative stress can have different effects on different membranes, we wished to determine which organelle or membrane was damaged the most by SA stress. We aimed to characterize these changes at the ultrastructural level using Transmission Electron Microscopy (TEM).
1.6 References


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Chapter 2: Data
Chapter 2: Investigation of a putative mitochondrial Twin Arginine Translocation pathway in *Arabidopsis thaliana* during salicylic acid (SA) stress

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Author contributions: CDS conceived and supervised this study; DP designed GFP constructs and completed preliminary work on the project; CDS, QM and TSW designed the experiments; TSW performed experiments; TSW and CDS analyzed the data; TSW wrote the draft and CDS edited the manuscript.
2.1 Abstract

The unique Twin Arginine Translocation (Tat) pathway transports fully folded proteins across the lipid bilayer using only a proton-motive force (PMF). A functional pathway has been found in bacteria, archaea and eukaryotic organelles such as the chloroplast, but not in mitochondria. However, a recent study confirmed the upregulation of a mitochondrial tatC gene in Nicotiana tabacum with salicylic acid (SA) stress. Here, we confirm the concentration and time dependent expression of the mtTATC transcript and mtTATC protein in Arabidopsis thaliana during SA treatment. This adds to the increasing evidence of the role of mitochondria as plant stress sensors. A minimal Tat pathway found in gram-positive bacteria contain both TatC and TatA (Tha4). Our confocal microscopy study shows the dual-localization of nuclear encoded cpTha4-GFP to the mitochondria, to possibly form a minimal Tat pathway during SA treatment. Our data suggests that a minimal mitochondrial Tat pathway is most likely assembled 4 h after treatment with high SA concentrations (>500 µM). Finally, we used Transmission Electron Microscopy to characterize ultrastructural changes in membranes due to SA mediated oxidative damage. We confirm significant damage to the mitochondrion at high SA concentrations. We then discuss the physiological implications of a SA dependent expression of a mitochondrial Tat pathway in Arabidopsis thaliana, especially in the context of plant pathology and the mitochondrial stress response.

2.2 Introduction

Mitochondria are ATP producing cellular powerhouses. However, increasing evidence suggests its role as a plant stress sensor, in both the initial plant hypersensitive response (HR) and delayed systematic acquired resistance (SAR)(Van Aken et al., 2009). Plant stress signaling molecules such as salicylic acid (SA) induce mtROS that regulate pathogen-resistant (PR) genes for mitochondrial proteins such as antioxidant defense enzymes or carrier proteins (Nie et al., 2015) (Gleason et al., 2011). Since mitochondria have lost ~ 99% of their genome to the nucleus, most of these proteins are nuclear encoded (Birky, 1995; Martin, 2003).

However, sequence analysis showed that the mitochondrial genome of higher order plants such as B. Vulgaris, A. thaliana and N. tabacum have an open reading frame (orfX) for TatC-like proteins
of the Tat pathway, although it is unknown whether they are pseudogenes or are actually expressed (Bogsch et al., 1998). A recent study in *N. tabacum* confirmed the expression of the mitochondrial orfX, *mtTATC* when treated with exogenous SA (van der Merwe and Dubery, 2007). For this study, we used qRT-PCR to investigate if a similar orfX in the mitochondrial genome of the model plant, *Arabidopsis thaliana* is responsive to exogenous SA. Since plants experience an initial HR and a prolonged SAR we performed this study over a 24-hour-time period and found that *mtTATC* expression levels changed over time (Alvarez et al., 1998; Nie et al., 2015).

The role of SA in the plant stress response is well described. However, the exact pathway and components for this complex plant-defense response are unknown. Furthermore, its ability to induce and more importantly change relative expression levels of a gene previously thought to be lost from the mitochondrial genome is a new area of research. Moreover, this stress responsive gene would code for *mtTATC*, a component of novel mitochondrial Tat protein transport pathway. Therefore this study provides insight into SA targets in higher order plants and the role of a putative mitochondrial translocase in the plant oxidative stress response.

A functional *mtTATC* which can insert substrate proteins into the mitochondrial IM was characterized in Oscarellidae (Gazave et al., 2013; Pett and Lavrov, 2013). A recent study showed the expression of *mtTATC* in *Arabidopsis thaliana* without stress conditions but its functionality was not confirmed (Carrie et al., 2016). Given the SA-mediated *MTTAC* transcript level changes over a 24-hour-time period, we used immunoblot detection to investigate the same for *mtTATC* protein. We performed this study at both low and high SA concentrations and found that *mtTATC* expression changed at high SA concentrations. We then analyze the implications of SA-mediated changes in *mtTATC* expression in the plant stress response.

Although a single gene (TatC) encoded mitoTat pathway is functional in Oscarellidae, certain prokaryotes require at least TatA and TatC to form a minimal functional pathway (Jongbloed et al., 2004). cpTatC recruits additional Tha4 components to form the dynamic pore essential for translocation of fully folded proteins of varying sizes (Aldridge et al., 2014). Therefore, we wished to investigate the presence of a mitochondrial TatA component which possibly associates with mtTatC to form a minimal mtTat pathway. However, since the *A. thaliana* mitochondrial genome does not have an OrfX similar to an existing *Tha4* or *TatA*, we studied the dual localization of the nuclear encoded cpTha4 to the mitochondrial during SA treatment. Over 100 proteins are dual
targeted to the mitochondria and chloroplast (Carrie and Small, 2013). In this chapter we present qualitative and quantitative confocal microscopy data for cpTha4-GFP dual localization during SA treatment. We then discuss how or when it may form a minimal mitochondrial Tat pathway during SA stress and its functional significance for plant defense.

A functional mitoTat pathway which translocates folded proteins would be on the selective mitochondrial inner membrane (IM) and not on the porous outer membrane. Previous studies have shown that SA-mediated oxidative stress causes significant morphological changes to plant organelles (Chandra et al., 2007; Huang et al., 2016). However, we wished to characterize the effect of SA on the ultrastructure of membranes which house the Tat pathway in higher order plants; the thylakoid membrane and mitochondrial IM. We used *Pisum sativum* since the thicker leaf of *A. thaliana* made TEM preparation challenging. We present Transmission Electron Microscopy (TEM) images which show that the mitochondrial membrane undergoes significant oxidative damage compared to the thylakoid membrane. This further suggests that mitochondria, as opposed to chloroplast, play a bigger role in the plant oxidative stress response (Van Aken et al., 2009).

### 2.3. Materials and Methods

#### 2.3.1 Plant Materials and Growth Conditions

Seeds of *Arabidopsis thaliana* wild type Col-0 were obtained from the Arabidopsis Biological Resource Center (https://abrc.osu.edu/order-stocks) (Alonso et al., 2003). Seeds were dried for 3 days at 28 °C and then stratified for 7 days at 4 °C. Seeds were surface-sterilized using 70% ethanol for 5 min followed by a solution of 20% bleach and 0.1% tween-20 for 5 min. Seeds were equally divided and grown on two separate plates of half-strength Murashige and Skoog (MS) medium (1% sucrose and 1 % agar pH 5.7~5.8). Plants were grown at 23 °C in a growth chamber under a 16h light/8 h dark photoperiod (light intensity 88 uE m$^{-2}$ s$^{-1}$) for two weeks.
2.3.2 Real Time Quantitative Reverse Transcription PCR (qRT-PCR)

i) One plate with two-week-old wild type Col-0 plants was treated with 25 ml of 400 µM solution of salicylic acid and the other plate which was the control was treated with 25 ml of water. Both plates were placed in the 23 °C growth chamber for 3 hours prior to RNA extraction (van der Merwe and Dubery, 2007). The experiment was repeated with Arabidopsis thaliana Hcf106 (At5g52440) gene T-DNA insertion mutants SALK_044421C (Alonso et al., 2003).

ii) Each experiment was repeated but instead with 500 µM solution of salicylic acid and RNA extracted along a 24 hour time-course. Two-week-old wild type Col-0 plants grown under the same conditions. One plate was treated with 25 ml of salicylic acid and the other plate, the control was treated with 25 ml of water. RNA extraction was performed at 0 hours after addition of solution. Subsequently, both plates were placed in the 23 °C growth chamber and RNA was extracted from each plate 1 hour, 4 hours and 24 hours post-treatment (Nie et al., 2015).

For each qRT-PCR reading, three biological replicates were used and each comprised leaf tissue from three separate plants. Additionally, three technical replicates were performed for each biological sample. Relative quantification calculations were performed as described by (Schmittgen and Livak, 2008). Statistical differences (p <0.05) were determined using student’s paired t-test.

For the quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) analysis of mRNA, total RNA was extracted from the plant leaves, using the Plant RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. The total RNA concentration was determined using a Nano Drop 2000C spectrophotometer (Thermal Scientific) and checked for RNA quality. DNA digestion was carried out on 200 ng of total RNA using the DNase I Kit (Quanta).

The quantitative real-time PCR was carried out in two steps. First, the total volume of 200 ng RNA from the DNA digestion step, was reverse transcribed to cDNA by using the qScript™ cDNA Synthesis Kit (Quanta) according to the manufacturer’s protocol. Second, aliquots of the reverse transcription reactions products were used as templates for the quantitative real-time PCR using the PerfeCta® SYBR® Green Supermix for iQ Kit (Quanta) according to manufacturer’s instruction. qRT-PCR was performed in a 96-well plate in the Bio-Rad iCycler, at an annealing
temperature of 52°C for 45 cycles until saturation point was reached. This step was performed in the Center for Bioinformatics and Functional Genomics at Miami University.

The mtTATC F1 and mtTATC R1 primers were used to amplify mtTATC cDNA. The mtTATC F2 and mtTATC R2 primer pair (Table 2.1) from the manufacturer failed to amplify the cDNA as intended. UBQ4 was amplified as an internal control to normalize the gene copy number of mtTATC (Brunner et al., 2004; Czechowski et al., 2005). Additionally, all primers used in this study are listed in Table 2.1. Gene-specific primers were identified from the literature or designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/).

Table 2.1. Summary of primers used in this study. The mtTATC and UBQ4 primers were used in section 2.3.2 for qRT-PCR. The 35 S 557 F (35S promoter specific 557 forward) and GFP R (GFP Reverse) primers were used separately as described in section 2.3.3 for agrobacteria DNA sequencing.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Melting temperature, Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtTATC F1</td>
<td>CTT CGACCAGCTCCTTCTTG</td>
<td>57</td>
</tr>
<tr>
<td>mtTATC R1</td>
<td>GCCAAGAAGCCTACACTCCA</td>
<td>59</td>
</tr>
<tr>
<td>mtTATC R2</td>
<td>CCCCAGTTGCTATGGAGAAC</td>
<td>57</td>
</tr>
<tr>
<td>mtTATC F2</td>
<td>GGGGAGGAGTTAGGAACAGG</td>
<td>59</td>
</tr>
<tr>
<td>UBQ4 F</td>
<td>GCT TGG AGT CCT GCT TGG ACG</td>
<td>63</td>
</tr>
<tr>
<td>UBQ4 R</td>
<td>CGC AGT TAA GAG GAC TGT CCG GC</td>
<td>65</td>
</tr>
<tr>
<td>35 S 557 F</td>
<td>CGGATACTTTACGTCACGTCTTGC</td>
<td>63</td>
</tr>
<tr>
<td>GFP R</td>
<td>CGATCATAGGCCGTCTCGCATATCT</td>
<td>60</td>
</tr>
</tbody>
</table>

2.3.3 Protein Extraction and Immunoblotting

Arabidopsis thaliana wild type Col-0 plants were grown on two separate plates as described. Plates with two-week-old plants were treated with 25 ml of increasing concentrations of salicylic acid solution (200, 400, 800, 2000 µM) and a separate control plate was treated with 25 ml of water. All plates were placed in the 23°C growth chamber prior to protein extraction at the indicated times post-treatment (0, 1, 4 and 24 hours).
For protein extraction, equal amounts of leaf or root tissue were homogenized for 2-3 minutes with a Bullet blender® (Next Advance) in 150 µl of lysis buffer (125 mM Tris-HCl, pH 6.8, 2% SDS and 10% glycerol). The homogenates were then centrifuged at 14000 rpm for 20 min. 100 µl of supernatant was mixed with 50 µl of 2x SSB solution (1:1) prior to adding 1 µl of Sigma Life Science Protease Inhibitor Cocktail.

Samples were heated for 2 minutes at 95 ºC and 10 µl of protein samples were loaded on a 12.5% SDS-PAGE gel. The protein markers, 4 µl of Fisher BioReagents™ EZ-RUN™ Pre-Stained Rec Protein Ladder and 2 µl of ThermoScientific SuperSignal Molecular Weight Protein Ladder were loaded into respective lanes on each gel and topped to 10 µl with 1x SSB. The gel was run for 1 hour (50 minutes if probing for cp-Thu4) at 200 V.

The gel is then assembled into a gel sandwich and the proteins are transferred to a nitrocellulose membrane in 1x transfer buffer (100 ml of 10x transfer buffer, 200 ml methanol and 700 ml water), run at 100 V for 1 hour. The proteins on the membrane were then visualized by the horseradish peroxidase-based Enhanced Chemi-Luminescence (ECL) system as previously described (Dabney-Smith et al., 2006).

The antibodies against cpTha4 (1:10000) and cpTatC (1:5000) are described in (Mori et al., 2001). Anti-Histone H3 (1:10000) was purchased from Agrisera (www.agrisera.com). The antibody against mtTATC (1:500), pre-immune serum (1:1000) and two separate mtTATC peptides (Table 2.2) were all obtained from GenScript. Each of these underwent optimization to determine which test bleed of antibody to use for future experiments.

For western blot testing, each peptide based on the desired peptide dilution (1:125, 1:250, 1:500) was pre-incubated with 100 µl of mtTATC antibody (1:500) for 2 hours at 4ºC. This peptide + antibody mixture was then added to 25 ml of a solution of 5% w/v nonfat dry milk, 1X TBS, 0.1% Tween® 20 prior to using it to treat the blocked membrane.
Table 2.2. mtTATC peptides summary. Peptide 1 sequence is the inverted sequence of Peptide 2. Both have similar molecular weights (MW) and purity. However, Peptide 2 was easily soluble in FA H₂O and was diluted to a much higher stock concentration prior to addition to the nitrocellulose membranes.

<table>
<thead>
<tr>
<th></th>
<th>Peptide 1</th>
<th>Peptide 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sequence</strong></td>
<td>NH₂-EISGSERMGSTWGEEC- COOH</td>
<td>H₂N-CEEGWTSGMRESGSIE- COOH</td>
</tr>
<tr>
<td><strong>MW (g/mol)</strong></td>
<td>1756.88</td>
<td>1756.86</td>
</tr>
<tr>
<td><strong>Purity (%)</strong></td>
<td>86.4</td>
<td>88.0</td>
</tr>
<tr>
<td><strong>Stock concentration</strong></td>
<td>2 µg/ml</td>
<td>4 mg/ml</td>
</tr>
<tr>
<td><strong>Solution</strong></td>
<td>DMSO, FA H₂O</td>
<td>FA H₂O</td>
</tr>
</tbody>
</table>

For mass spectroscopic analysis, the first peptide which was ordered (Peptide 1) was solubilized in DMSO solution to a stock concentration of 2 µg/ml. 1 µl of Peptide 1 was zip tipped to replace DMSO solution with high boiling point with volatile acetonitrile. This was mixed 1:5 with HCCA (α-cyano-4-hydroxycinnamic acid) matrix. 1 µl of this solution was loaded on spot, air dried for 15 minutes and tested for purity using the MALDI-TOF Berker autoflex III smartbeam mass spectrometer in the Miami University Mass Spectrometry Facility. The second peptide (Peptide 2) was easily solubilized in FA water to a high final stock concentration of 4.4 mg/ml. Since the solution did not contain DMSO solution, we directly mixed 1 µl of peptide in a 1:5 ratio with HCCA matrix prior to mass spectroscopic analysis.

2.3.4 Preparing and confirming GFP transformed plants

The following Green Fluorescent Protein (GFP) constructs amplified in pFGC5941 dsRNA binary vector, were obtained as prepared by (Pal, 2014). The constructs will be referred to by the names in parenthesis for easier reference in the future.

i) Empty pFG5941 vectors (Blank)
ii) GFP-pFGC5941 (GFP)
    pFGC5941 dsRNA binary vector was used as a vector to insert the green fluorescent protein
    (GFP) coding gene from the Coxtp-GFP-pGEM4Z plasmid.

iii) preAtTha4tp-GFP-pFGC5941 (Tha4-GFP)
    GFP was C terminally fused to the sequence for the Arabidopsis thaliana Tha4 presignal,
    preAtTha4tp found in GenBank (GenBank: AY091429.1)

iv) coxtp-GFP-pFGC5941 (Cox-GFP)
    GFP was C terminally fused to the mitochondria specific protein cytochrome C oxidase signal,
    Coxtp.

v) prSSU-GFP-pFGC5941 (prSSU-GFP)
    GFP was C terminally fused to the precursor of the small subunit of ribulose-1, 5-bisphosphate-
    carboxylase.

Wild type Col-0 Arabidopsis thaliana plants were grown at 23 °C in a growth chamber under a 16
h light/8 h dark photoperiod (light intensity 88 uE m⁻² s⁻¹ for six weeks or until they flowered.
Floral-dip method was used to introduce the transformed agrobacteria cells to the plant (Zhang et
al., 2006).

For Agrobacterium transformation, 2 µl of the individual plasmid constructs at 1 µg final
concentration were added to separate tubes containing the thawed competent agrobacteria cells on
ice. The tubes were then placed in liquid nitrogen for 5 minutes, then in a 37 °C water bath for 5
minutes and subsequently in an ice bucket for 30 minutes. 300 µl of Super Optimal broth with
Catabolite repression (SOC) medium were added to each tube to increase transformation
efficiency. The cultures grew for 1 hour at 28°C at 250-rpm on a shaker. 50 µl of each culture was
transferred to individual plates (55 °C pre-heated LB agar plates with 50 µg/µl kanamycin for
selection) and incubated at 28°C for 48 hours.

One colony was carefully picked up and transferred to 100 ml of liquid LB medium, again with
50 µg/µl kanamycin for more thorough selection. The flask was covered in aluminum foil and left
overnight at 28°C on a 250 rpm orbital shaker. Bacteria grew to stationary phase where the optical
density 600 nm (OD₆₀₀) was 1.5-2.0 (Jiang et al., 2006). 3 ml of solution from each flask was
extracted to carry out plasmid DNA elution using the PureYield™ MiniPrep System. The resulting
GFP clone from each colony was confirmed by sequencing the DNA on both strands, using 35S promoter specific 557 forward primer and GFP specific reverse primer at the Center for Bioinformatics and Functional Genomics at Miami University (Table 2.1).

The culture was then centrifuged at 3700g for 15 minutes. The supernatant was discarded and the pellet was suspended with an equal volume of dipping solution, 5% sucrose in solution. 0.02% concentration Silwet L-77 solution was added to help the culture solution to adhere to the plant leaf. Floral dip method was performed to coat the inflorescences and shorter rosette with the solution. (Zhang et al., 2006)

Plants were grown in the same conditions for 4 more weeks and then watering was withheld when siliques turned brown. Mature seeds were collected and incubated at 28°C for 3 days to allow seeds to dry, and then stratified at 4°C for 7 days. Transformed seeds and original Col-0 seeds were planted and grown once again treated with the Basta herbicide to select for transformed plants.

For Basta treatment, the seedlings were first allowed to develop up to four to six leaves. The seedlings were then sprayed from the top, with a solution of 0.1% Basta and 0.02% Silwet L-77. Treatment was repeated twice a week for the next 4 weeks until the non-transformed plants wilted and died while the transformed plants continued to grow healthily (Logemann et al., 2006). Mature seeds from the Basta selected plants were collected and incubated at 28°C for 3 days to allow seeds to dry, and then stratified at 4°C.

2.3.5 Confocal Microscopy Imaging & Analysis

Seeds from each confirmed transformed plant, namely GFP, At-Tha4, Cox-GFP, prSSU-GFP and wild type Col-0 were grown on two separate plates. The plants were grown for two weeks as described under section 2.3.1 “Plant Materials and Growth Conditions”. One of the plates with a construct type was treated with 25 ml of 400 µM solution of salicylic acid and the other plate which was the control, was treated with 25 ml of water. Both plates were placed in the 23 °C growth chamber for the rest of the experiment. Wild-type Col-0 was used as a biological control. Once again, one plate of Col-0 received the 400 µM solution of salicylic acid treatment while the other was treated with water.
At 1 hour, 4 hours and 24 hours after treatment, leaf and root tissue were each cut from each plant and placed on a glass microscope slide. Each sample was stained for 15 minutes in the dark with 50 µl of 50 nM MitoTracker® Orange CMTMRos solution, prepared as directed by the manufacturer. The leaves were gently rinsed of the dye using a few drops of Millipore water and a glass cover slip was placed on each sample. A fine brush was used to seal the cover slip with VALAP, a mixture of equal parts Vaseline, paraffin wax and lanolin heated on a hot plate at 60 ºC.

Samples were observed and imaged at 1, 4 and 24 hour post-treatment using the Zeiss 710 Laser Scanning Confocal System in the Center for Advanced Microscopy and Imaging (CAMI) at Miami University.

After detailed testing, three separate laser channels were chosen to image the samples (Jiang et al., 2006):

1. A GFP channel with a single wavelength excitation at 488 nm with emission characterized from 493 nm to 544 nm
2. A MitoTracker® Orange channel with single wavelength excitation at 543 nm with emission characterized between 544 nm to 601 nm
3. A Chlorophyll autofluorescence channel with single wavelength excitation at 633 nm and emission characterized from 647 nm to 721 nm.

These settings were chosen carefully to characterize GFP colocalization with either the chloroplast or mitochondria, while also accounting for the naturally occurring chlorophyll autofluorescence signal, but without masking the other signals. This was especially challenging in leaf tissue where chloroplasts and therefore pigments are abundant. We chose to account for chlorophyll a over chlorophyll b, as the absorption spectra for chlorophyll b was too close to that of MitoTracker® Orange with a wavelength excitation at 543 nm.

Channel pseudo colors (Table 2.3) were chosen based on ease and ability to distinguish between a colocalization signal and a single channel emission spectrum. On the image, a GFP and MitoTracker colocalization produces yellow colored pixels while a GFP and chlorophyll colocalization produces bright, cyan colored pixels.
Table 2.3. Summary of optimal parameters on the Zeiss LSM710 Confocal Microscope. These settings were saved as “Mito Project Configuration – TSW” on the software for future use.

<table>
<thead>
<tr>
<th>Channel</th>
<th>GFP</th>
<th>MitoTracker® Orange</th>
<th>Chlorophyll autofluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laser</td>
<td>Argon 488</td>
<td>HeNe543</td>
<td>HeNe633</td>
</tr>
<tr>
<td>Gain (Master)</td>
<td>1153</td>
<td>900</td>
<td>638</td>
</tr>
<tr>
<td>Excitation (nm)</td>
<td>488</td>
<td>543</td>
<td>638</td>
</tr>
<tr>
<td>Emission (nm)</td>
<td>493-544</td>
<td>544-601</td>
<td>647-721</td>
</tr>
<tr>
<td>Pinhole (~1 airy units)</td>
<td>34.1</td>
<td>34.1</td>
<td>34.1</td>
</tr>
<tr>
<td>Channel pseudo colour</td>
<td>Green</td>
<td>Red</td>
<td>Blue</td>
</tr>
</tbody>
</table>

Additional settings were used during focusing and image acquisition; a EC Plan-Neofluar 40x/0.75 M27 objective lens, 1024 x 1024 frame size, averaging number:1, mode: line, bit depth: 8-bit, and finally acquisition speed: 4, which resulted in a pixel dwell of 12.61 microseconds and a scan time of 44.89 seconds per image.

In addition to the qualitative information, quantitative data was acquired from each image as described by (Zinchuk and Grossenbacher-Zinchuk, 2011). Background correction was applied until the scatter gram showed pixels concentrating along a diagonal focal point or, as close to a diagonal as possible when there was no qualitative colocalization. Once a background correction setting was chosen, it was used consistently for all images.

To investigate and quantify GFP colocalization in chloroplast we collected signals from the GFP and Chlorophyll autofluorescence pair of channels. Accordingly, For GFP colocalization in mitochondria, we collected signals from the GFP and MitoTracker® Orange pair of channels. First, whole image colocalization data was acquired. Then, colocalization data from three separate regions of interest (ROI) within the same image were obtained. For each pair of channels, the data collected includes the Colocalization coefficients for each channel ($M_1$, $M_2$), Pearson’s Coefficient ($R_1$) for each channel and the Overlap coefficient ($R$) between the two channels according to Manders.
For each image, average values from the ROI’s were calculated and Quantitative Colocalization Analysis (QCA) along the 24 hour time series were conducted. In data analysis, the colocalization coefficients (\(M_1, M_2\)) and Manders values (R) were given higher priority since they reflect the true degree of signal overlap between the two channels. It is especially useful in comparison studies where the intensities of fluorescence differs; some GFP signals (prSSU-GFP) are stronger than others as discussed in the “Results” section (Zinchuk and Grossenbacher-Zinchuk, 2011).

2.3.6 Transmission Electron Microscopy (TEM)

To investigate any ultrastructural changes in subcellular organelles in response to salicylic acid stress, we performed high resolution TEM. Green pea plants, Pisum sativum (Little Marvel) were grown for 10 days. 25 ml of 400 µM salicylic acid solution was added to a plate with five plants and 25 ml of water was added to another plate as a control and incubated in ideal growing conditions for 6 hours. 0.5-1 mm\(^3\) pieces of leaf were cut and fixed overnight at room temperature in 2.0% glutaraldehyde/ 2.0% paraformaldehyde/1% sucrose in 0.05M sodium cacodylate buffer (pH 7.2) (Adamakis et al., 2008). Samples were rinsed three times for 15 minutes each with 0.05 M sodium cacodylate buffer and then fixed in 2% osmium tetroxide solution in 0.05M sodium cacodylate buffer for 8 hours. Samples were rinsed five times for 20 minutes each with distilled water and then fixed for 9 hours in the dark and at room temperature, in 0.05% Uranyl Acetate in aqueous solution, as it stains membranes (Pandithage, 2013). Samples were rinsed four times for 15 minutes each in distilled water and then dehydrated in increasing concentrations of acetone (25% for 15 minutes, 50% for 15 minutes, 75% for 30 minutes, twice at 95% for 30 minutes each and finally twice at 100% for an hour each). Infiltration was carried out at room temperature with a series of concentrations of Spurr’s resin: Acetone (1:3, 1:1, 3:1) with a minimum of 3 hours per step. Samples were then infiltrated with 100% Spurr’s resin for 48 hours and fresh 100% Spurr’s resin for 4 hours, and finally embedded in fresh resin molds for 72 hours at 60 °C. (Spurr, 1969)

90 nm thick sections were obtained using the Reichert Ultracut S Ultramicrotome and placed on TEM copper grids. Samples were observed and imaged using a JEOL JEM-1200EX II Transmission Electron Microscope at 100 KeV with 6 second camera exposure time, in the Center for Advanced Microscopy and Imaging (CAMI) at Miami University.
2.4 Results

2.4.1 Salicylic acid induced \textit{mtTATC} mRNA expression levels in wild type and thylakoid transport mutant of \textit{Arabidopsis thaliana}

2.4.1a. Salicylic acid upregulates \textit{mtTATC} transcript levels in wild-type \textit{Arabidopsis thaliana}

A study in \textit{Nicotiana tabacum} showed that an open reading frame (orfX) in the mitochondrial genome was transcriptionally active when treated with salicylic acid. This orfX was identified as \textit{mtTATC} as it had a 98.5\% conserved domain homology with \textit{E.coli} TatC (van der Merwe and Dubery, 2007). A similar orfX was found in the \textit{Arabidopsis thaliana} mitochondrial genome. In this study, we wished to investigate whether this orfX coded for a pseudogene or a real gene, and if its expression changed, with exogenous salicylic acid stress which mimics the plant hypersensitive response.

To examine this we applied 400 \textmu M exogenous salicylic acid or water (control) to wild type Col-0 leaves for 3 hours. We used qRT-PCR to measure expression levels of the putative \textit{mtTATC} gene against the internal control gene \textit{UBQ4}. Salicylic acid significantly increased \textit{mtTATC} levels (Figure 2.1). The \textit{mtTATC} orfX in \textit{Arabidopsis thaliana} is transcriptionally active and responsive to salicylic acid stress.
**Figure 2.1: mtTATC expression upregulated with salicylic acid stress.** Wild type Arabidopsis thaliana plants were grown on ½ MS medium for 2 weeks and treated with 400 µM salicylic acid stress treatment or control (water) for 3 hours. *mtTATC* cDNA were obtained from RNA isolation and reverse transcription. qRT-PCR measured expression levels. Data are given as the average of three biological replicates, with three technical repeats per biological sample. Error bar = SE. p < 0.05, significantly different from the control.

### 2.4.1b mtTATC transcript expressed in a time-dependent manner with salicylic acid stress

Nie et al., showed that 500 µM salicylic acid stress increased mitochondrial ROS and Aox1a transcript expression levels in a time dependent manner. The transcript reached its highest level at 4 hours post-treatment and declined to its original level after 24 hours. Plants experience an initial HR and a prolonged SAR. Therefore, we studied the time-dependent expression of the stress induced *mtTATC* transcript levels over a 24-hour time period. We limited our study to only 24 hours since even low SA concentrations of 20 µM applied over 3 minutes inhibits ATP synthesis; at the high 500 µM SA concentration beyond 24 hours, we would not get a reliable reading of SA induced *mtTATC* activity (Xie and Chen, 1999).

500 µM SA or water (control) was applied to wild type Col-0 plants. RNA isolation was carried out 0, 1, 4 and 24 hours post-treatment and levels of *mtTATC* internal control gene *UBQ4* levels were analyzed at each time (Nie et al., 2015). **Figure 2.2** shows that overall, salicylic acid increased *mtTATC* levels after 0 hours (moment of treatment). *mtTATC* transcript expression increased by over four fold 1 hour post-treatment and declined until 4 hours, before reaching its peak at 24 hours post-salicylic acid treatment. The two peaks in *mtTATC* expression levels suggest that it is SA concentration and time dependent and is affected by the plant stress response.
Figure 2.2: *mtTATC* transcript levels peak twice during 24-hour SA treatment. Wild type Arabidopsis thaliana plants were grown on ½ MS medium for 2 weeks and treated with 500 µM salicylic acid stress treatment or control (water) for 3 hours. *mtTATC* cDNA were obtained from RNA isolation and reverse transcription. qRT-PCR measured expression levels. All data on the graph is normalized to the internal control gene as well as the untreated control group. Data are given as the average of three biological replicates, with three technical repeats per biological sample. Error bar = SE. p < 0.05, significantly different from the control.

2.4.1c *mtTATC* levels upregulated in thylakoid transport mutant *Arabidopsis thaliana*

The mitochondrion and chloroplast exhibit cross-talk; for instance, lack of activity in one organelle is complemented by upregulation of transcripts or molecular re-routing for the same activity in the other organelle. For instance in diatoms which are unicellular eukaryotes, during iron-limiting conditions that disrupt mitochondrial respiration electrons are re-routed from the chloroplast to the mitochondria (Prihoda et al., 2012). This offers the mitochondria temporary protection from oxidative stress.

For this study we used a thylakoid TatB deficient mutant *hcf106*, produced by a T-DNA insertion to give the pale green and white phenotype SALK_044421C (Ma, 2016). We aimed to investigate whether mitochondrial *mtTATC* expression is affected by a defective chloroplast Tat system with decreased photosynthetic energy input. RNA isolation in mutant and wild type (control) plants was
followed by qRT-PCR analysis of *mtTATC* and internal control gene *UBQ4*. Figure 2.3 shows elevated *mtTATC* levels in the *hcf106* mutant plant. In the chloroplast Tat mutant, the mitochondrion maybe helping the cell compensate for decreased photosynthetic energy input (Ma, 2016).

![mtTATC expression upregulated in hcf106 mutant](image)

**Figure 2.3: mtTATC expression upregulated in hcf106 mutant.** Wild type and mutant *Arabidopsis thaliana* plants were grown on ½ MS medium for 2 weeks. *mtTATC* cDNA were obtained from RNA isolation and reverse transcription. qRT-PCR measured expression levels. Data are given as the average of three biological replicates, with three technical repeats per biological sample. Error bar = SE. p < 0.05, significantly different from the control.
2.4.2 mtTATC protein expression in *Arabidopsis thaliana* treated with salicylic acid

2.4.2a Testing and confirming a functional mtTATC antibody

The antibody was designed against amino acids 255 to 269 CEEGWTSGMRESGSIE from *Arabidopsis thaliana* mtTATC (GenBank: CAA69741.3) with a peptide-KLH immunogen (Figure 2.4A). The putative mitochondrial TATC amino acid sequence generated from the orfX sequence has 6 predicted transmembrane domains like known TatC structures (Berks, 2015). Given the parallel biochemistry, a functional mtTATC protein would have a similar structure and be incorporated into the membrane similarly too (Figure 2.4B).

![Diagram A](image1)

![Diagram B](image2)
Figure 2.4: Putative mitochondrial TATC has 6 predicted transmembrane domains. (A) The amino acid sequence of orfX, the putative mitochondrial TATC encoded by the *Arabidopsis thaliana* mitochondrial genome (GenBank: CAA69741.3). The sequence used as antigen to generate an antibody is underlined in bold and predicted transmembrane regions are highlighted in blue. (B) Predicted topology of the putative mtTATC protein by various algorithms. The amino acid sequence for mtTATC was used for topology predictions at the TOPCONS consensus topology predictor site (http://topcons.net/pred/result/rst_WIZpzC/) which compiles several algorithms [OCTOPUS (Viklund and Elofsson, 2008), Philius (Reynolds et al., 2008), PolyPhobius (Kall et al., 2005), SCAMPI (Bernsel et al., 2008), SPOCTOPUS (Viklund et al., 2008)] into one site comparing to the only known structure of a TatC protein (pdb:4b4a) from *Aquifex aeolicus* (Rollauer et al., 2012).

We received antiserum from multiple rabbits and therefore wished to identify one specific to our antigen. We compared membranes treated with the pre-immune serum, to those treated with the antibodies raised from the same rabbit. The western blots from only rabbit #984, showed bands absent when treated with its corresponding pre-immune serum (Figure 2.5B).

The ~26 kDa band, corresponds to the previously visualized 27 kDa mtTATC and 28.9 kDa cpTatC (Carrie et al., 2016; Robinson and Bolhuis, 2004). The heavier ~56 kDa band can be a TatC protein dimer. Finally, the ~42 band maybe a non-specific interaction or a tatC protein attached to a Hcf106 subunit (18.4 kDa) (Cline and Mori, 2001). We thus confirmed that antiserum from rabbit #984 is specific to the *Arabidopsis thaliana* mtTATC and this was used in future experiments.
Figure 2.5: Confirming a viable mtTATC antibody. Proteins extracted from two-week-old wild type Col-0 plant were separated by SDS-PAGE, transferred to nitrocellulose membrane and visualized by treatment with pre-immune serum or antibody from the same rabbit. (A), Rabbit #986 pre-immune serum (1:100) already contained antibodies against the proteins visualized with the antibody treatment (1:500) (orange lines) (B). Antibody from #984 (1:500) binds to proteins not detected by antibodies in the pre-immune serum (1:500). The bands represented by (*) at ~26 kDa, ~43 kDa and ~54 kDa represent bands specific to our antigen of interest.

2.4.2b Testing the mtTATC peptide and assessing antibody specificity

The mtTATC peptide was designed using the same antigen sequence as for the antibody (GenBank: CAA69741.3). The first peptide (Peptide 1) we generated had an inverted sequence. We first suspected this when we attempted to solubilize the peptide in aqueous buffer. The peptide demonstrated very low solubility in water and subsequent consultation with the manufacturers (GenScript) revealed the inverted sequence. Subsequently we re-ordered the same peptide but with the sequence in the correct orientation (Peptide 2). Peptide 2 was orders of magnitude more soluble in aqueous buffer (4.4mg/ml).

Furthermore, the mass spectroscopic analysis of both peptides confirmed that only Peptide 2 had the expected molecular weight of 1756.8 m/z for our mtTATC antigen sequence (Figure 2.7). The
highest peak for Peptide 1 was at 1695 m/z (potentially caused by the loss of an entire amino acid) while the highest peak for Peptide 2 was at the expected m/z.

Figure 2.6: Mass spectroscopy confirmation that Peptide 2 has the expected molecular weight. 1 µl of zip tipped Peptide 1 (A) and 1 µl of Peptide 2 (B) were each loaded 1:5 into an HCCA matrix and independently analyzed with mass spectroscopy. Orange star: expected weight (1756.8 m/z). Blue star: peak with highest signal. (Not shown on B: peaks to the left on the x-axis. They recorded as 7% or less of the signal intensity of the highest peak and therefore were considered irrelevant).
To determine non-specific binding of the antibody (#984) to the antigen (CEEGWTSGMRESGSIE), we conducted an immunizing peptide blocking experiment. One membrane was treated with only the antibody and the other was treated with a mixture of antibody and peptide, and they were compared side-by-side (Figure 2.7). We repeated the experiment with each individual peptide, as an additional way to confirm that Peptide 2 had the correct sequence. As expected for Peptide 1 which has an inverted sequence, the bands between the antibody and ab+ peptide mixture treatments are identical.

However, in the Peptide 2 blot at 1:3 concentration, the ~26 kDa bands (orange rectangle) are significantly fainter compared to with the antibody. Again, we show that Peptide 2 and not Peptide 1, is more likely to have the matching peptide sequence to the mtTATC #984 antibody. Furthermore, only the ~26 kDa band and not the ~43 kDa or ~56 kDa bands (Figure 2.5) appear to be fainter, indicating that only these bands are specific to our antibody.

Figure 2.7: Peptide blocking experiment confirms that mtTATC antibody is specific to ~26 kDa bands. Protein extraction from two-week-old wild type Col-0 plant and immunoblot detection analysis. Peptide 1 was diluted to 2 µg/ml or 4 µg/ml and Peptide 2 was diluted to very high concentrations of 44 µg/ml or 88 µg/ml. Each peptide was pre-incubated with #984 1:500 mtTATC antibody for 2 hours at 4°C prior to addition to the nitrocellulose membrane. Peptide 2 is the viable peptide. The antibody is specific to the ~26 kDa bands (orange rectangle). (+) indicates with salicylic acid and (−) indicates absence of salicylic acid but these results will be discussed in a subsequent section since two lanes were only included as test repeats.
2.4.2c Effect of different concentrations of SA on phenotype of wild type *Arabidopsis thaliana*

20 µM exogenous SA applied over 3 minutes impaired cellular functions at the biochemical level (Xie and Chen, 1999). However, we hoped to deduce when and at what salicylic acid concentrations, *Arabidopsis thaliana* shows *visible* oxidative damage. This is generally characterized by loss of rigidity, or light green or white leaf phenotype indicative of significant oxidative bleaching.

We treated two-week-old wild type plants grown on ½ MS plates with 200, 400, 800 and 2000 µM concentrations of salicylic acid. Water was applied as a control treatment (Nie et al., 2015). We imaged them after for 0, 1, 4 and 24 hours. Both groups appeared the same and healthy after an hour. After 4-hours, the 200 µM plants appeared healthy, while those at concentrations > 400 µM had lost rigidity and 2000 µM treated plants showed slight bleaching. After 24-hours, all SA treated plants had lost rigidity. Only the 800 µM and 2000 µM plants showed significant bleaching and oxidative damage (*Figure 2.8*).

Overall, our data indicates that SA concentrations > 400 µM produced visible phenotypic changes in *Arabidopsis thaliana* such as loss of rigidity. Very high SA concentrations of 800 µM and 2000 µM result in the most observable oxidative damage, as these plants showed the earliest and most oxidative bleaching.
Figure 2.8: Effect of different SA concentrations on wild type Arabidopsis thaliana phenotype. Wild type plants treated with various salicylic acid concentrations or water and imaged at 0 and 24 hours post-treatment. The 800 µM and 2000 µM SA treated plants showed the earliest and most oxidative bleaching and damage (yellow star).

2.4.2d Time-course study of the effect of SA on mtTATC protein expression levels in wild type Arabidopsis thaliana

qRT-PCR analysis confirmed expression and upregulation of a mtTATC transcript during SA treatment in wild type Arabidopsis thaliana (refer 2.4.1a). Transcript expression varied over time (Figure 2.2). We then wished to investigate the expression of the mtTATC protein during SA treatment and whether or how it may change over a 24-hour time period. This would help us better understand its role during the plant mitochondrial stress response.

We treated two-week-old wild type plants grown on ½ MS plates with 200, 400, 800 and 2000 µM SA concentrations. Water was used as a control treatment (Nie et al., 2015). We then extracted proteins at 0, 1, 4 and 24-hours post-treatment and carried out immunoblotting with #984 mtTATC antibody (1:500).
Although the \textit{mtTATC} transcript is upregulated at with SA, \textit{mtTATC} protein expression levels remained the same as control (C) at these concentrations (200-2000 µM). Furthermore, at these lower SA concentrations \textit{mtTATC} protein expression remained the same over 24-hours. However, at higher SA concentrations of 800 and 2000 µM, the \textit{mtTATC} protein is not visualized at 1 and 24 hours, but is seen at 0 and 4 hours (Figure 2.9). Therefore, our time-course study shows that \textit{mtTATC} protein expression varies at very high SA concentrations. This suggests that \textit{mtTATC} protein expression may play a role in the plant stress response at high SA concentrations.

\textbf{Figure 2.9: Time-course study of \textit{mtTATC} expression levels in \textit{Arabidopsis thaliana} with SA treatment.} Two-week old wild type \textit{Arabidopsis thaliana} plants were treated with 200, 400, 8000 or 2000 µM SA or water (control). Proteins were extracted at 0, 1, 4 and 24 hours post-treatment, separated by SDS-PAGE at equal protein loading, and visualized using an antibody against the ~26 kDa sized \textit{mtTATC} protein (yellow triangle). When expressed, the \textit{mtTATC} levels remained the same as control at all SA concentrations (200 to 2000 µM). At low or medium SA concentrations (200 µM, 400 µM) \textit{mtTATC} protein expression remained the same over time. However at very high SA concentrations (800 µM, 2000 µM) \textit{mtTATC} was expressed at 0 and 4 hours but not at 1 and 24 hours (yellow box).
2.4.3 cpTha4-GFP dual localization in *Arabidopsis thaliana* treated with salicylic acid

A minimal Tat pathway found in certain gram-positive bacteria comprises TatC and the Tha4 orthologue, TatA (Celedon and Cline, 2013). During stress conditions, cells can redistribute dual targeted proteins from one compartment to the other (Carrie et al., 2016; Stengel et al., 2010). The major goal of this study was to investigate the possible dual localization or redistribution of the nuclear encoded, cpTha4 component to the mitochondria under SA stress conditions. Together with a mtTATC component, this could then form a minimal functional Tat system in the plant mitochondria.

For this study, we used confocal microscopy to compare the localization patterns of Green Fluorescent Protein (GFP) fused cpTha4 over a period of 24-hours, with and without salicylic acid stress. We analyzed these protein localization patterns both qualitatively and quantitatively (Speth et al., 2009).

2.4.3a Confirming agrobacterium-mediated GFP transformation of *Arabidopsis thaliana*

We made a Green Fluorescent Protein (GFP) fused to pFGC5941 plasmid and then ligated this with the sequence for cpTha4 (Figure 2.10). The GFP-pFGC5941 construct then underwent additional modifications to create the controls for the study: the At-Tha4-GFP construct, the Cox-GFP construct and the prSSU-GFP construct as described in section 2.3.3. These GFP constructs and a blank pFGC5941 plasmid were incubated in separate competent agrobacteria cultures, selected for by kanamycin and confirmed with DNA sequencing at the Center for Bioinformatics and Functional Genomics at Miami University.
**Figure 2.10: Generation of the GFP constructs** A) pFGC5941 binary vector containing the 35S promoter. B) Full length GFP coding sequence was removed from **Cox**\textsubscript{ts}-GFP-pGEM4Z vector and ligated into pFGC5941 vector to make the GFP-pFGC5941 construct. Images adapted from (Pal, 2014).

Agrobacteria was applied to Col-0 wild-type *Arabidopsis thaliana* by floral-dip method. GFP-transformed plants which contain the *bar* gene for resistance to the herbicide Basta, were selected.
for by the same (Figure 2.11) (Rathore et al., 1993). A control pot of wild-type plants without agrobacteria treatment were grown as well and they all wilted and died after the first two Basta treatments (Figure 2.11, Control).

We successfully obtained healthy, transformed plants for all the different GFP-constructs except the blank one. The one surviving Blank-GFP plant rapidly extended its shoots within a week of Basta treatment; then its flower buds wilted, the seeds failed to dry and the plant died (Figure 2.11, Blank).

**Figure 2.11: Basta selection of transformed Arabidopsis thaliana plants.** Flowering wild-type plants underwent agrobacterium mediated floral-dip transformation and then grown on soil for several weeks with 2% Basta applications. All plants on the control plate without agrobacteria transformation withered and died (top left). About 2% of the agrobacterium transformed plants survived (red star) as expected for the Col-0 ecotype (Zhang et al., 2006).
The Basta selected transformed plants were grown for several more weeks. Their mature seeds were grown on separate plates of ½ MS according to the plant growth conditions given in section 2.3.1. The plants from this set of seeds were used in the future confocal microscopy experiments.

**Figure 2.12** shows the different phenotypes of the plants transformed by the different types of GFP constructs. The Tha4-GFP mutant had circular, dark leaves of above average size. Cox-GFP mutant had the biggest, lighter green leaves. prSSU-GFP mutants grew the quickest and appeared the healthiest. We obtained very few viable seeds from plants transformed with the GFP-only construct, but the ones that grew appeared healthy apart from a few wilted or abnormally small leaves.

![Tha4-GFP](image1.png) ![Cox-GFP](image2.png) ![GFP](image3.png) ![prSSU-GFP](image4.png)

**Figure 2.12. Different phenotypes of the GFP transformed plants.** Mature seeds from Basta selected transformed plants were grown on ½ MS medium and imaged. All plants appeared healthy except for the GFP-only construct which had smaller, pinched leaves.
2.4.3b Confocal microscopy qualitative analysis of GFP localization

In this study we aimed to use confocal microscopy to characterize the possible cpTha4 dual localization to both the chloroplast and the mitochondria during salicylic acid stress. We treated 2-week old wild-type and Tha4-GFP mutant plants with 400 µM SA since concentrations above this resulted in dramatic phenotypic changes (Figure 2.8). We also used the same SA treatment times as for the mtTATC protein expression study (0, 1, 4 and 24 hours) to better understand how both these components behave and possibly interact at the same time to form a minimal Tat pathway.

Prior to imaging, all samples (roots and leaves) were stained with MitoTracker® Orange CMTMRos dye to allow mitochondrial signal visualization. The MitoTracker® dye concentration and time had to be carefully optimized since we cannot use anti-fade; it causes interference during confocal microscopy image acquisition (Zinchuk and Grossenbacher-Zinchuk, 2011). The optimum MitoTracker® Orange dye parameters for our samples are 50 µl of 50 nM per slide, for exactly 15 minutes (Figure 2.13c). At lower concentrations or shorter times the mitochondria stained too weakly and the signal was absent (Figure 2.13a) and at higher concentrations or longer times the dye saturated the sample and we could not see distinguish the mitochondria (Figure 2.13b).

![Figure 2.13. MitoTracker® Orange dye optimization.](image)

**Figure 2.13. MitoTracker® Orange dye optimization.** Wild-type Col-0 roots were stained with different concentrations and times of MitoTracker® Orange to select the optimal conditions for our samples. A. Concentration and time too low. B. Concentration and time too high. C. Samples stained for 15 minutes at 50 nM concentration. The mitochondria are visible as little punctate dots.
Confocal microscopy imaging was performed at 1, 4 and 24 hours post-salicylic acid treatment to assess the pattern of dual localization over time. Three separate channels, each with a pseudo-colour to best visualize the localization were used. (1) Red colored channel for mitochondria stained with MitoTracker Orange, (2) Green colored channel for GFP and (3) Blue colored channel for chlorophyll autofluorescence (chloroplast). A GFP localization in mitochondria appears yellow (red & green combined) and a GFP localization in chloroplast appears a bright cyan (green & blue combined).

Before qualitatively assessing the cpTha4 confocal images, we studied the images of the other GFP controls first to (1) see whether the GFP signals localizes as expected at all times and are affected by SA chemical treatment and (2) gain a visual understanding of a ‘good’ colocalization signal for our GFP constructs and samples.

i) Wild-type *Arabidopsis thaliana*

We first attempted to see how the signals from each of the three channels appear in a non-transformed, wild-type plant (no GFP). The three-channel merged images for the leaves show signals for mitochondria (red) and chlorophyll (blue) but not for GFP (Figure 2.14, leaf). In the root where chlorophyll is absent, only mitochondrial signals can be seen as expected (Figure 2.14, root). Therefore, the confocal channels clearly show the signals from the organelles investigated in this study.
Figure 2.14: GFP signal is absent in wild type, non-transformed plants. Arabidopsis thaliana plants were treated with water (control). Their MitoTracker stained leaves and roots were imaged after 4 and 24 hours with a confocal microscope. Samples imaged at 40x with all three channels merged (n=3).

ii) prSSU-GFP Arabidopsis thaliana (chloroplast localization signal)

The prSSU-GFP protein is predicted to localize in the chloroplast. We wished to confirm this as well as study how this signal would behave when salicylic acid is applied. Figure 2.15 A, C show that prSSU-GFP works as expected when treated with water; it localizes in leaf chloroplast but not in the root where there is no chlorophyll. However colocalization (bright, cyan) decreases over 24 hours (Figure 2.15A). At all times no GFP signal was observed in leaves treated with salicylic acid. The chemical seems to hinder the GFP signal on prSSU (Figure 2.15B).
Figure 2.15: prSSU-GFP localization in *Arabidopsis thaliana* root and leaf. prSSU-GFP mutant plants were treated with SA or water (control) for 1, 4 and 24 hours. MitoTracker stained leaves and roots were imaged at 40x with a confocal microscope. (A) and (B) Imaged with the GFP and chlorophyll channels. (A) GFP signal seen in water treated leaves, but decreases over time. (B) No visible GFP signal in SA treated leaves. (C) Imaged first with the mitochondria channel and then the GFP channel. No clear GFP signal seen in root as expected (n=3).

iii) Cox-GFP *Arabidopsis thaliana* (mitochondrial localization signal)

Cox-GFP protein is predicted to localize in the mitochondria (positive control). Accordingly, with water (control) treatment the cox-GFP localized in the mitochondria as expected at all times; the yellow signal due to colocalization is seen throughout (Figure 2.16 A, B). High colocalization was also seen around the stomata, possibly since there is a lot of neighboring mitochondria to supplement its energy needs (Figure 2.16, leaf underside). However, no
colocalization or even a GFP signal was seen with salicylic acid treatment at all times, indicating that is hinders the GFP signal on Cox (Figure 2.16 C).

**Figure 2.16:** Cox-GFP localization in *Arabidopsis thaliana* root and leaf tissue. Cox-GFP plants were treated with SA or water (control) for 1, 4 and 24 hours. MitoTracker stained leaves and roots were imaged with a confocal microscope at 40x with all three channels merged. (A) (B). With water treatment, cox-GFP localizes in mitochondria (yellow) of leaves and roots at all times. (C) No GFP signal seen in roots treated with SA (n=3).
iv) GFP *Arabidopsis thaliana* (no localization signal)

In these plants the GFP signal is not fused to any protein and therefore should not localize on any specific cellular structure. Confocal microscopy images of water treated leaf samples confirmed this (Figure 2.17). The GFP signal was dispersed throughout the cell (Figure 2.17A) and was found on both the mitochondria (yellow) and chloroplast (cyan) in addition to other locations (Figure 2.17B). Hence, this GFP construct and signal work as expected in leaves. However, the GFP roots were very thin and brittle and there was no GFP observable signal (Figure 2.17C).

**Figure 2.17: GFP-only construct localization in *Arabidopsis thaliana* root and leaf.** GFP plants were treated with water. MitoTracker stained leaves and roots were imaged with a confocal microscope. (A) Imaged at 40x with the GFP channel. (B) Same as image A but imaged with all three channels merged. The GFP signal is dispersed everywhere as expected (C) Imaged at 10x with all three channels merged. No visible GFP signal in roots (n=3).
(v) cpTha4-GFP *Arabidopsis thaliana* (dual localization investigation)

cpTha4-GFP should be targeted to the chloroplast since cpTha4 is a component of the cpTat translocase. However, we could not make a qualitative interpretation of cpTha4-GFP localization in the leaves, since our confocal microscopy images did not show a clear GFP signal (Figure 2.18).

In the merged channels, the GFP signal may be masked by the chlorophyll or MitoTracker signal. However, when imaged with only the GFP channel, we did not see a visible green signal; this could be attributed to the structure of our GFP construct. The GFP signal is fused to the Tha4 protein pre-signal which is a very short amino acid sequence. However, if the GFP signal is expressed it will appear in a quantitative analysis of colocalization since the software is sensitive to each signal point. This will help us interpret cpTha4-GFP localization in leaf tissue more reliably.

![Figure 2.18: cpTha4-GFP localization in *Arabidopsis thaliana* leaf.](image)

Figure 2.18: cpTha4-GFP localization in *Arabidopsis thaliana* leaf. cpTha4-GFP leaves were treated with salicylic acid for 1, 4, and 24 hours. MitoTracker stained leaves were imaged at 40x with a confocal microscope with all three channels merged. The chlorophyll (blue) and mitochondrial (red) signals were seen clearly but a visible GFP signal was not observed (n=3).

Interestingly, we saw a cpTha4-GFP signal in the root tissue with both SA and water (control) treatment. Figure 2.19 shows this GFP signal as a yellow mitochondrial localization signal. The signal seems to decrease over time in the control treatment (Figure 2.19A), but increases or becomes clearer over time in SA treated plants. Quantitative analysis of the colocalization signal over time will provide more reliable information.
However, we still could not confirm whether cpTha4-GFP localizes in the root chloroplast as expected, since chlorophyll does not develop in the root.

![Figure 2.19: cpTha4-GFP localization in Arabidopsis thaliana root. Plants were treated with SA or water (control) for 1, 4 and 24 hours. MitoTracker stained roots were imaged at 40x with all three channels merged. (A) cpTha4-GFP localized in mitochondria at 1 hour and appeared to remain the same over time. (B) cpTha4-GFP localization in mitochondria appears to increase over time with SA treatment (more yellow).](image)

2.4.3c Confocal microscopy quantitative analysis of cpTha4-GFP dual localization

Although confocal microscopy images offer useful information, this qualitative data is biased as it depends on individual judgement of what the eye perceives. Therefore, this section offers a more reliable quantitative approach to investigating colocalization as explained by (Zinchuk and Grossenbacher-Zinchuk, 2011).

Two-week-old cpTha4-GFP plants were treated with either water or 400 µM SA for 1, 4 and 24 hours. Roots and leaves were stained with MitoTracker. Quantitative cpTha4-GFP co-localization data were collected with the confocal microscope software as explained in section 2.3.4 and the results analyzed according to Zinchuk et al.
cpTha4-GFP localization in chloroplast was lowest at 4 hours after SA treatment and then increased to a similar level as for mitochondria at 24 hours (Figure 2.20). Interestingly, cpTha4-GFP localized to mitochondria with and without SA. However, at all times mitochondrial localization was almost 11 times high with SA treatment, compared to the control treatment (too low to show on graph below). The highest mitochondrial cpTha4-GFP localization with SA stress is at 4 hours after treatment (three-fold increase since 1 hour) and then rapidly declines to original levels by 24 hours (Figure 2.20). Since mtTATC is also expressed at 4 hours post-SA treatment, a minimal Tat pathway composed of mtTATC and cpTha4 may exist in the mitochondria at this time.

![Figure 2.20](image)

**Figure 2.20:** cpTha4-GFP mitochondrial localization highest at 4 hours after SA treatment. GFP channel colocalization coefficient ($M_2$) values were averaged from > 5 Regions of Interest and expressed as a percentage (%). The red bars indicate the % of GFP pixels which co-localized with mitochondrial pixels and the blue bars indicate the % of GFP pixels which co-localized with chlorophyll/chloroplast pixels. cpTha4-GFP localizes preferentially in the mitochondria at 4 hours and then declines to original localization levels at 24 hours.
2.4.4 Salicylic acid induced ultrastructural changes in *Pisum sativum* mitochondria and chloroplast

Finally, we wished to characterize the physical manifestations of salicylic acid stress on the ultrastructure of plant organelles using Transmission Electron Microscopy (TEM). We especially focused on locations harboring a potential Tat system; the thylakoid membrane and mitochondrial inner membrane. Since the thick cell wall of *Arabidopsis thaliana* made TEM tissue preparation challenging, the experiment was performed on *Pisum sativum*. These results provide useful insight into salicylic acid induced ultrastructural changes in higher order plants.

![TEM ultrastructural analysis of mitochondria in *Pisum sativum* treated with 400 µM salicylic acid.](image)

Figure 2.21: TEM ultrastructural analysis of mitochondria in *Pisum sativum* treated with 400 µM salicylic acid. Prior to TEM treatment, leaf tissue was subject to either 400 µM salicylic acid or water (control) for 6 hours. Imaged with a TEM at 100KeV at 6 s exposure time, and images on the bottom are the same tissue location as in the maroon box, but imaged at a higher magnification. The star indicates an open region in the membrane where it appeared to be ruptured.
Salicylic acid stress was very detrimental to mitochondrial ultrastructure (Figure 2.21). The inner membrane was leaky or ruptured and had significantly reduced surface area due to distorted cristae. The matrix appeared swollen and lighter compared to control treatment, possibly due to loss of matrix proteins through the ruptured membranes. The intermembrane space was also narrower. Also, although mitochondria come in various shapes and sizes the SA treated samples consistently had more ‘stretched out’ mitochondria that were longer and thinner than usual.

In SA treated chloroplasts, the thylakoids appeared irregularly stacked and the grana were less compact. Also, the thylakoids had larger lumen space and appear inflated. However, the thylakoid membrane ultrastructure did not show any significant damage with SA treatment (Figure 2.22). Therefore, SA stress exerts more oxidative damage on the mitochondria and its inner membrane, compared to on the thylakoid membrane.

Figure 2.22: TEM ultrastructural analysis of chloroplast in Pisum sativum treated with 400 µM salicylic acid. Prior to TEM treatment, leaf tissue was subject to either 400 µM salicylic acid or water (control) for 6 hours. Imaged with a TEM at 100KeV at 6 s exposure time. Images on the bottom are the same tissue location as in the maroon box but imaged at a higher magnification.
2.5 Discussion

The mitochondrion and chloroplast are semi-autonomous organelles of prokaryotic origin which have their own internal genome and protein synthetic machinery (Birky, 1995). During pro-endosymbiotic evolution they lost most of their internal genome to the nucleus (Martin, 2003). In higher order plants the chloroplast has a unique protein transport pathway, the Twin Arginine Translocation (Tat) pathway which is entirely nuclear encoded and cytosolically synthesized (Cline and Dabney-Smith, 2008). The nuclear genes code for Tha4 (TatA), Hcf106 (TatB) and cpTatC (TatC) (Mori et al., 2001). In contrast mitochondria were thought to have lost the genes coding for these components.

But sequence analysis show that the mitochondrial genome of certain higher plants have an open reading frame (orfX) for TatC-like proteins although it is unknown whether it is actually expressed (Bogsch et al., 1998). A recent study in Nicotiana tabacum confirmed that the mitochondrial orfX for TatC was expressed when treated with salicylic acid stress, a chemical which mimics the plant hypersensitive response (van der Merwe and Dubery, 2007). In this study, we describe the salicylic acid stress induced expression of a putative mitochondrial Tat system in Arabidopsis thaliana.

Similar to N. tabacum, the mitochondrial genome of Arabidopsis thaliana contains an open reading frame with 98.5 sequence homology to E.coli TatC (Bogsch et al., 1998; Pal, 2014). We first performed qRT-PCR analysis to determine whether the orfX can be transcribed and/or if it is responsive to salicylic acid stress like the N.tabacum orfX. Our data confirms that the mitochondrial orfX in Arabidopsis thaliana is sensitive to exogenous stress (Figure 2.1).

Furthermore, salicylic acid upregulates mtTATC transcript levels in a time-dependent manner; expression peaks twice within 24-hours (Figure 2.2). The transcript increased almost five-fold in the first hour after SA treatment, similar to the upregulation of the pathogen-resistant AOX1 gene which helps the plant manage oxidative stress (Clifton et al., 2005; Nie et al., 2015). However, mtTATC expression declines ~4 hours and peaks again at ~24 hours unlike AOX1 which reaches its peak at 4 hours and then gradually declines until 24 hours. The two peaks in mtTATC can be explained by the two oxidative bursts and the biphasic accumulation of plant stress signals such as H₂O₂ and SA, which characterizes the initial hypersensitive response and later systemic acquired resistance in the plant defense response (Zhang and Xiao, 2015). The first oxidative burst is
immediate and maybe solely from exogenous SA stress while the second occurs about 24 hours later and may be from the accumulation of endogenous SA hormone as well (Alvarez et al., 1998; Baker and Orlandi, 1995). This increases the likelihood that mtTATC plays a role in the plant stress response and may explain why the mitochondrion retained this orfX despite losing most of its internal genome to the nucleus.

So far, only Oscarellidae expresses a functional mtTATC protein (Pett and Lavrov, 2013). We designed an antibody against the amino acid sequence of the mtTATC orfX to study if mtTATC protein is expressed in Arabidopsis thaliana treated with salicylic acid (Figure 2.4A). Our data show that a ~26 kDa mtTATC protein is expressed and that it can be recognized by the antibody (Figure 2.5). When expressed, the mtTATC levels remained the same as control at all SA concentrations (200 to 2000 µM) (Figure 2.9). Although the mtTATC transcript is upregulated compared to control, high SA concentrations maybe inhibiting cellular functions including mRNA translation (Silva et al., 2007). In fact, even 20 µM SA for 3 minutes impairs cellular biochemistry (Xie and Chen, 1999). SA could also be affecting post transcriptional control factors (Binder and Brennicke, 2003; Ho et al., 2008).

Furthermore, at lower SA concentrations mtTATC protein expression remained the same over 24-hours. However, at higher SA concentrations of 800 and 2000 µM, the mtTATC protein is not visualized at 1 and 24 hours but is seen at 0 and 4 hours (Figure 2.9). This discontinuous protein expression at high SA concentrations could be related to the biphasic accumulation of endogenous SA in the cell during stress (Baker and Orlandi, 1995). Furthermore, the first peak in mtTATC protein expression at 4 hours post-treatment is consistent with the first peak in mtTATC abundance at 1 hour (Figure 2.2) (Ndimba et al., 2005; Rhoads and McIntosh, 1992). Our Arabidopsis thaliana phenotypic studies also showed that plants treated with very high SA concentrations of 800 and 2000 µM showed the earliest and most significant signs of oxidative stress (Figure 2.8) (DeSalvo et al., 2008). Our data indicate that mtTATC protein expression varies at high SA concentrations suggesting it has a role in the plant stress response.

A minimal Tat system in gram-positive bacteria is composed of TatC and TatA (Tha4) (Barnett et al., 2008). In the chloroplast Tat system, cpTha4 forms the protein transporter pore and therefore is imperative for translocation (Aldridge et al., 2014; Ma, 2016). Therefore, we wished to study the presence of a Tha4 component in mitochondria. However, the mitochondrial genome does not
contain an orfX for *mtTHA4*. Interestingly, the chloroplast and mitochondrion display a high degree of cross-talk between them (Leister, 2005; Ma, 2016). For instance the mitochondrial *mtTATC* transcript was upregulated in a chloroplast Tat pathway mutant (Figure 2.3). Furthermore, over 100 proteins are dual targeted to both the chloroplast and mitochondrion (Carrie and Small, 2013). Therefore, after confirming mtTATC proteomic sensitivity to SA, we investigated the dual localization of the nuclear encoded cpTha4 component in mitochondria during stress.

We generated cpTha4-GFP constructs along with other controls to study dual localization (Figure 2.10). DNA sequencing and qualitative confocal microscopy studies showed that the GFP constructs worked as intended (Figure 2.15- 2.17). We confirmed that cpTha4 localized in the mitochondria during SA treatment. In roots, cpTha4-GFP localization in mitochondria increased over time with SA treatment (Figure 2.19). Quantitative analysis in leaves showed that cpTha4-GFP localization in mitochondria was highest at 4-hours after SA application. Conversely, chloroplast localization was at its lowest at this time (Figure 2.20).

This redistribution of a dual targeted protein between either component maybe a mechanism of environmental stress regulation (Carrie and Small, 2013; Stengel et al., 2010). In flowering plants, the mitochondrion, as opposed to the chloroplast is the site of most oxidative damage as well as protective stress mediation (Van Aken et al., 2009). TEM ultrastructural analysis too confirmed that salicylic acid causes significant damage to the mitochondrial inner membrane, but not to the thylakoid membranes. The mitochondria showed signs of mitophagy or early signs of apoptosis, such as ruptured membranes, loss of matrix proteins and swollen matrix (Figure 2.21) (Yang and Cortopassi, 1998). Therefore, during SA stress the dual-targeted cpTha4 maybe increasingly redistributed to the mitochondrion to form a minimal Tat pathway to mediate oxidative stress.

Salicylic acid is known to induce pathogen resistant (PR) genes which code for proteins that help mediate plant stress (Nie et al., 2015; Tiwari et al., 2002; Xie and Chen, 1999). However, as our data show, the expression of genes previously thought to be lost from the mitochondrial genome, especially ones that code for components of a novel mitochondrial protein transport system is a new area of research. Furthermore, mtTATC expression and cpTha4-GFP localization is SA concentration and time dependent, suggesting that a putative minimal mitochondrial Tat system may play an important role in the plant hypersensitive and immune responses.
2.6 References


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Chapter 3: Conclusions
3.1 Conclusions

3.1.1 Overview: Mitochondrial Tat system

The mitochondrion is a fascinating organelle with its own internal genome. Their origin, evolution, post-symbiotic genome loss and new functional roles are all being questioned through new scientific discoveries. New research focuses on the role of plant mitochondria as stress sensors; it coordinates the initial hypersensitive response (HR) and longer term plant immunity through systemic acquired resistance (SAR) (Bartoli et al., 2004; Carrie et al., 2009). The plant hormone salicylic acid (SA) upregulates pathogen-resistant (PR) genes, which code for mitochondrial proteins that mediate the plant defense response (Klessig et al., 2000; Nie et al., 2015). This complex process has many interconnected signals and regulatory pathways working together and much remains to be discovered.

In this study, we investigated the expression of a putative mitochondrial Twin Arginine Transport (Tat) system in Arabidopsis thaliana during salicylic acid stress. The unique Tat pathway, which is composed of three protein components TatA, TatB and TatC, transports fully folded proteins without the use of ATP energy. The Tat components have been well characterized in prokaryotes such as E.Coli and eukaryotic organelles such as the chloroplast, but not in mitochondria which share a similar evolutionary history. However, recent studies confirmed a salicylic acid stress inducible mtTATC gene in Nicotiana tabacum and a functional mtTATC protein in Oscarellidae (Pett and Lavrov, 2013; van der Merwe and Dubery, 2007).

3.1.2 mtTATC component during stress

We first confirmed that the mitochondrial orfX for mtTATC in Arabidopsis thaliana is expressed during salicylic acid stress. The expression of a mitochondrially encoded gene previously thought to be lost during mitochondrial evolution, especially one coding for a putative protein translocation system is significant (van der Merwe and Dubery, 2007). Furthermore, mtTATC expression over time is consistent with the biphasic accumulation of ROS and plant defense hormonal signals (Chamnongpol et al., 1998; Schneider et al., 2011; Zhang and Xiao, 2015). However, although low SA concentrations led to changes in mtTATC transcript levels, mtTATC protein expression only varied at high SA concentrations; it was expressed at 0 and 4 h but not at 1 or 24 h. SA induced oxidative damage to plant phenotype was also most visible at these high concentrations. Overall,
the SA concentration and time-dependent expression of mtTATC in Arabidopsis thaliana indicates that it is involved in the plant mitochondrial oxidative stress response.

Future experiments can study mtTATC protein levels in isolated mitochondria instead of whole leaf tissue to obtain a more accurate representation of expression. Furthermore, topology studies predict a six transmembrane domain structure for mtTATC which can be integrated into the membrane, much like known TatC structures (Figure 2.4A). Future work can investigate if this stress-responsive mtTATC protein is only expressed, or can also be incorporated in the mitochondrial IM to form a translocase, especially given the challenge of integrating its large transmembrane structure (Behrendt et al., 2004). It would also be interesting to understand how and what protein pathways are involved in integrating mtTATC in the inner membrane. The OXA pathway is a potential candidate as it co-translationally inserts mitochondrial encoded proteins into the IM (Funes et al., 2011; Lister et al., 2002; Stojanovski et al., 2012).

However, what would be the role of a putative mtTATC component and its physiological implications during plant stress conditions? Research suggests that mtTATC maybe functionally redundant to the mitochondrial Bcs1 transporter which exports and assembles Rieske Fe/S protein (Rip1) into mitochondrial complex III (Nobrega et al., 1992; Wagener and Neupert, 2012). In Arabidopsis thaliana, the cytochrome Bcs1 gene is upregulated with SA stress; its protein in turn amplifies endogenous SA levels which further upregulates defense genes coding for mitochondrial proteins (Ho et al., 2008). However, A. thaliana lacks the BCS1 AAA protein domain which is conserved in yeast and mammals and functions as an ATPase (Zhang et al., 2014). Instead, during stress conditions, a SA induced putative mtTATC may perform the role of the Bcs1 transporter. i.e it would export the fully folded C-terminal domain of Rip1, from the matrix to the intermembrane space for complex III assembly, without the use of ATP energy (Carrie et al., 2016; Pett and Lavrov, 2013; van der Merwe and Dubery, 2007)

Promisingly, in Oscarellidae, a functional mtTATC component alone is capable of transporting and inserting substrate proteins into the inner membrane (Pett and Lavrov, 2013). However, a functional mtTATC in higher order plants such as Arabidopsis thaliana is yet to be discovered. Future protein-protein interaction studies can characterize how exactly mtTATC and Rip1
substrate interact during SA stress. Such an understanding of the function and mechanism of Bcs1-like proteins in plants, can also be extended to human diseases caused by mitochondrial Bcs1 deficiencies (de Lonlay et al., 2001).

The Rieske Fe/S is a promising substrate for this pathway since it requires a ΔpH as found in the Tat pathway for assembly into complex III (Molik et al., 2001; Pett and Lavrov, 2013). For instance Tat-pathway dependent Rieske Fe/S substrate targeting has been confirmed in cyanobacteria (Aldridge et al., 2008). Rip1 also has a signal sequence with a conserved Tat domain across multiple eukaryotes (Pett and Lavrov, 2013). Furthermore, in yeast the gene for functional Rip1 can be successfully incorporated and expressed by the mitochondrial genome. This supports the existence of mitochondrial genes which can code for its transport machinery too (Golik et al., 2003).

The ability to transport Rip1 is physiologically important because during plant stress conditions, complex III and specifically Rip1 opens mitochondrial permeability transition (MPT) pores that release cytochrome C into the cytoplasm (Minibayeva et al., 2012). This begins the cascade of events related to autophagy and PCD which ultimately provide the plant with oxidative stress protection (Tiwari et al., 2002). Therefore a stress-induced mtTATC component would help facilitate release of apoptotic factors from the mitochondrion to protect the plant from oxidative damage.

3.1.3 cpTha4 component during stress

Although the mtTATC component alone can translocate substrates in Oscarallidae, a minimal Tat pathway found in gram-positive is composed of TatC and TatA (Tha4) (Barnett et al., 2008). Furthermore, the chloroplast Tat system in higher order plants require a Tha4 component, which functions as the pore and stabilizes the negative charge on TatC (Aldridge et al., 2014; Ma, 2016; Rollauer et al., 2012). Our quantitative data showed that cpTha4-GFP is dual localized to both the chloroplast and mitochondria during SA stress.

Interestingly, there was competing dual targeting since when mitochondrial localization increased, chloroplast localization decreased and vice versa. We propose that cellular cpTha4 levels remain
constant but it is redistributed to the mitochondria during stress, since it is both the main target of and source of oxidative stress regulation in flowering plants (Van Aken et al., 2009). Our TEM ultrastructural study also confirms that mitochondrial membranes as opposed to chloroplast membranes are damaged the most during SA stress. The IM was ruptured, its surface area was reduced and the matrix was lighter due to loss of protein; all signs of mitophagy which precede oxidative stress related apoptosis in plants (Kang et al., 2007; Yang and Cortopassi, 1998). qRT-PCR and cpTha4 immunoblotting must be executed to determine if cellular cpTha4 levels stay the same overt time with SA application. Additionally our qualitative data can be improved by designing cpTha4-GFP constructs with a longer amino acid sequence of cpTha4 instead of its presignal sequence.

3.1.4 Summary and model

When does a minimal Tat pathway exist and what would be the physiological implications of it during stress? In the chloroplast Tat system in the presence of a substrate and PMF, cpTha4 is dynamically recruited by cpTatC to the thylakoid membrane (Aldridge et al., 2014). Our 24-hour time course studies demonstrate that mtTATC is expressed at 4 h post-treatment with high SA concentration and that cpTha4 targeting to mitochondria peaks at the same time. Interestingly, the suggested substrate for the mitochondrial Tat pathway, Rip1 is targeted to both the chloroplast (cytochrome b6f complex) and mitochondrion (complex III) (Conte and Zara, 2011; Molik et al., 2001). However, during stress conditions Rip1 translocation in the mitochondrion may take precedence over the chloroplast, since it facilitates release of apoptotic factors (Minibayeva et al., 2012). Therefore, cpTha4 maybe increasingly targeted to the mitochondria during SA stress; at 4 hours after SA treatment, it may interact with mtTATC and assemble a minimal mitochondrial Tat pathway for Rip1 translocation. This timing is also consistent with the initial plant oxidative burst in the plant hypersensitive response (Morel and Dangl, 1997; Wojtaszek, 1997). Pull-down assays can be performed to determine protein-protein interactions between cpTha4 and mtTATC and its substrate(s) at various times after SA treatment.

A recent study also showed the constitutive expression of nuclear encoded TatB (Hcf106) in plant mitochondria (Carrie et al., 2016). Together, during SA stress these three components could form a functional mitochondrial Tat pathway to translocate fully folded or co-factor associated redox
proteins involved in the plant stress response, without the use of ATP energy. Apart from Rip1, it may also insert SA induced pathogen resistant (PR) proteins such as AOX1 in the mitochondrial IM, to undertake an alternate respiratory pathway when the ETC is compromised during oxidative stress (Cárdenas-Monroy et al., 2017; Nie et al., 2015; Smith et al., 2009).

This study investigated the expression of a novel, mitochondrial protein transport pathway in Arabidopsis thaliana with salicylic acid (SA) stress. We then discussed its biochemical and physiological role in the plant stress response. An understanding of such novel SA targets in plants, will help develop agricultural compounds that provide plants with systemic acquired resistance (SAR) against pathogens (Dempsey and Klessig, 2017). Furthermore, since mitochondrial SA pathways complement the animal kingdom there is high potential for pharmaceutical applications in humans, through drugs such as aspirin where SA is the main ingredient (Nulton-Persson et al., 2004; Paterson et al., 2008). More significantly it helps us understand how the powerhouse of the cell acts as a stress sensor, opening new avenues of research into mitochondrial energetics and biochemistry during stress.
3.2 References


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