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## Characterization of land plant-specific proteins required for mitochondrial translation initiation

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2023

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*Citation for published version (APA):*

Tran, H. C. (2023). *Characterization of land plant-specific proteins required for mitochondrial translation initiation*. [Doctoral Thesis (compilation), Department of Biology]. Lund University, Faculty of Science.

*Total number of authors:*

1

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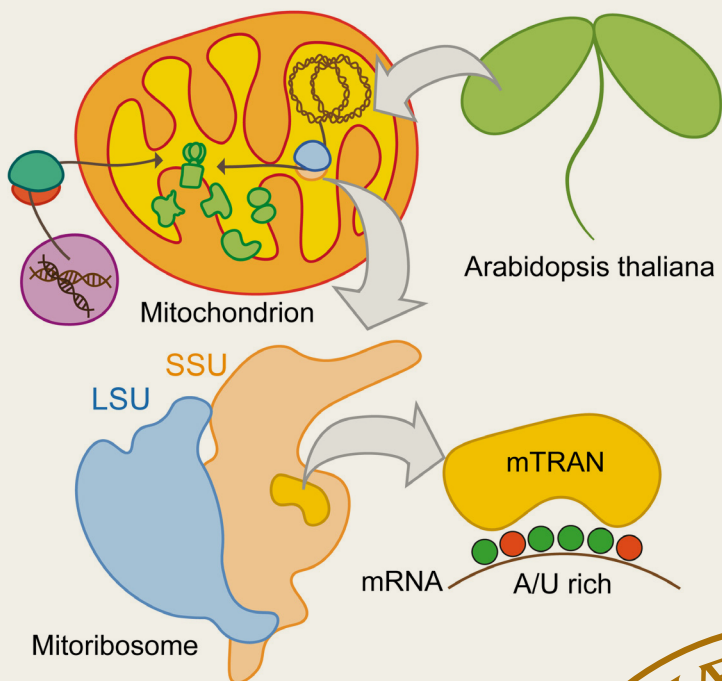
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# Characterization of land plant-specific proteins required for mitochondrial translation initiation

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# Characterization of land plant-specific proteins required for mitochondrial translation initiation

Huy Cuong Tran



**LUND**  
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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Science at Lund University to be publicly defended on the 5<sup>th</sup> of May at 13.00 in Biology Lecture Hall, Department of Biology, Sölvegatan 35, Lund, Sweden

*Faculty opponent*

Prof. Dr. Christian Schmitz-Linneweber

Humboldt-Universität zu Berlin, Berlin, Germany

**Organization:** LUND UNIVERSITY

**Document name:** DOCTORAL DISSERTATION

**Date of issue:** 2023-05-05

**Author:** Huy Cuong Tran

**Sponsoring organization:**

**Title:** Characterization of land plant-specific proteins required for mitochondrial translation initiation.

**Abstract:** Plant mitochondria produce the majority of adenosine triphosphate (ATP) – the cellular energy currency for metabolic reactions needed for plant growth, development, and maintenance. Despite a relatively thorough understanding of basic mitochondrial functions, many mitochondrial proteins and processes remain poorly understood. The aims of this thesis are to i) review and compare the mitochondrial unfolded protein response (UPR<sup>mt</sup>) and related signalling across eukaryotic kingdoms, to ii) describe an efficient isolation method of Arabidopsis mitochondria using continuous Percoll density gradients, and to iii) characterize two *Arabidopsis thaliana* genes, *AT4G15640* and *AT3G21465*.

Paper I summarizes the current knowledge of UPR<sup>mt</sup> across eukaryotic kingdoms, and describes a meta-analysis of UPR<sup>mt</sup> regulators and target genes. UPR<sup>mt</sup> is a mitochondria-to-nucleus “retrograde” response that regulates nuclear gene expression during mitochondrial dysfunction to maintain mitochondrial homeostasis. Although UPR<sup>mt</sup> has been extensively studied in animals, relatively little is known about the plant UPR<sup>mt</sup> and only few regulators have recently been identified. In yeast, very few unfolded protein responses that seem to be related to UPR<sup>mt</sup> have been described. Here, the UPR<sup>mt</sup> in animals, yeast and plants are compared. Our study indicates that each kingdom has evolved their own specific regulators, which however induce very similar groups of target genes. Our meta-analysis identifies homologs of known UPR<sup>mt</sup> regulators and responsive genes across eukaryotic kingdoms.

Paper II describes a strategy for efficient purification of Arabidopsis mitochondria using continuous Percoll density gradients. By using this method, the purity of isolated mitochondria is greatly improved. Obtained mitochondria can be either used for assays requiring highly intact and functional mitochondria, e.g. import assay or respiration measurement, or be stored for later use, e.g. BN-PAGE or western blot.

Paper III describes the characterization of two *Arabidopsis thaliana* genes, *AT4G15640* and *AT3G21465*. Here, *AT4G15640* and *AT3G21465* are shown to be land plant-specific mitochondrial proteins that are critical for plant performance, but they are unlikely to be adenyl cyclases as previously annotated. Interestingly, knocking out both *AT4G15640* and *AT3G21465* results in low abundance and activity of mitochondrial oxidative phosphorylation complexes. Using co-immunoprecipitation, *AT4G15640* and *AT3G21465* are confirmed to be part of the mitoribosome small subunit (mtSSU). Therefore, *AT4G15640* and *AT3G21465* were named as mitochondrial TRANslation factor 1 (mTRAN1) and 2 (mTRAN2), respectively. *In organello* protein synthesis and polysome fractionation assays suggest that mTRANs are required for mitochondrial translation initiation. Moreover, the RNA electrophoretic mobility shift assays suggest that mTRAN1 binds to the A/U-rich regions in the 5'-untranslated regions of mitochondrial mRNAs to mediate mtSSU-mRNA binding to initiate translation. Finally, our mitoribosome profiling suggests that mTRAN proteins are universal mtSSU-embedded translation initiation factors. Together, these findings suggest that mitochondrial translation initiation occurs via a unique mechanism in plants, as compared to in bacteria, yeast and mammals.

**Key words:** Mitochondrial unfolded protein response, Arabidopsis mitochondrial isolation, mitoribosome, mitochondrial translation initiation, mitoribosome small subunit, mTRAN, mTRAN-mRNA interaction.

Classification system and/or index terms (if any)

Supplementary bibliographical information

**Language:** English

**ISSN and key title:**

**ISBN:** 978-91-8039-598-4 (electronic), 978-91-8039-599-1 (print)

Recipient's notes

**Number of pages:** 67

Price

Security classification

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Paper 3 © by the Authors (Manuscript unpublished)

Faculty of Science

Department of Biology

ISBN 978-91-8039-598-4 (electronic)

ISSN 978-91-8039-599-1 (print)

Printed in Sweden by Media-Tryck, Lund University

Lund 2023



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*To my family (Gửi tới gia đình)*

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## List of papers

### *Paper I*

**Huy Cuong Tran**, Olivier Van Aken (2020). Mitochondrial unfolded protein-related responses across kingdoms: similar problems, different regulators. *Mitochondrion* 53: 166-177.

### *Paper II*

**Huy Cuong Tran**, Olivier Van Aken (2022). Purification of leaf mitochondria from *Arabidopsis thaliana* using Percoll density gradients. *Methods in Molecular Biology* 2363: 1-12.

### *Paper III*

**Huy Cuong Tran**, Vivian Schmitt, Sbatie Lama, Chuande Wang, Alexandra Launay-Avon, Katja Bernfur, Kristin Hofmann, Kasim Khan, Benoit Castandet, Fredrik Levander, Allan G. Rasmusson, Hakim Mireau, Etienne Delannoy, Olivier Van Aken. Mitochondrial translation in plants is initiated by a unique mTRAN-mRNA interaction mechanism. *In revision for Science*.

# My contributions to the papers

## *Paper I*

I participated in planning the project and performing the meta-analysis. I wrote the manuscript with input from my supervisor.

## *Paper II*

I participated in planning the project and collecting data. I wrote the manuscript with input from my supervisor.

## *Paper III*

I participated in conceiving and planning the project. I performed most of the experiments (with input from my supervisor), including phylogenetic analysis, confocal microscopy analysis, plant phenotyping, plant cAMP measurement, BN-PAGE following by Coomassie staining and activity measurement, oxygen consumption measurement, immunoblotting, co-immunoprecipitation, *in organello* protein synthesis, and polysome profiling. I also produced and collected the samples for RNA-seq and Ribo-seq. I co-supervised Vivian Schmitt doing her internship and master's thesis, in which she performed several immunoblot analysis, the bacterial cAMP synthase complementation assays, qRT-PCR on mitochondrial retrograde signalling genes, motif analysis of the 5'UTRs of mitochondrial mRNAs, recombinant protein expression and purification, and REMSAs. My supervisor and I wrote the manuscript with input from all co-authors.

## List of additional papers not included in this thesis

Essam Darwish, Ritesh Ghosh, Abraham Ontiveros-Cisneros, **Huy Cuong Tran**, Marcus Petersson, Liesbeth De Milde, Martyna Broda, Alain Goossens, Alex Van Moerkercke, Kasim Khan and Olivier Van Aken (2022). Touch signaling and thigmomorphogenesis are regulated by complementary CAMTA3- and JA-dependent pathways. *Science Advances* 8 (20): eabm2091.

## List of abbreviations

5'UTR	5' untranslated region
A	Adenine
AA	Antimycin A
ABI4	Abscisic acid-insensitive 4
Acetyl-CoA	Acetyl-Coenzyme A
AC	Adenyl cyclase
ADP	Adenosine diphosphate
ANAC017	Arabidopsis NAC domain containing 017
AOX	Alternative oxidase
ATP	Adenosine triphosphate
BLRP	Biotin ligase recognition peptide
BN-PAGE	Blue Native Polyacrylamide Gel-Electrophoresis
C	Cysteine
cAMP	3',5'-cyclic adenosine monophosphate
CI-V	Complex I-V
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
cpUPR	Chloroplast unfolded protein response
Cryo-EM	Cryogenic electron microscopy
D-GAP	D-glyceraldehyde-3-phosphate
Dox	Doxycycline
DXP	1-deoxy-D-xylulose-5-phosphate
DXS	1-deoxy-D-xylulose-5-phosphate synthase
ER	Endoplasmic reticulum
FADH <sub>2</sub>	Flavin adenine dinucleotide
G	Guanine
IF	Initiation factor
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IMTACT	Isolation of Mitochondria TAgged in specific Cell Types
Fe-S	Iron-sulfur
MAPK	Mitogen-activated protein kinase
MB	MitoBlock-6
MEP	Methylerythritol phosphate
MORF	Multiple Organellar RNA editing Factor
mRNA	Messenger RNA
mrp11-1	Mitochondrial ribosomal protein L1
mtDNA	Mitochondrial DNA
mtETC	Mitochondrial electron transport chain
mtIF	Mitochondrial initiation factor



mtLSU	Mitoribosome large subunit
mTRAN1	Mitochondrial translation factor 1
mTRAN2	Mitochondrial translation factor 2
MTS	Mitochondrial targeting sequences
mtSSU	Mitoribosome small subunit
NADH	Nicotinamide adenine dinucleotide
NDB4	NAD(P)H dehydrogenase B4
nPG	n-propylgallate
OAA	Oxaloacetate
OM64	Outer membrane 64
OM66	Outer membrane protein of 66 kDa
OMM	Outer mitochondrial membrane
ORRM	Organellar RNA Recognition Motif
OXPPOS	Oxidative phosphorylation
PEP	Phosphoenolpyruvate
PNPase	Polynucleotide phosphorylase
PPR	Pentatricopeptide repeat
PVP	Polyvinylpyrrolidone
REMSA	RNA electrophoretic mobility shift assay
RIP-seq	RNA Immunoprecipitation Sequencing
RIP	RNA-editing factor interacting protein
ROS	Reactive oxygen species
RpoT	RNA polymerase of the T-phage type
rRNA	Ribosomal RNA
SD	Shine-Dalgarno
SHAM	Salicylhydroxamic acid
SUBA	Subcellular Localization Database for Arabidopsis Proteins
Succinyl-CoA	Succinyl-Coenzyme A
TCA	Tricarboxylic acid
TIM	Translocase of the inner membrane
TM	Transmembrane
TOM	Translocase of the outer membrane
TPR	Tetratricopeptide repeat
tRNA	Transfer RNA
U	Uridine
UGT74E2	Uridine diphosphate glycosyltransferase 74E2
UPOX1	Up regulated by oxidative stress AT2G21640 1
UPR <sup>mt</sup>	Mitochondrial unfolded protein response
VDAC	Voltage-dependent anion channel

## Popular scientific summary

Plants are of key importance as their photosynthesis produces a massive amount of oxygen in the atmosphere, which is mandatory for respiration of many other organisms as well as plants themselves. Similar to in other eukaryotes, plant cellular respiration is carried out by mitochondria – organelles producing cellular energy needed for plant growth, development and maintenance. Nowadays, most scientists agree with the evolutionary hypothesis suggesting that mitochondria originated from endosymbiotic bacteria approximately 1.5 billion years ago. Nevertheless, in a cell, mitochondrial operation is not independent, but closely interacts with the nucleus – the central regulator of eukaryotic cells that contains most genes encoding the genetic information of an organism. The nucleus can send signals and materials to mitochondria to regulate mitochondrial activity. Vice versa, mitochondria are also able to send signals to the nucleus and affect nuclear gene expression. Gene expression is the process by which information of a gene is passed onto a messenger RNA (mRNA) during transcription, which is then used as a template to synthesize a corresponding protein during translation. This ultimately may affect the phenotype – the final effect on an individual living organism.

Due to their bacterial origin, mitochondria have their own sets of genes and have partially retained their own machinery for gene expression, including essential components required for transcription and translation. Mitochondrial gene expression is important to maintain mitochondrial operation. Thus, one could expect that the cellular energy would be affected if mitochondrial gene expression is perturbed. To date, many aspects of mitochondrial translation in plants are still enigmatic. In this work, we use the plant “mouse-ear cress” (scientific name: *Arabidopsis thaliana*) to study the land plant-specific proteins of unknown functions that we named as mitochondrial TRANslation factors (mTRAN). For many years, mTRAN proteins have been poorly characterized. During the course of our study, two recent studies suggested that mTRAN proteins are mitochondria-targeted and are components of the plant mitochondrial ribosome (hereafter referred to as mitoribosome) – the protein-RNA complex that translates the genetic information encoded in a mRNA into a corresponding protein. To study the functions of mTRAN proteins, *Arabidopsis* mutants lacking functional mTRAN proteins were generated, and they displayed a severe growth phenotype, indicating that mTRAN proteins are essential for plant growth and development. Using a combination of molecular, biochemical and bioinformatic approaches, we showed that the importance of mTRAN proteins for plant performance is due to their critical role in efficient mitochondrial translation in plants. In particular, mTRAN proteins are required for the mitoribosome – RNA binding in the initiation step of translation. Of note, this unique mTRAN-RNA interaction mechanism to initiate plant mitochondrial translation is fundamentally different from bacterial translation and mitochondrial translation in other eukaryotes. In conclusion, our study not only gains a deeper

insight into mitochondrial translation in plants, but also contributes to the knowledge of mitochondrial translation in eukaryotes.

# Introduction

## An overview of cellular respiration in plants

Plants are autotrophs that uptake nutrients in inorganic form and produce organic molecules themselves. Plant photosynthesis is carried out by chloroplasts to synthesize carbohydrates (sucrose and/or starch) from carbon dioxide (CO<sub>2</sub>) and water by using photon energy from sunlight. On the other hand, plant respiration consumes oxygen (O<sub>2</sub>) as the electron acceptor and releases CO<sub>2</sub> as a by-product. Similar to in other eukaryotes, plants have mitochondria, where aerobic respiration partially occurs to generate adenosine triphosphate (ATP) – the cellular energy currency to fuel metabolism required for their growth, development, and maintenance. Plant respiration is composed of glycolysis, the tricarboxylic acid (TCA) cycle (also known as the Krebs cycle or the citric acid cycle) and the oxidative phosphorylation (OXPHOS) of adenosine diphosphate (ADP) to ATP by the mitochondrial electron transport chain (mtETC) and ATP synthase complex. Plant cellular respiration strongly resembles their animal counterparts, but it has several unique features. As plants are sessile, they are unable to move away from extreme environmental conditions. Therefore, plant metabolism in general and plant respiratory metabolism in particular have evolved to adapt with environmental stress (O'Leary and Plaxton, 2016). The overall regulation of plant respiration is complicated and can occur “locally” in different processes of the respiratory pathway. The widespread use of plant mutant lines, which have important enzymes of respiration knocked out, gives us a broader insight not only into the function of key respiratory components, but also into the flexibility of plant respiratory pathway. Plant respiration is of great importance for the global carbon cycle as the amount of CO<sub>2</sub> used by photosynthesis of terrestrial plants is returned to the atmosphere by plant respiration, representing approximately 50% of the total annual CO<sub>2</sub> released from terrestrial ecosystems (Gifford, 2003; O'Leary and Plaxton, 2016).

## Mitochondrial origin

In 1967, Lynn Margulis proposed that eukaryotic organelles, including the chloroplast and the mitochondrion, originated from endosymbiotic bacteria in her

famous paper “On the origin of mitosing cells” (Sagan, 1967). Later, advanced phylogenomic analysis has shown that the mitochondrial endosymbiont is related to an  $\alpha$ -proteobacterium either within, or as a sister group to, the Rickettsiales. The host organism was likely an archaeon of the Asgard clade (Spang et al., 2015; Wang and Wu, 2014, 2015; Zaremba-Niedzwiedzka et al., 2017). The mitochondrion-containing ancestor, also known as the “last eukaryote common ancestor”, resembling the modern eukaryotes genetically and cellularly, evolved to all known eukaryotes nowadays. On one hand, the “mitochondria-early” evolutionary hypothesis proposes that the mitochondrial endosymbiotic event occurred very early in eukaryogenesis and/or even initiated the process (Martin et al., 2015). On the other hand, the “mitochondria-late” evolutionary hypothesis suggests that the mitochondrial endosymbiotic event took place when many other eukaryotic characteristics had already been established (Roger et al., 2017). Whether the former or the latter hypothesis is closer to what happened in the earliest stage of life, it is undeniable that the endosymbiotic origin of mitochondria is of key importance for the evolution of all modern eukaryotes.

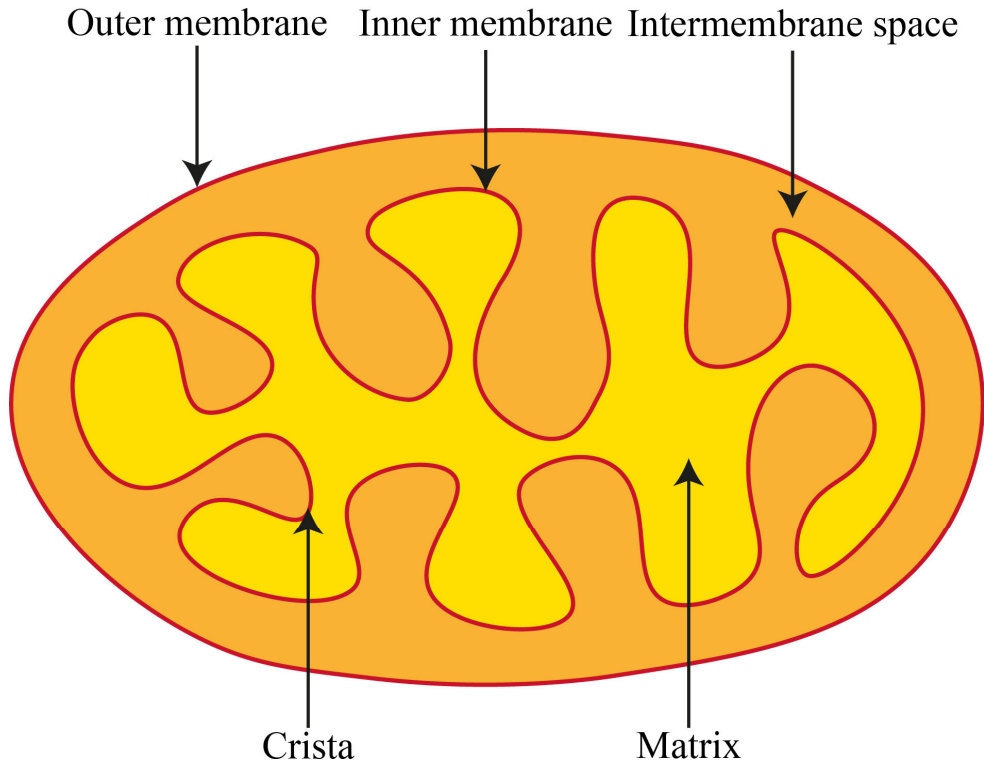
## A brief history of plant mitochondrial isolation

To study the structure and function of mitochondria, researchers attempted to isolate mitochondria from cells. The first attempt of mitochondrial isolation was carried out in guinea pig liver by Bensley and Hoerr in 1934 (Bensley and Hoerr, 1934) according to a recent review by Møller et al. (2021). Although mitochondria might be first described in plant cells in 1904 (Logan, 2012; Møller et al., 2021), the first plant mitochondria purification was done by Adele Millerd and her co-workers in 1951 (Millerd et al., 1951; Møller et al., 2021). At that stage, the crude mitochondria were isolated by differential centrifugations, thus they were however impure due to heavy contamination of thylakoid membranes from chloroplasts. Only in 1985, Day and his co-workers used differential centrifugations and Percoll-Polyvinylpyrrolidone (PVP) density gradients to successfully purify chlorophyll-free mitochondria from pea (*Pisum sativum*) leaves (Day et al., 1985). Also using the same approach, pure, intact and functional mitochondria of *Arabidopsis thaliana*, which became of great importance as the model dicot plant in the 1990s, were successfully purified from leaf tissues (Keech et al., 2005) and seedlings (Escobar et al., 2006). Nevertheless, this protocol of mitochondrial isolation requires a large amount of starting plant material and usually takes a few hours to accomplish. Recently, Boussardon et al. (2020) has developed a novel method of tissue-specific isolation of *Arabidopsis* mitochondria, named IMTACT (Isolation of Mitochondria TAGged in specific Cell Types). IMTACT, in which cell-specific biotinylated mitochondria are purified using streptavidin magnetic beads, yields pure and intact mitochondria in less than 30 min from sample preparation till final

extraction (Boussardon and Keech, 2022). This novel approach is much faster than traditional mitochondrial isolation methods using continuous or discontinuous gradients. To study plant mitochondrial compartments in depth, the protocols of isolating mitochondrial subfractions followed by biochemical approaches to study them have been developed and described in detail in the book “Plant Mitochondria: Methods and Protocols” edited by Whelan and Murcha (2015) and Van Aken and Rasmusson (2022).

## Structure and basis composition of plant mitochondria

Plant mitochondria are often spherical or rod-shaped organelles that are 1-3  $\mu\text{m}$  long and about 0.5  $\mu\text{m}$  in diameter (Møller et al., 2021). With the availability of electron microscopes after World War II, the structure of an individual plant mitochondrion was revealed by electron micrographs (Palade, 1952). Similar to in other eukaryotes, plant mitochondria are double membranous organelles comprised of an outer mitochondrial membrane (OMM) separated from an inner mitochondrial membrane (IMM) by the intermembrane space (IMS), and the mitochondrial matrix, which is an aqueous electron-dense phase enclosed by the IMM and cristae formed by invaginations of the IMM (Figure 1). The OMM was shown to contain the voltage-dependent anion channel (VDAC), also referred as porin (Hombler et al., 2012; Parsons et al., 1965; Zalman et al., 1980). Using electron tomography (available in the 1990s) to obtain 3D pictures of mitochondria, it turned out that the cristae look like narrow tubes running through the matrix (Frey and Mannella, 2000). A typical *Arabidopsis mesophyll* cell is comprised of many hundreds of mitochondria (Logan, 2006). Unlike mammalian and most yeast mitochondria, but resembling mitochondria in *Saccharomyces cerevisiae*, plant mitochondria move around mainly within the cytoplasm on the actin cytoskeleton (Logan, 2006; Olyslaegers and Verbelen, 1998; Van Gestel et al., 2002). Interestingly, mitochondrial fusion can occur, forming large, reticulated mitochondria. Mitochondrial fusion and fission can take place frequently to maintain the mitochondrial population in a cell homogenous (Logan, 2006; Møller et al., 2021). A recent review by Møller et al. (2021) has summarized the composition of mitochondrial compartments. In brief, the mitochondrial membranes are composed of lipids and proteins. The lipid in the mitochondrial membranes are usually phospholipids. The lipid composition of the OMM, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol, resembles that of the endoplasmic reticulum (ER) membrane. The IMM has a similar lipid composition as the OMM, but also contains a unique phospholipid, diphosphatidylglycerol (cardiolipin). The mitochondrial matrix does not contain phospholipids and has a very high concentration of proteins.



**Figure 1.** A simple diagram of plant mitochondrial structure.

## Plant aerobic respiration partially occurs in mitochondria

As in other aerobic eukaryotes, the aerobic respiration in plants includes three processes occurring in different subcellular compartments: glycolysis, the TCA cycle and the OXPHOS system. Together, these processes produce ATP – the cellular energy currency. Mitochondria are thus considered as the cellular power houses. The following description of these three stages of plant aerobic respiration is written to a large extent based on chapter 12 “Respiration and Photorespiration” by Millar et al. (2015) in the textbook “Biochemistry and Molecular Biology of Plants” edited by Buchanan et al. (2015).

## Glycolysis

Glycolysis takes place either in the cytosol or in the plastids, generating substrates that are subsequently used by the TCA cycle and the OXPHOS system, which occur within the mitochondria. Cytosolic glycolysis breaks down sucrose into glucose and fructose, which are converted into phosphoenolpyruvate (PEP). PEP can be turned either into pyruvate by pyruvate kinase and/or PEP phosphatase or into oxaloacetate (OAA) by PEP carboxylase. This glycolytic pathway generates reduced cofactor nicotinamide adenine dinucleotide (NADH) and ATP. Pyruvate is transferred directly into mitochondria whereas OAA is usually reduced to malate, which is then transported into mitochondria.

## The tricarboxylic acid cycle

Once pyruvate and malate are imported into the mitochondrial matrix via carriers located in the IMM, pyruvate is oxidized and decarboxylated to generate CO<sub>2</sub>, acetyl-Coenzyme A (acetyl-CoA) and NADH, while malate can be oxidized to either generate OAA and NADH by malate dehydrogenase or yield pyruvate, CO<sub>2</sub> and NADH by NAD<sup>+</sup>-linked malic enzyme. Once the TCA cycle starts occurring within the mitochondrial matrix, acetyl-CoA and OAA combine to form citrate, which is isomerized to isocitrate that is oxidized and decarboxylated to form CO<sub>2</sub>, 2-oxoglutarate, and NADH. 2-Oxoglutarate is further oxidized to produce CO<sub>2</sub>, succinyl-Coenzyme A (succinyl-CoA) and NADH. Succinyl-CoA synthetase converts succinyl-CoA into succinate. Associating with this reaction, ADP is phosphorylated to ATP. Succinate is oxidized to fumarate by succinate dehydrogenase - the only IMM-localized enzyme involved in both the TCA cycle and mtETC. In the final step of the TCA cycle, fumarate is hydrated to form NADH and malate, which is oxidized to OAA that subsequently combines with another acetyl-CoA to continue the cycle (Figure 2).

## The oxidative phosphorylation system

The OXPHOS system resides within the IMM and is based on the core of mtETC, which is composed of the four IMM-associated complexes: the NADH dehydrogenase (complex I), the succinate dehydrogenase (complex II), the cytochrome *bc<sub>1</sub>*/cytochrome *c* reductase complex (complex III) and the cytochrome *c* oxidase (complex IV) (Figure 2). Complex I is the largest respiratory complex of the mtETC (Wirth et al., 2016). The high-resolution structure of plant complex I has recently been described by Maldonado et al. (2020) and Klusch et al. (2021). Plant complex I has an L-like shape and is comprised of the membrane arm embedded into the IMM, the peripheral arm extending into the mitochondrial matrix and another matrix-exposed domain linking to the membrane arm at the central position.

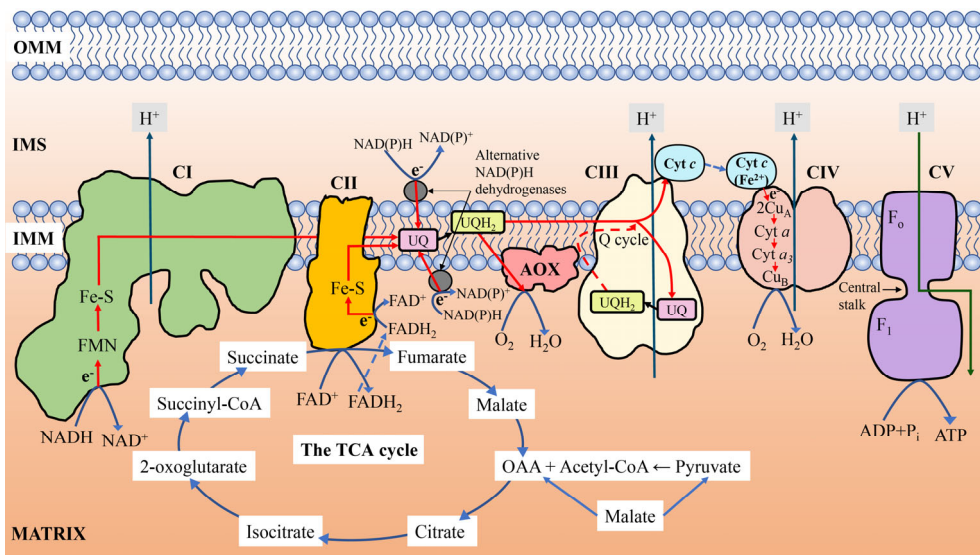


Complex I oxidizes NADH (formed by glycolysis and the TCA cycle) to regenerate  $\text{NAD}^+$  at the peripheral arm site. Complex II, the smallest complex of four respiratory complexes (Millar et al., 2004), oxidizes succinate (generated as an intermediate in the TCA cycle) to fumarate with the concomitant oxidation of flavin adenine dinucleotide ( $\text{FADH}_2$ ) to  $\text{FAD}^+$  at the membrane-spanning domain site. Both complex I and complex II contain iron-sulfur (Fe-S) clusters involved in the transfer of electrons onto ubiquinone – a mobile electron transporter located within the mitochondrial IMM. The electron transport from NADH to ubiquinone causes conformational change in the membrane arm of complex I, resulting in pumping four protons from the mitochondrial matrix to the IMS. Unlike complex I, complex II cannot translocate protons across the IMM. By accepting two electrons, ubiquinone is reduced to ubiquinol, which is released from complex I and complex II and transfers electrons onto complex III – the central segment of the mtETC.

The atomic structure of plant complex III has recently been described by Maldonado et al. (2021). Plant complex III consists of  $2 \times 10$  subunits, which can be functionally categorized as follows (Braun, 2020): subunits involved in the mtETC including the Rieske Fe-S protein, the heme-containing cytochrome  $c_1$  and cytochrome  $b$  subunits ( $b_{566}$  and  $b_{560}$ ); the large mitochondrial matrix-exposed domain of complex III containing the two subunits of mitochondrial processing peptidase ( $\alpha$ -MPP and  $\beta$ -MPP), which cleaves the targeting peptides of nuclear-encoded mitochondrial proteins; and five small subunits QCR6-QCR10, which participate in electron transport. At complex III, electrons are transferred from ubiquinol to cytochrome  $c$  and protons are translocated from the matrix to the IMS by the Q cycle. During the Q cycle, ubiquinol binds to the center P of complex III, where it is oxidized and releases two electrons, one of which is transferred to cytochrome  $c$  via the Rieske Fe-S center and cytochrome  $c_1$  and two protons are pumped into the IMS. The other electron is transferred by cytochrome  $b$ -type proteins within complex III to reduce ubiquinone (bound to the matrix side of complex III) to semiquinone at the center N. At this point, another ubiquinol attaches to the center P and passes through the same process, reduces semiquinone to ubiquinol, completes the Q cycle and pumps two more protons into the IMS. Once the Q cycle is completed, cytochrome  $c$ , a small heme-containing protein located on the outer surface of the IMM exposing it to the IMS, is reduced and released from complex III, carries one electron and transports it to complex IV - the terminal enzyme of the mtETC.

The molecular structure of plant complex IV has recently been described by Maldonado et al. (2021). At subunit II of complex IV, electrons from cytochrome  $c$  are transferred via  $\text{Cu}_A$  and cytochrome  $a$  then to the cytochrome  $a_3$ - $\text{Cu}_B$  center of the subunit I, where one oxygen molecule (the final electron acceptor) is reduced by one electron to form two water molecules. This redox reaction is coupled to the translocation of two protons across the IMM. The K, D, H pathways (named after crucial amino acid residues in each pathway) were suggested to contribute to the pumping of protons by plant complex IV but it has recently been suggested that the

H pathway is not involved in this process (Maldonado et al., 2021). During one “cycle” of the mETC, a total of 10 protons (4 from complex I, 4 from complex III and 2 from complex IV) are translocated from the mitochondrial matrix to the IMS, creating an electrochemical proton gradient, also known as the mitochondrial membrane potential. The protons in the IMS re-enter the mitochondrial matrix via the ATP synthase complex (also known as complex V) to dissipate the proton gradient. Complex V is composed of two major subcomplexes:  $F_0$  - the transmembrane subcomplex (“o” stands for oligomycin, which inhibits proton transfer through the subcomplex) and  $F_1$  - the matrix-exposed subcomplex, are connected by a central stalk. The  $F_0$  subcomplex allows protons to pass through, causing conformational changes within the  $F_1$  subcomplex, driving the phosphorylation of ADP to ATP. Generation of ATP – the final product of the OXPHOS system and more importantly, the cellular energy currency, is one of the prime functions of mitochondria.



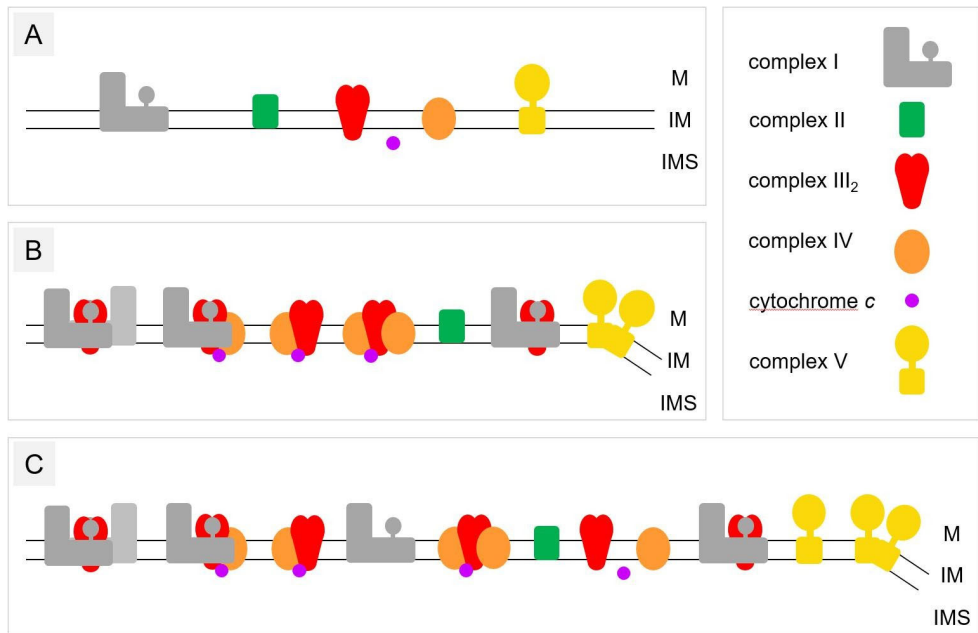
**Figure 2.** A diagram of the tricarboxylic acid (TCA) cycle and the oxidative phosphorylation (OXPHOS) system in plant mitochondria. Imported pyruvate is converted into acetyl-Coenzyme A (acetyl-CoA) whereas imported malate can be used to generate either pyruvate or oxaloacetate (OAA). As the TCA cycle starts in the mitochondrial matrix, acetyl-CoA and OAA combine to generate citrate, which is subsequently isomerized to isocitrate. Isocitrate is then converted into 2-oxoglutarate, which is in turn converted into succinyl-Coenzyme A (succinyl-CoA). Succinyl-CoA is turned into succinate, which is oxidized to fumarate by the inner mitochondrial membrane (IMM)-anchored succinate dehydrogenase (complex II (CII)). Concomitantly, flavin adenine dinucleotide ( $FADH_2$ ) is oxidized to  $FAD^+$ . Fumarate is converted into and malate, which is oxidized to OAA that subsequently combines with another acetyl-CoA to continue the cycle. The OXPHOS system occurs within the IMM. Complex I (CI) oxidizes nicotinamide adenine dinucleotide (NADH) to

generate  $\text{NAD}^+$  at the peripheral arm site and the electrons are transferred via flavin mononucleotide (FMN) and iron-sulfur (Fe-S) clusters onto ubiquinone (UQ). CII oxidizes  $\text{FADH}_2$  to  $\text{FAD}^+$  at the membrane-spanning domain site and the electrons are transported via Fe-S clusters to UQ. During the electron transport, CI pumps protons ( $\text{H}^+$ ) from the mitochondrial matrix into the mitochondrial intermembrane space (IMS) whereas CII is a non-proton pumping complex. By receiving two electrons, UQ is reduced to ubiquinol ( $\text{UQH}_2$ ), which transfers electrons to complex III (CIII). At CIII, electrons are transferred from  $\text{UQH}_2$  to cytochrome *c* (cyt *c*) and protons are translocated from the matrix into the IMS by the Q cycle. Because one cyt *c* can accept only one electron from  $\text{UQH}_2$  at a time, the second electron is transferred to UQ bound to CIII, which is reduced to form semi-UQ. During the second half of the Q cycle, another  $\text{UQH}_2$  enters the first half of the Q cycle, donating one electron to another cyt *c* and the second electron is transported to semi-UQ, which is fully reduced to  $\text{UQH}_2$  and this  $\text{UQH}_2$  will travel to the IMM and re-enter the Q cycle. Reduced cyt *c* (cyt *c* ( $\text{Fe}^{2+}$ )) transfers electrons from CIII to complex IV (CIV), where electrons are transferred via  $\text{Cu}_A$  and cytochrome *a* (cyt *a*) then to the cytochrome  $a_3$  (cyt  $a_3$ )- $\text{Cu}_B$  center to reduce oxygen ( $\text{O}_2$ ) to water ( $\text{H}_2\text{O}$ ). This redox reaction is coupled to the translocation of protons from the matrix to the IMS. During mitochondrial electron transport chain (mtETC), protons are pumped from the matrix to the IMS by CI, CIII and CIV, generating an electrochemical proton gradient. The protons in the IMS re-enter the mitochondrial matrix via the subcomplex  $\text{F}_o$  of the adenosine triphosphate (ATP) synthase (complex V (CV)). As a result, the subcomplex  $\text{F}_1$  connected to the subcomplex  $\text{F}_o$  by the central stalk changes its conformation and drives the phosphorylation of adenosine diphosphate (ADP) to ATP. In addition to CI, plant mitochondria contain the alternative nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) dehydrogenases located either on the outer surface of the IMM (exposed to the IMS) or on the inner surface of the IMM (exposed to the mitochondrial matrix). The alternative NAD(P)H dehydrogenases transport electrons from NAD(P)H to UQ, but do not pump protons across the IMM. Plants have an alternative respiratory pathway that bypasses cytochrome *c* oxidase and transfers electrons from  $\text{UQH}_2$  to the nuclear-encoded alternative oxidase (AOX) localized in the IMM to reduce  $\text{O}_2$  to  $\text{H}_2\text{O}$ . AOX does not pump protons, thus energy produced during the alternative respiratory pathway is lost as heat. OMM = outer mitochondrial membrane,  $e^-$  = electron. The figure is adapted from Millar et al. (2015) edited by Buchanan et al. (2015).

## **The respiratory complexes can associate into the respiratory supercomplexes in plants**

Analyses by Blue Native Polyacrylamide Gel-Electrophoresis (BN-PAGE) have revealed that the plant respiratory complexes can associate into the respiratory supercomplexes (Figure 3) (Dudkina et al., 2006; Eubel et al., 2004; Eubel et al., 2003; Krause et al., 2004). Of note, all respiratory supercomplexes contain dimeric complex III ( $\text{III}_2$ ). In supercomplexes, complex  $\text{III}_2$  can interact either with the membrane arm of complex I or with one or two copies of monomeric complex IV or can even interact with both complex I and IV. Respiratory supercomplexes formed by complex I,  $\text{III}_2$  and IV are referred as respirasomes because they can perform respiratory electron transport from NADH to reduce oxygen (Braun, 2020).

It is proposed that respirasomes can associate to form even larger structures, called respiratory megacomplexes, which are thought to be involved in defining the structure of the cristal membrane (Braun, 2020). Recently, the atomic structure of plant supercomplex III<sub>2</sub>-IV has been revealed at high resolution by cryogenic electron microscopy (cryo-EM) (Maldonado et al., 2021).



**Figure 3.** The respiratory complexes can associate into the respiratory supercomplexes in plants. Shapes and colors of the oxidative phosphorylation (OXPHOS) complexes and of cytochrome *c* are indicated in the legend at the top right. **A.** OXPHOS complexes in monomeric forms (except for complex III, which always occurs as a dimer). **B.** Respiratory supercomplexes in plant mitochondria. **C.** *In vivo*, monomeric OXPHOS complexes and supercomplexes are supposed to coexist within the inner mitochondrial membrane. M = matrix, IM = inner mitochondrial membrane, IMS = mitochondrial intermembrane space. The figure is taken from Braun (2020) with permission. Braun (2020) (DOI: 10.1016/j.mito.2020.04.007) is published under the terms of the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND).

### Plants have an alternative electron transport pathway

The respiratory pathway described above (see “the oxidative phosphorylation system”) is referred as the cytochrome *c* oxidase respiratory pathway, as it utilizes cytochrome *c* to carry electrons from complex III to complex IV. Plants have an alternative respiratory pathway that bypasses the cytochrome *c* oxidase and transfers electrons from ubiquinol to the nuclear-encoded alternative oxidase (AOX)

localized in the IMM to reduce oxygen to water. AOX is non-proton pumping, thus energy released during the alternative respiratory pathway is lost as heat. AOX is not only found in plants, but also found in many algae and fungi, animals and some protozoa (McDonald et al., 2009). The AOX pathway is resistant to classic inhibitors of complex III, such as antimycin A (AA; which inhibits the reduction of ubiquinone at the center N) and myxothiazol (which inhibits the oxidation of ubiquinol at the center P), and of complex IV, such as chemicals competing with oxygen for accepting electrons, including cyanide ( $\text{CN}^-$ ), azide ( $\text{N}_3^-$ ), carbon monoxide (CO) and nitric oxide (NO) (Millar et al., 2015). Nevertheless, AOX activity is inhibited by salicylhydroxamic acid (SHAM) and n-propylgallate (nPG) (Millar et al., 2015).

In addition to the NADH dehydrogenase/complex I, plant mitochondria contain the alternative (type II) nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) dehydrogenases located either on the outer surface of the IMM (exposed to the IMS) or on the inner surface of the IMM (exposed to the mitochondrial matrix) (Rasmusson et al., 2004). As the name implies, the alternative NAD(P)H dehydrogenases transport electrons from NAD(P)H to ubiquinone, but do not pump protons across the IMM (Rasmusson et al., 2004). As a result, if plants are totally dependent on the alternative NAD(P)H dehydrogenases, the theoretical number of ATP generated by complex V will be reduced. The alternative NAD(P)H dehydrogenases are insensitive to rotenone, which inhibits electron transfer from the Fe-S center of complex I to ubiquinone. Due to the presence of the alternative NAD(P)H dehydrogenases, plants can survive without complex I (Braun, 2020), although mutants defective in complex I showed severe growth and development deficiencies (Fromm et al., 2016; Kuhn et al., 2015). Interestingly, the hemiparasitic plant, mistletoe *Viscum album*, is the first reported multicellular eukaryote that completely lacks complex I due to the absence of mitochondrially-encoded *NAD* genes and other nuclear-encoded genes that encode subunits of complex I (Maclean et al., 2018; Senkler et al., 2018). Consequently, the abundance of complex IV and V are decreased whereas the abundance of alternative dehydrogenases and oxidases are increased in *Viscum album* as compared to in the model (non-parasitic) plant *Arabidopsis thaliana* (Maclean et al., 2018).

## The genome of plant mitochondria

Similar to in other eukaryotes, the plant mitochondrion has its own genome (hereafter referred as to the mitochondrial DNA (mtDNA) or the mitogenome) that originated from the endosymbiotic ancestor, an  $\alpha$ -proteobacterium (Sagan, 1967). Through evolution, the mitochondrial genome was decreased steadily through gene loss and/or transfer to the nuclear genome (Chevigny et al., 2020). Mitochondria

have partially retained their own genomes during evolution. On one hand, it is suggested that mitochondria need local regulation of gene expression to rapidly respond to the local redox state (Allen, 2015). On the other hand, it is proposed that mitochondrially-encoded membranous ETC proteins are highly hydrophobic, which causes a problem for importing the proteins into mitochondria (von Heijne, 1986). Indeed, Bjorkholm et al. (2015) proposed that hydrophobic membrane proteins could be targeted to the ER rather than to mitochondria. Nowadays, the plant mitogenomes can be diverse in size and structure depending on plant species, but the number of mitochondrially-encoded genes, including OXPHOS and mitochondrial ribosomal genes, transfer RNA (tRNA) and ribosomal RNA (rRNA), is relatively similar (Chevigny et al., 2020). In the model plant *Arabidopsis thaliana*, the mitogenome contains 57 genes, including OXPHOS proteins, ribosomal proteins, tRNAs and rRNAs (Table 1) (Unsel et al., 1997). To date, it is reported that the size of land plant mtDNA is the largest (Gualberto and Newton, 2017). The size of mammalian mtDNA is 15-17 kilobase pairs (kb), whereas the plant mitogenome is substantially larger: 13-96 kb in algae, usually 200-700 kb in angiosperms, but can be as enormous as 11 megabase pairs (mb) (in *Silene conica*) (Gualberto and Newton, 2017; Sloan et al., 2012). This is because the plant mitogenome mostly contains non-coding sequences that are not conserved across species rather than because the plant mtDNA encodes a few more genes and several genes possess introns (Gualberto and Newton, 2017). Although the plant mitogenome has non-coding sequences originating from chloroplastic, nuclear, or viral DNA by horizontal transfer, the origin of the vast majority of non-coding sequences are unknown (Berghthorsson et al., 2003; Chevigny et al., 2020; Gualberto and Newton, 2017). In addition to the high number of non-coding sequences, the plant mtDNA also contains a high abundance of repeated sequences, which can be categorized into large repeats (> 500 bp), intermediate-sized repeats (50-500 bp) and small repeats (< 50 bp) (Arrieta-Montiel et al., 2009; Davila et al., 2011; Gualberto et al., 2014). It is generally accepted to illustrate the plant mitogenome as a single circular chromosome. For example, the mitogenomes of *Marchantia polymorpha* and *Vicia faba* were observed as single circular DNA molecules (Negruk et al., 1986; Oda et al., 1992). However, using pulse field gel electrophoresis or electron microscopy failed to detect such a single circular chromosome (Backert et al., 1997; Oldenburg and Bendich, 1996). Rather, the plant mitogenome mainly contains a collection of circular subgenomes and linear and branched DNA molecules (Sloan, 2013). Due to the structural variability, the plant mitogenomes have distinct replication mechanisms. Replication of circular DNA molecules is likely to undergo rolling circle mechanisms whereas replication of linear DNA molecules is mediated by specific proteins that covalently interact with terminal inverted repeats (Gualberto and Newton, 2017; Handa, 2008). The plant mitogenome does not exist as naked DNA in the mitochondrial matrix but as part of nucleoids, which are regarded as the heritable units of mtDNA (Dai et al., 2005;

Gilkerson et al., 2013). In spite of the massive size of mitogenomes, the number of mitochondrially-encoded genes in plants is not much higher than that of animals and yeast (about 20 additional genes) (Chevigny et al., 2020). Interestingly, the plant mitogenomes are highly stable and conserved in most higher plant species as compared to in animals because of such lower mutation rates occurring in the plant mtDNA (Wolfe et al., 1987). This could be because plant mitochondria have efficient repair mechanisms, particularly homologous recombination, which is likely to be the main mtDNA repair mechanism in plants (Chevigny et al., 2020; Gualberto and Newton, 2017). Homologous recombination fixes the damaged copy by recruiting an undamaged DNA molecule, which can be brought by the continuous mitochondrial fusion and fission (Gualberto and Newton, 2017; Møller et al., 2021). Other DNA repair pathways existing in plant mitochondria, including direct repair, mismatch repair, nucleotide excision repair and base excision repair, are described in detail by Chevigny et al. (2020).

**Table 1.** The mitogenome of *Arabidopsis thaliana* contains 57 genes, encoding 25 proteins involved in the OXPHOS system (subunits of complex I, III, IV and V, and cytochrome *c* biogenesis), 7 ribosomal proteins, 22 transfer RNAs, and 3 ribosomal RNAs (5S, 18S, and 26S). The table is modified from Unseld et al. (1997). mtSSU = mitoribosome small subunit. mtLSU = mitoribosome large subunit.

Gene	Description
<i>nad1</i>	Subunit of complex I
<i>nad2</i>	
<i>nad3</i>	
<i>nad4</i>	
<i>nad4L</i>	
<i>nad5</i>	
<i>nad6</i>	
<i>nad7</i>	
<i>nad9</i>	
<i>cob</i>	Subunit of complex III
<i>cox1</i>	Subunit of complex IV
<i>cox2</i>	
<i>cox3</i>	
<i>atp1</i>	Subunit of complex V
<i>atp4</i>	
<i>atp6-1</i>	
<i>atp6-2</i>	
<i>atp8</i>	
<i>atp9</i>	
<i>ccmB</i>	Cytochrome <i>c</i> biogenesis
<i>ccmF<sub>c</sub></i>	
<i>ccmFN<sub>1</sub></i>	
<i>ccmC</i>	
<i>ccmFN<sub>2</sub></i>	

<i>rpL2</i>	mtLSU protein
<i>rpL5</i>	
<i>rpL16</i>	
<i>rpS3</i>	mtSSU protein
<i>rpS4</i>	
<i>rpS7</i>	
<i>rpS12</i>	
<i>rrn18</i>	mtSSU ribosomal RNA
<i>rrn26</i>	mtLSU ribosomal RNA
<i>rrn5</i>	
22 transfer RNAs	

## The proteome of plant mitochondria

Proteomic studies have shown that the number of mitochondrial proteins is diverse across eukaryotic organisms, e.g. ~1800 proteins in mammals (Palmfeldt and Bross, 2017), ~1000 proteins in yeast (Schmidt et al., 2010) and 2000-3000 proteins in plants, of which 98-99% are nuclear-encoded, translated by cytosolic ribosomes and imported into mitochondria (Rao et al., 2017). Of note, plants possess 200-300 mitochondrial pentatricopeptide repeat (PPR) proteins (Møller et al., 2021), which are substantially more than in other eukaryotes: yeast and human have only 12 and 7 mitochondrial PPR proteins, respectively (Lightowers and Chrzanowska-Lightowers, 2013). Plants contain 20-40 mitochondrially-encoded proteins (Møller et al., 2021; Mower, 2020). About 70% of nuclear-encoded mitochondrial proteins have N-terminal mitochondrial targeting sequences (MTS). MTS are removed and degraded once nuclear-encoded mitochondrial proteins are imported into different sub-mitochondrial compartments via the translocase of the outer membrane (TOM) and the translocase of the inner membrane (TIM) channels (Chacinska et al., 2009; Kmiec et al., 2014; Prokisch et al., 2004; Schleiff and Becker, 2011). The remaining 30%, e.g. mitochondrial proteins associating with the IMM and subunits of OXPHOS, do not contain canonical MTS (Huang et al., 2009; Kmiec et al., 2014; Schleiff and Becker, 2011; Senkler et al., 2017a). Of note, mitochondrial fusion and fission could mix individual mitochondrial proteomes (Fuchs et al., 2020; Møller, 2016; Møller et al., 2021). In order to find out if a protein is potentially targeted to mitochondria, information on its subcellular localization can be found in the Subcellular Localization Database for Arabidopsis Proteins – SUBA5 (suba.live), which is the central resource for Arabidopsis protein subcellular location data (Hooper et al., 2017). SUBA5 suggested that 2562 proteins are targeted to mitochondria by either large scale proteomics or fluorescent localization (accessed 4<sup>th</sup> January 2023).



# From genes to proteins in plant mitochondria

## Mitochondrial transcription

As the mitochondrion originated from an  $\alpha$ -proteobacterial ancestor, mitochondria have partially retained machinery for expression of their own genomes. Similar to nuclear-encoded genes, mitochondrially-encoded genes undergo transcription and translation, which however occur within mitochondria. In *Arabidopsis thaliana*, the 367 kb mtDNA encodes 25 OXPHOS proteins, 7 ribosomal proteins, 3 rRNAs (5S, 18S, and 26S) and 22 tRNAs (Table 1) (Unsel et al., 1997). Mitochondria have nuclear-encoded phage-type RNA polymerases, which are thought to have taken over from the original bacterial-type RNA polymerase (Cermakian et al., 1996; Gaspari et al., 2004; Gray and Lang, 1998; Greenleaf et al., 1986; Masters et al., 1987; Tiranti et al., 1997; Weihe, 2004; Weihe et al., 1997). Organellar phage-type RNA polymerases are highly similar to the bacterial T7 RNA polymerases (Liere and Börner, 2011). Plant phage-type RNA polymerases are encoded by RpoT gene family (RNA polymerase of the T-phage type) (Liere and Börner, 2011). The RpoT gene family of *Arabidopsis* contains mitochondria-targeted RpoTm, plastid-targeted RpoTp and dual-targeted RpoTmp (Hedtke et al., 1997, 2000; Hedtke et al., 2002; Hedtke et al., 1999; Kobayashi et al., 2002; Kobayashi et al., 2001). Kuhn et al. (2009) showed that RpoTm acts as the fundamental RNA polymerase in *Arabidopsis* mitochondria and is mandatory for the transcription of most, if not all, mitochondrially-encoded genes. Identified mitochondrial promoters in *Arabidopsis* contain the CRTA-type consensus motifs, loosely-unusual sequences for the tetranucleotide core-motif (e.g. ATTA and RGTA) and promoters without consensus (Kuhn et al., 2005). Of note, the study by Kuhn et al. (2009) also showed that RpoTmp activity is gene specific and promoter independent, suggesting that there is an RpoTmp-dependent transcriptional mechanism allowing mitochondria to regulate the expression of specific mitochondrially-encoded genes.

## Post-transcriptional processing of primary mitochondrially-transcribed RNA

Following transcription, mitochondrially-transcribed messenger RNA (mRNA), tRNA and rRNA are processed. According to Binder et al. (2011), processing of most 5' and 3' ends of mRNAs are accomplished via post-transcriptional modification, in which the mature 5' ends and 3' ends are achieved by endonucleolytic and/or exonucleolytic processing. Maturation of 5' and 3' ends of tRNAs recruits two nucleases RNase P and RNase Z, and tRNA processing is accomplished by the addition of 5'-CCA-3', by RNA editing (cysteine (C)-to-uridine (U)) and by normal base modification. Processing of the 5' ends of 18S and

5S rRNAs is likely to be done by endonucleolytic cleavage, however, very little is known about processing the 5' and 3' ends of rRNAs. The steady-state levels of functional mitochondrial RNAs are controlled by the rates of transcribing and degrading RNA. Plant mitochondrial RNAs can be polyadenylated for degradation and the Polynucleotide phosphorylase (PNPase) is a major player for RNA degradation (Holec et al., 2006).

Similar to primary nuclear-transcribed RNAs, mitochondrially-transcribed RNA precursors contain introns, which undergo splicing processes. Organellar introns in plants are categorized into group I and group II (Bonen, 2008; Bonen and Vogel, 2001; Lambowitz and Zimmerly, 2004; Michel et al., 1989; Zimmerly et al., 2001). In angiosperms (particularly vascular plants), most mitochondrial introns are group II introns, which are likely to be spliced by trans-acting cofactors (Brown et al., 2014). Identified proteins facilitating splicing of mitochondrial group II introns include a mitochondrially-encoded maturase MatR, nuclear-encoded maturases (nMATs), CRM-domain proteins (mCSF1 and mCSF2), a DEAD-box RNA-helicase (PMH2), PPR proteins (OTP43, BIR6 and ABO5), a PORR-domain protein (WTF9) and a RCC-domain protein RUG3 (Brown et al., 2014).

Another mitochondrial post-transcriptional modification is RNA editing – a process altering bases in transcript sequences relative to their corresponding genes (Knoop, 2011). About 80% of the plant mitochondrial transcripts have editing sites that are edited in high efficiencies (Bentolila et al., 2013). The most frequent RNA editing activity in plant organelles (both mitochondria and plastids) is C-to-U substitution in mRNAs (Chateigner-Boutin and Small, 2010; Small et al., 2020). Reverse U-to-C editing is however less frequent and has been observed in a few plant lineages, e.g. hornworts, lycophytes and ferns (Chateigner-Boutin and Small, 2010; Small et al., 2020). C-to-U editing also takes place in tRNAs and rRNAs (Binder et al., 1994; Fey et al., 2002; Grewe et al., 2011; Hecht et al., 2011; Marechal-Drouard et al., 1996; Marechal-Drouard et al., 1993; Schuster et al., 1991), in introns and in 5'- and 3'-untranslated regions of many mitochondrial mRNAs (Borner et al., 1995; Carrillo and Bonen, 1997; Farre et al., 2012; Lippok et al., 1994; Shi et al., 2017; Sutton et al., 1991; Wissinger et al., 1991; Zanlungo et al., 1995). RNA editing not only changes the amino acid sequence of a protein, but also influences the RNA structure, consequently affecting RNA splicing and stability and RNA-protein interaction (Bonen, 2008; Chateigner-Boutin and Small, 2010; Oldenkott et al., 2014; Small et al., 2020). Specific editing sites of plant organelles are recognized by site-specific binding of RNA-binding cofactors - PPR proteins containing repeats of a tandem domain of 35 amino acid motifs (a helix-turn-helix structure), the PPR domains (Small and Peeters, 2000).

Several studies on the structures of PPR protein–RNA complexes showed that PPR proteins bind to single-stranded RNAs in a sequence-specific manner (Coquille et al., 2014; Gully et al., 2015; Ke et al., 2013; Shen et al., 2016; Yin et al., 2013). Within one certain repeat, the amino acids at the 5<sup>th</sup> and 35<sup>th</sup> positions, which

correspond to residues 6 and 1' in Barkan et al. (2012) and residues 4 and ii in Yagi et al. (2013), specifically recognize RNA bases (Yan et al., 2019). These di-residues are termed as the PPR code. Several PPR codes have been identified: threonine and asparagine (TN), threonine and aspartic acid (TD), asparagine and serine (NS), and asparagine and aspartic acid (ND) at the 5<sup>th</sup> and 35<sup>th</sup> positions recognize the corresponding nucleotides adenine (A), guanine (G), cytosine (C) and uracil (U), respectively (Barkan and Small, 2014). Notably, the 5<sup>th</sup> position mainly determines the RNA base specificity. The presence of asparagine at this position prefers pyrimidine-binding whereas serine or threonine prefers purine-binding (Barkan and Small, 2014). The 35<sup>th</sup> position is the second main determinant. The presence of asparagine at this position prefers to bind to base A or C while aspartate results in preference of binding to base G or U (Barkan and Small, 2014). Recently, Yan et al. (2019) has further explored the correlation between PPR codes and RNA bases, which enriched the pool of PPR codes. Based on the results, Yan and co-workers developed the online PPRCODE web server (<http://yinlab.hzau.edu.cn/pprcode/>) to facilitate the prediction of PPR protein-RNA binding sites.

PPR proteins are categorized into two subclasses: the PPR-P subclass present in many eukaryotes, and the PPR-PLS subclass, which are land plant-specific and have long 35 or 36 amino acids and short 31 or 32 amino acids variants of the canonical 35 amino acid PPR motif (P) (Cheng et al., 2016; Lurin et al., 2004). The PPR-P subclass has been proposed to be involved in RNA transcription, splicing, editing, cleavage, stability and in mitochondrial translation (Barkan and Small, 2014; Haili et al., 2016; Rugen et al., 2019; Schmitz-Linneweber and Small, 2008; Uyttewaal et al., 2008; Waltz and Giege, 2020; Waltz et al., 2019; Waltz et al., 2020b). The PPR-PLS subclass is specific to C-to-U RNA editing (Barkan and Small, 2014; Small et al., 2020). The PPR-PLS proteins contain highly conserved domains E1, E2 and DYW (named after amino acid residues of the domain). E1 and E2 domains resemble PPR or tetratricopeptide repeat (TPR) motifs (Cheng et al., 2016) but their exact functions remain elusive. The DYW domain is likely to possess a cytidine deaminase activity that performs the nucleotide exchange process (Small et al., 2020). However, it remains unclear how many RNA-editing PPR proteins that do not contain the DYW domain can still perform editing (Small et al., 2020). In addition to the PPR-PLS subclass, non-PPR proteins, including Multiple Organellar RNA editing Factors (MORFs) (also known as RNA-editing factor Interacting Proteins (RIPs)), Other RNA Recognition Motif (ORRM) (e.g. ORRM4 and ORRM5) and NUWA-related proteins (a P-class PPR protein), are also found to be involved in plant mitochondrial RNA-editing (Andres-Colas et al., 2017; Bentolila et al., 2012; Guillaumot et al., 2017; Shi et al., 2017; Shi et al., 2016; Takenaka et al., 2012).

## Mitochondrial translation

Similar to nuclear gene expression, the last step of mitochondrial gene expression is translation, a process in which the mRNA sequence is used as a template to synthesize a corresponding protein chain by the mitochondrial ribosome (hereafter referred as to the mitoribosome). Translation is composed of four phases: initiation, elongation, termination and ribosome recycling. Like the cytosolic ribosome, a mitoribosome is also composed of rRNAs and two main subunits: the small subunit, which binds to mRNA and reads the genetic information, and the large subunit, which has tRNA aminoacyl (A), peptidyl (P) and exit (E) sites to synthesize the protein chain. In contrast to plastid translation, very little is known about plant mitochondrial translation. However, recent studies on mitoribosome structures of eukaryotes, including plants, help us to predict some aspects of plant mitochondrial translation.

Using cryo-EM, the structure of bacterial ribosome and mitoribosome structures of mammals, yeast, trypanosomes and plants were described at very high resolution (Figure 4) (Amunts et al., 2015; Desai et al., 2017; Greber et al., 2015; Noeske et al., 2015; Ramrath et al., 2018; Waltz et al., 2020b; Watson et al., 2020). As compared to bacterial ribosomes, mitoribosomes from eukaryotic kingdoms are protein-rich and 1.2-1.5 time larger (Waltz et al., 2020a; Waltz and Giege, 2020). This is because the mitoribosome small subunit (mtSSU) is substantially larger, and even surpasses the size of the mitoribosome large subunit (mtLSU) in trypanosomes and plants (Ramrath et al., 2018; Rugen et al., 2019; Waltz et al., 2019). In addition to the size difference, the structure and composition of eukaryotic mitoribosomes are also very different from the bacterial counterparts (Waltz and Giege, 2020). The diversity of mitoribosomes across eukaryotes suggests that eukaryotic mitoribosomes have evolved to adapt to particular niches during evolution (Waltz et al., 2020a). The composition of plant mitoribosome was revealed by Waltz et al. (2019) and Rugen et al. (2019), identifying 19 plant-specific mitoribosome proteins, of which half are PPR proteins. The study by Waltz et al. (2020b) described the atomic structure of the plant mitoribosome. Briefly, the plant mitoribosome is extended in rRNAs and protein contents. Most of plant-specific PPR proteins bind to the plant-specific rRNA expansion segments, which leads to reshaping the overall structure of plant mitoribosome. The central protuberance of plant mitoribosomes, where the mtLSU inter-connects with the mtSSU, consists of a 5S rRNA (like the bacterial counterpart) and mitochondrial plant-specific proteins. This specific structure thus contributes to increasing and reshaping the overall volume of the plant ribosome central protuberance. Similar to mammalian and yeast mitoribosomes, the plant mitoribosome is likely to be attached to the IMM and to interact with the insertase OXA1 through linker(s) (possibly protein(s)) (Waltz et al., 2020a; Waltz et al., 2020b).

	Fungi	Mammals	Plants	Kinetoplastids	Ciliates
	<i>Saccharomyces cerevisiae</i> , <i>Neurospora crassa</i>	<i>Homo sapiens</i> , <i>Sus scrofa</i>	<i>Brassica oleracea</i>	<i>Trypanosoma</i> spp., <i>Leishmania tarantolae</i>	<i>Tetrahymena</i> <i>thermophila</i>
Sedimentation coefficient:	70–74S	55S	77–78S	50S	80S
Molecular weight:	~3–3.3 MDa	2.7 MDa	–	~4.5 MDa	~4.0 MDa
Number of rRNAs:	2 (15/16S, 21/23S)	3 (12S, 16S, mtRNA <sup>Phe/Val</sup> )	3 (18S, 5S, 26S)	2 (9S, 12S)	2 (14S, 21S)
Number of mitoribosomal proteins:	~73–78	82	~86	~122	92

**Figure 4.** Advanced cryo-electron microscopy studies have recently revealed the structure of mitoribosomes and their diversification in fungi, mammals, plants and protozoans (kinetoplastids and ciliates). The figure is taken from Kummer and Ban (2021) (DOI: 10.1038/s41580-021-00332-2) with permission from Springer Nature.

In eukaryotes, (including yeast, mammals and plants), mitochondrial mRNAs do not have Shine-Dalgarno (SD) sequences – the ribosomal binding site in bacterial mRNAs (Ayyub and Varshney, 2020; Derbikova et al., 2018; Hazle and Bonen, 2007). Of note, yeast mitochondrial mRNAs have very long 5' untranslated regions (5'UTRs) while mammalian mitochondrial mRNAs have very short 5'UTRs (maximum three-nucleotide length) or even do not have 5'UTRs (Ayyub and Varshney, 2020; Derbikova et al., 2018). Therefore, mitochondrial translation initiation in yeast and mammals strongly relies on translation initiation factors and/or activators (Ayyub and Varshney, 2020; Derbikova et al., 2018). A unique feature of mitochondrial translation initiation in mammals has recently been described by Kummer et al. (2018). Translation initiation in mammalian mitochondria is mediated by initiation factors, IF2 and IF3, and the mtSSU PPR protein mS39, which binds to an U-rich domain in the mRNA downstream of its start codon AUG (Kummer et al., 2018). Such U-rich regions are conserved and found downstream of codon 7 (after AUG) in 11 mRNAs (out of 13) of mammalian mitochondria (Amunts et al., 2015; Bieri et al., 2018; Kummer et al., 2018). In yeast, mitochondrial translation initiation seems to recruit universal translation initiation factors and specific translation activators to help the mtSSU interact with the 5'UTRs of mRNAs (Derbikova et al., 2018). Plant mitochondrial mRNAs also contain long 5'UTRs without SD sequences and plant mitochondrial rRNAs do not have anti-SD sequences either (Waltz et al., 2020b). To date, the mechanism of translation initiation in plant mitochondria is still enigmatic. The structure study of plant mitoribosome by Waltz et al. (2020b) suggested that mS83/rPPR10 is a PPR protein sitting in a cleft where the incoming mRNAs may be recruited. Waltz et al.

(2020b) also hypothesized that the PPR motifs of mS83/rPPR10 recognize AxAAA-related motifs in the 5'UTRs (located about 19 nucleotides upstream of the start codon) of 17 (out of 33) mitochondrial mRNAs, thus acting as a SD-anti-SD-like recognition system.

## Mitochondrial retrograde signalling

Although mitochondria likely evolved from endosymbiotic  $\alpha$ -protobacteria, mitochondria do not function independently, but their activities are integrated with other cellular organelles. To operate efficiently, a continuous flow of information between the nucleus and the mitochondrion is required, and it is described as anterograde and retrograde signalling (Ng et al., 2014). Anterograde signals from the nucleus are transmitted to the mitochondrion, as the nuclear regulation of the mitochondrial activity. Retrograde signals derived from the mitochondrion are sent to the nucleus and affect nuclear gene expression. In plants, mitochondrial retrograde signalling was first discovered by inhibition of mitochondrial function, resulting in the upregulation of *AOX1* transcript in tobacco (*Nicotiana tabacum*) (Vanlerberghe and McIntosh, 1996). Subsequently, the promoter region of *AOX1a* has been used as a “classical” bait for identification of mitochondrial retrograde signalling regulators in plants, including ABSCISIC ACID-INSENSITIVE 4 (ABI4) (Giraud et al., 2009), WRKY transcription factors (Van Aken et al., 2013; Vanderauwera et al., 2012), and MYB transcription factors (Ivanova et al., 2014; Zhang et al., 2017a; Zhang et al., 2017b). Nevertheless, the most well-studied and specific transcriptional regulators of mitochondrial retrograde signalling belong to the ARABIDOPSIS NAC DOMAIN CONTAINING (ANAC) protein family, including ANAC017 functioning as the main regulator and ANAC013 contributing as a downstream target in positive feedback loops (De Clercq et al., 2013; Ng et al., 2013; Van Aken et al., 2016). Of note, the study by Ng et al. (2013) showed that ANAC017 is a dual-targeted protein. ANAC017 is anchored in the ER membrane via a C-terminal transmembrane (TM) domain containing a consensus rhomboid protease cleavage site, which is suggested to be truncated proteolytically by rhomboid-related proteases during mitochondrial dysfunction. This allows ANAC017 to be released from the ER while its C-terminal TM domain stays in the ER, leading to the translocation of the N-terminal fragment of ANAC017 into the nucleus to activate gene expression (Ng et al., 2013). However, the exact mechanism of ANAC017 translocation remains enigmatic. In the nucleus, ANAC017 stimulates expression of mitochondrial retrograde signalling marker genes, including *AOX1a*, *UP REGULATED BY OXIDATIVE STRESS AT2G21640 1 (UPOX1)*, *URIDINE DIPHOSPHATE GLYCOSYLTRANSFERASE 74E2 (UGT74E2)*, *NAD(P)H DEHYDROGENASE B4 (NDB4)*, *At12CYS-2*, *H<sub>2</sub>O<sub>2</sub> RESPONSE GENE 1 (HRG1)*

and *OUTER MEMBRANE PROTEIN OF 66 KDA (OM66)* (Van Aken et al., 2016; Van Aken et al., 2007). Reactive oxygen species (ROS) generated by the mtETC and peptides from degraded proteins possibly trigger the ANAC017 pathway (Møller and Sweetlove, 2010; Ng et al., 2014; Vestergaard et al., 2012), but the molecular signals from a dysfunctional mitochondrion and how they initiate this signalling pathway remain elusive. Surprisingly, when mitochondrial function was sufficiently impaired, a consistent downregulation of chloroplast-encoded transcripts was observed (Adamowicz-Skrzyrkowska et al., 2020; Zubo et al., 2014), suggesting that an as yet unknown (possibly retrograde) signalling pathway apparently can reduce chloroplast transcription.

## Mitochondrial unfolded protein response

The mitochondrial unfolded protein response (UPR<sup>mt</sup>) is described as the transcriptional response that regulates nuclear gene expression during mitochondrial malfunction to maintain mitochondrial homeostasis by inducing the mitochondrial protein quality control (chaperones and proteases). The UPR<sup>mt</sup> can be triggered by mtDNA mutations (Lin et al., 2016), malfunctioning mitochondrial membrane potential (Rolland et al., 2019), imbalance between nuclear- and mitochondrially-encoded proteins (Houtkooper et al., 2013) or overloading concentration of unfolded/misfolded proteins in mitochondria (Zhao et al., 2002). Recent studies have gained insight into the plant UPR<sup>mt</sup>. The study by Moullan et al. (2015) suggested that the plant UPR<sup>mt</sup> is at least partially conserved to the animal UPR<sup>mt</sup>. Wang and Auwerx (2017) showed that the UPR<sup>mt</sup> in *Arabidopsis thaliana* is activated by a transient oxidative burst, resulting in the activation of mitogen-activated protein kinases (MAPKs) and hormonal signalling (ethylene, auxin and jasmonate) to recover mitochondrial proteostasis. Kacprzak et al. (2020) showed that ANAC017 – the main transcriptional regulator of mitochondrial retrograde signalling, induces many classes of genes that are classic targets of UPR<sup>mt</sup> in response to AA treatment. Of note, ANAC017 induces “classical” mitochondrial retrograde signalling marker genes that have been also suggested as UPR<sup>mt</sup> marker genes by Moullan et al. (2015) and Wang and Auwerx (2017). This thus suggests that “classical” mitochondrial retrograde signalling and UPR<sup>mt</sup> in plants are most likely one and the same response (Kacprzak et al., 2020; Tran and Van Aken, 2020).

## Adenyl cyclases in plants

Adenyl cyclases (ACs), also known as adenylyl cyclases or adenylyl cyclases, are enzymes that convert ATP into 3',5'-cyclic adenosine monophosphate (cAMP), which is a secondary messenger that can affect different physiological and biochemical processes in the cells. The important roles of cAMP as well as identification of ACs have been well-established in bacteria, animals and fungi. In plants, cAMP is involved in ion homeostasis, stomatal opening, pollen tube development, seed germination and cell cycle progression (Blanco et al., 2020). Nevertheless, comprehensive knowledge of plant cAMP and ACs is still missing. This could be due to two reasons. Firstly, it could be difficult to detect plant cAMP because the cAMP concentration is lower in plants than in animals. Reported cAMP concentration in plants is less than 40 pmol g<sup>-1</sup> fresh weight in rye grass endosperm cell cultures, *Torenia* stem segments, and cultured cells of *Phaseolus vulgaris* and *Nicotiana tabacum*, 40-170 pmol g<sup>-1</sup> fresh weight in *Daucus carota*, *Triticum aestivum*, and *Lemna paucicostata*, 200–250 pmol g<sup>-1</sup> fresh weight in *Citrus*, *Lactuca*, *Hordeum*, and *Zea* (Sabetta et al., 2016). On the contrary, reported cAMP level in animals is usually greater than 250 pmol g<sup>-1</sup> wet weight (Gehring, 2010). Secondly, it could be challenging to identify plant ACs because plant ACs seem not to have conserved catalytic domains (Gehring, 2010). However, Gehring (2010) proposed that plant ACs might have the conserved motif [RKS]X[DE]X(9,11)[KR]X(1,3)[ED] in their catalytic domains. To date, experimentally identified ACs in plants are PsiP in *Zea mays* (Moutinho et al., 2001), HpAC1 in *Hippeastrum x hybridum* (Swiezawska et al., 2014), AtKUP7, ATPPR-AC, AtCIAP and LRRAC1 in *Arabidopsis thaliana* (Al-Younis et al., 2015; Bianchet et al., 2019; Chatukuta et al., 2018; Ruzvidzo et al., 2013), NbAC in *Nicotiana benthamiana* (Ito et al., 2014), and MpCAPE in *Marchantia polymorpha* (Kasahara et al., 2016). Of note, these plant ACs, except HpAC1 and MpCAPE, appeared to have the AC-motif proposed by Gehring (2010). Therefore, it seems to be difficult to identify plant ACs if just based on this motif search. It is also noticeable that *AT4G15640* and *AT3G21465* are annotated as ACs on The Arabidopsis Information Resource (TAIR), but there is no experimental confirmation.



# The aims of this thesis

The mitochondrial unfolded protein response (UPR<sup>mt</sup>) is described as the transcriptional response that regulates nuclear gene expression during mitochondrial malfunction to maintain mitochondrial homeostasis. The aim of paper I is to review and compare UPR<sup>mt</sup>-related signalling across eukaryotic kingdoms, including animals, fungi and plants.

The study of plant mitochondria usually requires isolation of pure, intact and functional mitochondria from plant tissues. The aim of paper II is to present a strategy for efficient isolation of mitochondria from leaf tissues of *Arabidopsis thaliana* by using continuous Percoll density gradients.

Despite a relatively thorough understanding of basic mitochondrial functions, many mitochondrial proteins and processes remain poorly understood. The aim of paper III is to characterize the two *Arabidopsis thaliana* genes, *AT4G15640* and *AT3G21465*, which are predicted as mitochondria-targeted proteins and have been poorly annotated as adenyl cyclases on TAIR.

# Summary of papers

Paper I summarizes the current knowledge of UPR<sup>mt</sup> across eukaryotic kingdoms, including animals, fungi and plants. UPR<sup>mt</sup> has been extensively studied in animals, whereas very few related-UPR<sup>mt</sup> have been described in yeast. In plants, little is known about the UPR<sup>mt</sup> and only few regulators have recently been identified. In this work, we compare the UPR<sup>mt</sup> across kingdoms. Our study indicates that each kingdom has evolved their own specific regulators, which however induce very similar groups of target genes. Our meta-analysis identifies homologs of known UPR<sup>mt</sup> regulators and their responsive genes across eukaryotic kingdoms, which could be of interest for future research.

Paper II describes two strategies for plant growth (in soil and in liquid half-strength Murashige and Skoog media) and an efficient method of mitochondrial isolation from leaf tissues of *Arabidopsis thaliana*. By using continuous Percoll density gradients, isolated mitochondria are relatively free from chloroplast contamination. Isolated *Arabidopsis* mitochondria obtained by this method can be either used for assays requiring intact mitochondria, e.g. import assays or respiration measurements, or be stored at -80°C for later use, e.g. BN-PAGE or western blot.

Paper III describes the characterization of two *Arabidopsis thaliana* genes, *AT4G15640* and *AT3G21465*. We show that *AT4G15640* and *AT3G21465* are unlikely to be ACs as previously annotated on TAIR. Instead, *AT4G15640* and *AT3G21465* are land plant-specific mitochondrial proteins that are essential for plant performance. Knocking out both *AT4G15640* and *AT3G21465* results in low abundance and activity of mitochondrial OXPHOS complexes, thus reducing capacity of oxygen consumption. Our immunoblotting and tandem mass spectrometry (MS/MS) analysis using isolated mitochondria show a severe perturbation of the mitochondrial proteome and reduction of many OXPHOS components due to loss of *AT4G15640* and *AT3G21465*. Using co-immunoprecipitation, we confirm that *AT4G15640* and *AT3G21465* are part of the mtSSU, in line with two proteomic studies of *Arabidopsis* mitoribosome during the course of our study. Therefore, we named *AT4G15640* and *AT3G21465* as mitochondrial TRANslation factor 1 (mTRAN1) and 2 (mTRAN2), respectively. Transcriptome profile of *mtran1 mtran2* mutant resembles most two mutants defective in a mitoribosomal protein and shows no clear effects on mitochondrial splicing and editing, thus indicating mitochondrial translation defects. *In organello* protein synthesis and polysome fractionation assays suggest that mTRAN proteins

are required for mitochondrial translation initiation. Our motif analysis suggests that the 5'UTRs of mitochondrial mRNAs contain two potential mitoribosome binding sites that are A/U-rich regions (CUUUxU and AAGAAx/AxAAAG). Moreover, the RNA electrophoretic mobility shift assays show that mTRAN1 binds to these A/U-rich domains in the 5'UTRs of mitochondrial mRNAs to initiate translation. Finally, our Ribo-seq analysis shows that not only a subset, but all mitochondrial mRNAs have lower ribosome loading levels in the *mtran* mutants compared to in the wild type, suggesting that mTRAN proteins are universal mtSSU-embedded translation initiation factors.

# Discussion

In paper I, the current knowledge of UPR<sup>mt</sup>-related signalling across eukaryotic kingdoms, including animals, fungi and plants, was summarized and compared. The comparison indicates that each kingdom has evolved their own specific regulators, which however induce very similar groups of target genes, such as mitochondrial (co-)chaperones and proteases. Homologs of known UPR<sup>mt</sup> regulators and responsive genes are identified across eukaryotic kingdoms, which might be interesting for future research. The authors hypothesized that such different UPR<sup>mt</sup> pathways occurring across kingdoms could be due to major evolutionary events, such as rise of multicellularity or colonization of land. Indeed, the ANAC017 group – one of the main UPR<sup>mt</sup> regulators in land plants, is suggested to be evolved from ancestral NACs (found in at least some green algae) during the period of land colonization by plants (Khan and Van Aken, 2022). In plants, “classical” mitochondrial retrograde signalling and UPR<sup>mt</sup> appear to be the same response during mitochondrial dysfunction. In *Arabidopsis*, UPR<sup>mt</sup> is activated in response to mitochondrial malfunction by treatment of Doxycycline (Dox) - a mitochondrial ribosome inhibitor, MitoBlock-6 (MB) - an inhibitor of protein import into mitochondria and AA, which inhibits electron transfer at the center N of complex III leading to an increase in ROS. However, the molecular signals from a dysfunctional mitochondrion and how they initiate this signalling pathway remain enigmatic.

In paper II, we described an efficient method of mitochondrial isolation from leaf tissues of *Arabidopsis thaliana* by using continuous Percoll-PVP density gradients. The most advantage of this protocol is that freshly isolated mitochondria are intact and pure (relatively free from chloroplast contamination), which is of great importance for following assays requiring functional mitochondria, e.g. *in organello* protein synthesis (Kwasniak-Owczarek et al., 2022), import assays (Zhang et al., 2012) and oxygen consumption measurement (Keech et al., 2005). However, this method usually requires a large amount of starting plant material and takes a few hours to complete. Another method of plant mitochondrial isolation, IMTACT (Boussardon et al., 2020), in which transgenic *Arabidopsis* carrying mitochondrial OUTER MEMBRANE 64 (OM64) - biotin ligase recognition peptide (BLRP) placed under tissue-specific promoters, enables cell-specific biotinylated mitochondria to be purified with streptavidin magnetic beads in less than 30 min (Boussardon and Keech, 2022). As compared to IMTACT, our described

mitochondrial isolation protocol is slower (timewise) and requires much more plant material. Nevertheless, IMTACT requires generation of transgenic plants, which would take time to produce a desired plant line. For instance, if we want to apply IMTACT on the *mtran* double mutants for mitochondrial isolation, we must cross the *mtran* double mutant line with the OM64-BLRP line, which would take at least several months to get a homozygous *mtran* double mutant line expressing OM64-BLRP. Depending on the purposes plus time management, we recommend you to wisely choose which method of Arabidopsis mitochondrial isolation will be suitable for your project.

In paper III, the two *Arabidopsis thaliana* genes, *AT4G15640* and *AT3G21465*, were characterized. It turned out that these genes are unlikely to be ACs as annotated on TAIR. The study shows that *AT4G15640* and *AT3G21465* are land-plant specific mitochondrial proteins that are critical for plant growth. Strikingly, *AT4G15640* and *AT3G21465* are shown to be part of the mtSSU and are of great importance for efficient mitochondrial translation initiation. Therefore, *AT4G15640* and *AT3G21465* are suggested to be annotated as mitochondrial TRANslation factor 1 (mTRAN1) and mitochondrial TRANslation factor 2 (mTRAN2), respectively. Of note, comparison of transcriptomic data (the *mtran1 mtran2* mutant versus *rps10* mutants, which have reduced expression of mtSSU ribosomal protein *RPS10*) showed that UPR<sup>mt</sup> was activated, leading to induction of target genes in ANAC017 pathway in both mutants. Wang and Auwerx (2017) showed that in the Arabidopsis *mitochondrial ribosomal protein L1 (mrpl1-1)* mutant, UPR<sup>mt</sup> was activated, which in turn induced ethylene responses as a systemic signal. Together, these suggest that UPR<sup>mt</sup> is activated by impairment of mitochondrial translation. Such a UPR<sup>mt</sup> activation was not only observed in plants, but also in animals, for instance, knockout of mitochondrial ribosomal protein triggered mitonuclear protein imbalance, which activated the UPR<sup>mt</sup> in *Caenorhabditis elegans* (*C. elegans*) (Houtkooper et al., 2013). Therefore, imbalance between nuclear- and mitochondrially-encoded proteins probably induces the plant UPR<sup>mt</sup> during malfunctioning mitochondrial translation. Upon UPR<sup>mt</sup> activation, ANAC017 and ethylene signalling pathways are independently induced. This suggests that there are possibly specific signal molecules inducing different signalling responses. However, the specific signals from mitochondria defective in translation and how they induce the UPR<sup>mt</sup> in plants remain elusive.

Knockout of *mTRAN1* and *mTRAN2* showed a reduction in OXPHOS complex abundance and activity, which resulted in a strong decrease in capacity of the cytochrome *c* oxidase respiratory pathway. Such a defect caused *mtran1 mtran2* mutants to rely on the alternative respiration. Rolland et al. (2019) proposed that the UPR<sup>mt</sup> in *C. elegans* was activated when most mitochondrial processes required for the maintenance of mitochondrial homeostasis, e.g mtETC, were compromised. A general signal that possibly triggers UPR<sup>mt</sup> is a decrease in mitochondrial membrane potential, which results in a decrease in mitochondrial protein import, likely leading

to accumulation of non-imported mitochondrial proteins (Tran and Van Aken, 2020). As loss of *mTRAN1* and *mTRAN2* impaired mtETC, one could expect that mitochondrial membrane potential was possibly reduced though it was not confirmed experimentally, causing activation of UPR<sup>mt</sup>. In addition, *mtran1 mtran2* mutants showed a defect in complex I and III, which could lead to an increase in the rate of superoxide production (Cadenas et al., 1977; Møller, 2001; Murphy, 2009). Although ROS measurement was not carried out in the study, superoxide – a signal that could trigger the plant UPR<sup>mt</sup> (Tran and Van Aken, 2020), could be expected to be increased and activate UPR<sup>mt</sup> due to loss of *mTRAN1* and *mTRAN2*.

Surprisingly, the RNA-seq data of *mtran1 mtran2* mutant showed a downregulation of 117 chloroplast-encoded transcripts, which was also observed in two previous studies (Adamowicz-Skrzyplowska et al., 2020; Zubo et al., 2014). Of note, the chloroplast transcriptome of *mtran1 mtran2* mutant showed that all chloroplast-encoded tRNAs were significantly downregulated, indicating that chloroplast gene expression was likely to be decreased, which could lead to a decrease in photosynthesis and an increase in ROS production in chloroplast (Arsova et al., 2010; Kindgren et al., 2012; Myouga et al., 2008). ROS can damage proteins inside the chloroplasts, causing activation of chloroplast unfolded protein response (cpUPR) (Kessler and Longoni, 2019). Indeed, *ClpB3* – a cpUPR marker gene (Llamas et al., 2017), was upregulated in the *mtran1 mtran2* mutant. ClpB3 is a nuclear-encoded chloroplastic Clp protease that helps refolding unfolded/misfolded/aggregated 1-deoxy-D-xylulose-5-phosphate synthase (DXS) – an enzyme that converts pyruvate and D-glyceraldehyde-3-phosphate (D-GAP) into 1-deoxy-D-xylulose-5-phosphate (DXP) in the first step of the methylerythritol phosphate (MEP) pathway occurring within the chloroplasts (Pulido et al., 2016). DXS aggregation was formed after chloroplast translation was specifically inhibited by lincomycin even though DXS transcript levels remained unchanged and only its protein levels increased (Llamas et al., 2017). The *mtran1 mtran2* mutant had all chloroplast-encoded tRNAs downregulated, suggesting that chloroplast translation was likely to be perturbed. Therefore, one could expect that DXS aggregation would occur, leading to the upregulation of *ClpB3*. Taken together, we suggest that loss of *mTRAN* genes not only induces UPR<sup>mt</sup> but also activates cpUPR.

All in all, compromised mitochondrial translation in plants could result in (i) mitonuclear protein imbalance, (ii) a decrease in mitochondrial membrane potential leading to accumulation of non-import protein in mitochondria, and (iii) impaired mtETC causing an increase in ROS production in mitochondria. These consequences could activate UPR<sup>mt</sup> and another as yet known (probably retrograde) signalling pathway that apparently can reduce chloroplast transcription, possibly leading to a decrease in chloroplast gene expression, which causes activation of cpUPR. Another hypothesis is that there is possibly a crosstalk between mitochondria and chloroplasts (but not via the nucleus), in which mitochondrial

gene expression could somehow affect transcription and/or even gene expression in chloroplasts.

To date, the full mechanism of mitochondrial translation in plants remains elusive. The atomic structure of cauliflower mitoribosomes revealed by Waltz et al. (2020b) helps predicting how plant mitoribosome initiates translation. Waltz and his co-workers suggested that mTRAN1 (named as mS83/rPPR10 by the authors) is a PPR protein, which functions as a RNA-binding protein. However, the PPR codes of mTRAN proteins could not be predicted by the PPRCODE web server (Yan et al., 2019). mTRAN1 resides in a cleft of the mtSSU, where its PPR domains might recognize and bind to AxAAA-related motifs of mRNAs to initiate translation because plant mitochondrial mRNAs do not contain SD sequences nor do plant mitochondrial rRNAs have anti-SD sequences. These AxAAA-related motifs were found in the 5'UTRs of 17 out of 33 mitochondrially-encoded mRNAs (Waltz et al., 2020b). In contrast, our polysome profiling showed that mTRAN1 and mTRAN2 are required for mitoribosome binding/translation initiation of not only mitochondrial mRNAs containing such an AxAAA 5'UTR motif (*NAD9*, *COX2* and *ATP9*) but also mitochondrial mRNAs that do not appear to contain this binding site (*NAD7*, *COB*, *ATP1* and *ATP8*). Moreover, the Ribo-seq analysis of mitochondrial genes further supports that mTRAN proteins are universal mtSSU-embedded translation initiation factors, which could recognize the motif CUUUxU/AxAAAG/AAGAAX in the 5'UTRs of mitochondrial mRNAs to initiate translation based on our motif analysis and RNA electrophoretic mobility shift assays (REMSAs). To verify the interaction between mTRAN1 and mitochondrial mRNAs *in vivo*, we will perform RNA Immunoprecipitation Sequencing (RIP-seq) using Arabidopsis cell culture transformed with mTRAN1-GFP and/or isolated mitochondria from a stably transformed Arabidopsis line expressing mTRAN1-GFP. Transcripts interacting with mTRAN1-GFP complexes will be purified using GFP-affinity purification and subsequently sequenced to find out if mTRAN1 interacts with the vast majority of mitochondrial mRNAs *in planta*, further supporting our finding that mTRAN1 is a universal translation initiation factor of mtSSU. In addition, RIP-seq could also allow us to identify the mitoribosomal binding sites to validate our REMSA results.

As mitochondria originated from the ancient endosymbiotic bacteria, mitochondrial translation could be expected to resemble bacterial translation, at least partially. However, comparison of bacterial ribosomes versus mitoribosomes of mammals, yeast and plants showed the diversity of mitoribosome structure, indicating that eukaryotic mitoribosomes have evolved to adapt to particular niches during evolution (Waltz et al., 2020a). This suggests that mitochondrial translation in general and mitochondrial translation initiation in particular could also be diverse across eukaryotic kingdoms. In bacteria, the ribosomal small subunit binds to the SD motif of mRNA using an anti-SD sequence of 16S rRNA (Shine and Dalgarno, 1974). In addition, bacterial translation initiation requires initiation factor (IF) 1, 2

and 3. Unlike bacteria, eukaryotes, including yeast, mammals and plants, do not have SD sequences in the 5'UTRs of mitochondrially-encoded mRNAs. In mammals (at least in humans), most of mitochondrial mRNAs do not have 5'UTRs (Montoya et al., 1981). Kummer et al. (2018) suggested that the PPR protein mS39, which is located in the mtSSU, binds to the U-rich motif downstream the start codon of mRNAs to initiate translation. Notably, the PPRCODE prediction tool–Yin Lab (Yan et al., 2019) predicts that mS39 has four PPR motifs that bind to RNA bases (G)(C>U)(C>U>A)(-) with low scores. Thus, it is possible that both mS39 and mTRAN proteins are PPR-like proteins that do not obey the “classical” PPR code rule. Mammals lack mitochondrial IF1 (mtIF1) and require mtIF2 and mtIF3 for translation initiation (Ayyub and Varshney, 2020). In yeast, the mtSSU contains a platform formed by mS35, mS46 and extensions of uS3m and uS5m, which is suggested to be the mtSSU-mRNA interaction site during translation initiation (Itoh et al., 2020). Mitochondrial translation initiation in yeast seems to recruit universal mtIFs and specific translation activators that are likely to recognize specific 5'UTRs of mitochondrial mRNAs (Derbikova et al., 2018). Therefore, the mtSSU-mRNA interaction site proposed by Itoh et al. (2020) is expected to act as a platform for mtIFs and translation activators to bind to the 5'UTRs of mitochondrial mRNAs to initiate translation. In fact, several translation activators were co-purified with the yeast mitoribosome (Desai et al., 2017). In plants, mTRAN1 is likely to be a PPR-like protein acting as a mRNA-binding factor of the mtSSU. Of note, both yeast and plant mitochondrial mRNAs have long 5'UTRs (Derbikova et al., 2018; Waltz et al., 2020a). Therefore, one could speculate that yeast and plant mitochondrial translation initiation/mtSSU-mRNA binding could somehow use a comparable mechanism. Our findings suggest that mTRAN proteins act as universal recognition factors for mitochondrial mRNAs, however, there is a high possibility that mtIFs and/or mitochondrial translation activators required for recognition of different specific motifs of mRNAs exist in plants. To date, very little is known about mtIFs in plants, e.g. only MTL1 and RFL8 have recently been identified as specific proteins mandatory for translation of *NAD7* and *ccmFN<sub>2</sub>*, respectively (Haili et al., 2016; Nguyen et al., 2021). We attempted to perform a BLAST search in *Arabidopsis thaliana* to identify proteins that are similar to bacterial translation IFs (unpublished data). The putative subcellular localization of identified proteins was searched in SUBA5 (Hooper et al., 2017). Bacterial translation IF1 has a similar protein, AT4G11175, which is targeted to chloroplasts and enables mRNA-binding (Bach-Pages et al., 2020; Millen et al., 2001; Trotta et al., 2019). Bacterial translation IF2 has 64 similar proteins with low scores of similarities, of which, 11 are targeted to mitochondria. Bacterial translation IF3 resembles 11 proteins, of which, AT1G34360 annotated as a translation IF3 family protein, is targeted to mitochondria (Senkler et al., 2017b). It is worth to mention that AT1G34360 did not give a high score of similarity by BLAST, but it is probably because the length of bacterial IF3 protein sequence (180 amino acids) is shorter as compared to that of AT1G34360 (520 amino acids). Atkinson et al. (2012) attempted to find functional



orthologues of mtIF3 in yeast by a sensitive PSI-Blast across eukaryotes, as BlastP searches alone could not detect mtIF3 homologues from distantly related eukaryotes. They found that Aim23p in yeast was a “missing” orthologue similar to mtIF3 in other eukaryotes and successfully showed that it functioned as the mtIF3 in yeast. Surprisingly, their phylogenetic analysis also revealed AL6G45350 as an orthologue of mtIF2 in *Arabidopsis lyrata*, which is a homologue to AT4G11160 in *Arabidopsis thaliana*. AT4G11160 was also found in our BLAST search but with a low score of similarity. According to SUBA5, it is predicted to be localized in cytosol, chloroplasts and mitochondria and no experimental studies have confirmed its subcellular localization. Atkinson et al. (2012) also identified SMO171G0119 as an orthologue of mtIF3 in *Selaginella moellendorffii* (an early vascular plant), which is a homologue to AT1G34360 in *Arabidopsis thaliana*.

Taken together, our findings suggest that AT4G11160 and AT1G34360 in *Arabidopsis thaliana* could function similarly to mtIF2 and mtIF3 in other eukaryotes, although AT4G11160 is only predicted to be targeted to mitochondria. If it is true, eukaryotic mitochondria seem to have lost mtIF1 and retain mtIF2 and mtIF3 for translation initiation during evolution. Mammalian mtIF2 has an extension domain (37 amino acids long) that functionally compensates for the absence of mtIF1 (Gaur et al., 2008; Yassin et al., 2011). This domain prevents tRNA from binding to the ribosomal A site, thus facilitating the fMet-tRNA to bind to the ribosomal P site during translation initiation (Kummer et al., 2018; Yassin et al., 2011). However, the conservation of this extension domain is limited to vertebrates (Atkinson et al., 2012). The role of mammalian mtIF3 is not clear. It is suggested that the mtSSU associates with mtIF2 and mtIF3 to form a translation initiation complex, ensuring that mRNA is stably bound to mtSSU prior to tRNA recruitment and before mtSSU and mtLSU assemble into a monosome (Kummer and Ban, 2021). In yeast, mtIF3 seems not to be a core component for translation of all mRNAs, but mitochondrial translation lacking mtIF3 is strongly imbalanced (Derbikova et al., 2018; Kuzmenko et al., 2016). In fact, yeast mitochondria are likely to recruit specific translation activator complexes to bind to the 5'UTRs of mRNAs to initiate translation (Derbikova et al., 2018). Therefore, it is difficult to predict whether plant mtIF2 and mtIF3 (if any) function in a comparable way as in other eukaryotes. In this study, we propose that mTRANs are PPR-like proteins that do not follow the “classical” PPR code rule and bind to the A/U-rich domains in the 5'UTRs of mRNAs to initiate translation. Perhaps due to their substantial length, the plant mitochondrial mRNA's 5'UTRs might form either the 2D or 3D structures to bring the binding sites physically closer to the start codon. Universal mtIFs and/or specific mtIFs/translation activators possibly help the mtSSU find the correct binding site and/or stably bind to mRNAs during the initiation step.

# Conclusion

In conclusion, the thesis work suggests that compromised mitochondrial translation results in the UPR<sup>mt</sup> activation in plants. In addition, we show that mTRAN proteins act as universal recognition factors of mtSSU for mitochondrial mRNAs to initiate translation. This indicates that translation initiation by mitochondrial ribosomes occurs in a fundamentally different way in plants as compared to in bacteria, yeast and mammals. mtSSU-mRNA binding in plants may also require mitochondrial translation IFs, which could be at least partially similar to in other eukaryotes. Therefore, further studies, such as characterizing *Arabidopsis* mutant(s) lacking functional mtIF2 and/or mtIF3 (see discussion), are required to demonstrate this hypothesis and to gain a broader insight into the mechanism of mitochondrial translation in plants.

# Acknowledgements

I would like to thank my main supervisor, Olivier Van Aken, for his guidance, support, and encouragement throughout my PhD study. I appreciate the fact that Olivier's office door is always open so I can bother him whenever I need his support. Thank you, Olivier, for everything you have done for me during my journey at Lund University (LU).

I also want to thank my co-supervisor, Allan Rasmusson, for his support and advice. I have to admit that I have "borrowed" quite many chemicals and equipment from Allan's lab, but he is always chill whenever I do it.

I would like to thank my examiner (the department representative), Klas Flärdh, and my scientific mentor, Courtney Stairs, for supporting me throughout my PhD study.

Regarding the mTRAN project, I would like to thank all the co-authors for their amazing work: Vivian Schmitt, Sbatie Lama, Chuande Wang, Alexandra Launay-Avon, Katja Bernfur, Kristin Hofmann, Kasim Khan, Benoit Castandet, Fredrik Levander, Allan Rasmusson, Hakim Mireau and Etienne Delannoy. I am grateful for the financial support from the Swedish Research Council (Vetenskapsrådet 2017-03854; 2021-04358), Crafoord Foundation (20170862; 20190868), Carl Trygger Foundation (CTS 17 487), Kungl. Fysiografiska Sällskapet i Lund, Jörgen Lindström's Foundation, Axel Hallströms donation and the Department of Biology at LU.

My sincere thanks go to all members (former and current) of Van Aken's lab: Abraham Ontiveros Cisneros, Vivian Schmitt, Kasim Khan, Oliver Moss, Jule Salfeld, Helene Röhricht, Essam Darwish, Sylwia Kacprzak, Ritesh Ghosh, Ameneh Khani, Berivan Mansuroglu and Alex Van Moerkercke. Thank you for always helping me and creating such a nice working environment that I have enjoyed a lot. I want to especially thank Abraham for being my best friend both at work and outside the lab. Thanks for all your support, advice and the BS talks we have had together. Without you, I would have never made the most important decision here (only you understand, for sure). My special thanks also go to Vivian Schmitt – the very first student that I have ever supported. Thanks for all memorable funny moment and your hardworking experiment, especially mitoprep (though I know you did not like it) and REMSAs. Thank you, Kasim Khan, for supporting me and having fun with me in the lab.

My special thanks go to Ewa Krupinska (LP3) for always supporting and giving me advice regarding the difficulty of mTRAN protein expression and purification.

I would like to thank the PhD students (former and current) at the Biology Building, LU. Thank you, Juan Manuel Orozco-Rodríguez, for helping with my protein expression, and I really enjoyed doing teaching with you. Thank you, Humberto Itriago, Katie Laschanky, Karla Iveth Aguilera Campos, Judith Matavacas, Dima Massri and Teresa Garibo Domingo, for having fun with me during the time I spent at LU. I also want to thank other PhD students: Veronica Ekdahl, David Stuart, Beer Sen, Vinardas Kelpas, Oscar Miguel Rollano Penalosa, Swati Aggarwal and Mengshu Hao.

I also want to thank the PIs, post-docs, administrative staffs, research engineerings and service technicians at the Biology Building, LU: Wolfgang Knecht, Mats Hansson, Lars Hederstedt, Sara Bengtsson, Lena Magnusson, Elisabeth Gauger Nilsson, Eva Svensson, Parminder Singh Mavi, Saritha Panthapulakkal Narayanan, Lars Fredriksson, Heather Sullivan, Nils Sundqvist, Sakthivel Kailasam, Shakhira Zakhrebekova, Chatarina Mattson, Torbjörn Säll, Carin Jarl-Sunesson, Rawana Alkhalili, Bradley Dotson, Deepak Anand, Claes von Wachenfeldt, Marita Cohn, Fredric Carlsson, Julia Lienard, Maria Gourdon, Céleste Sele and Anna Rasmussen.

Last but not least, I would like to say thanks to my family and friends, which will be written in my mother tongue, Vietnamese.

Trước hết, con muốn gửi lời cảm ơn đến gia đình: bố, mẹ, và chị. Con cảm ơn bố mẹ vì đã luôn luôn lo lắng, ủng hộ và yêu thương con vô điều kiện trong suốt quá trình học tập và làm việc nghiên cứu khoa học của con. Con đã đi xa nhà gần tám năm và bố mẹ cũng đã già đi nhiều nhưng vẫn luôn theo dõi và ủng hộ con. Con xin cảm ơn vì bố mẹ đã làm những điều tuyệt vời nhất cho con. Cảm ơn bà chị Thỏ vì đã luôn theo sát, kèm cặp, đốc thúc và cho tiền thàng em học hành chăm chỉ (và cả đi chơi) trong suốt những năm qua. Có chị gái luôn là số một! Con cũng gửi lời cảm ơn đến các ông, bà, bác, chú, cô, dì, cậu, mợ đã luôn hỏi thăm và động viên con.

Cảm ơn những người bạn ở Lund trong suốt quãng thời gian qua. Cảm ơn anh Thông và chị Âu vì những chuyến đi hiking, những bữa ăn vui vẻ, và đã luôn giúp đỡ em và Linh. Cảm ơn Hà và Đạt vì những bữa ăn và chuyến đi chơi vui vẻ. Đặc biệt, cảm ơn hai đứa vì đã giúp anh gặp được Linh. Cảm ơn Tâm vì đã chơi điện tử với tao mặc dù bây giờ tao đã bỏ rơi không chơi game với mày nữa. Cảm ơn Huy vì đã đến thăm và đi chơi với tao.

Gửi đến Võ Hoàng Yến Linh (tức Linh béo hay em béo, nhưng bây giờ gầy rồi): Cảm ơn em vì đã đến Lund để anh gặp được em và mình đã quen nhau. Cảm ơn em đã luôn chịu đựng và luôn ủng hộ anh trong suốt quãng thời gian qua. Em là động lực lớn để anh cố gắng trong suốt chặng đường của mình ở Lund. Anh mong rằng chúng mình sẽ không chỉ dừng lại ở đây mà sẽ còn đi xa hơn nữa cùng với nhau

trong mối quan hệ này và trong tương lai. Cho dù tương lai có thể nào, em vẫn luôn là kỉ niệm đáng nhớ và đáng trân trọng nhất mà anh đã có được ở Lund!

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