Plant type II NAD(P)H dehydrogenases

Structure, regulation and evolution of NDB proteins

Hao, Mengshu

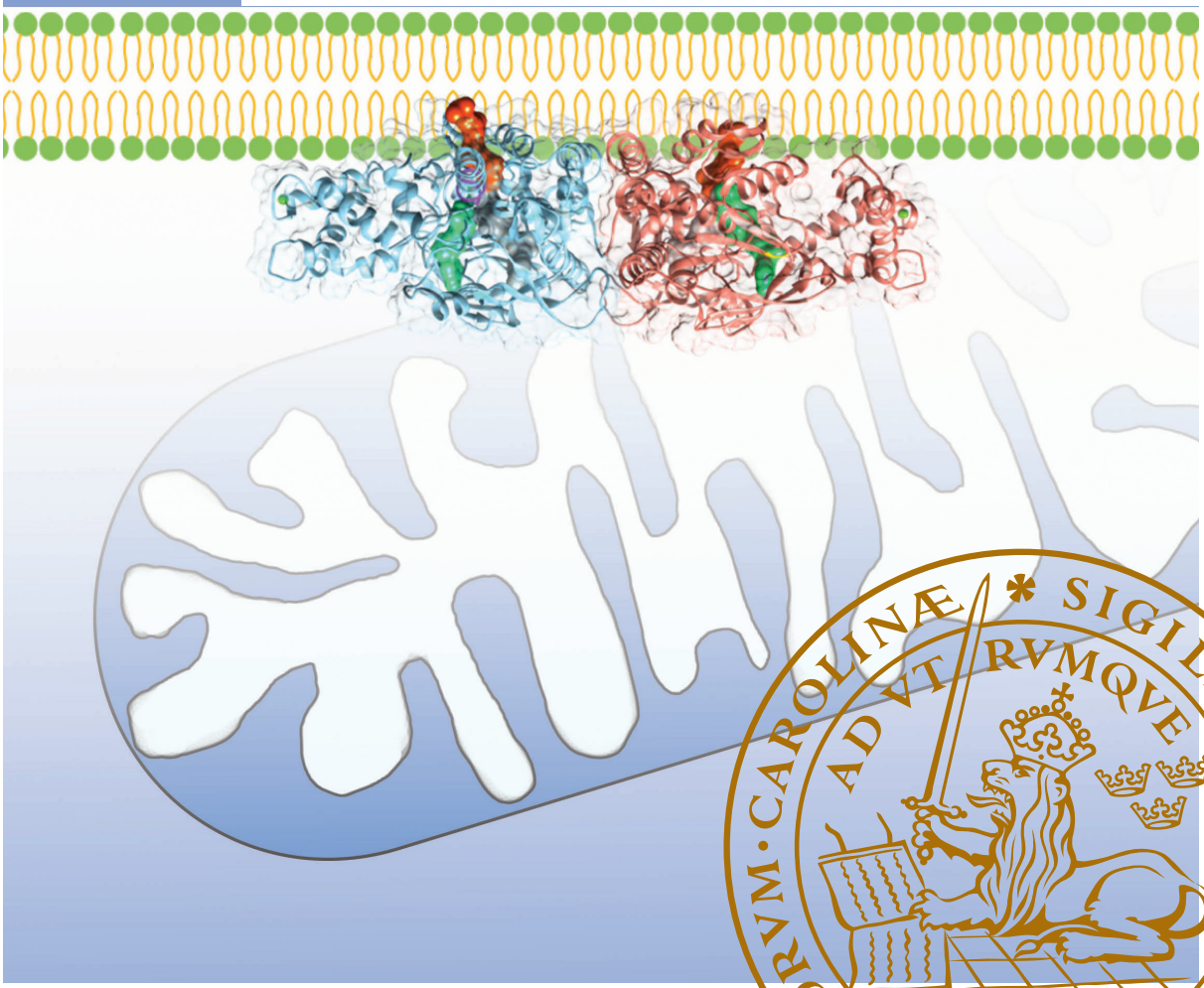
2019

Document Version:
Publisher's PDF, also known as Version of record

Plant type II NAD(P)H dehydrogenases

Structure, regulation and evolution of NDB proteins

MENGSHU HAO
DEPARTMENT OF BIOLOGY | FACULTY OF SCIENCE | LUND UNIVERSITY



Plant type II NAD(P)H dehydrogenases:

Structure, regulation and evolution of NDB proteins

Plant type II NAD(P)H dehydrogenases:

Structure, regulation and evolution of NDB proteins

Mengshu Hao

LUND
UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of Science, Lund University, Sweden.
To be defended in the Lecture hall A213, Biology Building, Sölvegatan 35B,
Lund, Sweden. Date 2019-05-29 and Time 09:30.

Faculty opponent
Dr. Tom Hamborg Nielsen
Department of Plant and Environmental Sciences
University of Copenhagen, Copenhagen, Denmark

Organization LUND UNIVERSITY	Document name: Doctoral Dissertation
	Date of issue: 2019-05-29
Author: Mengshu Hao	Sponsoring organization:

Plant type II NAD(P)H dehydrogenases: Structure, regulation and evolution of NDB proteins

Abstract:

In living organisms, respiration is a biological process degrading different carbon substrates, consuming O_2, and releasing the carbon as CO_2. Plants have several alternative enzymes that are involved in the respiratory processes, as compared to animals. These alternative respiratory enzymes allow electrons to be transferred to oxygen in the mitochondrial inner membrane, but bypassing ATP synthesis. The alternative enzymes, e.g., type II NAD(P)H dehydrogenases (NDH-2), affect cellular NAD(P)H redox status, which is of vital importance for energy metabolism, ROS production and removal, anti-oxidation and reductive biosynthesis.

Plant NDB-type proteins are NDH-2 enzymes located at the external mitochondrial inner membrane. It was earlier found that NDB1 oxidise cytosolic NADPH, and NDB2 oxidise cytosolic NADH. In this study, the regulatory mechanisms of *A. thaliana* and *Solanum tuberosum* NDB1 by cytosolic Ca^{2+} and pH were studied and compared to NDB2, using purified mitochondria and *E. coli*-produced proteins in a membrane-bound and a purified soluble state. Membrane bound NDB1 and NDB2 oxidised NADPH and NADH, respectively. Soluble forms of NDB1 oxidise both NADH and NADPH, with higher NADPH activity. Soluble forms of NDB2 oxidised only NADH like the membrane-bound enzyme. In solution, the active StNDB1 resided as oligomers of dimeric units, mainly hexamers, and recombinant AtNDB2 was highly oligomeric. Within a physiological pH range, an acidic pH was found to lower the Ca^{2+} demand for activation of the mitochondrial and *E. coli*-produced NADPH oxidation via NDB1, as compared to a more alkaline pH. Depending on pH, 3-82 μM Ca^{2+} was needed. In contrast, the sub-μM Ca^{2+} demand for activation of NADH oxidation was not linked to pH. Both soluble and mitochondrial StNDB1 (NADPH oxidation) could respond quickly to increased and decreased Ca^{2+}, whereas mitochondrial NADH oxidation responded quickly to Ca^{2+} increase but slowly to Ca^{2+} decrease. Overall, the results suggest that *in vivo*, the activity of NDB1 is rapidly controlled by pH-shift-associated Ca^{2+} spikes in the cytosol whereas NDB2 may be more continuously active.

Based on modelling of NDB1, the core catalytic parts and dimerization surface showed distinct similarities to the structures of yeast ScNDI1 and *Plasmodium falciparum* PfNDH-2. This analysis highlighted motifs that correlate with NAD(P)H substrate specificity, and which were followed by evolutionary analysis. Most eukaryotic species have NDB proteins that contain a non-acidic motif for NADPH binding. Angiosperms and liverworts contain NDB proteins of NDB1- and NDB2- type, i.e. they contain acidic and non-acidic motifs for NADH and NADPH binding, respectively. This indicates that plants have more flexibility for external NAD(P)H oxidation as compared to other eukaryotes. Based on the evolutionary analysis, Ca^{2+}-dependent external NADPH oxidation appears to be an ancient process as compared to NADH oxidation, and thus possibly has a more fundamental function in cellular redox metabolism.

Key words: Arabiposis; Ca^{2+}; electron transport; NDH-2; NDB; pH; NADH; NADPH; plant mitochondria; potato; type II NAD(P)H dehydrogenases

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language: English
ISSN and key title		ISBN: 978-91-7753-974-2
Recipient's notes	Number of pages 59	Price
	Security classification	

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature Mengshu Hao Date 2019-04-15

Plant type II NAD(P)H dehydrogenases:

Structure, regulation and evolution of NDB proteins

Mengshu Hao

LUND
UNIVERSITY

Cover photo by Mengshu Hao

Faculty of Science
Department of Biology

ISBN 978-91-7753-974-2 (Print)
ISBN 978-91-7753-975-9 (Pdf)

Printed in Sweden by Media-Tryck, Lund University
Lund 2019

Dedicated to my family

谨以此书献给我最爱的父母、家人。

Contents

Preface

This thesis is based on the following publications and a manuscript, referred to by their Roman numerals:

I. **Hao M-S,** Jensen AM, Boquist AS, Liu YJ, Rasmusson AG (2015) The Ca^{2+}-regulation of the mitochondrial external NADPH dehydrogenase in plants is controlled by cytosolic pH. PLoS One 10 (9):e0139224.

II. **Hao M-S**, Rasmusson AG (2016) The evolution of substrate specificity-associated residues and Ca^{2+}-binding motifs in EF-hand-containing type II NAD(P)H dehydrogenases. Physiologia Plantarum 157, 338-351.

III. **Mengshu Hao**, Trixie Chang, Lars Hederstedt, Allan Rasmusson, Purification and characterisation of mitochondrial external type-II NAD(P)H dehydrogenases after production in *E. coli* (Manuscript)

Publications not included in the PhD thesis:

IV. Wang HZ, Kanagarajan S, Han JL, **Hao M-S**, Yang YY, Lundgren A, Brodelius PE (2014) Studies on the expression of linalool synthase using a promoter-beta-glucuronidase fusion in transgenic *Artemisia annua*. Journal of Plant Physiology 171, 85-96.

V. Han JL, Wang HZ, Kanagarajan S, **Hao M-S**, Lundgren A, Brodelius PE, 2016. Promoting artemisinin biosynthesis in *Artemisia annua* plants by substrate channeling. Molecular Plant 9, 946-8.

VI. Glab B, Beganovic M, Anaokar S, **Hao M-S**, Rasmusson AG, Patton-Vogt J, Banas A, Stymne S, Lager I (2016) Cloning of glycerophosphocholine acyltransferase (GPCAT) from fungi and plants: a novel enzyme in phosphatidylcholine synthesis. Journal of Biological Chemistry 291, 25066-76.

Author contributions

In **paper I**, M-S Hao (MSH) performed the majority of measurements and data analysis. MSH wrote and edited the manuscript together with supervisor Allan Rasmusson (AGR). In **Paper II**, MSH performed the sequence searches, sequence alignments, evolutionary analysis, and first draft writing, whereas, most modelling work and writing of this paper were done by AGR. In **Paper III**, MSH independently performed the great majority of experimental work and data analysis. The draft manuscript of **Paper III** was written by MSH and edited jointly with AGR.

Popular science summary in English

Plant photosynthesis is a process where they capture energy from the sun to make carbohydrates from carbon dioxide and water with oxygen as a by-product. The carbohydrates are used as energy source for growth and maintenance in so-called respiration, that takes places in small membrane-surrounded compartments in each cell called mitochondria. In respiration energy is released, oxygen is consumed, and carbon dioxide is produced. Though some energy is released as heat, much is captured in special molecules by the help of highly controlled enzymatic steps taking place in the cells. Respiration is not unique to plants, but plants have several additional enzymes compared to animals that allow uncoupling of the breakdown from energy storage to give a more flexible process. The additional enzymes include several so-called NDB proteins that oxidize cellular NAD(P)H, the reducing equivalents that is important for respiration and many other cellular reactions. These enzymes are also believed to be important in plant defences, especially under stress conditions, such as drought, high salinity, heat and pathogen attack.

In this project, the NDB proteins were studied regarding their activity, structure and evolution. It was found that Ca^{2+} and pH conditions interacted in regulating the activities of NDB proteins. Therefore, the exact Ca^{2+} concentrations needed at different pH values within the physiological range were investigated. We found it was different for different type of NDB proteins. NDB1-type proteins oxidise only NADPH in isolated intact mitochondria and demands high Ca^{2+} and low pH. Whereas NDB2-type proteins oxidise only NADH, lower Ca^{2+} is needed and with little influence of the pH. Considering that cellular Ca^{2+} changes have features of spikes, we could predict that the NDB2-type proteins in cells should be permanently active whereas NDB1-type proteins would be active only when the Ca^{2+} concentration is high and pH is low. To understand better the regulation of NDB proteins, a 3D structure modelling of NDB1 and NDB2 from two model plants, potato and *Arabidopsis thaliana*, were made and in detail looked into. NDB proteins are modelled as a two-molecule unit, so-called dimer, with one side that associate with the inner mitochondrial membrane. Along the membrane side, there is a site where Ca^{2+} could bind. The binding of Ca^{2+} may change the structure of an NDB protein, and thus change the activity. NAD(P)H binding positions on the 3D-model helped us to predict the differences between NADH binding and NADPH binding. Based on two amino acids positions at the NAD(P)H binding position, we could predict what each NDB-type protein would oxidise, NADH or NADPH. Combined with evolutionary analysis, NDB1-type proteins were found present throughout most eukaryotic groups and evolutionarily ancestral as compared to NDB2-type proteins. These studies of NDB could improve understanding of the functions of NDB in plant energy regulation.

The view of biology department (in the left) from the tower of Faculty of Science, Lund University, Lund, Sweden & Mengshu Hao at 2015-11-23

Abstract

In living organisms, respiration is a biological process degrading different carbon substrates, consuming O_2, and releasing the carbon as CO_2. Plants have several alternative enzymes that are involved in the respiratory processes, as compared to animals. These alternative respiratory enzymes allow electrons to be transferred to oxygen in the mitochondrial inner membrane, but bypassing ATP synthesis. The alternative enzymes, e.g., type II NAD(P)H dehydrogenases (NDH-2), affect cellular NAD(P)H redox status, which is of vital importance for energy metabolism, ROS production and removal, anti-oxidation and reductive biosynthesis.

Plant NDB-type proteins are NDH-2 enzymes located at the external mitochondrial inner membrane. It was earlier found that NDB1 oxidise cytosolic NADPH, and NDB2 oxidise cytosolic NADH. In this study, the regulatory mechanisms of *A. thaliana* and *Solanum tuberosum* NDB1 by cytosolic Ca^{2+} and pH were studied and compared to NDB2, using purified mitochondria and *E. coli*-produced proteins in a membrane-bound and a purified soluble state. Membrane bound NDB1 and NDB2 oxidised NADPH and NADH, respectively. Soluble forms of NDB1 oxidise both NADH and NADPH, with higher NADPH activity. Soluble forms of NDB2 oxidised only NADH like the membrane-bound enzyme. In solution, the active StNDB1 resided as oligomers of dimeric units, mainly hexamers, and recombinant AtNDB2 was highly oligomeric. Within a physiological pH range, an acidic pH was found to lower the Ca^{2+} demand for activation of the mitochondrial and *E. coli*-produced NADPH oxidation via NDB1, as compared to a more alkaline pH. Depending on pH, 3-82 μM Ca^{2+} was needed. In contrast, the sub-μM Ca^{2+} demand for activation of NADH oxidation was not linked to pH. Both soluble and mitochondrial StNDB1 (NADPH oxidation) could respond quickly to increased and decreased Ca^{2+}, whereas mitochondrial NADH oxidation responded quickly to Ca^{2+} increase but slowly to Ca^{2+} decrease. Overall, the results suggest that *in vivo*, the activity of NDB1 is rapidly controlled by pH-shift-associated Ca^{2+} spikes in the cytosol whereas NDB2 may be more continuously active.

Based on modelling of NDB1, the core catalytic parts and dimerization surface showed distinct similarities to the structures of yeast ScNDI1 and *Plasmodium falciparum* PfNDH-2. This analysis highlighted motifs that correlate with NAD(P)H substrate specificity, and which were followed by evolutionary analysis. Most eukaryotic species have NDB proteins that contain a non-acidic motif for NADPH binding. Angiosperms and liverworts contain NDB proteins of NDB1- and NDB2-type, i.e. they contain acidic and non-acidic motifs for NADH and NADPH binding, respectively. This indicates that plants have more flexibility for external NAD(P)H oxidation as compared to other eukaryotes. Based on the evolutionary analysis, Ca^{2+}-dependent external NADPH oxidation appears to be an ancient process as compared to NADH oxidation, and thus possibly has a more fundamental function in cellular redox metabolism.

Abbreviations

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
NADH	Nicotinamide adenine dinucleotide, reduced form
NAD^+	Nicotinamide adenine dinucleotide, oxidized form
NAD	NADH and/or NAD^+
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
$NADP^+$	Nicotinamide adenine dinucleotide phosphate, oxidized form
NADP	NADPH and/or $NADP^+$
NAD(P)H	NADH and/or NADPH
$FADH_2$	Flavine adenine dinucleotide
ROS	Reactive oxygen species
UQ	Ubiquinone
DH	Dehydrogenase
$K_{0.5}(Ca^{2+})$	The Ca^{2+} concentration inducing half-maximal activity
$t_{1/2}$	The half-life of the activity after addition of Ca^{2+}/EGTA

1 Introduction to respiratory redox biology

1.1 Plant respiration

In respiration, carbon-containing substrates are oxidised to supply the organism with energy in the form of ATP, which is needed for growth and development. ATP is the universal 'energy currency' that supplies energy to most cellular metabolic activities. In plants, a large portion (35-80%) of carbohydrates assimilated by photosynthesis is consumed in respiration within the same period (Amthor 2000, Lambers et al 1998).

Plant respiration is controlled by e.g., energy demand, availability of substrates, O_2 supply, temperature and light conditions. In the absence of oxygen, fermentation will take place and lead to cytoplasmic acidification, which inhibits respiration and its ATP synthesis (Sakano 2001). Higher environmental temperature will also cause substantial increases in leaf respiratory carbon fluxes because more ATP is used for plant survival (Slot & Kitajima 2015). The major parts in plant respiration is briefly summarized below.

1.1.1 Plant cellular respiratory products and substrates

By completely degrading one glucose molecule, 30-32 ATP molecules can be regenerated through aerobic respiration (Browse et al 2014). Apart from ATP, respiration also produces metabolic intermediates that can be used for synthesis of amino acids, nucleic acids and fatty acids, which are needed for cell growth and maintenance. For example, 2-oxoglutarate is a respiratory intermediate exported from mitochondria to chloroplasts for glutamate synthesis (Lea & Miflin 2003).

Substrates for respiration are usually carbohydrates like glucose, sucrose, triose phosphates and organic acids, as well as protein, lipid and chlorophyll degradation products. Lipids, protein and amino acids can be degraded to maintain respiration under carbon starvation (Araujo et al 2011, Cavalcanti et al 2017) like under darkening-induced conditions (Slot & Kitajima 2015). Lipids are otherwise mainly used for respiration in germinating oilseeds, and they can also be metabolized into sucrose that plants can transport and utilize for growth (Graham 2008). Organic acids such as malate and fumarate may also be utilized as respiratory substrate in leaves by the end of the night. Therefore, depending on the growth conditions, cellular conditions and environmental conditions, plants utilize many different types

of respiratory substrates. Still, sugars are the most common respiratory substrates in plant cells.

1.1.2 Mitochondrial morphology

Based on transmission electron microscopy, plant mitochondria are usually spherically shaped with 0.5-1.0 μm in diameter and up to 3 μm in length, which is similar to animal mitochondria (Logan & Leaver 2000). *In vivo* studies of GFP-labelled plant mitochondria also showed elongated spherical structures (Logan & Leaver 2000). Additionally, the size, shape and distribution of mitochondria in a cell are not even (Scott & Logan 2011). The number and size of mitochondria depends on the mitochondrial fission and fusion rate (Scott & Logan 2011). Changes of mitochondrial size can also alter mobility and cellular distribution of the mitochondria.

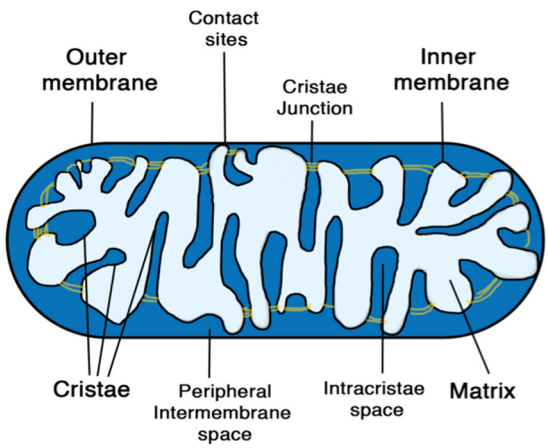


Figure 1. The general architecture of mitochondria.

A mitochondrion has two membranes, the outer membrane and the inner membrane (Figure 1). The outer membrane encloses the entire organelle. Ions and metabolites exchange efficiently across the outer membrane through voltage dependent anion channels (Duncan et al 2013). The inner membrane is highly folded, forming cristae. The formation of cristae allows a mitochondrion to contain large amount of proteins, and the protein-to-lipid ratio is high, about 3:1 (Douce 1985). The space between the outer membrane and the inner membrane is known as the intermembrane space, which can be subdivided into two compartments, the peripheral intermembrane space and the intracristae space (Figure 1). They are separated by ring-like cristae junctions, which separate cristae membrane from surrounding inner membrane.

Such have seen in yeast, plant and mouse (Barbot et al 2015, Hessenberger et al 2017). The compartment enclosed by the inner mitochondrial membrane is called the matrix. It has a very high content of macromolecules, and it is where the citric acid cycle takes place. The outer and inner membranes are connected at many sites by the translocase of the outer membrane complexes and the translocase of the inner membrane complexes, which are involved in protein translocation (Harner et al 2011).

1.1.3 Plant respiratory pathways

In a plant cell, the respiratory pathways are distributed between the cytosol, mitochondria and chloroplasts. Plant respiration can be divided into: glycolysis, oxidative pentose phosphate pathway, citric acid cycle and oxidative phosphorylation, which are located in different cell compartments (Figure 2). The final products from the joint action of glycolysis, oxidative pentose phosphate pathway, and citric acid cycle pathways are CO_2, H_2O and reduced compounds nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide ($FADH_2$) and a small amount of ATP.

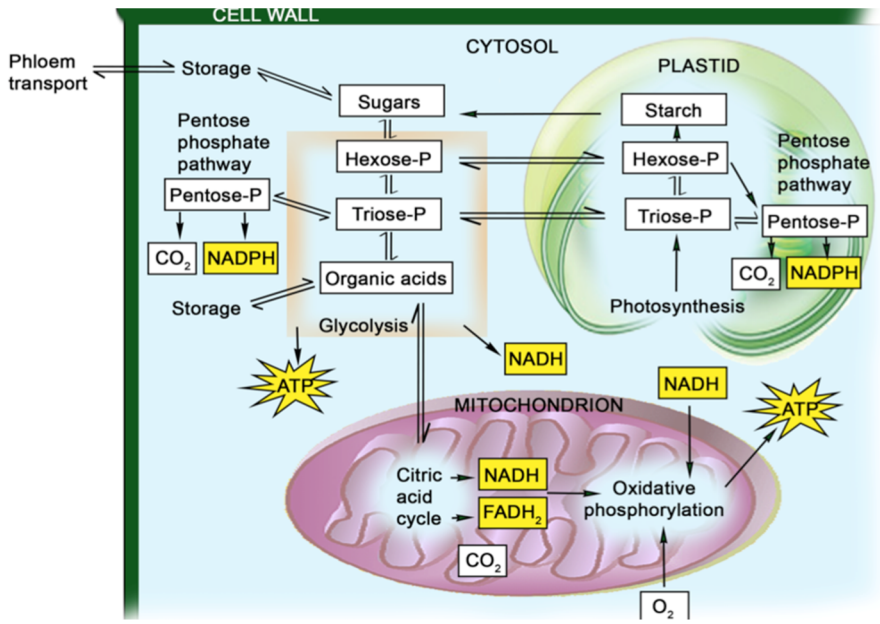


Figure 2. Overview of cellular respiration pathways. Modified from Browse *et al* (2014).

Glycolysis is an anaerobic pathway that provides pyruvate for the citric acid cycle and produce NADH and a small amount of ATP from oxidization of sugars in plants, mainly sucrose (Fernie et al 2004). The process mainly takes place in the plant cytosol, but also partly in plastids (Frehner et al 1990).

Plant oxidative pentose phosphate pathway is a metabolic pathway that works in parallel to glycolysis and that is also located both in the cytosol and in plastids. The pentose phosphate pathway provides NADPH and some precursors for nucleotide and amino acid biosynthesis. This pathway is rate controlled by glucose-6-phosphate DH, which is stimulated by the concentration of $NADP^+$ and strongly inhibited by cytosolic NADPH (Browse et al 2014, Kruger & von Schaewen 2003).

The *Citric acid cycle* is also called the tricarboxylic (TCA) cycle or Krebs cycle. It is located in the mitochondrial matrix. The citric acid cycle degrades acetyl-CoA generated from pyruvate and releases CO_2. A major amount of NADH and $FADH_2$ (16 NADH and 4 $FADH_2$ per sucrose) is synthesized together with a small amount of ATP. Synthesized NADH and $FADH_2$ are the primary substrates for the electron transport chain (ETC) of the oxidative phosphorylation in mitochondria (Sweetlove & Møller 2010).

Oxidative phosphorylation takes place in mitochondria and it is the process where most ATP is formed (Figure 3). Most aerobic organisms carry out oxidative phosphorylation. In this process, electrons are transferred from NADH to O_2 through the basic enzyme complexes I, III and IV in ETC. Complexes I, III and IV are embedded in the inner mitochondrial membrane and coupled to proton translocation. Complex I oxidize matrix NADH and transfer electrons to the ubiquinone (UQ) pool (Braun et al 2014). Complex III in turn accepts electrons from the UQ pool and transfers them to cytochrome *c*. Finally, complex IV transfers the electrons from cytochrome *c* to O_2, generating H_2O, and complex III and IV is jointly often called the cytochrome pathway. In the ETC process, an electrochemical gradient of protons is formed across the inner mitochondrial membrane by translocation of protons. Based on the chemiosmotic theory, the protons diffuse from the intermembrane space to the matrix through an ATP synthase, and drive the ATP synthase to produce ATP from ADP and $P_{i.}$ (Browse et al 2014, Mitchell 1961, Nicholls 2013, Walker 2013). ATP synthases are found as dimers at the cristae rims in eukaryotes, including plants (Seelert & Dencher 2011), whereas complex IV has been localized to the flat sides of the intracristae membranes in human cells (Rieger et al 2014).

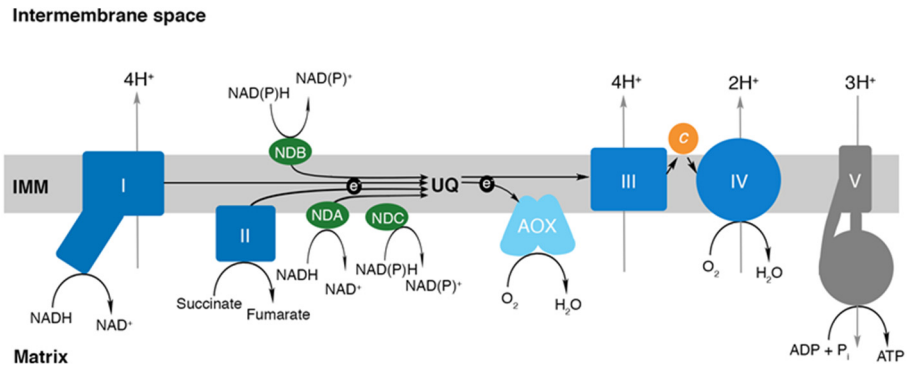


Figure 3. Schematic representation of oxidative phosphorylation in plants.
The inner mitochondrial membrane (IMM) has the classical electron transport chain (ETC) complexes I-IV and ATP synthase including the cytochrome pathway, and alternative pathways including AOX, NDA, NDB and NDC. Abbreviations: IMM: inner mitochondrial membrane, I: complex I, II: complex II, III: complex III, IV: complex IV, V: ATP synthase, AOX, alternative oxidase, *c*: cytochrome *c*, NDA and NDC: internal NAD(P)H dehydrogenases, NDB: external NAD(P)H dehydrogenases, UQ: ubiquinone. Adapted from Browse *et al* (2014).

Alternative pathways: Aerobic eukaryotes carry out mitochondrial oxidative phosphorylation by linking electron transport to ATP synthesis via proton translocation. However, unlike mammals, plants have several alternative enzymes involved in the electron transport processes, including alternative oxidase and type II NAD(P)H dehydrogenases (DHs) or in short NDH-2. None of the alternative oxidases or NDH-2 translocate protons, so they give no direct contribution to oxidative phosphorylation (Møller 2001). Therefore, NDH-2 could partially dissipate reductants without proton translocation in the electron transport chain, which is needed for ATP biosynthesis. The existence of NDH-2 enzymes may thus enhance the ability of balancing the mitochondrial membrane potential and reduction state, which turn down the efficiency of ATP production. Transgenic *A. thaliana* with suppressed NDH-2 expression have shown significant changes in cellular NADP(H) homeostasis, growth rate, respiratory metabolism and defence signalling (Wallström et al 2014a, Wallström et al 2014b).

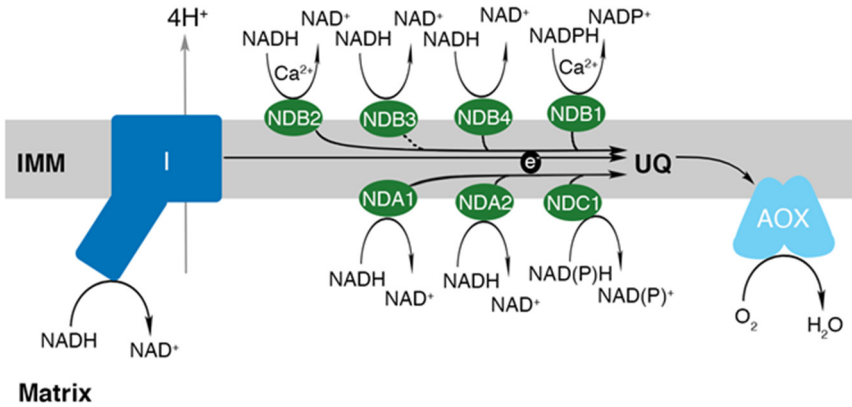


Figure 4. The mitochondrial alternative respiration pathways in *A. thaliana*.
The NDH-2 are in green color: NDA1-2, NDB1-4 and NDC1. They are specified with substrates and Ca^{2+} binding (Geisler et al 2007). Electron flow routes are shown as black solid arrow lines, and dash line constitutes a prediction for NDB3 as NADH DH. Complex I is shown for comparison. IMM: inner mitochondrial membrane.

NDH-2 enzymes exist in plants, fungi, protists and bacteria (Melo et al 2004, Rasmusson et al 2008). Seven predicted NDH-2 were found in *A. thaliana* and classified into 3 families: NDA, NDB and NDC, which contain 2, 4 and 1 members respectively (Michalecka et al 2003). Homologues of all 3 families were also found in rice, *Oryza sativa*, suggesting that both monocots and eudicots NDA, NDB and NDC proteins (Rasmusson et al 2004). In *A. thaliana* and potato, AtNDA1, AtNDA2, AtNDC1 and StNDA1 have been found localized to the inner surface of the inner membrane, whereas StNDB1, AtNDB1, AtNDB2 and AtNDB4 are situated on the outside of the inner membrane (Figure 4) (Elhafez et al 2006, Geisler et al 2004, Rasmusson & Agius 2001, Rasmusson et al 1999). AtNDB3 is possibly external but the gene has been less studied (Michalecka et al 2003). However, dual targeting of plant NDH-2 has been observed; besides mitochondria, NDH-2 may be targeted to peroxisomes or chloroplasts (Xu et al 2013).

Human apoptosis-inducing factor (AIF) and mitochondrion-associated inducer of death (AMID) have been claimed to be mitochondrial rotenone-sensitive NDH-2 enzymes (Elguindy & Nakamaru-Ogiso 2015). Homologs of AIF and AMID are also present in plants, e.g., *A. thaliana* homologs At5g22140 and At3g44190 (Michalecka et al. 2003). However, the NADH oxidation activity of the predicted proteins is not known.

Apart from above, plant mitochondria contain supplementary pathways that can oxidize carbon compounds and transfer electrons to UQ. Examples include D-lactate DH, dihydroorotate DH, glycerol-3-phosphate DH and also proline DH (Araujo et al 2010, Bartoli et al 2000, Rasmusson & Møller 2010, Schertl & Braun 2014, Szabados & Savoure 2010).

Uncoupling proteins bypass ATP synthesis because they mildly dissipate the electrochemical proton gradient, which leads to a decreased efficiency of oxidative

phosphorylation (Vercesi et al 2006). Uncoupling proteins are activated by superoxide, and they may protect cells against high production of reactive oxygen species (ROS) during biotic and abiotic stresses (Brandalise et al 2003, Fernie et al 2004, Maxwell et al 1999). Overexpression of StNDB1 in tobacco leads to higher levels of uncoupling proteins and alternative oxidases (Michalecka et al 2004). However, new research claimed that the uncoupling proteins in plants are not found to have uncoupling function, but instead play an role as mitochondrial transporters for aspartate, glutamate and dicarboxylates (Monne et al 2018). If it is true, alternative oxidase and NDH-2 would be the major components for dissipation of the electrochemical proton gradient in plant cells.

Fermentation is known as an alternative pathway to respiration when plants are under low O_2 conditions, such as hypoxia and anoxia that can happen under flooding or waterlogging. Under low O_2, the oxidative phosphorylation pathway is limited or inactivated (Møller 2001), and fermentation will take place. The ATP production efficiency per glucose is only 5-10 % compared to aerobic respiration (Drew 1997). Plant fermentation has three pathways, known as alcoholic fermentation, lactic fermentation and the alanine pathway, which are degrading pyruvate to ethanol, lactate and alanine respectively, and generating CO_2 and NADH. The cytosol pH is dramatically changed into acidification under lactic fermentation (Browse et al 2014).

In order to have a better understanding of alternative plant respiration, the knowledge on plant NDH-2 is extended in this study, including its structure, regulation, evolutionary analysis and possible molecular mechanisms.

1.1.4 Genes for mitochondrial proteins

In plant mitochondria, most polypeptides involved in the ETC (e.g., most subunits in Complex I-IV and all alternative oxidases and NDH-2) are nuclear-encoded (Rasmusson et al 2008). Most animal mitochondrial genomes are about 16.5. kbp in length, whereas plant mitochondrial genomes have more variation, which range from 200 to 7000 kbp (Gualberto et al 2014). Mitochondrial genes encode proteins that are mainly involved in basic processes, for example, oxidative phosphorylation and translation (Burger et al 2003). In human, the mitochondrial genome has 37 genes that encoding 13 proteins, 22 tRNAs, and 2 rRNAs (Anderson et al 1981). Model plant *A. thaliana* has 57 genes that encoding 33 proteins, 21 tRNAs, and 3 rRNAs (Unseld et al 1997). Moreover, plant mitochondrial DNA can be present as a collection of linear DNA, smaller circular and branched molecules (Morley & Nielsen 2017).

1.1.5 Oxidative stress caused by redox changes

During growth, plants have to face different environmental stresses such as a variable temperature, drought, nutrient deficiency, salt and metal toxicity, hypoxia, and pathogen attack (Vanlerberghe 2013). For example, high temperature can inhibit leaf respiration (Atkin et al 2006), salinity may decrease the cytochrome pathway and enhance alternative respiratory pathway (He et al 2019), heavy metals and O_2 limitation affects root respiration dramatically (Gupta et al 2009, Moyen & Roblin 2013). In response to many stresses, plants increase the production of ROS due to over-reduction. The balance of redox status may thus be shifted to oxidative stress. As a result of oxidative stress, plants trigger programmed cell death and/or acclimation and improved stress tolerance (Amirsadeghi et al 2007, Noctor & Mhamdi 2017). For example, studies have shown that defence hormone signalling is linked to glutathione status, that is changed by H_2O_2 (Han et al 2013a, Han et al 2013b). The stress-induced ROS may function in retrograde signalling, *i.e*. from mitochondria to nucleus, activating/repressing of nuclear genes, but ROS can also damage cellular components (Sweetlove & Møller 2010). With induced ROS, there are changes in mRNA and protein levels as well, e.g., under ROS-inducing stress conditions, genes encoding alternative oxidases are up-regulated together with *NDH-2* genes, such as *NDB2* with *AOX1a* and *NDB4* with *AOX1c* (Clifton et al 2005, Rasmusson et al 2009). Transcription factors ABI4, AtWRKY40 and AtWRKY63 can up- or down- regulate *AOX1* and *NDB2* genes (Giraud et al 2009, Van Aken et al 2013).

The alternative respiratory enzymes are believed to respond to plant stress, prevent over-reduction of the proton-translocating main respiratory chain and counteract deleterious short-term metabolic fluctuations, thus reducing ROS formation and damage (Giraud et al 2009, Møller 2001, Rasmusson et al 2009). It was shown that plant mitochondrial alternative oxidases decrease the reduction level of the electron transport chain and lower ROS production (Maxwell et al 1999). The tight redox regulation of the alternative respiratory enzymes may therefore be particularly important to balance the cellular redox status under stress.

1.2 Ca^{2+} in plant cells

Calcium is normally present at millimolar levels on a whole tissue basis, but the free intracellular Ca^{2+} concentration is much lower, around 100 nM (Thomas 1982). Cells actively and passively translocate Ca^{2+} between different organelles, cytosol, and extracellular compartments. Ca^{2+} is a key regulator for NDB-type NDH-2, such as AtNDB1 and AtNDB2 (Geisler et al 2007). Experiments of this thesis were done to understand the mechanisms of NDB activation under physiological Ca^{2+} levels *in vivo* (**Paper I & III**). Therefore, this section will briefly summarize the resting and

total Ca^{2+} concentrations of different cell compartments under physiological conditions and the Ca^{2+} signal decoding in plants that might related to the NDB protein activity.

1.2.1 Ca^{2+} concentrations in different cell compartments

In plant cells, the concentration of free Ca^{2+} responds to growth conditions, and frequency and amplitude are variable with different stimuli. Based on experiments using Ca^{2+} sensor proteins, Ca^{2+} concentration changes can be visualised. For example, when *A. thaliana* roots are under salt stimuli, Ca^{2+} waves move up to 400 µm/s through the cortical and endodermal cell layers, about several cells per second (Choi et al 2014b). The cytosolic Ca^{2+} concentration becomes elevated in an oscillatory manner in response to several factors, e.g., cell division, root cell elongation, heat or cold shock, drought, salinity, mechanical stimulation, pathogens attack (White & Broadley 2003).

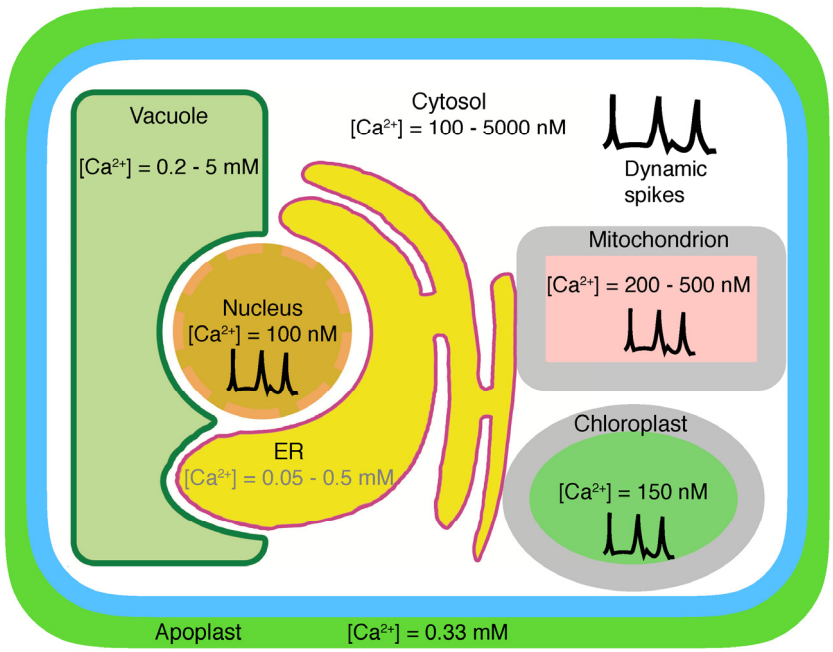


Figure 5. Free Ca^{2+} concentrations in a plant cell (collective from different cell types).
[Ca^{2+}] indicates free Ca^{2+} concentrations. Grey values are from animals. Different Ca^{2+} value sourses are listed: cytosol (Choi et al 2014a, Logan & Knight 2003), mitochondrion (Logan & Knight 2003), chloroplast (Johnson et al 1995, Nobel 1969), ER (Coe & Michalak 2009), Vacuole (Conn & Gilliham 2010), Nucleus (Mazars et al 2009) and Apoplast (Conn et al 2011, Hepler 2005).

Dynamic changes in concentrations and changes in Ca^{2+} gives plants an ability to 'sense' and 'respond' to different environments appropriately and specifically and to decode the Ca^{2+} signals induced by different responses. As summarised in Figure 5, a collection of free Ca^{2+} concentration data of different cell compartments has been determined. The main storage of Ca^{2+} in plant cells is in the vacuole, which has around 0.2-5 mM free Ca^{2+} and up to 80 mM of total Ca^{2+}, which is the sum of free and bound Ca^{2+} (Conn & Gilliham 2010). The apoplast is another place to store Ca^{2+}. Apoplastic Ca^{2+} is variable from 10 μM to 10 mM in total, and around 0.33 mM as free Ca^{2+}(Conn et al 2011, Hepler 2005). Cytosolic Ca^{2+} concentrations can reach 5 μM or higher at stress conditions, but the resting concentration is around 100 nM (Choi et al 2014a, Logan & Knight 2003), which is similar to the nuclear free Ca^{2+} level, around 100 nM (Mazars et al 2009). The estimated total and free Ca^{2+} of chloroplast are 15 mM and 150 nM respectively (Johnson et al 1995, Nobel 1969). Mitochondrial matrix take up Ca^{2+} actively through several Ca^{2+} uniporters (Wagner et al 2016). In plant mitochondrial matrix, the estimated free Ca^{2+} concentration at resting condition is around 200 nM, and under stress conditions it can reach 500 nM or even higher (Choi et al 2014b), e.g., the touch response of *A. thaliana* cause around 4-fold increase of free Ca^{2+} in mitochondria (Logan & Knight 2003). Ca^{2+} could pass the outer mitochondrial membrane freely through voltage-dependent anion channels. Therefore, in these investigations we have considered the Ca^{2+} level being similar in intermembrane space as in the cytosol and to follow the Ca^{2+} spikes in the latter compartment.

1.2.2 Decoding Ca^{2+} signals

Signals in the form of defined changes in Ca^{2+} are decoded by the cell through various Ca^{2+} sensor proteins and transferred into phosphorylation events, protein-protein interaction, and/or regulation of gene expression (Hashimoto & Kudla 2011). Ca^{2+} sensor proteins are calmodulin, calmodulin-like protein and calcineurin B-like protein (Figure 6). Ca^{2+} sensor proteins mostly contain EF-hand domain(s) that binds Ca^{2+}. Calmodulin is conserved in all eukaryotes, whereas calmodulin-like protein, calcineurin B-like protein and Ca^{2+}-dependent protein kinase are specific to plants and some protists (Batistič & Kudla 2009, Billker et al 2004, Harper & Harmon 2005, Hashimoto & Kudla 2011). For example, potato Ca^{2+}-dependent protein kinases (CDPK4 and CDPK5) activate the plasma membrane NADPH oxidase RBOHB, leading to elevated superoxide O_2^- production and a decreased $NADPH/NADP^+$ ratio (Kobayashi et al 2007, Pugin et al 1997). Plant NAD kinase (ATP:NADH 2′ -phosphotransferase) is activated by calmodulin and could modify the cytosolic NAD/NADP ratio (Turner et al 2005). Ca^{2+} signalling can also directly active EF-hand-containing enzymes by Ca^{2+} binding, such as *A. thaliana* NDB1 and NDB2 that oxidising cytosolic NAD(P)H (Geisler et al 2007), which may further

affect the NADNAD(P)H reduction level (Figure 6). The effect of the Ca^{2+} on the NDB enzymes are described below and in **Paper I & III**.

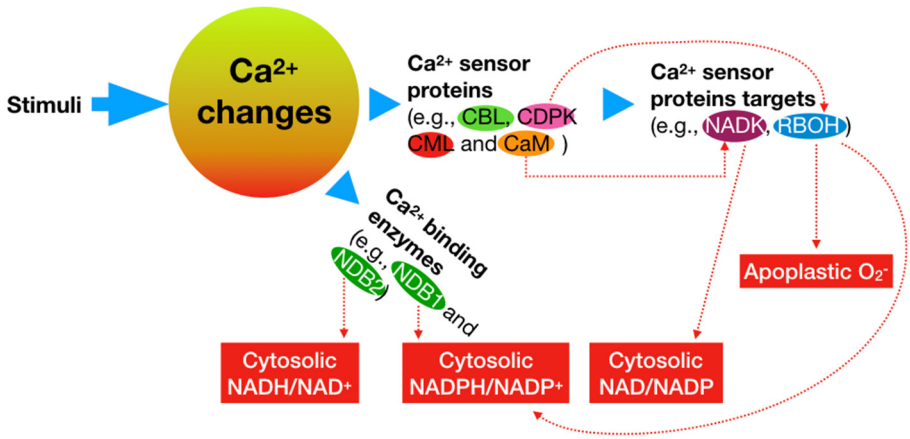


Figure 6. Responses to Ca^{2+} concentration changes in plant cells.
Ca^{2+} sensor proteins and Ca^{2+} binding enzymes are summarized above. Abbreviations: CaM, calmodulin. CML, calmodulin-like protein family. CDPK, the family of Ca^{2+}-dependent protein kinase. CBL, the calcineurin B-like protein family, NADK, NAD kinase. RBOH, respiratory burst oxidase homologues.

1.3 Cellular pH levels in plants

1.3.1 A summary of pH levels in plants

The pH varies between different compartments of plant cells. In *A. thaliana*, the cytosolic pH was found to be 6.5-7.6 in roots (Moseyko & Feldman 2001). When transfered to an acidic growth media, pH 3.8, the cytosol pH of plant cells drop 0.5-0.8 pH unit within 1 hour (Moseyko & Feldman 2001). In root hair of *Medicago sativa*, pH is around 7.3 at aerobic conditions and around 6.8 at anoxia (Felle 1996). The cytosol pH is also dramatically changed towards acidification under fermentation, e.g., in *Acer pseudoplatanus* cells under anoxia, cytoplasmic pH decreased from 7.5 to 6.8 within 5 min (Gout et al 2001). The central vacuole pH has been measured to be around pH 5.5 (Martiniere et al 2013, Otegui et al 2005). The extracellular cell wall space is more acidic than the cytosol, around pH 5-6 (Felle 2001). Shen measured cellular pH values of *A. thaliana* in different compartments as listed: pH 7.3 in the cytosol, pH 7.2 in the nucleus, pH 7.1 in the ER, pH 5.2 in the vacuole, pH 7.2 in the chloroplast stroma, pH 8.4 in the peroxisome, and pH 8.1 in the mitochondrial matrix (Shen 2017). However, the correctness of the mitochondrial matrix pH can be questioned because of the close

to solid environment with very high amount of proteins. A summary of pH estimations in a plant cells from above data is shown in Figure 7.

In human cells, the pH has been reported to be 7.6 in the cytosol, 6.9 in the mitochondrial intermembrane space (but it was not clear exactly where in the intermembrane space), and 7.8 in the matrix (Porcelli et al 2005). Within the intermembrane space of mammals, the local pH was found to be 0.3 units less acidic at the ATP synthases that are located at the edges of cristae, where protons are transported into the matrix by the ATP synthase, than close to complex IV, where protons are pumped into the intermembrane space (Rieger et al 2014). The locations of inner mitochondrial membrane proteins may thus constitute microenvironment at least in mammals. This may be similar in plants and thus important for activation of mitochondrial external NDB proteins.

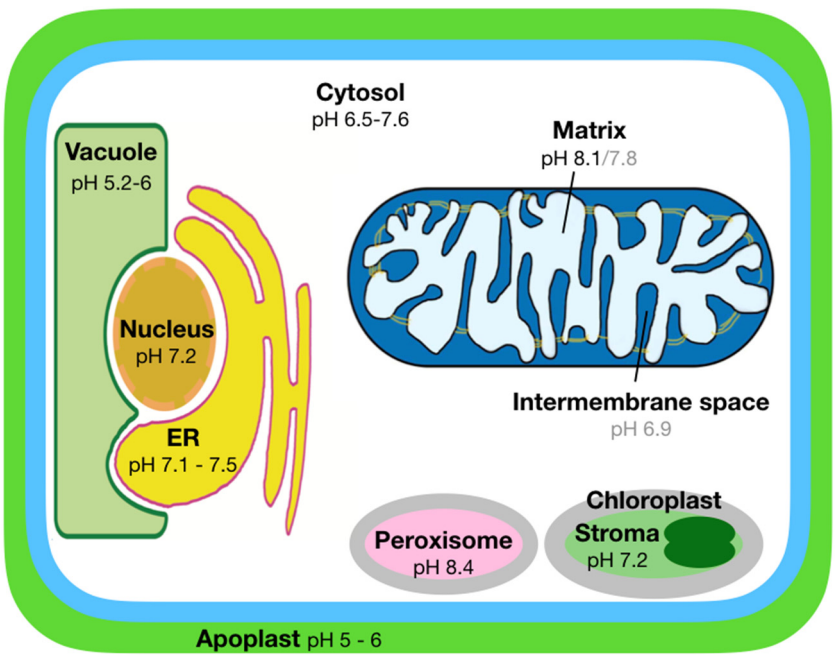


Figure 7. The pH within a plant cell.
Grey values are from data for human cells. Different pH value and sources are based on these pubulications: (Felle 1996, Gout et al 2001, Moseyko & Feldman 2001, Shen 2017)

1.3.2 Parallel changes of cellular Ca^{2+} and pH

Recent experiments have shown that changes in the pH and free Ca^{2+} in cells have some association in signalling in plant cells, e.g., Ca^{2+} spikes were found dynamically linked with pH changes in the cytosol (Behera et al 2018). Transiently enhanced Ca^{2+} concentrations was also linked with a lowered pH after wounding of

A. thaliana cells (Behera et al 2018). The parallel phenomenon of associated pH and Ca^{2+} changes was observed in relation to growth rates of plant pollen tubes as well (Hepler & Lovy-Wheeler 2006). Therefore, there is a link between cellular Ca^{2+} signals and pH signatures, which is possibly relevant for different developmental processes in plants. The combined effects of pH and Ca^{2+} on NDB proteins was investigated in Paper I and is described below.

1.4 NAD(P) in plant cells

1.4.1 NAD(P) cellular functions and biosynthesis

The pyridine nucleotides NAD and NADP occur in reduced and oxidized forms as shown in Figure 8. The reduced form is NAD(P)H and oxidized form is $NAD(P)^+$.

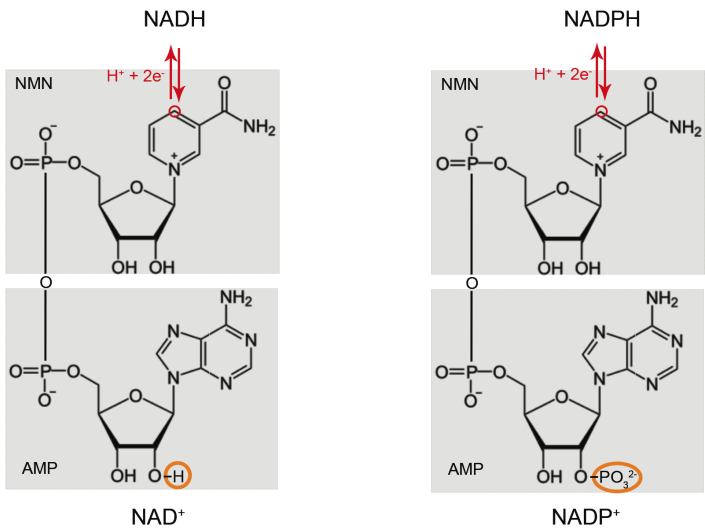


Figure 8. Molecular structures of NAD and NADP.
The position in the AMP part where NAD and NADP differ is marked with orange circles. The position of the hydrogen in the reduced form of NADH and NADPH are marked with a red circle at the NMN part.

NAD is strongly involved in respiratory energy metabolism in especially the cytosol and mitochondria but also has roles in chloroplasts (Noctor et al 2011). For example, chloroplasts of higher plants have NAD(P)-dependent malate DHs (Berkemeyer et al 1998). NADPH has a main role as a reducing agent in photosynthesis, where NADPH is generated in the light-dependent reactions. NADPH also reduces cellular antioxidants, such as the NADPH-requiring glutathione and thioredoxin systems that protect against oxidative stress but also regulates mitochondrial metabolism

(Fernandez & Wilson 2014, Møller 2015). Overall, NAD(P) plays an important role as a redox carrier in plant cells, and the redox status of NAD(P) affects respiration, photosynthesis, stress responses, reduction levels in cells and antioxidants.

NAD is synthesized in plants through two metabolic pathways, the *de novo* pathway from aspartic acid and the salvage pathway that recycles nicotinamide (Katoh et al 2006, Wang & Pichersky 2007). In plastids, aspartate is converted into nicotinate mononucleotide, which is transferred to the cytosol or nucleus for synthesis of NAD (Noctor et al 2011). NAD can be converted to NADP by NAD kinases that are active in cytosol, plastids and peroxisomes. NAD kinases may thus regulate NAD/NADP ratio (Noctor et al 2011, Turner et al 2005).

1.4.2 Plant cellular distribution of reducing equivalents

The amounts of NAD and NADP in individual cell compartments differ between plant species and tissues (Noctor et al 2011). The NAD(P) concentrations in plant organelles are generally within the range of 0.1 to 2.5 mM (Noctor et al 2011). A summary of NAD(P) distribution and redox ratios in cell sub-compartments is denoted in Table 1. Variations are, however, observed between compartments and with different conditions. The total NAD concentrations are generally highest in mitochondria and lowest in the chloroplast (Douce & Neuburger 1989). However, because the cytosol volume is about 6 times the total mitochondrial volume in mesophyll cells, the total NAD content in leaves is highly dominated by the cytosolic pool (Noctor et al 2011, Queval et al 2011).

Table 1. Pyridine nucleotide concentrations in three compartments of plant leaf cells and whole tissue.
Concentations are denoted in mM. n.m., not measured; n.d., not detected; The density of red colour indicates the relative value differences between ratios. The table is modifeied from Noctor *et al.* (2011)

Compartment	Condition	NAD^+	NADH	NADH/ NAD^+	Total NAD	$NADP^+$	NADPH	NADPH/ $NADP^+$	Total NADP	Reference
Chloroplast (stroma)	Light	0.21	0.05	0.24	0.26	n.m.	n.m.	-	n.m.	c
	Light	0.19	n.d.	-	0.19	0.59	0.29	0.49	0.88	a
	Dark	0.92	n.d.	-	0.92	0.51	0.12	0.24	0.63	a
Mitochondria	Light	0.68	1.76	2.59	2.44	n.m.	n.m.	-	n.m.	c
	Light (limiting CO_2)	1.55	0.46	0.30	2.01	0.08	0.24	3.00	0.32	b
	Light (saturating CO_2)	1.41	0.13	0.09	1.54	0.14	0.12	0.86	0.26	b
	Dark	1.52	0.08	0.05	1.6	0.27	0.05	0.19	0.32	b
Cytosol	Light	0.72	0.09	0.13	0.81	n.m.	n.m.	-	n.m.	c
	Light (limiting CO_2)	0.52	0.06	0.12	0.58	0.14	0.18	1.29	0.32	b
	Light (saturating CO_2)	0.62	0.02	0.03	0.64	0.14	0.14	1.00	0.28	b
	Dark	0.57	0.02	0.04	0.59	0.15	0.17	1.13	0.32	b
Whole tissue	Light			0.17				0.43		d
	Dark (4h)			0.9				1.5		e

a Data from Heineke *et al.* (1991); chloroplasts obtained by non-aqueous fractionation of spinach leaves.
b Data from Igamberdiev &Gardeström (2003); rapid fractionation of pea protoplasts by filtration.
c Data from Szal *et al.* (2008); rapid fractionation of cucumber protoplasts by filtration. The concentrations shown were recalculated from values given in nmol mg^{-1} chlorophyll assuming subcellular volumes reported for spinach leaves (Winter et al 1994).
d Data from Wallström *et al.* (2014b) whole tissue of *A. thaliana* leaves.
e Data from Liu *et al.* (2008); the first fully expanded leaf of 6-week-old tobacco.

When potato StNDB1 was overexpressed in tobacco, leaf NADPH/$NADP^+$ ratio was consistently lowered in light, and in one of three transgenic lines it was significantly lowered in darkness, whereas the NADH/NAD^+ ratio was not changed (Liu et al 2008). A similar pattern was observed in tobacco stem cells (Liu et al 2009). This means that NDB1 is able to modulate the cellular NADPH/$NADP^+$ ratio in living cells. The NAD pool is generally oxidized in the cytosol, with NAD^+ constituting at least 88% (Table 1). In contrast, the NADPH pool in the cytosol is generally about 50% reduced (Table 1). This is consistent with whole tissue studies of *A. thaliana* leaves, where about 10% of the NAD was in the reduced form under light conditions (Wallström et al 2014a, Wallström et al 2014b). In contrast, the NADP pool in *A. thaliana* leaves is about 30% reduced under light condition (Wallström et al 2014b). NDB1-suppressing lines of *A. thaliana* showed decreased $NADP^+$ (Wallström et al

2014a). Thus, NDB1 has the capacity to affect total amount of NADP pool and NADPH redox status in different cell compartments.

1.4.3 Transport of NAD(P) and reductant within plant cells

Within the cell, NAD(P) is transferred between some subcellular compartments through voltage-dependent anion channels on outer mitochondrial and chloroplast membranes (Hanning & Heldt 1993, Raghavendra et al 1994). The mitochondrial inner membrane is impermeable to NADH, but permeable for NAD^+ via NAD^+ transporters (Neuburger & Douce 1983). The electrons carried by NAD(P)H are transferred between separate pools of $NAD(P)^+$ across membranes via metabolite shuttles operating through metabolite carriers (Taniguchi & Miyake 2012). For example, the malate/oxaloacetate shuttle systems transport electrons across the inner mitochondrial membrane and inner chloroplast envelope membrane (Heineke et al 1991, Noctor et al 2011, Taniguchi et al 2002), and also between the cytosol and the peroxisomal compartment (Reumann et al 1994).

1.4.4 Consequences of NAD(P) redox changes in plant cells

There are more than 800 known enzymes that utilize NAD(P) (Enzyme Nomenclature; https://www.qmul.ac.uk/sbcs/iubmb/enzyme/). The majority of these enzymes are involved in energy metabolism. The NAD(P)H redox status reflects the total cell redox states and overall metabolic activities (Schäfer & Buettner 2001). In plants, $NADH/NAD^+$ redox status, $NADPH/NADP^+$ redox status, the ferredoxin/thioredoxin system and the glutathione/glutaredoxin system are key regulators of redox in cellular process (Buchanan & Balmer 2005, Møller 2015). In human cells, both oxidative and reductive stress can cause cellular redox changes involving many redox couples, such as $NADH/NAD^+$, $NADPH/NADP^+$ and glutathione disulphide-glutathione couples, which affects DNA-binding activity of some transcription factors, DNA repair, RNA synthesis, protein synthesis, enzyme activation and regulation of the cell cycle (Schäfer & Buettner 2001).

In plant cells, mitochondrial $NADH/NAD^+$ ratio and ATP/ADP ratio regulate the first citric acid cycle proper reaction that is catalysed by citrate synthase (Nunes-Nesi et al 2013). Higher matrix $NADH/NAD^+$ ratio will lead to more ROS production by generation of O_2^- by complex I and III (Lindsay et al 2015). In the cytosol, the plant oxidative pentose phosphate pathway is rate controlled by glucose-6-phosphate DH in a reaction that is stimulated by the concentration of $NADP^+$ and strongly inhibited by NADPH. Cytosolic NAD(P) redox status also affects starch storage and partitioning, lipid synthesis, nitrate assimilation and chlorophyll synthesis (Geigenberger & Fernie 2014).

For plant NDH-2 enzymes, a double RNAi suppression line of AtNDA1 and AtNDA2 showed higher total NADH content in rosettes tissues than the wild type (Wallström et al 2014b). The RNA modification also influenced the redox level of the NADPH pool, which indicates transhydrogenation between NAD and NADP can take place, at least within mitochondria (Wallström et al 2014b). However, NDB1-suppressing lines of *A. thaliana* instead showed specifically decreased $NADP^+$, and were affected in central metabolism, growth and defence signalling (Wallström et al 2014a). The consequences of pH and Ca^{2+}-regulation of NDB proteins and NAD(P)H reduction levels are discussed below and in Paper I.

Applied science, purposeful and determined, and pure science, playful and freely curious, continuously support and stimulate each other. The great nation of the future will be the one which protects the freedom of pure science as much as it encourages applied science.

— Edwin Herbert Land

2 Type II NAD(P)H dehydrogenases

The type II NAD(P)H DHs play an important role in respiration in mitochondria. They exist not only in plants, but are widely spread across the eukaryotic organisms. In this chapter, the overall knowledge of eukaryotic NDH-2 enzymes in substrate specificity, regulation, structure, molecular mechanism, distribution and evolution is covered, yet focused on plant NDB proteins.

2.1 Substrate specificity of type II NAD(P)H DHs

2.1.1 NADH and NADPH specificity

Analysis of potato StNDA1 indicated that NADH is the substrate for NDAs (Geisler et al 2004, Svensson & Rasmusson 2001). Additionally, an AtNDA1-lacking mutant was found to have a decreased internal rotenone-insensitive malate oxidation activity, which linked the protein to NADH oxidation (Moore et al 2003). The AtNDA2 sequence has high similarity with AtNDA1 and was likewise suggested to be an NADH DH (Michalecka et al 2003, Moore et al 2003). AtNDC1 has been claimed to oxidize both NADPH and NADH, but preferred NADH as substrate (Fatihi et al 2015).

Overexpression of potato StNDB1 in *N. sylvestris* leads to higher mitochondrial NADPH oxidation activity, whereas sense-suppression of both StNDB1 and the endogenous NsNDB1 leads to an undetectable mitochondrial NADPH oxidation activity, without affecting NADH oxidation, at least when measured at neutral pH (Liu et al 2008). This pattern was also seen at several pH values within the physiological range (Michalecka et al 2004)(**Paper I**). AtNDB1 in *A. thaliana* was also shown to be NADPH-specific when bound to *E. coli* membranes (Geisler et al 2007). Both AtNDB2 and AtNDB4 specifically oxidized NADH and could complement for the lack of NADH oxidation in an *E. coli* double mutant that lacks both the complex I-type and the NDH-2 NADH DH (Geisler et al 2007). The substrate for AtNDB3 is not known but has been predicted to be NADH (Michalecka et al 2003) (**Paper II**). The soluble recombinant AtNDB2 produced by *E. coli* had only NADH oxidation activity (**Paper III**). In contrast, the soluble recombinant StNDB1 had both NADH and NADPH oxidation activity, yet with

higher NADPH oxidation activity (**Paper III**). However, also the membrane bound AtNDB1 oxidise both NADH and NADPH at high substrate concentration (0.8 mM NAD(P)H), but mainly oxidise NADPH at a lower substrate concentrations (80 µM NAD(P)H), at pH 7.2 (Geisler et al 2007). This pattern is also observed for soluble recombinant StNDB1 (**Paper III)**. Hence, *in vivo*, NDB1 should mainly oxidize cytosolic NADPH and NDB2 only oxidize cytosolic NADH.

2.1.2 NDH-2 enzymes are UQ specific and has different activities for different UQ substrates

The motif AQxAxQ in NDH-2 proteins have been proposed to be a quinone binding site (Heikal et al 2014, Marreiros et al 2016). In a comparison of 131 eukaryotic NDH-2 homologues the collected sequence logo was consistent with this motif signature (Figure 9).

Figure 9. Sequence logo of quinone binding sites from 131 eukaryotic NDH-2 enzymes.
The conserved motifs (AQxAxQ) is marked out by red color. StNDB1a is used as an example to show the relative position of the quinone binding motif, $A_{467}Q_{468}VA_{470}XQ_{472}$. The 131 eukaryotic NDH-2 enzymes share the same species with the study in Paper II, but includes all types of NDH-2 enzymes, i.e., NDA, NDB and NDC protein sequences.

For analysed NDH-2 enzymes, quinones are standard electron acceptors. UQ is reduced by NAD(P)H DHs and then receive $2H^+ + 2e^-$ to form UQH_2, ubiquinol. The length of the side chain of common quinone and quinols found in energy-transducing membranes can vary, e.g., UQ_{10} in plants and mammals has 10 isoprenyl chemical subunits in the side chain, but UQ_6 in yeast has 6, and UQ_8 in *E. coli* has 8 (Okada et al 1996). In chloroplast, instead of UQ, plastoquinone is the redox carrier in the electron transport chain of photosynthesis (Allen et al 1981). The hydrocarbon side chain of ubiquinone UQ_{10} is highly hydrophobic. Because of the hydrophobic nature of UQ_{10}, it is difficult to use as a substrate *in vitro*. Therefore, quinones with shorter side chains are used. Decylubiquinone (DcQ) is water soluble and was applied as standard acceptor in **Paper I** and **Paper III**, whereas UQ_1, decylplastoquinone (DcPQ), duroquinone (DQ) and potassium ferricyanide (FeCN) were compared in **Paper III**. Among these electron acceptors, StNDB1 and AtNDB2 were relatively UQ-specific, *e.g.,* not accepting DcPQ. This means plant

NDB proteins is specifically active in mitochondria even though they could have a secondary location in peroxisomes or chloroplasts.

2.2 Regulation of external type II NAD(P)H DHs

NDH-2 enzymes locate at two side of the inner mitochondrial membrane, facing towards matrix and intermembrane space differently. Therefore, NDH-2 enzymes can be regulated differently. This section only summarises the regulations of plant external type II NAD(P)H DHs, i.e., NDB proteins. The summarised regulations include cytosolic conditions, such as cytosolic Ca^{2+}, pH and gene expression.

2.2.1 Ca^{2+} is the allosteric activator for NDB and the Ca^{2+} dependence is under pH regulation

From the study of isolated plant mitochondria, pH and Ca^{2+} are known to separately affect external NAD(P)H oxidation (Cowley & Palmer 1978, Møller 2001, Rasmusson & Møller 1991). Most external NDH-2 enzymes in *A. thaliana* are dependent on or stimulated by Ca^{2+}, except AtNDB4 (Geisler et al 2007). Based on our experiments with isolated potato mitochondria and membranes containing *E. coli* expressed *A. thaliana* NDB proteins, the activation of NDB1 is expected to require high Ca^{2+} concentrations and a low pH, and the halfway activated $K_{0.5}(Ca^{2+})$ is around 3 and 82 μM at pH 6.8 and 7.5, respectively (**Paper I**). Therefore, we proposed that the Ca^{2+} activation of NDB1 is strongly depending on the pH (**Paper I**). Thus, the parallel enhancement of Ca^{2+} and pH (see section 1.3.2.) could positively affect NDB1 activity *in vivo*. Transgenic experiments showed that NDB1 was active *in vivo* (Liu et al 2008), indicating a low pH and a high Ca^{2+} in the NDB1 microenvironment. Additionally, NDB1 is found to be rapidly responsive to Ca^{2+} transient simulations, and this was consistently seen *in situ* and with purified recombinant StNDB1 (**Paper I, III**). The measured $t_{1/2}$ (half-life of the activity after addition of Ca^{2+}/EGTA) of mitochondrial StNDB1 is less than 5 seconds for both enhanced and decreased Ca^{2+} concentrations (**Paper I**). For a comparison, NDB2 that is specific for NADH oxidation are likely constantly activated *in vivo*, based on the relatively low Ca^{2+} demand and slow deactivation properties. The $K_{0.5}(Ca^{2+})$ of the membrane bound AtNDB2 is 0.8 μM, independent of pH (**Paper I**), consistent with that the $K_{0.5}(Ca^{2+})$ of *Helianthus tuberosus* mitochondrial NADH oxidation is 0.2–1 μM at pH 7.2 (Moore & Åkerman 1982, Rugolo et al 1991). The $t_{1/2}$ of mitochondrial StNDB2 for Ca^{2+} increase is less than 5 sec but for Ca^{2+} decrease is around 35 seconds at pH 7.2 (**Paper I**). This is however not observed with soluble AtNDB2. A simplified illustration of the pH and Ca^{2+} regulations of NDB proteins is summarized in Figure 10. Therefore, Ca^{2+} signals and pH are essential for

mediating plant physiological changes via different Ca^{2+} responding paths and do have impact on NDB activity and cellular redox statues.

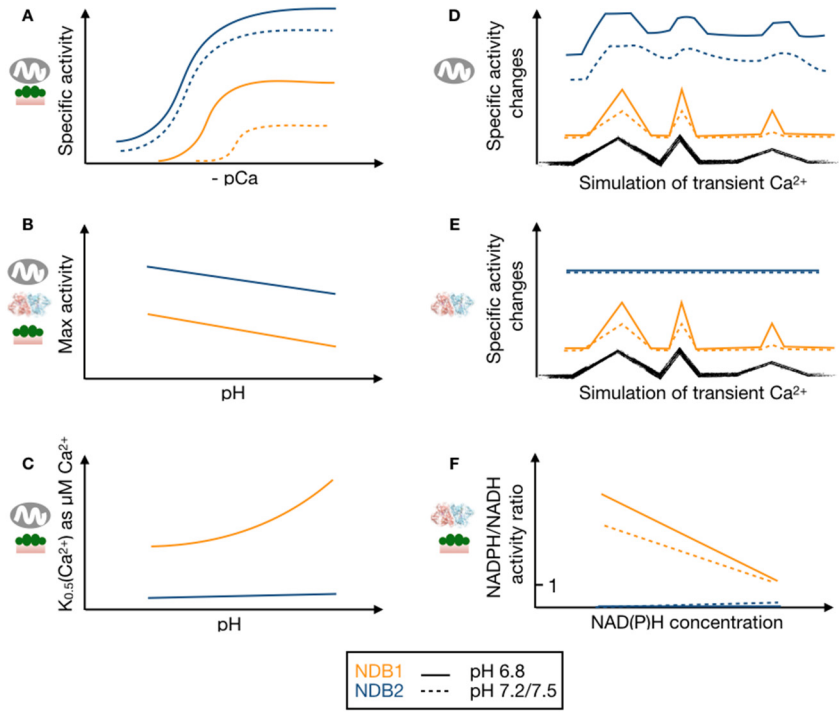


Figure 10. A simplified illustration of the regulation of plant NDB1 and NDB2.
Orange and blue curves indicate activities of NDB1 and NDB2, respectively. NADH is the substrate for NDB2 and NADPH is the substrate for NDB1. Normal and dashed lines indicates pH 6.8 and 7.2/7.5, respectively, and applies to all figure panels above except (B) and (C). NDB1 follows a sigmodal curve fit to pCa (A). The max activity of each related specific activity is decreasing with an enhanced pH within the physiological range (B). The $K_{0.5}(Ca^{2+})$ in µM is different between NDB1 and NDB2, and increasing with an enhanced pH for NDB1, but not for NDB2 (C). The simulations of transient Ca2+ changes within physiological range indicates a permeant active mitochondrial NDB2 and a Ca^{2+} elevated NDB1 activity for both mitochondrial and purified enzyme's study (D and E). Purified NDB1 protein could oxidise both NADH and NADPH, but it prefers NADPH as substrate and even more so at low substrate concentrations (F). In contrast, NDB2 only oxidise NADH (F). Simbles: indicate support by the mitochondrial study. indicates support by the study of purified enzymes. indicates support by the *E. coli* study of membrane-bound AtNDB1 and AtNDB2. Data of this figure is based on Paper I, Paper III and Geisler et al., 2007.

2.2.2 Gene regulation of type II NAD(P)H DHs expression

There is evidence that *NDH-2* genes such as *NDA1* and *NDC1* have a light-induced expression (Rasmusson & Escobar 2007). The gene *NDA2* and *NDB2* are also up-regulated by glucose (Clifton et al 2005, Price et al 2004, Rasmusson & Escobar 2007). Expression levels of plant genes encoding NDH-2 enzymes were strongly affected by the nitrogen sources in that supply of ammonium leads to an increased

expression level as compared to nitrate nutrition (Escobar et al 2006). A similar effect was observed for *AOX* genes (Escobar et al 2006), which indicated that there is an association between AOX and NDH-2 enzymes. Under ammonium toxicity stress, both wild type and *AtNDB1*-suppressed lines showed significant suppressed growth in *A. thaliana,* but a decreased mitochondrial ROS production and elevated glutathione-associated antioxidants were observed in the *AtNDB1*-suppressed lines (Podgorska et al 2018).

This may indicate a direct or indirect ROS production by NDB1, but it has not been tested. Moreover, there were no transcript changes found in all NDH-2 enzymes in *A. thaliana* with hyperosmotic stress (Clifton et al 2005). However, we found the hyperosmotic stress could lower StNDB1 and AtNDB2 activity. The summarized transcription changes of NDH-2 enzymes to different conditions is shown below (Figure 11). Hence, a different gene expression level of *NDH-2* genes could change the redox status of a plant, and consequently may vary plant growth.

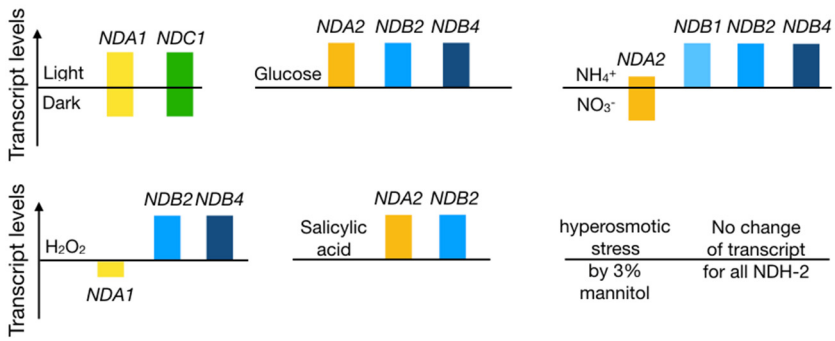


Figure 11. Transcription changes of *NDH-2* to different conditions
Upwards bars indicate increases and downwards indicate decreases in gene expression.(Clifton et al 2005, Podgorska et al 2018, Price et al 2004, Rasmusson & Escobar 2007).

2.3 Structure of type II NAD(P)H DHs

2.3.1 Primary structure of eukaryotic type II NAD(P)H DHs

Proteins in the plant NDB family have molecular masses of 60-65 kDa, whereas it is around 50-55 kDa for NDA and NDC proteins (Michalecka et al 2003). The proteins have mitochondrial targeting sequences (for NDC also targetting to plastids) and two Rossmann folds that bind FAD and NAD(P)H (Figure 12). Mitochondrial targeting sequences are formed in the first 10-90 N-terminal residues that has a high Arg content and few negatively charged residues (Schneider et al 1998). FAD is a redox prosthetic group that is usually non-covalently bound to

NDH-2 enzymes; an exception is *Chlamydomonas reinhardtii* CrNDA2, which has FMN as a flavin cofactor (Desplats et al 2009). The core FAD-binding motif is followed by an NAD(P)H binding motif. In general, NDH-2 enzymes found in eukaryotes show similarity to *A. thaliana* homologs (Figure 12). NDB-type proteins are defined by an inserted domain as compared to NDA and NDC-type proteins. The inserted domain in NDBs usually contains two EF-hand/EF-hand-like motifs, one or two of which binds Ca^{2+}. Exceptions are found for *A. thaliana* AtNDB4 and *Plasmodium falciparum* pfNDH-2, which lack EF-hand motifs and were found not to bind Ca^{2+} (Geisler et al 2007, Yang et al 2017). Additionally, the fungus *Ustilago maydis* NDB has an NDB-type enzymes with 2 EF-hand domains that was predicted to bind Ca^{2+} (**Paper II**), but a Ca^{2+} effect was not observed in membrane preparations (Matuz-Mares et al 2018). A quinone-binding motif has been described above, and it is located close to the inserted NDB-specific region (Figure 12). The C-terminus has been indicated to be essential for membrane interaction of NDA1 in potato and NDI1 in yeast (Feng et al 2012, Iwata et al 2012, Rasmusson et al 1999), and the domain is consistently found in all eukaryotic NDH-2 enzymes (**Paper II**).

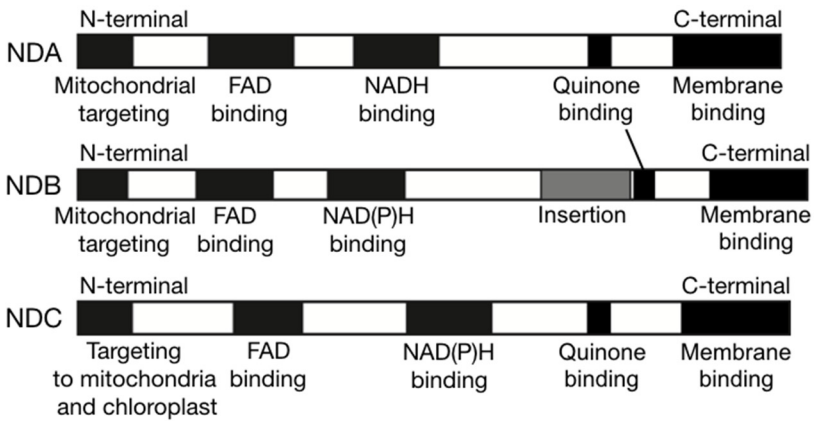


Figure 12. Overview of the primary structure of eukaryotic type II NAD(P)H dehydrogenases. The names are based on plant homologs.
The relative positions of domains are show in boxes. Black boxes denote mitochondrial targeting sequences, predicted FAD binding sites, NAD(P)H binding domains, quinone binding motifs, and membrane binding domains. The gray box denotes a piece of a sequence which looks like an insertion to NDA-type protein, usually a domain contain 1-2 EF-hand/EF-hand-like motifs. This figure is modified from a figure by Allan Rasmusson (personal communication).

2.3.2 Structural modelling of NDB proteins for substrate specificity predictions

There are four NDH-2 enzymes whose structures have been investigated. Budding yeast ScNDI1 (Feng et al 2012, Iwata et al 2012), which is an NDA-type protein, has 38% protein sequence identity with StNDB1. *Caldalkalibacillus thermarum* NDH-2 (Heikal et al 2014), an NDA-type protein, has 24% identity with StNDB1,

Staphylococcus aureus NDH-2 (Sena et al 2015), an NDA-type protein, has 27% identity with StNDB1, and *Plasmodium falciparum* NDH-2 (Yang et al 2017), an NDB-type protein, has 30% sequence identity with StNDB1. The structures of NDH-2 from the malaria parasite, *P. falciparum* (Figure 13) and budding yeast *S. cerevisiae* (**Paper II**) was used as templates for modeling the plant NDB proteins' structures.

From the modeling of NDB1 proteins, the core catalytic parts and the dimerization surface showed distinct similarities to the structure of crystalized yeast ScNDI1 and *P. falciparum* PfNDH-2. Based on AtNDB1 modelling in **Paper II**, we predicted the NAD(P)H binding motifs and classified them into acidic and non-acidic motifs, where an acidic motif correlated with NADH binding and non-acidic motif with NADPH binding. EF-hand structure was predicted separately (**Paper II)**, and its possible position in the enzyme is thought to be along the membrane (**Paper II**). The pfNDH-2 structure supports the prediction.

In brief, the substrate specificity for NAD(P)H in NDB proteins could be predicted by NAD(P)H binding motifs (**Paper II**). For example, for NDB proteins, an acidic motif (usually EA) binds the ribosyl group of NADH, whereas a non-acidic motif (usually QS) binds the phosphorylated ribosyl group of NADPH (**Paper II**).

2.3.3 Oligomeric features of purified and type II NAD(P)H DHs

The structures of all crystalized NDH-2 enzymes have a basic dimeric unit, and homo-oligomers were observed in all of them (Feng et al 2012, Heikal et al 2014, Iwata et al 2012, Sena et al 2015, Yang et al 2017). By BN-PAGE separation and mass spectrometry, AtNDA2, AtNDB2 and AtNDB4 from *A. thaliana* were found at a native molecular mass of 160 kDa and suggested to be heterotrimeric (Klodmann et al 2011). Potato NDA and NDB proteins showed several weight forms by BN-PAGE and western blotting, migrating at 150-200 kDa and 180-700 kDa, respectively (Michalecka et al 2004, Rasmusson & Agius 2001). A pattern of multiple oligomeric forms of recombinant StNDB1 and AtNDB2 was observed by native gel electrophoresis in this study, being consistent with the previous investigation of potato mitochondria (Rasmusson & Agius 2001). The minimal detected form of recombinant StNDB1 was approximately 160 kDa, and multi-oligomers of most likely dimeric units, mainly hexamers were observed (**Paper III**). The 160 kDa band would then correspond to a dimer of the 63.5 kDa monomer. Differently, the recombinant AtNDB2 had a highly oligomeric feature (**Paper III**). Therefore, *in vivo*, NDB proteins on inner mitochondrial membrane are highly possible having a dimeric structure as the smallest unit.

2.4 The molecular mechanism of type II NAD(P)H DHs

The catalytic mechanism of plant NDH-2 enzymes is currently not fully understood. Kinetic analyses of *S. cerevisiae*, *Yarrowia lipolytica, Mycobacterium tuberculosis* and *Caldalkalibacillus thermarum* claim it is either a 'ping-pong' scheme with a rate-limiting redox step (Blaza et al 2017, Eschemann et al 2005, Yamashita et al 2007, Yano et al 2014) or a ternary mechanism for bacterial NDH-2 enzymes (Blaza et al 2017). Blaza *et al.* explained the possible mechanism for a bacterial NDA-type enzyme from *C. thermarum* and showed that the reactions of two substrates, NADH and UQ, occur independently (Blaza et al 2017). All of these mechanisms are based on NDA-type proteins, whereas NDB-type enzymes have not been studied. A conformational change of StNDB1 was observed after a binding of Ca^{2+} by fluorescence microscopy, and this is possibly due to a tryptophan (Trp_{484}) in the helix containing the UQ binding motif (Figure 13), but not yet verified. We speculate that the Ca^{2+} binding of NDB1 may modify the efficiency of UQ binding (**Paper III**). However, kinetic measurements are needed to fully understand the molecular mechanism of NDB proteins and how Ca^{2+} may affect the rate .

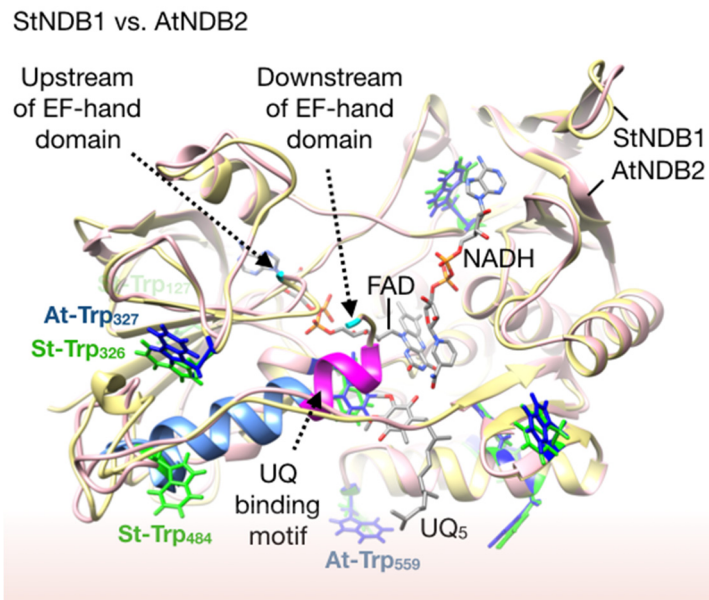


Figure 13. Molecular models of StNDB1 and AtNDB2 showing the active region.
An overlay of models for the StNDB1 (yellow) and AtNDB2 (pink) showing the catalytic parts without EF-hand domains. StNDB1 and AtNDB2 models were predicted by I-TASSER with PfNDH-2 (PDB: 5JWB) as template. FAD and NADH are predicted to bind to StNDB1 by I-TASSER. UQ5 is an overlay using the coordinates from the yeast homolog ScNDI1 (PDB: 4g73). Trp in StNDB1 and Trp in AtNDB2 are marked out in green and blue respectively. Because the EF-hand domain covers the substrates from this angle of the picture, only the two ends of the EF-hand domain of StNDB1 is shown, in light blue. The UQ binding motif (AQxAxQ) is shown in purple, A_{467}-Q_{472} in StNDB1, A_{472}-Q_{477} in AtNDB2.The alpha helix that contain the UQ-binding motif is shown in blue, but the StNDB1-specific Trp_{484} is marked in green. The models are shown with the predicted membrane-interaction sides downwards.

2.5 Type II NAD(P)H DHs distribution and evolution in eukaryotes

In our study, NDA-, NDB- and NDC-type NAD(P)H DHs in eukaryotes are classified based on homology to plant proteins to simplify the description of the evolutionary changes (Figure 14) (**Paper II**). Most plants, green and red algae contain the full set of alternative respiratory enzymes, including alternative oxidase, NDA-, NDB- and NDC-type proteins (**Paper II**).

The NDA and NDB proteins are homologs to alpha-proteobacterial NDA type NADH DHs and derive from an NDA-type enzyme that likely entered eukaryotes via the mitochondrial endosymbiosis event (Melo et al 2004, Michalecka et al 2003). NDC has a cyanobacterial origin via the plastid-forming endosymbiosis event (Michalecka et al 2003) (Figure 14). Phylogeny analysis combined with molecular structure analysis focused on substrate binding and Ca^{2+} binding of NDB proteins, and indicated that external NADPH oxidation (non-acidic motif) is an ancient process as compared to external NADH oxidation (acidic motif). However, switches between non-acidic and acidic motifs appear to have occurred multiple times in plant evolution, leading to changes in cytosolic NADH and NADPH oxidation **(Paper II)**. The plant NDB-type NADH DHs are thus descendants of NDB-type NADPH DHs and indicates that an expansion of NADH DHs has taken place in angiosperms, which parallels the expansion of AOX from a single gene in ferns into a multi-gene family in seed plants (Neimanis et al 2013). Thus, external NADPH oxidation should have a more fundamental importance for cellular redox metabolism, but not related to photosynthesis (**Paper II**).

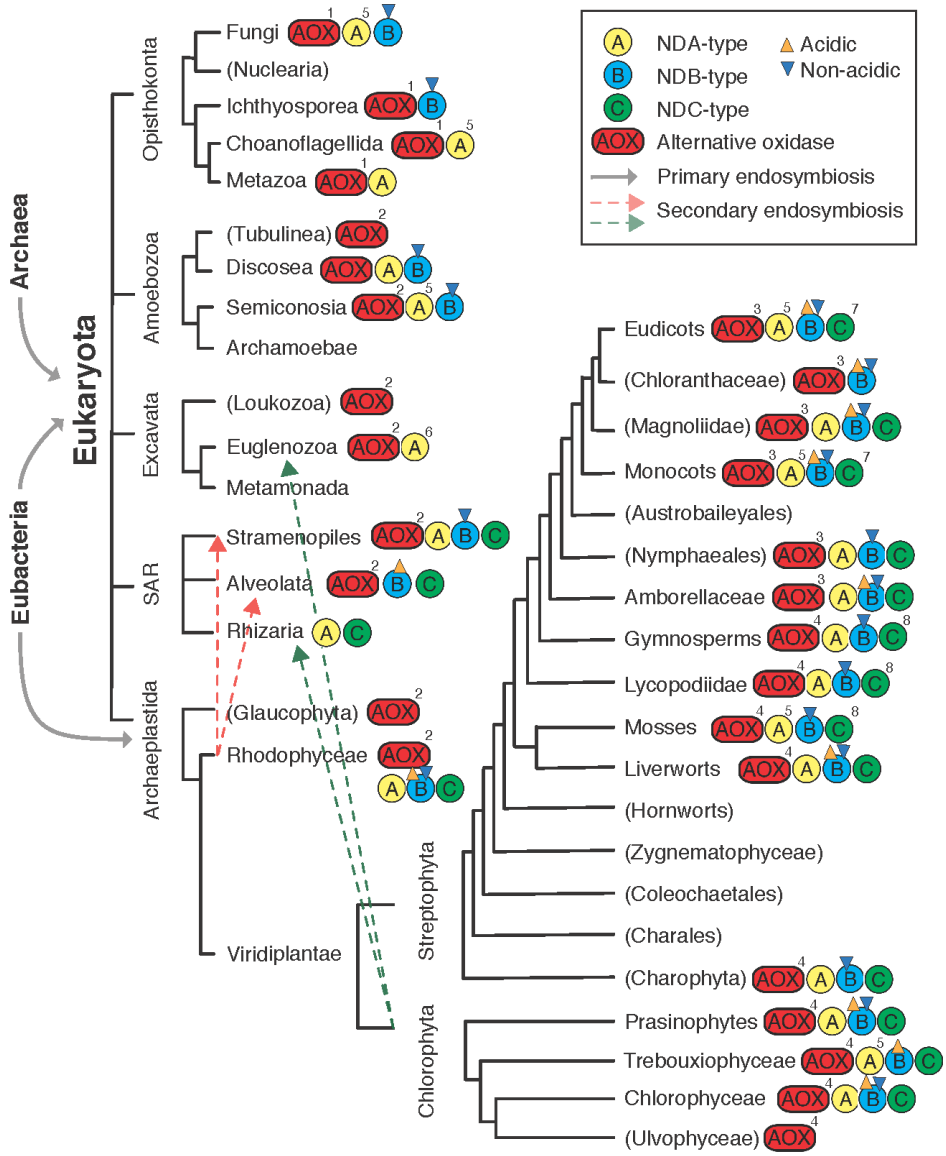


Figure 14. The distribution of NDH-2 and AOX proteins in eukaryotes, with emphasis on plants
NDA-type, NDB-type and NDC-type NDH-2 and AOXs are indicated with yellow, blue, green, and red boxes respectively. The NAD(P)H binding motifs of NDB, acidic and non-acidic type are denoted as triangles on NDB signs, which predict the substrate of NADH (acidic type)(yellow) and/or NADPH (non-acidic type)(blue) in the clade. Lineages for which less than 20000 protein entries are present in the Genbank database are denoted within parenthesis.

3 Conclusions and future perspectives

It was earlier known that, *A. thaliana* and potato external type II NADPH DH separately display pH and Ca^{2+} regulation (Michalecka et al 2004), but the Ca^{2+} concentrations needed for activation had not been determined. The results presented here indicate that the Ca^{2+} activation *in vivo* is controlled by pH, which means cytosolic acidification is a critical activator for NDB1 (**Paper I**). Purified NDB1 oxidise both NADH and NADPH, but with higher NADPH oxidation activity. Purified NDB2 is consistent with membrane-bound NDB2 that only oxidase NADH (**Paper III**). Ca^{2+} transient simulations predict a permanently active NDB2 *in vivo* and a transiently elevated NDB1 in response to Ca^{2+} and lowered pH.

Based on the NDB molecular modelling, we could connect substrate NAD(P)H specificity of characterised enzymes to sequence motifs. An acidic-type motif in NDB predicts binding of NADH, and a non-acidic-type motif NADPH. We followed the motifs in a phylogenetic analysis and discovered that external NADPH oxidation may be an ancient eukaryotic process as compared to external NADH oxidation (**Paper II**). NADPH oxidation may thus have a fundamental importance for cellular redox metabolism (**Paper II**).

For future perspectives, further studies of StNDB1 and AtNDB2 are needed for a better understanding of the molecular mechanisms of NDB proteins. For example, we still do not know whether there are one or two Ca^{2+} binding sites, and we have no explanation of how come the Ca^{2+} demand is lower for DcQ reduction than for O_2 reduction in mitochondria (Paper I). Therefore, determinations of Ca^{2+} binding affinities and kinetics with purified proteins are needed. Additionally, a kinetic study of different quinones may explain why the activities and especially Ca^{2+} demands are different when using different quinones and O_2 as electron acceptors. Two independent steps are needed for electrons to be transferred within the NDA-type protein in bacteria, and a reduced form of the protein could be stable detected (Blaza et al 2017). Comparing of reduced and oxidised NDB1 proteins could explain whether there is a stable reduced protein state where FAD is reduced. If so, there will be two apparent steps for electron transfer within a NDB1 protein, where the first step transfer electron from NADPH to FAD, and second step delivering the electrons from reduced FAD to quinone. If this would be the case, there are follow up questions, whether the Ca^{2+} is needed for first step or second step, or both. Additionally, when will then $NADP^+$ disassociate from the protein, before or after FAD is reduced to $FADH_2$, or even after the quinone is reduced? Furthermore, yeast

Ndi1 was found to generate O_2^- in solution but not when bound to submitochondrial particles (Yamashita et al 2007). Therefore, it was suggested the reduced FAD in NDB enzymes may leak electrons to O_2, and then an O_2^- will be formed. Accordingly, ROS detection assays of active soluble and membrane-bound NDB proteins could be analysed in the future to answer whether NDB proteins could lead to ROS production *in vitro* and *in vivo*. If ROS production is confirmed, it will be contrary to previous knowledge, where NDB proteins were considered to play an important role as ROS eliminator *in vivo* in plant mitochondria (Sanz et al 2010). Moreover, if we would build a permanent active external mitochondrial NADPH DH for oxidising cytosolic NADPH, the cytosolic redox homeostasis may switch to a more oxidised cytosolic environment as under stress condition. How will the genetic modified plants with continued-reduced cytosolic NADPH/$NADP^+$ pool grow under different normal and stress conditions? Would this provide more or less resistance to biotic and/or abiotic stresses? This would be very interesting to know in future by studying the according transgenic plants with a modified *ndb1* gene. If the results are positive, the knowledge of NDB proteins may help agriculture in a long perspective by enhancing the ability of crops to face different environmental stresses.

4 References

Allen JF, Bennett J, Steinback KE, Arntzen CJ. 1981. Chloroplast protein phosphorylation couples plastoquinone redox state to distribution of excitation energy between photosystems. *Nature* 291: 25

Amirsadeghi S, Robson CA, Vanlerberghe GC. 2007. The role of the mitochondrion in plant responses to biotic stress. *Physiologia Plantarum* 129: 253-66

Amthor JS. 2000. The McCree-de Wit-Penning de Vries-Thornley respiration paradigms: 30 years later. *Annals of Botany* 86: 1-20

Anderson S, Bankier AT, Barrell BG, Debruijn MHL, Coulson AR, et al. 1981. Sequence and organization of the human mitochondrial genome. *Nature* 290: 457-65

Araujo WL, Ishizaki K, Nunes-Nesi A, Larson TR, Tohge T, et al. 2010. Identification of the 2-Hydroxyglutarate and isovaleryl-CoA dehydrogenases as alternative electron donors linking lysine catabolism to the electron transport chain of *Arabidopsis* Mitochondria. *Plant Cell* 22: 1549-63

Araujo WL, Tohge T, Ishizaki K, Leaver CJ, Fernie AR. 2011. Protein degradation - an alternative respiratory substrate for stressed plants. *Trends in Plant Science* 16: 489-98

Atkin OK, Loveys BR, Atkinson LJ, Pons TL. 2006. Phenotypic plasticity and growth temperature: understanding interspecific variability. *Journal of Experimental Botany* 57: 267-81

Barbot M, Jans DC, Schulz C, Denkert N, Kroppen B, et al. 2015. Mic10 oligomerizes to bend mitochondrial inner membranes at cristae junctions. *Cell Metabolism* 21: 756-63

Bartoli CG, Pastori GM, Foyer CH. 2000. Ascorbate biosynthesis in mitochondria is linked to the electron transport chain between complexes III and IV. *Plant Physiology* 123: 335-43

Batistič O, Kudla J. 2009. Plant calcineurin B-like proteins and their interacting protein kinases. *Biochimica et Biophysica Acta* 1793: 985-92

Behera S, Zhaolong X, Luoni L, Bonza MC, Doccula FG, et al. 2018. Cellular Ca^{2+} signals generate defined pH signatures in plants. *The Plant cell* 30: 2704-19

Berkemeyer M, Scheibe R, Ocheretina O. 1998. A novel, non-redox-regulated NAD-dependent malate dehydrogenase from chloroplasts of *Arabidopsis thaliana* L. *Journal of Biological Chemistry* 273: 27927-33

Billker O, Dechamps S, Tewari R, Wenig G, Franke-Fayard B, Brinkmann V. 2004. Calcium and a calcium-dependent protein kinase regulate gamete formation and mosquito transmission in a malaria parasite. *Cell* 117: 503-14

Blaza JN, Bridges HR, Aragão D, Dunn EA, Heikal A, et al. 2017. The mechanism of catalysis by type-II NADH:quinone oxidoreductases. *Scientific Reports* 7: 40165

Brandalise M, Maia IG, Borecky J, Vercesi AE, Arruda P. 2003. Overexpression of plant uncoupling mitochondrial protein in transgenic tobacco increases tolerance to oxidative stress. *Bioenergetics Biomembranes* 35: 203-9

Braun HP, Binder S, Brennicke A, Eubel H, Fernie AR, et al. 2014. The life of plant mitochondrial complex I. *Mitochondrion* 19: 295-313

Browse JA, Møller IM, Rasmusson AG. 2014. Respiration and lipid metabolism In *Plant Physiology*, ed. L Taiz, E Zeiger, pp. 305-42. Sunderland: Sinauer Associates Inc.

Buchanan BB, Balmer Y. 2005. Redox regulation: a broadening horizon. *Annu Rev Plant Biol* 56: 187-220

Burger G, Gray MW, Lang BF. 2003. Mitochondrial genomes: anything goes. *Trends in Genetics* 19: 709-16

Cavalcanti JHF, Quinhones CGS, Schertl P, Brito DS, Eubel H, et al. 2017. Differential impact of amino acids on OXPHOS system activity following carbohydrate starvation in *Arabidopsis* cell suspensions. *Physiologia Plantarum* 161: 451-67

Choi J, Tanaka K, Cao Y, Qi Y, Qiu J, et al. 2014a. Identification of a plant receptor for extracellular ATP. *Science* 343: 290-94

Choi WG, Toyota M, Kim SH, Hilleary R, Gilroy S. 2014b. Salt stress-induced Ca^{2+} waves are associated with rapid, long-distance root-to-shoot signaling in plants. *Proceedings of the National Academy of Sciences of the United States of America* 111: 6497-502

Clifton R, Lister R, Parker KL, Sappl PG, Elhafez D, et al. 2005. Stress-induced co-expression of alternative respiratory chain components in *Arabidopsis thaliana*. *Plant Molecular Biology* 58: 193-212

Coe H, Michalak M. 2009. Calcium binding chaperones of the endoplasmic reticulum. *General Physiology and Biophysics* 28: F96-F103

Conn S, Gilliham M. 2010. Comparative physiology of elemental distributions in plants. *Annals of Botany* 105: 1081-102

Conn SJ, Gilliham M, Athman A, Schreiber AW, Baumann U, et al. 2011. Cell-specific vacuolar calcium storage mediated by CAX1 regulates apoplastic calcium concentration, gas exchange, and plant productivity in *Arabidopsis*. *The Plant Cell*: tpc. 109.072769

Cowley RC, Palmer JM. 1978. Interaction of citrate and calcium in regulating oxidation of exogenous NADH in plant mitochondria. *Plant Science Letters* 11: 345-50

Desplats C, Mus F, Cuine S, Billon E, Cournac L, Peltier G. 2009. Characterization of Nda2, a plastoquinone-reducing type II NAD(P)H dehydrogenase in Chlamydomonas chloroplasts. *Journal of Biological Chemistry* 284: 4148-57

Douce R. 1985. *Mitochondria in higher plants. Structure, function and biogenesis.*: Academic Press, Inc. USA.

Douce R, Neuburger M. 1989. The Uniqueness of Plant mitochondria. *Annual Review of Plant Physiology and Plant Molecular Biology* 40: 371-414

Drew MC. 1997. Oxygen deficiency and root metabolism: Injury and acclimation under hypoxia and anoxia In *Annual Review of Plant Physiology and Plant Molecular Biology*, ed. RL Jones, pp. 223-50

Duncan O, van der Merwe MJ, Daley DO, Whelan J. 2013. The outer mitochondrial membrane in higher plants. *Trends in Plant Science* 18: 207-17

Elguindy MM, Nakamaru-Ogiso E. 2015. Apoptosis-inducing factor (AIF) and its family member protein, AMID, are Rotenone-sensitive NADH:ubiquinone oxidoreductases (NDH-2). *The Journal of biological chemistry* 290: 20815-26

Elhafez D, Murcha MW, Clifton R, Soole KL, Day DA, Whelan J. 2006. Characterization of mitochondrial alternative NAD(P)H dehydrogenases in *Arabidopsis*: intraorganelle location and expression. *Plant and Cell Physiology* 47: 43-54

Eschemann A, Galkin A, Oettmeier W, Brandt U, Kerscher S. 2005. HDQ (1-hydroxy-2-dodecyl-4(1*H*)quinolone), a high affinity inhibitor for mitochondrial alternative NADH dehydrogenase. *Journal of Biological Chemistry* 280: 3138-42

Escobar MA, Geisler DA, Rasmusson AG. 2006. Reorganization of the alternative pathways of the Arabidopsis respiratory chain by nitrogen supply: opposing effects of ammonium and nitrate. *Plant Journal* 45: 775-88

Fatihi A, Latimer S, Schmollinger S, Block A, Dussault PH, et al. 2015. A dedicated type II NADPH dehydrogenase performs the penultimate step in the biosynthesis of vitamin K1 in synechocystis and *Arabidopsis*. *Plant Cell* 27: 1730-41

Felle HH. 1996. Control of cytoplasmic pH under anoxic conditions and its implication for plasma membrane proton transport in *Medicago sativa* root hairs. *Journal of Experimental Botany* 47: 967-73

Felle HH. 2001. pH: Signal and messenger in plant cells. *Plant Biology* 3: 577-91

Feng Y, Li W, Li J, Wang J, Ge J, et al. 2012. Structural insight into the type-II mitochondrial NADH dehydrogenases. *Nature* 491: 478-82

Fernandez J, Wilson RA. 2014. Characterizing roles for the glutathione reductase, thioredoxin reductase and thioredoxin peroxidase-encoding genes of *Magnaporthe oryzae* during rice blast disease. *PLoS One* 9: e87300

Fernie AR, Carrari F, Sweetlove LJ. 2004. Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. *Current Opinions in Plant Biology* 7: 254-61

Frehner M, Pozuetaromero J, Akazawa T. 1990. Enzyme sets of glycolysis, gluconeogenesis, and oxidative pentose-phosphate pathway are not complete in nongreen highly purified amyloplasts of Sycamore (*Acer Pseudoplatanus* L) Cell-Suspension Cultures. *Plant Physiology* 94: 538-44

Geigenberger P, Fernie AR. 2014. Metabolic control of redox and redox control of metabolism in plants. *Antioxidants & Redox Signaling* 21: 1389-421

Geisler DA, Broselid C, Hederstedt L, Rasmusson AG. 2007. Ca^{2+}-binding and Ca^{2+}-independent respiratory NADH and NADPH dehydrogenases of *Arabidopsis thaliana*. *Journal of Biological Chemistry* 282: 28455-64

Geisler DA, Johansson FI, Svensson AS, Rasmusson AG. 2004. Antimycin A treatment decreases respiratory internal rotenone-insensitive NADH oxidation capacity in potato leaves. *BMC Plant Biology* 4: 8

Giraud E, Van Aken O, Ho LH, Whelan J. 2009. The transcription factor ABI4 is a regulator of mitochondrial retrograde expression of *ALTERNATIVE OXIDASE1a*. *Plant Physiology* 150: 1286-96

Gout E, Boisson AM, Aubert S, Douce R, Bligny R. 2001. Origin of the cytoplasmic pH changes during anaerobic stress in higher plant cells. Carbon-13 and phosphorous-31 nuclear magnetic resonance studies. *Plant Physiology* 125: 912-25

Graham IA. 2008. Seed storage oil mobilization. *Annual Review of Plant Biology* 59: 115-42

Gualberto JM, Mileshina D, Wallet C, Niazi AK, Weber-Lotfi F, Dietrich A. 2014. The plant mitochondrial genome: Dynamics and maintenance. *Biochimie* 100: 107-20

Gupta KJ, Zabalza A, van Dongen JT. 2009. Regulation of respiration when the oxygen availability changes. *Plant Physiology* 137: 383-91

Han Y, Chaouch S, Mhamdi A, Queval G, Zechmann B, Noctor G. 2013a. Functional analysis of *Arabidopsis* mutants points to novel roles for glutathione in coupling H_2O_2 to activation of salicylic acid accumulation and signaling. *Antioxidants & Redox Signaling* 18: 2106-21

Han Y, Mhamdi A, Chaouch S, Noctor G. 2013b. Regulation of basal and oxidative stress-triggered jasmonic acid-related gene expression by glutathione. *Plant, Cell and Environment* 36: 1135-46

Hanning I, Heldt HW. 1993. On the function of mitochondrial metabolism during photosynthesis in spinach (*Spinacia oleracea* L.) leaves - partitioning between respiration and export of redox equivalents and precursors for nitrate assimilation products. *Plant Physiology* 103: 1147-54

Harner M, Neupert W, Deponte M. 2011. Lateral release of proteins from the TOM complex into the outer membrane of mitochondria. *Embo Journal* 30: 3232-41

Harper JF, Harmon A. 2005. Plants, symbiosis and parasites: a calcium signalling connection. *Nature Reviews* 6: 555

Hashimoto K, Kudla J. 2011. Calcium decoding mechanisms in plants. *Biochimie* 93: 2054-59

He Q, Wang XM, He L, Yang L, Wang SW, Bi YR. 2019. Alternative respiration pathway is involved in the response of highland barley to salt stress. *Plant Cell Reports* 38: 295-309

Heikal A, Nakatani Y, Dunn E, Weimar MR, Day CL, et al. 2014. Structure of the bacterial type II NADH dehydrogenase: a monotopic membrane protein with an essential role in energy generation. *Molecular Microbiology* 91: 950-64

Heineke D, Riens B, Grosse H, Hoferichter P, Peter U, et al. 1991. Redox transfer across the inner chloroplast envelope membrane. *Plant Physiology* 95: 1131-37

Hepler P, Lovy-Wheeler A. 2006. Calcium gradients and oscillations in growing pollen tube In *Plant Physiology*. Sunderland: Sinauer Associates Inc.

Hepler PK. 2005. Calcium: a central regulator of plant growth and development. *The Plant Cell* 17: 2142-55

Hessenberger M, Zerbes RM, Rampelt H, Kunz S, Xavier AH, et al. 2017. Regulated membrane remodeling by Mic60 controls formation of mitochondrial crista junctions. *Nature Communications* 8: 15258

Igamberdiev AU, Gardeström P. 2003. Regulation of NAD- and NADP-dependent isocitrate dehydrogenases by reduction levels of pyridine nucleotides in mitochondria and cytosol of pea leaves. *Biochimica* 1606: 117-25

Iwata M, Lee Y, Yamashita T, Yagi T, Iwata S, et al. 2012. The structure of the yeast NADH dehydrogenase (Ndi1) reveals overlapping binding sites for water- and lipid-soluble substrates. *Proceedings of the National Academy of Sciences of the United States of America* 109: 15247-52

Johnson CH, Knight MR, Kondo T, Masson P, Sedbrook J, et al. 1995. Circadian oscillations of cytosolic and chloroplastic free calcium in plants. *Science* 269: 1863-65

Katoh A, Uenohara K, Akita M, Hashimoto T. 2006. Early steps in the biosynthesis of NAD in *Arabidopsis* start with aspartate and occur in the plastid. *Plant Physiology* 141: 851-57

Klodmann J, Senkler M, Rode C, Braun HP. 2011. Defining the protein complex proteome of plant mitochondria. *Plant Physiology* 157: 587-98

Kobayashi M, Ohura I, Kawakita K, Yokota N, Fujiwara M, et al. 2007. Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. *Plant Cell* 19: 1065-80

Kruger NJ, von Schaewen A. 2003. The oxidative pentose phosphate pathway: structure and organisation. *Current Opinion in Plant Biology* 6: 236-46

Lambers H, Scheurwater I, Mata C, Nagel OW. 1998. Root respiration of fast- and slow-growing plants, as dependent on genotype and nitrogen supply: A major clue to the functioning of slow-growing plants. *Inherent Variation in Plant Growth*: 139-57

Lea PJ, Miflin BJ. 2003. Glutamate synthase and the synthesis of glutamate in plants. *Plant Physiology and Biochemistry* 41: 555-64

Lindsay DP, Camara AKS, Stowe DF, Lubbe R, Aldakkak M. 2015. Differential effects of buffer pH on Ca^{2+}-induced ROS emission with inhibited mitochondrial complexes I and III. *Frontiers in Physiology* 6: 10

Liu YJ, Norberg FE, Szilagyi A, De Paepe R, Åkerlund HE, Rasmusson AG. 2008. The mitochondrial external NADPH dehydrogenase modulates the leaf NADPH/$NADP^+$ ratio in transgenic *Nicotiana sylvestris*. *Plant Cell Physiology* 49: 251-63

Liu YJ, Nunes-Nesi A, Wallström SV, Lager I, Michalecka AM, et al. 2009. A redox-mediated modulation of stem bolting in transgenic *Nicotiana sylvestris* differentially expressing the external mitochondrial NADPH dehydrogenase. *Plant Physiology* 150: 1248-59

Logan DC, Knight MRJPP. 2003. Mitochondrial and cytosolic calcium dynamics are differentially regulated in plants. *Plant Physiology* 133: 21-24

Logan DC, Leaver CJ. 2000. Mitochondria-targeted GFP highlights the heterogeneity of mitochondrial shape, size and movement within living plant cells. *Experimental Botany* 51: 865-71

Marreiros BC, Sena FV, Sousa FM, Batista AP, Pereira MM. 2016. Type II NADH: quinone oxidoreductase family: phylogenetic distribution, structural diversity and evolutionary divergences. *Environmental Microbiology* 18: 4697-709

Martiniere A, Bassil E, Jublanc E, Alcon C, Reguera M, et al. 2013. *In vivo* intracellular pH measurements in tobacco and Arabidopsis reveal an unexpected pH gradient in the endomembrane system. *Plant Cell* 25: 4028-43

Matuz-Mares D, Matus-Ortega G, Cardenas-Monroy C, Romero-Aguilar L, Villalobos-Rocha JC, et al. 2018. Expression of alternative NADH dehydrogenases (NDH-2) in the phytopathogenic fungus *Ustilago maydis*. *FEBS Open Bio* 8: 1267-79

Maxwell DP, Wang Y, McIntosh L. 1999. The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proceedings of the National Academy of Sciences USA* 96: 8271-76

Mazars C, Bourque S, Mithöfer A, Pugin A, Ranjeva R. 2009. Calcium homeostasis in plant cell nuclei. *New Phytologist* 181: 261-74

Melo AM, Bandeiras TM, Teixeira M. 2004. New insights into type II NAD(P)H:quinone oxidoreductases. *Microbiol Mol Biol Rev* 68: 603-16

Michalecka AM, Agius SC, Møller IM, Rasmusson AG. 2004. Identification of a mitochondrial external NADPH dehydrogenase by overexpression in transgenic *Nicotiana sylvestris*. *Plant Journal* 37: 415-25

Michalecka AM, Svensson ÅS, Johansson FI, Agius SC, Johanson U, et al. 2003. Arabidopsis genes encoding mitochondrial type II NAD(P)H dehydrogenases have different evolutionary origin and show distinct responses to light. *Plant Physiology* 133: 642-52

Mitchell P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature* 191: 144-48

Møller IM. 2001. Plant mitochondria and oxidative stress: Electron transport, NADPH turnover, and metabolism of reactive oxygen species. *Annual Review of Plant Physiology and Plant Molecular Biology* 52: 561-91

Møller IM. 2015. Mitochondrial metabolism is regulated by thioredoxin. *Proceedings of the National Academy of Sciences of the United States of America* 112: 3180-1

Monne M, Daddabbo L, Gagneul D, Obata T, Hielscher B, et al. 2018. Uncoupling proteins 1 and 2 (UCP1 and UCP2) from *Arabidopsis thaliana* are mitochondrial transporters of aspartate, glutamate, and dicarboxylates. *Journal of Biological Chemistry* 293: 4213-27

Moore AL, Åkerman KEO. 1982. Ca^{2+} Stimulation of the External NADH Dehydrogenase in Jerusalem Artichoke (*Helianthus tuberosum*) Mitochondria. *Biochemical and Biophysical Research Communications* 109: 513-17

Moore CS, Cook-Johnson RJ, Rudhe C, Whelan J, Day DA, et al. 2003. Identification of AtNDI1, an internal non-phosphorylating NAD(P)H dehydrogenase in *Arabidopsis* mitochondria. *Plant Physiology* 133: 1968-78

Morley SA, Nielsen BL. 2017. Plant mitochondrial DNA. *Front Biosci (Landmark Ed)* 22: 1023-32

Moseyko N, Feldman LJ. 2001. Expression of pH-sensitive green fluorescent protein in *Arabidopsis thaliana*. *Plant Cell and Environment* 24: 557-63

Moyen C, Roblin G. 2013. Occurrence of interactions between individual Sr^{2+}- and Ca^{2+}-effects on maize root and shoot growth and Sr^{2+}, Ca^{2+} and Mg^{2+} contents, and membrane potential: Consequences on predicting Sr^{2+}-impact. *Journal of Hazardous Materials* 260: 770-79

Neimanis K, Staples JF, Huner NP, McDonald AE. 2013. Identification, expression, and taxonomic distribution of alternative oxidases in non-angiosperm plants. *Gene* 526: 275-86

Neuburger M, Douce R. 1983. Slow passive diffusion of NAD^+ between intact isolated plant mitochondria and suspending medium. *Biochemical Journal* 216: 443-50

Nicholls DG. 2013. *Bioenergetics*. Elsevier Science.

Nobel P. 1969. Light-induced changes in the ionic content of chloroplasts in *Pisum sativum*. *Biochimica et Biophysica Acta* 172: 134-43

Noctor G, Hager J, Li S. 2011. Biosynthesis of NAD and its manipulation in plants. *Advances in Botanical Research* 58: 154-201

Noctor G, Mhamdi A. 2017. Climate Change, CO_2, and Defense: The Metabolic, Redox, and Signaling Perspectives. *Trends in Plant Science* 22: 857-70

Nunes-Nesi A, Araujo WL, Obata T, Fernie AR. 2013. Regulation of the mitochondrial tricarboxylic acid cycle. *Current Opinion in Plant Biology* 16: 335-43

Okada K, Suzuki K, Kamiya Y, Zhu X, Fujisaki S, et al. 1996. Polyprenyl diphosphate synthase essentially defines the length of the side chain of ubiquinone. *Biochimica et Biophysica Acta - Lipids Lipid Metabolism* 1302: 217-23

Otegui MS, Noh YS, Martinez DE, Vila Petroff MG, Andrew Staehelin L, et al. 2005. Senescence-associated vacuoles with intense proteolytic activity develop in leaves of *Arabidopsis* and soybean. *Plant* 41: 831-44

Podgorska A, Ostaszewska-Bugajska M, Borysiuk K, Tarnowska A, Jakubiak M, et al. 2018. Suppression of external NADPH dehydrogenase NDB1 in *Arabidopsis thaliana* confers improved tolerance to ammonium toxicity via efficient glutathione/redox metabolism. *International Journal of Molecular Sciences* 19

Porcelli AM, Ghelli A, Zanna C, Pinton P, Rizzuto R, Rugolo M. 2005. pH difference across the outer mitochondrial membrane measured with a green fluorescent protein mutant. *Biochemical and Biophysical Research Communications* 326: 799-804

Price J, Laxmi A, St Martin SK, Jang JC. 2004. Global transcription profiling reveals multiple sugar signal transduction mechanisms in *Arabidopsis*. *Plant Cell* 16: 2128-50

Pugin A, Frachisse JM, Tavernier E, Bligny R, Gout E, et al. 1997. Early events induced by the elicitor cryptogein in tobacco cells: Involvement of a plasma membrane NADPH oxidase and activation of glycolysis and the pentose phosphate pathway. *Plant Cell* 9: 2077-91

Queval G, Jaillard D, Zechmann B, Noctor G. 2011. Increased intracellular H_2O_2 availability preferentially drives glutathione accumulation in vacuoles and chloroplasts. *Plant Cell and Environment* 34: 21-32

Raghavendra AS, Padmasree K, Saradadevi K. 1994. Interdependence of photosynthesis and respiration in plant cells - interactions between chloroplasts and mitochondria. *Plant Science* 97: 1-14

Rasmusson A, Møller I. 2010. Mitochondrial electron transport and plant stress In *Plant Mitochondria*, ed. F Kempken, pp. 357-81. New York: Springer

Rasmusson AG, Agius SC. 2001. Rotenone-insensitive NAD(P)H dehydrogenases in plants: Immunodetection and distribution of native proteins in mitochondria. *Plant Physiology and Biochemistry* 39: 1057-66

Rasmusson AG, Escobar MA. 2007. Light and diurnal regulation of plant respiratory gene expression. *Physiologia Plantarum* 129: 57-67

Rasmusson AG, Fernie AR, van Dongen JT. 2009. Alternative oxidase: A defence against metabolic fluctuations? *Physiologia Plantarum* 137: 371-82

Rasmusson AG, Geisler DA, Møller IM. 2008. The multiplicity of dehydrogenases in the electron transport chain of plant mitochondria. *Mitochondrion* 8: 47-60

Rasmusson AG, Møller IM. 1991. Effect of calcium ions and inhibitors on internal NAD(P)H dehydrogenases in plant mitochondria. *European Journal of Biochemistry* 202: 617-23

Rasmusson AG, Soole KL, Elthon TE. 2004. Alternative NAD(P)H dehydrogenases of plant mitochondria. *Annual Review of Plant Biology* 55: 23-39

Rasmusson AG, Svensson AS, Knoop V, Grohmann L, Brennicke A. 1999. Homologues of yeast and bacterial rotenone-insensitive NADH dehydrogenases in higher eukaryotes: two enzymes are present in potato mitochondria. *Plant Journal* 20: 79-87

Reumann S, Heupel R, Heldt HW. 1994. Compartmentation studies on spinach leaf peroxisomes: evidence for the transfer of reductant from the cytosol to the peroxisomal compartment via a malate shuttle. *Planta* 193: 167-73

Rieger B, Junge W, Busch KB. 2014. Lateral pH gradient between OXPHOS complex IV and F(0)F(1) ATP-synthase in folded mitochondrial membranes. *Nature Communications* 5: 3103

Rugolo M, Antognoni F, Flamigni A, Zannoni D. 1991. Effects of polyamines on the oxidation of exogenous NADH by Jerusalem Artichoke (*Helianthus tuberosus*) Mitochondria. *Plant Physiology* 95: 157-63

Sakano K. 2001. Metabolic regulation of pH in plant cells: Role of cytoplasmic pH in defense reaction and secondary metabolism. *International Review Cytology* 206: 1-44

Sanz A, Soikkeli M, Portero-Otin M, Wilson A, Kemppainen E, et al. 2010. Expression of the yeast NADH dehydrogenase Ndi1 in Drosophila confers increased lifespan independently of dietary restriction. *Proceedings of the National Academy of Sciences of the United States of America* 107: 9105-10

Schäfer FQ, Buettner GR. 2001. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radical Biology and Medicine* 30: 1191-212

Schertl P, Braun HP. 2014. Respiratory electron transfer pathways in plant mitochondria. *Frontiers in Plant Science* 5

Schneider G, Sjöling S, Wallin E, Wrede P, Glaser E, von Heijne G. 1998. Feature-extraction from endopeptidase cleavage sites in mitochondrial targeting peptides. *Proteins* 30: 49-60

Scott I, Logan DC. 2011. Mitochondrial Dynamics In *Plant Mitochondria*, ed. F Kempken, pp. 31-63. New York, NY: Springer New York

Seelert H, Dencher NA. 2011. ATP synthase superassemblies in animals and plants: two or more are better. *Biochim Biophys Acta* 1807: 1185-97

Sena FV, Batista AP, Catarino T, Brito JA, Archer M, et al. 2015. Type-II NADH:quinone oxidoreductase from *Staphylococcus aureus* has two distinct binding sites and is rate limited by quinone reduction. *Molecular Microbiology*

Shen J. 2017. The organelle pH estimate and measurement in plant secretory pathway. *Methods in Molecular Biology* 1662: 223-30

Slot M, Kitajima K. 2015. General patterns of acclimation of leaf respiration to elevated temperatures across biomes and plant types. *Oecologia* 177: 885-900

Svensson ÅS, Rasmusson AG. 2001. Light-dependent gene expression for proteins in the respiratory chain of potato leaves. *Plant Journal* 28: 73-82

Sweetlove LJ, Møller IM. 2010. Oxidation of proteins in plants – mechanisms and consequences. *Advances in Botanical Research* 52: 1-23

Szabados L, Savoure A. 2010. Proline: a multifunctional amino acid. *Trends in Plant Science* 15: 89-97

Szal B, Dabrowska Z, Malmberg G, Gardeström P, Rychter AM. 2008. Changes in energy status of leaf cells as a consequence of mitochondrial genome rearrangement. *Planta* 227: 697-706

Taniguchi M, Miyake H. 2012. Redox-shuttling between chloroplast and cytosol: integration of intra-chloroplast and extra-chloroplast metabolism. *Current Opinion in Plant Biology* 15: 252-60

Taniguchi M, Taniguchi Y, Kawasaki M, Takeda S, Kato T, et al. 2002. Identifying and characterizing plastidic 2-oxoglutarate/malate and dicarboxylate transporters in *Arabidopsis thaliana*. *Plant and Cell Physiology* 43: 706-17

Thomas MV. 1982. *Techniquesin calcium research*. Academic press.

Turner WL, Waller JC, Snedden WA. 2005. Identification, molecular cloning and functional characterization of a novel NADH kinase from *Arabidopsis thaliana* (thale cress). *Biochemical Journal* 385: 217-23

Unseld M, Marienfeld JR, Brandt P, Brennicke A. 1997. The mitochondrial genome of *Arabidopsis thaliana* contains 57 genes in 366,924 nucleotides. *Nature Genetics* 15: 57-61

Van Aken O, Zhang BT, Law S, Narsai R, Whelan J. 2013. AtWRKY40 and AtWRKY63 modulate the expression of stress-responsive nuclear genes encoding mitochondrial and chloroplast proteins. *Plant Physiology* 162: 254-71

Vanlerberghe GC. 2013. Alternative oxidase: A mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. *International Journal of Molecular Sciences* 14: 6805-47

Vercesi AE, Borecky J, de Godoy Maia I, Arruda P, Cuccovia IM, Chaimovich H. 2006. Plant uncoupling mitochondrial proteins. *Annual Review of Plant Biology* 57: 383-404

Wagner S, De Bortoli S, Schwarzlander M, Szabo I. 2016. Regulation of mitochondrial calcium in plants versus animals. *Journal of Experimental Botany* 67: 3809-29

Walker JE. 2013. The ATP synthase: the understood, the uncertain and the unknown. *Biochemical Society Transactions* 41: 1-16

Wallström SV, Florez-Sarasa I, Araujo WL, Aidemark M, Fernandez Fernandez M, et al. 2014a. Suppression of the external mitochondrial NADPH dehydrogenase, NDB1, in *Arabidopsis thaliana* affects central metabolism and vegetative growth. *Molecular Plant* 7(2): 356-68

Wallström SV, Florez-Sarasa I, Araujo WL, Escobar MA, Geisler DA, et al. 2014b. Suppression of NDA-type alternative mitochondrial NAD(P)H dehydrogenases in *Arabidopsis thaliana* modifies growth and metabolism, but not high light stimulation of mitochondrial electron transport. *Plant and Cell Physiology* 55: 881-96

Wang GD, Pichersky E. 2007. Nicotinamidase participates in the salvage pathway of NAD biosynthesis in *Arabidopsis*. *Plant* 49: 1020-29

White PJ, Broadley MR. 2003. Calcium in plants. *Annals of Botany* 92: 487-511

Winter H, Robinson DG, Heldt HW. 1994. Subcellular volumes and metabolite concentrations in spinach leaves. *Planta* 193: 530-35

Xu L, Law SR, Murcha MW, Whelan J, Carrie C. 2013. The dual targeting ability of type II NAD(P)H dehydrogenases arose early in land plant evolution. *BMC Plant Biology* 13: 100

Yamashita T, Nakamaru-Ogiso E, Miyoshi H, Matsuno-Yagi A, Yagi T. 2007. Roles of bound quinone in the single subunit NADH-quinone oxidoreductase (Ndi1) from *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* 282: 6012-20

Yang Y, Yu Y, Li X, Li J, Wu Y, et al. 2017. Target elucidation by cocrystal structures of NADH-ubiquinone oxidoreductase of *Plasmodium falciparum* (PfNDH2) with small molecule to eliminate drug-resistant malaria. *Journal of Medicinal Chemistry* 60: 1994-2005

Yano T, Rahimian M, Aneja KK, Schechter NM, Rubin H, Scott CP. 2014. *Mycobacterium tuberculosis* Type II NADH-Menaquinone Oxidoreductase Catalyzes Electron Transfer through a Two-Site Ping-Pong Mechanism and Has Two Quinone-Binding Sites. *Biochemistry* 53: 1179-90

Acknowledgements

I feel very lucky to have a very helpful supervisor. My deepest gratitude goes to my supervisor Allan Rasmusson. Thank you for teaching me with great patience and giving me the best possible supervision. You gave me great research guidance, encouraged and supported me during the whole length of my study time at the biology department at Lund University.

My sincere thanks go to my co-supervisor Lars Hederstedt for inspiring talks and guidance in my projects. I really like my PhD supervision committee that has four great professors from different fields in science, Cecilia Emanuelsson and Pål Axel Olsson together with my supervisors, who give me supports all these years while we are uncovering the mysteries of natural science.

Thanks to all supervisors and Susanne Widell, who give me comments on my thesis draft.

Thanks to Carin Jarl-Sunesson who gives me opportunities to teach in the department, the experiences of being teaching assistant are valuable for me.

Thanks to Abdelghafar Abu-Elsaoud, Oscar Rollano Peñaloza, Bradley Dotson, Sanjana Holla and Shichao Ren as colleagues in the same lab group. We shared knowledge of each other's research, happiness and sadness of daily life, which are valuable experience for me.

Thanks to LP3, Wolfgang Knecht, Katarina Koruza, Zoë Fisher and Therese Lindvall, who helped me to purify the NDB proteins.

Thanks for all my classes' and projects' students who keep reminding me that the younger students are still studying hard and pursuing the beauty of science.

Thanks to Ian Max Møller from Aarhus university, who is my idol scientist in our research field.

Thanks for everyone at department of biology, you are creating a great atmosphere for research. Thanks to professors Mats Hansson, Torbjörn Tyler, Oliver Van Aken, Klas Flärdh, Claes von Wachenfeldt, Marita Cohn, Nora Ausmees and Jure Piskur, for holding many seminars in molecular cell biology unit in biology department. Thanks to CMPS research school and GENECO research school, I learnt a lot during many activities you provided. Thanks professor Sara Linse for teaching me isothermal titration calorimetry. Thanks to Erik Alexandersson, who was my opponent at licentiate thesis defence. Thanks to Elisabeth Barane for the help in teaching. Thanks to Ida Lager for the collaboration on GPCAT project. Thanks to Plantlink, which was very nice organisation for 'plant people' to talk about, learn, support and discuss with each other on plant sciences. Thanks to my external mentor

Karolina Aloisi and Pawel Chrominski from the Nordic genetic resource centre (NordGen).

Thanks also goes to all you here are from biology department: Ahu Karademir Andersson, Ewa Bukowska-Faniband, Yusra Al-Eryani, Markus Fröjd, Vinardas Kelpšas, Beer Sen, Jakob Engman, Katsuya Fuchino, Yuan Li, Yihan Xia, Huan Zhang, Baojian Ding, HongLei Wang, Dandan Zhang, Xiaoqing Hou, Jian Li, Jing Zhang, Shakhira Zakhrabekova, Ewa Krupinska, David Stuart, Nick Sirijovski, Humberto Itriago, Anna Rasmussen. All of you are great.

Thanks to all my co-authors for the contributions.

Thanks to all friends that I did not mention here. Special thanks to Wubin Weng.

Thanks for the financial support from Lund University. As well as grants from SPPS, fysiografen and CFO Nordstedts fond.

Forever love you, my parents.

感谢郝延庆和张小萍，我永远最爱的父母。

After all these years of education, I think a pure scientist is a bio-artist, and an applied scientist is a bio-engineer. I enjoyed working as both.

2019-04-15, Lund, Sweden

Printed by Media-Tryck, Lund 2019 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

Lund University
Faculty of Science
Department of Biology

ISBN 978-91-7753-974-2