ALTERED EXPRESSION OF THE NON-PHOSPHORYLATION ELECTRON TRANSPORT CHAIN EFFECTS GROWTH AND STRESS TOLERANCE IN *ARABIDOPSIS THALIANA*

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ABBREVIATIONS

ADP	Adenosine Diphosphate
ANGIS	Australian National Genomic Information Service
AOX	Alternative Oxidase
At	*Arabidopsis thaliana*
ndb	external alternative NAD(P)H dehydrogenase
ATP	Adenosine Triphosphate
bp	Base Pairs
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
CAC	Citric Acid Cycle
CEI	Cell Intensity File
cDNA	Complementary DNA
DH	Dehydrogenase
DNA	Deoxyribose Nucleic Acid
DPI	Dipenyleneiodonium
dsRNA	double-stranded RNA
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol Bis(2-aminoethyl Ether)-N,N,N',N'-tetraacetic Acid
EPR	Eectron paramagnetic resonance
ETC	Electron Transport Chain
$FADH_2$	Flavin Adenine Dinucleotide [reduced]
FDR	false discovery rate
g / mg	grams / milligrams
µg / ng	micrograms / nanograms
GC-RMA	Guanine Cytosine Robust Multi-Array Analysis
gDNA	Genomic DNA
hpRNA	Hairpin RNA
IM	Inter Membrane Space
K^+	Potassium
Kb	Kilobase

KCN	Potassium Cyanide
kDa	kilodalton
L / ml / µl	litre / millilitres / microliters
LB	Luria Broth
M / mM	molar / millimolar
µM / nM	micromolar / nanomolar
MAS5	Affymetrix Microarray Suite version 5
MATDB	MIPS *Arabidopsis Thaliana* Database
mETC	Mitochondrial Electron Transport Chain
MIPS	Munich Information Centre for Protein Sequences
mRNA	Messenger RNA
MS	Murashige and Skoog
Na^+	Sodium
NaCl	Sodium Chloride
NAD+	Nicotinamide Adenine Dinucleotide
NAD(P)H	NADH and NADPH
NADH	Nicotinamide Adenine Dinucleotide [reduced]
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate [reduced]
NCBI	National Centre for Biotechnology Information
NDA	NAD(P)H dehydrogenase Internal
NDB	NAD(P)H dehydrogenase External
NDC	NAD(P)H dehydrogenase membrane spaning
NDE	NAD(P)H dehydrogenase External
NDI	NAD(P)H dehydrogenase Internal
NEB	New England Biolabs
NPT	Neomycin phosphotransferase
ORF	Open Reading Frame
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PTGS	Post Transcriptional Gene Silencing
QRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RE	Restriction Enzyme
REBASE	Restriction Enzyme Recognition Sites

RISC	RNA Induced Silencing Complex
RNA	Ribonucleic Acid
RNAi	RNA Interference
ROS	Reactive Oxygen Species
Rpm	Revolutions per Minute
RGR	Relative Growth Rate
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SAP	Shrimp Alkaline Phosphatase
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
siRNA	Small Interfering RNA
T_a	PCR cycle annealing temperature (°C)
T_m	PCR cycle melting temperature (°C)
UCP	Uncoupling Proteins
μE	Micro Einstein's
UQ	Ubiquinone
UTR	Untranslated Region
v / v	Volume / Volume
w / v	Weight / Volume
Wt	Wild Type

ABSTRACT

The branched respiratory pathway of plants contains a cyanide-insensitive alternative pathway. The alternative pathway is of particular interest due to the apparent wasteful activity of the non-phosphorylating NAD(P)H dehydrogenases. Seven putative alternative NAD(P)H dehydrogenase genes have been identified from the *Arabidopsis thaliana* genome however, there have only been five activities characterised experimentally. Thus it is not known which gene encodes which activity in *A. thaliana* mitochondria. The three main aims of this study were to generate a transgenic *A. thaliana* line with complete suppression of the *ndb4* gene to confirm that it encodes an external NAD(P)H dehydrogenase and its calcium dependence. It was also hoped to elucidate the role of these alternative NAD(P)H dehydrogenases by reducing or eliminating expression of all four external NAD(P)H dehydrogenases in one plant line. The third aim was to look at the response of the alternative pathway including Alternative Oxidase (AOX) to stress growth conditions, specifically salinity stress.

RNAi technology was used to generate transgenic *A. thaliana* plants. The *ndb4* lines showed a range of suppression which lead to the up-regulation of *Atnbd2* in some lines and *Aox1a* in all lines, which was confirmed at the protein level. It was determined that *ndb4* encodes a calcium independent NADH dehydrogenase, where *ndb2* is likely to have calcium dependent NADH activity. Silencing lines of an *Atndb* region indicated the plant was not viable without any of the external dehydrogenases present. Microarray data indicated an altered expression profile in *ndb4* knock-down lines which significantly impacted several anti-oxidant pathways, both in and outside the mitochondria indicating the altered *ndb4* resulted in a change in the global expression pattern of the cell.

The involvement of this alternative pathway in a stress response may be linked to its capacity to uncouple carbon metabolism from adenylate control and/or the minimisation of the formation of destructive reactive oxygen

species (ROS). Salinity stress is a widespread, adverse environmental stress, which leads to an ionic imbalance, hyperosmotic stress, and oxidative stress, the latter being the result of reactive oxygen species formation. We have shown that salinity stress of *A. thaliana* plants resulted in the formation of ROS, increased levels of Na^+ in both the shoot and the root and an increase in transcription of *Aox1a*, *ndb2* and *ndb4* genes, indicating the formation of a bridged non-phosphorylating electron transport chain in response to salinity stress. Furthermore, plants constitutively over-expressing *Aox1a*, with increased AOX capacity, showed lower ROS formation, 30-40% improved growth rates and lower shoot Na+ content compared to controls, when grown under salinity stress conditions, the *ndb4* knock-down plants responded to salinity stress with the same trend. This was even further supported when plants had both an external dehydrogenase and AOX up regulated. Thus, more active alternative pathway in roots and shoots can improve the salt tolerance of *A. thaliana* as defined by its ability to grow more effectively in the presence of NaCl, and maintain lower shoot Na^+ content. AOX does have an important role in stress adaptation in plants, and these results provide some validation of the hypothesis that AOX can play a critical role in cell re-programming under salinity stress.

DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Chevaun Smith

PUBLISHED WORK

Part of the work in this thesis has been published or is in preparation for publishing.

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1 INTRODUCTION

1.1 Mitochondria

Mitochondria are made up of two highly specialised membranes, which make up two compartments; the internal matrix and the inter membrane space, as seen in Figure 1.1. Within the inner membrane are the components of the Electron Transport Chain (ETC) that leads to the production of ATP. Mitochondria are the site of oxidative phosphorylation in eukaryotes.

1.2 The Electron Transport Chain

The primary features of the mitochondrial electron transport chain found in plants are similar to those of the electron transfer chain in mitochondria isolated from other eukaryotes, consisting of four integral multiprotein complexes (Siedow *et al.* 1995) (Figure 1.2). Complex I or NADH dehydrogenase, which is rotenone-sensitive (Finnegan et al., 2004), oxidizes NADH and transfers the electron to ubiquinones, a small lipid-soluble electron and proton carrier. For every electron pair oxidized from NADH by Complex I, four protons are pumped from matrix into the inter-membrane space. Succinate is oxidized to fumarate by Complex II or succinate dehydrogenase and then feed the electron to ubiquinone pool via FADH2 and a group of iron-sulfur protein. Complex II is inhibited by a substrate analog to succinate, malonate (Finnegan et al. 2004)(Finnegan et al. 2004)(Finnegan et al. 2004). Complex III or cytochrome bc_1 complex oxidized ubiquinol (the reduced form of ubiquinones) and distributes the electron to cytochrome *c* via an iron iron-sulfur centre, two *b*-type cytochrome (b_{565} and b_{560}) and a membrane-bound cytochrome c_1. Oxidation per pair electron by Complex III causes four protons pumped to the inter-membrane space. Antimycin A and myxothiazol are the inhibitors for Complex III (Finnegan *et*

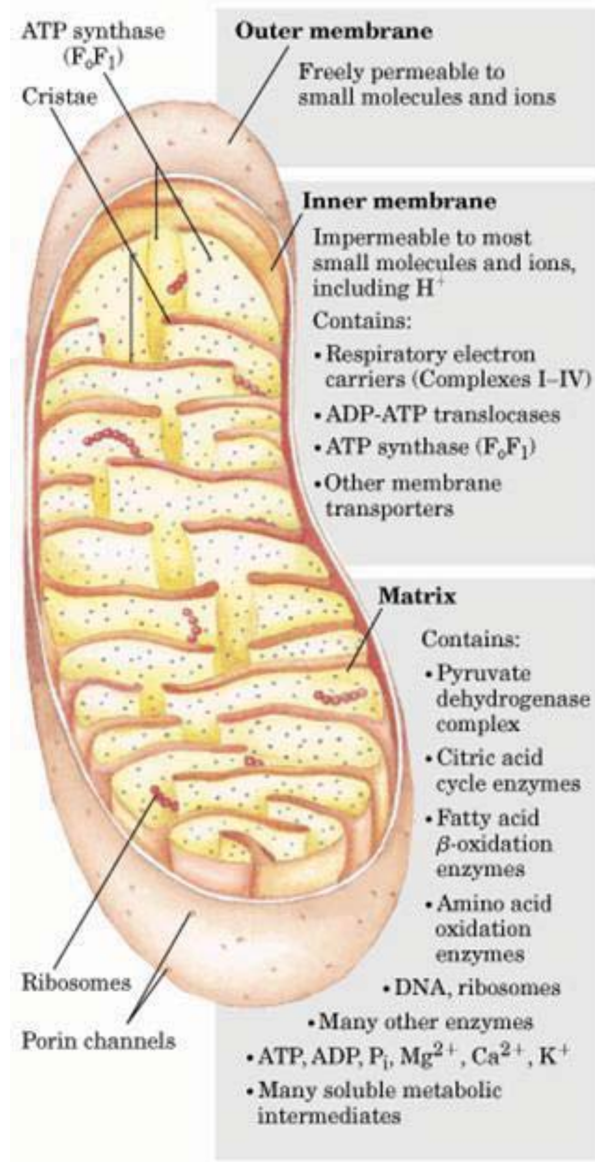

Figure 1.1 The Mitochondria (Nelson *et al.* 2000)

The following figure shows the three compartments the Matrix, the Inner membrane and the Outer membrane.

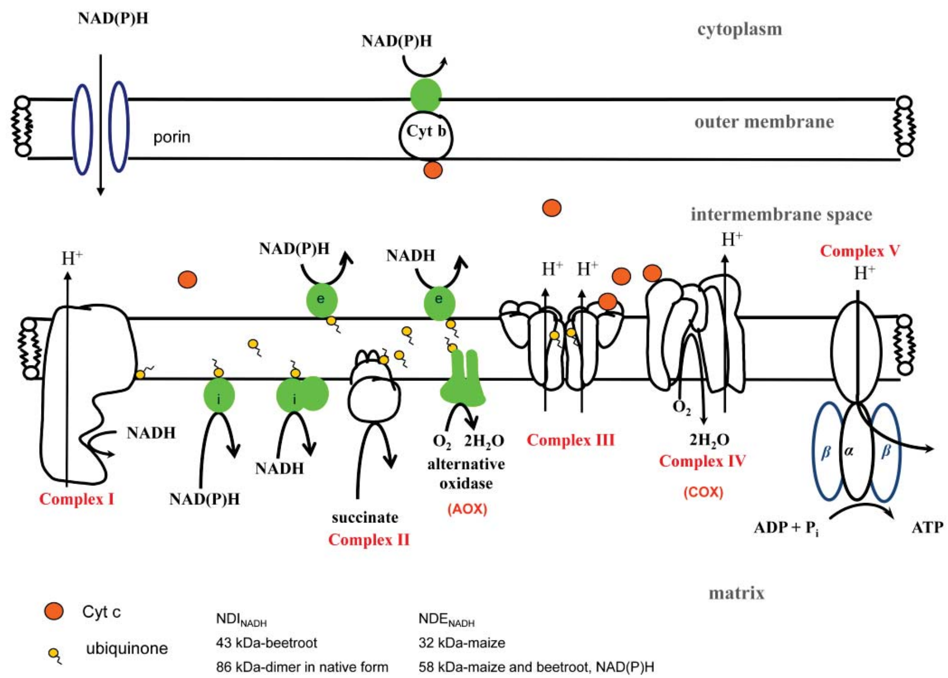

Figure 1.2 The electron transport chain containing the alternative enzymes

Organization of the electron transport chain and ATP synthesis in the inner membrane of plant mitochondria. In addition to the five standard protein complexes found in nearly all other mitochondria, the electron transport chain of plant mitochondria contains five additional enzymes marked in green. None of these additional enzymes pump protons.

al. 2004). Complex IV or cytochrome *c* oxidase transfers electron from cytochrome *c* to O_2 (oxygen) as the final electron acceptor, producing two molecules of H_20 for every four electron. When a pair of electron is transferred to O_2 by Complex IV, two protons are pumped across the inner membrane. The activity of Complex IV is inhibited by cyanide, carbon monoxide and azide (Finnegan *et al.* 2004). The series of proton pumping from matrix to the inter-membrane space creates proton-motive force that drives ATP production through ADP phosphorylation catalysed by F1F0 ATP synthase. The involvement of cytochrome *c* in this electron transport makes this pathway called cytochrome pathway.

This flow of electrons through the protein pumping complexes results in the generation of an electrochemical proton gradient across the inner mitochondrial membrane. When the gradient is established it is used to drive the synthesis by the mitochondrial ATP synthase or Complex V, through which protons flow passively back into the matrix via a proton pore associated with Complex IV. This is why oxygen consumption is said to be coupled to ATP synthesis.

1.3 Plant Mitochondria

Mitochondria play an essential role in diverse metabolic pathways in plants, including the production of ATP and the biosynthesis of carbon skeletons and metabolic intermediates, such as heme and amino acids. They also play roles in pathogen and stress responses and are intimately involved in the process of programmed cell death (Mackenzie *et al.* 1999; Lam *et al.* 2001).

Respiration is an important determinant of plant growth and development. As well as the cytochrome pathway (Phosphorylating pathway) mentioned above, plant mitochondria are unique in that they contain three unique non-phosphorylating respiratory pathways (Finnegan *et al.* 2004). These additional energy-dissipating components include the type II NAD(P)H dehydrogenases, the alternative oxidase (AOX) and uncoupling proteins

(UCP) (Picault *et al.* 2004; Rasmusson *et al.* 2004). The presence of AOX is characterised by the ability of plant mitochondria to respire in the presence of cyanide, which is a known potent inhibitor of cytochrome oxidase (Finnegan *et al.* 2004).

AOX is not limited to just plants, but also found in many algae, fungi and certain protozoa (Siedow *et al.* 1995), as well as recently being found in all kingdoms of life except the Archaebacteria (McDonald 2008). AOX has recently been found in 20 animal species, and as AOX has been identified in several sponges, members of the most primitive animal phylum, it suggests AOX was subsequently lost by vertebrates and arthropods (McDonald 2008). This enzyme branches from the respiratory chain at ubiquinone and catalyses the oxidation of ubiquinol, reducing oxygen to water with no proton translocation (Finnegan *et al.* 2004) (Figure 1.3). The type II NAD(P)H dehydrogenases provide an alternative entry point into the cytochrome pathway before ubiquinone. Both of these pathways are mediated by NAD(P)H: UQ oxidoreductases which are located on the inner mitochondrial membrane. There is a pathway associated with the oxidation of cytosolic NADH and NADPH, which is facilitated by at least two external NAD(P)H:UQ oxidoreductases, whose active sites face the cytoplasm (external). Matrix NADH and NADPH is oxidised via two internal NAD(P)H:UQ oxidoreductases which have their active sites facing the matrix (internal) (Figure 1.3).

The NAD(P)H:UQ oxidoreductases have been grouped into two classes, type I (NDH1) and type II (NDH2) NAD(P)H dehydrogenases (Yagi 1991). The two classes are characterized by differences in energy transduction, cofactors and polypeptide composition (Finnegan *et al.* 2004). The type I NAD(P)H dehydrogenases are Complex-like enzymes, and the type II NAD(P)H dehydrogenases unlike type I do not translocate protons, have non-covalent bound FMN, don’t have iron sulphur clusters and are usually comprised of one subunit, and are rotenone insensitive. These are referred to as the alternative NAD(P)H dehydrogenases, in this thesis. This does not mean that they only operate when Type I dehydrogenases are not functioning, but can oxidise NAD(P)H in the presence of a functional Complex I. Of the seven

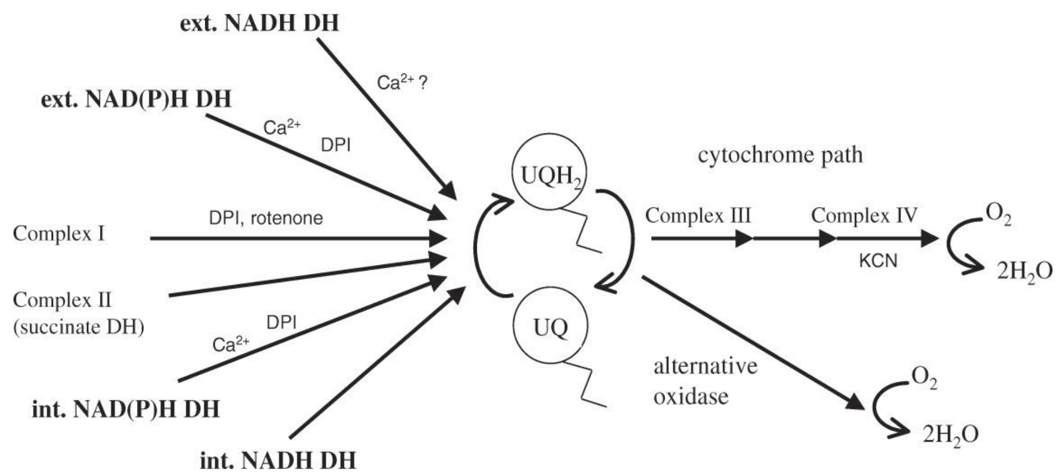

Figure 1.3 Schematic view of the plant mitochondrial electron transport chain (Rasmusson *et al.* 2004)

Multiple dehydrogenases reduce a common pool of ubiquinone, which is then oxidized by either the traditional cytochrome pathway or the alternative oxidase. Inhibitors are rotenone, DPI and KCN. Activators are Ca^{2+}.

genes encoding putative type II NAD(P)H dehydrogenases in *A. thaliana* three have been determined to be external dehydrogenases, (NDE)(*ndb1*, *2*, and *4*) the NDB family and three determined to be internal NAD(P)H dehydrogenases, (NDI) (*nda1* and 2 and *ndc1*) the NDA family(Michalecka *et al.* 2003; Elhafez *et al.* 2006), while the remaining cDNA, *ndb3* has not been able to be cloned and hence could be a pseudogene or have very restricted expression (Escobar *et al.* 2004). Using proteolytic digestion after in vitro import of each gene product, it has been confirmed that of the NAD(P)H dehydrogenases, the NDB family, are located on the external side of the inner membrane and that two NAD(P)H dehydrogenases (the NDA family) are located on the inner side of the inner membrane (Elhafez *et al.* 2005). The regulation of distribution of flux through each DH is not yet well understood, however the optimal conditions for each NAD(P)H DH activity is discussed in the relevant Sections below .

1.4 Pre genome analysis of the alternative pathway enzymes

1.4.1. Identification of alternative NAD(P)H dehydrogenases

External NAD(P)H oxidation was first demonstrated in lupin cotyledon mitochondria (Humphreys *et al.* 1956). This activity has now been detected in many plant species. The enzymes found in various tissue types have reported a wide range of Km values for external NAD(P)H activity depending on the assay conditions (Moller *et al.* 1993). External NAD(P)H oxidation has a total requirement for calcium (Coleman *et al.* 1971), hence the activity of external dehydrogenases can be inhibited by calcium chelators such as EGTA, EDTA and citrate. As NADPH oxidation is inhibited to a greater extent than NADH oxidation at the same inhibitor concentration for the sulfhydryl reagents, p-chloromercuribenzoate, n-ethylmaleimide and mersalyl inhibitors (Arron *et al.* 1979; Arron *et al.* 1980; Nash *et al.* 1983b) it has led to the proposal that NADH and NADPH were oxidised by separate enzymes. This was further supported by differential stimulation of NADH oxidation over NADPH oxidation during cold storage (Fredlund *et al.* 1991b; Zottini *et al.*

1993).

Fungal mitochondria contain combinations of internal and external rotenone-insensitive NADH and NADPH dehydrogenases, and these have been useful in helping to identify related bacteria enzymes (Michalecka *et al.* 2003). Additionally (Melo *et al.* 2001), identified an external NAD(P)H dehydrogenase in *Neurospora crassa*, as well an internal NADH dehydrogenase. These discoveries in yeast, fungi and bacteria have provided means of identifying related plant enzymes (Moore *et al.* 2003). The comparison of the NAD(P)H dehydrogenases between *N. crassa, Saccharomyces cerevisiae,* mammals and plants is compared in Figure 1.4.

1.4.2. Protein Purification

Attempts to purify the NDE enzymes in various species have resulted in the isolation of both NADH-specific (NDE_{NADH}) and NAD(P)H non-specific enzymes ($NDE_{NAD(P)H}$) suggesting the presence of multiple NDEs on the outer surface of the inner membrane (Finnegan *et al.* 2004). Several early purification efforts with different species indicated that 2 or 3 proteins with NAD(P)H dehydrogenase activities could be isolated from soluble fractions of plant mitochondria (Knudten *et al.* 1994). After several years, four different proteins emerged as potential alternative NAD(P)H dehydrogenases (Rasmusson *et al.* 2004). A 43 kDa protein which has NADH and NADPH activity has been purified from red beet root (Luethy *et al.* 1991a; Menz *et al.* 1996b), and thought to be the internal NAD(P)H DH.

A 58 kDa potential NAD(P)H dehydrogenase has been isolated from red beet root and maize, and was located to the external side of the inner membrane (Luethy *et al.* 1995). An antibody raised to this 58 kDa protein cross-reacted with several plant species and not with the rat liver mitochondria, further confirming it to be a potential candidate for the external NAD(P)H dehydrogenase. In 1996, Menz and Day supported that a 58 kDa protein, which was isolated from a soluble fraction from beetroot mitochondria had

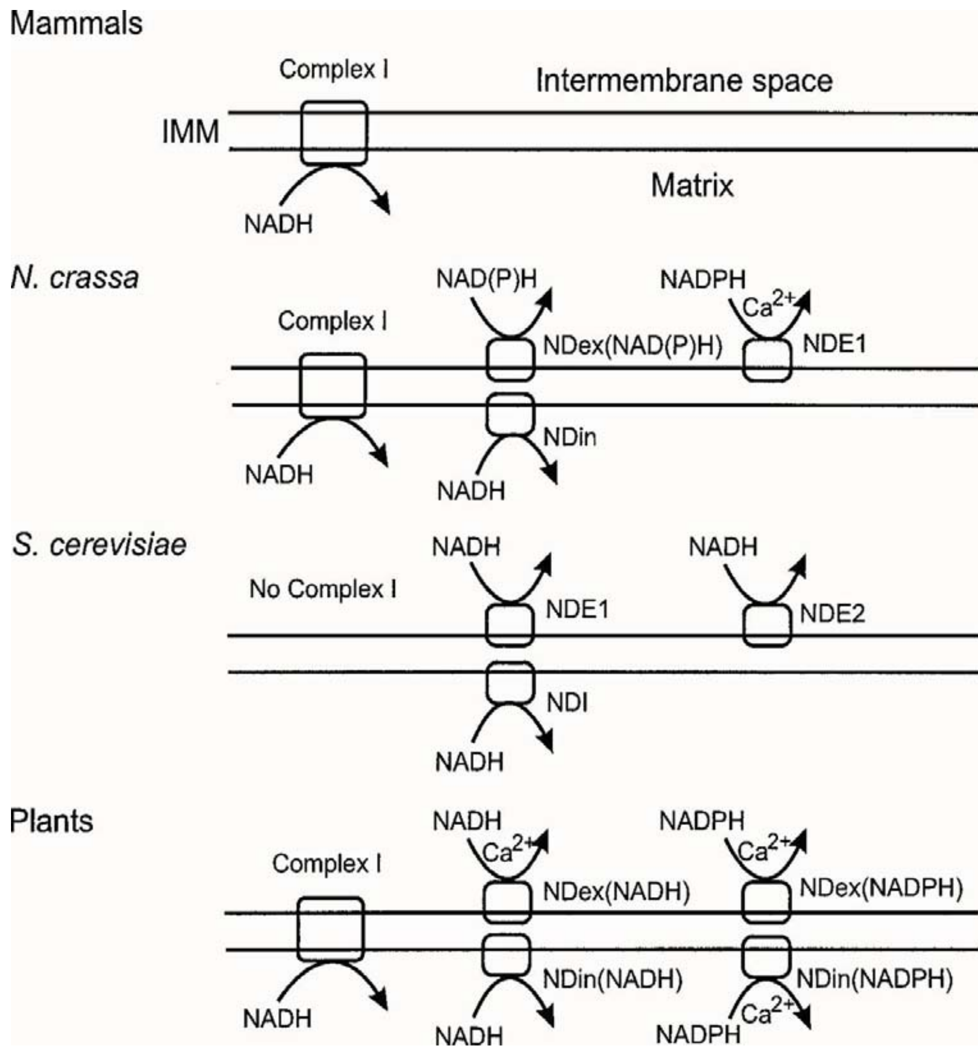

Figure 1.4 NAD(P)H dehydrogenases in the respiratory chain of mitochondria from mammals, *N. crassa*, *S. cerevisiae*, and plants (Moller 2001)

The NAD(P)H dehydrogenases of the alternative respiratory chain of mitochondria in mammals, *N. crassa, S. cerevisiae*, and plants.

NADH DH activity. In 1996 Menz and Day also purified a 43 kDa protein with NAD(P)H dehydrogenase activity in this soluble fraction of beetroot mitochondria. An antibody cross-linking experiment was done using an antibody raised to this protein, and located the 43kDa protein to the internal side of the inner membrane. This antibody also reacted with a protein in potato tuber and soybean mitochondria (Menz *et al.* 1996b). Using size exclusion chromatography, the 43 kDa protein was determined to be an 86 kDa dimer, in its native form. This study elucidated that there were two separate enzymes from NADH and NADPH oxidation as the protein was only capable of inhibition rotenone-insensitive NADH-specific oxidation.

1.4.2.1 Activators

Soole *et al.* (1990) have indicated that internal NADH dehydrogenase in plants are specific for NADH and are not reliant on calcium. On the other hand, in contrast to this, Moller (2001) provided evidence that the internal NAD(P)H dehydrogenase has a moderately high affinity for NADPH and is calcium specific. For the external NAD(P)H dehydrogenases, these are stimulated by calcium, indicating external oxidation is dependent on calcium, and hence have a specific requirement for calcium (Zottini *et al.* 1993; Finnegan *et al.* 2004). The stimulation of NAD(P)H oxidation by calcium has been shown to affect the enzymes activity (Soole *et al.* 1990).

1.4.2.2 Inhibitors

Currently for both internal and external NAD(P)H dehydrogenases there is no biological/ cellular function allocated to genes. The use of inhibitors is helpful in allocating function to protein, but unfortunately there is a lack of specific inhibitors for this enzyme family. External NAD(P)H dehydrogenases are inhibited by calcium chelators such as EGTA, EDTA and citrate. The effect with EGTA is more noticeable when mitochondria are incubated with EGTA before the addition of NAD(P)H, indicating that once activated, the calcium is tightly bound and therefore unavailable to EGTA or other chelators (Moller *et*

al. 1981). Inhibition of the external NAD(P)H dehydrogenases was also shown with sulhydryl reagents, although NADPH oxidation is inhibited to a greater extent that NADH oxidation with the same inhibitor concentration (Nash *et al.* 1983a). Dipenyleneiodonium (DPI) is a potent inhibitor of internal NAD(P)H dehydrogenases, and the use of the differential response to this inhibitor suggested that internal NAD(P)H dehydrogenase activity was facilitated by two separate enzymes. DPI also inhibits external NAD(P)H-dependent oxygen uptake, with inhibition at much lower concentrations for NADPH, compared to NADH (Melo *et al.* 1996). Flavone platanetin (Roberts *et al.* 1995), and dicumarol (Rasmusson *et al.* 1991) also have shown potential as NAD(P)H dehydrogenase inhibitors, but none of these specify either NADH or NADPH when inhibiting.

1.5 Alternative Oxidase

Alternative oxidase (AOX) is the sole component of the "alternative" electron transport pathway that accepts electrons from the UQ pool and it is tightly associated with the inner mitochondrial membrane (Dizengremel 1983; Siedow *et al.* 1992). AOX directly catalyses the two-electron oxidation of UQH_2 and the four electron reduction of oxygen to water (Moore *et al.* 1991). AOX is a non-proton pumping enzyme and hence the energy instead of adding to the proton motive force, is released as heat. This is why AOX is often associated with thermogenic plants. AOX in *A. thaliana* is encoded by a five member gene family with each gene displaying different spatial expression patterns (Thirkettle-Watts *et al.* 2003).

1.5.1. Identification of AOX

Alternative respiratory pathways were first noticed by scientists in specialised flowers due to their thermogenic metabolism, which was first reported in 1778 (Meeuse *et al.* 1969). It was Herk in 1938, who discovered this burst in respiratory pathway was due to a pathway other than the cytochrome

pathway (Finnegan *et al.* 2004). It was latter observed that mitochondrial respiration on D-day flowers was “uncoupled” and not associated with forming a proton gradient (Wilson *et al.* 1971). This observation was due to the presence of the alternative oxidase enzyme (AOX). Elthon *et al.* (1989) confirmed the observation of AOX protein in D-Day flowers, the cytochrome pathway decreased, and AOX increased. Since it was discovered, AOX has been identified in many plants, fungi, yeasts and several protozoans (Chaudhuri *et al.* 1998; Joseph-Horne *et al.* 2000). Recently AOX has been found in all kingdoms of life except the Archaebacteria (McDonald 2009).

1.5.2. Biochemistry

AOX is a quinol-oxygen oxidoereductase and does not pump protons, the oxygenase transfers electrons from ubiquinone to oxygen and generates water, and hence four electrons must be transferred to oxygen. AOX has the ability to reduce oxygen to water, which indicates the presence of a transition metal at the centre of its active site. Minagawa *et al.* (1990) have indicated that AOX requires iron. To confirm that AOX requires iron, Fe^{2+} was removed from the growth medium of a yeast culture, which resulted in the production of an inactive AOX protein. As well as biochemical approaches to confirm that iron was involved in AOX catalysis, gene sequence information was examined. Using sequence homology (Siedow *et al.* 1995) proposed a model that the AOX active site was predicted to be a four-helix bundle located in the C-terminal portion of the oxidase, as does other enzymes that bind iron. Recently using plant AOX expressed in *E. coli*, an EPR signal associated with AOX has confirmed the di-iron centre model (Berthold *et al.* 2002). The functional form of the enzyme is a dimer, with two polypeptides either covalently or non-covalently bound to each other. Since the this model was proposed (Siedow *et al.* 1995), another model has been proposed (Andersson *et al.* 1999), that like the (Siedow *et al.* 1995) model, postulates that the iron binding involves two antiparallel helix pairs combining to form a four helix bundle. To achieve the structure allowing for changes, the E-X-X-H motif (where iron binds) located in the intermembrane space in the

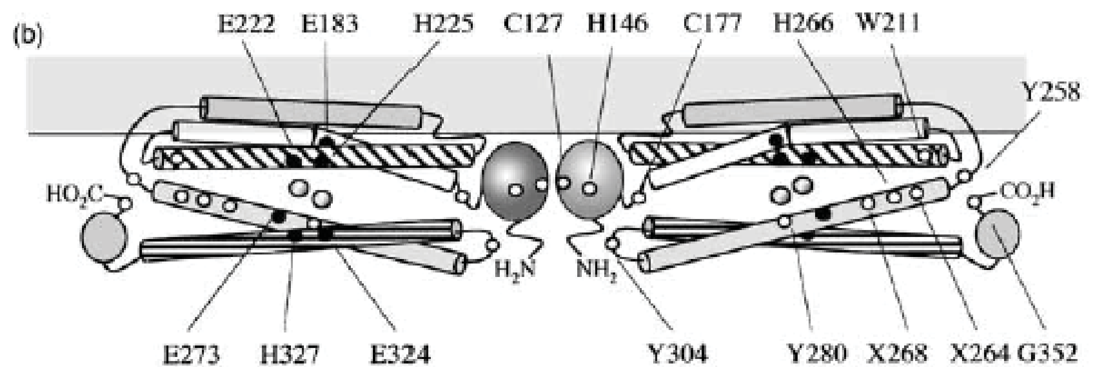

Figure 1.5 The evolution of AOX structural Models (Finnegan *et al.* 2004) Structural model of Andersson and Nordlund (1999). Proposed helical regions are shown as boxes. The unmodelled N- and C-terminal regions are depicted as ovals. Residues postulated to be involved in iron binding are shown by filled circles, with the positions specified in the left-hand subunit. The relative positions of other key residues mentioned in the text are indicated as open circles, with the positions specified in the right-hand subunit. Numbering is according to the AtAOX1a primary translation product. The drawings are not to scale.

Siedow et al. (1995) model had to be relocated to the matrix. The final model is presented in Figure 1.5 (Finnegan et al. 2004).

1.5.3. Physiological function

The heat generation of AOX activity in the thermogenic physiology of Aroid and other flowers was the first recognised function of AOX (Meeuse 1975). As well as AOX having a role in thermogenisis, it also found to be active during chilling stress as detected by microcalorimetry (Ordentlich *et al.* 1991). Confirming that AOX may play a role in chilling stress, arctic plants have been investigated, and found to have a higher level of alternative pathway activity than temperate species (McNulty *et al.* 1987).

AOX may also work as a bypass to oxidise NADH ad $FADH_2$ under conditions when ADP is a limiting factor. This allows for the glycolysis and citric acid cycle to continue functioning and provide the cell with biosynthetic precursors needed for other reactions.

Although plant mitochondria normally produce ROS, excessive ROS can have devastating effects, by directly inhibiting enzymes and damaging DNA, as well as causing the formation of lipid peroxidises from membrane lipids, causing loss of membrane integrity (Fridovich 1978). It has been suggested that the alternative pathway might also play a function in the reduction state of the UQ pool, by preventing or minimising ROS production (Chapter 5).

1.6 Relationship Between AOX and Alternative NAD(P)H dehydrogenases

Clifton *et al.* (2005) have suggested that there may be a link between the AOX and the alternative NAD(P)H dehydrogenases, in creating their own alternative electron transport chain. Neither the NAD(P)H dehydrogenases or AOX contribute to proton pumping or ATP synthesis and hence bypass

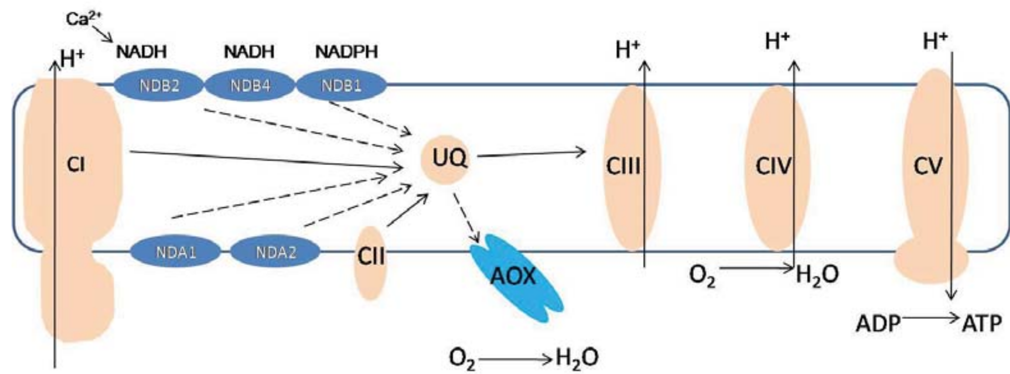

Figure 1.6 Diagrammatic representation of the plant mitochondrial electron transport chain (Clifton *et al.* 2005)

The multi subunit complexes I, II, III and IV and cytochrome c which comprise the cytochrome electron transport chain are shown in beige. The stress inducible alternative external NADH dehydrogenases (NDB) and alternative oxidase (AOX) are shown in blue. The flow of electrons is indicated by arrows with the proposed stress inducible pathway indicated by dotted lines. UQ = ubiquinone.

adenylate control and allow for respiration with flexible levels of energy conservation and respiration control (Figure 1.6)(Clifton *et al.* 2005). Although a linkage between AOX and NAD(P)H dehydrogenases is suggested, these genes display differences in response to the treatments applied, suggesting differential regulation occurs within the alternative gene family. Considine *et al.* (2002) have shown that AOX genes 1a-d in *A. thaliana* are induced by varying stress conditions, and relate to a need under a stress conditions. The AOX2 subfamily are not induced by stress, but display a constitutive or developmental expression, and hence are suggested to have a role in housekeeping in respiratory metabolism.

Co-expression of AOX and NDH has been observed, such as for *Aox1a* and *ndb2* (Clifton *et al.* 2005). This co-regulation may be due to the presence of common sequence elements in their promoters. Due to this co-expression, it has been suggested by Clifton *et al.* (2005) that the products of AOX and NDB2, a terminal oxidase and an external NAD(P)H dehydrogenase, are capable of forming a simple but functional alternative respiratory chain (Figure 1.6). This respiratory chain allows oxidation of cytosolic NAD(P)H in a manner uncoupled from oxidative phosphorylation. This self-contained pathway may be useful in helping maintain redox balance of the cell and turnover of carbon pathways.

1.7 Post genome analysis of the Alternative pathway enzymes

1.7.1. A. thaliana NAD(P)H Dehydrogenases

1.7.1.1 Sequence identification

After initial experiments to purify the alternative NAD(P)H dehydrogenases (Section 1.4.2), more recent work has been at the molecular level using a genome-based approach to identify the gene products and thus proteins responsible for these activities. The genes responsible for both internal and external NAD(P)H oxidation are nuclear in origin (Rayner *et al.* 1983). It was in *Escherichia coli* and *S. cerevisiae* that the alternative NAD(P)H

dehydrogenases were first identified at sequence level (Finnegan *et al.* 2004).

Rasmusson *et al.* (1991) discovered the initial sequence information about plant alternative NAD(P)H dehydrogenases, Rasmusson *et al.* (1999) screened a potato leaf cDNA library with a probe whose sequence similar to NDI from *E. Coli* and discovered two sequence with NADH DH-like features, these were named *St-nda* and *St-ndb*. From mitochondrial import experiments, Rasmusson *et al.* (1999) deduced St-NDA , which was 55 kDa in size, was oriented towards the internal face of the inner mitochondrial membrane, and that St-NDB, 60 kDa in size imbedded in the external face, where they face the inner mitochondrial membrane.

Moore *et al.* (2003) used sequence homology with *S. cerevisiae,* which is known to have at least three non-phosphorylating NAD(P)H dehydrogenases; ScNDE1, ScNDE2 and ScNDI1. The genome sequence database of *A. thaliana,* was blasted with these sequences to help identify *A, thaliana* genes. This approach identified seven putative NAD(P)H dehydrogenases (Table 1.1). Michalecka *et al.* (2003), used a similar strategy and grouped the *A. thaliana* NAD(P)H dehydrogenases based on their similarities to the potato homologs, so that putative internal dehydrogenases were termed NDA, and putative external dehydrogenases were termed NDB. These protein sequences of *A. thaliana* were aligned with the three yeast sequences ScNDE1, ScNDE2 and ScNDI1 and with two potato sequences StNDB and StNDA. An alignment of the internal NAD(P)H dehydrogenase sequences can be seen in Figure1.7.

These NAD(P)H dehydrogenase sequences have been separated into groups based on functional features in their sequences (Moore *et al.* 2003). There are two *A. thaliana* genes that when translated, show closer homology to *S. cerevisiae* internal NAD(P)H DH enzyme ScNDI1 and two that have closer homology to the external NAD(P)H DH enzymes ScNDE1 and ScNDE2 (Figure 1.8). The internal DH *A. thaliana* proteins are shorter than the external DH proteins. Also a difference between the internal and external

Table 1.1 Summary of the *A. thaliana* alternative NAD(P)H dehydrogenases

Gene nomenclature is taken from Mickalecka *et al.* (2003). Where the nda refers to genes encoding an internal NAD(P)H dehydrogenase, and ndb refers to genes encoding an external NAD(P)H dehydrogenase as confirmed Elhafez *et al.* (2006). Each of the *A. thaliana* (AT) NAD(P)H dehydrogenases were assigned a number based on order of identification. Expression is based on Elhafez *et al.* (2006) from Quantitative RT-PCR where tissue with most abundant expression for each gene is identified.

Gene	Locus	Accession No.	Protein Length	Expression
nda1	At1g07180	NM_100592[b]	510	Cotyldeons
nda2	At2g29990	AC004680[c]	508	Coyledons/Flowers
ndb1	At4g28220	NM_118962[b]	571	Buds
ndb2	At4g05020	AY039856[b]	582	Root
ndb3	At4g21490	AL161555[c]	581	Roots
ndb4	At2g20800	AC006234[c]	582	Roots
ndc1	At5g08740	NM_120955[b]	519	Flowers

```
                          *        20         *        40         *        60         *        80
AtNDI1     : MLWIKNLARISQTTSSSVGNVFRNPES----YTLSSRFCTALQKQQVTDTVQAKEDVVNALEPQRYDGLAPTKEGEKPRVLV :  78
AT2g29990  : MFMIKNLTRISPNTSSIIT-RFRNSGSSSLSYTLASRFCTAQETQ-----IQSPAKIPNDVDRSQYSGLPPTREGEKPRVVV :  76
NDA        : MPWFKNLIKISKTITNQSS------S-----YKSITPLASPLLTQ----FLQFTKQYS---TNDHVVGLEATKSDQKPRIVV :  64
ScNDI1     : -MTSKNLYSNKRLTTSTNT-------------TVRFASTRSTG------VENSGAGP---TSFKTMKVIDPQHSDKPNVTM :  58
             M6WIKNLT4IS4TT3S363NRFRNPSSSSLSYTL63RFC3AL2TQQVTDF62S3AQ6PNA6TSS4Y6G6APT4EG2KPR666

                      *       100         *       120         *       140         *       160
AtNDI1     : LGSGWAGCRVLKGIDTSIYDVVCVSPRNHMVFTPLLASTCVGTLEFRSVAEPISRIQPAISREPGSYYFLANCSKLDADNHE : 160
AT2g29990  : LGSGWAGCRLMKGIDTNLYDVVCVSPRNHMVFTPLLASTCVGTLEFRSVAEPISRIQPAISREPGSFFFLANCSRLDADAHE : 158
NDA        : LGSGWAGCRLMKDIDTNIYDVVCVSPRNHMVFTPLLASTCVGTLEFRSVAEPIGRIQPAVSTQPASYFFLANCNAIDFDNHM : 146
ScNDI1     : TGSGWGAISFLKHIDTKKYNVSIISPRSYFLFTPLLPSAPVGTVDEKSIIEPI--VNFATKKKGNVTYYEAEATSINPDRNT : 138
             LGSGWAGCR66KGIDTN6Y1VVC6SPRNHM6FTPLLASTCVGT6EF4S6AEPISR6QPA6S42PGS555LANC3461ADNHE

                  *       180         *       200         *       220         *       240
AtNDI1     : VHCETVTEGSSTLKP---------WKFKIAYDKLVLACGAEASTFGINGVLENAIFLREVHHAQEIRRKLLLNLMLSEVPGI : 233
AT2g29990  : VHCETLTDGLNTLKP---------WKFKIAYDKLVIASGAEASTFGIHGVMENAIFLREVHHAQEIRRKLLLNLMLSDTPGI : 231
NDA        : IECETVTEGVETLEA---------WKFNVSYDKLVIASGAHALTFGIKGVNEHATFLREVHHAQEIRRKLLLNLMLSDVPGV : 219
ScNDI1     : VTMKSLSAVSQTYQPENHTGTHQAEPAEIKYDYLISAVGAEPNTFGIPGVTDYGHFTKEIPNSLEMRRTFAANTEKANLLPK : 220
             6HCE363EGS2TLKPENHTGTHQAWKFK6AYDKL66ASGAEASTFGINGV6ENAIFL4E6HHAQE6RRKLLLNLMLS16PG6

                *       260         *       280         *       300         *       320
AtNDI1     : GEDEKKRLLHCVVVGGGPTGVEFSGELSDFIMKDVRQRYSHVKDDIRVTLIEARDILS-SFDDRLRHYAIKQLNKSGVKL-V : 313
AT2g29990  : SKEEKRRLLHCVVVGGGPTGVEFSGELSDFIMKDVRQRYAHVKDDIHVTLIEARDILS-SFDDRLRRYAIKQLNKSGVRF-V : 311
NDA        : SEEEKRRLLHCVVVGGGPTGVEFSGELSDFILKDVHQRYAHVKDYIHVTLIEANEILS-SFDDRLRVYATNQLTKSGVRL-V : 299
ScNDI1     : GDPERRRTTSIVVVGGGPTGVEAAGETQDYVHQDTRKFTPALAEEVQIHLVEATPIVLNMFEKKTSSYAQSHLENTSIKVHT : 302
             GEEE44RLLHCVVVGGGPTGVEFSGELSD566KDVRQRYAH6KDD6H6TL6EARDI6SNSFDD4LRSYAIKQLNK3G646HV

              *       340         *       360         *       380         *       400         *
AtNDI1     : RGIVKEVKP----QKLILDDG----TEVPYGPLVWSTG--VGPSSFVRSLDFPKD--PGGRIGIDEWMRVPSVQDVFAIGDC : 383
AT2g29990  : RGIVKDVQS----QKLILDDG----TEVPYGLLVWSTG--VGPSPFVRSLGLPKD--PTGRIGIDEWMRVPSVQDVFAIGDC : 381
NDA        : RGLVQHVQP----DNIILSDG----TNVPYGLLVWSTG--VGPSPFVNSLDIPK---AKGRIGIDEWLRVPSVQDVYSIGDC : 368
ScNDI1     : RTAVAKVEEKQLLAKTKHEDGKMTEETIPYGTLIWATGNKARPVITDTFKKIPEQNSSKRGLAVNDFLQVKGSNNIFAIGDN : 384
             RG6VKEV2PKQLLQK6ILDDGKMTETE6PYGLL6WSTGNKVGPSPFVRSLD6PKDNSPKGR6G61E56RVPSVQ165AIGDC

                    420         *       440         *       460         *       480         *
AtNDI1     : SGYLESTGKSTLPALAQVAEREGKYLANLFNVMGKAGGGRANSAKEMELG--EPFVYKHLGSMATIGRYKALVDLRESKEGK : 463
AT2g29990  : SGYLETTGKPTLPALAQVAEREGKYLANLLNAIGKGNGGRANSAKEIELG--VPFVYKHLGSMATIGRYKALVDLRESKDAK : 461
NDA        : SGFLESTGRQVLPALAQVAERQGKYLASLLNKVGKQGGGHANCAQNINLG--DPFVYKHLGSMATIGRYKALVDLRESKEAK : 448
ScNDI1     : AFAGLPPTAQVAHQEAEYLAKNFDKMAQMPNFQKNTSSRKDKIDTLFEENNFKPFKYNDLGALAYTGSERAIATMR-SGKRT : 465
             SG5LE3TG4QTLPALA2VAE42GKY6AN6LNA6GKAGGG4ANSAKE6ELGNFEPFVYKHLGS6ATIGRY4A6VD6RESKEAK

                  500         *       520         *       540
AtNDI1     : GISMAGFLSWFIWRSAYLTRVVSWRNRFYVAINWLTTFVFGRDISRI- : 510
AT2g29990  : GISMTGFVSWFIWRSAYLTRVISWRNRFYVAINWFTTFVFGRDISRI- : 508
NDA        : GVSLAGFTSFFVWRSAYLTRVVSWRNKIYVLINWLTTLVFGRDISRI- : 495
ScNDI1     : FYTGGGLMTFYLWRILYLSMITSARSRLKVFFDWIKLAFFKRDFFKGL : 513
             G636AGF63556WRSAYL3R66SWRN4FYVAI1W6TTFVFGRDIS4IL
```

Figure 1.7 An alignment of internal NAD(P)H dehydrogenase amino acid sequences (Finnegan *et al.* 2004)

The sequences from S. cerevisiae (ScNDI1, accession X61590), potato (NDA, accession AJ245861), and *A. thaliana* (AT1g07180, accession AC067971; AT2g29990, accession AC004680) proteins were aligned using ClustalW. Regions of high homology (75% or more) are boxed in black. Dashes represent gaps in the sequence introduced to optimize the alignment.

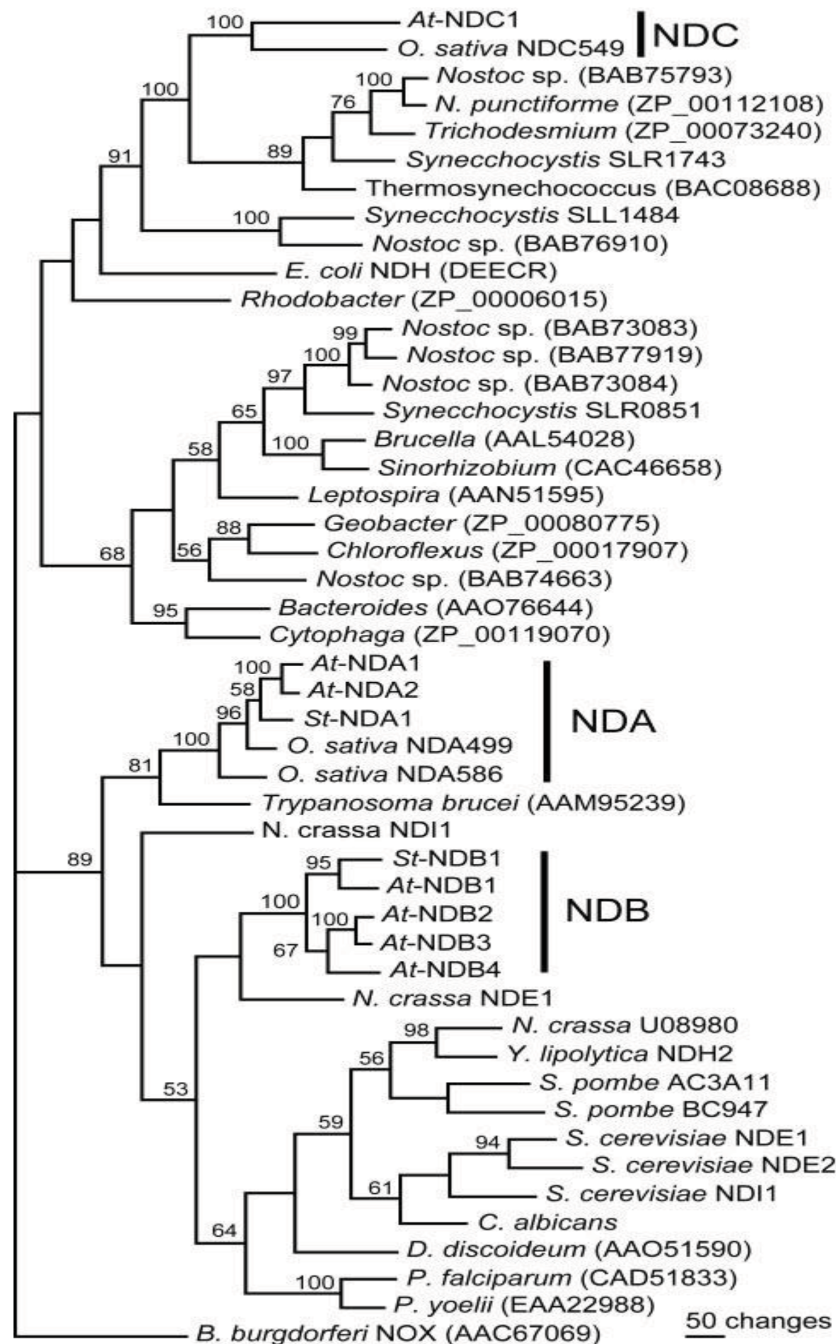

Figure 1.8 Phylogenetic analysis of bacterial, plant, fungal, and protest NAD(P)H dehydrogenase-like protein sequences

Sequences were aligned using ClustalW in MacVector 7.1 software (Accelrys Inc., San Diego). Alignments were manually inspected and edited, assuring correct matches of conserved regions (Michalecka *et al.* 2003).

is that the internal DH proteins have a higher degree of homology with an average of 74% identity, with the external proteins having an average identity of only 58% (Moore *et al.* 2003). The nomenclature for these alternativedehydrogenases changed for the internal dehydrogenases from NDI to what they are now referred to as NDA's and the external dehydrogenases from NDE's to NDB, and the third group known as NDC (Elthon *et al.* 1989). As well as the putative internal NDA and putative external NDB groups Michalecka *et al.* (2003) indicated that there may be a third group of NAD(P)H dehydrogenases with one gene and named NDC. These *A. thaliana* NDI and NDE sequences contain two dinucleotide fold motifs, as does the potato sequence. Of the four NDE three of them contain an insert, hence why the internal genes are shorter. These inserts contain a Ca^{2+} binding EF hand motif which is also found in potato NDB1 (Michalecka *et al.* 2003) (Figure 1.9). This EF hand motif is not found in the internal dehydrogenase sequences.

Recently it has also been discovered that several of the alternative DHs proteins are dual targeted not only to the mitochondria but also to the peroxisomes. There have been reports of over 30 proteins in plants which are dual targeted to the mitochondria and chloroplasts (Millar *et al.* 2006). For the internal dehydrogenases one of these NDC1 is targeted to both the mitochondria and the chloroplast. Three other proteins, the remaining two internal proteins (NDA1 and NDA2) as well as one external protein (NDB1) were found to be dual targeted between the mitochondria and the peroxisome. The remaining proteins did not display peroxisomal targeting (Carrie *et al.* 2008).

1.7.1.2 Internal NAD(P)H dehydrogenases

Internal NAD(P)H dehydrogenases in *A. thaliana* had their sequences identified by (Moore *et al.* 2003) based on homologous sequences with yeast. Using a t-DNA insertion mutation line the expression of an internal NAD(P)H dehydrogenase (AT1g07180) was disrupted. QRT-PCR on

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AtNDB2 TINQRKVMEDVSAIFSKAD.....KDKSGTLTLKEFQEAMDDICVRYPQVELYLKSKRMRGIADLLKEAETDDVSKNNIELKIEEFKSALSQVDSQVKF 467
AtNDB3 TINQRKVMEDIAAIFKKAD.....KENSGTLTMKEFHEVMSDICDRYPQVELYLKSKGMHGITDLLKQAQAENGSNKSVELDIEELKSALCQVDSQVKL 466
AtNDB4 TINQRRVMEDIAAIFNKAD.....KGNTGTLKKKDFNSVVKDICQRYPQVELYLKKNKLKNIANLLKSANGED.....TQVNIEKFKQALSEVDSQMKN 467
AtNDB1 SIAQRKILGDIANIFKAAD.....ADNSGTLTMEELEGVVDDIIVRYPQVELYLKSKHMRHINDLLADSEGNAR....KEVDIEAFKLALSEADSQMKT 456
StNDB1 SVDQHKVMEDISTIFEAAD.....KDDSGTLSVEEFRDVLEDIIIRYPQVDLYLKNKHLLEAKDLFRDSEGNER....EEVDIEGFKLALSHVDSQMKS 462
Pf     KIQPKLLHEHTNEIIKILT.....GNK...LTSEALKLKQSELTKTFPQLS........ISKWDYEKNKKG........EMTPQQFHDYLFEIDKNYKS 557
Py     QISPINSHEHVNEIINCLG.....NSK...ITSDVLKQKSKELSNIFPQLS........DTKWDYNKNKKS........EMSIKELQEYLFMIDKNYKS 431
NcNDE1 TIQNN.VADHIITFLRNLAWKHGKDPESLELHFSDWRDVAQQIKKRFPQATAHLKR..LDKLFEEYDKDQNG.......TLDFGELRELLKQIDSKLTS 478
Trop-C MLGQNPTKEELDAIIEEVD.....EDGSGTIDFEEFLVMMVRQMKEDAKGK...SEEELEDCFRIFDKNADG.......FIDIEELGEILRATGEHVTE 131
                          *       * ** **   **                                   # # ##          ##  ##
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Figure 1.9 Amino acid sequence denoting comparison of EF-hand motif of external NAD(P)H dehydrogenases from various species (Michalecka *et al.* 2003)

The EF hand motif in *A. thaliana* NDB proteins (NDB2, NDB3, NDB4, NDB1) Potato NDB1 (StNDB1), *Plasmodium falciparum* (Pf), *Plasmodium yoelli* (Py), Neurospora crassa (NcNDE1), and *Gallus gallus* troponin C (Trop-C). The known potato EF hand motif (*) is conserved amongst *A. thaliana* NDB sequences except for two position in NDB4. The two *plasmodium* sequences contain no EF hand motifs.

transcripts indicated this gene had been completely silenced but the highly homologous gene (AT2g9990) was still transcribed (Moore *et al.* 2003). When mitochondrial activities were done on this mutant there was loss of matrix NAD(P)H activity. A protein import experiment indicated this internal NAD(P)H dehydrogenase named AtNDI had a molecular weight of approximately 56 kDa (Moore *et al.* 2003). Expression profiles of internal NAD(P)H dehydrogenase genes has indicated that *nda1* and *ndc1* respond to light (Michalecka *et al.* 2003; Escobar *et al.* 2004; Elhafez *et al.* 2006). An examination of tissue specific expression has indicated that nda1 expression is absent from the roots, but that *nda2* and *ndc1* are expressed in most plant tissues (Michalecka *et al.* 2003).

1.7.1.3 External NAD(P)H dehydrogenases

There had been no published data on knockouts or t-DNA inserts in the external dehydrogenase family, at the start of this research project presented in this thesis. Tissue specific expression had been examined for the external dehydrogenases and it was found from mRNA transcripts of *A. thaliana* ndb genes, that *ndb1* was expressed mostly in the buds, *ndb2,3 & 4* in the roots, *nda1* were expressed in the cotyledons, *nda2* in the cotyledons and flowers and *ndc1* in the flowers. However *ndb3* and *ndb4* mRNAs were mostly absent in leaves and *ndb4* transcript was absent in the stems, indicating there is potential for spatial regulation of these genes at the transcript level (Michalecka *et al.* 2003).

1.8 *A. thaliana* Alternative Oxidase

The AOX antibody has proven useful for detecting AOX from many plants and other organisms, including numerous fungi, *Trypanasoma brucei* (Chaudhuri *et al.* 1998), *Acanthamoeba castellanii* and *Dictyostelium discoideum* (Jarmuszkiewicz *et al.* 2002). The AOX antibody detects either one or a few proteins at 35–37 kDa. AOX has the potential to be detected

immunologically in many species there is a highly conserved binding site on the denatured AOX protein, within the sequence R321-A-D-E-A-H-H-RD-V-N-H332 of the *A. thaliana* AOX1a (AtAOX1a) protein (Finnegan *et al.* 2004). The AOX antibody led to the isolation of the *Sauromatum guttatum* SgAox1 cDNA that encodes a 39 kDa protein (Rhoads *et al.* 1991). Since this there have been AOX cDNAs sequences isolated from many species including 28 Animal species (McDonald 2009).

First discovered in soybean (Whelan *et al.* 1996), AOX multi-gene families have also been reported in tobacco (Whelan *et al.* 1996), *A. thaliana* (Saisho *et al.* 1997), rice (Ito *et al.* 1997), mango (Considine *et al.* 2001), maize (Karpova *et al.* 2002) and tomato (Holtzapffel *et al.* 2003). The existence of multiple AOX genes explained why multiple bands have been visualised on immunoblots when using the AOX antibody. Commonly, each gene family member has a specific tissue and developmental stage-dependant expression pattern (Finnegan *et al.* 1997; Ito *et al.* 1997; Saisho *et al.* 1997; McCabe *et al.* 1998; Karpova *et al.* 2002; Holtzapffel *et al.* 2003; Thirkettle-Watts *et al.* 2003). This differential specific expression and tissue patterns for expression of the various AOX genes, may also explain the multiple gene family found.

1.8.1. Physiological Significance of the alternative pathway

Although external NAD(P)H oxidation has been known for quite some time, its physiological importance has yet to be determined (Rasmusson *et al.* 2004). Even for the most studied system, the red beet root, the physiological relevance is not known. However, there are currently many hypotheses for the role that these NAD(P)H dehydrogenases may play in plants.

1.8.1.1 Thermogenesis

The plant *Arum maculatum*, has a thermogenic respiratory burst, where

external NADH oxidation increases along with AOX activity (Leach *et al.* 1996). A closer measurement of the NAD(P)H dehydrogenases indicated that internal NADPH oxidation and external NAD(P)H oxidation increased concomitantly with AOX, but internal NADH remained high during the post thermogenic phase (Finnegan *et al.* 2004). The heat generated during this respiratory burst is for the purpose of pollination and floral development.

1.8.1.2 Turnover of matrix $NADP^+$ and its link to AOX capacity.

Moller *et al.* (1998) and (Agius *et al.* 2001) have determined that $NADP^+$ is present at lower levels in the matrix than $NAD^{+,}$ thus there is need for the capacity to turnover NADPH in the plant mitochondrial matrix. The NAD(P)H/NADP ratio could play a role in the suggested AOX activation thus the presence and activity of the NADPH dehydrogenase will potentially influence AOX capacity. The level of matrix or internal NADPH will depend on the relative NAD(P)H DH dehydrogenase activity which in turn may be regulated by matrix/internal calcium levels, as the protein is suspected to be dependent on calcium (Moller *et al.* 1998).

1.8.1.3 Stress Adaptation

Plants are often put under various stresses, depending on growth conditions, and environmental conditions, such as cold, heat, too much salt and phosphorus. Reactive oxygen species (ROS) are often produced in the mitochondria. This ROS production occurs when oxygen interacts with the reduced form of the electron transport components such as flavones and ubiquinone. Accumulation of ROS in plants will clearly result in a wide variety of deleterious effects through oxidation reactions involving proteins, lipids and nucleic acids (Taylor *et al.* 2002). Alternative NAD(P)H dehydrogenases are flavoproteins, and along with ubiquinone are potential sites of ROS production.

It has been observed that external NADH oxidation only, i.e. not NADPH

oxidation increases during long term chilling stress, such as after storing beetroot tubers at 4°C for 10 weeks (Fredlund *et al.* 1991a). On the contrary external NAD(P)H oxidation did not change in potato leaf mitochondria which were exposed to chilling stress of 2-6 days, but the internal NADH oxidation decreased, as did *Stnda* transcript levels (Svensson *et al.* 2002). It has been suggested that Internal NAD(P)H oxidation decreases due to the ROS production produced during chilling stress to further minimize ROS production.

1.8.1.4 Photosynthetic Metabolism

Svensson *et al.* (2001) have suggested that internal NADH oxidation decreases in the absence of light and increases through leaf growth, suggesting a role for internal NADH oxidation in photosynthesis. It is suggested that the external NADH dehydrogenases operate with alternative oxidase to oxidise excess NADH that is produced under photosynthesis and bypass ATP synthesis, and this allows for continued flux occurring through the photorespiratory cycle (Rasmusson *et al.* 2004).

1.8.1.5 Maintaining Redox Balance

Although the function of these alternative NAD(P)H dehydrogenases is unclear, other roles may be linked to maintaining the redox balance as these dehydrogenase can compensate for when the ETC is compromised. (Zhang *et al.* 1996) confirmed this by showing that external NADH and internal NADH oxidation rates are increased when Complex I is compromised by various inhibitors. There have been instances where mutants have shown increased external NADH oxidation, i.e. in Complex 1 mutants from *Nicotiana sylvestr*is indicate a role of these dehydrogenases in maintaining the redox balance of the cell (Gutierres *et al.* 1997). There has also been observed dramatic increases in external NAD(P)H dehydrogenase activity in an *nda1* T-DNA insertion line of *A. thaliana*. This could be suggesting that this enzyme is acting as a compensatory mechanism with matrix-reducing equivalents

being exported via a malate/oxaloacetate shuttle (Day *et al.* 1981; Moore *et al.* 2003). It has been postulated that the alternative pathway for NAD(P)H oxidation only operates when complex I and the phosphorylating pathway is compromised or saturated. Thus, the non-phosphoryalting pathway would become active and cycle to remove excess NAD(P)H both matrix and/or cytosolic. It is now thought that both may act at the same time with electrons passing through both pathways and thus a better understanding of what regulates electron flux through complex I and the non-phosphorylating dehydrogenases is needed.

1.9 ROS signaling

Plants contain a branched respiratory network containing antioxidant enzymes, antioxidants and enzymes capable of producing ROS. This system is said to be responsible for maintaining and tightly regulating ROS levels (Gechev *et al.* 2006). As well as causing cellular damage, ROS have been suggested to function as signalling molecules which are able to coordinate a range of plant processes (Apel *et al.* 2004; Gechev *et al.* 2006). Some of these plant processes, which ROS could coordinate, are growth and development, stress adaptation or programmed cell death. The oxidised products of ROS damage can also be important secondary signalling molecules (Rinalducci *et al.* 2008). This has lead to a concept of oxidative signalling, rather than oxidate stress. As plants can use ROS as a signalling pathway it suggests that over evolution, plants were able to control ROS toxicity and now use ROS as signalling molecules for more specialised process. (Vanlerberghe *et al.* 2002) have suggested that AOX plays a role in the cell signalling pathway generated by ROS which can lead to cell death. It is thought AOX plays a role in mitochondrial-generated ROS levels, which impacts on the cellular level of anti-oxidant defences resulting from signalling.

1.10 Aims

Moller (2001) provided evidence that the internal NAD(P)H dehydrogenase was allegedly calcium dependent, but none of the internal dehydrogenases or NDC have the EF-hand motif, suggesting the possibility of one of the external dehydrogenases moving internally. Hence further work is needed to confirm the calcium dependence of these dehydrogenases and confirm previous experiments. The other dehydrogenase with speculation is NDC, as experiments indicated it was imported to the matrix but spans the membrane as determined by proteolytic digestion (Elhafez *et al.* 2006), leaving the question open as to which side of the membrane does its active site face? Does it have internal or external NAD(P)H DH activity?

Since sequence information has become available there has also been questions raised to the mis-match between the purified protein sizes for these activities and the predicted sizes for the protein. It is hoped that these questions can be answered by using RNAi knockdown plants and western blots to confirm protein sizes, as have previously been determined, Hence there is still a lot to be determined about these dehydrogenases and it is thought that a transgenic approach towards altering the expression of all these NAD(P)H DH genes may help to resolve these issues.

The overall aims of this work is to help to further understand the role of these alternative dehydrogenases by focusing on the external dehydrogenases and specifically *ndb4*, as little work has been done on this gene. Then to help understand the role between these dehydrogenases and AOX and its response to abiotic stress and specifically salinity stress as little is known about salinity stress and the relationship with AOX and the non-phosphorylating pathway.

To do this I have taken a transgenic approach, which could use either T-DNA lines or RNAi knockdown. I have chosen to use RNAi knockdown lines for this work as it allows to specifically target one individual gene family member

or multiple at the same time. T-DNA lines do not offer this, and further, RNAi often does not result in complete elimination of gene expression and this may be important in genes that are critical for growth and development. This may be why there are few T-DNA lines available for the NAD(P)H dehydrogenase genes.