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AN INVESTIGATION OF ALTERNATIVE OXIDASE PRESENCE, EXPRESSION, AND REGULATION IN THE MOSS PHYSCOMITRELLA PATENS

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AN INVESTIGATION OF ALTERNATIVE OXIDASE PRESENCE, EXPRESSION, AND REGULATION IN THE MOSS *PHYSCOMITRELLA PATENS*

by

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Abstract

Alternative oxidase (AOX) is an inner mitochondrial membrane protein that introduces a branch point at ubiquinone within the respiratory electron transport system (ETS). The AOX protein bypasses two sites of proton translocation within the ETS and as a result the yield of ATP per oxygen consumed is significantly reduced. Although AOX appears to be energetically wasteful, recent studies have revealed that AOX has a wide taxonomic distribution. AOX multigene families, transcripts, protein levels, and enzymatic activity have been most thoroughly characterized in many angiosperm (flowering) plants. Given the data available for angiosperm AOXs, evidence of non-angiosperm AOXs in the primary literature is scarce and therefore is a logical starting point for comparative studies. The bioinformatics results suggest that AOX is an ancient character in the Viridiplantae, as it is present within algae, a liverwort, a moss, lycopods, ferns, and many gymnosperms. Most interestingly, it appears that the moss *Physcomitrella patens* possesses a single AOX gene; a characteristic unobserved in all angiosperm AOXs studied to date. This finding has been validated by sequence analysis of a cloned *P. patens* AOX cDNA that was amplified by PCR using specific genomic primers. Reverse transcriptase PCR has demonstrated that AOX is expressed in *P. patens* and sequencing analysis suggests that AOX has all the residues to be catalytically active. At the protein level, moss AOX was heterologously expressed in the yeast *Saccharomyces cerevisiae* and protein expression was analyzed by performing mitochondrial isolations, SDS-PAGE, and immunoblots. Preliminary studies using respirometry and site-directed mutagenesis of key residues were undertaken, and it is anticipated that the optimization of these procedures will yield useful data in future studies. A greater understanding of the AOX pathway in non-angiosperm plants is of importance as it will

contribute to understanding the evolutionary history of AOX and may help determine its physiological function(s).

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CHAPTER 1

Introduction

1. Introduction

1.1. Cellular Respiration and the Alternative Oxidase Pathway

All living cells require energy to carry out essential metabolic processes. Cellular respiration is the biochemical process by which cells obtain energy from the breakdown of organic molecules such as glucose. Several metabolic pathways are involved in the storage of energy in the form of adenosine triphosphate (ATP), these include: glycolysis, pyruvate oxidation, the citric acid cycle (TCA), and oxidative phosphorylation. The first three stages produce substrates (NADH and $FADH_2$) that donate electrons to protein complexes and mobile carriers used in the final pathway. Oxidative phosphorylation is the major site of ATP synthesis and involves two components, an electron transport system (ETS) and ATP synthase (Brooker et al., 2008).

The ETS is located in the inner mitochondrial membrane (IMM) and consists of four multisubunit protein complexes (I to IV) and small electron carriers (ubiquinone and cytochrome c) (Figure 1.1). In a linear manner, electrons are transferred to each complex via redox reactions. At the beginning of the chain, electrons are donated to complex I and II from the oxidation of NADH and $FADH_2$, respectively. Electrons transfer from complex I or II to ubiquinone which becomes reduced to ubiquinol. Electrons are transported from ubiquinol to complex III, to cytochrome c, and terminate at complex IV where oxygen is the final electron acceptor and is reduced to water (Brooker et al., 2008; Figure 1.1). Simultaneously, the energy released during electron transport is coupled to the translocation of protons across the IMM by three of the four complexes (Figure 1.1). This generates an electrochemical gradient that can be used to synthesize ATP by the enzyme ATP synthase. However, the presence of specific compounds can

render the terminal complexes of the ETS inactive. Antimycin A and cyanide are examples of inhibitors that will restrict electron flow at complexes III and IV, respectively (Siedow and Umbach, 1995; Albury et al., 2002). Despite this, it has been observed that respiration will still occur even under the influence of these inhibitors; this is due to a second pathway using the alternative oxidase (AOX) (Bendall and Bonner, 1971).

The AOX pathway is insensitive to all known inhibitors of the last two complexes of the ETS, and as a result it is referred to as 'cyanide-resistant' respiration (McDonald and Vanlerberghe, 2006). However, it has been demonstrated that AOX is sensitive to the compounds n-propyl gallate (nPG) and salicylhydroxamic acid (SHAM) (Siedow and Umbach, 1995; Figure 1.1). AOX is a non-proton motive, terminal quinol oxidase protein that dissipates energy in the form of heat through the reduction of oxygen to water (Berthold et al., 2002; Figure 1.1). This pathway introduces a branch point at ubiquinone, bypassing two sites of proton translocation within the ETS and as a result fewer ATP are generated per oxygen consumed (Guy et al., 1989; McDonald and Vanlerberghe, 2006; Figure 1.1).

1.2. Taxonomic Distribution of AOX

Despite the fact that AOX appears to be energetically wasteful, recent studies have revealed that AOX has a wide taxonomic distribution and is found in 2 of the 3 domains of life, excluding the Archaea. AOX is present in many plants, fungi, proteobacteria, protists, and has been recently identified in several animal species (McDonald, 2008). However, the most extensive distribution of AOX has been observed in the Viridiplantae. In basal plants, AOX has only been characterized in a single green algal species, *Chlamydomonas reinhardtii* (Dinant et al., 2001). The majority of AOX research has been conducted on a number of angiosperms

(flowering plants) (McDonald et al., 2002). AOX multigene families, gene expression, protein levels, and enzymatic activity have been thoroughly characterized in many angiosperm species such as *Arabidopsis thaliana*, soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), rice (*Oryza sativa*), and voodoo lily (*Sauromatum guttatum*) (Vanlerberghe et al., 1998; Saika et al., 2002; Frederico et al., 2009).

1.3. Physiological Role(s) of AOX

Even though AOX is widely distributed in nature, its physiological function(s) still remains unclear (Saika et al., 2002). The only confirmed function of AOX has been identified in thermogenic plant species such as the sacred lotus (*Nelumbo nucifera*) and dead horse arum (*Helicodiceros muscivorus*) (Angioy et al., 2004; Watling et al., 2006). Recent work has demonstrated that when AOX is active it will heat the plant during thermogenesis, thereby allowing it to survive at low temperatures (Watling et al., 2006). In addition, the heat that is generated will promote pollination by increasing the emission of an odour that is attractive to insect pollinators (Angioy et al., 2004). However, this function does not explain why AOX is present in non-thermogenic plants, a group which comprises the majority of plant species (McDonald, 2008).

Many alternative hypotheses have been made in an attempt to explain why a large group of taxa, particularly non-thermogenic plants, would possess this seemingly wasteful pathway. It has been proposed that AOX may play a key role in controlling oxidative damage (Maxwell et al., 1999), and in balancing carbon metabolism and electron transport (McDonald, 2008). Previous studies have also revealed that AOX activity increases when organisms are subjected to

biotic and abiotic stress (McDonald, 2008). Therefore, AOX may be advantageous to plants as they are sessile and constantly experience changing environmental conditions.

1.4. AOX Gene Structure and Expression

AOX is a nuclear-encoded gene and exists as a multigene family (typically three to five genes) in most monocot and dicot angiosperm species (Vanlerberghe and McIntosh, 1997; Van Aken et al., 2009). Two AOX subfamilies have been identified in higher plants, AOX1 and AOX2. The AOX1-type is present in both monocots and dicots, whereas the second subtype, AOX2, is found in eudicot species but is absent in all monocots (Considine et al., 2002). The physiological importance of these subtypes has yet to be determined, however, it has generally been observed that accumulation of AOX1 is induced in response to environmental cues such as low temperature and anoxic conditions (Costa et al., 2010). In contrast, AOX2 is often expressed during different stages in plant development and is the prevalent form expressed in eudicots (Considine et al., 2002). However, a recent study by Costa et al. (2010) revealed that both subtypes were expressed when subjected to stress, indicating for the first time that AOX2 gene expression in cowpea (*Vigna unguiculata*) is both constitutive and inducible.

The exon/intron structure of AOX1 and AOX2 has been investigated in several angiosperms. A study by Considine et al. (2002) revealed that both subtypes in Arabidopsis and rice generally had four exons that were interrupted by three introns (Figure 1.2). Similar gene structure was also seen in both AOX genes of cowpea and soybean (Costa et al., 2010). Furthermore, it was observed that the intron positioning in three regions along the terminal portion of the gene in Arabidopsis and rice AOX was highly conserved among these species (Considine et al., 2002; Figure 1.2).

1.5. AOX Protein Structure and Regulation

AOX is a homodimeric protein that has been categorized within the group of di-iron carboxylate proteins (Berthold et al., 2002). Structurally, AOX is made up of a di-iron centre and four helices that associate with the IMM (McDonald, 2008). Highly conserved amino acids have been identified within the four helices that are thought to play a role in AOX enzymatic function and regulation.

Several glutamate (Glu) and histidine (His) residues are required for AOX activity, as they are responsible for coordinating the di-iron centre (Siedow et al., 1995). This has been demonstrated using site-directed mutagenesis of the AOX sequence in species such as the voodoo lily (*Sauromatum guttatum*) (Albury et al., 2002) and *Arabidopsis thaliana* (Berthold et al., 2002). The iron-ligands (based on the numbering in *A. thaliana* AOX1a) are: Glu-183, Glu-222, His-225, Glu-273, Glu-324, and His-327 (Berthold et al., 2002).

Cysteine 127 (Cys-127) is another highly conserved amino acid residue within angiosperm AOX sequences (Vanlerberghe et al., 1998; Berthold et al., 2000). This cysteine is responsible for the redox regulation of AOX and its requirement for AOX regulation in some plant species has been verified using site-directed mutagenesis (Vanlerberghe et al., 1998). In plants, AOX exists as a dimer due to a non-covalent association between the monomers (Figure 1.3). The dimeric subunits of the AOX protein have the capability to interconvert between covalently linked (oxidized) and non-covalently linked (reduced) forms (Vanlerberghe et al., 1998). The oxidized dimer forms an intersubunit disulfide bridge resulting from the interaction of Cys-127 residues in each monomer (Umbach and Siedow, 1993; Figure 1.3). This protein state displays little activity, whereas the reduction of the disulfide-linked subunits produces the

active form of the protein (Vanlerberghe et al., 1998; Figure 1.3). The conversion between these two forms *in vitro* can be achieved with the addition of a reducing agent, such as dithiothreitol (DDT), or an oxidizing agent, such as diamide (Umbach and Siedow, 1993) and visualized using non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Additionally, treatment with α–keto acids, such as pyruvate, can further stimulate the reduced dimer activity by possibly interacting with the sulfhydryl group of Cys-127 (Umbach and Siedow, 1993; Figure 1.3). Holtzapffel et al. (2003) has demonstrated that some angiosperm AOX proteins are regulated and activated by succinate rather than pyruvate, due to the substitution of Cys-127 by a serine residue.

1.6. Non-angiosperm Plant AOXs

Although AOX is said to be ubiquitous in the plant kingdom, evidence for non-angiosperm (non-flowering plant) AOXs in the primary literature is scarce. These were the earliest plants to emerge onto land and originated from the Zygnematales green algae (Timme et al., 2012; Figure 1.4). Non-angiosperms can be classified into three different groups based on their morphology, physiology, and reproduction (Figure 1.4). The first group consists of the non-vascular seedless plants collectively known as the bryophytes and includes liverworts, hornworts, and mosses (Figure 1.4). The development of vascular tissue characterizes the second group that is comprised of lycophytes (clubmosses, spikemosses, and quillworts) and pterophytes (ferns and horsetails) (Figure 1.4). Lastly, the origin of seeds marked an important evolutionary development by the gymnosperms, a group that includes cycads, conifers, *Ginkgo*, and Gnetales (Figure 1.4).

Of the few studies that have investigated non-angiosperm AOX, the majority of the data have been collected from gymnosperms. Previous mitochondrial studies have suggested the presence of AOX in the gymnosperms *Araucaria angustifolia* (Mariano et al., 2008) and *Picea glauca* (Johnson-Flanagan and Owens, 1986; Weger and Guy, 1991). Additionally, putative gymnosperm AOX sequences have been identified using bioinformatics in a wide variety of pines and spruces such as *Cryptomeria japonica, Picea engelmannii, Picea glauca, Picea sitchensis, Pinus pinaster, Pinus pinea, Pinus taeda,* and *Pseudotsuga menziesii* (McDonald and Vanlerberghe, 2006; Frederico et al., 2009). To date, only a limited number of AOXs have been reported in basal plants such as *Marchantia polymorpha, Physcomitrella patens*, *Selaginella moellendorffii, Selaginella lepidophylla*, and *Adiantum capillus-veneris* (Frederico et al., 2009).

1.7. *Physcomitrella patens* as a Model System

The moss *Physcomitrella patens* has been extensively used in genetic studies over the past decade. *P. patens* is a widely distributed moss that is abundant in temperate regions including regions of North America, Europe, Africa (Prigge and Bezanilla, 2010). Mosses show alternation of generations where they interchange between the haploid (gametophyte) and diploid (sporophyte) stages of their life cycle to produce spores (Figure 1.5). The diploid sporophyte is generally dominant in the majority of plants that display this life cycle; however, the haploid gametophyte phase is dominant in moss (Cove, 2005; Smidkova et al., 2010; Figure 1.5). This feature makes *P. patens* an ideal model system over other plants because mutations can be detected and analyzed earlier in development than species with a dominant diploid phase (Cove, 2005). *P. patens* is an attractive model organism for plant molecular biology studies due to the availability of the complete genome sequence; the fact that it can grow rapidly and easily in a

laboratory setting; and its ability to incorporate foreign DNA by way of homologous recombination (Hohe et al., 2004; Rensing et al., 2008). As an expression system, *P. patens* has been exploited to investigate gene function by knock-out analysis, plant regulatory mechanisms, and recombinant protein expression (Hohe et al., 2004; Cho et al., 2007; Chater et al., 2011).

2. Purpose

The purpose of this study is to determine the similarities and differences between angiosperm and non-angiosperm AOXs by making comparisons at the levels of gene characteristics, transcript expression, and protein. Since angiosperm AOXs are encoded by a multigene family, they are challenging to study in these systems due to potential redundancy. The aim of this thesis is to characterize AOX for the first time in a non-angiosperm plant that can then be used as a model organism for future AOX studies. Ultimately, this research will contribute to understanding the evolutionary history of plant AOXs and may help determine the physiological function(s) of this protein.

2.1. Rationale

Given that there is little known about AOX in non-angiosperm plants, it is important to perform an initial characterization of this protein in a model system. Therefore, the objectives of this study are to:

1. Assess the taxonomic distribution of AOX in non-angiosperm members of the Viridiplantae using bioinformatics. Comparisons will be made at the amino acid level to identify similarities and differences between all plant AOXs.

2. Investigate *P. patens* AOX gene structure, expression and subtypes (AOX1/AOX2). Intron/exon structure will be examined using bioinformatics and comparisons will be made to angiosperm AOX gene (intron/exon) structure. Polymerase chain reaction (PCR) will be used to confirm the presence of AOX gene(s) in *P. patens* and amplified fragments will be cloned into a bacterial vector for sequencing. Bioinformatic analysis and PCR will be used to determine the AOX gene copy number and to verify what AOX1/AOX2 subtype(s) are present in moss.
3. Examine *P. patens* AOX transcript copy number and expression. AOX transcript copy number will be determined using bioinformatics. Expression will be assessed by reverse transcriptase polymerase chain reaction (RT-PCR).
4. Analyze *P. patens* AOX protein structure, size, conformation, localization, and functionality. Bioinformatics will be employed to determine if the amino acid residues that are essential for angiosperm AOX activity are conserved in moss. Recombinant *P. patens* AOX proteins will then be expressed in a heterologous system to analyze protein size and the oxidized/reduced forms will be detected in moss mitochondria by performing SDS-PAGE and immunoblotting. Finally, AOX functionality will be investigated by mutating key amino acid residues through site-directed mutagenesis and activity will be assessed through respirometry.

2.2. Hypotheses

I predict that AOX is widespread in the Viridiplantae based on its presence in the green alga *Chlamydomonas reinhardtii* and many angiosperms. Based on previous bioinformatics work, I believe that I will be able to verify the presence of a single AOX gene within the moss *P.*

patens using PCR.Previous *in silico* analyses of *P. patens* AOX indicates that there are likely three transcript copies due to alternative splicing and I will confirm AOX transcript expression by RT-PCR. These hypotheses will be tested in Chapter 2. It is also hypothesized that the AOX enzyme is active in moss due to the presence of the conserved glutamate, histidine, and cysteine residues necessary for protein activity as determined from previous bioinformatics results. Due to the previous success of heterologous expression of non-plant AOXs in yeast, I also expect that recombinant moss AOX will be detected in isolated yeast mitochondria by an AOX specific antibody as described by Lang et al. (2011). Through respirometry, I anticipate that wild-type AOX will be functional and that activity will be abolished in mutant AOX strains. These hypotheses will be tested in Chapter 3.

References

Albury MS, Affourtit C, Crichton PG, Moore AL. 2002. Structure of the plant alternative oxidase. J Biol Chem. 277: 1190-1194.

Angioy AM, Stensmyr MC, Urru I, Puliafito M, Collu I, Hansson BS. 2004. Function of the heater: the dead horse arum revisited. Proc R Soc Lond [Biol]. 271: S13-S15.

Bendall DS, Bonner WD. 1971. Cyanide-insensitive respiration in plant mitochondria. Plant Physiol. 47: 236-245.

Berthold DA, Andersson ME, Norlund P. 2000. New insight into the structure and function of the alternative oxidase. Biochim Biophys Acta. 1460: 241-254.

Berthold DA, Voevodskaya N, Stenmark P, Graslund A, Norlund P. 2002. EPR studies of the mitochondrial alternative oxidase. J Biol Chem. 277: 43608- 43614.

Brooker RJ, Widmaier EP, Graham LE, Stiling PD. 2008. Biology, 1st edition. New York (NY): McGraw-Hill. 135-142 p.

Chater C, Kamisugi Y, Movahedi M, Fleming A, Cuming AC, Gray JE, Beerling DJ. 2011. Regulatory mechanism controlling stomatal behavior conserved across 400 million years of land plant evolution. Curr Biol. 21: 1025-1029.

Cho SH, Quatrano RS, Shin JS. 2007. Transgenesis of *Physcomitrella patens*. Transgenic Plant J. 1: 99-103.

Considine MJ, Holtzapffel RC, Day DA, Whelan J, Millar AH. 2002. Molecular distinction between alternative oxidase from monocots and dicots. Plant Physiol. 129: 949-953.

Costa JH, Mota EF, Cambursano MV, Lauxmann MA, de Oliveira LMN, Lima MDS, Orellano EG, de Melo DF. 2010. Stress-induced co-expression of two alternative oxidase (*VuAox*1 and *2b*) genes in *Vigna unguiculata*. J Plant Physiol. 167: 561-570.

Cove D. 2005. The moss *Physcomitrella patens*. Annual Review of Genetics. 39: 339-358.

Dinant M, Baurain D, Coosemans N, Joris B, Matagne RF. 2001. Characterization of two genes encoding the mitochondrial alternative oxidase in *Chlamydomonas reinhardtii*. Curr Genet. 39: 101-108.

Frederico AM, Zavattieri MA, Campos MD, Cardoso HG, McDonald AE, Arnholdt-Schmitt B. 2009. The gymnosperm *Pinus pinea* contains both AOX gene subfamilies, AOX1 and AOX2. Physiol Plant. 137: 566-577.

Guy RD, Berry JA, Fogel ML, Hoering TC. 1989. Differential fractionation of oxygen isotopes by cyanide-sensitive respiration in plants. Planta. 177: 483-491.

Hohe A, Egener T, Lucht JM, Holtorf H, Reinhard C, Schween G, Reski R. 2004. An improved and highly standardised transformation procedure allows efficient production of single and multiple targeted gene-knockouts in a moss, *Physcomitrella patens*. Current Genetics. 44: 339-347.

Holtzapffel RC, Castelli J, Finnegan PM, Millar AH, Whelan J, Day DA. 2003. A tomato alternative oxidase protein with altered regulatory properties. Biochim Biophys Acta. 1606: 153-162.

Johnson-Flanagan AM, Owens JN. 1986. Root respiration in white spruce (*Picea glauca* [Moench] Voss) seedlings in relation to morphology and environment. Plant Physiol. 81: 21-25.

Lang EG, Mueller SJ, Hoernstein SNW, Porankiewicz-Asplund J, Vervliet-Scheebaum M, Reski R. 2011. Simultaneous isolation of pure and intact chloroplasts and mitochondria from moss as the basis for sub-cellular proteomics. Plant Cell Rep. 30: 205-215.

Mariano AB, Valente C, Maurer JBB, Cadena SMSC, Rocha MEM, de Oliveira MBM, Salgado I, Carnieri EGS. 2008. Functional characterization of mitochondria isolated from the ancient gymnosperm *Araucaria angustifolia*. Plant Sci. 175: 701-705.

Maxwell DP, Wang Y, McIntosh L. 1999. The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. Proc Natl Acad Sci USA. 96: 8271-8276.

McDonald AE. 2008. Alternative oxidase: an inter-kingdom perspective on the function and regulation of this broadly distributed 'cyanide-resistant' terminal oxidase. Funct Plant Biol. 35: 535-552.

McDonald AE, Sieger SM, Vanlerberghe GC. 2002. Methods and approaches to study plant mitochondrial alternative oxidase. Physiol Plant. 116: 135-143.

McDonald AE, Vanlerberghe GC. 2006. Origins, evolutionary history, and taxonomic distribution of alternative oxidase and plastoquinol terminal oxidase. Comp Biochem Physiol D. 1: 357-364.

Prigge MJ, Bezanilla M. 2010. Evolutionary crossroads in developmental biology: *Physcomitrella patens*. Development. 137: 3535-3543.

Rensing SA, Land D, Zimmer AD, Terry A, Salamov A, Shapiro H, Nishiyama T, Perroud P, Lindquist EA, Kamisugi Y, Tanahashi T, Sakakibara K, Fujita T, Oishi K, Shin T, Kuroki Y, Toyoda A, Suzuki Y, Hashimoto S, Yamaguchi K, Sugano S, Kohara Y, Fujiyama A, Anterola A, Aoki S, Ashton N, Barbazuk WB, Barker B, Bennetzen JL, Blankenship R, Cho SH, Dutcher SK, Estelle M, Fawcett JA, Gundlach H, Hanada K, Heyl A, Hicks KA, Hughes J, Lohr M, Mayer K, Melkozernov A, Murata T, Nelson DR, Pils B, Prigge M, Reiss B, Renner T, Rombauts S, Rushton PJ, Sanderfoot A, Schween G, Shiu S, Stueber K, Theodoulou FL, Tu H, Van de Peer Y, Verrier PJ, Waters E, Wood

A, Yang L, Cove D, Cuming AC, Hasebe M, Lucas M, Mishler BD, Reski R, Grigoriev IV, Quatrano RS, Boore JL. 2008. The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. Science. 319: 64-69.

Saika H, Ohtsu K, Hamanaka S, Nakazono M, Tsutsumi N, Hirai A. 2002. AOX*1c*, a novel rice gene for alternative oxidase; comparison with rice AOX*1a* and AOX*1b*. Genes Genet Syst. 77: 31-38.

Siedow JN, Umbach AL. 1995. Plant mitochondrial electron transfer and molecular biology. Plant Cell. 7: 821-831.

Siedow JN, Umbach AL, Moore AL. 1995. The active site of the cyanide-resistant oxidase from plant mitochondria contains a binuclear iron center. FEBS Lett. 362: 10-14.

Smidkova M, Hola M, Angelis KJ. 2010. Efficient biolistic transformation of the moss *Physcomitrella patens*. Biol Plantarum. 54: 777-780.

Timme RE, Bachvaroff TR, Delwiche CF. 2012. Broad phylogenetic sampling and the sister lineage of land plants. PLOS ONE. 7: e29696.

Umbach AL, Siedow JN. 1993. Covalent and non-covalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. Plant Physiol. 103: 845-854.

Van Aken O, Giraud E, Clifton R, Whelan J. 2009. Alternative oxidase: a target and regulator of stress responses. Physiol Plant. 137: 354-361.

Vanlerberghe GC, McIntosh L. 1997. Alternative oxidase: from gene to function. Annu Rev Plant Physiol Plant Mol Biol. 48: 703-734.

Vanlerberghe GC, McIntosh L, Yip JYH. 1998. Molecular localization of a redox-modulated process regulating plant mitochondrial electron transport. Plant Cell. 10: 1551-1560.

Watling JR, Robinson SA, Seymour RS. 2006. Contribution of the alternative pathway to respiration during thermogenesis in flowers of the sacred lotus. Plant Physiol. 140: 1367-1373.

Weger HG, Guy RD. 1991. Cytochrome and alternative pathway respiration in white spruce (*Picea glauca*) roots- effects of growth and measurement temperature. Physiol Plant. 83: 675-681.

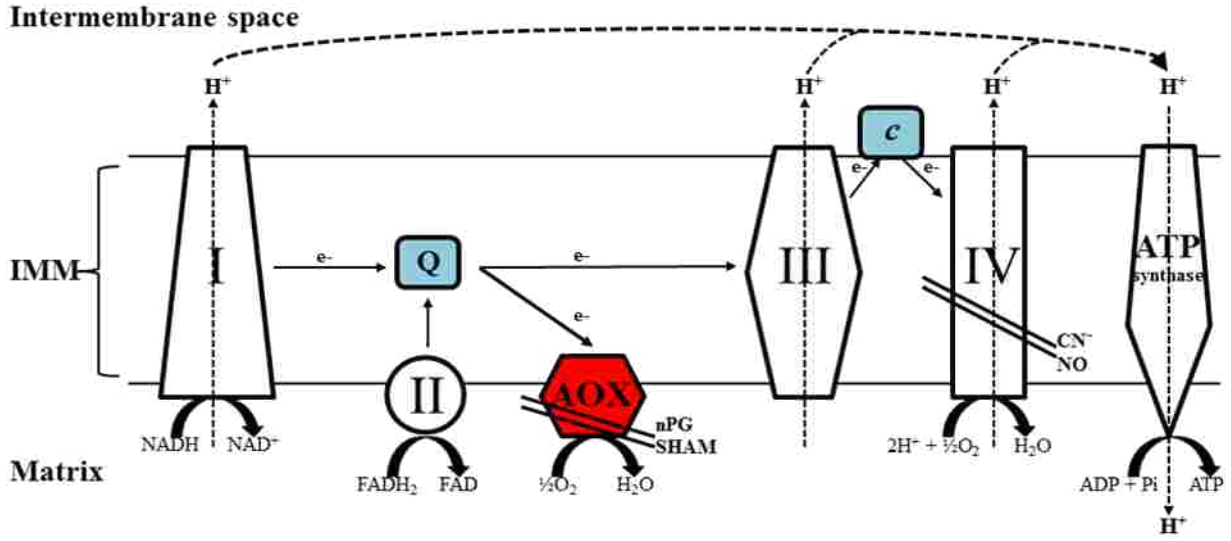

Figure 1.1. A representative diagram of the alternative oxidase (AOX) pathway within the respiratory electron transport system (ETS). The four protein complexes (I to IV) within the ETS sequentially transfer electrons (e-) by way of redox reactions. High-energy electrons are transferred to complex I and II by the oxidation of NADH and $FADH_2$, respectively. One-by-one, electrons are passed to ubiquinone (Q), complex III, cytochrome c (*c*), and lastly to complex IV. Simultaneously, protons (H^+) are pumped across the inner mitochondrial membrane (IMM) and into the intermembrane space by complexes I, III, and IV. The translocation of protons creates an electrochemical gradient that can be used to generate ATP by the enzyme ATP synthase. The AOX pathway branches at ubiquinone and bypasses two proton-pumping sites. AOX is resistant to all inhibitors of complex IV, such as cyanide (CN^-) and nitric oxide (NO). However, AOX can be inhibited by the compounds n-propyl gallate (nPG) and salicylhydroxamic acid (SHAM). The AOX protein is a terminal quinol oxidase and dissipates energy as heat by the reduction of oxygen to water (Berthold et al., 2002). Unlike the complexes within the ETS, AOX is non-proton pumping and as a result yields less ATP. Figure derived from McDonald, 2008.

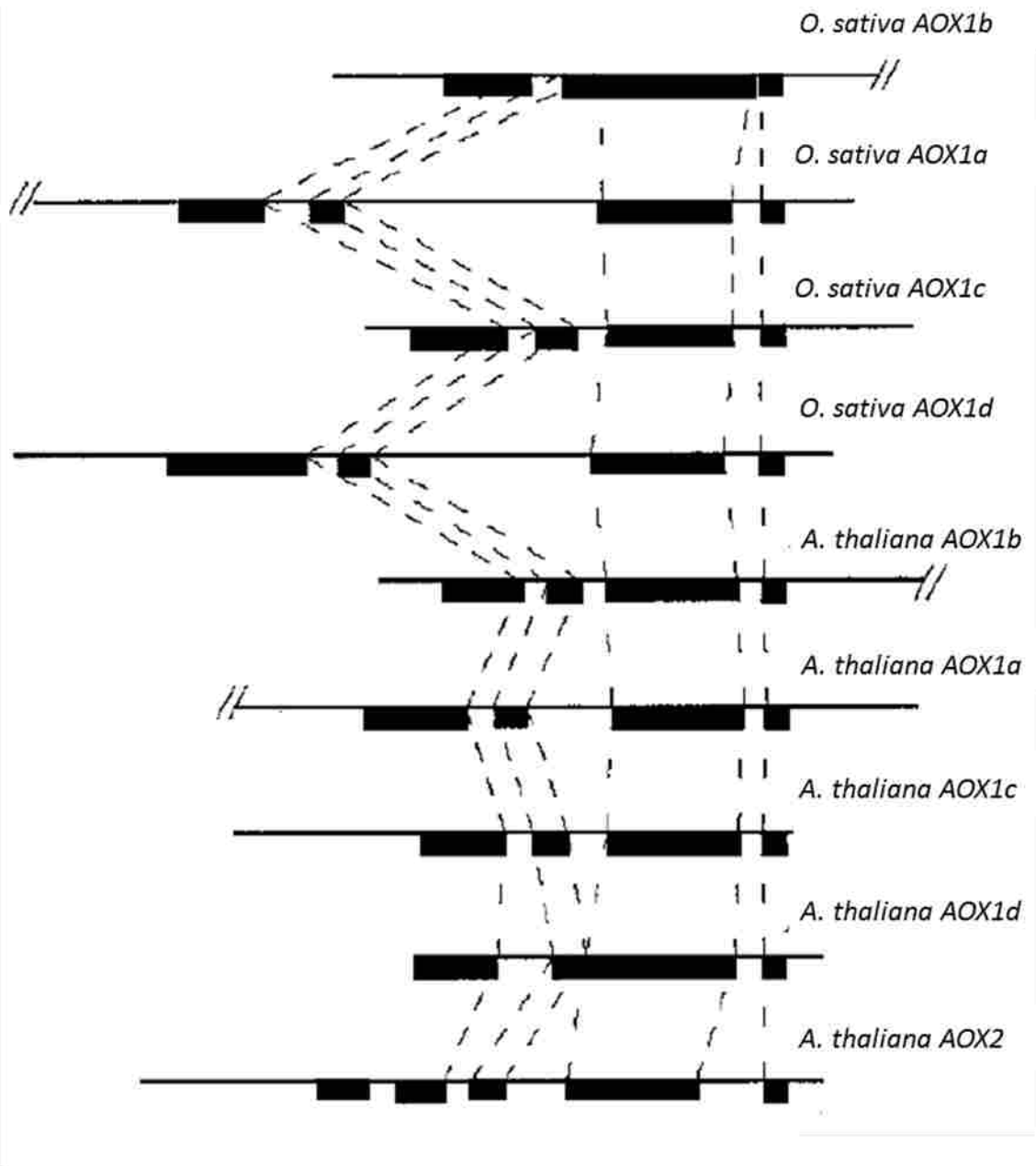

Figure 1.2. Intron/exon structure of AOX genes in rice (*Oryza sativa*) and *Arabidopsis thaliana*. Straight black line, intron; black rectangle, exon; dashed lines, conserved regions. Figure obtained from Considine et al., 2002.

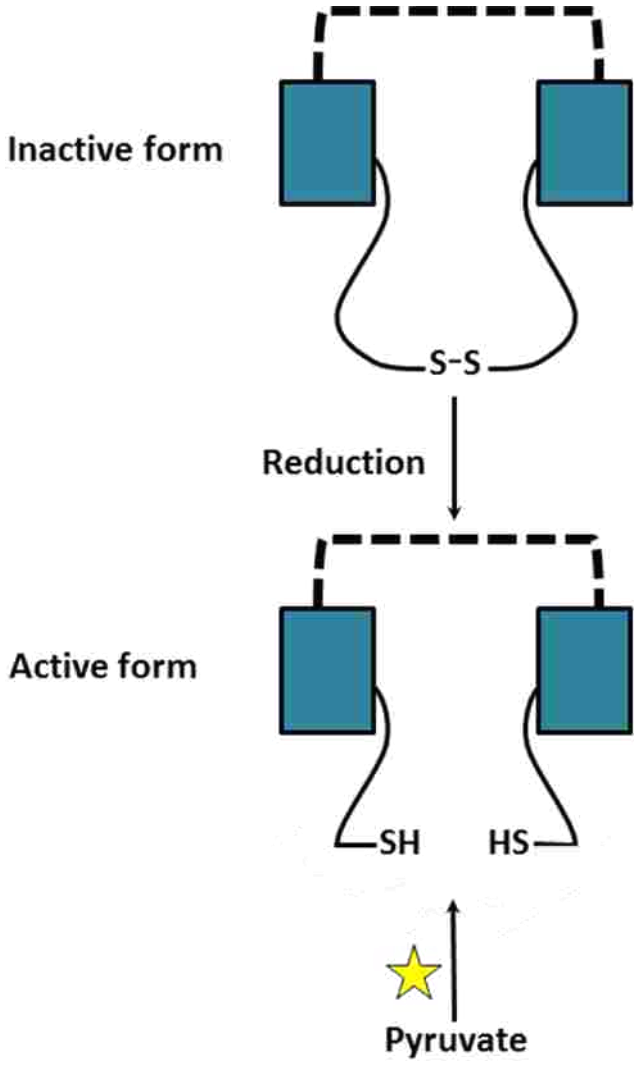

Figure 1.3. Post-translational regulation of angiosperm AOX dimeric proteins. A disulfide bridge covalently links the monomeric subunits (blue rectangles) of the inactive protein. AOX is activated when the disulfide-linked subunits are reduced. Further activation of the reduced dimer can be achieved in the presence of pyruvate. Hashed black line, the non-covalent association of the AOX dimer.

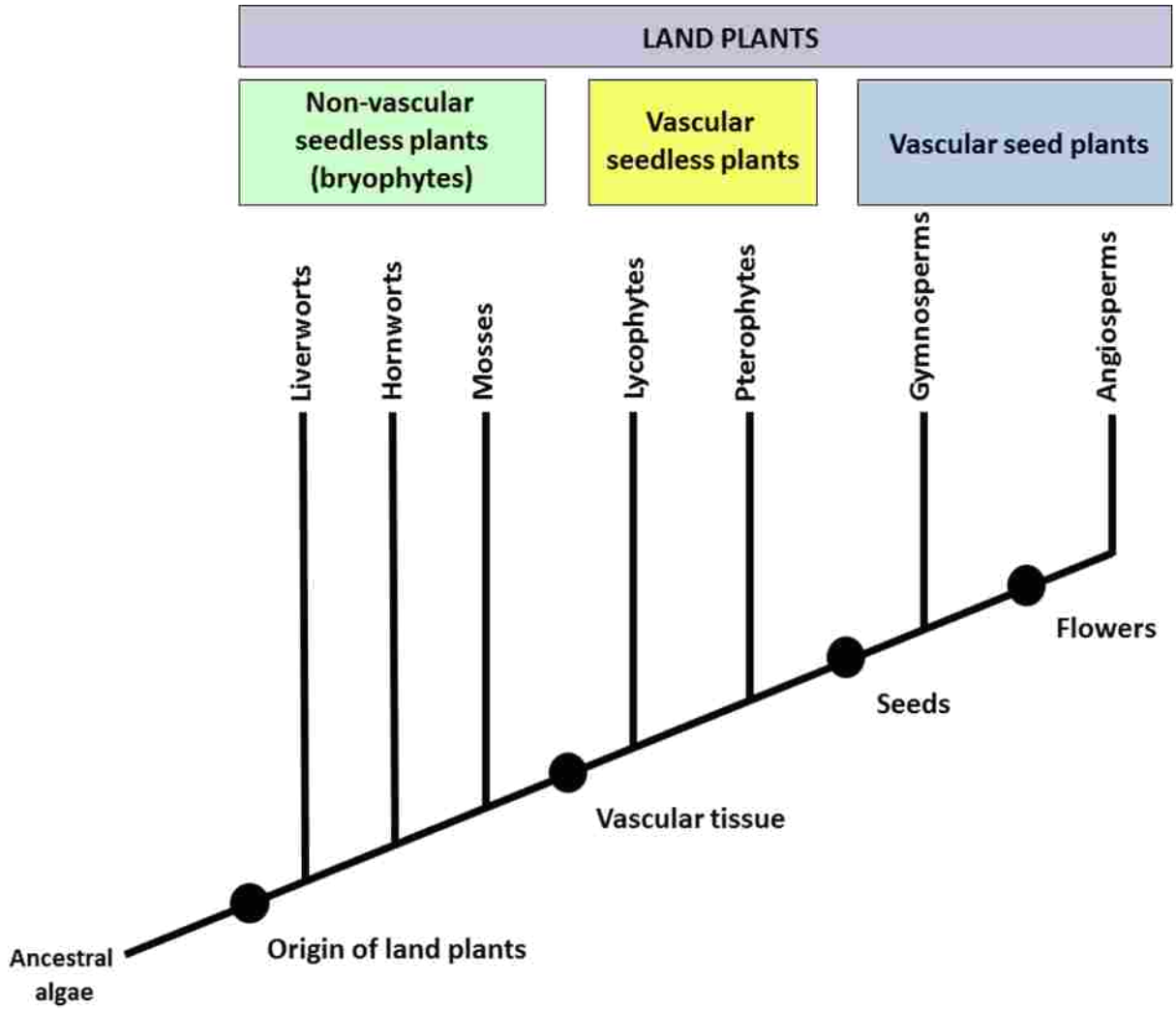

Figure 1.4. Evolutionary history of land plants showing the development of key evolutionary traits.

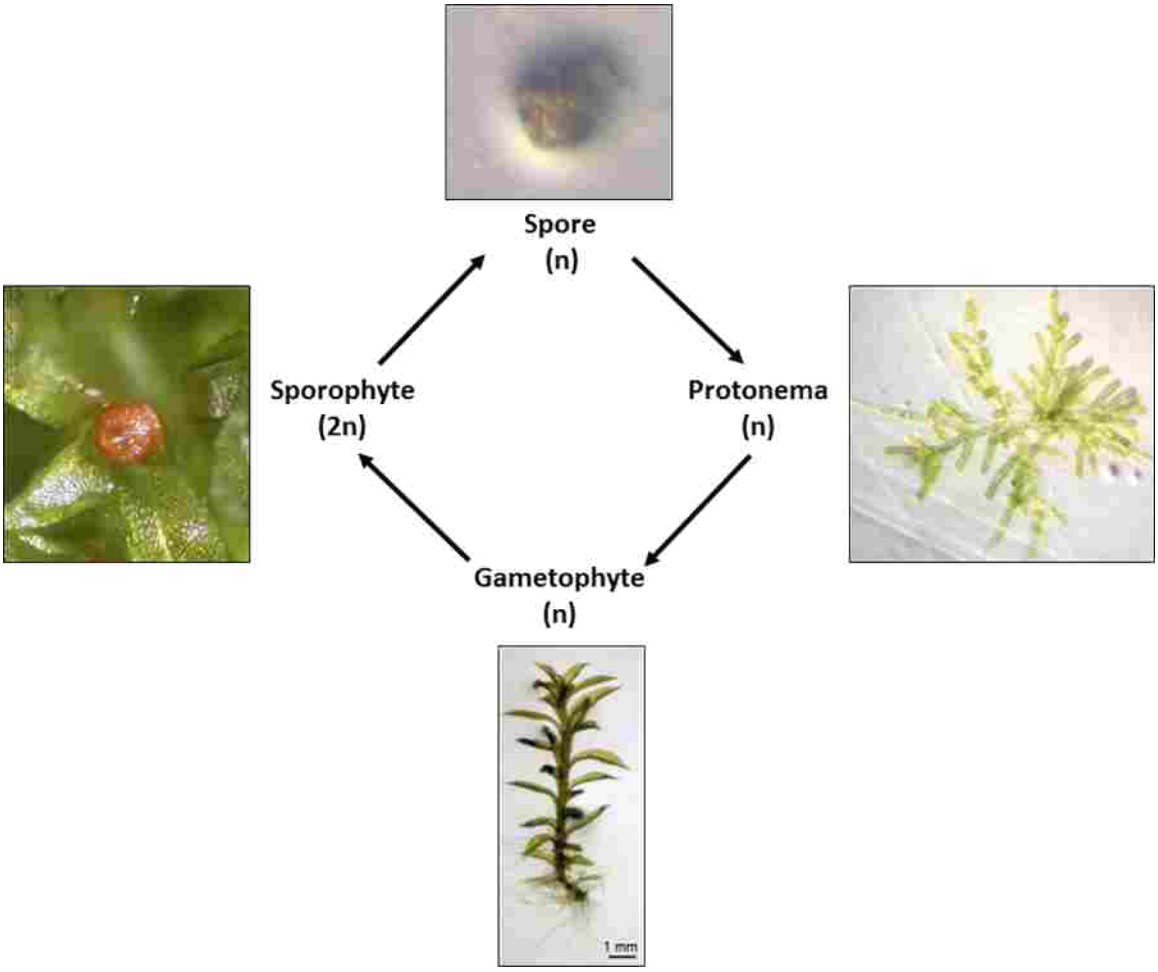

Figure 1.5. *P. patens* life cycle starting with the germination of spores. n, haploid number; 2n, diploid number. Photos were obtained from Cho et al., 2007 and Prigge and Bezanilla, 2010.

CHAPTER 2

Identification, expression, and taxonomic distribution of alternative oxidases in non-angiosperm plants

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Author contributions:

Karina Neimanis: collection of the non-angiosperm land plant AOX sequences, wet lab experiments, and co-writing the manuscript.

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Dr. Allison McDonald: collection of the non-angiosperm algal and land plant AOX sequences and co-writing the manuscript.

Abstract

Alternative oxidase (AOX) is a terminal ubiquinol oxidase present in the respiratory chain of all angiosperms investigated to date, but AOX distribution in other members of the Viridiplantae is less clear. The taxonomic distribution of AOX was assessed using bioinformatics. Multiple sequence alignments compared AOX proteins and examined amino acid residues involved in AOX catalytic function and post-translational regulation. Novel AOX sequences were found in both Chlorophytes and Streptophytes and it is concluded that AOX is widespread in the Viridiplantae. AOX multigene families are common in non-angiosperm plants and the appearance of AOX1 and AOX2 subtypes pre-dates the divergence of the Coniferophyta and Magnoliophyta. Residues involved in AOX catalytic function are highly conserved between Chlorophytes and Streptophytes, while AOX post-translational regulation likely differs in these two lineages. The presence of an AOX gene has been demonstrated experimentally in the moss *Physcomitrella patens* and that the gene is transcribed. The findings suggest that AOX will likely exert an influence on plant respiration and carbon metabolism in non-angiosperms such as green algae, bryophytes, liverworts, lycopods, ferns, gnetophytes, and gymnosperms and that further research in these systems is required.

1. Introduction

The phenomenon of cyanide-resistant respiration was first described in plant mitochondria (Bendall and Bonner, 1971) and results from the action of the enzyme alternative oxidase (AOX). AOX is a terminal oxidase that introduces a branch point in the respiratory electron transport system (ETS) at the level of ubiquinone. Electron flux through the AOX pathway bypasses two of the three proton pumping complexes in the ETS and thereby lowers ATP production per oxygen consumed and, as such, has been described as energetically wasteful (Moore and Siedow, 1991). The physiological role of AOX is still under investigation, but it is known to play a role in thermogenesis and pollination regimes in some angiosperms (Grant et al., 2009; Seymour and Matthews, 2006). AOX may also reduce the generation of reactive oxygen species (ROS) which can result in oxidative damage to the mitochondria and other cellular components (Maxwell et al., 1999). AOX may aid in balancing carbon metabolism and electron flow in plants (McDonald, 2008). Despite the fact that AOX activity is energetically costly, it has a wide taxonomic distribution and is present in some plants, fungi, animals, protists, and bacteria (McDonald and Vanlerberghe, 2006).

AOX has been most thoroughly studied within the Viridiplantae, also known as the green lineage, which is a monophyletic group that is comprised of the green algae and land plants (Leliaert et al., 2012). This clade is thought to have originated more than 1.5 billion years ago (Bya) as a result of a primary endosymbiotic event involving a cyanobacterium and a eukaryotic host which led to the formation of plastids (Sanderson et al., 2004). Molecular clock studies estimate that this group of oxygenic, photosynthetic organisms diverged into two distinct clades, the Chlorophyta and Streptophyta, approximately 725-1200 million years ago (Mya) (Becker and

Marin, 2009). The Chlorophytes, which include the majority of green algal species, inhabit freshwater, marine, and some terrestrial environments and can be divided into four distinct classes: Prasinophyceae, Trebouxiophyceae, Chlorophyceae, and Ulvophyceae (Becker and Marin, 2009; Leliaert et al., 2012). The divergence of the Streptophyta into streptophyte green algae and terrestrial plants (Embryophytes) is estimated to have occurred 646-792 Mya (Sanderson et al., 2004). The streptophytes are freshwater algae that can be classified into six groups based on morphological differences: Mesostigmatophyceae, Chlorokybophyceae, Klebsormidiophyceae, Zygnematophyceae, Coleochaetophyceae, and Charophyceae (Becker and Marin, 2009; Leliaert et al., 2012).

It is widely accepted that Streptophyte algae are the closest living relatives to terrestrial plants. Becker and Marin (2009) speculate that the freshwater environment inhabited by Streptophytes made it much easier to transition onto dry land compared to the high saline marine environments inhabited by many Chlorophytes. The earliest land plants (non-angiosperms) originated from green algae approximately 432-476 Mya (Bhattacharya and Medlin, 1998; Yoon et al., 2004) and the Zygnematales are the likely sister group to land plants (Leliaert et al., 2012). Land plants can be separated into three groups; the first group consists of the non-vascular seedless plants collectively known as the bryophytes and includes liverworts (Marchantiophyta), hornworts (Anthocerotophyta), and mosses (Bryophyta). The development of vascular tissue characterizes the second group that is comprised of lycophytes (clubmosses, spikemosses, and quillworts) and pterophytes (ferns and horsetails). Lastly, the origin of seeds by the gymnosperms marked an important evolutionary development in plants. Gymnosperms can be

further classified into four divisions: Cycadophyta, Ginkgophyta, Gnetophyta, and Coniferophyta.

The majority of AOX research in plants has focused on the Streptophyta, and specifically, a small number of angiosperm species such as *Arabidopsis thaliana* (Saisho et al., 1997), tobacco (*Nicotiana tabacum*) (Vanlerberghe and McIntosh, 1994), soybean (*Glycine max*) (Whelan et al., 1996), rice (*Oryza sativa*) (Ito et al., 1997), maize (*Zea mays*) (Karpova et al., 2002) and several species of thermogenic aroids (Onda et al., 2008). The only Chlorophyte in which AOX has been characterized in any depth is *Chlamydomonas reinhardtii* (Dinant et al., 2001).

No previous investigation of AOX has focused on the Streptophyte algae. Within the Streptophyte land plants, reports of AOX in basal plants and gymnosperms are scarce, but cyanide-resistant mitochondrial respiration has been reported in white spruce (*Picea glauca*) (Johnson-Flanagan and Owens, 1986; Weger and Guy, 1991) and *Araucaria angustifolia* (Mariano et al., 2008). It is also hypothesized that AOX might be responsible for the thermogenesis seen in various cycad species (Skubatz et al., 1993). Previous work has demonstrated that AOX has a higher ability to discriminate against ^{18}O compared to cytochrome c oxidase (COX) (Robinson et al., 1992). Work examining the soil discrimination for ^{18}O indicates that a high fraction of AOX respiration occurs in a boreal site dominated by *Picea mariana* (black spruce) and the moss *Hylocomium* (Angert et al., 2003a). Further work examining the isotopic composition of atmospheric O_2 (^{18}O vs. ^{16}O) indicates that the global rate of the AOX pathway has been underestimated (Angert et al., 2003b). Previous bioinformatics work indicates that AOX is present in Embryophyte lineages other than angiosperms (Frederico

et al., 2009; McDonald and Vanlerberghe, 2006). Only a limited number of basal plant AOXs have been identified, these include: *Physcomitrella patens*, *Selaginella lepidophylla*, and *Selaginella moellendorffii* (Frederico et al., 2009). The majority of non-angiosperm AOX sequences have been identified from a number of gymnosperm species such as *Cryptomeria japonica, Picea engelmannii, Picea glauca, Picea sitchensis, Pinus pinaster, Pinus pinea, Pinus taeda,* and *Pseudotsuga menziesii* (Frederico et al., 2009; McDonald and Vanlerberghe, 2006). Despite this somewhat limited assessment, AOX is often described as being ubiquitous in plants, however no focused study has ever been conducted to assess the taxonomic distribution of AOX in the Viridiplantae.

AOX in angiosperms is encoded by a multigene family that typically contains three to five gene copies (Saisho et al., 1997; Whelan et al., 1996). Dicots contain two subtypes, AOX1 and AOX2, while monocots contain only the AOX1 subtype; the physiological relevance of this distinction remains to be investigated (Considine et al., 2002). Recent work has demonstrated that the AOX1 and AOX2 subtypes are present in gymnosperms (Frederico et al., 2009), but it is unknown whether other non-angiosperms contain multiple AOX genes and what their complement of AOX1 and/or AOX2 genes might be.

Electron paramagnetic resonance technology has confirmed that AOX belongs to the family of di-iron carboxylate proteins (Berthold et al., 2002; Moore et al., 2008). These proteins are characterized by a series of highly conserved glutamate (Glu) and histidine (His) residues that are required for the co-ordination of the di-iron centre of the enzyme (Siedow et al., 1995). These Glu and His residues are conserved in AOXs in organisms from a wide variety of kingdoms (McDonald, 2008). In addition, a conserved tyrosine residue (TyrII/Tyr-275) is

required for AOX activity (Moore and Albury, 2008). Albury et al. (2010) observed a hydrophobic region of the protein that is thought to play a role in the binding of ubiquinol. The side groups, size and charge properties associated with conserved tyrosine (TyrI/Tyr-253), glutamine (GlnI/Gln-242), serine (Ser-256), histidine (HisIII/His-261), and arginine (Arg-262) residues are thought to play an important role at this site. A study by Crichton et al. (2009) identified a conserved tryptophan (TrpI/Trp-206) residue that is thought to play a role in anchoring AOX to the inner mitochondrial membrane (numbering as per the *S. guttatum* sequence). Recently, ThrI/Thr-179 and CysII/Cys-172 have been proposed to affect the catalytic cycle of the enzyme with respect to its interactions with oxygen (Crichton et al., 2010). Recent work examining the crystal structure of AOX in the protist *Trypanosoma brucei* has led to important insights about the structure and catalytic cycle of the enzyme (Shiba et al., 2013).

All angiosperms investigated to date contain an AOX protein that is present as non-covalently associated dimers (Onda et al., 2007; Umbach and Siedow, 1993). The AOX protein of most angiosperms is subject to post-translational regulation via redox control of an inter-subunit disulfide bond that has been localized to a key cysteine residue (CysI) in the N-terminal region (Rhoads et al., 1998; Vanlerberghe et al., 1998). Upon reduction of this disulfide bond, the enzyme is subject to additional regulation by pyruvate leading to a further increase in its activity (Rhoads et al., 1998). Some angiosperms possess a naturally occurring serine residue in this position (SerI) (Holtzapffel et al., 2003) causing the enzyme to be regulated by succinate instead of pyruvate (Grant et al., 2009; Holtzapffel et al., 2003). A second N-terminal cysteine residue, CysII, may be involved in AOX regulation by the α-keto acid glyoxylate (Umbach et al., 2006). It is not known whether non-angiosperm plants possess CysI or SerI and/or CysII or SerII

residues in these positions and this may have implications for the post-translational regulation of these AOXs.

The purpose of this study was to: 1) assess the taxonomic distribution of AOX within non-angiosperm plants, 2) determine whether particular species contain a single AOX gene copy or multiple AOX genes and what complement of AOX1 and AOX2 subtypes are present, 3) determine if amino acid residues that are involved in AOX post-translational regulation and are required for catalytic function in angiosperms are conserved in all members of the Viridiplantae, and 4) experimentally confirm the presence of AOX gene(s) and the expression of AOX mRNA in a non-angiosperm species. Bioinformatics was used to perform a molecular database search to identify AOX sequences from members of the non-angiosperms in order to address the above questions. To validate the bioinformatics results, polymerase chain reaction (PCR) was used to amplify AOX genes and reverse transcriptase PCR (RT-PCR) to examine AOX transcript levels in the moss *Physcomitrella patens*.

The results demonstrate that AOX is an ancestral protein in the Viridiplantae and that it is likely widespread in plants. I demonstrate that AOX multigene families are present in some members of the Chlorophyta and Streptophyta, and that the moss *Physcomitrella patens* most likely possesses a single AOX gene copy. The data confirm the claim that the AOX1 and AOX2 subtypes arose prior to the divergence of the Coniferophyta and Magnoliophyta. An analysis of AOX proteins in the Viridiplantae indicates that amino acids involved in the catalytic function of AOX are highly conserved in all lineages, but that differences exist at the amino acid level that are likely to confer different post-translational modes of regulation for AOXs of different groups. Specifically, it is highly probable that SerI is the ancestral residue in this position and that the

CysI mutation has arisen independently several times in different plant lineages and is an example of convergent evolution. AOX in *P. patens* was further analyzed experimentally and it was demonstrated that an AOX gene is present and that AOX transcripts are expressed in this species.

2. Materials and Methods

2.1. *In silico* Recovery of Non-Angiosperm AOXs

Molecular database searches to identify putative non-angiosperm AOX sequences were conducted using the BLASTn and tBLASTn software available online at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) using previously identified angiosperm AOX sequences (McDonald and Vanlerberghe, 2006) and searching the non-redundant, trace archive, and expressed sequence tags (EST) databases at NCBI and the databases at the Department of Energy Joint Genome Institute (www.jgi.doe.gov/). As new non-angiosperm AOX sequences were identified they were then used to search for other novel non-angiosperm AOX sequences in the above databases. In addition to the above molecular databases, a putative *P. patens* AOX sequence was retrieved from the complete *P. patens* genome at the Phytozome website (http://www.phytozome.net/) (Goodstein et al., 2012), a database that provides public access to over forty fully sequenced plant genomes.

2.2. *In silico* Analyses- Identification and Verification of Non-angiosperm AOX Sequences

Retrieved AOX DNA sequences were translated into protein sequences using the Translate tool located on the ExPASy server (http://ca.expasy.org/tools/dna.html). Putative AOX protein sequences had their identity verified using multiple sequence alignments with other plant AOX

sequences and the presence of one or more iron-binding sites was used as a means of positive identification (McDonald et al., 2003). Multiple sequence alignments of AOX proteins were generated using the Clustal X program (Thompson et al., 1997).

The retrieved *P. patens* AOX peptide sequence were placed in the mitochondrial targeting prediction programs MitoProt II- v1.101 (http://ihg.gsf.de/ihg/mitoprot.html) and TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/) to evaluate the probability that this protein is mitochondrially targeted in moss.

2.3. Plant Materials and Growth Conditions

Physcomitrella patens subsp. *patens* (Gransden; IMSC#40001) wild-type isolates were obtained from the International Moss Stock Centre in Germany. Somatic gametophyte tissue was grown in Petri plates with BCD solid medium supplemented with 5 mM diammonium tartrate (Cove et al., 2009; Appendix 1) and sealed with 3M™ Micropore™ tape. Cultures were incubated at room temperature (25°C) with constant white light (6.0 Wm^{-2}) under long-day conditions (16h light: 8h dark photoperiod) and sub-cultured every 6-8 weeks in a sterile flow hood.

2.4. Nucleic Acid Isolations and Amplification Parameters

Moss tissue was covered with liquid nitrogen in a chilled mortar and was ground into a fine powder using a pestle. Genomic DNA was extracted from 100 mg of moss gametophyte tissue with the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich Co., St. Louis, MO, USA) according to the manufacturer's instructions. Before processing, *P. patens* plants were subjected to cold treatment at 4°C for 1 hour to increase AOX transcript expression. Total

RNA was isolated from 300-400 mg of chilled gametophore tissue using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) as outlined by the manufacturer's directions. Prior to reverse transcriptase (RT)-PCR, *P. patens* RNA was treated with RQ1 RNase-Free DNase (Promega Corporation, Madison, WI, USA) to remove residual DNA from the sample. The concentration and purity of isolated genomic DNA and total RNA were assessed using a NanoPhotometer™ (Implen GmbH, München, Germany).

Custom primers were designed using the program Primer3 (http://frodo.wi.mit.edu/primer3/) to amplify *P. patens* AOX. The Access RT-PCR Introductory System (Promega Corporation, Madison, WI, USA) was used in 50 µL reactions for both PCR and RT-PCR. For each PCR, 20 ng genomic DNA was used with the *P. patens* full-length genomic AOX coding region forward (5′-ATGTTGGGAAGGGTAGGATC-3′) and *P. patens* full-length genomic AOX coding region reverse (5′-CTAGTGGTACCCGACGGGAG-3′) primers. The thermal cycler was programmed for 30 rounds of amplification where each cycle consisted of 30 sec. at 94°C for denaturation, 1 min. at 53°C for annealing, and 1 min. at 68°C for elongation where the final elongation step was extended for 7 min. during the last cycle.

For each RT-PCR reaction, the internal moss transcript forward (5′-GGATCGGAGGAAAAATTCGT-3′) and internal moss transcript reverse (5′-GGCGAAATGGTTAACGTCAC-3′) primers or the full-length coding forward (5′-ATGTTGGGAAGGGTAGGATC-3′) and full-length coding reverse (5′-CTAGTGGTACCCGACGGGAG-3′) primers were used with 2 µg of total RNA template. Identical amplification conditions to those described above were used to carry out RT-PCR with the addition of an initial reverse transcription step at 45°C for 45 min. and a 55°C or 53°C

annealing temperature for the internal moss transcript primers or full-length coding primers, respectively. All amplification products were separated using gel electrophoresis on a 1.2% agarose gel stained with ethidium bromide.

2.5. cDNA Cloning and Sequence Analysis

Amplified cDNA fragments were purified using the QIAquick® Gel Extraction Kit (QIAGEN Sciences, Maryland, USA) and quantified with the NanoPhotometer™ (Implen GmbH, München, Germany) before ligation into the pGem®-T Easy Vector (Promega Corporation, Madison, WI, USA), according to the manufacturer's directions. The insert: vector ratio was maximized in a 3:1 ratio using the equation:

$$\frac{\text{vector (ng)} \times \text{insert size (kb)}}{\text{vector size (kb)}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

Reactions were stored overnight at 4˚C to optimize ligation efficiency. Transformations were carried out in 14 mL polypropylene round-bottom Falcon™ tubes **(**BD Biosciences, Mississauga, ON**)** containing 2 µL ligation reaction and 50 µL of One Shot® TOP10 chemically competent *Escherichia coli* (Invitrogen, Carlsbad, CA, USA). Cells were kept on ice for 30 min. followed by a 30 sec. incubation at 42˚C the AccuBlock™ Digital Dry Bath (Labnet International, Inc., NJ, USA), and then immediately placed back onto ice. SOC medium (250 µL) was added to each tube and then incubated for 1 h. at 37˚C with 225 rpm shaking. Two different volumes (50 µL and 100 µL) of transformed cells were plated onto Luria-Bertani (LB) solid media supplemented with 100 µg/mL ampicillin that was overlaid with 40 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in dimethyl sulfoxide (DMSO), and 23 mg/mL isopropyl-β-D-thio-galactoside (IPTG). Plates were inverted and incubated overnight at 37˚C. Blue/white selection

was used to identify positive transformants that were prescreened using colony PCR (identical amplification parameters were used as described in Section 2.4.). Positive transformants were sub-cultured into 5 mL of liquid LB containing 100 µg/mL ampicillin and incubated for 24 h. at 37˚C with 150 rpm shaking. Recombinant plasmid DNA was then purified using the QIAprep® Spin Miniprep Kit (QIAGEN Sciences, Maryland, USA) before sequencing at the Centre for Applied Genomics at the Hospital for Sick Children in Toronto, Ontario.

3. Results

3.1. Recovery of Novel AOX Sequences Using Bioinformatics

Viridiplantae AOX sequences recovered using molecular database searches, exclusive of those found in angiosperms, are shown in Table 2.1. Within the Chlorophyta, AOX was present in several members of the Chlorophyceae including several species of the genus *Chlamydomonas* and a single species of *Volvox* (Table 2.1). *Chlamydomonas reinhardtii* was the only Chlorophyte to exhibit an AOX multigene family as it encodes two AOX genes in its genome (Table 2.1). AOX was present in the Mamiellophyceae in the species *Bathycoccus prasinos*, in the Prasinophyceae in several species of *Ostreococcus* and in the Trebouxiophyceae in *Prototheca wickerhamii* and *Chlorella vulgaris* (*Coccomyxa sp.* C-169) (Table 2.1). In the Ulvophyceae, AOX was present in *Ulva linza* and in *Acetabularia acetabulum* (Table 2.1). AOX was also recovered from two uncultured phototrophic eukaryotes (Table 2.1). No AOX was recovered from a member of the Pedinophyceae.

AOX was recovered from two Streptophyte algae within the Zygnemophyceae in the species *Spirogyra pratensis* and *Penium margaritaceum* (Table 2.1). AOX was present in the

Chlorokybophyceae in *Chlorokybus atmophyticus* and in the Klebsormidiophyceae in *Klebsormidium faccidum* and *Klebsormidium subtile*. AOX sequences were recovered from the Charophyceae in *Nitella hyalina* and in Coleochaetophyceae in the species *Chaetosphaeridium globosum* and *Coleochaete orbiculans*. No AOX was found in the Mesostigmatophyceae, but several AOX sequences were recovered from members of the Embryophyta. Within the Marchantiophyta, AOX is present in the liverwort *Marchantia polymorpha* (Table 2.1). Within the Bryophyta, an AOX sequence was found in the genome of the model moss species *Physcomitrella patens* (Table 2.1). No evidence of AOX in the Anthocerotophyta (hornworts) or for a multigene family in any of the above Streptophyte groups was recovered.

AOX is present in several species belonging to the Tracheophyta (Table 2.1). Within the Lycopodiophyta, AOX is found in two different species of Isoetopsida, *Selaginella lepidophylla* and *Selaginella moellendorffii* (Table 2.1). Both species of *Selaginella* present EST data for multiple putative AOX mRNA products, indicating that a multigene family may be present (Table 2.1). No evidence for AOX was found in the Isoetales or in the Lycopodiopsida.

Within the Euphyllophyta, members of both the Moniliformopses and Spermatophyta contain AOX. Within the Moniliformopses, the Polypodiopsida (ferns) contain AOX. AOX is present in the ferns *Adiantum capillus-veneris* and *Ceratopteris richardii* (Table 2.1). No evidence of AOX was found within the Equisetopsida (horsetails), Marattiopsida, Ophioglossopsida, or Psilotopsida (whisk-ferns).

Within the Spermatophyta, evidence for AOX exists for one member of the Gnetophyta in the species *Ephedra distachya* and in several members of the Coniferophyta (Table 2.1). No evidence for AOX was detected in the Ginkgophyta or Cycadophyta.

Within the Coniferales, AOX is present in the Cupressaceae in *Cryptomeria japonica* (Table 2.1). Within the Pinaceae, AOX is present in the genuses *Picea*, *Pinus*, and *Pseudotsuga* (Table 2.1). *Picea glauca* and *Picea sitchensis* contain mRNA encoding two putative different AOX products (Table 2.1). Several different AOX transcripts were also detected in *Pinus taeda* and *Pinus pinea* (Table 2.1). No evidence for AOX was found in the Araucariaceae, Cephalotaxaceae, Podocarpaceae, Sciadopityaceae, or the Taxaceae.

3.2. Characteristics of Non-Angiosperm AOX Proteins

All of the AOXs recovered displayed the extended N-terminal region described in other eukaryotic AOX sequences, in contrast to the short N-termini of prokaryotic AOXs (McDonald, 2008). The most N-terminal part of the protein was highly variable in all of the AOXs recovered (Figure 2.1). An examination of the N-terminal region directly upstream from the first iron-binding site of AOX identified several clear differences between the AOXs of Streptophytes and Chlorophytes (Figure 2.1). All of the Chlorophyte AOX proteins contain an insertion eight amino acids upstream of the L-E-T motif (Figure 2.1). The insertion is 7-9 amino acids in size and shares some sequence similarity within the different Chlorophytes examined (Figure 2.1). Streptophyte AOXs contain two motifs that are lacking in the Chlorophytes: Y-W-G and P-X-E-X-Y (Figure 2.1).

An examination of the two residues that play a role in the post-translational regulation of most angiosperm AOXs (i.e. CysI and CysII) revealed differences in these residues in non-angiosperms. With regards to CysII, a cysteine residue is only found in Streptophytes, but not Chlorophytes (Figure 2.1), however, in several Streptophyte species a cysteine residue was not present at this location (e.g. *P. patens*) (Figure 2.1). An examination of the residue that is

analogous to CysI in angiosperms revealed that in Chlorophytes belonging to the Mamiellophyceae, Prasinophyceae, and Trebouxiophyceae, this residue is a tyrosine (Y) (Figure 2.1). Tyrosine is an amino acid that is encoded by two codons (TAT and TAC). After examining this position at the DNA level, it was observed that both of these codons are used to code for tyrosine in these groups. In Chlorophytes belonging to the Chlorophyceae, this residue is a cysteine or a serine (Figure 2.1). *C. reinhardtii* contains two different AOX proteins, one of which contains a serine and one of which contains a cysteine in this position (Figure 2.1). Within the Streptophytes, all streptophyte algae, except for *P. margaritaceum* which possesses a cysteine, possess an AOX with a serine (Figure 2.1). Within the Embryophyta, a cysteine is found in this position in *P. patens*, *M. polymorpha*, *E. distachya*, and one AOX protein from *S. moellendorffii* (Figure 2.1). In contrast, the other AOXs from *Selaginella*, pines, spruces, and a fern all contain a serine in this position (Figure 2.1). Cysteine is encoded by the codons TGT and TGC, whereas serine has six different possible codons (TCT, TCC, TCA, TCG, AGT and AGC). After analyzing these putative AOX sequences at the DNA level, it was observed that all but one codon (TCT) is used to encode serine at this position in the Viridiplantae. Interestingly, it was observed that all Chlorophyte and Streptophyte species that possess CysI are encoded exclusively by the codon TGC.

An examination of the core of the AOX protein from a variety of Viridiplantae species indicates that the glutamate (E) and histidine (H) residues required for AOX activity are absolutely conserved. Other residues that may be important in AOX catalysis including T179/ThrI, W206/TrpI, Q242/GlnI, Y253/TyrI, H261/HisIII, Y275/TyrII, and Y299/TyrIII (numbering as per the *S. guttatum* sequence) are conserved in the Chlorophytes and

Streptophytes examined (Figure 2.2). The predicted sequence required for AOA antibody recognition (McDonald, 2009) is present in the majority of AOX sequences examined (Figure 2.2). The Chlorophyte AOXs also contain an indel that is not present in Streptophyte AOX proteins (Figure 2.2).

The C-terminal regions of AOX proteins differ between Chlorophytes and Streptophytes (Figure 2.2). The longest C-termini are seen in green algae belonging to the Mamiellophyceae, Prasinophyceae, and Ulvophyceae (Figure 2.2) which contain an extension not seen in members of the Chlorophyceae (Figure 2.2). Despite this, the C-termini of Chlorophytes do exhibit a high degree of sequence similarity to each other (Figure 2.2). The C-termini of Streptophytes also share a high degree of sequence similarity with each other (Figure 2.2). In some AOX sequences from *P. engelmannii*, *P. sitchensis*, *P. menziesii*, and *P. glauca* the last 3 amino acids (D-Y-R) vary from those seen in other Streptophytes (G-Y-H) (Figure 2.2).

3.3. AOX of the Moss *Physcomitrella patens*

A search of the Phytozome database revealed that the moss *P. patens* contains a single AOX gene (Figure 2.3). The genomic sequence comprised of the 5′ untranslated region (UTR), coding DNA sequence (CDS) including introns and exons, and 3′ UTR is predicted to be 3426 nucleotides in length (Figure 2.3A). The *P. patens* AOX transcript sequence is comprised a 5′ UTR, CDS (exons only), and 3′ UTR is predicted to be 2137 nucleotides in length (Figure 2.3B). One primary transcript and two alternative transcripts are predicted (Figure 2.3C). The gene is predicted to contain four exons and three introns and encodes a 365 amino acid protein (Figure 2.3D). MitoProt II software analysis gives a probability of export to the mitochondria for this

AOX of 0.9126, while TargetP 1.1 yielded a probability of 0.856 for mitochondrial targeting (Claros and Vincens, 1996; Emanuelsson et al., 2000).

PCR using AOX gene specific primers and genomic DNA from moss gametophore tissue amplified the expected ~ 2 kB product (Figure 2.4A). RT-PCR using DNase treated total RNA from cold treated gametophore tissue and internal AOX moss transcript specific primers amplified the expected ~700 bp product (primers were designed to amplify a portion of the AOX transcript) (Figure 2.4B) or the full-length coding specific primers to amplify the entire 1098 bp AOX coding region (Figure 2.4C). Cloning and sequencing of the genomic DNA and cDNA products identified AOX sequences that shared 100% identity with the sequences predicted by the Phytozome database. The second ~1.7 kB product (Figure 2.4A) was sequenced and was determined to be a hypothetical protein unrelated to AOX that is encoded by scaffold 111 of the moss genome project.

4. Discussion

4.1. The Taxonomic Distribution of AOX in the Viridiplantae

The Viridiplantae are composed of the Chlorophyta and the Streptophyta which are hypothesized to have diverged from a common ancestor between 725 to 1200 million years ago (Becker and Marin, 2009; Figure 2.5). The Chlorophyta are comprised of members of the Mamiellophyceae, Pedinophyceae, Prasinophyceae, Ulvophyceae, Trebouxiophyceae, and Chlorophyceae (Pombert et al., 2005; Figure 2.5), and AOX was detected in at least one species from 5 of these 6 these groups (Table 2.1). The Prasinophyceae are the most basal Chlorophyte group (Pombert et al., 2005) and AOX is present in several species within the *Ostreococcus*

(Table 2.1). Combined with the presence of AOX in other primary plastid lineages (i.e. red algae and glaucocystophytes) (McDonald, 2008), the AOX gene is likely an ancient character of the Viridiplantae. Therefore, it is predicted that AOX will be found in most species within the Chlorophyta, a hypothesis that can be tested as data from additional sequencing projects becomes accessible.

The Streptophyte algae are composed of the Mesostigmatales, Chlorokybales, Klebsormidiales, Zygnematales, Charales and Coleochaetales (Becker and Marin, 2009). Within the Streptophyta, it has been widely accepted that the algal group Mesostigmatales is the most basal (Becker and Marin, 2009; Rodríguez-Ezpeleta et al., 2007). AOX sequences were recovered from at least one species belonging to 5 of the 6 algal groups (Table 2.1). Given the fact that AOX is present in the Chlorophyta and members of the Embryophyta (Table 2.1; Figure 2.5), it is predicted that AOX will be found in other Streptophyte algae. This prediction can be tested as more sequencing data becomes available from this group.

AOX is present in the Embryophyta within the Bryophyta (mosses), Marchantiophyta (liverworts), and Tracheophyta (Table 2.1; Figure 2.5). Although no data are available for the Anthocerotophyta (hornworts) I predict that AOX will also be present in this group due to its distribution in other Embryophytes. Within the Tracheophyta, AOX is present in the Lycopodiophyta (within the Isoetopsida in two species of *Selaginella*), but no data exist for members of the Isoetales or the Lycopodiopsida (Table 2.1; Figure 2.5). Within the Euphyllophyta, in the Moniliformopses AOX is present in several ferns (Filicophyta), but no data were available for the Equisetophyta or the Psilotophyta (Table 2.1; Figure 2.5). Within the Spermatophyta (seed plants), AOX was detected in several members of the Coniferophyta and in

one member of the Gnetophyta (Table 2.1; Figure 2.5). No AOX data were available for the Cycadophyta or Ginkgophyta (Figure 2.5). Previous work has suggested that the distribution of AOX in the Magnoliophyta (angiosperms) appears to be ubiquitous (McDonald, 2008; Figure 2.5). The distribution of AOX in the Embryophyta therefore appears to be broad and I believe it likely that all members of this group will contain AOX.

4.2. The AOX Multigene Family and AOX1 and AOX2 Subtypes

Within the Chlorophyta, *Chlamydomonas reinhardtii* contains two AOX genes (Dinant et al., 2001). It is likely that these genes represent a gene duplication event within the Chlorophyceae. The AOX1 of *C. reinhardtii* shares a high degree of sequence similarity with AOX proteins found in *C. incerta*, *C. reinhardtii* S1D2 and *Volvox carteri*, while the AOX2 of *C. reinhardtii* shares sequence similarity with an AOX protein from *C. reinhardtii* CW15 (Figure 2.1). It is therefore possible that other members of the Chlorophyceae may contain two AOX genes, but additional sequence information will be required to test this hypothesis. No evidence of a multigene family was recovered in any other member of the Chlorophyta (Table 2.1; Figure 2.5). It has been previously noted that these protein sequences have a higher sequence similarity to the AOX genes of fungi and yeast than to those of angiosperms (Dinant et al., 2001). Although these genes are named AOX1 and AOX2, they do not reflect the presence of the two different subtypes seen in angiosperms in *C. reinhardtii* (Figure 2.1; Figure 2.2).

No evidence for an AOX multigene family was found within the Anthocerotophyta (hornworts), Bryophyta (mosses), or Marchantiophyta (liverworts) (Table 2.1). Within the Tracheophyta, EST data indicate that within the Lycopodiophyta AOX multigene families might exist in the species *Selaginella moellendorffii* and *Selaginella lepidophylla* as several different

mRNAs were detected (Table 2.1). Genome data will be required in order to determine whether these mRNAs are the result of multiple genes, alternative splicing, or allelic variation. Due to the evolutionary distance from the angiosperm AOXs it is difficult to determine whether these proteins should be classified as AOX1 or AOX2 subtypes.

Within the Euphyllophyta, no evidence for a multigene family was present in the Moniliformopses (Table 2.1). Within the Spermatophyta, potential AOX multigene families were detected in members of the Coniferophyta in the species *Picea glauca* and *Picea sitchensis* (Table 2.1). Previous work established the presence of a multigene family in *Pinus pinea* that contained both AOX1 and AOX2 subtypes (Frederico et al., 2009; Table 2.1) and AOX multigene families appear to be common in the Magnoliophyta (McDonald, 2008; Saisho et al., 1997; Whelan et al., 1996). Based on the current evidence, I hypothesize that the duplication of a single AOX gene in Streptophytes into a multigene family occurred after the separation of the Tracheophyta from the rest of the Embryophyta approximately 707 million years ago (Hedges et al., 2004; Figure 2.5). I therefore predict that AOX multigene families will be found in other members of the Lycopodiophyta, and in the Euphyllophyta in members of the Moniliformopses and Spermatophyta (Figure 2.5). Based on the results, only one non-angiosperm AOX2 sequence was found in the Streptophyta, in the species *Cryptomeria japonica* (Table 2.1; Figure 2.5). Recent work supports this result and also indicates that an AOX2 is present in the stone pine *Pinus pinea* (Frederico et al., 2009). Therefore, results to date indicate that the AOX1 and AOX2 subtypes are limited to members of the Spermatophyta and therefore predate the divergence of the Coniferophyta from the Magnoliophyta (Figure 2.5) and suggest that the lack of the AOX2 subtype in monocots is due to a secondary gene loss event.

4.3. AOX Protein Similarities and Differences in the Viridiplantae

4.3.1. N-terminal Region

Sequence analysis indicates that the AOX sequences of Chlorophytes and Streptophytes contain similarities and differences in their N-terminal regions. At the level of protein sequence, the AOXs of these two groups differ. The Streptophyte AOXs contain two N-terminal motifs, Y-W-G and P-X-E-X-Y that are absent in the Chlorophytes (Figure 2.1). It has recently been hypothesized that these regions might be involved in the non-covalent dimerization of angiosperm AOXs (McDonald, 2009). If this hypothesis is correct, it suggests that the Chlorophyte AOXs will be monomeric, similar to the situation seen in other protists and fungi, as opposed to the AOX dimers seen in angiosperms (McDonald, 2008). This would also mean that all Streptophytes (from bryophytes to angiosperms) would be expected to contain AOX dimers.

In terms of the post-translational regulation of the enzyme, the Chlorophyte AOXs contain an indel in the N-terminal region that is not present in the Streptophyte AOXs (Figure 2.1). Variations of this particular indel are also present in AOXs of protists, fungi, and animals (McDonald, 2009). It has been hypothesized that this indel may be responsible for the regulation of these AOXs by AMP/GMP or pH (McDonald, 2009). The Chorophyte insertion and the Streptophyte specific motifs now make it possible to differentiate between Chlorophyte and Streptophyte AOX proteins using an examination of only the N-terminus (Figure 2.1). This information may prove useful when analyzing metagenomic datasets or determining whether newly identified species are Streptophyte or Chlorophyte algae.

Further evidence exists that the post-translational regulatory mode that is typical for most angiosperm AOXs will not hold true for the Chlorophyte AOXs and several Streptophyte AOXs. The CysI residue that is important for the redox and pyruvate regulation of AOX in the majority of angiosperms is often absent in Chlorophyte AOXs (Figure 2.1; Table 2.2). Most basal Streptophyte AOXs recovered from the algae contained a serine in this position (Table 2.1; Figure 2.1; Table 2.2; Figure 2.5). While the CysI residue is present in the liverwort *M. polymorpha*, the moss *P. patens*, the gnetophyte *E. distachya*, and a protein from the lycopod *S. moellendorffii*, all other non-angiosperm Streptophytes contain a serine in this position (Figure 2.1; Table 2.2). Recent work in *Nelumbo nucifera* has demonstrated that a serine in this position results in an AOX that is responsive to succinate (Grant et al., 2009). I can hypothesize by extrapolation that the majority of Streptophyte AOXs may be subject to succinate regulation since they possess a SerI residue in this position. In addition, although CysI is present in Streptophyta, it was evident that the majority of non-angiosperm AOXs possess a serine at this position instead (Figure 2.5). A widespread abundance of CysI is not observed until the emergence of angiosperms (Magnoliophyta). At the DNA level, there are six different possible codons for serine; the majority of these codons are used in the species that have this residue. In contrast, those species that possess CysI are encoded by a single codon (TGC). This change from serine to cysteine is the result of a non-synonymous substitution that is most likely due to a single point mutation or multiple base substitutions. The fact that only a single codon encodes CysI likely suggests that there is codon usage bias that may have resulted from selection. In addition, the presence of cysteine residues in this position in plants from different lineages may represent a case of convergent evolution. However, the ancestral residue in this position cannot be confirmed until more sequences from basal plants become available.

With regards to the CysII residue, the AOXs of Chlorophytes do not have a cysteine in this position (Figure 2.1; Table 2.2). The vast majority of Streptophyte AOXs have a cysteine in this position, with some having an isoleucine, valine, or tyrosine present (Figure 2.1; Table 2.2). The role of the CysII residue in regulation of AOX activity in angiosperms is still under investigation (Umbach et al., 2006) and it has recently been proposed that it may play a role in influencing the catalytic cycle of the enzyme via interactions with oxygen (Crichton et al., 2010).

Overall, these results indicate that the AOXs of the majority of Chlorophytes will differ in their mode of post-translational regulation from those of Streptophytes (i.e. regulation by AMP/GMP and pH vs. regulation by pyruvate or succinate) and that the affinity of AOX for oxygen may differ in these systems. It is also likely that the post-translational regulation of non-angiosperm AOXs will differ from those of angiosperms due to the presence of a SerI residue instead of a CysI residue in the majority of sequences (Figure 2.1; Table 2.2). I hypothesize that the CysI residue present in the AOXs of some basal members of the Embryophyta represents multiple independent mutations that are lineage specific (Figure 2.1; Figure 2.5; Table 2.2). Within the Magnoliophyta it appears that some CysI residues have reverted back to SerI in some AOX proteins of tomato (*Lycopersicon esculentum*), *Nelumbo nucifera*, corn (*Zea mays*), rice (*Oryza sativa*), and wheat (*Triticum aestivum*) (Grant et al., 2009; Holtzapffel et al., 2003; Umbach et al., 2006; Figure 2.5).

4.3.2. Core Region

All of the iron-binding residues required for AOX activity are conserved in the sequences that were examined (Figure 2.2; Table 2.2) indicating that these proteins should be catalytically active. A key tyrosine residue involved in AOX activity (TyrII/Y-280) is conserved in all

Viridiplantae AOXs examined (Figure 2.2; Table 2.2). Therefore, at the functional level, it is likely that the AOXs of all Chlorophytes and Streptophytes share a similar catalytic mechanism to the AOXs of all other kingdoms (McDonald, 2009). At a structural level, it is evident that a tryptophan residue that is thought to play a role in AOX structure (TrpI/W-206) is conserved in all species of Viridiplantae (Figure 2.2). Amino acid residues that are thought to be involved with ubiquinol-binding (GlnI/Q-242, TyrI/Y-253, S-256, HisIII/H-261, and R-262) are conserved among most members of Viridiplantae. However, S-256 has been replaced in some land plants by phenylalanine (F-256) and R-262 has been replaced with an alanine (A-262) in several species of *Chlamydomonas* (Figure 2.2; Table 2.2). These changes may affect the ability of the enzyme to interact with ubiquinol and render it inactive.

4.3.3. C-terminal Region

The AOA antibody was generated against the AOX of the voodoo lily plant and has proved to be a valuable resource for the AOX community due to its cross-reactivity with the AOX proteins from a broad range of species and kingdoms (Elthon et al., 1989; McDonald, 2009). Recently a scheme was proposed for predicting whether the AOA antibody will cross-react with an AOX protein based on a 12 amino acid sequence located in the AOX C-terminus (McDonald, 2009). Based on a comparison with this consensus sequence for antibody binding, the majority of non-angiosperm AOXs that were recovered will likely cross-react with the AOA antibody (Figure 2.2; Table 2.2). It is likely that the antibody will continue to be a powerful tool for investigating the presence of AOX proteins in the Viridiplantae.

The extreme C-terminal regions are quite different between Chlorophytes and Streptophytes (Figure 2.2). The other interesting difference that was observed in the C-terminal

regions occurred in some members of the family Pinaceae. In most AOXs from Streptophytes the last 3 amino acids are G-Y-H (Figure 2.2). In some AOX sequences from *P. engelmannii*, *P. sitchensis*, *P. menziesii*, and *P. glauca* the last 3 amino acids are D-Y-R (Figure 2.2). Whether these differences translate into any effect on protein structure or regulation remains to be investigated.

4.4. AOX of the Moss *Physcomitrella patens*

AOX is encoded by a multigene family in all angiosperms studied to date. This attribute makes it particularly challenging to study its physiological function in these systems due to the possible functional redundancy of the protein isoforms. However, through the use of molecular databases, evidence has been provided that only a single AOX gene copy is present in the moss *Physcomitrella patens* (Figure 2.3A). This makes *P. patens* an attractive system for future research using antisense or RNAi technologies to examine the physiological role of AOX in plants. Structurally, this gene is made up of four exons that are interrupted by three introns; a characteristic seen in the majority of angiosperm AOXs studied to date (Considine et al., 2002; Ito et al., 1997; Figure 2.3A). The full-length gene sequence and full-length transcript sequence retrieved from the Phytozome database were used to design specific primers for use in PCR and RT-PCR, respectively (Figure 2.3A; Figure 2.3B). A region of the AOX gene was amplified and its presence and identity have been confirmed in moss (Figure 2.4A) after sequencing. The data available on Phytozome reveals that the *P. patens* AOX gene may be expressed as three putative transcript variants (Figure 2.3C). The RT-PCR results demonstrate that AOX is expressed in *P. patens* (Figure 2.4B; Figure 2.4C). An analysis of the cloned cDNA sequences revealed that the

amino acid iron-ligands are conserved which allows me to predict that AOX is catalytically active in moss.

The cloned moss AOX sequences exhibited identical sequence similarity with the sequences retrieved from the Phytozome database. Using the predicted AOX sequence from the Phytozome database, the N-terminus of the protein was able to be examined (Figure 2.3D). Dissimilar to most non-angiosperm AOXs, *P. patens* AOX has CysI which allows for the prediction that it is post-translationally regulated in a manner that is similar to angiosperm AOXs (Table 2.2; Figure 2.1). MitoProt II and TargetP 1.1 software programs predicted a high probability of mitochondrial targeting of the protein and suggests that organelle isolations will be required to analyze AOX proteins. A 35 kDa protein that cross reacts with an AOX1/2 antibody has been detected in Western blots of moss mitochondrial fractions (Lang et al., 2011).

5. Conclusions and Future Directions

This work demonstrates for the first time that AOX is broadly distributed in the Viridiplantae, both within the Chlorophyta and the Streptophyta (Table 2.1; Figure 2.5). Future work should endeavor to increase the sequence data available within the Anthocerotophyta, Lycopodiopsida, Psilotophyta, Equisetophyta, Ginkgophyta, and Cycadophyta since no AOX data are currently available for these lineages (Figure 2.5). Better sampling of these lineages will allow for the testing of hypotheses and predictions made in this work with respect to the taxonomic distribution of AOX, the origins of the AOX multigene family and AOX1 and AOX2 subtypes, and the post-translational regulation of the enzyme. Thus far, the majority of work on AOX in the Viridiplantae has occurred in the Magnoliophyta. This work indicates that a more detailed investigation of AOX in non-angiosperm lineages is warranted.

References

Albury MS, Elliott C, Moore AL. 2010. Ubiquinol-binding site in the alternative oxidase: mutagenesis reveals features important for substrate binding and inhibition. Biochim Biophys Acta. 1797: 1933-1939.

Angert A, Rachmilevitch S, Barkan E, Luz B. 2003a. Effects of photorespiration, the cytochrome pathway, and the alternative pathway on the triple isotopic composition of atmospheric O_2. Global Biogeochem Cy. 17: 1030.

Angert A, Barkan E, Barnett B, Brugnoli E, Davidson EA, Fessenden J, Maneepong S, Panapitukkul N, Randerson JT, Savage K, Yakir D, Luz B. 2003b. Contribution of soil respiration in tropical, temperate, and boreal forests to the ^{18}O enrichment of atmospheric O_2. Global Biogeochem Cy. 17: 1089.

Becker B, Marin B. 2009 Streptophyte algae and the origin of embryophytes. Ann Bot. 103: 999-1004.

Bendall DS, Bonner WD. 1971. Cyanide-insensitive respiration in plant mitochondria. Plant Physiol. 47: 236-245.

Berthold DA, Voevodskaya N, Stenmark P, Gräslund A, Nordlund P. 2002. EPR studies of the mitochondrial alternative oxidase. Evidence for a diiron carboxylate center. J Biol Chem. 277: 43608-43614.

Bhattacharya D, Medlin L. 1998. Algal phylogeny and the origin of land plants. Plant Physiol. 116: 9-15.

Claros MG, Vincens P. 1996. Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur J Biochem. 241: 779-786.

Considine MJ, Holtzapffel RC, Day DA, Whelan J, Millar AH. 2002. Molecular distinction between alternative oxidase from monocots and dicots. Plant Physiol. 129: 949-953.

Cove DJ, Perroud P, Charron AJ, McDaniel SF, Khandelwal A, Quatrano R. 2009. Emerging model organisms: a laboratory manual, First ed. Cold Spring Harbor Laboratory Press, New York.

Crichton PG, Albury MS, Affourtit C, Moore AL. 2009. Mutagenesis of the *Sauromatum guttatum* alternative oxidase reveals features important for oxygen binding and catalysis. Biochim Biophys Acta. 1797: 732-737.

Dinant M, Baurain D, Coosemans N, Joris B, Matagne RF. 2001. Characterization of two genes encoding the mitochondrial alternative oxidase in *Chlamydomonas reinhardtii*. Curr Genet. 39: 101-108.

Elthon TE, Nickels RL, McIntosh L.1989. Monoclonal antibodies to the alternative oxidase of higher plant mitochondria. Plant Physiol. 89: 1311–1317.

Emanuelsson O, Nielsen H, Brunak S, von Heijne G. 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol. 300: 1005-1016.

Frederico AM, Zavattieri MA, Campos MD, Cardoso HG, McDonald AE, Arnholdt-Schmitt B. 2009. The gymnosperm *Pinus pinea* contains both AOX gene subfamilies, AOX1 and AOX2. Physiol Plant. 137: 566-577.

Goodstein DM, Shu S, Howson R, Neupane R, Hayes RD, Fazo J, Mitros T, Dirks W, Hellsten U, Putnam N, Rokhsar DS. 2012. Phytozome: a comparative platform for green plant genomics. Nucleic Acids Res. 40: D1178-D1186.

Grant N, Onda Y, Kakizaki Y, Ito K, Watling J, Robinson S. 2009. Two Cys or not two Cys, that is the question? Alternative oxidase in the thermogenic *Nelumbo nucifera*. Plant Physiol. 150: 987-995.

Hedges SB, Blair JE, Venturi ML, Shoe JL. 2004. A molecular timescale of eukaryote evolution and the rise of complex multicellular life. BMC Evol Biol. 4: 2.

Holtzapffel RC, Castelli J, Finnegan PM, Millar AH, Whelan J, Day DA. 2003. A tomato alternative oxidase protein with altered regulatory properties. Biochim Biophys Acta. 1606: 153-162.

Ito Y, Saisho D, Nakazono M, Tsutsumi N, Hirai A. 1997. Transcript levels of tandem-arranged alternative oxidase genes in rice are increased by low temperature. Gene. 203: 121-129.

Johnson-Flanagan AM, Owens JN. 1986. Root respiration in white spruce (*Picea glauca* [Moench] Voss) seedlings in relation to morphology and environment. Plant Physiol. 81: 21-25.

Karpova OV, Kuzmin EV, Elthon TE, Newton KJ. 2002. Differential expression of alternative oxidase genes in maize mitochondrial mutants. Plant Cell. 14: 3271-3284.

Lang EG, Mueller SJ, Hoernstein SNW, Porankiewicz-Asplund J, Vervliet-Scheebaum M, Reski R. 2011. Simultaneous isolation of pure and intact chloroplasts and mitochondria from moss as the basis for sub-cellular proteomics. Plant Cell Rep. 30: 205-215.

Leliaert F, Smith DR, Moreau H, Herron MD, Verbruggen H, Delwiche CF, De Clerck O. 2012. Phylogeny and molecular evolution of the green algae. Crit Rev Plant Sci. 31: 1-46.

Mariano AB, Valente C, Maurer JBB, Cadena SMSC, Rocha MEM, de Oliveira MBM, Salgado I, Carnieri EGS. 2008. Functional characterization of mitochondria isolated from the ancient gymnosperm *Araucaria angustifolia*. Plant Sci. 175: 701-705.

Maxwell DP, Wang Y, McIntosh L. 1999. The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. Plant Biol. 96: 8271-8276.

McDonald AE. 2008. Alternative oxidase: an inter-kingdom perspective on the function and regulation of this broadly distributed 'cyanide-resistant' terminal oxidase. Funct Plant Biol. 35: 535-552.

McDonald AE. 2009. Alternative oxidase: what information can sequence comparisons give us? Physiol Plant. 137: 328-341.

McDonald AE, Amirsadeghi S, Vanlerberghe GC. 2003. Prokaryotic orthologues of mitochondrial alternative oxidase and plastid terminal oxidase. Plant Mol Biol. 53: 862-876.

McDonald AE, Vanlerberghe GC. 2006. Origins, evolutionary history, and taxonomic distribution of alternative oxidase and plastoquinol terminal oxidase. Comp Biochem Phys. *D* 1: 357–364.

Moore AL, Siedow JN. 1991. The regulation and nature of the cyanide-resistant alternative oxidase of plant mitochondria. Biochim Biophys Acta. 1059: 121-140.

Moore AL, Albury MS. 2008. Further insights into the structure of the alternative oxidase: from plants to parasites. Biochem Soc T. 36: 1022-1026.

Moore AL, Carré JE, Affourtit C, Albury MS, Crichton PG, Kita K, Heathcote P. 2008. Compelling EPR evidence that the alternative oxidase is a diiron carboxylate protein. Biochim Biophys Acta. 1777: 327-330.

Onda Y, Kato Y, Abe Y, Ito T, Ito-Inaba Y, Morohashi M, Ito Y, Ichikawa M, Matsukawa K, Otsuka M, Koiwa H, Ito K. 2007. Pyruvate-sensitive AOX exists as a non-covalently associated dimer in the homeothermic spadix of the skunk cabbage, *Symplocarpus renifolius*. FEBS Lett. 581: 5852-5858.

Onda Y, Kato Y, Abe Y, Ito T, Morohashi M, Ito Y, Ichikawa M, Matsukawa K, Kakizaki Y, Koiwa H, Ito K. 2008. Functional coexpression of the mitochondrial alternative oxidase and uncoupling protein underlies thermoregulation in the thermogenic florets of skunk cabbage. Plant Physiol.146: 636-645.

Pombert JF, Otis C, Lemieux C, Turmel M. 2005. The chloroplast genome sequence of the green alga *Pseudendoclonium akinetum* (Ulvophyceae) reveals unusual structural features and new insights into the branching order of chlorophyte lineages. Mol Biol Evol. 22: 1903-1918.

Rhoads DM, Umbach AL, Sweet CR, Lennon AM, Rauch GS, Siedow JN. 1998. Regulation of the cyanide-resistant alternative oxidase of plant mitochondria. Identification of the cysteine residue involved in alpha-keto acid stimulation and intersubunit disulfide bond formation. J Biol Chem. 273: 30750-30756.

Robinson SA, Yakir D, Ribas-Carbo M, Giles L, Osmond CB, Siedow JN, Berry JA. 1992. Measurements of the engagement of cyanide-resistant respiration in the Crassulacean acid metabolism plant *Kalanchoë daigremontiana* with the use of on-line oxygen isotope discrimination. Plant Physiol. 100: 1087-1091.

Rodríguez-Ezpeleta N, Philippe H, Brinkmann H, Becker B, Melkonian M. 2007. Phylogenetic analyses of nuclear, mitochondrial, and plastid multigene data sets support the placement of *Mesostigma* in the Streptophyta. Mol Biol Evol. 24: 723-731.

Saisho D, Nambara E, Naito S, Tsutsumi N, Hirai A, Nakazono M. 1997. Characterization of the gene family for alternative oxidase from *Arabidopsis thaliana*. Plant Mol Biol. 35: 585-596.

Sanderson MJ, Thorne JL, Wikstrom N, Bremer K. 2004. Molecular evidence on plant divergence times. Am J Bot. 91: 1656-1665.

Seymour RS, Matthews PG. 2006. The role of thermogenesis in the pollination biology of the Amazon waterlily *Victoria amazonica*. Ann Bot. 98: 1129-1135.

Shiba T, Kido Y, Sakamoto K, Inaoka DK, Tsuge C, Tatsumi R, Takahashi G, Balogun EO, Nara T, Aoki T, Honma T, Tanaka A, Inoue M, Matsuoka S, Saimoto H, Moore AL, Harada S, Kita K. 2013. Structure of the trypanosome cyanide-insensitive alternative oxidase. Proc Natl Acad Sci U S A. 110: 4580-5.

Siedow JN, Umbach AL, Moore AL. 1995. The active site of the cyanide-resistant oxidase from plant mitochondria contains a binuclear iron center. FEBS Lett. 362: 10-14.

Skubatz H, Tang W, Meeuse BJD. 1993. Oscillatory heat-production in the male cones of cycads. J Exp Bot. 44: 489-492.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25: 4876-4882.

Umbach AL, Ng VS, Siedow JN. 2006. Regulation of plant alternative oxidase activity: a tale of two cysteines. Biochim Biophys Acta 1757: 135-142.

Umbach AL, Siedow JN. 1993. Covalent and noncovalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. Plant Physiol. 103: 845-854.

Vanlerberghe GC, McIntosh L. 1994. Mitochondrial electron transport regulation of nuclear gene expression. Studies with the alternative oxidase gene of tobacco. Plant Physiol. 105: 867-874.

Vanlerberghe GC, McIntosh L, Yip JY. 1998. Molecular localization of a redox-modulated process regulating plant mitochondrial electron transport. Plant Cell. 10: 1551-1560.

Weger HG, Guy RD. 1991. Cytochrome and alternative pathway respiration in white spruce (*Picea glauca*) roots- effects of growth and measurement temperature. Physiol Plant. 83: 675-681.

Whelan J, Millar AH, Day DA. 1996. The alternative oxidase is encoded in a multigene family in soybean. Planta. 198: 197-201.

Yoon HS, Hackett JD, Ciniglia C, Pinto G, Bhattacharya D. 2004. A molecular timeline for the origin of photosynthetic eukaryotes. Mol Biol Evol. 21: 809-818.

Table 2.1. Chlorophyte and non-angiosperm streptophyte alternative oxidase (AOX) sequences retrieved from molecular database searches. Database searches are current as of July 1, 2013.

Group	Species	Accession Number	Conditions
Chlorophyta			
Chlorophyceae	*Chlamydomonas incerta*	DQ122873, EC114320	EST, cDNA
	Chlamydomonas reinhardtii AOX1	AF047832	mRNA
	Chlamydomonas reinhardtii AOX2	AF314255	mRNA
	C. reinhardtii CC-1690	BI997629, BI720614, BE453335, BE725120	EST, stress conditions
	Chlamydomonas sp. W80	AB009087	mRNA, anti-stress gene
	Chlamydomonas reinhardtii S1D2	FC095258, FC107788, FC089790, FC096794, FC101139	EST
	Chlamydomonas reinhardtii CW15 mt-	AF285187	mRNA
	Volvox carteri f. nagariensis	FD838977, XM_002955135	
Mamiellophyceae	*Bathycoccus prasinos*	FO082264 204834- 204196	DNA
Prasinophyceae	*Ostreococcus lucimarinus CCE9901*	XM_001419431	Genome
		XM_001422917	mRNA
		ES326082, ES336319, ES339063, ES336379, ES334493, ES330134, ES333378, ES337984, ES329658, ES336433, ES338640,	EST-likely a couple of genes but difficult to determine from ESTs

		ES330847, ES337415, ES332883, ES326357, ES343407, ES326174, ES340252, ES339340, ES338210	
	Ostreococcus sp. RCC809	GR817110, GR823881, GR817852, GR821051, GR821050	mRNA
	Ostreococcus tauri	CAID01000009	genome
Trebouxiophyceae	*Coccomyxa subellipsoidea* C-169	GW223177, GW238474, GW221449, GW232312, GW230086, GW220993, GW229529, GW226608, GW231106, GW223480, GW224325, GW218354, GW227179	mRNA
	Prototheca wickerhamii	EC181905, EC180273	EST
Ulvophyceae	*Acetabularia acetabulum*	CF258325, CF258617	EST, late adult and early reproductive cells
	Ulva linza	AJ892187, AJ891330, AJ891574	EST, sporulating thallus
Unclassified	uncultured phototrophic eukaryote	CAFX01005208	DNA
	uncultured phototrophic eukaryote	CAFY01001175	DNA
Streptophyta			
Zygnemophyceae	*Spirogyra pratensis Transeau*	GW599090, GW598494,	EST

		GW601827, GW600845	
	Penium margaritaceum	HO650634, HO623842	EST, cDNA
Chlorokybophyceae	*Chlorokybus atmophyticus*	HO408459, HO422383	EST, cDNA
Klebsormidiophyceae	*Klebsormidium faccidum*	HO471043, HO480760	EST, cDNA
	Klebsormidium subtile	JG441912	EST, cDNA
Charophyceae	*Nitella hyaline*	HO503263	EST, cDNA
Coleochaetophyceae	*Chaetosphaeridium globosum*	HO367913	EST, cDNA
	Coleochaete orbiculans	GW591598	EST, cDNA
Marchantiophyta	*Marchantia polymorpha*	BJ864156, BJ872239	EST, sexual organ
Bryophyta	*Physcomitrella patens*	XP_001774858	Genome, Gransden ecotype
		FC444005, FC455056	EST, Gransden ecotype, dessicated 7 day old protonema
		FC366080, FC356806, FC423252, FC387448, FC407449	EST, Villersexel ecotype, asexual protonema
		AW561656	EST, 7 day old protonemata
Tracheophyta			
Lycopodiophtya			
Isoetopsida	*Selaginella lepidophylla* AOX subtype 1	EH093479, EH093273, EH091901	EST, adult microphyll fronds and roots
	Selaginella lepidophylla AOX subtype 2	EH093745	EST, adult microphyll fronds and roots under a dehydration-rehydration cycle
		EH093135	EST, adult microphyll fronds and roots under a dehydration-rehydration cycle
		EH091642, EH092545,	EST, adult microphyll fronds

		EH092495	and roots
	Selaginella moellendorffii AOX subtype 1	XP_002963435, XP_002971569	Hypothetical protein
	Selaginella moellendorffii AOX subtype 2	XP_002972804, XP_002984380	Hypothetical protein
	Selaginella moellendorffii AOX subtype 3	XP_002981994, XP_002986074	Hypothetical protein
	Selaginella moellendorffii AOX subtype 4	FE468243	EST, whole juvenile plants and reproductive stems with developing strobili
	Selaginella moellendorffii AOX subtype 5	FE497473, FE490594, FE516656, FE439170, FE490593, FE497472, FE439171, FE450180, FE429925, FE508717, FE465252, FE516655, FE446706, FE465253	EST, whole juvenile plants with roots and reproductive stems with developing strobili
Moniliformopses			
Filicophyta	*Adiantum capillus-veneris*	DK948381	EST, prothallia, gametophyte, light-brown
	Ceratopteris richardii	BE642413	EST, spore 20 hours after germination initiation
Spermatophyta			
Gnetophyta	*Ephedra distachya*	JG722268, JG721633	mRNA, cell culture
Coniferophyta	*Cryptomeria japonica* AOX	BW993935, BY910481, BY898792	EST, male cone
	Picea engelmannii x Picea glauca	CO207489, CO213112, DR464823, DR464925, CO214470, CO208811	EST, bark (with phloem and cambium attached) from one year old trees

	Picea glauca AOX subtype 1	BT119347, GO371345, GO371284, EX306539, EX442167, GE477243	EST, actively elongating root tips free of mycorrhizae; EST, dormant shoot tips dissected from buds;
	Picea glauca AOX subtype 2	BT104039, EX313454, DR555875, CO479724, DR562015, DR569129, DR566922	EST, phloem; EST, cambium and phloem region from 35 year old trees; EST, male cones from mature trees; EST, whole root from seedlings, predominantly free of mycorrhizae
	Picea sitchensis subtype 1	BT123906, GT124975	
	Picea sitchensis subtype 2	EF084004, GT121657, DR504859, DR492349, ES256697, ES661756, FD730742, FD731057, ES662384, FD736777, ES861806	EST, leader bark (phloem and cambium attached) induced with *Pissodes strobi* (white pine weevil) feeding; EST, leader bark (phloem and cambium attached) induced with wounding; EST, bark (with phloem and cambium attached) from two year old trees; EST, green leader tissue from one year old trees
	Pinus banksiana	GW754219	Previous year's bark (phloem and cambium attached) from mechanically wounded trees
	Pinus pinaster AOX1	CT583387	EST, developing buds of adult trees
		BX678396	EST, 6 week old seedling root (drought stress

			treatment)
	Pinus pinea	ACV60631, ACV60632, ACV60633, ACV60634, ACV60635, ACV60636, ACV60637, ACV60638	Mitochondrial alternative oxidase 1a, partial
		ACV60639, ACV60640	Mitochondrial alternative oxidase 1b, partial
		ACV60641	Mitochondrial alternative oxidase 2, partial
	Pinus taeda AOX1	CF394475	EST, drought-stressed roots
		DT629136, DN613935	EST, subtracted pine embryo library
		DR120631, DR116916, DR116838	EST, roots minus magnesium
		DR059844	EST, roots minus nitrogen
		CO366022, CO367936, CO367860	EST, roots minus potassium
		CO166870	EST, flooded roots
		DR095420, DR099771, DR095336	EST, stems (shoot tips) challenged with the fungus *Fusarium circinatum* (agent of pitch canker disease)
		DR110126	EST, roots minus sulfur
		DR069321	EST, roots, 24 hour dark treatment of 1 year old trees
	Pseudotsuga menziesii AOX	CN636314	EST, 1 month old seedlings

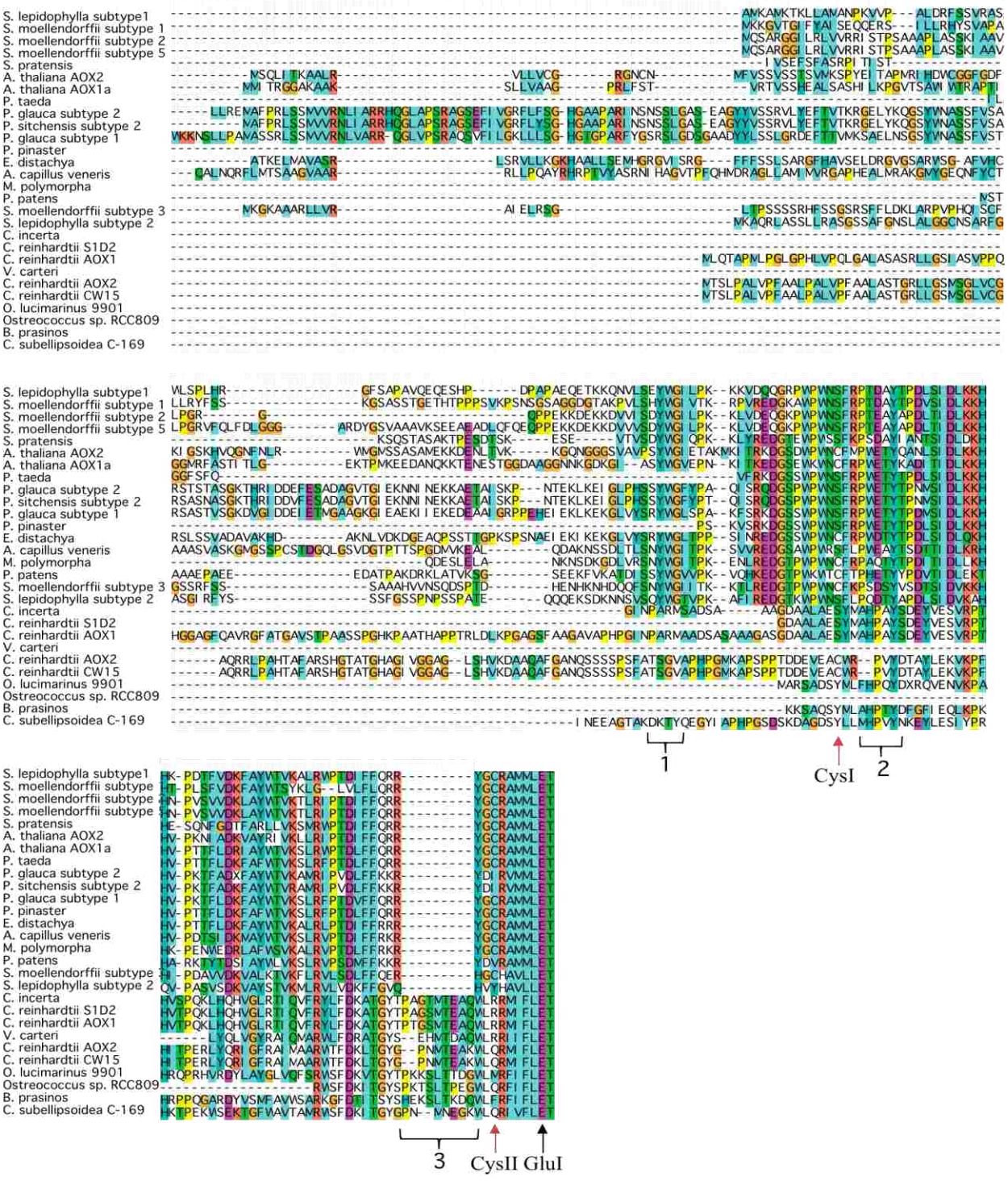

Figure 2.1. A multiple-sequence alignment of the N-terminal region of AOX proteins from non-angiosperm members belonging to Viridiplantae. Brackets 1 and 2 refer to motifs seen only in

Streptophytes, while bracket 3 highlights an insertion seen only in Chlorophytes. Residues analogous to CysI and CysII are indicated by red arrows. The first iron-binding residue (GluI) is indicated by a black arrow.

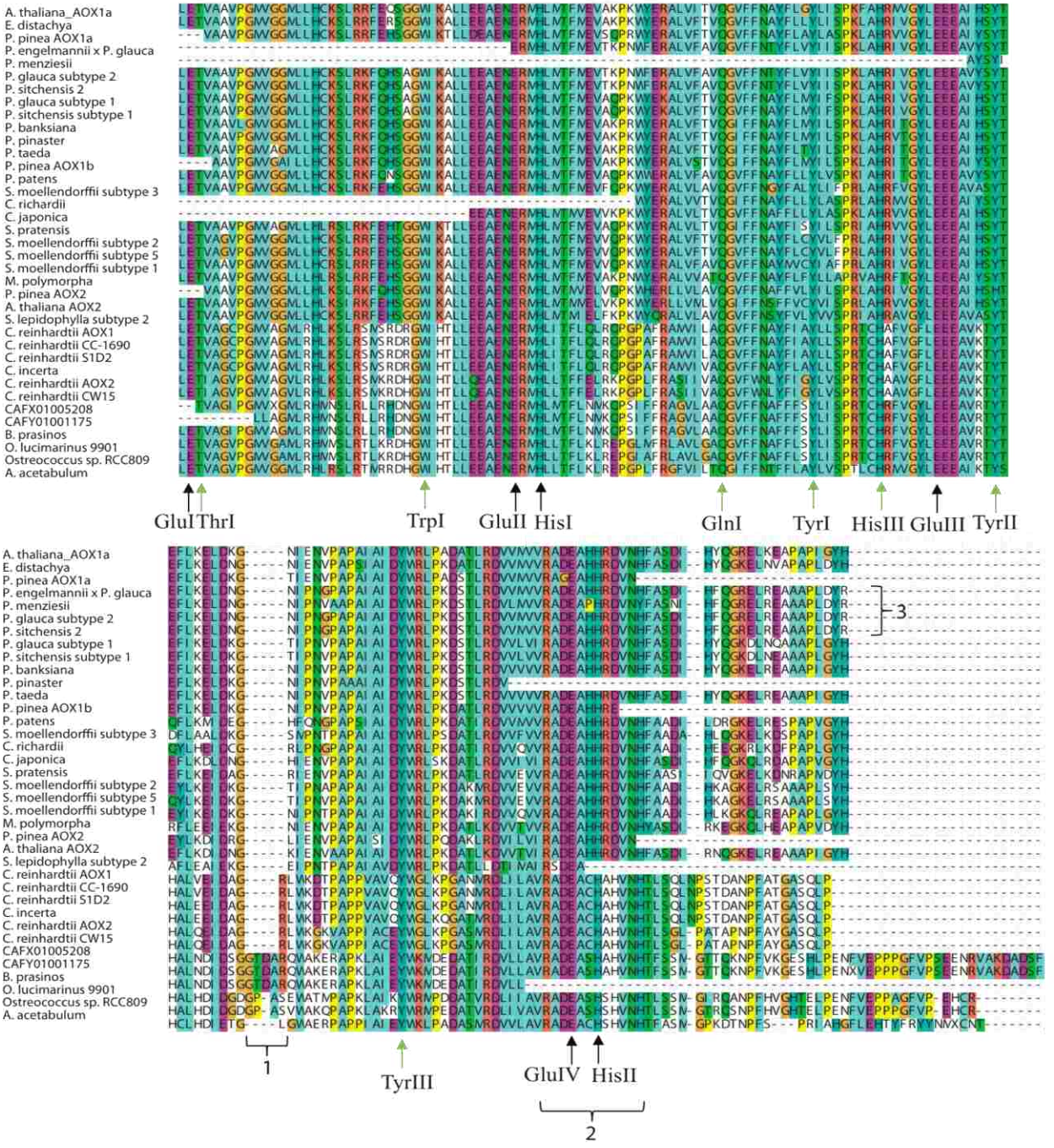

Figure 2.2. A multiple-sequence alignment from the first iron-binding site to the end the AOX protein from a variety of non-angiosperm members belonging to the Viridiplantae. The black arrows denote the iron-binding residues (GluI, GluII, GluIII, GluIV, HisI, HisII), the green

arrows point residues important for AOX activity (ThrI, TrpI, GlnI, TyrI, HisIII, TyrII, TyrIII). Bracket 1 shows an insertion seen only in Chlorophytes and bracket 2 highlights the epitope recognized by the AOA antibody, and bracket 3 shoes a unique C-terminal motif found in some gymnosperms.

A

TGAAGCTTCTCTCGTTTTATTATTCAAGCAGTTGGAATTTCATTATTAATGCGAATGTATGCCTGTTTCGCCATGAATTCCACACCGTGACCTAGTTGGT
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CTCGAGTGCAGTTTATTGTCATTGGAATTAGGAATCTCATGTATTATTTGCTCTCGCAGAAGCGGTATGATGTTCGTGCAATGATGCTGGAGACCGTGGC
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AACAGCTCTGGGTGGGGCTCACCAGACCTGGTCTGCAAATTTACACAATTCTGAGGCACTTTTCACATGTTCTGACGCAATTGGAATGAGATGGGGATGG
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TGTAGGCCGTTTCGCCTCCACAGGAGTAGATAGATTCATTCTACGAAATGCTGTTTTCAAGGTCCGTTACTTCTCCGCTCACTTGTGGCCGGATTTGTAT
CGAACCATTTGGTAGCGGAATGCTGATTTTCGAGAAGTCATAAATGTTAACATTCTGAGAAGAATTCATGTTATGCTTCATTTTTGATATTGGGTAAAAT
CCCTCATTGCAGACTAGAAGTAAACA

Figure 2.3. *Physcomitrella patens* AOX gene structure, transcript structure, transcripts, and peptide sequence retrieved from the Phytozome database. A) The 3426 nt length AOX genomic sequence is predicted to be comprised of a 5′ UTR (light and dark green underline), a coding region comprised of four exons (light and dark blue) interrupted by three introns (non-underlined regions), and a 3′ UTR (red underline). Forward and reverse genomic coding region primers (black boxes) were designed to amplify the full-length AOX gene in a PCR.

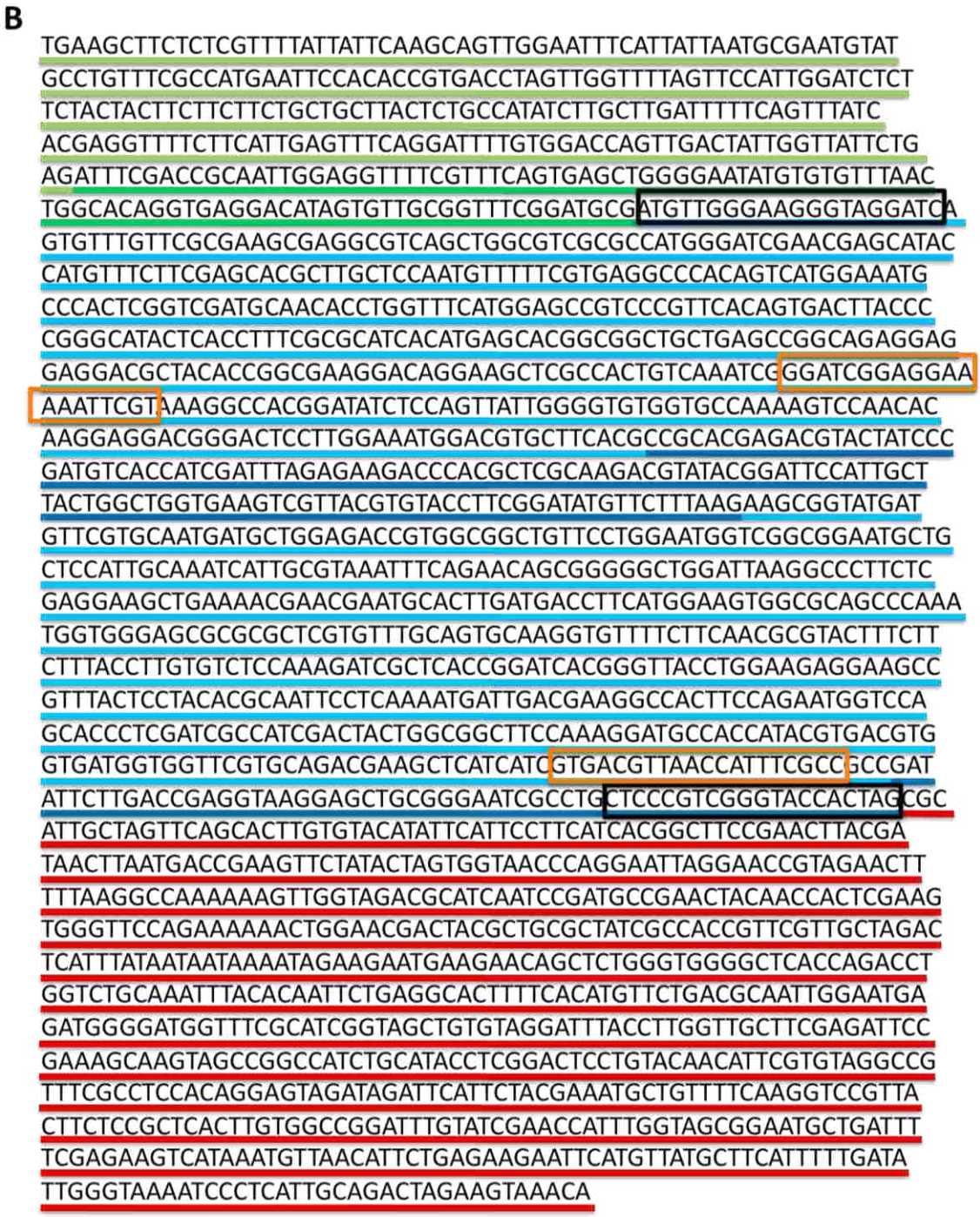


Figure 2.3. *Physcomitrella patens* AOX gene structure, transcript structure, transcripts, and peptide sequence retrieved from the Phytozome database. B) The 2137 nt length AOX transcript is predicted to be comprised of a 5′ UTR, a coding region (introns spliced), and a 3′ UTR. The

internal moss transcript primers (orange boxes) and the full-length coding primers (black boxes) were designed to amplify the AOX coding region in RT-PCRs.

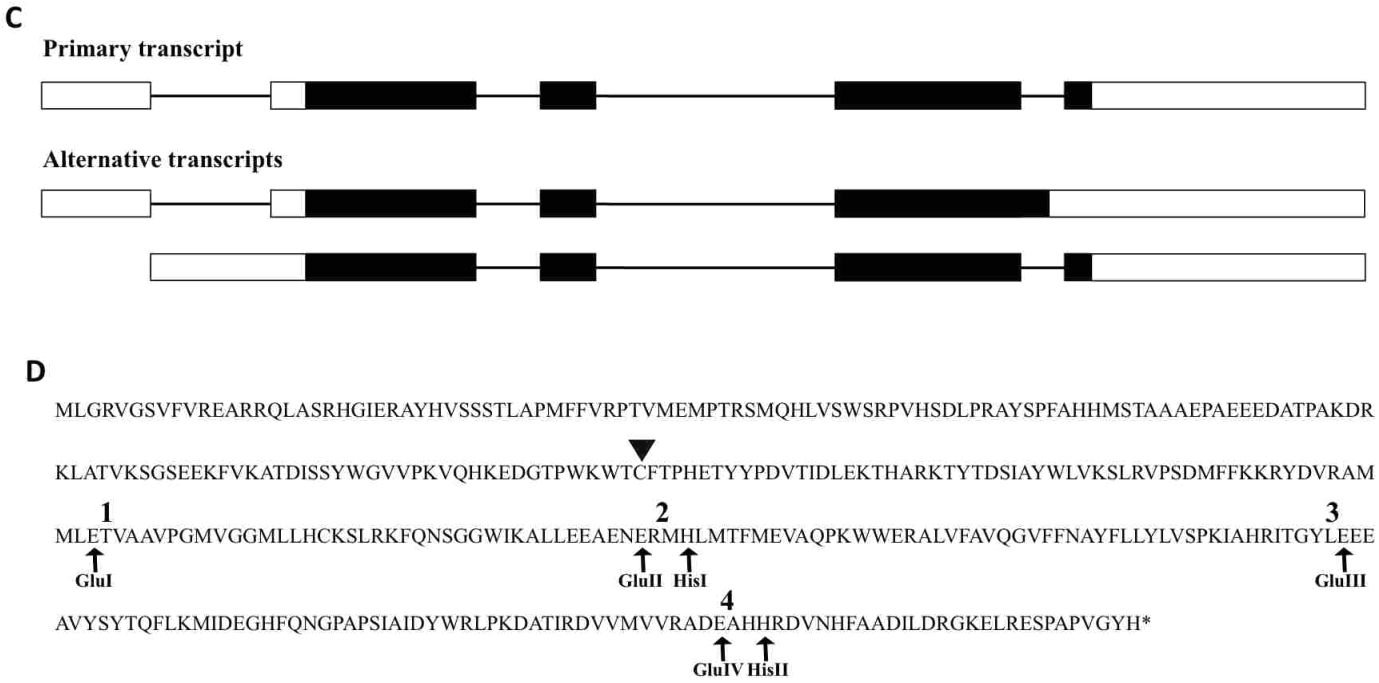


Figure 2.3. *Physcomitrella patens* AOX gene structure, transcript structure, transcripts, and peptide sequence retrieved from the Phytozome database. C) Software predicts that three AOX transcripts are expressed in moss (one primary and two alternatives) due to alternative splicing. The white boxes represent the untranslated regions, the black lines represent introns and the black boxes represent exons. D) The predicted AOX peptide sequence. The black triangle denotes the position of CysI and the four iron-binding site regions are numbered 1 through 4. The black arrows highlight the Glu (E) and His (H) residues that are important for the co-ordination of the di-iron centre. This figure was compiled from data modified from the Phytozome website.

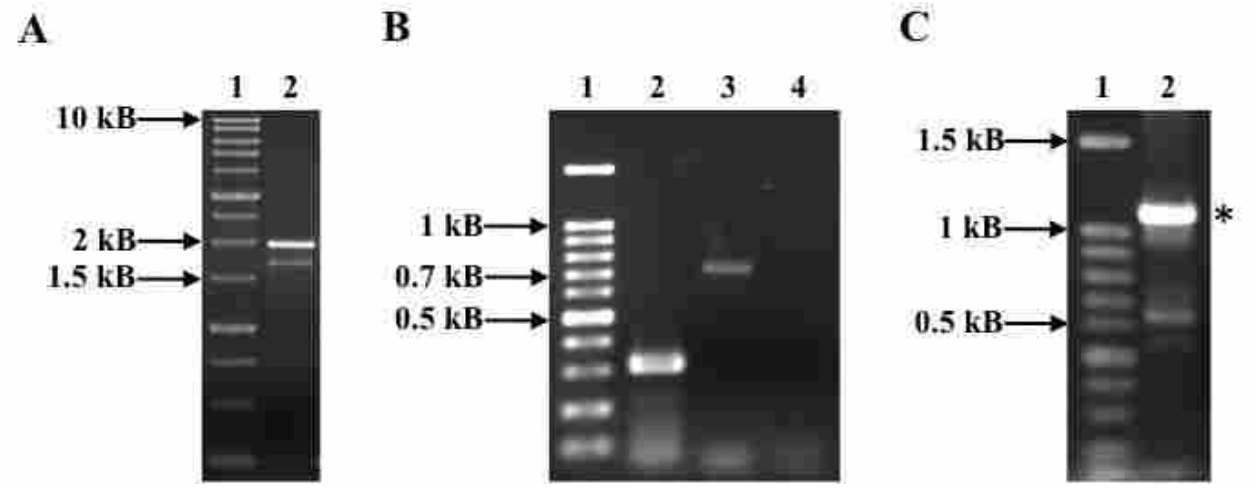


Figure 2.4. Detection of amplified AOX products from *P. patens* separated by DNA electrophoresis. A) The full-length 1988 bp AOX gene (introns and exons) was amplified from 20 ng *P. patens* genomic DNA by PCR using the full-length genomic coding region AOX primers (lane 2). B) Detection of AOX expression in *P. patens* by RT-PCR. Lane 2, 323 bp positive kit control; lane 3, 700 bp amplified AOX transcript using 2 µg total RNA and internal moss transcript primers; lane 4, negative kit control (omitted reverse transcriptase from reaction). C) Detection of the reamplified full-length 1098 bp AOX coding region cDNA in *P. patens* (Lane 2; band indicated by *). The original RT-PCR product was poorly amplified using the full-length coding primer set (not shown), and was therefore reamplified in a PCR (gel shown) to increase levels for extraction. Lane 1, DNA molecular size marker. Figure 4B was modified to remove a lane between lanes 3 and 4 that contained an unamplified product from *Selaginella.*

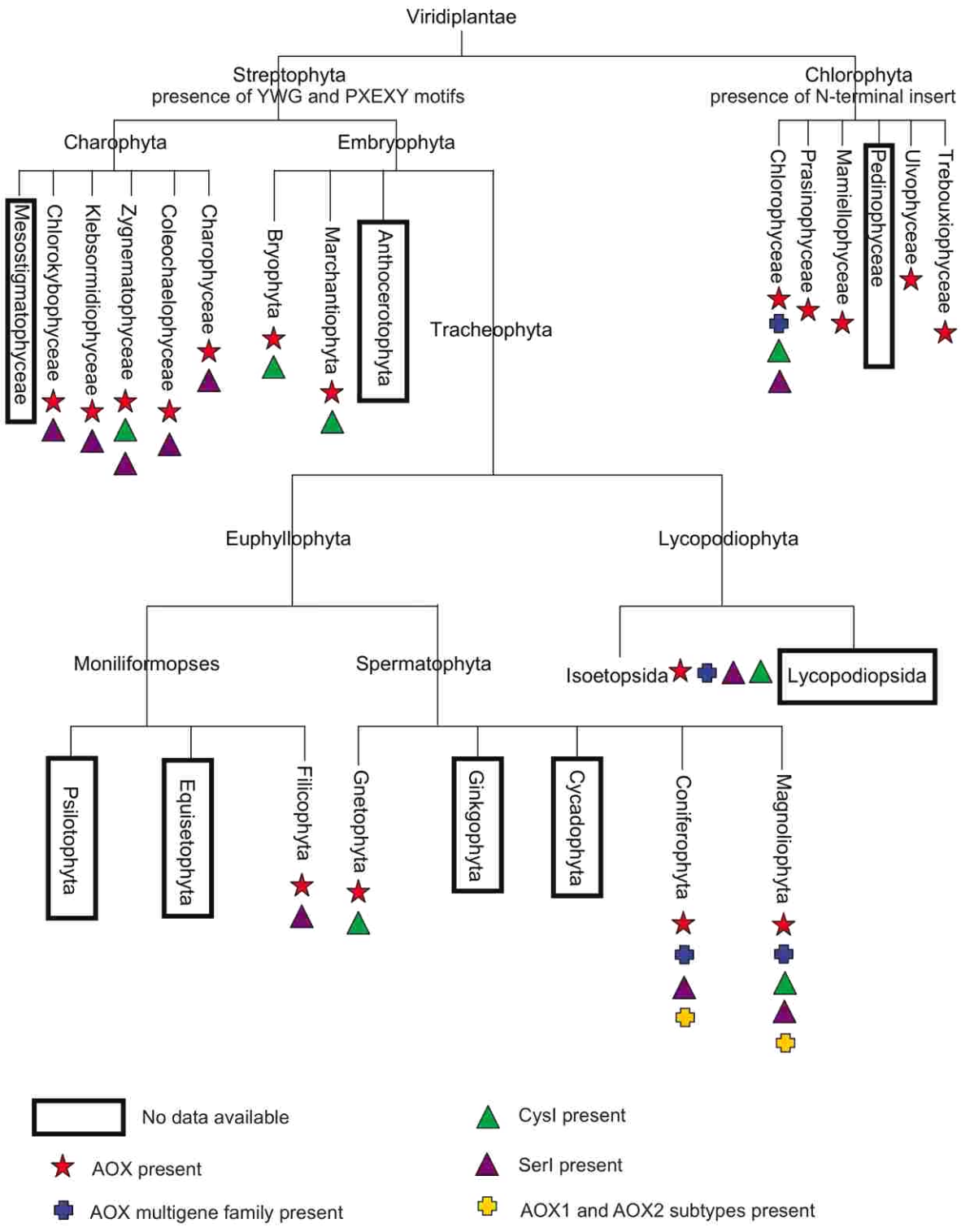


Figure 2.5. The evolutionary history and taxonomic distribution of AOX among non-angiosperm members of the Viridiplantae.

Table 2.2. Identification of amino acid residues that are involved in AOX regulation, characterization, or activity in non-angiosperm members of the Viridiplantae. *Arabidopsis thaliana* AOX1a (accession #AEE76627) is used as a reference for what is commonly seen in angiosperm AOXs and all residues are numbered according to this sequence. "Unknown" indicates that data is not available due to partial sequences.

Species	Accession number	CysI (C126)	CysII (C177)	IBS1 E-x_n-	IBS2 -E-x-x-H-x_n-	TyrI (Y258)	IBS3 -E-x_n-	TyrII (Y280)	IBS4 -E-x-x-H	'AOA' antibody recognition
Arabidopsis thaliana	AEE76627	Cys	Cys	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Chlamydomonas reinhardtii AOX1	AF047832	Ser	Arg	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Chlamydomonas reinhardtii AOX2	AF314255	Cys	Gln	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Bathycoccus prasinos	FO082264 204834-204196	Tyr	Phe	Yes	Yes	Yes	Yes	Yes	Unknown	Unknown
Ostreococcus lucimarinus CCE9901	XM_001419431	Tyr	Gln	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Spirogyra pratensis	GW599090	Ser	Cys	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Marchantia polymorpha	BJ864156/BJ872239	Cys	Cys	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Physcomitrella patens	XP_001774858	Cys	Val	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Selaginella lepidophylla	EH092495	Ser	Tyr	Yes	Yes	Yes	Unknown	Unknown	Unknown	Unknown
	EH093273	Ser	Cys	Yes	Yes	Unknown	Unknown	Unknown	Unknown	Unknown
Selaginella moellendorffii	XP_002981994	Cys	Cys	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	XP_002963435	Ser	Cys	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	XP_002984380	Ser	Cys	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Adiantum capillus-veneris	DK948381	Ser	Cys	Yes	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
Ceratopteris richardii	BE642413	Unknown	Unknown	Unknown	Unknown	Yes	Yes	Yes	Yes	Yes
Ephedra distachya	JG722268	Cys	Cys	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Picea engelmannii x *Picea glauca*	CO207489	Unknown	Unknown	Unknown	Yes	Yes	Yes	Yes	Yes	Yes
Picea glauca	DR569129	Ser	Iso	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	GO371284	Unknown	Unknown	Unknown	Yes	Yes	Yes	Yes	Yes	Yes
	EX442167	Ser	Cys	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Picea sitchensis	ABK23336	Ser	Iso	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Pinus pinaster	CT583387/BX678396	Ser	Cys	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Pinus banksiana	GW754219	Unknown	Unknown	Yes	Yes	Yes	Yes	Yes	Yes	yes
Pinus pinea	ACV60638	Unknown	Unknown	Yes (partial)	Yes	Yes	Yes	Yes	Yes	Yes
	ACV60641	Unknown	Unknown	Yes (partial)	Yes	Yes	Yes	No	Yes	Yes
Pinus taeda	DT629136	Ser	Cys	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Pseudotsuga menziesii	CN636314	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Yes	Yes	Yes

CHAPTER 3

Heterologous expression of *P. patens* AOX in the yeast *Saccharomyces cerevisiae*

Abstract

Despite being a non-energy conserving pathway, AOX has a vast taxonomic distribution and is present in many plants, fungi, and some protists. It has been most thoroughly characterized in a number of angiosperm plants; however, AOX distribution in other members of the Viridiplantae is less clear. An initial characterization of AOX at the gene and transcript level has been recently conducted in the moss *Physcomitrella patens*. In this study, I have extended that characterization by examining moss AOX at the protein level. I have heterologously expressed *P. patens* AOX in the yeast *Saccharomyces cerevisiae* and protein expression was analyzed by performing mitochondrial isolations, SDS-PAGE, and Western blots. The immunoblot results indicate that moss AOX is targeted to the mitochondria, and for the first time, I have demonstrated that both oxidized and reduced protein forms are present in a non-angiosperm plant. Although oxygen consumption was measured using respirometry, the results indicated that AOX was not functional in transformed yeast mitochondria using NADH as a substrate. I was unable to mutate several conserved amino acid residues in moss AOX by site-directed mutagenesis. Despite the lack of success in respirometry and site-directed mutagenesis, I believe that an examination of AOX protein characteristics in the *S. cerevisiae* expression system will be a valuable tool for future comparative studies once these procedures have been optimized.

1. Introduction

The alternative oxidase (AOX) is an inner mitochondrial protein that creates an alternative pathway for electrons at the ubiquinone pool in the respiratory electron transport system and bypasses the last two complexes in the cytochrome c oxidase (COX) pathway (Figure 1.1). Respiration by the AOX pathway has been termed as 'cyanide-resistant' because of its tolerance to all COX inhibitors, such as cyanide and nitric oxide (Vanlerberghe et al., 1994; Huang et al., 2002; Figure 1.1). However, it has been demonstrated that AOX activity is compromised by salicylhydroxamic acid (SHAM) and n-propyl gallate (nPG) (Vanlerberghe et al., 1994; Yip and Vanlerberghe, 2001; Figure 1.1). Similar to complex IV of the COX pathway, the AOX protein is a terminal oxidase and reduces oxygen to water (Berthold et al., 2000; Figure 1.1). However, unlike the COX complexes, AOX is non-proton motive and as a result, fewer ATP molecules are yielded per oxygen consumed during its activity (Moore and Siedow, 1991).

Despite being a non-energy conserving pathway, AOX has a vast taxonomic distribution and has been predominately characterized in the plant kingdom. AOX multigene families, transcript expression, protein regulation, and enzymatic activity have been thoroughly investigated in several angiosperm plants (McDonald, 2008). A number of amino acid residues have been identified in all angiosperm AOX sequences and have been shown to play a crucial role in protein regulation and activity by site-directed mutagenesis. In plants, AOX exists as a dimeric protein that is typically modulated by the redox state of a disulfide bond that is formed by a conserved cysteine residue (CysI) (Vanlerberghe et al., 1998). The reduction of the sulfhydryl groups renders AOX in an active state and can be further stimulated by pyruvate (Rhoads et al., 1998). Key glutamate (GluI, II, III, and IV) and histidine (HisI, II, and III)

residues have been confirmed to play a role in iron-binding (Berthold et al., 2000), and a tyrosine (TyrII) residue has been shown to contribute to AOX activity in all angiosperm AOXs studied to date (Moore and Albury, 2008). Angiosperm AOX protein levels have been detected in isolated mitochondria by AOX-specific antibodies using Western blot analyses. Wild-type and mutant angiosperm AOX functionality have also been assessed by respirometry to measure oxygen consumption rates after the addition of respiratory inhibitors (McDonald et al., 2002).

Although AOX is said to be widespread in the Viridiplantae, AOX has not been thoroughly investigated in non-angiosperms. A recent bioinformatics study by Neimanis et al. (2013) assessed the taxonomic distribution of AOX in this group and revealed that AOX is present in many chlorophytes and non-angiosperm streptophytes. Sequence analysis revealed that AOX is likely active in these plants due to the presence of conserved amino acids that are essential for angiosperm AOX function. For the first time, AOX was experimentally characterized at the gene and transcript levels in a non-angiosperm plant, *Physcomitrella patens* (moss) (Neimanis et al., 2013). It was demonstrated that the AOX gene is present in moss and an AOX transcript is produced. More interestingly, it is likely that *P. patens* possesses only a single AOX gene (Neimanis et al., 2013); a characteristic unseen in all angiosperm AOXs studied to date, which are encoded by multigene families. This lack of AOX gene copies and protein redundancy coupled with the genetic tools available in *P. patens* makes this moss an attractive system to study AOX in future experiments in order to elucidate the physiological significance of AOX in plants (Neimanis et al., 2013).

The moss *Physcomitrella patens* has been used as a model system in plant biology studies in recent years. The recent development of a mitochondrial isolation protocol by Lang et al.

(2011) has made it possible to investigate mitochondrial proteomics in moss. However, large-scale moss propagation is needed to carry out this procedure (Lang et al., 2011) and would require the use of costly bioreactors (Reutter and Reski, 1996). This type of culture set-up is not feasible in every laboratory setting. As an alternative solution, moss mitochondrial proteins can be easily studied using a heterologous eukaryotic expression system.

The yeast *Saccharomyces cerevisiae* has been extensively used as a model system in protein expression studies due to its rapid growth, the fact that it possesses eukaryotic protein processing mechanisms (Domínguez et al., 1998), and the availability of its complete genomic sequence (Goffeau et al., 1996). This system is particularly useful for AOX research as *S. cerevisiae* is one of the two known fungal species that naturally lack AOX (Minagawa and Yoshimoto, 1986). Thus, *S. cerevisiae* has been exploited to heterologously express AOX from angiosperms such as tomato (*Lycopersicon esculentum* L. Mill cv. Sweetie) (Holtzapffel et al., 2003) and other fungal species such as *Hansenula anomala* (Mathy et al., 2006) and *Candida albicans* (Huh and Kang, 1999).

The purpose of this study was to investigate *P. patens* AOX at the protein level to examine AOX size, localization, conformation, and functionality in isolated mitochondria from transformed *S. cerevisiae*. The Western blot analyses demonstrate that recombinant AOX protein is expressed in yeast and that *P. patens* AOX is targeted to the mitochondria; for the first time it has been have revealed that the oxidized and reduced AOX forms are both present in moss. I attempted to assess AOX functionality using respirometry. My results indicate that wild-type moss AOX is not functional using NADH as a substrate in transformed yeast mitochondria. Attempts to mutate amino acid residues required for AOX function using site-directed

mutagenesis were unsuccessful and I have yet to verify if conserved amino acid residues are essential for *P. patens* AOX activity in mutant strains. However, since this is a preliminary investigation, I anticipate that AOX respiratory activity and mutant protein functionality will be measured in future studies by modifying the experimental procedures described below.

2. Materials and Methods

2.1. AOX Primer Design for Yeast Transformation and Amplification Parameters

Custom DNA primers were designed to amplify the full-length, native *P. patens* AOX coding sequence using the program Primer3 (http://frodo.wi.mit.edu/primer3/). The identical forward "Stop" and "NoStop" primers (5′-CGGTTTCGGATGCGATGT-3′) included a Kozak consensus sequence and the "ATG" start codon and was designed to ensure translation initiation in the heterologous host once the cDNA product was cloned into the expression vector. The reverse-complement for the stop codon "TAG" was incorporated into the reverse "Stop" primer (5′-ACTAGCAATGCGCTAGTGG-3′) to end translation of the native moss AOX protein when expressed in the vector. The reverse "NoStop" primer (5′-ACTAGCAATGCGCT**T**GTGG-3′) included a point mutation (in bold and underlined) within the stop codon of the native AOX sequence, which converted it to lysine, to allow translation to continue until it reaches a stop codon designed within the vector. This moss AOX protein was thereby engineered to contain a C-terminal multi-histidine tag.

To amplify the "Stop" and "NoStop" cDNA products, a first-round of amplification was carried out using the Access RT-PCR Introductory System (Promega Corporation, Madison, WI, USA) using *Tfl* Polymerase. RT-PCR contained 2 μg of *P. patens* gametophyte total RNA that

was pre-treated with RQ1 RNase-Free DNase (Promega Corporation, Madison, WI, USA). The thermal cycler was programmed with an initial reverse transcription step for 45 min. at 45°C, followed by a first cycle of denaturation for 2 min. at 94°C. Next, for 30 rounds of amplification each cycle included 30 sec. at 94°C for denaturation, 1 min. at 53°C for annealing, and 1 min. at 68°C for elongation. The final extension time was prolonged for 7 min.

Next, 2 µL of the RT-PCR products were reamplified using the *iTaq* Polymerase kit (iNtRON Biotechnology, Inc.) in order to acquire 3′A-overhangs that are required for successful ligation into the pYES2.1 vector (Figure 3.1). The thermal cycler was programmed with an initial denaturation step for 2 min. at 94°C, followed by 30 cycles of amplification. Each cycle consisted of 20 sec. at 94°C for denaturation, 20 sec. at 53°C for annealing, and 1.15 min. at 72°C for elongation (the elongation step was extended for 7 min. during the last cycle). All cDNA products were analyzed on a 1.2% agarose gel stained with ethidium bromide. For yeast transformation, the inserts known as "Stop" and "NoStop" were selected for ligation into the pYES2.1/V5-His-TOPO® vector (Invitrogen, Carlsbad, CA) (Figure 3.1).

2.2. Ligation of PCR Products into pYES2.1 and Transformation into Competent *E.coli*

The pYES2.1 TOPO® TA Expression Kit (Invitrogen, Carlsbad, CA) was used to ligate the "Stop" and "NoStop" PCR products into the pYES2.1/V5-His-TOPO® vector (Figure 3.1). The following 6 µL reactions were prepared: 4 µL PCR reaction, 1 µL salt solution, and 1 µL vector. The samples were incubated at room temperature for 30 min. to increase ligation efficiency. The reactions were then placed on ice before transformation into competent *E. coli*.

One Shot® TOP10F′ chemically competent *E. coli* were transformed with 2 µL of the "Stop" or "NoStop" ligation reactions or with the pYES2.1/V5-His/*lacZ* plasmid (positive control provided with the kit), as outlined by the supplier (Invitrogen, Carlsbad, CA). Selective plates were inoculated with the transformed cells at two different volumes (50 µL or 100 µL). The plates were grown overnight at 37˚C on Luria-Bertani (LB) agar plates supplemented with 100 µg/mL ampicillin. Blue/white screening selection was not possible; therefore, colonies that appeared to have sufficient growth were randomly selected and restreaked onto LB ampicillin plates. Plasmids were pre-screened by colony PCR using the internal moss transcript forward (5′-GGATCGGAGGAAAAATTCGT-3′) and internal moss transcript reverse (5′-GGCGAAATGGTTAACGTCAC-3′) primers (see Chapter 2, Section 2.4) to verify the presence of the AOX insert. Positive transformants were subcultured into 5 mL LB broth with 100 µg/mL ampicillin and grown overnight at 37˚C with 100 rpm shaking. Glycerol stocks using 0.5 mL transformed cells with 0.5 mL 100% glycerol were prepared and stored at -80˚C. Plasmids were isolated using the QIAprep® Spin Miniprep Kit (QIAGEN Sciences, Maryland, USA) according to the manufacturer's instructions and eluted with 40 µL nuclease-free water. Five of the "Stop" and "NoStop" constructs were then sent for sequencing at the Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Canada) to confirm that the insert was in the correct orientation and in-frame, before proceeding to yeast transformation.

2.3. Transformation of *Saccharomyces cerevisiae* with *P. patens* AOX cDNA

2.3.1. Initiation of Yeast Growth

The wild-type uracil auxotrophic yeast strain *Saccharomyces cerevisiae* INV*Sc*1 cells (Invitrogen, Carlsbad, CA, USA) were streaked onto Yeast Extract Peptone Dextrose (YPD)

plates and grown for 4 days at 30˚C. A single colony was inoculated into 10 mL YPD liquid medium and incubated overnight at 30˚C with 150 rpm shaking. The next day, 9 mL of overnight culture was added to 50 mL fresh YPD medium and incubated overnight at 30˚C with 150 rpm shaking. A glycerol stock of wild-type INV*Sc*1 (0.5 mL culture: 0.5 mL 100% glycerol) was prepared and stored at -80˚C.

2.3.2. Transformation of INVSc1 Cells

The Stop *P. patens* AOX and positive *lac*Z control (pYES2.*1*/V5-His/*lacZ*) vectors were transformed into INV*Sc*1 yeast cells according to the supplier's instructions (Invitrogen, Carlsbad, CA, USA). An OD_{600} of 0.4 was required for the protocol and the absorbance of the overnight culture was determined by a spectrophotometer using the equation below:

$$(\text{Volume inoculum})(OD_{600}\ \text{inoculum}) = (\text{Volume required})(OD_{600}\ \text{required})$$

The calculated volume of overnight culture was then diluted into 50 mL YPD and grown for an additional 2 h. at 30˚C with shaking. Equal volumes of the yeast suspension were poured into two 50 mL centrifuge tubes and the cells were pelleted at 2500 rpm for 3 min. The supernatant was carefully decanted and the tubes were inverted onto paper towel to remove residual liquid. The pellets were resuspended in 20 mL 1X Tris-EDTA (TE) buffer by inverting the tubes and the cells were centrifuged at 2500 rpm for 3 min. and the supernantant was discarded. The pellets were then resuspended in 2 mL 1X lithium acetate (LiAc)/ 0.5X TE solution by inverting and incubated at room temperature for 10 min. Duplicate transformations for each sample (two tubes Stop and two tubes *lac*Z) were set-up to contain 1 µg plasmid DNA, 100 µg denatured sheared salmon sperm DNA (Sigma-Aldrich D9156), and 100 µL yeast suspension. Next, 700 µL of

fresh 1X LiAc/ 40% PEG-3350/ 1X TE solution was added to each tube and was mixed by inverting. The samples were incubated at 30˚C for 30 min. and then heat shocked at 42˚C for 7 min. The cells were centrifuged at 2500 rpm for 3 min. and the supernatant was carefully discarded. Pellets were resuspended in 1 mL 1X TE and repelleted. The supernatant was decanted and the cells were resuspended in 100 µL 1X TE. Two volumes (50 µL and 100 µL) were plated from each sample along with negative control wild-type INV*Sc*1 onto selective synthetic complete medium without uracil with 2% glucose (SC-U glucose) plates and incubated at 30˚C for 2 days. Three individual colonies from both the Stop and *lac*Z plates were randomly selected and restreaked onto SC-U glucose solid medium at 30˚C for 2 days.

2.4. Induced Protein Expression in Transformed *S. cerevisiae*

For the following procedure, all incubation steps were carried out at 30˚C with 90 rpm shaking. To initiate cultures, one loopful of transformed cells was inoculated into 50 mL SC-U glucose liquid medium and incubated for 26.5 h. The cells were spun at 3000 x g for 5 min., the supernatant was decanted, and the pellet was resuspended into 250 mL SC-U glucose for 20.5 h. The cells were repelleted, washed with 250 mL sterile Milli-Q water, and spun down again. The washed pellet was then resuspended into 250 mL SC-U with 2% filter-sterilized galactose (SC-U galactose) induction medium for recombinant protein expression or into 250 mL repression medium (SC-U glucose). Cultures were then incubated until mitochondrial isolations were performed.

2.5. Mitochondrial Isolations and Protein Quantification

The yeast mitochondria isolation procedure was carried out through differential centrifugation as described by Meisinger et al. (2006). Yeast mitochondria were isolated at various time points (0, 4, 8, 12, and 16 h.) in order to determine the maximal protein expression time after galactose induction. All time-course experiments were conducted at the same sampling times with cells grown in two different media conditions: one in SC-U glucose to ensure recombinant proteins are not expressed and the second in SC-U galactose in order to induce expression. All isolations were performed in duplicate.

To begin, the yeast culture was poured into a 400 mL centrifuge tube and spun at 3000 x g for 5 min. at room temperature, the supernatant was decanted and the pellet was resuspended in 250 mL water; this was repeated once in order to remove residual growth media. The cells were then centrifuged at 3000 x g for 5 min. and the mass of the wet pellet was recorded. The cells were resuspended in dithiothreitol (DTT) buffer (100 mM Trisma base pH 9.4, 10 mM DTT; 2 mL buffer: g wet mass cells), transferred to a 40 mL centrifuge tube, and shaken at 70 rpm for 30 min. at 28˚C. The culture was then pelleted at 3000 x g for 5 min. and was resuspended in Zymolyase buffer (20 mM potassium phosphate pH 7.4, 1.2 M sorbitol; 7 mL buffer: g wet mass cells); this was repeated once. The resuspension was then transferred to a beaker containing 20T Zymolyase (3 mg powder: g wet mass cells) and was incubated for 30 min. at 28˚C with 70 rpm shaking in order to lyse the cells. After this point, all remaining centrifugation steps were performed at 4˚C and all solutions and tubes were kept on ice. The culture was spun at 2200 x g for 8 min. in a 40 mL centrifuge tube and then resuspended in homogenization buffer (10 mM Tris-HCl pH 7.4, 0.6 M sorbitol, 1 mM EDTA; 6.5 mL buffer: g wet mass cells); this was

repeated once. The cells were then placed in a 50 mL glass potter, followed by 15 up and down strokes with a pestle in order to homogenize them. The mixture was then topped off with one volume of homogenization buffer and poured into a 40 mL centrifuge tube and spun at 1500 x g for 5 min. The supernatant was poured into a new tube and spun at 3000 x g for 5 min. The supernatant was transferred to a fresh tube and spun at 12000 x g for 15 min. The pellet was then resuspended in homogenization buffer (6.5 mL buffer: g wet mass cells) and centrifuged at 3000 x g for 5 min. The supernatant was transferred into a new tube and spun for 15 min. at 12000 x g. The remaining pellet containing the crude mitochondrial fraction was carefully resuspended in 1 mL SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS/KOH pH 7.2) and transferred to a microcentrifuge tube before a final spin at 12000 x g for 15 min. The pellet was then diluted in 250 µL SEM buffer and stored at -80˚C. Protein concentrations were determined using the Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories, Canada) with bovine serum albumin (BSA) standards quantified with the Ultraspec 1100 pro UV/visible spectrophotometer (Fisher Scientific).

2.6. Whole Cell Protein Extraction from Yeast

S. cerevisiae proteins were extracted from whole cells as outlined by Kushnirov (2000). Before proceeding with the mitochondrial isolation procedure (outlined above), 1 mL of whole yeast cells was taken from each culture, centrifuged at 5000 x g for 5 min. and stored at -80˚C in cryogenic vials until processing. Frozen yeast pellets (as described in Section 2.5), that were grown in SC-U galactose medium, were thawed at room-temperature and 2.3 mg of wet weight was resuspended in 100 µL distilled water and 100 µL 0.2 M sodium hydroxide. Cells were incubated for 5 min. at room temperature before centrifugation at 5000 x g for 5 min. The pellet

was resuspended in 50 µL modified Laemmli buffer (0.06 M Tris-HCl (pH 6.8), 5% glycerol, 2% SDS, 4% β-mercaptoethanol, 0.0025% bromophenol blue) and then boiled for 3 min. before centrifugation at 5000 x g for 5 min. The supernatant, which contained the yeast proteins, was then ready for analysis.

2.7. Theoretical Molecular Weight Determination, SDS-PAGE and Western blotting

Prior to performing SDS-PAGE and Western blotting, the predicted *P. patens* AOX peptide sequence retrieved from the Phytozome database (http://www.phytozome.net/) was placed into the compute pI/Mw tool (Gasteiger et al., 2005) available on the ExPASy server (http://web.expasy.org/compute_pi/) to estimate the theoretical molecular mass (kDa). This value was used to predict the approximate region of localization of the AOX protein in the gel and membrane.

Recombinant proteins from isolated mitochondria or whole cell yeast protein extracts were analyzed by SDS-PAGE and immunoblotting. Two 12% acrylamide gels were loaded with 5 µl Precision Plus Protein™ prestained ladder (Bio-Rad Laboratories, Canada), 5 µg of mitochondrial protein or 6 µL whole cell yeast proteins (as suggested by Kushnirov [2000]) per well, and 4.7 µg phosphate starved (low Pi) tobacco mitochondrial protein (provided by Dr. G.C. Vanlerberghe) as a control. Proteins were separated using reducing SDS-PAGE in the presence of Laemmli loading buffer (Laemmli, 1970) for mitochondrial proteins or modified Laemmli buffer (Kushnirov, 2000) for yeast protein extracts, both containing the reductant β-mercaptoethanol. To ensure proper separation and equal loading of the protein samples, the first acrylamide gel was stained with Coomassie brilliant blue R-250 for 30 min. and then incubated overnight on a shaker in destain solution.

For Western blot analysis, the resolved proteins in the second acrylamide gel were blotted onto a nitrocellulose membrane using a cassette holder that was placed into the Mini Trans-Blot® cell (Bio-Rad Laboratories, Canada) containing protein blotting buffer and transferred for 1 h. at 100 V constant. The membrane was blocked overnight using 0.5% milk powder in tris-buffered saline with Tween 20 (TBS-T). The blot was probed with a primary monoclonal antibody specific to AOX (AOX1/2 product number: AS04054; Agrisera AB, Vannas, Sweden) in a 1:5000 dilution of antibody to TBS-T, or with a primary monoclonal porin antibody specific to *S. cerevisiae* (product number: 459500; Invitrogen, Carlsbad, CA, USA) in a 1:1000 dilution of antibody to TBS-T, and was placed on a shaker for 1 h. at room temperature. The membrane was washed three times in TBS-T (once for 15 min. and twice for 10 min.) before incubation in the secondary antibody. The secondary AOX antibody was a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H&L) (product number: AS09602; Agrisera AB, Vannas, Sweden), whereas the secondary porin antibody was an HRP-conjugated goat anti-mouse IgG1 (g1) (product number: A10551; Invitrogen, Carlsbad, CA, USA), both of which were both applied in 1:10000 dilutions in TBS-T. Both secondary antibodies were combined with a 1:10000 dilution of the Precision Protein StrepTactin-HRP Conjugate (Bio-Rad Laboratories, Canada) to TBS-T and then incubated at room temperature for 1 h. on a shaker, followed by three washes in TBS-T as described above. Lastly, the membrane was covered with a 1:1 ratio of luminol/enhancer and peroxide buffer solutions from the Immun-Star™ WesternC™ Chemiluminescent Kit (Bio-Rad Laboratories, Canada) for 5 min. before being visualized on the Bio-Rad Molecular Imager Gel-Doc XR.

2.8. Oxygen Uptake Measurements

High-resolution respirometry was performed with intact, isolated mitochondria from *S. cerevisiae* to assess AOX functionality. Oxygen consumption rates were measured using a Clark-type oxygen electrode in the Oroboros Oxygraph-2k (Oroboros® Instruments GmbH, Innsbruck Austria). Each trace was maintained at 30˚C in a closed-glass chamber containing 0.1 mg mitochondrial proteins in 2 mL of MiR05 respiration medium (Appendix 2). Cytochrome c oxidase (COX) and AOX activity was assessed using two different assay conditions with a combination of respiratory substrates, AOX activators, and inhibitors (Table 1). In the first assay (assay A), the compounds were added to the chamber in the following order: NADH, ADP, potassium cyanide (KCN), and SHAM (Table 3.1). The second assay (assay B) included NADH, ADP, DTT, pyruvate, KCN, and SHAM (Table 3.1). Oxygen consumption rates (nmol O_2 min^{-1} mg^{-1} protein) and standard errors were calculated from the average of three independent mitochondrial isolations.

2.9. Site-directed Mutagenesis Primer Design and Construct Preparation

Six amino acids within the *P. patens* AOX were selected for site-directed mutagenesis (Table 3.2). The QuikChange® Primer Design program, located on the Agilent Technologies website (https://www.genomics.agilent.com/HomePage.aspx), was used to design primers to introduce the following individual mutations: Tyr107 to Phe, CysI to Ala, Tyr134 to Phe, GluII to Ala, TyrII to Phe, and HisII to Ala (Table 3.2).

The QuikChange® Lightning Site-directed Mutagenesis Kit (Agilent Technologies, Mississauga, ON) was used to generate mutant plasmids, as described by the supplier. For each

mutation, a 50 µL PCR reaction was prepared using 50 ng of 1Stop plasmid DNA and 125 ng of forward and reverse oligonucleotide primers. To optimize amplification conditions, an initial denaturation step was programmed for 2 min. at 95˚C, followed by 18 cycles consisting of: 20 sec. at 95˚C for denaturation, 10 sec. at 60˚C for annealing, and 3.5 min. at 68˚C for elongation (with a final extension time of 5 min. for the last cycle). PCR products were digested with 2 µL of the *Dpn* I restriction enzyme for 5 min. at 37˚C to remove the non-mutated, parental plasmid DNA. Next, XL10-Gold ultracompetent *E. coli* cells were chemically transformed with the mutant plasmids (Figure 3.2). Plasmid DNA was purified using the QIAprep® Spin Miniprep Kit (QIAGEN Sciences, Maryland, USA) and sent to the Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Canada) for sequencing (Figure 3.2).

3. Results

3.1. Sequence Analysis of Plasmid Constructs and Yeast Transformation

Five Stop and five NoStop plasmids were sequenced using the *GAL1* forward primer and the V5 C-term reverse primer (Invitrogen, Carlsbad, CA, USA) for N-terminal and C-terminal sequencing of the insert, respectively. A single construct, called "1Stop", contained the *P. patens* AOX insert in the correct orientation and in-frame; all of the "NoStop" sequencing results came back negative. The N-terminal and C-terminal products obtained through sequencing were translated into amino acids using the Translate tool located on the ExPASy server (http://ca.expasy.org/tools/dna.html) and then placed into a multiple-sequence alignment against the predicted *P. patens* AOX peptide sequence (Figure 3.3). The alignment revealed that the full-length AOX insert was successfully cloned into the pYES2.1 vector and that it shared 100% shared sequence identity to the predicted AOX protein (Figure 3.3).

INV*Sc*1 yeast cells were transformed with the *lacZ* positive kit control vector or the 1Stop construct. Two volumes of each transformation reaction and negative control wild-type INV*Sc*1 cells were plated onto SC-U glucose solid media (Figure 3.4). Sufficient colony growth on selective media demonstrated successful chemical transformation of yeast with the pYES2.1 vector (Figure 3.4A; Figure 3.B). No growth was observed on the negative control plates, as wild-type yeast do not possess the *URA*3 gene (Figure 4C).

3.2. Recombinant Protein Expression in Yeast

3.2.1. Mitochondrial Protein Analysis

Western blotting was used to analyze proteins in mitochondria isolated from yeast that were transformed with the pYES2.1 vector containing the 1Stop or *lac*Z gene. To assess recombinant protein expression, mitochondria were isolated at specific time points from cells that were grown in either repression (GLU) or induction (GAL) medium. Pilot experiments were carried out using 0 h., 4 h., and 8 h. GLU and GAL 1Stop mitochondria. Protein expression was not observed in the 0 h. samples and very low signals were seen in the 4 h. and 8 h. GAL samples (data not shown). Thus, transformed cells were incubated for longer time points (8 h., 12 h., and 16 h.) in order to achieve higher levels of protein expression (Figure 3.5).

The compute pI/Mw software predicted a theoretical AOX protein size of ~42 kDa (Gasteiger et al., 2005). Effective protein separation by electrophoresis was analyzed in acrylamide gels that were stained with Coomassie brilliant blue (Appendix 3). Experimentally, the reduced (~35 kDa) and oxidized (~75 kDa) forms of AOX were detected as double bands in 8 h., 12 h., and 16 h. 1Stop GAL mitochondria by the AOX antibody (Figure 3.5A). The highest

protein signals (most intense bands) were observed at 16h. of expression (Figure 3.5A). The porin antibody, specific to *S. cerevisiae*, was used as a positive control to validate the mitochondrial isolation procedure. Porin is a ~30 kDa outer mitochondrial membrane protein, and was recognized at comparable levels in all 1Stop GLU and GAL mitochondria (Figure 3.5B). A signal was not detected in any of the *lac*Z mitochondrial samples by the AOX antibody (Figure 3.5C); however, porin was detected in all *lac*Z samples (Figure 3.5D). The presence of porin in all 1Stop and *lac*Z samples indicated that mitochondria were successfully isolated. Low Pi tobacco mitochondria were as a positive or negative control in each experiment respectively. The reduced (~35 kDa) and oxidized (~75 kDa) forms of AOX were present as single bands in low Pi tobacco mitochondria (Figure 3.5A; Figure 3.5C); there was no signal detected by the yeast-specific porin antibody in this sample (Figure 3.5B; Figure 3.5D).

3.2.2. Whole Cell Protein Analysis

Whole cell yeast protein extracts were isolated from 16 h. 1Stop GAL and INV*Sc*1 GAL whole cell cultures and analyzed by Western blots probed with the AOX or porin antibody (Figure 3.6). The AOX antibody was used to detect recombinant AOX (Figure 3.6A). The reduced (~35 kDa) AOX protein was recognized in the 1Stop sample only (Figure 3.6A). Porin (~30 kDa) was present in the 1Stop and INV*Sc*1 samples (Figure 3.6B). These results indicate that residual AOX and porin proteins are detectable in whole cell extracts. Interestingly, two intense bands (~41 kDa and ~125 kDa) were recognized in 1Stop and INV*Sc*1 mitochondria by both antibodies (Figure 3.6A; Figure 3.6B). The identity of these proteins has yet to be determined.

3.3. Oxygen Uptake via the AOX Pathway

Oxygen consumption by the COX and AOX pathways was analyzed using isolated mitochondria from wild-type or AOX expressing *S. cerevisiae* (Appendix 4 & 5). For each assay, 0.1 mg mitochondrial proteins were added to the chamber containing 2 mL respiratory medium and equilibrated for 5 min. and this rate was used for background correction. In all samples, little activity was observed from isolated mitochondria until 2 mM NADH was added to the chamber (Table 3.3). A noticeable increase in the oxygen consumption rate in both wild-type and AOX mitochondria was observed after the addition of ADP in both assay conditions (Table 3.3). In "assay B" runs, the uptake rates decreased after the sequential addition of DTT and pyruvate (AOX activators) in both mitochondrial samples (Table 3.3). Resistance to COX inhibitors by AOX was measured by titration of KCN. Respiratory rates reached below zero levels after the addition of KCN in all samples (Table 3.3). Lastly, complete shutdown of AOX activity was to be achieved by titration with SHAM; however, inhibition was not possible since oxygen consumption rates were already well below zero (Table 3.3).

3.4. *P. patens* AOX Mutants

Six AOX amino acid residues were selected for mutation (Table 3.1). The plasmid containing the 1Stop insert was used as template DNA for site-directed mutagenesis. Amplification, restriction digest, and transformation procedures were performed in accordance with the kit manufacturer's instructions. No blue colonies were detected on selective plates, which implied 100% transformation efficiency. Since this is an unlikely result, only two of the six site-directed mutagenesis plasmids (GluII to Ala and HisII to Ala) were sequenced to determine if the mutation had been successfully incorporated. Sequencing analysis revealed that

the AOX insert was unaltered in both constructs. Therefore, site-directed mutagenesis experiments were halted.

4. Discussion

4.1. Expression of Moss AOX in *S. cerevisiae*

S. cerevisiae cannot perform cyanide-resistant respiration due to the absence of AOX (Minagawa and Yoshimoto, 1986). *P. patens* AOX was introduced into *S. cerevisiae* to examine non-angiosperm AOX localization and functionality. Prediction software estimated a high probability of mitochondrial targeting for moss AOX (Neimanis et al., 2013). Therefore, mitochondrial isolations from transformed *S. cerevisiae* cells were carried out to investigate this protein. Western blot analysis revealed that the reduced (~35 kDa) and oxidized (~75 kDa) forms of *P. patens* AOX were only expressed in cells that had been grown in induction media (Figure 3.5A). The most intense levels of expression were observed in 16 h. isolated mitochondria (Figure 3.5A). AOX expression was highly repressed in the presence of glucose (Figure 3.5A), and therefore suggests that the pYES2.1 yeast expression vector is not leaky. AOX was not recognized by the AOX antibody in mitochondria extracted from yeast transformed with the *lac*Z containing vector (Figure 3.5C), confirming that AOX is absent in *S. cerevisiae*. The detection of AOX in immunoblots confirms that AOX was successfully targeted to the mitochondrion, as predicted by MitoProt software. The 30 kDa porin protein was detected at equal levels in mitochondria isolated from AOX and *lac*Z transformed yeast cells grown in repression and induction media (Figure 3.5B; Figure 3.5D). Porin was not recognized in low Pi tobacco mitochondria, which confirmed the specificity of the porin antibody to *S. cerevisiae* (Figure

3.5B; Figure 3.5D). The presence of porin in the isolated yeast mitochondria authenticates the mitochondrial isolation procedure (Meisinger et al., 2006).

4.2. Characteristics of Moss AOX Protein

The experimental size of moss AOX (35 kDa) did not coincide very well with the mass predicted (~42 kDa) using *in silico* methods. This higher theoretical value may be due to the presence of an N-terminal signal peptide in the AOX peptide sequence that was used in the prediction program. The smaller 35 kDa sized protein observed in the immunoblot (Figure 3.5A) is likely due to the cleavage of the targeting peptide upon mitochondrial import (Whelan et al., 1993). Therefore, the predicted moss AOX peptide sequence was placed into the program TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/) which predicted an N-terminal presequence length of 34 amino acids. When the 34 amino acid targeting sequence was removed from the moss AOX protein sequence, the compute pI/mW software predicted a ~38 kDa protein, which coincides more strongly with the experimental 35 kDa protein. The detection of reduced AOX in moss mitochondrial fractions has been previously reported by Lang et al. (2011). The immunoblot results indicate that the reduced and oxidized forms of AOX are both present in moss, suggesting that it exists as a dimer that is redox-modulated at a conserved CysI in a similar manner to angiosperm AOXs (Neimanis et al., 2013). More prominent bands were observed at 35 kDa than at 75 kDa in all 1Stop GAL mitochondria (Figure 3.5A). The detection of the oxidized form suggests that as AOX is being overexpressed, the amount of β-mercaptoethanol (reductant) in the sample buffer is not sufficient to fully reduce all AOX proteins in the sample. The size of both AOX forms in moss is highly comparable to what is seen in most angiosperms (refer to the low Pi tobacco mitochondrial sample in Figures 3.5A & 3.5C).

However, the reduced and oxidized AOX forms in moss are present as doublets (Figure 3.5A). AOX doublets in Western blots have been previously reported in angiosperms such as transgenic maize (*Zea mays*) (Karpova et al., 2002) and potato (*Solanum tuberosum*) (Hiser et al., 1996). Hiser et al. (1996) speculates that doublet formation may be due to the accumulation of precursor proteins if AOX is overexpressed. Alternatively, Rhoads et al. (1998) proposes that double banding is attributable to a post-translational modification event. It has also been observed that the presence of doublets is tissue-specific (Hiser et al., 1996). This could explain why the double bands were absent in AOX in mitochondria isolated from moss protonema (Lang et al., 2011), but are present in the mitochondrial fractions containing AOX from moss gametophytes used in this study. Further investigation of AOX doublets is warranted.

Proteins were extracted from whole cells of wild-type and AOX expressing *S. cerevisiae* that were grown for 16 h. in induction medium in order to determine if AOX could be detected without the requirement for mitochondrial isolation. Western blot analysis using the AOX antibody revealed that only the reduced AOX protein was present in these extracts (Figure 3.6A). Porin was also detected in whole cell extracts (Figure 3.6B). Interestingly, two bands at 41 kDa and ~125 kDa were recognized by both antibodies in all samples (Figure 3.6A; Figure 3.6B). The detection of these bands is likely attributable non-specific antibody recognition of cytosolic proteins (Trinkle-Mulcahy et al., 2008). The identity of these proteins should be determined by mass spectrophotometry or N-terminal sequencing in future experiments (Trinkle-Mulcahy et al., 2008). The presence of the 35 kDa protein shows that all AOX proteins had been completely reduced in the whole cell lysate sample buffer, since the oxidized form was undetected. The presence of AOX in the whole cell extracts suggests that at 16 h. large quantities of AOX are

expressed and that organelle isolations may not be necessary for AOX detection in future experiments. However, it is unknown whether AOX is sufficiently expressed in cells grown for shorter induction times (8 h. and 12 h.) to be detectable. Future work should examine AOX protein levels in whole cell extracts from a range of induction times to determine if mitochondrial isolations are required for AOX detection on shorter time scales. The non-specific binding of the antibody to other proteins is also a concern in whole cell lysates.

4.3. Investigation of Moss AOX Functionality in the Yeast Expression System

Inhibitor studies have been extensively used to investigate cyanide-resistant respiration by the AOX pathway in a number of angiosperms, some of which include maize (*Zea mays* L.) (Van de Venter, 1985), *Arabidopsis thaliana* (Watanabe et al., 2010), tobacco (*Nicotiana tabacum*) (Vanlerberghe and McIntosh, 1992), and voodoo lily (*Sauromatum guttatum*) (Crichton et al., 2010). Despite the vast amount of data collected from both monocots and dicots, scarce information is available on AOX function in non-angiosperm plants. Of the few studies that have investigated non-angiosperm alternative respiration, data is limited to gymnosperms such as the ancient conifer *Araucaria angustifolia* (Mariano et al., 2008) and white spruce (*Picea glauca*) (Johnson-Flanagan and Owens, 1986; Weger and Guy, 1991). However, there has yet to be a study that has assessed cyanide-resistant respiration due to AOX in a basal plant. For the first time, I have attempted to measure the respiratory activity of moss AOX from isolated mitochondria of transformed yeast. Oxygen consumption rates of AOX were compared to those measured in AOX-deficient mitochondria from wild-type *S. cerevisiae*.

Respiratory activity was evaluated in the presence of substrates and inhibitors since very low levels of oxygen consumption were detected from the isolated mitochondria alone. In

contrast to most eukaryotic mitochondria, *S. cerevisiae* lacks complex I of the respiratory electron transport chain (Fang and Beattie, 2003; Figure 3.7). Instead, this yeast possesses internal and external NADH dehydrogenases, which are located on the inner and outer faces of the inner mitochondrial membrane, respectively (Fang and Beattie, 2003; Figure 3.7). Therefore, in respirometry runs using yeast mitochondria, the substrate NADH can be added directly to the chamber for oxidation by the external enzymes to initiate electron flow directly to ubiquinol. After the addition of NADH, the rate at which oxygen was consumed significantly increased, indicating that external NADH dehydrogenases were functional in both AOX expressing and wild-type isolated mitochondria (Table 3.3). Assay A rates were somewhat comparable to those observed by Crichton et al. (2010) in isolated mitochondria from *S. pombe* transformed with *S. guttatum* AOX (~132 nmol O_2 min^{-1} mg^{-1}); however, rates measured in Assay B, were significantly higher than what is observed in the literature (Crichton et al., 2010). The variation may be due to the difference in plant phylogeny (angiosperm vs. non-angiosperm enzyme) so it is difficult to make a direct comparison. Since electron transfer by the cytochrome pathway is directly coupled to proton translocation which drives ATP synthesis (Vanlerberghe et al., 1994), the rate of oxygen consumption is expected to greatly increase after ADP is added. An increase in rate by all samples after the addition of ADP suggests that the uptake of oxygen is a direct result of the coupling of electron transport to proton pumping (Table 3.3). However, a much higher rate was expected than what was measured, suggesting that the isolated mitochondria were only partially coupled. This provides evidence that the mitochondrial isolation protocol has not been optimized. To measure cyanide-resistant respiration, the complex IV inhibitor KCN was added to shut down the COX pathway. Interestingly, oxygen consumption rates measured below zero (indicating that oxygen was being generated) in all samples and continued to decrease after

the AOX inhibitor (SHAM) was added (Table 3.3). This implies that oxygen was unexpectedly introduced into the chamber. Previous respirometry experiments by other groups indicate that oxygen consumption will continue after the addition of KCN if AOX is active, but that this activity is lost (no oxygen uptake) after SHAM addition (Crichton et al., 2010; Johnson-Flanagan and Owens, 1986; Weger and Guy, 1991; Mariano et al., 2008). For these reasons, statistical analysis was not performed, as it would not be valid. The results suggest that moss AOX is not active in transformed yeast mitochondria that were isolated when NADH is used as a respiratory substrate. This also explains why the AOX activators DTT and pyruvate exhibited unusual effects on the respiration rate in Assay B conditions (Table 3.3) and it remains to be determined whether the same activators for angiosperm AOXs (Vanlerberghe et al., 1998) will stimulate non-angiosperm AOXs. However, I predict that since CysI is conserved in moss AOX it will be redox-modulated and stimulated by pyruvate in a similar manner as angiosperm AOXs (Neimanis et al., 2013).

I speculate that AOX-transformed yeast could not perform cyanide-resistant respiration due to incorrect targeting of the mitochondrial protein, protein degradation, disrupted mitochondrial membranes due to the isolation procedure, or because the AOX protein was not functionally active. The possibility of incorrect targeting can be ruled out because the Western blot analysis shows that AOX is present in isolated mitochondria from transformed *S. cerevisiae* (Figure 3.5A). Immunoblots also show that proteolysis was unlikely because smaller band fragments were not seen, which would be representative of degradation, in any of the transformed samples (Figure 3.5A). It is possible that that the inner mitochondrial membrane may have been disrupted during the mitochondrial isolation procedure as the respirometry results

indicated that the mitochondria were not fully coupled. However, the presence of porin in the immunoblots (Figure 3.5B & 3.5D) reveals that the outer mitochondrial membrane was not compromised, suggesting that the isolated mitochondria are intact. A recent study by Petrussa et al. (2008) investigated the respiratory capacity of *Picea abies* and *Abies cephalonica*. Their results demonstrated that the two conifers were incapable of cyanide-resistant respiration (zero oxygen consumption) when NADH was used as a substrate; interestingly, AOX capacity was measurable when succinate or malate combined with glutamate were used as substrates (Petrussa et al., 2008). Therefore, I predict that the AOX-transformed yeast cells possess a functionally active AOX, however, AOX activity was undetected because NADH was used as a substrate. I anticipate that with the adjustment of certain parameters, such as the substrate and inhibitor concentrations, I will be able to successfully assess *P. patens* AOX functionality in future experiments.

4.4. Functionality of *P. patens* AOX Mutants

A number of conserved amino acid residues have been identified as playing an essential role in angiosperm AOX activity and regulation, and their importance has been confirmed through site-directed mutagenesis. These key amino acid residues are also conserved in all non-angiosperm AOX sequences (Neimanis et al., 2013); however, the effects of mutations in these key residues on AOX activity has yet to be investigated in this group. For this reason, I have attempted to influence *P. patens* AOX functionality by introducing six individual mutations through site-directed mutagenesis. Four of the six mutations (CysI, GluII, TyrII, and HisII) (Table 3.2) have been previously investigated in angiosperm AOXs from tobacco (Rhoads et al., 1998; Vanlerberghe et al., 1998), voodoo lily (Albury et al., 2002), and *Arabidopsis thaliana*

(Berthold et al., 2002). The remaining amino acid residues (Tyr107 and Tyr134) that were selected for mutagenesis have yet to be investigated in plant AOXs (Table 3.2); thus, their effect on AOX activity has yet to be determined (Neimanis et al., 2013). My goal was to successfully mutate these residues in moss AOX and then assess protein functionality via respirometry with mitochondria isolated from transformed *S. cerevisiae*. Site-directed mutagenesis reactions were carefully performed as per the manufacturer's instructions and mutant constructs were transformed into *E. coli*. However, sequencing results from two selected constructs (GluII and HisII mutations) indicated that the full-length *P. patens* AOX sequence was unaltered; therefore, I felt that sequencing of the other constructs would be unnecessary. I speculate that mutant constructs were not generated due to a source of experimental error, which most likely occurred during amplification. Therefore, future site-directed mutagenesis experiments should modify the amplification parameters and attempt to improve the protocol. Alternatively, pre-made mutant constructs can be prepared by a manufacturer (Life Technologies), which will help reduce experimental error and troubleshooting. Further research is warranted to determine if conserved amino acids in non-angiosperm AOXs play an essential role in activity and in post-translational regulation as observed in angiosperm AOXs.

5. Conclusions and Future Directions

The overall goal of this study was to investigate *P. patens* AOX at the protein level in transformed *S. cerevisiae*. Through Western blot analysis, I have demonstrated that *P. patens* AOX is successfully targeted to the mitochondria. The presence of the reduced and oxidized forms indicates that moss AOX is likely dimeric and is redox-regulated in a manner similar to angiosperm AOXs. Preliminary respiratory data suggest that *P. patens* AOX is inactive in

transformed yeast mitochondria when NADH is used as a respiratory substrate and it has yet to be determined if conserved amino acid residues play a key role in moss AOX functionality and regulation. Despite this, I predict that AOX respiratory activity and mutant protein functionality will be successfully analyzed in future studies by using different respirometry substrates and improving the site-directed mutagenesis protocols. I believe that once these procedures have been optimized, investigation of *P. patens* AOX protein characteristics in the yeast expression system will be a useful tool for comparative studies.

References

Albury MS, Affourtit C, Crichton PG, Moore AL. 2002. Structure of the plant alternative oxidase. J Biol Chem. 277: 1190-1194.

Berthold DA, Andersson ME, Norlund P. 2000. New insight into the strcture and function of the alternative oxidase. Biochim Biophys Acta. . 1460: 241-254.

Berthold DA, Voevodskaya N, Stenmark P, Graslund A, Norlund P. 2002. EPR studies of the mitochondrial alternative oxidase. J Biol Chem. 277: 43608- 43614.

Crichton PG, Albury MS, Affourtit C, Moore AL. 2010. Mutagenesis of the *Saurmatum guttatum* alternative oxidase reveals features important for oxygen binding and catalysis. Biochim Biophys Acta. 1797: 732-737.

Domínguez A, Fermiñán E, Sánchez M, González FJ, Pérez-Campo FM, García S, Herrero AB, San Vicente A, Cabello J, Prado M, Iglesias FJ, Choupina A, Burguillo FJ, Fernández-Lago L, López MC. 1998. Non-conventional yeasts as hosts for heterologous protein production. Internatl Microbiol. 1: 131-142.

Fang J, Beattie DS. 2003. External alternative NADH dehydrogenase of *Saccharomyces cerevisiae*: a potential source of superoxide. Free Radic Biol Med. 34: 478-488.

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A. 2005. (In) John M. Walker (ed): The Proteomics Protocols Handbook. Humana Press. 571-607 p.

Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H, Oliver SG. 1996. Life with 6000 genes. Science. 274: 563-567.

Hiser C, Kapranov P, McIntosh L. 1996. Genetic modification of respiratory capacity in potato. Plant Physiol. 110: 277-286.

Holtzapffel RC, Castelli J, Finnegan PM, Millar AH, Whelan J, Day DA. 2003. A tomato alternative oxidase protein with altered regulatory properties. Biochim Biophys Acta. 1606: 153-162.

Huang X, von Rad U, Durner J. 2002. Nitric oxide induces transcriptional activation of the nitric oxide-tolerant alternative oxidase in *Arabidopsis* suspension cells. Planta. 215: 914-923.

Huh WK, Kang SO. 1999. Molecular cloning and functional expression of alternative oxidase from *Candida albicans*. J Bacteriol. 181: 4098-4102.

Johnson-Flanagan AM, Owens JN. 1986. Root respiration in white spruce (*Picea glauca* [Moench] Voss) seedlings in relation to morphology and environment. Plant Physiol. 81: 21-25.

Karpova OV, Kuzmin EV, Elthon TE, Newton KJ. 2002. Differential expression of alternative oxidase genes in maize mitochondrial mutants. Plant Cell. 14: 3271-3284.

Kushnirov VV. 2000. Rapid and reliable protein extraction from yeast. Yeast. 16: 857-860.

Lang EG, Mueller SJ, Hoernstein SNW, Porankiewicz-Asplund J, Vervliet-Scheebaum M, Reski R. 2011. Simultaneous isolation of pure and intact chloroplasts and mitochondria from moss as the basis for sub-cellular proteomics. Plant Cell Rep. 30: 205-215.

Mariano AB, Valente C, Maurer JBB, Cadena SMSC, Rocha MEM, de Oliveira MBM, Salgado I, Carnieri EGS. 2008. Functional characterization of mitochondria isolated from the ancient gymnosperm *Araucaria angustifolia*. Plant Sci. 175: 701-705.

Mathy G, Navet R, Gerkens P, Leprince P, De Pauw E, Sluse-Goffart CM, Sluse FE, Douette P. 2006. *Saccharomyces cerevisiae* mitoproteome plasticity in response to recombinant alternative ubiquinol oxidase. J Proteome Res. 5: 339-348.

McDonald AE. 2008. Alternative oxidase: an inter-kingdom perspective on the function and regulation of this broadly distributed 'cyanide-resistant' terminal oxidase. Funct Plant Biol. 35: 535-552.

McDonald AE, Sieger SM, Vanlerberghe GC. 2002. Methods and approaches to study plant mitochondrial alternative oxidase. Physiol Plant. 116: 135-143.

Meisinger C, Pfanner N, Truscott KN. 2006. Isolation of yeast mitochondria. Methods Mol Biol. 313: 33-40.

Minagawa N, Yoshimoto A. 1986. The induction of cyanide-respiration in *Hansenula anomala*. J Biochem. 101: 1141-1146.

Moore AL, Albury MS. 2008. Further insights into the structure of the alternative oxidase: from plants to parasites. Biochem Soc T. 36: 1022-1026.

Moore AL, Siedow JN. 1991. The regulation and nature of the cyanide-resistant alternative oxidase of plant mitochondria. Biochim Biophys Acta. 1059: 121-140.

Neimanis K, Staples JF, Huner NPA, McDonald AE. 2013. Identification, expression, and taxonomic distribution of alternative oxidases in non-angiosperm plants. Gene. http://dx.doi.org/10.1016/j.gene.2013.04.072.

Petrussa E, Bertolini A, Krajnakova J, Casolo V, Macri F, Vianello A. 2008. Isolation of mitochondria from embryogenic cultures of *Picea abies* (L.) Karst. and *Abies cephalonica* Loud.: characterization of K^{+}_{ATP} channel. Plant Cell Rep. 27: 137-146.

Reutter K, Reski R. 1996. Production of a heterologous protein in bioreactor cultures of fully differentiated moss plants. Plant Tissue Cult & Biotech. 2: 142-147.

Rhoads DM, Umbach AL, Sweet CR, Lennon AM, Rauch GS, Siedow JN. 1998. Regulation of the cyanide-resistant alternative oxidase of plant mitochondria. J Biol Chem. 273: 30750-30756.

Trinkle-Mulcahy L, Boulon S, Lam YW, Urcia R, Boisvert FM, Vandermoere F, Morrice NA, Swift S, Rothbauer U, Leonhardt H, Lamond A. 2008. Identifying specific protein interaction partners using quantitative mass spectrometry and bead proteomes. J Cell Biol. 183: 223-239.

Van de Venter HA. 1985. Cyanide-resistant respiration and cold resistance in seedlings of maize (*Zea mays* L.) Ann Botany. 56: 561-563.

Vanlerberghe GC, McIntosh L. 1992. Coordinate regulation of cytochrome and alternative pathway respiration in tobacco. Plant Physiol. 100: 1846-1851.

Vanlerberghe GC, McIntosh L, Yip JYH. 1998. Molecular localization of redox-modulated process regulating plant mitochondrial electron transport. Plant Cell. 10: 1551-1560.

Vanlerberghe GC, Vanlerberghe AE, McIntosh L. 1994. Molecular genetic alteration of plant respiration. Plant Physiol. 106: 1503-1510.

Watanabe CK, Hachiya T, Takahara K, Kawai-Yamada M, Uchimiya H, Uesono Y, Terashima I, Noguchi K. 2010. Effects of AOX1a deficiency on plant growth, gene expression of

respiratory components and metabolic profile under low-nitrogen stress in *Arabidopsis thaliana*. Plant Cell Physiol. 51: 810-822.

Weger, HG, Guy RD, 1991. Cytochrome and alternative pathway respiration in white spruce (*Picea glauca*) roots- effects of growth and measurement temperature. Physiol Plant. 83, 675-681.

Whelan J, Hugosson M, Glaser E, Day DA. 1993. Studies on the import and processing of the alternative oxidase precursor by isolated soybean mitochondria. Plant Mol Biol. 27: 769-778.

Yip JYH, Vanlerberghe GC. 2001. Mitochondrial alternative oxidase acts to dampen the generation of active oxygen species during rapid respiration induced to support a high rate of nutrient uptake. Physiol Plant. 112: 327-333.

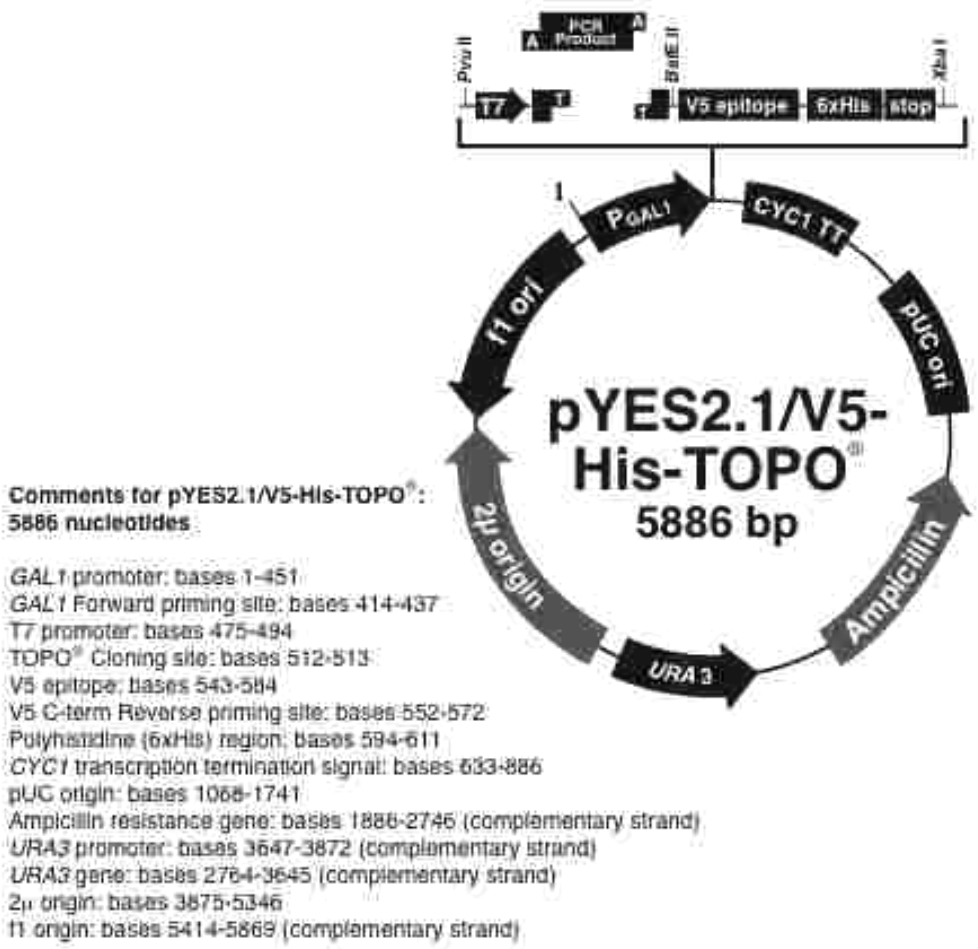


Figure 3.1. pYES2.1/V5-His-TOPO® vector map. Key features of this plasmid include: the *GAL1* promoter for recombinant protein expression by galactose induction and repression by glucose in yeast, a multiple cloning site, an ampicillin resistance gene for selection in transformed *E. coli*, and the *URA3* gene for selection on uracil deficient media. Figure obtained from: www.invitrogen.com.

Table 3.1. Substrate and inhibitor concentrations for respirometry.

Substrates	**Final concentration (mM) in 2 mL chamber**	**Purpose**
NADH	2	- External NADH dehydrogenase oxidizes NADH to NAD^+ - Initiates electron flow through the electron transport chain
ADP	1	- ATP synthesis
DTT	1	- Angiosperm AOX activator - Reduces AOX to the active form
Pyruvate	5	- Angiosperm AOX stimulator - Further activation of the reduced AOX form
Inhibitors		
KCN	1	- Complex IV inhibitor - Shuts down cytochrome c oxidase pathway
SHAM	3	- AOX inhibitor - Shuts down AOX pathway

Table 3.2. Site-directed mutagenesis primers.

Key AOX amino acid	Mut.	Forward primer	Reverse primer
Tyr107	Phe	5'-ggccacggatatctccagtttttggggtgtgg-3'	5'-ccacaccccaaaaactggagatatccgtggcc-3'
CysI (Cys127)	Ala	5'-ccttggaaatggacggccttcacgccgcacga-3'	5'-tcgtgcggcgtgaaggccgtccatttccaagg-3'
Tyr134	Phe	5'-gccgcacgagacgttctatcccgatgtca-3'	5'-tgacatcgggatagaacgtctcgtgcggc-3'
GluII (Glu222)	Ala	5'-cgaggaagctgaaaacgcacgaatgcacttgatga-3'	5'-tcatcaagtgcattcgtgcgttttcagcttcctcg-3'
TyrII (Tyr280)	Phe	5'-ggaagccgtttactccttcacgcaattcctcaaaa-3'	5'-ttttgaggaattgcgtgaaggagtaaacggcttcc-3'
HisII (His327)	Ala	5'-gttcgtgcagacgaagctcatgctcgtgacgttaaccattt-3'	5'-aaatggttaacgtcacgagcatgagcttcgtctgcacgaac-3'

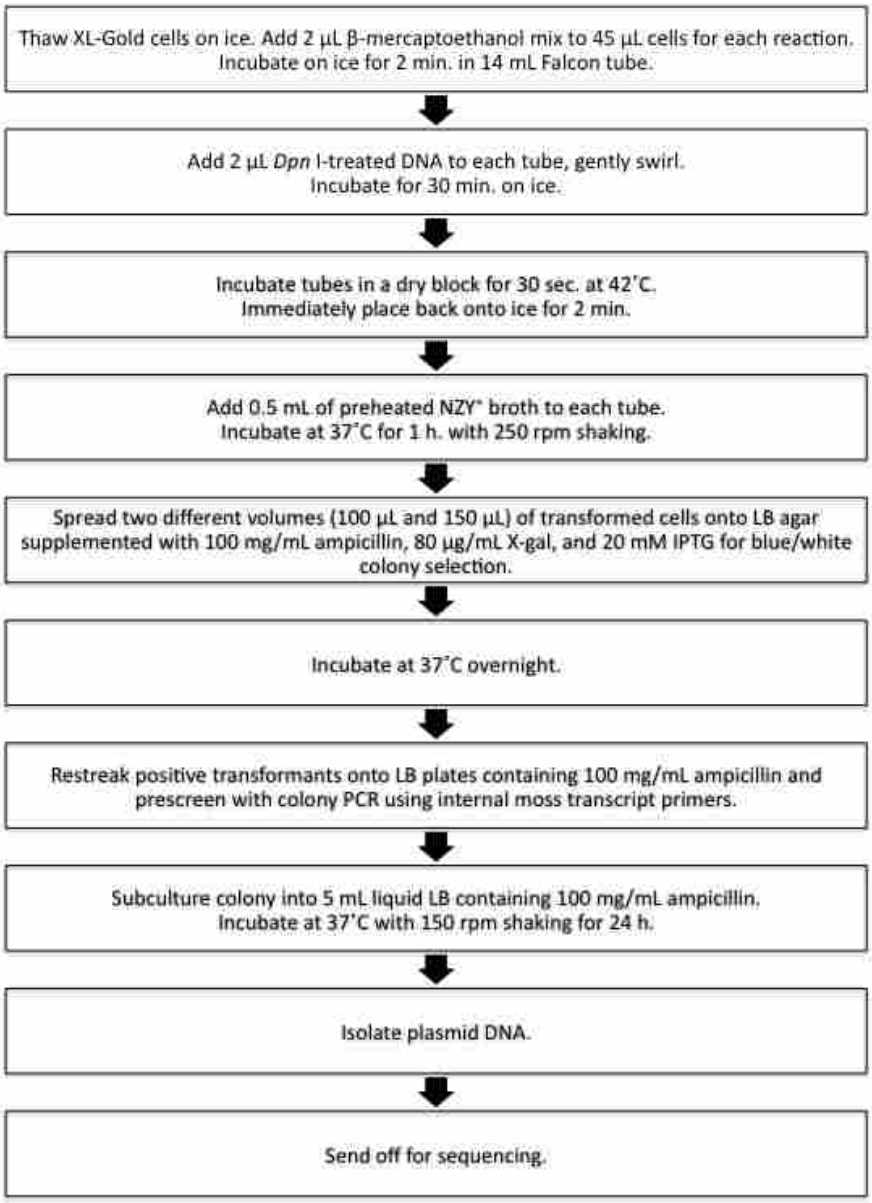


Figure 3.2. Experimental procedure for chemical transformation of *E. coli* with site-directed mutagenesis AOX constructs.

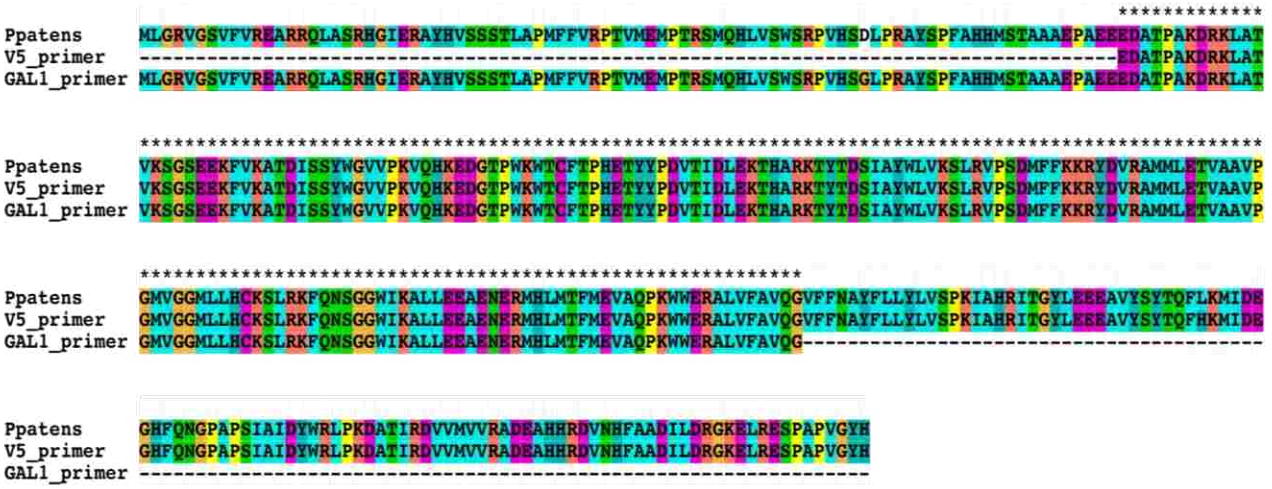


Figure 3.3. Multiple sequence alignment of the cloned 1Stop AOX insert with the predicted AOX peptide sequence. The forward *GAL*1 primer and the reverse V5 C-term reverse primer were used to sequence the N-terminal region and the C-terminal region of the insert DNA, respectively. Cloned DNA sequences were converted into amino acid sequences and were aligned to the predicted *P. patens* peptide sequence derived from the Phytozome database.

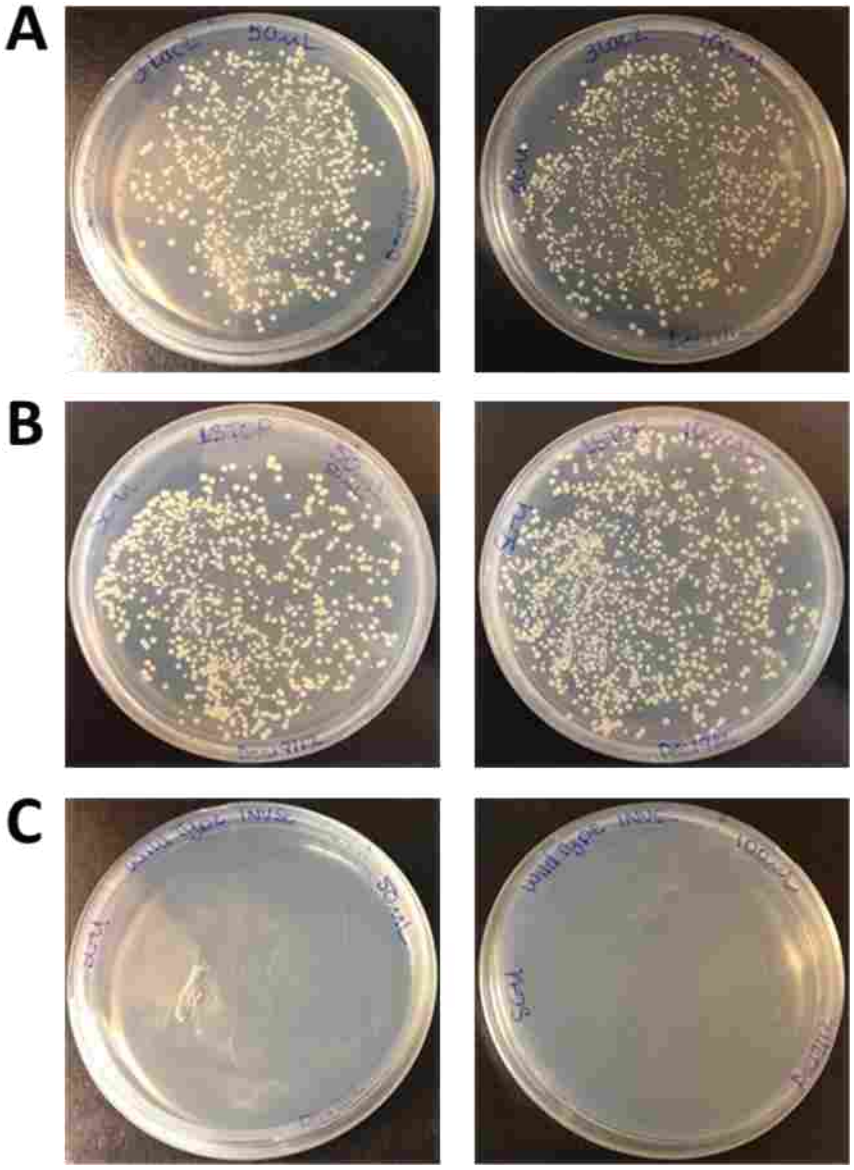


Figure 3.4. Chemically transformed *Saccharomyces cerevisiae* INV*Sc*1 plated onto selective synthetic complete medium without uracil (SC-U). Left, 50 µL; right, 100 µL. A) Yeast cells containing the *lac*Z positive kit control vector. B) Transformed yeast with the 1Stop *P. patens* AOX construct. C) Untransformed wild-type *S. cerevisiae* INV*Sc*1 cells (negative control) without pYES2 vector.

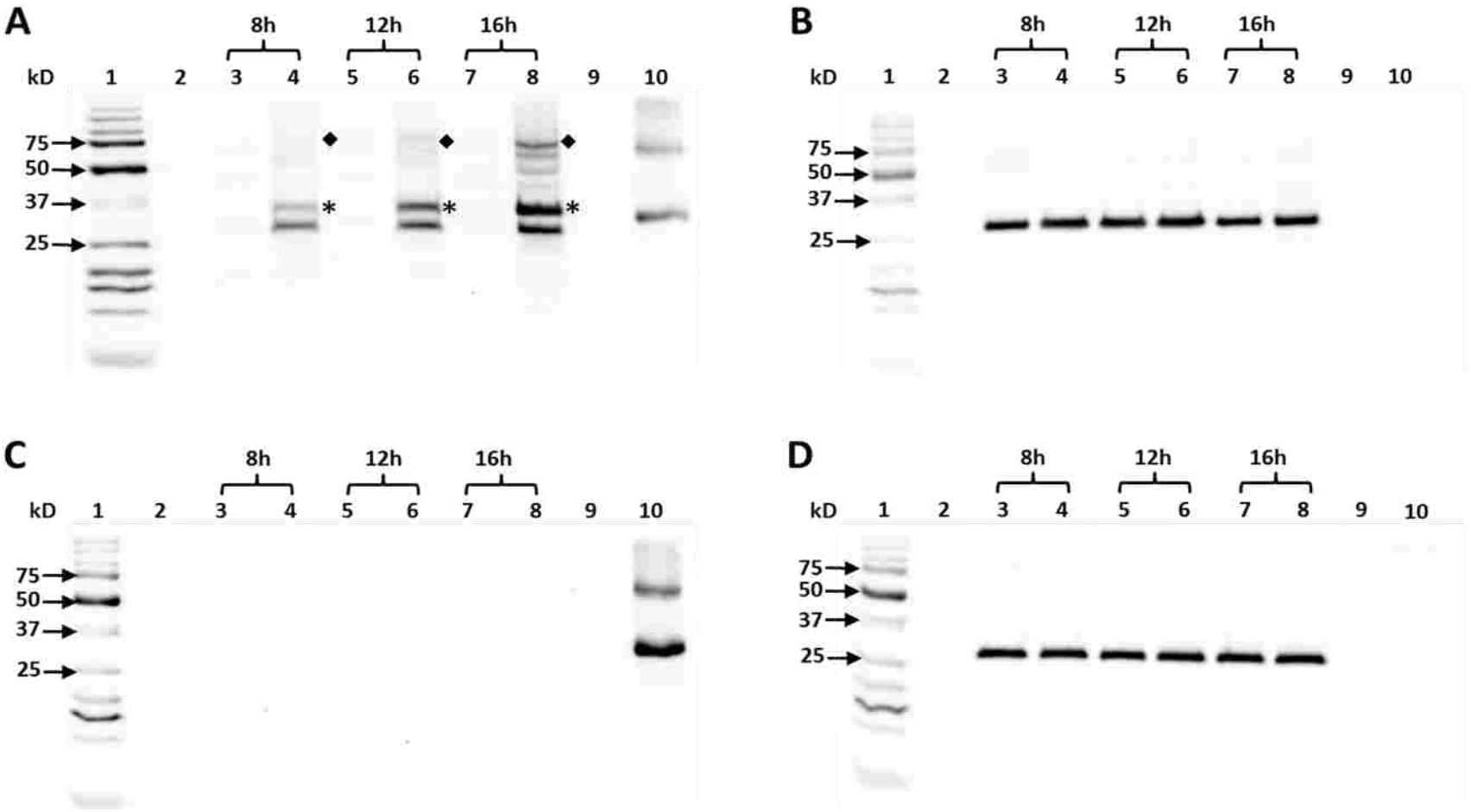


Figure 3.5. Western blot analysis of isolated *S. cerevisiae* mitochondrial proteins probed with the AOX antibody (A & C) or the yeast-specific porin antibody (B & D). Lane 1, protein ladder; lane 2, blank; lanes 3-8, mitochondrial protein (5 µg); lane 9, blank; lane 10, low Pi tobacco mitochondria (4.8 µg). Mitochondria that were grown in the presence of glucose (lanes 3, 5, and 7) or galactose (lanes

4, 6, and 8) were extracted at three different time points (8, 12, and 16 h.). A, B) 1Stop isolated mitochondrial proteins (lanes 3-8). *, 35 kDa reduced band; ◆, 75 kDa oxidized band. C, D) *lac*Z mitochondrial protein samples (lanes 3-8). Western blots are representative results that were obtained from two independent mitochondrial isolations for each sample.

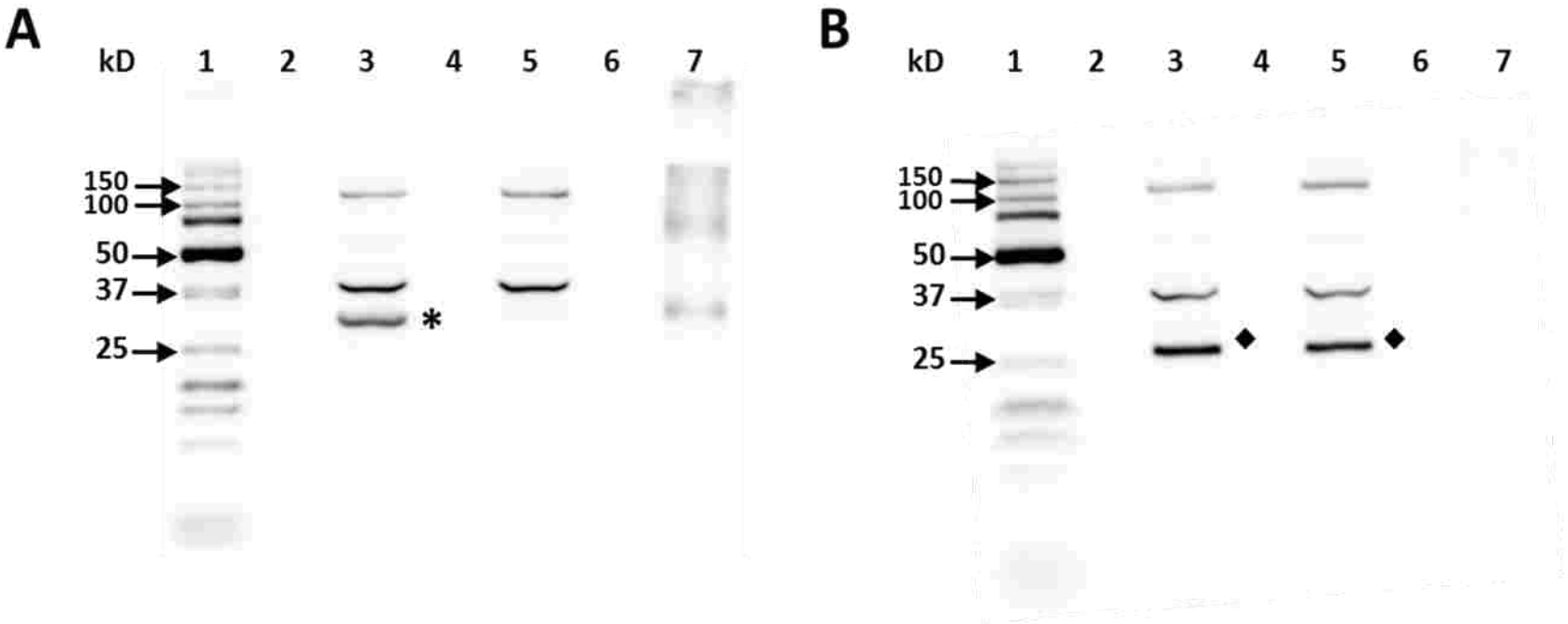


Figure 3.6. Western blot analysis of whole cell *S. cerevisiae* protein extracts. Lane 1, protein ladder; lane 2, blank; lane 3, (6 µL) 16 h. GAL 1Stop protein extract; lane 4, blank; lane 5, (6 µL) 16 h. GAL INV*Sc*1 protein extract; lane 6, blank; lane 7, low Pi tobacco mitochondria (4.8 µg). A) Nitrocellulose membrane probed with AOX antibody. *, 35 kDa reduced AOX. B) Nitrocellulose membrane probed with the porin antibody. ◆, 30 kDa corresponding porin band.

Table 3.3. Oxygen consumption rates of wild-type (WT) or AOX expressing mitochondria isolated from *S. cerevisiae*. Rates are the average from 3 individual mitochondrial isolations and were calculated after the addition of each substrate and inhibitor with ± the standard error. Data was background corrected with the initial mitochondrial respiratory rate before substrates were added.

	Rate (nmol O_2 consumed min^{-1} mg^{-1})					
	NADH	**ADP**	**DTT**	**Pyruvate**	**KCN**	**SHAM**
Assay A						
AOX	163.9 ±32.1	211.1 ±72.3	-	-	-6.0 ±6.4	-9.6 ±7.4
WT	170.3 ±7.7	264.5 ±19.1	-	-	-14.4 ±4.8	-17.8 ±5.0
Assay B						
AOX	225.2 ±17.5	308.9 ±17.8	254.4 ±10.2	167.8 ±3.9	-10.3 ±15.2	-19.5 ±14.5
WT	198.3 ±35.4	280.3 ±35.2	262.4 ±22.3	201.3 ±29.1	-7.9 ±5.5	-9.2 ±4.7

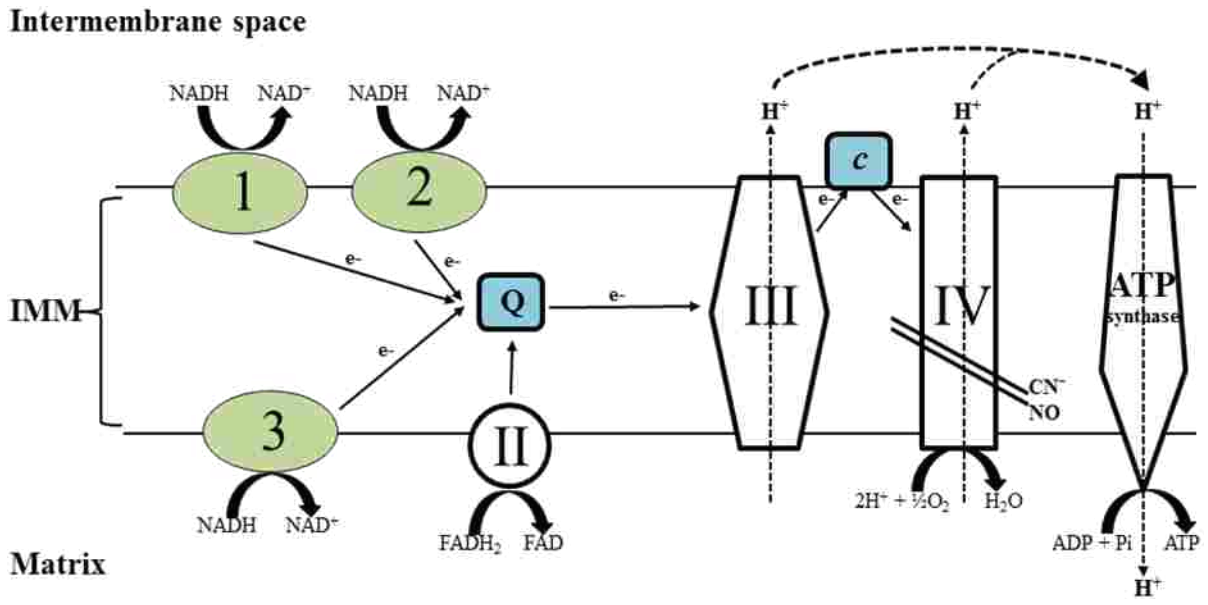


Figure 3.7. Representative electron transport system of *S. cerevisiae*. Electrons (e-) are transferred from external or internal NADH dehydrogenases (1-3) or from complex II to ubiquinone (Q). Electrons then flow to complex III, to cytochrome c (C), and lastly to complex IV. Protons (H^+) are translocated across the inner mitochondrial membrane (IMM) and into the intermembrane space by complexes III and IV. An electrochemical gradient is created which is used to drive ATP synthesis by the enzyme ATP synthase.

CHAPTER 4

General Discussion

1. Summary

1.1. Major Findings and Future Directions

The goal of this research project was to characterize AOX for the first time in a non-angiosperm plant. The taxonomic distribution of AOX in non-angiosperm members of the Viridiplantae was examined in order to assess the evolutionary history of the protein. Comparisons were made to identify similarities and differences between all plant AOXs. I have experimentally investigated AOX in the moss *Physcomitrella patens* at the DNA (gene), mRNA (transcript expression), and protein levels. An integrative approach was employed to examine AOX across a diverse group of plants differing with respect to their reproductive strategies, physiology, and morphology, and at different levels of biological organization (from the molecular level to the organism) using various interdisciplinary techniques. The major findings of this research are as follows:

The first major discovery of this research is that AOX is widespread in the Viridiplantae and is likely an ancient character in this group (Chapter 2, Section 4.1; Table 2.1). Evidence of multigene families in one chlorophyte and some streptophyte land plants suggests that an AOX gene duplication event occurred before the divergence of the Magnoliophyta (Chapter 2, Section 4.2). Variation at the amino acid level among chlorophyte and streptophyte AOXs was observed in the N-terminal and C-terminal regions of the protein, however, the protein sequence was highly conserved in the core-region of the enzyme in all species (Chapter 2, Section 4.3). This sequence analysis suggests that all AOXs in plants will be functional, but that chlorophyte AOXs are monomeric, whereas streptophyte AOXs are dimeric. Thus, major differences are expected to occur in the manner by which AOX will be post-translationally regulated in these two groups.

In this thesis, I have provided evidence that AOX has a widespread distribution in the Viridiplantae, as it is present in many chlorophyte and non-angiosperm streptophyte plants. I anticipate that as more sequencing data become available, it will be revealed that AOX is ubiquitous in the plant kingdom. Within the Chlorophyta, AOX data is unavailable from the Pedinophyceae (Figure 2.5). Streptophyte AOX sequence data is missing from the algal group Mesostigmatophyceae and from the embryophyte groups Anthocerotophyta, Lycopodiopsida, Psilotophyta, Equisetophyta, Ginkgophyta, and Cycadophyta (Figure 2.5). I propose that the Cycadophyta would be the logical starting point for AOX research in groups from which there is currently no sequence data. Cycad tissue is easily accessible from greenhouses and would be an interesting system to explore AOX activity as thermogenesis has been previously reported in the male cones of some cycad species (Skubatz et al., 1992). I propose that the lycophyte *Selaginella moellendorffii* should be used in future investigations of multigene families in basal plants, as my results indicate that it likely possesses two AOX genes based on the expression of two different mRNAs. This system would be advantageous in molecular biology studies as it has been recently described as a model plant system and its genome has been sequenced (Banks et al., 2011). In addition, a D-Y-R motif that is present in the C-terminal region of *P. engelmannii*, *P. sitchensis*, *P. menziesii*, and *P. glauca* should be explored as this may indicate the presence of different isoforms (Figure 2.2). It was apparent that differences exist among chlorophyte and streptophyte AOXs at the amino acid level. The majority of angiosperm AOXs are post-translationally regulated by the redox state of a conserved CysI residue and are stimulated by pyruvate (Vanlerberghe et al., 1998). After analyzing the non-angiosperm sequences, it is evident that some streptophyte AOXs will be post-translationally regulated in a similar manner due to the presence of CysI. However, most streptophyte AOXs contain a serine (SerI) residue in this

position, and I predict that these AOXs will be regulated by succinate (Grant et al., 2009). More research is warranted on this group to determine if AOX activity is stimulated in a similar manner as in angiosperms. Again, I suggest that additional characterization of streptophyte AOXs should be conducted using the model plant *S. moellendorffii* as the bioinformatics work has demonstrated that the CysI and SerI residues are present in different genes. I propose that future AOX research with this species should examine not only non-angiosperm AOX multigene families but also investigate different modes of post-translational regulation (pyruvate vs. succinate). Future respirometry and site-directed mutagenesis experiments can be conducted to assess the functionality of AOX in this species. Based on the presence of an indel located in the N-terminus of chlorophyte AOXs, I expect that these proteins will be regulated similarly to protist, fungal, and animal AOXs as opposed to those of angiosperms (McDonald, 2008). Future studies should investigate whether chlorophyte AOXs will be regulated by GMP/AMP or pH (McDonald, 2009). With the recent availability of complete genome sequences from *Volvox carteri* (Prochnik et al., 2010) and *Bathycoccus prasinos* (Moreau et al., 2012), I propose that these species would be a logical starting point for future respirometry work to assess AOX functionality within the chlorophytes. The streptophyte algae have not been investigated in any great detail, and represent another group where significant research contributions could be made. The presence of two conserved motifs (Y-W-G and P-X-E-X-Y) in the N-termini of streptophyte AOXs suggests that they will be dimeric. The absence of this motif in chlorophytes suggests that AOX is likely monomeric similar to protists and fungi. Further investigation of the protein form in these groups is required and can be examined by non-reducing SDS-PAGE and native PAGE analyses by subjecting mitochondria from the species mentioned above to various reducing and oxidizing agents.

The second major finding of this research is that my molecular database searches have revealed that a single AOX gene is present in the moss *Physcomitrella patens*. This work marks the first time that only one AOX gene has been identified in a plant species. This is in contrast to the gene structure seen in angiosperms which exist in multigene families. An analysis of the moss AOX gene indicates that the number of introns and exons that are present (Chapter 2, Section 4.4; Figure 2.3A) are highly comparable to what is commonly seen in angiosperm AOXs (Considine et al., 2002; Costa et al., 2010; Figure 1.3). The PCR results demonstrated that I have successfully amplified the full-length coding region from the genomic AOX sequence and sequencing analysis has confirmed that AOX is present in moss (Chapter 2, Section 4.4; Figure 2.4A).

For the first time, AOX has been characterized in a non-angiosperm plant. I demonstrated that a single AOX gene is present in moss using PCR; however, future work using this system should validate the AOX gene copy number by Southern blot analysis. The identification of only a single AOX gene in the moss *Physcomitrella patens* indicates that this species provides an excellent platform for future research to explore questions about the physiological role of the enzyme as this system will be amenable to using RNAi and anti-sense technologies without having to worry about possible redundant functions of different AOX genes. Future studies should focus on other basal land plant groups to determine if other species possess a single AOX gene copy (e.g. bryophytes such as hornworts and other mosses). Within plants, further investigation of AOX gene structure is warranted, in particular focusing on the N- and C-terminal intron/exon positioning of the genes to determine if they are comparable to angiosperms. Future research in this area can explore the possibility of alternative splicing of

mRNA and regulatory regions within introns. Future studies should explore the AOX genomic gene structure in other basal plants in order to contribute information to the areas described above.

The third significant outcome from this research is that the data available on the Phytozome website provided the full-length AOX transcript sequence and predicted that three different variants may be expressed in moss (Chapter 2, Section 4.4; Figure 2.3B; Figure 2.3C). RT-PCR results indicate that AOX is expressed in moss (Chapter 2, Section 4.4; Figure 2.4B; Figure 2.4C) and sequencing analysis of the cloned cDNA revealed that the key amino acids residues that are required for AOX activity are completely conserved in moss.

The information available on the Phytozome website revealed that there are three possible AOX transcripts in moss, and using RT-PCR, I have demonstrated that one was expressed. In future studies, changes in AOX expression levels should be examined at different developmental stages to determine if other predicted alternative transcripts are expressed at specific stages of the moss life cycle. My preliminary data revealed that AOX transcript expression increased when moss plants were subjected to cold temperature (data not shown). Therefore, I suggest that cold stress might be another area to explore further in moss.

Lastly, an analysis of the predicted AOX peptide sequence from moss indicated that it would be post-translationally regulated similarly to angiosperms due to the presence of a conserved cysteine (CysI) residue (Chapter 2, Section 4.4; Figure 2.3D). Western blot analyses provided evidence for the first time that the reduced (~35 kDa) and oxidized (~75 kDa) protein forms are present in moss AOX from isolated yeast mitochondria that were grown in induction media, suggesting that similar to angiosperms, AOX exists as a dimer in moss (Chapter 3,

Section 4.1; Figure 3.5A). Respirometry trace data have revealed that yeast transformed with moss AOX are incapable of performing cyanide-resistant respiration using NADH as a substrate under the conditions tested (Chapter 3, Section 4.3; Table 3.3).

Protein analysis has confirmed that moss AOX is dimeric from the detection of reduced and oxidized forms in isolated yeast mitochondria and is likely due to a conserved cysteine (CysI) that is responsible for the interconversion between the two forms (Vanlerberghe et al., 1998). Lastly, further work is warranted to assess wild-type and mutant moss AOX functionality in transformed yeast mitochondria by respirometry or cyanide-containing agar plate assays. Based on previous site-directed mutagenesis studies conducted in angiosperms, I predict that modification of key residues will render AOX inactive (Albury et al., 2002; Berthold et al., 2002). Once the mitochondrial isolation and site-directed mutagenesis procedures have been optimized, future work will be able to assess cyanide-resistant respiration rates.

Overall, this thesis has contributed significant information about non-angiosperm AOXs and has identified new areas for AOX research. Since previous AOX work in plants has primarily focused on angiosperms, many opportunities exist for comparative studies. This thesis has identified that chlorophyte and streptophyte AOXs exhibit significant differences at the amino acid sequence level that will likely have major impacts on enzyme dimerization and regulation. As more AOX data becomes available from non-angiosperm plants, it could provide insight as to why such an extensive group of organisms have retained this seemingly wasteful pathway and may help determine the physiological role(s) of AOX in plants. The identification of only a single AOX gene in moss also offers us an advantage over other systems as future knockout experiments can examine the physiological effects that AOX has on these plants.

1.2. Real-world Applications

Previous studies have revealed that AOX activity increases when plants experience stressful conditions such as temperature change, oxidative stress, drought, and pathogen invasion (bacterial, viral, etc.) (McDonald, 2008; Feng et al., 2010) Therefore, AOX may serve as a potential gene target to improve plant resistance to a wide range of environmental and biotic stresses (Purvis and Shewfelt, 1993). It may be advantageous for future biotechnology and agricultural research to manipulate this gene by designing plants that overexpress AOX or to breed plants that are more stress tolerant in order to decrease plant susceptibility to these factors. A better understanding of the physiological role of AOX in plants will aid in these efforts.

AOX research also has the potential to provide alternative treatments of human diseases. The parasite *Trypanosoma brucei brucei*, which causes African sleeping sickness in humans, is entirely dependent on AOX respiration during its infectious stage and therefore, AOX inhibition has been a target for drug design (Clarkson et al., 1989; Ott et al., 2006). Although it is rare, certain species of green algae have been known to cause human infections, primarily in immunosuppressed individuals (Krcmery, 2000). For example, *Prototheca wickerhamii* causes human chlorellosis, and patients are often treated with antifungal agents; however, resistance has been documented (Krcmery, 2000). The bioinformatics results have demonstrated that AOX is present in *P. wickerhamii* and it may serve as a potential target for drug therapy as an alternative treatment method. Therefore, further investigation of AOX regulation and/or inhibitors in basal plants is required.

Lastly, the potential impacts that climate change will have on the economy and the environment has made it a topic of special interest. In particular, Canada's economy will be

significantly impacted by climate change in the areas of forestry and agriculture (Natural Resources Canada, 2007). One potential way of reducing the impact of climate change is to exploit primary productivity (converting carbon dioxide into organic compounds) in plants. Trees are often discussed as the principal carbon sinks through primary productivity; however, moss is abundant in forest regions, such as the Boreal forest (Angert et al., 2003). Moss may be very effective for carbon sequestration due to its rapid growth and biomass accumulation in these regions (Hohe et al., 2004). Therefore, having a greater understanding of respiration and electron flow in these plants may give us a better idea of carbon sequestration processes. In addition, a more thorough investigation of AOX may aid in the development of more realistic models for plant respiration and global climate change.

References

Albury MS, Affourtit C, Crichton PG, Moore AL. 2002. Structure of the plant alternative oxidase. J Biol Chem. 277: 1190-1194.

Angert A, Barkan E, Barnett B, Brugnoli E, Davidson EA, Fessenden J, Maneepong S, Panapitukkul N, Randerson JT, Savage K, Yakir D, Luz B. 2003. Contribution of soil respiration in tropical, temperate, and boreal forests to the ^{18}O enrichment of atmospheric O_2. Global Biogeochem Cy. 17: 1089.

Banks JA, Nishiyama T, Hasebe M, Bowman JL, Gribskov M, dePamphilis C, Albert VA, Aono N, Aoyama T, Ambrose BA, Ashton NW, Axtell MJ, Barker E, Barker MS, Bennetzen JL, Bonawitz ND, Chapple C, Cheng C, Correa LGG, Dacre M, DeBarry J, Dreyer I, Elias M, Engstrom EM, Estelle M, Feng L, Finet C, Floyd SK, Frommer WB, Fujita T, Gramzow L, Gutensohn M, Harholt J, Hattori M, Heyl A, Hirai T, Hiwatashi Y, Ishikawa M, Iwata M, Karol KG, Koehler B, Kolukisaoglu U, Kubo M, Kurata T, Lalonde S, Li K, Li Y, Litt A, Lyons E, Manning G, Maruyama T, Michael TP, Mikami K, Miyazaki S, Morinaga S, Murata T, Mueller- Roeber B, Nelson DR, Obara M, Oguri Y, Olmstead RG, Onodera N, Petersen BL, Pils B, Prigge M, Rensing SA, Riaño-Pachón DM, Roberts AW, Sato Y, Scheller HV, Schulz B, Schulz C, Shakirov EV, Shibagaki N, Shinohara N, Shippen DE, Sørensen I, Sotooka R, Sugimoto N, Sugita M, Sumikawa N, Tanurdzic M, Theißen G, Ulvskov P, Wakazuki S, Weng J, Willats WWGT, Wipf D, Wolf PG, Yang L, Zimmer AD, Zhu Q, Mitros T, Hellsten U, Loqué D, Otillar R, Salamov A, Schmutz J, Shapiro H, Lindquist E, Lucas S, Rokhsar S, Grigoriev IV. 2011.The Selaginella genome identifies genetic changes associated with the evolution of vascular plants. Science. 332: 960-963.

Berthold DA, Voevodskaya N, Stenmark P, Graslund A, Norlund P. 2002. EPR studies of the mitochondrial alternative oxidase. J Biol Chem. 277: 43608- 43614.

Clarkson AB, Bienen EJ, Pollakis G, Grady RW. 1989. Respiration of bloodstream forms of the parasite *Trypanosoma brucei brucei* is dependent on a plant-like alternative oxidase. J Biol Chem. 264: 17770-17776.

Considine MJ, Holtzapffel RC, Day DA, Whelan J, Millar AH. 2002. Molecular distinction between alternative oxidase from monocots and dicots. Plant Physiol. 129: 949-953.

Costa JH, Mota EF, Cambursano MV, Lauxmann MA, de Oliveira LMN, Lima MDS, Orellano EG, de Melo DF. 2010. Stress-induced co-expression of two alternative oxidase (*VuAox*1 and *2b*) genes in *Vigna unguiculata*. J Plant Physiol. 167: 561-570.

Feng HQ, Sun K, Li MQ, Li HY, Li X, Li Y, Wang YF. 2010. The expression, function and regulation of mitochondrial alternative oxidase under biotic stresses. Mol Plant Pathol. 11: 429-440.

Grant N, Onda Y, Kakizaki Y, Ito K, Watling J, Robinson S. 2009. Two Cys or not two Cys, that is the question? Alternative oxidase in the thermogenic *Nelumbo nucifera*. Plant Physiol. 150: 987-995.

Hohe A, Egener T, Lucht JM, Holtorf H, Reinhard C, Schween G, Reski R. 2004. An improved and highly standardised transformation procedure allows efficient production of single and multiple targeted gene-knockouts in a moss, *Physcomitrella patens*. Current Genetics. 44: 339-347.

Kcmery V. 2000. Systemic chlorellosis, an emerging infection in humans caused by algae. Int J Antimicrob Ag. 15: 235-237.

Natural Resources Canada. 2007. Costing climate change. Date accessed: July 2, 2013.

McDonald AE. 2008. Alternative oxidase: an inter-kingdom perspective on the function and regulation of this broadly distributed 'cyanide-resistant' terminal oxidase. Funct Plant Biol. 35: 535-552.

McDonald AE. 2009. Alternative oxidase: What information can sequence comparisons give us? Physiol Plant. 137: 328-341.

Moreau H, Verhelst B, Couloux A, Derelle E, Rombauts S, Grimsley N, Van Bel M, Poulain J, Katinka M, Hohmann-Marriott MF, Piganeau G, Rouze P, Da Silva C, Wincker P, Van de Peer Y, Vandepoele K. 2012. Gene functionalities and genome structure in *Bathycoccus prasinos* reflect cellular specializations at the base of the green lineage. Genome Biol. 13: R74.

Neimanis K, Staples JF, Huner NPA, McDonald AE. 2013. Identification, expression, and taxonomic distribution of alternative oxidases in non-angiosperm plants. Gene. http://dx.doi.org/10.1016/j.gene.2013.04.072.

Ott R, Chibale K, Anderson S, Chipeleme A, Chaudhuri M, Guerrah A, Colowick N, Hill GC. 2006. Novel inhibitors of the trypanosome alternative oxidase inhibit *Trypanosoma brucei brucei* growth and respiration. Acta Tropica. 100: 172-184.

Prochnik SE, Umen J, Nedelcu AM, Hallmann A, Miller SM, Nishii I, Ferris P, Kuo A, Mitros T, Fritz-Laylin LK, Hellsten U, Chapman J, Simakov O, Rensing SA, Terry A, Pangilinan J, Kapitonov V, Jurka J, Salamov A, Shapiro H, Schmutz J, Grimwood J, Lindquist E, Lucas S, Grigoriev IV, Schmitt R, Kirk D, Rokhsar DS. 2010. Genomic analysis of organismal complexity in the multicellular green alga *Volvox carteri*. Science. 329: 223-226.

Purvis AC, Shewfelt RL. 1993. Does the alternative pathway ameliorate chilling injury in sensitive plant tissues?. Plant Physiology. 88: 712-718.

Skubatz H, Tang W, Meeuse BJD. 1993. Oscillatory heat-production in the male cones of cycads. J Exp Bot. 44: 489-492.

Vanlerberghe GC, McIntosh L, Yip JYH. 1998. Molecular localization of redox-modulated process regulating plant mitochondrial electron transport. Plant Cell. 10: 1551-1560.

Appendix

Appendix 1. BCD moss media recipe as described by Cove et al., 2009. All reagents and quantities are to be autoclaved when combined, unless otherwise specified.

Reagent	Amount
Phytoblend™ agar (Caisson PTP01)	9 g
1 M $CaCl_2$ [a]	1 mL
$FeSO_4•7H_2O$	12.5 mg
Stock Solution B (0.1 M $MgSO_4•7H_2O$ solution) [b]	10 mL
Stock Solution C (184 mM KH_2PO_4 solution) [b]	10 mL
Stock Solution D (1 M KNO_3 solution) [b]	10 mL
Hoagland's A-Z Trace Element Solution (TES) [b c]	1 mL
0.5 M Diammonium tartrate [b]	10 mL
Distilled water	Top to 1 L

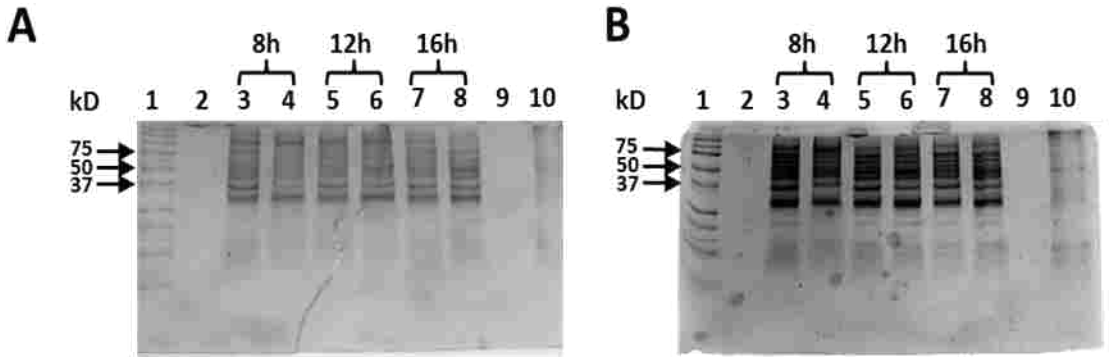
[a] Add after other media components have been autoclaved

[b] Autoclave solutions before preparation of BCD medium

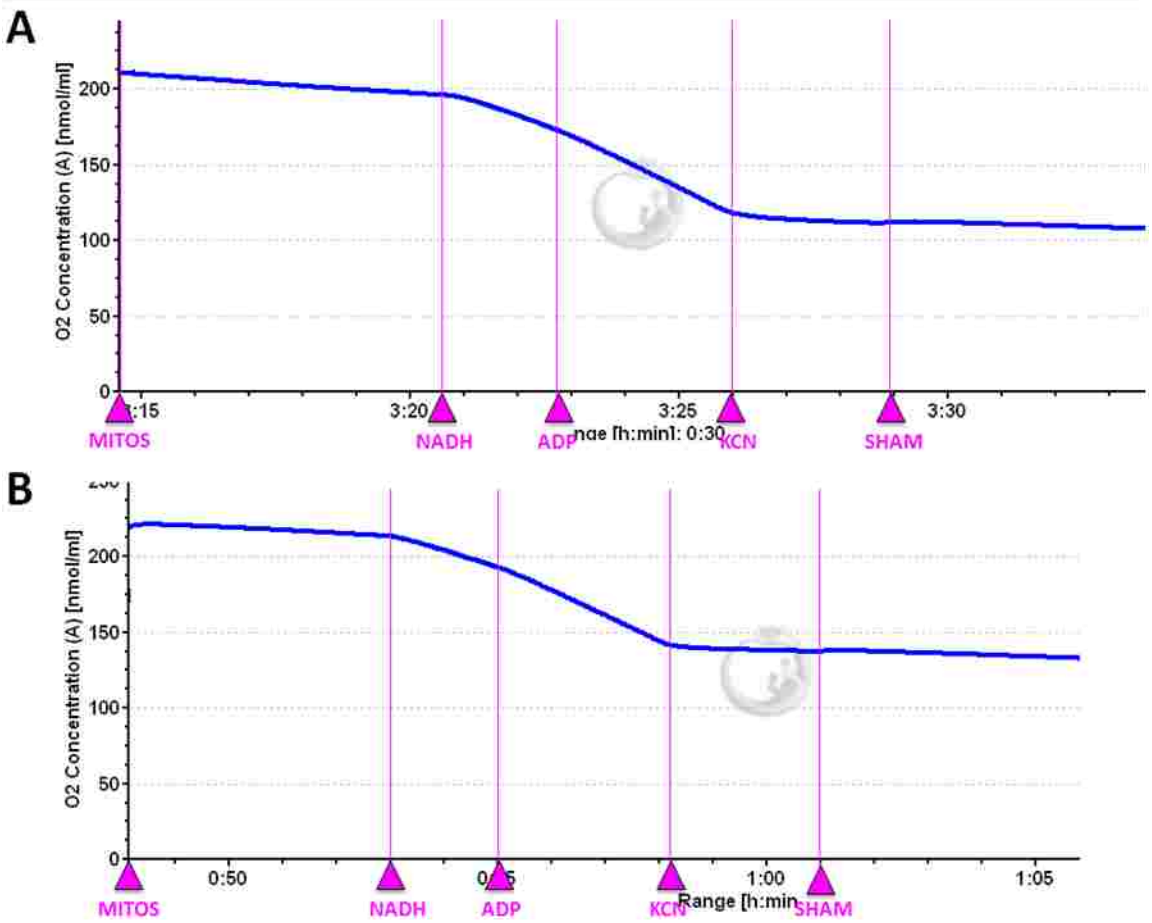
[c] TES: 0.006% (w/v) $Al_2(SO_4)_3•K_2SO_4•24H_2O$, 0.006% (w/v) $CoCl_2•6H_2O$, 0.006% (w/v) $CuSO_4•5H_2O$, 0.061% (w/v) H_3BO_3, 0.003% (w/v) KBr, 0.003% (w/v) KI, 0.003% (w/v) LiCl, 0.039% (w/v) $MnCl_2•4H_2O$, 0.003% (w/v) $SnCl_2•2H_2O$, and 0.006% (w/v) $ZnSO_4•7H_2O$ in 1 L distilled water

Appendix 2. MiR05 mitochondrial respiration medium recipe. Store in 15 mL portions at -20˚C.

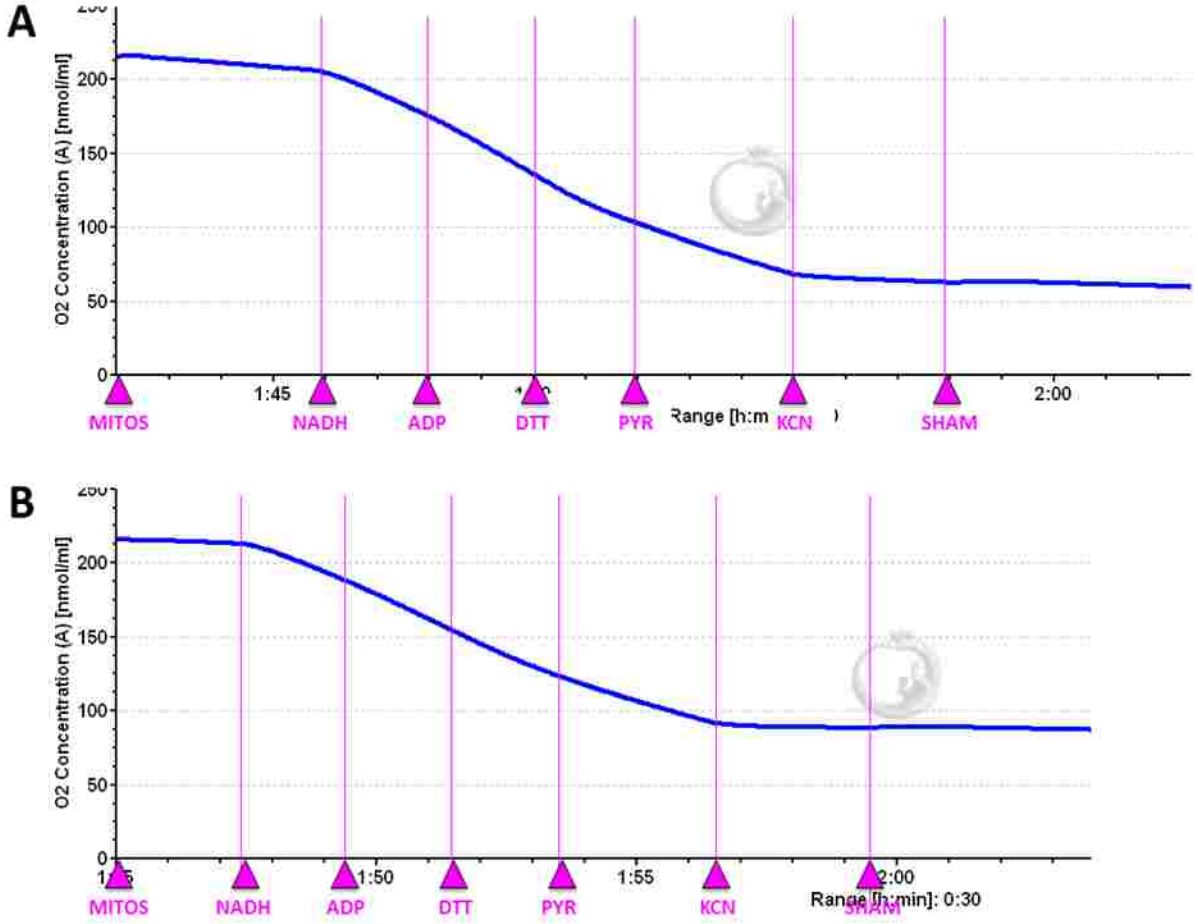
Reagent	Amount
EGTA	0.19 g
$MgCl_2•6H_2O$	0.61 g
0.5 M K-lactobionate stock (pH 7.0)	120 mL
Taurine	2.502 g
KH_2PO_4	1.361 g
HEPES	4.77 g
Sucrose	37.65 g
BSA	1.0 g
Distilled water	Top to 1 L (pH 7.1)



Appendix 3. Representative Coomassie blue stained acrylamide gels of isolated mitochondrial proteins from *S. cerevisiae*. Lane 1, protein ladder; lane 2, empty; lanes 3-8, mitochondrial protein (5 µg); lane 9, empty; lane 10, low Pi tobacco mitochondria (4.8 µg). Mitochondria were isolated at three time points (8, 12, and 16 h.) and were grown in glucose (lanes 3, 5, and 7) or galactose (lanes 4, 6, and 8) media. A) 1Stop mitochondrial protein samples (lanes 3-8). B) *lac*Z isolated mitochondrial proteins (lanes 3-8).



Appendix 4. Representative oxygen consumption (blue line) traces from *S. cerevisiae* isolated mitochondria under Assay A conditions. Additions to the chamber were: Mitos, mitochondria (0.1 mg per 2 mL of MiR05 respiration medium); 2 mM NADH; 1 mM ADP; 1 mM KCN; 3 mM SHAM. A) 1Stop isolated mitochondria. B) *lac*Z isolated mitochondria.



Appendix 5. Representative oxygen consumption (blue line) traces from *S. cerevisiae* isolated mitochondria under Assay B conditions. Additions to the chamber were: Mitos, mitochondria (0.1 mg per 2 mL of MiR05 respiration medium); 2 mM NADH; 1 mM ADP; 1 mM DTT; 5 mM PYR, pyruvate; 1 mM KCN; 3 mM SHAM. A) 1Stop isolated mitochondria. B) *lac*Z isolated mitochondria.