The Role of Mitochondrial Alternative Oxidase in Plant-Pathogen Interactions

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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Abstract

Alternative oxidase (AOX) is a non-energy conserving branch of the mitochondrial electron transport chain (ETC) which has been hypothesized to modulate the level of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in plant mitochondria. The aim of the research presented herein is to provide direct evidence in support of this hypothesis and to explore the implications of this during plant-pathogen interactions in *Nicotiana tabacum*. We observed leaf levels of ROS and RNS in wild-type (Wt) tobacco and transgenic tobacco with altered AOX levels and we found that plants lacking AOX have increased levels of both NO and mitochondrial $O_2^-$ compared Wt plants. Based on the results we suggest that AOX respiration acts to reduce the generation of ROS and RNS in plant mitochondria by dampening the leak of electrons from the ETC to $O_2$ or nitrite.

We characterized multiple responses of tobacco to different pathovars of the bacterial pathogen *Pseudomonas syringae*. These included a compatible response associated with necrosis (pv *tabaci*), an incompatible response that included the hypersensitive response (HR) (pv *maculicola*) and an incompatible response that induced defenses (pv *phaseolicola*). We show that the HR is accompanied by an early mitochondrial $O_2^-$ burst prior to cell death. Also, we found
that the appearance of HR and the appearance of the mitochondrial \( \text{O}_2^- \) burst are delayed in transgenic plants lacking AOX. A similar delay is seen in transgenic plants treated with the complex III inhibitor antimycin A. In Wt plants, expression of \( Aox1a \) is suppressed during the HR response to \( pv \) \textit{maculicola} despite the accumulation of signaling molecules known to induce \( Aox1a \) transcription. Also, MnSOD activity declined during the HR. We suggest that the mitochondrial ROS burst controlled by AOX and MnSOD is an important component for the induction and coordination of the HR during plant-pathogen interactions.
Acknowledgments

I am very thankful to my supervisor, Dr. Greg Vanlerberghe, for the guidance and support during my time in his lab. His enthusiasm and belief in my abilities have motivated me to continue the struggle even at times when the work seemed overwhelming. I think that his approach in providing me with just the right amount of guidance and independence has helped me to grow as a scientist. I would also like to thanks my supervisory committee members, Dr. Rene Harrison and Dr. Keiko Yoshioka, for their guidance in my research work and invaluable suggestions during the writing of my thesis. Finally, I would like to thank the examiners, Dr. Malcolm Campbell and Dr. Andrei Igamberdiev, for their involvement in my work.

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I am also very grateful to my parents, Biljana and Cvetan, and to my sisters, Vedrana and Sara. Their encouragement has been more valuable to me than even they have suspected. Finally, I would like to thank my husband, David, who has learned more about plants in the past six years than he ever wanted. I could not have made it this far without his love and support.
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<tbody>
<tr>
<td>4-HBA</td>
<td>4-hydroxybenzoic acid</td>
</tr>
<tr>
<td>$\Delta \Psi$</td>
<td>Membrane Potential</td>
</tr>
<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AOX</td>
<td>Alternative oxidase</td>
</tr>
<tr>
<td>APF</td>
<td>Aminophenyl fluorescein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Avr</td>
<td>Avirulence factors</td>
</tr>
<tr>
<td>B7</td>
<td>Aox1a overexpressing tobacco transgenic lines</td>
</tr>
<tr>
<td>BTH</td>
<td>Benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester</td>
</tr>
<tr>
<td>CMSII</td>
<td>Cytoplasmic male sterile II</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>cPTIO</td>
<td>2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>DAF-FM</td>
<td>4-amino-5-methylamino- 2',7'-difluorescein</td>
</tr>
<tr>
<td>ET</td>
<td>Ethylene</td>
</tr>
<tr>
<td>ETI</td>
<td>Effector triggered immunity</td>
</tr>
<tr>
<td>FADH$_2$</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FLS2</td>
<td>Flagellin sensing2 receptor</td>
</tr>
<tr>
<td>FW</td>
<td>Fresh weight</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
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GSNO	S-nitrosoglutathione
GSNOR	GSNO reductase
H$_2$O$_2$	Hydrogen peroxide
Hb	Hemoglobin
HIN1	Harpin-induced 1 tobacco gene
HR	Hypersensitive response
HSR203J	Hypersensitive-response 203J marker gene
hrp	Hypersensitive response and pathogenicity
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
ISC	Isochorismate synthase
JA	Jasmonic acid
KCN	Potassium cyanide
M1, M2	Manders' coefficients
MAMP	Microbe associated molecular pattern
MAPK	Mitogen activated protein kinase
mETC	Mitochondrial electron transport chain
MeSA	Methyl salicylate
NADH	Nicotinamide adenine dinucleotide
NDex	External NAD(P)H dehydrogenase
NDin	Internal NAD(P)H dehydrogenase
NiNOR	Plasma membrane bound nitrite:NO reductase
NR	Nitrate reductase
NO	Nitric oxide
NO$_2^-$	Nitrogen dioxide
NO$_2^-$	Nitrite
NO₃⁻  Nitrate
N₂O₃  Dinitrogen trioxide
N₂O₄  Dinitrogen tetroxide
NOS   Nitric oxide synthase
O₂    Molecular oxygen
O₂⁻   Superoxide
O₂¹   Singlet oxygen
OH⁺   Hydroxyl radical
OMM   Outer mitochondrial membrane
ONOO⁻ Peroxynitrite
PAL   Phenylalanine ammonia-lyase
PAMP  Pathogen associated molecular pattern
PCD   Programmed cell death
Pi    Phosphate
PR-1  Pathogenesis-related 1 gene
PRR   Pattern recognition receptors
PTI   PAMP triggered immunity
PTP   Permeability transition pore
PVP   Polyvinylpyrrolidone
R proteins  Receptor proteins
Rboh  Respiratory burst oxidase homologs
RI9, RI29  Aox1a RNA interference tobacco transgenic lines
RIN4  Resistance to Pseudomonas syringae 1-interacting protein 4
RNS   Reactive nitrogen species
ROS   Reactive oxygen species
RPS2  Resistance to P. syringae 2 protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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</thead>
<tbody>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SA</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>SAG</td>
<td>Salicylic acid β-glucoside</td>
</tr>
<tr>
<td>SAR</td>
<td>Systemic acquired resistance</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>S.E.</td>
<td>Standard error</td>
</tr>
<tr>
<td>SIN1</td>
<td>3-morpholino sydnonimine</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SOD-PEG</td>
<td>Superoxide dismutase polyethylene glycol</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TMV</td>
<td>Tobacco mosaic virus</td>
</tr>
<tr>
<td>TTSS</td>
<td>Type III secretion system</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage dependant anion channel</td>
</tr>
<tr>
<td>VPE</td>
<td>Vacuolar processing enzyme</td>
</tr>
<tr>
<td>XOR</td>
<td>Xanthine oxidoreductase</td>
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CHAPTER 1

Mitochondrial signaling and homeostatic maintenance during plant-pathogen interactions

Literature Review and Thesis Introduction
Chapter 1
Mitochondrial Signaling and Homeostatic Maintenance during Plant-Pathogen Interactions

Literature review

To provide appropriate background to the topic of my thesis, I will begin by outlining the plant mitochondrial electron transport chain (mETC), concentrating specifically on the position and role of alternative oxidase (AOX). I will follow with a brief introduction to plant-pathogen interactions, especially incompatible infections that lead to defense or programmed cell death (PCD) responses. I will summarize the current knowledge about signaling and induction of pathways leading to these responses, concentrating on the mitochondrial involvement in these processes. The specific position and possible roles of AOX will be emphasized, especially in relation to reactive oxygen species (ROS) and reactive nitrogen species (RNS) signaling.

1.1 The plant mitochondrial electron transport chain (ETC)

The mitochondrial ETC, located at the inner mitochondrial membrane, is an important component of primary carbon metabolism. The reducing equivalents generated by the tricarboxylic acid (TCA) cycle are used by the mETC to generate a proton motive force used to power ATP production. In general terms, electrons from NADH or succinate pass through a series of spatially separated redox reactions from a donor molecule to an acceptor molecule, ultimately received by a terminal oxidase where they are used to reduce molecular oxygen (O$_2$) to water (H$_2$O). The passage of electrons through this pathway is accompanied by a translocation of protons into the inner-membrane space, and the resulting electrochemical gradient is used to drive ATP synthesis. See Figure 1.1 for a diagrammatic representation of the plant mitochondrial ETC.

The phosphorylating pathway of the mETC is composed of 5 complexes (I-V). Complex I is a NADH:ubiquinone oxidoreductase which removes two electrons from NADH and transfers them to the lipid-soluble carrier ubiquinone, forming the reduced product ubiqinol (Fernie et al., 2004;
Dudkina et al., 2006). Electrons also enter the mETC through Complex II, a succinate ubiquinone oxidoreductase, where the initial source of electrons is FADH₂, rather than NADH. From ubiquinol the electrons are transferred to Complex III, a ubiquinol cytochrome c reductase, which oxidizes ubiquinol to ubiquinone and simultaneously reduces the water-soluble electron carrier cytochrome c. Finally, in Complex IV, or cytochrome c oxidase (COX), electrons are removed from cytochrome c and transferred to molecular oxygen producing water. The electrochemical gradient produced due to the pumping of protons by Complexes I, III and IV out of the mitochondrial matrix is used by Complex V (F₅F₁ATP synthase complex) to drive the phosphorylation of ADP, thus producing ATP. The F₅ subunit acts as an ion channel that allows for protons to flow into the matrix from the inner membrane space along their electrochemical gradient, which releases free energy that drives the formation of ATP by the F₁ subunit of this complex (Fernie et al., 2004; Dudkina et al., 2006). This process is referred to as oxidative phosphorylation and it is a major source of energy in the cell.

1.2 Non-phosphorylating pathways of the mETC

In addition to the phosphorylating ETC, plant mitochondria have non-phosphorylating components as well, composed of at least four additional dehydrogenases and an additional terminal oxidase. These proteins do not contribute to the electrochemical gradient, and subsequently passage of electrons through this part of the pathway does not lead to ATP production. They allow for electron transport to continue even when the mitochondrial membrane potential (ΔΨ) is high, thereby uncoupling it from ATP synthesis. Due to its non-energy-conserving nature, passage of electrons through these ETC components may appear to be wasteful; however it is thought that the additional components create branching points in the mETC providing plant mitochondria with greater metabolic flexibility and environmental adaptability (Dudkina et al., 2006; Noguchi and Yoshida, 2008; Vanlerberghe et al., 2009). The proportion of electrons passing through either pathway is modulated mainly by three factors: the environment (temperature, nutrient and CO₂ availability), physiology (age of the plant, wounding, pathogen infection) and the reduction state of the ubiquinone pool.
1.2.1 Alternative NAD(P)H dehydrogenases

The alternative NAD(P)H dehydrogenases provide more entry points for electrons in the mETC and are found on both the exterior (NDex) and the interior (NDin) sides of the inner mitochondrial membrane. They are considered part of the non-phosphorylating pathway because they bypass the proton-pumping Complex I and do not pump protons into the intermembrane space (IMS) themselves (Rasmusson et al., 2004). It is hypothesized that these alternative pathways of electron input provide means for the plant to remove excess reducing power and maintain the redox balance of plant cells. For example, silencing of one alternative NADPH dehydrogenase was found to lead to changes in the mETC composition, patterns of plant growth under stress conditions, NAPDH/NADP\(^+\) ratios and responses to oxidative stress (Smith et al., 2011; Liu et al., 2008).

1.2.2 Alternative Oxidase

Alternative oxidase (AOX) is another unique component of the plant mETC. This terminal oxidase accepts electrons directly from ubiquinol and catalyzes the reduction of oxygen to water. Passage of electrons through this protein is not coupled to proton pumping and does not contribute to the generation of proton motive force. Furthermore, electron flow through AOX bypasses Complexes III and IV thus dramatically reducing the energy yield of respiration (Finnegan et al., 2004). It is a disulfide-linked dimeric protein and it is found on the interfacial side of the inner mitochondrial membrane (IMM) located on the matrix side. Many members of the AOX family contain a conserved Cys residue which confers a tight biochemical control on the activity of this enzyme. The dimer can exist in two forms, as a noncovalently linked dimer (reduced form) or a covalently linked dimer (oxidized form) by a regulatory disulfide bond between the two conserved Cys residues of the monomers. The noncovalently linked dimer is the more active form of the enzyme and it can be brought about by reduction of the disulfide bond by the oxidation of specific TCA cycle substrates (Umbach and Siedow, 1993). Once reduced, the activity of this enzyme can be stimulated by certain \(\alpha\)-keto acids, particularly by pyruvate through the interaction with the exposed sulfhydryl of the regulatory Cys residue (Day et al., 1994).
AOX has been identified in all plants, many fungi and protists, α-proteobacteria and in a broad range of animal species (McDonald 2008; McDonald and Vanlerberghe, 2006). In plants, AOX is encoded by a small nuclear gene family generally comprised of several members that display different expression patterns and have different functions. For example, the Arabidopsis genome contains at least five AOX genes with clear gene-specific roles in different organs, stages of development and under stress. For example, AtAox1a seems to be present in all tissues and is stress responsive, while AtAox1b, AtAox1d and AtAox2 are predominantly found in organs and developmental stages associated with high metabolic rates, such as flowering and seed development (Clifton et al., 2006). In tobacco, there have been at least three AOX genes identified, however most of the research to date has been done on one stress responsive AOX gene (Vanlerberghe et al., 1994, Whelan et al., 1995, Wang and Vanlerberghe, unpublished results).

As mentioned above, Aox1a seems to be one of the most stress-responsive genes in the plant genome, indicating that the induction of this gene is a common response to many stresses. For example, this gene is induced by treatment with various ETC inhibitors such as antimycin A, KCN and rotenone (Clifton et al., 2005; Vanlerberghe and McIntosh 1996) or in plants with various mitochondrial mutations such as the CMSII mutation in tobacco which results in an inactive Complex I (Dutilleul et al., 2003). In addition to this, AOX is induced by a wide variety of biotic and abiotic stresses, which do not necessarily target the mitochondria directly (Kilian et al., 2007; Escobar et al., 2004; Escobar et al., 2006; Finnegan et al., 2004; Ho et al., 2008; Ederli et al., 2006; Rhoads and McIntosh 1992). It should be mentioned at this point, that the exact relationship between the amount of AOX and the activity of the alternative pathway in vivo is still not clear. For example, several studies have found a lack of relationship between AOX amount and activity when levels of AOX were altered by salicylic acid (SA) treatment (Lennon et al., 1997), genetic modification of AOX levels (Guy and Vanlerberghe, 1995) or mitochondrial mutations (Juszczuk et al., 2007; Vidal et al., 2007). Furthermore, changes in AOX did not cause changes in metabolite levels, respiration, photosynthetic performance or growth in plants under without the application of stress (Umbach et al., 2005; Giraud et al., 2008; Watanabe et al., 2008; Florez-Sarasa et al., 2011). These results suggest that the conditions of growth and the biochemical regulatory properties of AOX itself are the main factor regulating the activity of the alternative pathway under given conditions.
Due to its non-energy conserving nature, there has been a lot of research trying to understand the function of AOX in respiration. The energy generated as a result of the passage of electrons through AOX is dissipated as heat. In certain species of thermogenic plants, such as the sacred lotus, this heat generation is involved in volatilization of scent compounds that attract insect pollinators (Watling et al., 2006). However, AOX is present in all plant species, including non-thermogenic species (McDonald and Vanlerberghe, 2006), which suggests that AOX has a more general function in plants.

It has been proposed that AOX has a role in optimizing respiratory metabolism, since the alternative pathway is not associated with energy conservation. This would enable a certain level of metabolic flexibility and would allow a broader range of conditions under which respiration could function. For example, under ADP and/or phosphate (Pi) limitation the cyt pathway is inhibited due to lack of substrates, however the presence of AOX allows NADPH oxidation and electron transfer to continue, therefore maintaining TCA cycle function and the supply of carbon intermediates for biosynthesis (Millenaar and Lambers 2003). In support of this hypothesis, Pi-deficient bean roots have increased AOX activity and decreased cyt pathway activity (Juszczuk et al., 2001). Similarly, tobacco suspension cells grown in a Pi-deficient environment have increased AOX protein and alternative pathway capacity (Parsons et al., 1999) and transgenic cells lacking AOX have altered morphology, cellular composition and amino acid synthesis patterns (Yip and Vanlerberghe, 2001; Sieger et al., 2005). Similarly, AOX could also help integrate respiratory metabolism with other cellular processes. For example, AOX activity might oxidize the excess reducing power associated with photosynthesis or photorespiration (Raghavendra and Padmasree 2003; Yoshida et al., 2007). Since AOX can modulate energy yield, it might also play a role in optimizing the rate of high-energy processes such as plant growth to match it with the amount of available key resources (Hansen et al., 2002; Moore et al., 2002).

One possible function of AOX could be to support metabolism and respiration in cases when the cyt pathway has been suppressed. For example, AOX expression and capacity are strongly induced in cases where plants are treated with ETC inhibitors such as antimycin A, nitric oxide (NO) and sulfide (Vanlerberghe and McIntosh 1996). Similarly, AOX is induced by salicylic acid (SA), a plant hormone associated with biotic stress that is known to disrupt mitochondrial function by an unknown mechanism (Norman et al., 2004; van der Merwe and Dubery, 2006).
Interestingly, AOX induction occurs in response to different biotic and abiotic stress treatments, such as ozone treatment (Ederli et al., 2006), cold treatment (Popov et al., 2001; Wang et al., 2011), anoxia (Amor et al., 2000; Millar et al., 2004) and pathogen infection (Simons et al., 1999). This suggests that AOX might act as a general “survival protein” under stress conditions that affect the mETC.

Over-reduction of the mETC can occur during periods of increased metabolic activity resulting in high membrane potential or as a result of cyt pathway disruption due to stress. In these cases there is an increased chance of a single electron transfer to O₂ generating superoxide (O₂⁻). AOX might act to dampen the production of mitochondrial ROS in two ways. First, the passage of electrons from ubiquinone to O₂ through AOX is not coupled to proton translocation and therefore does not contribute to increases in membrane potential. Also, the reduction of O₂ to H₂O by AOX lowers the amount of free O₂ available for O₂⁻ production (Møller 2001). The hypothesis that AOX dampens ROS production is supported by studies with transgenic plants silenced for AOX expression. For example, transgenic tobacco suspension cells accumulated more ROS when treated with antimycin A as compared to Wt cells (Maxwell et al., 1999). Similarly, in Arabidopsis plants treated with the Complex IV inhibitor KCN, more oxidative damage was detected in AOX knockdown lines as compared to Wt plants (Umbach et al., 2005). However, it should be mentioned that these are very artificial conditions due to the presence of inhibitors, and the effects of these inhibitors on systems other than the mitochondria can not be ruled out. Several studies have also found that plants lacking AOX had an increased expression of ROS scavenging enzymes (Amirsadeghi et al., 2006; Watanabe et al., 2008). Conversely, overexpression of AOX was found to decrease the expression of certain ROS scavengers (Maxwell et al., 1999; Pasqualini et al., 2007) as well as the level of cellular ROS under stress (Maxwell et al., 1999; Sugie et al., 2006). These studies can be taken as indirect evidence in support of the proposed hypothesis.

In summary, the existence of the alternative pathway can balance carbon metabolism and electron transport with other cellular processes and can maintain the energetic and redox poise of the cell, especially under stress conditions. However, our knowledge regarding these complex functions is far from complete, and more research needs to be done to clarify the function of this terminal oxidase.
### 1.2.3 Uncoupling proteins

Uncoupling proteins (UCPs) are mitochondrial anion carrier proteins that transport protons across the IMM, from the IMS to the mitochondrial matrix, following the electrochemical gradient. This process decreases $\Delta \Psi$ thus effectively uncoupling the activity of the mitochondrial ETC from ATP synthesis, and the potential energy is dissipated as heat (Vercesi et al., 2006). The activity of UCPs is tightly regulated by pH status, redox poise of the ubiquinone pool, $\text{Mg}^{2+}$ concentration, free fatty acids as well as the concentration of ATP and ADP. It is interesting that ROS have also been demonstrated to activate UCPs through ROS-induced lipid peroxidation products, suggesting that UCPs might also have a role in protecting the cell from excessive mitochondrial ROS generation. Also, the activity of UCPs might stimulate the oxidation of reducing equivalents thus increasing metabolic flux and maintaining glycolysis and TCA cycle activity during situations of high respiratory activity (Smith et al., 2004; Sweetlove et al., 2006).

Both AOX and UCPs dissipate $\Delta \Psi$ as heat and uncouple ATP production from electron transport; however they are differently regulated and respond to different stimuli. The most important difference between these two proteins is that AOX prevents the generation of $\Delta \Psi$ by affecting the reduction state of the ubiquinone pool and the concentration of available $O_2$, in contrast with UCP which regulates electron transport indirectly by dissipating $\Delta \Psi$ before it can be used to generate ATP. It has been suggested that AOX might modulate fast changes in respiratory metabolism while UCP is involved in more long-term energy status maintenance (Rasmusson et al., 2009).
**Figure 1.1:** The mitochondrial electron transport chain in plants. Components of the non-phosphorylating pathways are shown in green. During oxidative phosphorylation, the electron carrier NADH is oxidized at Complex I, and FADH$_2$ is oxidized at Complex II. The electrons are passed to the carrier ubiquinone, and subsequently transferred to Complex III. Electrons are finally passed to Complex IV where they are used to reduce O$_2$ to H$_2$O. Passage of electrons through this pathway is accompanied by pumping of protons across the inner mitochondrial membrane (IMM) by Complexes I, III and IV, which establishes an electrochemical gradient used as a driving force for ATP synthesis. In contrast, passage of electrons through the alternative pathway, type II NADH dehydrogenases and alternative oxidase (AOX) does not contribute to the electrochemical gradient and ATP synthesis. Similarly, uncoupling proteins (UCP) allows for proton flow across the IMM along the gradient, uncoupling electron transfer from ATP synthesis.
1.3 The *Pseudomonas syringae*-plant model interaction

Plants have evolved highly regulated defense systems to face the challenges due to microbial pathogen infections. In general, plant defense consists of both preformed and induced resistance responses that can either prevent the pathogen from entering or from obtaining nutrients for growth and reproduction. Preformed, also called housekeeping resistance, does not require pathogen recognition. In general terms, this type of resistance provides a physical barrier to invading pathogens such as the cell wall and the cell cytoskeleton, which prevents most pathogens from penetrating into host tissues. In addition, plants also contained preformed chemicals that have anti-pathogenic properties (Hardham et al., 2007; Thordal-Christensen 2003).

In contrast, induced resistance mechanisms are typified by specific recognition of the invading pathogen that ultimately lead to the production of plant proteins and metabolites that are antagonistic to the pathogen. Basal resistance refers to non-specific defense mechanisms induced upon the perception of some generic microbe or pathogen associated molecular patterns (MAMPs or PAMPs), which are generally highly conserved and important for bacterial function. This so-called PAMP triggered immunity (PTI) is initiated by PAMP recognition by plant pattern recognition receptors (PRRs). Probably the most studied PAMP-PRR system involves the perception of a 22 amino acid stretch of the bacterial flagellin (flg22) by a concomitant FLAGELLIN SENSING2 (FLS2) receptor (Boller and He, 2009; Bent and Mackey 2006; Gomez-Gomez and Boller, 2000; Chinchilla et al., 2006). This suggests that plants evolved to activate defense against a widest array of potential pathogens, while giving the bacteria the lowest chance of avoiding detection through mutation of the PAMP. Upon successful recognition, a large number of molecular, physiological and morphological changes occur in host plants. Early changes include ion flux across the plasma membrane, the appearance of an oxidative burst, MAP kinase activation and protein phosphorylation, as well as significant genetic reprogramming. Later changes include callose deposition and stomatal closure, as forms of physical barrier to prevent further pathogen penetration (Altenbach and Robatzek, 2007; Schwessinger and Zipfel, 2008).

Host plants have evolved a second level of induced immunity to counteract microbial attack from pathogens adapted to overcome the PTI. This so called effector triggered immunity (ETI) is
mediated by resistance (R) proteins that can perceive pathogen effector molecules, called avirulence (Avr) factors. R proteins can detect the presence of effectors either directly, by physical contact, or indirectly by detecting modifications of host proteins affected by the Avr factors (van der Hoorn and Kamoun, 2008). A well studied example of indirect effector perception involves the Arabidopsis RIN4 which is an accessory protein to the immune receptor RPS2. RPS2 can activate defense signaling cascades as a result of RIN4 loss due to cleavage by the \textit{P. syringae} protein AvrRpt2 (Axtell et al., 2003; Mackey et al., 2003). Some of the downstream effects of this immune response include a prolonged biphasic oxidative burst, changes in plant hormones involved in plant defense signaling, most notably SA, jasmonic acid (JA) and ethylene (ET), as well as transcriptional programming (Jones and Dangl, 2006).

It is interesting to note that ETI and PTI extensively share downstream signaling machinery and seem to be mediated by an integrated signaling network. For example, PAMPs and R proteins trigger the induction of an overlapping set of genes (Tao et al., 2003; Navarro et al., 2004). Similarly, ROS production is a common response to both types of immunity, however PTI triggers a rapid and transient oxidative burst, while ROS induction during ETI appears to be more robust, prolonged and with a biphasic pattern with the first peak probably triggered by PAMPs and the second peak being the result of R-Avr recognition (Torres et al., 2006). This has led to the suggestion that common machinery is used in both types of immune responses, albeit in a different way. For example, in the case of low pathogen specificity during PTI it would be beneficial for the plant not to induce a strong immune response which would negatively impact plant fitness. In contrast, during ETI, strong pattern recognition can lead to a strong immune response induction early in the infection achieving a robust and persistent disease resistance (Tsuda and Katagiri, 2010). A representation of possible plant-pathogen interactions can be seen in Figure 1.2.

\textit{Pseudomonas syringae} is bacterial species composed of various strains that collectively infect hundred of plant species causing a wide variety of symptoms. \textit{P. syringae} strains are known for their diverse and host-specific interactions with different plants and may be assigned to more than 50 pathovars depending on the host range. This pathogen can be described as a hemi-biotroph. During such pathogenic infections the most aggressive phase of intercellular bacterial growth occurs in the absence of host cell death. In later stages, pathogens switch to a more destructive necrotrophic phase and infected tissues show extensive necrotic death (Hirano and
All of the *P. syringae* strains examined to date contain a *hypersensitive response and pathogenicity (hrp)*-gene-encoded type III protein secretion system (TTSS), a molecular syringe that is used by the bacteria to inject a number of virulence effector proteins in the host cells (Jin and He, 2001; Greenberg and Vinatzer, 2003; Chang et al., 2005). In addition to the TTSS, *P. syringae* strains are known to produce various phytotoxins, including cell-wall degrading enzymes, exopolysaccharides, siderophores and adhesins, which are necessary for the full virulence in host plants (Buell et al., 2003).
**Figure 1.2:** A model describing the resistance or susceptibility of a host plant to a bacterial pathogen. The bacterial type III secretion system (TTSS) delivers bacterial avirulence proteins (Avr) into the plant cell. An interaction of the Avr protein with a host pathogenicity target protein (P) can modulate the function of the P protein for the benefit of the bacterial pathogen (e.g., recognition avoidance mechanism). This type of interaction will end up in the development of disease (right-hand side). In a resistant plant, a pathogen associated molecular pattern (PAMP) is recognized by a PAMP receptor which can activate the PAMP triggered immunity (PTI). Alternatively, a resistance (R) protein receptor can recognize the Avr protein either directly (receptor-ligand model) or indirectly through its interaction with a target protein (guard model) and activate effector triggered immunity (ETI). Both defense pathways share the downstream signaling machinery, however the type of resistance induced (PTI or ETI) depends on the specific plant-pathogen interaction.
1.3.1 Compatible plant-pathogen interactions

Recent studies have firmly established that suppression of various plant defenses, including both PTI and ETI mechanisms, is a major virulence function of the bacterial TTSS effectors and toxins. In this case the host plant develops a disease, where the plant host cells are killed either directly by pathogen-derived toxins, or by a necrotic cell death process triggered by specific signaling molecules. This interaction is said to be compatible and the plant is considered susceptible to the pathogen. The exact mechanism of suppression of various plant defenses is still under study, however it is generally believed that virulence factors work by stabilizing negative regulators of plant defenses (for example, by stabilizing RIN4, see above – Kim et al., 2005). Another hypothesis is that the effectors function as programmed cell death suppressors and act downstream of resistance-protein-mediated recognition (Nomura et al., 2005) (Figure 1.2). Further studies are needed to fully understand the mechanism of pathogen virulence on host plants.

1.3.2 Incompatible plant-pathogen interactions

In a so-called incompatible plant-pathogen interaction, the plant recognizes the presence of the microorganism, and this recognition process triggers the induction of biochemical pathways that result in defense against the pathogen. In this case, the plant is considered to be resistant to the invading pathogen. This type of host resistance is very often governed by a single R gene, the product of which interacts directly or indirectly with a specific bacterial virulence effector (product of an Avr gene), and induces downstream defense pathways. This interaction is very specific and generally protects the plant from one type of pathogen. Typically, this type of plant resistance is associated with the appearance of the hypersensitive response (HR), a type of plant programmed cell death at the site of infection (Greenberg and Yao, 2004; Hofius et al., 2007) (Figure 1.2)
1.3.3 Non-host plant-pathogen interactions

Non-host resistance is one of the most important, and least understood, plant resistance mechanisms. This type of resistance is non-specific and it allows plant species to resist infection by majority of plant pathogens that would normally infect other species. Normally, this scenario happens when the pathogen is not fully adapted to the host plant and fails to overcome induced defenses (Thordal-Christensen, 2003). There are two types of non-host resistance that are generally recognized: type I that does not produce any visible symptoms and type II which is generally associated with the appearance of PCD. The type of non-host resistance is dependant on the specific host-pathogen interaction, in which one pathogen can cause a type I response in one plant species but a type II response in another species (Mysore and Ryu, 2004).

Many defense responses induced by non-host interactions are similar to those induced during gene-for-gene interactions, especially during the appearance of the HR. For example, ROS are produced during both types of interactions, even though the relative amounts and timing of accumulations might differ (Huckelhoven et al., 2001; Able at al, 2003). Also, expression profiles of resistance genes induced after infection with a host and a non-host pathogens exhibited significant overlap in Arabidopsis leaves (Tao et al., 2003). It is possible that both types of resistance have separate signal transduction pathways with a significant amount of cross-talk and downstream convergence.

1.4 Programmed cell death in the plant immune response

Pathogen recognition generally leads to induction of defense responses and inhibition of plant growth, which is often accompanied by the HR. This response is defined as plant PCD localized at the site of attempted pathogen entry. Morphologically, this type of cell death is unique. It shares certain hallmarks with animal apoptosis, such as cytoplasmic shrinkage, chromatin condensation and mitochondrial swelling, but it also has some plan-specific characteristics such as vacuolization and chloroplast disruption (Mur et al., 2008).

Despite recent advances in this field, the specific reason for the appearance of HR during biotic stress is still not clear. Traditionally, the HR was thought to be a mode to prevent pathogen spread and to induce further defense responses. However, this view has been challenged by
several studies that have shown that cell death does not inhibit pathogen proliferation, and that plant defenses can be induced without the appearance of death (Kiraly et al., 1972; Jakobek and Lindgren, 1990; Schifer et al., 1997; Coll et al., 2010). It has been proposed that the HR may occur as a consequence of the increased intensity and duration of the signaling due to incompatible plant-pathogen interactions, and that it is due to the rise in toxic intermediates that lead to both pathogen and host cell death (Coll et al., 2011). Another hypothesis is that even though the appearance of the HR might be uncoupled from disease resistance, it is required for the generation of long distance signaling mediated by ROS and SA, that induce the systemic acquired resistance (SAR) in plants (Durrant and Dong, 2004; Torres et al., 2005). The cause for the appearance of the HR is still not clear and requires further research.

Many recent studies have focused on the role of the mitochondria during HR induction and execution, as this organelle is an important player during mammalian apoptosis (Wang and Youle, 2009; Keeble and Gilmore, 2007; Ly et al., 2003). Animal apoptosis is primarily achieved by the activation of aspartate-specific cysteine proteases (caspases). These proteins are synthesized as dormant proenzymes, that upon proteolytic activation acquire the ability to cleave target proteins and activate further pro-apoptotic pathways. This activation is achieved by the release of mitochondrial IMS proteins into the cytosol, most notably the mETC carrier protein cytochrome c. Cyt c combines with other cytosolic proteins to form a caspase activating complex that induces the caspase cascade. The mechanism of cyt c release is still under debate (Ly et al., 2003), however it is hypothesized that it might involve the formation of a permeability transition pore (PTP), which would cause loss of $\Delta\Psi$, influx of water and solutes in the mitochondrial matrix, followed by outer mitochondrial membrane (OMM) rupture and IMS protein release. Another hypothesis states that certain proteins recruited to the OMM produce a pore that releases IMS proteins. A voltage-dependant anion channel (VDAC) might be a component of this pore. Alternatively, the VDAC might close due to death stimuli, depleting the mETC from ADP. This would cause an initial increase in $\Delta\Psi$, followed by an increase in mROS. These conditions are damaging to the mitochondria and lead to an influx of water and solutes, and eventually rupture of the OMM (Ly et al., 2003). Regardless of the mechanism of cyt c release, it is clear that this process has to be tightly regulated, mainly by a family of proteins called the Bcl-2 family. It is hypothesized that the anti-apoptotic members of this family, such as Bcl-2 and Bcl-XL, interact with the OMM and prevent the release of cyt c. Conversely, pro-apoptotic proteins, such as Bid,
Bax and Bak, can promote the release of cyt c in the cytosol (Wang and Youle, 2008; Keeble and Gilmore, 2007).

It is not clear how many of these processes occur during plant PCD initiation. During the HR there is mitochondrial swelling and a loss of mitochondrial function (Bestwick et al., 1995; Naton et al., 1996) as well as a rapid decrease in ΔΨ that could be suppressed by PTP inhibition (Yao et al., 2004), which suggests pore formation in these cells (Arpagaus et al., 2002). Also, several studies have detected cyt c release preceding cellular death (Krause and Durner, 2004; Kiba et al., 2006). However, it has not been demonstrated that cyt c interacts with other pro-apoptotic factors or that it activates caspase cascades. In fact, no plant homologue of animal caspases has been identified. Several plant alternatives have been proposed, such as metacaspases and vacuolar processing enzymes (VPE), both of which exhibit some homology to caspases, however their exact function and mode of action remain to be established (Watanabe and Lam, 2004; Bonneau et al., 2008).

There is also a debate regarding the relative importance of caspase activation versus mitochondrial dysfunction in promoting PCD. The rupture of OMM and the loss of cyt c will have severe consequences in mitochondrial function, electron flow, mitochondrial ROS generation and ultimately ATP production. Dramatic changes in these functions could have a profound effect on the cell as a whole and could potentially lead to cellular death. The functional significance of such changes in mitochondrial function in terms of promoting PCD requires further research.

As mentioned above, plant mitochondria have several additional components which are absent in their animal counterparts. These alternative proteins increase the points of entry and exit of electrons thus potentially increasing the flexibility of the ETC. These proteins, the alternative NADPH dehydrogenase AOX, could fundamentally alter the role of mitochondria during PCD. For example, in plant systems the existence of the alternative pathway can allow for continued respiration, limited ATP production and dampening of ROS generation upon cyt c release and cyt pathway disruption. In other words, the alternative pathways could act as anti-apoptotic components of the mitochondria which could play an important role in defining cellular fate (death or defense). In support of this hypothesis, lack of AOX promoted PCD in tobacco suspension cells with a down-regulated cyt pathway (Vanlerberghe et al., 2002). Also,
Numerous studies have shown increases in AOX transcript and protein levels under various biotic and abiotic stresses that potentially target the mitochondria (Amor et al., 2000; Lacomme and Roby, 1999; Simons et al., 1999; Ordog et al., 2002; Takahashi et al., 2003; Krause and Durner, 2004; Huang et al., 2002). A model for the role of mitochondria in the initiation and execution of PCD and defense responses during biotic stress can be seen in Figure 1.3.

In plants, the events that lead to the appearance of HR include the accumulation of plant hormones, accumulation of ROS and RNS, activation of MAPK cascades and changes in intracellular calcium levels. These cellular events are generally followed by transcriptional reprogramming and the synthesis of antimicrobial compounds. In the following section, some of these signaling events will be considered in more detail, with special consideration of the role of the mitochondria in the production and relay of these signals.
Figure 1.3: A model for the role of mitochondria in the activation and execution of PCD and defense responses during plant-bacteria interactions. According to this model, upon pathogen recognition, various signaling molecules produced by the host plant such as SA, ROS and RNS, promote defense or PCD responses by altering mitochondrial function. By inhibiting the cytochrome pathway of the mETC (Complexes I, III and/or IV) these factors can lead to the production of ROS in a self-amplifying loop eventually leading to mitochondrial membrane rupture, loss of membrane potential and release of intermembrane proteins (including cytochrome c) in the cytosol. This type of catastrophic mitochondrial dysfunction would lead to collapse of respiration, further production of ROS and eventually the initiation of PCD pathways. However, the unique components of the mETC, such as AOX, might allow for a functional respiratory pathway in case of mitochondrial dysfunction thus effectively dampening the production of mitochondrial ROS. In this way, the alternative components of the ETC could play an anti-PCD role during biotic stress.
1.4.1 Reactive Oxygen Species

A rapid and transient production of ROS, termed “the oxidative burst”, is one of the hallmarks of successful recognition of pathogen attack by host cells. This ROS generation is typically biphasic, with a first non-specific and transient phase that occurs rapidly upon pathogen recognition, and a second more sustained phase that is usually associated with the appearance of the HR. ROS generation typically results from the excitation of $O_2$ to form singlet oxygen ($O_2^1$) or from the reduction of $O_2$ to form superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$) or the hydroxyl radical ($OH^\cdot$). The most research on biotic stress to date has been done on the importance and signaling mechanisms of $O_2^-$ and $H_2O_2$, while the involvement of other ROS is still questioned (Foyer and Noctor, 2005).

There are several sources of ROS in plants. Plasma membrane NADPH oxidases, also known as Respiratory Burst Oxidase Homologs (Rboh), are the source for the apoplastic oxidative burst during most plant pathogen interactions (Torres and Dangl, 2005). Several studies done in Arabidopsis (Torres et al., 2002; Zhang et al., 2007) and tobacco (Simon-Plas et al., 2002; Yoshioka et al., 2003), have shown that these enzymes are responsible for the production of nearly all apoplastic ROS during incompatible bacterial infections. Several alternative enzymes such as diamine oxidases and cell wall peroxidases have also been implied in the production of ROS during biotic stress (Yoda et al., 2009) and it has been suggested that the enzymatic origin of apoplastic ROS can vary depending on the specific plant-pathogen interaction. Although the primary oxidative burst associated with the HR occurs in the apoplast, intracellular ROS may also play a role in defense and signaling. A growing body of evidence is implicating the chloroplasts and mitochondria as significant contributors to the appearance of the HR, possibly through modifying the redox status of the cell. More specifically, the chloroplast can produce ROS by overreduction of the photosynthetic ETC and reduction of $O_2$ by photosystem I to produce $O_2^-$ and subsequently $H_2O_2$ and $OH^\cdot$. Several studies have shown that at least some forms of HR are influenced by light (Zeier et al., 2004; Montillet et al., 2005).

There are several lines of evidence that point to the mitochondria as a major source of ROS during biotic stress. Krause and Durner (2004) have shown that treatment of Arabidopsis cell cultures with the bacterial elicitor harpin elicited a large ROS burst emanating specifically from the mitochondria, followed by a decrease in $\Delta\Psi$ and ATP production. Similarly, the fungal
Phytotoxin victorin elicited a rapid ROS burst in oat leaves from specific sites on the OMM (Yao et al., 2002). It has been proposed that this oxidative burst disrupts mitochondrial function and forms a self-amplifying loop where increased ROS leads to mitochondrial damage which further increases ROS accumulation. The culmination of this process would be mitochondrial dysfunction, membrane rupture and the release of mitochondrial proteins in the cytosol (Amirsadeghi et al., 2007).

A lot of the information on the mechanism of ROS production in the mitochondria comes from animal studies, where the mETC is considered to be the major source of cellular ROS. In the mammalian ETC, the one electron reduction of $O_2$ to $O_2^-$ happens mainly at Complexes I and III (Murphy 2009; Poyton et al., 2009), and there is evidence that this is also the case in plant mitochondria (Møller 2001; Sweetlove and Foyer, 2004). In both Complexes, electron transport is a multi-step process that involves the generation of the semiquinone radical ($Q^-$), which can spontaneously reduce $O_2$. The mechanism of $O_2^-$ generation at Complex I is still poorly understood, however this Complex is considered to be the major mETC ROS source and ROS produced at this location is released in the mitochondrial matrix. Complex III can generate $O_2^-$ at two different sites within the Complex, and $O_2^-$ can be released in the intermembrane space or the matrix (Murphy, 2009). The rate of mETC production is highly dependant on the mitochondrial activity, $\Delta \Psi$ and the reduction state of electron carriers. For example, actively respiring mitochondria have a high rate of electron transport and lower $\Delta \Psi$ which allows for the majority of the $O_2$ to be consumed. In contrast, mitochondria in a resting state (not making ATP), or during mitochondrial dysfunction, will have low rates of $O_2$ consumption, much higher $\Delta \Psi$ and highly reduced mETC components, which would favor the production of $O_2^-$. As mentioned above, it is hypothesized that one of the functions of AOX in plant mitochondria is to prevent the occurrence of this situation by enabling continuous electron flow even in unfavorable conditions, thus dampening excessive ROS production.

It should be noted that total cellular ROS levels depend not only on the rate of ROS production, but also on the rate of ROS scavenging. Plant mitochondria contain one Manganese Superoxide Dismutase (MnSOD) isoform which is the sole means of enzymatic conversion of $O_2^-$ to the less reactive $H_2O_2$ (Alcher et al., 2002). This enzyme is not inhibited by KCN or inactivated by $H_2O_2$ and it provides means of ROS control within the mitochondria. It has recently been shown that overexpression of this enzyme (targeted to either the mitochondria or the chloroplasts) leads to
better tolerance of oxidative stress and enhanced resistance against various abiotic stresses (Bowler et al., 1991; Wang et al., 2005; Wang et al., 2010), either by increased scavenging of O$_2^-$ or disruption of the O$_2^-$ / H$_2$O$_2$ balance. The effect of MnSOD on biotic stress has not been studied in great detail so far. It is hypothesized that the O$_2^-$ produced in mitochondria might also traverse to the cytosol through the VDAC or the PTP during PCD initiation, where it can be scavenged by other SOD isoforms or contribute to cell signaling (Alcher et al., 2002; Poyton et al., 2009). In contrast, H$_2$O$_2$ is an uncharged molecule and can move through the mitochondrial membranes into the cytosol where it can be scavenged by several systems, including ascorbate peroxidase, glutathione peroxidase and catalase (Mittler, 2002). Table 1.1 summarizes the production and scavenging of ROS in various locations in plant cells.

It has been known for some time that excess ROS can oxidize and damage proteins, nucleic acids, polysaccharides and lipids thus it was proposed that the function of the oxidative burst is to damage pathogens directly. However, recent evidence suggests that intracellular ROS can orchestrate both the establishment of defense systems to avoid cellular death and the appearance of PCD. For example, ROS could modify gene expression during biotic stress, either directly through redox regulation of transcription factors or indirectly, by interacting with other signaling cascades such as phosphorylation and hormone pathways (Kovtun et al., 2000; Kotchoni and Gachomo, 2006). Also, ROS have been suggested to interact with plant hormones during biotic stress during the establishment of long distance signaling and SAR (Durrant and Dong, 2004). In most plant-pathogen interactions, ROS are positively correlated with the HR. For example, elimination of ROS in transgenic Arabidopsis plants silenced for Rboh expression displayed reduced appearance of HR lesions in response to incompatible pathogen infection (Torres et al., 2002). Their role in inducing PCD could be either through elevated toxicity and reactivity of these molecules or by releasing other pro-death signals that mediate the HR. One example of this would be the production of mitochondrial ROS that can lead to cyt c release. It has also been noticed that in many incompatible plant pathogen interactions, the level of antioxidants is decreased due to the infection suggesting a programmed approach of increased ROS levels in cells undergoing PCD (Yang et al., 2004). These examples clearly show the need for a tight regulation of ROS production and scavenging during biotic stress.
Table 1.1: The production and scavenging of reactive oxygen species in plants (table adapted from Mittler, 2002).

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Localization</th>
<th>ROS type</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Production</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photosynthetic ETC</td>
<td>Chloroplast</td>
<td>$\text{O}_2^-$</td>
</tr>
<tr>
<td>Excited Chlorophyll</td>
<td>Chloroplast</td>
<td>$\text{O}_2^1$</td>
</tr>
<tr>
<td>Respiratory ETC</td>
<td>Mitochondria</td>
<td>$\text{O}_2^-$</td>
</tr>
<tr>
<td>NADPH oxidase</td>
<td>Plasma membrane</td>
<td>$\text{O}_2^-$</td>
</tr>
<tr>
<td>Peroxidases</td>
<td>Cell Wall</td>
<td>$\text{O}_2^-, \text{H}_2\text{O}_2$</td>
</tr>
<tr>
<td><strong>Scavenging</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>Chloroplast, Cytosol, Mitochondria, Apoplast, Peroxisome</td>
<td>$\text{O}_2^-$</td>
</tr>
<tr>
<td>Ascorbate Peroxidase</td>
<td>Chloroplast, Cytosol, Mitochondria, Apoplast, Peroxisome</td>
<td>$\text{H}_2\text{O}_2$</td>
</tr>
<tr>
<td>Catalase</td>
<td>Peroxisome</td>
<td>$\text{H}_2\text{O}_2$</td>
</tr>
<tr>
<td>Glutathione Peroxidase</td>
<td>Cytosol</td>
<td>$\text{H}_2\text{O}_2$</td>
</tr>
<tr>
<td>Glutathione</td>
<td>Chloroplast, Cytosol, Mitochondria, Apoplast, Peroxisome</td>
<td>$\text{H}_2\text{O}_2$</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>Chloroplast</td>
<td>$\text{O}_2^1$</td>
</tr>
</tbody>
</table>
1.4.2 Reactive nitrogen species

Nitric oxide (NO) is an important signaling molecule with various roles in plant systems, including an important signaling function during biotic stress. It is a gaseous molecule which can freely diffuse through membranes and the cytosol. It is a very reactive species which can form other oxides in the presence of atmospheric O$_2$, including NO$_2^-$, N$_2$O$_3$ and N$_2$O$_4$. It also rapidly reacts with O$_2^-$ forming the peroxynitrite ion (ONOO$^-$), another RNS with a potential role during plant-pathogen interactions (Arasimowicz and Floryszk-Wieczorek, 2007).

In plants, there is evidence for two major routes of NO synthesis: a reductive pathway where the electrons needed to reduce nitrite (NO$_2^-$) to NO are provided by the mitochondrial ETC, NAD(P)H or an acidic environment, and an oxidative pathway where NO is produced from substrates such as arginine and polyamines (Moreau et al., 2010; Gupta et al., 2010; Arasimowicz and Floryszk-Wieczorek, 2007; Blokhina and Fagerstedt, 2009). In animals, it is known that the majority of NO is produced by the oxidation of arginine to citrulline catalyzed by the enzyme nitric oxide synthase (NOS). Even though a NOS enzyme with sufficient homology to animal NOS has not been identified in plants to date, several candidates with NOS-like activity have been detected and analyzed but their function in NO production remains unconfirmed (Zemojtel et al., 2006). Arginine could be also involved in the production of NO via an alternative pathway, since it is a substrate in the pathway that produces polyamines such as spermine and spermidine which generates NO as a side-product. The biochemical mechanism of NO release in this situation is not known (Yamasaki and Cohen, 2006). The best characterized pathway for NO production in plants is the nitrate reductase (NR) pathway. This enzyme is localized in the cytosol and it primarily catalyzes the conversion of nitrate (NO$_3^-$) to nitrite (NO$_2^-$) using NADH as an electron donor. This enzyme also has the ability to reduce NO$_2^-$ to NO, however under steady-state conditions the nitrite reductase activity accounts for only ~1% of total enzyme activity and it generally requires high nitrite concentrations. The NO generation via this pathway might be increased under stress conditions, including plant-pathogen interactions (Shi and Li, 2008; Yamamoto-Katou et al., 2006). Other sources of NO include a putative plasma membrane-bound nitrite:NO reductase (NiNOR) and a peroxisomal xanthine oxidoreductase (XOR), however the relative importance, electron source and activity of these enzymes in vivo is still not clear (Gupta et al., 2010). A summary of NO producing pathways can be seen in Figure 1.4.
Recently, the mitochondria have emerged as a source of NO as well, however the details of NO synthesis at this organelle are still elusive. The mitochondrial NO$_2^-$ reductase activity is increased in the presence of high NO$_2^-$ concentrations and decreasing pH, conditions usually found during hypoxia. This reaction is oxygen-independent and generally activated under hypoxic or anoxic conditions (Planchet et al., 2005). Even though the exact mechanism of mitochondrial NO generation is not fully elucidated, it is hypothesized that this reaction is catalyzed by enzyme complexes downstream of ubiquinone. The evidence for this comes from studies that have used the respiratory inhibitors myxothiazol (Complex III inhibitor) and KCN (Complex IV inhibitor) to decrease the NO$_2^-$ reduction reaction under hypoxic conditions (Stoimenova et al., 2007). Currently there is no evidence that Complex III can function as a NO$_2^-$ reductase, however enzyme activity has been detected in purified Complex IV (COX) from yeast and rat mitochondria (Castello et al., 2006). It is thought that NO$_2^-$ reacts with a heme center in COX and NO is generated with the following reaction:

$$\text{NO}_2^- + \text{Fe}^{2+} + \text{H}^+ \rightarrow \text{NO} + \text{Fe}^{3+} + \text{OH}^-$$

It has been suggested that the production of NO by the mETC has a role in regulation of respiration, since COX can use NO$_2^-$ as a final acceptor to drive ATP synthesis under hypoxic conditions. It is interesting to note that NO is a COX inhibitor when oxygen is present and COX might even convert some of the bound NO to NO$_2^-$ under these conditions (Poyton et al., 2009). It should also be mentioned that even though NO is a COX inhibitor, this molecule does not inhibit the activity of AOX (Gupta et al., 2010).

NO can interact with O$_2^-$ to produce ONOO' (Blokhina and Fagerstedt, 2009). This reaction most likely happens in the mitochondria since both NO and O$_2^-$ generation has been linked to this organelle. Since NO can cross cellular membranes (which is not the case with O$_2^-$), it is possible that even NO that is not mitochondrial in origin can contribute to mitochondrial ONOO' production. This reaction might be an important scavenging mechanism for both NO and O$_2^-$ in the cell.
Figure 1.4: A representation of the two major routes for NO production in plant cells. In the reductive pathway, electrons for the reduction of NO$_2^-$ are provided by the mitochondrial ETC, NADPH via the enzyme nitrate reductase or in an acidic environment. During the oxidative pathway, several substrates can be used to generate NO (hydroxylamine, arginine, polyamines), however the mechanism of NO release in this case is largely unknown (adapted from Moreau et al., 2010).
There are several other ways that the concentration of NO can be buffered and decreased in plant cells. NO binds to glutathione (GSH) and forms S-nitrosoglutathione (GSNO), the concentration of which can be regulated by the enzyme GSNO reductase (GSNOR). GSNO is considered to be a cellular reservoir for NO and the biochemical method of its production has not been fully elucidated, however studies in mammalian cells have suggested that COX might be the catalyst for the reaction between GSH and NO (Sakamoto et al., 2002, Basu et al., 2010). It is interesting that the expression of GSNOR is upregulated by SA treatment (Diaz et al., 2003) and points to a potential role of this enzyme during biotic stress. Non-symbiotic haemoglobins (Hb) are proteins that hold an iron heme and are capable of binding not only oxygen but several other diatomic molecules as well, including NO. The efficiency and specificity of this scavenging method still remains questionable, since plants overexpressing Hb managed to reduce NO produced by hypoxic stress, but higher Hb levels did not have an effect on NO produced due to pathogen attack (Perazzolli et al., 2004).

Evidence that RNS play an important role during biotic stress, individually or by interacting with other molecules, is accumulating. For example, the application of exogenous NO induced cell death in Arabidopsis suspension cells similar as seen when cells were challenged with avirulent bacteria (Clarke et al., 2000). Similarly, both soybean and tobacco suspension cells induced PCD when treated with NO and H\textsubscript{2}O\textsubscript{2} simultaneously, whereas increasing the levels of only one of these molecules did not have an effect on these cells (Delledonne et al., 1998; de Pinto et al., 2002). The mechanism of interaction between these molecules during the induction of cell death is currently not known but it has been observed that a chemical reaction between NO and H\textsubscript{2}O\textsubscript{2} results in the production of singlet oxygen or hydroxyl radicals (Noronha-Dutra et al., 1993), both of which can be highly toxic and cause cell death. Furthermore, NO can also interact with \textit{O}_2^- to produce ONOO\textsuperscript{-}. This molecule can induce apoptosis in animals but it has been found to be relatively non-toxic in plants (Dalledonne et al., 2001). However increased ONOO\textsuperscript{-} concentrations have been linked to increased protein tyrosine nitration during incompatible plant-pathogen interactions. It has been suggested that tyrosine nitration might have a role during the induction of defense responses (Vandelle and Dalledonne, 2011); however the mechanism of action is still unclear.
1.4.3 Hormonal signaling during biotic stress

While various signals discussed above are relatively conserved between animal and plant kingdoms, plant specific hormones might play a unique role during plant-pathogen interactions. SA, ET and JA are the three plant hormones that are mainly involved in defense responses in infected plants. These three hormones are involved in signaling in a complex web of interactions, both synergistic and antagonistic. For example, it is hypothesized that while SA is mainly involved in signaling for the induction of defense and PCD during biotrophic pathogen infections, ET and JA play a role in defense against necrotrophic pathogens. However, it should be kept in mind that this is a very broad generalization, and the interplay between these hormones is more complex (reviewed in Rojo et al., 2003). The role of SA during plant defense and PCD signaling is the most studied so far.

SA is synthesized via two pathways: the phenylalanine ammonia-lyase (PAL)-mediated phenylalanine pathway and the isochorismate synthase (ICS)-mediated isochoristmate pathway, both of which are involved in SA accumulation during plant-pathogen interactions (An and Mou, 2011). Free SA is often conjugated to other molecules. For example, during pathogen attack, most of the free SA produced is glycosylated to form salicylic acid β-glucoside (SAG), which is usually sequestered in vacuoles and provides a quickly accessible SA source (Dean et al., 2005). SA can also be methylated to form the volatile ester methyl salicylate (MeSA), thought to be the long distance signal during the establishment of SAR (Park et al., 2007). Free SA can influence the development of defense or PCD responses in several different ways. For example, SA can bind to a variety of plant proteins affecting their activity, such as the H₂O₂ scavenger catalase, as well as activate gene expression via its influence on various transcription factors (Feys and Parker, 2000; Pieterse et al., 2001; Shah, 2003).

It is interesting that the action of SA has been linked to mitochondrial function as well. It has been known for a long time that SA controls heat production during flowering of thermogenic plants by inducing the expression of AOX. Since then it has been observed that AOX expression is induced by SA in non-thermogenic species as well (Raskin et al., 1987; Rhoads and McIntosh, 1993; van der Straeten et al., 1995; Maxwell et al., 2002). Furthermore, SA applied at lower concentrations (< 1 mM) to isolated mitochondria acts as an uncoupler of electron transport, while at higher concentrations blocks electron transport by preventing interactions between...
dehydrogenases and the Q pool (Norman et al., 2004). The mechanism of AOX expression due to SA application is still unknown, but it is possible that it could be due to the effect of SA as a respiratory inhibitor.

1.5 The Role of AOX during plant-pathogen interactions

As mentioned above, there are several hypotheses about the role of the alternative pathway in plant mitochondria, mostly relating to the potential of AOX to provide metabolic flexibility under stress. Several studies have lent support to this theory (see above), however the specific role of AOX in plant-pathogen interactions is still a matter of debate. There is strong evidence for the involvement of this protein in biotic stress. For example, SA was found to dramatically induce the expression of AOX and to increase its protein levels (Rhoads and McIntosh, 1992; Van Der Straeten et al., 1995; Djajanegara et al., 2002; Ho et al., 2008; Lei et al., 2010). As mentioned above, SA was also found to have an impact on the mitochondrial ETC (Norman et al., 2004; de Souza et al., 2011) and so far it is not clear whether the induction of AOX is due to mitochondrial dysfunction during this treatment. Similarly, application of NO leads to increases in AOX gene expression (Huang et al., 2002; Zottini et al., 2002; Ederli et al., 2006), however whether this induction is the effect of the action of NO as a respiratory inhibitor or as a signaling molecule (independent of cyt oxidase inhibition) is still not clear. Exogenous H$_2$O$_2$ can also induce AOX gene expression (Vanlerberghe and McIntosh, 1996) and H$_2$O$_2$ responsive elements are present in the stress-responsive Aox1a gene of Arabidopsis (Ho et al., 2008). From these examples, it is clear that AOX is influenced by biotic stress, however how would this impact the development of defense and PCD is still not clear.

Most of the research done in the field of biotic stress and the involvement of AOX is based on observing the role of AOX in viral infections, and even so, it is somewhat contradictory. One line of evidence supports the theory that AOX is directly involved in defense responses and it comes mainly from studies with transgenic tobacco expressing SA hydroxylase (NahG), an enzyme that degrades SA. A key role for SA in plants is the activation of disease resistance and therefore these transgenic plants have an increased susceptibility to pathogen infections (Alvarez, 2000). A study done by Chivasa and Carr (1998) used NahG plants infected with tobacco mosaic virus to observe the involvement of AOX in the resistance of tobacco to this pathogen. It was noticed that
treatment of KCN (a potent inhibitor of cyt oxidase) induced viral resistance through elevating the levels of AOX in plants. Moreover, a study done on transgenic tobacco plants with modified AOX levels infected with tobacco mosaic virus (TMV) used SA and antimycin A pretreatments in order to cause initial milder stress and to induce accumulation of AOX transcript. It was concluded that alterations in AOX expression did in fact affect the induction of resistance to TMV, with AOX being a regulator rather than trigger of defensive signaling (Gilliland et al., 2003). In addition to this, it has also been proposed that elevated levels of AOX may be an element of the SAR in tobacco (Lennon et al., 1997).

However, the second line of evidence suggests that AOX, even though it undoubtedly plays a role in the defense responses, is not a critical component of tobacco viral resistance. Ordog et al. (2002) used transgenic plants with elevated levels of AOX, as well as plants lacking AOX, to observe the disease response to TMV. The researchers concluded that overexpression of the protein can only dampen the progression of PCD at the lesion periphery but not heighten the resistance to the virus. Similarly, lack of AOX did not compromise the restriction of the virus by PCD. Moreover, another study found that susceptibility of tobacco cells (Wt and transgenic plants lacking AOX) to SA, did not correlate directly with the presence or absence of AOX, but only with the cellular levels of ROS (Amirsadeghi et al., 2006).

When referring to these previous studies, it has to be noted that all of them have observed the plant-virus interaction. The literature is not very extensive on fungal and bacterial pathogens and how these interactions relate to AOX levels and activity. Several studies have observed enhanced expression of the AOX gene in response to bacterial pathogens (Simmons et al., 1999; Lacomme and Roby, 1999), but it has to be noted that these studies were done on Arabidopsis thaliana plants, and not in tobacco. Harpin (a bacterial derived elicitor) induced HR associated with altered mitochondrial function, but this study did not explore the levels or involvement of AOX in the disease response (Xie and Chen, 1999)
Thesis introduction

Mitochondria are emerging as a “signaling organelles” during many biotic and abiotic stresses (see above). Based on the information presented previously, it is hypothesized that the mitochondria can produce an integrated signal (probably ROS or RNS-based) in response to stress and in this way control the downstream fate of the cell. A major focus of the work presented in this thesis is to investigate the role of the mitochondria during defense and PCD in plant-pathogen interactions. More specifically, this work concentrates on the role of AOX in controlling the generation of mitochondrial ROS and RNS, thus influencing the outcome of a pathogen infection. We suggest that the level of AOX activity is pivotal in determining the strength of the cell signaling pathway induced after bacterial infection. In this way, the level of AOX could help control the intensity of the response (weak or strong induction of defense pathways) and the type of the response (survival or PCD) (Figure 1.5). In my work we try to answer several questions in regards to the role of AOX in plant-pathogen interactions: (1) What is the nature of the signal being produced in the mitochondria? (2) Is AOX a positive or a negative regulator of this signaling pathway? (3) What aspects of cellular function are influenced by this signaling pathway? The current research on this topic does not give definite answers, but it does suggest that AOX negatively regulates the generation of a ROS based signal. The ROS scavenging mechanism could be at least one of the cellular functions immediately affected by this pathway. We hope that the work presented here will shed more light on these questions.
Figure 1.5: A working hypothesis for the role of AOX during biotic stress responses in plant cells. It is suggested that the mitochondria can produce an integrated signal (ROS or RNS based) under biotic stress conditions, in order to induce the appropriate cellular response. The level of AOX in the system could modulate the strength of this signal in terms of intensity (weak vs strong response) or type or response (defense responses vs PCD). This model can be tested by using various bacterial strains causing different responses in infected tobacco leaves thus providing a range of experimental conditions.
The work presented here has been performed almost exclusively on *Nicotiana tabacum* L. cv Petit Havana SR1 plants and suspension cells. In order to better observe the role of AOX during biotic stress, we have also used transgenic tobacco plants overexpressing AOX or silenced for AOX expression. We used three *Pseudomonas syringae* pathovars to induce a range of different responses in plants in order to compare the role of AOX under different conditions. In this work we try to further confirm the role of AOX as a ROS avoidance mechanisms and describe the impact of AOX on the levels of ROS and RNS during biotic stress. These results are further related to downstream effects on the cell survival or PCD induction. The research is described in three result-based chapters and is summarized in a model describing the role of AOX under steady state and biotic stress conditions. A brief summary of the findings in each chapter is as follows:

In **Chapter 3**, laser scanning fluorescent confocal microscopy and biochemical methods were used to directly estimate *in planta* leaf levels of $O_2^-$, NO, ONOO$^-$ and $H_2O_2$ in Wt and transgenic tobacco plants with altered levels of AOX. We found that plants lacking AOX had increased levels of leaf mitochondria-localized $O_2^-$ and leaf NO in comparison to Wt, while leaf levels of $H_2O_2$ were similar or lower in the AOX-suppressed plants. Based on the results, it is suggested that AOX respiration acts to reduce the generation of ROS and RNS in plant mitochondria by dampening the leak of single electrons from the electron transport chain to $O_2$ or NO$_2^-$, and a hypothesis is put forth describing the possible mechanism for the generation of these molecules.

In **Chapter 4** multiple distinct responses of *Nicotiana tabacum* to different pathovars of the bacterial pathogen *Pseudomonas syringae* were characterized. These include a compatible response associated with necrotic cell death (pv *tabaci*), an incompatible defense response that include the HR and cell death (pv *maculicola*) and an incompatible response that induce defenses but lacked cell death (pv *phaseolicola*). We showed that the HR (but not the response to pv *phaseolicola* or pv *tabaci*) was accompanied by an early mitochondrial $O_2^-$ burst prior to cell death. We also demonstrated that several signaling molecules known to induce the typically-stress responsive tobacco Aox1a gene (SA, NO, $H_2O_2$) accumulated preferentially during the HR. Despite this, expression of Aox1a was suppressed during this response, while strongly induced
during the defense response that lacked cell death. Also, MnSOD activity declined in response to *pv maculicola*. This was distinct from the response of MnSOD to the other pathovars, as well as being distinct from the response of other SOD isozymes. We proposed that a coordinated response of key ROS-avoiding (AOX) and ROS-scavenging (MnSOD) components in the mitochondria is important in the determination of cell fate during different responses to pathogens.

In **Chapter 5**, we demonstrated that a strong mitochondrial $O_2^-$ burst was seen in transgenic plants unable to induce AOX infected with *pv phaseolicola*. This response is similar to that normally seen in Wt plants infected with *pv maculicola*. This interaction did not however result in HR. These findings indicate that induction of AOX is the key event preventing the mitochondrial $O_2^-$ burst after inoculation of wild-type plants with *pv phaseolicola*, but also that a mitochondrial superoxide burst is, in itself, insufficient to induce the HR. The mitochondrial superoxide burst normally seen in response to *pv maculicola* was delayed in transgenic plants lacking AOX. This delay was associated with a delay in the HR, suggesting that the mitochondrial $O_2^-$ burst does, when in combination with other factors associated with *pv maculicola* inoculation, promote the HR. Interestingly, a mitochondrial $O_2^-$ burst could also be induced in wild-type plants by antimycin A (a Complex III inhibitor), and such a burst was similarly delayed in transgenic plants lacking AOX. The similar mitochondrial response induced by *pv maculicola* and antimycin A suggests that the plant mitochondrial electron transport chain is a target during HR-inducing biotic interactions.
CHAPTER 2

Materials and Methods
Chapter 2
Materials and Methods

2.1 Growth conditions

Tobacco plants (*Nicotiana tabacum* L. cv Petit Havana SR1) were grown in a controlled-environment growth chambers (Model PGR-15, Conviron, Winnipeg, Canada) with a 16 h photoperiod, a temperature of 28ºC/22ºC (light/dark) and a relative humidity of 60%, under a relatively low light irradiance of approximately 130 µmol m$^{-2}$ s$^{-1}$. Plants were grown in general purpose growing medium (Pro-mix BX, Premier Horticulture Ltd., Rivière-du-Loup, Quebec, Canada). Plants were watered daily and fertilized with 10x Hoagland’s solution three times a week. *Arabidopsis thaliana* ecotype Columbia was grown similarly but with a 9 h photoperiod, constant temperature (22ºC), relative humidity of 65%, and photosynthetic photon flux density of 200 µmol m$^{-2}$ s$^{-1}$. Plants were grown in the same medium as above and irrigation alternated between water and general purpose fertilizer (Plant Products, Brampton, Canada) as required. Plants were used 5-6 weeks after initiating germination in vermiculite (tobacco) or soil (*Arabidopsis*).

Three AOX1a transgenic *N. tabacum* lines were used, two of which contained an AOX1a-interfering construct (RI9 and RI29). The third line contained a construct of AOX1a in the sense direction with a constitutive promoter (B7). These AOX-silencing and AOX-overexpressing transgenic lines have been previously characterized and the levels of AOX protein have been determined (Vanlerberghe et al., 1994; Amirsadeghi et al., 2006; Wang et al., 2011). These lines do not show phenotypic differences under steady-state conditions.

Suspension cells derived from Wt tobacco leaves (Vanlerberghe et al., 2004) were grown in axenic batch culture in a previously described medium (Linsmaier and Skoog, 1965), with 88 mM sucrose as a carbon source. The cultures (200 ml) were maintained in the dark on a rotary shaker (140 rpm) at 28ºC and were subcultured every 7 days by dilution in fresh growth medium. Unless stated otherwise, cells were used 2 days after subculture (early exponential growth phase) and each individual treatment was performed on a separately grown 200 ml cell culture.
2.2 Imaging of cellular ROS and RNS

Imaging of $\text{O}_2^{-}$ was performed using the MitoSOX Red mitochondrial $\text{O}_2^{-}$ indicator (M36008; Invitrogen, Carlsbad, CA) that is selectively accumulated by mitochondria, where it is oxidized by $\text{O}_2^{-}$ and becomes fluorescent upon binding nucleic acid (Robinson et al., 2008). Leaf NO was detected using DAF-FM diacetate NO indicator (D23844; Invitrogen). After crossing the plasma membrane, this dye is cleaved by esterases to generate intracellular DAF-FM, which is then oxidized specifically by NO to produce a fluorescent product (Kojima et al., 1999). Leaf peroxynitrite (ONOO$^-$) was detected using APF (A36003; Invitrogen), that becomes fluorescent after reacting with ONOO$^-$ (Setsukinai et al., 2003). In some cases, leaf tissue was also labeled with MitoTracker Red or MitoTracker Green (M7512 and M7514; Invitrogen), that selectively accumulate in mitochondria.

Tobacco leaves were removed and the lower epidermis peeled off. The remaining leaf segments were then floated with the peeled surface exposed to the liquid (30 min, room temperature (RT), dark) on either 3 µM MitoSOX Red (in water), 10 µM DAF-FM diacetate (10 mM KH$_2$PO$_4$, pH 7.4), or 10 µM APF (in 10 mM KH$_2$PO$_4$, pH 7.4) In some cases, leaf segments were double-labeled by also including 0.35 µM MitoTracker Red or MitoTracker Green in the floating solution. Samples were then mounted on microscope slides and the mesophyll cell layer was immediately examined with a LSM510 META laser-scanning confocal microscope (Carl Zeiss, Jena, Germany) with appropriate excitation/detection settings (MitoSOX Red, 488/585-615 nm; MitoTracker Red, 543/585-615 nm; MitoTracker Green 488/500-530 nm; DAF-FM diacetate, 488/500-530 nm; APF, 488/500-530 nm).

All images were acquired under similar acquisition settings. Z-stacks, typically 8-16 µm in depth with 2 µm step size, were combined in a maximum intensity projection image using LSM510 imaging software. In this case the intensity values for each pixel in all individual images are collapsed into a single image, without altering the transparency of the layers. Colocalization (after double-labeling experiments) was analyzed after thresholding for image background noise using JACoP, a plugin for the software Image J (Bolte & Cordelieres, 2006). Two coefficients of corelation were used to quantify the degree of overlap between pixels in two channels. In this case, Pearson’s coefficient of 1 corresponds to complete colocalization of two fluorophores, while a value of 0 indicates random localization. In this analysis, the Manders’ coefficients...
represents the fraction of pixels from the red fluorescence channel that overlap with the green fluorescence channel (M1) and vice versa (M2), and ranges from 0 (random localization) to 1 (full colocalization). All images were analyzed as Z-stacks to ensure that the areas of colocalization existed in all three dimensions.

2.3 SOD activity

All steps were performed at 4°C. Leaf tissue (0.2 g fresh weight (FW)) was flash frozen in liquid N₂ and homogenized in 0.2 ml protein extraction buffer (50 mM KH₂PO₄, 0.2% Triton X-100, 0.05% β-mercaptoethanol, 1% [w/v] PVP-25, pH 7.8). Samples were incubated (10 min, 4°C), centrifuged (13,000 g, 10 min, 4°C), and then aliquots of the supernatant stored at -80°C until use. Protein concentration was determined using the Lowry assay with BSA standards (Lowry et al., 1951).

A non-denaturing running gel (7.5% (w/v) acrylamide, 0.2% (w/v) bis-acrylamide, 0.375 M Tris) was prepared by de-aerating the solution and adding 300 µl of 10% (w/v) ammonium persulfate and 20 µl TEMED. After pouring in a gel apparatus, the running gel was sealed with n-butanol for 1 h to aid in polymerization. A non-denaturing stacking gel (4% (w/v) acrylamide, 0.1% (w/v) bis-acrylamide, 0.125 M Tris) was de-aerated, followed by the addition of 100 µl of 10% (w/v) ammonium persulfate and 10 µl TEMED. Following the removal of n-butanol, the stacking gel was poured on top of the resolving gel and polymerized for 30 min. 100 µg of isolated protein was combined with 3x loading buffer (0.125 M Tris, 30% (v/v) glycerol) and 6 µl of 2% (w/v) bromophenol blue. Samples were loaded into gels, submerged in running buffer (25 mM Tris, 0.192 M glycine). Gel electrophoresis was conducted overnight (15 mA, 4°C).

SOD activity gel staining was performed as previously described (Beauchamp and Fridovich, 1971) with some modifications. Following gel electrophoresis, the non-denaturing gels were pre-equilibrated in a solution containing 0.05 M KH₂PO₄, 1 mM EDTA, pH 7.8, along with the appropriate inhibitors when required (2 mM KCN to inhibit copper zinc SOD [CuZnSOD], 5 mM H₂O₂ to inhibit CuZnSOD and iron SOD [FeSOD]). After 30 min, the pre-equilibration solution was replaced with a fresh solution containing 0.24 mM nitro-blue tetrazolium, 33.2 µM riboflavin and 0.2% TEMED. After incubation (30 min, dark, shaking), the gel was illuminated
with fluorescent light (400 µmol m\(^{-2}\) s\(^{-1}\)) for 5 min, or until the gel became uniformly blue except in positions with SOD activity. The individual SOD activities were then analyzed using ImageJ software (National Institutes of Health, USA).

### 2.4 NO analysis

NO was assayed using the oxyhaemoglobin method as described by Murphy and Noack (1994), with some modifications. Leaf tissue (0.15 g FW) was flash frozen, ground under liquid N\(_2\) and homogenized with 100 mM KH\(_2\)PO\(_4\) buffer (pH 7.0) containing 0.6% (w/v) insoluble polyvinylpyrrolidone (PVP). The sample was clarified by adding powdered activated carbon and centrifuged (11,000 g, 10 min, 4ºC). The clear supernatant was collected and pre-treated with catalase (100 U) and SOD (50 U) for 5 min at RT to remove ROS. Oxyhaemoglobin was prepared fresh on the day of the experiment, by converting 20 mg metHb to Hb using an excess of sodium dithionite (2mM) in 50mM Tris pH 7.0. The solution was oxidized by blowing a stream of O\(_2\) and desalted by passing it through Sephadex G-25 column (GE Healthcare, Mississauga, Ontario Canada). Following elution with 50 mM Tris pH 7.0, the concentration of \(\text{HbO}_2\) was determined by absorbance at 415 nm (extinction coefficient 131 mM\(^{-1}\)cm\(^{-1}\)). \(\text{HbO}_2\) (10 µM) was added directly to the pre-treated samples (see above) and incubated (5 min, RT). The conversion of \(\text{HbO}_2\) to metHb was measured by absorbance at 401 nm and 421 nm. The concentration of NO was calculated as \((A_{401} \text{HbO}_2 - A_{421} \text{metHb})\) using the extinction coefficient of 77 mM\(^{-1}\)cm\(^{-1}\).

### 2.5 \(\text{H}_2\text{O}_2\) determination

\(\text{H}_2\text{O}_2\) was extracted and measured as previously described (Jiang et al., 1990) with some modifications. Leaf tissue (0.2 g FW) was flash frozen, ground under liquid N\(_2\) and homogenized in 1 ml of 0.2 M HClO\(_4\). The homogenate was incubated (5 min, 4ºC), centrifuged (10,000 g, 10 min, 4ºC) and the supernatant then removed and neutralized to pH 7-8 with 0.2 M NH\(_4\)OH, pH 9.5. After centrifugation (3,000 g, 2 min, 4ºC) the supernatant was passed through an AG 1-X8 resin column (Bio-Rad Laboratories, Mississauga, Ontario, Canada). The sample was eluted from the column with ice-cold distilled water and 0.2 ml of the eluate was combined with 0.5 ml
of an assay reagent (0.5 mM (NH₄)₂Fe(SO₄)₂, 50 mM H₂SO₄, 0.2 mM xylenol orange, 200 mM sorbitol). Following incubation (45 min, RT), the absorbance at 560 nm was measured using a spectrophotometer. To evaluate the % recovery of H₂O₂ during extraction and purification, 5 µmol of H₂O₂ was added directly to the tissue prior to extraction. Recovery ranged from 90-95%.

2.6  Bacterial cultures and plant inoculations

*Pseudomonas syringae pv tabaci*, *pv maculicola* ES4326, *pv phaseolicola* NP3121 and *pv tomato* strains DC3000, DC3000 (avrRpt2) and DC3000 (avrRps4) were cultured in King’s B medium (King et al., 1954) either without antibiotics (*pv maculicola*), with 50 µg/ml rifamycin (*pv phaseolicola*) or with 50 µl/ml of each rifamycin and kanamycin (*pv tabaci* and *pv tomato*). Bacterial cultures were grown overnight with rotation (28ºC, ~20 rpm), washed once and resuspended in distilled water (tobacco) or 10 mM MgCl₂ (*Arabidopsis*). The density of the bacterial suspension was determined at 600 nm with a spectrophotometer (HP 8452, Agilent Technologies, Canada). Unless stated otherwise, the density of the bacterial culture was adjusted to 10⁷ cfu/ml for plant inoculations.

Tobacco plants were inoculated 5 h into the light period by completely infiltrating the abaxial side of the second to lowest true leaf with liquid, using a syringe with a needle. Mock-inoculated plants were infiltrated with distilled water. *Arabidopsis* plants were inoculated 1 h into the light period by completely infiltrating the abaxial side of rosette leaves using a syringe without a needle. Mock-inoculated plants were infiltrated with 10 mM MgCl₂.

2.7  Leaf cell death and bacterial proliferation *in planta*

The symptoms of the infection were observed and compared visually, and leaf cell death was quantified using the ion leakage method. For each measurement, 10 leaf discs (d = 1 cm) were floated abaxial side up on 10 ml of distilled water for 4 h in the dark at RT. After incubation, the conductivity of the bathing solution was measured with a conductivity meter (CON510, Oakton Instruments, Vernon Hills, IL, USA).
Bacterial growth was determined by harvesting 3 discs from each infected leaf (d = 1 cm) homogenizing them in 10 mM MgCl$_2$ and plating them as dilution series on King’s B medium agar plates supplemented with the appropriate antibiotics. The plates were incubated at 28°C for 2 days after which the number of colonies was determined.

2.8 SA determination

SA levels were determined by the method described by Mosher et al. (2010). Leaf tissue (0.1 g FW) was flash frozen in liquid N$_2$ and extracted twice with 0.8 ml extraction buffer (acetone:50 mM citric acid [70:30] and 5 µl of internal standard ([$^2$H$_6$]SA; CDN isotopes, Point-Claire, Quebec, Canada)) in 1.5 ml screw-cap tubes containing 1.4 mm ceramic beads (Qbiogene, Carlsbad, CA, USA), using a FastPrep FP120 homogenizer (Qbiogene). After centrifugation (14,000 g, 3 min, RT), the acetone was dried and the remaining supernatant was extracted with 0.75 ml diethyl ether and centrifuged (14,000 g, 1 min, RT), after which the upper organic phase containing free SA was collected. SAG was extracted from the remaining aqueous phase by acidification with 5 µl 1M HCl and hydrolysis at 80°C for 1 h, followed by diethyl ether extraction, as described above. All samples were passed through a Supelclean LC-NH$_2$ solid phase extraction column (Supelco, Bellefonte, PA, USA) equilibrated with ether. The column was washed with 1.2 ml chloroform:N-propanol (2:1) and the samples were eluted with 1.5 ml ether:formic acid (98:2). After drying under a stream of nitrogen, the pellet was resuspended with 0.1 ml dichloromethane:methanol (80:20) and derivatized with 2 µl trimethylsilyldiazomethane (Sigma-Aldrich, Oakville, Canada) for 20 min at RT. The reaction was terminated by adding 2.1 µl acetic acid in hexane (1:7.5) and incubating for 30 min at RT. The resulting methyl esters were analyzed on a gas chromatograph / mass spectrometer (6890N GC connected to a 5975 mass selective detector; Agilent Technologies, Palo Alto, CA, USA) using a DB-5 capillary column (flow rate = 1.2 ml/min, helium carrier gas; Agilent Technologies). The methyl esters were measured using selected-ion monitoring with m/z 153 (SA) and m/z 157 ([$^2$H$_6$] SA).
2.9 RNA extraction and transcript determination

RNA was extracted from leaves and suspension cells using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. In short, leaf tissue (~0.6 g FW) was flash frozen, ground under liquid N\textsubscript{2} to a fine powder and homogenized with TRIZOL (1 ml per 0.15g tissue). The samples were incubated for 5 min at RT, followed by the addition of 1 part chloroform: 5 parts tissue homogenate. Samples were mixed well, incubated for 3 min at RT and centrifuged (12,000 g, 15 min, 4\textdegree C). The upper aqueous phase was transferred to a new tube, mixed with an equal volume of isopropanol and incubated for 10 min at RT. The sample was centrifuged (12,000 g, 15 min, 4\textdegree C) and the supernatant was removed. The remaining RNA pellet was vigorously washed with 75\% ethanol (0.1\% (v/v) DEPC), and vacuum dried for 10 min at RT. The pellets were resuspended in dH\textsubscript{2}O (1\% (v/v) DEPC), followed by incubation at 60\textdegree C for 10 min. The resulting RNA was diluted in distilled water and the absorbance of the sample was used to calculate the concentration. All samples were stored at -80\textdegree C until use.

In all experiments, 15 \mu g of RNA were combined with RNA loading buffer (41.6\% formamide, 8.3\% formaldehyde, 1.3x MOPS, 74 \mu M bromophenol blue) and incubated at 60\textdegree C for 10 min prior to addition of 1 \mu l of 1\% (w/v) ethidium bromide. Samples prepared in this way were loaded on a 1.25\% agarose gel (1x MOPS, 1.8\% (v/v) formaldehyde) and submerged in 1X MOPS running buffer (0.1 M MOPS-NaOH, 5 mM sodium acetate, 1 mM EDTA-N\textsubscript{2}, 0.1\% (v/v) DEPC). Gel electrophoresis was conducted for approximately 2.5 h at 70 V. The resulting RNA gel was incubated in 1x SSC (0.75 M NaCl, 1.5 mM trisodium citrate, 1\% (v/v) DEPC) containing 6.5 mM NaOH for 20 min at RT, and transferred to Hybond-N nylon membranes for 18 h at RT. The membrane was cross-linked under UV light and used immediately or stored at -20\textdegree C.

Partial cDNAs (to be used as hybridization probes in Northern blot analyses) were amplified from tobacco or \textit{Arabidopsis} leaf RNA using an RT-PCR kit (Access RT-PCR, Promega, Madison, WI, USA) and cloned into pGEM-T Easy plasmid (Promega). The cDNAs were excised from the plasmid and gel purified for use as hybridization probes. The primer sequences used for RT-PCR can be found in \textbf{Table 2.1}. In the case of tobacco \textit{Aox1a}, a cloned full-length cDNA was used as a probe (Vanlerberghe and McIntosh 1996).
### Table 2.1. Primer sequences for various *Nicotiana tabacum* and *Arabidopsis thaliana* genes used for RT-PCR intended for the production of hybridization probes in Northern blotting.

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>Gene</th>
<th>Accession Number</th>
<th>Primers</th>
</tr>
</thead>
</table>
| *Nicotiana tabacum* | PAL | D17467 | fwd 5’-GCTGAATCCTTAAGAGGGAGTCATTGG-3’  
                      |      |       | rev 5’-CAAGCCATTGTGGAGATGTTGAG-3’ |
|                 | HSR203J | X77136 | fwd 5’-CCCGTCATTCTCTACTCC-3’  
          |      |       | rev 5’-GATAAAAGCTATGCTTCCACTCC-3’ |
|                 | HIN1 | AF212183 | fwd 5’-CACCAGCCTAAAATCTATCACC-3’  
           |      |       | rev 5’-GATGATGATCTGCTCCATTAGACC-3’ |
|                 | PR-1 | X06361 | fwd 5’-ATTGCTTCTTTAATTCTTGTCTT-3’  
            |      |       | rev 5’-GACCTTGCTCTTATAATTACCTG-3’ |
|                 | GRAS | DQ449940 | fwd 5’-CCATCGACGAAGATGATGACC-3’  
            |      |       | rev 5’-CTGTAATCTTCAAACCCTCCTGAC-3’ |
| *Arabidopsis thaliana* | Aox1a | NM113135 | fwd 5’-GAGTTCTCTTACAAATTTC-3’  
                               |      |       | rev 5’-ATGCTTCTTCAGATCTATCG-3’ |
|                 | Aox1b | D89875 | fwd 5’-CTCACAGCCAATCTTGAATCC-3’  
|                 |      |       | rev 5’-CTAGCATCATTGCTCTGCATCC-3’ |
|                 | Aox1d | NM102968 | fwd 5’-AAACTCGTTGATACACATCACC-3’  
|                 |      |       | rev 5’-CAATCGCTCGTCCATGACC-3’ |
|                 | Aox2 | NM125817 | fwd 5’-TCATTGAAAGCAGCTTTACG-3’  
|                 |      |       | rev 5’-GCATTGTCTCCACCAGATCG-3’ |
RNA membranes were incubated with prehybridization buffer (0.25 M Na$_2$HPO$_4$ pH 7.2, 7% SDS, 0.1% (v/v) DEPC) containing 5 µg/ml denatured salmon sperm DNA at 65°C for 5 h prior to radioactive blotting. The cDNA probes were diluted in TE buffer pH 8.0 (10 mM Tris-HCl, 1 mM EDTA) to a final concentration of 25 ng/ml, denatured and combined with Rediprime II Random Prime Labelling System reaction (GE Healthcare) and 5 µl $^{32}$P (PerkinElmer, Woodbridge, ON, Canada). The probe was incubated at 37°C for 20 min and the unlabelled DNA was removed using a ProbeQuant G-50 Micro Column (GE Healthcare). The radioactively labeled probe prepared in this way was denatured, added to the RNA membranes together with fresh prehybridization buffer, and incubated at 65°C overnight with spinning. Following incubation, the radiolabelled membranes were washed twice with Church Wash I (20 mM Na$_2$HPO$_4$ pH 7.2, 5% SDS, 0.1% (v/v) DEPC) for 30 min at 65°C with spinning. Depending on the radioactivity, the membranes were washed 1-2X with Church Wash I (20 mM Na$_2$HPO$_4$ pH 7.2, 1% SDS, 0.1% (v/v) DEPC). The membranes were stored in cassettes with x-ray film at -80°C until developing. The developed blots were quantified by densitometry using ImageJ (National Institutes of Health, USA).

2.10 Mitochondria extraction and protein determination

Several fully developed leaves (~35 g) were harvested, immediately placed at 4°C and used on the same day. The midvein of each leaf was removed and the remaining tissue was shredded with a hand-blender and ground with acid-washed sand for ~ 10 min in the presence of homogenization buffer (0.3 M sucrose, 25 mM TES, 1 mM EDTA, 10 mM KH$_2$PO$_4$, 1% (w/v) PVP-40, 1% (w/v) BSA, pH 7.5 containing 20 mM ascorbic acid and 4 mM cysteine added just before use). The resulting sample was filtered through 2 layers of Miracloth and centrifuged (3,000 rpm, 5 min, 4°C). The supernatant was transferred to a new tube and centrifuged (12,000 rpm, 20 min, 4°C). The pellet obtained in this way was resuspended in Wash I (0.3 M sucrose, 10 mM TES, 0.1% (w/v) BSA, pH 7.2), and centrifuged (3,000 rpm, 5 min, 4°C). Once again, the supernatant was transferred to a new tube and centrifuged (12,000 rpm, 20 min, 4°C). The resulting pellet was resuspended in Wash I, applied on a Percoll gradient (heavy solution: 0.15 M sucrose, 5 mM TES, 0.05% (w/v) BSA, 28% (v/v) Percoll, 4.4% (v/v) PVP-25; light solution: 0.15 M sucrose, 5 mM TES, 0.05% (w/v) BSA, 28% (v/v) Percoll) and centrifuged (18,000 rpm,
40 min, 4ºC). The supernatant above the mitochondrial fraction was removed with a vacuum and the fraction itself (~7 ml) was removed to a new tube and washed with Wash I. After centrifugation (15,000 rpm, 15 min, 4ºC), the supernatant was removed and the pellet containing mitochondria was washed with Wash I without BSA. Once again, the sample was centrifuged (15,000 rpm, 15 min, 4ºC) and the resulting pellet was resuspended in 300 µl of Wash I without BSA, containing 5% (v/v) DMSO. Protein concentration was determined by the method described by Lowry et al. (1951) with BSA standards and the sample was stored at -80ºC until use.

Resolving gel was poured in a gel apparatus using a gradient maker (heavy solution: 0.4 M Tris, 0.2% (w/v) SDS, 9% (w/v) sucrose, 17.5% acrylamide, 0.05% (w/v) ammonium persulfate, 0.05% (v/v) TEMED; light solution: 0.37 M Tris, 0.2% (w/v) SDS, 0.9% (w/v) sucrose, 10% acrylamide, 0.05% (w/v) ammonium persulfate, 0.05% (v/v) TEMED). Gels were sealed with n-butanol and polymerized for 1 h. Following the removal of n-butanol, a stacking gel (0.125 Tris, 0.2% SDS, 5% acrylamide, 0.06% (w/v) ammonium persulfate, 0.01% (v/v) TEMED) was poured on top and polymerized for 1 h. A total of 100 µg of protein was combined with 3X sample buffer (0.125 M Tris, 6% (w/v) SDS, 6% (v/v) β-mercaptoethanol, 30% (v/v) glycerol), boiled for 2 min and immediately stored on ice. The samples were then combined with 6 µl of 2% (w/v) bromophenol blue, loaded on the gel and submerged in running buffer (25 mM Tris, 0.192 M glycine, 0.1% (w/v) SDS). Gel electrophoresis was conducted for 6 h at 50 mA at RT. Following electrophoresis, proteins were transferred on nitrocellulose membrane for 1 h at 0.75 A at 4ºC. The membranes were washed twice with 1x PBS-Tween (10 mM NaH₂PO₄, 0.15 M NaCl, 0.3% (v/v) Tween-20), dried and stored until use.

Membranes were incubated with blocking buffer for 1.5 h with shaking, followed by incubation with primary antibodies raised against AOX and COXII both prepared as a 1:1000 dilution in PBS/Tween, for 1 h. Membranes were washed with 1x PBS/Tween 6X for 10 min with shaking, followed by the addition of a secondary antibody at 1:25,000 dilution in PBS/Tween (Goat Anti-Mouse IgG Horseradish Peroxidase, Pierce, Rockford, IL, USA). Membranes were once again washed 6X with 1x PBS/Tween for 10 min with shaking. Equal volumes of West Pico Stable Peroxide and West Pico Luminol/Enhancer solution (Thermo Scientific, Rockford, IL, USA) were combined and added to the membrane for 15 min in the dark with shaking. The membranes were dried, placed on an x-ray film and exposed.
2.11 Chemical treatment of suspension cells and culture viability

Chemical treatment of suspension cells involved addition of filter-sterilized 1 M stock solutions of SA and 4-HBA (Sigma-Aldrich, Oakville, Ontario, CA), or 0.2 M BTH (Actigard 50 WG, Syngenta Crop Protection, Guelph, CA) in distilled water directly to the culture. All stock solutions were made fresh on the day of use. Viability of suspension cells was determined by microscopic observation of cells treated with 0.025% Evan’s Blue, which accumulates in dead cells as a blue protoplasmic stain (Baker and Mock, 1994). In each experiment, at least 400 cells were scored to establish % viability.

2.12 DNA extraction

Isolation of genomic DNA from suspension cells was done as previously described (Lin et al., 2001) with some modifications. Tissue (0.2 g FW) was harvested by vacuum filtration, transferred to eppendorf tubes, and flash frozen in liquid N\textsubscript{2}. The tissue was homogenized in 0.7 ml extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 500 mM NaCl, 2% [v/v] SDS, 2% [v/v] β-mercaptoethanol, containing 1% [w/v] PVP-10, pH 8.0 added after autoclaving). The homogenate was incubated (15 min, 65°C) and centrifuged (12,000 g, 10 min, 4°C). The upper aqueous layer was collected, treated with 10 µg/ml DNase-free RNase A (10 min, 37°C), and then an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added. After thorough mixing, the sample was centrifuged (12,000 g, 5 min, 4°C) and the upper aqueous layer collected. The procedure was repeated a second time and the pooled collected layer was mixed with an equal volume of ice-cold isopropanol. The sample was then incubated (20 min, -20°C), centrifuged (12,000 g, 15 min, 4°C) and the pellet of DNA washed twice with 70% ethanol, dried briefly, and suspended in TE buffer pH 8.0 (10 mM Tris-HCl, 1 mM EDTA). The DNA was diluted in distilled water and the absorbance of the sample was used to calculate the concentration. DNA (10 µg) was then separated on a 2% (w/v) agarose gel containing ethidium bromide, visualized on a UV-transilluminator and photographed. A 100-bp DNA ladder (New England Biolabs, Mississauga, Canada) was also separated on the gel.
2.13 Statistical analysis

Where experiments were repeated several times, the mean the standard error (S.E) were calculated. Where appropriate, analysis of variance (ANOVA) followed by a Bonferroni multiple comparison test was used to determine whether differences between means were statistically significant, and the level of significance was indicated by asteriks (*P<0.05; **P<0.01; ***P<0.001). All statistical analysis were done by GraphPad Prism 5.
CHAPTER 3:

Alternative oxidase modulates leaf mitochondrial levels of superoxide and nitric oxide

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Chapter 3
Alternative oxidase modulates leaf mitochondrial levels of superoxide and nitric oxide

3.1 Introduction

The plant mitochondrial electron transport chain is unique due to the presence of two terminal oxidases. In addition to the usual cytochrome (cyt) oxidase (Complex IV), an alternative oxidase directly couples the oxidation of ubiquinol with reduction of oxygen to water. AOX is an interfacial membrane protein oriented toward the matrix side of the inner mitochondrial membrane. AOX dramatically reduces the energy yield of respiration since it is non-proton pumping and bypasses proton-pumping Complexes III and IV (Finnegan et al., 2004). AOX is encoded by a small nuclear gene family (Considine et al., 2002). In both *Arabidopsis* and tobacco (*Nicotiana tabacum*), the expression of a single gene family member (*Aox1a*) is strongly responsive to stress conditions such as cold or drought, while other gene family members display tissue and/or developmental specificity in their expression (Considine et al., 2002; Vanlerberghe et al., 2009).

Since AOX reduces the otherwise tight coupling between carbon metabolism, electron transport and ATP turnover, it could have a general role in the optimization of respiratory metabolism, as well as the integration of this metabolism with other pathways that impact the supply of or demand for carbon skeletons, reducing power and ATP (Finnegan et al., 2004). In addition, Purvis & Shewfelt (1993) suggested that AOX could act to dampen ROS generation by the ETC. The rate of such ROS generation is strongly dependent upon membrane potential and the reduction state of ETC components (Møller, 2001). Increased membrane potential correlates with more highly reduced ETC components and this increases the probability for single electron leak to O$_2$, generating O$_2^-$, which can then act as substrate for the generation of other ROS species such as H$_2$O$_2$ and hydroxyl radical. The magnitude of membrane potential is dependent upon the activity of the energy-dissipating systems, particularly oxidative phosphorylation. Hence, when ADP is being actively phosphorylated, membrane potential and ROS generation are lower than when ADP is limiting. Increased energy dissipation, resulting in reduced ROS generation, can
similarly be achieved by artificial uncouplers or by the action of uncoupling proteins (Møller, 2001). Since electron flow from ubiquinol to O$_2$ via AOX is not coupled to proton translocation (hence dissipating the energy as heat) and since this activity also reduces electron flow via the energy-conserving cyt pathway, AOX represents another potential means to control O$_2^-$ generation by the ETC (Purvis & Shewfelt, 1993).

More recently, AOX has also been implicated to influence the generation of NO, a RNS. A number of studies have shown that NO is produced by animal and plant mitochondria from nitrite and that its synthesis involves the ETC (Modolo et al., 2005; Planchet et al., 2005; Poyton et al., 2009; Gupta et al., 2011). Further, at least three different mechanisms have been suggested for means by which NO level in plant mitochondria could be modulated by AOX.

Here, we used transgenic tobacco with modified levels of AOX to directly test the hypotheses that AOX modulates the generation of ROS and RNS. For the first time, we establish that a lack of AOX increases the steady-state in planta levels of both leaf mitochondrial O$_2^-$ and leaf NO. Our findings also establish the most likely of the suggested mechanisms by which AOX influences NO generation. Our work indicates an important and perhaps universal role of AOX to manage ROS and RNS generation by mitochondria.

3.2 Results:

3.2.1 Various controls ensuring the validity of confocal images

Several controls were observed prior to all experiments, to confirm the validity of the images obtained by confocal microscopy. To ensure no confounding effect due to autofluorescence, images of unlabeled plant tissue were obtained using the acquisition settings and longest exposure time being used for labeled samples. Chlorophyll autofluorescence was detected through a 650 nm long-pass filter but no signal was detected in the 585-615 and 500-530 nm range (Figure 3.1). Furthermore, the fluorescence of cell-free mixture of dye and loading media was examined over an extended period of time (~2 h) without the observance of a rise in fluorescence levels due to dye auto-oxidation (data not shown). Finally, in cases where the tissue was labeled with two dyes simultaneously, single-labeled controls experiments established that there was no significant bleed-through and cross-talk between channels (data not shown).
MitoSOX Red fluorescence was used to directly visualize mitochondrial $\text{O}_2^-$ level in the tobacco leaf. As a positive control specific for this set of experiments, leaf segments with the lower epidermis removed were treated with the Complex III inhibitor antimycin A (10 µM, 1 h, RT) prior to loading with MitoSOX Red and, as expected, this dramatically elevated MitoSOX Red fluorescence. Further, a co-treatment with antimycin A and the cell-permeable $\text{O}_2^-$ scavenger superoxide dismutase-polyethylene glycol (SOD-PEG, 100 U, 1 h, RT) dramatically reduced the subsequent MitoSOX Red signal (Figure 3.2).

To confirm that MitoSOX Red was localizing to the mitochondria, we double-labelled leaf tissue treated with antimycin A (10 µM, 1 h, RT) with this dye and the mitochondria-specific dye MitoTracker Green (Figure 3.3). There was strong colocalization between the two dyes visible as yellow signal. This was further explored with colocalization analysis (Image J, National Institute of Health). We constructed a colocalization map, where the pixels overlapping in the red and green channels are shown in white, showing a significant colocalization between the channels. Colocalization between these two dyes was further analyzed using the Pearson’s coefficient which measures the overlap between red and green pixels in the image and varies between 0 (for random localization) and 1 (total colocalization). The Pearson’s coefficient in this experiment was 0.619, showing a significant overlap between channels. However, this coefficient is highly dependent on image noise and variations in fluorescence intensities between channels. To avoid these effects we also used the two Manders’ coefficients for the proportion of the signal in the red channel (MitoSOX) coincident with the signal in the green channel (MitoTracker Green) – M1, and vice versa – M2, regardless of the differences in the intensities of the two channels (Manders et al., 1992). These coefficient confirmed a strong colocalization between these two dyes (Manders’ coefficient M1 = 0.892; Manders’ coefficient M2 = 0.720). There are reports that MitoSOX Red can cause mitochondrial rupture and signal relocation to the nucleus at higher concentration (Robinson et al., 2008) and we did observe labeling of nuclei. However, we observed no visible difference in the number of mitochondria in antimycin A-treated leaf labeled with either MitoSOX Red or Mitotracker Red (Figure 3.4), suggesting that the mitochondria are not rupturing and failing to emit a fluorescent signal due to the addition of MitoSOX.

DAF-FM fluorescence was used to directly visualize NO level in tobacco leaf. Several controls specific for this experiment were first observed. When leaf samples were treated with the NO
donor sodium nitroprusside (2 mM, 1 h, RT, 130 µmol m\(^{-2}\) s\(^{-1}\)), it dramatically increased subsequent DAF-FM fluorescence, while co-treatment with both sodium nitroprusside and the cell-permeating NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 200 µM) reduced the subsequent DAF-FM signal (Figure 3.5). It should be noted that cPTIO has been shown to produce N\(_2\)O\(_3\) when reacting with NO, potentially increasing DAF-FM fluorescence due to its reaction with this molecule (Arita et al., 2006). Indeed, a slightly higher fluorescence can be observed in plants treated with cPTIO alone as compared with non-treated control samples; however this does not seem to interfere significantly with the observed decrease in fluorescence when both cPTIO and SNP were applied.

ONOO\(^-\) (a product of the reaction of O\(_2^-\) and NO), can be visualized in cells using APF fluorescence. As expected, treating leaf samples (0.5 mM, 1 h, RT) with 3-morpholino sydnonimine (SIN1) that spontaneously releases NO and O\(_2^-\) increased APF fluorescence, while a co-treatment with the ONOO\(^-\) scavenger Ebselen (40 µM, 1 h, RT) reduced the signal (Figure 3.6). However, it should be noted that the concentration of Ebselen used had to be carefully optimized and maintained during experiments. We found that concentrations lower than 30 µM did not scavenge ONOO\(^-\) successfully and higher concentrations of this compound (>50 µM) had the opposite effect and increased the APF fluorescence when used alone or together with SIN1 (results not shown). We currently do not known the cause of this effect, however this showed that the concentration of Ebselen has to be very carefully maintained throughout experiments.
**Figure 3.1:** Images of untreated Wt tobacco mesophyll cells. The images show a lack of fluorescence signal in the 500-530 nm range (used for observing MitoTracker Green, DAF-FM and APF fluorescence) and in the 585-615 nm range (used for observing MitoSOX Red and MitoTracker Red fluorescence). Chlorophyll autofluorescence is seen at wavelengths higher than 650 nm (blue false coloration). All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative of at least four independent experiments, each of which showed similar results. Scale bar = 20 µm.
**Figure 3.2:** Levels of mitochondrial $\text{O}_2^-$ (MitoSOX Red fluorescence) in Wt tobacco mesophyll cells either left untreated (control) or treated with 10 µM antimycin A and/or 100 U SOD-PEG for 1h. All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative of at least three independent experiments, each of which showed similar results. Scale bar = 20 µm.
Figure 3.3: Images of Wt tobacco mesophyll cells treated with 10 µM antimycin A, double labeled with MitoTracker Green (detecting mitochondria) and MitoSOX Red (detecting mitochondrial O$_2^-$). In the merged image, yellow color represents colocalization between the two fluorescent dyes. A colocalization map is also shown, where the pixels overlapping between the green and red channels are shown in white for better clarity. All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative of at least three independent experiments, each of which showed similar results. Scale bar = 20 µm.
Figure 3.4: Images of Wt tobacco mesophyll cells labeled with MitoTracker Red and MitoSOX Red. The right-most image shows MitoSOX Red fluorescence due to an increase in superoxide levels in leaves treated with antimycin A. The image in the middle shows MitoTracker Red fluorescence accumulating in active mitochondria in leaves treated with antimycin A. The left-most image shows MitoTracker Red fluorescence in plants mock-treated with H$_2$O. The number of visible mitochondrial signals is comparable in all cases. All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative of at least three independent experiments, each of which showed similar results. Scale bar = 20 µm.
Figure 3.5: Cellular NO in Wt tobacco mesophyll cells either left untreated (control) or treated with 2 mM SNP and/or 200 µM cPTIO for 1 h. The images in (a) show Mitotracker Red fluorescence (red), localizing in the mitochondria. The images in (b) show DAF-FM fluorescence (green), indicating NO level. The images in (c) are the merged images. All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative of three independent experiments, each of which showed similar results. Scale bar = 20 µm.
Figure 3.6: Cellular ONOO− in Wt tobacco mesophyll cells either left untreated (control) or treated with 0.5 mM SIN1 and/or 40 µM Ebselen for 1 h. The images in (a) show Mitotracker Red fluorescence (red), localizing in the mitochondria. The images in (b) show APF fluorescence (green), indicating ONOO− level. The images in (c) are the merged images. All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative of three independent experiments, each of which showed similar results. Scale bar = 20 µm.
3.2.2 Superoxide levels and SOD activity in transgenic plants with altered AOX levels

MitoSOX Red was used to directly compare the levels of mitochondrial $\mathbf{O}_2^-$ in transgenic plants with very low levels of AOX (RI9), no detectable AOX protein (RI29) and increased levels of AOX (B7) in comparison to Wt plants. We found that under steady-state conditions, both knockdown lines had higher levels of $\mathbf{O}_2^-$ as compared to Wt. The relative fluorescence levels were difficult to accurately compare in B7 and Wt lines, since $\mathbf{O}_2^-$ levels were very low in both cases (Figure 3.7).

Three major SOD enzymes (MnSOD, CuZnSOD, FeSOD) were identified on activity gels according to their sensitivity to different inhibitors. CuZnSOD activity is inhibited by the addition of KCN, while both CuZnSOD and FeSOD are inhibited by the addition of $\mathbf{H}_2\mathbf{O}_2$ (Figure 3.8). Plant cell have only one MnSOD isoform and several FeSOD and CuZnSOD isoforms (Alscher et al., 2002), which is what was detected in this experiment. Since the localization of these enzymes could not be determined (with the exception of MnSOD which localizes to the mitochondria), they were arbitrarily designated based on their relative size (eg. CuZnSOD1 is a larger protein than CuZnSOD2). This method was further used to compare the activity of these enzymes in Wt, R19 and RI29 lines under steady-state growth conditions. In this case the CuZnSOD and FeSOD isoforms with the strongest activity (CuZnSOD1 and FeSOD2) were measured. While MnSOD activity levels were slightly lower (but not significantly different) in knockdown plants, the levels of CuZnSOD and FeSOD were similar or slightly increased, but not significantly different, in these lines as compared to Wt plants (Figure 3.9).
Figure 3.7: Mitochondrial O$_2^-$ in mesophyll cells of Wt and transgenic plants with suppressed levels of AOX (RI9, RI29) or elevated AOX (B7) under steady-state conditions. All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative of four independent experiments, each of which showed similar results. Scale bar = 20 µm.
Figure 3.8: SOD activity gels in tobacco leaf of Wt and transgenic plants with suppressed levels of AOX (RI9, RI29) under steady-state conditions. Activity gels treated with different inhibitors were used to separate the activity of MnSOD, FeSOD and CuZnSOD. 2 mM KCN was used to inhibit CuZnSOD and 5 mM H$_2$O$_2$ was used to inhibit both CuZnSOD and FeSOD. The quality of protein and conformation of equal loading between lanes was routinely checked by Coomassie staining (not shown). The experiment was performed at least 3 times with similar results; a representative gel is shown.
**Figure 3.9:** Densitometry analysis of SOD activity in tobacco leaf of Wt and transgenic plants with suppressed levels of AOX (RI9, RI29). An in-gel activity assay was used to separate the activity of MnSOD, CuZnSOD and FeSOD. Gels that were not treated with SOD inhibitors were used to determine the activity by densitometry. The quality of protein and conformation of equal loading between lanes was routinely checked by Coomassie staining (not shown). Data are the mean ± S.E. of three independent experiments each of which showed similar results. In all cases, the activity level in Wt plants was arbitrarily set to 1. Each set of data were analyzed by one-way ANOVA followed by a Bonferroni multiple comparison test to determine if they are significantly different from Wt data (ie. P < 0.05), no significant difference was found between lines.
3.2.3 Nitric oxide levels in transgenic plants with altered AOX levels

In this set of experiments it was found that both knockdown lines each had consistently higher levels of NO than Wt. In this case, the level of NO was also considerably higher in the stronger knockdown (RI29) than the slightly leaky AOX knockdown (RI9). The AOX overexpressor (B7) had similar or lower NO than Wt although, similar to the $O_2^-$ results, distinguishing between these two lines was difficult due to the relatively low signal in both (Figure 3.10).

As can be seen in these confocal microscopy experiments, signal due to DAF-FM fluorescence is observed throughout the cell. This is not surprising since DAF-FM, unlike MitoSOX Red, does not localize to a specific organelle and can detect NO in various cellular locations. Furthermore, NO can readily traverse membranes and move from its compartment of origin. A certain level of colocalization was observed between NO and mitochondria in these experiments observed as the presence of yellow signal (Figure 3.10c, inset). A colocalization map, where the pixels overlapping the red and the green channels are shown in white clearly illustrated that some of the NO was localized to the mitochondria and that NO levels in the mitochondria were higher in the knockdowns than in the Wt plants (Figure 3.11a). To further confirm these results, colocalization between NO and mitochondrial signals was compared using the Pearson’s coefficient and the Manders' coefficient for the overlap between the signal in the green channel coinciding with signal in the red channel (M2). Both coefficients clearly showed that a greater fraction of the mitochondria in the knockdowns colocalized with NO signal (Figure 3.11b and 3.11c).

To confirm the above results, leaf NO level was measured by an independent, biochemical method. Similar to the microscopy results, NO level in RI29 was much higher than Wt, while mean NO level in RI9 was only slightly higher (and not significantly different) than Wt (Figure 3.12a).
Figure 3.10: Levels of cellular NO in mesophyll cells of Wt and transgenic plants with suppressed levels of AOX (RI9, RI29) or elevated AOX (B7) under steady-state conditions. The images in (a) show Mitotracker Red fluorescence (red), localizing in the mitochondria. The images in (b) show DAF-FM fluorescence (green), indicating NO level. The images in (c) are the merged images, showing that NO localizes to both mitochondria (yellow) and other parts of the cell (green). The images in the bottom right corners in (c) are a close-up of the boxed areas. All images are maximum intensity projections of Z-stacks (8-16 μm in depth) and are representative of three independent experiments, each of which showed similar results. Scale bar = 20 μm.
Figure 3.11: Colocalization analyses of DAF-FM and Mitotracker Red in Wt and transgenic plants with altered AOX levels. The images in (a) show a colocalization map where the pixels overlapping in the red and green channels are shown in white, for better clarity. The Pearson’s coefficient (b) and the Manders’ coefficient (c) indicate that plants with decreased levels of AOX (RI9 and RI29) have a greater fraction of mitochondrial signal overlapping with NO signal. Data are the mean +/- S.E. of three independent experiments. Scale bar = 20 μm.
Figure 3.12: Leaf level of NO (a) and H$_2$O$_2$ (b) in Wt and transgenic plants with suppressed levels of AOX protein (RI9, RI29) under steady-state conditions. Data are the mean +/- S.E. of three independent experiments. Each set of data were analyzed by one-way ANOVA followed by a Bonferroni multiple comparison test to determine if they were significantly different from one another. Bars not sharing a common letter (a or b) are significantly different from one another (ie. P < 0.05).
3.2.4 Hydrogen peroxide levels in transgenic plants with altered AOX levels

A biochemical assay was used to compare \( \text{H}_2\text{O}_2 \) level between Wt and transgenic plants. This analysis showed that the knockdowns tended to have less \( \text{H}_2\text{O}_2 \) (though not statistically different) than Wt (Figure 3.12b).

3.2.5 Peroxynitrite levels in transgenic plants with altered AOX levels

Using APF, we could only detect very low levels of ONOO\(^-\) under the steady-state conditions used in our experiments, with no observable differences between Wt and transgenic lines (Figure 3.13).
Figure 3.13: Levels of cellular ONOO\(^{-}\) in mesophyll cells of Wt and transgenic plants with suppressed levels of AOX (RI9, RI29) or elevated AOX (B7) under steady-state conditions. The images in (a) show Mitotracker Red fluorescence (red), localizing in the mitochondria. The images in (b) show APF fluorescence (green), indicating ONOO\(^{-}\) level. The images in (c) are the merged images. All images are maximum intensity projections of Z-stacks (8-16 \(\mu m\) in depth) and are representative of three independent experiments, each of which showed similar results. Scale bar = 20 \(\mu m\).
3.3 Discussion:

ROS and RNS are important signaling molecules during various plant processes and it is clear that their production must be executed in a tightly regulated fashion in order for the proper cellular program to be induced. The generation of both ROS and RNS has been linked to the mitochondria, and it has been suggested that AOX has an impact on the production of these molecules. To critically evaluate this hypothesis, the levels of $\text{O}_2^-$, $\text{H}_2\text{O}_2$, NO and ONOO$^-$ were studied in tobacco plants with either increased (B7) or decreased (RI9, RI29) levels of AOX under steady-state conditions.

One of the key studies to date showed that suspension cells lacking AOX had increased levels of ROS (observed using the general ROS fluorescent probe DCF-DA) emanating specifically from the mitochondria (Maxwell et al., 1999). Efforts have been made to extend this finding to whole plants, however so far most of the evidence in support of this hypothesis has been indirect. For example, several studies have shown that lack of AOX was found to increase the levels of ROS scavenging systems and ROS-associated damage of macromolecules, which suggests higher ROS levels in modified plants (Amirsadeghi et al., 2006, Watanabe et al., 2008; Giraud et al., 2008, Wang et al., 2011). The work shown here is the first to directly establish a mitochondria-localized increase in $\text{O}_2^-$ due to the lack of AOX.

Mitochondrial $\text{O}_2^-$ is thought to originate from both Complexes I and III in the ETC. It is thought, at least in animal mitochondria, that Complex I releases all of the $\text{O}_2^-$ in the matrix (Murphy, 2009) while Complex III can generate $\text{O}_2^-$ on both sides of the mitochondrial inner membrane (Muller et al., 2004). It has also been suggested that under physiological conditions, the production of $\text{O}_2^-$ from Complex III is negligible compared to the rates of $\text{O}_2^-$ production from Complex I (Murphy, 2009). The research on the exact localization of $\text{O}_2^-$ production in the mitochondria is very limited in animal systems, and especially so in plant systems which contain additional ETC components. One possible additional source of $\text{O}_2^-$ in the matrix and intermembrane space of plant mitochondria, are the plant-specific external NAD(P)H dehydrogenases (de Oliveira et al., 2008). Having this in mind, it should be noted that in the work presented in this chapter $\text{O}_2^-$ was visualized specifically in the mitochondrial matrix using the fluorescent dye MitoSOX, and it is possible that the concentration of this molecule is higher in the intermembrane space as well.
Furthermore, it should be kept in mind that AOX might also have a role in indirectly controlling ROS production from the chloroplast by means of transport and dissipation of excess reducing power (Zhang et al., 2011; Pastore et al., 2007; Bartoli et al., 2005). Increase in whole leaf O$_2^-$ (visualized using the nitroblue tetrazolium staining method) under stress occurs in plants lacking AOX, and it was suggested that this O$_2^-$ is of chloroplastic origin due to the strong impact that AOX was having on photosynthesis (Giraud et al., 2008). There is a possibility that the total cellular levels of O$_2^-$ are higher even under steady-state conditions than what is observed in the results shown here, due to the inability of MitoSOX to detect O$_2^-$ outside of the mitochondrial matrix.

As previously mentioned, increases in antioxidant systems have been observed in plants lacking AOX under stress conditions (Amirsadeghi et al., 2006; Watanabe et al., 2008). It is interesting that in the work presented here the activity levels of CuZnSOD and FeSOD were found to be slightly elevated in transgenic plants lacking AOX, but this was not the case with the mitochondria-specific MnSOD and the activity of this enzyme was slightly lower in the transgenic plants. It has been proposed the CuZnSOD accounts for most of the O$_2^-$ scavenging activity in the cell (Kliebenstein et al., 1999; Jabs et al., 1996; Yahraus et al., 1995), possibly controlling the overall ROS levels in the cell, however in our work we observe only minor differences in the activities of the different SOD enzymes in Wt and transgenic plants lacking AOX under steady-state conditions. How much do these small differences contribute to the overall ROS balance in the cell is not clear at this point.

The mitochondria have been established as sources for RNS as well; however the details of synthesis of these molecules remain elusive (Gupta et al., 2010). Several lines of evidence implicate the mETC as a source of electrons for the reduction of nitrite to NO and AOX might control this reaction in three distinct ways. The work presented here aims to distinguish between these proposed mechanisms. The first hypothesis states that AOX may be directly involved in the catalysis of nitrite to NO, based on studies that used AOX inhibitors such as SHAM and n-propyl gallate to inhibit the nitrite-dependent NO production in *Chlorella* (Tischner et al., 2004) and higher plants (Gupta et al., 2005; Planchet et al., 2005). In this situation we would expect to observe lower levels of NO in transgenic plants lacking AOX (RI9 and RI29), which was not the case in the experiments presented here.
The second hypothesis states that AOX is indirectly responsible for the levels of NO in the cell, by influencing the scavenging of this molecule. A proposed mechanism for NO scavenging in the mitochondria is its non-enzymatic reaction with $O_2^-$ originating from the ETC to form ONOO$. It has been proposed that this mechanism contributes to reactivation of the NO-sensitive COX and the maintenance of electron flow (Wulff et al., 2009). However, under the assumption that this hypothesis is correct under steady-state conditions, we would expect to observe less NO in the knockdown plants, since more $O_2^-$ is available to scavenge NO. Once again, the opposite was observed in this work.

Finally, NO could be produced by the ETC in a similar way to $O_2^-$ production, namely an electron leak to nitrite (Kozlov et al., 1999; Igamberdiev et al., 2010). If this is the case, AOX could indirectly control the generation of NO by preventing over-reduction of the ETC and moderating membrane potential. Furthermore, electron flow through the alternative pathway would bypass Complex III, cyt c and COX, all three which have been proposed to contribute to NO generation in the mitochondria (Igamberdiev et al., 2010). In this scenario, the lack of AOX would lead to higher levels of NO present in RI9 and RI29 due to a higher reduction state of the ETC. The results presented here lend support to this hypothesis since NO levels were shown to be higher in transgenic plant lines lacking AOX by two independent methods.

There are several considerations that should be kept in mind when critically accessing these experiments. First of all, DAF-FM is not a compartment-specific probe and it can interact with NO throughout the cell. Furthermore, NO is readily membrane-permeable and thus able to diffuse away from the site of synthesis. These facts, taken together with the fact that multiple sites and mechanisms of production of NO have been proposed (Gupta et al., 2010), increase the uncertainty with which the exact site of NO production can be determined in planta. Nonetheless, a significant amount of NO signal in the images presented above (Figure 3.11) overlaps with mitochondrial signal, and AOX knockdown lines show a higher degree of colocalization between these two fluorescent signals as compared to Wt or AOX overexpressor plants. These results infer a higher level of NO in the absence of AOX under steady-state conditions, however the possibility that AOX can indirectly influence the generation of NO from sites other than the mitochondrial ETC can not be excluded.
It should also be stressed that the experiments performed here were done under normoxic conditions in leaves, where the plants were not experiencing any applied stress. In contrast with this, several studies have suggested that AOX might have a role in the production of mitochondrial NO primarily under anoxia or hypoxia, conditions possibly localized to the roots of higher plants (Igamberdiev et al., 2010; Gupta et al., 2005). This raises the intriguing possibility of a dual role of AOX to either generate or dampen NO production depending on oxygen tension. Furthermore, while an AOX-controlled mechanism of mitochondrial NO generation under steady-state conditions has been proposed here, this does not exclude the possibility that several different pathways might be operational (separately or concurrently) under stress conditions. For example, arginine-dependent NO production by a NOS-like enzyme has been proposed in response to abiotic stress (Besson-Bard et al., 2009; De Michele et al., 2009). Several pathways of NO production have been suggested in plants under biotic stress, including an arginine-dependent oxidation pathway (Asai and Yoshioka, 2009; Besson-Bard et al., 2008) as well as ETC-dependent nitrite reduction pathway (Modolo et al., 2006).

H$_2$O$_2$ is a secondary ROS, produced enzymatically by the dismutation of O$_2^-$ by SOD (Møller, 2001). Given the higher level of O$_2^-$ in AOX knockdown plants, it could be expected that these plants would have higher levels of H$_2$O$_2$, however, the opposite was shown here. The RI9 and RI29 lines tended to have less H$_2$O$_2$ compared to Wt plants, as measured by a biochemical assay. This finding is consistent with what has been previously shown in both tobacco and Arabidopsis (Amirsadeghi et al., 2006; Watanabe et al., 2008) and it is attributed to an induction of H$_2$O$_2$ scavenging systems due to the lack of AOX in transgenic plants. This appears to be an overcompensation of these scavenging mechanisms leading to lower H$_2$O$_2$ in AOX knockdowns as compared to Wt plants during steady-state conditions. We attempted use of the fluorescent probe DCF-DA (often used as an indicator of H$_2$O$_2$ level) to confirm the results of the biochemical assay of H$_2$O$_2$. Two problems with this approach became clear. First, DCF-DA has poor specificity for H$_2$O$_2$ and other oxygen species (including NO, O$_2^-$ and ONOO$^-$) are actually able to oxidize the probe much faster than H$_2$O$_2$ (Hempel et al., 1999). Second, even at concentrations as low as 2 µM, we surprisingly found that the probe’s fluorescence emission was strong across a wide range of the visible spectrum (~500-650 nm), overlapping strongly with the emission signal of Mitotracker Red. This fluorescent dye was not used further. Previous studies have also attempted the use of this dye to show an increase in ROS specifically from the
mitochondria in AOX knockdown cell lines (Maxwell et al., 1999), however in light of the experiments described here, caution must be exercised in interpreting results involving this probe.

ONOO$^-$ is the product of the diffusion-limited reaction between NO and $\text O_2^-$, and it is thought to be produced mainly in locations of $\text O_2^-$ generation, since this molecule does not readily diffuse across membranes (Botti et al., 2010, Quijano et al., 2005). The production of ONOO$^-$ is limited only by the availability of the precursors, which would suggest that transgenic plants that lack AOX would accumulate higher levels of this RNS in the mitochondria as compared to Wt plants. However, it was found that despite higher levels of $\text O_2^-$ and NO in the AOX knockdown plants, ONOO$^-$ remained low in all lines (near the limit of detection). It has been proposed that this molecule has a function during non-stress conditions in animals (Pacher et al., 2007), however is whether this is also true in plants is currently unknown. The results presented here would suggest that much higher levels of NO and $\text O_2^-$, as produced during biotic stress, are required to produce significant levels of ONOO$^-$ that can be detected by confocal microscopy.

In conclusion, the fact that AOX can impact the levels of ROS and RNS in the mitochondria has broad implications to plant stress biology and signal transduction, given the range of different plant processes that involve these molecules. For example, during stress both ROS and RNS can act as signaling molecules when produced in a tightly controlled manner, or as cell damaging factors when produced in an uncontrolled fashion (Apel and Hirt, 2004; Foyer and Noctor, 2005; Beaudouin, 2011; Moreau et al., 2010; Mittler et al., 2011). Since the mitochondria are involved in the production of these ROS, they are being recognized as important organelles during stress signaling. It has been suggested that mitochondrial oxidative burst is an important player during the induction and execution of PCD (Yao and Greenberg, 2006; Amirsadeghi et al., 2007). Mitochondria-dependent NO production has been linked to the appearance of the HR due to bacterial infection (Modolo et al., 2005). Furthermore, glycine decarboxylase, a key mitochondrial matrix-localized enzyme involved in photorespiration, is one of the few enzymes whose activity has been shown to be modulated by S-nitrosylation (Palmieri et al., 2010). If the mitochondria are a source of NO, this would put NO in direct proximity to glycine decarboxylase, thus controlling its activity. The implications of the work presented here open a rich area for future studies on the effect of AOX and the mitochondria on various aspects of plant signaling and metabolism.
CHAPTER 4

Coordination of a mitochondrial superoxide burst during the hypersensitive response to bacterial pathogen in *Nicotiana tabacum*

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4.1 Introduction

Infection of a plant with a pathogen can have two possible outcomes – disease (compatible interaction) or resistance (incompatible interaction). In a typical compatible interaction, the pathogen is thought to act by suppressing preformed and induced plant defenses, thus successfully colonizing the host and causing disease (Nomura et al., 2005). During incompatible interactions, specific recognition by the host plant can trigger the appearance of the HR, a localized PCD appearing at the site of pathogen entry that is thought to delay pathogen spread (Hofius et al., 2007; Coll et al., 2011). It has been suggested that even though this host-controlled process is non-essential for the development of disease resistance, it is required for the rapid and strong activation of host defense responses (Heath et al., 2000). A second type of incompatible interaction occurs if the pathogen is poorly adapted to the host physiology, fails to overcome even basal defense barriers and does not proliferate. Consequently, such an interaction does not induce the HR. However, despite the lack of visible symptoms there is ample evidence that during such interactions, defense responses are activated at the molecular and genetic level (Mysore and Ryu, 2004).

Recent research has established the mitochondrion as an important player in plant PCD, including the HR, however the details on the specific role of this organelle are not well understood (Lam et al., 2001; Amirsadeghi et al., 2007; Noctor et al., 2007; Kusano et al., 2009; van Doorn et al., 2011). Several events happen during this process, including changes in the mitochondrial membrane potential, the mitochondrial permeability transition, cytochrome c release and changes in mitochondrial morphology, however the exact role of these events in the appearance of PCD is not clear. Mitochondria have also been shown to be an early source of intracellular ROS during the so-called mitochondrial oxidative burst. Various ROS have been shown to be involved in the induction of PCD, and this places mitochondria at a central position...
for the coordination and execution of various PCD pathways (Naton et al., 1996; Arpagaus et al., 2002; Tiwari et al., 2002; Yao et al., 2002; Weir et al., 2003; Takahashi et al., 2004; Yao et al., 2004; Yao and Greenberg, 2006; Ashtamker et al., 2007; Scott and Logan, 2008).

AOX is a second terminal respiratory oxidase in the plant mitochondrial electron transport chain, which branches from the main respiratory chain at the level of the ubiquione pool and catalyzes the reduction of O$_2$ to H$_2$O. Unlike COX, AOX is non-proton pumping and bypasses proton-pumping Complexes III and IV, thus reducing the energy yield of respiration (Finnegan et al., 2004). Several hypotheses have been suggested to explain the role of such a non-energy conserving pathway (Vanlerberghe et al., 2009). It was proposed that the AOX pathway provides flexibility of the mitochondrial respiratory metabolism and broadens the conditions under which respiration can be effectively maintained, especially when the functioning of the cyt pathways has been compromised. AOX might also act to dampen the production of mitochondrial ROS, since it bypasses Complex III (an important source of O$_2^-$) and since its energy-dissipating nature will act to moderate membrane potential. This will lower the overall reduction state of ETC components, reducing the electron leak that produces O$_2^-$ (Møller, 2001).

Strong links exist between the mitochondria and several important signaling molecules that initiate and coordinate the HR and defense responses. SA, RNS such as NO and ONOO$^-$ as well as ROS (H$_2$O$_2$ and O$_2^-$) have all been shown to play a role during incompatible pathogen interactions (Leitner et al., 2009; Vlot et al., 2009; Torres, 2010; Spoel and Loake, 2011; Vandelle and Delledonne, 2011). SA has been shown to dramatically induce the expression of AOX at the gene and protein levels (Rhoads and McIntosh, 1992; Van Der Straeten et al., 1995; Djajanegara et al., 2002; Ho et al., 2008; Lei et al., 2010) possibly through its disruptive effect on the ETC function (Xie and Chen, 1999; Norman et al., 2004; de Souza et al., 2011). O$_2^-$ is a well-established by-product of ETC activity and, once produced, can be scavenged and converted to H$_2$O$_2$ by a mitochondria-specific and matrix-localized MnSOD (Møller, 2001; Mittler et al., 2004). Exogenous H$_2$O$_2$ can induce AOX gene expression (Vanlerberghe and McIntosh, 1996) and H$_2$O$_2$ responsive elements are present in the stress-responsive Aox1a gene of Arabidopsis (Ho et al., 2008). NO production has also been linked to the mitochondria (Gupta et al., 2010; Igamberdiev et al., 2010) and significantly, this molecule has been shown to be a competitive inhibitor of COX but not AOX (Millar and Day, 1996). Furthermore, application of NO leads to increases in AOX gene expression (Huang et al., 2002; Zottini et al., 2002; Ederli et al., 2006),
however whether this induction is the effect of the action of NO as a respiratory inhibitor or as a signaling molecule (independent of COX inhibition) is still not clear. Finally, ONOO$^-$ is the product of the reaction between NO and $O_2^-$ and it is thought to act as a mediator of NO signaling during plant-pathogen interactions through protein tyrosine nitration (Romero-Puertas et al., 2007; Cecconi et al., 2009). Interestingly, AOX was found to be a target of tyrosine nitration in sunflower hypocotyls (Chaki et al., 2009) however the significance of this finding during defense responses is not clear.

Based on this evidence, we proposed that AOX might be an important determinant of cell fate during responses to pathogens. The work presented here aims to test this hypothesis by characterizing three distinct responses of *Nicotiana tabacum* to different pathovars of the bacterial pathogen *Pseudomonas syringae*: one a compatible response associated, at a late stage, with disease-causing cell death (*pv tabaci*), one an incompatible response that includes the HR (*pv maculicola*), and finally an incompatible response without cell death (*pv phaseolicola*). We show that the HR response is accompanied by an early mitochondrial $O_2^-$ burst prior to cell death. We also show that the expression of the typically stress-responsive tobacco AOX gene (*Aox1a*) is suppressed during the HR response and that this is accompanied by a specific loss of MnSOD activity. Based on our results, we propose that a coordinated response of ROS-avoiding (AOX) and ROS-scavenging (MnSOD) components in the mitochondria, is important in the determination of cell fate during different responses to pathogens.
4.2 Results

4.2.1 Compatible and incompatible responses of tobacco leaf to *P. syringae* infection

To study the role of the mitochondria in responses to biotic stress, tobacco leaf was inoculated with three different *P. syringae* pathovars. An initial characterization established that the pathovars resulted in three distinct sets of symptoms. *P. syringae pv tabaci* is naturally occurring virulent pathogen of tobacco that proliferated rapidly over the first two days (Figure 4.1A) accompanied by a significant loss of membrane integrity (a measure of cell death) by Day 2 (Figure 4.1B). Visual observations indicated the appearance of water-soaked tissue and necrosis-like cell death lesions by Day 2, both classic symptoms of the wildfire disease (Figure 4.2). The *pv maculicola* also proliferated rapidly, however bacterial numbers reached a maximum by the first day (Figure 4.1A) and this was accompanied by an appearance of cell death as early as Day 1 (Figure 4.1B). In this case, the leaf displayed dry lesions (the typical appearance of HR lesions) beginning at Day 1 and the tissue was not water-soaked (Figure 4.2). In the case of *pv phaseolicola*, it did not proliferate after inoculation into the leaf and there was no loss of membrane integrity or visual symptoms of cell death or disease (Figures 4.1, 4.2). The results suggest that the interaction of tobacco with *pv tabaci* is a compatible (disease-causing) interaction, that *pv maculicola* induces a resistance response (incompatible interaction) that includes the HR, and that *pv phaseolicola* also represents an incompatible interaction but one not inducing cell death.
Figure 4.1. Bacterial proliferation in tobacco leaf (A) and conductivity (ion leakage) of tobacco leaf (B) at different times post-inoculation with different pathovars of *P. syringae* (closed circle, *tabaci*; closed square, *maculicola*; closed triangle, *phaseolicola*). The bacterial density was adjusted to $1 \times 10^5$ cfu/ml prior to inoculation when determining bacterial proliferation. Data are the mean +/- S.E. of at least three independent experiments, each of which showed similar results. In some cases error bars are smaller than the data symbols.
Figure 4.2. Visual appearance of tobacco leaf at 2 days post-inoculation with different pathovars of *P. syringae* (pv *tabaci*, pv *maculicola* or pv *phaseolicola*). See text for details.
4.2.2 Dynamic changes in SA levels in response to *P. syringae* infection

To further characterize the plant responses to *P. syringae*, the levels of SA (a well-known biotic stress signaling molecule) and salicylic acid β-glucoside (SAG; SA storage form) were quantitatively measured. In the case of infection with HR-inducing *pv maculicola*, levels of leaf SA increased rapidly between 3 and 6 h post-inoculation (Figure 4.3A). Interestingly, the SA accumulation seen with *pv maculicola* was clearly biphasic (very high at 6 h, back to control levels at 12 h, and higher again by 24 h. The compatible interaction (*pv tabaci*) had no effect on SA level at 6 h, although low accumulation of SA was seen by 24 h. In the case of infection with the incompatible (but not HR-inducing) *pv phaseolicola*, some accumulation of SA occurred by 6 h, but to only 36% of the level seen with *pv maculicola*. No changes were seen in the level of SAG at 6 h post-inoculation with any of the pathovars, but the level of this biological inactive form did accumulate to high levels by 24 h in response to both of the incompatible interactions (Figure 4.3B).
Figure 4.3. Levels of SA (A) and SAG (B) in tobacco leaf at different times post-inoculation with H₂O (open circles) or different pathovars of *P. syringae* (closed circle, *tabaci*; closed square, *maculicola*; closed triangle, *phaseolicola*). For each treatment, data are the mean +/- S.E. from three separate plants, each of which showed similar results. In some cases error bars are smaller than the data symbol.
4.2.3 Dynamic changes in ROS levels in response to different *P. syringae* pathovars

Mitochondrial O$_2^-$ levels in tobacco leaf mesophyll cells was assessed by using confocal microscopy to image MitoSOX Red fluorescence (Figure 4.4). In leaves inoculated with the HR-inducing *pv maculicola*, high levels of O$_2^-$ were clearly evident by 4 h post-inoculation (the earliest time point examined) and persisted through 24 h. In contrast, there was little change in O$_2^-$ level (compared to mock-inoculated leaves) at any time point following inoculation with *pv phaseolicola*. Finally, *pv tabaci* induced only a late (24 h) increase in O$_2^-$ but not the early mitochondrial O$_2^-$ burst seen in response to *pv maculicola*.

Just as the two incompatible pathovars had different impacts on mitochondrial O$_2^-$ they also had distinct impacts on total leaf H$_2$O$_2$ levels, as measured by a biochemical assay. Inoculation with the HR-inducing *pv maculicola* resulted in a general increase in H$_2$O$_2$, with a first and transient peak at 2 h post-inoculation and a second and sustained peak at 8 h (Figure 4.5A). This pattern is reminiscent of the classical two-phase oxidative burst previously reported (Huang et al., 2003); however variability of the data in these experiments only allows us to conclude that leaf H$_2$O$_2$ levels were high. In the case of *pv phaseolicola*, there was no evidence of the oxidative burst of H$_2$O$_2$ but rather a general decline in H$_2$O$_2$ level after inoculation.
Figure 4.4: Laser-scanning confocal microscope images of mitochondrial $O_2^-$ in mesophyll cells, observed at different times post-inoculation with $H_2O$ or with different pathovars of *P. syringae*: pv *phaseolicola*, pv *maculicola*, pv *tabaci*. All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative results of four independent experiments, each of which showed similar results. Scale bar = 20 µm.
Figure 4.5. Levels of H$_2$O$_2$ (A) and NO (B) in tobacco leaf at different times post-inoculation with different pathovars of P. syringae (closed square, maculicola; closed triangle, phaseolicola). Data are the mean +/- S.E. of four independent experiments, each of which showed similar results.
4.2.4 Dynamic changes in RNS levels in response to different *P. syringae* pathovars

Leaf NO levels were measured with two separate methods, to ensure specificity and reproducibility. When measured with a biochemical assay, inoculation with the HR-inducing *pv maculicola* resulted in a rapid 10-fold accumulation of NO over the first 6 h and further accumulation through 24 h (Figure 4.5B). During inoculation with *pv phaseolicola*, there was no rapid accumulation of NO, but rather just a steady and modest accumulation over the full 24 h period. Attempts were made to use this assay to measure NO levels during infection with *pv tabaci*, however the data proved to be very variable (data not shown).

To confirm these results, confocal microscopy was performed on leaves inoculated with three *P. syringae* pathovars (Figure 4.6). DAF-FM is a cell-permeant dye that is oxidized specifically by NO inside the cell to form a fluorescent product. A similar pattern as described above was observed in leaves infected with *pv maculicola*, where an early and rapid increase in fluorescent signal due to NO was visible by 4 h, and high levels of this molecule persisted through 24 h. Once again, inoculation with *pv phaseolicola* did not cause NO levels to increase significantly, and only a modest increase in fluorescence signal was observed by 24 h. Finally, *pv tabaci* did not induce an early NO burst similar to *pv maculicola*, however a significant increase in fluorescence was observed by 24 h.

When analyzing these results, it has to be kept in mind that DAF-FM does not localize to a specific organelle and can detect NO in various cellular locations. Furthermore, NO can readily traverse membranes and move from its compartment of origin thus contributing to fluorescent signal throughout the cell. A colocalization analysis was performed on the images obtained at 4 h and 24 h post-inoculation in order to explore the possible origin of NO production. A colocalization map of images at 24 h post-inoculation, where the pixels overlapping in the red and green channels are shown in white, clearly illustrates that some of the NO was localized to the mitochondria during infection with both *pv maculicola* and *pv tabaci* (Figure 4.7A). This was further analyzed using the Manders’ coefficient at 4 h and 24 h, for the proportion of the signal in the green channel (NO) coincident with the signal in the red channel (mitochondria) (M1; Figure 4.7B) and vice versa (M2; Figure 4.7C) regardless of the differences in the intensities of the two channels (Manders et al., 1992). It is interesting to note that in the case of *pv maculicola*, there is a high level of NO observed and a high percentage of mitochondria
(~60% at 4 h and ~80% at 24 h) colocalize with the DAF-FM signal (M2); however it is also evident that only ~30-50% of the total NO localizes to the mitochondria (M1) suggesting alternate sources of NO during these interactions. The pattern was opposite in the case of pv phaseolicola. In this case, there was very little NO observed, however most of it seemed to localize to the mitochondria. Finally, during infection with pv tabaci there is ~50% of the NO signal was colocalizing with the mitochondrial signal (M1), however the percentage of mitochondria colocalizing with NO increased from ~20% at 4 h, to ~70% at 24 h. This pattern suggests that early in the infection there are several sources of NO, at later points the mitochondria become the major source of this molecule.

ONOO\(^{-}\) is the product of the reaction between O\(_2\)\(^{-}\) and NO, and can be visualized using the fluorescent dye APF. Just as the three pathovars had different impacts on leaf NO, they also had distinct impacts on leaf ONOO\(^{-}\) levels. Infection with the HR-inducing pv maculicola and the disease-inducing pv tabaci led to early increases in fluorescence due to ONOO\(^{-}\) by 4 h, and high levels of this molecule persisted through 24 h. In contrast, the non-host pv phaseolicola did not cause an early rise in the levels of ONOO\(^{-}\), however higher levels of fluorescent signal were observed by 24 h (Figure 4.8).

A colocalization analysis was performed in this case as well, in order to gain insight of the origins of ONOO\(^{-}\) production, since the fluorescent dye APF does not localize to specific organelles. A colocalization map clearly showed that there was a certain level of localization between ONOO\(^{-}\) and mitochondria 24 h post-infection (Figure 4.9A), and this was further confirmed using Manders’ coefficients M1 and M2 (Figure 4.9B, 4.9C). The level of colocalization appears to be similar between the three pathovars at both 4 h and 24 h post-inoculation, however it is clearly evident that not all of the cellular ONOO\(^{-}\) localizes to the mitochondria.
Figure 4.6: Laser-scanning confocal microscope images of cellular NO in mesophyll cells, observed at different times post-inoculation with H₂O (A) or with different pathovars of *P. syringae*: pv *phaseolicola* (B), pv *maculicola* (C), pv *tabaci* (D). The images shown depict DAF-FM fluorescence (green), Mitotracker Red fluorescence (red) and merged images where the colocalization between DAF-FM and Mitotracker Red can be observed as yellow signal. All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative results of four independent experiments, each of which showed similar results. Scale bar = 20 µm.
**Figure 4.7:** Colocalization analyses of DAF-FM and Mitotracker Red in plants mock-inoculated with dH2O or infected with the three *P. syringae* pathovars. The images in (A) show a colocalization map of images obtained 24 h post-inoculation where the pixels overlapping in the red and green channels are shown in white, for better clarity. The Manders’ coefficients M1 (B) and M2 (C) measure the relative amount of DAF-FM signal overlapping Mitotracker signal (M1) and vice versa (M2) at 4 h and 24 h post-inoculation. Data are the mean +/- S.E. of three independent experiments.
Figure 4.8: Laser-scanning confocal microscope images of cellular ONOO⁻ in mesophyll cells, observed at different times post-inoculation with H₂O (A) or with different pathovars of *P. syringae*: pv *phaseolicola* (B), pv *maculicola* (C), pv *tabaci* (D). The images shown depict APF fluorescence (green), Mitotracker Red fluorescence (red) and merged images where the colocalization between DAF-FM and Mitotracker Red can be observed as yellow signal. All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative results of four independent experiments, each of which showed similar results. Scale bar = 20 µm.
Figure 4.9: Colocalization analyses of APF and Mitotracker Red in plants mock-inoculated with dH₂O or infected with the three P. syringae pathovars. The images in (A) show a colocalization map of images obtained 24h post-inoculation where the pixels overlapping in the red and green channels are shown in white, for better clarity. The Manders’ coefficients M1 (B) and M2 (C) measure the relative amount of DAF-FM signal overlapping Mitotracker signal (M1) and vice versa (M2) at 4 h and 24 h post-inoculation. Data are the mean +/- S.E. of three independent experiments.
4.2.5 Leaf Aox1a transcript levels are dynamic and experimental conditions have to be clearly defined

AOX is one of the most stress-responsive genes in the plant genome, with transcript levels changing in a response to a multitude of developmental, physiological and environmental conditions or perturbations (Vanlerberghe et al., 2009). To critically examine AOX gene expression in response to inoculation with different bacterial pathovars, it was therefore important to carefully characterize the system of study. In this regard, we found that the transcript level of tobacco Aox1a was extremely dynamic over a diurnal cycle, with transcript levels clearly rising during the early part of the light period and then falling during the later part of the light period and through the dark period (Figure 4.10A, 4.10B). In contrast, transcript encoding PAL (phenylalanine ammonia-lyase), a gene with a known diurnal rhythm of expression, peaked during the dark period and decreased during the light period (Figure 4.10C, 4.10D). Since leaves mock-inoculated with water were used as controls, it was also critical to determine that water injection alone was not having a significant effect. To confirm this, Aox1a and PAL transcript levels were compared in leaves either injected with water or left untreated. Based on these experiments it was determined that the water injection itself was having little if any impact (Figure 4.10). Based on these results, it was determined that it was critical to always perform inoculations at a defined time (5 h into the light period) and to normalize all transcript data against control samples (leaves mock-inoculated with dH₂O) taken in parallel with all treatment samples.
Figure 4.10: Diurnal changes in the level of *Aox1a* (A, B) and *PAL* (C, D) transcript in tobacco leaf either left untreated (open squares) or after injection with H$_2$O (open circles) at time 5 h (as denoted by the arrow in [B] and [D]). Note that 0-16 h represents the light period while 16-24 h is the dark period. (A) and (C) show representative Northern blots. The data in (B) and (D) are the mean +/- S.E. of densitometer analysis of Northern blots from two independent experiments, each of which showed similar results. For these data, the transcript level in the untreated sample at 6 h has been arbitrarily set to 1. Quality of RNA and confirmation of equal loading between lanes was routinely checked by ethidium bromide staining (representative blot shown).
4.2.6 Distinct changes in Aox1a transcript level and AOX protein level occur in response to different P. syringae pathovars

The level of Aox1a transcript was compared up to 48 h after inoculation with different pathovars (Figure 4.11A, 4.11B). These results were also compared with changes in AOX protein level in isolated tobacco leaf mitochondria (Figure 4.11C). Three distinct patterns emerged. In the case of pv phaseolicola, there was a strong and transient increase in Aox1a transcript at 12 h post-inoculation and this was accompanied by a dramatic increase in AOX protein, as measured at 24 h. In contrast, in the case of the HR-inducing pv maculicola, there was little if any change in Aox1a mRNA or protein in comparison to water-injected controls. Innoculation with the virulent pv tabaci did not result in an early accumulation of Aox1a transcript, although a late accumulation (at 48 h, when disease symptoms and necrotic cell death were already prevalent) was clearly evident. In this case, some increase in AOX protein was also already evident at 24 h.

For comparison, we examined the expression of AOX in Arabidopsis thaliana in response to P. syringae pv tomato infections (Figure 4.12). In this case, both DC3000 (a virulent strain that caused necrotic cell death by 48 h) and DC3000 (AvrRpt2) (an incompatible strain that induced the HR by 24 h) caused similar and modest induction of Aox1a, particularly at later stages (24-48 h). In the case of DC3000 (AvrRps4) (an incompatible strain that did not induce HR), there was little change in Aox1a expression in comparison to the control. We also examined the expression of several other A. thaliana AOX genes (not shown). The expression pattern observed for Aox1d in response to the three bacterial strains was generally similar to Aox1a, although the relative amount of transcript was much lower. The expression of Aox1b, Aox1c and Aox2 was not significantly impacted by the treatments.
Figure 4.11. Level of Aox1a transcript (A, B) and AOX protein (C) in tobacco leaf at different times post-inoculation with different pathovars of *P. syringae* (closed circles, *tabaci*; closed square, *maculicola*; closed triangle, *phaseolicola*). Data in (A) is a representative Northern blot. Data in (B) is the mean +/- S.E. of densitometer analysis of Northern blots from three independent experiments normalized for diurnal changes of AOX expression, each of which showed similar results. For these data, the level of mRNA in the mock-inoculated plants has been arbitrarily set to 1 and used to normalize the data for the plants inoculated with bacteria. Quality of RNA and confirmation of equal loading between lanes was routinely checked by ethidium bromide staining (representative blot shown). For (C), mitochondria were isolated from tobacco leaf at 24 h after treatments. The mitochondrial proteins (100 µg) were then separated by SDS-PAGE, transferred to nitrocellulose and probed with an antibody against AOX. Confirmation of sample quality and equal loading between lanes was routinely checked by also examining levels of cytochrome oxidase subunit II (COXII) protein in the isolated mitochondria, as shown. The lane labeled H₂O are leaves injected with H₂O while the lane labeled +ve control is a mitochondrial sample isolated from a transgenic tobacco line overexpressing AOX. Representative results are shown.
Figure 4.12: Level of Aox1a transcript in A. thaliana rosette leaves at different times post-inoculation with different pathovars of *P. syringae* - DC3000, DC3000 (*AvrRpt2*), DC3000 (*AvrRps4*). Mock-inoculated plants were treated with 10 mM MgCl₂. Data in (A) is a representative Northern blot. Data in (B) are the mean +/- S.D. of densitometer analysis of Northern blots from two independent experiments, each of which showed similar results. For these data, the level of mRNA at 0 h post-inoculation has been arbitrarily set to 1. Quality of RNA and conformation of equal loading between lanes was routinely checked by ethidium bromide staining (representative blot shown).
4.2.7 Other biotic stress-related genes are also differentially expressed in response to different *P. syringae* pathovars

Changes in *Aox1a* transcript were compared with that of other tobacco genes previously shown to respond to biotic stress. *HSR203J* and *HIN1* have each been previously judged as good marker genes for the HR response (Pontier et al., 1994; Gopalan et al., 1996; Krzymowska et al., 2007). Similarly, increased *PAL* gene expression is often associated with the HR (Vlot et al., 2009). As expected, these genes were all strongly induced within 4 h of inoculation with *pv maculicola* (*Figure 4.13A-C*). In the case of *HSR203J* and *HIN1*, there was also a later (12 h) induction by *pv phaseolicola*. All three genes were only induced by *pv tabaci* at a much later stage (24-48 h). Increased ethylene production is a common response to biotic stress (van Loon et al., 2006). The gene encoding 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (which catalyzes the last step in ethylene biosynthesis) increased transiently (at 12 h) in response to each of the bacteria but most strikingly in response to *pv maculicola* (*Figure 4.13D*). Pathogenesis-related-1 (*PR-1*) is a well-known late-induced, SA-responsive defense gene to incompatible pathogens (Eulgem, 2005). As expected, *PR-1* transcript did not change significantly until 24 h, at which time it was induced by the incompatible interactions (strongly by *pv phaseolicola* and more moderately by *pv maculicola*), and not induced at all by the compatible interaction with *pv tabaci* (*Figure 4.13E*). *GRAS* likely encodes a transcription factor and its transcript level has previously been shown to respond to treatments (such as antimycin A, NO, SA and H$_2$O$_2$) in a manner very similar to that of *Aox1a* (Maxwell et al., 2002; Czikkel and Maxwell, 2007). *GRAS* transcript was transiently induced (at 12 h) by *pv phaseolicola*, not induced at all by *pv maculicola*, and induced only late by *pv tabaci* (*Figure 4.13F*). The pattern of *GRAS* transcriptions mirrors that seen with *Aox1a* transcript (*Figure 4.11B*).
Figure 4.13: Level of HSR203J (A), PAL (B), HIN1 (C), ACC oxidase (D) PR-1 (E), and GRAS (F) transcript in tobacco leaf at different times post-inoculation with different pathovars of *P. syringae* (closed square, *maculicola*; closed triangle, *phaseolicola*; closed circle, *tabaci*). Data are the mean +/- S.E. of densitometer analysis of Northern blots from two independent experiments, each of which showed similar results. For these data, the level of mRNA in the mock-inoculated plants has been arbitrarily set to 1 and used to normalize the data for the plants inoculated with bacteria. Quality of RNA and confirmation of equal loading between lanes was routinely checked by ethidium bromide staining (not shown).
4.2.8 Qualitatively similar changes in Aox1a transcript abundance can be seen in tobacco suspension cells treated with different concentrations of SA

Treatment of tobacco suspension cells with different concentrations of SA led to differences in the timing, extent and type of cell death in the culture. It was therefore of interest to see what change in Aox1a gene expression accompanied these distinct responses and whether these mirrored the changes seen during the response of tobacco leaf to the different bacterial pathovars.

A high concentration of SA (3 mM) led to rapid cell death, with complete death of the culture within 24 h (Figure 4.14A). This was accompanied by a distinct pattern of breakdown of high molecular weight DNA to generate the low molecular weight “DNA ladder” that is typical of a programmed response, analogous to the HR (Figure 4.14B). In contrast, treatment with a moderate concentration of SA (0.5 mM) led to a slower death of the culture, with only ~30% of cells dead by 24 h and 85% by 48 h. This cell death was also accompanied by the breakdown of high molecular weight DNA but did not generate the DNA ladder typical of PCD, indicating that in this case the death was necrotic. Low (0.1 mM) SA had little impact on cell viability (Figure 4.14A) and hence cultures maintained high molecular weight DNA through 120 h (Figure 4.14B).

The SA treatments led to distinct changes in Aox1a gene expression. In the case of the treatment that did not induce significant cell death (0.1 mM SA), there was a rapid (within 2 h), large and transient induction of Aox1a transcript (Figure 4.14C, 4.14D). In the case of the treatment generating PCD (3.0 mM SA), there was no induction, but rather a slight decline in Aox1a transcript. In the case of the treatment causing necrotic cell death (0.5 mM), there was also no short-term induction by 2 h but rather an induction in the longer-term (8 h).

In order to confirm that the observed effects on the suspension cells were due to SA, the extent and type of cell death were monitored during treatment with benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH; a compatible SA analog) and 4-hydroxybenzoic acid (HBA; an incompatible SA analog). BTH caused similar responses as SA. The highest concentration (3 mM) caused 90% cell death by 48 h and the formation of the “DNA ladder”. Moderate concentration of this chemical (0.5 mM) led to a more delayed cell death (60% by 48
h) and non-specific DNA degradation. Finally, treatment with a low concentration (0.1 mM) did not lead to cell death or DNA degradation (Figure 4.15A, 4.15B). In contrast, treatment with the inactive analog 4-HBA did not have an effect on cell viability or DNA stability at any concentration (Figure 4.15C, 4.15D).
Figure 4.14: Effects of SA on tobacco suspension cells. (A) Viability of suspension cell cultures following treatment with 0.1 mM (closed triangles), 0.5 mM (closed circles) or 3 mM (closed squares) SA. Data are the mean +/- S.E. of two independent experiments. Note that in some cases, error bars were smaller than the data symbol. (B) Representative agarose gel analysis of DNA extracted from suspension cells at different times after treatment with 0.1, 0.5, or 3.0 mM SA. The lane marked L is a 100 bp low-molecular weight DNA ladder. (C) Representative Northern blot showing changes in the level of Aox1a transcript in suspension cells at different times after treatment with 0.1, 0.5 or 3.0 mM SA. Quality of RNA and confirmation of equal loading between lanes was routinely checked by ethidium bromide staining (representative blot shown). (D) Level of Aox1a transcript in suspension cells at different times after treatment with 0.1, 0.5 or 3.0 mM SA. Data are the mean +/- S.E. of densitometer analysis of Northern blots from three independent experiments, each of which showed similar results. Note that the level of Aox1a in control (untreated) cells (time 0 h) was arbitrarily set at 1.
**Figure 4.15:** Effects of active (BTH) and inactive (4-HBA) analogs of SA on tobacco suspension cells. (A) Viability of suspension cell cultures following treatment with 0.1 mM (closed triangles), 0.5 mM (closed circles) or 3 mM (closed squares) BTH. Data are the mean +/- S.E. of two independent experiments. Note that in some cases, error bars were smaller than the data symbol. (B) Representative agarose gel analysis of DNA extracted from suspension cells at different times after treatment with 0.1, 0.5, or 3.0 mM BTH. (C) Viability of suspension cell cultures following treatment with 0.1 mM (closed triangles), 0.5 mM (closed circles) or 3 mM (closed squares) 4-HBA. Data are the mean +/- S.E. of two independent experiments. Note that in some cases, error bars were smaller than the data symbol. (D) Representative agarose gel analysis of DNA extracted from suspension cells at different times after treatment with 0.1, 0.5, or 3.0 mM 4-HBA. In all agarose gels the lane marked L is a 100 bp low-molecular weight DNA ladder.
4.2.9 SOD isoforms show distinct changes in activity in response to different *P. syringae* pathovars

The major SOD enzymes (MnSOD, CuZnSOD, FeSOD) were identified on activity gels according to their sensitivity to different inhibitors (see Chapter 3). The activity gels were then used to monitor the activity of each enzyme over a 48 h period following inoculation of tobacco leaf (*Figure 4.16A*) All three enzymes (but particularly CuZnSOD) increased in activity by 24 h (and further by 48 h) following inoculation with *pv phaseolicola*. In the case of *pv tabaci*, none of the enzymes changed by 24 h. A small increase in MnSOD and CuZnSOD activity did appear however by 48 h, with no change in FeSOD. In response to *pv maculicola*, the activity of CuZnSOD and FeSOD showed a slight increase over the 48 h period, while MnSOD clearly declined by 24 h and continued declining by 48 h. In summary, the activities of all three isoforms tended to increase or remain unchanged in response to all three pathovars, with the exception of MnSOD activity following inoculation with the HR-inducing *pv maculicola*, in which case activity declined dramatically (*Figure 4.16B*). It is interesting to note that both *pv maculicola* and *pv tabaci* induced different FeSOD isoforms activity late in the experimental period (24-48 h, *Figure 4.16A*), however the fact that these isoforms were not active during earlier time periods (or in the control samples) made analysis difficult.
Figure 4.16. SOD activity in tobacco leaf at different times post-injection with H₂O (open circles) or post-inoculation with different pathovars of *P. syringae* (closed circle, *tabaci*; closed square, *maculicola*; closed triangle, *phaseolicola*). An in-gel activity assay (A) was used to separate the activity of MnSOD (B), CuZnSOD (C) and FeS (D). Data are the mean +/- S.E. of three independent experiments, each of which showed similar results. Note that in some cases, error bars were smaller than the data symbol.
4.3 Discussion

Recent research suggests that mitochondria can be an important intracellular source of ROS during plant PCD, generating a so-called mitochondrial oxidative burst. This organelle has also been implicated in the generation of RNS, which are important players for the induction of this response. Since AOX has emerged as an important regulator of ROS and RNS generation by the ETC, several studies have examined the relationship between AOX and susceptibility to PCD induced by various biotic or abiotic stimuli. For example, overexpression of AOX in transgenic Arabidopsis protoplasts protected against aluminum-induced PCD (Li and Xing, 2011) while overexpression in tobacco resulted in slightly smaller TMV-induced HR lesions (Ordog et al., 2002). Conversely, suppression of AOX in transgenic tobacco suspension cells increased their susceptibility to SA or H₂O₂-induced PCD (Robson and Vanlerberghe, 2002; Amirsadeghi et al., 2006). Chemical inhibition of AOX has also been shown to increase the susceptibility of potato cells to β-glucan elicitor (Mizuno et al., 2005), soybean cells to H₂O₂ (Amor et al., 2000), tomato protoplasts to ethylene (Lei et al., 2003), and lettuce leaves to compatible and incompatible bacteria (Kiba et al., 2008; Kiba et al., 2009), although such studies should be interpreted with caution given the potential side-effects of AOX inhibitors. Collectively, the above studies suggest that AOX may provide a level of protection against PCD.

Given the above, it can be hypothesized that during natural examples of PCD that are likely of benefit to the plant (such as the HR), AOX with its potential to act as a negative regulator of PCD, would not be induced or might even decline, such as to promote the programmed response. To test this hypothesis, we compared responses to three different P. syringae pathovars. The differential responses to these pathovars were carefully characterized by examining the kinetics of cell death, changes in key biotic stress signaling molecules, and changes in established biotic stress related genes. These analyses established three distinct plant responses to the pathovars: a compatible response that did not elicit typical defenses (such as increased SA) and which was associated, at a late stage, with disease-causing cell death (pv tabaci); an incompatible response associated with well-known defense responses (such as increased SA and PR-1 transcript) but which did not include any HR and cell death (pv phaseolicola); and an incompatible response that included strong defense responses (such as rapid accumulation of SA, NO and H₂O₂; induction of HR-associated genes) and the HR, which was then able to restrict any further proliferation of the pathogen (pv maculicola).
Our results show that mitochondrial $\text{O}_2^-$ increases dramatically during the response of tobacco to the HR-inducing pv *maculicola*, something that has previously not been shown directly (to the best of our knowledge). This leads to the important question of whether mitochondrial $\text{O}_2^-$ is in fact an important signal for the HR. While it is generally accepted that $\text{H}_2\text{O}_2$, $\text{O}_2^-$ and NO are important players in the initiation and execution of the HR and defense responses, it is not clear what their individual roles are or how they interact with each other. The specific importance of $\text{O}_2^-$ has been suggested by several studies. For example, the *Arabidopsis* spontaneous cell death mutant *lsd1*, which does not induce CuZnSOD, has an increased susceptibility to SA-induced PCD (Mazel and Levine, 2001; Epple et al., 2003). Our group has previously suggested that complex III-derived $\text{O}_2^-$ could induce PCD in tobacco suspension cells (Robson et al., 2008). This was based on the differential response of cells to two different respiratory inhibitors, one that induces $\text{O}_2^-$ at Complex III (antimycin A) and one that does not (myxothiazol).

The role of ROS and RNS in the induction of PCD is still not fully understood; however it is clear that these molecular signals are involved in an intricate web of interactions. There are several hypotheses that might suggest the means by which mitochondrial $\text{O}_2^-$ might be acting mechanistically to promote PCD. For example, it has been suggested that mitochondria are a potential source of NO and that AOX might influence its rate of synthesis by the ETC (Modolo et al., 2005; Gupta et al., 2011). In this work, we have shown that NO is accumulated early in the interaction between tobacco and pv *maculicola*, and that at least some NO colocalized with the mitochondria. $\text{O}_2^-$ can react in diffusion-controlled reaction with NO to produce ONOO$^-$.

Indeed, we observed an early and rapid increase in ONOO$^-$ in tobacco plants infected with pv *maculicola*, in part localizing to the mitochondria. In plant systems this molecule does not directly induce PCD, however higher levels have been reported in plants infected with HR-inducing pathogens (Gaupels et al., 2011). It has been proposed that ONOO$^-$ could impact the function of proteins via the nitration of key tyrosine residues and studies have just begun to identify those proteins subject to tyrosine nitration during the HR (Cecconi et al., 2009).

It has been previously suggested that induction of the HR depends on the balanced production of NO and $\text{H}_2\text{O}_2$ (Delledonne et al., 2001) but potential pro-survival functions have been described for these molecules as well (Yun et al., 2011; Torres et al., 2006). Some of these functions might
be attenuated if NO is being scavenged by $O_2^-$, or if the balance of mitochondrial $O_2^-$ to $H_2O_2$ is changed during the HR. Furthermore, during infection with the defense-inducing pv \textit{phaseolicola} the lower overall mitochondrial-generated $O_2^-$, along with perhaps a shift toward $H_2O_2$ (at the expense of $O_2^-$) and increased NO levels, might be an important pro-survival signal to prevent PCD and/or induce defense gene expression. Elucidating the functional importance of the mitochondrial $O_2^-$ burst clearly represents a rich area for further study.

The signaling molecules SA, NO and $H_2O_2$ have each been shown to strongly induce AOX in numerous species, including tobacco, and these molecules all showed a strong and early accumulation in response to the HR-inducing pv \textit{maculicola}. Ethylene is another signaling molecule shown to be accumulated during the HR response in tobacco to \textit{P. syringae} (Mur et al., 2008) and it has also been strongly implicated in the induction of AOX by stress conditions (Ederli et al., 2006; Wang et al., 2010). The strong induction of ACC oxidase gene expression in tobacco plants infected with pv \textit{maculicola}, as seen in our work, can be taken as indirect evidence that ethylene was likely also being produced. However, despite the increase of these signaling molecules, our results clearly indicated no increase in Aox1a transcript or protein in response to pv \textit{maculicola}. Hence, during the HR program, AOX expression was uncoupled from several of the signaling molecules thought to otherwise promote its induction. In contrast, pv \textit{phaseolicola} (which induced defense responses, but in the absence of PCD), did elicit a strong transient increase in Aox1a transcript, accompanied by increased AOX protein. In the case of pv \textit{tabaci}, AOX expression was induced but only much later after infection, just prior to necrotic death of the diseased tissue.

Similar to our findings, a few other reports have suggested that AOX is in fact not induced during a PCD response. In tobacco cells challenged by metabolic products of a fungal pathogen that induced PCD, it was noted that AOX capacity was reduced (Cheng et al., 2011). Similarly, AOX was not induced by harpin in \textit{Nicotiana sylvestris} leaf (Bocca et al., 2001). Also, several studies have noted that AOX expression can be strongly induced at the late stages of compatible bacterial interactions that culminate in a disease-associated cell death or other cases of necrotic cell death. Examples are reported in \textit{Arabidopsis} (Simons et al., 1999), lettuce (Kiba et al., 2009) and wheat (Sugie et al., 2007). The late AOX induction may represent an extreme response to a collapse of energy metabolism or may simply be a non-specific response to cellular dysfunction. To our knowledge, no studies have specifically addressed this issue.
We tried to extend our findings to *Arabidopsis* however the results differed between the two species compared in this work. We observed an increase in *Arabidopsis* Aox1a transcript by both compatible (DC3000) and incompatible HR-inducing *P. syringae* (DC3000 AvrRpt2) and furthermore, transcription of this gene was not induced during an incompatible response that lacked the HR (DC3000 AvrRps4). It should be noted that the absolute changes in Aox1a transcript that we observed in *Arabidopsis* were much more subtle than we would see, for example, in tobacco responding to *pv phaseolicola*. Lacomme and Roby (1999) also identified Aox1a as a gene induced in *Arabidopsis* suspension cells by an avirulent (but not virulent) strain of *Xanthomonas campestris* and suggested it as an early marker of the HR. Simons et al. (1999) showed that both compatible and incompatible HR-inducing *P. syringae* were able to induce Aox1a, but with the earliest and strongest induction occurring during the HR. Studies in citrus and lettuce leaf have also noted AOX induction in response to HR-inducing bacteria (Daurelio et al., 2009; Kiba et al., 2008), while harpin (an HR-inducing bacterial elicitor) was shown to increase Aox1a expression in *Arabidopsis* suspension cells (Krause and Durner, 2004) and AOX activity in *Nicotiana sylvestris* (Vidal et al., 2007). Overall, the contrasting results suggest that there may be fundamental differences between species regarding the role of AOX in cell death programs, factors regulating AOX gene expression, or baseline AOX level between species. Some differences between studies may also be due to differences in experimentation and interpretation. Our study had the advantage of being able to directly compare multiple distinct responses to different bacterial pathovars, accompanied by direct measures of cell death, changes in signaling molecules (SA, NO, ONOO\(^-\), \(\text{H}_2\text{O}_2\)), changes in mitochondrial \(\text{O}_2^-\), and careful characterization of Aox1a expression against a background diurnal rhythm. Also, our studies have been done *in planta* (rather than suspension cells where oxidative poise may differ; Halliwell, 2003), and in response to bacteria rather than an HR-inducing elicitor, that might not “elicit” the same or complete plant response as that of bacteria (Mur et al., 2005).

At present, we do not know the mechanism by which Aox1a expression is controlled in various conditions. It is especially intriguing that Aox1a is suppressed during the HR, despite the dramatically increased level of potential inductive signaling molecules. A clue to this might be provided by the experiment on suspension cells in which it was clear that while low concentrations of SA were highly effective at inducing Aox1a, higher concentrations of SA had no effect. This suggests that either high concentrations of SA itself, or some downstream
consequence of high SA triggers a suppression rather than induction of Aox1a expression. Significantly, the results seen with cells mirror well the relationship between Aox1a expression, SA level and cell fate seen in leaf during the incompatible interactions. The HR-inducing pv maculicola showed the strongest early induction of SA, lacked any Aox1a induction and resulted in PCD. Alternatively, pv phaseolicola showed less early accumulation of SA, a strong transient Aox1a induction and no PCD.

There are also means by which the low constitutive level of AOX protein that still exists in tobacco leaf after pv maculicola infection could be inactivated. For example, high levels of H$_2$O$_2$ (such as are seen specificity with pv maculicola) have the potential to convert AOX to its oxidized (low activity) form (Vanlerberghe et al., 1999). AOX activity is also sensitive to the lipid peroxidation product 4-hydroxy-2-nonenal (Winger et al., 2005) and, interestingly, AOX was identified as a target of tyrosine nitration in sunflower hypocotyls (Chaki et al., 2009), although that study was not focused specifically on the HR or PCD.

Our results clearly showed that MnSOD activity declines in tobacco leaf in response to the HR-inducing pv maculicola. This response was distinct from the response of MnSOD activity to the other pathovars, as well as being distinct from the response of the other SOD isozymes. All other activities tended to either increase or remain unchanged. Particularly the increase in CuZnSOD is consistent with previous studies (Kliebenstein et al., 1999). Only few studies to date have examined changes in MnSOD activity during PCD or in response to biotic stress. A microarray analysis of senescence in Arabidopsis suspension cells did report a decrease in both AOX and MnSOD transcript (Swidzinski et al., 2002). PCD of barley aleurone cells is proceeded by a loss of SOD activity, with MnSOD appearing to be the first to decline (Fath et al., 2001). Evidence that MnSOD can significantly impact the leaf redox state comes from experiments with transgenic Arabidopsis (Morgan et al., 2008). Knocked-down expression of MnSOD increased the oxidation of a mitochondrial-targeted redox-sensitive green fluorescent protein, as well as impacting whole leaf antioxidant activities and the pool sizes and oxidation states of ascorbate and glutathione. At present, we do not know the mechanism responsible for the loss of MnSOD activity in response to pv maculicola. A previous study showed that loss of MnSOD activity during fruit senescence correlated with carbonylation of the protein (Qin et al., 2009). Interestingly, human MnSOD is inactivated by peroxynitrite (ONOO$^-$) via tyrosine nitration (Castro et al., 2011). However, initial studies to identify potential target proteins subject to either
S-nitrosylation or tyrosine nitration during the HR, have not identified MnSOD (Romero-Puertas et al., 2008; Cecconi et al., 2009).

A major finding of our study is that the HR of tobacco leaf is preceded by an early and persistent mitochondrial burst of superoxide. We suggest that AOX and MnSOD are uniquely positioned to impact both the strength and specificity of ROS signals in the mitochondria. AOX will impact the *strength* of the ROS signal by modulating the rate of $\text{O}_2^-$ generation by the mitochondrial ETC. Then, the activity of MnSOD (the sole enzymatic means to scavenge matrix $\text{O}_2^-$ and convert it $\text{H}_2\text{O}_2$) will determine the *specificity* of the signal by determining the relative levels of $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ in the matrix. In this way, MnSOD may have a major influence on the distribution of mitochondrial-generated ROS between mitochondria and cytosol, since $\text{H}_2\text{O}_2$ is readily membrane-permeable (while $\text{O}_2^-$ is not). Furthermore, $\text{O}_2^-$ could potentially regulate the levels of mitochondrial generated NO and ONOO⁻, thus acting as a key regulator of several signaling pathways.

Numerous studies have shown a suppression of different ROS-scavenging systems during the HR and other forms of PCD, and it has been suggested that this aspect of the cellular response is necessary to allow a sufficient accumulation of ROS needed to promote the PCD (Foyer and Noctor, 2005; Vlot et al., 2009). Most studies to date have examined suppression of the ROS-scavenging systems of the cytosol, chloroplast and peroxisome, relating this to ROS sources such as NADPH oxidase, photosynthesis and photorespiration. Consistent with this theme, we suggest that the coordinated activity of AOX and MnSOD as a potential means to construct distinct mitochondrial ROS signatures that lead to distinct cellular responses. Elucidating the relative importance of this mechanism in the overall response of the plant to biotic stress would be a rich area for further study.
CHAPTER 5

Alternative oxidase coordinates the appearance and timing of a mitochondrial oxidative burst during the hypersensitive response in *Nicotiana tabacum*

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Chapter 5
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5.1 Introduction

Alternative oxidase (AOX) is a plant protein located in the mitochondrial inner membrane that acts as a second terminal oxidase branching at the level of the ubiquinone pool, which catalyzes a four-electron reduction of $O_2$ to $H_2O$. The operation of this reaction is non-proton pumping, and bypasses two sites of proton-pumping (Complexes III and IV) thus reducing the energy yield of respiration (Finnegan et al., 2004). It is believed that the presence of this alternative pathway allows for certain flexibility of plant respiratory metabolism and moderation of membrane potential, especially under stress conditions. Furthermore, AOX might also reduce the production of mitochondrial ROS since it by-passes Complex III, one of the known sites of $O_2^-$ production (Vanlerberghe et al., 2009). In support of this, we have shown that transgenic plants suppressed for AOX expression accumulate more $O_2^-$ under steady-state conditions (see Chapter 3). It is interesting that AOX has also been linked to NO production (Igamberdiev et al., 2010, Wulff et al., 2009) and we have also shown that AOX knockdown lines accumulate more NO during steady state conditions (see Chapter 3).

We have previously demonstrated that a strong mitochondrial $O_2^-$ burst precedes the appearance of HR-like lesions in *Nicotiana tabacum* plants infected with the incompatible pathogen *Pseudomonas syringae* pv *maculicola*. This oxidative burst was accompanied by low levels of AOX and a general decrease in MnSOD activity (the only means of scavenging $O_2^-$ enzymatically in the mitochondria). On the other hand, infection with another incompatible pathogen, pv *phaseolicola*, was associated with a rapid increase in AOX level and MnSOD activity, no mitochondrial $O_2^-$ burst and infected leaves failed to develop HR-like lesions. Based on this data we proposed that AOX and MnSOD can impact both the strength and the specificity of the ROS signal by modulating both the rate of $O_2^-$ production and scavenging and thus can determine cell fate under biotic stress (Chapter 4).
It is well known that incompatible pathogens (and especially ones that induce the HR) cause the rapid accumulation of not only $O_2^-$, but other ROS and RNS as well, most notably $H_2O_2$, NO and ONOO$^-$ (Torres et al., 2006; Arasimowicz et al., 2007; Besson-Bard et al., 2009; Blokhina et al., 2010; Torres 2010; see Chapter 4). The specific roles of these molecules in the initiation and coordination of defense responses are currently not known, however it is clear that they are involved in an intricate web of interactions. For example, several cellular locations have been proposed for the production of both ROS and RNS during biotic stress (Mittler 2002; Foyer and Noctor, 2005; Moreau et al., 2010; Blokhina and Fagerstedt, 2009) suggesting that there are different targets and interactions between these molecules depending on their cellular origin. This brings us to the important question of how important is the mitochondrial $O_2^-$ burst specifically for the execution of the HR? There are several lines of evidence in support of the importance of $O_2^-$. One of the pioneering studies on the link between ROS and HR observed an accumulation of $O_2^-$ prior to the appearance of HR in potato tubers infected with *Phytophthora infestans* and in tobacco infected with TMV, and noted that in this case the HR could be delayed by the addition of scavenging enzymes (Doke 1982; Doke and Ohashi, 1988). Similarly, the appearance of HR-like lesions in TMV-infected tobacco plants could be suppressed by the application of SOD enzymes (Király et al., 2008). A second line of evidence comes from the *Arabidopsis* spontaneous cell death mutant *lsd1*, which does not induce CuZnSOD and is susceptible to PCD inducing compounds (Kliebenstein et al., 1999; Mazel and Levine, 2001; Epple et al., 2003). However, at this point it would be difficult to determine if the effects observed are due to the signaling function of $O_2^-$ itself or through its role as a source of $H_2O_2$ through dismutation. The evidence presented above suggests that the appearance of the HR could be promoted by increased $O_2^-$ levels, decreased $H_2O_2$ levels or an imbalance between these two molecules.

Another important question arising from our previous work is how crucial is AOX specifically in the execution of the mitochondrial $O_2^-$ burst, and thus the HR? Studies have shown that manipulation of AOX gene expression has an effect on the susceptibility of transgenic plants to stress. For example, overexpression of AOX lead to a decrease in lesion size in tobacco plants infected with TMV (Ordog et al., 2002). Furthermore, plants and suspension cells lacking AOX have increased susceptibility to PCD-inducing treatments, different ROS balance and antioxidant levels (Amirsadeghi et al., 2006; Robson and Vanlerberghe, 2002). At this point it is not known...
if these effects are due to altered mitochondrial ROS levels directly or reflect the ability of AOX
to influence distal parts of the cell through modulation of mitochondrial signaling by other
means.

The aim of this study is to further explore the link between AOX, ROS and incompatible plant-
pathogen interactions. Based on the evidence presented in this study we propose that AOX is an
important determinant of cell fate during biotic stress, mainly through the control of ROS
production. We used transgenic *Nicotiana tabacum* plants with altered AOX levels infected with
two incompatible *Pseudomonas syringae* pathovars: *pv maculicola* which induces the HR; and
*pv phaseolicola* that induces defense responses without the appearance of cell death in infected
leaves. We show that transgenic plants lacking AOX show a strong mitochondrial O$_2^-$ burst when
infected with *pv phaseolicola* (which is not seen in Wt plants), however this interaction does not
result in the appearance of the HR. We found that the appearance of HR symptoms as well as the
appearance of the typical mitochondrial O$_2^-$ burst is delayed in transgenic plants lacking AOX
and infected with *pv maculicola*. A similar delay is seen in transgenic plants treated with the
complex III inhibitor antimycin A. We suggest that the mitochondrial ROS burst controlled by
AOX is an important component for the induction and coordination of the HR and that the
mitochondrial ETC is a direct target during incompatible biotic interactions.
5.2 Results

5.2.1 The lack of AOX influences the kinetics of cell death during incompatible P. syringae infection

To study the role of AOX in response to biotic stress, tobacco plants silenced for AOX expression were inoculated with two different incompatible P. syringae pathovars. P. syringae pv maculicola caused the appearance of HR-like cell death lesions in Wt tobacco as early as Day 1 post-infection, measured as a loss of membrane integrity. Infection with this pathogen caused the appearance of cell death in the two AOX knockdowns as well, however the appearance of typical symptoms (such as dry HR-like lesions) and the progression of cell death were significantly delayed. RI29 (the stronger knockdown) showed similar loss of membrane integrity as Wt by Day 3, while RI9 (the slightly leaky AOX knockdown) reached similar levels by Day 4 post-infection. (Figure 5.1A). Infection with pv phaseolicola did not cause the appearance of visible cell death or the loss of membrane integrity in all three lines. (Figure 5.1B). It was determined that the lack of AOX did not affect the proliferation of these two pathovars in planta, at least with the bacterial concentrations used in this study. The incompatible pv maculicola proliferated rapidly, with maximal bacterial numbers reached by Day 1 post-infection in all three lines while pv phaseolicola failed to proliferate in tobacco leaves after inoculation (Figure 5.2)
Figure 5.1: Measurements of conductivity (ion leakage) of Wt (open circle) and transgenic tobacco leaves silenced for AOX expression (RI9, open square; RI29, open triangle) at different times post-inoculation with P. syringae pv *maculicola* (A) and pv *phaseolicola* (B). Data are the mean +/- S.E. of at least three independent experiments, each of which showed similar results. In some cases error bars were smaller than the data symbols. Each set of data were analyzed by two-way ANOVA followed by a Bonferroni multiple comparison test to determine if they were significantly different from data obtained for Wt plants. The number of asterisks indicates the level of significant difference (* P < 0.05; ** P<0.01; *** P<0.001).
Figure 5.2: Bacterial proliferation in Wt (open circle) and transgenic tobacco leaves silenced for AOX expression (RI9, open square; RI29, open triangle) at different times post-inoculation with P. syringae pv *maculicola* (A) and pv *phaseolicola* (B). The bacterial density was adjusted to 1 x 10^5 cfu/ml prior to inoculation when determining bacterial proliferation. Data are the mean +/- S.E. of at least two independent experiments, each of which showed similar results. In some cases error bars were smaller than the data symbols. Each set of data were analyzed by two-way ANOVA followed by a Bonferroni multiple comparison test to determine if they were significantly different from data obtained for Wt plants (P < 0.05).
5.2.2 Transgenic plants lacking AOX accumulate mitochondrial O$_2^-$ at different rates after infection with incompatible *P. syringae* pathovars

Mitochondrial O$_2^-$ level in tobacco leaf mesophyll cells was observed using confocal microscopy to image MitoSOX Red fluorescence. As expected, in Wt leaves infected with the HR-inducing *pv maculicola*, high levels of O$_2^-$ were present by 4 h post-inoculation and persisted through 24 h, while plants inoculated with dH$_2$O did not accumulate mitochondrial O$_2^-$ due to the mock treatment. As previously shown (see Chapter 3), the two knockdown lines, RI9 and RI29, had higher levels of O$_2^-$ in mock treated plants as compared to Wt; however these two lines showed a marked delay in the accumulation of O$_2^-$ when inoculated with *pv maculicola*. By 4 h post-inoculation, these lines did not accumulate significant amount of mitochondrial O$_2^-$ and had similar levels of this molecule as mock treated plants (in contrast to Wt plants, which showed a marked increase by this time point). By 24 h post-inoculation (the last time point examined) the levels of O$_2^-$ were similar between lines (Figure 5.3).

Following inoculation with *pv phaseolicola*, there was little change in O$_2^-$ level compared to mock-inoculated leaves at any time point in Wt plants. In contrast, RI9 and RI29 plants accumulated higher levels of mitochondrial O$_2^-$ due to inoculation with this pathovar, compared to both Wt plants infected with *pv phaseolicola* and mock-inoculated transgenic plants (Figure 5.4). This increase was evident by 4 h post-inoculation and persisted through the experimental period.
**Figure 5.3:** Laser-scanning confocal microscope images of mitochondrial O$_2^-$ in tobacco mesophyll cells, observed at different times post-inoculation with *P. syringae pv maculicola* in Wt plants, and transgenic plant silenced for AOX expression (RI9, RI29). All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative results of three independent experiments, each of which showed similar results. Mock-inoculated plants were observed over 24 h, without a change in O$_2^-$ levels, representative images taken at 24 h post-inoculation are shown here. Scale bar = 20 µm.
Figure 5.4: Laser-scanning confocal microscope images of mitochondrial O$_2^-$ in tobacco mesophyll cells, observed at different times post-inoculation with *P. syringae pv phaseolicola* in Wt plants, and transgenic plant silenced for AOX expression (RI9, RI29). All images are maximum intensity projections of Z-stacks (8-16 μm in depth) and are representative results of three independent experiments, each of which showed similar results. Mock-inoculated plants were observed over 24 h, without a change in O$_2^-$ levels, only representative images taken at 24 h post-inoculation are shown here. Scale bar = 20 μm.
5.2.3 SOD isoform activities and H$_2$O$_2$ levels show minor differences between lines in response to the two incompatible *P. syringae* pathovars

Three major SOD enzymes (MnSOD, CuZnSOD, FeSOD) were identified on activity gels according to their sensitivity to different inhibitors and used to monitor the activity of each enzyme over a 48 h period following inoculation with the two incompatible *P. syringae* pathovars. In response to *pv maculicola* (*Figure 5.5A, 5.5B, 5.5C*) the activity of CuZnSOD and FeSOD remained mostly unchanged over the experimental period in all three lines, while MnSOD clearly started declining by 12 h and further by 24 and 48 h post-inoculation. It is interesting to note that the two knockout lines showed a more rapid (albeit not statistically significant) decrease in activity as compared to Wt plants. The activities of all three isoforms tended to increase slightly in response to *pv phaseolicola* (*Figure 5.5D, 5.5E, 5.5F*), however this increase seemed to be slightly delayed in RI9 and RI29 as compared to Wt plants.

The two different incompatible pathogens had a distinct impact on total leaf H$_2$O$_2$ levels in infected plants. Inoculation with the HR-inducing *pv maculicola* resulted in a general increase in H$_2$O$_2$, with a first and transient peak at 2 h post-inoculation and a second and sustained peak at 8 h (*Figure 5.6A*), suggesting a classical two-phase oxidative burst generally associated with the appearance of the HR. This pattern of accumulation was observed in all three lines. In the case of *pv phaseolicola*, there was no evidence of the oxidative burst of H$_2$O$_2$ but rather a general decline in H$_2$O$_2$ level after inoculation in Wt plants, however both knockdown lines accumulated a 2-fold higher level of H$_2$O$_2$ by 4 h post-inoculation, as compared to the levels of this molecule at 0 h. In this case, we did not observe a two-phase response, but rather a sustained peak of this ROS (*Figure 5.6B*). However due to the variability of the data in these experiments, the results were not statistically significant.
Figure 5.5: SOD activity in tobacco leaf at different times post-injection with P. syringae pv maculicola (MnSOD - A, CuZnSOD - B, FeSOD - C) and pv phaseolicola (MnSOD - D, CuZnSOD - E, FeSOD - F). An in-gel activity assay was used to separate the activity of the three isoforms. Three lines were used in these experiments, Wt (open circle) RI9 (open square), RI29 (open triangle) Data are the mean +/- S.E. of three independent experiments, each of which showed similar results. Note that in some cases, error bars were smaller than the data symbol. The level of SOD in mock-inoculated Wt plants (at time 0 h) was arbitrarily set to 1, and all other values were normalized in relation to this activity.
Figure 5.6: Levels of H$_2$O$_2$ in tobacco leaf at different times post-inoculation with *P. syringae* pv *maculicola* (A) and pv *phaseolicola* (B). Three lines were used in these experiments, Wt (open circle) RI9 (open square), RI29 (open triangle). Data are the mean +/- S.E. of three independent experiments, each of which showed similar results.
5.2.4 Dynamic changes in RNS levels in Wt and AOX knockdown plants during infection with incompatible *P. syringae* pathovars

DAF-FM is a cell-permeant dye that is oxidized specifically by NO inside the cell to form a fluorescent product which can be observed using confocal microscopy. This method was used to compare the levels of NO between Wt and AOX knockdown plants infected with the two *P. syringae* pathovars. As observed before (see Chapter 4) Wt plants infected with the HR-inducing *pv maculicola* showed early and rapid increase in fluorescent signal due to NO visible by 4 h, which persisted through 24 h. RI9 and RI29 plants accumulated similar amounts of NO early in the infection period (4 h), however the levels of this molecule were lower by 24 h in the stronger knockdown RI29, as compared to Wt plants (Figure 5.7). Infection with *pv phaseolicola* caused a very modest increase in fluorescence signal by 24 h in all three lines (Figure 5.8).

To ensure reproducibility, NO levels were also measured using a biochemical assay (Figure 5.9). Once again, infection of Wt and RI9 plants with *pv maculicola* resulted in a rapid accumulation of NO over the first 6 h and further accumulation through 24 h, when compared to steady state conditions in these lines. RI29 accumulated only a modest amount of NO when compared to steady state levels (measured at 0 h). The second *pv phaseolicola* did not induce such a rise in NO levels in all three lines.

ONOO\(^{–}\) (the product of the interaction between O\(_2\)\(^{–}\) and NO) can be visualized by confocal microscopy using the fluorescent dye APF. Inoculation with *pv maculicola* induced an increase in the levels of this molecule by 4 h in all three lines, however the two knockdown lines (and especially the stronger knockdown RI29) accumulated more ONOO\(^{–}\) than Wt plants by 24 h (Figure 5.10). Inoculation with *pv phaseolicola* lead to a small increase in the levels of this RNS by 24 h (Figure 5.11).
Figure 5.7: Levels of cellular NO in tobacco mesophyll cells of Wt and transgenic plants with suppressed AOX levels (RI9, RI29) infected with *P. syringae* pv *maculicola*. The images shown depict DAF-FM fluorescence (green), Mitotracker Red fluorescence (red), where the colocalization between DAF-FM and Mitotracker Red can be observed as yellow signal. All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative results of three independent experiments, each of which showed similar results. Mock-inoculated plants were observed over 24 h, without a change in NO levels. Only representative images taken at 24 h are shown here. Scale bar = 20 µm.
Figure 5.8: Levels of cellular NO in tobacco mesophyll cells of Wt and transgenic plants with suppressed AOX levels (RI9, RI29) infected with *P. syringae pv phaseolicola*. The images shown depict DAF-FM fluorescence (green), Mitotracker Red fluorescence (red), where the colocalization between DAF-FM and Mitotracker Red can be observed as yellow signal. All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative results of three independent experiments, each of which showed similar results. Mock-inoculated plants were observed over 24 h, without a change in NO levels. Only representative images taken at 24 h are shown here. Scale bar = 20 µm.
Figure 5.9: Levels of total leaf NO in tobacco leaf at different times post-inoculation with P. syringae pv maculicola (A) and pv phaseolicola (B). Three lines were used in the experiments, Wt (open circle) R19 (open square) and R129 (open triangle). Data are the mean +/- S.E. of three independent experiments, each of which showed similar results.
**Figure 5.10**: Cellular ONOO\(^-\) levels in tobacco mesophyll cells of Wt and transgenic plants with suppressed AOX levels (RI9, RI29) infected with *P. syringae pv maculicola*. The images shown depict APF fluorescence (green), Mitotracker Red fluorescence (red), where the colocalization between APF and Mitotracker Red can be observed as a yellow signal. All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative results of three independent experiments, each of which showed similar results. Mock-inoculated plants were observed over 24 h, without a change in NO levels. Only representative images taken at 24 h are shown here. Scale bar = 20 µm.
Figure 5.11: Cellular ONOO⁻ levels in tobacco mesophyll cells of Wt and transgenic plants with suppressed AOX levels (RI9, RI29) infected with P. syringae pv phaseolicola. The images shown depict APF fluorescence (green), Mitotracker Red fluorescence (red), where the colocalization between APF and Mitotracker Red can be observed as yellow signal. All images are maximum intensity projections of Z-stacks (8-16 μm in depth) and are representative results of three independent experiments, each of which showed similar results. Mock-inoculated plants were observed over 24 h, without a change in NO level. Only representative images taken at 24 h are shown here. Scale bar = 20 μm.
5.2.5 Qualitatively similar changes in ROS and RNS accumulation patterns can be seen in Wt and transgenic tobacco leaves treated with antimycin A

We have previously shown that transgenic tobacco plants silenced for AOX expression (RI9, RI29) accumulated higher levels of both NO and O$_2^-$, while transgenic plants with increased AOX expression (B7) showed similar or lower levels of these molecules under steady state conditions as compared to Wt plants (see Chapter 3). In attempt to make a connection between these findings and the work presented in this chapter, we treated Wt, RI29 and B7 plants with the mitochondrial Complex III inhibitor antimycin A, and observed the levels of O$_2^-$, NO and ONOO$^-$. As expected, this treatment dramatically elevated the MitoSOX fluorescence in Wt plants after 1 h incubation, however this increase appeared to be transient and levels of O$_2^-$ decreased to nearly control levels by 4 h (Figure 5.12A). In contrast, the accumulation appeared to be delayed in the knockdown line RI29 and we did not observe an increase in fluorescence after 1 h. Higher levels of O$_2^-$ were visible only after 4 h incubation with antimycin A (Figure 5.12B). The AOX overexpressor line B7, had significantly lower amounts of mitochondrial O$_2^-$ over the entire experimental period as compared to Wt plants (Figure 5.12C).

Treatment with antimycin A also had an effect on cellular NO levels. As before, DAF-FM was used to observe NO, however since this fluorescent dye does not localize specifically to the mitochondria, colocalization analysis was also performed in order to gain some insight in the origin of NO production. Wt plants accumulated high levels rapidly (1 h) after being exposed to this inhibitor and higher levels of NO persisted even after 4 h treatment (Figure 5.13A). Once again, RI29 accumulated NO at lower levels early into the experimental period (1 h) and higher levels as compared to Wt at a later time point (4 h) (Figure 5.13B). B7 did not produce NO due to the antimycin A treatment after 1h, and only modest amounts of fluorescence could be observed by 4 h (Figure 5.13C). Colocalization between NO and mitochondria was analyzed using the Manders’ coefficients. M1 (Figure 15.4A) represents the amount of the signal in the green channel (NO) coincident with the signal in the red channel (mitochondria). From this analysis it was evident that the proportion of NO signal did not coincide strongly with the mitochondria signal, possibly due to different compartments contributing to cellular NO as well as the fact that this molecule can traverse membranes and move away from its compartment of origin. However by observing the Manders’ coefficient M2 (Figure 5.14B), namely the amount
of signal in the red channel (mitochondria) colocalizing with the signal in the green channel (NO), the colocalization was much stronger suggesting that this organelle is a source of NO in the cell. Similarly as seen before (Chapter 3), in mock treated plants incubated with H$_2$O the strong AOX knockdown line had a larger fraction of mitochondria (~20%) coinciding with the NO signal, while Wt and the overexpressor line B7 had similar low levels of overlap between the two signals (<5%). By 1 h exposure to antimycin A, nearly 50% of mitochondria in Wt plants coincided with NO compared to less that 30% in Ri29 and 5% in B7. By 4 h this proportion stayed similar or slightly lower in Wt plants (~40%) and it increased in the RI29 plants (~55%). The number of mitochondria coinciding with NO also increased in B7 plants at this point (~20%) however this was still lower than Wt plants.

As expected, antimycin A treatment also had an effect on ONOO$^-$ levels in plant cells and the pattern of ONOO$^-$ accumulation was similar as seen with O$_2^-$ and NO. Wt plants started accumulating this molecule by 1 h in the experimental treatment with similar amounts seen by 4 h as well (Figure 5.15A). RI29 plants appeared to have a delayed response to antimycin A, with only modest accumulation of ONOO$^-$ by 1 h, however later in the treatment (4 h) levels of this molecule appeared to be higher that what was seen in Wt plants (Figure 5.15B). Once again, in B7 plants antimycin A did not cause the accumulation of large amounts of this RNS, and instead only a modest increase was seen by 4 h (Figure 5.15C). The Manders’ coefficients were once again used in order to analyze the level of overlap between the mitochondrial and the ONOO$^-$ signal (Figure 5.16). Similarly as seen with NO, a comparison between M1 and M2 suggested that this molecule had multiple sites of production or high mobility in the cell. In this case, both coefficients showed a similar pattern. When analyzing M2 more closely, it was evident that there was very little overlap (and very little observable signal) in mock treated plants while 1 h treatment with antimycin A resulted in a strong overlap between the mitochondrial and the ONOO$^-$ signal in Wt and RI29 plants (~50-60%) with a very small percentage in B7 plants (~5%). By 4 h into the treatment, the amount of overlap was similar in Wt and RI29 plants, however this number increased in B7 plants (~40%).
Figure 5.12: Levels of mitochondrial O$_2^-$ in tobacco mesophyll cells treated with antimycin A and observed at different times. In this experiment three lines were used: Wt plants, AOX knockdown RI29 plants and AOX overexpressor B7 plants. All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative results of three independent experiments, each of which showed similar results. Mock-treated plants incubated with H$_2$O were monitored over the entire experimental period, without significant changes in O$_2^-$ levels observed. Only representative images taken at 1 h are shown here. Scale bar = 20 µm.
Figure 5.13: Levels of cellular NO in tobacco mesophyll cells treated with antimycin A and observed at different times. Three lines were used: Wt plants, AOX knockdown RI29 plants and AOX overexpressor B7 plants. The images shown depict DAF-FM fluorescence (green), Mitotracker Red fluorescence (red) where the colocalization between DAF-FM and Mitotracker Red could be observed as yellow signal. All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative results of three independent experiments, each of which showed similar results. Mock-treated plants incubated with H$_2$O were monitored over the entire experimental period, without significant changes in NO levels observed. Only representative images taken at 1 h are shown here Scale bar = 20 µm. Images from three experiments were used for colocalization analyses of DAF-FM and Mitotracker Red in Wt, RI29 and B7 plants mock-inoculated with dH$_2$O or treated with antimycin A. The Manders’ coefficients M1 (A) and M2 (B) measure the relative amount of DAF-FM signal overlapping Mitotracker signal (M1) and vice versa (M2). Data are the mean +/- S.E. of three independent experiments. Each set of data were analyzed by two-way ANOVA followed by a Bonferroni multiple comparison test to determine if they were significantly different from data obtained for Wt plants. (* P < 0.05).
Figure 5.14: Levels of cellular ONOO' in tobacco mesophyll cells treated with antimycin A and observed at different times. Three lines were used: Wt plants, AOX knockdown RI29 plants and AOX overexpressor B7 plants. The images shown depict APF fluorescence (green), Mitotracker Red fluorescence (red) where the colocalization between APF and Mitotracker Red could be observed as yellow signal. All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative results of three independent experiments, each of which showed similar results. Mock-treated plants incubated with H2O were monitored over the entire experimental period, without significant changes in ONOO' levels observed. Only representative images taken at 1h are shown here. Scale bar = 20 µm. Images from three independent experiments were used for colocalization analyses of APF and Mitotracker Red in Wt, RI29 and B7 plants mock-inoculated with dH2O or treated with antimycin A. The Manders’ coefficients M1 (A) and M2 (B) measure the relative amount of APF signal overlapping Mitotracker signal (M1) and vice versa (M2). Data are the mean +/- S.E. of three independent experiments. Each set of data were analyzed by two-way ANOVA followed by a Bonferroni multiple comparison test to determine if they were significantly different from data obtained for Wt plants (* P < 0.05; *** P<0.001).
Antimycin A

\[
\begin{array}{ccc}
H_2O & 1h & 4h \\
\hline
Wt & & \\
RI29 & & \\
B7 & & \\
\end{array}
\]

Manders' Coefficient (M1)

\[
\begin{array}{ccc}
\text{Control} & 1h & 4h \\
\hline
Wt & & \\
RI29 & & \\
B7 & & \\
\end{array}
\]

Manders' Coefficient (M2)

\[
\begin{array}{ccc}
\text{Control} & 1h & 4h \\
\hline
Wt & & \\
RI29 & & \\
B7 & & \\
\end{array}
\]

A.

B.
5.3 Discussion:

There is ample evidence for the involvement of plant mitochondria in the production of ROS during biotic stress, with perhaps the overall reduction level of the ubiquinone pool being the primary determinant of ROS output (Lam et al., 2001; Amirsadeghi et al., 2007; Yao et al., 2004; Yao and Greenberg, 2006; Ashtamker et al., 2007; Scott and Logan, 2008). This study examines the role and relative importance of AOX in tobacco plants under stress in relation to ROS accumulation and signaling in two ways. Two AOX knockdown lines, RI9 and RI29, were observed in their responses to infection with two different incompatible pathogens: *pv maculicola* that triggers the HR and *pv phaseolicola* that induces defense responses without the appearance of cell death. Bacterial infections typically affect plants in many different ways (Garcia-Brugger et al., 2006; Pritchard and Birch, 2011, Hann et al., 2010) making the study of a single system difficult to perform in isolation. In attempt to circumvent this difficulty, we tried to study the production of mitochondrial ROS and RNS and their relation to AOX, by treating Wt and transgenic plants with altered AOX levels with the Complex III inhibitor antimycin A. Keeping in mind that one of the proposed functions of AOX is to modulate the production of mitochondrial ROS by controlling ubiquinone reduction status and the mitochondrial membrane potential (Vanlerberghe et al., 2009, Chapter 3) it could be hypothesized that transgenic plants lacking AOX would generate more ROS and RNS due to stress, while plants overexpressing AOX would accumulate less of these molecules.

One of the major findings of this study is the observation of higher levels of mitochondrial O$_2^-$ in the AOX knockdown lines RI9 and RI29 infected with *pv phaseolicola*, which is not normally not seen in Wt plants infected with the same pathogen. However, despite a strong mitochondrial O$_2^-$ burst reminiscent of the HR, we did not observe the appearance of cell death lesions in these lines. This is not entirely surprising since it is widely recognized that ROS and RNS form a complex web of interactions during plant-pathogen interactions. It is possible that despite elevated mitochondrial O$_2^-$, PCD is not induced in transgenic plants due to lack of other components necessary to initiate this response. In our work we did observe elevated H$_2$O$_2$ levels as well, however the accumulation of this molecule did not follow the typical biphasic pattern generally associated with the appearance of PCD (Huang et al., 2003). Similarly, we did not observe differences between lines in the accumulation patterns of NO and ONOO$^-$. It has been suggested that the HR is triggered only when ROS and RNS are produced at properly balanced
ratios (Dalledonne et al., 2001; Beers and McDowell 2001), and our work demonstrated that these ratios are changed in transgenic plants lacking AOX. It should also be mentioned that there are several instances where it has been observed that an oxidative burst was uncoupled from the appearance of PCD (Glazener et al., 1996, Yano et al., 1999).

Surprisingly, AOX knockdown infected with the HR-inducing *pv maculicola*, showed a marked delay in both mitochondrial $\text{O}_2^-$ accumulation and the appearance of HR-like lesions. Interestingly, this effect was also observed when these transgenic lines were treated with antimycin A and these plants failed to generate an early mitochondrial $\text{O}_2^-$ burst. The similarity between these two responses suggests that certain parallels can be drawn between *pv maculicola* infection and treatment with antimycin A. It is well established that antimycin A is a potent inhibitor of the mitochondrial ETC that binds to Complex III, thereby preventing the oxidation of ubiquinol, blocking electron transport and leading to the overproduction of mitochondrial ROS (Moller, 2001; Sweetlove and Foyer, 2004). The mode of action of bacterial pathogens is not established in such detail, however there are several studies that link incompatible infections to mitochondrial dysfunction. Large scale studies have predicted that nearly all *P. syringae* effectors target mitochondria or chloroplast (Guttmann et al., 2002; Greenberg and Vinatzer, 2003). An example studied in more depth is HopG1, a type III effector broadly conserved in different *P. syringae* pathovars. It was recently shown that HopG1 localizes to the mitochondria, leads to decreased respiration rates and increased levels of mitochondrial ROS (Block et al., 2010). The bacterial elicitor harpin was also found to cause mitochondrial dysfunction by lowering of the membrane potential and decreasing ATP production (Krause and Durner, 2004). Furthermore, incompatible bacterial infections generally lead to accumulation of high levels of SA and NO, both of which are known inhibitors of the mitochondrial ETC (Norman et al., 2004; Huang et al., 2002; Zottini et al., 2002; Ederli et al., 2006).

As mentioned above, there are several lines of evidence linking $\text{O}_2^-$ accumulation with the appearance of HR. Our results seem to add additional weight to these arguments, since delays in the mitochondrial $\text{O}_2^-$ burst were accompanied with delays in the HR symptoms in AOX knockdowns infected with *pv maculicola*. There could be two possible explanations for this effect. First, $\text{O}_2^-$ could act as a promoter of cell death, with SOD being an important mediator of this role. Second, $\text{O}_2^-$ could suppress the HR by scavenging NO, one of the known inducers of PCD (Beers and McDowel, 2001). Our results seem to suggest that the effect observed here is a
combination of these two possibilities. In Wt plants infected with pv *maculicola*, a strong and sustained mitochondrial O$_2^-$ and cellular NO bursts preceded the appearance of HR-like lesions. In contrast, transgenic plants silenced for AOX did not show rapid accumulation of mitochondrial O$_2^-$ early after infection, and had lower levels of NO late in the infection. It is interesting to note that these plants also had higher levels of ONOO$^-$ at later time points, possibly due to the scavenging effect of O$_2^-$ on NO later in the infection (Wullf et al., 2009). Several other studies have pointed out the importance of timing and the right balance between different ROS and RNS for the proper initiation of PCD (Clarke et al., 2000; de Pinto et al., 2002; Delledonne et al., 2001; Delledonne et al., 1998).

We currently do not know the reason for the transient delay in O$_2^-$ generation in transgenic plants lacking AOX treated with pv *maculicola* or antimycin A. We previously demonstrated that the lack of AOX disrupts the ROS and RNS homeostasis under steady state conditions, where RI9 and RI29 lines accumulated more O$_2^-$ and NO (but less H$_2$O$_2$) and it is possible that this imbalance leads to increased resistance to accumulation of ROS under stress conditions by an unknown mechanism. However, it should be pointed out that this delay is not due to higher SOD activity in AOX knockdown plants, since we did not observe this in our study. Several studies have suggested that AOX levels are coordinated with the levels of other mitochondrial ETC components, particularly with energy dissipating enzymes such as uncupling proteins or the alternative NAD(P)H dehydrogenases (Yoshida et al., 2008; Elhafez et al., 2006; Clifton et al., 2005). So far small changes in these ETC components or in the genes encoding them have been observed (Umbach et al., 2005; Giraud et al., 2008; Watanabe et al., 2008), however how much these changes would affect the ROS and RNS balance under stress is still not clear.

An example of ETC imbalance that has been studied in more detail is the CMSII mutant that lacks a functional Complex I (Guttieres et al., 1997). Despite the lack of Complex I activity, this mutant does not show decreased respiration rates under steady state, however mutant plants were found to have lower mitochondrial H$_2$O$_2$ levels, possibly through increased capacity of the antioxidant systems, and an increased resistance to pathogen attacks (Dutilleul et al., 2003b). Furthermore, the diurnal rhythm of AOX expression in this mutant is completely reversed. These effects were shown to have an impact on metabolism and nuclear gene expression downstream of the mitochondria (Noctor et al., 2004). Based on this example, it is clear that changes in the components of the ETC may lead to ETC rearrangement and imbalance in various signaling
molecules. Transgenic plants lacking AOX would have to be characterized in more detail to determine if this is the reason behind differences in ROS and RNS accumulation in transgenic versus Wt plants under stress. For example, it would be interesting to observe the levels of other non-proton pumping proteins in the ETC and to relate their levels to ROS and RNS generation.

One of the findings of this study that lends support to the hypothesis that AOX controls the rate of ROS generation comes from the fact that AOX overexpressor plants generate less $O_2^-$, NO and consequently ONOO$^-$ when treated with antimycin A as compared to Wt plants. Rapid accumulation of $O_2^-$ does happen in Wt plants however this oxidative burst is transient and it decreases to control levels over time. It is well established that AOX is one of the most stress-responsive genes in the plant genome, and it is especially sensitive to treatment with inhibitors of the cyt pathway (Clifton et al., 2006; 2005; Mittler et al., 2004; Vanlerberghe and McIntosh 1994, 1992). Increase in AOX levels and activity can presumably decrease the reduction level of the mETC and thus lead to decreased ROS generation over time. From our study it is clear that the level of AOX transcript is rapidly increased following antimycin A treatment. Similarly, several other studies have found that AOX capacity and protein levels are similarly increased during treatment with this cyt pathway inhibitor. In transgenic plants that constitutively express AOX (and have high AOX capacity and protein levels even before the addition of antimycin A), this mechanism would be operational at the beginning of the treatment and thus over-production of ROS would be delayed or completely abolished. It is generally thought that the biological significance of decreased ROS production in transgenic plants is to increase the resistance of such plants to various abiotic and biotic stresses. For example overexpression of AOX in Arabidopsis protected against aluminium induced PCD (Li and Xing, 2011) and enhanced shoot growth at low temperatures (Florani et al., 2005). Similarly, infection of tobacco AOX overexpressor lines with TMV resulted in smaller HR-like lesions (Ordog et al., 2002). However, Gilliland et al. (2003) found that genetic manipulation of AOX levels had little effect on basal susceptibility to TMV and Pasqualini et al. (2007) observed increased levels of ozone-induced damage due to AOX overexpression in tobacco. Our study also did not observe any beneficial effect due to AOX overexpression in plants infected with the HR-inducing P. syringae pv maculicola. In this case plants overexpressing AOX did not differ significantly from Wt plants in terms of the timing of appearance and extent of the HR-like lesions (results not shown). It is
clear that the ROS and RNS network is very complex and requires further study in different experimental systems in order to be fully understood.

In summary, based on the results presented here we propose that mitochondrial ROS burst controlled by AOX is an important component for the induction and coordination of the HR. The mitochondrial $O_2^\cdot$ burst which is one of the first symptoms of infection with an HR-inducing incompatible bacterial pathogen might play a major role during triggering of PCD pathways. Furthermore, $O_2^\cdot$ could potentially regulate the levels of mitochondrial generated NO and ONOO$^-$, thus acting as a key regulator of several signaling pathways. This work suggests that the mitochondrial AOX holds a central place in regulating the appearance, extent and timing of the HR and potentially of defense responses as well. Further research into the mechanisms of AOX control and of the biological significance of the HR would be a rich area for further study.
CHAPTER 6

Summary and Future Directions
Chapter 6
Summary and future directions

Recently, the mitochondrion has emerged as an important player in plant-pathogen interactions, especially in initiating plant PCD and defense responses, however the details of the involvement of this organelle are still not fully known (Lam et al., 2001; Amirsadeghi et al., 2007; Noctor et al., 2007; Kusano et al., 2009; van Doorn et al., 2011). One hypothesis is that mitochondria act as an early source of ROS during pathogen infections and that this ROS is one of the signals for the activation and execution of plant responses (Naton et al., 1996; Arpagaus et al., 2002; Tiwari et al., 2002; Yao et al., 2002; Weir et al., 2003; Takahashi et al., 2004; Yao et al., 2004; Yao and Greenberg, 2006; Ashtamker et al., 2007; Scott and Logan, 2008). However, the exact role, or even the chemical nature of mROS ($O_2^-$ or $H_2O_2$) in activating cellular death and survival pathways is still not known. Furthermore, the interaction of ROS and other signaling molecules originating in the mitochondria, such as RNS, or affecting the mitochondria, such as plant hormones, is currently not clear. The major focus of the work presented in this thesis has been to investigate the role of the mitochondria, and more specifically the mitochondrial AOX, during defense and PCD in plant-pathogen interactions.

We hypothesized that AOX plays a role in the generation of mROS (and potentially RNS) by modulating mitochondrial membrane potential and preventing the overreduction of ETC components. In this way the level of AOX could help control the intensity of the response (weak or strong induction of defense pathways) and the type of the response (survival or PCD) during biotic stress. In this work, we tried to test the hypothesis that one of the functions of AOX is to modulate the generation of ROS and RNS. We also related AOX function with the determination of cell fate during response to bacterial pathogens, by exploring the level of AOX and various signaling molecules in different plant-bacteria interactions. Finally, we addressed the question of what chemical signal is necessary for the initiation of cell death during bacterial infections, and how important is AOX during the generation of this signal. From the findings presented in this work, a model was constructed that explains some of the aspects of mitochondrial signaling during defense and PCD pathways (Figure 6.1).
Figure 6.1: ROS and RNS are important signaling molecules for the initiation and execution of PCD and defense responses during biotic stress. In the model outlined here, it is suggested that AOX and MnSOD are uniquely positioned to control the strength and specificity of the mitochondrial ROS signal, thus determining cell fate. AOX could control the strength of the signal (total amount of ROS and RNS generated) by dampening the electron leak from the ETC. MnSOD could control the chemical signature of the signal and the balance between different signaling molecules (H₂O₂ or O₂⁻) by controlling the rate of O₂⁻ scavenging. MnSOD might also influence the distribution of the ROS signal between the mitochondria and the cytosol, since H₂O₂ is membrane permeable and O₂⁻ is not. The mitochondrial ETC could also act as a source of electrons for the production of NO, in a way analogous to the production of O₂⁻. The interaction between NO and O₂⁻ removes these molecules from the system and produces ONOO⁻, another potential cellular signal. We propose that AOX holds a central position in the mitochondria for the control of the levels of various ROS and RNS, which act as signals for the induction of various plant responses during biotic stress.
We used transgenic tobacco plants with modified AOX levels grown under steady-state conditions in order to directly test the hypothesis that AOX modulates the generation of ROS and RNS. Based on data, we established that a lack of AOX increases the levels of both mitochondrial O$_2^-$ (but not H$_2$O$_2$) and leaf NO and we proposed that this is due to a higher reduction level of components within the mitochondrial ETC. However, there were several considerations that should be kept in mind when critically accessing these experiments. First, even though the level of O$_2^-$ and NO in transgenic plants lacking AOX was higher than in Wt plants, this did not have an effect on the viability of the plants under steady-state conditions. Second, we proposed an AOX-controlled mechanism of mitochondrial ROS and RNS generation under steady-state conditions, however this did not exclude the possibility that several different pathways might be operational (mitochondrial or otherwise) under stress conditions.

A large part of the work presented here was done in order to determine the role of AOX in modulating a mitochondrial signaling pathway leading to different symptoms in plants under biotic stress. Numerous studies have been performed to determine what signals are required for the activation of the HR (Reviewed in Mur et al., 2008). While it is generally accepted that a ROS and RNS burst are associated with PCD activation, the exact mechanism and importance of these processes is still not known. For example, the chemical nature and interactions between these signaling molecules are still a matter of debate. Also, the distinction between the activation of PCD and the activation of defense responses is rarely made in the same system, even though both are components of incompatible plant-pathogen interactions. The results presented in this work suggest that both cell death and defense responses share a common signaling pathway involving mitochondria, however they become divergent and activated by chemically different signaling molecules downstream of this organelle. Based on this work it can be hypothesized that mitochondrial O$_2^-$ is the signal for the initiation of PCD while cell survival pathways are potentially mediated by H$_2$O$_2$. In this model, AOX activity would modulate the strength of the mitochondrial signal (or the total amount of ROS being produced) while MnSOD, the only means of enzymatically scavenging O$_2^-$ in the mitochondria, would determine the specificity of the signal, thus modulating the relative levels and ratios between O$_2^-$ and H$_2$O$_2$. It appears that AOX is in a key position to regulate mitochondrial RNS production as well, either directly by influencing the membrane potential, or indirectly via the scavenging reaction between O$_2^-$ and
NO. Our results suggest that AOX is a key regulator of several signaling pathways influencing the downstream cell fate during stress.

It is intriguing that there are differences in AOX expression between the two species used in this work. While AOX was downregulated due to infection with the HR-inducing pv *maculicola* in tobacco, we showed that AOX transcript levels were increased in *Arabidopsis* plants infected with the incompatible HR-inducing pv *tomato* DC3000 AvrRpt2. Such discrepancies between species have been noted before (Lacomme and Roby, 1999; Simmons et al., 1999; Boccara et al., 2001; Krause and Durner, 2004; Kiba et al., 2008; Cheng et al., 2011) and suggest that there might be fundamental differences in the regulation of AOX expression and activity between plant species, as well as in the role of AOX during the induction of PCD. Observing some of the crucial events during PCD signaling, such as SOD activity and ROS accumulation, in *Arabidopsis* might be a good way to expand on this research topic.

The hypothesis that O$_2^-$ is an important signal for the induction of PCD was confirmed in our work with transgenic plants, where the lack of AOX lead to a delayed mitochondrial O$_2^-$ burst, an imbalance on ROS and RNS accumulation and a delay in the appearance of the HR. It is interesting that such a delay was also seen in transgenic plants silenced for AOX when treated with antimycin A. The reason for this delay in the oxidative bursts is currently unknown however it can be assumed that the lack of AOX in transgenic plants is directly linked with the observed delay in the generation of mitochondrial ROS. One possibility is that knockdown plants are ‘primed’ to withstand increases in O$_2^-$ and NO without any detrimental effects on plant viability due to higher levels of these molecules under steady-state conditions. The nature of this ‘priming’ is not clear as of yet (especially since only small differences in SOD activities were observed in these lines under steady-state conditions and biotic stress) but it promises to be a rich area for future studies. For example, it would be interesting to observe the levels of other ROS and RNS scavengers in these systems both during steady-state and stress conditions, such as the activity of the H$_2$O$_2$ scavenges APx, GPx and CAT, as well as the activity of GSNOR, the enzyme that regulates NO scavenging by GSNO.

Another intriguing possibility is that the imbalance of ROS and RNS in transgenic plants lacking AOX is due to a permanent rearrangement of components of the ETC. One of the better studied mitochondrial mutants is the CMSII mutant that lacks a functional Complex I. A certain
rearrangement in the mitochondrial ETC has been reported for this mutant (Noctor et al., 2004; Dutilleul et al., 2003b) however how these changes affect the levels of various signaling molecules is still not clear. It is possible that the situation is similar in transgenic plants that lack AOX and a careful study of gene expression and activity of components of the ETC might prove to be fruitful. It would be especially interesting to observe the levels of other non-proton pumping proteins in the ETC and to relate their levels to ROS and RNS generation.

One conclusion that can be taken from the data presented in this thesis is that the level of mitochondrial \( \text{O}_2^- \) is an important determinant of the fate of the cell downstream of a stress response, either directly or through the interactions of this molecule with other molecular signals, since a delay in \( \text{O}_2^- \) accumulation was associated with a delay in the appearance of HR symptoms in transgenic plants lacking AOX challenged with \( \text{pv maculicola} \). My work suggests that this delay is due to the lack of AOX; however attempts can be made to test the hypothesis in a different manner. If we assume that the mitochondrial \( \text{O}_2^- \) is one of the key signals for the appearance of HR-like lesions in plants infected with incompatible bacteria, then removing this molecule from the system would also potentially cause delays, or even the abolishment, of PCD. In such an experiment, one could treat leaves with an \( \text{O}_2^- \) scavenger and observe the effect that a bacterial infection has in this scenario. Similarly, transgenic plants overexpressing MnSOD would potentially be able to scavenge \( \text{O}_2^- \) more successfully and thus cause a delay in the appearance of death under biotic stress. Based on my data it is also clear that \( \text{O}_2^- \) is not the only signal required for PCD activation, since transgenic plants that lack AOX had elevated \( \text{O}_2^- \) levels (but without an increase in RNS levels) when challenged with \( \text{pv phseolicola} \) that does not induce the HR. This is an interesting observation that could be further studied to fully understand the interactions between signaling molecules during stress. Experiments where RNS are scavenged from the system prior to bacterial infection could provide us with the information needed to fully elucidate these pathways. Experiments such as these might be very beneficial in further characterizing the mechanism responsible for cell death activation versus activation of other defense responses.

Another important question stemming from this work concerns the signal regulating AOX expression during stress. While previous studies have shown that signaling molecules such as NO and SA can induce AOX transcription (Rhoads and McIntosh, 1992; Van Der Straeten et al., 1995; Djajanegara et al., 2002; Ho et al., 2008; Lei et al., 2010, Huang et al., 2002; Zottini et al.,
2002; Ederli et al., 2006), my work clearly shows that this is not the case in all situations. For example, AOX transcript was not detected during the HR even though ROS, RNS and SA were all accumulated at high levels during this response. Furthermore, scavenging \( \text{O}_2^- \) or NO did not have a large effect on the induction of AOX by antimycin A (see Appendix I). The reason for this effect is still not clear, but it is possible that during situations where PCD is induced the expression of AOX is actively suppressed either by a currently unknown plant mechanism or even by the pathogen. This could be especially true in the case of necrotrophic or hemi-necrotrophic pathogens (such as \( P. \text{syringae} \) used in this study) that require host cell death for successful proliferation and colonization. The mechanism of this suppression is currently not known, but it is an interesting possibility to consider.

The definition of a cell death pathway dependant on the balance of ROS and RNS may not be exclusively associated with the HR. PCD is a process found in several developmental processes and environmentally-induced stresses. The ability to modulate this type of cell death in response to the severity of the stress applied via the mitochondria and AOX can provide the plant with a great deal of flexibility in responding to a variety of stress situations. This could suggest that modulating the level of AOX (and possibly MnSOD) represents a global response to stress. As such, it would be beneficial to determine if abiotic stresses, such as chilling, drought, heat or light stress have a similar effect on the levels of AOX, and furthermore if they induce similar pathways of cell death involving mitochondria-generated ROS. It would also be interesting to determine if transgenic plants silenced for AOX show a similar delay in exhibiting symptoms of cell death when challenged with an abiotic stress, as they show during biotic stress. I believe that further work in this field would be a promising target for developing more successful strategies for plant stress tolerance.
References


Buell CR, Joardar V, Lindeberg M, Selengut J, Paulsen IT, Gwinn ML, Dodson RJ, Deboy RT, Durkin AS, Kolonay JF et al. (2003) The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. tomato DC3000. *PNAS USA* **100**: 10181-10186


Doke N (1983) Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. *Physiological and Molecular Plant Pathology* **23**: 345-357
Doke N, Ohashi Y (1988) Involvement of an O$_2^-$ generating system in the induction of necrotic lesions on tobacco leaves infected with tobacco mosaic virus. *Physiological and Molecular Plant Pathology* **32**: 163-175


Gupta KJ, Stoimenova M, Kaiser WM (2005) In higher plants, only root mitochondria, but not leaf mitochondria reduce nitrite to NO, in vitro and in situ. *Journal of Experimental Botany* **56**: 2601-2609


**Jeong SY, Seol DW (2008)** The role of mitochondria in apoptosis. *BMB Reports* **41**: 11-22


Ordog SH, Higgins VJ and Vanlerberghe GC (2002). Mitochondrial alternative oxidase is not a critical component of plant viral resistance but may play a role in the hypersensitive response. *Plant Physiology* **129**: 1858-1865


Vanlerberghe GC, Yip JYH, Parsons HL (1999) In organello and in vivo evidence of the importance of the regulatory sulphydryl/disulfide system and pyruvate for alternative oxidase activity in tobacco. Plant Physiology 121: 793-803


Yip JYH, Vanlerberghe GC (2001). Mitochondrial AOX acts to dampen the generation of active oxygen species during a period of rapid respiration induced to support a high rate of nutrient uptake. *Physiologia Plantarum* **112**: 327-333


Appendix I
Leaf AOX transcript levels are affected by antimycin A

We have previously shown that infection of tobacco leaves with *P. syringae pv maculicola* did not lead to accumulation of *Aox1a* transcript despite a strong increases in ROS and RNS levels during this interaction (see Chapter 4). ROS (primarily H$_2$O$_2$) and RNS (primarily NO) have each been previously shown to induce AOX gene expression in various plant species and tissues (Vanlerberghe and McIntosh 1996; Huang et al., 2002; Ederli et al., 2006; Ho et al., 2008). Such studies have led to the development of a general model whereby overproduction of these ROS or RNS by the respiratory chain acts as a retrograde signal from mitochondria to the nucleus to increase *Aox1a* expression (Rhoads and Subbaiah 2007; Schwarzländer et al., 2012). However, this model is based primarily upon experiments involving the exogenous addition of ROS or RNS and it remains unclear the degree to which mitochondrial-generated ROS and RNS might act as retrograde signals to control AOX gene expression.

We explored the validity of this model by monitoring *Aox1a* levels in leaves treated with antimycin A (shown to cause a rapid increase in ROS and RNS in Wt plants, see Chapter 5) as well as leaves co-treated with this compound and various scavengers of ROS and RNS.

**Materials and Methods**

Tobacco leaves were removed and the lower epidermis peeled off. The remaining leaf segments were then floated (RT, dark) on water or 10 µM antimycin A (AA) (A8674; Sigma-Aldrich, Oakville, Ontario, CA). In some cases, the floating solution also contained a scavenger of ROS or RNS. These scavengers included: the cell-permeating O$_2^-$ scavenger superoxide dismutase-polyethylene glycol (SOD-PEG, 100 U) and the cell-permeating NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 200 µM). We have previously shown the effectiveness of these scavengers in tobacco leaf (Chapter 3). After the
floating treatments (1 h or 4 h), leaves were processed for either confocal microscopy imaging or for transcript determination (see Chapter 2 for detailed procedure description).

**Results**

SOD-PEG is a cell permeable $\text{O}_2^-$ scavenger (Mukhopadhyay et al., 2007) that does not have an effect on the leaf when applied alone but it can successfully reduce the amount of $\text{O}_2^-$ in Wt plants treated with antimycin A (**Figure S1A**). cPTIO is a NO scavenger (Pfeiffer et al., 1997) that successfully delayed the accumulation of NO in plants treated with antimycin A. After 1h co-treatment with both compounds leaf levels of NO were significantly lower than in leaves treated with antimycin A alone (**Figure S1B**). However, at 4 h after treatment, the difference was not as pronounced, indicating less effective scavenging by this later time point.

Leaf $Aox1a$ transcript levels were compared in Wt plants treated with antimycin A and co-treated with this compound and both ROS and RNS scavengers (**Figure S2**). The mock treatment with $H_2O$ and treatment with the scavengers alone had no effect on transcript levels. $Aox1a$ transcript levels did not respond strongly to a short-term treatment (1 h) however there was a strong increase at 4 h due to antimycin A treatment. We observed a similar increase when plants were co-treated with antimycin A and either cPTIO or SOD-PEG for 4 h, with the expression of $Aox1a$ being slightly lower when plants were co-treated with an inhibitor.
Figure S1: Wt tobacco plants treated with antimycin A (AA) and scavengers for ROS and RNS (A) Mitochondrial O$_2^-$ levels observed at two time points treated with 10 µM antimycin A and/or 100 U SOD-PEG. (B) Leaf NO levels observed at two time points treated with 10 µM antimycin A and/or 200 µM cPTIO. The images shown depict DAF-FM fluorescence (green), Mitotracker Red fluorescence (red) where the colocalization between DAF-FM and Mitotracker Red can be observed as yellow signal. All images are maximum intensity projections of Z-stacks (8-16 µm in depth) and are representative of at least three independent experiments, each of which showed similar results. Scale bar = 20 µm.
Figure S2: Levels of *Aox1a* transcript in Wt tobacco leaf at different times treated with 10 µM antimycin A and/or 100 U SOD-PEG and 200 µM cPTIO. Data in (A) is a representative Northern blot. Data in (B) are the mean +/- S.E of at least four independent experiments, each of which showed similar results. All data sets are compared to the level of mRNA in control non-treated leaves which was arbitrarily set to 1. Quality of RNA and confirmation of equal loading between lanes was routinely checked by ethidium bromide staining (Representative blot shown).
**Discussion**

We previously showed that antimycin A treatment rapidly increased WT leaf levels of \( \text{O}_2^- \), NO and ONOO\(^-\), as well as the expression of \( \text{Aox1a} \). To further test the retrograde model of \( \text{Aox1a} \) expression, we used scavenging compounds to control the level of specific ROS and RNS, examining then the effectiveness of the different scavengers to block \( \text{Aox1a} \) induction by antimycin A. SOD-PEG and cPTIO are scavengers of \( \text{O}_2^- \) and NO respectively. Importantly, we were able to use our confocal microscopy methods to confirm the effectiveness of each scavenger to maintain low levels of specific ROS and RNS in the presence of antimycin A.

Based on our results with AOX overexpressors and knockdowns so far, it does appear that AOX is an important player in the regulation of ROS and RNS production and cellular balance. Even though our work with transgenic plants has yielded some important information about the function of AOX, at present we do not know the mechanism by which Wt plants regulate the expression of AOX under different conditions. It is especially surprising that AOX gene expression is not induced in plants undergoing the HR due to pathogen infection, despite dramatically induced levels of various ROS and RNS (Chapter 4), most of which have been shown to induce AOX under certain conditions. For example, several studies have found that application of \( \text{H}_2\text{O}_2 \) induced AOX expression (Wagner 1995, Vanlerberghe and McIntosh, 1996) and recent work has shown that the AOX promoter in *Arabidopsis* has \( \text{H}_2\text{O}_2 \) responsive elements (Ho et al., 2008). NO application has also been linked to increases in AOX expression (Huang et al., 2002; Zottini et al., 2002; Ederli et al., 2006). Currently, it is not clear whether these molecules induce AOX expression due to their role as signaling molecules, or this induction is the effect of ETC inhibition and mitochondrial dysfunction brought about by the application of ROS and RNS. It is possible that \( \text{H}_2\text{O}_2 \) and NO are positive regulators of AOX expression, however during situations where PCD is induced the expression of AOX is actively suppressed ether by a currently unknown plant mechanism or even by the pathogen. The mechanism of this suppression is currently not known, but it is an interesting possibility to consider. To date, the signals regulating AOX expression under biotic stress are still unclear. Our work shows that scavenging \( \text{O}_2^- \) and NO from the experimental system did not have a significant effect on AOX expression due to antimycin A treatment. The mechanisms of this induction is still not clear, however if AOX is an important component for the plant response to biotic stress it is expected
that it would possess a flexible level of transcriptional control. Future studies are needed to provide more evidence for the regulation of AOX expression.
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Statement of Publications
The research presented in this thesis has appeared or has been submitted as a series of original publications in refereed journals.

Chapter 3


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