Investigating the Role of Alternative Oxidase in *Nicotiana tabacum* during Light Acclimation

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science,

Graduate Department of Cell and Systems Biology and Graduate Department of Biological Sciences

University of Toronto

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Abstract

Photosynthetic electron transport produces ATP and NADPH which support carbon fixation by the Calvin Cycle. To avoid over-reduction of the electron transport chain, plants must balance absorption and consumption of light energy. Mitochondrial alternative oxidase (AOX) is a non-energy-conserving electron sink, making it an ideal candidate to oxidize excess reductant and regulate chloroplastic redox state. Wild-type (WT) and transgenic *Nicotiana tabacum* lines with enhanced or suppressed AOX protein levels were grown under low light (LL) and moderate light (ML). LL-grown plants were also shifted to ML. AOX transcript and protein levels were enhanced in WT plants under ML. Chlorophyll fluorescence, gas exchange, and contents of chlorophyll, carbohydrate, and malondialdehyde were measured. Lack of AOX protein decreased Photosystem II (PSII) quantum efficiency and CO$_2$ assimilation rates while enhancing PSII excitation pressure compared to WT. These findings suggest a role for AOX in mediating the chloroplast-mitochondrion relationship during acclimation to higher irradiance.
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1-q<sub>P</sub>  PSII excitation pressure
O<sub>2</sub>  Singlet oxygen
3-PGA  3-phosphoglycerate
Φ<sub>PSII</sub>  Photosynthetic efficiency of PSII
ADP  Adenosine diphosphate
AL  Actinic light
AMG  Amyloglucosidase
ANOVA  Analysis of variance
AOX  Alternative oxidase
APX  Ascorbate peroxidase
ATP  Adenosine triphosphate
B7, B8  AOX1a over-expressing transgenic plant lines
CAT  Catalase
CMSII  Cytoplasmic male sterile II
CoA  Coenzyme A
COX  Cytochrome c oxidase
COXII  Cytochrome c oxidase subunit II
CuZnSOD  Copper zinc superoxide dismutase
DHAP  Dihydroxyacetone phosphate
DNA  Deoxyribonucleic acid
F<sup>’</sup>  Steady-state fluorescence
FADH<sub>2</sub>  Flavin adenine dinucleotide
FeSOD  Iron superoxide dismutase
F<sub>m</sub>  Maximum fluorescence in the dark
F<sub>m’</sub>  Maximum fluorescence in the presence of actinic light
F<sub>o</sub>  Minimum fluorescence in the dark
F<sub>o’</sub>  Minimum fluorescence in the presence of actinic light
FR  Far-red light
F<sub>v</sub>  Variable fluorescence, calculated by F<sub>m</sub>-F<sub>o</sub>
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>G3P</td>
<td>Glyceraldehyde-3-phosphate</td>
</tr>
<tr>
<td>G6PDH</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDC</td>
<td>Glycine decarboxylase complex</td>
</tr>
<tr>
<td>GPX</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HK</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>INV</td>
<td>Invertase</td>
</tr>
<tr>
<td>LHC</td>
<td>Light harvesting complex</td>
</tr>
<tr>
<td>LHCII</td>
<td>Light harvesting complex associated with Photosystem II</td>
</tr>
<tr>
<td>LL</td>
<td>Low light (120 µmol m$^{-2}$ s$^{-1}$)</td>
</tr>
<tr>
<td>Mal</td>
<td>Malate</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>ML</td>
<td>Moderate light (700 µmol m$^{-2}$ s$^{-1}$)</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NAD-MDH</td>
<td>NAD$^+$-dependent malate dehydrogenase</td>
</tr>
<tr>
<td>NAD(P)-G3PDH</td>
<td>NAD(P)$^+$-dependent glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NDH</td>
<td>NADH dehydrogenase</td>
</tr>
<tr>
<td>NPQ</td>
<td>Non-photochemical quenching</td>
</tr>
<tr>
<td>OAA</td>
<td>Oxaloacetate</td>
</tr>
<tr>
<td>pETC</td>
<td>Photosynthetic electron transport chain</td>
</tr>
<tr>
<td>$P_1$</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PGA</td>
<td>Phosphoglycerate</td>
</tr>
<tr>
<td>PGI</td>
<td>Phosphoglucone isomerase</td>
</tr>
<tr>
<td>PPFD</td>
<td>Photosynthetic photon flux density</td>
</tr>
<tr>
<td>PQ</td>
<td>Plastoquinone</td>
</tr>
<tr>
<td>PSI</td>
<td>Photosystem I</td>
</tr>
<tr>
<td>PSII</td>
<td>Photosystem II</td>
</tr>
<tr>
<td>$q_E$</td>
<td>Non-photochemical quenching, PsbS-dependent</td>
</tr>
</tbody>
</table>
$q_l$ Non-photochemical quenching, PsbS-independent
$q_{Pl}$ Photochemical quenching ("lake" model)
$q_N$ Non-photochemical quenching
$q_P$ Photochemical quenching ("puddle" model)
$q_T$ Non-photochemical quenching associated with state transitions
RI29, RI9 $AOX1a$ RNA-interference transgenic plant lines
RNA Ribonucleic acid
ROS Reactive oxygen species
SHAM Salicylhydroxamic acid
SHMT Serine hydroxymethyltransferase
SOD Superoxide dismutase
SP Saturating pulse
TCA Tricarboxylic acid
TPI Triose-phosphate isomerase
TPT Triose-phosphate/phosphate transporter
WT Wild-type, non-transgenic plant lines
VDE Violaxanthin de-epoxidase
Chapter 1: Introduction

1.1 Respiration

Respiration describes the process utilized by plants and animals in which carbohydrates are oxidized, which leads to the conversion of these organic substrates into energy. In organisms undergoing aerobic respiration, respiration consists of glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation.

The tricarboxylic acid (TCA) cycle, also known as the Krebs cycle, is an important feature of mitochondrial respiration. Upon input of acetyl coenzyme A (CoA) to the TCA cycle, oxaloacetate (OAA) is oxidized in a series of steps (Figure 1). The TCA cycle results in the regeneration of OAA, the release of CO₂, and the generation of the reducing equivalents nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂). NADH and FADH₂ generated through the TCA cycle are oxidized through the process of oxidative phosphorylation.

Oxidative phosphorylation occurs in the mitochondrial electron transport chain. The mitochondrial electron transport chain in plants is composed of five protein complexes (Dudkina et al., 2006) (Figure 2). Four of these complexes, Complexes I to IV, are oxidoreductases, whereas the fifth complex, Complex V, is an ATP synthase (Dudkina et al., 2006).
Figure 1 The tricarboxylic acid (TCA) cycle. Acetyl CoA, typically generated from glycolysis through pyruvate oxidation, is required for operation of the cycle. Reaction of oxaloacetate and acetyl CoA commences a series of oxidation reactions, generating CO$_2$ and the reductant nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH$_2$).
**Figure 2** The mitochondrial electron transport chain in plants. Components of the phosphorylating pathway are depicted in red, whereas components of the non-phosphorylating pathway are shown in blue. During oxidative phosphorylation, the electron carrier nicotinamide adenine dinucleotide (NADH) is oxidized at NADH-ubiquinone oxidase (Complex I) and flavin adenine dinucleotide (FADH$_2$) is oxidized by a succinate-ubiquinone oxidase (Complex II). Subsequent electrons are passed to ubiquinone (Q). Electrons from the ubiquinol pool are transferred to ubiquinol-cytochrome c oxidase (Complex III), which contributes towards the generation of an electrochemical gradient across the mitochondrial inner membrane. Electrons are then passed to cytochrome c oxidase (Complex IV), further contributing towards a proton gradient. The flow of protons back into the mitochondrial matrix supports ATP generation through ATP synthase (Complex V). In contrast to the phosphorylating pathway, the Type II NADH dehydrogenase (NDH) or alternative oxidase (AOX) do not directly contribute towards ATP synthesis. For clarity, activity of only one Type II NDH is depicted.
Complex I is a NADH-ubiquinone oxidoreductase which functions to oxidize NADH to NAD\(^+\), thus reducing ubiquinone into ubiquinol (Dudkina et al., 2006). Electrons may also enter the mitochondrial electron transport chain through Complex II, a succinate-ubiquinone oxidoreductase (Yankovskaya et al., 2003). Although both Complexes I and II contribute electrons towards the ubiquinone pool, the initial source of electrons in Complex II is FADH\(_2\) rather than NADH (Dudkina et al., 2006). Complex II is a monomer consisting of two subunits, making it the smallest complex of the oxidative phosphorylating pathway (Yankovskaya et al., 2003). From ubiquinol, the electrons are transferred to Complex III, a ubiquinol-cytochrome c oxidase (Berry et al., 2000; Hunte et al., 2000). This enzyme functions as a dimer to oxidize ubiquinol to ubiquinone, while simultaneously reducing cytochrome c (Dudkina et al., 2006). Crystallography analyses have revealed the monomer structure of Complex III to be comprised of 10-11 subunits (Hunte et al., 2000). A portion of Complex III is located in the inner mitochondria membrane, although parts of the enzyme also extend into both the mitochondrial inner membrane space and the mitochondrial matrix (Hunte et al., 2000). Finally, Complex IV, cytochrome c oxidase, catalyzes the reduction of \(\text{O}_2\) to \(\text{H}_2\text{O}\) (Dudkina et al., 2006). Cytochrome c oxidase functions as a monomer with 12-13 subunits in the mitochondrial membrane (Michel et al., 1998; Stock et al., 2000). Finally, the electrochemical gradient produced due to the pumping of protons out of the mitochondrial matrix into the intermembrane space is utilized to turn subunits of the F\(_1\) head of Complex V, which is an ATP synthase enzyme (Dudkina et al., 2006). The rotation of these subunits facilitates the phosphorylation of ADP, thus producing ATP (Dudkina et al., 2006).

From this discussion of the mitochondrial electron transport chain, it is apparent that respiration through oxidative phosphorylation is an important process that produces energy in the form of ATP from NADH and FADH\(_2\).
1.2 Non-Phosphorylating Pathways of the Mitochondrial Electron Transport Chain

In addition to the “classical”, phosphorylating pathway of the mitochondrial electron transport chain, plant mitochondria also possess a non-phosphorylating pathway. This pathway is composed of several proteins which do not contribute to the electrochemical gradient, and subsequently do not generate ATP (Dudkina et al., 2006). A number of such proteins have been identified, including NADH dehydrogenases and an enzyme entitled alternative oxidase (AOX) (Dudkina et al., 2006).

Since the non-phosphorylating pathway facilitates non-energy-conserving reactions, it may appear to be wasteful (Dudkina et al., 2006). However, research has indicated that the non-phosphorylating pathway is up-regulated under certain stressful conditions, and therefore may play a role in protecting the mitochondria under adverse conditions (Noguchi and Yoshida, 2008). The proportion of electrons flowing through either pathway is modulated by three main factors: the environment, such as temperature and availability of nutrients and CO₂; physiological characteristics, which include tissue age, injury and wounding; and the reduction state of the ubiquinone pool (Padmasree et al., 2002).

1.2.1 Type II NADH Dehydrogenases (NDH)

Several NADH dehydrogenases (NDH) exist in plant mitochondria which contribute electrons to the ubiquinol pool. These include Complex I of the phosphorylating pathway as well as Type II NDH (Rasmusson et al., 2004). Type II NDHs are considered part of the non-phosphorylating pathway because they do not contribute to the generation of the electrochemical gradient, or subsequent ATP production (Rasmusson et al., 2004). The activity of these NDH is insensitive to the Complex I inhibitor rotenone (Rasmusson et al., 2004). It is currently hypothesized that there are at least two internal and two external alternative NADH
dehydrogenases in plant mitochondria (Rasmusson et al., 2004). Based on sequence homology to yeast, three internal and three external Type II NDHs have been identified in Arabidopsis (Elhafez et al., 2006).

1.2.2 Alternative Oxidase (AOX)

As previously mentioned, transfer of electrons through the cytochrome c oxidase from the ubiquinone pool to O$_2$ contributes to the electrochemical gradient that drives ATP synthesis. However, cytochrome c oxidase is not the only such protein which catalyzes the reduction of O$_2$ to H$_2$O using electrons from the ubiquinone pool; another terminal oxidase exists in plants named alternative oxidase (AOX) (Millenaar and Lambers, 2003).

AOX is a di-iron carboxylate protein and is located in the inner mitochondrial membrane (Berthold and Stenmark, 2003). This 32 to 39 kDa protein functions as a dimer (Day and Wiskich, 1995). In its inactive form, the two monomers are covalently linked through a disulfide bridge (Vanlerberghe et al., 1999). Enhanced AOX activity has been reported for dimers with reduced bridges compared to those that are oxidized (Umbach and Siedow, 1993).

Similar to cytochrome c oxidase, AOX catalyzes the transfer of electrons from the ubiquinone pool, leading to the reduction of O$_2$ to H$_2$O (Millenaar and Lambers, 2003). The source of these electrons is the same as in cytochrome c oxidase, specifically from Complexes I and II, as well as alternative NDH (van Lis and Atteia, 2004). However, AOX is a member of the non-phosphorylating pathway because electron flow through AOX is not coupled to ATP synthesis (Millenaar and Lambers, 2003). Therefore, AOX appears to catalyze an energy-wasteful respiration process; this has led to intriguing speculation over the precise function of this mitochondrial protein.
To understand the function of AOX, it is useful to consider the implications of its independence from ATP synthesis. Similar to the dependence of the photosynthetic electron transport chain upon NADPH consumption through the Calvin Cycle, the movement of electrons through the mitochondrial electron transport chain depends on ATP consumption (Berthold and Stenmark, 2003). Under conditions where the supply of ADP is limiting, the activity of cytochrome c oxidase decreases, leading to the over-reduction of the mitochondrial electron transport chain (Berthold and Stenmark, 2003). The resulting accumulation of reductant may facilitate the generation of superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), particularly at Complexes I and III (Kowaltowski et al., 2009). Since AOX enables electron flow through the mitochondrial electron transport chain under ADP-limiting conditions, AOX may act as an energy overflow pathway under adverse environmental conditions (Amirsadeghi et al., 2007; Giraud et al., 2008). Enhanced levels of mitochondrial ROS have been observed in transgenic AOX1a knockout tobacco cell lines, suggesting that AOX may contribute towards suppression of ROS accumulation (Maxwell et al., 1999).

Studies investigating the relationship between AOX and redox status of the chloroplast under various abiotic conditions have been published. These studies frequently study the role of AOX by comparing the effects of the AOX chemical inhibitor salicylhydroxamic acid (SHAM) to untreated plants. Of particular interest is the role of AOX during light acclimation, since changes in photosynthetic photon flux density (PPFD) represent one of the most common oxidative stresses in plants (Gechev et al., 2003). Previous studies involving AOX demonstrate its putative importance in light responses. In Arabidopsis (Yoshida et al., 2007) and pea mesophyll protoplasts (Dinakar et al., 2010), enhanced AOX protein levels in response to higher PPFD have been observed. In addition to enhanced protein levels, SHAM-sensitive O$_2$ uptake was observed to increase in Arabidopsis shifted to higher PPFD (Bartoli et al., 2006), providing further evidence for a role of AOX under changing PPFD. Given this evidence for a role of
AOX in modulating responses to changes in PPFD, it would be interesting to consider whether this mitochondrial protein affects photosynthesis, an important light-dependent process.

1.3 Photosynthesis

Photosynthesis describes the process in which light energy is harvested by pigments in the thylakoid membrane of the chloroplast to produce ATP and NADPH (Figure 3). These products are then used to fix atmospheric CO$_2$ through the Calvin Cycle to produce carbohydrates, which are then utilized through respiration (Takahashi and Murata, 2008; Noguchi and Yoshida, 2008). Thus, photosynthesis and carbon metabolism are intimately linked through the cycle of absorption and consumption of light energy in the forms of ATP and NADPH.
**Figure 3** The photosynthetic electron transport chain. The absorption of light energy, particularly at 680 nm wavelengths, by the photocenter of Photosystem II (PSII) results in the splitting of O$_2$ and the accumulation of protons in the thylakoid lumen. Subsequent electrons are passed from PSII to plastoquinone (PQ), reducing plastoquinone and generating plastoquinol. The electrons are then transferred to the cytochrome b$_{6}$f complex (cyt b$_{6}$f). The cyt b$_{6}$f complex contributes towards the generation of an electrochemical gradient across the thylakoid membrane. Electrons passed to plastocyanin are relayed to Photosystem I (PSI), which maximally absorbs light energy at 700 nm wavelengths. Electrons then pass from PSI to ferredoxin (Fd). Fd transfers electrons to the ferredoxin NADP$^+$-reductase (FNR). The final electron acceptor is NADP$^+$, which is reduced to NADPH during the light-dependent reactions of photosynthesis. In addition to generation of nicotinamide adenine dinucleotide phosphate (NADPH), the proton gradient across the thylakoid membrane supports ATP generation through ATP synthase (ATP syn). NADPH and ATP generated through photosynthetic electron transport supports the Calvin Cycle.
1.4 Photoinhibition and the Importance of Energy Balance

Exposure of PSI and PSII to illumination excites chlorophyll $a$ molecules bound within the light harvesting complexes (LHC), raising their electronic state (Müller et al., 2001). When the excited chlorophyll $a$ molecules return to the ground state, the energy can be transferred to drive the reactions of photochemistry (Müller et al., 2001).

During the light-dependent reactions of photosynthesis, photodamage is a common and unavoidable consequence (Takahashi and Murata, 2008). Photodamage describes the inactivation of photocenters by light (Aro et al., 1993). The rate of this process is proportional to the incoming PPFD (Takahashi and Murata, 2008). Reaction center proteins of PSII, such as the D1 protein, are particularly susceptible to photodamage (Aro et al., 1993). When photodamaged, D1 proteins are rapidly removed, degraded, and replaced by newly synthesized D1 proteins (Ohira et al., 2005). This repair process, known as the D1 repair cycle, limits photodamage and contributes to the maintenance of photosynthetic electron transport (Ohira et al., 2005; Takahashi and Murata, 2008).

Photoinhibition describes the situation in which the rate of photodamage exceeds the capacity of repair processes (Aro et al., 1993). In this situation, photodamaged PSII photocenters cannot be repaired quickly enough to avoid their inactivation (Aro et al., 1993). The generation of reactive oxygen species (ROS) can result from the reaction between redox components of the photosynthetic electron transport chain and oxygen (Vass and Aro, 2008). For example, reaction of excited chlorophyll pigment molecules in the triplet state with molecular $O_2$ can generate singlet oxygen ($^1O_2$), a very reactive ROS intermediate (Krieger-Liszkay, 2005). Electrons from PSI can also be directly transferred to $O_2$ rather than ferredoxin-NADP$^+$-reductase, leading to the generation of the ROS superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$) (Takahashi and Murata, 2008). Superoxide is a particularly potent oxidant of a variety of
molecules, including lipids, nucleic acids, and proteins (Apel and Hirt, 2004). If allowed to remain in the chloroplast, ROS have the potential to inhibit repair mechanisms, which can subsequently lead to oxidative damage, or programmed cell death (Padmasree et al., 2002; Scheibe et al., 2005; Takahashi and Murata, 2008). For example, H$_2$O$_2$ inhibits the synthesis of D1 protein, thus contributing to photoinhibition (Ohira et al., 2005; Takahashi and Murata, 2008). While excess ROS accumulation may lead to oxidative damage, a number of ROS including O$_2^-$, H$_2$O$_2$, and nitric oxide have also been implicated as important redox signalling molecules (Scheibe et al., 2005).

The development of photoinhibition is enhanced under environmental conditions where CO$_2$ fixation is limited (Takahashi and Murata, 2008). These conditions include drought, high salt, nutrient deficiency, and high and low temperature stresses (Takahashi and Murata, 2008; Noguchi and Yoshida, 2008; Li et al., 2009). Excess PPFD is another common cause of this energy imbalance, since plants must acclimate to changes in their light environment on both short and long-term scales (Wagner et al., 2008). A discussion of the relationship between CO$_2$ fixation, excess excitation energy, and ROS follows.

Under normal conditions, the uptake of light energy and the consumption of reductant subsequently produced are in balance. However, when plants experience excess excitation energy, or are under conditions where CO$_2$ fixation is limited, energy absorption may exceed energy utilization (Takahashi and Murata, 2008; Li et al., 2009). Since the utilization of reductant through CO$_2$ fixation in the Calvin Cycle is insufficient compared to the amount of light energy absorbed, a limitation of NADP$^+$ in the chloroplast ensues (Takahashi and Murata, 2008). However, light capture continues even when other metabolites are limiting (Aro et al., 1993). This leads to the over-reduction of the plastoquinol (PQ) pool and, as previously described, the generation of ROS (Aro et al., 1993; Murata et al., 2007; Li et al., 2009).
In conclusion, the balance between absorption and consumption of light energy is critical for maintenance of the redox state of the photosynthetic electron transport chain. Under excess excitation energy, or conditions that limit CO₂ fixation, mechanisms must exist which enable plants to adapt and acclimate to their changing environment. A number of these protective processes will be described in the following section.

1.5 Strategies to Avoid the Effects of ROS Accumulation

Several strategies exist in plants to maintain the redox state in the chloroplast under conditions where absorption of excess excitation energy exceeds utilization. These can be broadly grouped into four general categories: the reduction of excitation energy reaching photocenters, the redirection of electrons from photosynthetic electron transport, the redirection of generated reductant from the chloroplast, and the scavenging and metabolism of ROS (Table I).
Table I Strategies utilized by plants to maintain redox status in the chloroplast under excess excitation energy. Strategies can be broadly classified into four main categories. First, plants may reduce excitation energy reaching photocenters. Second, plants may adapt to increased excitation energy by redirecting photosynthetic electrons from entering the photosynthetic electron transport chain. Third, the export of reductant out of the chloroplast contributes to maintenance of chloroplastic redox state. Finally, the metabolic scavenging of ROS contributes towards maintaining photosynthetic activity under excess energy conditions.

<table>
<thead>
<tr>
<th>Category</th>
<th>Strategy</th>
<th>Examples</th>
</tr>
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<tbody>
<tr>
<td>Reduce excitation energy reaching photocenters</td>
<td>Decrease light absorption</td>
<td>Chloroplast avoidance movement, light harvesting complex (LHC) monomerization</td>
</tr>
<tr>
<td></td>
<td>Dissipate excess energy as heat</td>
<td>Non-photochemical quenching, xanthophyll cycle, conformational changes in LHC proteins</td>
</tr>
<tr>
<td>Redirect electrons from photosynthetic electron transport</td>
<td>Use alternative electron sinks</td>
<td>Photorespiration, Mehler reaction</td>
</tr>
<tr>
<td>Redirect reductant of photosynthetic origin</td>
<td>Export excess reductant</td>
<td>Malate valve, triose-phosphate/phosphate transporter</td>
</tr>
<tr>
<td>Scavenging of reactive oxygen species (ROS)</td>
<td>Scavenging and metabolism of generated ROS</td>
<td>Superoxide dismutase (SOD) enzymes, ascorbate-gluthathione cycle</td>
</tr>
</tbody>
</table>
Plants have developed several strategies to cope with excess excitation energy. First, plants may reduce excitation energy reaching photocenters, which alleviates the imbalance between absorption and utilization of light energy. This may be accomplished by the chloroplast avoidance movement. In this strategy, chloroplasts will reorient themselves to be parallel to the direction of incoming excitation energy, thus reducing light absorption (Li et al., 2009). Light harvesting complex (LHC) proteins associated with PSII can be degraded to reduce excitation energy absorption or moved between photosystems to balance excitation energy (Kargul et al., 2008).

Excess excitation energy can also be dissipated through the process of non-photochemical quenching (NPQ). This process, also known as antenna quenching, describes the dissipation of excess excitation energy as heat (Baker, 2008). NPQ is a complex process that includes the xanthophyll cycle, the PSII subunit PsbS, and LHCs. It has been estimated that more than 75% of absorbed light energy can be removed through NPQ (Vass and Aro, 2008). NPQ consists of three major components: q_T, q_I, and q_E. q_T represents non-photochemical quenching due to state transitions (Müller et al., 2001). Since q_T is considered negligible in most plants experiencing excess excitation energy, it will not be discussed further (Müller et al., 2001). q_I has been described as the PsbS-independent pathway contributing to NPQ (Dall’Osto et al., 2005). This component of NPQ is long-lasting and slowly reversible (Müller et al., 2001). It is believed that q_I is the major component of NPQ under prolonged or severe high light conditions. In contrast to q_I, q_E is a comparatively fast component of NPQ and can act within minutes or seconds to protect against short-term high light (Müller et al., 2001; Dreuw et al., 2005). This quenching process is the major component that contributes towards NPQ (Müller et al., 2001). q_E is regulated by the thylakoid lumen pH and depends on the presence of functional PsbS protein, a PSII subunit with unclear function (Müller et al., 2001; Dreuw et al., 2005; Li et al., 2009). A further discussion of the role of the lumen pH and the PsbS subunit in q_E follows.
During light absorption, protons accumulate in the thylakoid lumen, creating a proton gradient across the thylakoid membrane (Müller et al., 2001). As previously mentioned, excess excitation energy may limit CO₂ fixation, thus decreasing the availability of ADP and NADP⁺ (Takahashi and Murata, 2008). The decreased availability of ADP disables the transfer of electrons across the thylakoid membrane through ATP synthase, resulting in a decrease in pH in the lumen (Müller et al., 2001). There are two major consequences that result from the change in pH. First, the change in pH changes the conformation of the thylakoid membrane enzyme violaxanthin de-epoxidase (VDE) (Vass and Aro, 2008; Li et al., 2009). The protonation promotes the binding of VDE to the thylakoid membrane, thus bringing the enzyme closer to its substrate, violaxanthin (Li et al., 2009). VDE converts violaxanthin into antheraxanthin, which is then converted into zeaxanthin (Müller et al., 2001). Zeaxanthin is a xanthophyll with a high energy-quenching capacity (Gruszecki, 2010). In addition to its role in energy quenching, zeaxanthin may also help to protect the thylakoid membrane from lipid peroxidation by scavenging ROS or stopping lipid peroxidation reactions (Müller et al., 2001). Zeaxanthin is largely absent under low light conditions, but accumulates under conditions in which energy absorption and utilization are no longer balanced (Demmig-Adams et al., 1990). The importance of the xanthophyll cycle is evidenced by the observation that all organisms that show qₑ have a functional xanthophyll cycle (Müller et al., 2001). When the pH in the lumen increases, zeaxanthin is reverted back to violaxanthin by zeaxanthin epoxidase (Vass and Aro, 2008).

In addition to the activation of the xanthophyll cycle, the drop in the lumen pH also induces the protonation of LHC proteins of the PSII antennae (Li et al., 2009). It has been suggested that the protonation occurs on PsbS, a subunit of PSII that does not bind pigments (Müller et al., 2001; Ivanov et al., 2008). LHC are also altered by the binding of zeaxanthins (Müller et al., 2001). Together, the binding of both protons and xanthophylls to PSII subunits induces a conformational change in the photosystem, effectively decreasing the lifetime of
excited chlorophyll $a$ molecules (Müller et al., 2001). This speeds the relaxation of excited chlorophyll a molecules back to the ground state (Müller et al., 2001).

Plants may also maintain the chloroplast redox state under excess excitation energy by redirecting electrons from the photosynthetic electron transport chain to other electron sinks. For example, photorespiration provides an alternative source of 3-phosphoglycerate (3-PGA) through the oxygenation of Rubisco (Takahashi and Murata, 2008; Ivanov et al., 2008). The consumption of ATP and NADPH through this process may also contribute to protection of the photosynthetic electron transport chain from over-reduction (Ivanov et al., 2008).

In addition to redirecting electrons from the photosynthetic electron transport chain, reductant produced through photosynthetic electron transport can also be redirected. This concept will be discussed in a later section.

The generation of ROS is a natural phenomenon occurring in plant chloroplasts as a result of photosynthetic activity (Alscher et al., 2002). From the previous discussion, it is clear that the accumulation of ROS in the chloroplast could be detrimental to the cell. However, under normal or unstressed conditions, ROS formed are rapidly removed, meaning that they do not accumulate in the chloroplast. To accomplish this, several mechanisms are present which enable the scavenging and metabolism of ROS, thus limiting their potentially damaging effects. The glutathione-ascorbate cycle and carotenoid pigments of the xanthophyll cycle both act to metabolize ROS (Gruszecki, 2010). Additionally, a number of enzymes exist with ROS-metabolizing activities, including catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX), and several superoxide dismutase (SOD) enzymes (Apel and Hirt, 2004). SOD enzymes are classified by their metal co-factors and include iron (FeSOD), manganese (MnSOD), and copper-zinc (CuZnSOD). The SOD enzymes have a particularly important role in scavenging ROS, as they are present in the ROS-generating mitochondria, chloroplasts, cytosol, and peroxisomes (Gechev et al., 2003; Alscher et al., 2002). The wide intercellular
distribution of SOD enzymes is required due to the impermeable nature of lipid bilayer membranes to $O_2^-$, which means that these ROS must be scavenged and metabolized in the cellular component of their generation (Alscher et al., 2002).

1.6 Relationship between Chloroplast and Mitochondrion

As discussed, a variety of mechanisms exist in which plants may regulate the redox state of the chloroplast under challenging environmental conditions. Intriguingly, plants may also maintain redox status by transporting excess reductant from the chloroplast to other cellular components, such as the cytosol or mitochondrion (Noguchi and Yoshida, 2008). Therefore, under circumstances where NADPH oxidation through CO$_2$ fixation is limited, this strategy enables plants to remove excess reductant for its consumption elsewhere.

Shipping of reductant from the chloroplast is accomplished by transporter proteins in the inner chloroplast envelope (Heineke et al., 1991). No transporter exists to transport excess NADPH directly (Heineke et al., 1991). Rather, excess NADPH is oxidized to NADP$^+$; this reaction reduces another metabolite, which is subsequently transported (Heineke et al., 1991). The transport between chloroplast and cytosol is made possible by two shuttles, the triose phosphate-phosphate transporter (TPT) and the malate valve.

CO$_2$ assimilation in the Calvin Cycle involves the uptake of inorganic phosphate ($P_i$) from ATP (Flügge et al., 1980). This process results in the generation of triose phosphates, such as glyceraldehyde-3-phosphate (G3P). The TPT facilitates the exchange of triose phosphates and 3-phosphoglycerate (3-PGA) with inorganic phosphate ($P_i$) (Flügge, 1995; Noguchi and Yoshida, 2008). Thus, the TPT enables the balance of production and export of metabolites generated through the Calvin Cycle (Flugge et al., 1980). The TPT has also been implicated as a means for export of excess reductant from the chloroplast. In the proposed function of the TPT,
the reduction of 3-PGA to G3P consumes NADPH, but subsequent reoxidation of G3P to 3-PGA regenerates NADPH in the cytosol (Fliege et al., 1978). Thus, an indirect transport of NADPH from chloroplast to cytosol is facilitated by the TPT.

The malate valve, also known as the oxaloacetate-malate antiporter, is considered an important transporter of reducing equivalents (Noguchi and Yoshida, 2008). In a similar manner as the TPT, the malate valve acts to transport excess reductant out of the chloroplast. Excess NADPH is used to reduce oxaloacetate (OAA) into malate, which regenerates the electron acceptor NADP⁺ (Scheibe et al., 2005) (Figure 4B). This reaction is catalyzed by the light-dependent enzyme NADP⁺-dependent malate dehydrogenase (NADP-MDH) (Noguchi and Yoshida, 2008). In concurrence with its putative role in reductant transport, previous studies have shown that over-expression of the NADP-MDH enzyme helps to maintain the oxidation state of PSI (Noguchi and Yoshida, 2008). Once generated, malate is reduced back into oxaloacetate by cytosolic NAD⁺-dependent malate dehydrogenases (NAD-MDH) to generate NADH, which may be subsequently consumed through mitochondrial respiration, photorespiration, or nitrate reduction (Scheibe et al., 2005; Noguchi and Yoshida, 2008). The non-phosphorylating pathway has also been implicated in malate metabolism without ATP generation (Scheibe et al., 2005).
Figure 4 Transport mechanisms from the chloroplast. (A) The triose-phosphate transporter. Glyceraldehyde-3-phosphate (G3P) generated through the Calvin Cycle during photosynthesis is converted to dihydroxyacetone phosphate (DHAP) by triose phosphate isomerase (TPI). DHAP is subsequently converted 3-phosphoglycerate (3-PGA), then shuttled through the triose-P transporter into the cytosol. G3P can be phosphorylated by NAD(P)+-dependent glyceraldehyde-3-phosphate dehydrogenase (NAD(P)-G3PDH), generating 3-PGA and NAD(P)H. The majority of 3-PGA is metabolized through glycolysis, whereas a smaller proportion is shipped back to the chloroplast for use in the Calvin Cycle. (B) The malate valve. NADP+-malate dehydrogenase (MDH) enzymes catalyze the reduction of OAA to malate. This reaction regenerates the electron carrier NADP+. Generated malate is transported through the malate valve to the cytosol, where it may subsequently be converted back into OAA by NAD-MDH. This process generates NADH, which may be metabolized in the mitochondrion.
1.7 The Role of Mitochondria in Photorespiration

The importance of the relationship between chloroplasts and mitochondria is highlighted in the process of photorespiration, which is considered to be an important sink for electrons from the photosynthetic electron transport chain (Ivanov et al., 2008). Glycine formed through photorespiration is exported from the peroxisome into the mitochondrial matrix, where it is converted to serine using the mitochondrial glycine decarboxylase complex (GDC) and serine hydroxymethyltransferase (SHMT) enzymes (Douce et al., 2001). NADH formed through the conversion of glycine to serine in the matrix can then be consumed by the mitochondrion (Douce et al., 2001). In addition, the oxidation of glycine in the mitochondria provides glycerate to the Calvin Cycle, supporting carbon assimilation (Raghavendra and Padmasree, 2003). Taken together with the potential of chloroplast to export excess reductant to the mitochondrion, this suggests that regulation of the redox state of the chloroplast is mediated by the mitochondrion providing sinks for oxidizing reductant.

1.8 Studies Using Mitochondrial Inhibitors and Mutants

It is now clear that a complex relationship exists between the chloroplast and mitochondrion, where the mitochondrion may provide sinks for reductant and electrons of chloroplastic origin. To further study these interactions, previous studies have examined the effect upon photosynthesis when the mitochondrial electron chain was impaired through mutants or chemical inhibitors. The aim of these studies was to further elucidate the role of the mitochondrion in optimizing photosynthesis.

Studies based on the cytoplasmic male sterile II (CMSII) mutant in tobacco, which lacks a functional mitochondrial Complex I, have been extensively used to investigate the relationship
between respiration and photosynthesis in plants. The CMSII mutant was shown to have slower induction of photosynthesis, and a lower optimal rate (Dutilleul et al., 2003; Noctor et al., 2004). Since the CMSII mutant represents the lack of a major NADH sink, these studies highlight the importance of mitochondrial redox state on the chloroplast.

In addition to the oxidative phosphorylating pathway, a role of AOX has been suggested as a contributing factor in the chloroplast-mitochondrion interaction. Dinakar et al. (2010) demonstrated enhanced accumulation of malate in SHAM-treated pea mesophyll protoplasts during acclimation from darkness to moderate or high PPFD. This result suggests a role AOX in the malate valve. Specifically, it is possible that chloroplasts shuttle excess reductant through the malate valve to mitochondria, where they may be subsequently consumed by AOX.

To further investigate the role of AOX in optimizing photosynthesis, studies reducing functional AOX through chemical inhibition or in mutants lacking functional AOX have been conducted. For example, the AOX inhibitor SHAM in rice was reported to decrease CO₂ assimilation rates compared to untreated plants (Feng et al., 2007). Treatment of pea mesophyll protoplasts with SHAM generated similar results, particularly when protoplasts were subjected to higher PPFD (Dinakar et al., 2010). Similarly, decreases in PSII quantum efficiency (ΦPSII) of wheat plants under combined high light and drought conditions were enhanced following application of SHAM (Bartoli et al., 2005). Yoshida et al. (2006) also reported decreases in photochemical quenching in SHAM-treated plants at saturating light intensities.

Despite the large number of studies utilizing chemical inhibitors of AOX, it has been suggested that SHAM is not entirely reliable (Raghavendra and Padmasree, 2003). Therefore, Padmasree et al. (2002) have suggested that studies using transgenic plants would be beneficial for elucidation of the interaction between chloroplasts and mitochondria. One such study was conducted by Giraud et al. (2008), whereby the researchers generated an AOX1a mutant in Arabidopsis and subjected it to combined light and drought conditions. In AOX1a mutants, non-
photochemical quenching was enhanced compared to WT plants. In addition, glycerate content in \textit{AOX1a} mutants was markedly decreased under light and drought compared to mutants under unstressed conditions, suggesting that AOX contributes towards photorespiration under abiotic stress conditions (Giraud \textit{et al.}, 2008).

1.9 Purpose of Research Project

From this discussion, it is apparent that a complex relationship between chloroplasts and mitochondria exists, wherein the mitochondria may assist in the mediation of the chloroplast redox state through provision of ATP, consumption of excess reductant, and contribution towards photorespiratory processes. Evidence from previous studies suggests that this interaction may involve AOX, although its putative functions in photosynthesis have largely been determined through chemical inhibition studies rather than using transgenic plants with altered AOX protein levels. Therefore, the objective of this research project is to investigate the role of AOX in mediating the acclimation response of plants to high-light treatment. As previously described, the chloroplast may avoid excess ROS accumulation by shipping excess reductant to the mitochondrion. If AOX is involved in non-ATP-limiting consumption of this reductant, it is hypothesized that AOX may play a role in protecting the photosynthetic electron transport chain from over-reduction under excess light conditions.

To investigate the relationship between the mitochondrion and chloroplast during light acclimation, and the role of AOX in mediating this relationship, measurements of chlorophyll content and chlorophyll \textit{a/b} ratios, AOX gene expression and protein levels, chlorophyll fluorescence, gas exchange, carbohydrate content, and lipid peroxidation were conducted. Transgenic \textit{Nicotiana tabacum} lines differing in AOX protein levels were utilized to compare the putative role of AOX during acclimation to higher PPFD.
Chapter 2: Materials and Methods

2.1 Plant material and growth conditions

Tobacco seeds were sown on vermiculite wetted with tap water and then placed under 24-hour fluorescent light (85 µmol m\(^{-2}\) s\(^{-1}\) PPFD). Beginning 1 week after germination, seedlings were watered daily with 10-times-diluted Hoagland’s solution (2 mM MgSO\(_4\)•7H\(_2\)O, 4 mM KH\(_2\)PO\(_4\), 10 mM KNO\(_3\), 10 mM Ca(NO\(_3\))\(_2\)•4H\(_2\)O, 18 µM EDTA, 17.99 µM FeSO\(_4\)•7H\(_2\)O, 92.51 µM H\(_3\)BO\(_3\), 18.29 µM MnCl\(_2•4H_2O\), 1.61 µM ZnSO\(_4\), 0.59 µM CuSO\(_4\), and 0.24 µM Na\(_2\)MoO\(_4•2H_2O\)). At the age of 2.5 weeks, seedlings were transferred to a soil mix (4 parts potting mix: 1 part vermiculite) wetted with 10 times diluted Hoagland’s solution contained in 5”-diameter pots. The seedlings were then moved to growth chambers set for 16-hour days and 8-hour nights under low-light (LL) conditions (120 µmol m\(^{-2}\) s\(^{-1}\) PPFD) or moderate-light (ML) conditions (700 µmol m\(^{-2}\) s\(^{-1}\) PPFD) for four weeks. For short-term ML acclimation experiments, LL-grown plants were transferred to ML conditions for 24, 48, or 72 hours. Temperature was maintained at 28°C during the day and 22°C during the night under both growth conditions. Relative humidity was equivalent between chambers at 60%. All plants were watered daily with 10-times diluted Hoagland’s solution 13 hours into the photoperiod.

Four AOX1a transgenic N. tabacum lines were used in this project. Two of these lines, termed RI29 and RI9, contained an AOX1a-RNA interfering construct. The B7 and B8 lines, which contained a construct with AOX1a in sense direction with constitutive promoter, were included to examine the role of enhanced AOX protein levels. Wild-type (WT) plants were used as a non-transgenic control. Confirmation of the protein levels in AOX1a-silencing and AOX1a-overexpressing lines has been previously described (Vanlerberghe et al., 1994; Vanlerberghe et al., 1995; Amirsadeghi et al., 2006; Wang, 2009).
Unless otherwise noted, tissue used was collected from fifth-developed leaves of six-week-old tobacco plants. Harvesting was conducted 12.5 hours following the beginning of the photoperiod.

2.2 RNA Extraction

Frozen leaf tissue was ground in liquid nitrogen with a mortar and pestle to a fine powder. Approximately 7 g of resulting powder was transferred to a pre-cooled mortar and homogenized again with cold guanidine hydrochloride extraction buffer (6.5 M GHCL, 1 M Tris-HCl pH=8.0, 1 M sodium acetate pH=5.5, β-mercaptoethanol 0.1% (v/v) DEPC). Samples were stored in liquid nitrogen until completion of grinding for all samples. Following thawing on ice, 0.2 M sodium acetate (0.1% (v/v) DEPC) was added to each sample, vortexed thoroughly, and incubated at room temperature for 10 minutes. Samples were centrifuged at 4°C for 10 minutes at 12 000 rcf. Resulting supernatants were combined with TRIZOL reagent at 2 parts supernatant: 3 parts TRIZOL and incubated at room temperature for 5 minutes prior to addition of 1 part chloroform: 5 parts supernatant. Samples were inverted by hand for 15 seconds, then centrifuged at 4°C for 10 minutes at 12 000 rcf. Aqueous supernatants were transferred to new microtubes. Samples were diluted 2 times with isopropyl alcohol, immediately vortexed, and incubated at -20°C for 1 hour to precipitate RNA. Following incubation, samples were centrifuged at 4°C for 20 minutes at 12 000 rcf. Isopropyl alcohol was removed from samples. RNA pellets were vigorously washed with 80% ethanol (0.1% (v/v) DEPC). Ethanol was removed and pellets were dried in vacuum dryer for 6 minutes. RNA pellets were resuspended in 20 µl 0.1% (v/v) DEPC-H₂O at 4°C for 20 minutes, then at 60°C for 10 minutes.
RNA samples were 100-times-diluted with DEPC-H$_2$O prior to quantification in spectrophotometer. The absorbance at 260 and 280 nm were determined with a spectrophotometer blanked with an empty quartz cuvette. RNA samples were stored at -80°C.

RNA concentration was calculated as follows: $C \, (\mu g/ml) = 4 \times A$, where $A$ = absorbance at 260 nm. To determine the volume required for 15 µg, the following formula was used: $V \, (\mu l) = 15 \, \mu g / C$, where $C$ = RNA concentration (µg/ml).

2.3 Northern Blots

Based on concentration of isolated RNA, 15 µg of RNA was combined with 0.1% (v/v) DEPC-H$_2$O and RNA loading buffer (41.6% formamide, 8.32% formaldehyde, 1.3X MOPS, 74.62 µM bromophenol blue). Samples were incubated at 60°C for 10 minutes before addition of ethidium bromide (25.36 mM EtBr, 0.1% (v/v) DEPC). RNA samples were loaded into 1.24% agarose gels (0.98X MOPS, 0.02% (v/v) formaldehyde) submerged in 1X MOPS (0.1 M MOPS-NaOH, 5mM sodium acetate, 1 mM EDTA-Na$_2$, 0.1% (v/v) DEPC). Gel electrophoresis was conducted for approximately 90 minutes at 70 V. Gels were visualized in Gel-Doc to ensure RNA quality, then washed with 1X SSC (0.75 M NaCl, 15 mM trisodium citrate, 0.1% (v/v) DEPC) and 6.56 mM NaOH for 20 minutes. RNA was allowed to transfer onto Hybond-N nylon membranes wetted with 10X SSC for 18 hours. Following transfer, membranes were cross-linked with ultraviolet light for 2 minutes, then wrapped in saran wrap and stored at -20°C until probed.

Propylene tubes were prepared for radioactive blotting by washing with 0.1% (v/v) DEPC-H$_2$O at 37°C overnight in a spinning hybridizer. The next day, membranes were removed from saran wrap and placed in propylene tubes such that gel-contacting side of membrane faced inside of tube. Membranes were washed with prehybridization buffer (0.18 Na$_2$HPO$_4$, 70 mM
NaH$_2$PO$_4$, 7% (w/v) SDS, 0.1% (v/v) DEPC) and placed in 37°C spinning hybridizer for 15 minutes. 5 mg/ml salmon sperm DNA was boiled for 3-4 minutes and placed on ice. Following removal of prehybridization buffer, 300 µl salmon sperm DNA and fresh prehybridization solution were applied to each membrane. Membranes were incubated in 37°C spinning hybridizer for 4-5 hours.

Final DNA content of Northern probes was equalized with TE buffer (1 mM EDTA pH=8.0, 10 mM Tris pH=8.0). Northern probes against AOX1a were boiled for 3 minutes, immediately placed on ice for 3 minutes, then pulsed in centrifuge. Probes and 5 µl $^{32}$P were added to reaction tube and gently mixed by pipetting before incubation at 37°C for 20 minutes. To remove unlabelled DNA, columns were used. Columns were vortexed to resuspend resin, then centrifuged at room temperature for 10 minutes at 3 000 rpm. Columns were placed in fresh tubes before application of probes. Subsequent probe-containing columns were centrifuged at room temperature for 2 minutes at 3 000 rpm. Probes were boiled for 3 minutes, cooled on ice for 3 minutes, then pulsed in centrifuge. Probes were added to propylene tubes and incubated at 65°C overnight in a spinning hybridizer.

The following day, probes and hybridization buffer were removed from propylene tubes into Falcon tubes and stored in -20°C freezer behind Lucite shield. Membranes were washed with Church Wash I (0.14 M Na$_2$HPO$_4$, 56 mM NaH$_2$PO$_4$, 5% (w/v) SDS, 70°C) and incubated at 65°C for 30 minutes in a spinning hybridizer. Church Wash I was removed and the washing procedure was repeated once more. Radioactivity of membranes was measured with Geiger counter in cpm. Depending on activity, membranes were washed with Church Wash II (0.14 M Na$_2$HPO$_4$, 56 mM NaH$_2$PO$_4$, 1% (w/v) SDS) 1-2 times. Following washing, membranes were wrapped in saran blot and placed in cassette. In darkroom, film was placed under membrane and amplifying sheet in cassette. Films and membranes were stored in cassettes at -80°C until developing.
2.4 Mitochondria Extraction

Fully-developed leaves from six-week-old plants were harvested, then placed in cold distilled H₂O. At 4°C, midveins were removed with a razor blade. Leaves were dried with paper towel, then measured to a final fresh weight of 40 g. Homogenization buffer (0.3 M sucrose, 25 mM TES, 2 mM EDTA, 10 mM KH₂PO₄, 1% (w/v) PVP-40, 1% (w/v) BSA pH=7.5) was added to samples prior to shredding with hand blender for 7-8 minutes. Resulting samples were homogenized with 4 grams acid-washed sand and homogenization buffer in a pre-cooled mortar and pestle for 5 minutes. Samples were poured through 2 layers of Miracloth, divided into centrifuge tubes, then centrifuged at 3 000 rpm at 4°C for 5 minutes. Supernatants were transferred to new centrifuge tubes, then centrifuged at 12 000 rpm at 4°C for 20 minutes. Resulting pellets were resuspended in 1X Wash (0.15 M sucrose, 5 mM TES, 0.05% (w/v) BSA pH=7.2) using a paintbrush. Samples were pooled and centrifuged at 3 000 rpm at 4°C for 5 minutes. Supernatants were transferred to new centrifuge tubes, then centrifuged at 12 000 rpm at 4°C for 20 minutes. Resulting pellets were resuspended in 1X Wash using a paintbrush.

Samples were applied to cold Percoll gradient (heavy solution: 0.15 M sucrose, 5 mM TES, 0.5% (w/v) BSA pH=7.2, 28% (v/v) Percoll, 4.4% (w/v) PVP-25; light solution: 0.15 M sucrose, 5 mM TES, 0.5% (w/v) BSA pH=7.2, 28% (v/v) Percoll, 22% (v/v) ddH₂O), then centrifuged at 18 000 rpm at 4°C for 40 minutes with centrifuge brake turned off. Supernatant above mitochondria fraction was removed with vacuum. 7 mL of mitochondria fraction was collected, transferred to new centrifuge tubes, then washed with 1X Wash. Samples were centrifuged at 15 000 rpm at 4°C for 15 minutes. Supernatant was removed from pellet with vacuum. Pellets were washed with 1X Wash minus BSA (0.15 M sucrose, 5 mM TES pH=7.2), then centrifuged at 15 000 rpm at 4°C for 15 minutes. Supernatant was removed from pellet
with vacuum. Pellets were resuspended in 1X Wash minus BSA to total volume of approximately 200-300 µl. DMSO was added to final concentration of 5% (v/v). Mitochondria protein samples were stored at -80°C.

Protein concentration was determined using a Lowry assay with BSA standards of 0, 2, 5, 10, 15, and 20 mg/ml conducted with spectrophotometer. The equation of resulting standard curve was used to calculate sample protein concentration. To determine the volume required for 100 µg, the following formula was used: \( V (\mu l) = \frac{100 \mu g}{C} \), where \( C \) = mitochondria protein concentration (mg/ml).

2.5 Western Blots

Resolving gel was poured into gel apparatus using gradient maker (heavy solution: 0.404 M Tris, 0.1% (w/v) SDS pH=8.8, 9.7% (w/v) sucrose, 0.1% (w/v) SDS, 16.73% (w/v) acrylamide, 0.05% (w/v) ammonium persulfate, 0.054% TEMED; light solution: 0.376 M Tris, 0.1% (w/v) SDS pH=8.8, 0.934% (w/v) sucrose, 0.1% (w/v) SDS, 9.94% (w/v) acrylamide, 39% dH₂O, 0.05% (w/v) ammonium persulfate, 0.05% TEMED). Gels were sealed with n-butanol and polymerized for 1 hour. Following removal of n-butanol, stacking gel (0.125 M Tris-HCl, 0.1% (w/v) SDS pH=6.8, 0.1% (w/v) SDS, 5% (w/v) acrylamide, 0.066% ammonium persulfate, 0.096% TEMED) was added on top of resolving gel. Gels were polymerized for 2 hours. Based on concentration of isolated protein, 100 µg of protein was combined with ddH₂O and 3X sample buffer (125 mM Tris-HCl, 6% (w/v) SDS, 6% (v/v) β-mercaptoethanol, 30% (v/v) glycerol). Samples were immediately boiled for 2 minutes, then cooled on ice. 6 µl 2% (w/v) bromophenol blue was added to each sample. Samples were pulsed in centrifuge prior to loading into gels submerged in running buffer (25 mM Tris, 0.192 M glycine, 0.1% (w/v) SDS). Gel electrophoresis was conducted for approximately 5 hours at 50-60 mA.
Following electrophoresis, gels were removed and placed into transfer cages. Proteins were allowed to transfer onto nitrocellulose membrane for 1 hour at 0.5-1.0 A at 4°C for 1-2 hours in Towbin buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol). Following transfer, membranes were submerged in 1X PBS-Tween (10 mM NaH₂PO₄, 150 M NaCl, 0.3% (v/v) Tween-20) and incubated on shaker for 15 minutes. This wash step was repeated. Membranes were dried with blotting paper, then stored in plastic sheet at room temperature until probed. Gels were incubated in Coomassie stain on shaker overnight.

The next day, Coomassie stain was removed from gels. Coomassie destain solution was added to each gel, incubated on shaker until clear background was obtained, then photographed with scanner. Membranes were incubated in blocking buffer on shaker for 1-2 hours. Primary antibodies raised against AOX or COXII were diluted 1 000 times into 1X PBS-Tween, then added to membranes on shaker for 1 hour. Membranes were washed with 1X PBS-Tween for 10 minutes to a total of 6 washes. Secondary antibodies were diluted 25 000 times into 1X PBS-Tween, then added to membranes on shaker for 1 hour. Membranes were washed with 1X PBS-Tween for 10 minutes to a total of 6 washes. Equal volumes of West Pico Stable Peroxide and West Pico Luminol/Enhancer solution were combined, then added to membrane on shaker for 15 minutes in dark. Following drying on blotting paper, membranes were placed on film and exposed in darkroom.

2.6 Chlorophyll Content

The chlorophyll content was measured using an acetone extraction method adapted from Ni et al. (2009). 1.5 cm-diameter leaf discs were harvested from between major veins to a final fresh weight of 300 mg. After weighing, leaf discs were immediately frozen in liquid nitrogen. Samples were then ground in liquid nitrogen with a mortar and pestle in a darkened fume hood.
100 mg of the frozen tissue powder was collected into a pre-cooled microtube. 1 mL of cold buffered aqueous acetone (80% (v/v) acetone, 2.6 mM NaH$_2$PO$_4$, 22.4 mM Na$_2$HPO$_4$) was added to each sample. Samples were vortexed, then incubated in the dark on ice for 15 minutes. Following incubation, samples were then centrifuged at 4°C for 15 minutes at 3000 rcf. The resulting supernatant was collected into new microtubes and 10-times-diluted with 80% buffered acetone. The absorbance of the samples at 663, 664, and 750 nm was determined using a spectrophotometer blanked with buffered aqueous acetone. Quartz cuvettes were used for all blanks and samples.

Total chlorophyll content and chlorophyll $a$ and $b$ ratios were calculated from the absorbance values according to Porra et al. (1989). Total chlorophyll content was calculated as follows: $C_{chl_{a+b}}$ (µmol/m$^2$) = $10 \times [19.54 \times (A_{646} - A_{750}) + 8.29 \times (A_{663} - A_{750})]/SA$, where SA = surface area (cm$^2$). For calculation of chlorophyll $a$ and $b$ concentrations, the following equation was used: $C_{chl\ a}$ (nmol/ml) = 13.71 $A_{663}$ – 2.85 $A_{646}$ and $C_{chl\ b}$ (nmol/ml) = 22.39 $A_{646}$ – 5.42 $A_{663}$.

2.7 Chlorophyll Fluorescence and Gas Exchange

Chlorophyll fluorescence (Figure 5) and gas exchange were measured on the fifth-developed leaves of six-week-old tobacco plants using the PAM GFS-3000 (Walz). Separate programs were developed for measurements of chlorophyll fluorescence and concomitant fluorescence and gas exchange under varying actinic light. The wavelengths of light were as follows: measuring light: 470 nm; actinic light: 640 nm red light and 470 nm blue light; far-red light: 740 nm.

For chlorophyll fluorescence measurements, a program of approximately 31 minutes in length was developed. Dark-adaptation was initiated 4-5 hours following start of photoperiod.
Plants were dark-adapted in a box with ambient PPFD of 0.5 µmol m\(^{-2}\) s\(^{-1}\) prior to insertion of fifth-developed leaf into the cuvette and commencement of measurement. After 5 minutes, plants were subjected to a saturating pulse (4 500 µmol m\(^{-2}\) s\(^{-1}\)) for measurement of the minimum (\(F_o\)) and maximum (\(F_m\)) fluorescence values in the absence of light. Actinic light (900 µmol m\(^{-2}\) s\(^{-1}\)) was applied for 20 minutes prior to application of far-red light and saturating pulses for determination of the minimum (\(F_o'\)) and maximum (\(F_m'\)) fluorescence values in the presence of actinic light. All measurements were conducted in an 8 cm\(^2\) cuvette at 28°C, 400 ppm CO\(_2\), 14 000 ppm H\(_2\)O, and 750 µmol s\(^{-1}\) flow speed.
Figure 5 A typical chlorophyll fluorescence profile. In dark-adapted leaves, PSII photocenters are maximally oxidized and minimum fluorescence ($F_o$) can be measured. Application of a saturating pulse (SP) of light fully reduces PSII photocenters, enabling measurement of the maximum fluorescence in the dark ($F_m$). The difference between $F_m$ and $F_o$ is termed variable fluorescence ($F_v$). Application of actinic light (AL) causes a transient increase in chlorophyll fluorescence, which is subsequently quenched by photochemical and non-photochemical processes to a steady-state level ($F'$) (Baker, 2008). The application of far-red (FR) light is used to oxidize PSII photocenters, generating a measurement of minimum fluorescence ($F_o'$). A pulse of saturating light is used to measure maximum fluorescence ($F_m'$). Removal of actinic light restores fluorescence to dark-adapted levels. Figure adapted from Baker (2008).
Calculations of fluorescent parameters were conducted by the GFS-3000 software. Maximum quantum efficiency of PSII was calculated as follows: $F_v/F_m = (F_m - F_o)/F_m$. The quantum efficiency of PSII ($\Phi_{PSII}$) was calculated as $(F_m' - F')/F_m'$ (Genty et al., 1989). Non-photochemical quenching was calculated as follows: $q_N = (F_m' - F_m)/(F_m - F_o')$ (Schreiber et al., 1986). Photochemical quenching ($q_P$) was calculated as $(F_m' - F')/(F_m' - F_o')$. As a comparison between the “puddle” and “lake” models of photosynthesis, $q_L$ was also calculated as $(q_P)(F_o'/F')$ (Kramer et al., 2004).

For gas exchange measurements, plants were subjected to a program of approximately 2 hours in length. Plants were dark-adapted as previously described; dark-adaptation was initiated 3-7 hours following start of photoperiod. Following measurement of $F_o$ and $F_m$, actinic light ranging from 40-2000 µmol m$^{-2}$ s$^{-1}$ was applied for 15 minutes each, beginning at the lowest PPFD. Before proceeding to the next PPFD, far-red light and saturating pulses were applied to the plants for determination of $F_o'$ and $F_m'$. All measurements were conducted in an 8 cm$^2$ cuvette at 28°C, 400 ppm CO$_2$, 20 000 ppm H$_2$O, and a flow speed of 500 µmol s$^{-1}$.

The CO$_2$ assimilation rate was calculated as follows: $A = [\mu_e \times (c_e - c_o)]/LA - (E \times c_o)$ where $A =$ assimilation rate (µmol m$^{-2}$ s$^{-1}$), $\mu_e =$ molar flow rate at the inlet of the cuvette (µmol s$^{-1}$), $c_o =$ CO$_2$ mole fraction at the outlet of the cuvette (ppm), $c_e =$ CO$_2$ mole fraction at the inlet of the cuvette (ppm), LA = leaf area (cm$^2$), and $E =$ transpiration rate (mmol m$^{-2}$ s$^{-1}$).
2.8 Carbohydrate Assay

Soluble sugars were extracted from frozen leaf tissue and quantified using an enzymatic cycling assay (Figure 6). Frozen intact leaves were ground in liquid nitrogen with a mortar and pestle to a fine powder. Approximately 6 g of frozen leaf tissue was lyophilized at -50°C for 5 hours. Following freeze-drying, 6 mg powder was transferred to screw-cap tubes and stored at -80°C until extraction.

For extraction of soluble sugars, samples were incubated with 80% ethanol at 80°C. Samples were then centrifuged at 4°C for 5 minutes at 13 200 rpm. This procedure was repeated for a total of three times. Ethanol-insoluble pellets were stored at -80°C until starch extraction. Supernatants were pooled, applied to activated charcoal, and then centrifuged at 4°C for 10 minutes at 13 200 rpm. Charcoal pellets were resuspended with 80% ethanol and centrifuged again as previously described. Finally, pooled supernatants were dried in a vortex-centrifuge at 40°C for 2 hours. Resulting samples were dissolved in 1 ml ddH₂O.

To assay starch content, AG reagent (0.18 M CH₃COONa•3H₂O pH=5.0, 1.82 U/µl amylglucosidase, and 9.10 U/ml α-amylase) was dialyzed 3-4 times in 0.2 M CH₃COONa•3H₂O pH=5.0 for 24 hours prior to starch assay. Ethanol-insoluble pellets were washed twice with 1 ml ddH₂O and centrifuged at 4°C for 10 minutes at 13 200 rpm. Following repetition of washing, starch pellets were incubated with 0.1M NaOH at 95°C for 1 hour. Pellets were acidified with 3.5 µl acetic acid. Samples were incubated with 1 ml AG reagent at 55°C for 16 hours.

Enzymes were prepared for cycling assay as follows: 1 U/µl glucose-6-phosphate dehydrogenase (G6PDH) in G6PDH buffer (0.1 M Tris-HCl pH=8.1, 0.5 µM MgCl₂); 0.25 U/µl hexokinase (HK) in 50:50 G6PDH buffer: glycerol; 1 U/µl phosphoglucone isomerase (PGI) in 0.1 M Tris-HCl pH=8.1, 10 U/µl invertase (INV) in 50:50 G6PDH buffer: glycerol, and 20 U/µl
amyloglucosidase (AMG) in ddH$_2$O. Aliquots of G6PDH and PGI were stored at -80°C, whereas HK, INV, and AMG were stored at -20°C.

For quantification of soluble sugars, soluble samples were 20-times-diluted in assay medium (89.29 mM imidazole pH=6.9, 1.34 mM MgCl$_2$, 0.98 mM ATP, 0.49 mM NADP$^+$). The change of absorbance at 340 nm was measured with a spectrophotometer blanked with an empty plastic cuvette; absorbance was recorded after sequential addition of 1 U G6PDH, 0.5 U HK, 2 U PGI, and 20 U INV; for each enzyme, activity was allowed to saturate before further addition.

Concentrations of glucose and fructose were calculated as follows: $C$ (mol/L) = $V \times \frac{\Delta A}{(\varepsilon \times l \times W)}$, where $V$ = spectrophotometer volume (ml) $\times$ total volume (ml)/sample volume (ml); $\Delta A$ = change in absorbance at 340 nm; $\varepsilon$ = 6 300 M$^{-1}$ cm$^{-1}$; $l$ = path length (cm); and $W$ = lyophilized dry weight (mg). Sucrose content was calculated using above formula, but the result was halved to account for the 2:1 stoichiometry between glucose and sucrose.

To quantify starch content, insoluble samples were 20-times-diluted with ddH$_2$O, then 40-times-diluted with assay medium. The absorbance at 340 nm was measured with a spectrophotometer after sequential addition of 1 U G6PDH and 0.5 U HK. Concentration of starch was calculated as for glucose.
Figure 6 Schematic of enzymatic cycling assay for determination of carbohydrate content. Change in absorbance at 340 nm, corresponding to reduction of NAD$^+$ to NADH is used to determine enzyme activity. (A) Determination of sucrose content is accomplished by measuring invertase (INV) activity for conversion of sucrose to fructose and glucose. (B) Fructose content is determined through activity of hexokinase (HK) and phosphoglucose isomerase (PGI). (C) The conversion of glucose to 6-phosphogluconate through the enzymes HK and glucose-6-phosphate dehydrogenase (G6PDH) is used to determine glucose content. (D) The enzymatic conversion of starch by α-amylase and amyloglucosidase (AMG) allows determination of starch content.
2.9 Lipid Peroxidation

Malondialdehyde (MDA) content was determined using a thiobarbituric acid assay developed by Fryer et al. (1998). Approximately 300 g of fresh leaf discs were harvested from fifth and sixth-developed leaves. The fresh tissue was homogenized with 0.4 g acid-washed sand and 5 mM potassium phosphate buffer (1.93 mM KH₂PO₄, 0.31 mM K₂HPO₄ pH=7.8) with an ice-cold mortar and pestle for 2 minutes. Samples were centrifuged at 4°C for 5 minutes at 3000 rpm before addition of extraction buffer (0.45% (w/v) SDS, 8.33% (v/v) acetic acid, 0.33% (w/v) thiobarbituric acid). To act as a spectrophotometric blank, 5 mM potassium phosphate buffer in an equivalent volume as supernatant was similarly treated with extraction buffer. Samples and blank were incubated at 98°C for 1 hour, cooled to room temperature for 5 minutes, then centrifuged at room temperature for 5 minutes at 12 000 rcf. The absorbance of the samples at 535 and 600 nm were determined using a spectrophotometer blanked with 5 mM potassium phosphate buffer. Plastic cuvettes were used for all blanks and samples.

The concentration of MDA was calculated as follows: \[ C \text{ (nmol/g)} = \frac{10^9 \times \Delta A \times V}{(\varepsilon \times 1 \times FW)} \], where \( \Delta A = A_{535} - A_{600} \); \( V \) = total volume of incubated extract (L); \( \varepsilon = 1.56 \times 10^9 \text{ M}^{-1} \text{ cm}^{-1} \); \( l \) = path length (cm); and \( FW \) = sample fresh weight (g).

2.10 Statistical Analyses

Where applicable, analysis of variance (ANOVA) was used to evaluate whether differences between means were statistically significant, as determined by \( p < 0.05 \). Each AOX transgenic line was compared against WT using Bonferroni post-tests.
Chapter 3: Results

3.1 The Role of AOX in Long-Term Acclimation Response

3.1.1 AOX Protein Levels

To investigate long-term acclimation response, *N. tabacum* plants were grown under two different conditions for four weeks. Plants were grown under either low light (LL) conditions (120 µmol m$^{-2}$ s$^{-1}$ PPFD) or moderate light (ML) conditions (700 µmol m$^{-2}$ s$^{-1}$ PPFD) for a period of four weeks. To determine whether AOX plays a role in mediating long-term acclimation to ML, mitochondria were isolated from leaves of WT tobacco grown under LL or ML conditions for four weeks. Subsequent mitochondrial proteins were separated by gel electrophoresis and probed for AOX protein using an appropriate antibody.

For plants grown under LL conditions, very low levels of AOX protein were detected (Figure 7A). In plants undergoing long-term acclimation, AOX protein levels were higher than those subjected to LL conditions. However, the levels of another mitochondrial protein, COXII, were not markedly affected by growth under ML conditions (Figure 7B). The equivalence of the protein bands observed in the Coomassie-stained gel also confirmed that protein levels were equal among samples (Figure 7C).
Figure 7 Western blot analysis of mitochondrial protein extracted from WT plants grown under two different PPFD for four weeks. Isolated mitochondria were extracted from WT leaf tissue. Subsequent mitochondrial protein were separated through SDS-PAGE, transferred to nitrocellulose membranes, and probed with appropriate antibodies. (A) AOX protein levels were determined under different PPFD. (B) Protein levels of cytochrome c oxidase subunit II (COX II) were also determined in LL-grown and ML-grown WT plants. (C) Coomassie-stained gel demonstrates equal protein loading.
The observed up-regulation of AOX protein levels suggests that long-term acclimation to increases in PPFD may involve AOX. Therefore, transgenic lines with decreased or enhanced AOX protein levels were analyzed under long-term LL and ML conditions. Of the two AOX-silencing lines used, RI29 and RI9, it was observed that RI29 was the stronger suppresser of AOX protein levels (Amirsadeghi et al., 2006; Wang, 2009). The over-expressing line B8 had higher protein levels than B7. Therefore, subsequent data has been ordered to reflect increasing AOX protein levels, namely: RI29, RI9, WT, B7, and B8.

3.1.2 Chlorophyll Content

As a general determination of ML acclimation strategies, total chlorophyll content and chlorophyll $a/b$ ratios were measured in WT, AOX-silencing, and AOX-overexpressing plants grown under either LL or ML conditions. In order to determine whether leaf developmental stage had an effect, leaf discs were taken from the fourth-, fifth-, and seventh-developed leaves, where the first developed leaf was considered the first true leaf to develop following the cotyledons.

In leaves at all developmental stages studied, total chlorophyll content was not markedly different among AOX genotypes grown under LL conditions (Figure 8A-C). Growth under ML conditions resulted in the marked decrease of chlorophyll content. However, there appeared to be no correlation between AOX protein levels and chlorophyll content in leaves grown under these conditions.

In fourth-, fifth-, and seventh-developed leaves, chlorophyll $a/b$ ratios were enhanced in ML-grown plants compared to LL-grown plants (Figure 8D-F). Differences between AOX genotypes were not discernible with respect to chlorophyll $a/b$ ratios.
For all subsequent experiments examining the role of AOX in long-term acclimation response to enhanced PPFD, data was collected from fifth-developed leaves of LL-grown and ML-grown tobacco plants.
Figure 8 Chlorophyll content in WT plants and transgenic plants with suppressed or enhanced levels of AOX protein grown under two different PPFD for four weeks. (A-C) Total chlorophyll content and (D-F) chlorophyll \(a/b\) ratios in fourth-, fifth-, and seventh-developed leaves were determined. Chlorophyll content and chlorophyll \(a/b\) ratios were determined according to Porra et al. (1989). Error bars represent the standard deviation of the mean of two to six independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. No significant differences were observed.
3.1.3 Chlorophyll Fluorescence

Chlorophyll fluorescence is a technique used to assay photosynthetic capacity and activity (Baker, 2008). One of the greatest advantages of using this technique is its non-invasive nature, which allows for measurements in vivo (Baker, 2008). The chlorophyll fluorescence method relies on taking measurements of the minimal fluorescence of a dark-adapted plant, referred to as $F_o$. The value of $F_o$ represents the fully oxidized state of the photocenters of Photosystem II (PSII). Following this measurement, the leaf is subjected to a saturating pulse that fully reduces PSII photocenters, yielding the maximal fluorescence ($F_m$). Measurements of minimum and maximum fluorescence are repeated in the presence of actinic light to give values of $F_o'$ and $F_m'$. The variable fluorescence, $F_v$, was determined by $F_m - F_o$.

From the values $F_o$, $F_m$, $F_o'$, and $F_m'$, various photosynthetic parameters can be calculated. Some of the most commonly studied fluorescence parameters include the maximal photosynthetic efficiency of PSII ($F_v/F_m$), the PSII quantum efficiency ($\Phi_{PSII}$), and the proportion of light energy which contributes towards photochemistry (photochemical quenching, or $q_P$) or heat dissipation (non-photochemical quenching, or $q_N$) (Baker, 2008). For detailed calculations of each parameter, please see the Materials and Methods section.

To test the putative role of AOX during light acclimation, the photosynthetic characteristics of plants subjected to long-term ML acclimation was determined. In LL-grown plants, the maximum photosynthetic efficiency of PSII ($F_v/F_m$) did not appear to differ among AOX genotypes. Rather, the value of 0.8 was very stable among the different lines tested (Figure 9A). ML-grown plants exhibited moderately lower $F_v/F_m$ ratios compared to LL-grown plants. In addition to the $F_v/F_m$ ratio, which measures the maximum or intrinsic efficiency of PSII, the operating efficiency of PSII ($\Phi_{PSII}$) was also determined. Values of $\Phi_{PSII}$ were moderately enhanced in plants grown under ML conditions compared to those grown under
lower PPFD, although differences between these lines were not notable (Figure 9B).
Figure 9 Chlorophyll fluorescence measurements of PSII efficiency in WT plants and transgenic plants with suppressed or enhanced levels of AOX protein grown under two different PPFD for four weeks. Measurements of (A) maximum PSII efficiency ($F_v/F_m$) and (B) quantum efficiency of PSII ($\Phi_{PSII}$) were conducted using the GFS-3000 system (Walz). Plants were dark-adapted for one hour prior to measurement. Actinic PPFD used was 900 µmol m$^{-2}$ s$^{-1}$. Error bars represent the standard deviation of the mean of four independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. No significant differences were observed.
Photochemical quenching ($q_P$) is a measure of the relative proportion of light energy which contributes towards photochemistry (Baker, 2008). Although similar to $\Phi_{PSII}$, $q_P$ is slightly different because it measures the number of photochemical centers which are open, or oxidized (Maxwell and Johnson, 2000). Under LL-conditions, the AOX-silencing lines RI29 and RI9 showed slightly decreased $q_P$ compared to WT, although the differences were not statistically significant (Figure 10A). Enhanced AOX protein levels in B7 and B8 plants did not affect $q_P$. Compared to LL-grown plants, ML-grown plants showed increased $q_P$ compared to plants grown under LL conditions. However, differences among AOX transgenic lines compared to WT were not noted under ML conditions.

As a further investigation of photochemical quenching, the parameter $q_L$ was also determined. The value of $q_L$ represents photochemical quenching under the “lake” model of PSII (Kramer et al., 2004). Values of $q_L$ showed similar patterns to $q_P$, indicating that the choice of interpreting the chlorophyll fluorescence data under the “puddle” model of PSII did not affect the general patterns observed (Figure 10B).

Measurements of photochemical quenching are commonly reported as PSII excitation pressure, which is given as $1-q_P$. PSII excitation pressure represents the accumulation of closed, or reduced, PSII photocenters (Huner et al., 1998). Under conditions where light energy intake exceeds the capacity for utilization, PSII excitation pressure is expected to increase as photochemistry becomes limited (Huner et al., 1998). Measurements of $1-q_P$ were determined in LL-grown plants and ML-grown plants (Figure 10C). In keeping with the observed pattern in $q_P$, plants grown under LL conditions had markedly higher $1-q_P$ than ML-grown plants. Notable differences between AOX-silencing, AOX-overexpressing, and non-transgenic plants were not observed.
Figure 10 Chlorophyll fluorescence measurements of photochemical quenching in WT plants and transgenic plants with suppressed or enhanced levels of AOX protein grown under two different PPFD for four weeks. (A) Measurements of photochemical quenching were determined under the “puddle model” of PSII (qP). (B) Photochemical quenching according to the “lake” model of PSII (qL). (C) PSII excitation pressure (1-qP) was also calculated. All fluorescence measurements were conducted using the GFS-3000 system (Walz). Plants were dark-adapted for one hour prior to measurement. Actinic PPFD used was 900 µmol m^{-2} s^{-1}. Error bars represent the standard deviation of the mean of four independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. No significant differences were observed.
Finally, non-photochemical quenching (q\textsubscript{N}) was also measured using chlorophyll fluorescence. Values of q\textsubscript{N} represent the proportion of light energy which is dissipated as heat, rather than contributing towards photochemistry. For LL-grown plants, q\textsubscript{N} was not markedly different among transgenic lines and non-transgenic lines (Figure 11). ML-grown plants showed slight decreases in q\textsubscript{N} values compared to LL-grown plants. Major differences between AOX transgenic lines were not noted.
Figure 11 Chlorophyll fluorescence measurements of non-photochemical quenching in WT plants and transgenic plants with suppressed or enhanced levels of AOX protein grown under two different PPFD for four weeks. Measurements of non-photochemical quenching ($q_N$) were conducted using the GFS-3000 system (Walz). Plants were dark-adapted for one hour prior to measurement. Actinic PPFD used was 900 $\mu$mol m$^{-2}$ s$^{-1}$. Error bars represent the standard deviation of the mean of four independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. No significant differences were observed.
3.1.4 Carbohydrate Content

The final measurement of photosynthetic activity for comparison of LL-grown and ML-grown plants was assays of carbohydrate content. Specifically, the content of glucose, fructose, sucrose, and starch were measured using a multi-step enzymatic assay (Figure 6).

In LL-grown plants, glucose levels were very low (Figure 12A). In ML-grown plants, glucose content was increased compared to plants grown under LL conditions. The accumulation of fructose showed a very similar pattern to glucose (Figure 12B). Growth under ML conditions tended to promote fructose accumulation compared to growth under LL conditions.

In addition to glucose and fructose, sucrose content was also assayed. In LL-grown plants, sucrose content appeared to be correlated to AOX protein levels, although this pattern was not observed in ML-grown plants (Figure 12C). Sucrose content in ML-grown plants was slightly decreased compared to plants grown under lower PPFD. Therefore, it was concluded that long-term acclimation to ML conditions results in an enhancement of glucose and fructose, but moderate decreases of sucrose.

The accumulation of starch was also measured in plants undergoing long-term ML acclimation. Starch levels in LL-grown plants under LL conditions were very low (Figure 12D). A dramatic induction of starch accumulation was observed in ML-grown plants compared to LL-grown plants. The level of AOX did not appear to affect this response.
Figure 12 Carbohydrate content in WT plants and transgenic plants with suppressed or enhanced levels of AOX protein grown under two different PPFD for four weeks. Contents of (A) glucose, (B) fructose, (C) sucrose, and (D) starch were determined using a cycling enzymatic assay. Error bars represent the standard deviation of the mean of three independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. No significant differences were observed.
3.1.5 Lipid Peroxidation

As previously mentioned, ROS accumulation may result in plants subjected to excess excitation energy which exceeds their capacity for photosynthesis. To determine the potential role of AOX in mediating levels of ROS during ML acclimation, lipid peroxidation, as determined by the content of malondialdehyde (MDA), was assayed. The content of MDA provided an indirect measure of ROS accumulation.

For LL-grown plants assayed under LL conditions, MDA content did not significantly differ among AOX genotypes (Figure 13). MDA content was increased by approximately two-fold in ML-grown plants compared to LL-grown plants. No significant differences were observed between AOX-silencing, AOX-overexpressing, or WT plants.
Figure 13 Lipid peroxidation in WT plants and transgenic plants with suppressed or enhanced levels of AOX protein grown under two different PPFD for four weeks. As an indirect measure of ROS content, malondialdehyde (MDA) content was determined using a thiobarbituric acid assay. Error bars represent the standard deviation of the mean of three independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. No significant differences were observed.
From the results generated above, it appeared that long-term acclimation to ML decreased chlorophyll content but increased chlorophyll $a/b$ ratios. Chlorophyll fluorescence measurements suggested that ML-grown plants had fewer reduced PSII photocenters but similar non-photochemical quenching compared to plants grown under LL conditions. The content of several carbohydrates, particularly starch, was also enhanced in plants grown under ML conditions compared to LL-grown plants.

Despite these general differences noted among LL-grown and ML-grown plants, specific differences between AOX genotypes were not noted. Rather, comparison of AOX-silencing, AOX-overexpressing, and WT lines suggested that AOX does not directly affect long-term ML acclimation. Therefore, the role of AOX in modulating short-term acclimation to increased PPFD was also investigated.
Chapter 3: Results

3.2 The Role of AOX in Short-Term Acclimation Response

Long-term acclimation did not yield interesting differences among AOX genotypes. Therefore, another experimental approach was applied to investigate the role of AOX during short-term acclimation response. In these experiments, plants were grown at LL conditions (120 µmol m\(^{-2}\) s\(^{-1}\)) and then shifted to ML conditions (700 µmol m\(^{-2}\) s\(^{-1}\)) for 24, 48, or 72 hours.

3.2.1 AOX Gene Expression

To investigate the role of AOX during shift to higher PPFD, RNA was extracted from fifth-developed leaves of WT plants and radioactively probed for the expression of AOX1a. Northern blots of AOX demonstrated induction of AOX gene expression during ML acclimation, particularly after 48 and 72 hours (Figure 14A). Densitometry analysis of subsequent blots showed similar induction of AOX in response to the transfer to ML conditions (Figure 14B). Quality of RNA from extraction and equal loading during gel electrophoresis are shown by a representative agarose gel stained with ethidium bromide (Figure 14C).
Figure 14 Northern blot analysis of RNA extracted from WT plants grown under LL conditions for four weeks, then transferred to higher PPFD under ML conditions. RNA were isolated from WT leaf tissue, separated through gel electrophoresis, transferred to nitrocellulose membranes, and probed with radioactive cDNA probes. (A) AOX transcript levels were determined in LL-grown WT plants transferred to higher PPFD. (B) Densitometry analysis of Northern blots was conducted using ImageJ software. Bars represent the mean of two independent experiments. (C) Ethidium bromide-stained gel demonstrates quality of RNA and equal RNA loading.
3.2.2 AOX Protein Levels

To complement AOX gene expression data, mitochondria were also isolated from plants subjected to short-term light acclimation conditions. Resulting mitochondrial protein were analyzed using the Western blotting technique. Western blots depicted the induction of AOX protein in LL-grown WT plants shifted to ML conditions, beginning at 24 hours and continuing to 48 hours (Figure 15A). However, application of ML treatment did not significantly affect COXII protein levels at any of the time periods examined (Figure 15B). Densitometry analyses of Western blots from two independent experiments depict the induction of AOX protein levels after 48 hours of ML treatment (Figure 15C). The quantitative analysis revealed that AOX protein levels were markedly induced in plants shifted to ML conditions compared to LL-grown plants under lower PPFD. Coomassie staining of gels demonstrated equivalence of protein loading (Figure 15D).
Figure 15 Western blot analysis of mitochondrial protein extracted from WT plants grown under LL conditions for four weeks, then transferred to higher PPFD under ML conditions. Isolated mitochondria were extracted from WT leaf tissue. Subsequent mitochondrial proteins were separated through SDS-PAGE, transferred to nitrocellulose membranes, and probed with appropriate antibodies. (A) AOX protein levels were determined in LL-grown WT plants transferred to higher PPFD. The 0 hrs data is equivalent to the LL-grown value depicted in Figure 7. (B) Protein levels of cytochrome c oxidase subunit II (COX II) were used as a loading control. (C) Densitometry analysis of Western blots was conducted using ImageJ software. Bars represent the mean of two independent experiments. (D) Coomassie-stained gel demonstrates equal protein loading.
For further study of the influence of AOX during short-term ML acclimation, transgenic *N. tabacum* lines silencing or over-expressing AOX were used. These transgenic lines were generated and characterized by Amirsadeghi *et al.* (2006).

### 3.2.3 Chlorophyll Content

Upon application of ML treatment for 24 to 48 hours, chlorophyll content in fourth-developed leaves remained similar to LL-grown plants, but decreased in fifth- and seventh-developed leaves (*Figure 16A-C*). The trend of decreasing chlorophyll content during ML acclimation persisted to 72 hours ML treatment. Similar to the conclusion generated from the long-term acclimated plants, a relationship between AOX protein levels and chlorophyll content in either fourth-, fifth-, or seventh-developed leaves was not observed at this level.

Chlorophyll *a/b* ratios were also determined for fourth-, fifth-, or seventh-developed leaves undergoing short-term acclimation to ML. Chlorophyll *a/b* ratios were not notably variable among fourth- or fifth-developed leaves of LL-grown plants shifted to ML conditions (*Figure 16A-B*). However, seventh-developed leaves showed a gradual increase in chlorophyll *a/b* ratios during ML acclimation (*Figure 16C*). For the remaining experiments, fifth-developed leaves were analyzed to measure the role of AOX protein during transfer to higher PPFD.
Figure 16 Chlorophyll content in WT plants and transgenic plants with suppressed or enhanced levels of AOX protein grown under LL conditions for four weeks, then transferred to higher PPFD under ML conditions. (A-C) Total chlorophyll content and (D-F) chlorophyll a/b ratios in fourth-, fifth-, and seventh-developed leaves were determined. Chlorophyll content and chlorophyll a/b ratios were determined according to Porra et al. (1989). The 0 hrs data is equivalent to the LL-grown value depicted in Figure 8. Error bars represent the standard deviation of the mean of two to six independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. No significant differences were observed.
3.2.4 Chlorophyll Fluorescence and Gas Exchange

Measurements of chlorophyll fluorescence were determined to further investigate the reduction state of the photosynthetic electron transport chain during short-term acclimation to ML conditions. After both 24 and 48 hours of ML treatment, \( F_v/F_m \) ratios decreased (Figure 17A). However, no differences among AOX genotypes were observed for plants during the shift to higher PPFD conditions.

Similar to the \( F_v/F_m \) ratios, the quantum efficiency of PSII (\( \Phi_{PSII} \)) was relatively equivalent among AOX genotypes under LL conditions (Figure 17B). After 24 hours of acclimation to ML, an interesting pattern was observed. Specifically, the AOX-silencing lines RI29 and RI9 plants had lower \( \Phi_{PSII} \) values than WT plants, whereas the two AOX-overexpressing lines B7 and B8 exhibited higher \( \Phi_{PSII} \) values. Therefore, there appeared to be a positive correlation between AOX protein levels and \( \Phi_{PSII} \). The pattern observed after 24 hours of ML exposure became even more evident as the length of ML treatment progressed. Although a general trend was noted, the absolute differences from either the AOX-silencing or AOX-expressing lines were not found to be statistically significant when analyzed with a two-way ANOVA and Bonferroni post-test.
Figure 17 Chlorophyll fluorescence measurements of PSII efficiency in WT plants and transgenic plants with suppressed or enhanced levels of AOX protein grown under LL conditions for four weeks, then transferred to higher PPFD under ML conditions. Measurements of (A) maximum PSII efficiency ($F_{v}/F_{m}$) and (B) quantum efficiency of PSII ($\Phi_{PSII}$) were conducted using the GFS-3000 system (Walz). Plants were dark-adapted for one hour prior to measurement. Actinic PPFD used was 900 µmol m$^{-2}$ s$^{-1}$. The 0 hrs data is equivalent to the LL-grown value depicted in Figure 9. Error bars represent the standard deviation of the mean of four independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. No significant differences were observed.
In addition to $F_v/F_m$ and $\Phi_{PSII}$, measurements of photochemical quenching ($q_P$) were also conducted to investigate the photosynthetic characteristics of PSII during ML acclimation. Similar to $\Phi_{PSII}$, $q_P$ values were also positively correlated with AOX protein levels in plants undergoing short-term acclimation to ML (Figure 18A). Specifically, AOX-silenced lines RI29 and RI9 had lower $q_P$ values than WT, whereas the AOX-overexpressing lines B7 and B8 had higher photochemistry. The relationship between AOX protein levels and photochemical quenching was also reflected in values of $q_L$, which are determined according to the “lake” model of PSII (Figure 18B). Since the values of both $q_P$ and $q_L$ showed the same patterns among AOX genotypes, it was concluded that the choice of PSII model did not affect the observed correlation between AOX protein levels and the number of oxidized PSII reaction centers. For plants subjected to short-term light acclimation, PSII excitation pressure ($1-q_P$) was inversely proportional to AOX protein levels (Figure 18C). These results suggest that AOX protein helps to alleviate PSII excitation pressure in tobacco plants undergoing acclimation to higher PPFD. Although the pattern among lines became more enhanced as the length of ML application period increased, none of the AOX transgenic lines showed significant differences compared to WT plants.
Figure 18 Chlorophyll fluorescence measurements of photochemical quenching in WT plants and transgenic plants with suppressed or enhanced levels of AOX protein grown under LL conditions for four weeks, then transferred to higher PPFD under ML conditions. (A) Measurements of photochemical quenching ($q_P$) were determined under the “puddle” model of PSII. (B) Photochemical quenching according to the “lake” model of PSII ($q_L$). (C) PSII excitation pressure ($1-q_P$) was also calculated. All fluorescence measurements were conducted using the GFS-3000 system (Walz). Plants were dark-adapted for one hour prior to measurement. Actinic PPFD used was 900 µmol m$^{-2}$ s$^{-1}$. The 0 hrs data is equivalent to the LL-grown value depicted in Figure 10. Error bars represent the standard deviation of the mean of four independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. No significant differences were observed.
When LL-grown plants were shifted to ML conditions for 24 hours, a decrease in q_N was observed in all lines (Figure 19). However, q_N began to increase after 48 hours, and by 72 hours had reached levels similar to those observed under LL conditions. Measurements of q_N did not reveal a significant relationship between AOX protein levels and non-photochemical quenching. This data suggests that q_N does not directly function to alleviate enhanced 1-q_p in plants silenced for AOX gene expression.
Figure 19 Chlorophyll fluorescence measurements of non-photochemical quenching in WT plants and transgenic plants with suppressed or enhanced levels of AOX protein grown under LL conditions for four weeks, then transferred to higher PPFD under ML conditions. Measurements of non-photochemical quenching ($q_N$) were conducted using the GFS-3000 system (Walz). Plants were dark-adapted for one hour prior to measurement. Actinic light level used was 900 µmol m$^{-2}$ s$^{-1}$. The 0 hrs data is equivalent to the LL-grown value depicted in Figure 11. Error bars represent the standard deviation of the mean of four independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. No significant differences were observed.
Given the observation that AOX protein levels was inversely correlated with 1-q_P in plants shifted to ML conditions, the fluorescence experiment was redefined to focus on the role of AOX during short-term acclimation to higher PPFD. An AOX-silencing line (RI29) and an AOX-overexpressing line (B8) were selected for further study of the role of AOX under these light conditions. The concomitant measurement of gas exchange and fluorescence parameters was conducted on RI29, WT, and B8 LL-grown plants shifted to ML conditions for 0, 48, or 72 hours. Fluorescence and gas exchange measurements were conducted over a range of actinic PPFD, from 40 to 2000 μmol m$^{-2}$ s$^{-1}$.

Similar to the original chlorophyll fluorescence experiment, F$_{v}$/F$_{m}$ decreased in LL-grown plants shifted to ML conditions (Figure 20). However, no notable differences were noted between WT plants and either the AOX-silencing line RI29 or the AOX-overexpressing line B8. F$_{v}$/F$_{m}$ ratios of LL-grown plants under these experimental conditions were similar to values observed in the original chlorophyll fluorescence experimental conditions (Figure 19A).
Figure 20 Chlorophyll fluorescence measurements of maximum PSII efficiency ($F_v/F_m$) in WT plants and transgenic plants with suppressed or enhanced levels of AOX protein grown under LL conditions for four weeks, then transferred to higher PPFD under ML conditions. Chlorophyll fluorescence measurements were conducted using the GFS-3000 system (Walz). Plants were dark-adapted for one hour prior to measurement. Error bars represent the standard deviation of the mean of three independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. No significant differences were observed.
In contrast to the stability of the $F_v/F_m$ ratio, the quantum efficiency of PSII ($\Phi_{\text{PSII}}$) showed an interesting difference between WT and AOX-silencing RI29 plants. In LL-grown RI29 and WT plants, significant decreases in $\Phi_{\text{PSII}}$ in RI29 plants compared to WT plants were noted at PPFD of 700 and 900 $\mu$mol m$^{-2}$ s$^{-1}$ (Figure 21A). The differences observed between these two genotypes persisted when WT and RI29 plants were acclimated to higher PPFD for 48 hours (Figure 21B) and 72 hours (Figure 21C). In contrast, the AOX-overexpressing line B8 did not significantly differ from WT plants under higher PPFD at any of the time points of ML treatment examined (Figure 21D-F).
Figure 21 Chlorophyll fluorescence measurements of PSII efficiency ($\Phi_{\text{PSII}}$) in WT plants and transgenic plants with (A-C) suppressed or (D-F) enhanced levels of AOX protein grown under LL conditions for four weeks, then transferred to higher PPFD under ML conditions. Chlorophyll fluorescence measurements were conducted using the GFS-3000 system (Walz). Plants were dark-adapted for one hour prior to measurement. Actinic PPFD used was 0-2000 µmol m$^{-2}$ s$^{-1}$. Error bars represent the standard deviation of the mean of three independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. * represents $p < 0.05$; ** represents $p < 0.01$. Points without an asterisk are not significantly different from WT.
The original chlorophyll fluorescence experiment suggested that 1-q_P and AOX protein levels are negatively correlated during ML acclimation (Figure 18). Therefore, it was of interest to determine whether the concomitant gas exchange and fluorescence experiment would confirm these results. In LL-grown plants under LL conditions, AOX-silencing RI29 plants had significantly higher 1-q_P values compared to WT plants (Figure 22A). When RI29 and WT plants were shifted to higher PPFD, this pattern became even more evident (Figure 22B-C). Similar to the patterns observed in Φ_PSII, the greatest differences between RI29 and WT plants were noted at PPFD of 700 and 900 µmol m^-2 s^-1.

Although PSII excitation pressure appeared to be inversely correlated to AOX protein level, this relationship was not observed for the AOX-overexpressing B8 plants at any ML treatment studied (Figure 22D-F).
Figure 22 Chlorophyll fluorescence measurements of PSII excitation pressure (1-qP) in WT plants and transgenic plants with (A-C) suppressed or (D-F) enhanced levels of AOX protein grown under LL conditions for four weeks, then transferred to higher PPFD under ML conditions. Chlorophyll fluorescence measurements were conducted using the GFS-3000 system (Walz). Plants were dark-adapted for one hour prior to measurement. Actinic PPFD used was 0-2000 µmol m\(^{-2}\) s\(^{-1}\). Error bars represent the standard deviation of the mean of three independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. * represents p < 0.05; ** represents p < 0.01. Points without an asterisk are not significantly different from WT.
In addition to q_P, the contribution of fluorescence quenching through non-photochemical processes (q_N) was also measured. Intriguingly, significant differences between AOX genotypes and WT were only observed at LL conditions. Specifically, RI29 plants, which were silenced for AOX protein levels, showed an enhanced q_N values compared to WT at PPFD ranging from 700 and 900 µmol m⁻² s⁻¹ (Figure 23A). However, this observed trend did not persist when LL-grown WT and RI29 plants were shifted to higher PPFD, since both lines exhibited very similar q_N values when shifted to ML conditions for 48 or 72 hours (Figure 23B-C). The application of ML treatment did not appear to cause significant induction of non-photochemical processes.

LL-grown AOX-overexpressing B8 and WT plants did not markedly differ with respect to q_N values under LL conditions (Figure 23D). Indeed, values of q_N remained nearly identical among WT and B8 plants during the shift to higher PPFD (Figure 23E-F).
Figure 23 Chlorophyll fluorescence measurements of non-photochemical quenching (q_N) in WT plants and transgenic plants with (A-C) suppressed or (D-F) enhanced levels of AOX protein grown under LL conditions for four weeks, then transferred to higher PPFD under ML conditions. Chlorophyll fluorescence measurements were conducted using the GFS-3000 system (Walz). Plants were dark-adapted for one hour prior to measurement. Actinic PPFD used was 0-2000 µmol m^{-2} s^{-1}. Error bars represent the standard deviation of the mean of three independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. * represents p < 0.05; *** represents p < 0.001. Points without an asterisk are not significantly different from WT.
As a final investigation of photosynthetic activity in plants undergoing ML acclimation, net CO$_2$ assimilation rates were measured through the gas exchange of the GFS-3000 (Walz). LL-grown RI29, WT, and B8 plants were measured under LL conditions, then shifted to ML conditions for 48 or 72 hours. Gas exchange data was measured simultaneously with the previously presented chlorophyll fluorescence data.

The CO$_2$ assimilation rates increased with PPFD until approximately 700 µmol m$^{-2}$ s$^{-1}$, at which point the CO$_2$ uptake appeared to be saturated. RI29 plants under LL conditions demonstrated consistently lower CO$_2$ assimilation rates compared to WT plants, although this pattern was most marked at PPFD higher than the saturating point (Figure 24A). However, at light intensities above this point, an interesting pattern was detected between AOX-silencing RI29 and WT plants. When LL-grown RI29 and WT plants were subjected to ML conditions for 48 hours, a significant reduction in CO$_2$ assimilation rate in RI29 lines compared to WT was noted (Figure 24B). Similar results were observed in RI29 plants acclimated to ML conditions for 72 hours (Figure 24C). In contrast, CO$_2$ assimilation rates in LL-grown B8 plants under LL conditions were similar to WT (Figure 24D). When shifted to ML conditions, B8 plants showed slightly decreased CO$_2$ assimilation rates compared to WT, although analysis with the two-way ANOVA and Bonferroni post-test determined these differences were not statistically significant (Figure 24E-F).

Together, the chlorophyll fluorescence and gas exchange data suggest that AOX protein may play a role in supporting photochemical quenching and CO$_2$ assimilation in LL-grown plants transferred to higher PPFD.
Figure 24 Net CO$_2$ assimilation rate in WT plants and transgenic plants with (A-C) suppressed or (D-F) enhanced levels of AOX protein grown under LL conditions for four weeks, then transferred to higher PPFD under ML conditions. Gas exchange measurements were conducted using the GFS-3000 system (Walz). Actinic PPFD used was 0-2000 µmol m$^{-2}$ s$^{-1}$. Error bars represent the standard deviation of the mean of three independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. * represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$. Points without an asterisk are not significantly different from WT.
3.2.5 Carbohydrate Content

From the chlorophyll fluorescence experiments, there appeared to be a positive correlation between photochemical quenching and AOX protein levels. Therefore, to investigate whether presence of AOX protein contributed toward the maintenance of photosynthetic activity under ML conditions, the carbohydrate content was measured. Glucose accumulation among AOX genotypes did not significantly differ under these conditions.

Following transfer to ML conditions for 24 hours, a three to four-fold increase in glucose content was observed in all AOX genotypes analyzed (Figure 25A). Despite an enhancement of glucose content observed in RI9 and AOX-overexpressing lines compared to WT plants, statistical analyses concluded that these differences were not significant. After the initial induction at 24 hours ML treatment, glucose levels remained relatively constant and did not continue to increase. AOX-silencing, AOX-overexpressing, and WT plants were not significantly different under these conditions. Fructose accumulation during short-term ML acclimation was very similar to the observed pattern in glucose (Figure 25B).

Compared to LL-grown plants under LL conditions, sucrose content was moderately induced following transfer to higher PPFD for 24 hours (Figure 25C). Unlike glucose and fructose, sucrose content appeared to decrease following 48 hours of ML treatment. This observed response persisted to 72 hours ML treatment, and corresponded with observations in ML-grown plants (Figure 12C). No marked differences among AOX genotypes were noted with respect to sucrose content for the remainder of the short-term ML acclimation experiment. Therefore, it appears that transfer to higher PPFD enhances content of glucose and fructose, but decreases sucrose content.

Starch accumulation was also measured in plants undergoing short-term ML acclimation. Starch levels in LL-grown plants were very low, and consistently remained at this
level despite increases in PPFD (Figure 25D). Notable differences among AOX-silencing, AOX-overexpressing, or WT plants were not observed at the level of starch accumulation.
Figure 25 Carbohydrate content in WT plants and transgenic plants with suppressed or enhanced levels of AOX protein grown under LL conditions for four weeks, then transferred to higher PPFD under ML conditions. Contents of (A) glucose, (B) fructose, (C) sucrose, and (D) starch were determined using a cycling enzymatic assay. The 0 hrs data is equivalent to the LL-grown value depicted in Figure 12. Error bars represent the standard deviation of the mean of three independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. No significant differences were observed.
3.2.6 Lipid Peroxidation

To determine whether AOX plays a role in mediating cellular ROS levels during acclimation to higher PPFD, lipid peroxidation was measured in plants undergoing short-term acclimation to ML conditions. It was of interest to determine whether the reduction state of the chloroplast would affect total lipid peroxidation, as measured by MDA content. Transfer of LL-grown plants to higher PPFD for 24 hours enhanced lipid peroxidation (Figure 26).

Surprisingly, B7 plants showed higher MDA content compared to WT plants. Although this pattern persisted throughout the remainder of the short-term ML acclimation experiment, analysis with the two-way ANOVA and Bonferroni post-test concluded that these were not significant. Enhanced levels of lipid peroxidation were noted after 24 hours ML treatment, but further application of higher PPFD did not result in further increases in MDA content.
Figure 26 Lipid peroxidation in WT plants and transgenic plants with suppressed or enhanced levels of AOX protein grown under LL conditions for four weeks, then transferred to higher PPFD under ML conditions. As an indirect measure of ROS content, malondialdehyde (MDA) content was determined using a thiobarbituric acid assay. The 0 hrs data is equivalent to the LL-grown value depicted in Figure 13. Error bars represent the standard deviation of the mean of three independent experiments. Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare each transgenic line against the WT. No significant differences were observed.
Chapter 4: Discussion

Increases in PPFD have been shown to cause imbalances in the redox state of the chloroplast, which may contribute to photoinhibition and ROS accumulation (Li et al., 2009). To avoid these effects, excess NADPH may be exported out of the chloroplast. In this process, NADPH is oxidized by the enzyme NADP-MDH to regenerate the electron carrier NADP$^+$; this process simultaneously reduces OAA to malate (Noguchi and Yoshida, 2008). Malate is shipped out of the chloroplast through the malate valve, where it can then be converted into pyruvate through the TCA cycle and consumed through the AOX and COX pathways (Noguchi and Yoshida, 2008) (Figure 1). It has been suggested that the mitochondrial protein AOX facilitates an energy-overflow pathway, which may contribute towards balance the production and consumption of reductant in the chloroplast (Scheibe, 2004). From this discussion, it was of interest to investigate whether AOX plays a role in the interaction between chloroplast and mitochondrion. Therefore, this project aimed to determine whether differences in AOX protein levels could affect acclimation response in *N. tabacum* under changes in PPFD.

Two experimental approaches were used in this study. In the first approach, plants were grown under either LL or ML conditions and analyzed for their long-term acclimation under either treatment. In the second approach, LL-grown plants were transferred to higher PPFD for determination of short-term ML acclimation response.

Although similar approaches to study the role of AOX in maintaining photosynthesis under changes in PPFD have been conducted, the majority of these studies have been focused on the use of the AOX chemical inhibitor SHAM. However, the current research is novel in its use of transgenic *N. tabacum* plants with decreased or enhanced AOX protein levels during acclimation to higher PPFD.
4.1 Induction of AOX during Light Acclimation Response

To examine the putative importance of AOX during both long-term and short-term ML acclimation response, the response of AOX during acclimation was determined in WT plants. RNA was extracted from leaves of LL-grown plants transferred to ML conditions and analyzed for AOX gene expression (Figure 14A). Levels of AOX transcripts were induced in LL-grown WT plants subjected to ML conditions for 48 and 72 hours. To complement gene expression data, Western blots were conducted on protein from mitochondria isolated from leaf tissue. Growth under ML conditions enhanced AOX protein levels in WT plants (Figure 7A), as did the transfer of LL-grown plants to ML conditions for 24 or 48 hours (Figure 15A). The enhancement of AOX protein levels in response to higher PPFD has been previously described in *Arabidopsis* (Yoshida *et al.*, 2007) and pea mesophyll protoplasts (Dinakar *et al.*, 2010). In addition, SHAM-resistant O$_2$ uptake has been shown to increase in *Arabidopsis* plants (Bartoli *et al.*, 2006), and rice plants (Feng *et al.*, 2007), and pea mesophyll protoplasts (Dinakar *et al.*, 2010) shifted to higher PPFD. Enhanced respiration through AOX has also been reported in soybean during the greening process of etiolated seedlings (Ribas-Carbo *et al.*, 2000).

Although AOX protein levels responded to changes in PPFD, COXII protein levels were not affected by ML treatment (Figures 7B, 15B). The induction of AOX protein levels, but not those of COXII, suggests that the AOX pathway may play a greater role during light acclimation response than the cytochrome pathway. Taking the gene and protein data together, the induction of AOX in WT plants suggests the importance of this mitochondrial protein during acclimation to increases in PPFD.

4.2 Modulation of Photosynthetic Antennae under Moderate Light Treatment
The light-harvesting complex associated with PSII (LHCII) is composed of the chlorophyll \( a/b \)-binding protein and pigments (Allen, 1992; Li et al., 2009). This pigment-protein complex contains 7 chlorophyll \( a \) molecules and 5 chlorophyll \( b \) molecules (Paulsen, 1995). Under conditions where excitation energy exceeds the ability of use in the chloroplast, LHCII is removed through proteolysis (Lindahl et al., 1995). This proteolytic activity decreases light harvesting and limits the absorption of excess excitation energy under ML conditions. Since LHCII contains half of the chlorophyll in the thylakoid membrane of green plants (Allen, 1992), removal of LHCII results in decreased chlorophyll content. In addition, LHCII removal causes a relatively greater proportion of the core PSII antenna, CP43 and CP47. Since CP43 and CP47 bind chlorophyll \( a \), whereas LHCII binds both chlorophylls \( a \) and \( b \), proteolysis of LHCII results in the enhancement of chlorophyll \( a/b \) ratios in response to light acclimation (Anderson, 1986).

In ML-grown plants, total chlorophyll content was decreased in all AOX genotypes compared to plants grown under LL conditions (Figure 8A-C). Similar results were obtained for LL-grown plants transferred to ML conditions (Figure 16A-C). These results correspond with Arabidopsis plants transferred to higher PPFD (Dall’Osto et al., 2010). Increases in chlorophyll \( a/b \) ratios were noted in ML-grown plants compared to LL-grown plants (Figure 8D-F). However, short-term acclimation to ML conditions did not substantially alter the chlorophyll \( a/b \) ratios except in seventh-developed leaves (Figure 16D-F). Enhanced chlorophyll \( a/b \) ratios in response higher PPFD has been reported in both spinach (Yang et al., 1998) and Lemna perpusilla (Yang et al., 2001).

Despite these general trends, no relationship between chlorophyll content or chlorophyll \( a/b \) ratios and AOX protein level was observed. In support of these findings, the application of the AOX chemical inhibitor SHAM caused only moderate decreases in chlorophyll content compared to untreated control in rice leaves (Feng et al., 2007).
The lack of alteration in chlorophyll \(a/b\) ratios in fourth and fifth-developed leaves, but the gradual enhancement of these ratios in seventh-developed leaves, suggests that LHCII proteolysis is sensitive to developmental stage. Analysis of proteolytic activity in spinach thylakoid membranes indicated a 36 to 48 hour lag between application of higher PPFD and induction of LHCII proteolysis (Lindahl et al., 1995). From this result, it is possible that proteolytic activity in more mature tobacco leaves experiences a similar lag. Thus, increases in chlorophyll \(a/b\) ratios could not be measured in fourth- and fifth-developed leaves at the time points measured in the current research study.

### 4.3 Silencing of AOX Decreases PSII Photochemistry

The observed similarity among AOX genotypes with respect to the light-harvesting antennae prompted a more specific investigation of photosynthetic electron transport in plants during ML acclimation. Therefore, measurements of chlorophyll \(a\) fluorescence were conducted to determine the effect of AOX protein levels on the oxidation state of the photosynthetic electron transport chain. Changes in PPFD have been reported to modulate photosynthetic electron transport, which can be measured through chlorophyll \(a\) fluorescence (Miyake et al., 2009).

Growth under ML conditions decreased maximum PSII efficiency (\(F_v/F_m\)) compared to LL-grown plants (Figure 9A). Transfer of LL-grown plants to higher PPFD also decreased \(F_v/F_m\) ratios (Figures 17A, 20A). Length of ML acclimation period did not appear to affect this response. The observed decrease in \(F_v/F_m\) ratios in response to ML conditions corresponds to studies in Arabidopsis (Karpinski et al., 1999; Yoshida et al., 2007; Dall’Osto et al., 2010), wheat (Behera and Choudhury, 2003; Bartoli et al., 2005), spinach protoplasts (Ohira et al., 2005) and tobacco (Miyake et al., 2009). Differences between AOX transgenic lines and WT
were not notable with respect to \(F_v/F_m\) ratios. This corresponds to results in isolated chloroplasts, where SHAM addition did not affect \(F_v/F_m\) (Bartoli et al., 2005; Yoshida et al., 2006).

In contrast to \(F_v/F_m\), the quantum efficiency of PSII (\(\Phi_{PSII}\)) was increased in ML-grown plants compared to LL-grown plants (Figure 9B), although shift to ML conditions decreased \(\Phi_{PSII}\) (Figures 17B, 21). This finding corresponds with results generated in wheat, where transfer to higher PPFD decreased \(\Phi_{PSII}\) (Behera and Choudhury, 2003). The observation that \(\Phi_{PSII}\) was decreased in LL-grown plants transferred to ML conditions, but enhanced in ML-grown plants, may reflect the length of acclimation period.

Intriguingly, when shifted to ML conditions, AOX-silencing lines had consistently lower \(\Phi_{PSII}\) than WT or AOX-overexpressing plants (Figure 17B). This result was also repeated in the concomitant chlorophyll fluorescence and gas exchange experiment conducted (Figure 21A-C). The observed decrease in \(\Phi_{PSII}\) in plants lacking AOX protein corresponds with the observation that SHAM addition further decreases \(\Phi_{PSII}\) in bean (Yoshida et al., 2006). Under combined drought and high PPFD, SHAM application also enhanced decreases in \(\Phi_{PSII}\) compared to untreated plants under the same conditions (Bartoli et al., 2005).

When measured at equivalent actinic PPFD, PSII excitation pressure (1-\(q_P\)) was decreased in ML-grown plants compared to LL-grown plants, suggesting the successful acclimation response of the former (Figure 10C). Another research study in tobacco reported similar decreases in 1-\(q_P\) in ML-grown plants compared to ones grown under LL conditions (Miyake et al., 2009). 1-\(q_P\) was generally increased when plants were shifted to higher PPFD (Figures 18C, 22). Of greatest interest was the observed correlation between AOX protein levels and 1-\(q_P\), where AOX-silencing lines showed consistently higher values than WT or AOX-overexpressing plants. At saturating light intensities, SHAM application has been demonstrated to decrease \(q_P\), which corresponds to enhanced 1-\(q_P\) (Yoshida et al., 2006). These
results suggest that the lack of AOX in plants transferred to higher PPFD results in a more reduced state of the photosynthetic electron acceptors in PSII.

Decreases in Fv/Fm have been suggested to be a consequence of enhanced contribution of non-photochemical processes towards heat dissipation (Maxwell and Johnson, 2000; Behera and Choudhury, 2003). Therefore, measurements of non-photochemical quenching (qN) were conducted. In plants undergoing long-term ML acclimation, qN was slightly elevated compared to LL-grown plants (Figure 11). This suggests that the decrease in Fv/Fm ratios observed in ML-grown plants was partially due to enhanced non-photochemical quenching. Data generated for LL-grown plants shifted to higher PPFD was less clear. In these experiments, qN was initially decreased after 24 hours ML treatment, but became increasingly enhanced over the period of ML acclimation (Figures 19, 23). The observed increases in non-photochemical quenching during ML treatment were consistent with work conducted in Arabidopsis (Yoshida et al., 2007) and tobacco (Miyake et al., 2009). The effect of AOX silencing on qN was only apparent in LL-grown plants at PPFD between 500-900 µmol m⁻² s⁻¹ (Figure 23A). At these PPFD, RI29 plants had higher qN values compared to WT, although the pattern was lost as the transfer to ML conditions proceeded (Figure 23B-C). A previous study using SHAM in wheat found that increasing PPFD increased non-photochemical quenching, but with no differences between SHAM-treated and untreated plants (Bartoli et al., 2005). These results are in accordance with the current research study.

Given the discussion of non-photochemical quenching and its role in mediating Fv/Fm ratios, the observation in this study that decreases in Fv/Fm did not translate into enhanced qN was surprising. In addition to non-photochemical quenching, it has been suggested that decreases in Fv/Fm may also be attributed to loss of PSII photocenters due to photoinactivation (Baker, 2008). The results generated in this study suggest that, at least in N. tabacum plants under these experimental conditions, decreases in maximum PSII efficiency are attributed to
photoinactivation rather than non-photochemical quenching, and that this inactivation is not modulated by AOX.

When considering all of the photosynthetic parameters studied, it appears that AOX does not play a direct role in modulating the long-term ML acclimation response. In contrast, AOX appears to play a significant role in mediating the oxidation state of the PSII photocenters during short-term acclimation to higher PPFD. AOX-silencing lines consistently showed significantly decreased $\Phi_{\text{PSII}}$ and enhanced $1-q_p$ than WT plants. Therefore, the current research study suggests that AOX plays an important role in optimizing photosynthesis during short-term acclimation to higher PPFD, although AOX does not directly function in mediating non-photochemical quenching processes during this acclimation process.

4.4 Lack of AOX Decreases CO$_2$ Assimilation

Chlorophyll fluorescence experiments suggested a role of AOX maintaining the oxidation state of PSII photocenters in the photosynthetic electron transport chain. To further elucidate the role of AOX in photosynthesis, the CO$_2$ assimilation rate was measured in LL-grown plants transferred to higher PPFD, particularly after 72 hours ML treatment (Figure 24). Transfer to ML conditions generally decreased assimilation rates. These results correspond to previous studies in pea mesophyll protoplasts (Dinakar et al., 2010) and Alocasia odora (Noguchi et al., 2001). In contrast, a study by Miyake et al. (2009) in tobacco showed no significant declines in CO$_2$ assimilation rates following increase of PPFD. This discrepancy may reflect differences in sampling conditions, since no mention was given of the leaf developmental stage selected for measurements. The importance of leaf developmental stage for measurements of net carbon assimilation rates has been demonstrated in tobacco (Willekens et al., 1997).
In LL-grown plants transferred to higher PPFD, the lack of AOX in RI29 lines significantly decreased net CO$_2$ assimilation rate compared to WT (Figure 24A-C). Studies using the AOX inhibitor SHAM in pea mesophyll protoplasts demonstrated decreased CO$_2$ assimilation rates compared to untreated cells (Dinakar et al., 2010). SHAM treatment also decreased net CO$_2$ assimilation in rice, even when grown under a variety of light regimes (Feng et al., 2007). Therefore, the decrease in CO$_2$ assimilation rates observed in RI29 plants is supported by previous research in both protoplasts and plants.

The observation that silencing of AOX gene expression had a detrimental effect on CO$_2$ assimilation rates suggests that AOX not only contributes towards sustaining PSII operating efficiency ($\Phi_{\text{PSII}}$) and reducing PSII excitation pressure, but also functions to maintain photosynthetic carbon assimilation during short-term acclimation response to increased PPFD.

4.5 Carbohydrate Content Is Not Affected by Silencing of AOX

The chlorophyll fluorescence and gas exchange data suggested a role of AOX in maintaining the oxidation state of the photosynthetic electron transport chain, as well as the enhancement of carbon assimilation during acclimation to short-term changes in PPFD. Therefore, it was of interest to determine whether observed differences in these photosynthetic parameters would translate into differences in carbohydrate content among AOX genotypes.

In ML-grown plants, glucose and fructose were moderately accumulated, although sucrose content decreased (Figure 12A-C). Starch content was markedly enhanced in ML-grown plants compared to plants grown under LL conditions (Figure 12D). Growth under higher PPFD has also been shown to increase starch content in lettuce (Zhou et al., 2009).

LL-grown plants transferred to higher PPFD showed an increase in glucose and fructose (Figure 25A-B). This increase in soluble sugar content coincides with data generated in wheat
(Behera and Choudhury, 2003). Similar to results obtained in ML-grown plants, the transfer to ML conditions decreased sucrose content (Figure 25C). Finally, starch content was moderately enhanced under higher PPFD than in plants under LL conditions (Figure 25D). It was originally hypothesized that suppressed CO$_2$ assimilation in AOX-silencing plants compared to WT would translate into lower accumulation of carbohydrates. However, the data do not support this hypothesis, since the differences among AOX genotypes were not observed under either experimental approach. Rather, the data suggest that the previously described correlation between AOX protein levels and photosynthetic capacity did not translate into higher carbohydrate content.

4.6 The Relationship between Photosynthetic Electron Transport and CO$_2$

Assimilation under Excess Excitation Energy

The balance between absorption and consumption of excitation energy is central to maintaining the redox state in the chloroplast (Takahashi and Murata, 2008). Specifically, the generation of NADPH and ATP through photosynthetic electron transport and their consumption through metabolism must be balanced (Paul and Foyer, 2001).

Under excess excitation energy conditions, more light is captured through photosynthetic electron transport than can be utilized in the Calvin Cycle (Takahashi and Murata, 2008; Li et al., 2009). Consequently, levels of ATP and NADPH are in excess of their requirement (Takahashi and Murata, 2008). Limitation of ADP and P$_i$ for photophosphorylation leads to the acidification of the thylakoid lumen, and may possibly lead to the enhancement of non-photochemical quenching processes (Demmig-Adams, 1990).

The imbalance between production and consumption of NADPH and ATP can be ameliorated by restriction of photosynthetic electron transport (Hald et al., 2008). In addition,
the over-reduction of PSII photocenters has already been described, and contributes towards further loss of PSII activity (Aro et al., 1993; Murata et al., 2007). Thus, the photosynthetic electron transport chain appears able to modulate the balance between production and consumption of NADPH and ATP under excess excitation energy conditions in order to maintain the redox state of the chloroplast.

When the AOX-silencing RI29 plants are shifted to higher PPFD, a lower quantum efficiency of PSII (ΦPSII) and lower CO₂ assimilation were observed than in WT plants. The decreased ΦPSII in RI29 plants compared to WT suggests a greater loss of PSII activity through photoinhibition in plants lacking AOX protein. This may indicate that the chloroplast stroma is initially more reduced in RI29 plants than in WT. However, non-photochemical quenching was not affected by AOX protein levels (Figure 23).

Several important regulators of CO₂ assimilation have been described. For example, the supply of inorganic phosphate (Pᵢ), which is required for ATP synthesis through photophosphorylation, is implicated as an important regulator of photosynthetic activity (Paul and Foyer, 2001). Dujardyn and Foyer (1989) suggest that NADPH was the limiting factor for Calvin Cycle activity, since ATP levels were not affected by shift of chloroplasts to higher PPFD (Dujardyn and Foyer, 1989). Further experiments are required to discern the regulatory mechanism responsible for the decreased CO₂ assimilation in RI29 compared to AOX-expressing plants.

4.7 AOX Protein Does Not Directly Affect Lipid Peroxidation

Excitation energy that exceeds the capacity of plants’ use for photosynthesis can result in the over-reduction of the plastoquinol pool, and ROS generation (Aro et al., 1993; Murata et al., 2007; Li et al., 2009). To determine whether AOX plays a role in mediating ROS levels during
acclimation to higher PPFD, lipid peroxidation was measured in plants undergoing both long-term and short-term acclimation to ML conditions. From the fluorescence data, the PSII photocenters of AOX-silencing lines appeared to be more reduced, resulting in higher PSII excitation pressure. Therefore, it was of interest to determine whether the reduction state of the chloroplast would affect lipid peroxidation, as measured by MDA content.

Growth under ML conditions resulted in increased MDA content compared to growth under LL conditions (Figure 13). Increases in levels of lipid peroxidation have also been reported in lettuce plants grown under moderate and high PPFD compared to LL conditions (Zhou et al., 2009). In LL-grown plants shifted to ML conditions, an increase in MDA content was noted after 24 hours of treatment (Figure 26). However, MDA levels remained at this level for the remainder of the ML treatment. This suggests that, after 24 hours under higher PPFD, plants are able to acclimate to higher levels, and therefore limit further ROS accumulation. Previous research by Dall’Osto et al. (2010) described no increases in MDA content in Arabidopsis plants experiencing short-term acclimation to higher PPFD. This may reflect the shorter period elapsed prior to measurement of lipid peroxidation compared to the current research study, since MDA content was measured by Dall’Osto et al. (2010) after 14 hours of ML treatment rather than 24 hours.

Data generated from chlorophyll fluorescence measurements demonstrated enhancement of PSII excitation pressure in plants lacking AOX. This suggested that ROS accumulation may be enhanced in AOX-silencing lines compared to WT. However, the data presented do not support this conclusion. Rather, it appeared that all AOX genotypes studied showed insignificant differences in lipid peroxidation when MDA content was measured in acclimating plants undergoing long-term or short-term ML acclimation. This finding did not correspond with the proposed function of AOX in suppressing ROS accumulation (Vanlerberghe et al., 2009). Experimental evidence has also been provided for the role of AOX in limiting ROS
accumulation in tobacco cell lines, where AOX-silencing cells had much higher levels of ROS than non-transgenic WT cells (Maxwell et al., 1999). Conversely, AOX-overexpressing cells demonstrated lower ROS accumulation than WT (Maxwell et al., 1999).

The observed equivalence in MDA content between RI29, RI9 and WT plants may result from the activity of a ROS-scavenging mechanism in the AOX-silencing lines. For example, components of the ascorbate-glutathione cycle, CAT, and SOD enzymes all have ROS-dampening effects (Apel and Hirt, 2004). Previous studies using AOX-silencing transgenic tobacco cell lines have demonstrated an induction of several antioxidant enzymes, including GPX and CAT (Amirsadeghi et al., 2006). Therefore, it is postulated that up-regulation of antioxidant enzymes in the AOX-silencing RI29 and RI9 lines could compensate for lack of AOX function in these plants. This hypothesis could be further investigated through Northern blot analysis of antioxidant genes, specifically GPX and CAT.

MDA content was also equivalent to WT in the AOX-overexpressing plants B7 and B8. This suggests that AOX protein levels in WT are sufficient for suppressing ROS accumulation, since additional protein levels did not affect MDA content.

4.8 Proposed Model and Conclusions

The role of AOX in mediating the interaction between chloroplast and mitochondrion during acclimation to higher PPFD was investigated in this research project. A summary of this research study has been presented in a model (Figure 27).

Application of higher PPFD causes the re-modulation of chloroplastic PSII antennae, particularly in plants grown under higher PPFD. These modifications are likely due to proteolysis of the LHC, resulting in decreased total chlorophyll content and enhanced chlorophyll a/b ratios. Excitation energy that exceeds the capacity for use results in the
accumulation of reduced PSII photocenters, thus leading to the decrease in PSII quantum efficiency ($\Phi_{\text{PSII}}$) and enhanced PSII excitation pressure ($1-q_P$).

ATP and NADPH generated through photosynthetic electron transport are utilized in the Calvin Cycle for assimilation of inorganic carbon. As a result, glucose and fructose contents are increased, although sucrose synthesis decreases. Under excess excitation energy conditions, excess reductant may be transported to the mitochondria through the malate valve. To do so, NADP-MDH enzymes reduce oxaloacetate to malate, regenerating NADP⁺ from NADPH. Exported malate can then be converted by malic enzyme into pyruvate and subsequently metabolized through the TCA cycle.

When AOX protein is present and functional, as is the case in WT, B7, and B8 mitochondria, AOX is hypothesized to facilitate the consumption of excess reductant. However, when AOX is silenced, as occurs in RI29 and RI9 plants, excess reductant cannot be consumed through the AOX pathway. In this case, excess reducing power is suggested to cause greater reduction of the chloroplast stroma in AOX-silencing plants. The accumulation of excess reductant may contribute towards loss of photosynthetic electron transport.
Figure 27 Model summarizing the role of AOX during short-term acclimation to increased PPFD. Shift to ML conditions does not notably affect PSII antennae, as determined by chlorophyll a/b ratios. If excitation energy is in excess, the photosynthetic electron transport chain is over-reduced and PSII excitation pressure increases. Excess reductant is converted to malate through the activity of NADP-MDH. Malate is then converted to pyruvate and metabolized through the TCA cycle. When AOX protein is present (WT, B7, B8), the AOX pathway can consume excess reductant. In AOX-silencing (RI29, RI9) plants, lack of AOX enhances stroma reduction, contributing towards down-regulation of photosynthetic activity. pETC: photosynthetic electron transport chain; TCA: tricarboxylic acid cycle; COX: cytochrome oxidase; AOX: alternative oxidase.
The importance of AOX in the malate valve was demonstrated by results generated in pea mesophyll protoplasts Padmasree and Raghavendra (1999). In this study, treatment with the AOX inhibitor SHAM causes a marked increase in the malate/OAA ratio (Padmasree and Raghavendra, 1999). In contrast, the COX pathway inhibitors antimycin A and oligomycin did not affect the malate/OAA ratio as dramatically (Padmasree and Raghavendra, 1999). Thus, AOX inhibition through SHAM showed the accumulation of excess reductant in the chloroplast, as shown by the high malate/OAA ratios. This data suggests that AOX promotes oxidation of malate to OAA and contributes to the transport of reductant through the malate valve (Padmasree and Raghavendra, 1999).

In conclusion, the current research project demonstrated a role of AOX in mediating acclimation responses to higher PPFD in *N. tabacum* plants. The lack of AOX through RNA-silencing *in vivo* was demonstrated to significantly decrease quantum efficiency of PSII (ΦPSII) and enhance PSII excitation pressure (1-qP), but without significant effect on non-photochemical quenching processes. Net rates of CO₂ assimilation were also decreased in AOX-silencing lines compared to WT and AOX-overexpressing lines. However, the mechanism regulating this response remains unclear.

Taken together, this project supports and progresses current ideas regarding the role of AOX in mediating the complex interaction between mitochondrial respiration and chloroplastic photosynthesis under excess excitation energy conditions.

4.9 Future Directions

The present study suggests a role of AOX in regulating photosynthesis with respect to both photosynthetic electron transport and CO₂ assimilation. Based on the findings of this research project, several future directions are proposed. For example, it would be of interest to
determine the importance of photorespiration in the observed relationship between AOX and photosynthesis during acclimation to higher PPFD. Two main reasons exist for the interest in examining photorespiration. First, photorespiration acts as an electron sink under excess excitation energy conditions. Since this function as an electron sink is similar to that of AOX, it would be of interest to determine whether simultaneous restriction of both AOX and photorespiration would result in detrimental effects to photosynthesis during acclimation to higher PPFD. Second, photorespiration is of interest because several of its components are located in the mitochondrion (Bauwe et al., 2010). Regulation of the TCA cycle by photorespiratory intermediates has been described (Bauwe et al., 2010). Therefore, it would be of interest to determine whether the link between photorespiration and respiration also involves AOX. Future directions could include subjecting AOX-silencing and WT plants to elevated CO₂ conditions to limit photorespiration. Under these conditions, chlorophyll fluorescence and gas exchange measurements could be conducted to determine the relationship between AOX and photorespiration during light acclimation.

In addition, the observation that F₉/Fₑ ratios did not significantly decrease following shift to higher PPFD suggests that the plants were relatively unstressed under ML conditions. Therefore, future studies could utilize even higher PPFD than ML, or combine the current ML PPFD with conditions that limit CO₂ assimilation, such as drought or extreme (high or low) temperature.
References


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