The Phytoglobin-Nitric Oxide Cycle: A Transgenic Approach to Studying Hypoxic Stress in Plants

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Abstract

Hypoxic stress is one of the most common abiotic stresses plants face. Without sufficient oxygen to drive mitochondrial respiration, plants undergo an energy crisis. To combat this, a form of nitrite driven respiration called the phytoglobin-nitric oxide cycle is induced to maintain minimal ATP production. I studied the effects of overexpression and knockdown of the class-1 phytoglobin (Pgb) gene in barley (*Hordeum vulgare* L.) and the alternative oxidase (AOX) gene in tobacco (*Nicotiana tabacum*) in low-oxygen conditions to gain a better understanding of their roles in the hypoxic stress response. NO production was significantly lower in AOX knockdown plants compared to wild type and AOX overexpressing plants. The knockdown of AOX resulted in the increased level of protein *S*-nitrosylation under normoxia and the decreased *S*-nitrosylation under hypoxia. Under both conditions, there were complex differences in the pool size of amino and organic acids, depending upon AOX amount. Specifically under hypoxia, AOX amount strongly influenced the mitochondrial, but not cytosolic, activity of aconitase, which is known to be inactivated by NO and superoxide. Under normoxia and during reoxygenation after hypoxia, the knockdown of AOX increased the amount of superoxide, lipid peroxidation (malondialdehyde content) and total antioxidant reducing power. Hence, AOX has pervasive and oxygen concentration-dependent effects on NO production and protein *S*-nitrosylation, respiratory carbon and nitrogen flow, as well as on the metabolism of reactive oxygen species. The overexpression of phytoglobin lowered the amount of NO released, while knockdown significantly stimulated NO emission. The overexpression of phytoglobin corresponded to higher ATP/ADP ratios, pyrophosphate levels and aconitase activity under anoxia, while knockdown of phytoglobin resulted in the increased level of protein nitrosylation, elevation of alcohol dehydrogenase and nitrosoglutathione reductase activities. The overexpressing plants showed various signs of stunted growth under normoxia, but were the only type to germinate and survive under hypoxia. These results show that overexpression of phytoglobin protects plant cells via NO scavenging and improves their low-oxygen stress

survival. However, it may not be useful for cereal crop improvement since it comes with a significant interference with normoxic NO signalling pathways.

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List of Abbreviations

ADH: Alcohol dehydrogenase

ADP: Adenosine diphosphate

AOX: Alternative oxidase

AOX +: Plants overexpressing alternative oxidase

AOX-: Plants with knockdown alternative oxidase

ATP: Adenosine triphosphate

CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

COX: Cytochrome *c* oxidase

EDTA: Ethylenediaminetetraacetic acid

EGTA: Egtazic acid

GSH: Glutathione

GSNO: *S*-nitrosoglutathione

GSNOR: Glutathione reductase

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

MDA: Malondialdehyde

NO: Nitric oxide

Pgb: Phytoglobin

Pgb+: Plants overexpressing phytoglobin

Pgb-: Plants with knockdown phytoglobin

ROS: Reactive oxygen species

SHAM: Salicylhydroxamic acid

SNP: Sodium nitroprusside

WT: Wild type

Chapter 1: General introduction

1.1 Plant stress

Plants are exposed to a wide range of abiotic and biotic stresses throughout their life cycle. Due to their immobile nature, plants have no means to escape such stress factors. Without the option of fight or flight, they have adapted various physiological and metabolic advantages to endure these stresses. The common strategy among plant stress responses is to temporarily prolong survival by protecting crucial life processes until the stress subsides. This can be seen in responses such as the compartmentalization of excess salt to lower toxicity (Zhu, 2003), stomatal closure to reduce water loss (Neill *et al.*, 2008), and an oxidative burst to kill pathogen infected cells (Delledonne, 1998). With plants prospering in a diverse range of environments and ecosystems across the world, it is clear this strategy is successful.

One predominant stress factor encountered by plants occurs during times of flooding when the plant is exposed to an anaerobic environment due to oxygen diffusing through water 10,000 times slower than through air (Cussler, 2009). An environment with low levels of oxygen can be called hypoxic, whereas the absence of oxygen entirely can be called anoxic (Sasidharan *et al.*, 2017). Hypoxia can be caused by the restricted diffusion of oxygen through a substrate such as mud, or by the compact plant tissues found during early germination. This means that even plants that grow in environments with minimal water can experience hypoxic stress. Oxygen is required for aerobic

respiration, and when insufficient oxygen is present to drive mitochondrial oxidative phosphorylation, the plant undergoes an energy deficiency and eventually cell death (Gupta & Igamberdiev, 2011). This thesis will focus on various mechanisms relating to the mitochondria and how plants have adapted to combat hypoxic stress.

1.2 Mitochondrial electron transport chain

The mitochondrion is a double membrane bound organelle found exclusively in eukaryotic cells (Taiz & Zeiger, 2010). It is the primary site of energy production for all life processes in the form of adenosine triphosphate (ATP). This energy is generated via oxidative phosphorylation from the mitochondrial electron transport chain, also called the respiratory chain. The mitochondrial electron transport chain is located in the inner mitochondrial membrane and consists of the primary pathway and the alternative pathway.

The primary pathway consists of five membrane-bound proteins termed complexes I-V as well as various plant-specific alternative NADH and NADPH dehydrogenases (NDs) (Møller *et al.*, 2001). Products from the tricarboxylic acid (TCA) cycle and glycolysis donate electrons to complex I and NDs. These electrons enter a ubiquinone pool where they are then passed through the subsequent complexes via a series of redox reactions. Finally they pass through complex IV, also called cytochrome oxidase, which catalyzes the reduction of O_2 to H_2O (Figure 1). The passage of electrons

through the intermediary complexes results in protons being actively pumped into the intermembrane space, generating a proton motive force. This electrochemical gradient is used by ATP synthase to phosphorylate adenosine diphosphate (ADP) into ATP (Figure 1).

The secondary pathway, commonly referred to as the alternative pathway, was once considered to be unique to plants but has been shown to reside in some fungi, protists and animals (McDonald *et al.,* 2009). It is similar to the primary pathway with the addition of an alternate terminal oxidase termed alternative oxidase (AOX). Electrons are passed from the ubiquinone pool directly to AOX, bypassing complexes III and IV (Figure 1). Without the proton pumping activity of these bypassed complexes, significantly less proton motive force, and therefore less ATP, is generated (Panda *et al.,* 2013). The primary pathway links respiratory oxidation, electron transport and ATP production very tightly whereas the alternative pathway allows these processes to be unlinked, providing a mechanism to maintain metabolic homeostasis (Lambers, 1982). It also acts as a means to control reactive oxygen species (ROS) production (Maxwell *et al.,* 1999). Mitochondria are a source of ROS due to electrons leaking from the transport chain to free oxygen producing superoxide (Vanlerberghe, 2013). While electron leakage may occur anywhere along the transport chain, complex I and III are commonly seen as the primary sites (Jastroch *et al,* 2010). By bypassing a large portion of the electron transport chain, including complex III, the alternative pathway exhibits far less electron leakage and therefore less ROS production.

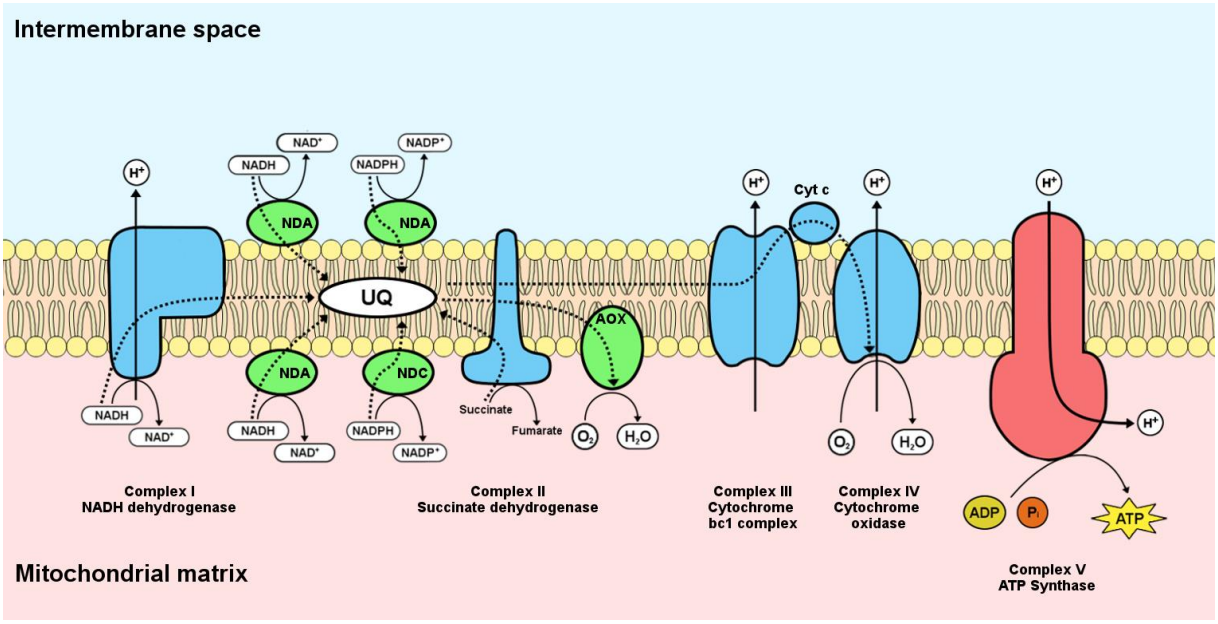

Figure 1: Mitochondrial electron transport chain. Dashed lines represent electron flow and solid black lines represent proton movement. (NDA and NDC – NAD(P)H dehydrogenase, UQ – ubiquinone, AOX – alternative oxidase). Created in GNU image manipulation program.

1.3 Reactive oxygen species

The most common ROS in plants are superoxide, hydroxyl radical and peroxides. Naturally present in low levels, these ROS play an important role in homeostasis and signalling intracellular responses such as stomatal closure, gravitropism, programmed cell death, hormone signalling, as well as biotic and abiotic stress responses (Miller *et al.,* 2008; Sharma *et al.,* 2012). However, their overproduction under stress can be seen as either a stressor or a stress response. Under high light or heat exposure, an excess of ROS are produced in a non-controlled manner due to the degradation of photosystems and over-reduction of mitochondria (Suzuki & Mittler, 2005). As ROS are highly reactive molecules, they can inactivate key enzymes, damage DNA and RNA, as well as cause lipid peroxidation of bilayers resulting in cell death. This is called oxidative stress. When a plant is infected by a pathogen however, ROS are deliberately overproduced as a stress response to kill the infected cell, restricting the pathogen's ability to reproduce and spread (Bolwell *et al.,* 2002). The unsaturated lipids of plant membranes are one of the primary targets of oxidative stress. The lipid hydroperoxides produced by this oxidative reaction are instable and highly reactive, thus malondialdehyde (MDA), a secondary product resulting from the breaking down of hydroperoxides, is commonly used as a biomarker to determine oxidative stress (Davey *et al.,* 2005). The careful balance between the production of ROS to ensure proper signalling and their scavenging to mitigate cellular damage is easily perturbed by abiotic stress and should always be considered when assessing the homeostatic state of a plant.

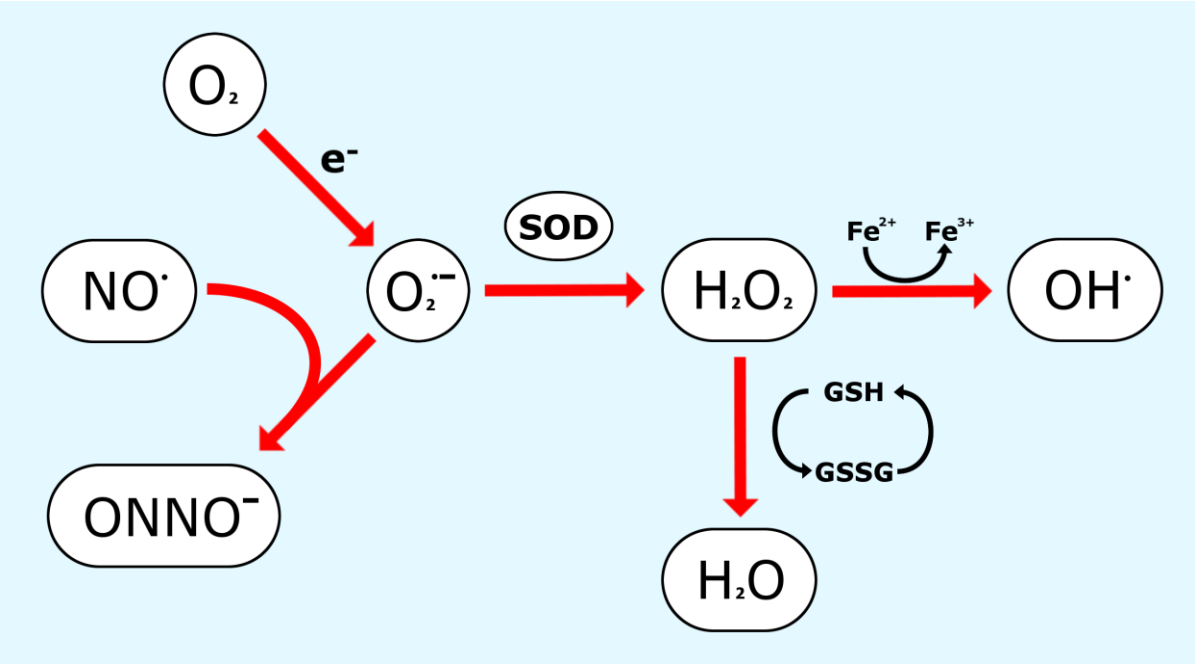

Figure 2: Reactive oxygen species and their transformations. Oxygen (O_2) receives a single electron to produce superoxide (O_2-), which can either freely react with nitric oxide ($NO^{\cdot}$) to produce peroxynitrite (ONNO-), or undergo dismutation via superoxide dismutase (SOD) to generate hydrogen peroxide (H_2O_2). H_2O_2 can be converted to hydroxyl radical ($OH^{\cdot}$) by the Fenton reaction or be detoxified into water (H_2O) by antioxidants such as glutathione (GSH) being converted into glutathione disulfide (GSSG). Created in GNU image manipulation program.

1.4 Nitric oxide

Nitric oxide (nitrogen monoxide) is a diatomic molecule with the chemical formula NO. It is one of the smallest diatomic molecules with a molar mass of 30.01 Da, has a relatively long half-life of 3-5 seconds when compared to other free radicals, and has a neutral charge (Henry *et al.*, 1997). Due to these physio-chemical properties, it has high diffusibility in both aqueous media and across cell membranes, making it an ideal signalling molecule. It has been shown that NO acts as a signal for various processes in growth and development, senescence, stomatal aperture, flowering and stress responses (Mur *et al.*, 2013; Neill *et al.*, 2003), and is a metabolic byproduct of alternative forms of mitochondrial respiration (Igamberdiev *et al.*, 2005). Its signalling and metabolic importance has garnered increased interest among the scientific community over the past decade.

Many forms of cellular nitrogen are converted into NO, and the pathways which accomplish this can be divided into two groups; the reductive and the oxidative (Gupta *et al.*, 2012). The reductive pathways consist of the best characterized sources of NO production. Nitrite (NO_2^-) can be reduced within the cytoplasm, mitochondria, chloroplasts, peroxisomes and apoplast of the plant cells (Roszer, 2012). Nitrate reductase can carry out this reduction when nitrite accumulates in the cell under stress conditions (Gupta & Igamberdiev, 2011). Similarly, the mitochondrial electron transport chain can use electrons from the ubiquinone pool to reduce nitrite.

Very little is understood about the oxidative pathways of NO production. Nitric oxide synthase is a well characterized source of NO production in animals, oxidizing L-arginine into L-citrulline and NO (Bruckdorfer, 2005), however no direct homologue protein has been identified in plants. The term NOS-like activity is more commonly used when observing the consumption of L-arginine and consequent production of NO in plants. As this reaction requires oxygen, it is unlikely to be a major source of NO under hypoxic stress.

1.5 The nitric oxide signal

While the sources of NO are well established, little is known about how the NO signal is sensed to cause the alteration of gene expression and protein activity. A NO receptor has not been identified in plants, but it has been suggested that NO may accomplish this through cGMP pathways, MAPK cascades or the direct *S*-nitrosylation, metal-nitrosylation or tyrosine nitration of target proteins (Arasimowicz & Floryszak-Wieczorek, 2007; Baudouin, 2011). There's no doubt that cells do sense NO but the downstream transduction signal they facilitate remains a mystery.

The increased production of NO under a wide range of abiotic stresses is attributed to it being a stress response signal. Similar to ROS however, the overproduction of reactive nitrogen species (RNS) such as NO can be damaging to the cell. This is called nitrosative stress. Aconitase, the enzyme responsible for catalyzing

citrate to isocitrate as part of the citric acid cycle, is inhibited by NO and commonly used as a biomarker to determine intracellular nitrosative stress (Navarre *et al.*, 2000). NO can also be considered an antioxidant as it interacts with various ROS to form inactive products (Corpas & Juan, 2013). The modulation of ROS and RNS as both signals and damaging reactive molecules is a balancing act between their production and scavenging which is further complicated under stress.

1.6 Phytoglobin

Plant hemoglobins, hence-forth referred to as phytoglobins, are a group of heme proteins that can be classified as either symbiotic or non-symbiotic. Symbiotic phytoglobins, or leghemoglobin, work in symbiosis with the nitrogen-fixing bacteria *Rhizobium* most commonly found in the root nodules of legumes (Gupta *et al.*, 2011). The bacteria fix nitrogen for the plant, and in return the plant provides an efficient environment to support the bacteria's life cycle. One crucial aspect of this environment is maintaining the oxygen levels needed for nitrogen-fixation by nitrogenase enzymes. Symbiotic phytoglobins are suited for this role as they possess a high affinity to oxygen.

Non-symbiotic phytoglobins earned their name due to their inability to act symbiotically within root nodules. They are present in all plants and are expressed in seed, root and stem tissue (Dordas, 2009). Divided into two classes, non-symbiotic phytoglobins have been found to function in seed development and germination, root

development, flowering and stress response (Hebelstrup *et al.*, 2007). Class 1 possesses a high oxygen affinity and low oxygen dissociation rate constant, indicating that it is unlikely they play a role in either electron or oxygen transport (Gupta *et al.*, 2011). They are present in very low concentrations *in vivo*, but are highly expressed under low oxygen stress in which they function as nitric oxide scavengers to help maintain ATP and NADH ratios via the phytoglobin/nitric oxide cycle (Gupta & Igamberdiev, 2011). Class 2 non-symbiotic phytoglobin have similar physio-chemical properties as class 1, but are induced under cold stress rather than hypoxia. They have been theorized to play a role in seed oil production by promoting oxygen supply in developing seeds (Gupta *et al.*, 2011). The widespread presence of non-symbiotic phytoglobins in the plant kingdom suggests that they may have multiple key roles in plant metabolism.

1.7 The phytoglobin-nitric oxide cycle

The phytoglobin-nitric oxide (Pgb-NO) cycle (Figure 2) is crucial to the short-term survival of plant tissues under hypoxic stress. When insufficient oxygen is present to drive mitochondrial oxidative phosphorylation, ATP levels dwindle causing a cell wide energy crisis. Plants have been shown to substitute oxygen with nitrite as the terminal electron acceptor of the electron transport chain, converting nitrite to NO (Gupta & Igamberdiev, 2011). This maintains electron flow in diminished quantities, and the small amount of ATP being produced when compared to normoxia is enough to prolong plant

survival. As NO is a gas, it escapes the mitochondria into the cytosol. With nitrite being in far lower supply than atmospheric oxygen, nitrogen stores would quickly deplete if not for the scavenging of NO by phytoglobin. Class 1 non-symbiotic phytoglobin are induced under hypoxic stress and use what little oxygen is present to form oxyphytoglobin [Pgb(Fe^{2+})O_2]. This oxyphytoglobin prevents NO from escaping the tissue by oxidizing it to nitrate [NO_3^-]. Nitrate reductase (NR) can then reduce nitrate into nitrite to be reused in the resulting Pgb-NO cycle. The oxidation of NO by oxyphytoglobin results in the formation of oxidized ferric metPgb [Pgb(Fe^{3+})] which can then be reduced back into its ferrous form by metPgb reductase. Both nitrate reductase and metPgb reductase oxidize excess NAD(P)H which accumulates under hypoxia, further alleviating metabolic stress.

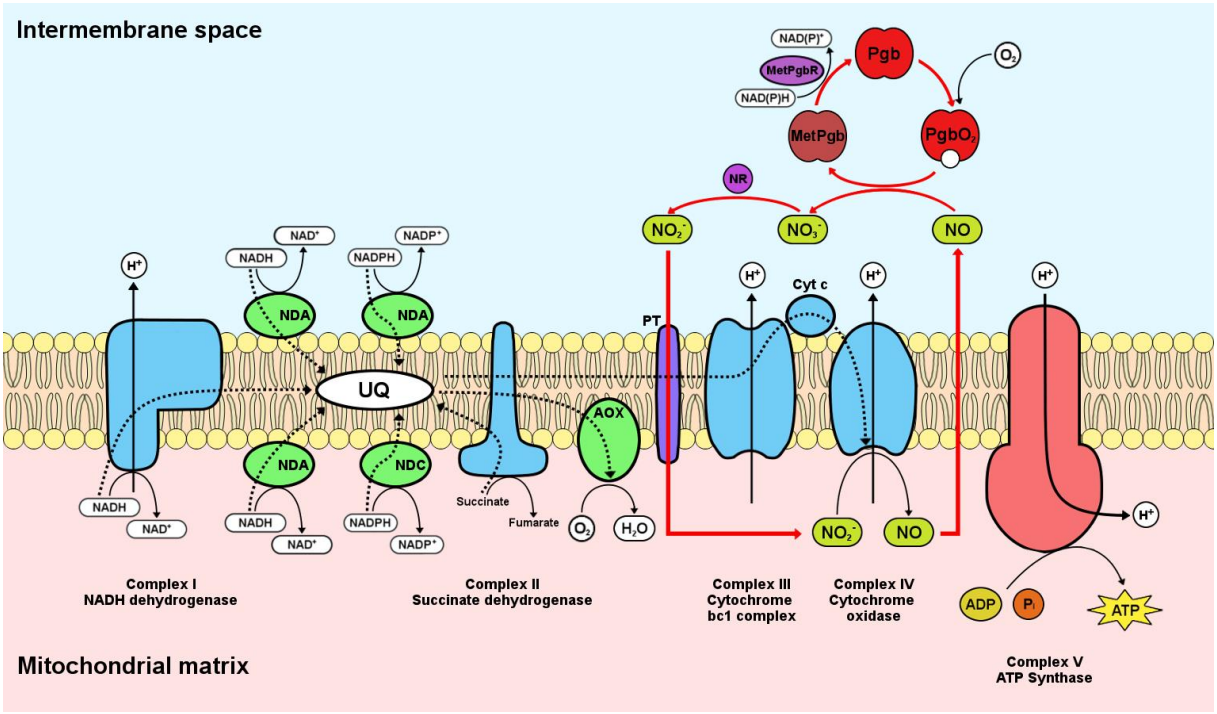

Figure 3: Mitochondrial electron transport chain with the phytoglobin/nitric oxide cycle. Dashed lines represent electron flow, solid black lines represent proton movement and solid red lines represent the phytoglobin/nitric oxide cycle. Structures in blue are present in all mitochondrial electron transport chains, whereas structures in green are unique to plants. (NDA and NDC – NAD(P)H dehydrogenase, UQ – ubiquinone, AOX – alternative oxidase, NR – nitrate reductase, Pgb – phytoglobin, $PgbO_2$ – oxyphytoglobin, metPgb – oxidized ferric phytoglobin form, metPgbR – metPgb reductase). Created in GNU image manipulation program.

1.8 Fermentation

The Pgb-NO cycle is far from the only stress response plants deploy under hypoxic stress. The fermentation enzymes lactate dehydrogenase (LDH) and alcohol dehydrogenase (ADH) are induced under hypoxic stress and work in tandem to maintain the $NAD(P)^+$/NAD(P)H ratio. As hypoxia results in a loss of respiratory function, NAD(P)H oxidation is blocked, causing it to accumulate (Sairam *et al.*, 2008). Lactate dehydrogenase catalyzes the conversion of pyruvic acid to lactate as it oxidizes NADH. Similarly, alcohol dehydrogenase catalyzes the conversion of acetaldehyde to ethanol while oxidizing NADH. Activity is modulated between the two to prevent significant pH change in the cell. LDH is often the first to be induced, producing lactate (lactic acid) which lowers cellular pH. Once the pH drops to just below neutral, LDH is inactivated and ADH is activated which subsequently produces ethanol, thereby raising pH and preventing any major changes. While the Pgb/NO cycle is critical in maintaining electron acceptors, fermentation works at the opposite end of the mitochondrial electron transport chain in continuing glycolytic pathways and maintaining electron donors.

1.9 The role of phytoglobin and nitric oxide in morphogenesis

NO is a crucial signalling molecule in various morphological processes such as shoot and root growth and development (Pagnussat *et al.*, 2002), flowering (He *et al.*,

2004) and senescence (Mishina *et al.,* 2007). The effects of NO can be seen throughout the entire life cycle of a plant. While phytoglobin is present in much higher concentrations under stress, members of class 1 and class 2 phytoglobin are upregulated by cytokinin and abscisic acid under non-stress conditions. This has been observed in the early stages of development, as well as in the primary locations of growth such as root tips, the hypocotyl, leaf hydathodes and the auxiliary meristems (Hebelstrup *et al.,* 2013). As both bind oxygen very tightly, it is unlikely they function in oxygen transport and are more likely tasked with modulating NO levels. As previously stated, balancing the signalling properties and destructive capabilities of NO is done by controlling its production and scavenging. Phytoglobin could be a crucial scavenger to help maintain this balance throughout all stages of development.

1.10 Research objectives

While our understanding of plant metabolism under hypoxic stress has greatly expanded over the past decade, many questions remain unanswered. My thesis will focus on the Pgb-NO cycle and aim to expand our knowledge of both its function and effectiveness. In chapter 2, I will use transgenic tobacco plants with modified amounts of AOX to determine if AOX participates in the Pgb-NO cycle, as well as to gain a better understanding of its overall role under hypoxic stress. In chapter 3, I will use transgenic barley plants with modified amounts of Pgb to determine the impact of Pgb expression on

hypoxic energy maintenance and plant survivability. This will be done in the early stages of plant development to identify when during development the Pgb-NO cycle begins to impact energy mobilization. Chapter 4 will build on identifying the role of Pgb during early growth by investigating how altering Pgb expression impacts NO signalling and morphogenesis. My thesis will add to the ever growing pool of research into hypoxic metabolism and hopefully identify a transgenic plant of agricultural importance. A mutation that results in a positive impact on hypoxic metabolism could yield flood resistant crops.

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Chapter 2: Differential expression of alternative oxidase modulates normoxic and hypoxic metabolism

2.1 Introduction

The alternative oxidase (AOX; EC 1.10.3.11) of plant mitochondria is a terminal oxidase that helps maintain metabolic homeostasis. Similar to cytochrome *c* oxidase (COX, EC 1.9.3.1), AOX acts as a terminal electron donor in a pathway called the alternative pathway. This pathway bypasses the proton pumping activity of complex III and IV and thus produces less ATP and reactive oxygen species (ROS) (Vanlerberghe & McIntosh, 1997). Modulating respiratory electron flow between the primary and alternative pathways allows for control of cellular redox and energy balance (Vanlerberghe & McIntosh, 1992; Vanlerberghe *et al.*, 2009; Dahal *et al.*, 2017). While this has mainly been observed under non-stressed conditions, AOX expression has been shown to increase during and post hypoxic stress (Szal *et al.*, 2003) as well as during pathogen infection, cold, water deficit, oxidative stress and nitrosative stress (Vanlerberghe & McIntosh, 1997). Since AOX has a much lower affinity for oxygen than COX (Millar *et al.*, 1994; Affourtit *et al.*, 2001), it is usually considered to play a minor role under low oxygen stress. The nitric oxide (NO) produced under hypoxia however, lowers the affinity of COX for oxygen (Millar *et al.*, 1994) but does not affect AOX (Cooper, 2002). Expression of AOX has even been shown to increase in the presence of NO (Huang *et al.*, 2002), hypoxia (Gupta *et al.*, 2012), and ROS such as superoxide, hydrogen peroxide, singlet oxygen and the hydroxyl radical (Feng *et al.*, 2008; Feng *et*

al., 2010; Minagawa *et al.*, 1992; Wagner, 1995). These ROS accumulate when the mitochondrial primary pathway is restricted, either by inhibitors (Minagawa *et al.*, 1992) or by abiotic stress (Low & Merida, 1996). These observations hint at AOX having an increased role under hypoxic conditions or during the subsequent period of re-oxygenation. It has been suggested that similar to COX, AOX could generate NO by directly donating electrons to nitrite as part of the Pgb/NO cycle. Tobacco cell suspensions under anoxia have had their NO emissions reduced by half due to the addition of salicylhydroxamic acid (SHAM), an inhibitor of the AOX pathway (Planchet *et al.*, 2005). It may be the case that AOX operates independently of NO turnover, and rather helps maintain a limited rate of respiration under NO accumulation. Under hypoxia, the intracellular concentration of oxygen may remain sufficient for at least partial AOX operation, especially during the subsequent reoxygenation period when ROS are intensely produced.

In this chapter, I used transgenic tobacco plants with modified amounts of AOX to gain a better understanding of the role of AOX under hypoxic stress. I observed how altering AOX expression would influence the metabolic flux of organic and amino acids, as well as the balance between formation and scavenging of ROS and RNS under normoxia and hypoxia.

2.2 Materials and methods

2.2.1 Plant growth and sampling

Tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) wild type (WT), AOX knockdown mutant R19 and AOX overexpressor mutant B7 (Wang *et al.,* 2011; Wang *et al.,* 2013) were grown in a growth chamber (Forma Environmental Chamber; Thermo Scientific). The chamber was set to 14 h day and 10 h night cycle. The day temperature was set to 26°C and the night temperature was set to 20°C. The plants were moisture sensitive and care was taken to ensure that moisture was set to 50% saturation and never allowed to exceed 60%. Plants were watered every alternate day and 20-20-20 fertilizer was added once every two weeks as prescribed by the supplier, Plants Products, Brampton, Ontario, Canada.

2.2.2 Hypoxic stress treatment

To test plants under hypoxic stress, an entire individual plant was placed in a custom built, sealed chamber with a small opening on each side. One opening had a tube leading to a gas source, allowing a steady flow (about 5 psi) of gas to enter the chamber. The gas used was either pure nitrogen (Alphagaz 1 grade), nitrogen mixed with 0.1% oxygen or nitrogen mixed with 3% oxygen. The other opening allowed for the gas to escape, creating either a constant anoxic or hypoxic environment at normal atmospheric pressure. This chamber was placed in total darkness inside a black plastic bag to prevent

photosynthetic oxygen production. The chamber was opened after 3 hours and the plant was quickly tested using the following described methods.

2.2.3 NO measurements

A single leaf was detached at the base of the petiole and immediately placed in 20 mM Hepes buffer with 50 mM sodium nitrate. When inhibitors were used, this buffer also contained either 5 mM potassium cyanide (KCN) or 100 µM sodium tungstate (Na_2WO_4). Extra care was taken to ensure the cut end spent as little time as possible in open air to prevent bubbles from forming in the vascular tissue. The leaf was then placed in an air tight chamber with a constant inflow of nitrogen at 120 mL min^{-1}. NO was measured by chemiluminescence as described by Planchet *et al.*, (2005). In brief, a constant flow of measuring gas (nitrogen) at 120 mL min^{-1} was pulled through the chamber and subsequently through the chemiluminescence detector (CLD 770 AL ppt; Eco-Physics, Durnten, Switzerland; detection limit 20 ppt; 20 s time resolution) by a vacuum pump connected to an ozone destroyer. The ozone generator of the chemiluminescence detector was supplied with dry oxygen (99%). The inflowing gas (nitrogen) was made NO free by passing it through a NO scrubber supplied by Eco Physics Ltd, Switzerland. Flow controllers (Fisher Scientific) were used to observe and adjust all gas flows (Figure 1). The emission slope of 5 leaves were averaged for each plant line.

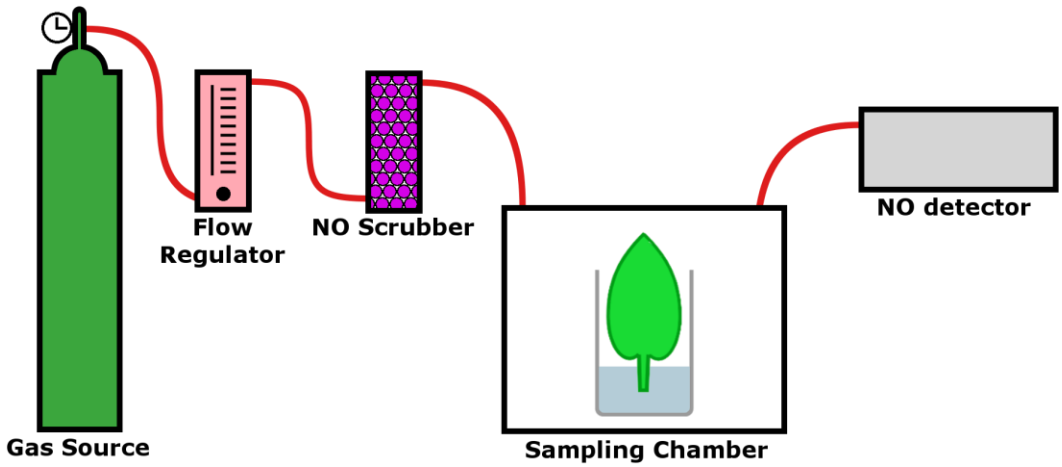

Figure 1: Experimental setup for NO measurements from leaf tissue. Red lines represent medical tubing used to connect each component.

2.2.4 Aconitase activity

Fresh leaf biomass (100 mg) was crushed with 1 mL of extraction buffer using a mortar and pestle over ice. The extraction buffer consisted of 50 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, 5 mM $MgCl_2$, 2 mM trisodium citrate and 0.4 M mannitol. The extract was centrifuged at 3000 × *g* for 5 min at 4°C before having the supernatant removed and centrifuged again at 10000 × *g* for 20 min at 4°C. The mitochondria-containing pellet from this centrifugation was then resuspended in a buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM trisodium citrate, 5 mM dithiothreitol and 100 mM $MgCl_2$. The cytoplasmic supernatant from this centrifugation and the resuspended pellet were then assayed individually. The assay buffer consisted of 50 mM Tris-HCl (pH 7.4) and 40 mM citrate. Extract (100 μL) was added to 900 μL of assay buffer and aconitase activity was measured spectrophotometrically at 240 nm for 10 min using Ultraspec 4300 (Biochrom). An extinction coefficient of 3.6 $mM^{-1}cm^{-1}$, which corresponds to cis-aconitate, was used (Baumgart and Bott, 2011). The cross-contamination between the mitochondria-containing pellet and the cytosol was identified using the marker enzymes succinate dehydrogenase for mitochondria, and lactate dehydrogenase for cytosol (Eprintsev *et al.,* 2014). The level of cross-contamination did not exceed 10%.

2.2.5 Superoxide assay

Fresh leaf biomass (100 mg) was crushed with 1 mL of 8 M potassium hydroxide using a mortar and pestle over ice. The extract was then centrifuged at 15000 × *g* for 10 min at 4°C. The level of superoxide anion present in the supernatant was measured by the method described in Ma et al. (2016) using the reduction of cytochrome *c* at 550 nm.

2.2.6 Hydrogen peroxide assay

Fresh leaf biomass (100 mg) was crushed in the same way as superoxide, and the extract was homogenized in 6% (v/v) trichloroacetic acid for 30 min before being centrifuged at 15000 × *g* for 10 min at 4°C. The level of hydrogen peroxide in the supernatant was measured spectrophotometrically by using a Pierce Quantitative Peroxide Assay kit provided by Thermo Scientific.

2.2.7 Malondialdehyde assay

Lipid Peroxidation was measured in terms of malondialdehyde (MDA) content by the thiobarbituric acid (TBA) method described by Heath and Parker (1968) with some modifications. Fresh leaf biomass (250 mg) was homogenized in 5 mL of 0.1% (v/v) TCA using a mortar and pestle before being centrifuged at 10 000 × *g* for 5 min. A 1 mL aliquot of supernatant was added to 3 mL of 20% (v/v) TCA containing 0.5% (w/v) TBA.

The mixture was incubated at 95°C for 30 min, quickly cooled in crushed ice and centrifuged at 10 000 × *g* for 10 minutes. The supernatant then had its absorbance measured at 532 nm and the concentration of MDA was calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.2.8 Total radical scavenging capacity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was conducting according to the method described by Brand-Williams et al. (1995) with some modifications. The stock solution of 1 mM DPPH in methanol was diluted to 60 µM, 1.9 mL of which was mixed with 0.1 mL of leaf extract. The mixture was shaken vigorously and left in the dark for 20 min. The absorbance was read at 515 nm. Total scavenging capacity was expressed as percentage of inhibition of DPPH consumption. The gallic acid standard curve was used to express the results as gallic acid equivalent (GAE).

2.2.9 Total phenolic content

Soluble phenolics and other compounds were extracted from leaves by homogenizing in 80% (v/v) acetone with 0.2% (v/v) formic acid in a ratio of 1:10. The homogenate was then shaken for 8h at 4°C before being centrifuged at 20000 × *g* for 20 min. The subsequent pellet was extracted twice under the same conditions and the supernatants were mixed together and further diluted to make the working concentration

1 g L^{-1}. Total soluble phenolic content was determined using Folin-Ciocalteu reagent as described by Chandrasekara and Shahidi (2011) with modifications described by Vyas et al. (2013). Total soluble phenolic content was determined using the gallic acid standard curve and expressed as GAE per leaf fresh weight.

2.2.10 Total flavonoid content

Total flavonoid content was measured by aluminum chloride colourimetric assay as described by Zhishen et al. (1999). Either 1 mL of extract or 0.5 mL of standard catechin solution was mixed with 4 mL of water. After 5 min, 0.3 mL of 5% (w/v) $NaNO_2$ and 0.3 mL of 10% (w/v) $AlCl_3$ were added. After another 1 min, 2 mL of 1 M NaOH was added and the final volume was adjusted with water to 10 mL. The absorbance was measured at 510 nm. Total flavonoid content was expressed as catechin equivalent (CE) per leaf fresh weight.

2.2.11 Total tannin content

Total tannin content was determined by the method described by Chandrasekara and Shahidi (2011). The 0.5% (w/v) vanillin-HCl reagent (5 mL) was added to 1 mL of planet extract, mixed thoroughly and incubated at room temperature for 20 min. A separate blank for each sample was read with 4% (v/v) HCl in methanol. The absorbance

was read at 500 nm and the content of proanthocyanidins was expressed as CE per leaf fresh weight.

2.2.12 Sugars, organic acids and amino acids

Metabolomic studies were performed using nuclear magnetic resonance (NMR) analysis (Psychogios et al, 2011; Kruger *et al.,* 2008). Leaf tissue was flash frozen in liquid nitrogen and powdered using ceramic beads and a table top centrifuge. The resulting powder was then homogenized in 2 M perchloric acid and incubated at room temperature for 1 h. Homogenates were neutralized on ice to pH 7 using 3 M potassium hydroxide and the subsequent potassium perchlorate precipitate was removed by centrifuging at 15 000 x *g* for 10 min at 4 °C . Samples were then freeze-dried for 48 hours before being homogenized in heavy water (D_2O) using a ceramic mortar. After incubation for 24 h at room temperature, the solutions were centrifuged and an aliquot of the supernatant (aqueous extract) with the volume of ~0.6 mL was placed in a vial for NMR analysis performed by The Metabolomics Innovation Centre. Subsequently, 140 µL of a standard buffer solution (54% (v/r) D_2O: 46% (v/v) 150 mM KH_2PO_4 pH 7.0 containing 5.0 mM DSS-d6 (2,2-dimethyl-2-silcepentane-5-sulphonate), 5.84 mM 2-chloropyrimidine-5 carboxylate, and 0.1% (w/v) NaN_3 in H_2O) was added to the sample.

2.2.13 Statistical analyses

At least three individuals for each plant line were selected for experimentation allowing for biological and experimental triplicates. Results were averaged and standard deviation bars were calculated using Microsoft Excel. Statistical analyses were performed using Prism 5.0 (GraphPad Software). Two-way ANOVA analyses were followed by a Bonferroni post-test to identify statistically significant differences between plant lines within a treatment.

2.3 Results

2.3.1 Nitric oxide emission rates

The rate of NO emission was measured from leaves incubated in nitrate solution, in darkness and under different oxygen concentrations (Figure 2). Under near anoxic conditions (0.001% O_2) and hypoxic conditions (0.1% O_2), the rate of NO emission was almost half in knockdown plants compared to WT and overexpressing plants. NO emission rates were much higher in all plant lines under near anoxia. In 3% O_2 and ambient air, no NO emission could be detected by this chemiluminescent method. The addition of cyanide to the nitrate solution resulted in no NO emissions under each environmental condition. The addition of tungstate to the nitrate solution resulted in an 80% decrease in NO emission under each environmental condition.

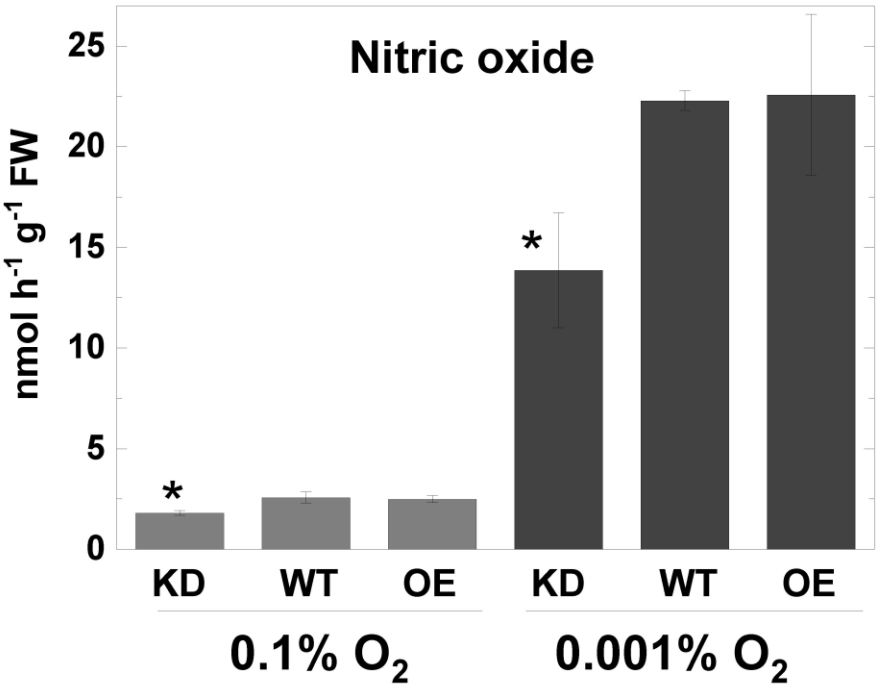

Figure 2. Nitric oxide emission rates measured from the leaves of tobacco plants with differentially expressing AOX. KD (knockdown plants, transgenic line R19); WT (wild type); OE (overexpressing plants, transgenic line B7). Light grey bars – 0.1% oxygen atmosphere in nitrogen (hypoxia); dark grey bars – 0.001% oxygen atmosphere in nitrogen (anoxia). Asterisks indicate significant difference between AOX mutants compared to WT at $P < 0.05$.

2.3.2 Aconitase activity

Cytosolic and mitochondrial aconitase activity was measured under both normoxic conditions and within a 2 min period of re-oxygenation following a 3 h hypoxic condition (Figure 3). Under normoxia, both cytosolic and mitochondrial aconitase activity was similar between the WT, knockdown and overexpressing plants. While hypoxia had no significant impact on cytosolic aconitase activity across all plants lines, it did reduce mitochondrial activity and this effect differed for each plant line. The least severe decline occurred in the knockdowns and the most severe decline occurred in the ovexpressors, while the WT showed an intermediate response. When cyanide was present, total aconitase activity lowered as a result of both cytosolic and mitochondrial activity lessening. The hypoxic treatment had much less impact on the mitochondrial aconitase activity in the presence of cyanide, regardless of plant line.

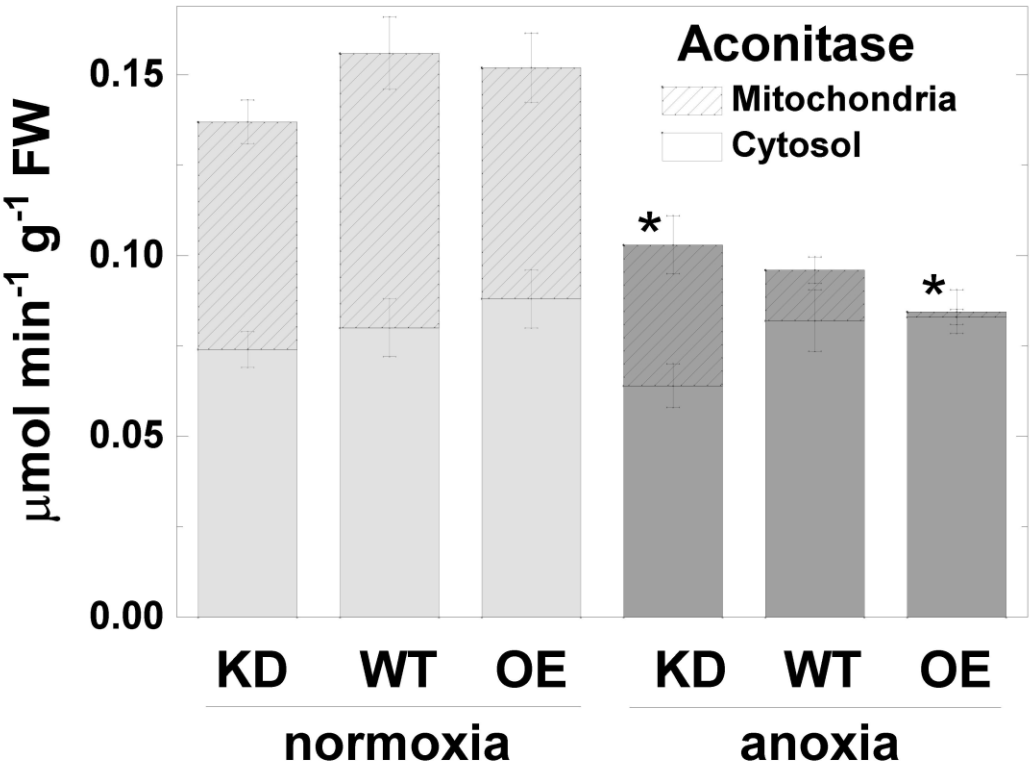

Figure 3. Level of aconitase activity in leaf mitochondria and cytosol of tobacco plants with differentially expressing AOX. Abbreviations are the same as Figure 2. Light grey bars – normal aeration, dark grey bars – after 3 h of incubation in nitrogen atmosphere (reoxygenation). Asterisks indicated significant difference between AOX mutants compared to corresponding WT within mitochondria at $P < 0.05$.

2.3.3 Superoxide, hydrogen peroxide and malondialdehyde levels

The amounts of superoxide, hydrogen peroxide and malondialdephyde were measured under both normoxic conditions and during re-oxygenation (Figure 4). In WT plants, the amount of superoxide, hydrogen peroxide and malondialdehyde was significantly increased by the hypoxic (re-oxygenation) condition. In knockdown plants, the amount of superoxide was approximately 2-times higher under normoxia and 1.5-times higher under hypoxia, compared to the corresponding WT plants. Malondialdehyde was slightly higher in these knockdown plants when compared to WT. The amount of hydrogen peroxide however, was similar between the WT and knockdown plants, regardless of condition. The overexpressing plants showed similar superoxide, hydrogen peroxide and malondialdehyde amounts as the WT, regardless of condition. Nearly no malondialdehyde was present under normoxic conditions for any plant line.

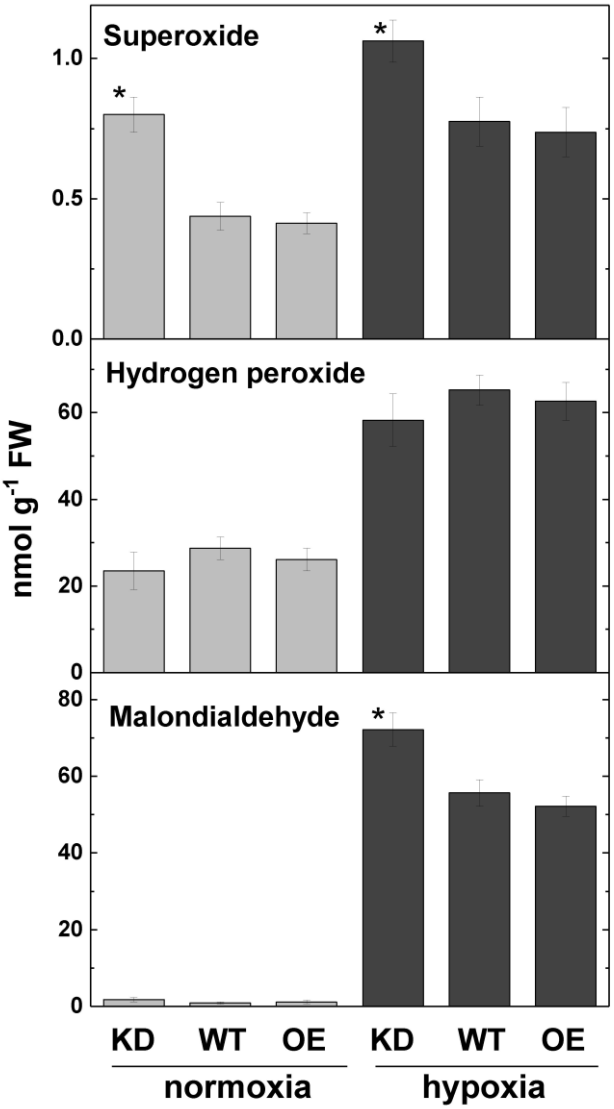

Figure 4. Levels of superoxide, hydrogen peroxide and malondialdehyde in the leaves of tobacco plants with differentially expressing AOX. Abbreviations are the same as Figure 2. Light grey bars – normal aeration, dark grey bars – after 3 h of incubation in nitrogen atmosphere (reoxygenation). Asterisks indicated significant differences between AOX mutants as compared to WT at $P < 0.05$.

2.3.4 Antioxidant capacity

The total radical scavenging capacity, amount of phenolics and amount of flavonoids were significantly higher in WT plants following a 3 h hypoxic condition, compared to normoxia (Figure 5). Under normoxia, the knockdown plants maintained a higher radical scavenging capacity, phenolics and flavonoids than the WT plants. Each of these amounts increased in the WT plants from normoxia to hypoxia, but the knockdown plants were unaffected. Tannins however, did not increase in the WT plants from normoxia to hypoxia, nor were they higher in the knockdowns under either condition when compared to WT plants. Overexpressing plants showed a different pattern. They had a higher radical scavenging capacity and amount of phenolics than WT under normoxia, while the amount of flavonoids and tannins were similar to WT. However, in response to hypoxia, total radical scavenging capacity dropped dramatically, while the amount of phenolics, flavonoids and tannins rose dramatically.

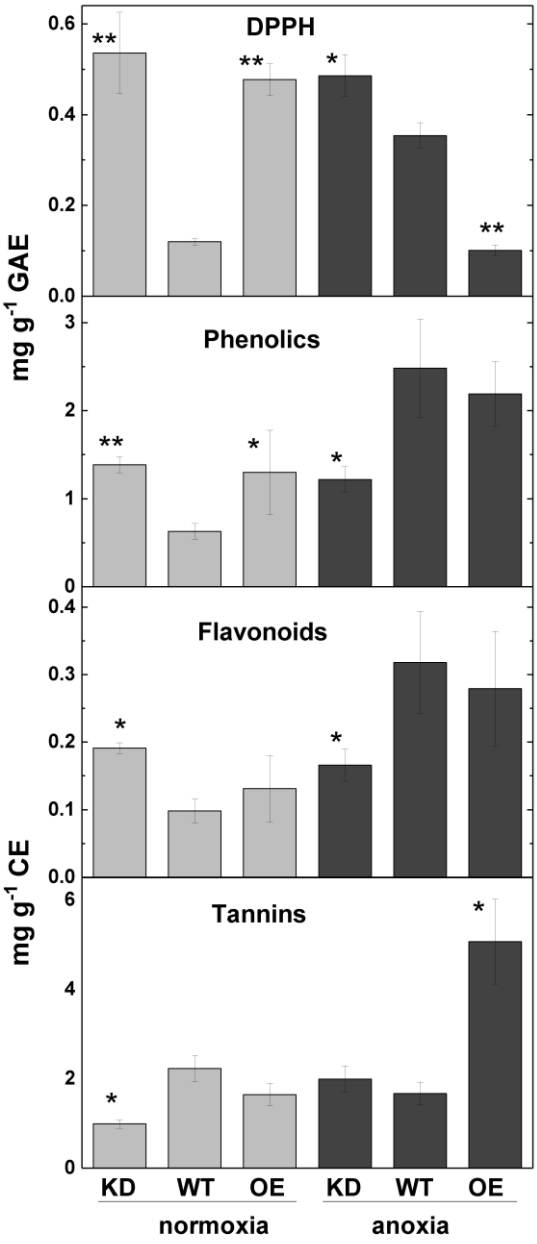

Figure 5. Total phenolics, flavonoids, tannins and radical scavenging capacity (DPPH assay) in the leaves of tobacco plants with differentially expressing AOX. GAE (gallic acid equivalent), CE (catechin equivalent), all other abbreviations are the same as Figure 2. Asterisks indicated significant differences between AOX mutants as compared to WT at *P < 0.05, **P < 0.005.

2.3.5 Sugars and organic acids

Under normoxic conditions, the amount of sugars (glucose and galactose) and organic acids (lactate, acetate, pyruvate, citrate and malate) were similar between WT and overexpressing plants, but higher in knockdown plants (Figure 6). The knockdown plants maintained much higher sugar and organic acid amounts when compared to WT under both normoxia and hypoxia. While hypoxia had little to no effect on organic acids (pyruvate, citrate, succinate and malate), there was a decrease in sugars (glucose and galactose) in the knockdown plants. Overexpressor plants showed a different pattern following hypoxia. While sugar amounts were unchanged by hypoxia and remained similar to that of the WT plants; lactate, acetate, succinate and malate were increased by hypoxia and higher than in WT plants. Pyruvate and citrate showed a small decrease following hypoxia, to amounts similar to that of WT plants under hypoxia.

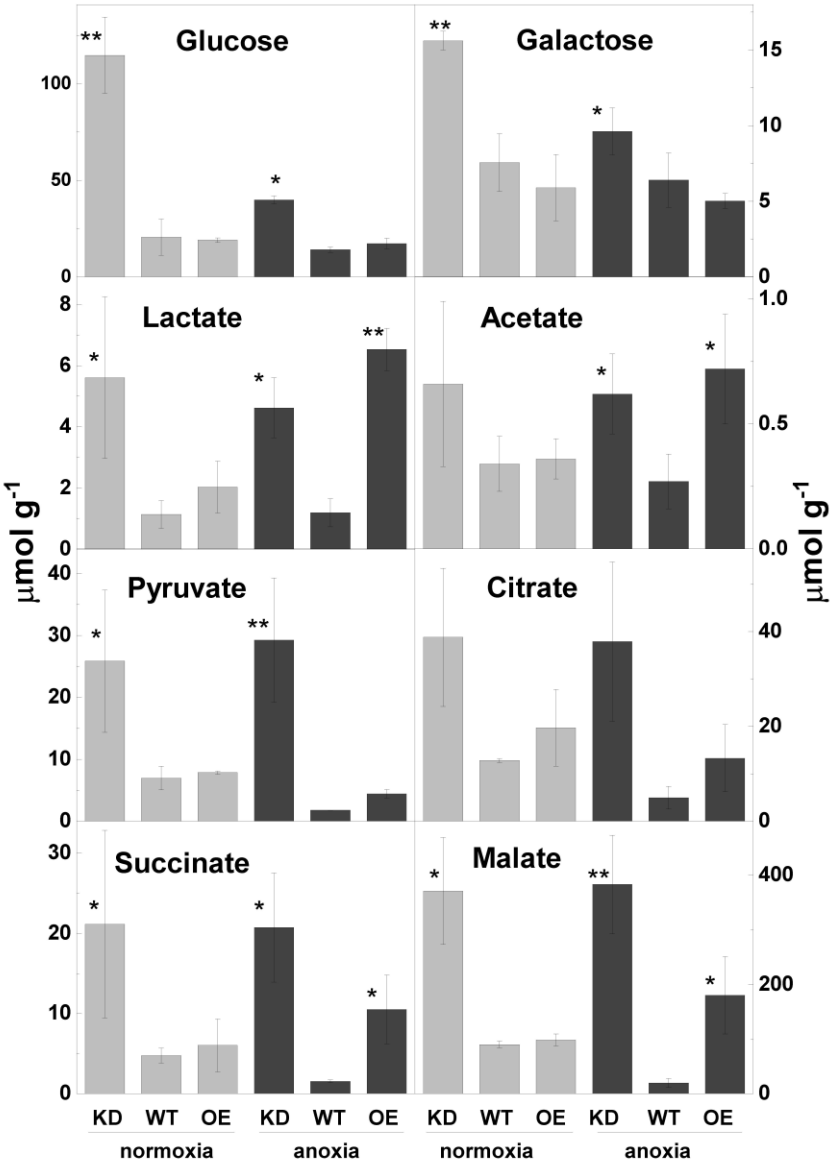

Figure 6. Level of sugars and organic acids in the leaves of tobacco plants with differentially expressing AOX. Abbreviations are the same as Figure 2. Light grey bars – normoxia, dark grey bars – after incubation in nitrogen atmosphere. Asterisks indicated significant differences between AOX mutants as compared to WT at *P < 0.05, **P < 0.005.

2.3.6 Amino acids

Under normoxia, the amount of glycine and serine was much higher in the knockdown plants when compared to WT (Figure 7). For serine this was a several-fold difference, whereas for serine it was only 2-fold. Following hypoxia, both glycine and serine were reduced to similar levels as WT plants under the same conditions. Overexpressing plants maintained slightly higher levels of glycine and slightly lower levels of serine when compared to WT plants under normoxia and hypoxia. The amount of alanine was similar across all plants lines under normoxia. Hypoxia caused an increase in alanine across all plant lines, but this change was most significant in the overexpressing plants. Similarly, both GABA and threonine amounts were higher following hypoxia, however there was no significant difference between plant lines under normoxia or hypoxia. Under normoxia, leucine, isoleucine and asparagine were all lower in the overexpressing plants, compared to the WT. These amino acids increased under hypoxia in the WT and overexpressing plants, but decreased in the knockdown plants.

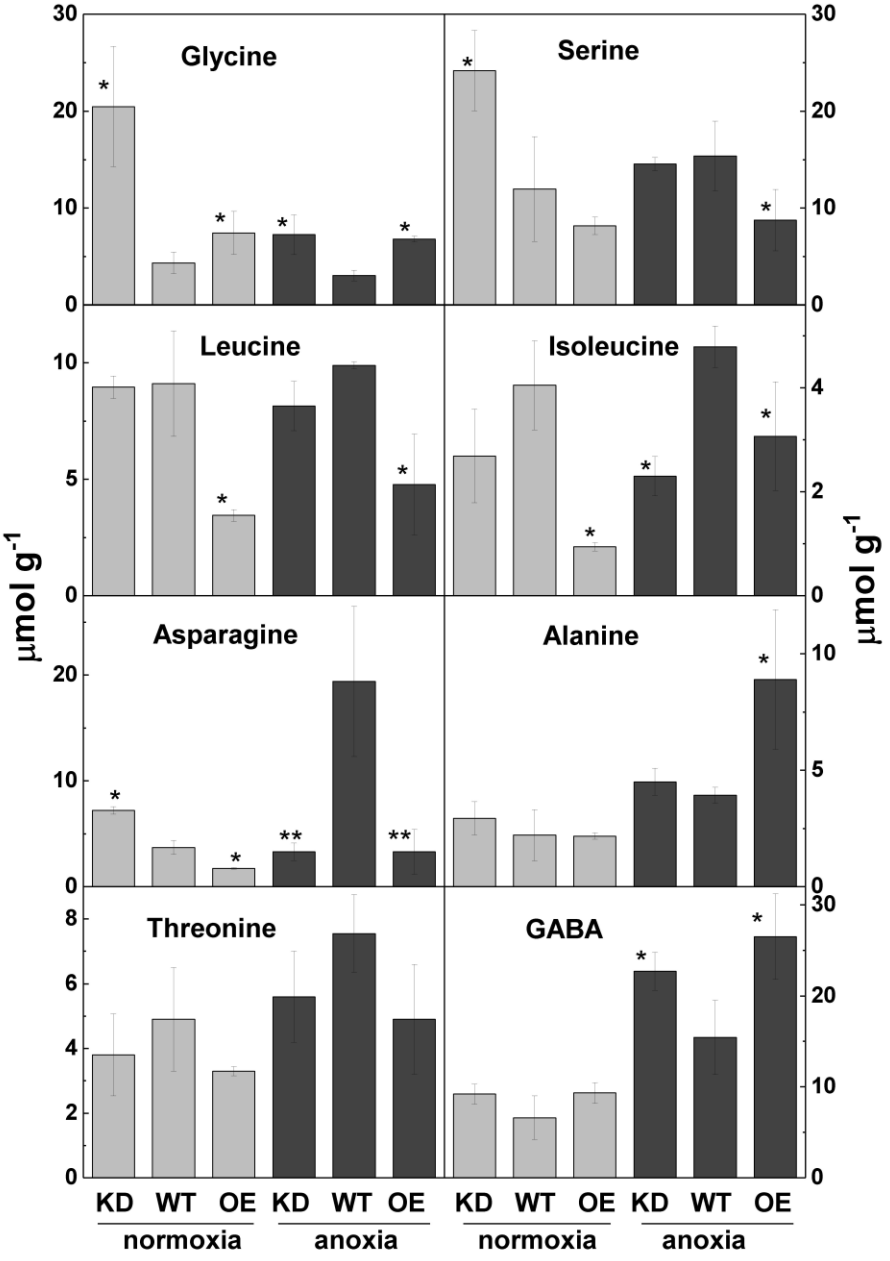

Figure 7. Level of amino acids in the leaves of tobacco plants with differentially expressing AOX. Abbreviations are the same as Figure 2. Light grey bars – normoxia, dark grey bars – after incubation in nitrogen atmosphere. Asterisks indicated significant differences between AOX mutants as compared to WT at *P < 0.05, **P < 0.005.

2.4 Discussion

2.4.1 AOX and metabolic homeostasis under stress

AOX plays an important role in maintaining metabolic homeostasis between the respiratory chain, TCA cycle and glycolysis (Vanlerberghe, 2013). Carbohydrate oxidation, electron transport and ATP production are tightly linked through the primary pathway, but use of the alternative pathway largely unlinks these processes. Due to the alternative pathway producing less ATP than the primary pathway, modulating its use gives the mitochondrion independent control over the quantity of ATP produced. Under normoxia, the AOX knockdown plants display significantly higher amounts of carbohydrates (glucose, galactose) and organic acids, suggesting that a lack of AOX limits the respiratory carbon oxidation pathways (Figure 6). This trend is seen to a lesser degree in the knockdowns under hypoxia, suggesting that AOX impacts the flux of carbohydrate oxidation in this condition as well. Lactate, normally an end product of glycolysis under hypoxia due to fermentation, as well as its precursor pyruvate are elevated in knockdowns under normoxia. This shows that the flux via TCA cycle is limited in these plants and that fermentation is active, even under normoxia. Such normoxic fermentation was also observed in plants with impaired complex I (Shah *et al.,* 2013) suggesting that impairment of mitochondrial activity at any site may result in a stress response. A similar explanation may account for the accumulation of acetate in the knockdowns. Acetate is formed from acetyl-CoA by the enzyme acetyl-CoA hydrolase (Zeiher and Handall, 1990) when flux through the TCA cycle is restricted. In

combination with an accumulation of pyruvate and lactate, the knockdown plants under hypoxia show signs of restricted TCA cycle activity. This suggests that even at near anoxia, AOX supports continued TCA cycle carbon oxidation, making use of the remaining oxygen. The AOX knockdowns show an accumulation of citrate, succinate and malate under both normoxic and hypoxic conditions which is also explained by limitation of the TCA flux. When the TCA cycle is restricted, such as by hypoxia or in this case by suppression of AOX, a partial reverse from malate to succinate may be responsible for subsequent succinate accumulation (Vanlerberghe *et al.,* 1989). While these sugars and organic acids only cover a small portion of total carbohydrate metabolism, it is clear that the detrimental effects of hypoxic stress are often further amplified by knocking down AOX and partially alleviated by overexpressing AOX.

Knocking down AOX results in a distinct phenotype. Plants without AOX activity are smaller with poorly developed shoots and lighter coloured leaves compared to the WT (Figure 8). The presence of AOX, whether overexpressed or not, results in similar plants which appear healthy. This observation is unquantified and future research should focus on the ability of AOX to help mobilize energy during early development, as well as whether or not the disturbance of metabolic and redox homeostasis is the cause of this deviation in growth.

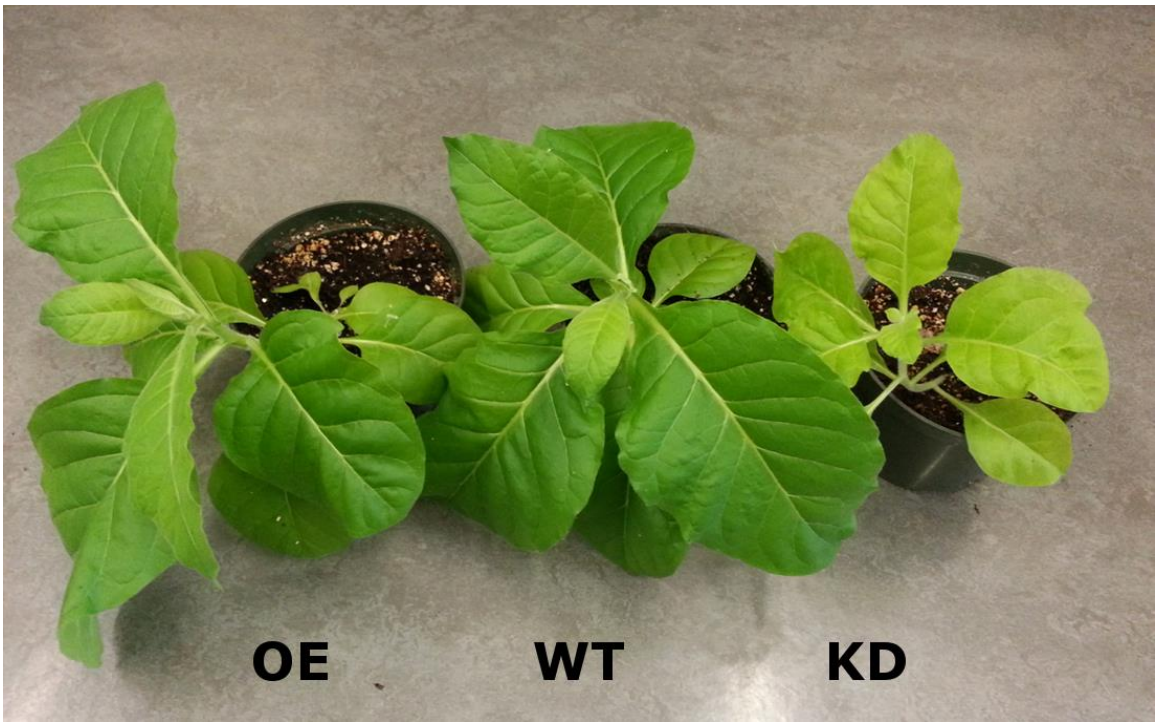

Figure 8: Observed phenotype in tobacco plants with varying expression of AOX. Abbreviations are the same as Figure 2.

2.4.2 AOX and redox homeostasis under stress

AOX is known to aid in lowering mitochondrial reactive oxygen species production as the secondary pathway circumvents the major sites of electron leakage in the primary pathway. Knocking down AOX in tobacco results in increased ROS and RNS production under normoxic conditions. This ability to influence redox homeostasis appears to be important during the reoxygenation period after hypoxic stress. It has been suggested that exposure to oxygen after hypoxic stress can be more damaging than hypoxic stress itself (Subbaiah and Sachs, 2003). Due to the low energy availability but high energy demand of reactivated metabolic pathways, increased oxygen uptake and accelerated mitochondrial activity cause a burst of ROS (Pavelic *et al.*, 2000; Shingaki-Wells *et al.*, 2014; Tamang and Fukao, 2015). This rapid and dramatic increase in ROS production results in lipid peroxidation, membrane leakage and metabolic inhibition (Fukao *et al.*, 2011). The level of superoxide, hydrogen peroxide and malondialdehyde were determined under normoxia and within 2 min of reoxygenation following near anoxic conditions. The amount of hydrogen peroxide increased upon reoxygenation, but this was similar across all plant lines. Superoxide however, not only increased under reoxygenation but was higher in all cases for the AOX knockdown plants. This shows that not only does AOX dampen superoxide production under normoxia, but also during the critical reoxygenation period following hypoxic stress. Increased levels of malondialdehyde and total reducing power after hypoxia indicate that these knockdowns are experiencing a greater oxidative load. The level of phenolics and flavonoids increase under hypoxia in wild type and AOX overexpressing plants but not in AOX knockdown

plants. This may indicate that these antioxidant systems attempt to compensate for the lower AOX capacity.

While higher levels of superoxide contribute to greater oxidative stress, they also lower nitrosative stress. Superoxide can bind with the NO produced within the mitochondria to form peroxynitrite, which is further scavenged by peroxiredoxin and thioredoxin. While peroxynitrite does bind to aconitase similarly to NO, it does not inhibit aconitase activity, meaning superoxide acts as a NO scavenger and protects aconitase from NO in the knockdown plants (Tórtora *et al.,* 2007). Similarly, when AOX is overexpressed, lower superoxide levels prevent NO removal via this pathway which results in stronger inhibition of aconitase by NO. The almost nil amount of mitochondrial aconitase activity in these plants can explain the elevated levels of acetate and lactate. This is primarily seen in the mitochondria where both superoxide and NO are being produced by the electron transport chain, and this effect is eliminated in the presence of cyanide which inhibits COX and thus suppresses NO formation.

2.4.3 AOX as a possible nitrite:NO reductase

While various sources of NO have been identified under stress, the largest source still appears to be the reduction of nitrite via the electron transport chain as part of the Pgb/NO cycle. The inhibition of nitrate reductase via tungstate dramatically decreased NO emissions due to it being the primary source of nitrite in the Pgb/NO cycle. This indicates that the reductive phase of the Pgb/NO cycle is the primary source of NO

production under hypoxia. The complete absence of NO emission in the presence of cyanide indicates that cytochrome c oxidase is the primary site of nitrite reduction and that AOX likely does not participate. While this elevated NO production does induce AOX, this is not to increase the nitrogen turnover within the cycle as initially theorized, but is instead to combat ROS under low oxygen and during reoxygenation.

2.4.4 Conclusions

While NO acts as an intermediary metabolite under hypoxic stress, it also acts as an initial signal of that stress, triggering a regulatory chain that cascades through aconitase, the TCA cycle, citrate and pyruvate to alter AOX expression and activity. The overexpression of AOX does not appear to significantly change this multifaceted regulatory chain when compared to the wild type, but knocking down AOX expression has a dramatic impact on plant metabolism and the ability of plants to combat hypoxic stress.

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Chapter 3: The effects of phytoglobin expression on nitric oxide metabolism and energy state of barley plants

3.1 Introduction

Exposure to an anaerobic environment is one of the predominant stresses encountered by plants. Whether due to flooding or compact substrate, the resulting low concentration of oxygen in the immediate environment is insufficient to support aerobic respiration within the cell. This effect is two-fold as these stresses often hinder exposure to light, limiting photosynthetic oxygen production. Cell death ensues due to the lack of ATP production via mitochondrial oxidative phosphorylation (Limami *et al.,* 2014). This stress is especially prevalent during the early stages of germination when the plant is small enough to be easily encompassed by this environment.

Under hypoxic stress, plants substitute oxygen with nitrite as the terminal electron acceptor for respiration (Gupta & Igamberdiev, 2011). This nitrite is reduced to nitric oxide (NO), which escapes the mitochondria into the cytoplasm. Hypoxically induced phytogobin (Pgb) (Taylor *et al.,* 1994) uses nanomolar concentrations of oxygen to scavenge this NO and form nitrate, which can be converted back into nitrite via nitrate reductase (Gupta *et al.,* 2011). Transport of this nitrate back into the mitochondria allows for continued NO production by conserving cellular nitrogen. This cycle of nitrate-dependent anaerobic respiration is called the phytoglobin-nitric oxide (Pgb-NO) cycle (Stoimenova *et al.,* 2007). It has been shown that plants increase nitrate intake under

hypoxia (Morard *et al.,* 2004) and this increased nitrate results in increased tolerance under hypoxia (Horchani *et al.,* 2010). Along with this, there is a 2.5 fold activation of cytoplasmic nitrate reductase expression during exposure of plant roots to hypoxia (Botrel and Kaiser, 1997). While nitrate reductase typically catalyzes the reduction of nitrate to nitrite, it can further reduce nitrite to NO in the presence of excess NADH (Yamasaki *et al.,* 1999). By allowing respiration to continue in low oxygen conditions, the Pgb-NO cycle plays a key role in energy and redox homeostasis as it facilitates the production of small amounts of ATP to sustain life processes.

The Pgb-NO cycle has primarily been studied in adult plants and cell cultures but little is known of the role Pgb plays during early development. In this chapter I observed how differentially expressing phytoglobin in barley (*Hordeum vulgare* L.) would affect NO levels, energy state in the cell and plant survivability. Barley seedlings with overexpressed and knockdown phytoglobin genes were compared to their wild type counterpart across a variety of metabolic parameters to determine how changing phytoglobin expression would impact the effects of hypoxic stress.

3.2 Materials and methods

3.2.1 Plant growth

Barley (*Hordeum vulgare* L. var. Golden Promise) plants with an overexpressing phytoglobin gene (HvPgb1.1+) or a knockdown phytoglobin gene (HvPgb1.1-) were provided by Aarhus University as described previously (Hebelstrup *et al*., 2010, Hebelstrup *et al*., 2014, Sorensen *et al*., 2019, Zhang *et al*., 2019). Seeds were germinated on moist filter paper on a 14 h day and 10 h night cycle in a controlled growth chamber (Forma Environmental Chamber; Thermo Scientific). The day temperature was set to 25 °C and the night temperature to 20 °C.

3.2.2 Anoxic stress

To test wild type and mutant plants under anoxic stress, entire 6-day-old-seedlings were placed in a custom-built 2 L chamber for 3 h. The chamber was opaque to obscure any light, and contained two openings on either side. A steady inflow of nitrogen gas at 120 mL min^{-1} was maintained and unidirectional air valves facing outwards were placed in the openings to maintain ambient pressure within the chamber while preventing ambient air from entering.

3.2.3 NO emission

To measure NO emission from a whole seedling, it was placed in an air-tight flask with 20 mM Hepes buffer (pH 7.0) with 50 mM sodium nitrate as a nitrogen source. The 250 mL flask, which contained two openings at the top, had a steady 120 mL min^{-1} inflow of nitrogen gas through one opening, and the other was attached to a chemiluminescent detector (CLD 88 p; Eco-Physics, Durnten, Switzerland). The measuring gas was made NO free by a NO scrubber (EcoPhysics, Switzerland) and gas flow was regulated by flow controllers (Fisher Scientific) (Figure 1). Nitrate reductase was inhibited by adding 0.1 mM tungstate (Na_2WO_4) and complex IV was inhibited by adding 5 mM of sodium cyanide.

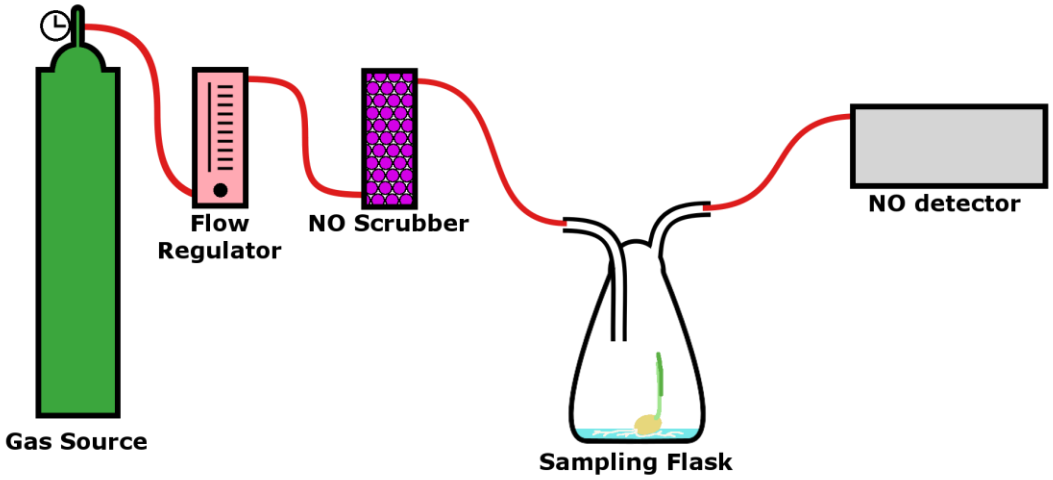

Figure 1: Experimental setup for NO measurements from seedlings. Red lines represent medical tubing used to connect each component.

3.2.4 Aconitase activity

Fresh shoot biomass (100 mg) was homogenized in 1 mL of extraction buffer using a mortar and pestle over ice. The extraction buffer consisted of 50 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, 5 mM $MgCl_2$ and 2 mM trisodium citrate. The homogenate was centrifuged at 10 000 × *g* for 5 min at 4 °C. An assay buffer was prepared that consisted of 50 mM Tris-HCl (pH 7.4) and 40 mM trisodium citrate. Supernatant extract (100 µL) was added to 900 µL of assay buffer and aconitase (EC 4.2.1.3) activity was measured by spectrophotometer (Ultraspec 4300l; Biochrom) at 240 nm. The extinction coefficient of 3.6 mM^{-1} cm^{-1} (Vásquez-Vivar *et al.*, 1999) was used to calculate total enzymatic activity.

3.2.5 Alcohol dehydrogenase activity

A similar protocol as the one used to measure aconitase activity was followed, with some modifications to the buffers. The extraction buffer consisted of 50 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol and 5 mM $MgCl_2$. Alcohol dehydrogenase (EC 1.1.1.1) activity was measured in the ethanol to acetaldehyde direction using an assay buffer of 0.5 mM Tris-HCl (pH 9.0), 0.1 M ethanol, and 2 mM NAD^+ at 340 nm. The extinction coefficient of 6.22 mM^{-1} cm^{-1} (Aalbers & Fraaije, 2017) was used to calculate total enzymatic activity.

3.2.6 ATP/ADP ratio

Extraction of ATP and ADP was conducted according to Dordas et al. (2003) with minor modifications. Fresh biomass (100 mg) was frozen in liquid nitrogen and then homogenized with 2.4 M perchloric acid using a mortar and pestle over ice. The pH of the resulting homogenate was then observed using pH paper and neutralized using 5 M KOH. The final solution was then centrifuged at 16 000 × *g* for 10 min at 4 °C. The supernatant was used to measure ATP by a luciferase based assay kit (Invitrogen). ADP was converted to ATP using pyruvate kinase following to the manufacturer's instructions. (Calzyme Laboratories). ADP levels were determined by subtracting total ATP before and after pyruvate kinase treatment.

3.2.7 Pyrophosphate

Measuring pyrophosphate (PPi) concentration was conducted according to Smyth et al. (1984). Fresh biomass (100 mg) was frozen in liquid nitrogen and homogenized in a mortar and pestle with 1 mL of 0.45 N $HClO_4$. The homogenate was then centrifuged at 27 000 × *g* for 15 min at 4 °C before being neutralized with KOH. The perchlorate precipitate was removed using a mini-centrifuge for 30 s. PPi was determined using a pyrophosphate reagent (Sigma-Aldrich, P-7275). The assay was done in a 1 mL final volume and measured spectrophotometrically at 340 nm.

3.2.8 Statistical analyses

All experiments were repeated at least three times. Results were averaged and standard deviation bars were calculated using Microsoft Excel. Statistical analyses were performed using Prism 5.0 (GraphPad Software). Two-way ANOVA analyses were followed by a Bonferroni post-test to identify statistically significant differences between plant lines within a treatment.

3.3 Results

3.3.1 NO emission

Young barley seedlings emitted nitric oxide when placed under nitrogen gas and immersed in nitrate solution (Figure 2). Before radicle protrusion (first 15-20 h), NO emission was not detectable but it became significant as early as 1 day post-imbibition. The emission was highest in knockdown plants, showing a 2-2.5 times higher rate than the wild type and overexpressing plants. The difference between wild type and overexpressing plants became statistically significant on the 5^{th} day. While knockdown plants showed a higher accumulation and lower duration than the wild type, the overexpressing plants showed the inverse with the lowest accumulation and longest

duration (Figure 3). Tungstate (0.1 mM) decreased NO emission by 80%, whereas cyanide (5 mM) inhibited all detectable emission (Figure 4).

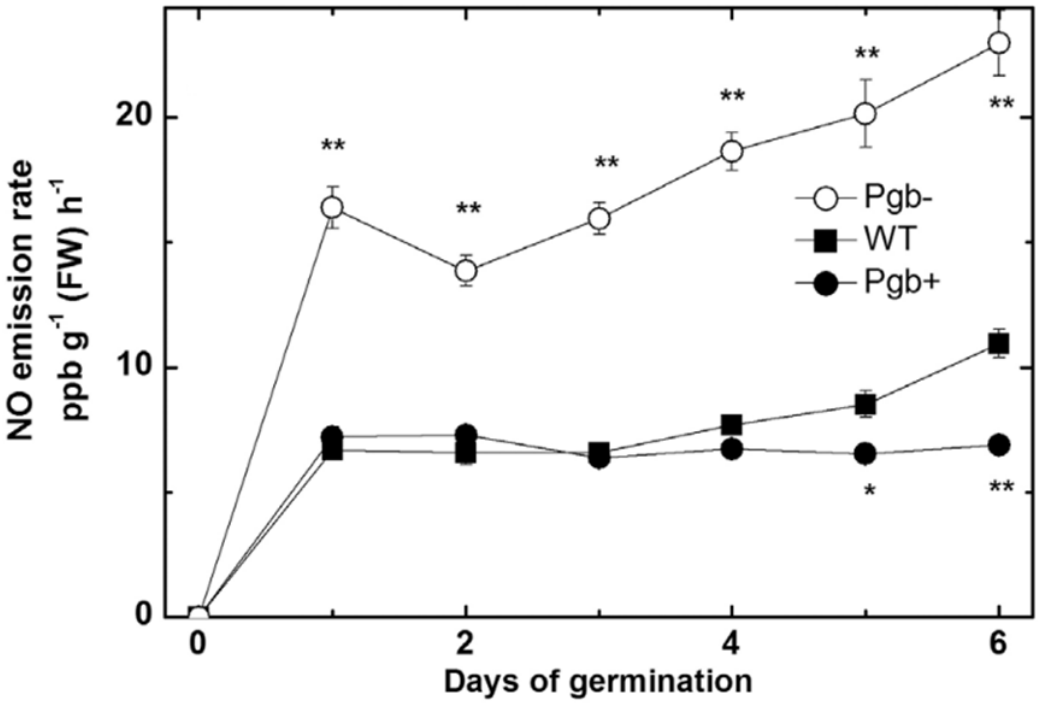

Figure 2. Nitric oxide emission rates measured from barley seedlings with differentially expressing phytoglobin. WT – wild type plants, Pgb+ - plants overexpressing phytoglobin, Pgb- - plants with knockdown phytoglobin. Asterisks indicate significant differences in NO emissions by Pgb+ and Pgb- plants as compared to WT plants: *P < 0.05, **P < 0.005.

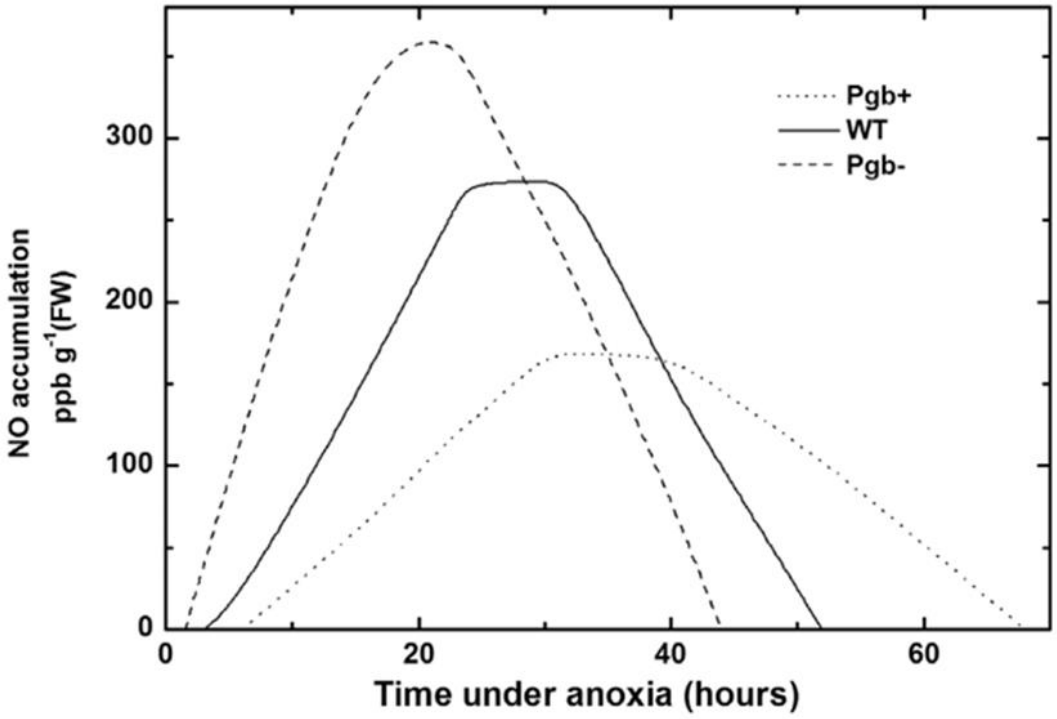

Figure 3. Profiles of nitric oxide accumulation by 6-day-old barley seedlings with differentially expressing phytoglobin. Total NO accumulation was averaged every 30 min for 5 individual plants. Abbreviations are the same as Figure 1.

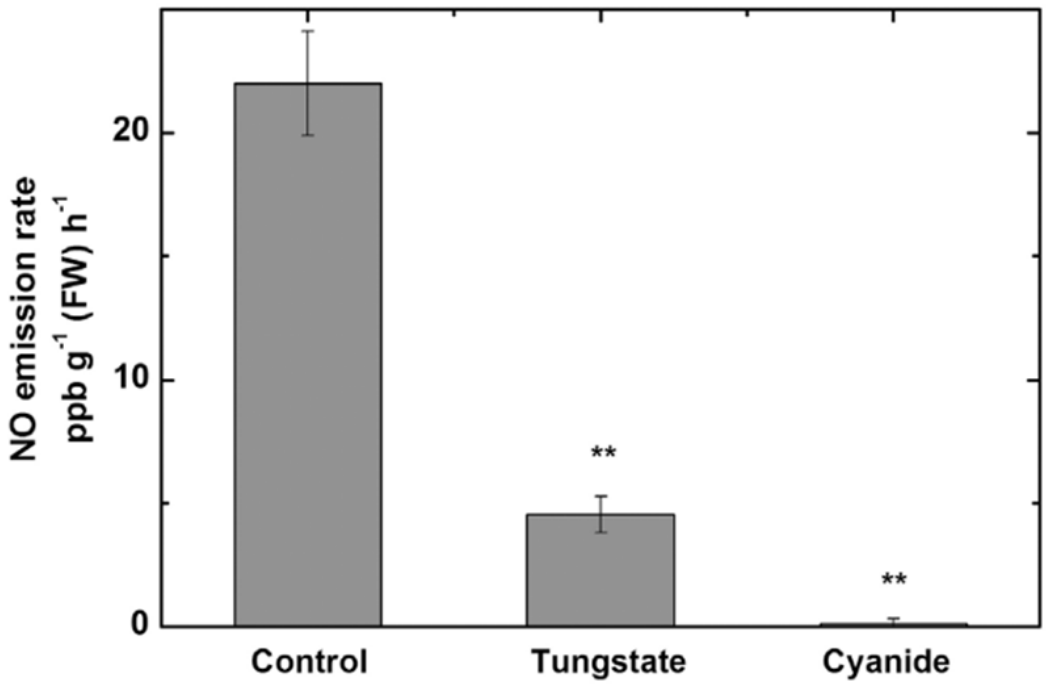

Figure 4. Inhibition of nitric oxide emission from 6-day-old Pgb- plants in 0.1 mM tungstate or 5 mM cyanide. Asterisks correspond to the same statistical significance as in Figure 1.

3.3.2 Aconitase activity

Aconitase activity decreased in both shoots and roots exposed to anoxia (Figure 5). This decrease was far more dramatic in roots. Overexpressing shoots showed little decrease under anoxia and were both significantly higher than their wild type counterparts. Despite overexpressing shoots also being significantly higher than wild type, the dramatic decrease under anoxia remained.

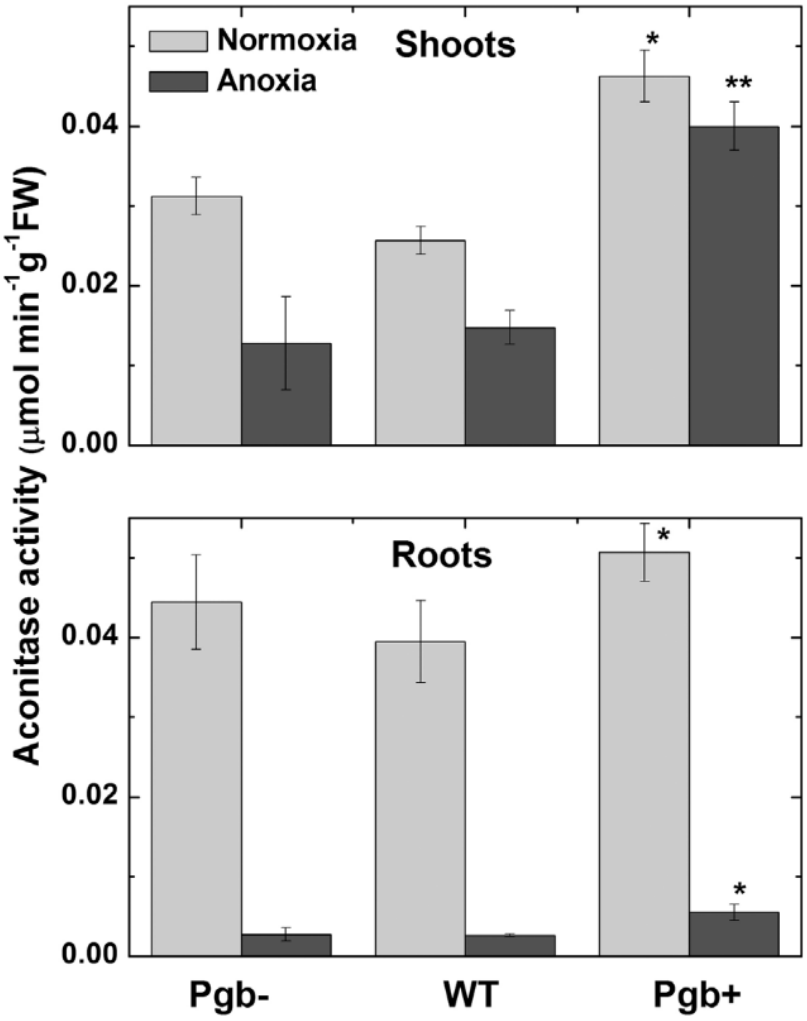

Figure 5. Aconitase activity in shoots and roots of 6-day-old barley seedlings with differentially expressing phytoglobin in normoxic and anoxic conditions. Abbreviations and asterisks values are the same as in Figure 1.

3.3.3 Alcohol dehydrogenase activity

ADH activity was significantly induced under anoxic conditions in only the knockdown plants (Figure 6). This induction was twofold stronger in shoots, while in roots it was ~20%. Knockdown roots similarly showed increased ADH activity under normoxia when compared to wild type.

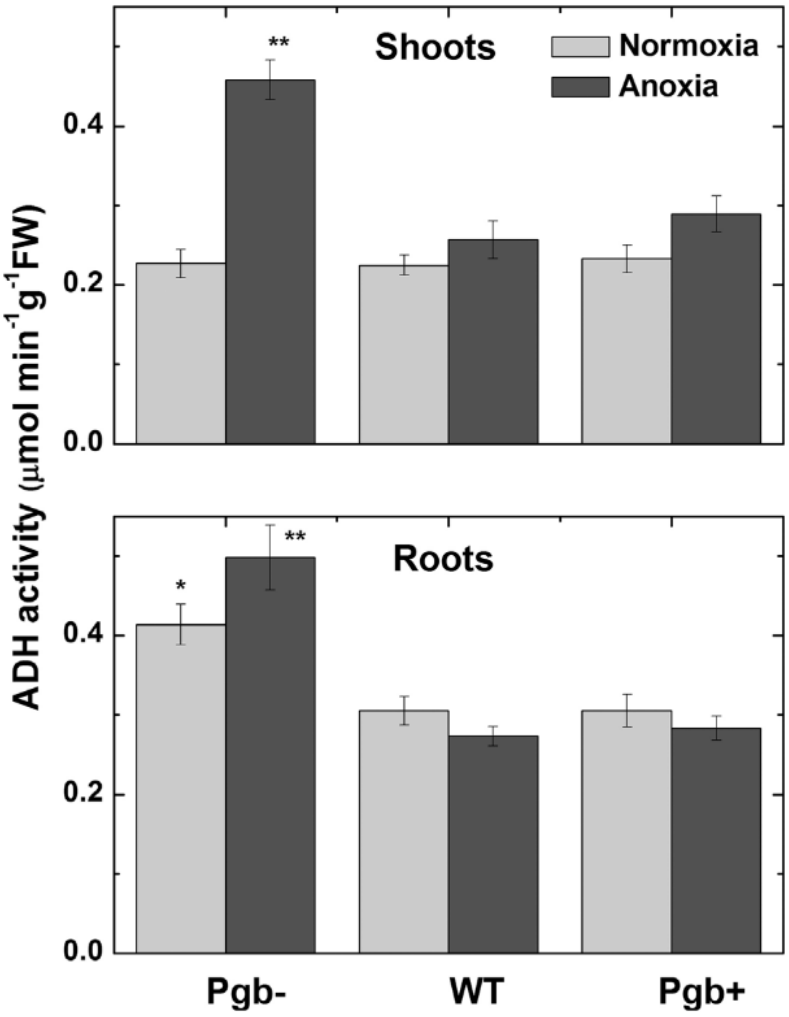

Figure 6. Alcohol dehydrogenase (ADH) activity under normoxic and anoxic conditions in the shoots and roots of 6 day old barley plants with differentially expressing phytoglobin. Abbreviations and asterisks values are the same as in Figure 1.

3.3.4 ATP, ADP and pyrophosphate

ATP/ADP ratios in shoots were similar under normoxia regardless of expression. Under anoxia they decreased by ~25% in overexpressing plants, ~50% in wild type plants and ~75% in knockdown plants (Figure 7). The total pools of ATP + ADP showed a similar pattern with similar levels under normoxia as well as overexpressing plants in anoxia, while wild type plants decreased by ~20% and knockdown plants by ~40%. This trend carried over to pyrophosphate levels with a marked decreased under anoxia (Figure 7). This was minor in overexpressing plants (~20%), major in wild type plants (~45%) and profound in knockdown plants (~70%).

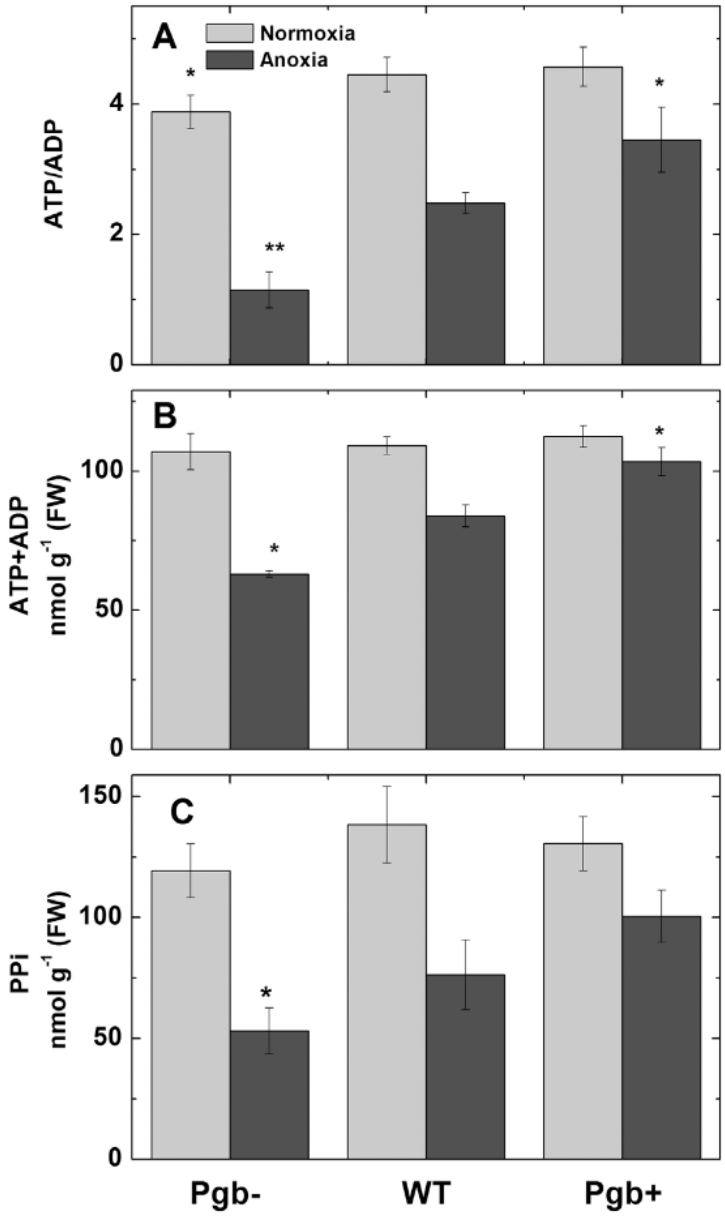

Figure 7. ATP/ADP ratios (A), adenylate (ATP + ADP) levels (B) and pyrophosphate (PPi) content (C) under normoxic and anoxic conditions in the shoots of 6 day old barley plants with differentially expressing phytoglobin. Abbreviations and asterisks values are the same as in Figure 1.

3.4 Discussion

3.4.1 NO emission

The emission of NO by developing barley plants (Figure 2) exhibited a clear dependence on Pgb expression. The wild type and overexpressing plants only differed after day 5 which coincides with the transition of coleoptile to true leaves. This timeframe suggests that leaf tissue likely has a stronger induction of Pgb than the developing shoot. The knockdown plants showed a significantly higher rate of NO emission due to the absence of Pgb-related NO scavenging. The NO emitted is a result of the reductive pathway of NO formation since cyanide inhibited it completely (Figure 4). This means that NO is formed by cyanide sensitive proteins, likely associated with the ETC of mitochondria (Gupta & Igamberdiev, 2011). Nitrate reductase plays a key role in converting nitrate to nitrite for continued NO production (Gupta *et al.,* 2012). Tungstate, a nitrate reductase inhibitor, significantly reduced NO emission, indicating that the primary source of NO is the reduction of nitrite. While it has been suggested in previous studies that nitrate reductase itself can contribute to NO formation (Yamasaki et al., 1999), recent estimations show that the main source of reductive NO under hypoxic stress is the mitochondria (Igamberdiev at al., 2014). All evidence suggests that the NO measured is the byproduct of the Pgb-NO cycle.

Despite NO emissions not being detected for day 0, recent studies have shown NO production in the tissues of barley seedlings hours post-imbibition (Ma *et al.*, 2017). It is likely that this early NO production is not present in detectable quantities capable of diffusing through the unbroken seed coat and emitted into the environment. These findings indicate that Pgb is expressed or induced by incubation in anoxic medium during the early stages of development.

While cytochrome *c* oxidase (COX) is credited with the mitochondrial production of NO, it has been shown that NO is a potent inhibitor of COX reduction of oxygen (Millar & Day, 1996). Similarly, aconitase is reversibly inhibited by NO (Tortora *et al.*, 2007). The rapid scavenging of NO via phytoglobins would not only help protect COX activity under stress, but also aconitase activity. High levels of NO in Pgb knockdown plants resulted in the suppression of aconitase activity (Figure 5). Very strong suppression in roots and less significant in shoots can be explained by the higher emission of NO in anoxic roots, which may even be related to the properties of root mitochondria (Gupta *et al.*, 2005). The protection of aconitase by Pgb can be seen in both roots and shoots but only in shoots is activity kept near normoxic levels. Only a slight, statistically insignificant difference between WT and Pgb knockdown plants indicates that NO may not be the only cause of aconitase inhibition under hypoxia. Other inhibitors of aconitase include reactive oxygen species and various hypoxic metabolites (e.g. the accumulation of organic acids) (Navarre *et al.*, 2000). Inhibition of aconitase should affect operation of the TCA cycle and related pathways, which can lead to a shift to fermentation (Shah *et al.*, 2013). At the stage of barley development I studied however,

anoxia only caused an increase in ADH activity in Pgb knockdown plants indicating that fermentation does not play a significant role at this stage of development. A low importance of fermentation at this stage is supported by the lack of induction of lactate dehydrogenase (data not shown).

3.4.2 The Pgb-NO cycle and survivability

The correlation between NO emission and Pgb expression is supported by the difference in NO accumulation (Figure 3). Accumulation was first detected after an initial lag phase. Likely, this is due to NO production needing to loosely exceed cellular scavenging capacity before NO emission could be detected. The duration of this lag phase correlated with Pgb expression indicating that as NO scavenging capacity increases, so does the threshold of detection. NO was then emitted at a linear rate as previously described (Figure 2) until it reached a plateau where accumulation ceased to increase. This may be the result of NO production and scavenging reaching peak turnover. Plants with greater Pgb expression peaked later and at a lower total accumulation due to higher NO scavenging and recycling. Finally, NO accumulation steadily decreased until completely ceasing. This can be seen as cellular death from prolonged exposure to anoxia. When considering the end of NO emission as the point of plant death, Pgb expression had a direct impact on plant survivability. Overexpressers emitted NO and therefore survived for ~30% longer than wild type plants and ~50%

longer than knockdown plants. This increased survivability is supported by the ratios and totals of ATP and ADP (Figure 7).

3.4.3 Energy maintenance

Both ATP/ADP ratios and total pools are strongly dependent on Pgb expression under anoxia indicating that Pgb operation in the cell leads to the maintenance of energy state. This is achieved by the Pgb-NO cycle stabilizing redox level and supporting limited rates of ATP production in mitochondria (Igamberdiev & Hill, 2004). While PPi under low oxygen stress becomes an important alternative energy currency (Igamberdiev & Kleczkowski, 2011), the maintenance of its levels is important for survival under hypoxia. A fall in total ATP under anoxia leads to an increase in Mg^{2+} which activates various enzymes normally limited by low Mg^{2+}. Two such enzymes, pyruvate kinase (PK) and pyruvate, phosphate dikinase (PPDK), produce PPi which substitutes ATP in glycolysis (Huang *et al.,* 2008). The role of Pgb in maintaining PPi levels is evident from the obtained data (Figure 7). By maintaining ATP levels under stress, Pgb lowers the glycolytic requirement for Mg^{2+} dependent metabolic assistance resulting in less PPi being consumed. A lack of Pgb causing the further inhibition of aconitase (Figure 5) likely creates an unfavorable condition for the citric acid cycle and the subsequent accumulation of pyruvate would further drive the activity of PK/PPDK. Overall, Pgb expression is related to the improved energy maintenance during energy crisis and results in increased survivability.

3.4.4 Conclusions

The obtained metabolic data unequivocally shows that the overexpression of Pgb during the early stages of plant development does improve energy maintenance under low-oxygen stress. This improved energy state under increased Pgb expression can be directly linked with improved plant survivability.

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Chapter 4: The effects of phytoglobin expression on the growth and development of barley plants

4.1 Introduction

A large number of physiological processes in plants such as dormancy, germination, root growth, stomatal closure, gravitropic bending, flowering and senescence are regulated by nitric oxide (NO) (Delledonne, 2005). Due to the integral processes NO regulates, understanding this signalling molecule from perception and production to transduction and translation has been a key field of research in plant biology over the last two decades.

As a readily diffusible free radical, NO encounters and reacts with a variety of intercellular and extracellular targets. It forms complexes with transition metal ions and heme groups, as well as acts as a precursor for other harmful N-oxides such as nitrogen dioxide, peroxynitrite, dinitrogen trioxide and nitrosyl cation (Hausladen & Stamler, 1999). One would expect a signalling molecule to be stable, specific and harmless to the cell, but NO does not follow these conventions. Several studies have shown that increasing exposure to NO will suppress the transition from vegetative to generative growth in plants (He *et al.*, 2004), stimulate biomass accumulation (Antonia et al, 2018), increase the number of lateral roots (Correa-Aragunde *et al.*, 2004) and decrease differentiation zone length (Fernandez-Marcos *et al.*, 2011). Many of the ever growing

number of effects resultant to NO signalling are well categorized but the processes in which NO causes these changes remains unclear.

While it has been made clear that NO plays a key role in plant growth and development, most research into NO signalling has been done under normoxic conditions using sodium nitroprusside (SNP). The validity of SNP as a NO donor has been brought into question as it inconsistently releases NO, is light-sensitive, and can produce cyanide as a byproduct (Bethke *et al.*, 2006; Floryszak-Wieczorek *et al.*, 2006). As the NO signal is unique and plays a role in regulating such a wide variety of early-life processes, I investigated how altering the expression of a NO scavenger such as phytoglobin would affect barley seedling growth and development.

4.2 Materials and methods

4.2.1 Plant growth

The same barley (*Hordeum vulgare* L. cv. Golden Promise) plants with an overexpressing or silenced phytoglobin gene were used as Chapter 3. Seeds were germinated on moist filter paper on a 14 h day and 10 h night cycle in a controlled growth chamber (Forma Environmental Chamber; Thermo Scientific). The day temperature was set to 25 °C and the night temperature to 20 °C.

4.2.2 Total protein *S*-nitrosylation

The measurement of protein *S*-nitrosylation was carried out according to Ma et al. (2016) by reducing nitrosothiols (R-SNO) to thiols (R-SH) in the presence of ascorbate and then assaying free thiol groups using 5,5'-dithiol-bis (2-nitrobenzoic acid) (DTNB). Fresh biomass (100 mg) was homogenized in 1 mL of extraction buffer using a mortar and pestle. The extraction buffer consisted of 50 mM HEPES (pH 8.0), 1 mM EDTA, 0.1 mM neocuproine, 0.2% (w/v) SDS and 0.5% (w/v) CHAPS. The homogenate was centrifuged at 15,000 × *g* for 10 min at 4 °C and proteins were precipitated from the supernatant by two volumes of ice cold acetone (-20 °C) overnight. Protein precipitate was separated again by centrifuging at 15,000 × *g* for 10 min at 4 °C and the subsequent pellet was washed four times with chilled 70% acetone before being resuspended in the same volume of extraction buffer. Protein solution was separated into two 0.9 mL samples, adding 50 µL of 100 mM ascorbate to the experimental sample and the same volume of distilled water to the control. After incubating for 1 h at 25 °C, 50 µL of 10 mM DTNB in 75 mM phosphate buffer (pH 7.0) was added and both samples were measured by spectrophotometer at 412 nm. The same procedure was followed with an absence of plant tissue to set up blanks for both treatment and control groups. The difference between experimental sample and control was used to calculate quantity of R-SNO.

4.2.3 *S*-nitrosoglutatione reductase activity

S-nitrosoglutatione reductase (GSNOR) activity was measured using a modified method as described by Sakamoto et al. (2002). Fresh biomass (100 mg) was homogenized using a mortar and pestle with the addition of 50 mM HEPES (pH 8.0) containing 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine and 1 mM aminocaproic acid. The homogenate was centrifuged at 15,000 × *g* for 10 min at 4 °C and the supernatant was desalted using a protein desalting spin column (Thermo Fischer Scientific). Protein concentration was determined and 100 µL of desalted protein was added to 900 µL of assay mix consisting of 20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA and 0.2 mM NADH. Change in absorbance at 340 nm was measured via spectrophotometer for 75 s after the reaction was started by adding 10 µL of 40 mM *S*-nitrosoglutathione (GSNO).

4.2.4 Morphological studies

Seeds were germinated on moist, nutrient free potting soil inside a 1 L glass beaker. For hypoxic treatment, a specialized plug with two adjustable airways was used to seal the beaker. Twice a day the airways were opened, one to allow a 1 L min^{-1} flow of 3% oxygen-nitrogen mix, the other to maintain atmospheric pressure by allowing gas to escape. After 5 min of flushing the beaker, the airways were sealed and the beaker was returned to the growth chamber. Plants were germinated on a 14 h day and 10 h night cycle, with a day temperature 25 °C and a night temperature of 20 °C. For normoxic treatment, the beaker was flushed using atmospheric air instead.

Any seed that formed a radicle protrusion was considered germinated. The shoots and roots of 6 day old plants were separated from what remained of the seed body and lightly dried using paper towel before being weighed and measured.

4.2.5 Statistical analyses

All experiments were repeated at least three times. Results were averaged and standard deviation bars were calculated using Microsoft Excel. Statistical analyses were performed using Prism 5.0 (GraphPad Software). Two-way ANOVA analyses were followed by a Bonferroni post-test to identify statistically significant differences between plant lines within a treatment.

4.3 Results

4.3.1 Total protein *S*-nitrosylation

The level of *S*-nitrosothiols was 2 to 3 orders of magnitude higher under anoxia in all investigated plants when compared to normoxia (Figure 1). The anoxic level of nitrosothiols was 30 to 40% higher in Pgb knockdown plants than in the wild type and Pgb overexpressing plants.

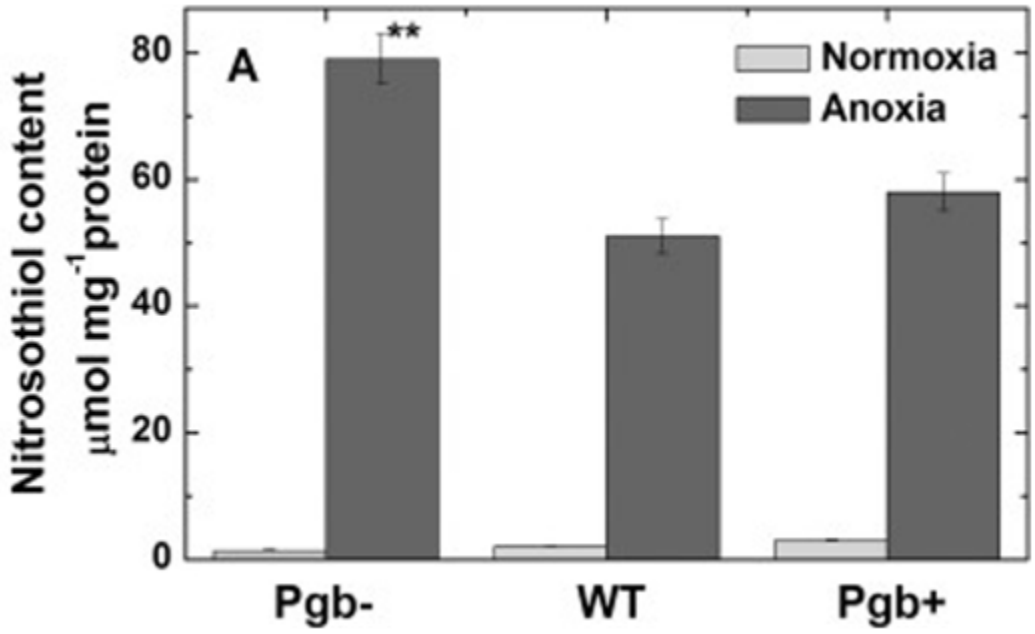

Figure 1. The level of nitrosylation under normoxic and anoxic conditions in the shoots of 6 day old barley plants with differentially expressing phytoglobin. WT – wild type plants, Pgb+ - plants overexpressing phytoglobin, Pgb- - plants with knockdown phytoglobin. Asterisks indicate significant differences in NO emissions by Pgb+ and Pgb- plants as compared to WT plants: *$P < 0.05$, **$P < 0.005$.

4.3.2 *S*-nitrosoglutathione reductase activity

The activity of GSNOR increased under anoxia in all plants when compared to normoxia (Figure 2). This increase was more profound in Pgb knockdown plants and lower in Pgb overexpressing plants when compared to wild type.

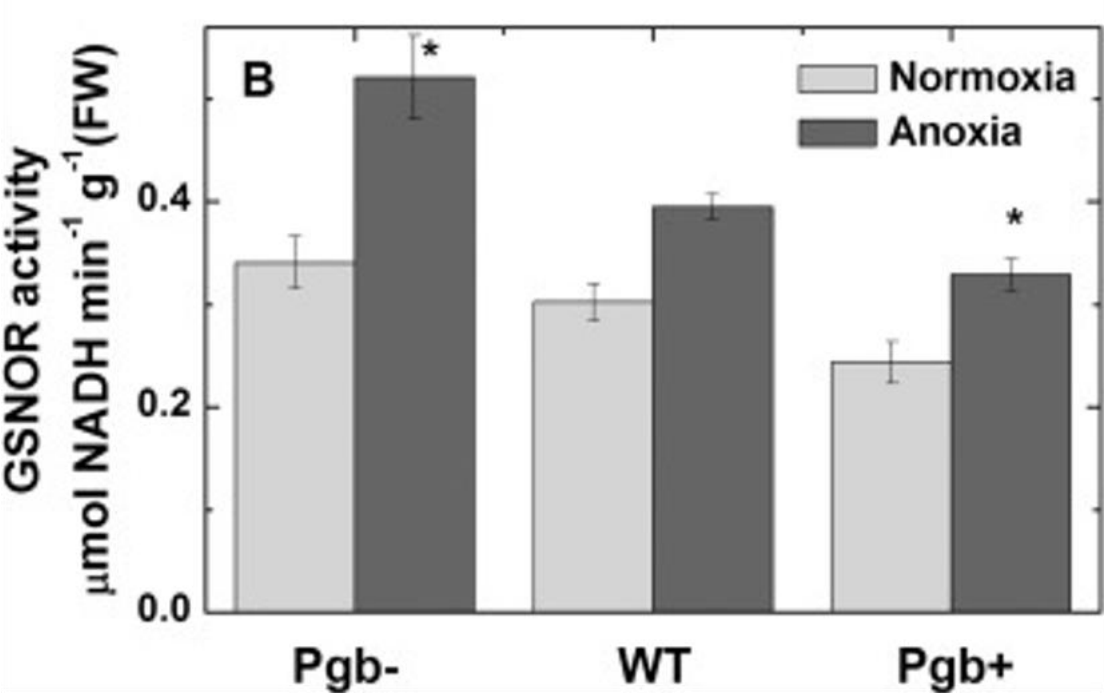

Figure 2. The level of *S*-nitrosoglutathoine activity under normoxic and anoxic conditions in the shoots of 6 day old barley plants with differentially expressing phytoglobin. Abbreviations and asterisks values are the same as in Figure 1.

4.3.3 Morphological studies

Under normoxia, the overexpressing plants showed stunted growth and decreased rate of germination (Figure 3). They weighed half as much as wild type and knockdown plants (Figure 4), with 35% shorter shoots and 15% shorter roots (Figure 5) after 6 days of growth. However, under 3% oxygen (hypoxia) only overexpressing plants germinated. Not only did they survive the entire seven days at 3% oxygen, but they only exhibited a partial growth deficit (more profound in shoots than in roots). The differences between overexpressing, knockdown and wildtype plants were clearly visible in 3-day-old plants (Figure 6).

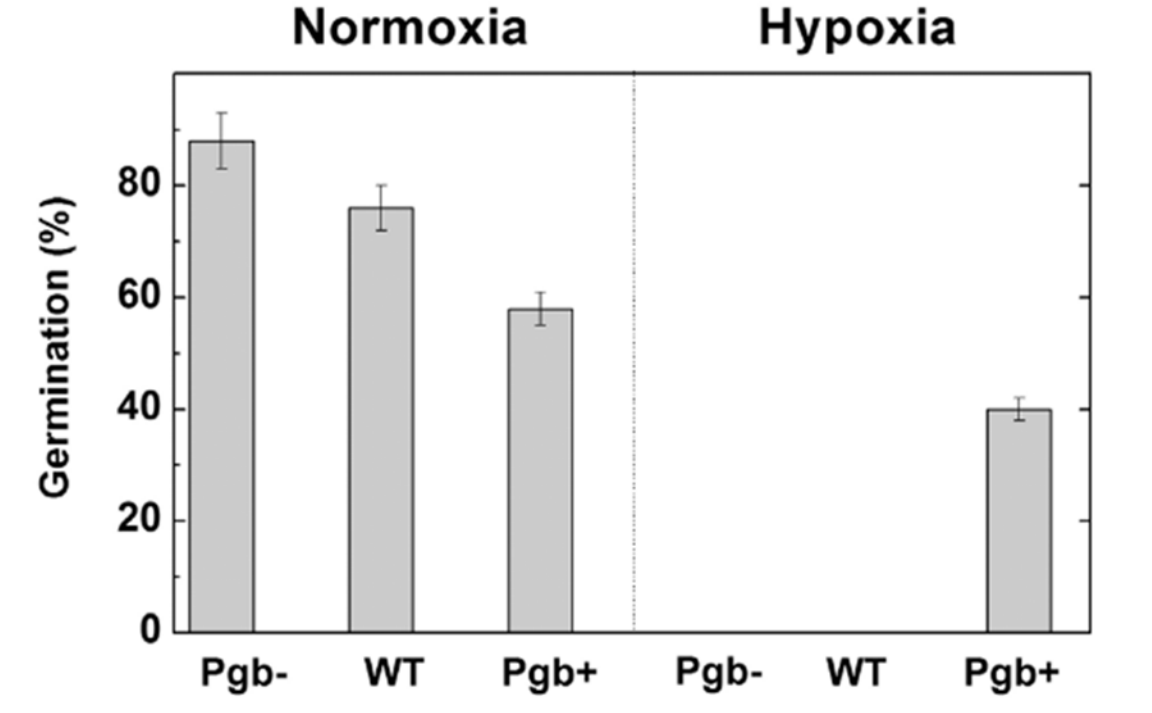

Figure 3. Germination rate of barley seeds with differentially expressing phytoglobin. Abbreviations are the same as in Figure 1.

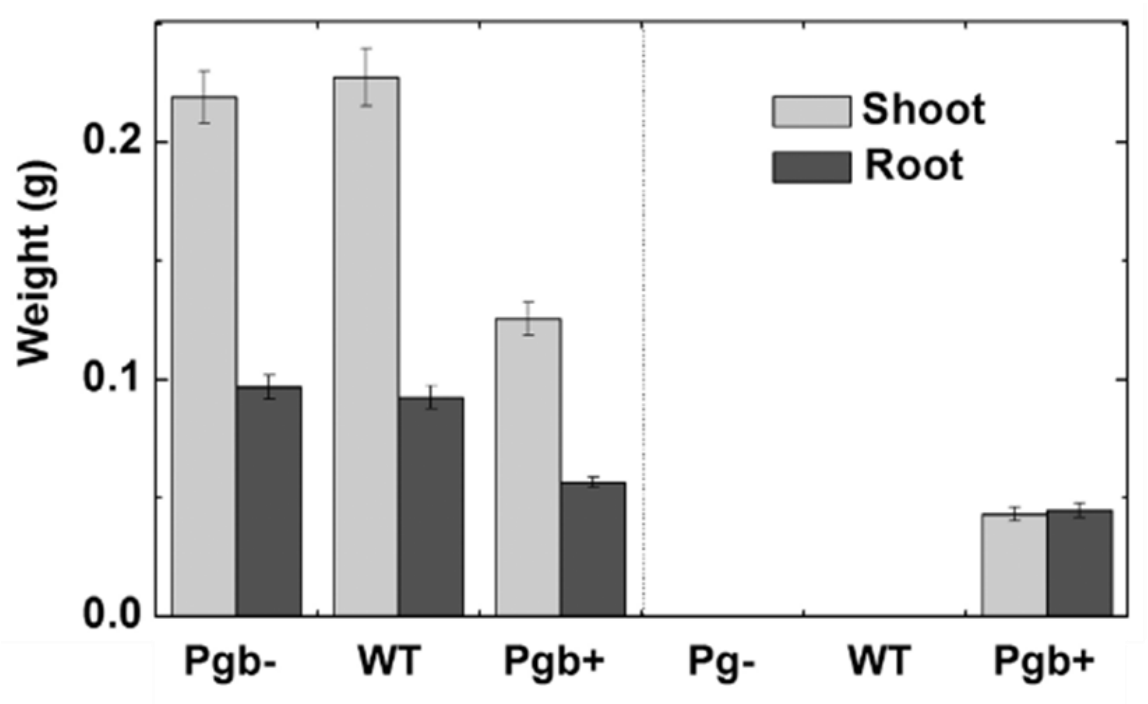

Figure 4. Shoot and root weight of barley seedlings under normoxia and hypoxia with differentially expressing phytoglobin. Abbreviations are the same as in Figure 1.

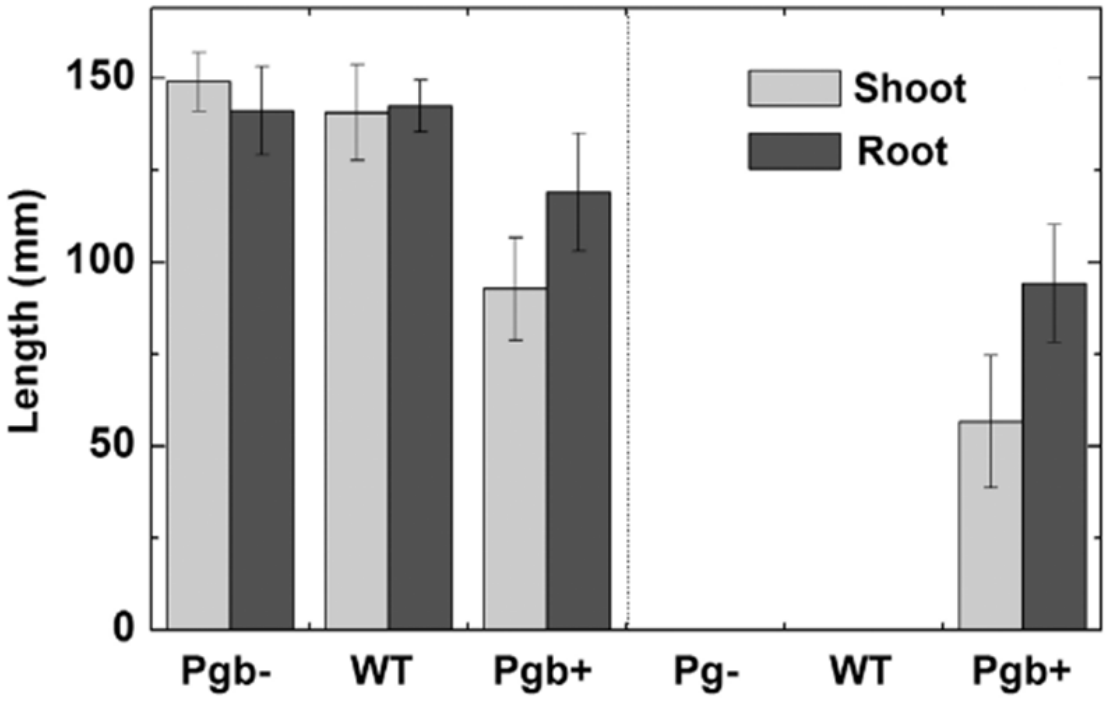

Figure 5. Shoot and root length of barley seedlings under normoxia and hypoxia with differentially expressing phytoglobin. Abbreviations are the same as in Figure 1.

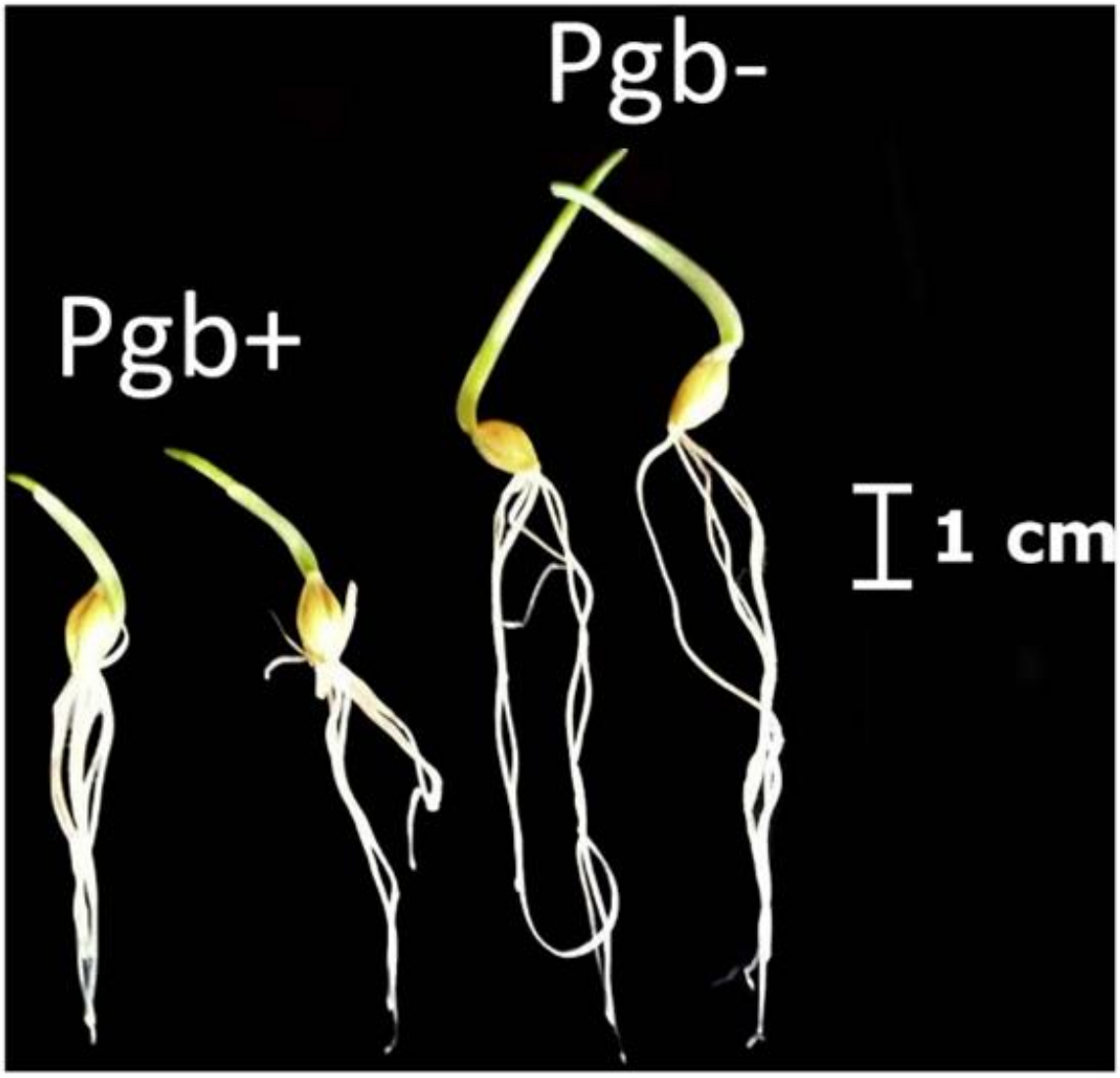

Figure 6. Photo of 3 day old barley seedlings with overexpressed (Pgb+) and knockdown (Pgb-) phytoglobin.

4.4 Discussion

4.4.1 Protein nitrosylation

To exert a cellular effect, the NO signal must nitrosylate a thiol group of a target protein (Neill et al, 2003). A correlation between the level of protein nitrosylation and Pgb expression was observed, however it was much higher in anoxia as compared to normoxia (Figure 1). Glutathione (GSH) reacts with NO to form *S*-nitrosoglutathione (GSNO), a stable and more long-lived molecule which acts as a mobile reservoir of NO and as such is one of the major endogenous nitrosylated compounds (Airaki *et al.*, 2011). To mediate NO signalling, GSNOR scavenges GSNO and forms non-donor molecules such as oxidized GSH and hydroxylamine or *S*-hydroxylaminoglutathione. It appears that GSNOR activity is influenced by Pgb expression with the knockdown of Pgb under anoxia resulting in overcompensation from GSNOR to maintain control of the NO signal and combat nitrosative stress (Figure 2). While GSNO achieves protein *S*-nitrosylation via transnitrosylation, the direct *S*-nitrosylation of proteins by free roaming NO is of far greater concern under anoxic conditions.

Endogenous NO saturation exceeding the capabilities of denitrosylating systems should severely impact the NO signal. Recent studies have shown that endogenous *S*-nitrosylation has a high degree of spatio-temporal specificity (Benhar *et al.*, 2009). This specificity is lost in Pgb knockdown plants under anoxia as they experience severe nitrosative stress resulting in a likely higher degree of non-specific protein binding and

increased nitrosothiols (Figure 1). While GSNOR activity did increase under anoxia in Pgb overexpressing plants, total nitrosothiols did not. This may indicate that GSNOR has loosened its control over the NO signal, lowering the amount of GSNO being scavenged as less GSNO is produced in the presence of low NO levels.

4.4.2 Growth and Development

A significant suppression of the germination of these plants in normoxia indicates that the presence of Pgb is interfering with the NO signal responsible for breaking dormancy (Figure 3). This interference may explain low weight, low shoot length and low root length in the Pgb overexpressing seedlings (Figure 4, 5). In the wild type plants, Pgb is not expressed under normoxia. In the overexpressing plants however, the presence of an additional NO scavengers under normoxia appears to be causing a significant portion of the NO signal to be lost. Only Pgb overexpressing plants were capable of germinating under hypoxia, likely due to their increased survivability and energy maintenance as outlined in Chapter 3. Their diminished growth and development when compared to their normoxic counterparts is the result of persistent low-oxygen stress.

4.4.3 Conclusions

The transgenic modification of Pgb gene expression provides great insight into the role of NO and Pgb in metabolism and signalling (Figure 7). While the overexpression of Pgb has been shown to increase the survivability of barley seedlings under stress, this comes with a trade-off of poor normoxic signalling resulting in the interruption of various growth and developmental processes. As barley has agricultural importance, I entered this research questioning whether or not the overexpression of Pgb would yield a crop with improved hypoxic tolerance and therefore flood resistance. While this does appear to be the case, the trade-off of poor seed yields and decreased biomass rules out the economic significance of these specific plants.

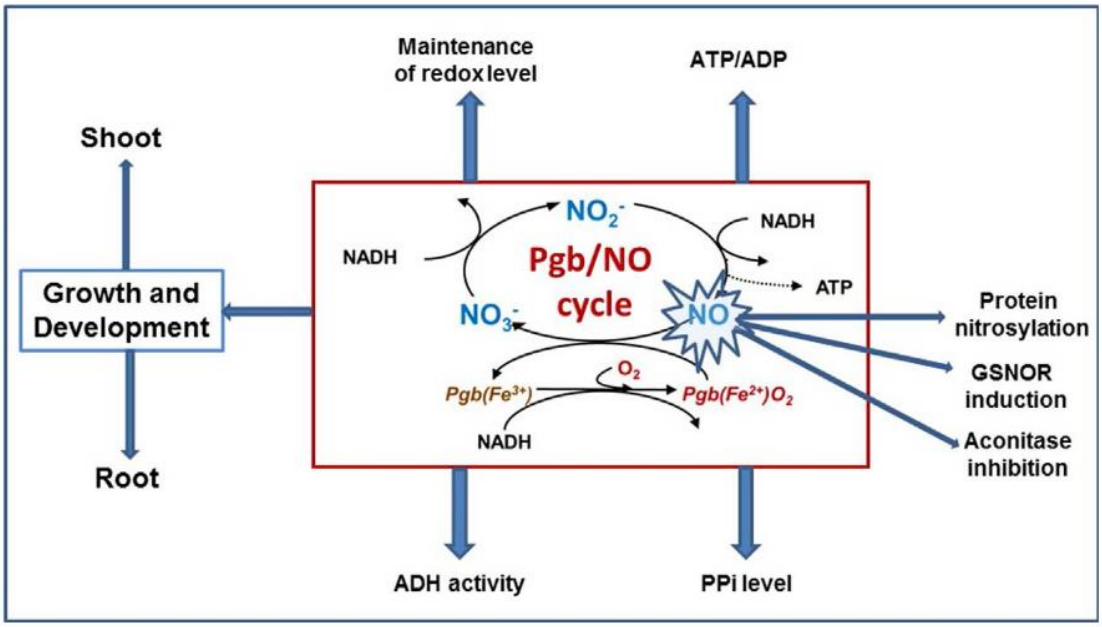

Figure 7. Scheme showing the influence of Pgb expression on metabolic pathways, bioenergetics and signalling in the hypoxic plant cell.

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Chapter 5: Future Research Directions and Conclusions

5.1 Conclusions

Mutant plants with varying expression of a study gene present a unique opportunity to not only gain a better understanding of that gene but to potentially discover a plant line with agricultural or economic importance. In the case of AOX expression, this study found that AOX does not directly participate in the Pgb-NO cycle and therefore varying its expression does not impact the ability of a plant to combat the energy crisis resulting from hypoxic stress. It does, however, help facilitate continued carbohydrate metabolism and plays a key role in maintaining redox homeostasis during reoxygenation. The overexpression of Pgb had a much more novel effect, improving energy maintenance under hypoxic stress and increasing plant survivability during early development. This significant enhancement comes with a tradeoff, however, as overexpressing Pgb hampers NO signalling causing a decrease in seed yield and vegetative growth. It is unlikely that either of these plants hold any agricultural or economic importance but they are still valuable study specimens for future research into plant metabolism.

5.2 Future directions

In the future, I hope to see the Pgb-NO cycle studied with a similar approach to that which I took but comparing various crop species and their flood resistant vs. non-flood resistant lines. Some species, such as rice, are naturally more flood resistant when compared to others, such as barley, tobacco or corn. I've shown that increasing turnover of the Pgb-NO cycle improves plant survivability while under hypoxia but little is known about whether or not this strategy is used naturally by plants that have evolved to survive in frequently hypoxic environments. Many crop lines are genetically uncharacterized as they are bred to carry favorable traits such as flooding resistance. It would be valuable to understand how the Pgb-No cycle functions in species or plant lines that show varying degrees of resistance to hypoxic stress.

My study helped develop the use of NO emission analysis for measuring the turnover rate of the Pgb-NO cycle. This approach was complimented with quantitative assays, nuclear magnetic resonance and phenotypic measurements. While this did expand the collective understanding of the Pgb-NO cycle, AOX and hypoxic plant stress, many questions remain unanswered. I would like to see my methodology expanded in the future to include fluorescent imaging or electron-spin resonance to allow for quantification of NO turnover in seeds with varying expression of Pgb before the 1 day mark. This would give us a clearer understanding of how, when and where the Pgb-NO cycle helps mobilize energy during the first stages of development. I would also like to see real-time PCR utilized to measure popular developmental and stress-response genes. While I have shown that modulating Pgb expression influences both morphology and *S*-nitrosylation,

the targets of NO signalling remain relatively unknown. Determining which genes have their expression altered by varying levels of Pgb would expand our understanding of which developmental processes and stress responses are signaled by NO and how the Pgb-NO cycle may moderate these processes.