The Coordination of Phytoglobin-Nitric Oxide Cycle and Alternative Oxidase in Plant Adaptation to Hypoxia

By

Somaieh Zafari Ph.D.

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Department of Biology

Memorial University of Newfoundland, St. John's, Newfoundland and Labrador, Canada

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Abstract

The non-energy conserving protein alternative oxidase (AOX) of the mitochondrial respiratory electron transport chain (ETC) is hypothesized to regulate nitric oxide (NO), reactive oxygen species (ROS), and ethylene levels in plants under stress. The purpose of this research is to provide direct evidence in favor of this hypothesis, as well as to investigate the implications of this regulation during plant adaptation to hypoxic stress. We studied NO metabolism and the involvement of key components of the phytoglobin (Pgb1) -NO cycle in imbibed transgenic barley seeds with altered Pgb1 levels during germination process as well as in transgenic tobacco seedlings with altered AOX levels exposed to nitrogen atmosphere. NO emission increased more in overexpressing lines of tobacco under hypoxia, although the quantity of nitrosylated proteins was higher in *AOX* knockdown plants. There was a significant increase in *Pgb1* expression which upregulates the turnover of the Pgb1-NO cycle in imbibed barley seeds and tobacco leaves under hypoxic condition. The cycle's operation not only controls NO metabolism and redox homeostasis, but it improves the efficiency of energy metabolism. The current study demonstrates that AOX which contributes positively to the operation of the Pgb1-NO cycle, regulates NO generation under hypoxia and leads to a shift towards biosynthesis of amino acids. It demonstrates that hypoxia results in the upregulation of fermentation pathways in the plants expressing *AOX*. The plants lacking *AOX* exhibited the increased levels of ROS as compared to wild type and *AOX* overexpression plants during hypoxia, suggesting that AOX induces a fine-tuned balance in ROS levels by the regulation of ROS production and scavenging. I found that ethylene biosynthesis genes are induced during

hypoxia and correlate with AOX and NO levels. I conclude that AOX is involved in NO turnover and plays a protective role by reducing ROS, regulating the ethylene levels, and sustaining energy requirements during hypoxia.

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AA	Ascorbic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	1-aminocyclopropane-1-carboxylic acid oxidase
ACS	1-aminocyclopropane-1-carboxylic acid synthase
ACT	Actin
ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AOX	Alternative oxidase
AP	Alternative pathway
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
B7, B8	Aox1a overexpressing tobacco transgenic lines
CaCl2	Calcium chloride
CAT	Catalase
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CO_2	Carbon dioxide
COX	Cytochrome oxidase
DHA	Dehydroascorbic acid
DHAR	Dehydroascorbate reductase

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTNB	5,5’-dithiol-bis 2-nitrobenzooic acid
DTT	Dithiothreitol
EDTA	2,2',2",2'"-(Ethane-1,2-diyldinitrilo) tetraacetic acid
EIN3	Ethylene insensitive 3
ERF	Ethylene response factor
ET	Ethylene
FADH2	Flavin adenine dinucleotide
GABA	γ-aminobutyric acid
GABA-T	γ-aminobutyrate transaminase
GAD	glutamate decarboxylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPX	Guaiacolperoxidase
GR	Glutathione reductase
GSNO	S-nitrosoglutathione HA Hydroxylamine
GSNOR	GSNO reductase
GSH	Glutathione
GSSG	Glutathione disulfide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O_2	Hydrogen peroxide
KCl	Potassium chloride

KCN	Potassium cyanide
KI	Potassium iodide
KOH	Potassium hydroxide
MDHAR	Monodehydroascorbate
mETC	Mitochondrial electron transport chain
NAD^+	Nicotinamide adenine dinucleotide oxidized form
NADH	Nicotinamide adenine dinucleotide reduced form
$NADP^+$	Nicotinamide adenine dinucleotide phosphate oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form
NBT	Nitro-blue tetrazolium
NDB	Rotenone-insensitive NADH dehydrogenase
NiR	Nitrite reductase
NR	Nitrate reductase
NO	Nitric oxide
NO_2	Nitrogen dioxide
N_2O_3	Dinitrogen trioxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
$O_2^{\cdot -}$	Superoxide
$OH^{\cdot}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite

PA	Polyamine
PDC (chapter 2)	Pyruvate dehydrogenase complex
PDC (chapter 4)	Pyruvate decarboxylase
PGDH	3-phosphoglycerate dehydrogenase
Pgb	Phytoglobin
PM NiNOR	Plasma membrane bound nitrite: NO reductase
PPSB	Phosphorylated pathway of serine biosynthesis
PSAT	3-Phosphoserine aminotransferase
PSP	3-Phosphoserine phosphatase
PVP	Polyvinylpyrrolidone
Rboh	Respiratory burst oxidase homolog
RI9, RI29	*Aox1a* RNA interference (knockdown/ downregulating) tobacco transgenic lines
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAM	S-Adenosyl-L-Methionine
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulfate
SD	Standard deviation
SHAM	Salicylhydroxamic acid
SNO	Nitrosothiol
O_2^-	Superoxide

$^1O_2$	singlet oxygen
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Tricarboxylic acid
WT	Wild type
XOR	Xanthine oxidoreductase

Chapter 1

Maintenance of energy and metabolic acclimations to low oxygen supply

Literature review and thesis introduction

Literature review

To give a proper context for my thesis, I start by describing the plant mitochondrial electron transport chain (mETC), with a focus on the site and function of alternative oxidase (AOX). I will then give a quick overview of nitric oxide-stress interactions, focusing on the processes that regulate NO emission and amount under stress. I will l review what we know about ROS signaling and induction in response to oxygen deficiency. Then, the importance of AOX's potential roles will be highlighted, particularly in relation to NO, ROS, and ethylene signaling.

1.1 The plant mitochondrial electron transport chain (mETC)

When glucose is converted to pyruvate by glycolysis, just a small part of the total free energy is released. Pyruvate is transported into the mitochondrion where the tricarboxylic acid (TCA) cycle oxidizes it to CO_2 and H_2O. Organic compounds generated by the TCA cycle are used by the mitochondrial respiratory electron transport chain (mETC) to provide accessible chemical energy in the form of ATP. In general words, electrons from NADH or $FADH_2$ proceed through a sequence of spatially separated redox reactions from a donor to an acceptor molecule before being received by a terminal oxidase and used to reduce molecular oxygen to water.

The phosphorylating pathway of the ETC is composed of 5 complexes (I-V): the NADH dehydrogenase (complex I), the succinate dehydrogenase (complex II), the cytochrome c reductase (complex III), the cytochrome c oxidase (complex IV), and ATP synthase complex (complex V) (Fernie et al., 2004; Dudkina et al., 2006). The electron transfer

reactions of three of the four oxidoreductase complexes (complexes I, III, and IV) are coupled with proton translocation across the inner mitochondrial membrane. As a result, a proton motive force is generated used to power ATP production by the complex V.

The electron transfer from the oxidation of the substrate is not 100 percent efficient. Due to electron and proton leaks, not all ETC electrons can be passed to the final electron acceptor oxygen (Mitchell, 1961). Consequently, the released energy by the transferred electrons cannot be fully coupled with ATP output. As a result, it is important to know how cellular respiration is controlled, which is governed primarily by the need for ATP.

1.2 Alternative Oxidase: Non-phosphorylating pathways of the mETC

Another characteristic component of the plant mETC is alternative oxidase (AOX) which catalyzes the reduction of oxygen to water by accepting electrons directly from ubiquinol. See **Figure 1.1** for a diagrammatic representation of the plant mETC and the presence of AOX.

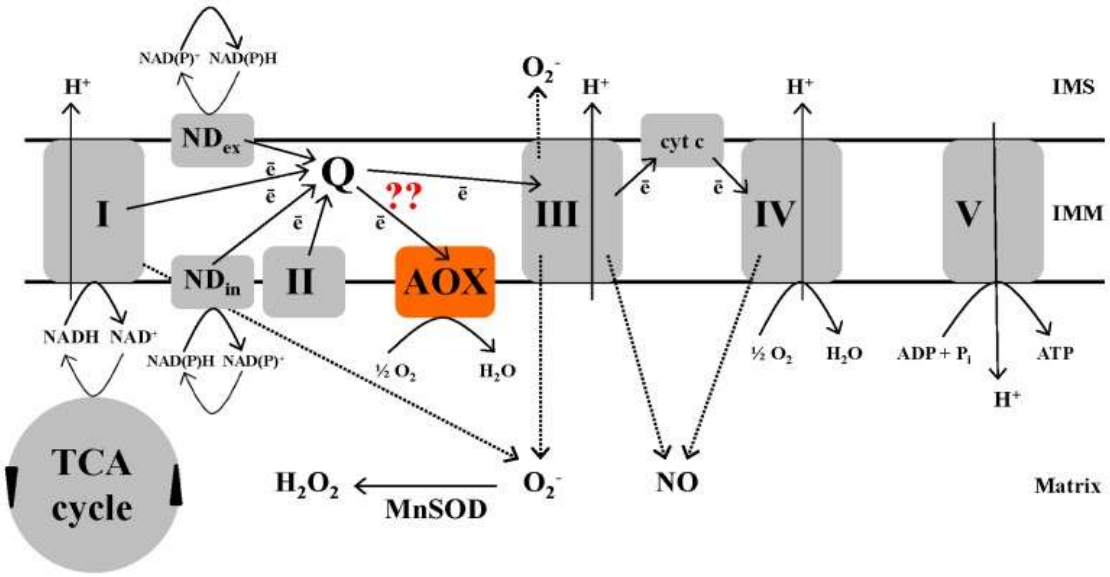

Figure 1.1 The mitochondrial electron transport chain in plants. The electrochemical gradient and ATP production are not affected by electrons passing through the alternative oxidase (AOX) (Adapted from Vanlerberghe et al., 2013).

The passage of electrons through this protein is uncoupled from proton pumping and does not lead to proton motive force production. Furthermore, since electron flow through AOX bypasses Complexes III and IV, the energy yield of respiration is drastically reduced.

AOX is a disulfide-bonded dimeric protein located on the matrix side of the membrane. AOX monomers may be connected noncovalently (reduced form) or covalently (oxidized form) by the formation of a disulfide bridge. The conserved Cys residue found in several members of the AOX family confers strict biochemical control on the enzyme's activity. The more active form of the enzyme is the noncovalently linked dimer, which is formed by reducing the disulfide bond by oxidation of unique TCA cycle substrates (Umbach and Siedow, 1993). Some oxo-acids, particularly pyruvate, can stimulate the activity of this

enzyme once it has been reduced by interacting with the exposed sulfhydryl of the regulatory Cys residue.

AOX is a nuclear-encoded multigene family found in most monocot and dicot angiosperm species, which is comprised of several members (3 to 5 genes) exhibiting various expression patterns and functions (Vanlerberghe and McIntosh, 1997; Van Aken et al., 2009). In land plants, two AOX subfamilies, *AOX1* and *AOX2*, have been found. *AOX1* is found in both monocot and dicot species, but *AOX2* is found only in eudicots (Considine et al., 2002). The Arabidopsis genome, for example, contains at least five *AOX* genes with distinct gene-specific functions in various organs, stages of growth, and under stress conditions.

Aox1a appears to be one of the most stress-responsive genes in the plant genome, implying that its induction is a typical response to a wide range of stresses such as low temperature, anoxic conditions, light and drought stress (Giraud et al., 2008; Costa et al., 2010). This gene is upregulated in plants treated by various ETC inhibitors such as antimycin A, KCN and rotenone (Clifton et al., 2005; Vanlerberghe and McIntosh 1996). *AOX2*, on the other hand, is frequently expressed at various plant developmental process and is the most common type found in eudicots (Considine et al., 2002). Costa et al. (2010) found that both *AOX1* and *AOX2* are expressed in cowpea (*Vigna unguiculata*) under stress, suggesting for the first time that the expression of *AOX2* is both constitutive and inducible.

1.3 Alternative oxidase and plant stress tolerance

AOX in mitochondria does play crucial roles in both abiotic and biotic stress responses. AOX induction is triggered by a variety of stressors, including nutrient deficiency (Noguchi and Terashima, 2006), ozone exposure (Ederli et al., 2006), heavy metal toxicity (Keunen et al., 2016), drought (Vanlerberghe et al., 2016), cold (Grabelnych et al., 2014), low oxygen (Millar et al., 2004; Szal et al., 2004), and pathogen infection (Cvetkovska and Vanlerberghe, 2012; Ordog et al., 2002). Plants treated with ETC inhibitors such as nitric oxide (NO), and sulphide, showed a strong induction of *AOX* expression and activity (Vanlerberghe and McIntosh, 1996). This suggests that AOX as a component of the stress response affects the mETC.

Since the alternative pathway is not associated with energy conservation, it has been proposed that AOX plays a role in improving respiratory metabolism. For example, when the respiratory process is interrupted by an unfavourable growing condition or stress, one hypothesis is that AOX regulates energy metabolism and maintains metabolic homeostasis by adjusting respiration (Moore et al., 2002; Fiorani et al., 2005). In cases where the cytochrome pathway has been suppressed, one potential role of AOX may be to promote metabolism and respiration. The cytochrome pathway is hindered by shortage of ADP and/or phosphate (Plaxton, 2004), which impairs electron transport and prevents the conversion of NADH to NAD^+. The TCA cycle, which provides carbon skeletons for biosynthesis, requires NAD^+ to continue (Millenaar et al., 2003). AOX maintains the function of the TCA cycle and the availability of carbon intermediates for biosynthesis by preventing the interruption of NADH oxidation and electron transport (Millenaar et al.,

2003). Since AOX can modulate energy, it could help optimize the rate of high-energy processes like plant growth with the required amount of critical resources available (Hansen et al., 2002). AOX regulates the energy balance when the phosphate potential (ATP/ADP ratio) or NADPH status are high (Jarmuszkiewicz et al., 2001). Although the electron flow from ubiquinone to AOX is not coupled with oxidative phosphorylation, the electron transfer in the upstream complex I produces proton motive force, implying that when the cytochrome pathway is disrupted, AOX assists in maintaining ATP production, albeit inefficiently, by maintaining the electron flow to oxygen and allowing some energy production through respiration to support plant growth (Vanlerberghe et al., 1997).

Over-reduction of the mETC may occur as a result of high membrane potential caused by increased metabolic activity or as a result of cytochrome pathway disruption caused by stress. A single electron transfer to O_2, resulting in superoxide (O_2^-) formation, is more likely in these situations. Under various stress conditions, the high level of cellular ROS can damage the biomolecules and can cause serious metabolic problems (Das et al., 2014). AOX can be induced by an increase in intracellular oxidative stress, ROS, and reactive nitrogen species (RNS) during stressful situations (Vanlerberghe and McIntosh, 1997). Under KCN treatment, Arabidopsis leaf tissue of wild-type and *AOX* overexpressors showed no increase in oxidative damage, whereas knockdown lines showed a higher level of oxidative damage (Umbach et al., 2005).

These findings strongly suggest that AOX plays a critical role in regulating ROS production under adverse situations. AOX has the potential to reduce mitochondrial ROS activity in two ways. First, the passage of electrons from ubiquinone to O_2 through AOX

is uncoupled from proton translocation and therefore does not lead to membrane potential increases. AOX reduces the amount of free O_2 available for O_2^- processing by converting O_2 to H_2O. Tobacco cells lacking AOX displayed higher ROS accumulation in response to antimycin A treatment (Maxwell et al., 1999). Overexpression of *AOX*, on the other hand, was found to have lower expression of genes encoding ROS-scavenging enzymes as well as ROS formation under stress (Tripathy et al., 2012; Pasqualini et al., 2007). These findings strongly suggested that AOX plays a critical function in regulating ROS production under stress.

Studies show that AOX function is not just constrained to mitochondria, but impacts other metabolic processes in other compartments (Umbach et al., 2005; Giraud et al., 2008; Yoshida et al., 2007). Inhibition of AOX resulted in an over-reduction of photosystem II in drought-treated wheat (Bartoli et al., 2005), and a decrease in the photosynthetic rate and operating efficiency of photosystem II in broad bean (Yoshida et al., 2007). AOX may be responsible for these phenomena because it can consume excess reducing power (NADPH) produced by photosynthesis, preventing ROS generation and photoinhibition in chloroplast, allowing chloroplast to function even under stressful conditions.The malate-oxaloacetate (OAA) shuttle and other shuttle mechanisms are known to carry surplus NADPH from chloroplasts into the mitochondria, where they are effectively oxidised by AOX (Zhang et al., 2012). Furthermore, its ability to control energy imbalances in the metabolism has been proven to be critical in maximizing photosynthetic activity during drought (Dahal et al., 2015), and high light (Vishwakarma et al., 2015). Thus, the presence of AOX helps to balance carbon metabolism and electron transport with other cellular

processes, as well as keep the cell energetic and redox balance under stress. However, further research is needed to fully understand the role of this terminal oxidase.

1.4 Nitric Oxide metabolism

Nitric oxide (NO) is a small molecule that has emerged as an important biomolecule in plant growth, development, and stress physiology. NO regulates a number of plant growth and development processes including seed germination, cell wall synthesis, root development, flower transition, fruit ripening, senescence, and mineral nutrition, as well as plant responses and adaptations to unfavorable environmental conditions, host-pathogen interactions, and promote cell death (Yan et al., 2007; Neill et al., 2008; Siddiqui et al., 2011; Mur et al., 2013; Wendehenne and Hancock, 2004; Gupta et al., 2011a; Bellin et al., 2013; Sun et al., 2015).

Nitric oxide possesses physicochemical properties that make it an ideal candidate for a short-term regulator or signal molecule. Through the liquid and lipid phases, the formed nitric oxide easily diffuses out of its origin cells and into nearby target cells (Siddiqui et al., 2011). In the presence of oxygen, NO is highly reactive. The reaction of NO with oxygen, termed autoxidation, produces nitrogen dioxide (NO_2). Dinitrogen trioxide (N_2O_3) can be formed from NO_2 reaction with NO and is considered an important intermediate in the autoxidation of NO. Peroxynitrite ($OONO^-$) is a powerful one- and two-electron oxidant which is formed from the diffusion-controlled reaction between superoxide (O_2^-) and NO (Blokhina and Fagerstedt, 2010). At low NO and O2 concentrations, the halflife of NO will be considerably longer than 10 s, and consequently the path of NO diffusion much greater. The lower the concentration, the higher is its capacity of diffusion.

Chloroplasts, mitochondria (Gupta et al., 2005), peroxisomes (Corpas et al., 2009), cytosol (Rockel, 2002), and plasma membranes (Stöhr et al., 2001) can all produce nitric oxide. In contrast to the numerous NO synthesis mechanisms and sites proposed, researchers typically point to two primary routes for NO synthesis in plants: an oxidative pathway in which NO is formed from substrates such as arginine and polyamines and a reductive pathway in which the electrons required to reduce nitrite (NO_2^-) to NO (Moreau et al., 2010; Gupta et al., 2011b; Blokhina and Fagerstedt, 2010). The majority of NO in animals is generated by the oxidative pathway in which the enzyme nitric oxide synthase (NOS) catalyzes the oxidation of arginine to citrulline. Even though no plant NOS enzyme with sufficient homology to animal NOS has yet been discovered, several candidates with NOS-like activity have been found and investigated. Still, their function in NO formation is unknown (Crawford et al., 2006). The most well-studied pathway for NO production in plants is the nitrate reductase (NR) pathway. This enzyme, located in the cytosol, converts nitrate (NO_3^-) to nitrite (NO_2^-), and then NO_2^- to NO, using NADH as an electron donor. NO production through this pathway might be increased under stressful conditions (Fu et al, 2018; Ziogas et al., 2013). Nitrite reduction may generate NO via the activities of membrane-bound nitrite reductase (Ni:NOR; Stöhr et al., 2001); and xanthine oxidoreductase (Godber et al., 2000). Mitochondrial electron transport has also been shown as a possible important site for nitrite reduction under hypoxic or anoxic conditions (Planchet et al., 2005; Stoimenova et al., 2007).

The presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), by blocking the Q_B plastoquinone binding site of photosystem II, reduced nitrite-dependent NO generation in

isolated chloroplasts, implying that photosynthetic electron transport is involved (Jasid et al., 2006). Non-enzymatic pathways are an appealing alternative hypothesis for NO synthesis. The protonation of nitrite to yield NO is favoured under acidic conditions, and the presence of ascorbic acid or phenolic compounds accelerates this conversion, where the apoplast fulfils these requirements (Bethke et al., 2004). Changes in apoplastic pH are associated with many physiological processes in plants, including development, growth, leaf movement, gas exchange, and pathogen defence (Amtmann, 1999). An increase in reductants such as ascorbic acid (Urzica et al., 2012) and a drop in apoplastic pH due to plasma membrane H^+-ATPase activity (Młodzińska et al., 2015) shows that a non-enzymatic mechanism for NO production may be active under different physiological or stress situations. **Figure 1.2** shows a summary of NO-production and removal mechanisms in plants.

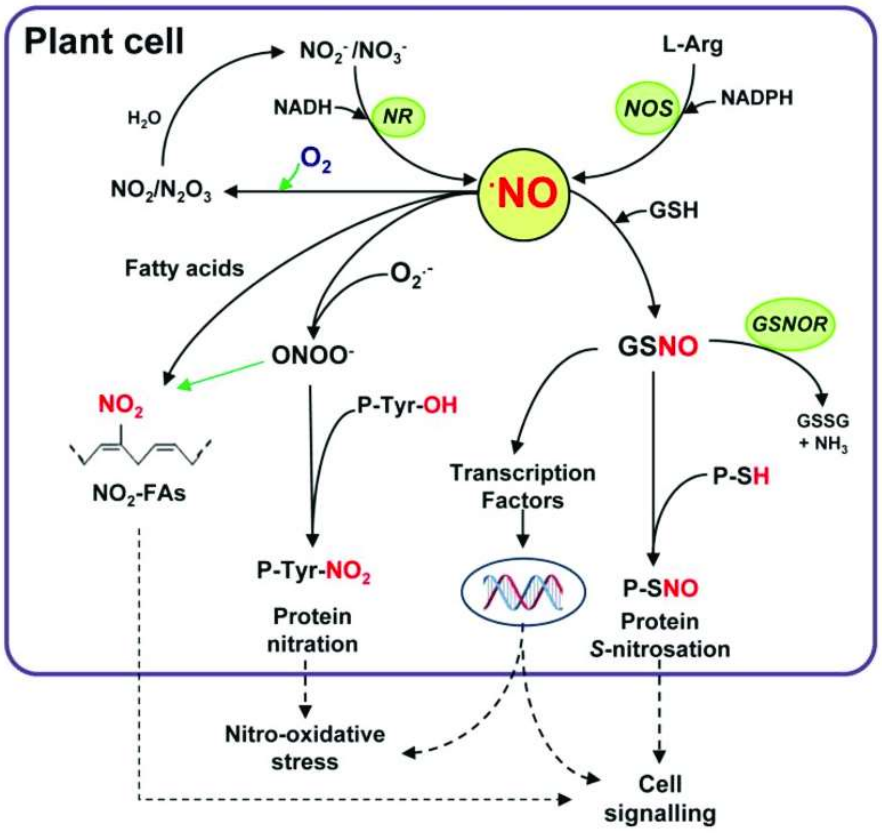

Figure 1.2 Nitric oxide (NO) metabolism in plant cells. Nitric oxide can be generated by either nitrate reductase (NR) or by L-arginine-dependent nitric oxide synthase (NOS). Then, NO can react with reduced glutathione (GSH) to form S-nitrosoglutathione (GSNO) through a process of S-nitrosation (S-nitrosylation). This metabolite can be converted by the enzyme S-nitrosoglutathione reductase (GSNOR) into oxidized glutathione (GSSG) and NH_3. GSNO and other S-nitrosothiols can interact with specific protein sulfhydryl groups (P-SH) to produce S-nitrosated proteins (P-SNO) in a process called S-transnitrosation, which can mediate signaling processes. Nitric oxide also interacts rapidly with superoxide radicals ($O_2^{\bullet-}$) to generate peroxynitrite ($ONOO^-$), a powerful oxidant molecule that can mediate the tyrosine nitration of proteins (P-TyrNO_2) and also the nitration of fatty acids (NO_2-FAs). Alternatively, NO in the presence of oxygen is converted into dinitrogen trioxide (N_2O_3) and nitrogen dioxide (NO_2) which, in aqueous solutions, are transformed into nitrite and nitrate. Nitric oxide and related molecules could be part of cell signaling or nitro-oxidative stress processes (adapted from Corpas and Palma, 2018).

Although the mitochondria have recently been discovered to be a source of NO, little is known about NO synthesis in this organelle. In the presence of elevated NO_2^- concentrations and falling pH, as occurs during hypoxia, mitochondrial NO_2^- reductase activity is increased. This reaction is oxygen-independent and is usually triggered in hypoxic or anoxic environments (Planchet et al., 2005). Even though the exact mechanism of mitochondrial NO generation is unknown, it is thought that enzyme complexes downstream of ubiquinone catalyze the reaction. Stoimenova et al. (2007) showed that the respiratory inhibitors of the complex III and IV (myxothiazol and KCN, respectively) reduced the NO_2^- reduction reaction in hypoxia. Following a nitrite reaction with a heme centre in Complex IV (COX), COX may function as a nitrite reductase, generating NO (Castello et al., 2006). Since COX may use nitrite as a final acceptor in the synthesis of ATP under low oxygen, it has been suggested that NO plays a role in respiration regulation. It is worth noting that when oxygen is present, NO inhibits COX, and COX may convert some of the bound NO to nitrite under normoxia (Poyton et al., 2009).

In plant cells, NO concentrations can be buffered and diminished in several ways. The presence of target molecules, such as superoxide radicals, thiols, and Fe-containing molecules, helps in the maintenance or reduction of NO levels. There are specific scavenging mechanisms. Non-symbiotic phytoglobin (Pgb) proteins contain iron heme and are capable of binding not only oxygen but many other diatomic molecules such as NO. Class 1 phytoglobin (Pgb1) are non-symbiotic, having an extremely high avidity for oxygen (Hargrove et al., 2000), which is optimal for oxygen dependent NO scavenging under hypoxia (Perazzolli et al., 2004). The interaction of Pgb1 with NO is aided by

MDHAR and ascorbate, which convert NO to nitrate and eliminates NO (Igamberdiev and Hill, 2004; Igamberdiev et al., 2005; Poole, 2005). Igamberdiev et al. (2005) proposed an Pgb1-NO cycle (**Figure 1.3**), in which oxyphytoglobin [Pgb(Fe_2^+)O_2] oxygenates NO and converts it to nitrate before being reduced to metPgb [Pgb(Fe_3^+)] (MetPgb). MetPgb reductase consumes half a molecule of NAD(P)H to catalyze the conversion of MetPgb to [Pgb(Fe_2^+)O_2]. Hypoxia-inducible non-symbiotic Pgbs in barley seeds play a key role in preserving the energy state of cells under oxygen deficiency (Sowa et al., 1998). That is, when the internal environment in barley seeds is hypoxic during the germination, Pgbs produced will scavenge excess NO and higher levels of ascorbate and active MDHR will help the reaction. In this scenario, monodehydroascorbate reductase uses NADH to reduce monodehydroascorbate back to ascorbate to sustain the cycle.

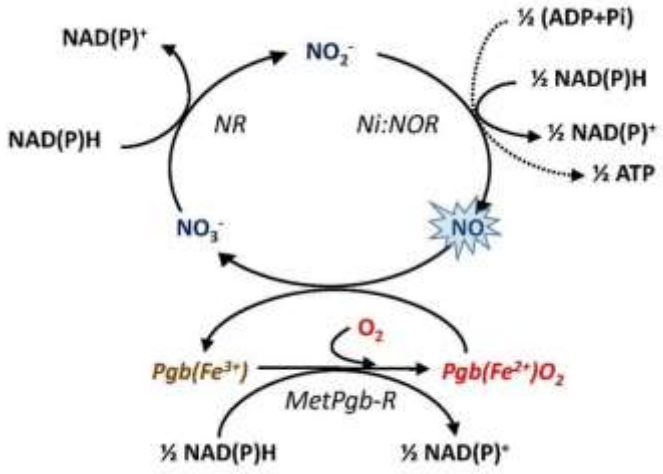

Figure 1.3 Phytoglobin-nitric oxide (Pgb1-NO) cycle. A sequence of reactions of NO scavenging by oxyphytoglobin followed by its regeneration via metphytoglobin reductase (MetPgb-R) protein. The cycle is linked to the oxidation of NADH (Adapted from Ma et al., 2017).

In addition to the reduction of NO by Pgb1, the denitrosylation of S-nitrosoglutathione (GSNO) is another way of metabolizing NO, which can be regulated by the enzyme GSNO reductase (GSNOR) (**Figure 1.2**). (Sakamoto et al., 2002; Holzmeister et al., 2011; Kwon et al., 2012). GSNO is a form of storage and transport of NO in plants and seeds and has a decisive effect on intracellular NO content and on seed germination (Sakamoto et al., 2002). When GSNO is reduced by GSNOR, one source of NO is eliminated, allowing the content of NO in tissue to be regulated (Singh et al., 1996).

1.5 NO and AOX interaction

During hypoxia, COX has a limited capacity to donate electrons to oxygen, whereas AOX does not function (Igamberdiev and Hill, 2009). The oxygen Km for the AOX is 10 μM which limits the AOX function under low oxygen conditions, while the Km value of COX for oxygen is in the range from 0.1 to 0.15 μM (Igamberdiev and Hill, 2009). When exposed to low oxygen levels, mitochondrial oxygenic respiration decreases below the oxygen level required to saturate terminal oxidases (Igamberdiev and Hill, 2009). Under this condition, COX can produce NO from nitrite (Gupta and Igamberdiev, 2011). NO production under hypoxia reduces COX efficiency due to irreversible inhibition by NO (Parihar et al., 2008).

NO binds to the Fe_2^+ -haem group at the oxygen binding site of COX's binuclear core $Fe_{a3}Cu_B$ (Cleeter et al., 1994), establishing the framework for an autoregulatory mechanism in which increasing NO reduces oxygen consumption under hypoxia. In normoxic barley roots, overexpression of *Pgb1* improved NO scavenging, increased the respiration rate, and

decreased the internal oxygen content (Gupta et al., 2011b). The normoxic NO signaling pathways in barley were likewise altered by *Pbg1* overexpression via post-translational modification (Cochrane et al., 2017). Studies have shown that nitrite reduction at complex III reversibly reduces COX, allowing the mitochondria to maintain a steady-state oxygen concentration (Borisjuk et al., 2007; Benamar et al., 2008).

However, changes in AOX activity may potentially affect mitochondrial activity when NO is generated under hypoxic conditions. There are well-established link between AOX and NO, with AOX inhibiting excess NO generation in tobacco leaves (Cvetkovska & Vanlerberghe, 2012) and NO stimulating AOX in hypoxia (Gupta et al., 2012). Under normoxia, Vishwakarma et al. (2018) found that AOX reduces excess NO, $ONOO^-$, and tyrosine nitration. However, it was shown that AOX can produce NO in the presence of hypoxia, and that the NO is then oxidized via the Pgb1-NO cycle (Vishwakarma et al., 2018). Under hypoxia, inhibiting AOX resulted in reduced ATP production, whereas AOX overexpressing lines produced more. These findings suggested that AOX-mediated NO generation promotes ATP synthesis in hypoxia by facilitating proton translocation via complex I. Extra NO produced under hypoxia did not result in the generation of $ONOO^-$ or tyrosine nitration, as it did in normoxia (Jayawardhane et al., 2020). As a result, the relationship between AOX and NO is different in normoxia and hypoxia and is likely much different than that of COX.

1.6 ROS metabolism

ROS are highly reactive chemical species generated when molecular O_2 is depleted in stages (Das and Roychoudhury, 2014). Superoxide ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH) are examples of ROS (Mittler, 2017) (**Table 1.1**). ROS are byproducts of a variety of metabolic pathways, and they can be created either enzymatically or nonenzymatically. Non-enzymatic ROS can be produced by ETC in chloroplasts and mitochondria (via Complexes I and III). Oxygen can be partially reduced by the occasional leaking of electrons during electron transport in both organelles, resulting in $O_2^{\cdot-}$, which can subsequently be used to generate other types of ROS (Asada, 2006). Mitochondria perform a dual role in oxidative stress as both ROS generators and antioxidant defence system integrators (Rhoads and Subbaiah, 2007). About 1% of metabolically consumed O_2 is thought to be used in the production of ROS in aerobic cells (Puntarulo et al., 1988).

Enzymatic ROS can be created in a variety of sites in the cell, including peroxisomes, cell walls, plasma membrane, and apoplast (Mignolet-Spruyt et al., 2016). Peroxisomes are involved in a number of oxidative metabolic activities that may generate ROS, such as B-oxidation of fatty acids and photorespiration (Del Río and López-Huertas, 2016). ROS are created by the peroxisome's xanthine oxidase and glycolate oxidase reactions, which produce $O_2^{\cdot-}$ and H_2O_2 (Bolwell and Wojtaszek, 1997; Del Río and López-Huertas, 2016). Plasma membrane bound NADPH oxidases, well-known as respiratory burst oxidase homologs (Rbohs), are another enzymatic ROS generator. Rbohs use cytosolic NADPH to convert extracellular O_2 to O_2^- (Møller, 2001). The Arabidopsis genome has ten Rboh

genes, each of which plays a unique role in biological processes (Foreman et al., 2003). In the immunological response of *A. thaliana*, the Rboh gene contributes to the generation and signaling of Rboh-dependent ROS (Morales et al., 2016). The functions of Rbohs in hypoxic or anoxic circumstances, on the other hand, are little understood.

Given that ROS are product of O_2, it is reasonable to assume that under hypoxic condition, ROS generation will be reduced as well. Several investigations, however, have found enhanced ROS formation under hypoxia/anoxia (Pucciariello et al., 2012; Paradiso et al., 2016). ROS production in mitochondria, chloroplasts, and peroxisomes is expected to continue in the absence of oxygen. Anoxia inhibits the mitochondrial ETC, resulting in the production of mitochondrial ROS (Chang et al., 2012), and chloroplastic ROS may be produced in a similar way. Hypoxia can alter the antioxidant system, upsetting the finely regulated balance between scavenging and production (Lasanthi-Kudahettige et al., 2007). When the balance between the creation and detoxification of active oxygen species is disrupted, the formation of toxic oxygen species increases. ROS is produced in a controlled manner by Rbohs during hypoxia. The membrane localized *RbohD* gene is one of the main hypoxia genes in Arabidopsis, and it is increased specifically during flooding and hypoxia (Mustroph et al., 2009; Pucciariello et al., 2012). After waterlogging, *rbohd*, *rbohf*, and *rbohdf* mutants collected less H_2O_2 and O_2^- than wild type, and waterlogging reduced *rbohd*, *rbohf*, and *rbohdf* mutants' growth (Guan et al., 2019; Chen et al., 2015). This shows that in waterlogged Arabidopsis roots, RbohD and RbohF are necessary for ROS buildup and the ROS signaling pathway. ROS signaling via regulated ROS synthesis is expected to

improve hypoxia signaling and adaptive responses to stress (Steffens et al., 2012; Pucciariello et al. 2012; Yamauchi et al., 2017). **Table 1.1** summarizes the production and scavenging of ROS in various locations in plant cells.

Table 1.1 The production and scavenging of reactive oxygen species in plants (adapted from Mittler, 2002).

Mechanism	**Localization**	**ROS type**
Production		
Photosynthetic ETC	Chloroplast	O_2^-
Excited Chlorophyll	Chloroplast	O_2^1
Respiratory ETC	Mitochondria	O_2^-
NADPH oxidase	Plasma membrane	O_2^-
Peroxidases	Cell wall	O_2^-, H_2O_2
Scavenging		
Superoxide dismutase	Chloroplast, Cytosol, Mitochondria, Apoplast, Peroxisome	O_2^-
Ascorbate Peroxidase	Chloroplast, Cytosol, Mitochondria, Apoplast, Peroxisome	H_2O_2
Catalase	Peroxisome	H_2O_2
Glutathione Peroxidase	Cytosol	H_2O_2
Glutathione	Chloroplast, Cytosol, Mitochondria, Apoplast, Peroxisome	H_2O_2
Carotenoids	Chloroplast	O_2^1
flavonoids	Chloroplast, Cytosol, Mitochondria	O_2^1, H_2O_2, $OH^{\cdot}$
Ascorbic acid	Chloroplast, Cytosol, Mitochondria, Apoplast, Peroxisome	H_2O_2
Proline	Chloroplast, Cytosol, Mitochondria	O_2^1, $OH^{\cdot}$

1.7 Antioxidative system in plants

To offset the harmful effects of oxygen radicals by avoiding ROS formation or detoxifying ROS to overcome stress circumstances, all aerobic organisms display higher levels of both enzymatic and non-enzymatic antioxidant activities (Arbona et al., 2003; Kim et al., 2017; Belinskaia et al., 2020; Irato and Santovito, 2021). The first line of defence in the mitochondrial inner membrane comprises AOX, alternative NAD(P)H dehydrogenases, and plant uncoupling proteins that limit ROS creation owing to mETC overreduction (Czarna and Jarmuszkiewicz, 2005; Umbach et al., 2005). Activating AOX reduces ROS generation, while inhibiting it has the opposite effect (Czarna and Jarmuszkiewicz, 2005; Umbach et al., 2005).

The enzymatic components of the antioxidant machinery include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR) (ascorbate–glutathione cycle); and non-enzymatic antioxidants include ascorbic acid, reduced glutathione (GSH), α-tocopherol, carotenoids, flavonoids, and the osmolyte proline (Chew et al., 2003; Das and Roychoudhury, 2014; Huang et al., 2019; Hasanuzzaman et al., 2020). Ascorbate and glutathione, on the other hand, are thought to be the principal redox cell buffers and redox sensors (Hasanuzzaman et al., 2019; Hasanuzzaman et al., 2020) (**Figure 1.4**).

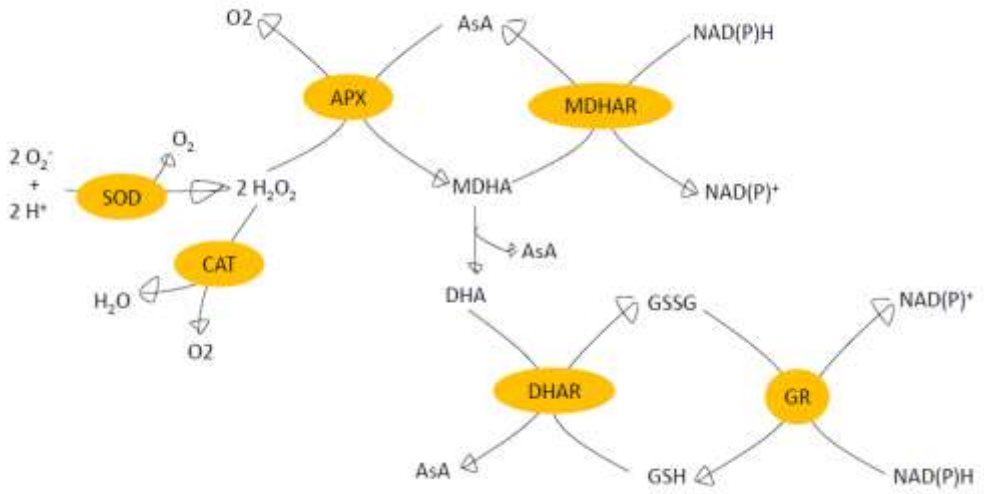

Figure 1.4 scavenging of H_2O_2 by the ascorbate-glutathione cycle (adapted from Hasanuzzaman et al., 2019).

SODs are the first line of defense against ROS which catalyze the conversion of superoxide into oxygen and H_2O_2. Cu/Zn SODs, Mn SOD/Fe SODs, and Ni SODs are three types of SOD that have developed in organisms with differing catalytic metal ions (Duttaroy et al., 1997; Antonyuk et al., 2009; Jung et al., 2011; Blackney et al., 2014). SOD enzymes have unique subcellular localizations in addition to metal ion cofactor needs. Cu/Zn SODs (in the chloroplast, cytoplasm and extracellularly), Fe SODs (in the chloroplast), and Mn SODs are the only SODs expressed by eukaryotes (in the mitochondria; Miller, 2012). Several enzyme systems (e.g., CAT, GPX, and APX) regulate the elimination of H_2O_2 (Blokhina *et al*., 2000), but this could be for the purpose of regulating its signaling function rather than its potential toxicity.

1.8 Ethylene (ET) and Ethylene response factors (ERFs)

The amino acid methionine is converted to S-adenosyl-L-methionine (SAM) in the first step of ET synthesis, which is mediated by SAM synthetase (**Figure 1.6**). After that, the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase catalyzes the formation of ACC from SAM, which is then oxidized to ET by ACC oxidase. Both ACC synthase (ACS) and ACC oxidase (ACO) are members of large multigene families in plants that are controlled by a variety of external and internal stimuli (Voesenek and Sasidharan, 2013). ACS and ACO have been found to be induced by hypoxia (Peng et al., 2005).

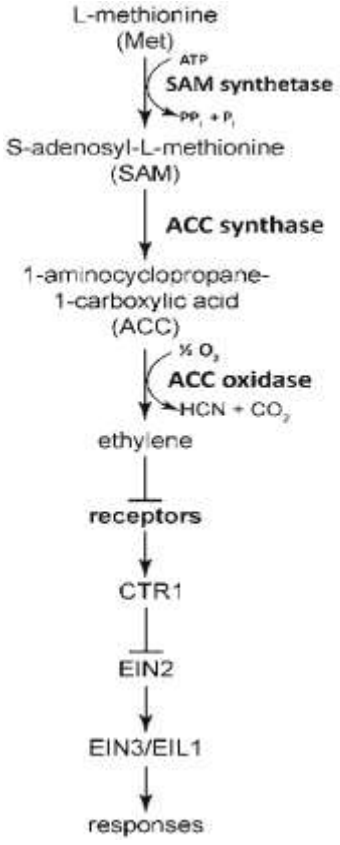

Figure 1.5 General diagram of pathways for ethylene (ET) biosynthesis and signaling showing the sites of action for several chemical inhibitors

ET has been found to regulate aerenchyma production in the root tips of maize plants that have been subjected to hypoxic circumstances (Yamauchi et al., 2016). According to recent studies, ET accumulates quickly under flooding-induced hypoxia (Banga et al., 1996), and this leads to fast ET-dependent signaling. In hypoxic Arabidopsis root tips, nuclear accumulation of Ethylene Insensitive 3 (EIN3), the main transcriptional regulator of ET signaling, increased in the early hours of stress (Hartman et al.,2019). When O_2 levels dropped, the rapid induction of ET signaling in a short period of hypoxia was found to be critical for better hypoxia tolerance via increased expression of a core hypoxia gene set (Hartman et al., 2019). Induction of essential hypoxia genes is thought to be adaptive for surviving longterm hypoxia. This core group of genes includes genes involved in fermentation, energy management, oxidative stress, NO scavenging, O_2 sensing, and ET recognition and biosynthesis (Mustroph et al., 2010). The effect of ET in the stimulation of fermentation genes was investigated previously. In Arabidopsis, Peng et al. (2001) reported that aminooxyacetic acid, an ET inhibitor, could partially reduce the hypoxia induction of alcohol dehydrogenase (ADH). This inhibition was abolished by the direct precursor of ET, ACC. In the marsh plant Rumex palustris, 4 hours of ET treatment resulted in a minor but significant activation of the major hypoxia genes ADH and Pgb1 (van Veen et al., 2013). Recent research suggests that ET has a favourable effect on both the transcriptional response to hypoxia and subsequent survival (Hartman et al., 2019). The ET signaling appears to be connected with the plant's O_2-sensing mechanism.

ET Response Factors (ERFs) are the last downstream components of the ET transduction pathway (Benavente and Alonso, 2006; Chang, et al., 2013). ERFs are only found in plants and play a critical role in the plant's response to diverse biotic and abiotic environmental challenges. These factors belong to the AP2/ERF multi-gene family, which has a highly conserved DNA-binding domain known as the AP2 domain, which consists of 58 or 59 amino acids and is involved in binding to various cis-acting elements located in the promoter regions of ET-responsive genes (Riechmann and colleagues, 2000). ERF genes have various expression patterns and can influence transcriptional activity in a positive or negative way. ERFs have the necessary features to direct ET signaling to a specific group of genes required for the proper developmental process or the desired responses to environmental stressors (Pirrello et al., 2012). Transcription factors belonging to the AP2/ERF family have been reported to be able to up-regulate *ADH* expression in Arabidopsis (Licausi et al., 2010). ET activates the ERFs transcription factors SNORKEL1 and 2 in deep water rice, which in turn enhance internode elongation to escape hypoxia through restoration of above-water gas exchange (Hattori et al., 2009).

Based upon the number of AP2 domains and sequence similarities, Members of the ERF family are further classified into 12 groups, yet the biological functions of the majority remain unknown (Nakano et al., 2006). The IX group has been studied the most, and includes ERFs such as ERF1, ERF14 and ORA59, which play roles in plant innate immunity (Moffat et al., 2012). ERFs play a key part in stress responses, therefore keeping them under tight control ensures that plants respond effectively to environmental stresses. ET or environmental mechanical stress upregulate *NtERF3* expression quickly and

transiently (Nishiuchi et al. 2002). ERF protein stability is regulated by ubiquitin-mediated protein degradation via the 26S proteasome pathway. The N-end rule pathway as a proteolytic system, is responsible for some ERF-VIIs' stability (Gibbs et al., 2014). ERFS stability, localization, and activity are influenced by a variety of variables. Oxygen and NO increase ERF proteolysis, while ET promotes their stability (Hartman et al., 2019; Kim et al., 2018).

1.9 ET interaction with NO and ROS

The interaction of ET and NO has been described numerous times, although it has rarely been explored mechanistically. Plant responses are influenced by chemical treatment or genetic manipulation of one of these pathways (Asgher et al., 2016; Manjunatha et al., 2012). As a result, waterlogged Arabidopsis and cotton plants produced more ET and signaling, while their NO amount emitted from aerial regions decreased (Zhang et al., 2017). Exogenous NO treatment enhanced ET synthesis in a range of plant species, most likely due to increased ACO activity (Magalhaes et al., 2000; Manac'h-Little et al., 2005). NO levels, on the other hand, decreased in ET-deficient or constitutive mutants (Magalhaes et al., 2000; Liu et al., 2017). As a result, both gases may interact with one another during the hypoxic condition.

Crosstalk between NO and ET could be facilitated by NO-scavenging phytoglobins. Phytoglobin mRNA levels increased in response to waterlogging and hypoxia (Hebelstrup et al., 2012; Mira et al., 2016). In Arabidopsis and maize, silencing Pgb1 elevated ET and NO emissions and activated ET biosynthesis and signaling genes (Hebelstrup et al., 2012;

Mira et al., 2016). In turn, NO increased Pgbs mRNA abundance in cotton, barley, Arabidopsis (Qu et al., 2006; Zhang et al., 2019; Kuruthukulangarakoola et al., 2017), suggesting a feedback mechanism. Following a brief ET treatment, Pgb1 transcript levels increased in the wetlands species *Rumex palustris*, likely lowering NO levels in these tissues (van Veen et al., 2013). Thus, Pgbs influence the amounts of NO and ET, and these gases influence the quantity of Pgbs. As previously indicated, crosstalk and feedback between NO and ET may occur at the level of ERFVIIs. ERFVII stability and action are substantially influenced by NO and O_2 levels (Giuntoli and Perata, 2018; Gibbs et al., 2015; Vicente et al., 2017), therefore ET regulating *Pgb1* expression to control NO levels may have an impact on ERFVII stability (Gasch et al., 2016).

ET elevated ROS production by increasing the activity and levels of NAPDH oxidase (Desikan et al., 2006). In Arabidopsis, ET is implicated in ROS detoxification during reoxygenation, according to one study (Tsai et al., 2014) Furthermore, ET-regulated oxidative stress tolerance in various plant species has been widely examined for a variety of abiotic stimuli, including drought, heat, cold, and salt stress (Wu et al., 2008; Peng et al., 2014). Heat, drought, and salt stress tolerance have all been linked to increased ERFVII stability (Vicente et al., 2017), and ERFVIIs regulate a number of genes involved in oxidative stress tolerance (Gonzali et al., 2015; Papdi et al., 2015). Many genes coding for proteins involved in ROS scavenging, such as carbonic anhydrase, catalase, peroxidase, and ascorbic acid biosynthesis, were found to be directly controlled by ET, EIN3, and EIN3-target transcription factors across diverse plant species (Wu et al., 2008; Peng et al., 2014; Zhang et al., 2016). These findings support the idea that ET may play a role in ROS

detoxification and oxidative stress tolerance during hypoxic condition and reoxygenation. ET encourages adventitious root formation in deep water rice by prompting NADPH oxidases to create ROS, a signal of cell death in epidermal cells above the root primordia (Steffens and Sauter 2009; Steffens et al. 2012). ET and H_2O_2 promote the development of constitutive aerenchyma in deep water rice (Steffens et al. 2011), as well as aerenchyma production in Arabidopsis in response to hypoxia (Mühlenbock et al. 2007).

It is worth noting that a relationship between O_2 sensing and ROS signaling was recently discovered (Gonzali et al. 2015). The transcription factor RELATED TO APETALA (RAP) RAP2.12, one of the group VII ERFs, transcriptionally induces the hypoxia responsive universal stress protein 1 (HRU1), which interacts with RbohD (Gonzali et al. 2015). HRU1 appears to act as a hub, connecting low O_2 sensing to ROS signaling. However, it is still unclear how the plant defense system interacts with redox changes that occur when oxygen levels are low.

1.10 ET and AOX interplay

AOX improves plant stress tolerance by reducing ROS production (Xu et al., 2012a). Kendrick and Chang (2008) and Xu et al. (2012a) both reported that increased AOX and ET contribute to plant abiotic stress tolerance. Furthermore, according to a study by Xu et al. (2012b) the ET production route was linked to the AOX capacity in tomato fruit ripening. The suppression of AOX and ET under abiotic stress conditions resulted in more dissipated excitation energy and a lower level of photochemical efficiency (Wei et al., 2015; Giraud et al., 2008). The mRNA levels of AOX and genes involved in ET production

were higher in the cucumber seedlings exposed to abiotic stress than in control seedlings (Wei et al., 2015). The quantity of ERF5 in aox1a and Col-0 plants differs, indicating that components involved in the response to oxidative stress in aox1a plants have changed (Giraud et al., 2008). Using pharmacological suppression of ET and NO, Ederli et al. (2006) reveal that both NO- and ET-dependent pathways are required for ozon-induced up-regulation of *AOX1a*.

In plant cells, both ET and H_2O_2 can elicit AP (Ederli 1t al., 2006; Wagner et al., 1995) In Arabidopsis, Simons et al. (1999) showed that ET is essential for AP induction and that AP operation is ET-dependent. H_2O_2 has been proposed as a second messenger for inducing AOX activity by directly oxidizing transcription factors or regulating phosphorylation pathways (Wagner et al., 1995; Neill et al., 2002). According to Wang et al. (2010a), H_2O_2 and ET altered salt-induced *AOX* gene (*AOX1a*) expression and pyruvate content. These findings suggest that ET and H_2O_2 are involved in the salt-induced elevation of the AP, which is required for WT callus salt tolerance, and that ET may act as a downstream regulator of H_2O_2. The expression of *AOX* genes (*AOX1a* and *AOX1c*) and the rise in pyruvate content induced by chilling stress were both regulated by ET (Wang et al., 2012). As demonstrated in transgenic potato lines with pyruvate kinase down-regulation, increasing pyruvate concentration in the stress treated cell activates the AOX by modifying its redox-sensitive regulatory sulfohydryl/disulfide group (Oliver et al. 2008). According to a significant body of studies (Ederli et al., 2006; Wang et al., 2009, 2010b; Xia et al., 2009), different stressors cause the production of H_2O_2 and ET, which increases AOX

capacity. Increased AOX activity can indirectly reduce excess H_2O_2 production, preventing oxidative damage in plant cells, and improving stress tolerance.

Both AOX and ET have been shown to be important adaptive responses to hypoxic stress (Igamberdiev and Hill, 2009; Gupta et al., 2012; Vishwakarma et al., 2018; Millar et al., 2004; Szal et al., 2004; Banga et al., 1996; Hartmanet al.,2019; Peng et al., 2001; van Veen et al., 2013; Bui et al. 2015). Although the link between AOX and ET in the absence of oxygen remain unclear.

1.11 Metabolite changes under low oxygen conditions

Hypoxia occurs as a result of insufficient oxygen availability and is detected by plants, which adapt their growth and metabolism accordingly. Excessive rain and soil waterlogging can cause plant hypoxia, which limits plant growth (Bailey-Serres and Voesenek, 2008). Under optimal growth conditions, plants experience hypoxia in roots, germinating and developing seeds and tubers due to the limited capacity of oxygen diffusion into these tissues through internal cell layers (Tschiersch et al., 2011).

Plants can endure hypoxic stress by going through metabolic changes that help them maintain energy and regulate oxidative damage. Indeed, as oxygen levels drop, mitochondrial respiration and ATP synthesis are impeded, forcing hypoxic cells to rely on glycolysis to generate substrate-level ATP (Geigenberger, 2003; Bailey-Serres et al., 2012). To keep the glycolytic flux continuing, fermentation pathways are activated. The goal of fermentative metabolism is to produce ATP via the glycolytic pathway by recycling

NAD^+ through the activity of two key enzymes, pyruvate decarboxylase (PDC) and ADH (Kumutha et al., 2008) (**Figure 1.6**). Carbohydrate degradation via glycolysis in conjunction with the fermentative pathway produces two moles of ATP rather than the normally produced during aerobic respiration (Saika et al., 2006). Despite its limitations, ATP production via fermentation is critical for hypoxia tolerance (Loreti et al., 2016).

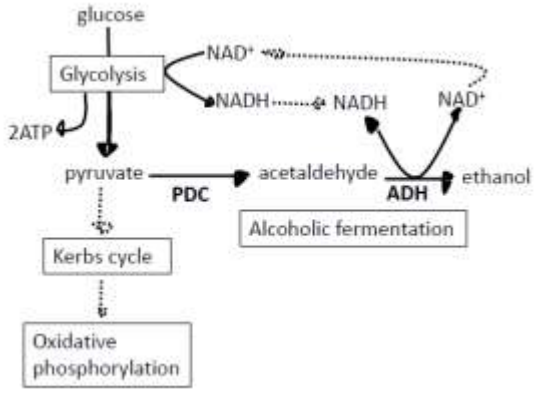

Figure 1.6 Alcoholic fermentation pathway in plants. The decarboxylation of pyruvate to acetaldehyde by PDC and the subsequent reduction of acetaldehyde to ethanol by ADH are the two reactions that occur during fermentation (adapted from Saika et al., 2006).

As fermentation uses up the plant's carbohydrates and causes cytosolic acidification, alanine and serine synthesis are increased to prevent carbon loss, and γ -aminobutyric acid (GABA) shunt is activated to enhance the cytosolic pH stabilization (Ricoult et al., 2006; Miyashita & Good, 2008; Felle, 1996). The increase of amino acids in rice and wheat coleoptiles was observed in response to anoxia or oxygen deficiency (Narsai et al., 2009; Rocha et al., 2010).

GABA and alanine production is a crucial adaptation process for storing carbon and nitrogen that might otherwise be wasted in low-oxygen environments (Ricoult et al., 2006; Mustroph et al., 2014). Furthermore, energy demanding processes such as protein synthesis are slowed, but sucrose and starch metabolism are changed in numerous plant species/tissues to meet the high glucose needs during hypoxia (Branco-Price et al., 2008; Loreti et al., 2018). Furthermore, several studies suggest that in response to hypoxia, mitochondrial respiration is actively downregulated, resulting in ROS generation (Ramrez-Aguilar et al., 2011). Multiple mechanisms are activated in plant cells to reduce oxidative stress damage caused by hypoxia and subsequent reoxygenation (Gonzali et al., 2015; Yeung et al., 2018).

Thesis introduction

Oxygen is required for energy production as it acts as a terminal electron acceptor in the ETC of mitochondria. Hypoxia affects plants at many stages of growth (seed germination, for example), as well as during flooding or waterlogging. Oxygen depletion causes an energy crisis, which stifles growth and development.

A major focus of the work presented herein is to investigate the role of AOX in the adjustment of energy requirements and metabolic fluxes during oxygen shortage. More specifically, this work concentrates on the role of AOX in controlling the generation of mitochondrial NO, ROS, and ET, thus influencing the plants response to low oxygen. I suggest that the level of AOX activity is pivotal in determining the levels of signaling molecules and stress hormone induced by anoxic condition.

In my work I try to answer several questions: Is there a link between the Pgb1-NO cycle and AOX production during germination process? Is Pgb1 required for redox and energy balancing prior to radicle protrusion when seeds have a low internal oxygen concentration? Is AOX a positive or negative regulator of NO generation? What role does AOX play in maintaining carbon, nitrogen, and energy metabolism in hypoxia? What role does AOX play in the regulation of the downstream defence system when it interacts with ROS and ET? I hope that the research presented here will help to clarify these questions.

In chapter 2, I have used transgenic barley plants with modified amounts of class 1 Pgb (Pgb+, Pgb-, wild type (WT)). I try to understand how the Pgb1 is involved in germination process (as a normal hypoxic situation) via the modulation of NO metabolism. In barley

seeds, the observed high level of S-nitrosylation of proteins indicated the elevated NO concentrations during the initial hours of germination before radicle protrusion when seed coat prevented oxygen exchange. The expression profiles of the *AOX* gene *AOX1a* revealed that, during imbibition of the *Pgb1* overexpression barley seeds, *AOX1a* is induced. The Pgb1-NO cycle is important not only during the anaerobic stage, but following radicle protrusion, when the TCA cycle becomes more active due to the coordinated expression of succinate and pyruvate dehydrogenases. As a result, these findings indicate the functioning of the Pgb1-NO cycle, which regulates NO turnover, lowers the reduction level in mitochondria and cytosol, and may contribute to ATP generation.

This research revealed that there is a link between the Pgb1-NO cycle and AOX in response to low oxygen levels. AOX involvement in tobacco exposed to anoxia was examined in detail in the following chapters.

In chapter 3, I have used transgenic tobacco plants with modified amounts of *AOX* (knockdown mutants: R19, R29; overexpressors: B7, B8 and WT). I have studied possible involvement of AOX in NO turnover and modulation of the phosphorylated pathway of serine biosynthesis (PPSB) and the GABA shunt in the lines of tobacco. The NO production was the highest in the overexpressing lines and lowest in the knockdown *AOX* lines. This corresponded to the levels of expression of *Pgb1* and GSNOR that participate in NO scavenging. The plants overexpressing AOX exhibited an increased transcript abundance of the genes encoding key enzymes of PPSB and GABA shunt. Based on our results, we suggest that possible AOX involvement in NO turnover results in the activation

of the PPSB and GABA pathway, which represent two important processes linking carbon and nitrogen metabolism and maintaining cellular energy levels under stress conditions. Considering the demonstrated link between AOX and NO in this chapter, and publications describing the NO, ROS and ET connection, I hypothesized that ROS and e may have a role in tobacco's response to anoxia which was investigated in the next chapter.

In chapter 4, I evaluated the physiological role(s) of AOX in regulating the hypoxic induced nitro-oxidative stress and metabolic changes after exposing tobacco to hypoxia. The presence of AOX was crucial in avoiding hypoxia-induced superoxide and H_2O_2, probably due to greater enzyme activity of CAT and GR and reduced expression of *Rbohs* in overexpressors compared to knockdowns. Plant lines expressing AOX accumulated lower pyruvate and had increased transcript levels and activity of PDC1 and ADH1 under hypoxia. This suggests that AOX contributes to ATP production and NAD^+ regeneration by increasing the overflow of pyruvate into fermentation pathways. We found that hypoxia increased the expression of genes encoding important ET biosynthesis enzymes and ERFs, which were linked more positively with AOX and NO levels. The synergistic interplay of AOX, NO, ROS and ET is important in inducing resistance to oxygen deficit by regulating the downstream defence system.

Chapter 2

Transcriptional and metabolic changes associated with phytoglobin expression during germination of barley seeds

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2.1 Abstract

To understand how the class 1 phytoglobin (Pgb1) is involved in germination process via the modulation of the nitric oxide (NO) metabolism, we performed the analysis of physiological and molecular parameters in the embryos of transgenic barley (*Hordeum vulgare* L. cv Golden Promise) plants differing in expression levels of the *Pgb1* gene during the first 48 h of germination. Overexpression of *Pgb1* resulted in a higher rate of germination, higher protein content and higher ATP/ADP ratios. This was accompanied by a lower rate of NO emission after radicle protrusion, as compared to the wild type and downregulating line, and a lower rate of S-nitrosylation of proteins in the first hours postimbibition. The rate of fermentation estimated by the expression and activity of alcohol dehydrogenase was significantly higher in the Pgb1 downregulating line, the same tendency was observed for nitrate reductase expression. The genes encoding succinate dehydrogenase and pyruvate dehydrogenase complex subunits were more actively expressed in embryos of the seeds overexpressing Pgb1. It is concluded that *Pgb1* expression in embryo is essential for the maintenance of redox and energy balance before radicle protrusion, when seeds experience low internal oxygen concentration and exerts the effect on metabolism during the initial development of seedlings.

2.2 Introduction

Seed germination represents an initial and critical phase of the life cycle in plants. It starts with water uptake, resulting in a transition from the quiescent state of metabolism in the dry seed to the high metabolic activity upon hydration (Qiu et al., 2010), and finishes with radicle protrusion, which is an observable indicator of the completion of germination (Steinbrecher and Leubner-Metzger, 2017). After imbibition, seeds develop highly hypoxic conditions limiting mitochondrial respiration (Bykova et al., 2015), which results in the increase of the reduction level of electron transferring components, triggering the generation of reactive species of oxygen (ROS) and of nitrogen (RNS) (Bethke, 2009; Ma et al., 2016). Under low oxygen conditions, plants have a limited capacity to substitute oxygen with nitrite as the terminal electron acceptor (Igamberdiev and Hill, 2004). This nitrite can be reduced to nitric oxide (NO) by various iron-containing proteins and molybdocofactors. The anoxically induced phytoglobin (Pgb1) converts this NO to nitrate, which is metabolized back into nitrite via nitrate reductase (NR) for the continuation of NO production (Gupta et al., 2011b). The cycle of the nitrate- and nitrite-driven redox reactions is defined as the phytoglobin-nitric oxide (Pgb1-NO) cycle, playing a key role in the maintenance of the energy status of the embryo under hypoxic conditions (Stoimenova et al., 2007). Besides NO oxygenation by Pgb1, denitrosylation of S-nitrosoglutathione (GSNO) catalyzed by S-nitrosoglutathione reductase (GSNOR) is another way to metabolize NO. GSNO is the storage and transport form for NO in seeds (Sakamoto et al., 2002), which has a crucial impact on seed germination (Kwon et al., 2012).

Germination has a high demand of energy, which is fulfilled due to the functional mitochondria that remain stable and efficient through the assembly of the mitochondrial protein complexes during imbibition (Luo et al., 2019; Rodríguez et al., 2015). Once seeds are imbibed, the tricarboxylic acid (TCA) cycle supplies intermediates and energy to support seed germination and seedling growth. This cycle couples with oxidative phosphorylation to produce ATP (Fernie et al., 2004). The mobilization of starch from the endosperm would seemingly proceed through glycolysis and the TCA cycle. The TCA cycle cannot function without the acetyl-CoA input produced by pyruvate dehydrogenase complex (PDC). Succinate dehydrogenase (SDH) activity in the scutellum of germinating cereal seeds reflects the need for succinate conversion for the continuous operation of the TCA cycle, and for the utilization of succinate produced in the glyoxylate cycle (Eprintsev et al., 2016). NO inhibits mitochondrial respiration by reversible binding to cytochrome c oxidase (Millar et al., 1996), while it does not affect the alternative oxidase (AOX) (Millar et al., 1996). NO action on the mitochondrial electron transport affects oxidative phosphorylation (Clerc et al., 2007). An interplay and flexible equilibrium between ROS, NO, and mitochondrial respiration is needed for the maintenance of energy status within the seed in the course of germination. Plants possess non-energy conserving electron transport pathways in mitochondria, which couple the oxidation of NADH and NADPH with the reduction of O_2 to H_2O without generating a proton motive force (Millar et al., 2011). These pathways include the type II NAD(P)H dehydrogenases (NDs) on both sides of the inner membrane of the mitochondria and AOX. AOX and NDs are encoded by multigene families. In barley, AOX is encoded by four genes: *HvAOX1a*, *HvAOX1c*,

HvAOX1d1 and *HvAOX1d2*. All three subfamilies of plant ND genes, *NDA*, *NDB* and *NDC*, were identified in barley (Wanniarachchi et al., 2018). In this study, we analyzed *AOX1a* and *AOX1d1*, and *NDB2* and *NDB3* encoding NDB proteins oxidizing NADH and facing the outer side of the inner membrane. Non-energy conserving electron transport, which includes rotenone-insensitive NADH and NADPH dehydrogenases and AOX, represents a tool for relaxing the coupling of the respiratory carbon oxidation pathways, electron transport, and ATP turnover, thus establishing metabolic homeostasis during germination (Wanniarachchi et al., 2018; Vanlerberghe, 2013). The involvement of these pathways was studied in plants mostly in relation to photosynthesis, while their role in germinating seeds is also important (Daley et al., 2003). It may be connected with NO metabolism and operation of the Pgb1-NO cycle (Igamberdiev et al., 2010), providing the possibility of oxidation of the cytosolic NADH and the prevention of ROS and RNS formation.

The apparent imbalance between the respiration levels (measured as oxygen uptake) of cereal species and the relative abundance of mitochondrial ATP suggests that energy charge may be supplied by alternative pathways (e.g., fermentation) during seed germination (Rosental et al., 2014). This is in accordance with a drastic increase in the ATP level in the first hours of imbibition (Benamar et al., 2003). Alcoholic fermentation is induced during germination of rice seeds to provide energy when oxygen is deficient for providing normal respiration (Ismail et al., 2009). The energy obtained via fermentation facilitates seed germination and radicles protrusion to overcome anaerobic stress. However, there is an alternative to the classic fermentation pathways (Igamberdiev and

Hill, 2004), associated with the turnover of NO in the Pgb1-NO cycle (Igamberdiev et al., 2005). This cycle can operate at the concentrations of oxygen two orders of magnitude lower than required to support the oxygenic respiration and oxidize NADH and produce ATP at the intensities comparable to or exceeding the glycolytic levels (Stoimenova et al., 2007).

While the role of NO in seed biology has been studied extensively, the information about the function of phytoglobin in NO interactions with other molecules involved in fermentation, TCA, and electron transport, including the alternative pathway, remains scarce. This work clarifies how the changes in the endogenous level of phytoglobin affect the underlying molecular features to control the onset of germination and manage the energy crisis, which dictate the tolerance to anoxic step of germination, and finally support the growth and development of embryonic axis.

2.3 Materials and Methods

2.3.1 Plant Growth

The transgenic lines of barley (*Hordeum vulgare* L. var. Golden Promise) seeds with overexpression and knockdown of the *Pgb1* (Pgb+, Pgb-) were obtained from Aarhus University, Denmark, where they were constructed as described earlier (Hebelstrup et al., 2010; Hebelstrup et al., 2014). Single independent transformants and the wild type plants were used for all experiments. Seeds were soaked with sterile deionized water on filter paper in Petri dishes in darkness at 25 °C. To gain insight into the biochemical and molecular changes during germination, an extensive time course was examined, from dry

seeds to radicle protrusion (at 15–20 h post imbibition) and up to 48 h. Embryos (usually 100 mg) were isolated and ground in liquid nitrogen using a mortar and pestle for studying gene expression and assaying several metabolic parameters.

2.3.2 ATP/ADP Ratio

Fresh biomass (100 mg) was homogenized with the addition of 1 mL of 2.4 M perchloric acid. The homogenate was then neutralized using 5 M KOH and centrifuged at 16,000 xg for 10 min at 4 °C (Dordas et al., 2003). The ADP/ATP ratio was measured by a luciferase-based assay kit (Enzylight TM ADP/ATP ratio assay kit; Bioassay Systems, Hayward, CA, USA), following the manual instructions.

2.3.3 NO Emission

NO emission was measured using a chemiluminescent detector (CLD 88 p; Eco-Physics, Dürnten, Switzerland), as described earlier (Cochrane et al., 2017; Planchet et al., 2005), and averaging total NO accumulation every 30 min. The measuring gas was kept NO free using a NO scrubber supplied by Eco Physics Ltd., Switzerland. Gas flow was regulated by flow controllers (Thermo Fisher Scientific,Waltham, MA, USA).

2.3.4 Measurement of protein concentration

The total concentration of proteins was measured using Bradford reagent (Sigma–Aldrich, St. Louis, MO, USA) and bovine serum albumin as a standard (Bradford, 1976).

2.3.5 Total Protein *S*-Nitrosylation

The measurement of the protein *S*-nitrosylation was performed following Ma et al. (2016). The method is based on the reduction of R-SNO to R-SH in the presence of ascorbate and detecting free thiol groups by 5,50-dithiol-bis (2-nitrobenzoic acid) (DTNB). Extraction was achieved using 50 mM HEPES (pH 8.0) containing 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℃, and the supernatant was incubated overnight in ice-cold acetone (-20℃). Then, protein precipitate was separated by centrifuging, the subsequent pellet was washed several times with chilled 70% acetone, before being resuspended in the same volume of the extraction buffer. Protein solution was separated into two 0.9 mL samples, adding 50 µL of 100 mM ascorbate to the experimental tube and the same volume of water to the control. After incubating for 1 h at room temperature, 50 µL of 10 mM DTNB in 75 mM potassium phosphate buffer (pH 7.0) was added, and the optical density of both samples was measured at 412 nm. The mixture of ascorbate and DTNB in the extraction buffer and DTNB in the same buffer were set up as a blank for the treatment and control groups, respectively. The difference of R-SH quantity between sample and control groups was taken for calculation of R-SNO level. The quantity of R-SH generated by ascorbate treatment corresponded to that of R-SNO in proteins. The evaluation of free SH-groups in proteins was performed without ascorbate treatment.

2.3.6 Enzymatic Activity Assays

Alcohol dehydrogenase (ADH; EC 1.1.1.1) activity was assayed by measuring the reduction of NAD^+ in the course of oxidation of ethanol at 340 nm, as previously described

(Molina et al., 1987). S-Nitrosoglutathione reductase (GSNOR or ADH3; EC 1.2.1.46) activity was assayed spectrophotometrically at 25℃ by monitoring the oxidation of NADH in the presence of S-nitrosoglutathione (GSNO) at 340 nm (Sakamoto et al., 2002).

2.3.7 Gene Expression

The primers of target genes for the quantitative real-time polymerase chain reaction (qRT PCR) were designed using the NCBI/Primer-BLAST, according to known cDNA sequences of the *Pgb1, NR*, *NiR*, *ADH1*, *ADH3, SDH-A*, *SDH-B*, *PDC-E1*, *PDC-E2*, *AOX1a*, *AOX1d1*, *NDB2* and *NDB3* genes. They encode correspondingly the class 1 phytoglobin, nitrate reductase (EC 1.7.99.4), nitrite reductase (EC 1.7.2.1), alcohol dehydrogenase (EC 1.1.1.1), GSNOR (or ADH3; EC 1.2.1.46), flavoprotein subunit A and iron–sulfur protein subunit B of succinate dehydrogenase (EC 1.3.5.1), pyruvate dehydrogenase (EC 1.2.4.1) and dihydrolipoyl transacetylase (EC 2.3.1.12) subunits of the pyruvate dehydrogenase complex, two forms of alternative oxidase (EC 1.10.3.11) and two forms of the externally facing rotenone-insensitive NADH dehydrogenase. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) was set up as a reference gene. Specific primers of the target genes are listed in **Table 2.1**.

Table 2.1 Primers used for qRT-PCR assays of germinating seeds of barley

Primer	Forward sequence	Reverse sequence	Accession number
Pgb 1	5'-ACCAACCCCAAGCTCAAGAC-3'	5'-CTGCCACGCCGTATTTCAAG-3'	U94968.1
NR	5'-CAACACCAACTCCGTCAT-3'	5'-CTGAGTATGCGTATCCCTTG-3'	X57844.1
NiR	5'-GACATCGGCTTCATGGGCT-3'	5'-GCACGGCCTTCTTGTACACC-3'	S78730.1
ADH1	5'-GATCTGCTCAGGATCAACAC-3'	5'-GTGGAAGTCCCTACGAAATG-3'	AF253472.1
ADH3	5'-GTCTCTCAACTGGACTTGGTG-3'	5'-TAGCTTGTTCGTATTTTGCAGG-3'	X12734.1
SDH-A	5'-CAGTGAAGGTGAGCGTTTCA-3'	5'-CACCAGCAAAAATAGCAGCA-3'	AK376855.1
SDH-B	5'-TGTACGAGTGCATCCTCTGC-3'	5'-TCGTCGTTGATGGAGTCAAG-3'	AK372209.1
PDC-E1	5'-ATTGAATTCCGCCCTTGACG-3'	5'-GCCAGTAAAACCAGCCTCTG-3'	AK353615.1
PDC-E2	5'-TGCAGGGATGGAAAGAGCTT-3'	5'-GGTTGGAGCTGCTTCATACG-3'	AK362954.1
AOX1a	5'-CGTCAACCACTTCGCATCGG-3'	5'-GCCCTCATTTCCTCGGAAGC-3'	AK363239.1
AOX1d1	5'-CACTACGCATCCGACATCCA-3'	5'-CAACAATCCATCCAAATTAACG-3'	AK365405.1
NDB2	5'-CGTCCACTGTCGCTCTGC-3'	5'-GGCATCCTCCACTTCCTTCAG-3'	AK367948.1
NDB3	5'-GCAAAATCCAGCTACTGGCG-3'	5'-TTCACGCACCCTTAGCCATT-3'	AK354220.1
GAPDH	5'-GCTCAAGGGTATCATGGGTTACG-3'	5'GCAATTCCACCCTTAGCATCAAAG-3'	AB120301.1

Extraction of total RNA was performed using the FastRNA® Pro Green Kit (MP Biomedicals, Irvine, CA, USA), according to the standard protocol of the manufacturer. Reverse transcription of RNA was conducted according to the protocol for the SuperScript. II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The single strand cDNA was used as a template in the following PCR. PCR was performed on an Applied Biosystems (Foster City, CA, USA) StepOnePlus Real-Time PCR System. The procedure followed the manufacturer's protocol for the SYBR Green qPCR Master Mixes (Thermo Fisher Scientific,Waltham, MA, USA): 0.4 µL 10 µM forward primer, 0.4 µL 10 µM reverse primer, 1 µL cDNA and 5 µL SYBR Green qPCR Master Mixes were mixed and adjusted to 3.2 µL using nuclease-free water. Biological replicates corresponded to independent RNA extracts, and three technical replications were run for each biological replicate. The program for the RT PCR reactions was set up as the initial activation at 95℃ for 30 s, followed by 40 cycles of 95℃ for 10 s and 61℃ for 45 s; followed by melting curve analysis obtained by heating to 95℃ for 15 s, cooling to 55℃ and incrementally heating to 95℃ at

the rate of 0.05 ℃ s^{-1}. Standard curves of target genes were made based on a 5-fold dilution series for the genomic barley cDNA (E = 1.3–1.8, R2 = 0.93–0.99). The amount of target genes in unknown samples was calculated from the cycle threshold (Ct) using the standard curve.

2.3.8 Statistical Analysis

All the experiments were repeated at least three times. The statistical analyses were performed using SPSS software (Statistical Package for Social Science; version 21, Chicago, IL, USA). A one-way ANOVA was performed to identify significant differences among different lines of barley ($p \leq 0.05$) and measured by using Duncan's multiple range. The data in the text, table and figures are expressed as means ± standard deviations of three replicates. The differences with $p \leq 0.05$ were considered as statistically significant.

2.4 Results

In this paper, we report the results of a wide-range study of the development of barley embryos differing in expression levels of the *Pgb1* gene (Pgb+ and Pgb–) during the first 2 days of germination. **Figure 2.1A** presents the images of germination of transgenic barley seeds, as observed at 24 h after imbibition (after radicle protrusion) and at 48 h. Radicle protrusion occurs mostly between 15 and 20 h postimbibition in all lines but the Pgb+ seeds develop a longer radicle by 24 h, as compared to WT and Pgb-.

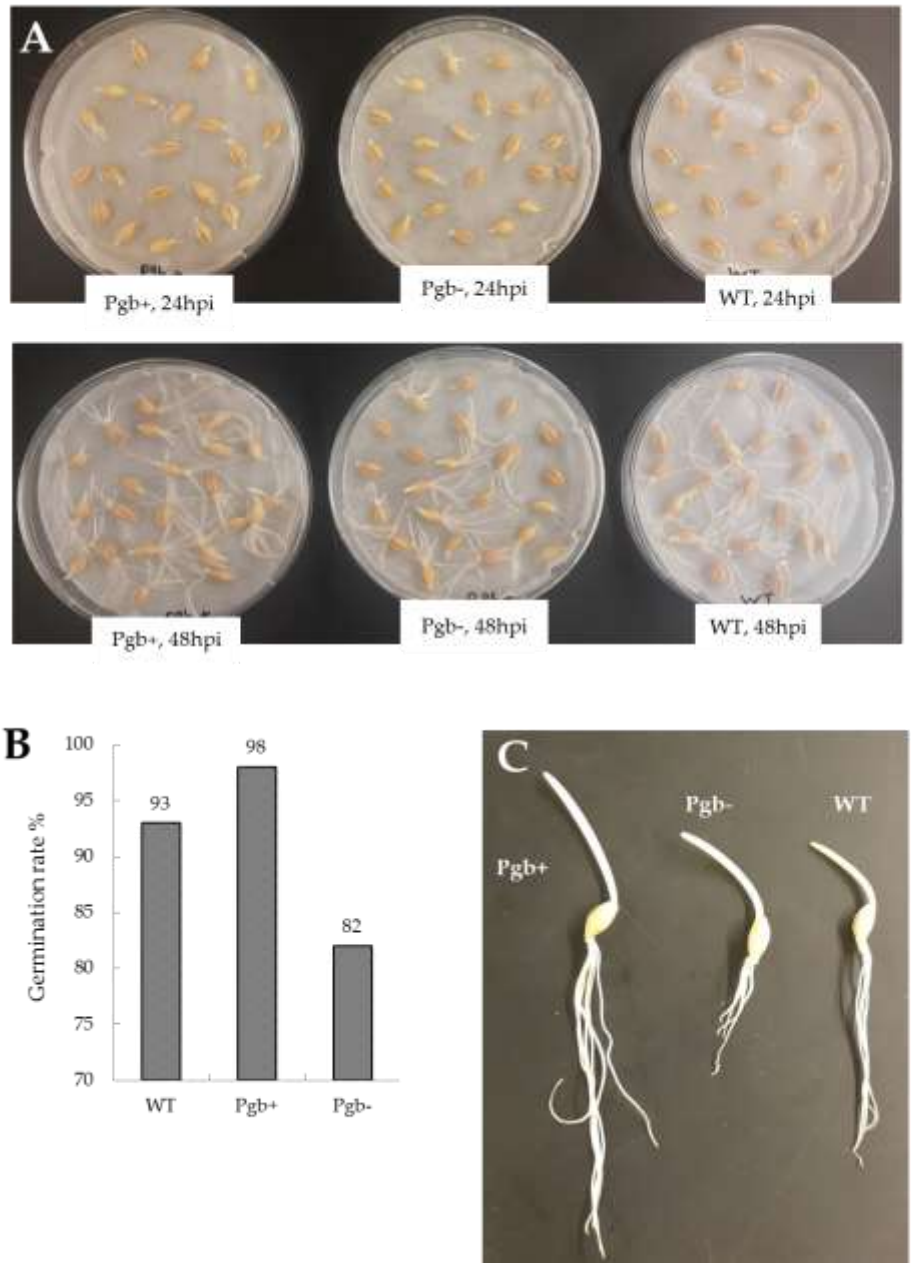

Figure 2.1 Germination of barley seeds differentially expressing class 1 phytoglobin (Pgb1). (**A**) Image of germinating seeds of Golden Promise cultivar of with overexpressed (Pgb+) and knockdown (Pgb-) phytoglobin compared to wild type (WT) at 24 and 48 h post imbibition. (**B**) Germination rate of barley seeds differentially expressing Pgb1. The typical result of the experiment with 250 seeds. (**C**) Image of 3-day-old barley seedlings with overexpressed (Pgb+) and knockdown (Pgb-) phytoglobin compared to wild type (WT).

By 48 h, the root system of the Pgb+ becomes more developed than that of WT and Pgb-. Germination rate of Pgb+ seeds (98%) was significantly higher than of WT seeds (93%) and then Pgb- (82%) (**Figure 2.1B**). After 3 days of germination, the Pgb+ seedling exhibited twice as long roots and 1.5 times longer shoots compared to Pgb-, while the WT seedling exerted the intermediate length of roots and shoots (**Figure 2.1C**).

The total protein content in the embryo started to decrease immediately after imbibition during the first 3 h (**Figure 2.2A**). The decrease was strong (by ~30%) in the WT and Pgb- seeds, while in Pgb+ seeds it was about 10% in the first three hours, and then showed the tendency to increase to the initial level at 48 h. In Pgb- seeds, the total protein remained at lower level, while in the WT it started to increase after 24 h, and reached almost the level as in dry seeds at 48 h. The expression of the *Pgb1* gene sharply increased during 10 h postimbibition (**Figure 2.2B**) from zero levels in the dry seeds of WT and Pgb-, and from well detectable levels in Pgb+. At 10 h, the level of expression of *Pgb1* was half in Pgb-, as compared to the WT, and about 20 times higher in Pgb+ seeds. At 48 h postimbibition, the level of Pgb1 mRNA was quite low in the Pgb- and WT lines but remained high in Pgb+.

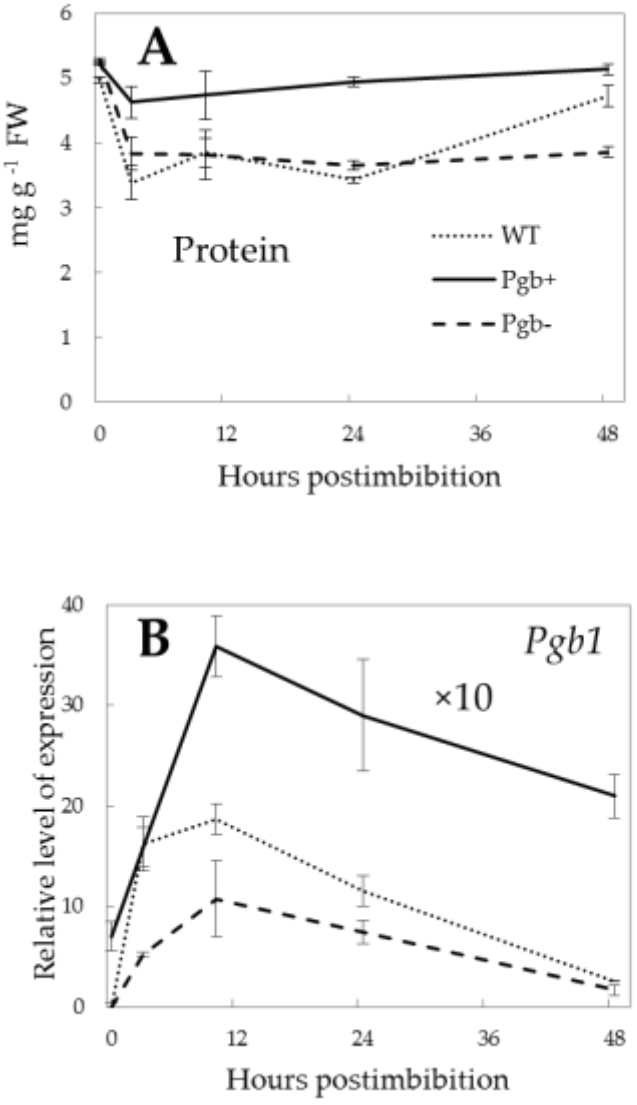

Figure 2.2 Changes in total protein content (**A**) and *Pgb1* expression (**B**) in the embryo of barley during germination. Solid line: Pgb+, overexpressed phytoglobin; dashed line: Pgb-, knockdown phytoglobin; dotted line: WT, wild type. The values for relative level of expression of Pgb+ should be multiplied by 10 times. The vertical bars represent the values of standard deviations.

The ATP/ADP ratio in the embryo strongly increased in the first 3 h after imbibition, then

maintained unchanged in Pgb- embryos and gradually but slightly increased in the WT (**Figure 2.3**). In Pgb+ embryos, the ATP/ADP ratio increased more significantly and remained ~1.5 times higher than in Pgb- and WT embryos at 24–48 h.

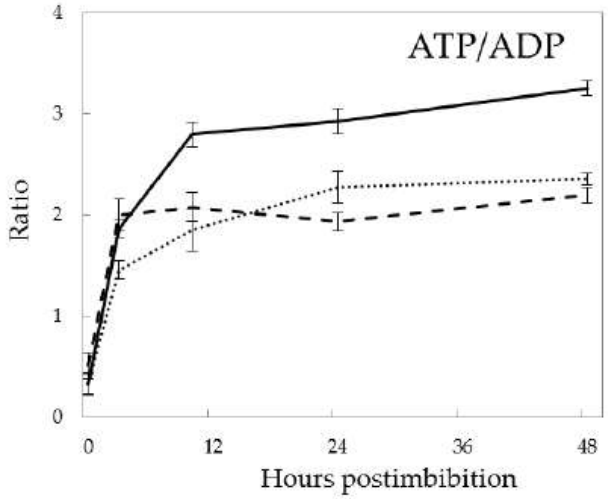

Figure 2.3 ATP/ADP ratio in barley embryo during germination depending on expression of *Pgb1*. The symbols are the same as in Figure 2.4.2.

It was possible to detect NO by the chemiluminescent method only after radicle protrusion (**Figure 2.4A**). The rate of NO emission was more than twice as high in Pgb- than in Pgb+ and the WT at 24 h, and remained almost at the same rate at 48 h. It was possible to detect a statistically significant difference between the WT and Pgb+, in which NO production was the lowest (**Figure 2.4A**). Although we could not detect NO emissions before radicle protrusion by the applied method, the rate of S-nitrosylation of proteins (**Figure 2.4B**) was less than half in Pgb+ than in the Pgb- and WT in dry seeds at 3 h of germination. At 10 h, the levels were not quite different between the lines, except a slightly higher level in the WT, while later, at 24 and 48 h, Pgb- exhibited the same level of nitrosothiols as at 10 h,

and the nitrosothiol level in the Pgb+ and WT gradually decreased to very low values at 48 h. While the level of nitrosothiols essentially differed depending on *Pgb1* expression, the concentration of SH-groups showed essential but smaller differences between the lines in the first hours and after two days postimbibition (**Figure 2.4C**). The reduced concentration of nitrosylated (-SNO) groups in the proteins of dry seeds corresponding to the overexpressing line can be related to the capacity of scavenging NO even in dry seeds, when Pgb1 exhibited a certain level of expression in the overexpressing line and was practically absent in the wild type and downregulating line (**Figure 2.2**). Before the decreasing trend, the increase of nitrosylation in the WT embryos was accompanied by the opposite trend of free SH-groups in proteins. In the Pgb+ embryos, the level of R-SNO, being markedly reduced after 10 h from imbibition, did not show a correlation with the level of RSH, which exhibited no significant changes.

The rate of fermentation estimated by the expression and activity of alcohol dehydrogenase (ADH) was strongly dependent on the Pgb1 expression. The expression of *ADH1* was lower almost by 1.3 times in Pgb+ seeds than in the Pgb- and WT seeds in the first 3 h after imbibition. It was almost the same in all lines at 24 h but became lower again in Pgb+ after 48 h (**Figure 2.5A**). The activity of ADH was higher in Pgb- embryo by more than seven times than in the Pgb+, and by five times than in the WT before radicle protrusion, in the first 10 h after imbibition (**Figure 2.5B**).

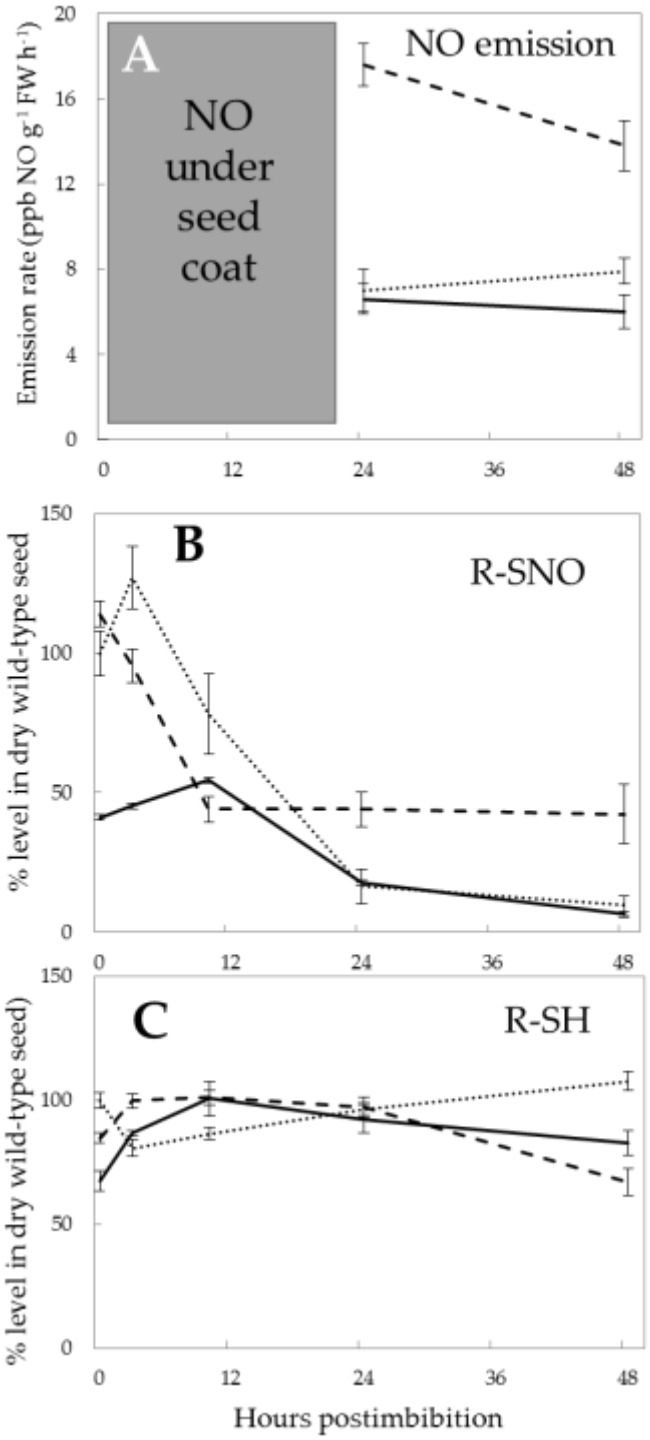

Figure 2.4 Nitric oxide (NO) emissions (**A**), changes in the quantity of nitrosylated (-SNO) groups in proteins (**B**), and the quantity of sulfhydryl groups in proteins (**C**) in the embryos of barley seeds differentially

expressing Pgb1 during germination. NO emission was recorded by chemiluminescent method as described in Methods; detection was possible after breakage of the seed coat (radicle protrusion). The symbols are the same as in Figure 2.2.

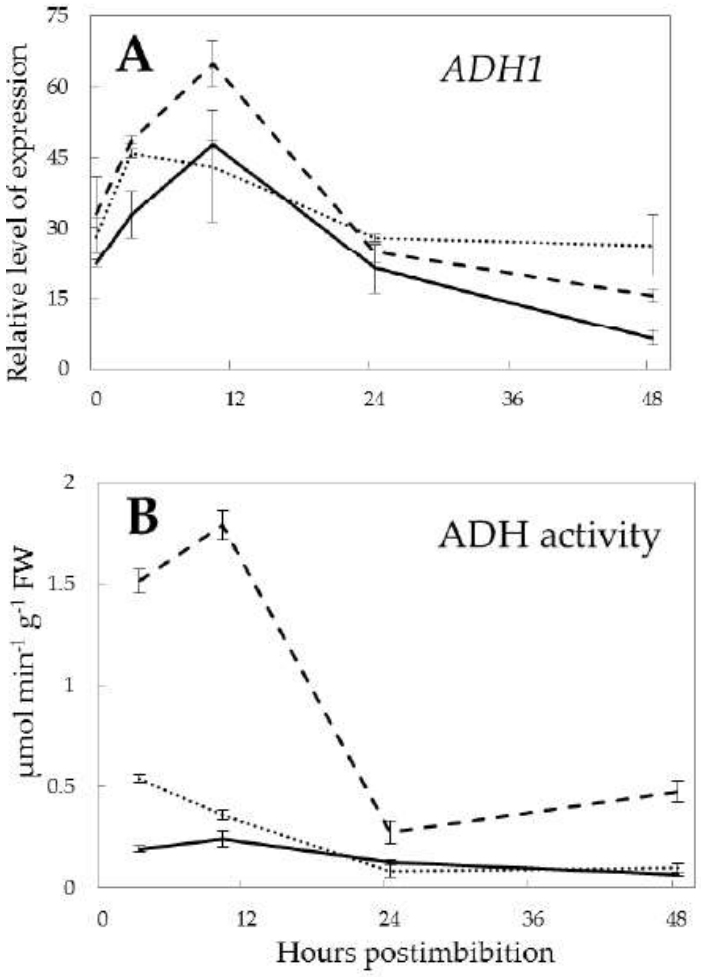

Figure 2.5 Expression of alcohol dehydrogenase (*ADH1*) (**A**) and its activity (**B**) in the embryos of barley seeds differentially expressing Pgb1 during germination. The symbols are the same as in Figure 2.2.

The expression profiles of nitrate reductase (*NR*) and nitrite reductase (*NiR*) genes revealed a higher expression of *NR* in the Pgb- line before radicle protrusion, with the highest

difference measured at 10 h postimbibition (**Figure 2.6A**). The profile of *NiR* expression was almost flat in Pgb+, while in the Pgb- and WT, the expression increased by 24 h being higher at that point than in the Pgb+ and then decreased (**Figure 2.6B**).

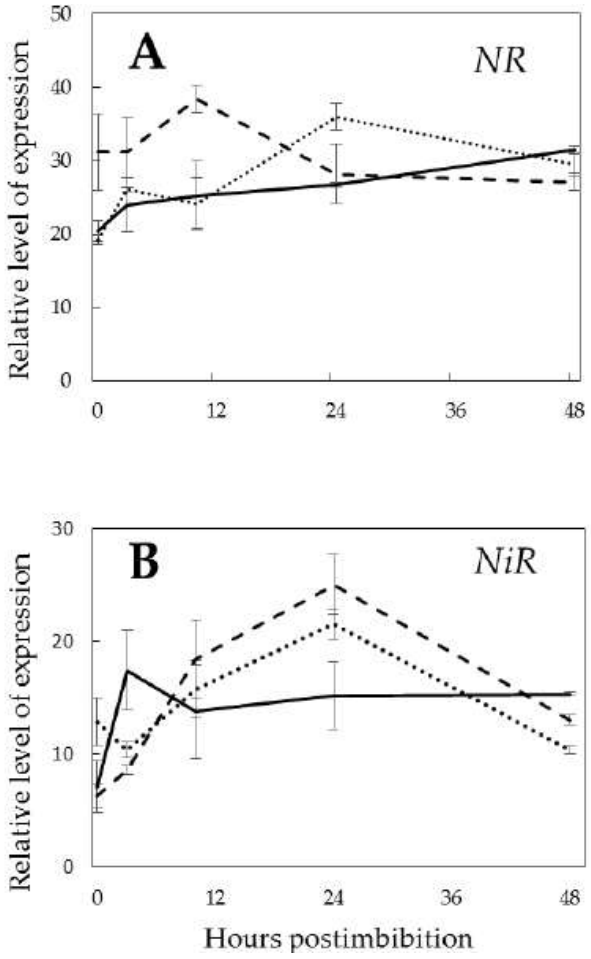

Figure 2.6 Expression of nitrate reductase (*NR*) (A) and nitrite reductase (*NiR*) (B) in the embryos of barley seeds differentially expressing Pgb1 during germination. The symbols are the same as in Figure 2.2.

The expression and activity of GSNOR followed a similar trend in all three types of the barley embryos (**Figure 2.7**). However, the expression of *GSNOR* in Pgb+ embryos was

slightly higher than that of the Pgb- embryos at 10 h from imbibition (**Figure 2.7A**), while the highest activity was observed on the Pgb- embryos (**Figure 2.7B**). Generally, the RNS-scavenging activity of GSNOR increased on the first day of seed germination, in concert with the decreasing trend of protein S-nitrosylation (**Figure 2.4B**).

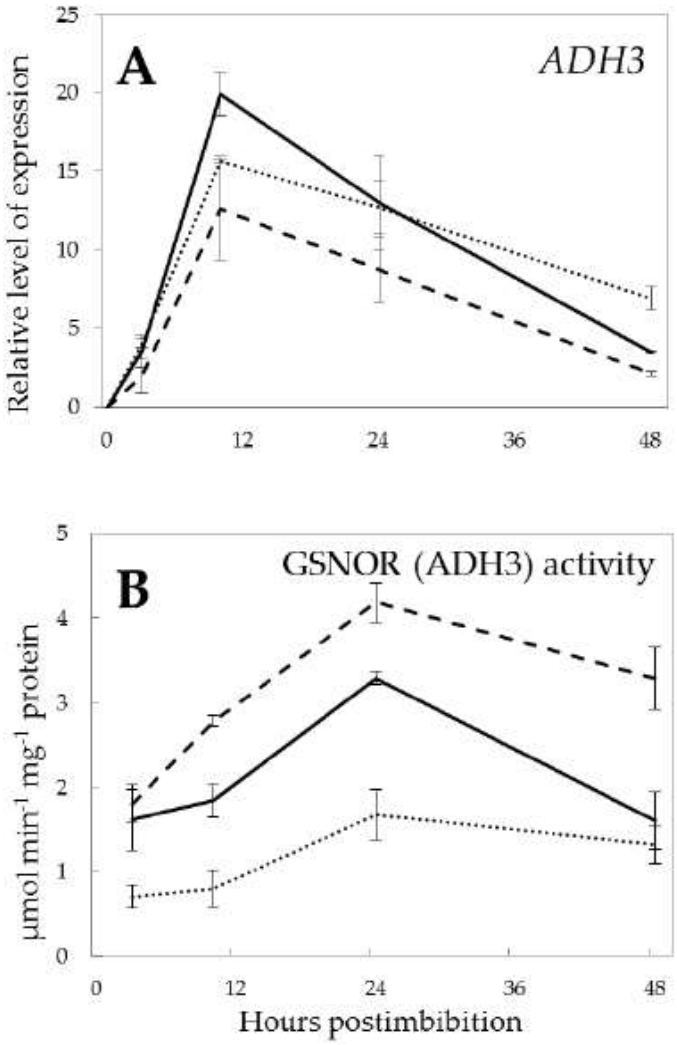

Figure 2.7 Expression of *ADH3* (**A**) and GSNO reductase activity (**B**) in the embryos of barley seeds differentially expressing Pgb1 during germination. The symbols are the same as in Figure 2.2.

We have studied the expression of genes encoding SDH subunits A (flavoprotein subunit) and B (iron–sulfur protein subunit) (**Figure 2.8A,B**) and PDC subunits E1 (pyruvate dehydrogenase) and E2 (dihydrolipoyl acetyl-transferase) (**Figure 2.8C,D**). Generally, all these genes were upregulated in all types of embryos after radicle protrusion when the aerobic metabolism becomes more intensive, and reached higher values in the Pgb+ embryos, although the differences in *SDH-B* and *PDC-E2* were less pronounced.

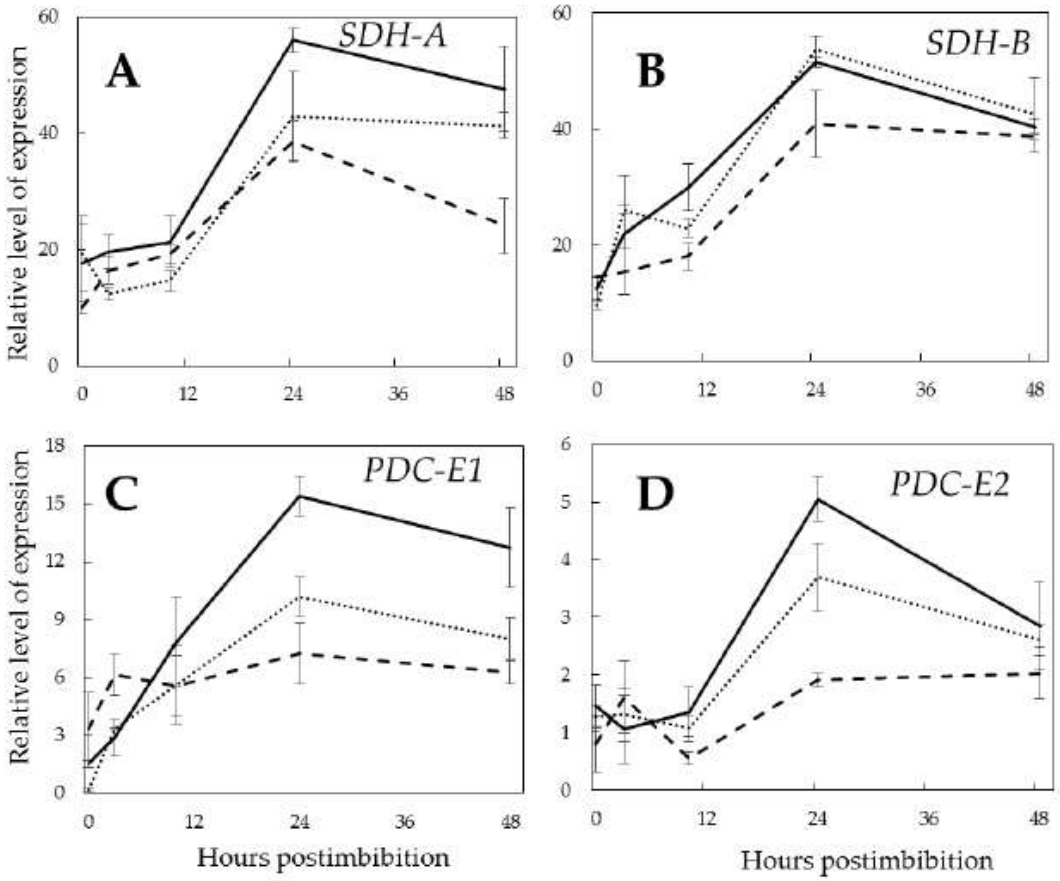

Figure 2.8 Expression of the genes encoding succinate dehydrogenase subunit A (*SDH-A*) (**A**) and subunit B (*SDH-B*) (**B**), and pyruvate dehydrogenase complex components E1 (*PDC-E1*) (**C**), and E2 (*PDC-E2*) (**D**) in the embryos of barley.

Expression of the genes encoding the enzymatic members of the non-coupled respiratory pathways, alternative oxidase (*AOX1a* and *AOX1d1*) and external NADH dehydrogenases (*NDB2* and *NDB3*) revealed a specific pattern in the course of germination (**Figure 2.9A–D**). The transcripts of *AOX1a* increased in the first 3 h of germination, with the slower increasing trend in Pgb- embryos. Expression of the gene encoding AOX1d1 was lower and did not show correlation with *Pgb1* expression. The expression of *NDB2* gradually increased during germination with no correlation with Pgb1, while the expression of *NDB3* in the dry and 3 h imbibed Pgb+ seeds was higher, then started to decrease during germination, and by 48 h reached s much lower level than in the WT and Pgb- lines.

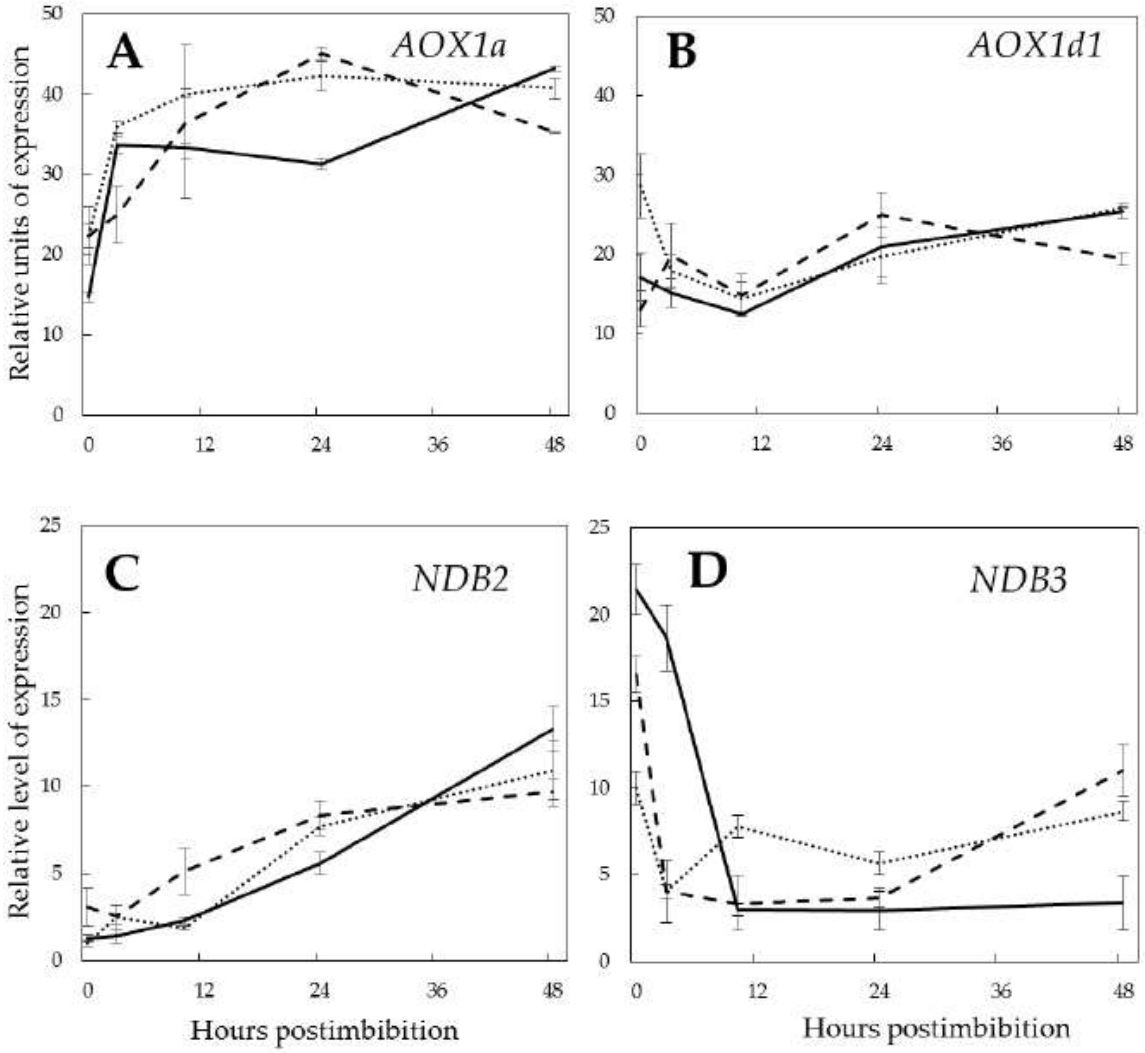

Figure 2.9 Expression of alternative oxidase genes *AOX1a* (**A**) and *AOX1d1* (**B**) and of external NADH dehydrogenase genes *NDB2* (**C**) and *NDB3* (**D**) in the embryos of barley seeds differentially expressing Pgb1 during germination. The symbols are the same as in Figure 2.2

2.5 Discussion

2.5.1. Anaerobic conditions in germinating seeds and expression of class 1 phytoglobin

Seed germination is a complex process that begins by imbibition and leads to anatomical structure protrusion initiating seedling development. During germination, rapid oxygen depletion makes the environment inside the seed close to anaerobic (Bewley and Black, 1994). After radicle protrusion, oxygen concentrations return gradually to aerobic, resulting in the active mobilization of storage reserves, followed by seedling development. Based on the previous estimations (Bewley and Black, 1994), we assume that by 3–5 h from imbibition, most of oxygen is depleted, leading to mostly anaerobic conditions until the protrusion of a radicle. In our study, the first radicles appeared between 15 and 20 h, and by 24 h, all the seeds developed radicles (**Figure 2.1A**)

Anaerobic conditions are characterized by the development of fermentation (Kennedy et al., 1992), and by the conversion of nitrite to NO (Igamberdiev et al., 2010). NO is metabolized by the induced Pgb1 to nitrate, the latter is reduced to nitrite by NR (Igamberdiev et al., 2010). The sequence of reactions called the phytoglobin-nitric oxide (Pgb-NO) cycle (Igamberdiev and Hill, 2004) operates as a substitute to classic fermentation pathways at low oxygen, and is efficient in keeping the redox level under control, as well as in generation of limited quantities of ATP (Stoimenova et al., 2007). Expression of *Pgb1* under the hypoxic conditions developed in seeds is an important prerequisite of their successful development. The germination rate of the seeds with *Pgb1* knockdown is decreased as compared to the wild type, and especially to the seeds

overexpressing Pgb1 (**Figure 2.1B**). The seedlings from Pgb+ seeds are characterized by a better growth with longer roots and shoots (**Figure 2.1C**). This means that the expression of *Pgb1* and efficient NO turnover are important for metabolism of seeds during germination, which is confirmed by the recently published metabolomics and proteomic data obtained on 8-day old seedlings of barley (Andrzejczak et al., 2020). It was suggested that NO scavenging by Pgb activates transcription factors that are regulated by levels of O_2 and NO in the N-end rule pathway (Andrzejczak et al., 2020), which is an evolutionarily conserved pathway for protein degradation (Holman et al., 2009). It relates the regulation of the in vivo half-life of a protein to the composition of its N-terminal residue (Holman et al., 2009). Germination is characterized by the continuing utilization of stored proteins that are used as an important source of amino acids, and for energy production (Angelovici et al., 2011). The protein level decreased in the first three hours postimbibition (**Figure 2.2A**), and the drop was minimal in the Pgb+ seeds, indicating that they have an energy source independent on protein utilization. The level of expression of *Pgb1* increases sharply upon imbibition (**Figure 2.2B**), reaching the maximum close to 10 h, exhibiting the differences depending on *Pgb1* expression in the lines.

2.5.2. Energy Production During Seed Germination and the Role of NO

A sharp increase in the ATP/ADP ratio was detected at 3 h post imbibition (**Figure 2.3**), despite of the depletion of oxygen during this period. The overexpression of *Pgb1* resulted in further increase of ATP/ADP ratio which was stabilized in the Pgb- embryos at a lower level, similarly to the wild type. This indicates that, even under highly anoxic conditions,

there exist the pathways promoting ATP synthesis, and *Pgb1* expression is important for its buildup and maintenance.

The main role of Pgb1 is determined to be NO scavenging (Igamberdiev and Hill, 2004; Hebelstrup et al., 2007) which is confirmed in this study by measuring NO emissions from germinating seeds after radicle protrusion (**Figure 2.4A**). Although this method does not make it possible to measure NO before radicle protrusion, as it does not leave seeds protected by seed coat, and the hemoglobin method applied in the previous study (Ma et al., 2016) is not sufficiently precise, so we can assume high NO concentrations in the first hours postimbibition from S-nitrosylation profiles (**Figure 2.4B**). From the graph, it is evident that Pgb1 protects from nitrosylation already in the first hours of imbibition. At 10 h, the difference in the Pgb+ line disappears or becomes less pronounced, which means that other mechanisms can be important, including GSNO reductase and other scavenging pathways.

The contribution of Pgb1 to a buildup of ATP (Gupta et al., 2011b; Stoimenova et al., 2007) is apparent from the data on ATP/ADP ratio (**Figure 2.3**), indicating that Pgb1 operation considerably supports the physiological performance of the germinating seed. Previous studies showed that *Pgb1* gene expression during hypoxia has proven important for improving energy status in maize cell culture and alfalfa roots (Sowa et al., 1998; Igamberdiev et al., 2004). The production of NO at the initial stages of germination, when the seeds develop anoxic conditions, was reported earlier (Ma et al., 2016). It results in the nitrosylation of SH-groups in peptides such as glutathione, many proteins, free cysteine

and its derivatives, the processes controlled by the fine balance of NO manufacturing and scavenging mechanisms (Bykova et al., 2015).

2.5.3 Fermentation and the Pgb-NO Cycle

The development of anaerobic conditions in seeds leads to the induction of fermentation within the first hours post imbibition. Our previous work showed that the higher ATP/ADP ratio is characterized by higher activities of fermentation enzymes (ADH and LDH) in the first hours of the germination of barley seeds (Ma et al., 2016). It was also established that the highest activities of fermentation enzymes are observed in the plants downregulating Pgb1 (Cochrane et al., 2017). We observed the anoxia-triggered increase of *ADH1* expression and activity in barley embryos (**Figure 2.5**), confirming that fermentation does play a significant role at the stage when plants rely on the seed's energy stores. In accordance with our results, the upregulation of fermentation-related genes in barley and Arabidopsis seeds was reported during early imbibition (Sreenivasulu et al., 2008; Weitbrecht et al., 2011).

A higher expression of *ADH1* and a several-fold higher ADH activity in the *Pgb1* knockdown line points on the higher use of fermentation for producing energy in the absence of Pgb1. In this case, Pgb- embryos attempt to use the fermentation pathway to a higher extent than the Pgb+ and wild type embryos. This supports the statement that NO turnover in the Pgb-NO cycle represents an alternative to fermentation pathways (Igamberdiev and Hill, 2004; Igamberdiev et al., 2005; Gupta and Igamberdiev, 2011). Both metabolic processes operate during seed germination, and the reported dependence

of *Pgb1* expression on the hypoxic conditions indicates the deficiency of oxygen in germinating seed before radicle protrusion (Igamberdiev and Hill, 2004).

The Pgb1-NO cycle, which is based on the turnover of NO, nitrate and nitrite, is essential for controlling O_2 homeostasis and for supporting low redox and a high energy level. The Pgb1-NO cycle relies on the participation of electron transport complexes using nitrite, Pgb1, and NR. *NR* expression before radicle protrusion was higher in the *Pgb1* knockdown line, which may be considered as a compensation for lower nitrate production in these seeds from the reaction of NO with Pgb1 (**Figure 2.6A**). Contrarily, expression of *NiR* in the first hours postimbibition was higher in the Pgb+ embryos (**Figure 2.6B**). This may indicate that nitrite is utilized not only for NO production, but also for the buildup of ammonia for the synthesis of amino acids, which remains high in anoxia and increases upon NO production (Gupta et al., 2012).

2.5.4 S-Nitrosoglutathione Reductase

While the Pgb1-dependent NO scavenging can efficiently suppress the rate of nitrosylation, GSNO, which represents the major pool of nitrosylated compounds (Corpas et al., 2013), is metabolized via GSNOR. Our data demonstrate that GSNOR exhibits the dependence on *Pgb1* expression (**Figure 2.7A**). The induction of GSNOR activity in Pgb- seeds may be linked to the necessity of controlling GSNO levels in this line. The more pronounced increase of GSNOR activity in early hours of germination in Pgb- embryos could be attributed to a compensational strategy for the insufficient presence of Pgb to modulate NO.

Its higher activity, despite lower expression in the Pgb- plants, may be due to the post-translational mechanisms of activation, or higher level of translation of existing mRNAs, although our data do not provide evidence for concrete mechanisms. GSNOR controls the intracellular levels of S-nitrosylated proteins and, in turn, it is post-translationally regulated by S-nitrosylation, and this modification has been suggested to regulate allosterically the enzyme activity (Frungillo et al., 2014; Bignon et al.,2018). Thus, NO concentration, depending on the balance between pgb1 level, GSNOR and NR activity, determines the extent of S-nitrosylation inside the cell. A low level of nitrosylated proteins on the second day of germination (**Figure 2.4B**) can be in accordance with a higher activity of GSNOR and still high expression of *Pgb1*.

2.5.5 Pgb1 and Operation of the TCA Cycle

The TCA cycle is a vital metabolic pathway supplying intermediates and energy for the maintenance of seed germination and seedling growth (Botha et al., 1992). SDH catalyzing the conversion of succinate to fumarate is the only enzyme joining both the TCA cycle and ETC (Oyedotun and Lemire, 2004). It also utilizes succinate formed in the glyoxylate cycle (Eastmond and Graham, 2001). The mitochondrial PDC, representing an assembly of three respiratory enzymes, links glycolysis and the TCA cycle. Besides being the entry to TCA cycle, PDC supplies the C intermediate (acetyl-CoA) and NADH for the anabolic processes during seedling development (Weraduwage et al., 2016).

The high expression of *SDH* and *PDC* in embryos, where Pgb1 is upregulated, on the second day of germination is in accordance with their enhanced mitochondrial respiratory

activity (**Figure 2.8A–D**). The studies showed that NO is involved in the inhibition of SDH (Simonin and Galina, 2013), possibly via nitrosylation of protein thiols and removing iron from the iron-sulfur centers (Brown, 1999). NO inhibits the upregulation of *SDH* and *PDC* in Pgb- embryos on the second day of germination, when the high metabolic demands are met by aerobic respiration, while Pgb1, by scavenging NO, protects SDH from inactivation. Thus, the importance of the Pgb1-NO cycle is evident not only at the anaerobic stage, but also after radicle protrusion, when the TCA cycle operation becomes more active. It may prevent the suppression of cytochrome oxidase by NO, which is considered an important mechanism of the regulation of respiratory metabolism (Gupta and Igamberdiev, 2011).

2.5.6 Pgb1 and the Non-Coupled Respiration

The capacity of seeds to germinate might be related to the regulation of ROS levels generated in the mitochondrial consumption of oxygen, and to the detoxification of the products of fermentation (Ismail et al., 2009; Pergo and Ishii-Iwamoto, 2011). The non-coupled pathways of the mitochondrial electron transport that include AOX and rotenone-insensitive NAD(P)H dehydrogenases, decrease the reduction level of NAD(P)H and ubiquinone, and prevent excessive ROS and RNS formation (Møller, 2001). Among the NAD(P)H dehydrogenases, the NDB-type enzymes oxidize NADPH (NDB1) and NADH (NDB2 and NDB3) from the outer side of the inner mitochondrial membrane, and can be involved in the oxidation of glycolytic NADH and redox equivalents formed in other cytoplasmic processes. They can participate in seed germination and seedling development

by controlling ROS homeostasis during the germination process, and by promoting the cellular redox balance during post-germination development (Wanniarachchi et al., 2018; Vanlerberghe, 2013; Velada et al., 2016). Participation of the NDB-type dehydrogenases in NAD(P)H-dependent scavenging of NO via its reaction with superoxide anion shows their role in NO homeostasis (De Oliveira et al., 2008).

The expression profiles of *AOX1a* and *AOX1d1*, which differ in the regulation by oxo-acids (Selinski et al., 2018), show differences upon germination, indicating that *AOX1a* is inducible upon imbibition, while *AOX1d1* is not. However, both forms, as well as *NDB2*, do not show any significant dependence on expression of *Pgb1*. However, *NDB3* is highly upregulated in Pgb+ embryos in the first hours after imbibition, and downregulated at 48 h. This may indicate its particular role in supplying the cytosolic NADH to the mitochondrial ETC upon the depletion of oxygen, and its lower contribution during the aerobic phase.

2.6 Conclusions

Pgb1 expression in embryo is essential for the maintenance of redox and energy balance before radicle protrusion, when seeds experience low internal oxygen concentration. It also exerts the effect on metabolism and gene expression during the initial development of seedlings. Overall, Pgb1, by participating in NO turnover in the Pgb1-NO cycle, influences the ability of the embryo to maintain the delicate balance of energy production and utilization, which is of fundamental importance for the growth and development of germinated seeds.

Chapter 3

Nitric oxide turnover under hypoxia results in the rapid increased expression of the plastid-localized phosphorylated pathway of serine biosynthesis

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3.1 Abstract

The plant mitochondrial electron transport chain influences carbon and nitrogen metabolism under near anoxic conditions through its involvement in the phytoglobin-nitric oxide cycle, where the respiratory chain reduces nitrite to nitric oxide (NO), followed by NO conversion to nitrate by class 1 phytoglobin. Wild type (WT) and transgenic tobacco (*Nicotiana tabacum* L.) with differing amounts of alternative oxidase (AOX) were used to manipulate NO generation under hypoxia, and to examine whether this in turn influenced the gene expression of two stress-related amino acid biosynthetic pathways, the plastid-localized phosphorylated pathway of serine biosynthesis (PPSB), and the γ-aminobutyric acid (GABA) shunt. Under hypoxia, leaf NO emission rate was highest in AOX overexpressors and lowest in *AOX* knockdowns, with WT showing an intermediate rate. In turn, the rate of NO emission correlated with the degree to which amino acids accumulated. This amino acid accumulation was associated with the increased expression of the enzymes of the stress-related amino acid biosynthetic pathways. However, induction of the PPSB occurred much earlier than the GABA shunt. This work shows that high rates of NO turnover associate with rapid gene induction of the PPSB, establishing a clear link between this pathway and the maintenance of carbon, nitrogen and energy metabolism under hypoxia.

3.2 Introduction

Plant acclimation to oxygen deficiency involves increased glycolytic fermentation (Kennedy et al., 1992) and upregulation of nitrate reductase (*NR*) (Botrel and Kaiser, 1997). The latter relates to nitrogen turnover by the class 1 phytoglobin-nitric oxide (Pgb1-NO) cycle, an alternative to the classical fermentation pathways (Igamberdiev and Hill, 2004, 2018; Gupta and Igamberdiev, 2011; Hebelstrup and Møller, 2015). In this cycle, the mitochondrial electron transport chain (mETC) reduces nitrite to NO, Pgb1 converts the NO to nitrate, and NR converts the nitrate back to nitrite. The mETC activity associated with this cycle allows for limited ATP production when oxygen is limiting.

In addition to cytochrome c oxidase, the plant mETC includes a second terminal oxidase, an ubiquinol oxidase termed alternative oxidase (AOX) (Selinski et al., 2018; Vanlerberghe et al., 2020). Under normoxic conditions, AOX activity can dampen NO generation by preventing the over-reduction of mETC components (Cvetkovska and Vanlerberghe, 2012; Gupta et al., 2014; Alber et al., 2017). However, under hypoxic (near anoxic) conditions, AOX activity accelerates NO generation. This conclusion is based on chemical inhibitor studies (Tischner et al., 2004; Planchet et al., 2005) and studies using *AOX* knockout/knockdown and overexpression plants of *Arabidopsis thaliana* (Vishwakarma et al., 2018) and *Nicotiana tabacum* (Jayawardhane et al., 2020). The AOX protein contains non-heme iron, making possible its involvement in various redox reactions. Nonetheless, a direct demonstration of the catalysis of nitrite to NO by AOX remains lacking. Hence, it is possible that the effect of AOX on NO metabolism under

hypoxia is by some indirect mechanism, as discussed elsewhere (Jayawardhane et al., 2020).

Studies have shown that hypoxia can result in the accumulation of amino acids such as alanine and γ-aminobutyric acid (GABA) (Miyashita and Good, 2008; Rocha et al., 2010; Mustroph et al., 2014; António et al., 2016; Wu et al., 2021). This likely reflects specific interactions of carbon and nitrogen metabolism to support energy metabolism under hypoxia. For example, GABA accumulation may contribute to the maintenance of cytosolic pH (Crawford et al., 1994; Bown and Shelp, 2016; Igamberdiev and Hill, 2018). Interestingly, the profile of amino acids accumulating under hypoxia may differ depending upon AOX amount (Gupta et al., 2012; Jayawardhane et al., 2020). This hints that the Pgb1-NO cycle, whose function also depends upon AOX amount, may influence the specific pathways of carbon and nitrogen metabolism that result in amino acid accumulation under hypoxia.

This study used wild type (WT) and transgenic plants with differing amounts of AOX to manipulate the rate of NO generation under hypoxia. The rate of NO generation effected the gene expression of enzymes involved in NO turnover, as well as the energy status and amino acid pool size of the tissue under hypoxia. Further, high rates of NO turnover resulted in the rapid induction of the plastid-localized "phosphorylated pathway of serine biosynthesis" (PPSB) (Ros et al., 2014). This reveals a previously unrecognized role of the PPSB in carbon and nitrogen metabolism under hypoxia.

3.3 Materials and Methods

3.3.1 Plant material, growth condition, and hypoxia treatment

All experiments included wild type (WT) tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1), two *AOX* overexpression lines (B7, B8), and two *AOX* knockdown lines (RI9, RI29), all of which are previously described (Wang et al., 2011; Wang and Vanlerberghe, 2013). Tobacco wild type (WT), *AOX* knockdown and overexpression plants were grown in controlled-environment growth chambers for 4 weeks prior to experiments, as previously described (Jayawardhane et al., 2020). To gain a comprehensive insight into how AOX interconnects to the PPSB and GABA shunt under low oxygen, plants were subjected to nitrogen atmosphere for an extensive time course, and the samples were taken at 0 h (normoxia), 3, 6, 12, 24, and 48 h of the anoxic condition. For experimentation, the plants were placed in a custom-built, sealed, and dark chamber, to which the air supply could be replaced by nitrogen gas containing 0.001% oxygen (Cochrane et al., 2017). This treatment limits both aerobic respiration and photosynthesis by depriving plants of oxygen and light. The plants endure a progressive decrease in oxygen availability and some cells may reach a state of anoxia. The control plants were treated with normal air in the same chamber. After treatment, the fourth leaf from the top of each plant was removed, quickly frozen in liquid nitrogen, and stored at −80°C. The images of overexpressors B8 and B7, WT, and knockdowns RI9 and RI29 under normoxic conditions and after anoxic treatment were provided in the earlier study (Jayawardhane et al., 2020).

3.3.2 Nitric Oxide Emission

Gas phase NO was measured by the chemiluminescence detection method as described (Planchet et al., 2005). The leaves were detached from the plants and placed in 20 mM HEPES buffer (pH 7.0) with 50 mM sodium nitrate as nitrogen source. The leaves in the buffer were immediately placed in glass chamber with a continuous constant inflow of nitrogen at 120 mL min^{-1}. The air-tight chamber was filled with measuring gas (purified air or nitrogen) which was pulled through the chemiluminescence detector (CLD 88 p; Eco-Physics, Dürnten, Switzerland) by vacuum pump connected to ozone destroyer. The measuring gas was made NO free by a NO scrubber supplied by Eco Physics Ltd., Switzerland. Chemiluminescence analyzer was calibrated daily with NO-free air and with various NO concentrations (1–35 ppb) adjusted by mixing the calibration gas (500 ppb NO in nitrogen, Messer Griesheim, Darmstadt, Germany) with NO-free air. Flow controllers (Fisher Scientific) were used to adjust all gas flows.

3.3.3 Metabolites

About 100 mg of fresh frozen biomass were immediately gently homogenized in 1 mL of 2.4 M perchloric acid. The homogenate was then neutralized using 5 M KOH and centrifuged at 16,000 × *g* for 10 min at 4 °C (Dordas et al., 2003). The ADP/ATP ratio was measured by luciferase-based assay kit (Enzylight™ ADP/ATP ratio assay kit; BioAssay Systems, Hayward, CA, United States) on FB 12 Single Tube Luminometer (Berthold Detection Systems, GmbH, Germany).

The content of free amino acids was calculated using the approach of Lee and Takahashi (1966). 0.5 g of plant material was extracted by 70% ethanol, incubated overnight and then centrifuged at 14,000 × *g* for 15 min at 4°C. 1 mL extract was mixed with 0.5 M citrate buffer (pH 5.6), 55% glycerol, and ninhydrin solution, and then heated at 100°C for 30 min. The test tubes were immediately chilled and gently shaken. The absorbance at 570 nm was measured using a spectrophotometer. Glycine in citrate buffer was used as a standard.

3.3.4 Enzyme Assays

Nitrate reductase (NR, EC 1.7.1.1-3) activity of tobacco leaves was measured by the method of Ferrario-Mery et al. (1998). The activity was assayed in the reaction mixture consisting of 50 mM HEPES-KOH, pH 7.5, 0.1 mM NADH, 5 mM KNO_3, and 2 mM EDTA. After 15 min, the reaction was stopped by adding equal volumes of 1% sulfanilamide and 0.02% naphthylethylene-diamine dihydrochloride and nitrite concentration was determined at 540 nm.

Phosphoglycerate kinase (PGK, EC 2.7.2.3) activity was evaluated by measuring NADH oxidation, in a coupled reaction with glyceraldehyde phosphate dehydrogenase (GAPDH) (Rosa-Téllez et al., 2018). The activity was assayed in the reaction mixture containing 100 mM HEPES-KOH, 1 mM EDTA, 2 mM $MgSO_4$, 0.3 mM NADH, 6.5 mM PGA, 1 mM ATP, and 3.3 units of GAPDH. The reaction was initiated by adding protein extracts and the absorbance was read at 340 nm.

3.3.5 Transcript Amounts

RNA was extracted from frozen leaf using the FastRNA® Pro Green Kit (MP Biomedicals), according to the manufacturer's instructions. RNA (5 μg) was reverse transcribed using the Superscript III reverse transcriptase kit (Invitrogen). Quantitative PCR was carried out with an Applied Biosystems StepOnePlus Real-Time PCR System, using SYBR Green qPCR Master Mixes (Thermo Scientific) and gene-specific primers (**Table 3.1**). Comparative quantification was performed by the $\Delta\Delta C_t$ method, with actin as the reference gene. All transcript amounts are relative to the amount in WT at time 0 (log2 fold-change). For a subset of genes (those encoding *PGDH* and *PSP*) we also confirmed the gene expression patterns using a second reference gene encoding GAPDH.

Table 3.1 Primers used for qRT-PCR assays of tobacco leaves

Primer	Forward sequence	Reverse sequence	Accession no/References
NtACT	TTGGCTTACATTGCTCTTG	TCATTGATGGTTGGAACAG	XM_033660572.1
NtPGD	GGCCTCGGTATGCATGTAAT	CCTGCTCAAAGGAAACCAAATC	XM_009612696.3
NtPSA	CCACCCTCACCTCTACCTATAA	GAAGTTGAAGACCCGATCTGAG	XM_016597132.1
NtPSP	CTGGAAAGGCTGTAGCAGAA	CCTGGAGTTGGGACAATGAA	XM_016601311.1
NtGABAT	AGTCCCATAGTCGGAGAGATTAG	GGGAAAGGATCATTGGGAGATT	NM_001324662.1
NtGAD	TGAAGTGGAGCTAAAGGAAGTAAA	TTGAGTGTGGAACCCAAGATAG	U54774.1
NtHb1	TGCCAAACTCAAGCCTCAT	TCGAATCCCTCACCACTACT	KJ808726.1
NtGSNOR	TCTGCCACACTGATGCTTATAC	CTCCTTCACCGACACTTTCTAC	XM_009631265
NtNR	CACTCGAGGTTGAGGTGTTAG	AGCTTCTCGGGTTGAGTATTG	X14059.1

3.3.6 Statistical Analysis

All experiments were repeated three times. To statistically analyze the data of tobacco leaves, the software package SPSS V. 21.0 (Statistical Package for Social Science; Chicago, IL, United States) was used. To determine significant differences between the WT, AOX overexpressors, and *AOX* knockdowns, one-way ANOVA was conducted by using Duncan's multiple range. Data in the text and figures are presented as mean value ± standard deviations of three replicates. The differences with $p \leq 0.05$ were considered as statistically significant. Only statistically significant differences are discussed in the manuscript.

3.4 Results

3.4.1 Nitric oxide emission, energy state, and amino acid content of plants under hypoxia

Tobacco leaves in a hypoxic (near anoxic, ~0.001% O_2) atmosphere emitted NO, while no emission was detected from leaves in air (**Figure 3.1A**). The NO emission rate depended upon AOX amount. Rates were 50% higher in AOX overexpressors (B7, B8) than WT throughout the first 24 h of hypoxia. The *AOX* knockdown lines (RI9, RI29) had much lower NO emission rates than WT. In all plant lines, NO emission rates declined by 48 h of hypoxia, perhaps due to the poor physical condition of the plants by this time point (**Figure 3.1A**).

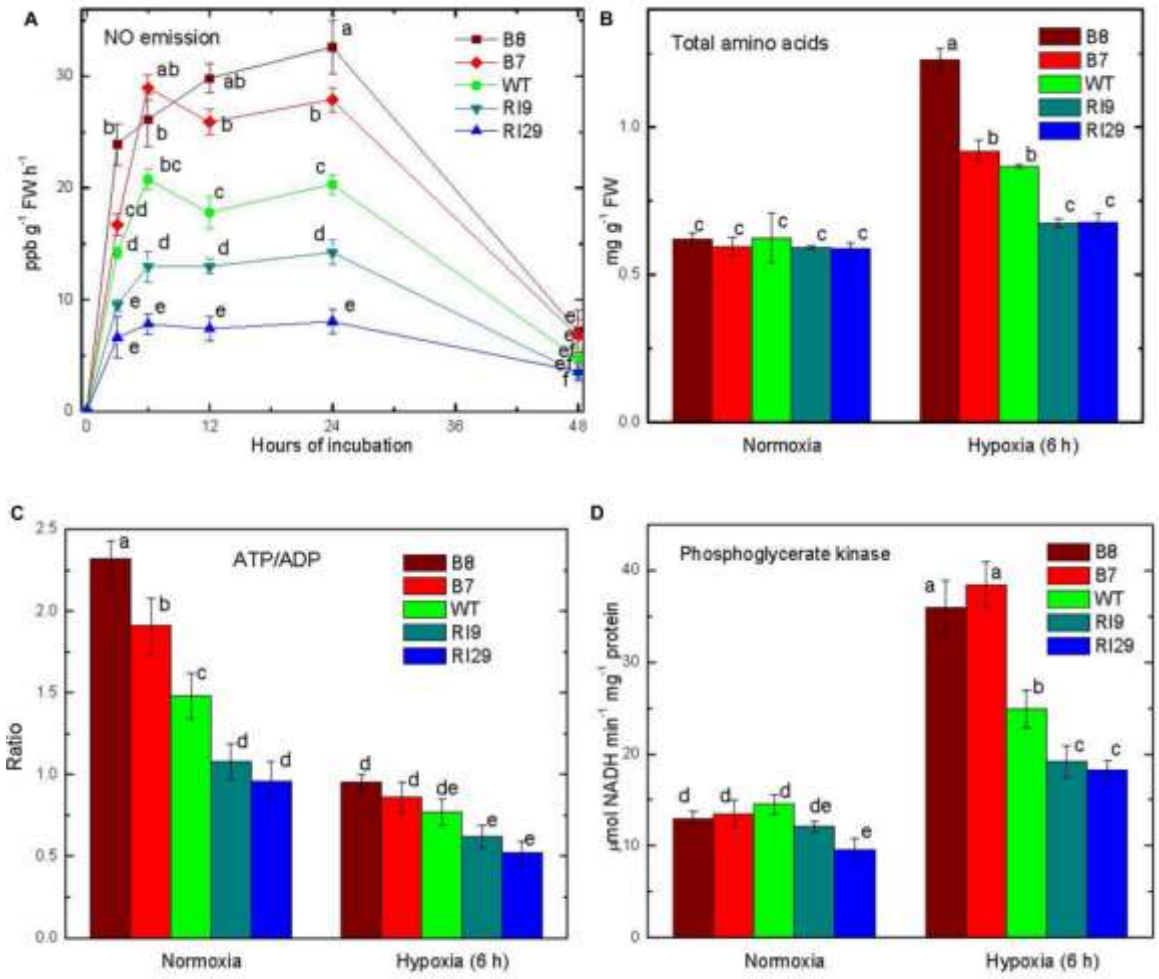

Figure 3.1 Leaf nitric oxide emission (A), total amino acid content (B), ATP/ADP ratio (C), and phosphoglycerate kinase activity (D) in tobacco plants with differing amounts of alternative oxidase, and exposed to hypoxia (at time 0 in A). The plant lines used included wild type (WT), two alternative oxidase overexpressors (B7, B8), and two alternative oxidase knockdowns (RI9, RI29). Vertical bars indicate SD from three to four independent experiments (n = 3–4); different letters indicate significant differences between five tobacco lines and the time points.

In air, all of the plant lines had a similar total pool size of amino acids in the leaf (**Figure 3.1B**). The pool size of amino acids increased following 6 h of hypoxia, and was now

highest in the overexpressors (particularly B8) and lowest in the knockdowns, with WT showing an intermediate amount. In air, the leaf ATP/ADP ratio was higher by 1.5 times in the overexpressor B8 and lower by more than 50% in the knockdown RI29 as compared to WT (**Figure 3.1C**). Overall, ATP/ADP ratios decreased following 6 h hypoxia, but the relative differences across plant lines persisted. In hypoxia, B8 had 1.2-fold greater ATP/ADP ratio than WT, while RI29 had a 1.5-fold lower ratio. In air, the maximum PGK activity, which represents a measure of the glycolytic flux capacity (Rosa-Téllez et al., 2018), was similar across plant lines (**Figure 3.1D**). The PGK activity increased in all plant lines following 6 h of hypoxia. However, the activity was now highest in the overexpressors and lowest in the knockdowns, with WT showing an intermediate activity (**Figure 3.1D**).

3.4.2 Components of Nitric oxide turnover

NR transcript amount increased rapidly in all plant lines in response to hypoxia (**Figure 3.2A**). NR activity showed no significant changes across plant lines under normal conditions. The activity increased is substantially in response to a 6 h hypoxia treatment and was now highest in the overexpressors and lowest in the knockdowns, with WT showing an intermediate activity (**Figure 3.2B**). The overexpressor line B8 had a 2.1- and 3.3-fold higher activity than knockdowns, RI9 and RI29, respectively.

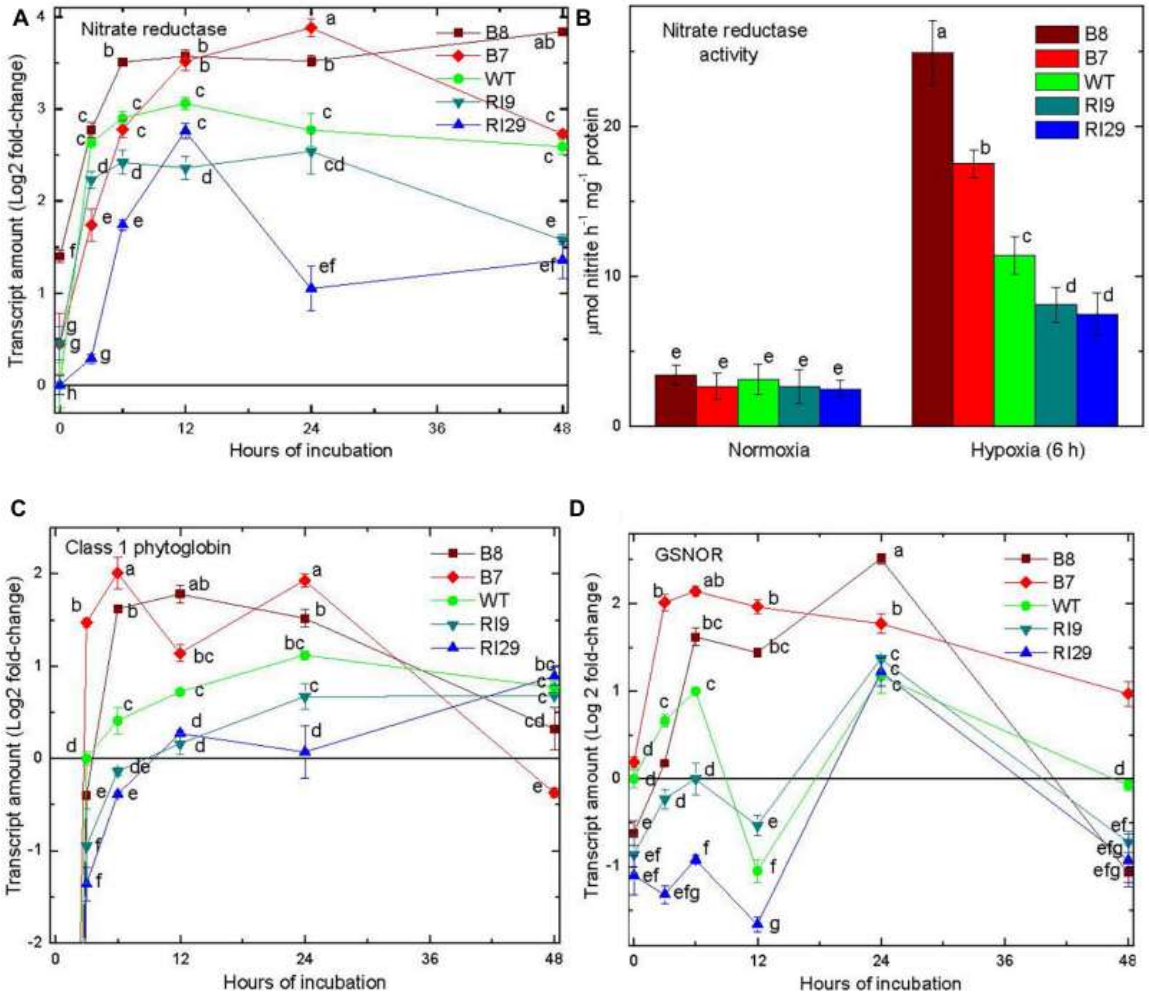

Figure 3.2 Leaf nitrate reductase transcript amount (A), nitrate reductase activity (B), class 1 phytoglobin transcript amount (C), and S-nitrosoglutathione reductase transcript amount (D) in tobacco plants with variable amounts of alternative oxidase, and exposed to hypoxia (at time 0 in A,C,D). In (C), there was no detectable transcript at time 0. Vertical bars indicate SD from three independent experiments (n = 3); different letters indicate significant differences between five tobacco lines and the time points.

In air, there was no detection of the transcript encoding Pgb1 in any plant line. However, this transcript was readily detectable following 3 h of hypoxia (**Figure 3.2C**). Further, the transcript amounts over the first 24 h under hypoxia were highest in the overexpressors and lowest in the knockdowns, with WT showing an intermediate amount. By 48 h, transcript amounts declined and were similar across plant lines (**Figure 3.2C**).

Following 6 h of hypoxia, the transcript amount of *S*-nitrosoglutathione reductase (GSNOR) was highest in the overexpressors and lowest in the knockdowns, with WT showing an intermediate amount (**Figure 3.2D**). Interestingly, the WT and knockdown lines displayed a clear oscillation of transcript amount (high at 6 h, lower at 12 h, up again by 24 h) that was not evident in the overexpressors, where transcript amount remained high through 24 h. All plant lines showed a decline in GSNOR transcript amount between 24 and 48 h (**Figure 3.2D**).

3.4.3 The Phosphorylated Pathway of Serine Biosynthesis

We examined the gene expression of enzymes within the plastid-localized PPSB (Ros et al., 2014). In response to hypoxia, the transcript amounts for 3-phosphoglycerate dehydrogenase (*PGDH*), 3-phosphoserine aminotransferase (PSAT), and 3-phosphoserine phosphatase (*PSP*) increased in the WT and *AOX* overexpression plants, with the overexpressors clearly showing the greatest increases (**Figure 3.3**). Depending upon the plant line and enzyme, transcript abundance peaked between 3 and 12 h of hypoxia treatment. Thereafter, transcript amount either remained steady (*PSP*) or gradually declined (*PGDH, PSAT*). The knockdown plants acted very differently. In this case, the

hypoxia treatment either had no effect on transcript amount (*PGDH*) or resulted in declines in transcript amount (*PSAT, PSP*) (**Figure 3.3**). The results of *PGDH* and *PSAT* expression obtained by using the second reference gene encoding GAPDH were quite similar confirming the results with the actin reference gene. The data for the transcript amounts of *PGDH* and *PSAT* are shown with actin and *GAPDH* as the reference genes (**Figures 3.3A,C**).

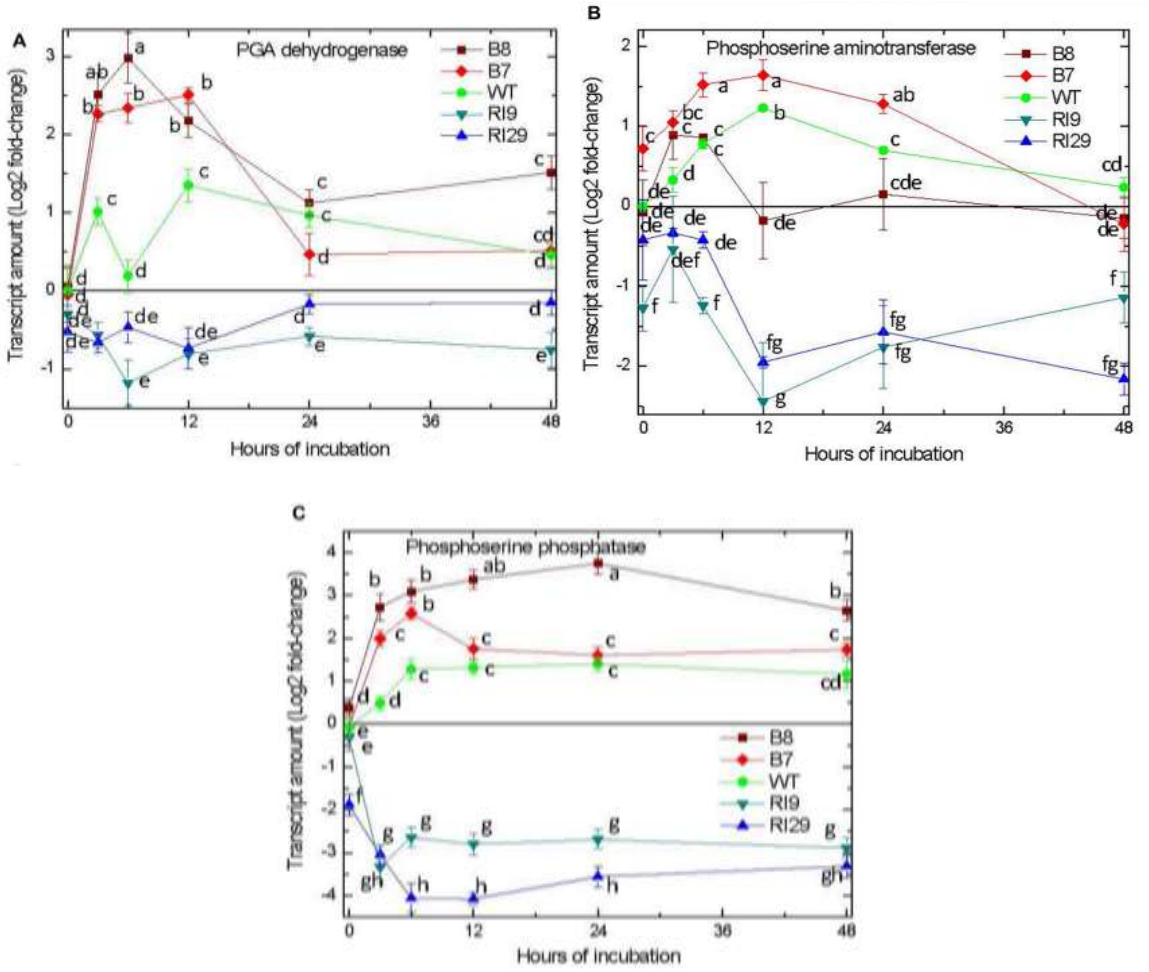

Figure 3.3 Leaf transcript amount for enzymes of the phosphorylated pathway of serine biosynthesis, including 3-phosphoglycerate dehydrogenase (A), 3-phosphoserine aminotransferase (B), and 3-phosphoserine phosphatase (C) in tobacco plants with differing amounts of alternative oxidase and exposed to hypoxia at time 0. For 3-phosphoglycerate dehydrogenase (A) and 3-phosphoserine phosphatase (C) the normalized transcript levels are shown using two reference genes, actin and GAPDH. Vertical bars indicate SD from three independent experiments (n = 3); different letters indicate significant differences between five tobacco lines and the time points.

3.4.4 The γ-Aminobutyric Acid Shunt

We examined the gene expression of enzymes within the GABA shunt, including glutamate decarboxylase (GAD; responsible for GABA synthesis), and γ-aminobutyrate transaminase (GABA-T; catalyzing conversion of GABA to succinic semialdehyde) (Bown and Shelp, 1997). The abundance of these transcripts did increase in response to hypoxia, but with an obvious lag compared to the transcripts associated with the PPSB (**Figure 3.4**). In the case of these GABA shunt enzymes, transcript increases were relatively moderate within the first 12 h of hypoxia, and without any clear differences in abundance across the plant lines. However, following 24–48 h of hypoxia, there were clear increases in transcript abundance, and transcript amounts were highest in the overexpressors and lowest in the knockdowns (**Figure 3.4**).

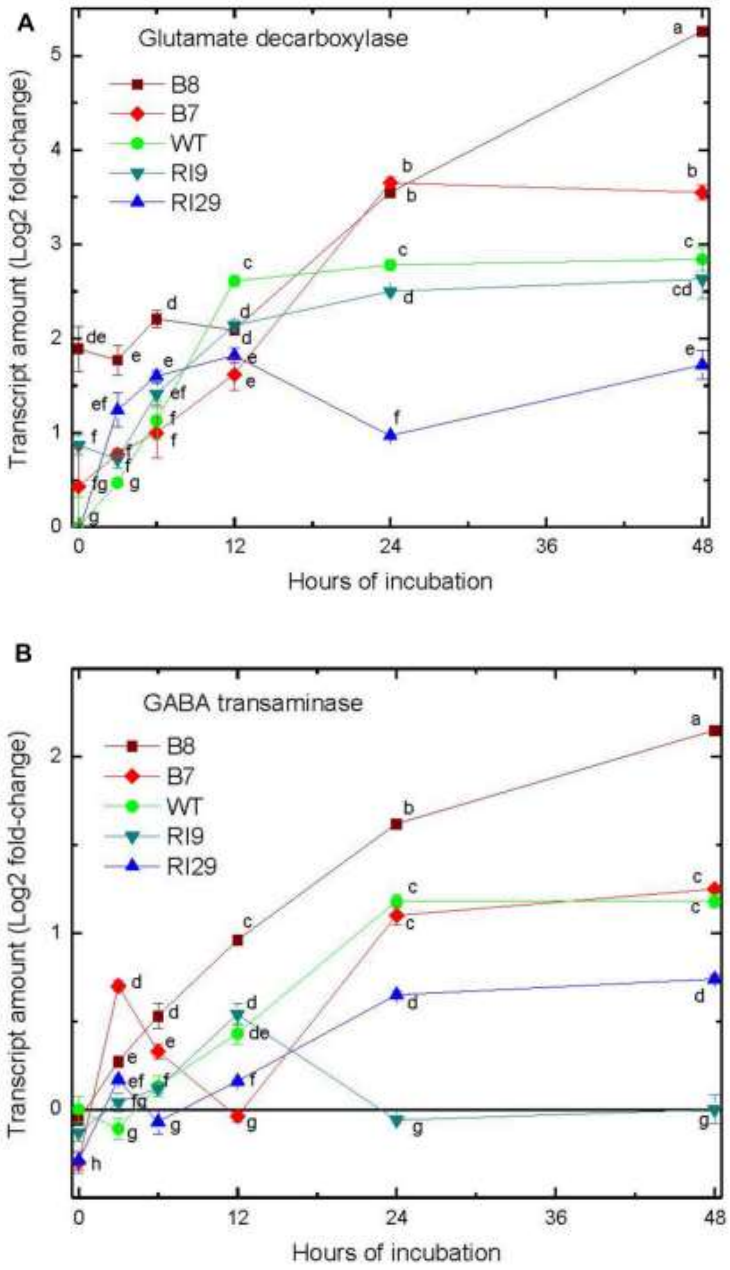

Figure 3.4 Leaf transcript amount for enzymes of the γ-aminobutyric acid shunt, including glutamate decarboxylase (A) and γ-aminobutyrate transaminase (B) in tobacco plants with differing amounts of alternative oxidase, and exposed to hypoxia at time 0. Vertical bars indicate SD from three independent experiments (n = 3); different letters indicate significant differences between five tobacco lines and the time points.

3.5 Discussion

3.5.1 Interaction of alternative oxidase and the Pgb1-Nitric oxide cycle in energy metabolism under hypoxia

AOX is a non-energy conserving ubiquinol oxidase of the plant mETC (Vanlerberghe et al., 2020). This study confirms previous work in *Arabidopsis* (Vishwakarma et al., 2018) and tobacco (Jayawardhane et al., 2020) that AOX amount is an important determinant of NO generation under hypoxia. Overexpression of *AOX* increased NO generation, while knockdown of *AOX* decreased NO generation, relative to WT. These differences in NO generation corresponded with differences in the expression/activity of key enzymes involved in the Pgb1-NO cycle including NR and Pgb1 (**Figures 3.2A–C**). In both cases, expression/activity were greatest in the plant lines with highest NO emission (*AOX* overexpressors) and lowest in the plant lines with lowest NO emission (*AOX* knockdowns). This is further evidence that the source of NO generation under hypoxia is the Pgb1-NO cycle (Jayawardhane et al., 2020). Interestingly, *GSNOR* expression also increased under hypoxia (**Figure 3.2D**). GSNOR can buffer against high tissue concentrations of NO by degrading *S*-nitrosoglutathione, a bioavailable cellular reservoir of NO (Sakamoto et al., 2002; Gupta et al., 2011a; Li et al., 2021). The differential expression of this important player in NO metabolism across plant lines is another indication that AOX amount is an important determinant of NO generation under hypoxia (Jayawardhane et al., 2020).

An intriguing observation arising from an earlier study (Gupta et al., 2012) is that NR activity is required for increased *AOX* expression under hypoxia and from this study, it follows that vice versa AOX activity determines *NR* expression under hypoxia. This

strongly supports the conclusion that AOX participates in the Pgb1-NO cycle and indirectly indicates that AOX may be involved in the production of NO from nitrite as suggested by Vishwakarma et al. (2018). While NR and Pgb1 are well known as essential components of the Pgb1-NO cycle (Igamberdiev et al., 2005), AOX appears to be another important factor, whose contribution may be comparable to that of other electron transport chain components.

The AOX-dependent changes in Pgb1-NO cycle activity under hypoxia impacted leaf energy status. Under normoxia, induction of AOX in response to diverse stressors can decrease electron transfer through complexes III and IV, preventing electron leakage to nitrite and consequent NO buildup (Cvetkovska and Vanlerberghe, 2012). Excess NO can block complex IV (lowering the ATP/ADP ratio); as a result, the AOX reduces nitrite-dependent NO synthesis (raising the ATP/ADP ratio) that would otherwise result from increased electron leakage in the cytochrome pathway. In fact, even under normoxia, AOX had a positive impact on energy status, despite its non-energy conserving nature. This indicates that tobacco AOX must optimize leaf respiration, perhaps by preventing bottlenecks in carbon metabolism and/or controlling the generation of reactive oxygen and nitrogen species (Cvetkovska and Vanlerberghe, 2012; Dahal and Vanlerberghe, 2017; Selinski et al., 2018; Jayawardhane et al., 2020). In contrast to its function under normal air, AOX has a unique role in hypoxia, when it can boost nitrite-dependent NO generation (Vishwakarma et al., 2018). This reaction drives the Pgb1-NO cycle to increase energy efficiency under hypoxia (Kumari et al., 2019).

The observation that the ATP/ADP ratio in AOX overexpressors substantially higher than in WT under normal conditions is also notable. One potential explanation is related to the amount of NO in these transgenic lines under normoxia and is due to prevention of overreduction of the components of the mitochondrial ETC, in particular at the level of one-electron leakage at the site of Complex III. The difficulty in comparing NO between the WT and overexpressors under normoxia is that it is very low in both, so a difference cannot be easily measured. It is known that NO has a strong inhibitory effect on cytochrome *c* oxidase. Lower levels of NO in the mitochondria of the overexpression lines may promote cytochrome oxidase activity and thus raise the ATP/ADP ratio, whereas greater levels of NO in the mitochondria of the knockdown lines under normoxia suppress cytochrome *c* oxidase and thus reduce the ATP/ADP ratio. Furthermore, it was previously demonstrated that reduced NO levels in barley roots caused by non-symbiotic phytoglobin overexpression increased respiration rates in normoxia (Gupta et al., 2014). We previously demonstrated that when hypoxic leaves were pre-treated with the AOX inhibitor SHAM, NO emission rates fell in all plant lines (Jayawardhane et al., 2020). However, NO emission rates under hypoxia were highest in overexpressors and lowest in knockdowns, with WT plants responding in a middle ground. It suggests the AOX role in the Pgb1-NO cycle. An increased operation of the Pgb1-NO cycle in the AOX-overexpressing lines is evidenced by NO production and greater expression/activity of Pgb1 and NR, suggesting that AOX can feed the Pgb1-NO cycle, which can contribute to ATP generation under hypoxia (Stoimenova et al., 2007; Vishwakarma et al., 2018). However, further research is needed

to determine the difference in ATP/ADP ratios between WT and overexpressors under normoxia.

3.5.2 A role for the phosphorylated pathway of serine biosynthesis in amino acid metabolism under hypoxia

In plants and green algae, amino acid metabolism can be supportive of energy metabolism when oxygen is limiting or absent (Reggiani et al., 1988; Rocha et al., 2010; Narsai et al., 2011; Mustroph et al., 2014; Shingaki-Wells et al., 2014; António et al., 2016; Nakamura and Noguchi, 2020; Solhaug et al., 2021). Such amino acid metabolism may reduce lactate and ethanol accumulation, in this way lessening cytosolic acidification (in the case of lactate) and tissue carbon loss (in the case of ethanol). For example, in the green alga *Selenastrum minutum*, there is a stoichiometric decline in aspartate and increases in succinate and alanine at the onset of anoxia (Vanlerberghe et al., 1990). This suggests that aspartate provided the nitrogen for alanine accumulation (with pyruvate providing the carbon skeleton) and the carbon skeleton for succinate accumulation (by a partial reductive tricarboxylic acid cycle). This process continued until the aspartate pool was depleted. However, if inorganic nitrogen was also available for assimilation by glutamine synthase and glutamate-oxoglutarate aminotransferase (the GS-GOGAT cycle), then alanine could continue to accumulate, while maintaining energy and redox balance (Vanlerberghe and Turpin, 1990; Vanlerberghe et al., 1991).

In the current study, we found that high rates of Pgb1-NO cycle activity under hypoxia favored an increased accumulation of amino acids (**Figure 3.1B**). This suggests that some of the inorganic nitrogen (nitrate, nitrite) cycling within the Pgb1-NO cycle becomes

assimilated by the GS-GOGAT cycle following reduction to ammonium. The increased accumulation of amino acids corresponded to the increased PGK activity, which may indirectly reflect higher glycolytic capacity to provide the necessary carbon skeletons (**Figure 3.1D**). Further, the elevated sequestration of carbon and nitrogen into the amino acid pool corresponded with a rapid increased expression of genes encoding each of the enzymes within the PPSB (**Figure 3.3**). The pattern of higher gene expression in *AOX* overexpression lines and decreased expression in *AOX* knockdowns provides confidence that the transcript regulation patterns observed are likely coordinated. Still transcripts themselves do not provide evidence of a substantial change to metabolism, which might impact upon hypoxia or carbon nitrogen interactions. Therefore, it is worth to compare the data obtained in this study to the profiles of amino acids obtained in the previous investigation (Jayawardhane et al., 2020). They show that in response to hypoxia, the amount of GABA increased in all plant lines, but was much higher in AOX overexpressor. The amount of serine in the knockdown was substantially higher than in WT and overexpressor under normoxia. The level of serine in the knockdown plant lines dropped from the higher values after placing the plants to hypoxic conditions and became identical to the other plant lines. This has a relation to the changes in expression of several genes involved in the serine and GABA biosynthesis observed in this study. The changes in the patterns of several metabolites are linked to the alterations of the transcript regulatory patterns of the enzymes participating in metabolism of these compounds.

Besides generating serine in the chloroplast, the PPSB would also release 2-oxoglutarate that might be necessary to support continued GS-GOGAT activity (Benstein et al., 2013).

In *A. thaliana*, the activity of different PGDH isoforms is inhibited by serine but activated by pyruvate-family and aspartate-family amino acids such as alanine, valine, homoserine, homocysteine, and methionine (Okamura and Hirai, 2017; Okamura et al., 2021). This suggests a close integration of the PPSB with other pathways of carbon and nitrogen metabolism, as recently highlighted (Anoman et al., 2015, 2019; Igamberdiev and Kleczkowski, 2018; Watanabe et al., 2021). Under hypoxia, this integration could allow the nitrogen in serine to be readily distributed to other amino acids, such as through transamination reactions.

The increases in transcript amount of the PPSB enzymes was already near maximal by 3 h, the first time point examined following the hypoxic treatment. By comparison, gene transcripts associated with the GABA shunt, a recognized player in low oxygen amino acid metabolism (Bown and Shelp, 1997; Miyashita and Good, 2008; Igamberdiev and Hill, 2018) increased in abundance only at much later time points (**Figure 3.4**). In *A. thaliana*, only roots accumulated GABA during a short-term hypoxia treatment, while both roots and shoots accumulated alanine (Mustroph et al., 2014). Hence, the lack of early induction of GABA shunt genes shown here could be a leaf-specific response.

This study identifies the PPSB as an important player in amino acid metabolism under hypoxia, at least when the Pgb1-NO cycle is highly active. In fact, gene transcripts associated with the PPSB enzymes were only induced in the WT and *AOX* overexpression plants. In the *AOX* knockdowns, where Pgb1-NO cycle activity was being suppressed, the transcript amount of the PPSB genes actually declined substantially under hypoxia. Serine is positioned at the central node linking the biosynthetic flux from glycolysis to glutathione

synthesis and to one-carbon metabolic cycle, which are closely related to the antioxidant capacity (Anoman et al., 2019). Glycine that can be formed from serine, in turn, can generate glyoxylate, which is reduced to glycolate, the latter being an important hypoxic metabolite (Narsai et al., 2009). In the sequence of these reactions, the redox level can be efficiently regulated (Igamberdiev and Kleczkowski, 2018). While the route ending in serine generates NADH, the metabolism beyond that point could oxidize the pool again, e.g., when glycine is produced from serine and glycolate is formed, the whole pathway can efficiently contribute to the redox status during stress (Ho and Saito, 2001; Ros et al., 2014).

Serine serves as a precursor for a variety of essential biomolecules, including nucleic acid bases, phospholipids, and sphingolipids, as well as providing the carbon skeleton for the synthesis of l-tryptophan, l-cysteine and, glutathione. In PPSB-deficient lines, serine lowered the local Cys and GSH pools (Anoman et al., 2019), which, in turn, affects the ascorbate-glutathione cycle. The latter is involved in redox metabolism that results in the oxidation of NADPH to $NADP^+$ (Noctor and Foyer, 1998). We can conclude that PPSB is most likely involved in NAD $(P)^+$ regeneration in an indirect way. Glycolytic fermentation and the Pgb1-NO cycle both help to regenerate NAD^+ when oxygen levels are low. In turn, PPSB and the consequent reactions of glycine, glyoxylate and formate metabolism involve NAD turnover, affecting the cellular $NAD(P)^+$/NAD(P)H balance and redox status.

Future studies should establish the signal (s) that is controlling PPSB gene expression under hypoxia. It seems unlikely that NO itself is the signal since, even in the *AOX* knockdowns, NO amounts did increase with hypoxia, while the PPSB gene transcripts declined. On the

other hand, a recent study provides evidence that NO is an important signal intermediate controlling the expression of *A. thaliana PGDH1*. *PGDH1* was amongst 23 genes whose increased expression was linked to NO-dependent changes in histone acetylation (Ageeva-Kieferle et al., 2021). Another study showed that a depleted amount of leaf serine could induce the PPSB genes in *A. thaliana* (Modde et al., 2017).

While oxygen deficiency is usually associated with roots and other bulky tissues, it is evident that leaves can also experience this condition, such as during pathogen infection. Interestingly, *A. thaliana* leaf *PGDH1*, *PGDH2*, and *PSAT1* gene expression all increased at the site of *Botrytis cinerea* infection (Benstein et al., 2013), a site recently shown to experience near-anoxic conditions (Valeri et al., 2021). In conclusion, by using AOX transgenic plants as a means to manipulate Pgb1-NO cycle activity, we have identified the PPSB as a previously unrecognized player supporting carbon and nitrogen metabolism under hypoxia.

Chapter 4

The role of alternative oxidase in the interplay between nitric oxide, reactive oxygen species, and ethylene in tobacco (*Nicotiana tabacum* L.) plants incubated under normoxic and hypoxic conditions

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4.1 Abstract

Reactive nitrogen and oxygen species (RNS and ROS) are part of plant metabolism, and variations in the availability of oxygen (O_2) can lead to the altered RNS and ROS levels. To prevent the nitro-oxidative damage, the steady state levels of nitric oxide (NO) and ROS must be tightly regulated. In this study, we used transgenic tobacco (*Nicotiana tabacum* L.) plants with the modified levels of alternative oxidase (AOX) to evaluate physiological roles of AOX in regulating the hypoxically induced nitro-oxidative stress and metabolic changes after exposing tobacco plants to hypoxia for 6 h. Under normoxia, *AOX* expression resulted in the decrease of NO levels and in the rate of *S*-nitrosylation of proteins, while under hypoxia *AOX* overexpressors exhibited higher NO levels than knockdowns but the rate of *S*-nitrosylation changed to a less extent. The expression of *AOX* was essential in avoiding hypoxia-induced superoxide and H_2O_2 levels, which was achieved via higher activities of catalase and glutathione reductase and the reduced expression of respiratory burst oxidase homolog (*Rboh*) in overexpressors as compared to knockdowns. The plant lines overexpressing *AOX* accumulated less pyruvate and exhibited the increased transcript levels and a higher activity of pyruvate decarboxylase and alcohol dehydrogenase under hypoxia. This suggests that AOX contributes to energy state of hypoxic tissues by stimulating the increase of the flow of pyruvate into fermentation pathways. Ethylene biosynthesis genes encoding 1-aminocyclopropane 1-carboxylic acid (ACC) synthase, ACC oxidase and ethylene-responsive factors (ERFs) were induced during hypoxia and correlated with AOX and NO levels. We propose that AOX, as a key centre in the

mitochondria, controls the interaction of NO, ROS, and ethylene, triggering a coordinated downstream defensive response against hypoxia.

4.2 Introduction

Nitric oxide (NO) appears to be a critical signaling and metabolic molecule in a wide range of environmental crises such as hypoxia. When the ambient oxygen (O_2) is low, NO production and its turnover are involved in the phytoglobin-nitric oxide pathway (Pgb1-NO cycle) for the maintenance of redox and energy levels (Zafari et al., 2020, 2022). NO represents the main reactive nitrogen species (RNS) and it can interact with reactive oxygen species (ROS) such as superoxide anion (O_{2^-}) forming other reactive nitrogen species, e.g. peroxynitrite ($ONOO^-$). The main sources of hydrogen peroxide (H_2O_2) in cells are the plasma membrane NADPH oxidases, or respiratory burst oxidase homologs (Rbohs) (Morales et al., 2016). They catalyze the apoplast generation of O_{2^-} which is then converted into H_2O_2. Even though NO and ROS are produced under normal physiological conditions, they are prone to uncontrolled overproduction in stressful situations, resulting in cellular nitro-oxidative damage that can compromise cell survival.

Plant cells have evolved sophisticated defence mechanisms to avoid organelle damage as a result of redundant NO and ROS formation. For example, excessive electron flow is dissipated by the alternate respiratory pathway, such as AOX, to preserve mitochondrial redox state. AOX maintains energy stability and keeps the flow through the mitochondrial electron transport chain (mETC) by minimizing the generation of mitochondrial NO and ROS (Cvetkovska and Vanlerberghe, 2012; Wang et al., 2012). However, under hypoxic

conditions, AOX activity accelerates NO generation in tobacco, followed by NO conversion to nitrate by the upregulated Pgb1 (Zafari et al., 2022). AOX may also play a crucial role in ROS avoidance via activating antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) as revealed in brassinosteroids-treated *N. benthamiana* (Wei et al., 2015). Despite the current knowledge regarding the AOX contribution to hypoxia, the NO and ROS interplay in plants expressing variable levels of AOX under hypoxic conditions is little understood.

Plants have evolved a plethora of plant-specific transcription factors to precisely control the expression of their genes. The ethylene response factors (ERFs) superfamily is one of the largest families of transcription factors that respond to a variety of biotic and abiotic stressors by binding directly to promoter regions of defense-related genes (Zhuang et al. 2011). Overexpression of hypoxia-inducible *ERF* genes promotes tolerance to low oxygen environments in Arabidopsis and rice (Licausi et al., 2010; Fukao et al., 2008). Since many ERFs are involved in stress responses, the tight control of ERFs ensures that plants respond to environmental stressors effectively. Ubiquitin-mediated protein degradation regulates ERFs proteins stability via the 26S proteasome pathway. The N-end rule route is also responsible for the stability of ERFs belonging to the group VII ethylene response factors (ERF-VII) (Gibbs et al., 2014). ERF-VII proteolysis is facilitated by oxygen and NO (Hartman et al., 2019), while ethylene (ET) promotes their stability (Kim et al., 2018). ET was found to be critical for the improved hypoxia tolerance by increasing the expression of genes encoding proteins involved in fermentation, energy maintenance, oxidative stress, NO scavenging, and O_2 sensing (Dubois et al., 2018; Hartman et al., 2019). Studies have

provided solid evidence that Et-signaling pathway is essential for stress-induced up-regulation of AOX (Wang et al., 2010; Ederli et al., 2006). In this work, the effects of different amounts of AOX on the expression of *ERFs* and ET biosynthesis enzymes (1-aminocyclopropane-1-carboxylic (ACC) synthase (*ACS*) and ACC oxidase (*ACO*)) were investigated in transgenic tobacco under hypoxia. Furthermore, the molecular mechanism by which AOX impacts the interaction of NO, ROS, and ET in alleviating hypoxic-induced stress in tobacco was also examined.

4.3 Methods

4.3.1 Plant material, growth condition and hypoxia treatment

Tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) wild type (WT) and transgenic lines with the suppressed levels of AOX protein (RI9 and RI29) and elevated levels of AOX protein (B7 and B8) were used for all experiments. The transgenic lines used in this work have been described earlier (Wang et al., 2011; Wang and Vanlerberghe, 2013). The plants were grown in a cultivation chamber under controlled environmental conditions for 4 weeks before tests, as previously described (Jayawardhane et al., 2020). To gain a full understanding of how AOX impacts the ROS metabolism under oxygen deficiency, plants were exposed to nitrogen atmosphere, and the samples were taken at 0 h (normoxia), and 6 h of the hypoxic condition. To test the plants under low oxygen, an entire individual plant was placed in a custom-built sealed dark chamber, where the air supply could be replaced with nitrogen gas having ~0.001% oxygen (Cochrane et al., 2017). By depriving plants of oxygen and light, the treatment eliminates both aerobic respiration and photosynthesis. The

control plants were treated with normal air in the same chamber. The fourth leaf from the top of each plant was taken after treatment, instantly frozen and kept in liquid nitrogen before being transferred to the –80˚C freezer. The images of *AOX* overexpression (B7, B8), WT and knockdowns plants (RI9 and RI29) under normoxia and after anoxic treatment were presented in the previous study (Jayawardnahe et al., 2020).

4.3.2 Reactive nitrogen and oxygen species

NO was measured in the gas phase using the chemiluminescence detection method, as described before (Planchet et al., 2005). The excised leaves were placed in 20 mM HEPES buffer (pH 7.0) containing 50 mM sodium nitrate before being transferred to a glass chamber with a continuous steady input of nitrogen at 120 mL min^{-1}. A vacuum pump coupled to an ozone destroyer drew the measuring gas (purified air or nitrogen) through the chemiluminescence detector (CLD 88 p; Eco-Physics, Dürnten, Switzerland). The measuring gas was made NO free by a NO scrubber (Eco Physics Ltd, Switzerland). All gas flows were adjusted using flow controllers (Fisher Scientific).

NO in the liquid phase was measured by the oxyhemoglobin assay. NO was extracted from frozen leaves of tobacco with 1 mL of cooled buffer (50 mM Tris-HCl, pH 7.0, 0.6% (w/v) PVP). The homogenates were centrifuged at 15,000 ×*g* for 10 min at 4°C. The supernatant was pretreated for 3 min at room temperature with superoxide dismutase (SOD; 4,000 U/ml) and catalase (10,000 U/ml) to remove ROS. NO was quantified in cleared extracts spectrophotometrically measuring the conversion of oxyhemoglobin to methemoglobin (Ma et al., 2016).

To measure the amount of superoxide anion (O_2^-), 200 mg of fresh leaf biomass was crushed using a pestle and mortar in 2 mL of 8 M KOH under chilled conditions, and then centrifuged for 15 min at 12,000 ×*g* at 4 °C. The amount of O_2^- in the supernatant was then evaluated at 550 nm by reduction of cytochrome *c* as described in Ma et al. (2016). The H_2O_2 content of leaves was measured as described by Xu et al. (2012a). Approximately 200 mg fresh leaf biomass was homogenized in an ice bath with 5 mL 0.1% TCA and centrifuged for 20 min, at 12,000 ×*g* and 4 °C. Then, 0.5 mL supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1M KI and the absorbance was read at 390 nm.

4.3.3 Assays for antioxidant enzymes

Fresh leaf tissue (300 mg) was homogenized with 3 ml of ice-cold 25 mM HEPES buffer (pH 7.8) containing 0.2 mM EDTA, 2 mM ascorbate and 2% PVP. The homogenates were then centrifuged for 20 min at 12,000 ×*g* at 4 °C. The resulting supernatants were used to determine the enzymatic activities of SOD, CAT, GPX, APX and glutathione reductase (GR). SOD (EC 1.15.1.1) activity was assayed by measuring the inhibition of the photochemical reduction of NBT following the method of Wu et al. (2015). CAT (EC 1.11.1.6) activity was determined as the decline in the absorbance at 240 nm due to the decrease of extinction of H_2O_2 using the method of Huang et al. (2006). GPX (EC 1.11.1.7) activity was measured as the increase in the absorbance at 470 nm due to guaiacol oxidation (Nickel and Cunningham 1969). APX (EC 1.11.1.11) activity was measured by tracking the drop in absorbance at 290 nm as ascorbate was oxidized (Nakano and Asada, 1981).

GR (EC 1.8.1.7) activity was monitored at 340 nm for 3 min by the rate of conversion of NADPH to $NADP^+$ (ε_{340} = 6.2 mM^{-1} cm^{-1}) (Ma *et al*., 2016).

4.3.4 Assays of fermentation enzymes and metabolites

Pyruvate decarboxylase (PDC; EC 4.1.1.17) activity was evaluated by measuring NADH oxidation in a coupled reaction with lactate dehydrogenase (LDH) (Laszlo and Saint Lawrence, 1983). The reaction mixture consisted of 50 mM MES-KOH buffer, pH 6.5, 5 mM $MgCl_2$, 0.5 mM TPP, 6 mM pyruvate, 0.67 mM NADH, 1 mM DTT, and 3.5 units mL^{-1} of yeast ADH (Sigma). The reaction was initiated by adding protein extracts and the absorbance was read at 340 nm.

Alcohol dehydrogenase (ADH; EC 1.1.1.1) activity was measured by monitoring at 340 nm the reduction of NAD^+ during ethanol oxidation using an assay buffer of 0.5 M Tris-HCl, pH 9.0, 0.1 M ethanol, and 2 mM NAD^+ (Molina et al., 1987).

To measure the amount of pyruvate, freshly frozen biomass (100 mg) was gently homogenized in 1 mL 70% $HClO_4$. The homogenate was then neutralized using 5 M KOH and then centrifuged at 7,000 ×*g* for 10 min at 4 °C. The clear supernatant was used for spectrophotometric determination of pyruvate by the enzymatic assay coupled to NADH oxidation as described in Dinakar et al. (2010).

4.3.5 Protein *S*-nitrosylation

Leaf biomass (100 mg) was homogenized in 1.8 mL of 50 mM HEPES-KOH buffer (pH 8.0) containing 0.2% (w/v) SDS, 0.5% (w/v) CHAPS, 1 mM EDTA, and 0.1 mM neocuproine (which inhibits denitrosylation). The homogenate was centrifuged (15,000

×*g*, 10 min, 4 °C) and protein *S*-nitrosylation was determined by reducing R-SNO to R-SH in the presence of ascorbate and then measuring free thiol groups using 5,5'-dithiol-bis (2-nitrobenzoic acid) (Ma et al., 2016).

4.3.6 Electrolyte leakage rate

The relative electrolyte leakage rate was measured as described by Guo et al. (2017) . The leaf discs were placed in 10 ml distilled water and incubated for 2 h. After measuring the initial electrical conductivity (EC1) of the leaves, the samples were boiled for 30min to achieve the final electrical conductivity (EC2). The electrolyte leakage rate (ELR) was calculated by using the formula ELR = (EC1/EC2) × 100.

4.3.7 Transcript amounts

Total RNA was extracted from frozen leaf using the FastRNA® Pro Green Kit (MP Biomedicals). First strand of cDNA was synthesized from 5 µg total RNA using the Superscript III reverse transcriptase kit (Invitrogen). Quantitative PCR was carried out with an Applied Biosystems StepOnePlus Real-Time PCR 128 System, using SYBR Green qPCR Master Mixes (Thermo Scientific) and gene-specific primers (**Table 4.1**). Triplicate reactions were performed with three biological replicates, and the relative RNA expression was analyzed using the $2^{-\Delta Ct}$ method. The relative transcript abundance of target genes was normalized against geometric mean of the CT value of two reference genes, NtACT and glyceraldehyde phosphate dehydrogenase (GAPDH). Transcripts levels of *NtADH1* (alcohol dehydrogenase 1), *PDC1* (pyruvate decarboxylase), *NtACO1* (1-

aminocyclopropane-1-carboxylic acid oxidase), *NtACS1* (1-aminocyclopropane-1-carboxylic acid synthase), *NtRbohA,B,D* (plasma membrane NADPH oxidase, or respiratory burst oxidase), and *NtERF1,3,4,5* (ethylene-responsive factors belong to groups IX and VIII (only ERF3)) were analyzed in this study.

Table 4.1 Primers used for qRT-PCR assays of tobacco leaves

Primer	Forward sequence	Reverse sequence	Accession no/References
NtACT	TTGGCTTACATTGCTCTTG	TCATTGATGGTTGGAACAG	XM_033660572.1
GAPDH	GGCAGCATCCTTCAACATTATTC	CTCGGAAAGCCATTCCAGTTA	KR007670.1
NtADH1	AAGCTGGAGGAATTGTGGAGAG	ACCAGTGAACACAGGAAGAACA	Tan et al; 2019
NtPDC1	CCCGAACACCATTCACATTTC	CATCAGCAGATTCCACGATTTC	Tan et al; 2019
NtASC1	AAACGAGCCATTGCAACAAAGA	TGAATCCTGGTAAGCCCATGTC	Tan et al; 2019
NtACO1	GATTACACAAACAGACGGGACT	TTGATTCCACCACACACAATAC	Zhou et al., 2008
NtRbohA	AAGGTGTTATGAACGAAGTG	CTGGTGCCTGATACGATA	Xu et al., 2018
NtRbohB	CTATGCTTCAGTCTCTTCAC	GGCGTGTTGTCTTAGTTC	Xu et al., 2018
NtRbohD	ACCAGCACTGACCAAAGAA	TAGCATCACAACCACAACTA	Wu et al., 2008
NtERF3	AGGAATTGATCTTGATCTTAAC	ACAAAATTCAACCATTAGTCTC	Ogata et al., 2012
NtERF5	GCCCAAAGTTCCAATCTTGA	AACCTCCTGCTGCTTCATT	XM_009596177.3
NtERF4	CGGGTCTGGTTAGGTACTTTC	GTGACTTGGGCTCTGATTCT	XM_009611511.3
NtERF1	GGAAGGCATTACAGAGGTGTTA	CCAAACCCTAGCTCCATTCTT	XM_009613506.3

4.3.8 Statistical analysis

The software package SPSS V. 21.0 (Statistical Package for Social Science; Chicago, IL, USA) was used for statistical analysis. One-way ANOVA with Duncan's multiple range was used to identify significant differences between different lines of tobacco. The data in the text and on figures are the means of three biological repeats ± SD. The statistically significant differences at $P < 0.05$ are discussed.

4.4 Results

4.4.1 NO production and protein *S*-nitrosylation under hypoxia

Tobacco leaves when placed under hypoxia emitted NO, while NO emission from leaves incubated in air could not be detected using the method of chemiluminescence due to technical features of this method (Gupta and Igamberdiev, 2013). From 3 to 6 h of hypoxia, we observed an increase in NO emission in all plant lines (for B8 and RI29 the increase was insignificant). AOX overexpressors (B7, B8) had much greater NO rates than the WT, which exhibited an intermediate rate, while the NO emission rates of the *AOX* knockdown lines (RI9, RI29) were significantly lower than those of the WT (**Figure 4.1A**).

To confirm these results by the second method (Gupta and Igamberdiev, 2013) and to determine the amount of NO in the leaves exposed to normal air, we used an independent biochemical method to measure leaf NO content, which includes the interaction of NO with hemoglobin. Under normoxia, NO concentrations in RI29 and RI9 were nearly two times greater than in WT. NO amount in the B7 was lower than in the WT (and B8), while the mean NO amount in B8 was only slightly lower (and not significantly different) than in WT (**Figure 4.1B**). The level of NO in the overexpressors and WT increased significantly in hypoxic conditions but dropped in the knockdowns. Now, when compared to WT, NO levels were significantly higher in *AOX* overexpressors (particularly B8) and significantly lower in *AOX* knockdowns (particularly RI29), which is consistent with the chemiluminescence results (**Figure 4.1A**).

Under normoxia, the amount of protein *S*-nitrosylation (R-SNO) was significantly lower in the *AOX* overexpressors, compared to the WT and knockdowns (**Figure 4.1C**). The two knockdowns had consistently higher concentrations of RNO than the WT. In this case, the concentration of RNO was also considerably higher in the stronger knockdown (RI29) than the slightly leaky *AOX* knockdown (RI9). In response to hypoxia, R-SNO amount decreased in the WT and knockdown plants but did not change significantly in overexpressors. When compared to WT, the level of R-SNO in the overexpressors was only slightly lower and somewhat higher in the knockdowns (but not significantly different).

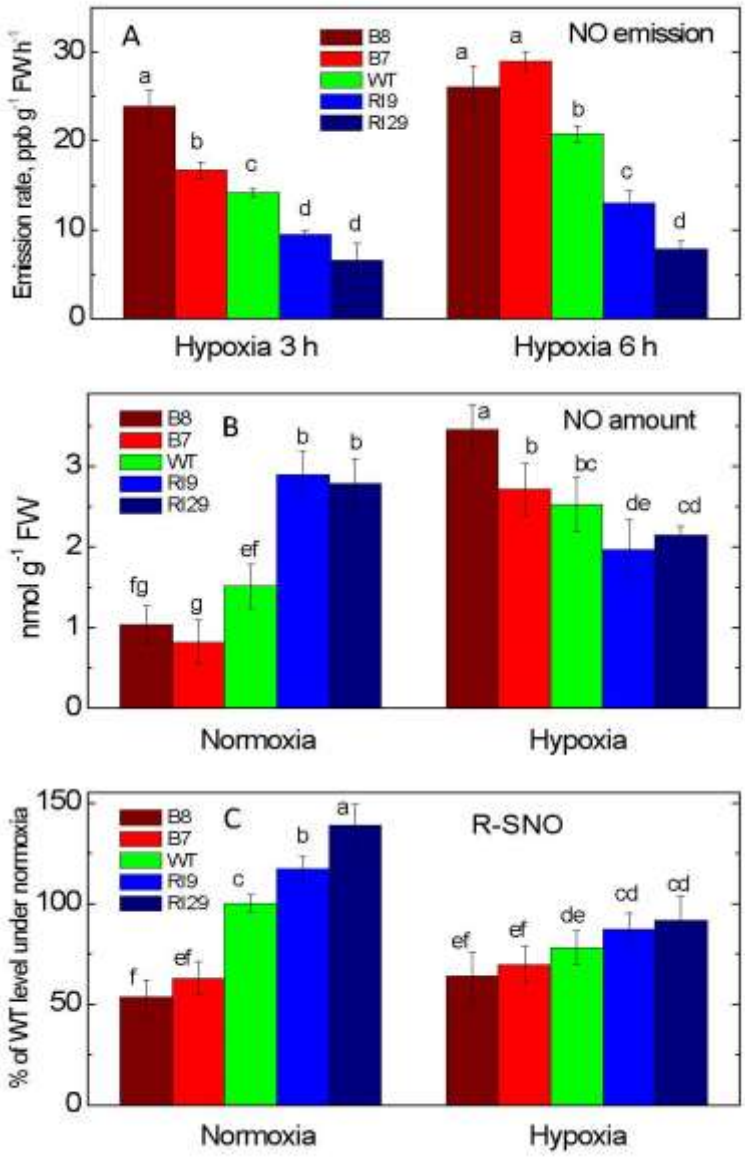

Figure 4.1 Leaf nitric oxide emission (A), nitric oxide amount (B), and the amount of protein S-nitrosylation (R-SNO) (C), in tobacco plants with differing amounts of alternative oxidase, and exposed to 6-hour of hypoxia.The plant lines used included wild type (WT), two alternative oxidase overexpressors (B7, B8), and two alternative oxidase knockdowns (RI9, RI29). Vertical bars indicate SD from three to four independent experiments (n = 3–4); different letters indicate significant differences between five tobacco lines and the time points.

4.4.2 ROS production and cell damage under hypoxia

To check the oxidative damage, we investigated the generation of ROS in plants subjected to a hypoxic (near anoxic, ~0.001% O_2) atmosphere. The level of O_2^- in tobacco leaves was significantly higher in the knockdowns than in other plant lines under normal conditions. The O_2^- level increased significantly in response to a 6 h hypoxia treatment and was the highest under this treatment in the knockdowns and the lowest in the overexpression plants, with WT showing an intermediate amount (**Figure 4.2A**). H_2O_2 levels were significantly higher in the overexpressor B8 and the knockdown RI9 than in the other lines under normoxia. Following hypoxia, *AOX* knockdowns exhibited greater H_2O_2 levels than WT, while the overexpression plants had similar H_2O_2 levels as WT (**Figure 4.2B**).

In addition to the burst of reactive oxygen species, the rate of electrolyte leakage, can also reflect the degree of oxidative damage in tobacco caused by hypoxic condition. AOX overexpressors, particularly B8, displayed less stress damage than other plant lines, according to our findings shown in **Figure 4.2C**. Higher ELR in AOX knockdowns revealed severe oxidative damage.

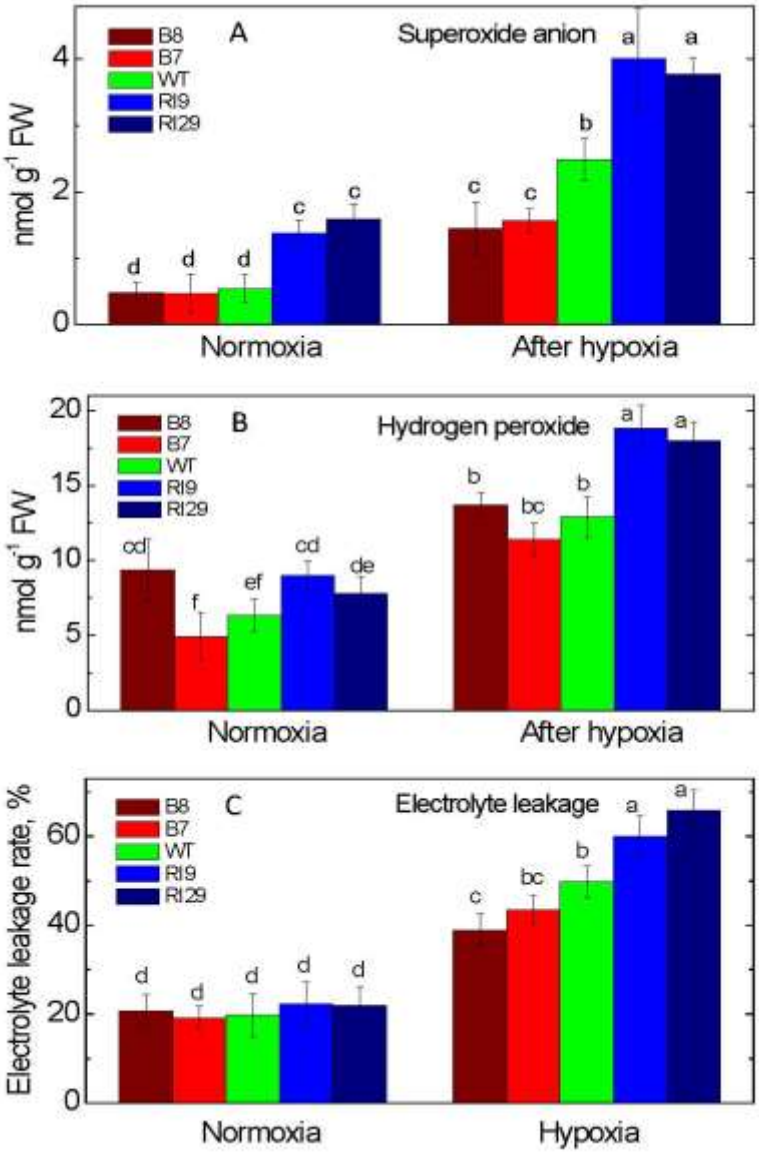

Figure 4.2 The level of superoxide anion (O_2^-) (A), hydrogen peroxide (H_2O_2) (B), and rate of electrolyte leakage (C), in tobacco plants with differing amounts of alternative oxidase, and exposed to 6 hours of hypoxia. The plant lines used included wild type (WT), two alternative oxidase overexpressors (B7, B8), and two alternative oxidase knockdowns (RI9, RI29). Vertical bars indicate SD from three to four independent experiments (n = 3–4); different letters indicate significant differences between five tobacco lines and the time points.

The transcript levels of the NADPH oxidase enzymes (Rbohs) generating ROS, determined by quantitative RT-PCR, revealed the differences between tobacco lines under normoxia and hypoxia. In air, *RbohB* expression was significantly higher in the knockdowns and then in the overexpressor B8, followed by other plant lines (**Figure 4.3A**). *RbohA* mRNA levels showed a similar pattern under normoxia with higher amount in the knockdowns and the overexpressor B7 (**Figure 4.3B**). However, the *RbohD* expression was similar across all plant lines relative to WT (**Figure 4.3C**). The transcript levels of these hypoxia-inducible genes were significantly increased after 6 h of hypoxia treatment and their transcripts were significantly higher in the knockdowns than WT and the overexpressors. The transcript abundance of *RbohD* and *RbohA* was similar between the WT and overexpression plants under hypoxia, but the level of *RbohA* was somehow lower only in the overexpressor B8 (**Figure 4.3B,C**). However, the transcript level of *RbohB* gene was affected under hypoxic stress with a similar level of increase in all plant lines (**Figure 4.3A**).

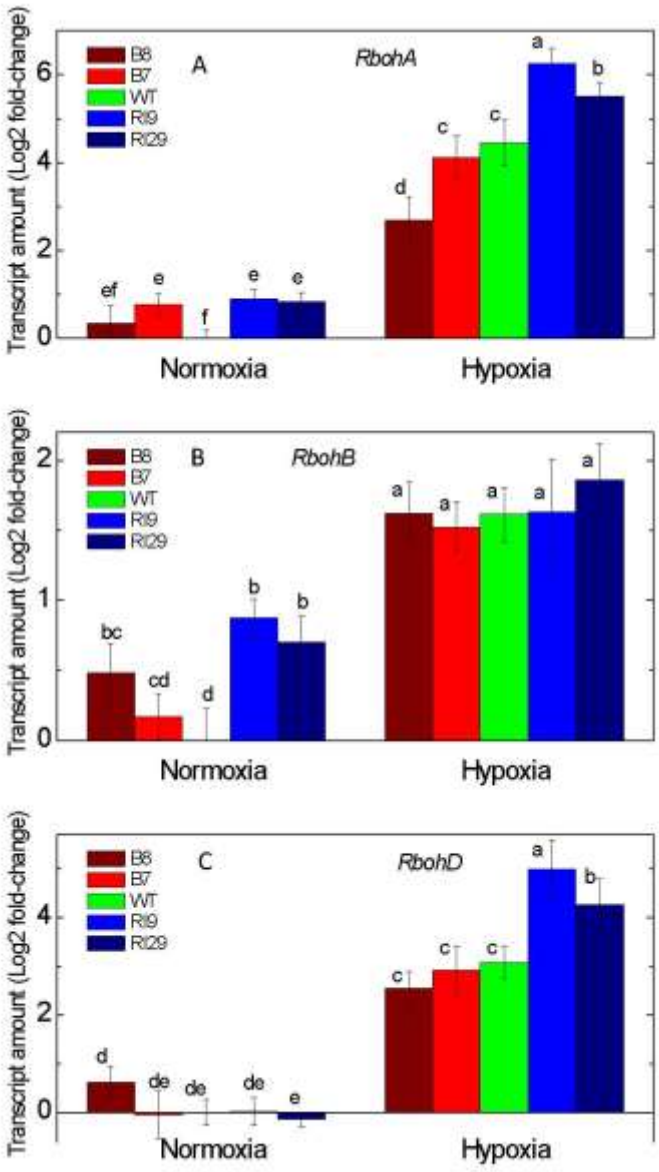

Figure 4.3 The transcript levels of the NADPH oxidase enzymes well-known as respiratory burst oxidase homologs (Rbohs A,B, and D)(A, B, and C, respectively) in tobacco plants with variable amounts of alternative oxidase, and exposed to 6-hour hypoxia. Vertical bars indicate SD from three independent experiments (n = 3); different letters indicate significant differences between five tobacco lines and the time points.

4.4.3 Antioxidant enzymes capacity of tobacco under hypoxia

We monitored the activities of antioxidant enzymes, which play important roles in plants to mitigate oxidative damage. In our study, we investigated the activities of SOD, CAT, GPX, APX, and GR (**Figure 4.4**). In air, the AOX upregulating plants and WT exhibited higher activities of CAT and APX as compared to the lower activity in the knockdowns; while SOD activity in the knockdowns was significantly higher than in overexpressors. Under normal air, no significant differences in GPX and GR activity were observed across all plant lines, with the exception of significantly higher GR activity in the overexpressor B7.

While the activity of SOD increased substantially in response to a 6 h hypoxia treatment in the knockdowns, the activity in the overexpressors and WT showed no significant changes (**Figure 4.4A**). The activity of GPX as a vital enzyme for the detoxification of H_2O_2, followed the similar pattern as SOD with the greatest activity in the knockdowns (**Figure 4.4B**). CAT, as a common antioxidant enzyme present almost in all living tissues, increased significantly under hypoxia in the overexpressors and WT, but no significant changes of CAT activity were observed in the knockdowns (**Figure 4.4C**). In response to 6 h hypoxia, the activities of APX and GR were drastically altered. APX activity in the overexpressors and WT decreased significantly and still they had higher activity compared to the knockdowns (**Figure 4.4D**). Under hypoxia, GR activity increased substantially in the overexpression plants while WT and the knockdowns revealed no significant changes and displayed the similar activity (**Figure 4.4E**).

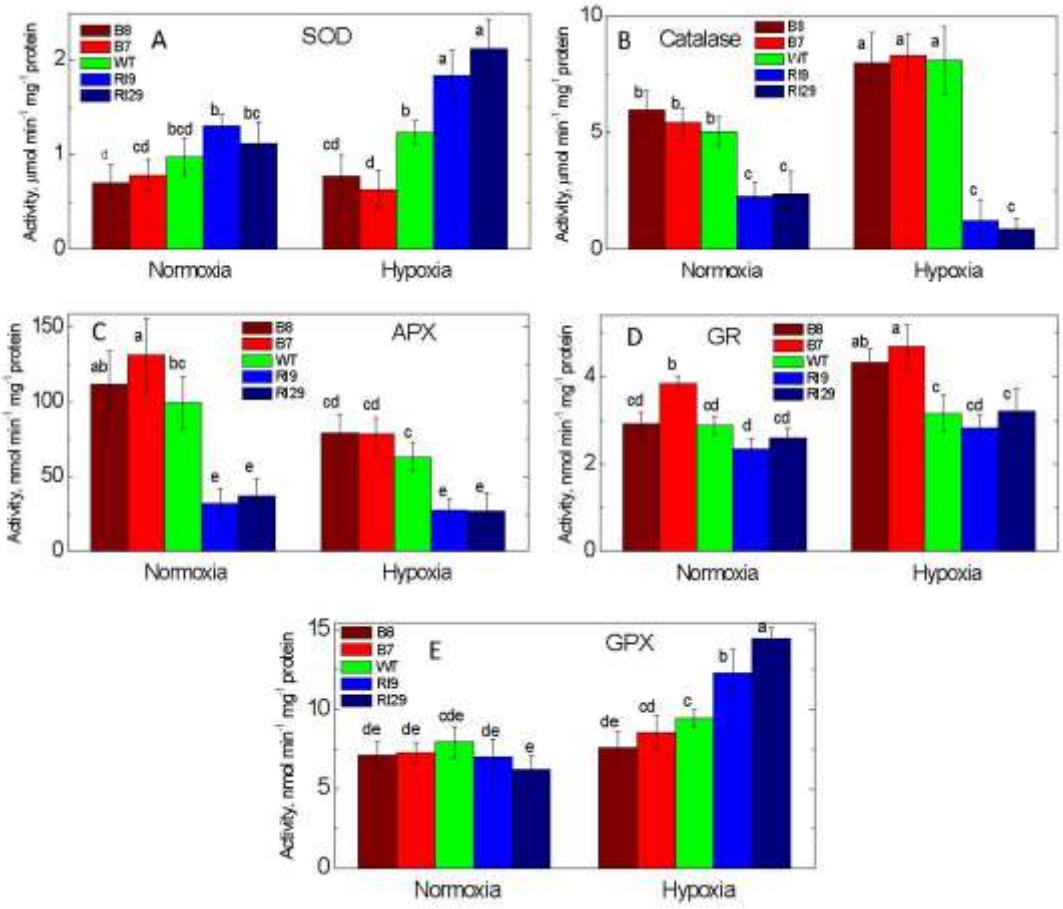

Figure 4.4 The activity of superoxide dismutase (SOD; A), catalase (B), ascorbate peroxidase (APX; C), glutathione reductase (GRD, D), and glutathione peroxidase (GPX; E) in tobacco plants with differing amounts of alternative oxidase, and exposed to 6 hours of hypoxia. The plant lines used included wild type (WT), two alternative oxidase overexpressors (B7, B8), and two alternative oxidase knockdowns (RI9, RI29). Vertical bars indicate SD from three to four independent experiments (n = 3–4); different letters indicate significant differences between five tobacco lines and the time points.

4.4.4 Fermentation under hypoxia

To examine the effect of the introduced AOX transgenes on the ethanol fermentation pathway, the mRNA levels (**Figure 4.5A,B**) and enzyme activities of PDC1 and ADH1 (**Figure 4.5C,D**) were analyzed in tobacco leaf under aerobic and hypoxic conditions. Under normal air, *ADH1* and *PDC1* expression and activity did not differ significantly between the WT and the other plant lines, except for *PDC1* expression which was marginally higher in the knockdowns RI29 (**Figure 4.5A**). In response to anaerobic condition, the activity and relative expression of the hypoxia-responsive genes *ADH1*, and PDC1 increased substantially. *PDC1* transcript abundance was higher in overexpression plants than in WT, while PDC1 activity was increased with a similar pattern (**Figure 4.5C**). The knockdowns (particularly RI29) had lower expression and activity of PDC1 in relative to WT. *ADH* expression was elevated to the same level in the WT and overexpressors, whereas ADH activity peaked in the overexpressor B7 (**Figure 4.5C,D**). *ADH1* in the knockdowns, on the other hand, showed lower levels of mRNA and enzyme activity (**Figure 4.5E**).

The amount of pyruvate was significantly higher in the knockdowns under both aerobic and anaerobic conditions (**Figure 4.5E**). Following oxygen deprivation, the level of pyruvate did not differ significantly, except for the knockdown RI29 where it increased as compared to its level in normal air.

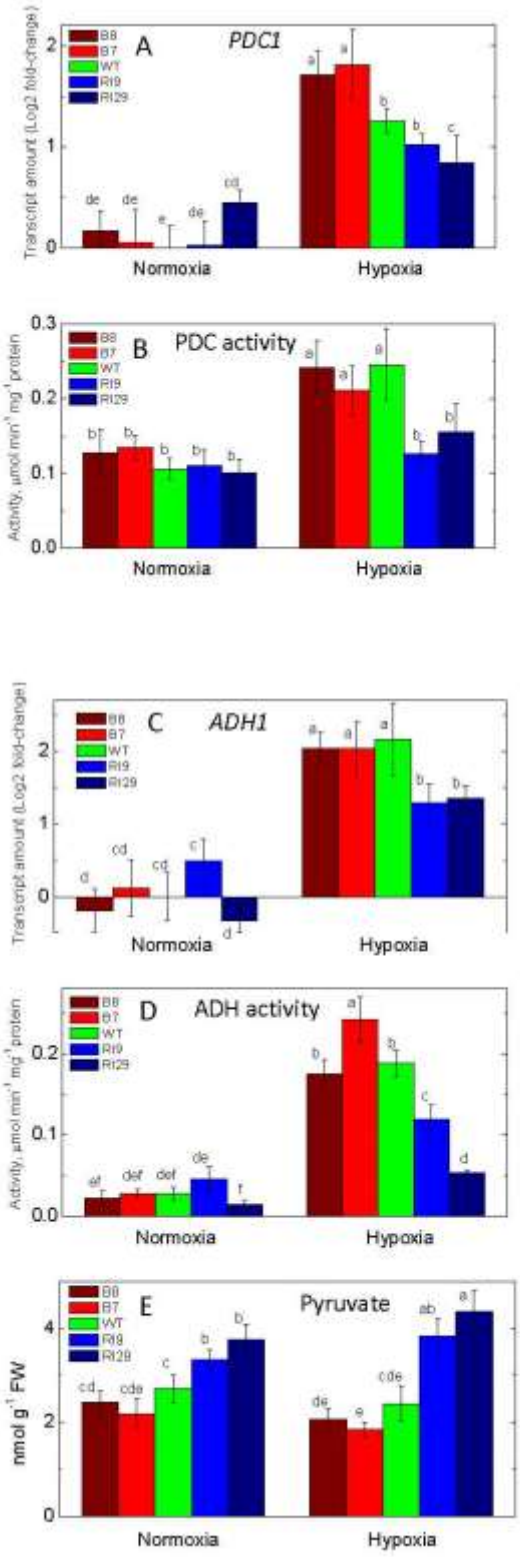
A
PDC1
Transcript amount (Log2 fold-change)
B8
B7
WT
RI9
RI29
Normoxia
Hypoxia
B
PDC activity
Activity, µmol min-1 mg-1 protein
C
ADH1
D
ADH activity
E
Pyruvate
nmol g-1 FW

Figure 4.5 The transcript levels and activities of pyruvate decarboxylase 1 (PDC1; A, B) and alcohol dehydrogenase 1 (ADH1; C, D), and amount of pyruvate (E) in tobacco plants with variable amounts of alternative oxidase, and exposed to 6-hour hypoxia. Vertical bars indicate SD from three independent experiments (n = 3); different letters indicate significant differences between five tobacco lines and the time points.

4.4.5 Enzymes of ethylene synthesis and ERF induction under hypoxia

In response to hypoxia, the transcripts abundance of the enzymes of ethylene synthesis 1-1-aminocyclopropane-1-carboxylic acid synthase (ACS1) and aminocyclopropane-1-carboxylic acid oxidase (ACO1) strongly increased in the WT and *AOX* overexpression plants to the similar levels, with the knockdowns (particularly RI29) showing slightly lower increases (**Figure 4.6A,B**).

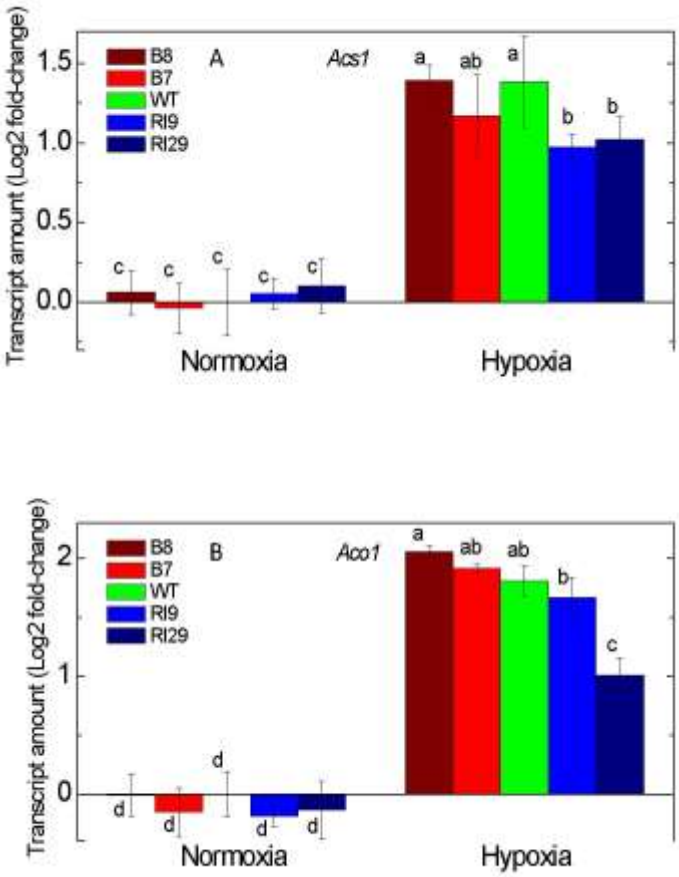

Figure 4.6 The transcript levels of 1-aminocyclopropane-1-carboxylic acid synthase (ACS1) and aminocyclopropane-1-carboxylic acid oxidase (ACO1) in tobacco plants with variable amounts of alternative oxidase, and exposed to 6-hour hypoxia. Vertical bars indicate SD from three independent experiments (n = 3); different letters indicate significant differences between five tobacco lines and the time points.

The expression of four factors of the ethylene response factor family (*ERF1, ERF3, ERF4, and ERF5*), which perceive low-oxygen signals and play a crucial role in determining survival in low-oxygen environments (**Figure 4.7**), also showed the response to hypoxia, which in some cases was affected by *AOX* expression. In air, all plant lines exhibited almost

the same low expression level of all ERFs tested in this study. The hypoxic treatment increased ERFs transcripts abundance in all plant lines, compared to normoxia. After 6 h hypoxia, ERF1 and ERF4 transcript amounts were significantly higher in the overexpression plants and WT, as compared to the knockdowns showing the lower transcript amount (**Figure 4.7A,C**). All plant lines showed similar high mRNA levels of *ERF3* and *ERF5* under hypoxia treatment (**Figure 4.7B,D**). Although the *ERF3* expression was slightly higher in the knockdown RI29 was higher than overexpressor B8.

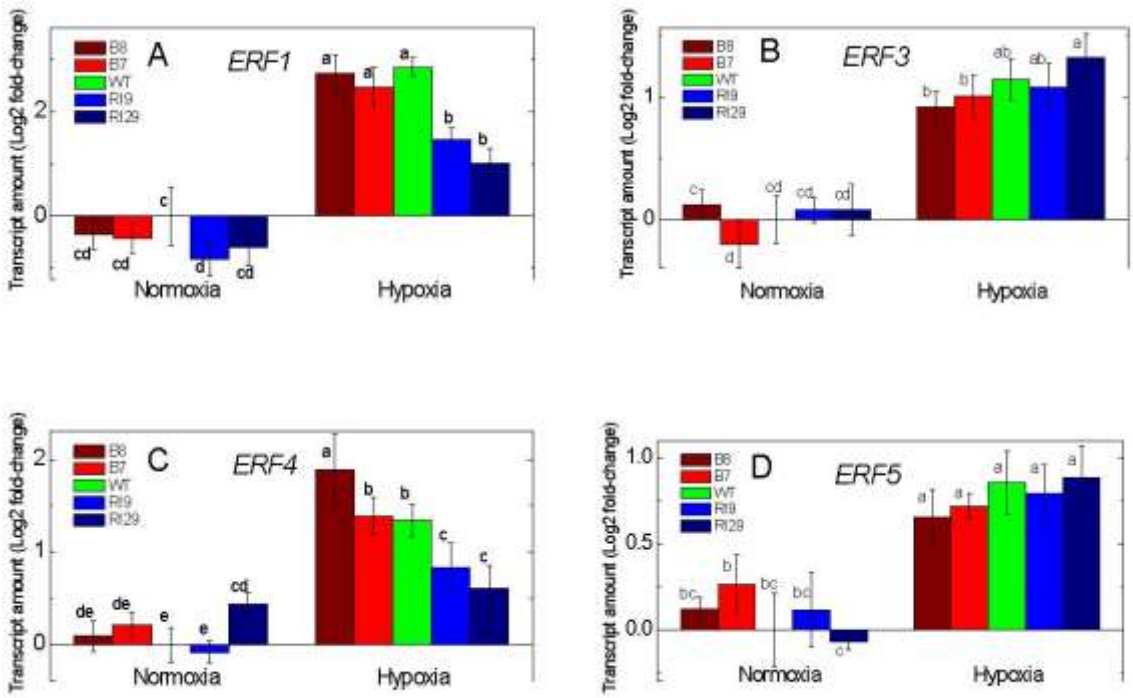

Figure 4.7 The transcript levels of ethylene response factors (ERF1,3,4,5) (A, B, C, D, respectively), in tobacco plants with variable amounts of alternative oxidase, and exposed to 6-hour hypoxia. Vertical bars indicate SD from three independent experiments (n = 3); different letters indicate significant differences between five tobacco lines and the time points.

4.5 Discussion

4.5.1 NO metabolism under hypoxia

Nitric oxide (NO) is a crucial signaling molecule in plants since it is quickly produced, stimulates particular activities within cells, and is rapidly scavenged. The AOX knockdown plants have higher levels of leaf NO in normoxia in relative to WT tobacco plants. The higher amount of protein *S*-nitrosylation can be linked to an increase in NO generation in this plant line. AOX presence can reduce electron transfer across complexes III and IV under normoxia, avoiding electron leakage to nitrite and subsequent NO accumulation in *AOX* overexpressors (Cvetkovska and Vanlerberghe, 2012).

In contrast to its regular function under normoxia, AOX plays a unique role in hypoxia, where it can increase nitrite-dependent NO production (Vishwakarma et al., 2018; Jayawardhane et al., 2020). *AOX* overexpression plants have higher NO production compared to *AOX* knockdown under hypoxia (**Figure 4.1A,B**). Endogenous NO plays an important role in the regulation of target proteins by post-translational modifications (PTMs). NO-mediated *S*-nitrosylation of various proteins, including ERF-VIIs, COX, aconitase, and APX, might be connected to flooding signaling and tolerance (Sasidharan et al., 2018; Gibbs et al., 2014). Furthermore, NO can activate ethylene production, potentially by *S*-nitrosylation of important enzymes such ACS and ASO (Li et al., 2016). The S-nitrosylation of RbohD reduces its function, limiting the cell death caused by stress-induced oxidative burst (Yun et al., 2011). Given the substantial role of NO in gene regulation, metabolism, and physiology, it's reasonable to assume that AOX supports continued NO production, maybe through the efficient operation of the Pgb1-NO cycle.

Pgb1 expression was shown to be higher in *AOX* overexpressors and lower in *AOX* knockdowns according to our recent study (Zafari et al., 2022). Under hypoxia, the crosstalk between AOX, and NO, drives the Pgb1-NO cycle, which modulates NO metabolism influencing the function of target genes/proteins (Zafari et al., 2022).

4.5.2 Oxidative damage under hypoxia

The ROS levels change in a dynamic and rapid manner in plant tissues under environmental stress. Under normal air, knockdown of *AOX* increased the leaf amount of O_2^- (**Figure 4.2A**), implying that AOX respiration is critical in limiting ROS production by the mitochondrial ETC. Apart from knockdowns, the overexpressor B8 enhanced the amount of H_2O_2 in the leaves under normoxia, revealing an intriguing and convoluted relationship between AOX and H_2O_2 levels (**Figure 4.2B**). NADPH oxidase, which is encoded by the Rbohs genes, is a major generator of ROS in plants (Morales et al., 2016). Under normal air, we found that *RbohA* and *RbohB* transcript levels were slightly greater in the knockdowns and, also in one of the overexpressors, which could explain the high amount of H_2O_2 under normoxia.

Following hypoxia, both reactive species rose, and O_2^- and H_2O_2 levels were substantially lower in the overexpressors and much higher in the knockdowns, as compared to WT, indicating the important role for AOX in keeping ROS levels under control by regulation the production and removal systems of ROS. After 6 h hypoxia, we observed the high expression of all *Rbohs* genes tested in this work. The pattern of H_2O_2 level across plant

lines was mirrored by the expression levels of *Rbohs* (especially *NtRbohD* and *NtRbohA),* which were also elevated in the knockdowns and reduced in the overexpressors in comparison to WT (**Figure 2B**). In our research, a higher level of AOX decreased the production of H_2O_2 in overexpressors under hypoxia through regulating the expression of the *Rbohs* genes. Both ROS can act as signaling molecules when produced in a tightly controlled manner, or as cell damaging factors when produced in an uncontrolled fashion (Moreau et al., 2010). Superoxide anion can interact with NO initiating the ROS-dependent NO degradation pathway that involves thioredoxins (Wulff et al., 2009). H_2O_2 can activate the expression of *ERF73* and *ADH*, via modulation of ET signaling under oxygen deprivation (Yang 2014). By controlling the expression of *Rbohs*, we assume that AOX determines the signal's strength and specificity by keeping H_2O_2 at a relative level, which has to be elucidated further in this hypoxic model. Electrolyte leakage is widely used as an indicator of membrane damage caused by produced ROS. ELR was lower in plants expressing AOX which clearly indicates that AOX alleviates anaerobic stress-induced oxidative damage by controlling ROS production.

4.5.3 Antioxidant defense system under hypoxia

Antioxidant enzymes scavenge superfluous ROS caused by stress conditions and protect plants from oxidative damage. The increases in antioxidant systems have been reported in plants lacking AOX under stress circumstances (Amirsadeghi et al., 2006; Watanabe et al., 2008). Following hypoxia, the activity levels of SOD and GPX were shown to be significantly greater in the transgenic plants lacking AOX, correlating with higher levels

of superoxide anion. Giraud et al. (2008) demonstrated that in the AOX1a T-DNA insertion lines, the expression of *FeSOD2, FeSOD3* and other antioxidant enzymes was up-regulated. These findings revealed that, whereas AOX may directly restrict ROS formation, other antioxidant defense systems can supplement this effect in knockdowns. H_2O_2 levels need constant control to prevent hydroxyl radical production via Fenton chemistry, in particular, when CAT levels are low in the conditions of hypoxia/anoxia. In our work, significant increase in CAT activity was observed in the plants overexpressing *AOX*. Other investigations observed the increases in the activity of numerous antioxidative enzymes under waterlogging/flooding, such as APX (Biemelt et al. 1998), GR and CAT (Ushimaru et al. 1997).

Following hypoxia, GR activity was considerably higher in the overexpressors than in the other plant lines. Under hypoxia, the highly reducing circumstances could be mirrored by the increased GR activity, keeping the antioxidants (ASA and GSH) in their physiologically active and reduced states through the oxidation of NADH and NADPH (Biemelts et al., 1998). We presume that the antioxidant defense systems can be induced by ROS generation when the lack of AOX takes place. However, the high basal activities of antioxidant defense systems in the overexpressors under any conditions, aerobic and anaerobic, indicates that AOX maintains the function of the antioxidant enzymes to some extent in favor of the antioxidant to oxidant ratio.

4.5.4 Fermentation under hypoxia

In hypoxia, a combination of glycolysis stimulation and the TCA cycle slowing may raise pyruvate levels (António et al., 2016). Pyruvate accumulation is hypothesized to trigger

AOX activity, which stimulates the TCA cycle carbon flow and so reduces pyruvate levels (Vanlerberghe et al., 1995). Our findings back this up by revealing that plants that upregulate AOX had lower pyruvate levels, meaning that pyruvate moved into fermentation pathways. The metabolic transition in plants may be caused by the acidification of cytoplasm during anoxia, and by pyruvate moving into the fermentation pathway (Fox et al., 1994). The up-regulation of fermentation pathways for energy production by boosting ADH and PDC activity is one of the earliest and best-studied responses to low oxygen (Mustroph et al. 2010; Narsai et al. 2009). Following hypoxia, *PDC1* and *ADH1* transcript increased considerably in all plant lines studied here, with highest level in overexpressors. It indicates that AOX improves the activity of PDC and ADH indirectly, presumably through the binding of hypoxically induced ERFs to their gene's promoters. In plants that overexpress *AOX*, this leads to the enhanced ATP and NAD^+ production.

4.5.5 ET synthesis enzymes and ERF induction under hypoxia

ET is synthesized from *S*-adenosyl-L-methionine (SAM) via 1-aminocyclopropane 1-carboxylic acid (ACC) in the reactions catalyzed by ACS and ACO.

Here, the transcript levels of *ACS1* and *ACO1* were higher in the plants expressing *AOX*. We believe that AOX-induced NO can boost ET production by inducing ACO and ACS activity via S-nitrosylation. In turn, the generated ET can stimulate Pgb1 production which regulates NO metabolism as revealed by van Veen et al., 2013 and Hartman et al., 2019.

The ubiquitin-mediated protein degradation regulates ERFs protein stability via the 26S proteasome pathway. NO targets ERF-VII for proteasomal degradation through an O_2^- dependent N-end rule pathway. Since NO-mediated PTMs have a strong relationship with ubiquitylation-mediated proteasomal degradation of proteins, we hypothesize that AOX-induced Pgb1 impacts the ubiquitination and degradation of other groups of ERFs by modulating NO levels.

Deprivation of oxygen increases the activity of ACSs, which increases ET synthesis and activates downstream genes such *ERF73* and *ADH1* (Peng et al., 2005). ET accumulation leads to *ERFs* (particularly *ERF-VII*) gene induction and ERFs participate in Arabidopsis defense downstream of ET signaling (Zhao et al., 2012; Brown et al., 2003). ERFs, on the other hand, they could be regulators of *ACO* expression as reported in tomato, banana, and rice (Zhang et al., 2009; Han et al., 2016; Iwamoto et al., 2010). AOX-induced ET production as a result of hypoxia-induced elevated redox levels leads ERFs activation and is controlled by ERFs feedback mechanisms which in turns control cellular Et level.

In this study, hypoxia increased the quantity of ERFs transcripts in all plant lines. The examined ERF1 and ERF4 positively correlated with the overexpression of AOX under hypoxic stress. Studies show that *ERFs* genes (Particularly ERF-VII) are key factors in improving plant tolerance to hypoxia by increasing anaerobic gene expression, *ADH*, *PDC*, and ROS metabolic enzymes (Licausi et al., 2010; Yin et al., 2019; Zhang et al., 2016). A lack of AOX may lead to leaf PCD in response to hypoxia, as indicated by the severe oxidative damage found in this study, with elevated ROS and ELR in the kockdowns. According to Ogata et al. (2012), PCD induction may be mediated by the tobacco

transcriptional repressor ERF3, which is somewhat higher in the *AOX* knockdown, RI29. We assume that ERFs particularly ERF1 and ERF4 as ET signaling pathway markers can either activate or suppress the genes encoding fermentation enzymes, and Rbohs, and antioxidant enzymes through directly binding to genes promoters.

4.6 Conclusion

From the findings presented in this work, a model can be proposed that explains some of the aspects of stress signaling during anoxia (**Figure 4.8**). In this model, AOX levels control the intensity and maybe specificity (H_2O_2 vs. O_2^-) of the generated ROS signal via modifying the level of Rboh and antioxidant systems. By damping the electron leak from the ETC and increasing Pgb1, AOX could modulate the intensity of the NO. AOX can induce ET biosynthesis genes and ERFs directly, or indirectly by modulated level of NO and ROS. Produced ET might control the NO production through Pgb1 activation, and induced ERFs can regulate Rboh and fermentation to keep energy levels stable during anoxia. We propose that AOX regulates the interaction of NO, ROS, and ET in the cell, which act as signals for the onset of tobacco responses in the absence of oxygen.

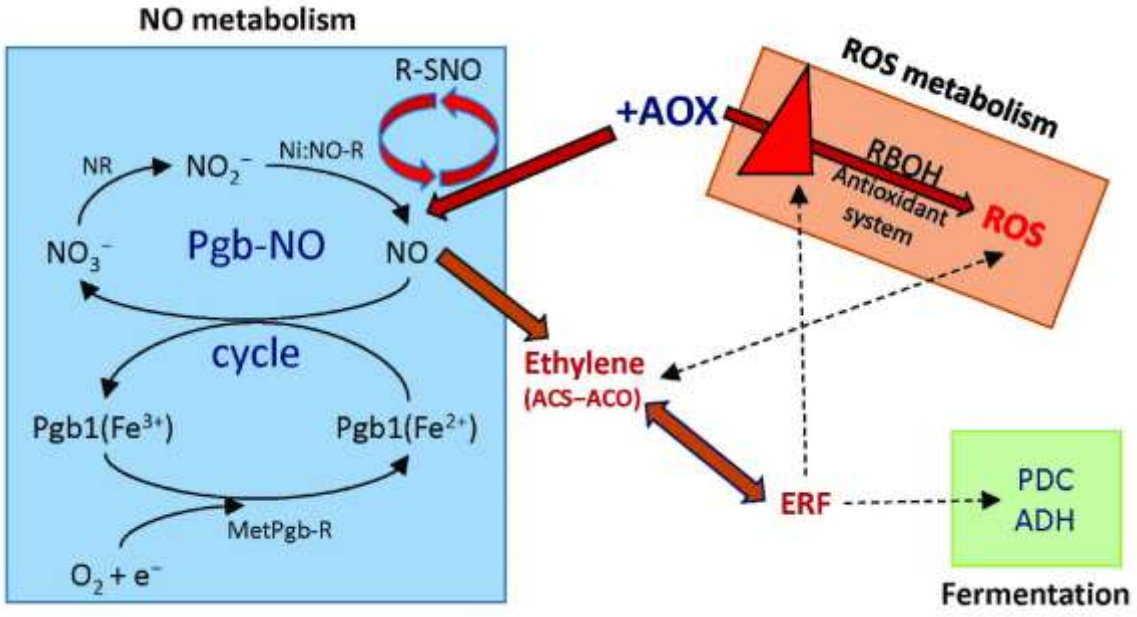

Figure 4.8 A model for the role of AOX in the activation and regulation of NO, ROS, ET to improve tobacco resistance to hypoxia.

Chapter 5

Summary and future directions

Summary and future directions

Plant mitochondria function under a variety of growth phases (e.g., seed germination) and adapt to stress situations. In the absence of sufficient oxygen content, nitrite appears to serve a significant role as a terminal electron acceptor in the mETC. NO is produced in mitochondria from nitrite, which diffuses into the cytoplasm. NO is toxic to cells at high concentrations, but it can also play a part in a variety of signaling systems that control and regulate plant growth and development. The increased turnover of NO generated by mitochondria, as well as its subsequent scavenging by Pgb1 and the enzymes that reduce methemoglobin, will have important consequences such as maintaining mitochondrial stability for NADH oxidation and possibly ATP generation.

The expression of non-symbiotic class 1 *Pgb* is induced under diverse biotic and abiotic stress conditions. Their primary function is to scavenge NO, resulting in an increase in the Pgb1-NO cycle's turnover. *Pgb1* upregulation in transgenic barley plants not only regulates NO metabolism and supports mitochondrial function, but it also influences the expression of many other genes, either directly or indirectly, through the signaling properties of NO during germination. The expression of several genes involved in the germination process is also influenced. During the anoxic stages of germination, changes in AOX and GSNOR gene expression and activity could contribute to a shift in ROS generation patterns and protein *S*-nitrosylation. The data presented in Chapter 2 provides evidence for a link between the Pgb1-NO cycle and AOX production during anoxia. Thus, as shown in chapters 3 and 4, the current work contributes to a better understanding of the role of mitochondrial AOX during anoxia in tobacco leaves expressing varied levels of AOX.

We hypothesized that AOX regulates the amount of NO and ROS by modulating their production and scavenging. The amount of AOX could mediate plant adaptation to stress by determining the degree of the response by weak or robust stimulation of defence pathways under abiotic stress. Recent evidence suggests that AOX regulates NO generation and signalling, and that NO is required to activate the AOX pathway under stress conditions (Kumari et al., 2019). However, the specific role of AOX under stress conditions, as well as whether AOX is directly implicated in NO generation, is still unknown and debated. In this study, we tested the idea that one of AOX's functions is to regulate NO production via influencing the expression and activity of Pgb1, NR, GSNOR, and protein S-nitrosylation.

In hypoxia, AOX increases NO levels, allowing S-nitrosylation to change target protein function; however, in normoxia, AOX is important for maintaining NO levels and possibly decreasing NO inhibition of cyt oxidase. In the presence of overexpressed AOX, the significantly enhanced NO production supports the Pgb1-NO cycle in maintaining the energy balance (ATP) in tobacco leaves under anoxia. We believe that AOX is involved in NO generation in tobacco leaves during anoxia based on our findings. However, AOX will not be the only pathway or mechanism involved in NO generation; other pathways and mechanisms may also be involved. Furthermore, *AOX*-overexpression helps tobacco leaves maintain the energy status more efficiently during anoxia than other plant lines by making effective use of the Pgb1-NO cycle and fermentation.

While it is widely established that AOX activation is linked to a NO and ROS burst, the specific mechanism and importance of this link is unknown. The chemical nature and

relationships between these signaling molecules, for example, are still up for debate. Based on this research, it is likely that AOX promotes hypoxia resistance via a regulated level of NO (as mentioned above) and ROS. In this model, AOX activity would modulate the strength of the signals, or the total amount of NO and ROS being produced.

According to the findings of this study, when oxygen levels are low, AOX aids in the generation of a modest amount of ROS in tobacco leaves. Elevated H_2O_2 levels in tobacco seedlings connected to Rboh-mediated electron transport from NADPH to O_2 resulting in H_2O_2 production shortly after anoxia is imposed. The subsequent ROS burst may enhance the antioxidative responses and trigger the expression of downstream beneficial targets including fermentation genes (e.g. *ADH*) required for hypoxia acclimation. When *AOX* is downregulated, the antioxidative system (particularly CAT, GR, and APX) is not fully engaged to lower ROS levels in tobacco leaves, resulting in elevated levels of ROS. Thus, for tobacco resistance to anoxia, the balance between ROS production via Rboh and scavengers is critical, which is apparently controlled by AOX levels in this study.

Both NO and ROS are influenced by AOX and have an impact on hypoxic ethylene signaling. While NO destabilizes ERF-VIIs, ethylene-inducible and N-end rule target, Pgb1 scavenges it, establishing a mechanistic relationship between ethylene and NO. During anoxia, ethylene gene biosynthesis and ERFs increase in tobacco expressing *AOX*, possibly making ethylene a reliable hypoxia indicator. We think that ethylene affects H_2O_2 signaling in the hypoxia pathway by regulating *Rboh* expression, which then activates downstream defence activities including fermentation and metabolic alterations.

Many metabolic processes, such as glycolysis, fermentation, the TCA cycle, and amino acid metabolism, are fine-tuned during anoxia. AOX helps driving the anoxic fermentation by raising ADH and PDC activity, which regenerates NAD+ and produces a low level of ATP. The activation of the non-photorespiratory serine metabolism and the GABA pathway as a result of AOX participation in NO turnover represents an important adaptive mechanism for storing carbon and nitrogen that would otherwise be lost under oxygen-deficient conditions.

According to our findings, NO and ROS are key signaling molecules for the initiation and execution of defence responses during anoxia. AOX levels control the intensity and specificity (H_2O_2 vs. O_2^-) of the generated ROS signal via modifying the Rboh and antioxidant systems, influencing tobacco tolerance to anoxia. By damping the electron leak from the ETC and boosting Pgb1 and NR, AOX could modulate the intensity of the NO. AOX-induced ethylene biosynthesis genes and ERFs might control the NO and ROS amount through Pgb1 and Rboh activation. ERFs and Rbohs may initiate fermentation to keep energy levels stable during anoxia. We propose that AOX plays a key role in the mitochondria for the control of NO, ROS and ethylene levels, which act as signals for the induction of tobacco responses during oxygen shortage.

A model (**Figure 4.8**) for plant metabolic pathways cannot be developed only by using one species, one plant part or one sort of stress. It would be beneficial if we check the role of AOX on defence signaling pathways in both anoxia/hypoxia-intolerant and tolerant species and in plant roots (in contrast to leaves). Mitochondria act as an early source of NO and

ROS production under stress, hence using mitochondrial suspensions would be more efficient.

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