Plant mitochondria under hypoxia: a cross-talk between nitric oxide, hemoglobin and oxygen

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Abstract:

The major goal of the present study is to describe the mechanisms by which plants adapt to deficiencies in the electron transport chain, either in terms of lack of the functional electron transport complex or lack of final electron acceptor (oxygen). In plants, the main function of class 1 hemoglobins is to scavenge nitric oxide (NO). Under hypoxia and anoxia, there is a significant increase in hemoglobin expression which upregulates the turnover of the Hb-NO cycle. This cycle not only participates in the electron transport chain, but NO influences various signalling processes which govern plant development, morphology and physiology. The current study demonstrates that NO produced under hypoxia induces *AOX1A* gene expression, which is translated to a higher AOX activity and leads to a shift towards biosynthesis of amino acids. It also demonstrates that respiratory complex I deficiency results in low NO levels and upregulation of fermentation pathways. The current study undertakes extensive efforts to characterise the morphological, biochemical and physiological effects of modified hemoglobin expression in barley. Results show a difference in observations when compared with other model organisms such as *Arabidopsis*. NO also influences the process of senescence. Hence we investigate the process of senescence under hypoxia and in plants with modified hemoglobin expression. The study also involves generating of three barley transgenic lines to further study the role of hemoglobin in plant development, physiology, pathology, nutrition and stress tolerance.

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This thesis is dedicated to my father Mr. Kumar Shah, who has been struggling with his life since 2012. He is living on a machine and I, his son need to fund it to keep it running for the family to survive. The thesis is stained with his sacrifice of not informing me about the details of his health to keep my moral high.

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List of Abbreviations:

ABA : Abscisic acid

ACO : 1-aminocyclopropane-1-carboxylate oxidase

ADH : Alcohol dehydrogenase

AOX : Alternative oxidase

ATF : Alanine amino transferase

COX : Cytochrome *c* oxidase

CPTIO : 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide

cNR : cytosolic nitrate reductase

GA : Gibberellic acid

Hb: hemoglobin

HvHb : *Hordeum vulgare* Hemoglobin

Kd : Dissociation constant

Km: Michaelis-Menten constant

MS medium : Murashige and Skoog medium

NOS : Nitric oxide synthase

nsHb: non-symbiotic hemoglobin

PM-NR: Plasma membrane bound nitrate reductase

RNS : Reactive nitrogen species

ROS : Reactive oxygen species

SD : Standard deviation

SHAM : salicylhydroxamic acid

sHb: symbiotic hemoglobin

SNP : Sodium nitro prusside

trHb: truncated hemoglobin

TCA: Tricarboxylic acid

Chapter 1: Introduction and Literature review

1.1. Mitochondria and the electron transport chain

One of the defining events in the evolution of eukaryotes is the origin of mitochondria. The serial endosymbiosis theory is the most widely accepted as the basis of mitochondrial evolution. The theory states that mitochondria are the direct descendants of a bacterial endosymbiont that became established at an early stage in a nucleus-containing host cell. Phylogenetic studies support the theory of monophyletic origin of the mitochondrion from a eubacterial ancestor shared with a subgroup of the α-Proteobacteria. The main role of this organelle is to generate energy and metabolic intermediates in a form which can be utilised to perform cellular functions.

Mitochondria generate cellular energy via the well-studied, complex electron transport chain. The mitochondrial electron transport chain is located in the inner mitochondrial membrane. The reducing equivalents (NADH and NADPH) generated by the tricarboxylic acid cycle (TCA) cycle, glycolysis and the pentose phosphate pathway and oxidation of fatty acids are used by the mitochondrial electron transport chain to generate a proton motive force, energy from which is used to generate ATP. Electrons from NADH or succinate pass through a series of spatially arranged redox reactions from an electron donor molecule to an electron acceptor molecule, ultimately received by a terminal oxidase that reduces molecular oxygen to water. The passage of electrons through the intermediary electron acceptors is accompanied by translocation of protons

into the intermembrane space of mitochondria, which results in the generation of electrochemical gradient, which is used to drive ATP synthesis via ATP synthase.

In addition to the complexes I-IV, the plant mitochondrial electron transport chain contains two unique features. A non-proton-pumping alternative oxidase and a rotenone insensitive, non-proton-pumping NAD(P)H dehydrogenases on each side of the inner membrane (Møller 2001). Figure 1.1 describes the plant mitochondrial electron transport chain. The structures in green represent the unique features of plant mitochondria and the structures in orange represent the components of the mitochondrial electron transport chain common to both plants and animals.

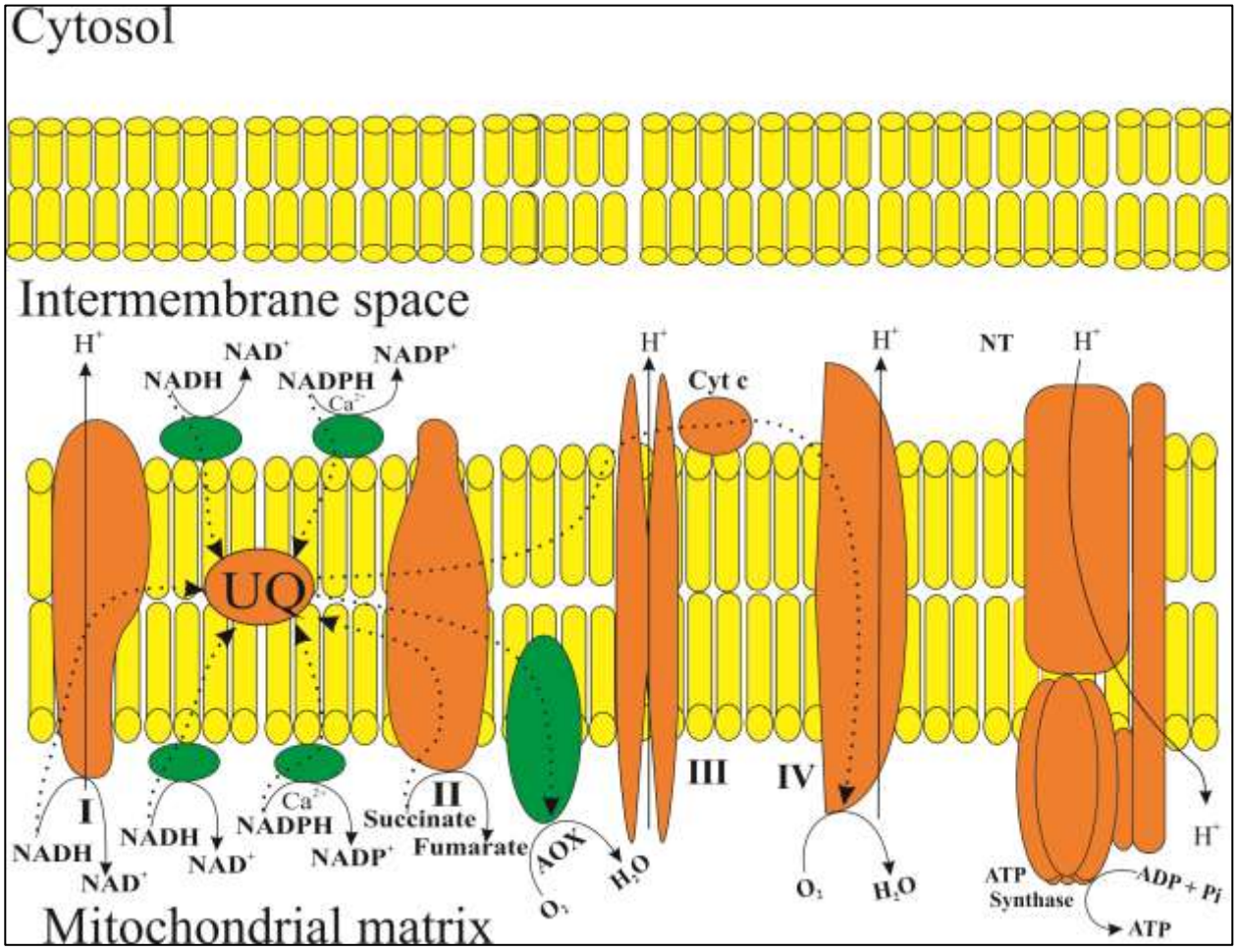


Figure 1.1: Mitochondrial electron transport chain. Dashed lines represent the path of electrons (adapted from Taiz and Zeiger, 2010). The structures in green represent the unique features of plant mitochondria and the structures in orange represent the general scheme of the mitochondrial electron transport chain.

1.2. Nitric Oxide

Nitric oxide (nitrogen monoxide) is a compound with the chemical formula NO. It was discovered by Joseph Priestley in 1772. It is a colourless and odourless gas. Its molar mass is 30.01Da, density 1.3402 g dm^{-3} and it is linear in shape. Neutral $NO^{\cdot}$ has a single electron in its 2p-π antibonding orbital (Marletta and Tayeh 1990). The removal of this electron forms NO^{+}; conversely the addition of an electron to this orbital forms NO^{-} (Rinden 1989). The charge neutrality of $NO^{\cdot}$ has been assumed to facilitate its free diffusibility in aqueous medium and across cell membranes (Goretski et al., 1988). Nitric oxide readily forms complexes with the transition metal ions, including those regularly found in metalloproteins. The reactions with heme-containing proteins have been widely studied, particularly in the case of hemoglobin (Doyle and Hoekstra 1981). The bond length between the nitrogen and oxygen atom is 115 pm, which is very similar to the oxygen molecule whose bond length is 121 pm. NO was proclaimed the "molecule of the year" by the journal *Science* in 1992 (Lancaster 1992). NO plays an important role in mammals as it participates in the operation of the nervous system, and is essential in mammalian physiology and immunology. Nitric oxide was first reported to play an important role in plants in 1998 (Delledonne 1998; Durner 1998). NO plays an important role in seed germination (Beligni 2000), senescence (Guo 2005), disease resistance (Delledonne and Xia 1998; Mur et al., 2012), cold stress tolerance (Zhao and Chen 2009), high salt stress tolerance (Corpas et al., 2009) and in organ development. Nitric oxide binds to Fe(III)-porphyrins. The association rate constants are influenced by the protein structure at the heme pocket and range from 10^{3} to 10^{7} $M^{-1}s^{-1}$ (Sharma et al., 1983). The

Fe(III)NO^{-}-heme complex appears to undergo a charge transfer reaction to form Fe(II)NO^{+}. Subsequent loss of NO^{+} occurs readily by attack of ambient nucleophiles (Gwost et al., 1973; Wade and Castro 1990). The net effect is that the Fe(III)-heme adduct exhibits rapid loss of NO, with rate constant in the range of 0.65-40 s^{-1}(Sharma et al., 1983). Nitric oxide also forms non-heme transition metal complexes. There is considerable interest focused on its reactivity towards iron-sulfur centers in proteins, which include several enzymes involved in mitochondrial electron transport chain and TCA, such as aconitase (Gupta et al., 2012). NO inhibits the electron transport chain via competitive inhibition of cytochrome *c* oxidase (complex IV) by binding to the oxygen-binding site (Millar and Day 1996, Brown et al., 2001). This is one of the reasons for the toxicity caused by NO, which is due to the suppression of the respiratory oxygen uptake and generation of superoxide due to over-reduction of the ubiquinone pool (Shiva et al., 2001).

1.3. Hemoglobin

The first thing that comes to our mind when we read the word hemoglobin is oxygen transport proteins present in erythrocytes in the circulatory system of blood. For many, it may be surprising that plants, which do not contain blood, encode hemoglobins. The name hemoglobin is derived from the globular structure associated with the heme prosthetic group, which binds to oxygen. Apart from oxygen, hemoglobins also bind other ligands, predominantly nitric oxide, carbon monoxide, hydrogen sulfide and other

organic molecules present in membrane lipids (D'Angelo and Lucarelli 2004; Rinaldi et al., 2006).

1.4. Evolution and groups of hemoglobins

Plant hemoglobins constitute a diverse group of hemeproteins and evolutionarily belong to three different classes, namely class 1, class 2 and class 3 hemoglobins. Class 1 hemoglobin possesses an extremely high affinity for oxygen, with a dissociation constant (K_d) on the order of 2 nM (Hargrove et al., 2000; Smagghe et al., 2009). Their main function is scavenging NO (Gupta and Igamberdiev 2011). This is a very important role, as nitric oxide is an essential molecule influencing signalling and biotic and abiotic stress response (Baudouin 2011). Class 2 hemoglobins have a lower oxygen affinity, with a K_d of the order of 150 nM (Dordas 2009). Its function is related to facilitating oxygen supply to developing tissues (Spyrakis et al., 2011). Most of the symbiotic hemoglobins have evolved from class 2 hemoglobins. Many consider them as a subclass of class 2 that have acquired specific structural properties to support oxygen symbiosis with nitrogen-fixing bacteria via buffering of oxygen concentration. In nitrogen-fixing root nodules, leghemoglobin (legHb) plays an important role in transporting free oxygen away from the oxygen sensitive nitrogenase enzyme. The characteristic red colour of the nodules is due to hemoglobin, which can be present at a concentration of up to 0.7 mM. Plants do produce hemoglobins with a non-symbiotic role, the concentrations of which are usually very low in the range of 2-20 μM. This is too low concentration for the plant tissues to appear red or pink in colour (Hebelstrup et al., 2007). It should be noted that a few

symbiotic hemoglobins (sHb), such as *Parasponia* Hb have originated from class 1, which are classically thought to have a non-symbiotic role (nsHb). Class 3 hemoglobins are also referred to as truncated hemoglobins (trHb) and represent a group with very low similarity to class 1 and 2 hemoglobins and a low affinity for oxygen, with K_d on the order of ~1500 nM. Their function is not well defined. A few reports suggest that they may be related to regulation of oxygen delivery at high oxygen concentrations (Watts et al., 2001).

1.5. Coordination chemistry of hemoglobins

Based on coordination chemistry of the heme iron, hemoglobins can be divided into two groups, namely hexacoordinate and pentacoordinate hemoglobins. The heme prosthetic group contains an iron atom with four of the six coordination sites occupied by the heme pyrrole nitrogen atoms. It is further attached to histidines of the globin moiety through coordination of either one or two histidine side chains (Trent and Hargrove 2001). The hemeproteins with one histidine coordinating the fifth site of the heme iron, leaving the sixth open for exogenous ligand binding are designated as pentacoordinate. Examples include the hemoglobin present in erythrocytes and other oxygen-transporting hemoglobins. The hemeproteins in which the iron coordinates with both the proximal and distal histidine are called hexacoordinate, the example is cytochrome b_5. Most class 1 hemoglobins have a single conserved cysteine residue located in the E-helix of the polypeptide chain. This helix is bent towards the center of the porphyrin ring due to a direct coordination of His(e7) with the iron atom (Hargrove et al., 2000). *Arabidopsis*

class 1 hemoglobin has two cysteine residues per monomer, located adjacent to one another in the same positions where one cysteine is found in the other class 1 hemoglobins (Trevaskis 1997). Most plant leghemoglobins do not contain cysteines at all (Uchiumi ; Stróżycki 2000). Human blood hemoglobin contains two cysteine residues in the β-chain and one in the α-chain. Cysteine-93 of the β-chain contributes to the maintenance of cooperativity and ligand binding (Cheng et al., 2002). Human myoglobin contains a single cysteine having a role in reduction of heme iron, a process during which the inter-molecular disulfide bond is likely to be formed (Hirota et al., 2005). The proximal histidine is bound tightly to the heme iron; the coordination of the distal histidine is reversible and differs significantly. This allows binding of exogenous ligands such as oxygen, nitric oxide and carbon monoxide. Pentacoordinate hemoglobins are adapted to the function of oxygen buffering and delivery because the pentacoordinate state allows oxygen to bind reversibly, hence giving optimal condition for storage and transportation (Gupta et al., 2011).

When we compare the class 1, class 2 and class 3 groups of hemoglobin to the two groups based on coordination chemistry, two of the classes, namely class 1 and class 2, exhibit the evolution from non-symbiotic hexacoordinate towards symbiotic pentacoordinate hemoglobins. This is observed more in class 2 and to a lesser extent for class 1 hemoglobins. The class 1 nsHb has the highest hexa-coordination level, whereas the degree of coordination in class 2 nsHb is much lower (Gupta et al., 2011).

1.6. Genes encoding hemoglobins

Gene sequence analysis among the three classes shows that class 1 and class 2 plant hemoglobins are distantly related to animal myoglobin and blood hemoglobin, whereas the primary structure of the class 3 hemoglobin suggests that this class is closer to bacterial hemoglobins (Watts et al., 2001). The class 3 hemoglobins share 40-50% sequence similarity with bacterial hemoglobins of the 2-on-2 structural motif. The presence of this class of hemoglobins in plants is suggested to be an event of horizontal gene transfer from bacteria. The class 1 and class 2 plant hemoglobins are characterised by a 3-on-3 sandwich model. In plants, class 3 hemoglobins have a longer sequence than class 2 and class 1 (Smagghe et al., 2009). The molecular structures of the class 2 barley and rice hemoglobin and of the class 2 soybean hemoglobin show a marked similarity with animal myoglobin and hemoglobin (Hoy et al., 2007). There is also a similarity at the exon-intron structural level of the respective genes encoding these proteins. Animal myoglobin and hemoglobin genes have two conserved introns which are common with nsHb and sHb. However nsHb and sHb have an additional third intron between the two conserved introns. It is interesting that such third intron is also found in genes of neuroglobins and cytoglobins of vertebrates and in hemoglobin genes from invertebrates (Burmester et al., 2004; Roesner et al., 2005). Human neuroglobin, which resembles plant class 1 hemoglobin in hexacoordination of heme and high affinity for oxygen and NO, contains three cysteine residues that participate in intra- and intermolecular disulfide bonds depending on the redox state of the environment (Dewilde et al., 2001).

1.7 Class 1 hemoglobins

Class 1 hemoglobins are non-symbiotic, having a low value of hexacoordination equilibrium constant (K_H), which is the binding constant of the distal histidine, allowing the equilibrium of pentacoordinated and hexacoordinated species and facilitating the binding of ligands. This weak hexacoordination causes an extremely high avidity for oxygen (Hargrove et al., 2000). The rapid oxygen binding results in a conformational change, which prevents fast dissociation from the heme site. Upon binding of a ligand, the distal histidine moves away from the iron atom, resulting in an overall stable confirmation. This allows a very tight and slowly reversible binding of oxygen. This is optimal for oxygen dependent NO scavenging under hypoxia (Perazzolli et al., 2004; Hoy et al., 2007). Class 1 hemoglobins are characterized by an increased rate of Fe^{3+} reduction. This reduction is mediated by a cysteine residue, which can form a reversible covalent bond between two monomers as shown by mass spectrometric analysis. This also protects the molecule from autoxidation (Bykova et al., 2006).

The above cited structural and biochemical properties of class 1 hemoglobins allow them to serve as soluble electron transport proteins in the enzyme system scavenging NO produced under hypoxic conditions, primarily via reduction of nitrite in plants (Dordas et al., 2003). Class 1 hemoglobins exist as homodimers with subunits of approximately 18 kDa. The equilibrium dissociation constant of the non-covalent dimerization of rice ferric Hb is 86 μM (Hargrove et al., 2000). The stability of the oxyferrous compound against autoxidation is provided by the interaction of distal histidine with the bound ligand

through a strong hydrogen bond (Smagghe et al., 2008). The large increase in autoxidation upon cysteine mutation could be related to the disruption of these interactions in combination with solvent entry into the distal heme pocket. Cysteine may contribute to the maintenance of the distal heme pocket through the formation of a stable conformation state. In human myoglobin, the cysteine residue may participate directly in reduction of heme iron, which results in intermolecular disulfide bond formation (Hirota et al., 2005). This bond is not permanent and appears as a part of the reduction-oxidation cycle of the myoglobin molecule. This could be also the explanation for the role of cysteine in barley and other class 1 plant hemoglobins.

Barley class 1 hemoglobin is a homodimer containing a single cysteine per monomer (Duff et al., 1994; Taylor et al., 1994). The concentrations of class 1 hemoglobins in plant tissue are very low even upon induction. In barley, they constitute roughly 0.04% of the total soluble protein, whereas in wheat and wild oat, concentrations are as low as 0.01% (Duff et al., 1998). Hb amounts are highest in the root (0.3%) and aleurone layer (0.12%) of barley seedlings. Concentrations in coleoptiles are very low at 0.02% (Hebelstrup et al., 2007). It is usually in the range of 5-20 μM (Hill et al., 1998; Hebelstrup et al., 2007). This concentration is two orders of magnitude lower than the concentration of leghemoglobin in root nodules and three orders lower than hemoglobin in erythrocytes (Igamberdiev et al., 2011).

1.8. Hemoglobin and nitric oxide cycle under hypoxia

At sufficiently low oxygen concentrations, hemeproteins are deoxygenated and become capable of reducing nitrite to NO in a reversal of the reaction in which NO is converted to nitrate or nitrite by oxygenated hemeproteins. Nitric oxide produced from nitrite at low pH is a common chemical phenomenon. This reaction is facilitated by reducing agents such as ascorbate and phenolics (Bethke et al., 2004). Hemeproteins can serve both as NO scavengers and NO producers. If oxygen concentration is low and the hemeprotein molecule is deoxygenated, NO can be formed from nitrite, while at oxygen concentrations at which hemeproteins are oxygenated, NO conversion to nitrate or nitrite occurs (Gladwin et al., 2008). If the affinity of hemeprotein to oxygen is extremely high, as in the case of hexacoordinated hemoglobins induced in plants under low oxygen conditions, it is unlikely that the hemoglobin will function to produce NO as the oxygen dissociation constant is in the order of 2 nM (Duff et al., 1997; Dordas et al., 2003, 2004). The ability of deoxygenated hemoglobin to reduce nitrite to NO was first described by Brooks (1937). Nitrite represents a biological reservoir of NO that can regulate major physiological processes (Gladwin et al., 2008). Maximum NO production is observed at approximately 50% hemeprotein oxygen saturation (Gladwin et al., 2008; Grubina et al., 2007). Following is the reaction

$$NO_2^- + Fe^{2+} + H^+ \longrightarrow NO + Fe^{3+} + OH^-$$

$$NO_3^- + NADH + H^+ \longrightarrow NO_2^- + NAD^+ + H_2O$$

The opposite reaction of NO scavenging is described by the following reaction

$$NO + Fe^{2+} + O_2 \longrightarrow NO_3^- + Fe^{3+}$$

The mechanism of NO scavenging by hemoglobin has been investigated in plants exposed to anoxic conditions and shown to have an important physiological role in the maintenance of redox and energy balance (Igamberdiev et al., 2004, 2006; Stoimenova et al., 2007). The opposite process leading to NO formation during anoxia has been demonstrated in plants (Dordas et al., 2003; Planchet et al., 2005).

Hemeproteins of the mitochondrial electron transport chain, which include cytochrome *c* oxidase (COX) (Castello et al., 2006, 2008), bc_1 complex (Kozlov et al., 1999), and cytochrome *c* (Basu et al., 2008) can participate in NO formation from nitrite. The reaction is not limited to hemeproteins. Molybdocofactors of nitrate reductase (Dean and Harper 1998), xanthine oxidase (Yamasaki and Sakihama 2000) and iron-sulfur clusters (Li et al., 2004, 2008) have also been shown to be capable of producing NO.

1.9. The Hb/NO cycle:

The basis of the hemoglobin/NO cycle is the conversion of nitrate to nitrite in the cytosol. Nitrite then enters the mitochondria and is converted to NO by various complexes of the electron transport chain as discussed above. The formed NO leaves the mitochondria and is converted to nitrate by class 1 hemoglobins, facilitated by reducing agents.

Conventional knowledge says that under oxygen-limiting conditions, ethanol and lactic acid formation provides a mechanism to produce ATP and to oxidise glycolytic substrates. There is now increasing evidence for a second process which involves class 1 hemoglobin and nitric oxide produced via nitrite reduction. NADH oxidation is achieved in the reactions forming NO and its subsequent oxidation back to nitrate. The extremely high affinity of hemoglobin for oxygen facilitates the high turnover of this cyclic process at extremely low oxygen concentrations (Igamberdiev and Hill 2004).

The fermentation pathway triggered under hypoxia leads to the formation of lactate. This is accompanied by a marked decline in cytosolic pH (Saint-Ges et al., 1991; Kennedy et al., 1992; Gout et al., 2001). As the pH lowers, it slows the rate of lactate formation (Hanson and Jacobson 1984). Subsequently, pyruvate decarboxylase and alcohol dehydrogenase are activated, which diverts glycolytic carbon flow towards ethanol (Kennedy et al., 1992). Alanine, formed via amination of pyruvate, is second only to ethanol as a product of glycolytic flux during hypoxia (Gibbs and Greenway 2003). The formation of alanine does not consume NADH, potentially leading to a decline in glycolytic flux due to the unavailability of electron acceptor (Igamberdiev et al., 2004). A portion of the requirement for an electron acceptor may be supplied via nitrate reduction, providing the ammonium ions required for the transamination reaction forming alanine. Activation of nitrate reductase during hypoxia as a result of pH decline suggests that nitrate reduction plays an important role under hypoxia (Botrel et al., 1997). Alkalization of the medium observed during nitrate uptake may be linked to the prevention of acidification of the cytosol under hypoxic conditions (Steffen et al., 2001).

Various reports have suggested that nitrate indirectly influences the functional state of mitochondria under hypoxia which leads to the inhibition of the mitochondrial electron transport chain (Vartapetian et al., 1999, 2003; Rawyler et al 2002).

Nitrate uptake can be regulated by plasma membrane redox activity (Steffen et al., 2001). The H^+-ATPase of the plasma membrane and cytosolic nitrate reductase are both regulated by 14-3-3 proteins (Finnie et al., 1999). Proton-motive force generated via a redox loop mechanism on the plasma membrane may induce NAD(P)H oxidase linked putatively to PM-NR and/or Ni-NOR via cytochrome b_5 and phylloquinone found in plasma membrane preparations (Bridge et al., 2000).

Anoxic maize cells overexpressing class 1 hemoglobin have lower ADH activity compared with the wild type lines and lines down regulating hemoglobin (Sowa et al., 1998). Higher hemoglobin levels would result in a greater turnover of NO in the Hb/NO cycle, which oxidizes NADH. In plants overexpressing hemoglobins, lower $NADH/NAD^+$ and $NADPH/NADP^+$ ratios have been reported (Igamberdiev et al., 2004). In plants with downregulation of Hb, these ratios increase drastically under low oxygen tensions. Hence it is evident that the expression of hemoglobin in hypoxic cells assists in maintaining the energy status of the cell and also assists in maintaining the redox status of the cell. It can be hypothesised that hemoglobin does it via influencing the fermentation pathways and the operation of Hb/NO cycle along with a membrane-associated electron transport.

1.10. Effect of hemoglobin and nitric oxide on growth and development

A review published by Kim Helebstrup, Jay K. Shah and Abir U. Igamberdiev titled “The role of nitric oxide and hemoglobin in plant development and morphogenesis” describes the role of NO and Hemoglobin in plant growth and development.

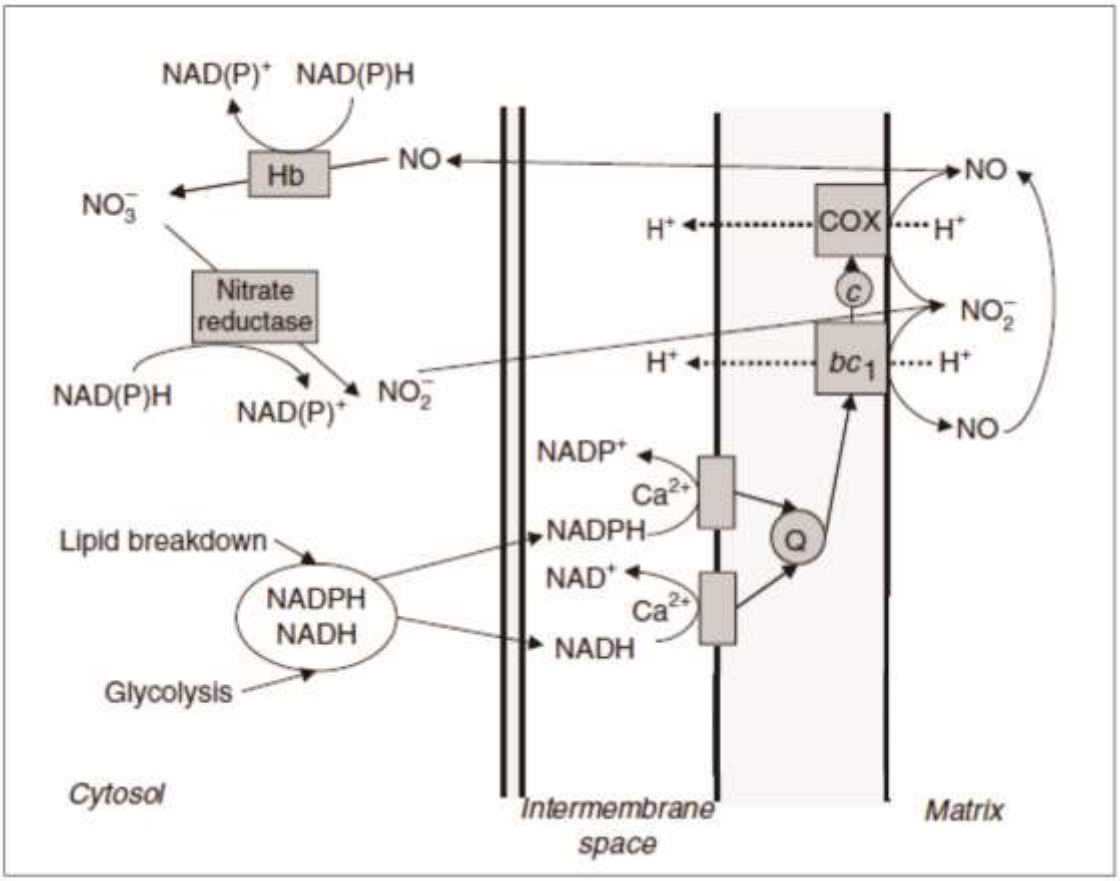


Figure 1.2: The Hb/NO cycle (adapted from Igamberdiev et al., 2009).

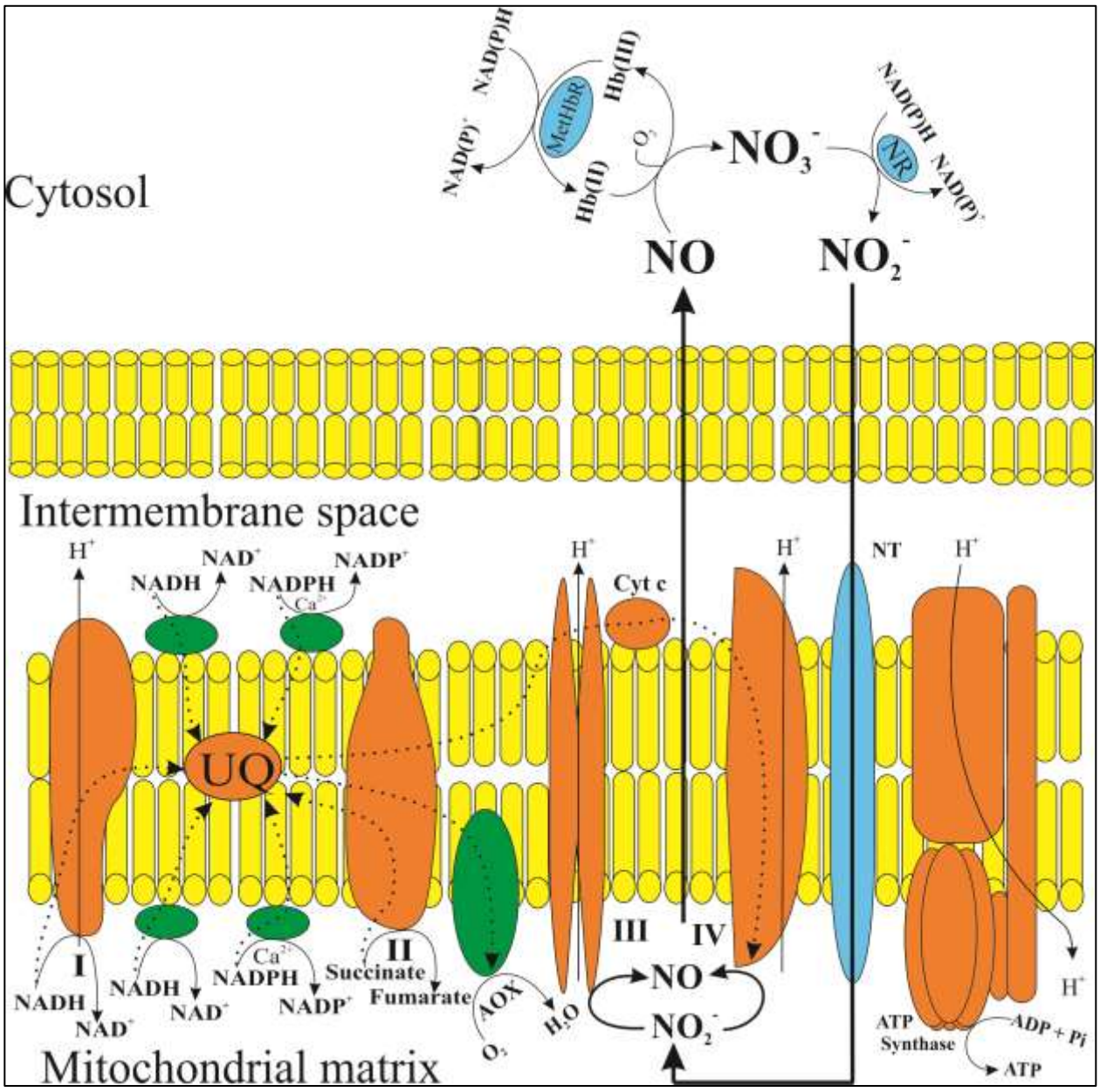


Figure 1.3: Hb/NO cycle incorporated in the mitochondrial electron transport chain. The structures in green represent the unique features of plant mitochondria and the structures in orange represent the general scheme of the mitochondrial electron transport chain. (Adapted from Hebelstrup et al., 2013)

1.10. Effect of hemoglobin and nitric oxide on growth and development

Nitric oxide influences various processes in plant growth and development starting from seed dormancy, seed germination (Beligni and Lamattina 2000; Bethke et al., 2004; Zhao et al., 2009; Gniazdowska et al., 2010), vegetative growth of shoots, cell division, xylem differentiation, root development, gravitropic bending (Leshem 1996), pollen tube growth (Guo 2003, Pardo 2004, Otvos 2005, Shapiro 2005), flowering and senescence (He 2004, Crawford 2005, Guo 2005) and iron homeostasis (Murgia 2004). Figures 1.4 and 1.5 summarise the effects of NO and Hb on plant growth and development.Nitric oxide interferes with seed dormancy and germination (Bethke 2004), which was demonstrated using NO donors and scavengers. In *Lactuca sativa*, which requires light for germination, NO treatment allowed seeds to germinate in the dark. External application of NO leads to greening and reduced elongation of the hypocotyl. NO partially restores etiolation and helps to perform normal growth of etiolated leaves (Beligni 2000). Involvement of NO has also been implicated in lateral root development and suppression of primary root growth (Shapiro 2005; Kolbert et al., 2008; Jin et al., 2011). A reduction of NO production with plant maturation and senescence has been observed (Shapiro 2005). Application of NO delays flowering time, maturation and senescence (Crawford 2005, Shapiro 2005).

There are various mechanisms by which NO modulates plant growth and development. NO has been reported to mediate the effect of plant hormones. Cytokinins help in

proliferation of hypocotyl cells and promote growth of seedlings. They also restore normal growth in etiolated plants (Beligni 2000). It is also reported that cytokinins induce nitric oxide synthesis in plants (Tun 2001). This suggests that NO may be a downstream signal of cytokinins. NO mediates the abscisic acid (ABA)-induced changes in water and ion transport of guard cells in the leaf epidermis (Bright 2006; Zhang 2011). NO administration causes closure of stomata via the effects of ABA (Shapiro 2005, Bright 2006). NO activates cGMP synthesis (Shapiro 2005) and also leads to S-nitrosylation (Gupta 2011) or tyrosine nitration of proteins (Moreau et al., 2010). Hence, NO activates cGMP, which influences ABA levels in plants and leads to a closure of stomata. ABA induces Ca^{2+} release from the endoplasmic reticulum and in turn opens Ca^{2+}-sensitive ion channels of the cell membrane (Shapiro 2005, Bright 2006, Sun 2010). Efflux of ions and water leads to cell shrinkage and consequently to stomata closure (Shapiro 2005). NO mediates the effect of auxin on cell division and root branching (Otvos 2005, Kolbert 2008, Jin 2011, Romera 2011).

The functional studies on hemoglobins in plant development have shown that hemoglobin gene expression is predominantly seen in meristematic tissues. Meristematic tissues are often depleted in oxygen due to its high O2 consumption and compact packing of cells. Hemoglobin gene expression in these tissues are involved in controlling development and morphogenesis. This effect is correlated to modulation of the endogenous level of NO. The mechanism behind plant hemoglobin function is based on its ability to directly control NO by oxidation in a biochemical cycle described as the Hb/NO cycle. The effect

of NO is present throughout the life of a plant starting from seed germination and finishing at plant senescence (Hebelstrup et al.,, 2013)

Chapter 2: The effects of nitric oxide on alternative oxidase and biosynthesis of amino acids

2.1. INTRODUCTION

2.1.1. Alternative oxidase

Plants and fungi possess a unique cyanide-resistant alternative mitochondrial respiratory pathway (Figure 1.1). It involves a single protein called alternative oxidase (AOX). In most organisms, this complex is encoded by a small family of nuclear genes (Vanlerberghe & McIntosh 1997; Millar et al., 2011;). Electron transfer via the AOX pathway branches at the ubiquinone pool and bypasses complex III and IV, which are sites for translocation of protons to the matrix, hence electron transport via AOX does not lead to the generation of ATP and the released energy is lost as heat. AOX activity can only lead to proton translocation when coupled to the electron flow through complex I acting as NADH dehydrogenase.

2.1.2. Expression of the alternative oxidase genes

AOX pathway is influenced by various stimuli such as stress (cold, oxidative, pathogenic attack) and by factors restricting electron flow through the cytochrome pathway. Expression of AOX is controlled both at the gene expression and at the post-translational modification levels. AOX reduces molecular oxygen to water in a single four electron transfer step (Day et al., 1991, 1995; Moore 1991). It is resistant to cyanide, but is inhibited by substituted hydroxamic acids such as salicylhydroxamic acid (SHAM) and n-

propylgallate (Schonbaum et al., 1971; Siedow 1981). *AOX1* gene expression responds to particular active oxygen species such as superoxide, hydrogen peroxide or the hydroxyl radical. The treatment of plant cells with hydrogen peroxide induces the alternative oxidase pathway (Vanlerberghe 1996, Wagner 1995). Inhibition of the cytochrome pathway by antimycin A results in generation of active oxygen species in the mitochondria (Minagawa 1992). Generation of active oxygen species is pronounced during biotic and abiotic stresses (Low 1996), and the levels of the alternative oxidase protein are sensitive to diverse stress treatments (Vanlerberghe and McIntosh 1997).

NO is known to participate in signal transduction mechanisms in plants and animals. It also mediates various cellular and physiological processes. Despite the fact that NO participates in signal transduction and regulates various developmental processes in plants, it is toxic to the cell at high concentrations. It inhibits the electron transport chain via competitive binding to the oxygen-binding site of the mitochondrial complex IV (cytochrome *c* oxidase or COX). This causes the suppression of the respiratory oxygen uptake and generation of superoxide due to the over-reduction of the ubiquinone pool (Brown 2001, Shiva 2001).

2.1.3. Nitric oxide and aconitase under hypoxia

In plants, NO is generated via either oxidizing or reducing pathways. In the oxidizing pathways NO is formed from reduced nitrogen compounds, such as arginine or hydroxylamine. In the reducing pathways NO produced by reduction of nitrate and nitrite. NO is produced under hypoxia and the reduction of nitrate and nitrite to NO participates

in the mitochondrial electron transport chain (Igamberdiev et al., 2005; Planchet et al., 2005; Gupta and Igamberdiev 2011). NO can bind irreversibly to many enzymes and modulate their activity. Aconitase is one such enzymes (Navarre et al., 2000). Aconitase contains a FeS cluster that participates in the mechanism of interconversion of three tricarboxylic acids namely citrate, cis-aconitate and isocitrate. It exhibits sensitivity towards both NO and ROS. In plants, aconitase is found both in mitochondria and the cytosol. In mitochondria, it participates in the TCA cycle (Zemiyanukhin et al., 1984). The activity of the cytosolic aconitase is relatively higher than the mitochondrial aconitase and it participates in supplying 2-oxoglutarate for amino acid biosynthesis in conjunction with NADPH-dependent isocitrate dehydrogenase (Igamberdiev and Gardestrom 2003). During stress, there is a high degree of accumulation of reactive oxygen species and reactive nitrogen species particularly in mitochondria. Hence, it can lead to the inhibition of aconitase, which can influence overall cellular metabolism. Aconitase inactivation by NO or superoxide hence decreases cellular energy metabolism and may have a protective effect against additional oxidative stress by acting as a reversible circuit breaker that prevents wasteful turnover of the TCA cycle and further increases in reduction level under stress conditions (Gardner 1991).

2.1.4. Link between alternative oxidase and nitric oxide

Alternative oxidase gene expression also responds to particular metabolites whose levels reflect key parameters of mitochondrial status. Increase in cellular levels of the TCA intermediate citrate is rapidly followed by increase in *AOX1A* mRNA (Vanlerberghe 1996). Citrate is the first intermediate in the TCA cycle and its accumulation is an

important physiological signal linking mitochondrial metabolism and nuclear gene expression. There are reports which show that regulation of gene expression for photosynthetic metabolism is also controlled by metabolites (Sheen 1994, Koch 1996,).

It has been hypothesised that NO produced during hypoxia can influence the expression of alternative oxidase and hence AOX-dependent respiration. The present study aims at providing evidence that NO produced under hypoxia can lead to upregulation of AOX expression and respiration. It also presents the results of the overall effect of hypoxic NO accumulation on amino acid metabolism.

2.2. Materials and methods

2.2.1. Plant material

Three-week old Arabidopsis seedlings from a WT and a NR double mutant (*nia*) (obtained from National Arabidopsis Stock Centre, UK) in the Col-0 background were grown on vertical agar plates containing half-strength Hoagland nutrient solution containing 5 mM potassium nitrate and 2% sucrose and treated with normal air containing 21% O_2 (normoxia) or 1% O_2 (hypoxia). For determination of aconitase and NR, seedlings were transferred from the plates to liquid medium that was in equilibrium with air containing either 21% or 1% O_2.

2.2.2. Nitric oxide measurements

NO was measured by gas phase chemiluminescence detection method (Planchet et al., 2005) A small glass beaker containing root slices (0.5 g of roots, 8 ml of buffer) was immediately placed in a glass cuvette (1 l air volume) which was mounted on a rotary shaker (150 rpm). A constant flow of measuring gas (purified air or nitrogen) of 1.3 l min^{-1} was pulled through the cuvette, through a cold moisture trap, and subsequently through the chemiluminescence detector (CLD 770 AL ppt, Eco-Physics, Dürnten, Switzerland, detection limit 20 ppt; 20 s time resolution) by a vacuum pump connected to an ozone destroyer. The measuring gas (air or nitrogen) was made NO-free by pumping it through a custom-made charcoal column (1 m long, 3 cm internal diameter, particle size 2 mm). Calibration was routinely carried out with NO-free air (0 ppt NO) and with various

concentrations of NO (1–35 ppb) adjusted by mixing the calibration gas (500 ppb NO in nitrogen, Messer Griessheim, Darmstadt, Germany) with NO-free air. Flow controllers (FC-260, Tylan General, Eching, Germany) were used to adjust all gas flows. The air temperature in the cuvette was 25 ºC. NO analyzer data were logged into a computer and processed using customized 'Visual designer'-based software (Intelligent Instrumentation, Inc.).

2.2.3. Aconitase assay

The wild-type and *nia* mutant of Arabidopsis were grown in flasks placed on a shaker at 80 rpm for 7 days. For hypoxic treatment, the flasks were pumped with 1% oxygen for 3 h. Biomass (0.1 g) was crushed in liquid nitrogen and the enzyme was extracted in 50 mM Tris-HCl

(pH 7.4), 1 mM dithiothreitol, 5 mM $MgCl_2$, and 2 mM citrate. Aconitase was assayed spectrophotometrically at 240 nm in the same buffer but containing 40 mM citrate (Racker, 1950). Protein concentration was estimated by the method of Bradford (1976).

2.2.4. Measurement of respiration

Respiration rates of roots from both WT and *nia* mutants under hypoxia and normoxia were measured using the oxygen electrode (Hansatech, UK) calibrated by a simple two-point calibration (100 and 0% air saturation). Root tissue (0.1-0.2 g) was cut from the plant, washed twice and blotted on tissue paper to remove extra water and then immediately cut into 4–6 mm slices, which were placed in a 2.5 ml oxygen electrode

chamber which was continuously circulated with thermostat-controlled water. To estimate the effect of citrate on the partitioning between COX and AOX and on AOX capacity, 0.2 mM citrate was fed in the medium of root cultures 3 h prior to the respiratory measurements.

2.2.5. AOX gene expression analysis

The total RNA from the WT and mutant plants was isolated using RNase kit (Qiagen) as per the manufacturer's instructions. Five µg of RNA was subjected to reverse transcriptase activity to form cDNA using Superscript III reverse transcriptase kit (Invitrogen) using random N9 primers. DNase was used as per manufacturer's instructions. Real Time PCR was performed using SYBR-green master mix (Applied Biosystems) on the ABI Prism 7900 sequence detection system. *Ubiquitin10* was used as the housekeeping gene to normalise the results. The relative expression of *AOX1A* was analysed using the comparative threshold cycle method. Melting curve of the reaction was checked for single peak

2.2.6. Metabolite analysis

This work was performed in collaboration with Department of Plant Physiology, University of Rostock, Rostock, Germany. GC-MS analysis was performed as described by Lisec et al. (2006) for six replicates each consisting of 30 pooled plants obtained from two independent experiments. Targeted metabolite analysis was performed using the Target Search software package (Cuadros-Inostroza et al., 2009). Metabolites were selected by comparing their retention indexes (62 s) and spectra (similarity >85%) against the compounds stored in the Golm Metabolome Database (Kopka et al., 2005). Each

metabolite is represented by the observed ion intensity of a selected unique ion, which allows for a relative quantification between groups. Metabolite data were log_{10}-transformed to improve normality (Steinfath et al., 2008) and normalized to show identical medium peak sizes per sample group.

2.7.7. Measurement of nitrate reductase activity

NR activity was measured using L-NaRA-100 assay kit (NECi Co, USA). The extraction medium was 100 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, 0.4% PVP and 0.05% cysteine. Hundred mg of root material was ground in ice cold mortar and pestle with 400 µL of chilled extraction buffer and centrifuged at 10,000 g for 10 min. The NR assay medium contained 25 mM potassium phosphate, pH 7.5, 0.025 mM EDTA; and 10 mM nitrate. 100 µl extract was added and reaction was stopped after 20 min with Zn acetate. Nitrite was determined colorimetrically as described earlier (Hageman & Reed, 1980).

2.3. Results

2.3.1. Nitric oxide emissions

Chemiluminescence-based gas measurements from slices of Arabidopsis roots showed that under normoxic conditions (21% oxygen), no NO was produced. But upon decreasing the oxygen concentration to 1%, NO production increased and after 30 min reached a steady state rate of 6.5 nmol (g WT^{-1}) h^{-1}. Both under conditions of normoxia and hypoxia, the roots of *nia* double mutants did not produce any NO. Under normoxic

conditions, the total NR activity in *nia* roots was 0.026 µmol (g FW)$^{-1}$ h^{-1} whereas WT roots exhibited an NR activity of 0.099 µmol (g FW)$^{-1}$ h^{-1} which is nearly 4 times higher than in *nia* plants.

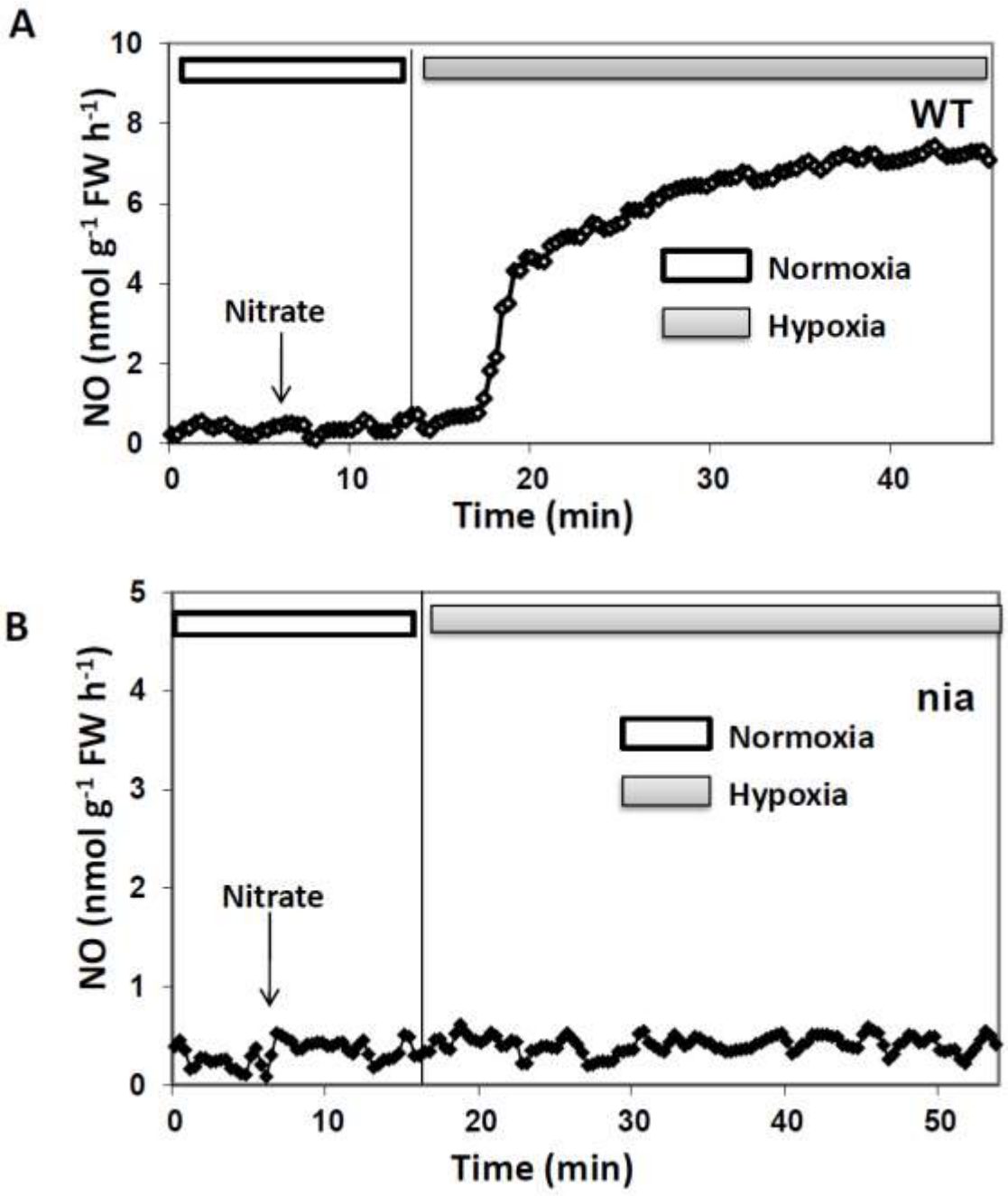


Figure 2.1. NO emissions from WT and *nia* plants under hypoxia and normoxia. (a) NO emissions from roots of WT plants under hypoxia and normoxia. (b) NO emissions from roots of *nia* mutants under hypoxia and normoxia. The figure shows that nitrite produced by nitrate reductase is a significant source of NO production under hypoxia.

2.3.2. Aconitase activity

We tested the effects of hypoxia and presence of NR on aconitase in plants by measuring aconitase activity in the WT and *nia* under normoxia and hypoxia. The results presented in Figure 2.2 show that under hypoxic conditions aconitase in WT plants is strongly suppressed when compared with normoxic conditions.

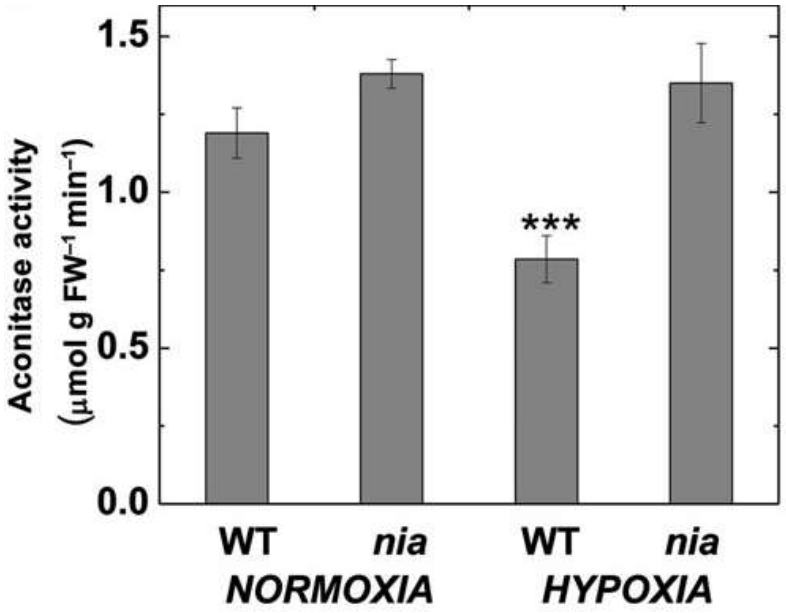


Figure 2.2. Aconitase activity in WT and *nia* mutants under hypoxia and normoxia. As it is seen, the aconitase activity was significantly inhibited by the NO produced in WT plants under hypoxia, whereas in *nia* mutants, the inhibition was non-significant, apparently due to the low NO production. Error bars indicate SD. Asterisks indicate significant differences between responses to oxygen conditions: *** $P < 0.001$, based on biological triplicates.

2.3.3. Citrate levels

Aconitase catalyses the interconversion between citrate and isocitrate. The inhibition of aconitase can lead to accumulation of citrate. To validate this experimentally, citrate levels were measured by GC-MS. As shown in Figure 2.3, citrate levels were only slightly higher in the WT in comparison to *nia* under normoxic conditions. However under hypoxic conditions, while in the WT citrate level increased by 70%, in *nia* plants it was reduced by twofold. This suggests that NO inhibits aconitase and thus stimulates citrate accumulation.

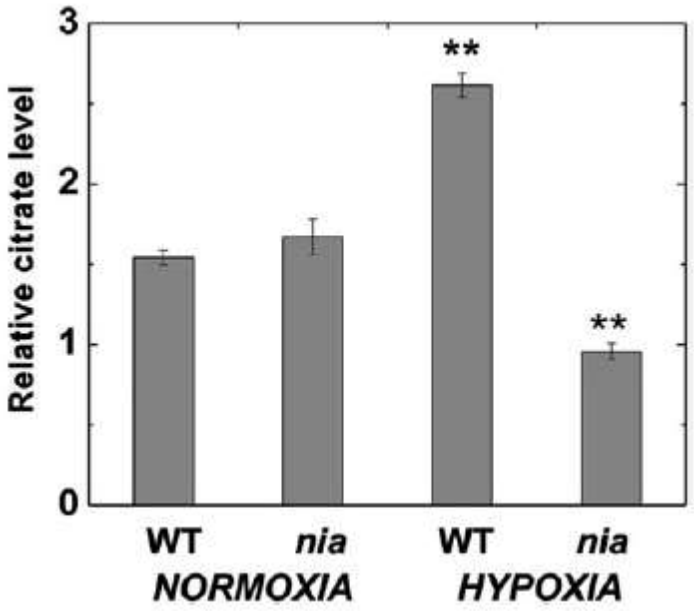


Figure 2.3. Citrate levels as measured by GC-MS analysis from WT and *nia* mutants. (A) Citrate levels under normoxic conditions. (B) Citrate levels under hypoxic conditions. Due to the inhibition of aconitase, WT plants under hypoxia have a significantly higher level of citrate as compared to *nia* lines. Error bars indicate SD from biological triplicates. Asterisks indicate significant differences between responses to oxygen conditions: ** $P < 0.01$.

2.3.4. AOX gene expression

To test whether there is a correlation between AOX gene expression and NO under hypoxia, the relative gene expression of *AOX1A* gene was estimated by quantitative reverse-transcription PCR. In WT, the expression of *AOX1A* was slightly higher than in *nia* under normoxic conditions, whereas under hypoxic conditions *AOX1A* gene expression was 3-fold higher (Figure 2.4). In order to check whether the transcript abundance and AOX respiratory capacity correlate with its protein amount, an immunoblot analysis was performed using polyclonal antibodies raised against Arabidopsis AOX (Figure 2.5).

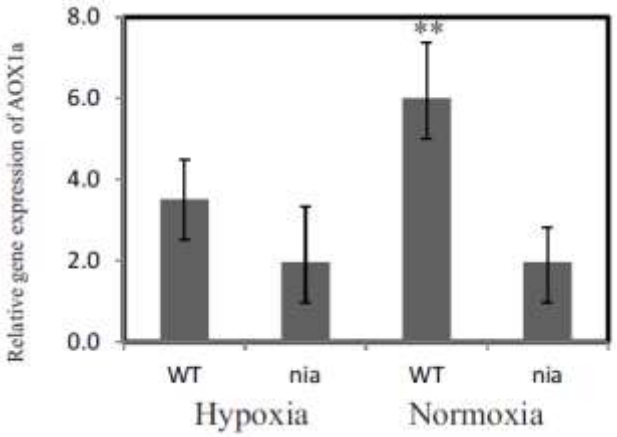


Figure 2.4: Relative gene expression of *AOX1A* gene in *nia* and WT plants under hypoxia and normoxia. This shows that NO mediates the induction of *AOX1A* gene expression under hypoxia. Error bars indicate SD. Asterisks indicate significant differences between *nia* and WT lines based on biological triplicates: ** $P < 0.01$.

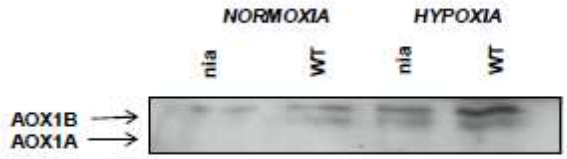


Figure 2.5: Immunobloting of proteins from WT and *nia* plants using AOX polyclonal antibodies. Results are indicative of two biological replicates. The 35-kDa band indicates AOX1A protein.

2.3.6. Respiration rate

The involvement of the increased citrate levels in AOX induction was tested by measuring AOX and COX capacities via incubation of root slices with and without salicylhydroxamic acid (SHAM), which is an inhibitor of AOX, and cyanide, which is an inhibitor for COX. The results were also verified with n-propylgallate (the inhibitor of AOX) and myxothiazol (the inhibitor of complex III). The total respiration (without inhibitors) in *nia* roots was 109 µmol (O_2) (g FW)$^{-1}$ h^{-1}, whereas the respiratory rate in WT roots was about 66 µmol (g FW)$^{-1}$ h^{-1}.

The capacity of the cytochrome pathway was 96.7 µmol (g FW)$^{-1}$ h^{-1} in *nia* roots whereas it was 51.7 µmol (g FW)$^{-1}$ h^{-1} in WT roots. A similar trend was observed when cytochrome capacity was measured in the presence of 100 nM n-propylgallate, suggesting that the absence of NO in *nia* roots triggers operation of the cytochrome pathway with a twofold higher intensity as compared to the presence of NO in WT roots. In order to check the AOX capacity, the respiration of WT and *nia* plants was measured in the presence of 1 mM KCN. The respiratory rate in *nia* roots was 9.5 µmol (g FW)$^{-1}$ h^{-1}, whereas in WT roots the respiratory rate was 14.3 µmol (g FW)$^{-1}$ h^{-1}. A similar trend was observed when using 25 µM myxothiazol: the AOX capacity in presence of citrate was 18.2 µmol (g FW)$^{-1}$ h^{-1} whereas in the absence of citrate it was 9.6 µmol (g FW)$^{-1}$ h^{-1}.

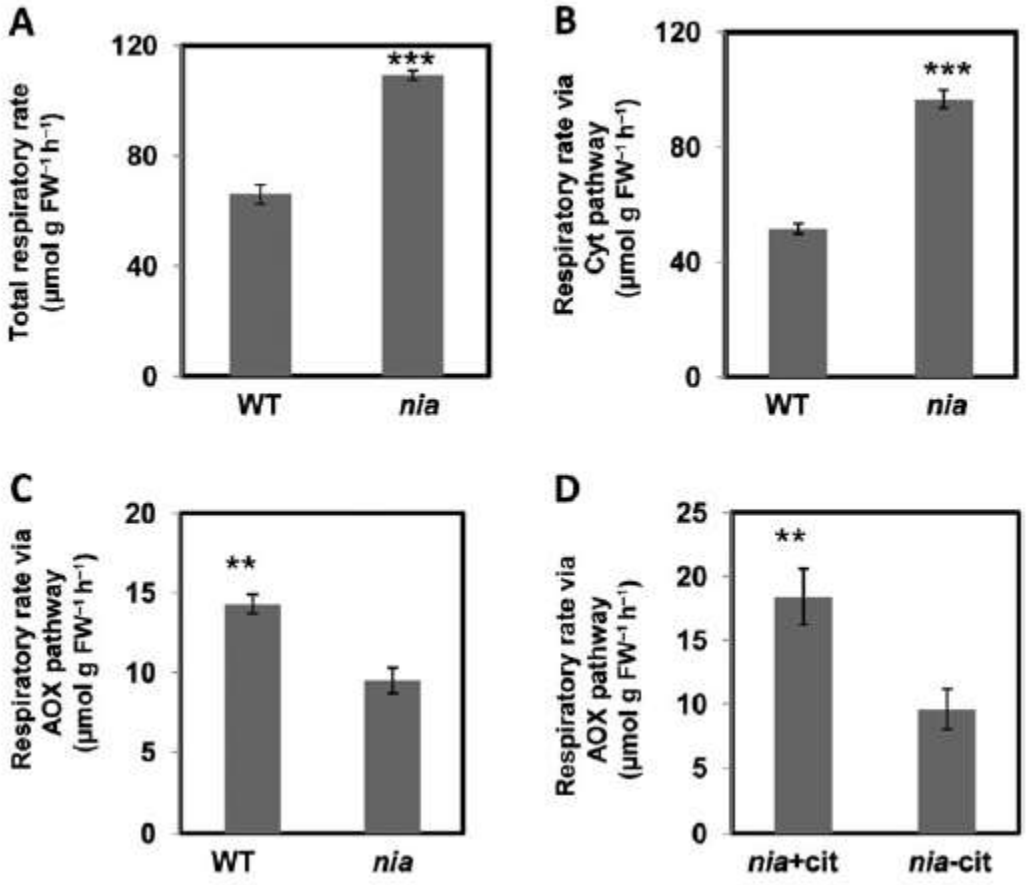
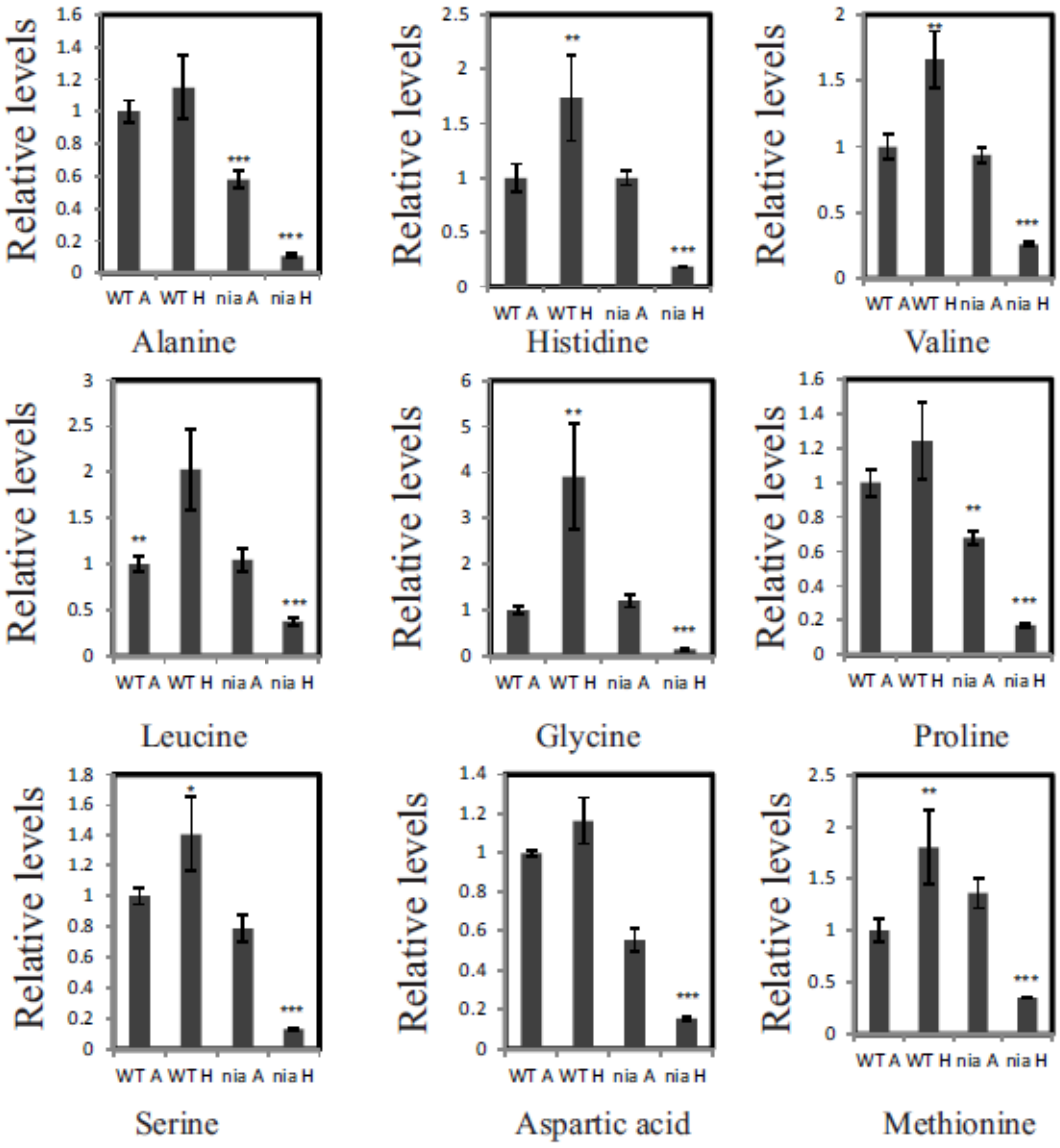


Figure 2.6. Respiration rate and cytochrome and AOX pathway capacities in roots of WT (control) and *nia* Arabidopsis plants. (A) Total respiration rate in absence of any inhibitors. (B) Rate of respiration in presence of 2 mM SHAM showing capacity of cytochrome pathway. (C) Rate of respiration in presence of 2 mM KCN showing AOX capacity. (D) AOX capacity of *nia* plants without citrate feeding (*nia*-cit) and with citrate feeding (*nia*+cit). Error bars indicate SD. Asterisks indicate significant differences between responses to oxygen conditions: ** $P < 0.01$; *** $P < 0.001$.

2.3.6. Metabolic analysis

A detailed metabolite analysis of WT and *nia* mutants grown under hypoxia and normoxia was performed to analyse the changes in overall cellular metabolism. Figure 2.7 reports the relative levels of various amino acids in each of the plants and conditions mentioned above relative to WT under normoxia. In general, it was observed that there is a marked increase in amino acid accumulation in WT under hypoxia. The levels of amino acids were significantly higher in the case of glycine, leucine, tyrosine, ornithine, citrulline and arginine (2 times) and marginally in valine, serine, methionine and histidine (1.5 times).


Relative levels
WT A WT H nia A nia H
Alanine
Histidine
Valine
Leucine
Glycine
Proline
Serine
Aspartic acid
Methionine

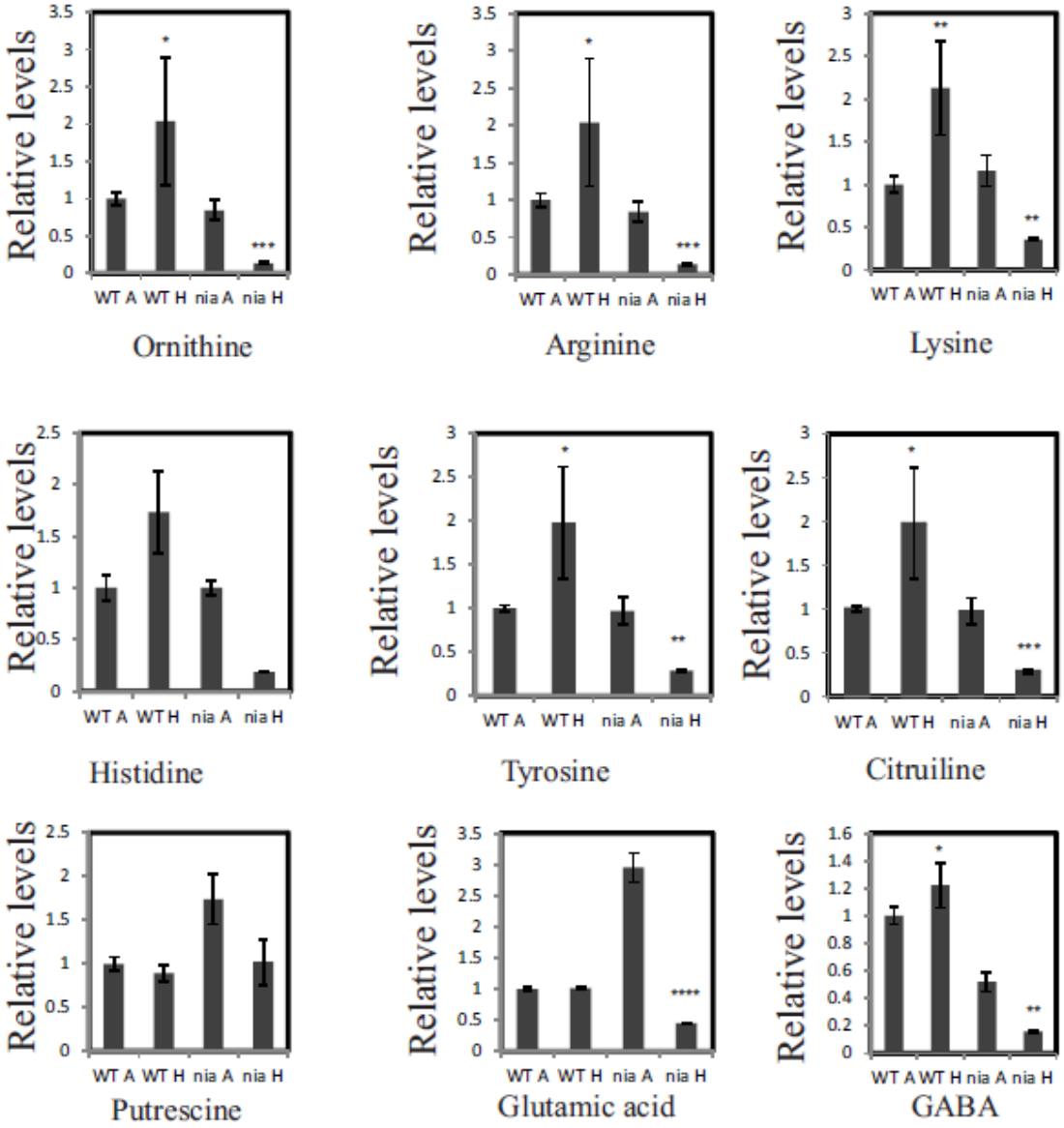


Figure 2.7: Relative levels of amino acids in WT and *nia* plants under normoxia (A) and hypoxia (H) as measured by GC-MS in sets of three biological replicates. All levels are relative to WT under normoxia. Error bars indicate SD. Asterisks indicate significant differences between responses to oxygen conditions: * ($P < 0.05$)** $P < 0.01$; *** $P < 0.001$.

Results in Figure 2.8 show that the TCA intermediates such as citrate, fumarate, malate, succinate and oxaloacetate accumulated under hypoxia in WT, whereas in *nia* mutants the levels of succinate, fumarate and malate were much lower in normoxia. The levels of citrate, 2-oxoglutarate and oxaloacetate remained almost the same in WT plants, whereas under hypoxia in *nia* mutants, except fumarate, there was a decrease in all the analyzed intermediates. The levels of 2-oxoglutarate in WT decreased by 20% under hypoxia, and increased in *nia* mutants.

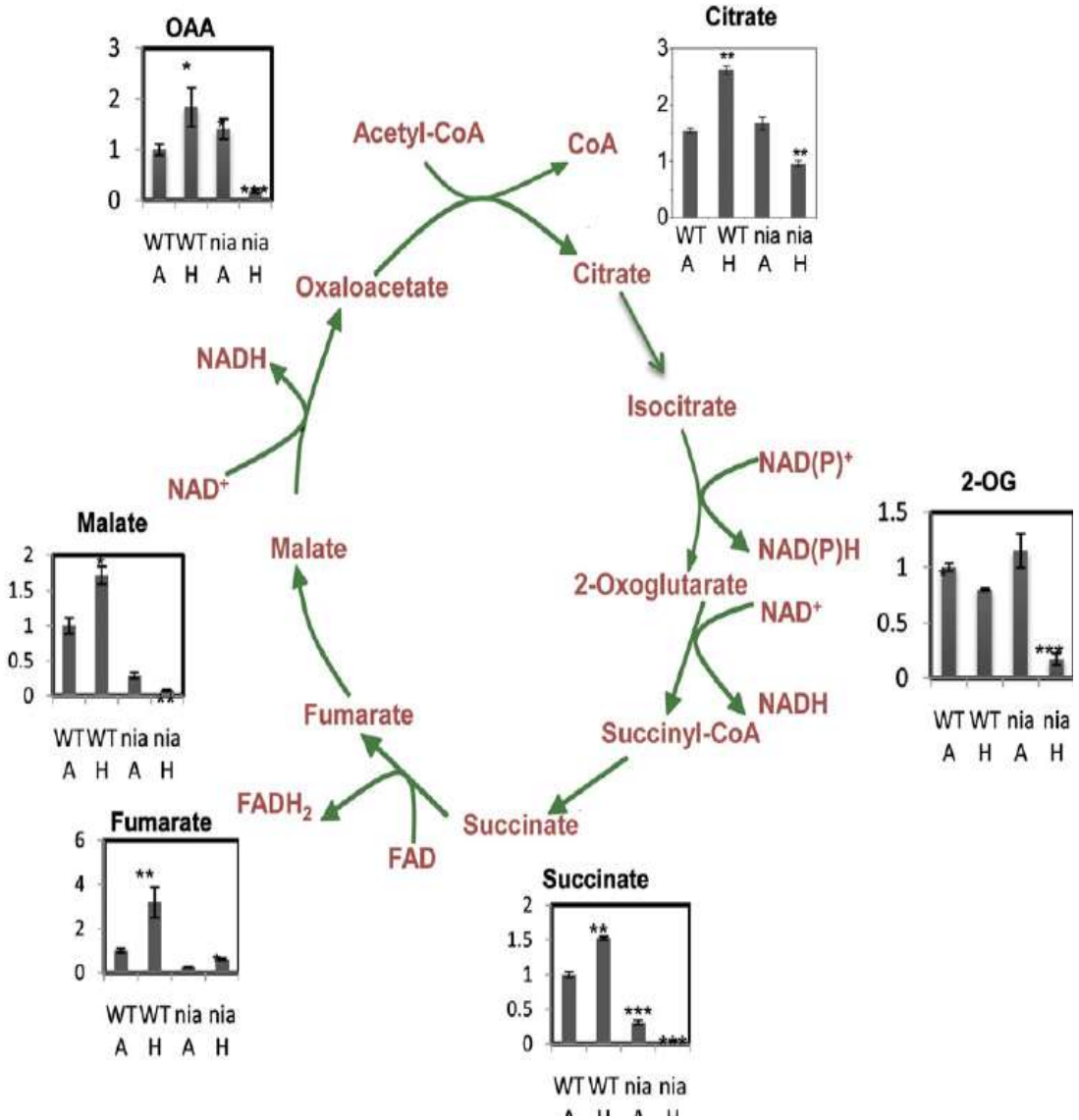


Figure 2.8: Relative levels of TCA intermediates in WT and *nia* mutants under normoxia (A) and hypoxia (H). All levels are relative to WT under normoxia. Error bars indicate SD. Asterisks indicate significant differences between responses to oxygen conditions: * ($P < 0.05$)** $P < 0.01$; *** $P < 0.001$.

The obtained results clearly suggest that in the WT, which exhibits elevated NO levels under hypoxia, the AOX capacity is increased. In the absence of NO, as seen in *nia*, respiration followed the cytochrome pathway. In order to verify that the *in vivo* increase in citrate levels is responsible for AOX induction, citrate was fed to the medium of root cultures 3 h prior to the respiratory measurements. These data suggest that citrate is responsible for the induction of AOX, which is in agreement with the results of (Degu et al., 2011) showing that the inhibition of aconitase by citramalate (2-hydroxy-2-methylbutanedioate) induces AOX.

2.4. Discussion

2.4.1. A link between NO, aconitase and AOX

The FeS clusters of enzymes such as aconitase are sensitive to NO (Verniquet 1991, Navarre 2000). Both in plants (Lindermayer et al., 2005) and animals (Scheving et al., 2012), aconitase is a target for S-nitroylation. NO is a strong inhibitor of aconitase especially at low pH (Gardner 1997) which is a phenomenon observed under hypoxia. Results show that there is a strong inhibition of aconitase in WT plants under hypoxia, where NO levels are much higher than in *nia* mutants. AOX participates to keep the ROS levels in check under various stress conditions by diverting the flow of electrons from ubiquinone to oxygen and thus preventing the formation of superoxide (Maxwell et al., 1999). The AOX activity is influenced both at the transcriptional and post-translational levels by citrate (Vanlerberghe and McIntosh 1999) and pyruvate respectively (Mackenzie 1999). Hence the accumulation of citrate due to inhibition of aconitase by

NO generated under hypoxia can lead to the induction of AOX gene expression. Our results provide evidence to prove the above mentioned hypothesis of the regulatory role of aconitase. The activation of AOX was observed not only at the transcript level, but also at the protein and activity levels. The induction of AOX can serve to counteract the increase in ROS levels under reoxygenation. Retrogade signalling that senses the metabolic state of the organelle and tranduces the signal to the nucleus and hence leads to the expression of corresponding genes can be achieved by inhibition of aconitase by NO, which leads to the accumulation of citrate, acting as a signal for the induction of the AOX gene in the nucleus. The AOX induction has also been shown to be associated with active photorespiratory flux that increases the redox level in mitochondria and stimulates nitrogen metabolism. Barley (Igamberdiev et al., 2001) and potato (Bykova et al., 2005) lines with decreased expression levels of glycine decarboxylase complex are characterised by a very low expression of AOX protein. Both photorespiration and hypoxia are linked to active nitrogen metabolism, therefore AOX may be involved in the evolutionary adaptation of plants as a part of a mechanism supporting metabolic switch to intensive nitrogen assimilation.

2.3.2. Effects of inhibition of aconitase by NO on amino acid biosynthesis

A significant decrease in the levels of all investigated amino acids under hypoxia in the nitrate reductase deficient mutants was observed. Hypoxic treatment resulted in significant increase in GABA levels in WT plants, whereas in *nia* mutants a marked decrease was observed. Citrate is accumulated in WT plants under hypoxia due to the inhibition of aconitase by NO. There is a distinct increase in amino acid accumulation in

such conditions. Under these conditions, because of the low affinity of AOX to oxygen, mitochondrial functioning is made possible by the induction of AOX, which supports a limited flux through mitochondria that contributes to the formation of 2-oxoglutarate for ammonium incorporation into amino acids and to significantly decreased ATP synthesis (Gupta and Igamberdiev 2011).

Chapter 3: Studies of the effects of modified expression of hemoglobins in barley under hypoxia

3.1. Introduction

Under hypoxic or anoxic conditions, there is a switch from the aerobic tricarboxylic acid cycle to fermentation pathways, forming lactate and ethanol. This is accompanied by a decline in pH (Saint-Ges et al., 1991; Kennedy et al., 1992; Ratcliffe 1995; Gout et al., 2001). The decline in pH slows the rate of lactate formation (Hanson and Jacobsen 1984) and activates pyruvate decarboxylase, which diverts glycolytic carbon flow to ethanol formation (Kennedy et al., 1992). Alanine is formed via the amination of pyruvate and is second only to the ethanol as a product of glycolytic flux during hypoxia. Estimates of the end products of nitrate reduction suggests that less than 4% of NADH recycled during hypoxia is connected with the reduction of nitrate to ammonium ion (Gibbs and Greenway 2003). Electron microscopy of rice mitochondria during anaerobisis shows a protective role of nitrate in maintaining membrane ultrastructure (Vartapetian and Polyakova 1999; Vartapetian et al., 2003). A higher level of ATP is maintained in potato cells at a higher level under hypoxia in nitrate as opposed to ammonium ion medium (Obrson et al., 1999). These findings suggest that during inhibition of the mitochondrial electron transport chain by lack of oxygen, the presence of nitrate may have an influence, either directly or indirectly on the functional state of mitochondria (Igamberdiev and Hill 2004). It is important to note that there is a 2.5 fold activation of cytoplasmic nitrate

reductase expression during exposure of plant roots to hypoxia (Bortel and Kaiser 1997). Along with this, nitrite reduction is supressed at the nitrite reductase step. The limitation of nitrite reduction is connected both with cellular acidification and with the increase of flux through nitrate reductase (Bortel et al., 1996). In aerated roots, cytoplasmic nitrate reductase is highly phosphorylated and inactive. It is partly dephosphorylated and hence activated by anoxia or by cellular acidification. NO is considered as a possible product of nitrite reduction from nitrate reductase and is only a minor part of nitrite formed during hypoxia will undergo reduction to ammonia, since nitrite reductase is inhibited under these conditions (Botrel et al., 1996; Igamberdiev and Hill 2004). This suggests that under hypoxia there is a switch from formation of ammonia via reduction by NR to formation of NO by mitochondria.

3.1.1. Aim of study

Hemoglobins participate in various stress responses. Their role is noteworthy under hypoxia and anoxia. Class I hemoglobins participate in the presence of traces of oxygen in scavenging NO, which is formed in particular in the mitochondrial electron transport chain at very low oxygen tensions.

The current study focuses on the generation and analysis of plants with modified expression of hemoglobin in the cultivar of barley Golden Promise. The project involves construction of lines with either silenced or overexpressing hemoglobin gene. Morphological studies of plants overexpressing hemoglobin were performed in greenhouse conditions. A hydroponic system was developed to study plants under

hypoxia. Morphological and gene expression analysis was performed on roots, shoots and seeds of wild type Golden Promise and of lines overexpressing hemoglobin grown under hypoxia and normoxia. The results of this study provide an insight in the potential role of hemoglobin in the induction of stress tolerance in plants.

3.2. Materials and Methods

3.2.1. Hydroponic systems to mimic hypoxia

Independent barley transgenic lines with expression of HvHb under the control of ubiquitin promoter were generated by Dr. Kim H. Hebelstrup at Flakkebjerg Research Centre, Slagelse, Denmark. Golden Promise wild type barley (WT) and hemoglobin overexpressing lines (UHb) were germinated on moist filter paper under dark conditions at 22 °C for three days. The seedlings were transferred to custom-made hydroponic chambers. The chamber consisted of a plastic box, 60 cm long and 30 cm widelong and 12 inches wide feet long and one foot wide. The outer side of the box was completely covered with a layer of aluminium foil such that no light could pass through the box. It was necessary to make the box totally opaque to avoid any algal growth in the water. Several rows of three holes (3.5 cmdiameter) per row were punched above the aluminum layer. Soft absorbent sponge was used to hold the seedlings over the holes in the box. The sponge was covered with play dough to ensure no air passes through into the water. The boxes were filled with half strength fertilizer (20-20-20) for first two weeks and then full strength fertilizer was used. The medium was changed every week. The pH of the

medium was maintained in the range of 6.2-6.4 throughout the growth stage. The pH was checked twice a day in the first two weeks, followed by once in two days henceforth. The medium was supplied with ambient air for growing plants under normoxia and with nitrogen gas to grow the plants under hypoxia. The oxygen levels in the medium were regularly monitored using an HQ40D oxygen electrode (Hatch Inc., Denmark). An oxygen level as low as 2% saturation was achieved in the hypoxic systems (Figure 3.1).

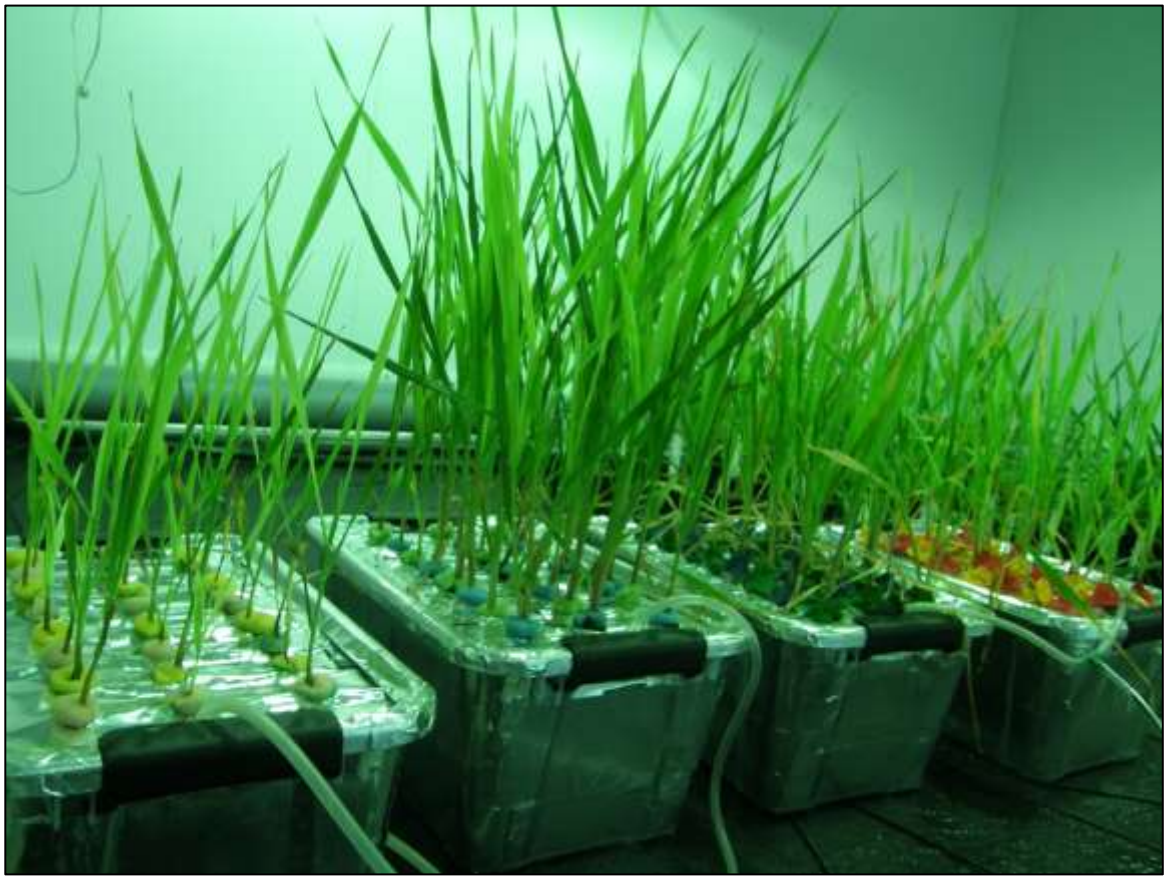


Figure 3.1: Hydroponic system to grow plants under hypoxia and normoxia. Golden Promise barley lines with overexpression of hemoglobin and wild type were grown in a 16/8 hour day/night regime at 23 °C.

3.2.2. Sampling and gene expression analysis

The plants were harvested after 6 weeks post germination. The plants were carefully removed from the hydroponic system and root and shoot length and weight were immediately measured. The shoots were cut and separately placed in 15 ml falcon tubes, each having two ¼ inch ceramic beads. Roots were collected in 20 ml plastic containers containing four 4 mm steel beads. They were immediately flash-frozen by immersing the tubes in liquid nitrogen. The tubes containing shoots were vortexed at maximum speed thrice for 20 s each. After each 20 s run, the tubes were immersed in liquid nitrogen for one minute. The roots were disrupted in the Geno/Grinder 2000 (SPEX CertiPrep, Metuchen, NJ) homogenizer at 1200 strokes per minute over a period of 40 s for three times. After each run, the tubes were immersed in liquid nitrogen for one minute. Total RNA from the samples was isolated using the Fast RNA green kit supplied by MP Biochemicals as per the instructions prescribed by the supplier. RNA quality and quantity was analyzed using a Nanodrop. One microgram of total RNA was used as template to synthesise cDNA using the Superscript Reverse transcriptase II (Invitrogen) and random hexamer oligonucleotide primers in a total reaction volume of 20 µl as per the manufacturer guidelines. The RNA samples were stored at -80 °C and the cDNA samples were stored at –20 °C for further analysis. The cDNA was used as a template to perform Real Time PCR. SYBR-Green PCR mastermix (Applied Biosystems) was used as a basis for the reaction in a final volume of 10 µl (5 µl PCR mastermix, 1 µl primer mix, 5 µM each primer, 3 µl H_2O, 1 µl cDNA). The reaction was performed on ABI PRISM 7900 HT sequence detection system (Applied Biosystems). Melting curve of the

reaction was checked for single peak. Glyceraldehyde-3-phosphate dehydrogenase gene (*HvGAPDH*) was used as the housekeeping gene for normalization of results. The list of primers for each gene analysed is provided in Table 3.1. Measurements were based on three technical triplicates. Relative quantification was calculated as described by Pfaffl (2001).

Table 3.1. List of primers to study plant response to Hb overexpression and hypoxia

Gene	Primer (5' to 3')
nitrate reductase	Fwd: GAGGACGAGACGGAGATGCA Rev: TCACAAACCCGACGCTGAA
monodehydroascorbate reductase	Fwd: AAGGCCTAGCGTTTGCCA Rev:CGTACAGGTTTATACAGGGTTTCC
alanine aminotransferase	Fwd:TGGTGAGTGTGGTAAAAGAGGTG Rev: GCCAGTGATATTGGAGCATAGGT
ADH1	Fwd: TATGGCGAAGGGGGAGG Rev: ACCGATGATCTGGTTCAGAAGA
ADH2	Fwd: CCGCCTTTGAATGCGTG Rev: GCCGTGTTGATCTGCGAGAA
ADH3	Fwd: GACCAATGGCGGAGTCGA Rev: GAAGGGCACGCTATGTGTGA
ADH4	Fwd: AGAAGACCCTGAAAGGCACC

	Rev: ACCTGAAAACAGCGAGAAACAC
GAPDH	Fwd:CCACCGGTGTCTTCACTGACAAGG Rev: TAGCATCAAAGATGCTGG
PP2C	Fwd: TCGCAATGTCACGGTCAATCGG Rev: ACGCTGACTTCAGGCTTTGGAATC
JRG1	Fwd: CTGGTTTGGGTTCGACTGTT Rev: AGCGTTGACCCCTTAAACCT
GAMyB	Fwd: TATGCACCACAGCTTTCAGC Rev: AGCCTGCTTGAGCTTCTCAG

3.2.3. Plant growth analysis

Fifty WT Golden Promise and UHb lines were grown in small pots (one plant per pot). Plants were grown in 16/18 h day/night regime at 23°C for three months. Three plants were harvested every week and the roots were cleaned using a water spray. The length (in cm) and weight (in mg) of the roots and shoots were separately measured and recorded. The growth stages of the plants were recorded every week following the Zadoks scale.

The Zadoks scale is a cereal development scale proposed by the Dutch phytopathologist Jan C. Zadoks that is widely used in cereal research and

agriculture (Zadoks et al., 1974). They are represented on a scale from 00 to 99, where 00 is the dry seed and stage 99 is when the secondary dormancy is lost. This scale follows the plant development through 10 primary development stages (first digit), subdivided into secondary growth stages (second digit) to produce a two digit scale number. Table 3.3 describe the primary growth stages and table 3.4 describes the growth stages in detailed which were used in the current study. A tiller is defined as a lateral shoot from the base of the stem.

Table 3.3 Primary growth stages for Zadoks scale

Code	Description
0	Dry seed
1	Seedling Growth
2	Tillering
3	Stem elongation
4	Booting
5	Inflorescence emergence
6	Anthesis
7	Milk development
8	Dough development
9	Ripening

Table 3.4. Zadoks scale in detail

Code	Description
10	First leaf emerged
11	First leaf unfolded
12	2 leaves unfolded
13	3 leaves unfolded
14	4 leaves unfolded
15	5 leaves unfolded
16	6 leaves unfolded
17	7 leaves unfolded
18	8 leaves unfolded
19	9 or more leaves unfolded
20	Main shoot only (no tiller emerged yet)
21	Main shoot and 1 tiller
22	Main shoot and 2 tillers
23	Main shoot and 3 tillers
24	Main shoot and 4 tillers
25	Main shoot and 5 tillers
26	Main shoot and 6 tillers
27	Main shoot and 7 tillers
28	Main shoot and 8 tillers
29	Main shoot and 9 or more tillers

3.2.4. Generation of Hb silencing lines

3.2.4.1. Generation of Hb silencing construct

The coding region of *HvHb* was amplified by PCR using Phusion polymerase as per the instructions of the manufacturer (Figure 3.2). The amplified region was cloned in pENTR4.0 vector at EcoRI and BamHI sites using conventional ligation. Resultant plasmid was isolated and digested with EcoRI to verify the construct (Figure 3.4). pENTR HvHB was cloned in Stargate silencing construct (Hebelstrup et al., 2010) via recombination using Clonase kit (Invitrogen) according to the manufacturer's instructions with the exception that the mixture was incubated overnight and proteinase K was not applied. Schematic representation of the method is given in Figure 3.4 (Source Invitrogen website) The Gateway Cloning System, invented and commercialized by Invitrogen, is a molecular biology method that enables researchers to efficiently transfer DNA-fragments between plasmids using a proprietary set of recombination sequences, the "Gateway" sites, and two proprietary enzyme mixes, called "LR Clonase". Gateway Cloning Technique allows transfer of DNA fragments between different cloning vectors while maintaining the reading frame. Using Gateway, one can clone or sub-clone DNA segments for functional analysis. The system requires the initial insertion of a DNA fragment into a plasmid with two flanking recombination sequences called "att L 1" and "att L 2", to develop a "Gateway Entry clone" (special Invitrogen nomenclature). Screening for clones involves digesting the expression clone with prescribed restricting enzymes. For the vector used in this study, the destination vector would form three bands with Sma*I* whereas the expression clone would digest to a single linear band (unless there

is a Sma*I* site in the gene of interest. Upon digestion with BamHI, both the destination vector and the expression vector would form three bands, but the expression vector would have a higher size of bands (unless there is a BamHI site in the insert). Lane 4 in figure 3.5 shows the destination vector digested with BamHI, whereas lane 2 shows the expression clone digested with the same enzyme. Similar lanes are shown in figure 3.6 where the plasmids were digested with SmaI. Primers Fwd: GAAATTCACGACCAACACGTA Rev: ACAAATTATGCACGAATCC. This set of primers would amplify part of the coding sequence of the hemoglobin gene.

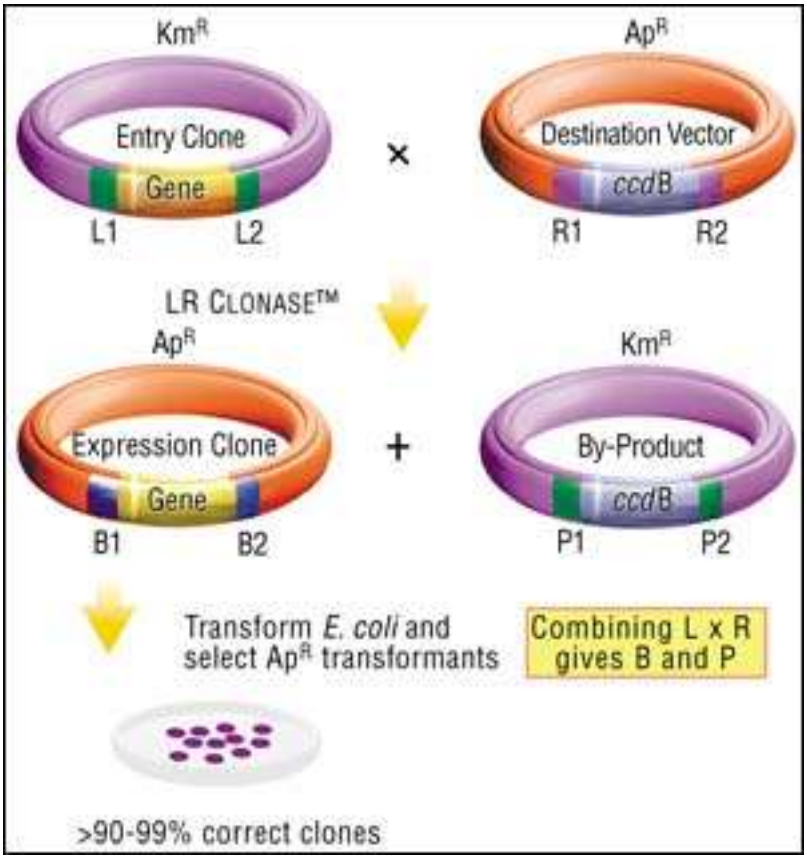


Figure 3.2: Description of gateway cloning (Source: Invitrogen website)

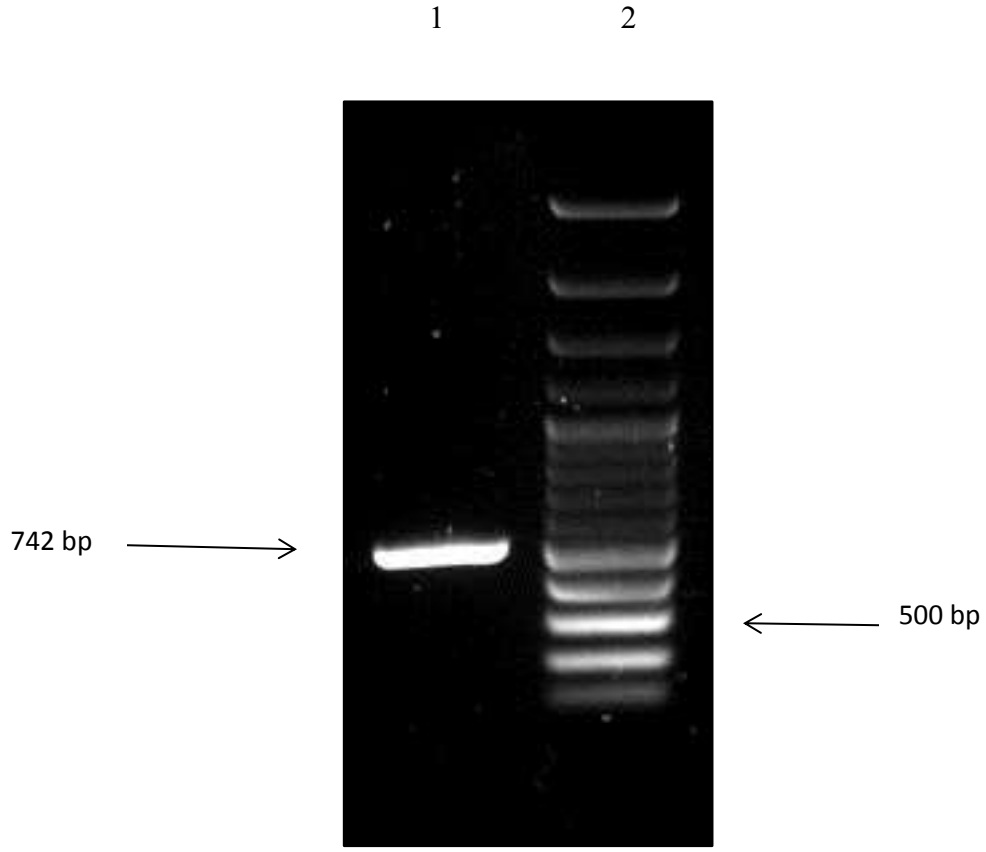


Figure 3.3: HvHb pcr product

Lane 1: PCR product

Lane 2: 100 bp+ marker

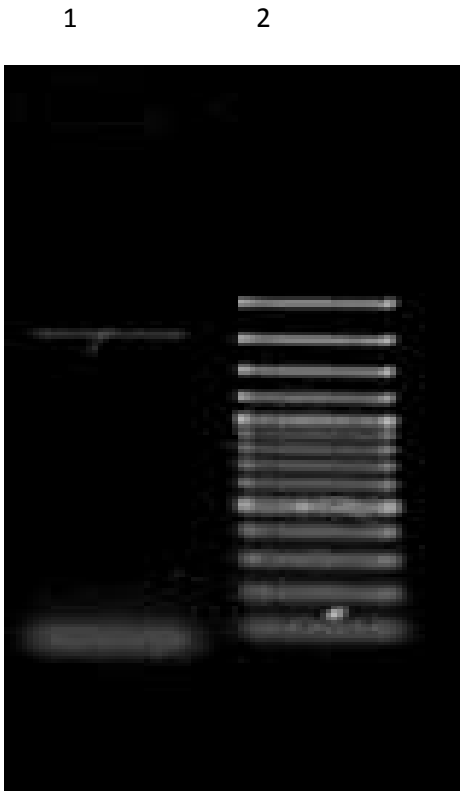


Figure 3.4: pENTR 4.0 vector digested with EcoRI

Lane 1: pENTR 4.0 digested with EcoRI

Lane 2: 10 bp+ marker

1 2 3 4 5

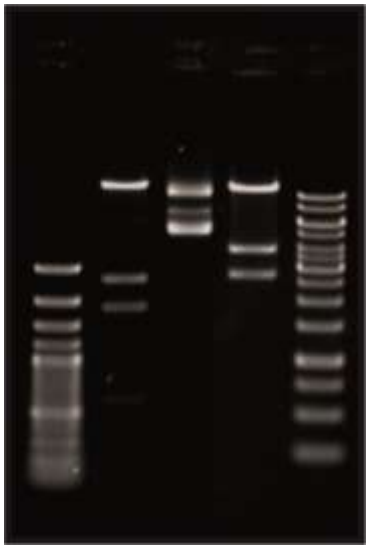


Figure 3.5: Putative clones digested with Bam*HI* for screening of HvHb silencing vector construct

Lane 1: Kb marker

Lane 2: Positive clone

Lane 3: Negative clone

Lane 4: Vector control

Lane 5: 100 bp+ ladder

1 2 3 4 5

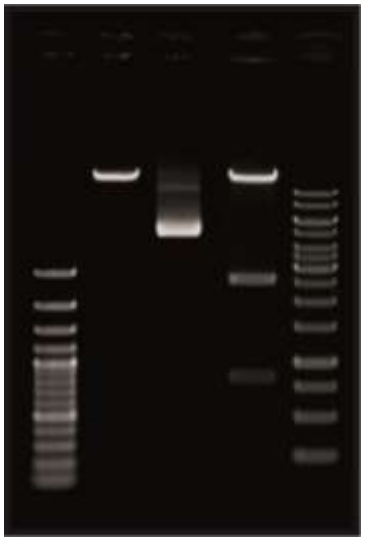


Figure 3.6: Putative clones digested with Sma*I* for screening of HvHb silencing vector construct

Lane 1: Kb marker

Lane 2: Positive clone

Lane 3: Negative clone

Lane 4: Vector control

Lane 5: 100 bp+ ladder

3.2.4.2. Transformation of barley by *Agrobacterium* infection

The *Agrobacterium*-mediated barley transformation was developed by modification of protocols described (Matthews et al., 2001; Bartlett et al., 2008). All the media were used according to Matthews et al., (2001) but with $CuSO_4$ addition (Table 3.5).

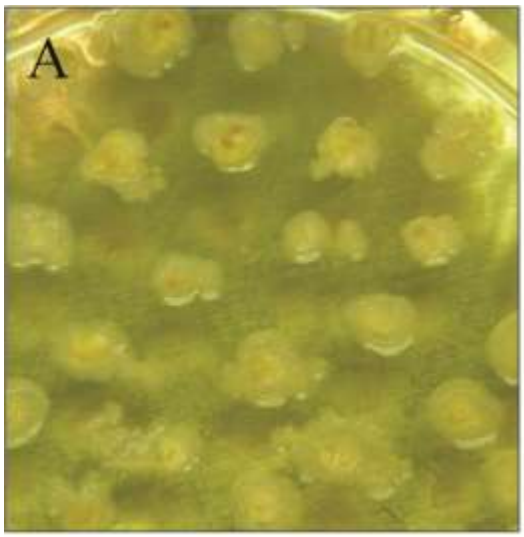
3.2.4.3. *Agrobacterium* infection and barley transformation

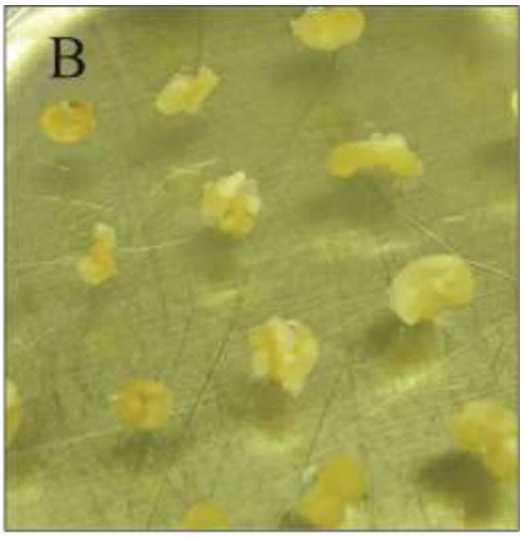
Fresh spikes from Golden Promise barley were harvested in the morning. Spikes were selected such that the embryo of the seeds was 1.5-2 mm in length and were in the stage of transition from translucent to white. The seeds were surface-sterilized by stirring them in a flask containing 10% v/v hypochlorite solution for one minute followed by three washes with sterile distilled water to remove the excess bleach. The *Agrobacterium* stock with the silencing plasmid was thawed on ice and later placed at room temperature. The seeds were dissected and individual embryos were collected. The tip of a scalpel was dipped in the *Agrobacterium* culture and used to cut out the scutellum of the embryo. The scutellum was discarded and the remaining embryo, which looked like a horseshoe, was placed on co-infection medium for two days. Embryos were placed in rows of 7-8 with no more than 30 embryos on one 90 cm plate. More than 300 embryos were dissected per batch. Two batches of transformation were performed in a week giving more than 600 embryos. Two days after infection, the embryos were carefully transferred to callus induction medium (Figure 3.7-A). The medium contained the antibiotics timentin and ampicillin to kill the *Agrobacterium*. The embryos with highly dense *Agrobacterium* growth were also discarded. Embryos that were too small and had turned dense brown were discarded. These are symptoms of excessive infection, which leads to

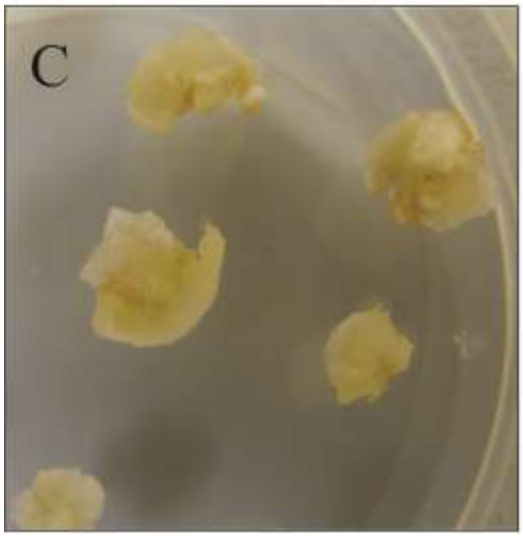
the death of the embryo (Figure 3.7-B). The embryos were placed on callus induction medium for two weeks and were subcultured once in two weeks (Figure 3.7-C). The embryos now gave rise to callus. Later, the callus was transferred to callus maturation medium for two weeks. At the end of two weeks, the callus was transferred to shooting medium. When the shoots on the callus were more than 3 cm (Figure 3.7-D), they were transferred to rooting medium in cylinders (Figure 3.7-E). A few shoots were colourless and they were discarded. Many of the plants underwent selection and in the probable non-transformants, the roots were seen trying to grow upwards, defying gravity. They were discarded. When plantlets had dense root system, they were transferred to small pots and were placed in a closed box in a plant growth chamber. After two weeks (Figure 3.7-F and 3.7-G), they were transferred to larger pots and placed in the greenhouse (Figure 3.7-H and 3.7-I). Composition of media used in each stage is reported in Table 3.5.

Table 3.5. Composition of media used for barley transformation and tissue culture

Ingredients	Co-cultivation	Callus induction	Callus maturation	Shooting	Rooting
(MS) Macro and micro nutrients + Vitamins	4.4 g	4.4g			4.4 g
Macro and micro nutrients w/o ammonium nitrate			2.7g	2.7g	
Ammonium nitrate		165 mg	165 mg		
$CuSO_4$		1.25 mg	1.25		
Thiamine			1 mg	1 mg	
Dicamba	2.5 mg	2.5 mg			
BAP			0.1 mg	0.1 mg	
2,4-D			2.5 mg		
Inositol	25. mg	250 mg	100 mg	100 mg	250 mg
Casein hydrosylate	1000 mg	1000 mg			1000 mg
Proline	690 mg	690 mg			690 mg
Glutamine			750 mg	750 mg	
Acetosyringone	98 mg				
Maltose	30 g	30 g	20 g	20 g	30 g
Timentine		160 mg			
Ampicillin		100 mg	100 mg	100 mg	
Hygromycin		50 mg	30 mg	30 mg	30 mg
Add ½ liter milliQ water and adjust pH and filter sterilize	5.8	5.8	5.8	5.8	5.8
Add ½ liter Phytagel to make final volume 1 liter	3.5 g	3.5 g	3.5 g	3.5 g	3.5 g


A


B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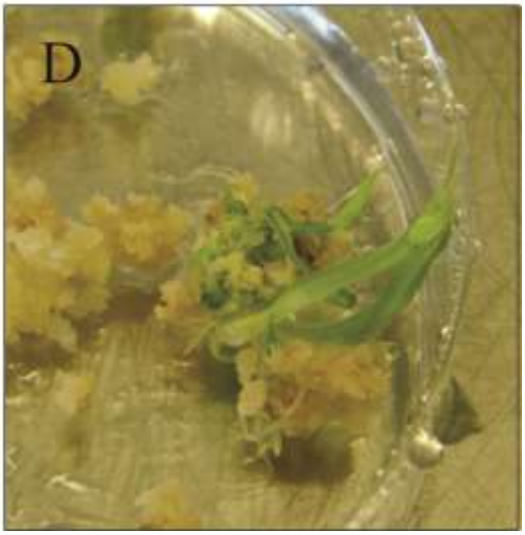
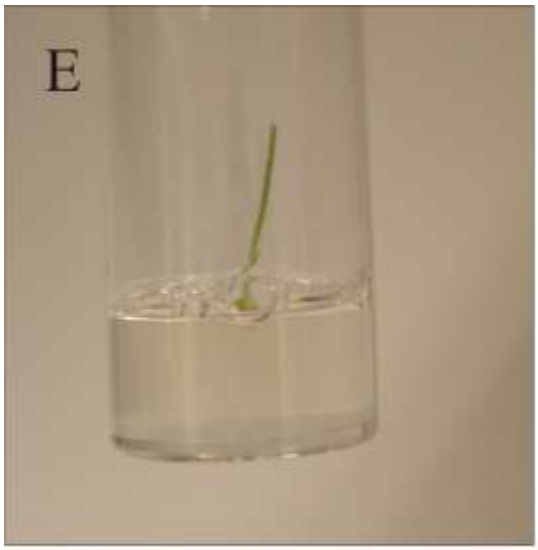
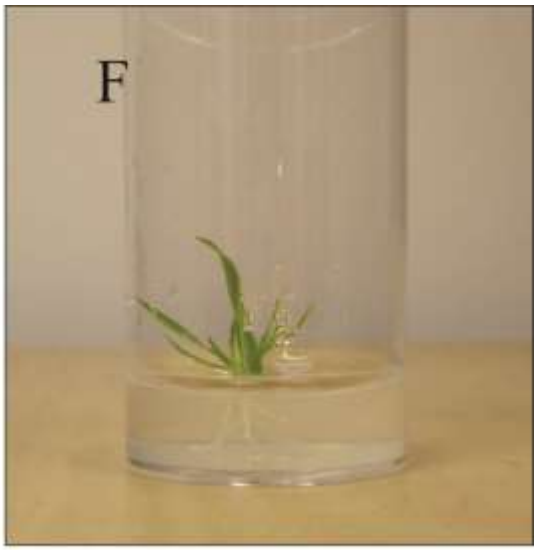

D


E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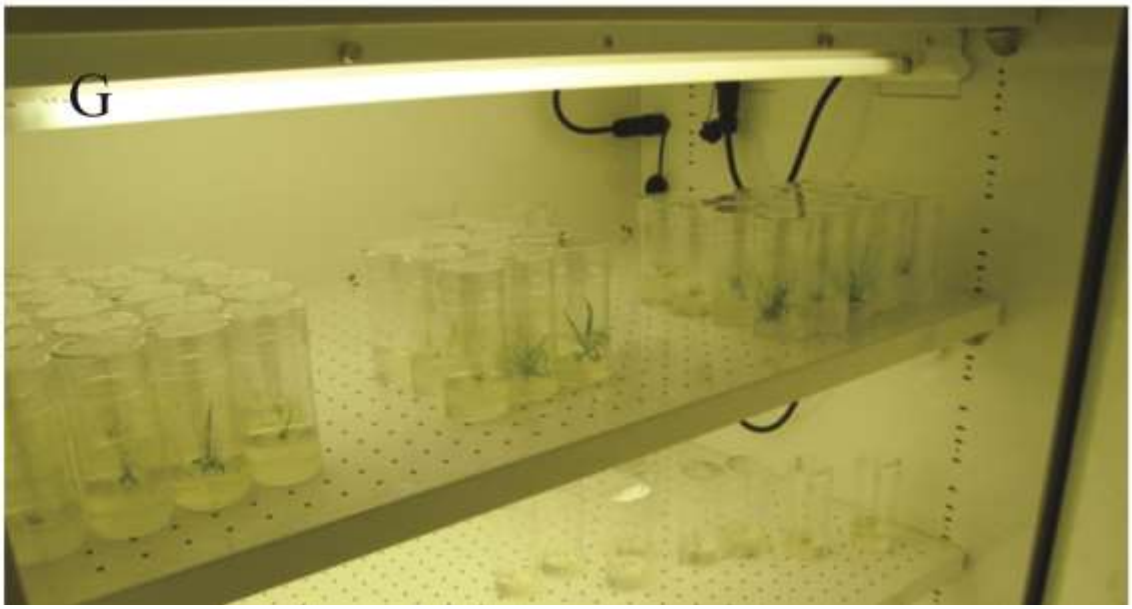
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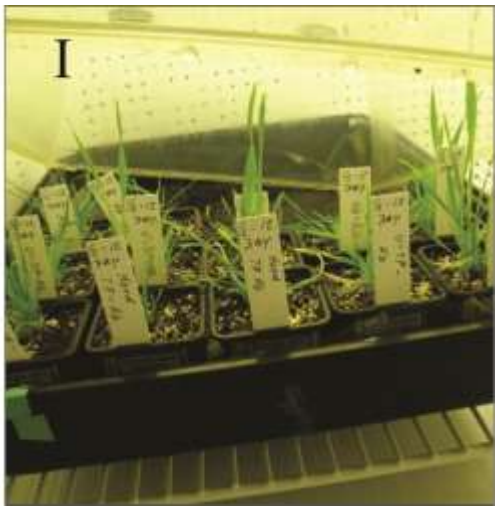


Figure 3.7. Different stages of barley transformation from infection to pots. (A) *Agrobacterium* infected barley embryos. (B) Callus on callus induction medium subcultured after two weeks. (C) Callus on callus maturation medium. (D) Callus with shoots ready for transfer to rooting medium. (E) Fresh putative transformant transferred to rooting medium. (F) Putative transformants with roots post two weeks incubation. (G) Putative transformants in cylinders on rooting medium. (H) Transfer of putative transformants to pots. (I) Putative transformants in pots incubated in growth chamber under covered conditions.

3.3. Results

3.3.1. Growth characteristics

Plant growth and development of wild type Golden Promise barley as compared to the hemoglobin overexpressing lines grown in greenhouse conditions were measured based on the Zadoks scale.

A box and whisker plot of the results was plotted (Figure 3.8). The notch marks the 95% confidence interval for the medians. 5% certain that the actual median for the underlying population is within the interval marked by the notches. If the notches from two box plots do not overlap, we can assume (0.05 significance level) that the medians are different. Confidence intervals provide a statistical measure for how reliably the sample data represent an underlying population. Results indicate that barley lines overexpressing hemoglobin grow at a significantly slower rate than wild type Golden Promise.

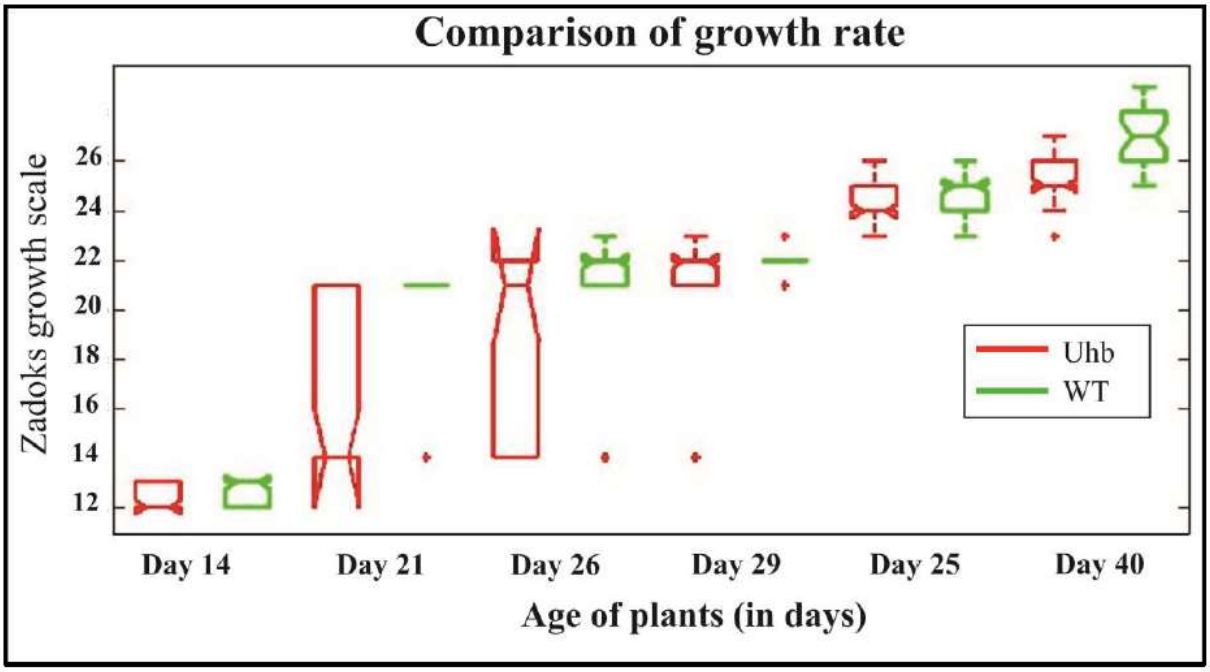


Figure 3.8: Box and whisker plot representation of plant growth stages based on Zadoks scale. Results indicate that barley plant overexpressing hemoglobin display slower growth rate as compared to wild type plants. The notch marks the 95% confidence interval for the medians. We are 95% certain that the actual median for the underlying population actually is within the interval marked by the notches. If the notches from two box plots don't overlap, we can assume at the (0.05 significance level) that the medians are different.

3.3.2. Morphological analysis

Height and weight of roots and shoots of wild type Golden Promise barley were significantly higher as compared to the hemoglobin overexpressing plants (Figure 3.9). It was observed that the height and weight of the plants follow the trend observed in the developmental stages of the plants (Figures 3.8 and 3.10).

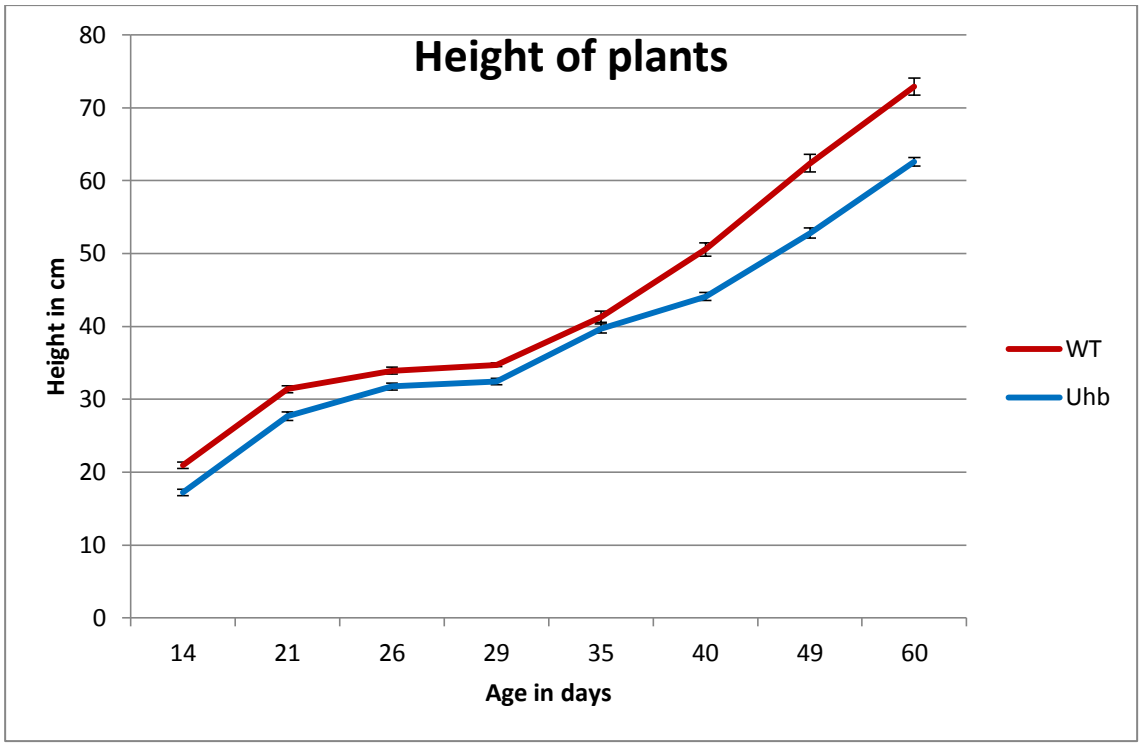


Figure 3.9: Height of the shoots of wild type Golden Promise and hemoglobin overexpressing lines 8 weeks post germination. Error bars indicate SD from the fifty plants analysed. Hemoglobin overexpressing lines show a slower growth rate, as compared to the wild type lines.

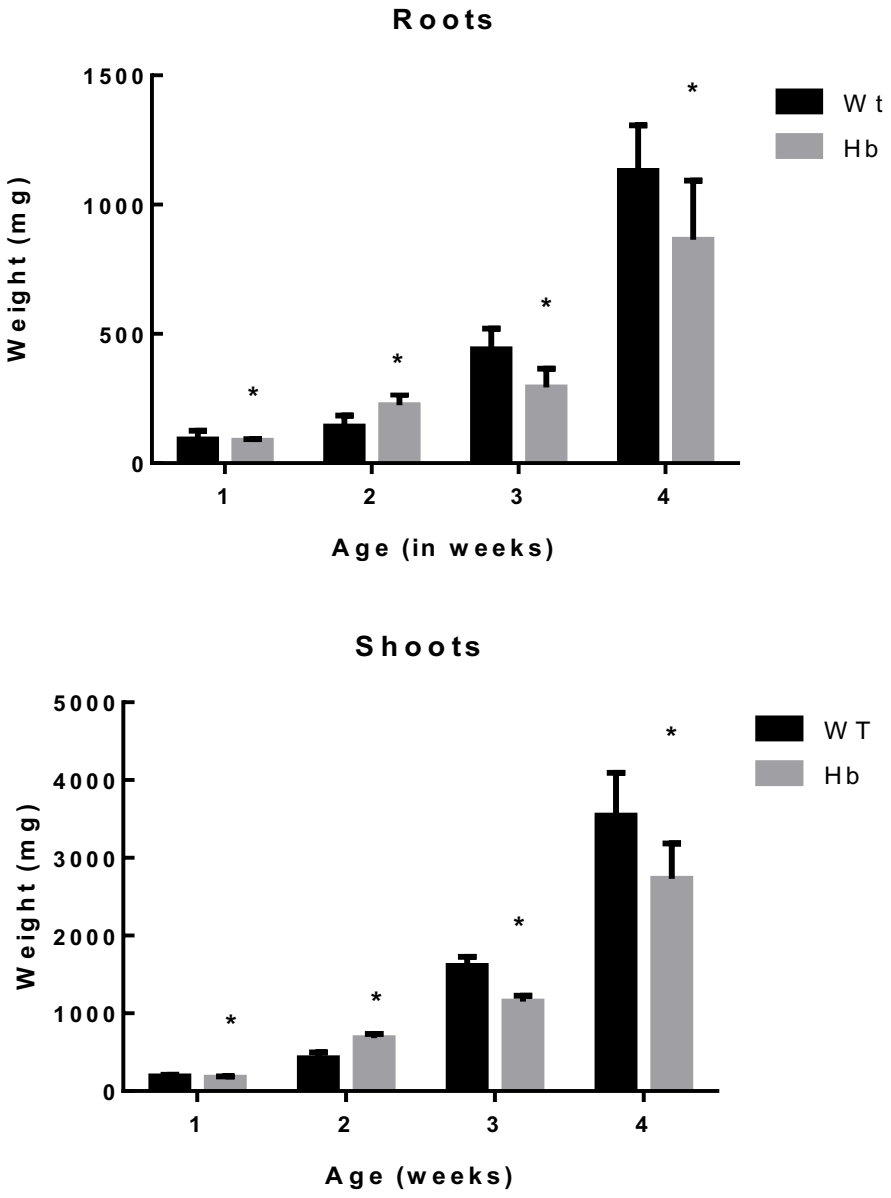


Figure 3.10: Weight of the shoots and roots of wild type Golden Promise and hemoglobin overexpressing lines at 4 weeks post germination. Error bars indicate SD based on five replicates. Asterisks indicate significant differences between wild type and hemoglobin overexpressing lines: *P<0.01.

3.3.3. Root analysis

Roots of the wild type Golden Promise and hemoglobin overexpressing plants grown under hypoxia were compared to analyze morphological differences. The roots were scanned and analysed using Lamnatech Scanalyzer. The image of the roots was divided into 9 equal segments and the density determined by image analyzer. Figure 3.11-A shows the image of the roots. Figure 3.11-B describes the sections of the roots for analysis. Figure 3.11-C describes the results of the image analysis. Results are based on three replicates. It was observed that the hemoglobin-overexpressing plants have denser adventitious roots as compared to the wild type Golden Promise plants.

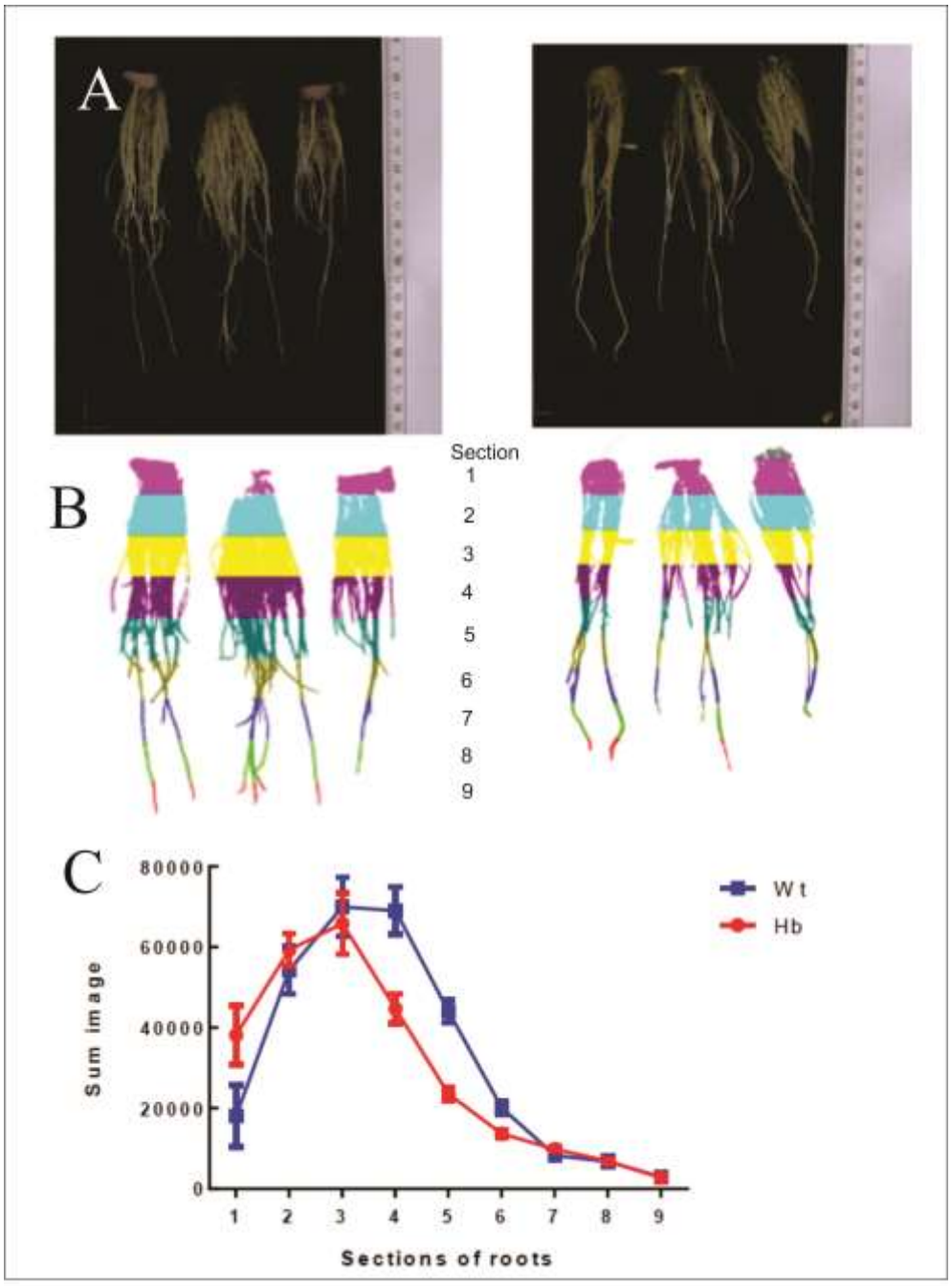


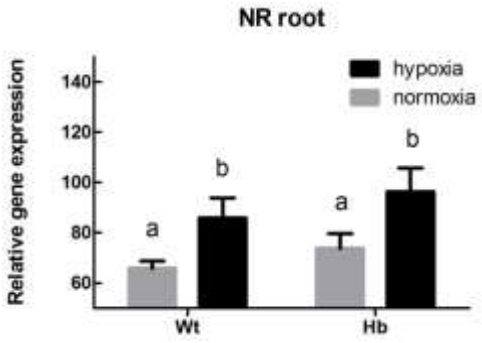
Figure 3.11. (A) Roots from wild type Golden Promise (right) and hemoglobin overexpressing plants (left) plants grown under hypoxia; (B) Sections of roots as the basis for root density analysis; (C) Root image analysis of the roots in terms of density of roots in sections of roots from crown to tip. Error bars indicate SD based on three replicates.

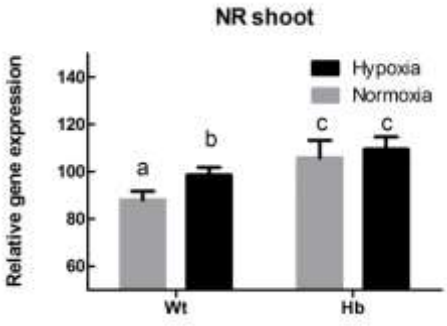
3.3.4. Gene expression analysis under hypoxia and normoxia

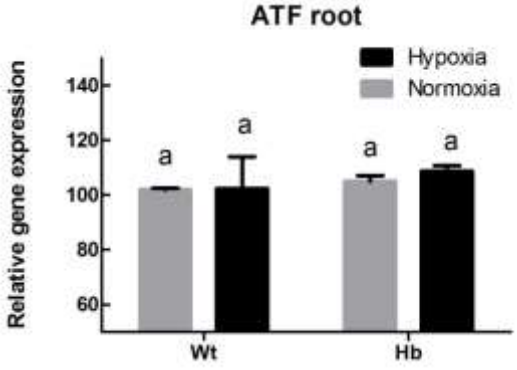
Figure 3.12 shows the results of the comparative gene expression analysis of nitrate reductase, MDHAR and alanine aminotransferase genes. It was observed that there is a trend of upregulation of all of these genes in hemoglobin overexpressing lines. The trend was distinct with expression of nitrate reductase in shoots and MDHAR and alanine aminotransferase in roots.

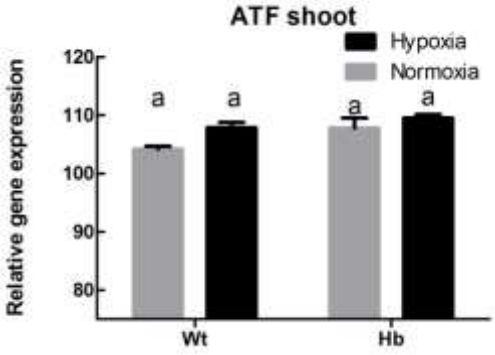
Figure 3.13 shows the results of the comparative gene expression analysis of JRG1, GaMYb and PP2C genes which are the marker genes for jasmonic acid, gibberellic acid and ABA levels. There is a significant upregulation of PP2C in shoots overexpressing hemoglobin and roots of plants under hypoxia. Jasmonic acid marker is upregulated under hypoxia in hemoglobin overexpressing plants as compared to wild type plants. Gibberellic acid marker is significantly upregulated in shoots of plants overexpressing hemoglobin as compared to wild type under hypoxia.

A detailed analysis of gene expression levels of all 4 isoforms of the *adh* gene in wild type and hemoglobin overexpressing plants from roots, shoots and seeds both under hypoxia and normoxia was performed. It was found that hypoxia and hemoglobin overexpression does not have a significant impact on the expression levels of *adh* compared to normoxia (p value 0.764) and wild type plants (p value 0.992), respectively. But there is a significant difference in expression levels of *adh* genes within roots, shoots and seeds under the conditions noted above (p value 0.034). Detailed gene expression analysis of isoforms of *adh* are described in Figure 3.14.


NR root
hypoxia
normoxia
Relative gene expression
140
120
100
80
60
a
b
a
b
Wt
Hb


NR shoot
Hypoxia
Normoxia
Relative gene expression
140
120
100
80
60
a
b
c
c
Wt
Hb


ATF root
Hypoxia
Normoxia
Relative gene expression
140
120
100
80
60
a
a
a
a
Wt
Hb


ATF shoot
Hypoxia
Normoxia
Relative gene expression
120
110
100
90
80
a
a
a
a
Wt
Hb

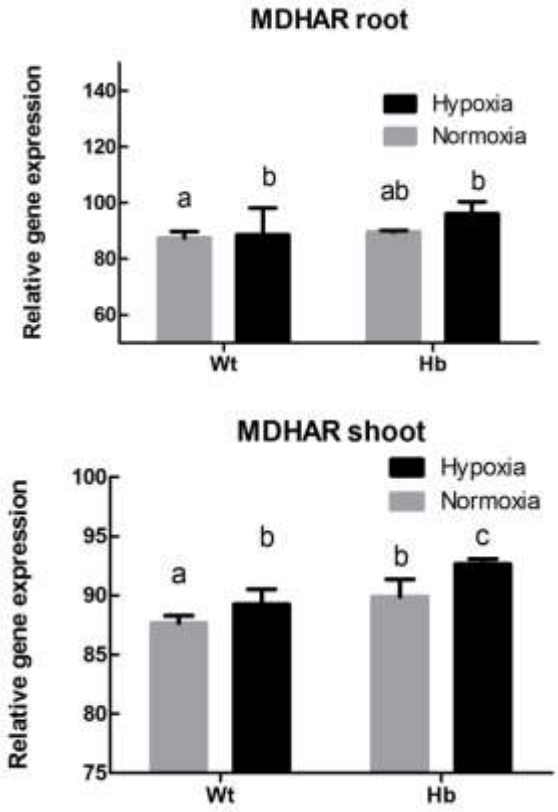
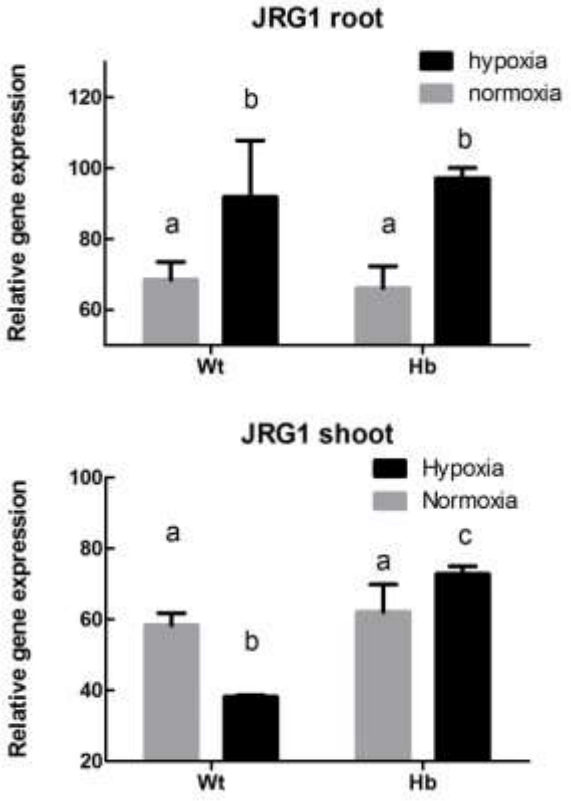
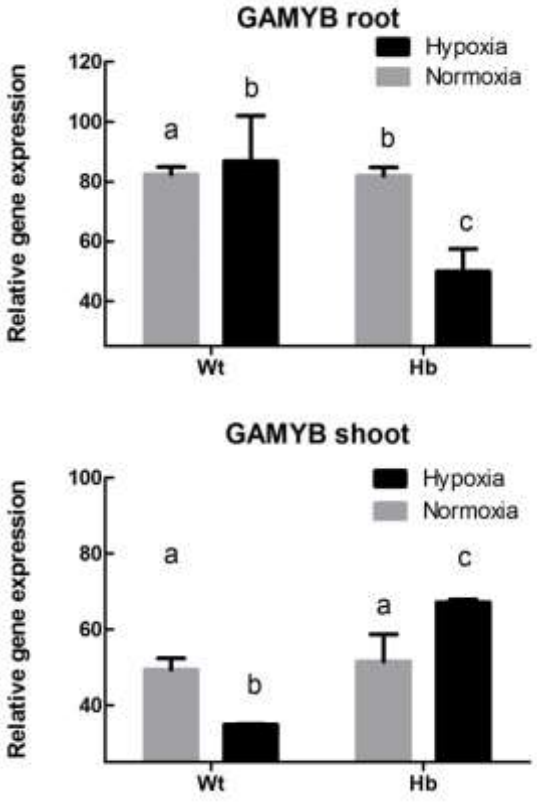


Figure 3.12. Relative expression of genes participating in NO synthesis and NO cycle in wild type Golden Promise and hemoglobin overexpressing barley plants grown under hypoxia and normoxia. Error bars indicate SD based on triplicates. The letters above the bars indicate whether the expression levels of the gene are significantly different in the barley lines and conditions described in the graph.


JRG1 root
hypoxia
normoxia
Relative gene expression
120
100
80
60
a
b
a
b
Wt
Hb
JRG1 shoot
Hypoxia
Normoxia
Relative gene expression
100
80
60
40
20
a
b
a
c
Wt
Hb


GAMYB root
Hypoxia
Normoxia
Relative gene expression
120
100
80
60
40
a
b
b
c
Wt
Hb
GAMYB shoot
Hypoxia
Normoxia
Relative gene expression
100
80
60
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Wt
Hb

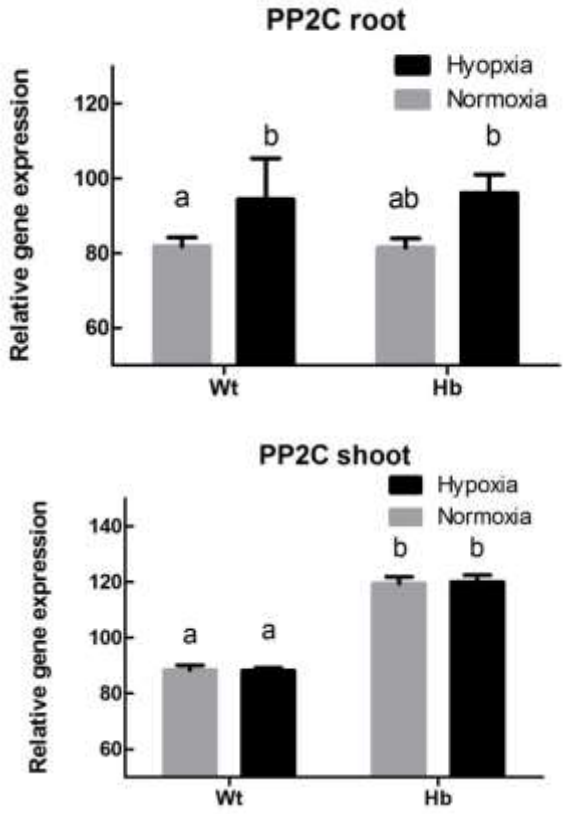

Figure 3.13: Relative expression of hormone marker in wild type Golden Promise and hemoglobin overexpressing barley plants grown under hypoxia and normoxia. Error bars indicate SD based on triplicates. The letters above the bars indicate whether the expression levels of the gene are significantly different in the barley lines and conditions described in the graph.

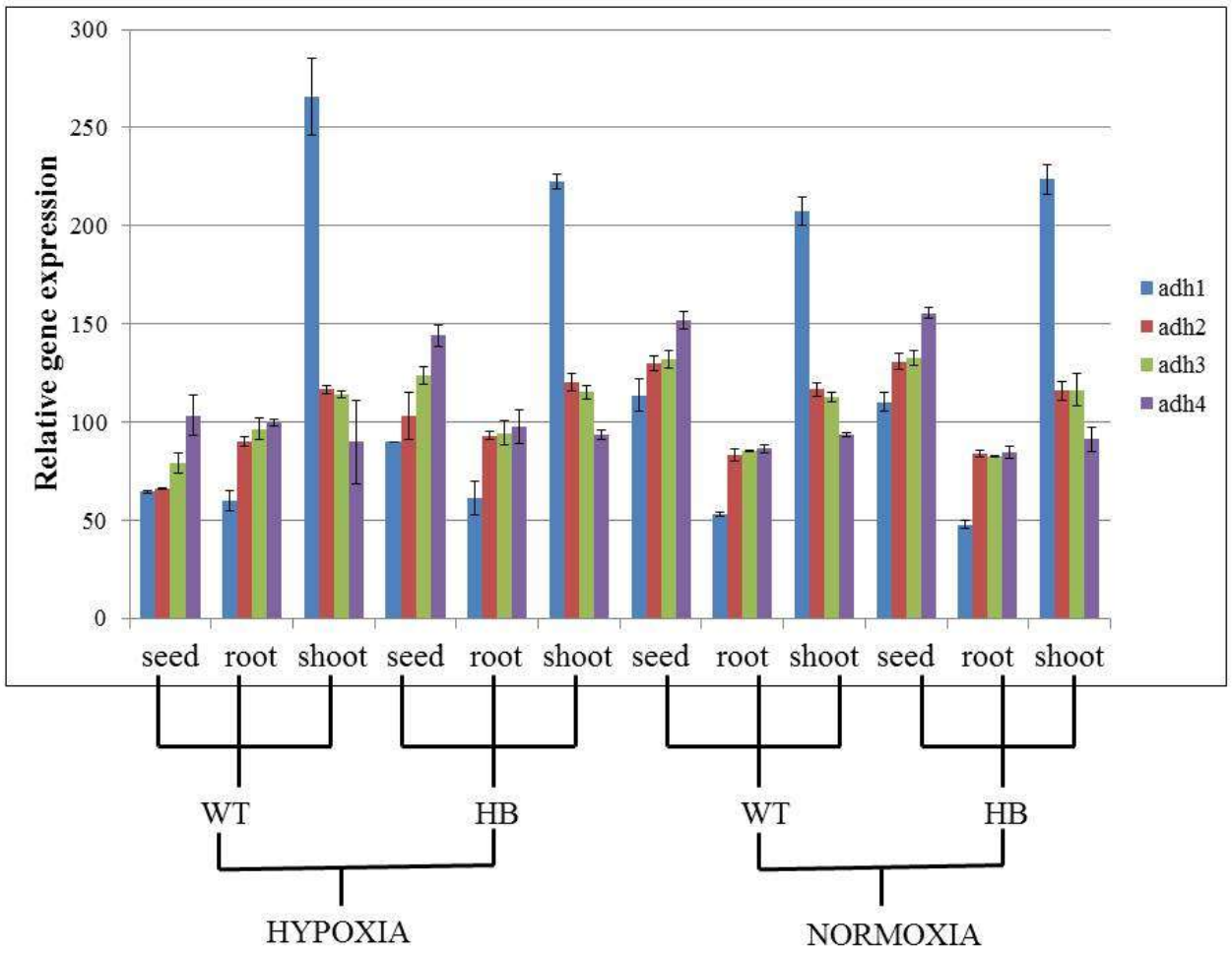

Figure 3.14: Relative gene expression of isoforms of alcohol dehydrogenase gene in hemoglobin overexpressing and wild type Golden Promise lines under hypoxia and normoxia. Error bars indicate SD based on biological triplicates.

3.3.5. Construction of Hb silencing lines

More than 300 embryos were infected with *Agrobacterium* transformed with the Hb silencing construct. 35 out of the 300 reached the shooting stage and 22 were transplanted to small pots. They are currently in T1 stage and further analysis is awaited.

3.4. Discussion

3.4.1. The model to study effects of NO in plants

There are several reports on the effect of NO on plant morphology based on *in vitro* studies employing NO donors such as SNP, GNSO and others along with NO scavengers such as CPTIO. There exist very few *in vivo* systems where NO levels have been modulated to demonstrate the effect of high or low NO levels. Most of the studies are using the nitrate reductase mutants (Rockel 2001; Desikan 2002,) or NOS mutants (Crawford 2003). The study mimicked flooding using hydroponic system of plant growth to demonstrate the effects of hemoglobin overexpression under hypoxia.

3.4.2. Nitric oxide cycle

The exogenous application of nitrate in rice, pea, wheat seedlings and maize root tips under strict anoxia has a protective role and results in a decrease in ethanol production during anoxia (Vartapetian 1999). Under hypoxia, there is an accumulation of nitrite due to the activation of cytosolic nitrate reductase, which can be metabolised by mitochondrial electron transport chain complexes. This may contribute towards mitochondrial functioning under hypoxia. Hb is also induced by nitrate, nitrite and NO treatment (Nie and Hill 1997, Ohwaki 2005). Induction of the Hb genes is closely associated with that of nitrate reductase gene (Ohwaki 2005). NR knockouts have shown that nitrate, nitrite and NO by themselves fail to induce Hb gene expression. Nitrate reductase and MDHAR participate in the NO cycle which is upregulated under hypoxia. Upregulation of these genes under hypoxia represents further evidence of their

involvement in the NO cycle supporting mitochondrial functioning under hypoxia. Alanine aminotransferase is another source of NO. It is upregulated in hemoglobin overexpressing plants both under normoxia and hypoxia in roots, but the expression levels are significantly unchanged in shoots. Results of the gene expression analysis show that there is a significant effect of hemoglobin overexpression in roots as compared to shoots. Roots face hypoxia under flooding and hence need a higher degree of adaptation.

3.4.3. Studies using hormone markers

Relative levels of jasmonic acid and gibberellic acid markers in wild type Golden Promise and hemoglobin overexpressing plants under hypoxia and normoxia show that both hemoglobin expression level and hypoxia have an influence on hormonal levels. It has been reported that NO is responsible for induction of stomatal closure (García-Mata 2001). Stomatal closure is induced by many different factors, such as osmotic stress, darkness, high CO_2 concentrations, and some mechanical stresses (Kearns and Assmann 1993). All responses are accompanied by an increase in intracellular Ca^{2+} concentrations in the guard cells. Increases of Ca^{2+} concentration precede stomatal closure (Grabov and Blatt, 1999). Ca^{2+} is released from mitochondria during hypoxia (Subbaiah 1998). During drought stress processes, stomata are induced to close as leaves sense water deficit, especially after the leaf water potential drops below some threshold level. The production of ABA triggers the increase of cytosolic Ca^{2+} concentration in guard cells both via IP3 signal transduction cascade and also via cADPR. These processes are correlated with a reduction in stomatal aperture (Muir and Sanders 1996; Grill and Himmelbach 1998;

Leckie et al., 1998). Hemoglobin overexpression is linked with increase in PP2C gene marker expression. This indicates that the levels of ABA in hemoglobin overexpressing plants could be higher as compared to wild type plants. Increased ABA levels trigger the reduction of stomatal aperture and hence reduce the rate of transpiration. This is another factor contributing towards the lower NO emissions in hemoglobin overexpressing plants. It can be hypothesised that the lower NO emissions from hemoglobin overexpressing plants is attributed to two factors. 1. Increase in NO scavenging and hence increase in the turnover of the NO cycle. 2. Decrease in rate of transpiration due to closure of stomata caused by elevated ABA levels. This indicates that hemoglobin overexpression may have a role to play in drought resistance. This demonstrates the potential for developing both flooding and drought tolerance via hemoglobin overexpression and hence provides evidence for the diverse role of hemoglobins in crop biotechnology.

3.4.4. Root morphology

NO is a second messenger in auxin signal transduction leading to root developmental processes. It has been reported that the NO donor sodium nitroprusside (SNP) applied to germinated tomato seeds was able to induce lateral root formation in the same way as auxin treatment (Lombardo 2006, Hebelstrup 2013). NO modulates the expression of cell cycle regulatory genes in tomato pericycle cells and leads, in turn, to induction of lateral root formation. NO mediates the induction of the *CYCD3* gene and the repression of the CDK inhibitor *KRP2* gene at the beginning of lateral root primordia formation (Hebelstrup 2013). In addition, auxin-dependent cell cycle gene regulation was dependent on NO (Correa-Aragunde 2006).

GA3 regulates nitrate reductase activity in root nodules by enhancing its synthesis (Ramanujam 1998). Our results suggest that gibberellic acid synthesis could be upregulated in hemoglobin overexpressing plants. Root nodules are highly anoxic. Induction of nitrate reductase synthesis via gibberellic acid can be a part of cellular adaptation to low oxygen stress. Reduction of endogenous NO levels in maize root apical cells caused an inhibition of root elongation upon external application of high nitrate levels (Zhao 2007). A high NO turnover and hence reduction of nitrate in hemoglobin overexpressing plants under hypoxia can lead to inhibition of root elongation. Our results show that under hypoxia, there is a significant difference in root length as compared to normoxia. NO has been reported to induce adventitious root formation (Yadav 2010, Liao 2012). Our results are in line with this hypothesis and demonstrate that there is a higher level of adventitious root formation in plants overexpressing hemoglobin as compared to wild type plants under hypoxia. NO and hydrogen peroxide alleviate drought stress and promote adventitious root development in marigold explants (Liao et al., 2012). Hence it further supports the role of hemoglobin overexpression and resultant increase in NO turnover in conditions of drought stress (Figure 3.15).

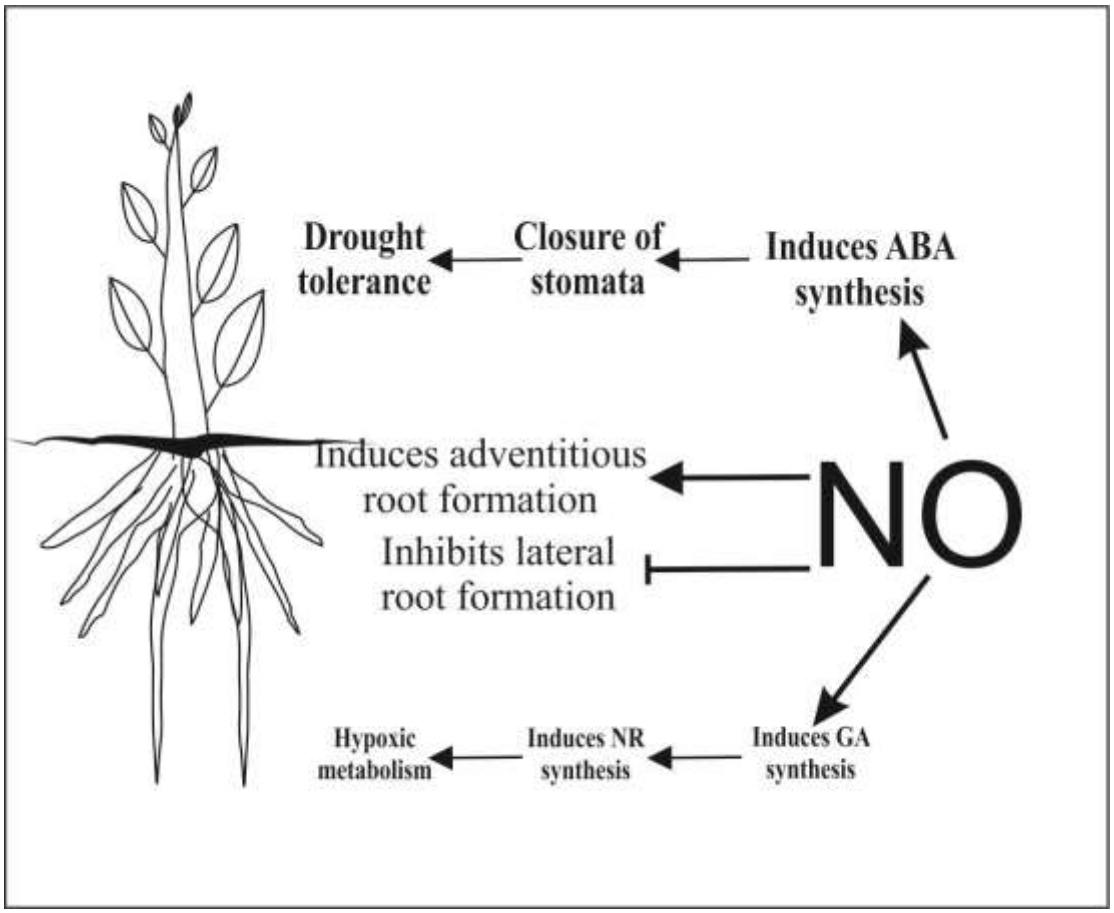

Figure 3.15. Schematic diagram showing the effect of increase of NO cycle turnover under hypoxia

3.4.5 Hemoglobin overexpression adapts quiescence strategy for flooding tolerance

Rice is the most extensively studied plant for hypoxia and anoxia tolerance. It adapts two exclusive strategies to cope with submergence: the first is called the escape strategy which is based on the avoidance of anaerobiosis through rapid growth and the second is called the quiescence strategy which is characterized by tolerance to anaerobiosis via reduced growth rate. Each strategy is relevant under appropriate conditions of flooding and submergence. The escape strategy is characterized by improving the elongation of the stem and/or leaf in order to break the water surface and establish contact with air. Deep-water rice displays an extremely fast internode elongation in response to increasing water levels (Narsai 2010). Internodes of deep-water rice elongate with a daily increase of 20-25 cm and even reach 7 m in height (Stunzi 1989). Ethylene along with ABA and GA was suggested to have a role in internode elongation (Metraux 1983). Escape strategy is characterised by increase in ethylene levels, inhibition of ABA, and hence GA response leading to elongation. Under the quiescence mechanism, growth is arrested under flooding (Bailey-Serres 2010). Plants tend to reduce growth and metabolic activity to a minimum, thus storing energy for re-growth. Quiescence strategy is characterised by downregulation of growth, continuation of fermentation, upregulation of ROS detoxification systems.

Hemoglobin influences ADH activity and lactate fermentation. Hemoglobin overexpression helps to maintain ATP levels under hypoxia, which indirectly influence starch synthesis. All this evidence suggests that overexpression of hemoglobin in cereals

can confer flooding and submergence tolerance via the quiescence strategy. Hemoglobin overexpression also leads to an upward leaf movement phenotype (Hebelstrup 2012). This further indicates that the leaves follow an upward movement, rather than rapid elongation while trying to break the water surface barrier, providing further evidence towards similarity to quiescence strategy. ABA-mediated stomatal closure in hemoglobin overexpressing plants can provide drought tolerance to plants. Submergence followed by drought and vice versa is a common occurrence in rain-fed lowlands. Hence crop improvement via a combined tolerance to flooding and drought by hemoglobin overexpression can help to increase plant survival and hence to increase yield.

3.4.6. Construction of Hb silencing lines

Throughout the length of the thesis, the importance of hemoglobin and NO in plant physiology, plant growth and development has been emphasised. This chapter described the morphological changes observed in barley overexpressing hemoglobin. Various reports have shown that over-expression of hemoglobin in plants can delay time to flowering, increase stress tolerance, seed yield and even biomass. These studies have been conducted in *Arabidopsis* and tobacco lines overexpressing and either silenced or knocked out hemoglobin expression. They are model organisms and there is a need to reproduce these results in crop plants. The current work aims to facilitate such a study to engineer crop plants with desired traits.

Chapter 4: The role of plant hemoglobin and nitric oxide during the hypoxic stress and senescence

4.1. Introduction

4.1.1. Senescence

Senescence is an age-dependent process in plants, but it can also be induced by various factors such as darkness, stress, temperature and hormones. It is a complex, well-coordinated process involving programmed cell death (PCD). Plants face hypoxia and anoxia during flooding and submergence. Under these conditions, accumulating nitrite can be reduced by the mitochondrial electron transport chain to form NO. Class 1 hemoglobin plays an important role to ensure the efficient scavenging of NO to nitrate. It has been reported that Hb modulates the levels of NO and ethylene under stress (Manac'h-Little et al., 2005). The present study investigates the role of hemoglobin in dark-induced senescence and its cross-talk with NO under hypoxia. We demonstrate that overexpression of hemoglobin in barley can delay dark-induced senescence. In hypoxic conditions, plants overexpressing hemoglobin display characteristics of quiescence strategy for survival. Hence, hemoglobin overexpression and the resultant increase in NO turnover can delay senescence, support mitochondrial function and facilitate plant survival in low oxygen conditions.

Leaf senescence is the final stage of leaf development and constitutes a coordinated degeneration program in which nutrients are mobilized from senescing leaves to other parts of the plant (Buchanan 1997). It is characterised by a decline in photosynthesis, chlorophyll degradation and yellowing of the leaves. It also leads to a decrease in protein and mRNA concentrations. It is accompanied by various changes in cell structure metabolism and gene expression (Nooden 1997). Senescence is controlled by various internal and external signals and the process of senescence can be delayed or accelerated by these signals (Nooden 1997). In Arabidopsis, senescence is characterized by a downregulation of photosynthetic genes and upregulation of a group of genes called senescence-associated genes or SAG (Lohman 1994; Buchanan 1997; Gepstein et al., 2003). Among the predominant SAG genes are those encoding the enzymes involved in degradation of different compounds, e.g. proteinases (Lohman 1994).

4.1.2. Nitric oxide and ethylene

There are various reports that describe a possible stoichiometric relationship between NO and ethylene. Decrease of NO in young immature tissues is a typical and overall trend in plant tissue, and exogenously applied NO could delay ripening and/or senescence. NO increment and ethylene decrement have been related to heat stress and water stress. In a wide a variety of immature or unripe plant organs such as fruits, vegetables and flowers, the endogenous levels of NO emissions significantly exceed that emitted in mature or senescing conditions (Leshem 1996; 1998). It has been independently reported by several authors that Hb influences ethylene levels (Hebelstrup et al., 2012; Manac'h-Little et al., 2005).

4.1.3. Aim of study

In this part of the project, we investigate the role of hemoglobin during senescence in barley. We show that in barley, hemoglobin gene expression is upregulated under dark-induced senescence. It has been reported that hemoglobin expression influences ethylene levels in plants. Considering these two facts, we investigate the role of hemoglobin in dark-induced senescence and under hypoxia.

4.2. Materials and methods

4.2.1. Plant material

The transgenic barley lines overexpressing hemoglobin and the wild type Golden Promise were grown under long-day conditions (16 h light and 8 h dark). To study the effect of dark-induced senescence on individual leaves, the second leaf of three plants was covered with aluminum foil for 4 days and then leaves were sampled and immediately frozen in liquid nitrogen. To study the effect of darkness, three plants each from wild type and hemoglobin overexpressing Golden Promise lines were uprooted and roots immersed in distilled water. The plants were placed in total darkness for 24 h. To study the effect of hypoxia, similar plants were placed in an enclosed chamber flushed with 0.01% oxygen mixed with nitrogen. Leaves were sampled and immersed in liquid nitrogen.

For each leaf sample, the total RNA was isolated from the plant material using the SpectrumTM Plant total RNA kit (Sigma) as per the manufacturers' recommendations. RNA concentration and purity was checked on nanodrop spectrophotometer. First-strand

cDNA synthesis was performed on 1 µg of RNA and a nonamer, random oligonucleotide primer (2.5 µM) by incubation at 65°C for 5 min followed by 10 min at room temperature in a volume of 18.4 µl. 200 U SuperscriptII (Invitrogen), 40 U RNAsin (Promega), 1× FS Buffer (Invitrogen), 10 µM dichlorodiphenyltrichloroethane 1,4-dithiothreitol (DDT), and 2 mM dNTPs (GE Healthcare) were added to make a final volume of 30 µl, which was incubated for 1 h at 42 °C and 10 min at 70 °C, followed by the addition of 70 µl of water. qRT-PCR was performed using the ABI Prism 7900HT Sequence Detection System with the Power SYBR Green PCR master mix (Applied Biosystems). The cDNA template used in the PCR was synthesized from senescent or green leaf tissue. Differential ct values of corresponding gene expressed under given conditions in the wild type Golden Promise and in the plants with modified hemoglobin expression were calculated. The expression level of 18S rRNA was used for normalization of the results. List of primers is the following (Table 4.1).

Table 4.1. List of primer sequences for study of senescence in Arabidopsis

Gene	Primer sequence
Sen4	Fwd: CCTGGTAACTCTGCAGGAACAGTCAC Rev: GCATTCCTTAGGAGCTCCCTGTGG
RBSC	Fwd: CGGCGGAAGAGTTAACTGCAT Rev: CGGGTGTTGTCGAATCCGAT
Sag21	Fwd: CCAATGCTATCTTCCGACGTG Rev: GAACCGGTTTCGGGTCTGTAA
Cab1	Fwd: GAGACTACGGATGGGACAC

	Rev: GATGGCTTGAACGAAGAAT
Sag13	Fwd: TCGTCAACAATGTGGGAACG Rev: CGACTCCAGCAGCAGAGGAT
Cab2	Fwd: GAGACTACGGATGGGACAC Rev: GATGGCTTGAACGAAGAAT
18S	Fwd: AAACGGCTACCACATCCAAG Rev: ACTCGAAAGAGCCCGGTATT

Table 4.2. List of primer sequences for study of senescence in barley

Gene	Primer sequence
Nac5	Fwd: CCATGTGAACAGCAGCGGCAAC Rev: CCGACGTTGAGGCTGGTGAATC
Nac13	Fwd: ATGCCGCCGCACATGATGTAC Rev: ACAGGTCGCCGGAATTAGCG
Nac27	Fwd: ACGGCTACGTGAACCACGACAC Rev: CAAGCTGCCGCTGGATCTCTTC
RUBISCO	Fwd: TCTTCCGTGAGCACAACAGC Rev: TCCAGTATCGGCCGTCGTAG
Cyspep	Fwd: AAAGGTGGCAAGGATTATTGG Rev: TGCCACAGATACCTGACGAT
18S	Fwd: ACGGCTACCACATCCAAGGA Rev: ACGGCTACCACATCCAAGGA

4.3. Results

4.3.1. Effects of dark-induced senescence

In barley leaves covered for three days, it was found that hemoglobin expression was significantly upregulated as compared to uncovered leaves (Figure 4.1). In plants overexpressing hemoglobin, senescence markers such as *cyspep*, *nac5*, *nac13* and *nac27* were downregulated in leaves covered for three days as compared to wild type leaves. Similarly, downregulation of Rubisco was significantly lower in hemoglobin overexpressing plants as compared to the wild type. This shows that hemoglobin overexpression can delay senescence in barley leaves covered for three days.

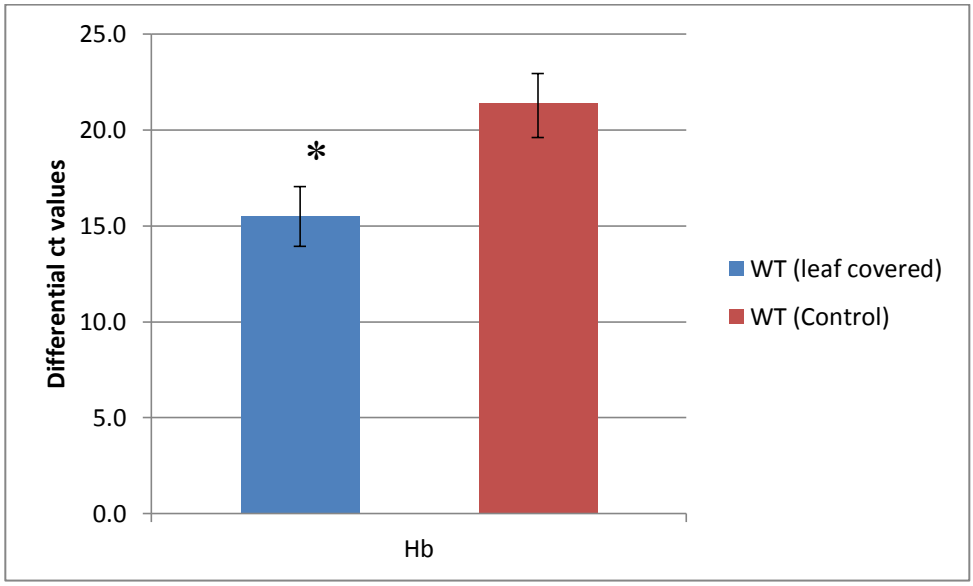

Figure 4.1. Expression of hemoglobin gene in a single leaf of WT Golden Promise plants covered for three days. Y-axis represents the differential ct values, hence the lower the ct value translates to higher level of gene expression. Error bars indicate SD based on biological and technical triplicates. Asterisks indicate significant differences between gene expression levels under described condition. * $P<0.01$.

4.3.2. Effect of darkness and hypoxia

The next step was to investigate the effect of hemoglobin overexpression on senescence markers in whole plants under darkness and hypoxia. Hemoglobin overexpressing and wild type plants were placed in darkness for 24 hours.

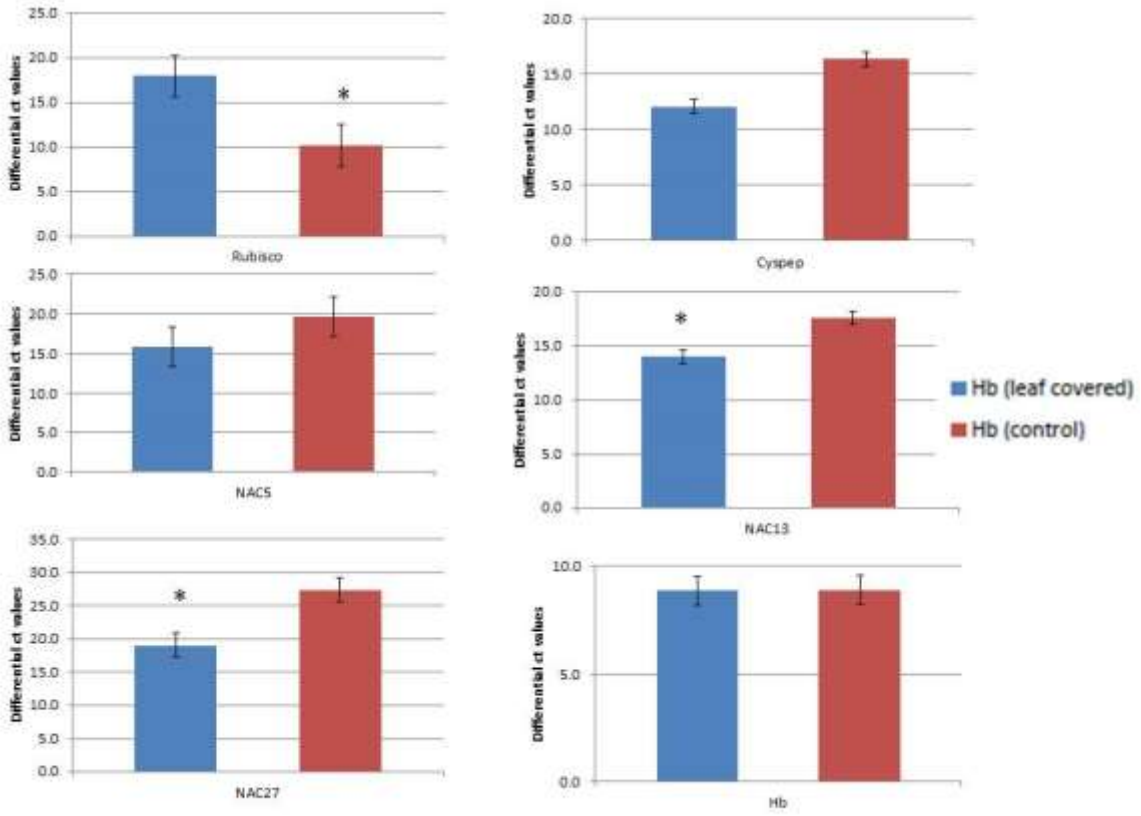

Figure 4.2: Effect of covering of a single leaf for 72 hours on hemoglobin overexpressing barley as compared uncovered hemoglobin overexpressing leaves. Y-axis represents the differential ct values, hence the lower the ct value translates to higher level of gene expression. Error bars indicate SD based on biological and technical triplicates. Asterisks indicate significant differences between gene expression levels under described condition. * $P<0.01$.

Results from Figure 4.2 that the downregulation of Rubisco is significantly lower in hemoglobin overexpressing plants as compared to wild type plants. Similarly cyspep and *nac13* were significantly downregulated in hemoglobin overexpressing plants. To investigate the effects of hypoxia as compared to darkness on senescence markets in whole plants, wild type and hemoglobin overexpressing plants were placed in darkness and in an enclosed chamber flushed with 0.01% oxygen in nitrogen gas for 24 hours.

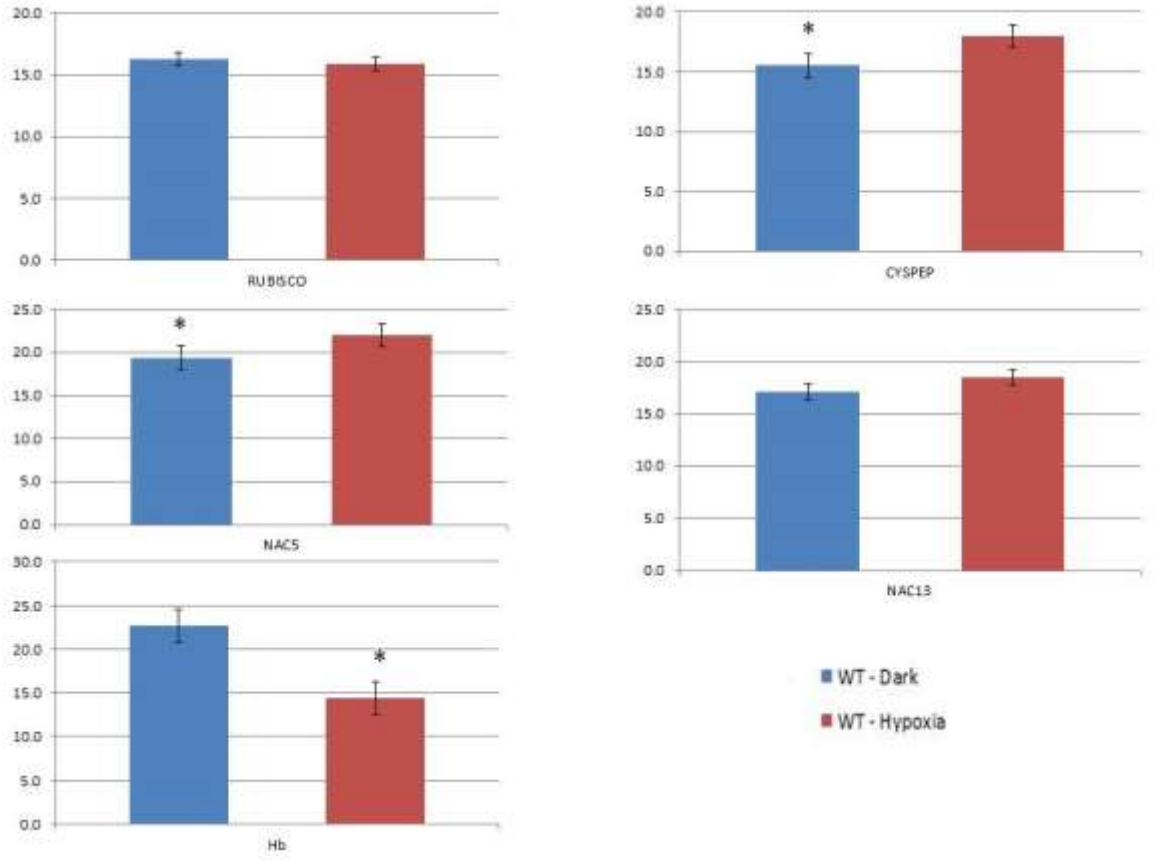

Figure 4.3: Effect of hypoxia under dark in wild type barley plants under dark and hypoxia. Y-axis represents the differential ct values, hence the lower the ct value translates to higher level of gene expression. Error bars indicate SD based on biological and technical triplicates. Asterisks indicate significant differences between gene expression levels under described condition. * $P<0.01$.

Results from Figure 4.3 suggests that hypoxia can influence dark induced senescence. Downregulation of CYSPEP and NAC5 in WT plants under hypoxia as compared to darkness indicate that under hypoxia, there could be a delay in the senescence.

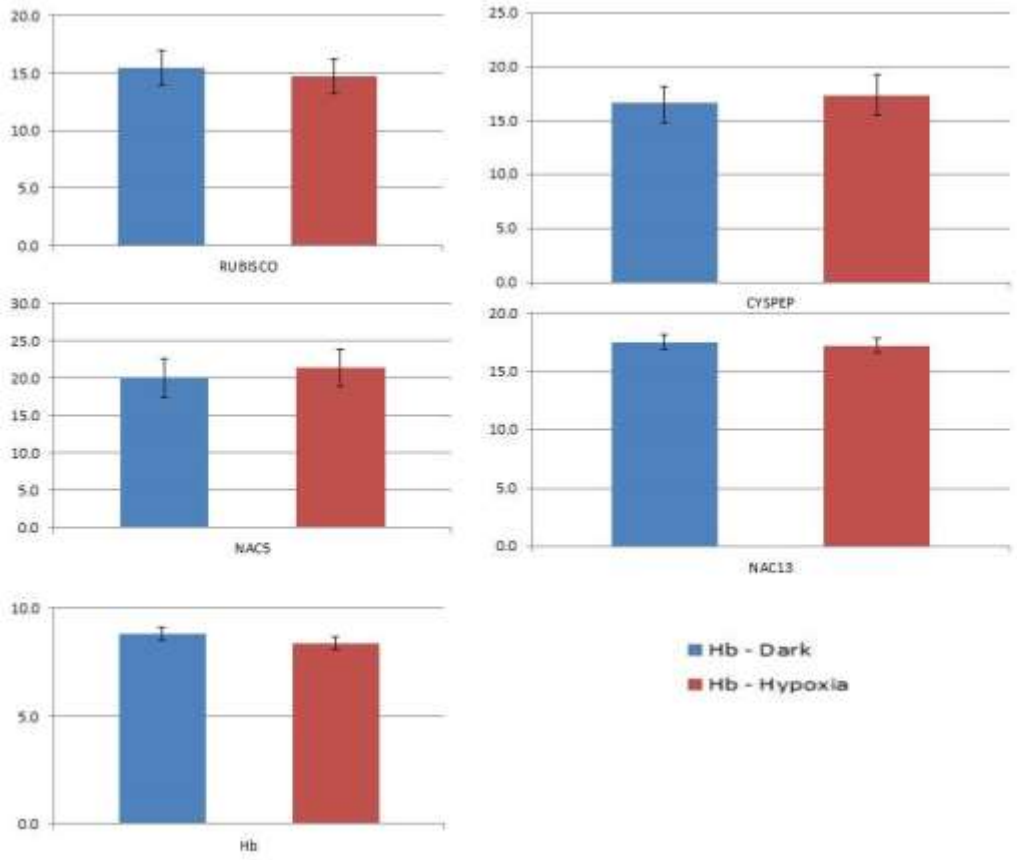

Figure 4.4: Effect of hypoxia under dark conditions on expression of hemoglobin and senescence markers in barley plants overexpressing hemoglobin. Y-axis represents the differential ct values, hence the lower the ct value translates to higher level of gene expression. Error bars indicate SD based on biological and technical triplicates. Asterisks indicate significant differences between gene expression levels under described condition. * $P<0.01$.

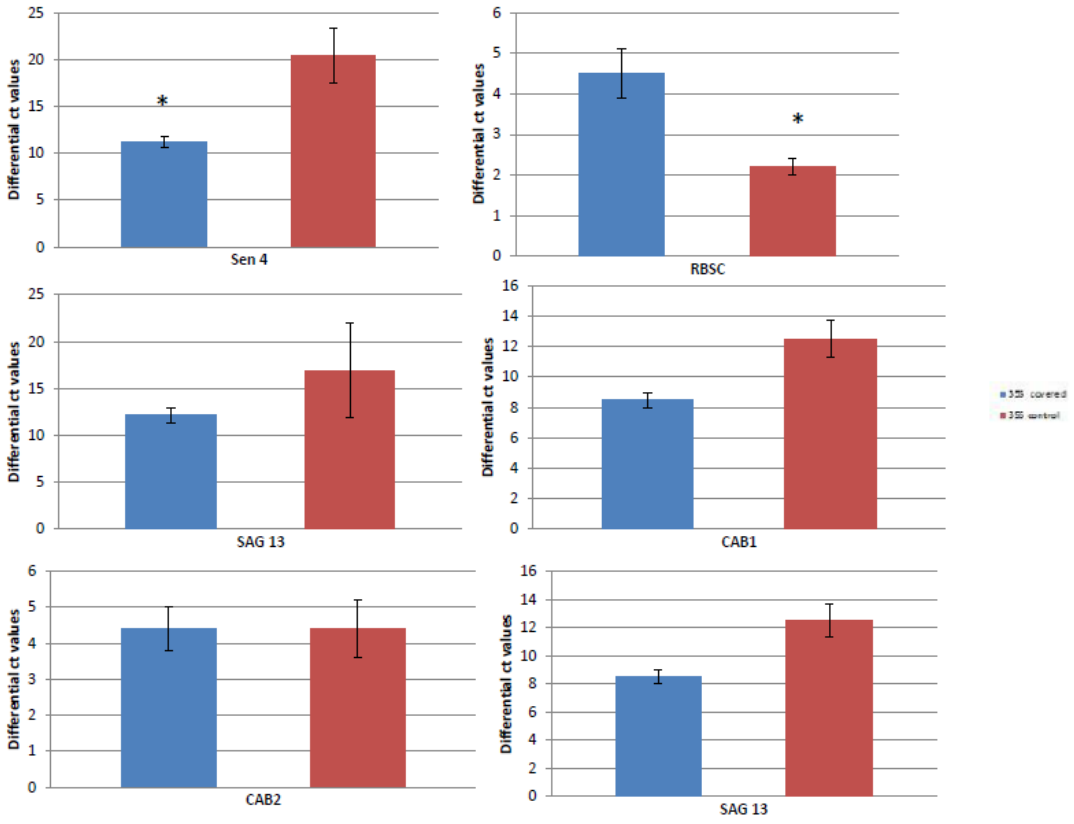

Figure 4.5: Effect of covering of a single leaf for 72 hours wild type Arabidopsis plants as compared to uncovered. Y-axis represents the differential ct values, hence the lower the ct value translates to higher level of gene expression. Error bars indicate SD based on biological and technical triplicates. Asterisks indicate significant differences between gene expression levels under described condition. * P<0.01.

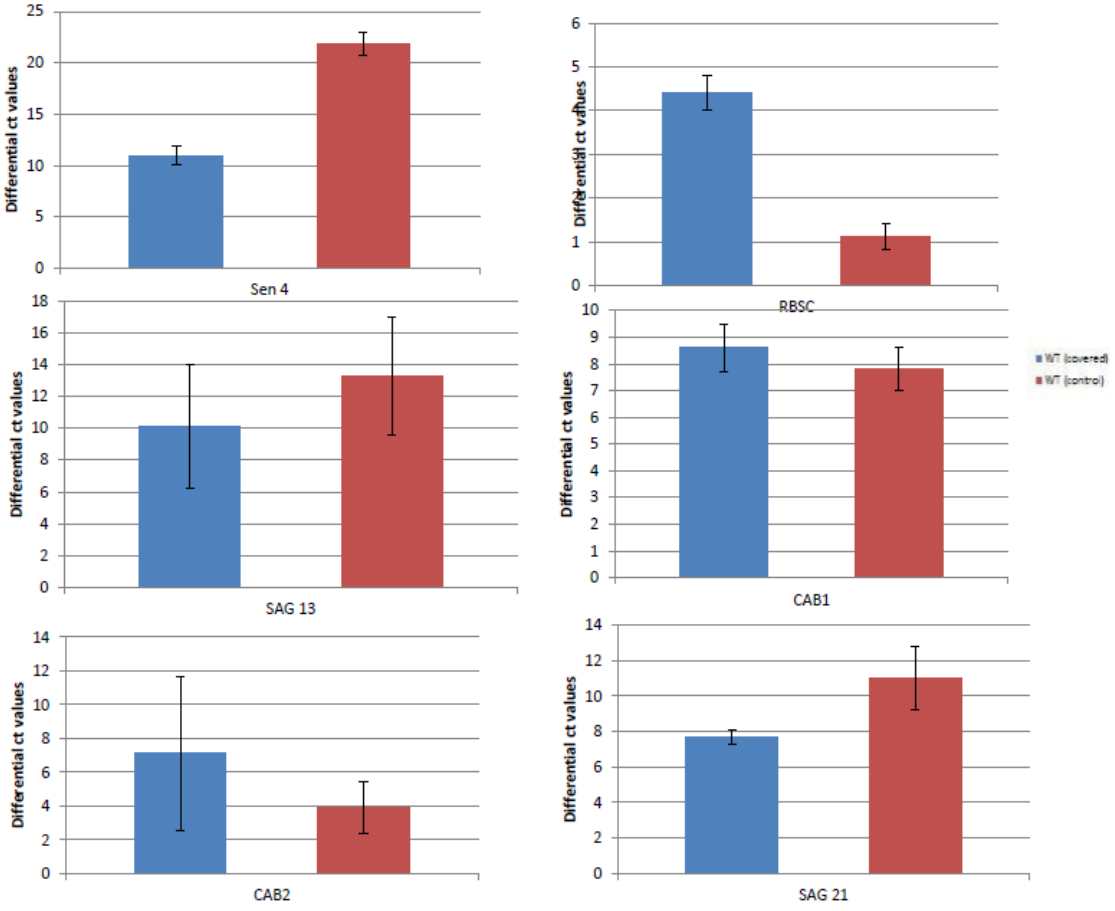

Figure 4.6: Effect of covering of a single leaf for 72 hours wild type Arabidopsis plants overexpressing hemoglobin as compared to uncovered. Y-axis represents the differential ct values, hence the lower the ct value translates to higher level of gene expression. Error bars indicate SD based on biological and technical triplicates. Asterisks indicate significant differences between gene expression levels under described condition. * $P<0.01$.

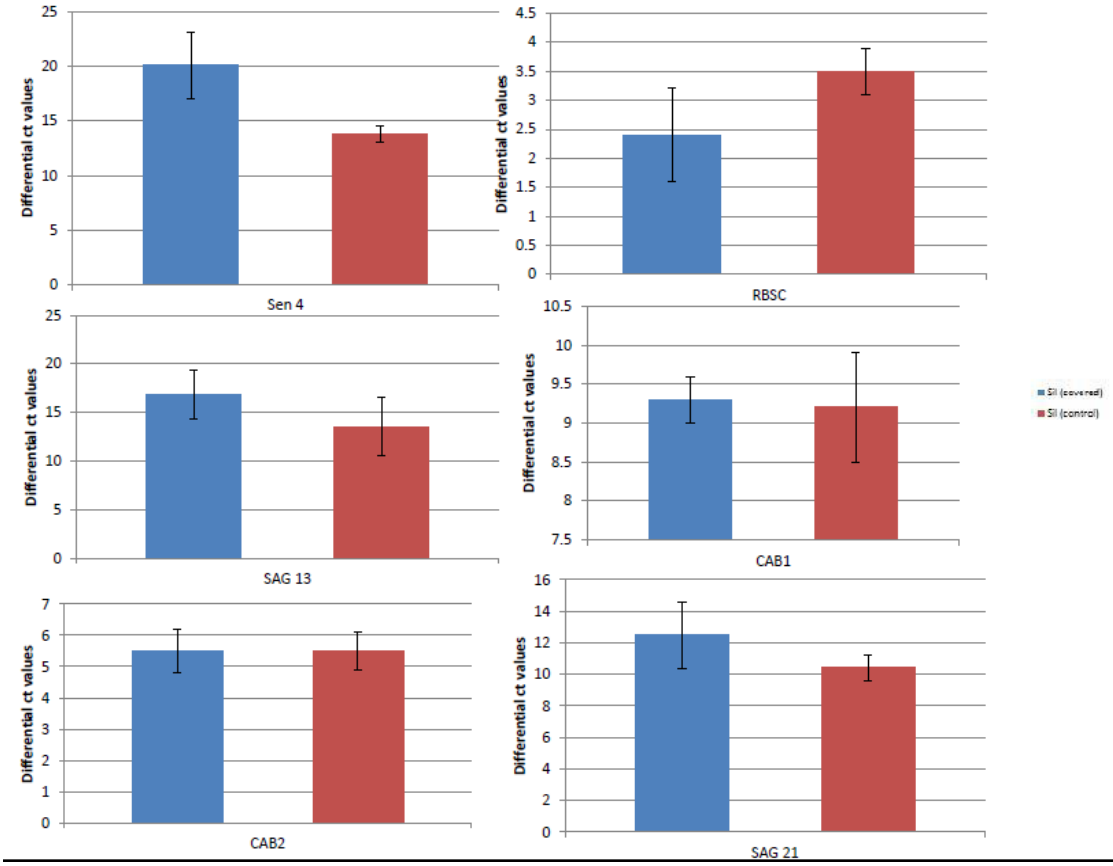

Figure 4.7: Effect of covering of a single leaf for 72 hours wild type Arabidopsis plants silencing hemoglobin as compared to uncovered. Y-axis represents the differential ct values, hence the lower the ct value translates to higher level of gene expression. Error bars indicate SD based on biological and technical triplicates. Asterisks indicate significant differences between gene expression levels under described condition. * $P<0.01$.

Results from Figure 4.4 suggest that overexpression of hemoglobin does not have an effect on dark induced senescence under hypoxia.

4.4. Discussion

4.4.1. Possible role of nitric oxide under senescence

The plant hormones ethylene and cytokinin have been shown to influence senescence, the first as a senescence-inducing factor and the second as a senescence-delaying factor (Gan 1995, Grbic 1995). Fruit maturation and floral senescence is associated with a significant decrease in NO emissions and application of NO donating compounds retards flower senescence and extends the post-harvest life of fruits and vegetables (Leshem 1998). Plants with silenced hemoglobin gene expression have delayed and abnormal development of flowering (Hebelstrup et al., 2006, 2008). It has been reported in a variety of immature or unripe fruits, vegetables and flowers that the endogenous NO emissions significantly exceed the levels emitted in mature conditions. NO emissions from Arabidopsis plants decrease significantly when they mature and leaves start to senesce. During natural ageing of Arabidopsis, plant NO emissions decrease continuously and reach a minimum value during senescence (Magalhaes 2000). Exogenous NO counteracts the promotion of leaf senescence caused by ABA and methyl jasmonate in rice (Hung 2003). Arabidopsis plants expressing the bacterial nitric oxide dioxygenase (NOD) under an inducible promoter resulted in NO deficient plants which underwent senescence-like process several days after the initiation of NOD expression The NOD-

induced senescence process shared similarities with natural senescence processes and exerted a senescence-inducing effect. The exogenous application of NO attenuated the effects of NOD resultant NO degradation. A similar phenotype was observed in *Atnos1* mutant, which showed a strong accelerated dark-induced senescence compared with the leaves of wild type plants (Guo 2003, 2005).

NO has been recognized to possess both pro- and antioxidant properties in plants. This is based on the relative ratios of ROS and NO in different physiological conditions. NO triggers a hypersensitive response elicited by avirulent pathogens in concert with ROS (Delledonne 2001). NO may contribute towards elevated ROS levels via inhibition of antioxidant enzymes such as catalase and ascorbate peroxidase (Clark 2000). The accelerated dark-induced senescence in *Atnos1* mutants is accompanied by increase in ROS levels and oxidation of proteins (Guo 2005).

Ethylene is capable of promoting senescence, and the ethylene levels raise when plants start to senesce (Smart 1994, Grbic 1995, Aharoni 1979, Magalhaes 2000). There are various reports that describe a possible stoichiometric relationship between NO and ethylene. NO increment and ethylene decrement have been related to heat stress and water stress (Leshem 1996, 1998). In a recent report from our collaborators, it has been suggested that there is an obvious link between ethylene and NO generation under hypoxia (Hebelstrup 2012). Cell cultures expressing Hb can modulate ethylene production and 1-aminocyclopropane-1-carboxylate oxidase (ACO) enzyme activity without affecting its protein or gene expression level and NO can directly stimulate ACO enzyme activity (Manac'h-Little et al., 2005). Arabidopsis plants with reduced

expression of hemoglobin and increased NO emission rates have increased rates of ethylene emissions.

NO has been reported to modulate the expression of ethylene biosynthetic genes ACC synthase and/or ACO, which influences the ethylene production (Mur 2012).

4.4.2. Role of hemoglobin during senescence

Results from the current study demonstrate that hemoglobin is overexpressed during dark-induced senescence. This modulates the NO levels in senescing leaves. Under hypoxia there is an upregulation of the Hb/NO cycle which leads to a higher turnover of NO in plants. There are reports that suggest that NO can delay senescence. Thus, the upregulation of NO cycle during hypoxia leads to a marginal delay in senescence. Downregulation of Rubisco and cysteine peptidase gene expression is significantly lower in hemoglobin overexpressing plants under darkness as compared to the wild type plants. This indicates that hemoglobin overexpression delays dark-induced senescence. Hypoxia also has an effect on *nac4* and Rubisco expression during hypoxia. With all this evidence along with the reported relation between hemoglobin and ethylene production, it can be suggested that overexpression of hemoglobin can delay darkinduced senescence. Further studies are required to prove the relationship between hypoxia, hemoglobin overexpression and senescence. This has important implications to submergence tolerance in plants.

Chapter 5. Nitric oxide metabolism in plants with the non-functional mitochondrial complex I

5.1. Introduction

5.1.1. Mitochondrial complex I

The complex I, which operates as NADH dehydrogenase, is the first of the four major membrane-bound complexes found in the mitochondrial respiratory chain. Electrons from NADH generated in the mitochondrial matrix in the TCA, formate dehydrogenation and other processes are transferred to ubiquinone by this complex. The electron carriers in this complex include a tightly bound cofactor flavin mononucleotide (FMN) and flavin has several iron-sulfur centres. Complex I transfers the electrons to ubiquinone. For every two electrons that pass through this complex, four protons are pumped from the matrix to the intermembrane space. As seen in all eukaryotic organisms, complex I is a multimeric enzyme made up of over 35 subunit polypeptides (Leterme & Boutry, 1993). Two of these subunits, named NAD7 and NAD9, are mitochondrially encoded in plants (Lamattina et al., 1993; Gäbler et al., 1994). The cytoplasmic male sterile (CMS) mutant of *Nicotiana sylvestris* has a 72-kb deletion in its mitochondrial genome that corresponds to the gene encoding the NAD7 subunit (Pineau et al., 2005). The absence of this subunit renders the entire complex disfunctional. The lack of complex I is lethal in animal cells, but plant mitochondria contain alternative respiratory pathways that act as bypasses to complex I. They consist of at least four additional NAD(P)H: ubiquinone

oxidoreductases (Sabar et al., 2000). While these oxidoreductases act as a way to bypass the electron donation requirement from complex I to the ubiquinone pool, they do not pump protons across the membrane and thus do not accommodate the contribution of complex I in creation of the proton-motive force.

5.2.2. Fermentation pathways

Anoxic conditions effectively block ATP synthesis in the mitochondria. In the absence of oxygen as a terminal electron acceptor, NADH oxidation is blocked. Respiration stops and the cell uses up most of its ATP-stored energy. To supplement the ATP levels, cells intensify fermentation by increasing the expression of fermentation enzymes. Glycolysis is relatively inefficient at energy production when compared to the mitochondrial respiration, and it generates a large quantity of pyruvate. This pyruvate is used by the fermentation enzymes (Sairam et al., 2008). Alcohol dehydrogenase (ADH) is one of the enzymes that can undertake anaerobic oxidation of NADH. Pyruvate is turned into acetaldehyde via pyruvate decarboxylase. This acetaldehyde is then reduced to ethanol with the oxidation of NADH by ADH. A similar redox reaction is performed by lactate dehydrogenase (L-lactic acid dehydrogenase; EC 1.1.1.27), which reduces pyruvate to lactate.

5.2. Materials and methods

5.2.1. Plant growth conditions

Nicotiana sylvestris Speg. & Comes wild type and the CMS mutant (previously called CMSII; Gutierres et al., 1997) plants were grown in a growth chamber supplied by Z-Sciences, Canada. The mutant seeds were provided by Dr. Rosine De Paepe (Université Paris-Sud, Paris, France). Plants were grown on a 14 h day and 10 h night cycle. The day temperature was set to 26 °C and the night temperature was at 20 °C. Moisture was set to 50% saturation. The CMS plants were moisture-sensitive and a care was taken to ensure that the moisture levels in the chamber did not increase above 60%. Plants were watered every alternate day and 20-20-20 fertilizer was added once every two weeks as prescribed by the supplier, Plant Products (Brampton, Ontario, Canada).

5.2.2. Nitric oxide emissions

NO was measured by chemiluminescence detection as described (Planchet et al., 2005). In brief, a constant flow of measuring gas (purified air or nitrogen) at 120 ml min^{-1} was pulled through the chamber and subsequently through the chemiluminescence detector (CLD 770 AL ppt; Eco-Physics, Dürnten, Switzerland; detection limit 20 ppt; 20 s time resolution) by a vacuum pump connected to an ozone destroyer. The ozone generator of the chemiluminescence detector was supplied with dry oxygen (99%). The inflowing gas (air or nitrogen) was made NO free by passing it through a NO scrubber supplied by Eco-Physics Ltd, Switzerland. Calibration was routinely carried out with NO free air (0 ppt

NO) and with various concentrations of NO (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.

Two-months old plants were individually placed in custom built chambers with holes on three sides. Nitrogen gas was pumped in the chamber under pressure from one of the holes on the sides of the chamber. The inlet of the nitric oxide analyzer was attached to the hole on top of the chamber and a one way exit valve was attached to the second and the third hole on the side of the chamber to avoid build-up of pressure within the chamber.

5.2.3. Hemoglobin expression

Leaves (100 mg) were ground in liquid nitrogen with a mortar and pestle, and extracted with 0.2 ml of ice-cold extraction buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride (PMSF)). The protein content of the extract was measured by the method of Bradford with BSA as a standard. SDS-loading buffer (0.05 M Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 3% β-mercaptoethanol, and a trace of bromophenol blue) was added, the samples were boiled for 10 min and the proteins separated by SDS-PAGE using a Bio-Rad Miniprotean II gel apparatus according to an established protocol (Bio-Rad bulletin 1721). Final acrylamide concentration was 15% (w/v) for the separating gel and 4% (w/v) for the stacking gel. Proteins were electroblotted onto polyvinylidene fluoride (PVDF) membranes and detected using a monoclonal antibody (dilution 1:5000) raised against Arabidopsis

recombinant class 1 hemoglobin. The protein concentration in different lines was calculated by densitometric comparison using Multi-Analyst PC software (Bio-Rad Laboratories, Mississauga, ON, Canada) of immunoblots.

5.2.4. Measurement of enzyme activities

Biomass (100 mg) was crushed in liquid nitrogen and the enzyme was extracted in 50 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, 5 mM $MgCl_2$, and (for aconitase) 2 mM citrate. The extracts were centrifuged at 10 000 g for 20 min at 4°C and the supernatant was assayed for enzyme activities and protein content. To study intracellular distribution of aconitase between cytosol and mitochondria the fractions of cytosol and mitochondria were separated by differential centrifugation. The homogenization buffer contained 0.4 M mannitol, cell walls were sedimented at 2000 g and the fraction of organelles containing mitochondria at 15000 g.

Aconitase (EC 4.2.1.3) was assayed spectrophotometrically at 240 nm in the same buffer but containing 40 mM citrate (Racker). The activity of alcohol dehydrogenase (ADH; EC 1.1.1.1) and lactate dehydrogenase (EC 1.1.1.28) were measured according to Hanson et al., (Hanson et al., 1984). The ADH assay buffer contained 0.1 M glycine-NaOH (pH 9), 0.1 M ethanol, and 2 mM NAD^+. Lactate dehydrogenase was measured in 0.1 M tris-HCl buffer pH 8.0 containing 0.15 mM NADH and 0.1 mM pyruvate.

5.3. Results

5.3.1. Nitric oxide production

As evident from Figure 5.1, nitric oxide emission from the leaves of complex I mutant plants was much lower than that of wild type individuals. The NO emissions from wild

type plants rapidly increased starting at around 165 min and continued up until roughly 500 min, at which point they reached a plateau. The mutant took over twice as long to begin showing NO emission and only reached 5% of the maximum emission seen from the wild type.

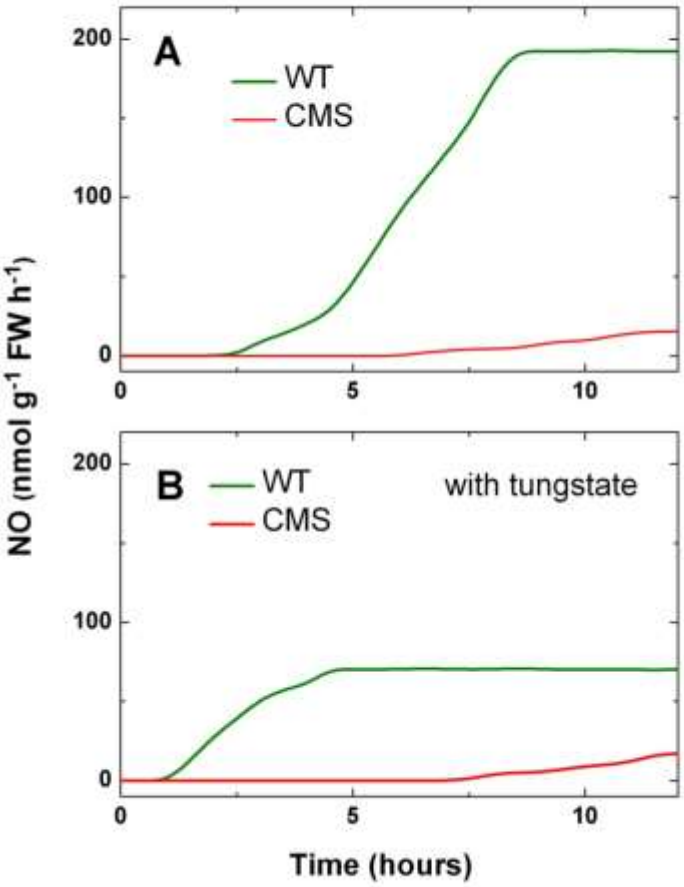

Figure 5.1: Nitric oxide emissions from wild type and CMS mutants under nitrogen atmosphere measured by chemiluminesence. Tungstate is an inhibitor of nitrate reductase The graph represents medium values obtained from triplicates.

5.3.2. Hemoglobin expression

The results from the western blot analysis are presented in Figure 5.2. It is evident that there is a higher expression of non-symbiotic class I hemoglobins in leaves of the CMS plant as compared to the wild type not only under hypoxic conditions when it is usually induced but also under normoxia when its level was lower but still clearly detectable as compared to the wild type where its expression was negligible. The expression of Hb protein was more than twice higher under hypoxia and under normoxia it was at a similar level in the CMS mutant as under hypoxia in the wild type. The experiment was repeated three times and the same pattern was obtained.

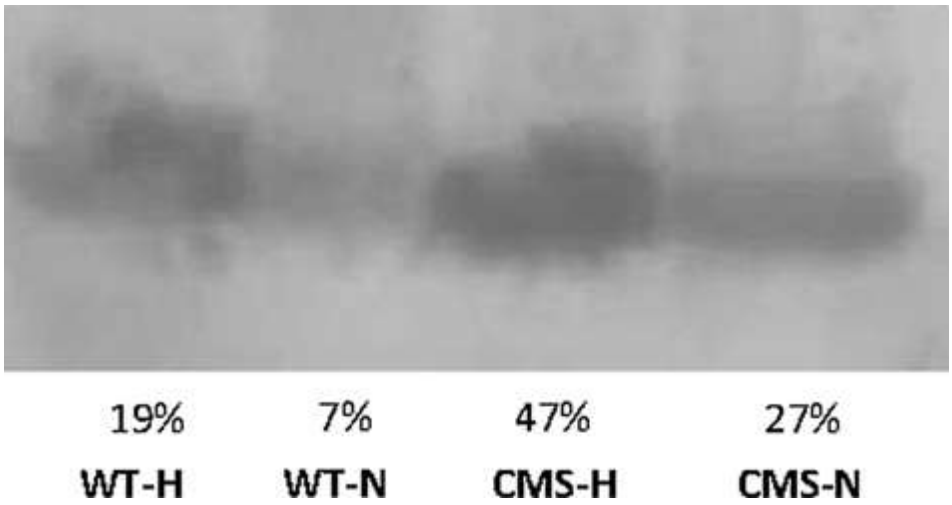

Figure 5.2: Levels of nonsymbiotic class 1 hemoglobins in wild type and CMS mutants unde hypoxia and normoxia. WT-H—wild type hypoxia, WT-N—wild type normoxia, CMS-H—CMS mutant hypoxia, CMS-N—CMS mutant normoxia. Levels were measured using the software Quantity One (BioRad). Parallel gel was run and stained with coomassie brilliant blue as loading control.

5.3.3. Fermentation enzymes

Lactate and alcohol dehydrogenase were measured under normoxic conditions in leaves and exhibited markedly higher activity in the CMS mutant as compared to the wild type (Figure 4). LDH was more than twice higher and ADH was about four times higher in the CMS mutant.

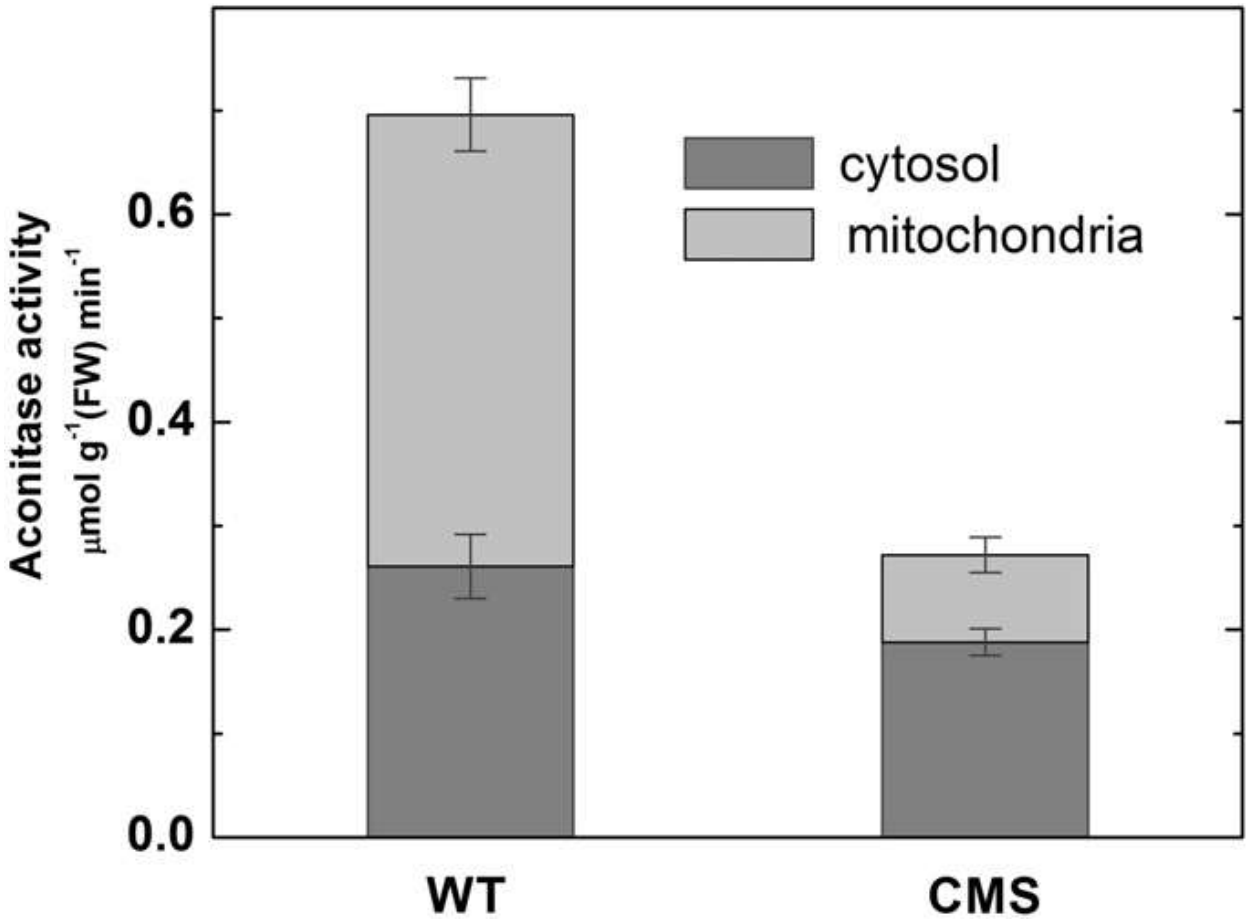

Figure 5.3: Aconitase activity in the cytosolic and mitochondrial compartments of leaves of wild type and CMS mutant tobacco plants. Means ± SD, n = 4.

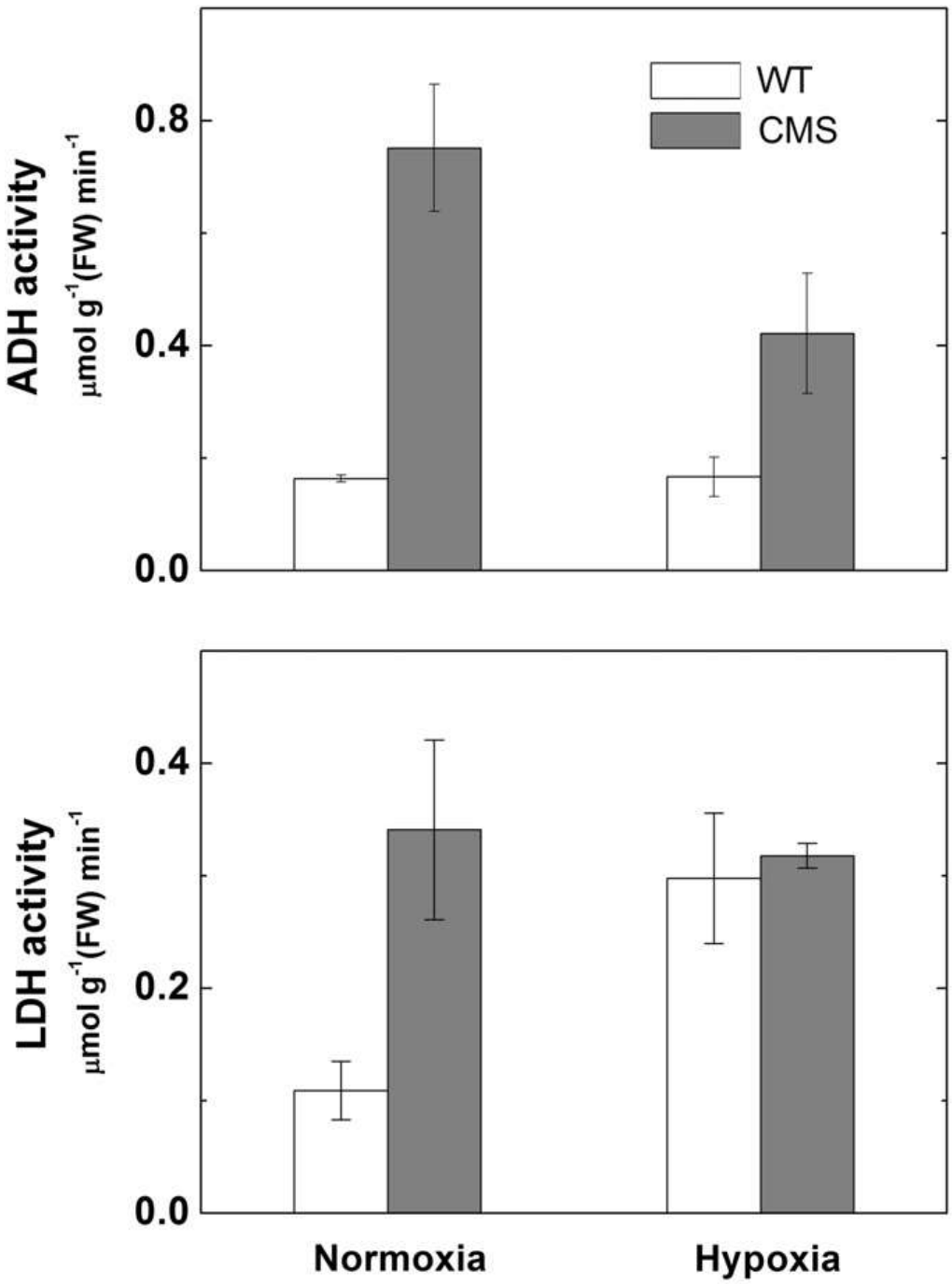

Figure 5.4 : Lactate dehydrogenase (LDH) and alcohol dehydrogenase (ADH) activities in leaves of wild type and CMS mutant plants under normoxia and hypoxia (3 h incubation under nitrogen). Means ± SD, n = 6.

5.4. Discussion

5.4.1. Nitric oxide emissions and hemoglobin expression

Despite its high nitrogen status, the CMS tobacco mutant showed a very low rate of NO emission under anoxic conditions. The formation of NO in plants occurs mainly via reduction of nitrite that takes place anaerobically in mitochondria, by nitrate reductase and possibly by the reductases of plasma membrane (Gupta and Igamberdiev 2011). While Garmier et al., (2008) showed clear differences in rotenone-treated cells as compared to the complex I mutant, which is acclimated, the observed high hemoglobin content is necessarily the result of the CMS mutation. Low NO emission may be explained by a high expression of the nonsymbiotic class 1 hemoglobin protein (Figure 5.2), which has the primary function of NO scavenging (Gupta et al., 2011). The expression of the Hb gene is related to alterations in the electron flux in mitochondria and triggered by the elevation of free calcium level (Nie et al., 2006). Most likely, the altered ability of CMS mitochondria to oxidize NADH due to the complex I impairment results in expression of the class 1 hemoglobin which is observed even in normoxic conditions, and this causes scavenging of most of NO produced in the cell. Additionally, the reported higher superoxide levels (Liu et al., 2006) can remove NO via the pathway involving peroxynitrite formation (Gupta and Igamberdiev 2011) The superoxide-dependent NO degradation (Kalmat et al., 2008; Wulff et al., 2008) and results in formation of peroxynitrite, which is further scavenged via cytochrome oxidase and other mechanisms (Gupta and Igamberdiev 2011) making it less toxic for plant than for animal cells.

However, the accumulation of peroxynitrite is harmful also for plants if its level is elevated, therefore a safer mechanism of NO scavenging to nitrate involving Hb may be important to maintain the low levels of NO and peroxynitrite in the CMS plant.

5.4.2. Morphogenesis and NO

The reduced levels of NO in the CMS mutant tobacco plants can explain some peculiarities in morphology observed for these plants. In tobacco CMS plants, the root system is not developed significantly. This was previously explained in relation to nitrate-dependent regulation of plant architecture (Pellny et al., 2008). Based on the NO data (obtained for leaves), we can extrapolate that the significant reduction of root system can be related to the decreased NO level. NO is known as an important factor in establishing root morphology, in particular, by promoting the growth of lateral roots (Correa-Aragunde et al., 2004) We observe their marked underdevelopment in the CMS tobacco plants. Other studies have shown that NO reduces the length of the elongation zone in roots, whereby cells differentiate faster and more root hairs are found closer to the tip of the root (Fernández-Marcos et al., 2011). NO is considered as a second messenger in auxin signal transduction (Lanteri et al., 2008). The observed low stomatal conductance (Sabar et al., 2000; Djebbar et al., 2012) and the high sensitivity of CMS tobacco mutant to humidity and sensitivity to drought (Djebbar 2012) is also related to low NO levels that participate in cross-talk with abscisic acid in regulation of stomatal conductance (García-Mata 2002).

5.4.3. Aconitase and NO

The CMS plants are characterized by a decreased activity of aconitase, which is most strikingly expressed for the mitochondrial compartment (Fig. 5.3). This may be associated with inhibition of aconitase by reactive oxygen species which might accumulate at a higher level at high redox state observed in the mitochondrial compartment, although superoxide and hydrogen peroxide did not accumulate in total leaf extracts (Dutilleul et al., 2003; Liu et al., 2008). Although NO inhibits aconitase (Navarre et al., 2000; Gupta et al., 2012), this is not the case for CMS plants exhibiting low NO levels, however the inhibition by superoxide radicals (Flint et al., 1993) may explain low aconitase activity especially in mitochondria. It was shown that inhibition of aconitase results in accumulation of citrate which in turn promotes expression of the alternative oxidase gene (Navarre et al., 2000; Cvetkovska et al., 2012). The accumulation of citrate reported for the CMS tobacco plants (Dutilleul et al., 2005) is not due to NO but most likely due to a higher redox state in mitochondria of CMS plants, which results in high expression of AOX. The role of AOX in avoidance of NO accumulation has been proposed recently (Cvetkovska et al., 2012). It is important to note that aconitase activity in the CMS mutant is affected most significantly in mitochondria (fivefold decrease), while in the cytosol the decrease is relatively small (~1.4 times). This means that the lack of the complex I function targets primarily mitochondria and then affects all other compartments. It was shown earlier that in the CMS mutant NAD-dependent isocitrate dehydrogenase is elevated while the NADP-dependent form is unchanged (Dutilleul et. al., 2005). It was suggested that modulation of isocitrate dehydrogenases by redox level (Igamberdiev and Gardeström 2003) could result in citrate accumulation. To a higher extent it can be caused by low activity of the mitochondrial aconitase.

5.4.4. Fermentation pathways

Activities of ADH and LDH are strongly enhanced in the CMS mutant (Fig. 5.4). This may be related to lower capacity of mitochondria to oxidize NADH which in turn induces fermentation pathways even under normoxic conditions. Earlier (Dutilleul et al., 2005) it was shown that alanine is accumulated in the CMS mutant, which is also related to activation of fermentation pathways. This means that the CMS mutant resembles hypoxic plant already under normoxic conditions. The Arabidopsis mutant lacking the alternative internal mitochondrial NAD(P)H dehydrogenases shows a clear shift to accumulation of lactate and other hypoxic metabolites (Florez-Sarasa et al., 2012), which is also in accordance with our observations. The induction of non-symbiotic hemoglobin (which is normally hypoxically induced) is the exhibition of the same tendency. High activities of ADH and LDH in the CMS plant can significantly lower NADH level in the cytosol and increase ATP/ADP ratio, which can explain some unexpected results that were obtained earlier on high ATP content and relatively low NAD(P)H in plants lacking functional complex I (reviewed in Juszczuk and Rychter 2012).

The CMS mutant having low capacity of mitochondria to NADH oxidation, is characterized by induction of enzymes of lactic and alcoholic fermentation and of the non-symbiotic hemoglobin, which participates in scavenging of nitric oxide. Basic features of plants expressed under hypoxia are developed in the CMS mutant under normoxic conditions. This partially substitutes the impaired function of the main mitochondrial electron transport chain and, in addition to alternative mitochondrial

NADH dehydrogenases, might contribute to NADH oxidation either in the terminal reactions of glycolysis or in the hemoglobin/nitric oxide cycle.

Chapter 6: Future directions

6.1. Adaptiveness and versatility of plant mitochondria

Plant mitochondria are capable of adapting and functioning under diverse stress conditions. Mitochondrial adaptation to the lack of oxygen or to its extremely low concentrations leading to a condition known as hypoxia has been studied in the present project. It is now evident that nitrite plays an important role as an intermediary electron acceptor participating in the mitochondrial electron transport chain in the absence of sufficient oxygen concentration. Mitochondria can produce NO from nitrite which diffuses into the cytoplasm. High concentrations of NO are toxic to the cell, but, on the other hand, NO can actively participate in various signal transduction processes that control and regulate plant morphology, physiology and development (Hebelstrup et al., 2013). Therefore, the increase in turnover of NO produced by mitochondria and its subsequent scavenging by Hb in conjunction with the enzymes reducing methemoglobin will have far-reaching consequences beyond simply maintaining mitochondrial stability for NADH oxidation and possibly ATP synthesis.

6.2 Morphological and physiological roles of Hb and NO

There is an abundant literature dealing with the effects of increased or decreased NO levels on plant morphology by using NO donors and scavengers. These effects have been well documented in the review titled “The role of nitric oxide and hemoglobin in plant development and morphogenesis” (Hebelstrup et al., 2013). The author of this thesis is a

co-author in the paper. There is a lot of debate on the relevance to *in vivo* conditions of the effects revealed in the studies based on application of NO donors and scavengers and performed *in vitro*. The aim of current study was to modify the NO cycle *in vivo* through overexpression and silencing of hemoglobin and treating plants with artificially induced hypoxia. This provided a unique system to study the effects of NO on morphology and physiology of barley. There is also abundant data on the effect of modified levels of expression of Hb in *Arabidopsis* and tobacco. It is important to study these effects on cereal crops which are practically used by farmers and agriculture-based industries. Results from the current study and reports by collaborators participating in papers published by the author of this thesis indicate that many of the observations reported in Arabidopsis and tobacco were not reproduced in barley in particular. Hence, further research on the effects of hemoglobin and NO on crops is required before the technology can be used on a commercial scale.

The author of the thesis has made extensive efforts to study the role of hemoglobin overexpression and NO on root hairs formation, aerenchyma formation and seed germination. More than 50 wild type and hemoglobin overexpressing seeds were germinated in two independent batches to study the effect of hemoglobin overexpression on seed germination. There was no statistically significant effects of hemoglobin overexpression on germination of barley, and further tests are required to make a firm conclusion whether hemoglobin overexpression has an effect on seed germination or not.

There is an abundant set of data obtained using NO donors and scavengers, available in the literature, which shows a significant effect of NO on the above mentioned

morphological and physiological processes. *In vitro* studies using SNP as NO donor have shown that NO functions as a positive regulator of root hair development (Lombard et al., 2006). Figure 6.1 demonstrates the difference in root development and root hair density in hemoglobin overexpressing and WT plants. Observations were not reproducible and require further studies to test the hypothesis derived from other model systems.

Along with the studies on effects of hemoglobin and NO on plant morphology, it is important to study the signalling pathways influenced by NO. Protocols based on animal systems could not be implemented on plant systems. Putative model of NO signal transduction mediated by H_2O_2-activated MPK6 has been reported in *Arabidopsis* (Want et al., 2010). It will be interesting to study the signalling pathways controlling various morphological and developmental processes in plants influenced by hemoglobin and NO as described in Hebelstrup et al., (2013) and compare the results with *in vitro* studies based on SNP and other NO donors and scavengers.

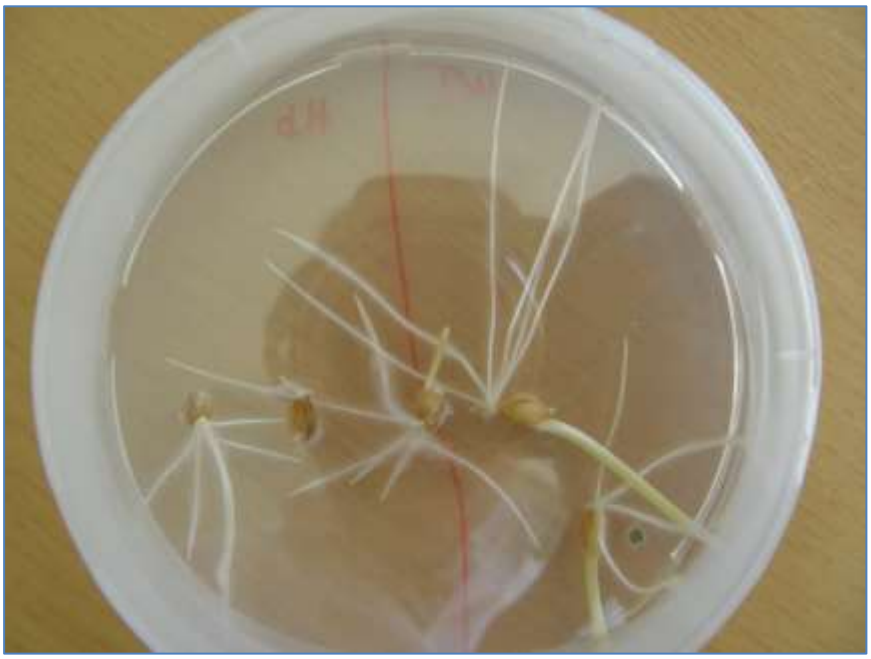

Figure 6.1: Preliminary analysis of root development and root hair density in hemoglobin overexpressing barley (left) and WT Golden Promise (right). Seeds were germinated under darkness at 20 °C for three days

6.3. The role of Hb and NO in dark-induced senescence

The results presented in this thesis indicate that the dark-induced senescence is delayed under hypoxia, but further tests are required to determine whether hemoglobin overexpression has a significant effect to further delay the process. It is essential to investigate the mechanism of delay of dark-induced senescence during hypoxia. The role of NO in delaying senescence is known, but further evidence is required to define its role in delaying senescence during hypoxia. Additional control condition where leaves are placed under hypoxia in the presence of light can suggest the role of hypoxia in dark-induced senescence. Hemoglobin silencing lines placed under hypoxia can serve as another standard to clarify the role of NO during the delay of dark-induced senescence under hypoxia. The hemoglobin silencing lines of barley developed by the author of this thesis, which are currently under characterisation, can be used in the future for the suggested studies.

6.4. Final conclusions

It is evident that plant mitochondria are capable of adjusting their functions in diverse stress conditions including the lack of oxygen, when mitochondria start using nitrite as an alternative electron acceptor. Non-symbiotic class 1 hemoglobins are upregulated in various biotic and abiotic stress conditions. Their main role is to scavenge NO. This leads to an increase in the turnover of the hemoglobin-NO cycle. The hemoglobin-NO cycle plays an important role in stress tolerance and mitochondrial functioning under low oxygen. Upregulation of the NO metabolism during hypoxia and under other biotic and abiotic stress conditions not only supports the mitochondrial function, but also influences plant morphology, physiology and expression of many other genes, either directly or indirectly via the signalling properties of NO. The predominant

influences of modulation in the levels of hemoglobin and NO include alteration in root morphology, induction of senescence and other characteristics. The expression of various genes that participate in stress tolerance is also affected. Change of alternative oxidase gene expression and its activity leads to a shift in amino acid biosynthesis patterns and hence has a profound effect on cellular biochemistry. The present study contributes to further understanding of the role of hemoglobins and NO in overall plant growth and development, which will provide critical information for engineering of stress tolerant plants to support the ever increasing demand of food in the world. It also provides the important preliminary information on the role of hemoglobin in cereal biotechnology.

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Appendix

List of presentations at international conferences:

- Poster titled "The role of NIA1 and NIA2 in nitric oxide production associated with survival under hypoxia" at IAPB 2010 in St. Louis, Missouri, USA

- Poster titled "Pathways of Nitric Oxide production in Tobacco and Arabidopsis plants" at Plant Biology 2010 conference in Montreal, Canada

- Poster titled "The role of nitric oxide and alternative mitochondrial electron transport in tobacco and Arabidopsis plants under hypoxia" at International conference on plant mitochondrial biology, Germany 2011.

- Oral presentation titled "The cross-talk between plant hemoglobin and nitric oxide during flooding stress and senescence" at the 8th Canadian society of plant biotechnology conference, University of Guelph

- Poster titled "The biotechnological applications of hemoglobin overexpression in cereal crops" at Plant Biology 2012, Austin, USA

- Poster titled "Biochemical and morphological characteristics of barley (Hordeum vulgare L.) plants overexpressing hemoglobin" at the 2013 Canadian Society of Plant Biologists Annual Meeting

List of publications

- Gupta KJ, Shah JK, Brotman Y, Jahnke K, Willmitzer L, Kaiser WM, Bauwe H, Igamberdiev AU (2012) Inhibition of aconitase by nitric oxide leads to induction of the alternative oxidase and to a shift of metabolism towards biosynthesis of amino acids. Journal of Experimental Botany 63: 1773-1784
- Igamberdiev AU, Bykova NV, Shah JK, Hill RD (2010) Anoxic nitric oxide cycling in plants: participating reactions and possible mechanisms. Physiologia Plantarum 138: 393-404
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- Hebelstrup KH, Shah JK, Igamberdiev AU (2013) The role of nitric oxide and hemoglobin in plant development and morphogenesis. Physiologia Plantarum 148: 457-469
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Co-authorship statement

This is to certify that the two chapters of my dissertation submitted to the school of graduate studies, Memorial University of Newfoundland towards the partial fulfilment of the requirement of the degree of Ph. D. are published in peer reviewed journals. Following are the papers along with a list of the co-authors and their respective affiliations.

Inhibition of aconitase by nitric oxide leads to induction of the alternative oxidase and to a shift of metabolism towards biosynthesis of amino acids

Journal of Experimental Botany. (2012) 63 (4): 1773-1784.

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Jay Shah along with Dr. Abir Igamberdiev formulated the hypothesis and performed literature search. He also performed hypoxic treatments of the plants, performed enzyme assays. Dr. Gupta along with Dr. Brotman, Dr. Willmitzer performed the GCMS analysis for metabolite analysis. Dr. Bauwe and Dr. Kaiser provided critical comments and reviews on the manuscript.

Respiratory complex I deficiency results in low nitric oxide levels, induction of hemoglobin and upregulation of fermentation pathways

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An assessment of the biotechnological use of hemoglobin in cereals (communicated) Physiologia Plantarum

Hebelstrup, Kim; Shah, Jay Kumar; Simpson, Catherine; Schjoerring, Jan K.; Mandon, Julien; Cristescu, Simona M; Harren, F.J.M.; Christiansen, Michael W; Mur, Luis; Igamberdiev, Andre (Abir) U.

K.H. Hebelstrup conceived of the idea and produced transgenic barley plants, confirmed overexpression of nsHb by qRT-PCR and western blotting, performed yield assessment.

J. Mandon, S.M. Cristescu and F.J.M Harren, Radboud University conducted NO measurements at Life Science Trace Gas Facility

J.K. Shah assisted on yield assessments and scored the development of plants.

C. Simpson and L.A.J Mur assessed the interactions of plants with Blumeria graminis and helped edit the final manuscript.

J.K. Schjoerring performed iron concentration analysis of grains.

A.U. Igamberdiev supervised on the design, conductance and interpretation of experiments.

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RESEARCH PAPER

Inhibition of aconitase by nitric oxide leads to induction of the alternative oxidase and to a shift of metabolism towards biosynthesis of amino acids

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Abstract

Nitric oxide (NO) is a free radical molecule involved in signalling and in hypoxic metabolism. This work used the nitrate reductase double mutant of *Arabidopsis thaliana* (*nia*) and studied metabolic profiles, aconitase activity, and alternative oxidase (AOX) capacity and expression under normoxia and hypoxia (1% oxygen) in wild-type and *nia* plants. The roots of *nia* plants accumulated very little NO as compared to wild-type plants which exhibited ~20-fold increase in NO emission under low oxygen conditions. These data suggest that nitrate reductase is involved in NO production either directly or by supplying nitrite to other sites of NO production (e.g. mitochondria). Various studies revealed that NO can induce AOX in mitochondria, but the mechanism has not been established yet. This study demonstrates that the NO produced in roots of wild-type plants inhibits aconitase which in turn leads to a marked increase in citrate levels. The accumulating citrate enhances AOX capacity, expression, and protein abundance. In contrast to wild-type plants, the *nia* double mutant failed to show AOX induction. The overall induction of AOX in wild-type roots correlated with accumulation of glycine, serine, leucine, lysine, and other amino acids. The findings show that NO inhibits aconitase under hypoxia which results in accumulation of citrate, the latter in turn inducing AOX and causing a shift of metabolism towards amino acid biosynthesis.

Key words: Aconitase, alternative oxidase, citrate, nitrate reductase, nitric oxide.

Introduction

Nitric oxide (NO) is a versatile free radical molecule in plants, animals, and microbes (Delledonne, 2005; Besson-Bard *et al.*, 2008; Gupta *et al.*, 2011) that mediates various physiological and developmental processes. These include seed germination (Beligni and Lamattina, 2000), senescence (Guo and Crawford, 2005), stomatal closure (Desikan *et al.*, 2002), and floral transition (He *et al.*, 2004). NO is also involved in response to various stresses such as low oxygen (Dordas *et al.*, 2003), cold (Zhao *et al.*, 2009; Gupta *et al.*, 2011), high salt (Corpas *et al.*, 2009), heavy metals (Besson-Bard *et al.*, 2009), UV-B stress (Zhang *et al.*, 2011), disease resistance (Delledonne *et al.*, 1998; Gupta, 2011), and apoptosis (Beligni *et al.*, 2002).

NO is an important regulator of plant respiration. Mechanistically, NO inhibits the respiratory chain by competitively binding to the oxygen binding sites of cytochrome *c* oxidase (COX, complex IV) (Brown, 2001). By doing so, it affects the mitochondrial electron transport chain and oxidative phosphorylation (Millar and Day, 1996; Yamasaki *et al.*, 2001). The inhibitory effect of NO on the electron transport chain is one of the reasons of its toxicity due to the suppression of respiratory oxygen uptake and generation of

superoxide resulting from over-reduction of the ubiquinone pool (Shiva *et al.*, 2001).

Besides the cytochrome pathway of mitochondrial electron transport, all plants and fungi and some protists contain an alternative respiratory pathway. This pathway comprises a single protein alternative oxidase (AOX), which in most species is encoded by a small family of nuclear genes (Millar *et al.*, 2011). The electron transfer via this protein bypasses two sites of proton translocation to the matrix and, therefore, does not lead to ATP production so the excess energy is lost as heat. AOX plays important roles when the cytochrome pathway gets inhibited under various stress conditions. The mechanism of AOX induction includes different mechanisms and could result from shifts in the level of reduced pyridine nucleotides, reactive oxygen species (ROS), or citrate (Vanlerberghe and McIntosh, 1996; Gray *et al.*, 2004). The increases in citrate and ROS act as signals to induce transcription of AOX, while pyruvate and other oxoacids are known to activate AOX allosterically at the protein level, and NADPH activates AOX via reduction of its disulphide bond (Mackenzie and McIntosh, 1999). Under conditions of hypoxia, NO is the major metabolite which can induce AOX in a similar way as shown when it was applied exogenously (Huang *et al.*, 2002), but this possibility has not been explored. While NO inhibits cytochrome oxidase (Cooper *et al.*, 1997), AOX is insensitive to NO and can provide electron flux in mitochondria even at elevated NO concentrations (Millar and Day, 1996; Gray *et al.*, 2004).

In plants, NO can be generated by various pathways and these are divided into oxidative and reductive categories. In oxidative pathways, NO is formed via oxidation of more reduced nitrogen compounds such as arginine or hydroxylamine, while in reductive pathways, NO appears as a product of reduction of nitrate and nitrite. NO synthase-like activity (Corpas *et al.*, 2009), the polyamine-mediated pathway (Tun *et al.*, 2006), and the hydroxylamine-mediated pathway (Rumer *et al.*, 2009) are oxidative pathways having limited capacity in plants and producing NO mainly for signalling purposes under normoxia (Igamberdiev and Hill, 2004; Gupta and Igamberdiev, 2011). In contrast, the nitrate reductase (NR) pathway (Sakihama *et al.*, 2002; Planchet *et al.*, 2005), the mitochondrial electron transport pathway (Planchet *et al.*, 2005; Gupta and Igamberdiev, 2011), and the plasma membrane nitrite–NO pathway (Stöhr *et al.*, 2001) are reductive pathways becoming major NO-generating mechanisms with decrease of oxygen.

The NO produced via the above-mentioned pathways under various stress conditions can induce AOX (Huang *et al.*, 2002; Fu *et al.*, 2010), which is insensitive to NO (Millar and Day, 1996). A major target of NO that has been recently identified is the enzyme aconitase (Navarre *et al.*, 2000). Aconitase contains a FeS cluster that participates in the mechanism of interconversion of three tricarboxylic acids (citrate, *cis*-aconitate, and isocitrate) and exhibits sensitivity to both NO and ROS. In plant cells, aconitase is found in both mitochondria, where it participates in the TCA cycle, and cytosol (Zemlyanukhin *et al.*, 1984), where its activity is higher and where in conjunction with NADP-dependent isocitrate dehydrogenase it participates in supplying 2-oxoglutarate for amino acid biosynthesis (Igamberdiev and Gardeström, 2003). Since the stress-related accumulation of reactive oxygen and reactive nitrogen species is particularly high in mitochondria, the inhibition of aconitase refers primarily to this compartment.

Under hypoxic conditions, NR produces high amounts of NO since the step of nitrite reduction to ammonia is inhibited and accumulating nitrite can serve as an alternative substrate for NR (Planchet *et al.*, 2005). Nitrite is also transported into mitochondria where complex IV (and possibly complex III) reduces it to NO (Gupta *et al.*, 2005). The function of hypoxically generated NO is attributed to the decrease of reduction level of pyridine nucleotides (Igamberdiev *et al.*, 2006) and limited ATP generation via the haemoglobin-NO cycle (Stoimenova *et al.*, 2007), and also to the hypoxic survival via prevention of complete depletion of oxygen (Borisjuk *et al.*, 2007; Benamar *et al.*, 2008).

The goal of the present study was to investigate the induction of AOX at low oxygen and to provide a rationale for how NO may be involved in this process. Using the wild type and the NR double mutant of *Arabidopsis*, it is demonstrated that NR is involved in hypoxically induced NO production. This NO generation correlates with the inhibition of aconitase in mitochondria which suggested that it is the increase in citrate levels that acts as a potent inducer of AOX expression. Citrate serves as AOX inductor and also as a precursor of 2-oxoglutarate for amino acid biosynthesis. The overall process involving NO production, inhibition of aconitase, and induction of AOX leads to a shift of plant metabolism towards amino acid biosynthesis.

Materials and methods

Plant material

Three-week old *Arabidopsis* seedlings from a wild type (WT) and a NR double mutant (*nia*) (obtained from National *Arabidopsis* Stock Centre, UK) in the Col-0 background were grown on vertical agar plates containing half-strength Hoagland nutrient solution containing 5 mM potassium nitrate and 2% sucrose and treated with normal air containing 21% O_2 (normoxia) or 1% O_2 (hypoxia). For determination of aconitase and NR, seedlings were transferred from the plates to liquid medium that was in equilibrium with air containing either 21% or 1% O_2.

Nitric oxide measurements

NO was measured by the gas-phase chemiluminescence detection method (Planchet *et al.*, 2005) A small glass beaker containing root slices (0.5 g root, 8 ml buffer) was immediately placed in a glass cuvette (1 l air volume) which was mounted on a rotary shaker (150 rpm). A constant flow of measuring gas (purified air or nitrogen) of 1.3 l min^{-1} was pulled through the cuvette, through a cold moisture trap, and subsequently through the chemiluminescence detector (CLD 770 AL ppt, Eco-Physics, Dürnten, Switzerland, detection limit 20 ppt, time resolution 20 s) by a vacuum pump connected to an ozone destroyer. The measuring gas (air or nitrogen) was made NO free by conducting it through a custom-made charcoal column (length 1 m, internal diameter 3 cm, particle

size 2 mm). Calibration was routinely carried out with NO-free air (0 ppt NO) and with various concentrations of NO (1–35 ppb) adjusted by mixing the calibration gas (500 ppb NO in nitrogen, Messer Griessheim, Darmstadt, Germany) with NO-free air. Flow controllers (FC-260, Tylan General, Eching, Germany) were used to adjust all gas flows. The air temperature in the cuvette was 25 °C. NO analyser data were logged into a computer and processed using customized 'Visual designer'-based software (Intelligent Instrumentation).

Measurement of NR activity

NR activity was measured using L-NaRA-100 assay kit (NECi Co, USA). The extraction medium was 100 mM potassium phosphate buffer (1 mM EDTA, 0.4% polyvinylpyrrolidone, and 0.05% cysteine; pH 7.4). Root material (100 mg) was ground in an ice-cold mortar and pestle with 400 µl of chilled extraction buffer and centrifuged at 10,000 *g* for 10 min. The NR assay medium contained 25 mM potassium phosphate (0.025 mM EDTA; pH 7.5), 10 mM nitrate and 0.1 mM NADH. An aliquot (100 µl) of extract was added and reaction was stopped after 20 min with Zn acetate. Nitrite was determined colorimetrically as described earlier (Hageman and Reed, 1980).

Real-time quantitative reverse-transcription PCR for determination of transcript abundance of AOX1A

Total RNA was extracted from the seedlings, which were grown as indicated in the figure legends, using the RNeasy kit (Qiagen), according to the manufacturer's instructions and subjected to DNase treatment using the TURBO DNA-free kit (Ambion). RNA (5 µg) was reverse transcribed into cDNA using the Superscript III reverse transcriptase kit (Invitrogen). Real-time PCR amplification was carried out with the ABI Prism 7900 sequence detection system (Applied Biosystems), using a power SYBR-green master mix (Applied Biosystems) and the primers described. *Ubiquitin10* (*At4G05320*) and *PDF2* (*At1g12320*) were used as housekeeping genes and the change in *AOX1A* was normalized to *Ubiquitin10*. The relative quantification of each individual gene expression of *AOX1A* was performed using the comparative threshold cycle method, as described in the ABI PRISM 7900 Sequence Detection System User Bulletin Number 2 (Applied Biosystems).

Measurement of respiration

Respiration of roots was measured using oxygen electrode (Hansatech, UK) calibrated by a simple two-point calibration (100 and 0% air saturation). Root tissue (0.1–0.2 g) were cut from the plant, washed twice and blotted on tissue paper to remove extra water, immediately cut into 4–6 mm slices, and placed in a 2.5 ml oxygen electrode chamber continuously circulated with thermostat-controlled water. To estimate the effect of citrate on the partitioning between COX and AOX and on AOX capacity, 0.2 mM citrate was fed in the medium of root cultures 3 h prior to the respiratory measurements.

Aconitase assay

Wild-type and *nia* mutant *Arabidopsis* were grown in flasks on shaker at 80 rpm for 7 days. For hypoxic treatment, the flasks were pumped with 1% oxygen for 3 h. Biomass (0.1 g) was crushed in liquid nitrogen and the enzyme was extracted in 50 mM TRIS-Cl (pH 7.4), 1 mM dithiothreitol, 5 mM $MgCl_2$, and 2 mM citrate. Aconitase was assayed spectrophotometrically at 240 nm in the same buffer but containing 40 mM citrate (Racker, 1950). Protein concentration was estimated by the method of Bradford (1976).

GC-MS analysis

GC-MS analysis was performed as described previously (Lisec *et al.*, 2006) for six replicates each consisting of 30 pooled plants obtained from two independent experiments. Metabolite levels were determined in a targeted fashion using the Target Search software package (Cuadros-Inostroza *et al.*, 2009). Metabolites were selected by comparing their retention indexes (±2 s) and spectra (similarity >85%) against the compounds stored in the Golm Metabolome Database (Kopka *et al.*, 2005). Each metabolite is represented by the observed ion intensity of a selected unique ion, which allows for a relative quantification between groups. Metabolite data were $\log_{10}$-transformed to improve normality (Steinfath *et al.*, 2008) and normalized to show identical medium peak sizes per sample group.

Results

Nitrate reductase-dependent NO increase under hypoxia

The NR double mutant (*nia*) has a deletion in *NIA2* gene and an insertion in *NIA1* gene. This mutant was previously shown to have no detectable NR activity (Wang *et al.*, 2004). The present study could detect very low NR activity in this plant, which can be explained by a background nitrite formation via possible alternative nitrate reduction pathways (Wienkoop *et al.*, 1999). In order to check NO production rates, NO emission from the WT *Arabidopsis* root slices was measured by the gas-phase chemiluminescence method. The slices did not produce NO under normoxia (21% O_2 in 100% air saturation), while upon imposing hypoxia (1% O_2 in air saturation) NO production from roots increased and reached a steady-state rate of 6.5 nmol (g FW)$^{-1}$ h^{-1} after 30 min (Fig. 1A). To determine whether NO production in WT roots under low oxygen is due to NR, the NO production from roots of the NR double mutant (*nia*) was measured. Roots of the *nia* double mutant produced very little NO and its emission did not increase even under hypoxia (Fig. 1B). Under normoxic conditions, the total NR activity in *nia* roots was 0.026 µmol (g FW)$^{-1}$ h^{-1} whereas WT roots exhibited an NR activity of 0.099 µmol (g FW)$^{-1}$ h^{-1} which is nearly 4-times higher than in *nia* plants. In hypoxic conditions, NR activity in the WT increased to 0.22 µmol (g FW)$^{-1}$ h^{-1} in comparison to the WT under normoxic conditions (Fig. 1C), i.e. more than 2-fold, while no detectable increase was observed in the mutant. This clearly suggests that NR is involved in the hypoxic NO production either directly or by supplying nitrite for nitrite:NO reductase activity of mitochondria and possibly other compartments.

Nitric oxide inhibits aconitase and increases citrate levels

The mitochondrial form of aconitase is a constituent of the TCA cycle. Its inactivation by NO or superoxide hence decreases cellular energy metabolism and may have a protective effect against additional oxidative stress by acting as a reversible 'circuit breaker' that prevents wasteful turnover of the TCA cycle and further increases in reduction level in stress conditions (Gardner and Fridovich, 1991; Gardner *et al.*, 1997). The present study tested the effects of hypoxia and presence of NR on aconitase in plants by measuring

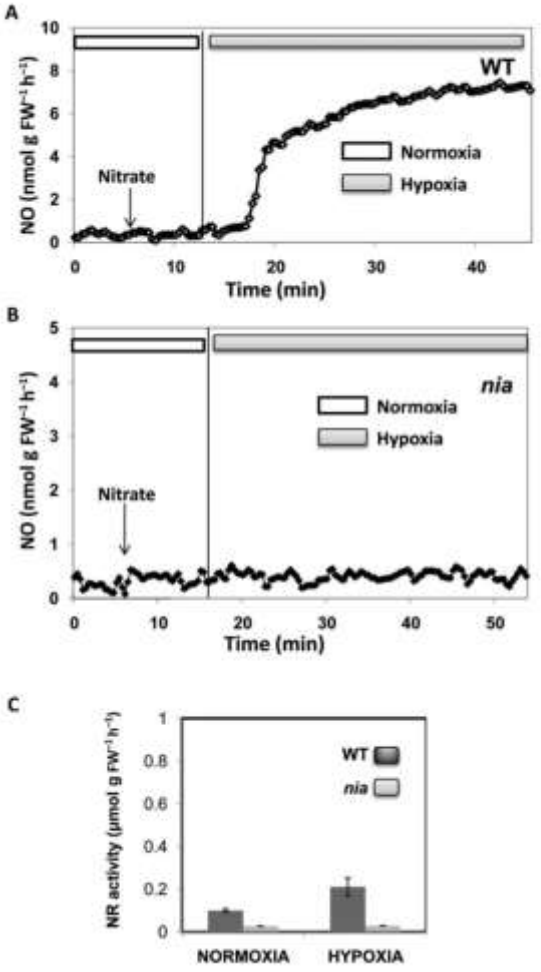

Fig. 1. Nitric oxide emissions and nitrate reductase activity in wild-type (WT) and *nia Arabidopsis* plants. (A) NO emission from WT roots under normoxia (21% O_2) and hypoxia (1% O_2): 0.5 mM nitrate was added as indicated. (B) NO emission from *nia* roots under normoxia and hypoxia. (C) Nitrate reductase (NR) activity in WT and *nia* roots under normoxic and hypoxic conditions.

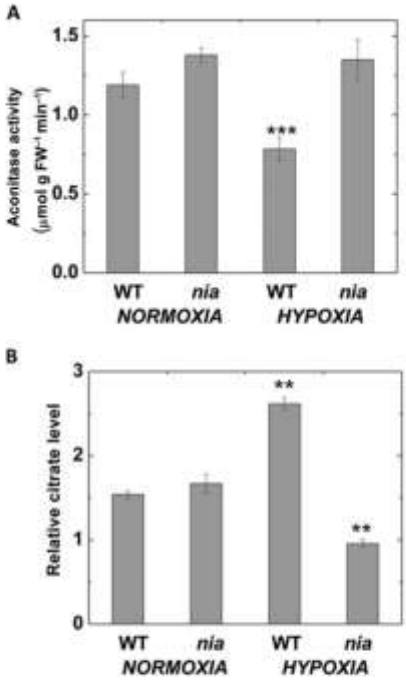

Fig. 2. Aconitase activity and citrate levels in wild-type (WT) and *nia Arabidopsis* plants under normoxia and hypoxia. (A) Aconitase activity. (B) Citrate levels measured as GC-MS values from WT and *nia* under normoxia and hypoxia. Levels are relative to WT under normoxic conditions. Error bars indicate SD. Asterisks indicate significant differences between responses to oxygen conditions: ** $P < 0.01$; *** $P < 0.001$.

aconitase activity in the WT and *nia* under normoxia and hypoxia and found that under hypoxic conditions aconitase in WT plants is strongly suppressed when compared with normoxic conditions (Fig. 2A). This is clearly in agreement with the earlier observation (Navarre *et al.*, 2000) that NO (which production is linked to NR activity, Fig. 1) is an inhibitor of aconitase. The inhibition of aconitase can lead to accumulation of citrate because aconitase mediates its conversion to isocitrate. In order to check this possibility, the citrate levels were measured by GC-MS. As shown in Fig. 2B, citrate levels were only slightly higher in the WT in comparison to *nia* under normoxic conditions. Under hypoxic conditions, while in the WT citrate level increased by 70%, in *nia* plants it was strongly (almost 2-fold) reduced. This suggests that NO inhibits aconitase and thus stimulates citrate accumulation.

AOX induction correlates with the increased levels of citrate

The involvement of the increased citrate levels in AOX induction was tested by measuring AOX and COX capacities via incubation of root slices with and without salicylhydroxamic acid, which is an inhibitor of AOX, and cyanide, which is an inhibitor for COX. The results were also verified with *N*-propylgallate (the inhibitor of AOX) and myxothiazol (the inhibitor of complex III). As shown in Fig. 3A, the total respiration (without inhibitors) in *nia* roots was 109 μmol (O_2) (g FW)$^{-1}$ h^{-1}, whereas the respiratory rate in WT roots was about 66 μmol (g FW)$^{-1}$ h^{-1}. Also, in Supplementary Fig. S1A (available at *JXB* online), the total respiratory rates of the WT and *nia* were comparable. To examine the capacity of the cytochrome pathway, the respiratory rates of WT and *nia* roots were measured in the presence of 2 mM salicylhydroxamic acid. The capacity of the cytochrome pathway was 96.7 μmol (g FW)$^{-1}$ h^{-1} in *nia*

roots whereas it was 51.7 μmol (g FW)$^{-1}$ h^{-1} in WT roots (Fig. 3B). A similar trend was observed when cytochrome capacity was measured in the presence of 100 μM *N*-propylgallate (Supplementary Fig. S1B) suggesting that the absence of NO in *nia* roots triggers operation of the cytochrome pathway with 2-times higher intensity as compared to the presence of NO in WT roots.

In order to check AOX capacity, the respiration of WT and *nia* plants was measured in the presence of 1 mM KCN (Fig. 3C). The respiratory rate in *nia* roots was 9.5 μmol (g FW)$^{-1}$ h^{-1}, whereas in WT roots the respiratory rate was 14.3 μmol (g FW)$^{-1}$ h^{-1}. A similar trend was observed when using 25 μM myxothiazol (Supplementary Fig. S1C). These results clearly suggest that in the WT, which exhibits elevated NO levels under hypoxia (Fig. 1A), AOX capacity is increased. In the absence of NO, as seen in *nia*, respiration followed the cytochrome pathway. In order to verify that the *in vivo* increase in citrate levels is responsible for AOX induction, citrate was fed to the medium of root cultures 3 h prior to the respiratory measurements. In the condition when citrate was fed to *nia* plants (Fig. 3D). AOX capacity was 18.2 μmol (g FW)$^{-1}$ h^{-1}, whereas in the absence of citrate it was 9.6 μmol (g FW)$^{-1}$ h^{-1}. These data suggest that citrate is responsible for the induction of AOX, which is in agreement with the results of Degu *et al.* (2011) showing that the inhibition of aconitase by citramalate (2-hydroxy-2-methylbutanedioate) induces AOX.

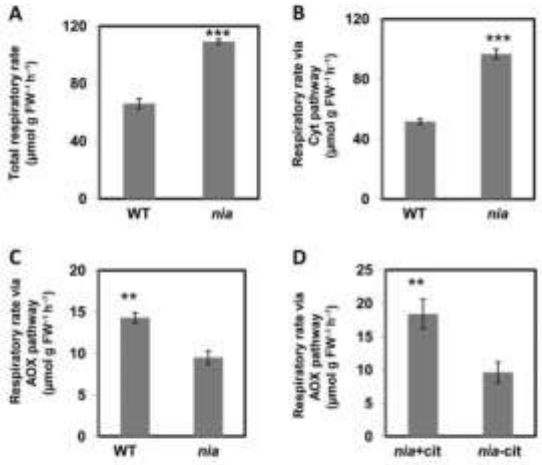

Fig. 3. Respiratory rates and capacities of the cytochrome and alternative oxidase (AOX) pathways in the roots of wild-type (WT) and *nia Arabidopsis* plants. (A) Total respiratory rates measured without inhibitors. (B) Respiratory rate in the presence of 2 mM salicylhydroxamic acid, indicating the capacity of the cytochrome pathway. (C) Respiratory rate in the presence of 2 mM KCN, indicating AOX capacity. (D) AOX capacity of *nia* plants without citrate feeding (*nia*–cit) as compared to *nia* plants fed with 0.2 mM citrate 3 h before measurements (*nia*+cit). Error bars indicate SD. Asterisks indicate significant differences between responses to oxygen conditions: ** $P < 0.05$; *** $P < 0.005$.

Increased AOX capacity in the presence of NO is correlated with gene expression and protein biosynthesis

A possible correlation between AOX respiratory capacity and its gene expression, was estimated by measuring the transcript abundance of the AOX gene by quantitative reverse-transcription PCR. In WT, the expression of *AOX1A* was slightly higher than in *nia* in normoxic conditions, whereas under hypoxic conditions *AOX1A* gene expression was 3-fold higher (Fig. 4A). In order to check whether the transcript abundance and AOX respiratory capacity correlate with its protein amount, an immunoblot analysis was performed using polyclonal antibodies raised against *Arabidopsis* AOX. The Western blot (Fig. 4B) clearly shows that *AOX1A* has low expression in normoxic conditions in both WT and

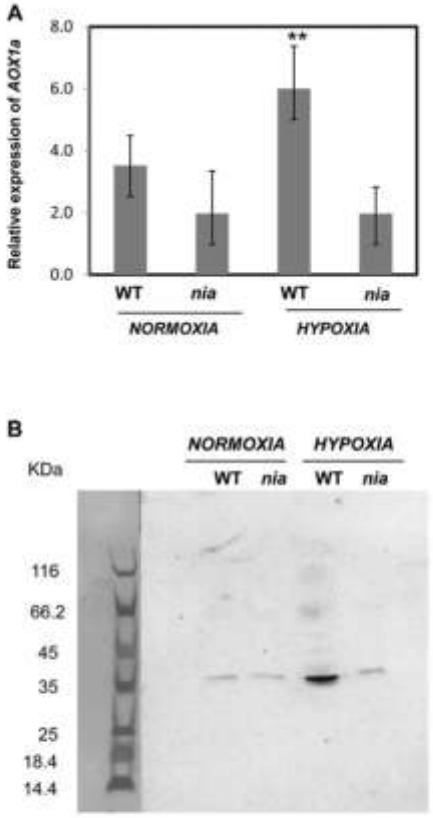

Fig. 4. Expression of alternative oxidase gene (*AOX1A*) and proteins under normoxia and hypoxia. (A) Relative gene expression of *AOX1A* in wild-type (WT) and *nia* plants under normoxic and hypoxic conditions. Error bars indicate SD. Asterisks indicate significant differences between responses to oxygen conditions: ** $P < 0.05$. (B) Immunoblotting of proteins from WT and *nia* with AOX polyclonal antibody. The 35-kDa band indicates the AOX1A protein.

nia. Under the hypoxic conditions, *AOX1A* is induced much stronger in the WT than in *nia*.

NO-induced inhibition of aconitase leads to increase in amino acid biosynthesis

The GC-MS analysis of WT and *nia* plants exposed to normoxia and hypoxia was performed for identification of the changes in various metabolites. Fig. 5 shows increased amino acid accumulation in the WT under hypoxia. The most marked increase was observed for glycine (4 times), less significant for leucine, lysine, tyrosine, arginine, ornithine, citrulline (2 times), valine, serine, methionine, histidine (~1.5 times), and only a marginal increase was observed for alanine, aspartate, and proline. Glutamate levels did not change and putrescine was slightly reduced in the WT under hypoxic conditions. The involvement of NR in the supply of amino groups for amino acid synthesis is supported by data obtained with the double NR mutant. Under normoxia, the NR deficiency did not result in a marked shift in the level of several amino acids including glycine, serine, valine, leucine, arginine, ornithine, citrulline, histidine, and tyrosine. A significant increase in glutamate and a decrease in aspartate revealed a marked shift between these two acidic amino acids. A clear decrease in alanine and proline was observed. The most striking effect of reduction in NR activity on amino acid levels was seen under hypoxia. A strikingly strong decrease was observed for all investigated amino acids, and this decrease was 5-fold or even more for several amino acids, including glycine, serine, and alanine. The hypoxic treatment also resulted in an elevation of γ-aminobutyric acid (GABA) in the WT, while in *nia* the level of GABA markedly decreased.

Changes in TCA cycle intermediates in response to NO

The data in Fig. 6 clearly show that the TCA intermediates citrate, succinate, fumarate, malate, and oxaloacetate accumulated under hypoxia in WT plants. In *nia* plants, the levels of succinate, fumarate, and malate were much lower under normoxic conditions, but the levels of citrate, 2-oxoglutarate, and oxaloacetate were kept at about the same level as in the WT. Under hypoxia in *nia* plants, a decrease was observed for all examined intermediates (except fumarate which still remained low). The 2-oxoglutarate levels in the WT were decreased under hypoxia by 20% and much increased in *nia*, but in the WT they were correlated with 60% accumulation of citrate while in *nia* the citrate levels strongly decreased under hypoxia.

Discussion

Nitrate reductase triggers amino acid metabolism under normoxia and hypoxia

The observed very low NO production in *nia* plants, which is not increased significantly even under hypoxia, clearly indicates that NR is a major source for NO production either directly by using nitrite as an alternative substrate upon its accumulation (Rockel *et al.*, 2002; Sakihama *et al.*, 2002) or indirectly by supplying nitrite for nitrite:NO reductase activity of mitochondria (Gupta and Igamberdiev, 2011) and possibly of other cell constituents such as the plasma membrane (Stöhr *et al.*, 2001). Under low oxygen, the increase of flux through NR limits the capacity of nitrite reductase (Botrel *et al.*, 1996), the activity of which can be repressed under hypoxia (Ferrari and Varner, 1971), causing a drastic accumulation of nitrite, a major substrate for reductive NO production at low oxygen tensions (Planchet *et al.*, 2005). Thus, the *nia* mutant of *Arabidopsis*, in which both genes encoding NR isoforms are affected, is indispensable in clarifying the role of NR in nitrogen and carbon metabolism, in particular in conditions of hypoxic stress, and it also reveals how the absence of NO production affects physiological processes.

The comparison of amino acid levels determined by GC-MS in wild-type and *nia* plants indicates a vital role of NR in nitrogen metabolism under oxygen deficiency. For the increase of amino acid biosynthesis, the supply of reduced nitrogen (via nitrate and nitrite reduction) should be complemented with an increased supply of carbon skeletons (via accumulation of citrate) and the latter can be achieved by the mechanism that involves inhibition of mitochondrial aconitase (Degu *et al.*, 2011) resulting in outflow of citrate from the TCA cycle.

The obtained results show that amino acid synthesis is strongly increased under hypoxia. This increase is dependent on NR activity and related to the induction of NR under hypoxia. In *Arabidopsis*, this shift does not lead to the preferential formation of alanine as it was shown for many species (Narsai *et al.*, 2011) but primarily results in a preferential formation of glycine which may be related to hypoxic accumulation of glycolate, considered as one of metabolic markers of anaerobiosis (Narsai *et al.*, 2009). While alanine is formed from pyruvate at the last step of glycolysis as an alternative to lactic and ethanolic fermentation (Rocha *et al.*, 2010), glycine formation may be linked to the deviation of glycolytic metabolism at the level of 3-phosphoglyceric acid, resulting in the non-photorespiratory serine pathway (Kleczkowski and Givan, 1988), the key enzymes of which were recently shown to be strongly upregulated under anoxia (Narsai *et al.*, 2009; Shingaki-Wells et al., 2011). This pathway may represent a major flux in stress, in particular hypoxic, conditions, leading to accumulation of glycine and glycolate and regulating another important hypoxically induced pathway of nitrogen metabolism, the GABA shunt, by supplying glyoxylate for transamination of GABA (Clark *et al.*, 2009) and by sharing the common reductase for reduction of glyoxylate and succinic semialdehyde (Allan *et al.*, 2008, 2009). The formation of GABA, according to the present data, is dependent on NR activity and increased nitrogen metabolism. The GABA shunt, which is important for pH regulation, is hypoxically induced (Fait *et al.*, 2007; Simpson *et al.*, 2010).

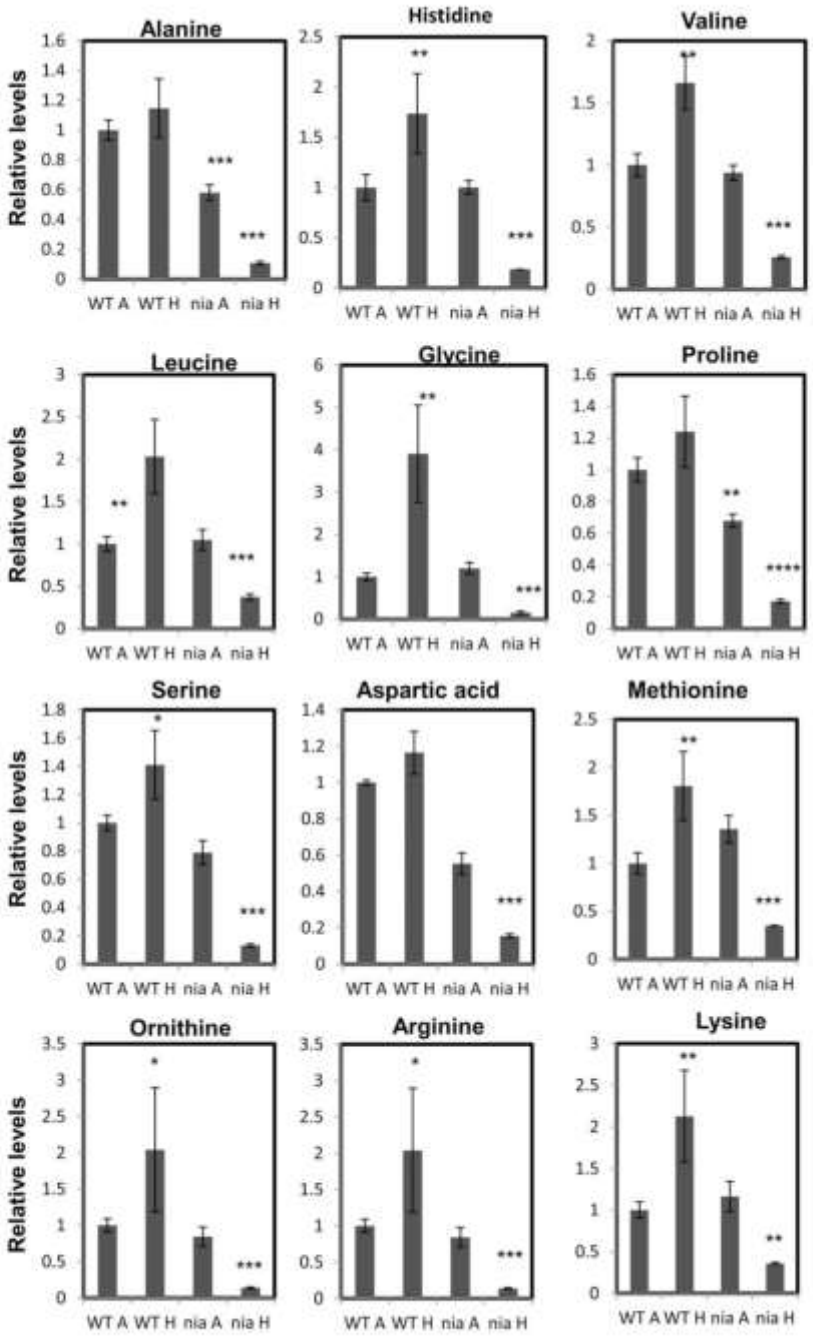

Fig. 5. Relative levels of amino acids in wild-type (WT) and *nia* plants under normoxia (A) and hypoxia (H) as measured by GC-MS. Levels are relative to WT under normoxic conditions. Asterisks indicate significant differences between responses to oxygen conditions: * $P < 0.05$; ** $P < 0.05$; *** $P < 0.005$.

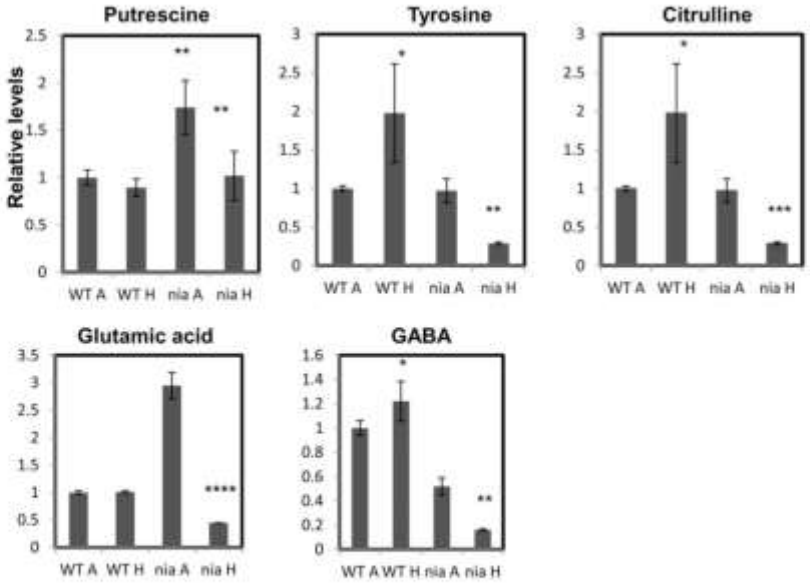

Fig. 5. Continued

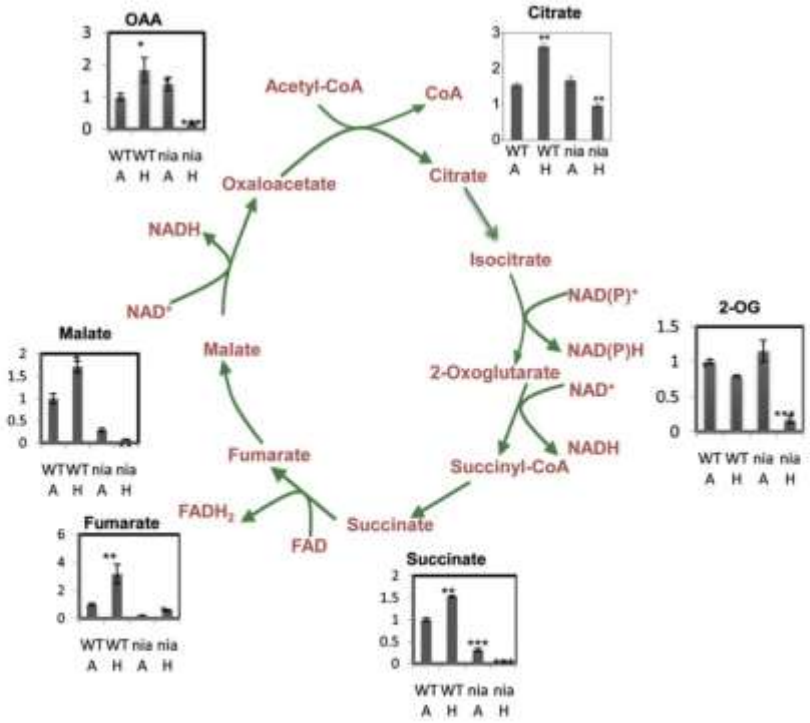

Fig. 6. Relative levels of the TCA cycle intermediates in wild-type (WT) and *nia* plants under normoxia (A) and hypoxia (H) as measured by GC-MS. OAA, oxaloacetate; 2-OG, 2-oxoglutarate. Levels are relative to WT under normoxic conditions. Asterisks indicate significant differences between responses to oxygen conditions: * $P < 0.05$; ** $P < 0.05$; *** $P < 0.005$.

The reductive pathway of NO formation under hypoxia

Nitrate and nitrite reductases can provide ammonium for both the serine biosynthetic pathway and the GABA shunt, while NO, being a co-product of nitrate/nitrite reduction, can facilitate ammonium assimilation by diverting carbon from the TCA cycle by inhibiting the mitochondrial aconitase. While ammonium formation results from complete reduction of nitrate, the incomplete reduction of nitrate (to NO) may induce a mechanism for supplying carbon skeletons to ammonium incorporation to form amino acids. The data on TCA cycle intermediates, in particular the accumulation of citrate in wild-type plants under hypoxia as compared to *nia* plants, support this suggestion. Another role of NO in such a system will consist in prevention of over-reduction and participating in some build up of ATP. NO can indirectly support metabolism via turnover of the haemoglobin-NO cycle (Igamberdiev and Hill, 2004). In this cycle, NO generated from nitrite by mitochondria is converted (oxygenated) to nitrate which in turn is supplied to NR and reduced again to nitrite. This cycle helps to prevent over-reduction of the hypoxic cell and contributes to a limited ATP synthesis (Stoimenova *et al.*, 2007), which can support assimilation of ammonium.

Under hypoxic conditions, NO originates from nitrate (Planchet *et al.*, 2005) in a reductive process that involves nitrite:NO reductase activity of mitochondria (Gupta and Igamberdiev, 2011), of other organelles such as the plasma membrane (Stöhr *et al.*, 2001), or of NR reductase itself (Rockel *et al.*, 2002; Sakihama *et al.*, 2002). The oxidative pathway of NO formation (Corpas *et al.*, 2009) will be inhibited at low oxygen. The existence of this pathway in plants, however, is still under debate (Moreau *et al.*, 2008, 2010) because neither a NO synthase gene nor a protein has been identified as yet. Therefore, most likely, the hypoxically accumulated nitrite is responsible for observed high NR activity and high rates of NO emission (Fig. 1C). The cytosolic acidification taking place at low oxygen further stimulates the NR (Kaiser and Brendle-Behnisch, 1995) and the NO production (Rockel *et al.*, 2002; Planchet *et al.*, 2005) Even if the major part of NO is formed in hypoxic roots by mitochondria as suggested (Planchet *et al.*, 2005), these organelles depend on NR for nitrite to produce NO.

Reliable methods are important for measurement of NO production in plants. Various methods have been developed for the NO measurement; however, there are numerous limitations for using the diaminofluorescent dye-based assays (Planchet and Kaiser, 2006; Mur *et al.*, 2011). The present measurements used the most reliable chemiluminescence method in which NO reacts with ozone and produces a chemiluminescence signal. Moreover, all plants were grown on sterile cultures to exclude any microbial interference in the assays.

A link between NO, aconitase, and AOX

Aconitase contains FeS clusters that are sensitive to hydrogen peroxide (Verniquet *et al.*, 1991) and NO (Navarre *et al.*, 2000) and, through this, aconitase is involved in modulation of the resistance to oxidative stress and in apoptosis (Moeder *et al.*, 2007). A strong inhibition of aconitase by NO is stronger at lower pH (Gardner *et al.*, 1997), which is commonly observed under hypoxia. The stronger inhibition of aconitase in the WT but not in NR mutant (*nia*) suggests that NO is directly responsible for the irreversible inhibition of aconitase under hypoxia.

In response to a range of stress factors, the induction of the alternative pathway resulting from high AOX activity is thought to keep ROS levels in check by increasing the capacity of mitochondrial electron transport and diverting electrons from reduced ubiquinone to oxygen, thus preventing formation of superoxide (Maxwell *et al.*, 1999). While at the protein level AOX is activated by pyruvate and by the increased reduction level (Mackenzie and McIntosh, 1999), the most powerful inducer of AOX at the gene level is citrate (Vanlerberghe and McIntosh, 1996). This may establish the link between aconitase, the enzyme of the TCA cycle that is most sensitive to reactive oxygen and nitrogen species, and the component of electron transport chain which can effectively prevent formation of reactive oxygen and cope with elevated NO levels.

The results provide substantial evidence that AOX is induced under hypoxic conditions via citrate production caused by the inhibition of aconitase by nitric oxide. AOX may lower the ROS produced during low oxygen (Blokhina *et al.*, 2001) and during re-oxygenation following hypoxia (Skutnik and Rychter, 2009). The induction of AOX can serve as a priming mechanism to counteract the anticipated increased ROS production during reoxygenation (Maxwell *et al.*, 1999). Retrograde signalling that senses the metabolic state of the organelle and transduces that signal to the nucleus to express corresponding genes can be achieved via inhibition of aconitase by NO leading to elevation of citrate, the latter acting as a signal for the induction of the AOX gene in the nucleus. The observed correlation between citrate accumulation and 2-oxoglutarate decrease in wild-type (but not *nia*) plants is in favour of the suggested regulatory chain. Therefore, induction of AOX by citrate is a part of retrograde signalling. Such induction was shown to be associated also with the active photorespiratory flux which increases the redox level in mitochondria and stimulates nitrogen metabolism. The plants of barley (Igamberdiev *et al.*, 2001) and potato (Bykova *et al.*, 2005) with low expression of the glycine decarboxylase complex are characterized by a very low expression of AOX protein. Since both photorespiration and hypoxia are linked to active nitrogen metabolism, AOX may be involved in the evolutionary adaptation of plants as a part of the mechanism supporting metabolic switch to intensive nitrogen assimilation.

The observed increase in amino acid accumulation in wild-type *Arabidopsis* under hypoxia and the lack of effect in the NR mutant showing the involvement of NR in amino acid production can be facilitated by inhibition of aconitase by NO, causing the supply of citrate for providing carbon skeletons for amino acid synthesis. Operation of mitochondria and their electron transport in these conditions becomes

possible due to the induction of AOX by accumulating citrate, which supports a limited flux through mitochondria that contributes to formation of 2-oxoglutarate for ammonium incorporation into amino acids and to a limited ATP synthesis (Gupta and Igamberdiev, 2011). Thus, under hypoxia, a retrograde interorganellar signalling pathway is induced in which NR activation triggers NO formation, which in turn inhibits aconitase and increases the level of citrate, which serves as an AOX inducer. This pathway leads to a shift of plant metabolism towards amino acid biosynthesis and provides an important adaptation strategy of plants to low oxygen conditions.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Respiratory rates and capacities of the cytochrome and AOX pathways in the roots of wild-type and *nia Arabidopsis* plants.

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Research article

Respiratory complex I deficiency results in low nitric oxide levels, induction of hemoglobin and upregulation of fermentation pathways

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ABSTRACT

The cytoplasmic male-sterile (CMS) mutant of *Nicotiana sylvestris* which lacks NAD7, one of the subunits of respiratory complex I (NADH: ubiquinone oxidoreductase, EC 1.6.5.3), is characterized by very low (~10 times lower as compared to the wild type plants) emissions of nitric oxide (NO) under hypoxic conditions. The level of the non-symbiotic class 1 hemoglobin, as shown by Western blotting, is increased compared to the wild type plants not only under hypoxia but this protein reveals its marked expression in the CMS mutant even under normoxic conditions. The activity of aconitase (EC 4.2.1.3) is low in the CMS mutant, especially in the mitochondrial compartment, which indicates the suppression of the tricarboxylic acid cycle. The CMS mutant exhibits the severalfold higher activities of alcohol dehydrogenase (EC 1.1.1.1) and lactate dehydrogenase (EC 1.1.1.27) under the normoxic conditions as compared to the wild type plants. It is concluded that the lack of functional complex I results in upregulation of the pathways of hypoxic metabolism which include both fermentation of pyruvate and scavenging of NO by the non-symbiotic hemoglobin.

1. Introduction

The cytoplasmic male-sterile (CMS) mutant of *Nicotiana sylvestris* lacking functional complex I [1,2] is characterized by altered redox state, energy level [3–8] and elevated nitrogen content [9]. The mutant has increased respiration but decreased photosynthesis which is linked to modified intracellular redox recycling [3,8,10]. It is also characterized by enhanced rates of nitrogen assimilation and realignment of metabolite profiles to a nitrogen-rich state.

The impairment of complex I in the CMS mutant is partially compensated by the upregulation of the cytochrome pathway [2], by higher capacity of the alternative internal NADH dehydrogenase (NDin) and the alternative oxidase [10,11]. Total cellular ATP content was higher in both the light and the dark, with unchanged ATP/ADP ratio [9,10]. Generally, the mutant is characterized by the increased pool of pyridine nucleotides, increased mitochondrial NADH/NAD ratio and elevated cytosolic and chloroplast NADPH/NADP [9], although this may depend on light conditions [6,12] and carbon/nitrogen supply [13]. For a cucumber mutant with complex I deficiency both the decreased ATP/ADP and increased NAD(P)H/NAD(P) ratios were reported [7,14,15]. The elevated redox level may explain higher leaf superoxide content [6], while concentrations of H_2O_2 are lower or unchanged [3,6]. Altered redox level may also explain the higher superoxide content previously reported in the *fro* Complex I mutant of Arabidopsis [16,17].

The high nitrogen content reported for the CMS mutant of tobacco is reflected in elevated levels of several amino acids including alanine, asparagine, glutamine, arginine, glycine and serine, but decreased aspartate [9,13]. The elevation of alanine may indicate upregulation of the fermentation glycolytic pathway in conditions when the capacity of mitochondria for the respiratory flux is suppressed at the level of complex I while the total respiratory flux (via alternative NADH and NADPH dehydrogenases) is increased in order to maintain ATP production [2,11]. While the nitrogen status is increased but maximal extractable nitrate reductase activity is slightly decreased [9], it is interesting to determine possible changes in nitric oxide (NO) production in CMS plants which in plants are linked to mitochondria with the contribution of nitrate reductase that supplies nitrite [18]. In this study we show that NO production by CMS tobacco plants under

Abbreviations: ADH, alcohol dehydrogenase; AOX, alternative oxidase; CMS, cytoplasm male sterile; LDH, lactate dehydrogenase; NO, nitric oxide; NR, nitrate reductase.

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hypoxic conditions is extremely low, while the expression of the NO-scavenging non-symbiotic hemoglobin is elevated. This indicates that the CMS mutant plant exhibits some features of hypoxic metabolism in normal air, which is reflected in the expression of the non-symbiotic hemoglobin, the upregulation of lactate and alcohol dehydrogenases and the suppression of aconitase activity making the tricarboxylic acid cycle less operative.

2. Results

2.1. NO production

The maximum rate of NO production from leaves of the CMS mutant was approximately ten times lower than from the wild type (Fig. 1A). When the leaves were incubated in nitrate solution under anoxic conditions, after the 2 h lag period, leaves started to produce NO and in 5–6 h the rate of NO emission reached 200 nmol NO g^{-1} FW h^{-1} which corresponds to the values for higher plants reported in Ref. [19]. For the mutant the lag phase was longer (near 5 h) and the rate of NO emission was close to 20 nmol NO g^{-1} FW h^{-1}. Rotenone did not affect the rate and the shape of curve of NO emission not only in the mutant but also in the wild type (data not shown). The inhibition of nitrate reductase by tungstate resulted in threefold decrease of NO emission in the wild type, shortening of the lag period a faster approaching the maximum level, while in the CMS mutant it did not affect the rate and profile of NO emission (Fig. 1B).

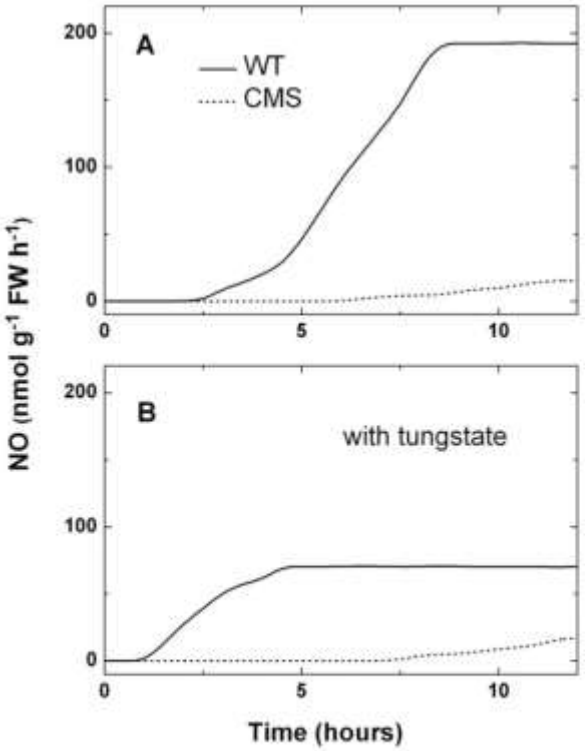

Fig. 1. Nitric oxide emissions from leaves of wild type (solid line) and CMS mutant (dashed line) under nitrogen atmosphere. Leaves were incubated in 50 mM $NaNO_3$ (A) and in 50 mM $NaNO_3$ plus 50 μM tungstate (B). The typical experiment is presented from 5 independent measurements showing similar results.

2.2. Hemoglobin blotting

Protein blotting of non-symbiotic hemoglobin revealed its higher expression in leaves of the CMS plant as compared to the wild type not only under hypoxic conditions when it is usually induced [20] but also under normoxia when its level was lower but still clearly detectable as compared to the wild type where its expression was negligible (Fig. 2). The expression of Hb protein was more than twice higher under hypoxia and under normoxia it was at a similar level in the CMS mutant as under hypoxia in the wild type. The experiment was repeated three times, the same pattern was obtained.

2.3. Aconitase

Aconitase activity was significantly (about threefold) lower in leaves of the CMS plants. In particular this low activity is related to strongly decreased level of aconitase in mitochondria (5–6 times lower than in wild type) than to the cytosol. This corresponded to a different distribution of the enzyme in CMS mutant and WT. In the WT, cytosol contained 35–40% of aconitase activity while mitochondria 60–65%. In CMS mutant, 65–70% of aconitase was in the cytosol and only 30–35% in the organellar fraction (Fig. 3).

2.4. Fermentation enzymes

Lactate and alcohol dehydrogenase were measured both under normoxic and hypoxic conditions in leaves and exhibited markedly higher activity in the CMS mutant in normoxia as compared to the wild type (Fig. 4). The ADH activity was more than 4 times higher in the CMS than in the wild type plants even under normoxia while the short-term (3 h) hypoxia is not sufficient to stimulate significantly the ADH activity in the wild type. In the CMS mutant, hypoxia resulted in some decrease of ADH activity which may be due to a decreased viability of these plants under stress conditions. LDH activity exhibited a threefold increase in the wild type plants under 3-h hypoxia and this corresponded to the observed level of LDH in the CMS mutant under normoxia and hypoxia (Fig. 4).

3. Discussion

3.1. NO emissions and hemoglobin content

Despite of its high nitrogen status, the CMS tobacco mutant shows very low rate of NO emission under anoxic conditions. The formation of NO in plants occurs mainly via reduction of nitrite that takes place anaerobically by mitochondria, nitrate reductase and possibly by the reductases of plasma membrane [18]. The NO emission takes place when leaves take nitrate [21] but this does not

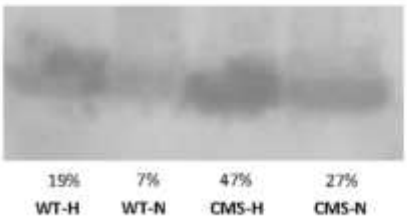

Fig. 2. The levels of non-symbiotic hemoglobin in wild type and CMS mutant under normoxic and hypoxic conditions. WT-H – wild type hypoxia, WT-N – wild type normoxia, CMS-H – CMS mutant hypoxia, CMS-N – CMS mutant normoxia. The result of a typical experiment is presented. Densitometry values for spots were determined after background subtraction and normalization by total spot intensity.

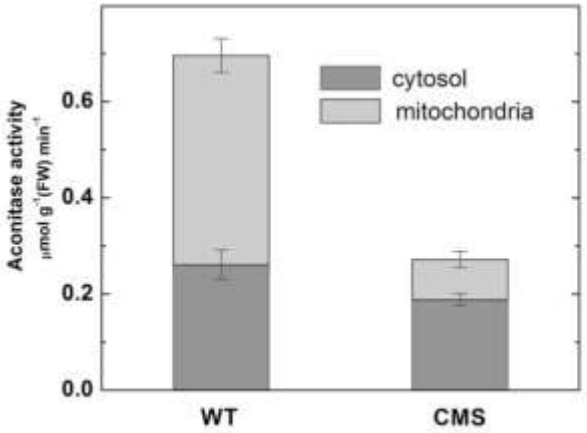

Fig. 3. Aconitase activity in the cytosolic and mitochondrial compartments of leaves of wild type and CMS mutant tobacco plants. Means ± SD, $n = 4$.

mean necessarily that nitrate reductase (NR) is a direct source of this NO. NR can supply nitrite which is further reduced to NO [18]. Its total extractable activity is slightly lower in the CMS mutant [9] but this cannot explain the observed tenfold lower rate of NO emissions in the mutant. The inhibitor of NR tungstate decreases NO emissions in the wild type threefold and this can be related to lower production of nitrite, while the inhibitor of complex I rotenone is inefficient in decreasing NO production suggesting that low NO level in the CMS mutant might not be a direct consequence of the complex I defect. While Garmier et al. [22] showed clear differences in rotenone treated cells as compared to complex I mutant, which is acclimated, the observed high hemoglobin content is necessarily the result of the CMS mutation. Low NO emission may be explained by a high expression of the non-symbiotic class 1 hemoglobin protein (Fig. 2), which primary function is NO scavenging [23]. The expression of Hb gene is related to alterations in the electron flux in mitochondria and triggered by the elevation of free calcium level [24]. Most likely, the altered ability of CMS mitochondria to oxidize NADH due to the complex I impairment results in expression of the class 1 hemoglobin even in normoxic conditions and this causes scavenging of most of NO produced in the cell. Additionally, the reported higher superoxide levels [6] can remove NO via the pathway involving peroxynitrite formation [18]. The superoxide-dependent NO degradation [25,26] likely involves the external NAD(P)H dehydrogenases [25] and results in formation of peroxynitrite which is further scavenged via cytochrome oxidase and other mechanisms [18] making it less toxic for plant than for animal cells. However, the accumulation of peroxynitrite is harmful also for plants if its level is elevated, therefore a safer mechanism of NO scavenging to nitrate involving Hb may be important to maintain the low levels of NO and peroxynitrite in the CMS plant.

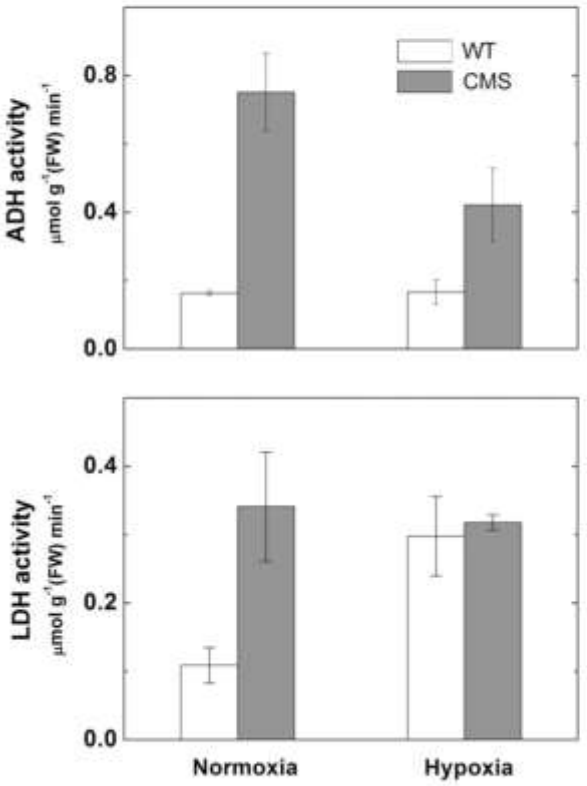

Fig. 4. Lactate dehydrogenase (LDH) and alcohol dehydrogenase (ADH) activities in leaves of wild type and CMS mutant plants under normoxia and hypoxia (3 h incubation under nitrogen). Means ± SD, $n = 6$.

3.2. Morphogenesis and NO

The reduced levels of NO in CMS mutant tobacco plants can explain some peculiarities in morphology observed for these plants. In tobacco CMS plants the root system is not developed significantly and this was previously explained in relation to nitrate-dependent regulation of plant architecture [27]. Based on the NO data (obtained for leaves), we can extrapolate that the significant reduction of root system can be related to the decreased NO level. NO is known as an important factor in establishing root morphology, in particular, by promoting the growth of lateral roots [28]. We observe their marked underdevelopment in the CMS tobacco plants. Other studies have shown that NO reduces the length of the elongation zone in roots, whereby cells differentiate faster and more root hairs are found closer to the tip of the root [29]. NO is considered as a second messenger in auxin signal transduction [30]. The observed low stomatal conductance [1,31] and the sensitivity of CMS tobacco mutant to humidity and drought tolerance [31] is also related to low NO levels that participate in cross-talk with abscisic acid in regulation of stomatal conductance [32].

3.3. Aconitase and NO

The CMS plants are characterized by a decreased activity of aconitase, which is most strikingly expressed for the mitochondrial compartment (Fig. 3). This may be associated with inhibition of aconitase by reactive oxygen species which might accumulate at a higher level at high redox state observed in the mitochondrial compartment, although superoxide and hydrogen peroxide did not accumulate in total leaf extracts [3,6]. Although NO inhibits aconitase [33,34], this is not the case for CMS plants exhibiting low NO levels, however the inhibition by superoxide radicals [35] may explain low aconitase activity especially in mitochondria. It was shown that the inhibition of aconitase results in a shift in the tricarboxylic acid cycle intermediates, in particular in accumulation of citrate which in turn promotes expression of the alternative oxidase gene [34,36]. The accumulation of citrate reported for the CMS tobacco plants [9] is not due to NO but

most likely due to a higher redox state in mitochondria of CMS plants, which results in high expression of AOX. The role of AOX in avoidance of NO accumulation has been proposed recently [37]. It is important to note that aconitase activity in the CMS mutant is affected most significantly in mitochondria (5 times decrease), while in the cytosol the decrease is relatively small (~1.3 times). This means that the lack of the complex I function targets primarily mitochondria, in particular via the increase of superoxide levels in them, and then (to a lesser extent) affects the level of reactive oxygen species in the cytosol. It was shown earlier that in CMS mutant NAD-dependent isocitrate dehydrogenase is elevated while the NADP-dependent form is unchanged [9]. It was suggested that modulation of isocitrate dehydrogenases by redox level [38] could result in citrate accumulation. To a higher extent it can be caused by low activity of the mitochondrial aconitase. The inhibition of aconitase indicates the restriction of the tricarboxylic acid cycle which resembles the hypoxic metabolism.

3.4. Fermentation enzymes

Activities of ADH and LDH are strongly enhanced in the CMS mutant under normoxic conditions (Fig. 4). The upregulation of ADH and LDH in the CMS mutant may be related to a lower capacity of mitochondria to oxidize NADH which in turn induces fermentation pathways even under normoxic conditions. Earlier [9] it was shown that alanine is accumulated in the CMS mutant, which is also related to activation of fermentation pathways. Our data showing hypoxic ADH and LDH activities demonstrate that the activities of the fermentative enzymes do not further increase in CMS leaves under hypoxia. This all means that the CMS mutant resembles hypoxic plant already under normoxic conditions. The Arabidopsis mutant lacking alternative internal mitochondrial NAD(P)H dehydrogenases shows a clear shift to accumulation of lactate and other hypoxic metabolites [39], which is also in accordance with our observations. The induction of non-symbiotic hemoglobin even under normoxia (which is normally hypoxically induced) is the exhibition of the same tendency. High activities of ADH and LDH in the CMS plant can significantly lower NADH level in the cytosol and increase ATP/ADP ratio, which can explain some unexpected results that were obtained earlier on high ATP content and relatively low NAD(P)H in plants lacking functional complex I (reviewed in Ref. [12]).

4. Conclusions

The CMS mutant having low capacity of mitochondria to NADH oxidation, is characterized by induction of enzymes of lactic and alcoholic fermentation and of the non-symbiotic hemoglobin, which participates in scavenging of nitric oxide. The suppression of aconitase activity in the CMS mutant, in a higher extent in mitochondria, indicates the restriction of the tricarboxylic acid cycle resembling the hypoxic metabolism and resulting in outflow of citrate to the cytosol to amino acid synthesis. Basic features of plants expressed under hypoxia are developed in the CMS mutant under normoxic conditions. This partially substitutes the impaired function of the main mitochondrial electron transport chain and, in addition to alternative mitochondrial NADH dehydrogenases, might contribute to NADH oxidation either in the terminal reactions of glycolysis or in the hemoglobin/nitric oxide cycle.

5. Materials and methods

5.1. Plant growth and sampling

N. sylvestris Speg. & Comes wild type and the CMS mutant were grown in the controlled growth chamber supplied by Z-Sciences. Mutant seeds were collected at the Université Paris-Sud, Paris, France (Dr. Rosine De Paepe). Plants were grown on a 14 h day and 10 h night cycle. The day temperature was set to 26 °C and the night temperature was at 22 °C, the humidity level was set to 50% saturation. The plants were moisture sensitive and care was taken to ensure that the humidity levels in the chamber did not increase above 60%. Plants were watered every alternate day and 20–20–20 fertilizer was added once every two weeks as prescribed by the supplier (Plant Products, Brampton, Ontario Canada). Wild type plants were sowed three weeks after the mutant seeds to ensure that the plants reach the same growth stage near maturity. Mature leaves were used for the experiments.

To test wild type and mutant plants under hypoxic conditions, the entire individual plant was placed for 3 h in a custom-built sealed chamber with a small opening on each side. A steady flow of nitrogen gas of 120 ml min^{-1} was maintained and unidirectional air valves facing outwards were placed in the openings to ensure the ambient air does not enter the chamber and the chamber does not develop high air pressure.

5.2. NO measurements

For the measurement of NO production from leaves, they were detached and immediately placed in 20 mM Hepes buffer (pH 7.2) containing 50 mM sodium nitrate. The leaves were then placed in an air tight chamber with a constant inflow of nitrogen at 120 ml min^{-1}. NO was measured by chemiluminescence detection as described [21]. In brief, a constant flow of measuring gas (purified air or nitrogen) of 120 ml min^{-1} was pulled through the chamber and subsequently through the chemiluminescence detector (CLD 88 p; Eco-Physics, Dürnten, Switzerland; detection limit 0.05 ppb; 20 s time resolution) by a vacuum pump connected to an ozone destroyer. The ozone generator of the chemiluminescence detector was supplied with dry oxygen (99%). The measuring gas (air or nitrogen) was made NO free by conducting it through a NO scrubber supplied by Eco-Physics, Switzerland. Flow controllers (Fisher Scientific) were used to adjust all gas flows. Nitrate reductase was inhibited by adding 0.05 mM tungstate (Na_2WO_4), complex I was inhibited by rotenone (dissolved in DMSO, final concentration 40 μM).

5.3. Western-blotting of the class 1 non-symbiotic hemoglobin

Leaves (100 mg) were ground in liquid nitrogen with a mortar and pestle, and extracted with 0.2 ml of ice-cold extraction buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride (PMSF)). The protein content of the extract was measured by the method of Bradford with BSA as a standard. SDS-loading buffer (0.05 M Tris–HCl, pH 6.8, 10% glycerol, 1% SDS, 3% β-mercaptoethanol, and a trace of bromophenol blue) was added, the samples were boiled for 10 min and the proteins separated by SDS-PAGE using a Bio-Rad Miniprotean II gel apparatus according to an established protocol (Bio-Rad bulletin 1721). Final acrylamide concentration was 15% (w/v) for the separating gel and 4% (w/v) for the stacking gel. Proteins were electroblotted onto polyvinylidene fluoride (PVDF) membranes and were detected using a monoclonal antibody (dilution 1:5000) raised against Arabidopsis recombinant class 1 hemoglobin [40]. The protein concentration in different lines was calculated by densitometric comparison using Multi-Analyst PC software (Bio-Rad Laboratories, Mississauga, ON, USA) of immunoblots.

5.4. Measurement of enzyme activities

Biomass (100 mg) was crushed in liquid nitrogen and the enzyme was extracted in 50 mM Tris–HCl (pH 7.4), 1 mM

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MINIREVIEW

The role of nitric oxide and hemoglobin in plant development and morphogenesis

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Plant morphogenesis is regulated endogenously through phytohormones and other chemical signals, which may act either locally or distant from their place of synthesis. Nitric oxide (NO) is formed by a number of controlled processes in plant cells. It is a central signaling molecule with several effects on control of plant growth and development, such as shoot and root architecture. All plants are able to express non-symbiotic hemoglobins at low concentration. Their function is generally not related to oxygen transport or storage; instead they effectively oxidize NO to NO_3^- and thereby control the local cellular NO concentration. In this review, we analyze available data on the role of NO and plant hemoglobins in morphogenetic processes in plants. The comparison of the data suggests that hemoglobin gene expression in plants modulates development and morphogenesis of organs, such as roots and shoots, through the localized control of NO, and that hemoglobin gene expression should always be considered a modulating factor in processes controlled directly or indirectly by NO in plants.

Introduction

Morphogenesis is the most basic biological phenomenon and a mostly ordered and complicated process (Igamberdiev 2012). While plant development requires cell division, growth and differentiation, various combinations of these three processes determine the size and shape of individual cells and of the whole plants (Palin and Geitmann 2012). Metabolic processes play an important role in morphogenesis. Kinetic parameters correspond to spatial geometric effects, and structures can be considered as morphological fixations of dynamical processes (Igamberdiev 1999). A biochemical cycle is a system of incomes and outcomes of participating compounds and can be considered as a basic structure relative to which biological patterns are organized. Even insignificant changes in the parameters of cycles can lead to morphological reconstructions. Generally, morphology is formed by the stable trajectories of the formation and deposition of compounds formed in biochemical cycles. The formation of new cycles leads to the formation of new metabolic trajectories, and this corresponds to morphological changes.

In these processes, reactive oxygen and nitrogen species (ROS and RNS) play an important role. Regulatory products of metabolism, e.g. hormones and ROS/RNS, affect the turnover of the cycle and therefore realize morphological transformations. Many of them are regarded to be mild uncouplers between different processes connected with energy conservation (Skulachev 1996). When the system realizes more futile recyclings via generation of ROS/RNS, living cycles will be faster

Abbreviations – ABA, abscisic acid; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF, diaminofluorescein; DAF-2DA, diaminofluorescein-2 diacetate; GSH, glutathione; GSNO, S-nitrosoglutathione; MDHAR, monodehydroascorbate reductase; NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; NT, nitrite transporter; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNAP, S-nitroso-N-acetyl-penicillamine; SNP, sodium nitroprusside; WT, wild-type.

A. thaliana and internodes of potatoes (Beligni and Lamattina 2000).

Plants respond to environmental factors to optimize root morphology for mineral and water uptake. Endogenous signaling molecules, which are synthesized locally or transported within the plant, play an important role in this process. The hormone auxin is particular important for root morphology, however, a number of experiments have also demonstrated an effect of NO on root morphology. Plant root grow at the tip, from a group of proliferating meristematic cells. However, non-proliferating cells further up the root may be able to redifferentiate and initiate a lateral meristem that penetrates the outer root cell layers and forms a lateral root. This mechanism is the basis of root branching and root architecture.

NO was shown to stimulate root segment elongation by classic dose response experiments using sodium nitrate and SNP as NO donors (Gouvea et al. 1997). SNP had an optimum at 0.1 n*M*, however a stimulatory effect was found up to 1 µ*M*. This is much lower than the optimum concentration for growth stimulation found in leaves (see above). When SNP was applied at higher concentrations than 50 µ*M*, root elongation was significantly inhibited and instead formation of lateral roots was stimulated (Correa-Aragunde et al. 2004). In 8-day-old tomato seedlings (*Lycopersicon esculentum*), the optimal concentration of SNP for stimulation of lateral roots was 200 µ*M*, at which the average number of lateral roots per seedling was increased from below 3 to more than 6. The effect was inhibited by co-treatment with the NO scavenger cPTIO, and when cPTIO was used alone, the number of natural roots decreased significantly to a number of lateral roots below that of untreated plants. Further evidence was found in untreated plants by using DAF-2DA to visualize endogenous NO. This showed increased NO in cells re-differentiating into new lateral meristems, which implies a role for NO as an internal signal in lateral root formation.

Adventitious roots are, similar to lateral roots, *de novo* organs formed by re-differentiation of non-proliferating cells. However, lateral roots are formed at the side of already existing roots, adventitious roots are formed at the hypocotyl, which connects the roots and the shoots. Formation of adventitious roots often occurs in situations where existing roots have been physically removed, damaged or otherwise significantly stressed. Several studies have shown that NO is also involved in formation of adventitious roots (Pagnussat et al. 2002, 2003, Lanteri et al. 2008). When the root system from cucumber (*Cucumis sativus*) seedlings was removed, addition of SNP or SNAP stimulated the number and length of *de novo* adventitious roots formed after 5 days (Lanteri et al. 2008). The optimal concentration for this effect was 10 µ*M*, at which the number of adventitious roots was around 12 compared to 4 on non-treated plants. Also this effect was prevented by co-treatment with cPTIO, and treatment with cPTIO alone actually reduced the number of adventitious roots to 2 in a similar experiment (Pagnussat et al. 2003).

In gravitropism root tips respond to gravity by redistribution of the hormone auxin toward the lower side of the root giving rise to a high local concentration, whereby cells on the lower side elongate less than cells on the upper side. This way the root starts to bend downwards. An interesting study using soybean (*Glycine max*) seedlings showed that NO plays a role in the gravitropic response in roots similar to that of auxin (Hu et al. 2005). Measurements of NO concentration in root tissues showed that endogenous NO accumulated specifically on the lower part of gravistimulated roots, and application of agar containing 5 µ*M* SNP on the upper side of a gravistimulated root inhibited the response. Also the gravitropic response in itself was inhibited by cPTIO. In summary, these studies show that NO contributes significantly to the regulation of plant development and morphogenesis (see left column of Table 1).

The role of hormones in the effect of NO on plant morphogenesis

Several effects of NO on plant growth and morphogenesis mentioned above are either similar to or have been shown to be directly related to actions of various plant hormones – in particular auxin. Auxin is mainly synthesized in the shoot meristems, and the action of this hormone is dependent on a downward transport through the stem and the roots. Segment elongation, apical dominance, formation of lateral and adventitious roots, and gravitropism are all stimulated by auxin. In some of the studies mentioned above it is suggested that NO may act in a signaling pathway downstream of auxin (Pagnussat et al. 2003, Hu et al. 2005, Lanteri et al. 2008). The induction of root gravitropism (Hu et al. 2005) or the formation of lateral (Correa-Aragunde et al. 2004) or adventitious roots (Pagnussat et al. 2002) could all be inhibited by application of cPTIO. Similarly NO donors were able to restore an effect, when auxin action was inhibited either by preventing endogenous formation of auxin by removal of the shoot meristem or by application of auxin transport inhibitors. And finally, application of auxin alone induced a transient increase of endogenous NO in all three responses. However, recent experiments in *A. thaliana* suggest that NO may act upstream of auxin (Fernandez-Marcos et al. 2011). These studies showed that application of NO through the donors SNP or SNAP

Table 1. Effects of NO and hemoglobin on morphogenesis.

Morphological process	Effect of NO	Effect of hemoglobin
Transition from vegetative to generative growth	In *Arabidopsis thaliana* the transition is delayed by endogenous NO (He et al. 2004). In *Lemna aquinoctialis* NO induces the transition (Khurana et al. 2011)	In *A. thaliana* hemoglobin stimulates the transition. Similarly, the transition is delayed in plants with silencing of hemoglobin (Hebelstrup and Jensen 2008, Figs 1 and 2)
De novo root formation	Increases number of lateral roots (Correa-Aragunde et al. 2004) and increases number of adventitious roots (Pagnussat et al. 2002)	No studies reported yet
Cell differentiation at root tips	NO decreases the length of the differentiation zone (Fernandez-Marcos et al. 2011)	Increases the length of the differentiation zone (Hunt et al. 2002)
Changes in stomatal aperture	Stimulates closure of stomata (Neill et al. 2002)	Inhibits closure of stomata (Shi et al. 2012)
Tropism	Asymmetric accumulation of NO stimulates gravitropism in vertical roots. The highest NO concentration is found on the lower side of the roots (Hu et al. 2005)	Silencing of hemoglobin increases the positive tropism of petioles, known as the hyponastic leaf response, in plants treated with ethylene (Hebelstrup et al. 2012)

inhibits the auxin transporter PIN1 at the protein level, resulting in a decreased auxin transport to the root meristem. However, these studies did not look into the formation of adventitious or lateral roots, but in a second report by the same authors, NO was found to inhibit cell elongation in the elongation–differentiation zone of roots (Fernandez-Marcos et al. 2012). Other studies have shown that NO enhances auxin signaling directly through S-nitrosylation of a cysteine residue in the auxin receptor TIR1 (Terrile et al. 2012). In contrast, NO interferes negatively with cytokinin signaling by inhibiting the phosphorelay activity through S-nitrosylation of a histidine phosphotransfer protein in the phosphorylation signaling cascade of cytokinins (Feng et al. 2013). Another study suggests that cytokinins can inhibit the activity of NO by a direct chemical reaction between NO and cytokinin in cells (Liu et al. 2013). However, NO may also act downstream of cytokinins, in the cytokinin-induced activation of expression of cell cycle genes in cell proliferation (Shen et al. 2012). NO also seems to stimulate formation of the gaseous hormone ethylene (Manac'h-Little et al. 2005, Hebelstrup et al. 2012). Moreover, NO has been shown to be part of the signaling cascade triggered by the stress hormone abscisic acid (ABA; Hancock et al. 2011).

The involvement of NO in photomorphogenesis is triggered by its antagonism with the effect of gibberellic acid (Lozano-Juste and Leon 2011). It was shown that phytochrome B-dependent NO production promotes photomorphogenesis by a mechanism based on the coordinated repression of growth-promoting PIF genes and an increase in the content of DELLA proteins. A complex interaction of NO, ROS and hormones is suggested to explain the mechanism of thigmomorphogenesis (plant response to mechano-stimulation) (Chehab et al. 2009).

Molecular actions of NO

In mammals NO is produced from L-arginine by nitric oxide synthases (NOSs). No homologue of NOSs has been identified in plants and recent studies indicate that mitochondria are the major cellular sources of NO in plants (Planchet et al. 2005, Gupta and Igamberdiev 2011). Cytochrome *c* oxidase may be a source of NO at low oxygen tensions using nitrite as electron acceptor instead of oxygen and reducing it to NO. The mechanism of reaction includes participation of the iron-copper center (Gupta and Igamberdiev 2011, Hematian et al. 2012). Plants with an impaired mitochondrial electron transport chain produce less NO (Shah et al. 2013), which causes changes in their morphological characteristics, e.g. underdeveloped root system. Another well-documented source of NO in plants is NR, which may reduce NO_2^- to NO by a NAD(P)H-dependent mechanism at high concentrations of NO_2^- (Kaiser et al. 2002).

NO can react with glutathione to form S-nitrosoglutathione (GSNO), which can subsequently be reduced by GSNO reductase – a mechanism whereby NO is removed (Frungillo et al. 2013). The reaction of NO with glutathione may trigger morphogenetic events via a mechanism that is affected by the glutathione reduction level. Nitrosylation of glutathione by NO significantly affects the glutathione redox potential because the scavenging of nitrosylated glutathione consumes two NADH and one reduced glutathione (GSH) (Mullineaux and Rausch 2005, Vyas et al. 2013). The importance of the glutathione pool size and redox potential for determination of cell division, growth and even apoptosis was mentioned in many works (reviewed in Noctor et al. 2012). The quiescent parts of plants, such as the root quiescent center and cells in organs such as seeds, maintain a highly oxidized intracellular state, in particular reflected in a low reduction level of the glutathione pool

(Kranner et al. 2006). Auxin accumulation in the root stem cells is dependent on the oxidized status of the cells (Jiang and Feldman 2010). The lowering of glutathione content results in a non-functional root meristem while the shoot meristem is largely unaffected (Vernoux et al. 2000). A subsequent increase in the total cellular GSH pool is essential for the cells to progress to cell division (Diaz-Vivancos et al. 2008). Generally, more negative glutathione redox potentials stimulate cell proliferation while less negative potentials lead to differentiation processes (Schafer and Buettner 2001). In line with the effect of NO on the glutathione redox potential, NO regulates the expression and activity of cell cycle genes during lateral root formation (Correa-Aragunde et al. 2006) and in cell cultures (Ötvös et al. 2005).

The plant proteome contains several proteins that are targeted for nitrosylation of cysteine residues by NO (Lindermayr et al. 2005) forming S-nitrosocysteine (S-nitrosylation). This suggests that there may not be one particular receptor, but that NO action may be mediated by modulation of several proteins. For example, NO influences auxin and cytokinin signaling in development by S-nitrosylation of either the auxin receptor TIR1 (Terrile et al. 2012) or the phosphotransfer protein AHP1 in the cytokinin phosphorelay signaling cascade (Feng et al. 2013). The transcription factor NPR1 and the salicylic acid binding protein SABP3 are involved in NO-mediated pathogenic responses. They are both modulated by S-nitrosylation. NPR1 is sequestered in the cytoplasm as an oligomer and S-nitrosylation of the protein facilitates its oligomerization (Tada et al. 2008). In SABP3 S-nitrosylation prevents SA binding (Wang et al. 2009). NADPH oxidase activity in Arabidopsis, which generates reactive oxygen intermediates in response to pathogens, is regulated by a negative feedback of NO mediated by S-nitrosylation (Yun et al. 2011).

Other functions of NO in plants related to plant development and adaptation

In addition to control of development NO is also involved in various responses to biotic and abiotic stresses. It is an important signaling molecule in plant defense against pathogens necessary for activation of signals leading to suppression of infection (Mur et al. 2005, Leitner et al. 2009). NO has also been shown to be a mediator of signaling in stomatal guard cells (Hancock et al. 2011). Plants respond to drought stress by closing stomatal guard cells, whereby transpiration is minimized. The plant hormone ABA plays a central role in this response. The involvement of NO in this process was originally observed in pea (*P. sativum*) by demonstrating that application of SNP alone was sufficient for reducing stomatal aperture. Also endogenous NO increased significantly in guard cells, when stomatal closure was induced by ABA (Neill et al. 2002). NO has also been reported to be involved in formation of root hairs (Lombardo et al. 2006). Other studies have shown that NO reduces the length of the differentiation zone in roots, whereby cells differentiate faster and more root hairs are found closer to the tip of the root (Fernandez-Marcos et al. 2011).

Other experiments suggest that NO plays a role in programmed cell death in xylem development (Gabaldon et al. 2005). Xylem cells are dead cells in the vascular tissue through which water and minerals are transported from roots to leaves. NO may have a similar role during aerenchyma formation in roots under anaerobic conditions (Igamberdiev et al. 2013). The involvement of NO and nitrosothiols in the process of H_2O_2-induced leaf cell death has been shown for rice (Lin et al. 2012). It has also been suggested that NO regulates the rate and orientation of pollen tube growth (Prado et al. 2004).

Finally, various studies show that NO can break seed dormancy in various plant species (Bethke et al. 2006a, 2006b, Sarath et al. 2006). This effect may be triggered by an induction of genes for ABA breakdown (Hancock et al. 2011) but may also be related to stimulation of nitrite-dependent respiration and changes in redox level in seeds. There are no reports on the effect of hemoglobin expression on dormancy, but hemoglobin expression increases in the embryos and seed coat (aleurone layer) of barley seeds during germination (Duff et al. 1998).

Plant hemoglobins as NO scavengers triggering morphogenetic events

Plant hemoglobins

Cloning and characterization of hemoglobin genes from plants, fungi, bacteria and protists have opened a door to a new world of possible primordial functions of hemoglobins. New members of the globin gene family have been identified in the human and in animal genomes – such as neuroglobin and cytoglobin (Storz et al. 2011). Gene expression studies show that actual hemoglobin subunits are expressed in avascular tissues, such as in embryoids during early development or in ocular lens cells under development and maturation (Mansergh et al. 2008). Altogether this suggests that there are universal functions of globin proteins, which are different from oxygen transport in the blood. Studies in plants suggest that some of these functions are involved in development and morphogenesis through modulation of the endogenous level of NO (Hebelstrup et al. 2006, 2007, Hebelstrup and Jensen 2008).

Some plant hemoglobins, including leghemoglobins and other symbiotic hemoglobins, have been known for a long time to be specifically located in root nodules in an isolated group of plants, which possess a specialized symbiotic interaction with nitrogen-fixing bacteria. These symbiotic hemoglobins play a role in oxygen binding; however this function is specific only to plants performing symbiotic nitrogen fixation and for the majority of plant hemoglobins their function is not related to symbiosis (Hebelstrup et al. 2007, Gupta et al. 2011). There are three phylogenetic classes of hemoglobins in plants: classes 1, 2 and 3, of which the structure of classes 1 and 2 more closely resembles that of the human and animal globins, whereas class 3 globins resemble that of truncated globins from prokaryotes (Watts et al. 2001). Most functional studies on plant hemoglobin have focused on class 1, whereas classes 2 and 3 are less well characterized (Gupta et al. 2011). The highest affinity for oxygen is observed in class 1 hemoglobins, class 2 hemoglobins have more moderate oxygen affinity, while the weakest oxygen binding are characteristic for truncated hemoglobins (Gupta et al. 2011). Class 2 hemoglobins may facilitate oxygen supply to developing tissues and the symbiotic hemoglobins in nodules have mostly evolved from them, while class 3 hemoglobins may regulate oxygen delivery at high O_2 concentrations (Gupta et al. 2011). Removal of NO is a well-documented function of class 1 hemoglobins (Perazzolli et al. 2004, Hebelstrup et al. 2006, Igamberdiev et al. 2006a, Gupta et al. 2011, Hill 2012). Also, there are several independent studies in different plant species showing that modulation of cellular class 1 hemoglobin levels affects development and morphogenetic processes in plants. In the following two sections we will go through these studies, and for each of them compare how the same process is also affected by NO. Some of these morphogenetic processes are summarized in Table 1.

Role of hemoglobin in plant development

Plant hemoglobins have been studied both in vivo and in vitro. Most in vivo methods have focused on gene and protein expression under development or stress, either by measuring endogenous gene and protein expression or by manipulating expression levels in transgenic lines. The in vitro studies have focused on protein structure and ligand binding kinetics. Most in vivo studies of plant hemoglobins have been carried out in the model plant *A. thaliana*. *Arabidopsis thaliana* contains three genes for hemoglobin: *Glb1*, *Glb2* and *Glb3*, one for each of the three classes (Trevaskis et al. 1997, Watts et al. 2001). Isolating the promoters, which control the gene expression of *Glb1* and *Glb2* and fusing each of them with a cDNA of the enzyme β-glucuronidase, which is identifiable by histochemical staining, revealed the expression pattern of each of these genes in transgenic plants into which this construct had been inserted (Hunt et al. 2001, Heckmann et al. 2006). Strong expression of *Glb1* was observed in root tips and in the cells surrounding lateral root branches. In seedlings it was also observed at the base of the hypocotyl from which the root system emerges. In the aerial parts of the plant, *Glb1* expression was seen in leaf hydathodes, which are the endpoints of vascular tissues in leaves, and also in the top and auxiliary meristems. Meristems are the sites of growth and emergence of new organs in plant shoots. Meristems may be dormant, and often one meristem identified as the top meristem is suppressing shooting from auxiliary meristems in a phenomenon known as apical dominance. The interplay between meristems has a central role in plant morphogenesis. The *Glb1* expression is strongest in dormant and young meristems and tends to disappear in the individual shoot along with the maturation of the shoot. *Glb2* has a much weaker expression pattern with no expression in roots and leaf hydathodes and only weak expression in meristems (Heckmann et al. 2006).

Shoot meristems, root tips and lateral branch points of roots all play a central role in plant development and morphology suggesting that expression of hemoglobin genes may be involved in morphogenesis. This was confirmed by further genetic studies in which expression of *Glb1* or *Glb2* was either increased by overexpression using a *CaMV 35S* promoter, or decreased by silencing with an RNAi construct (Hebelstrup et al. 2006, Hebelstrup and Jensen 2008). Silencing of *Glb1* resulted in plants with severe developmental deficiencies confirming the role of *Glb1* in development and plant morphology. Affected organs were co-incident with the observed gene expression pattern of *Glb1*. *Glb1* gene expression had been observed in hydathodes, and similarly in plants with silencing of *Glb1*, hydathode development was affected by enlargement and stunting. *Glb1* expression was seen in top and auxiliary meristems, and in plants with silencing of *Glb1* meristem coordination and control was affected with loss of apical dominance and a delay of the transition of the top meristem from vegetative to regenerative growth.

The transition from vegetative to regenerative growth is a central event in plant morphology, controlled by endogenous factors such as age and genotype as well as environmental factors such as day-length and temperature. In particular several species require a period of colder days (temperature depends on species) to induce transition to regenerative growth. This phenomenon

is known as vernalization. The morphology of the vegetative and generative phase can be quite distinct. In *A. thaliana* the vegetative phase is characterized by a very short stem without any laterals shoots, leaves are placed in a so-called rosette with very short internode lengths. Following transition to the generative phase, this morphology is changed to the opposite so that the stem is elongated because of a much longer internode lengths, leaves are not in a rosette and the stem is branching through lateral shoots. The very short internodes in the rosette formed before transition remains very short, so that the rosette leaves stay intact below the generative shoots. At the top of the generative shoots, lateral shoots develop into flowers. This particular morphological change from a rosette *habitus* to an elongated stem with flowers is called bolting and appears in several plant species. This transition – bolting – was affected in *A. thaliana* plants with silencing of *Glb1* expression (see Fig. 1). The plants remained in the vegetative stage for longer making more rosette leaves, and when the shoot finally made the transition to generative growth, auxiliary meristems would often revert the development back to the vegetative and make a rosette of leaves on the stem rather than a new shoot (Fig. 1B). The expression of hemoglobin, by modulating NO levels, modulates the time of flowering. Fig. 2 shows that hemoglobin overexpression in Arabidopsis induces the onset of flowering.

The developmental phenotypes that appeared in *Glb1* silenced plants were associated with accumulation of NO in the affected organs (Hebelstrup et al. 2006, Hebelstrup and Jensen 2008), suggesting that the function of plant hemoglobins in development and morphogenesis is associated with modulation of the NO level. In fact, the main function of class 1 hemoglobins is associated with modulation of NO levels and this class may be mostly responsible for morphogenetic effects, while class 2 hemoglobins may affect morphogenesis via regulation of oxygen levels and also through the involvement in NO metabolism at higher oxygen concentrations than the class 1 hemoglobin. Differential cell-specific expression of classes 1 and 2 hemoglobins may also contribute to their apparent different functions in development (Hebelstrup et al. 2006, Heckmann et al. 2006). While NO formation is triggered by low oxygen, rapidly dividing meristematic cells have a high respiration rate and often exhibit oxygen depletion (Bidel et al. 2000) which is followed by NO accumulation. The summarized effect of hemoglobin gene expression on plant development and morphology is shown in Table 1.

There are few studies on the effect of plant hemoglobin expression on root morphology. But one study reported that plants with overexpression of *Glb1* in *A. thaliana* had

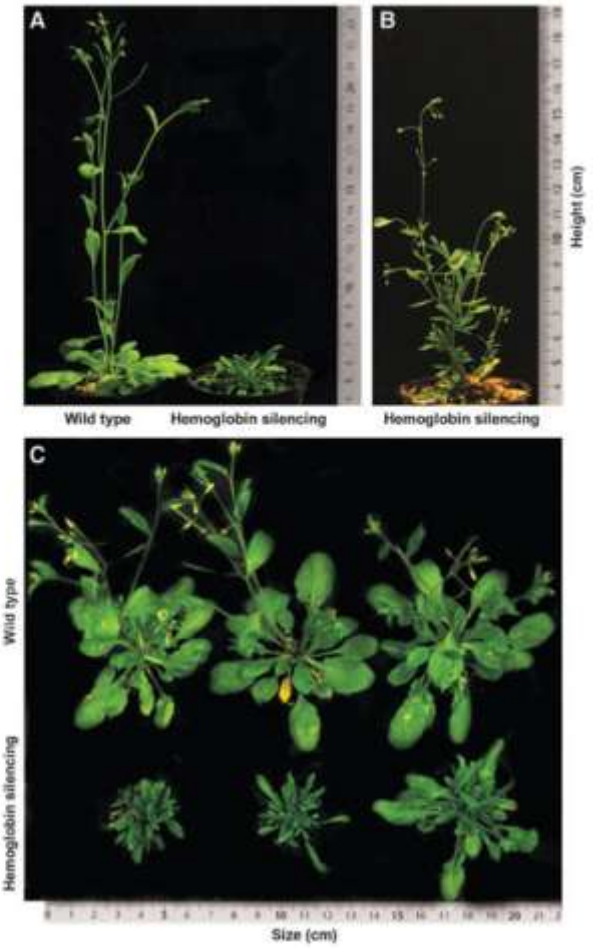

Fig. 1. The transition to flowering is affected in Arabidopsis plants with silencing of Glb1 hemoglobin expression. (A) Five-week-old WT plants were flowering, whereas silencing plants of similar age were still at the rosette stage. (B) When silencing plants were 10-week-old, they were at the generative phase. There was a loss of apical shoot dominance in the silencing lines. The 10-week-old WT plants were senescent and dead. (C) Comparison of rosettes in 5-week-old plants. (The photos are taken of plants that were originally characterized and presented in Hebelstrup et al. 2006 and Hebelstrup and Jensen 2008.)

an increased growth of primary roots and an elongated cell differentiation zone at the root tips (Hunt et al. 2002). Controlled morphogenesis with initiation of new meristems in plants can be forced by tissue culture techniques. By adding a cocktail of plant hormones, differentiated cells can be reset to start formation of new shoots or roots. In a tissue-culture study in *A. thaliana*, *Glb2* gene expression was significantly upregulated in roots that were forced by hormone treatments to initiate shoot organogenesis (Wang et al. 2011). Furthermore *glb2* mutant plants (with no *Glb2* expression), initiate significantly fewer shoots and plants with overexpression

Fig. 2. Hemoglobin overexpression in Arabidopsis induces the onset of flowering. Five-week-old plants with overexpression of Glb1 hemoglobin had flowered earlier and had more progressed inflorescences than WT plants. (The photos are taken of plants that were originally characterized and presented in Hebelstrup et al. 2006 and Hebelstrup and Jensen 2008.)

of either *Glb1* or *Glb2* initiate significantly more shoots during this type of induced organogenesis. A similar study with tissue-culture from leaves of chicory (*Cichorium intybus* × *Cichorium endivia*), where embryogenesis was forced by hormone treatments, also showed an upregulation of hemoglobin protein expression in the embryogenic cells (Smagghe et al. 2007). Interestingly, hemoglobin expression controls auxin synthesis through NO modulation of the formation of embryogenic cells during morphogenesis (Elhiti et al. 2013). Overexpression of class 1 hemoglobin in Arabidopsis also leads to cold-induced increase of content of long chain fatty acids, which are precursors of complex sphingolipids and cellular signals in eukaryotic cells, which results in impairment in cold-dependent root growth inhibition (Guillas et al. 2013).

Other consequences of NO scavenging by plant hemoglobin

The NO scavenging function of plant hemoglobin (of the class 1 in particular) also plays a role in different aspects of plant biology which may not be directly linked to development and morphogenesis. In particular, plant hemoglobins play a role in oxidation of NO into nitrate during hypoxic stress in plant tissues (Dordas et al. 2003a, 2003b, 2004, Igamberdiev et al. 2004, 2005, 2006a, 2006b, 2010). A class 1 hemoglobin gene from barley (*Hordeum vulgare*) was upregulated by hypoxia or flooding (Taylor et al. 1994). This gene was used for transgenic silencing or overexpression in liquid maize cell cultures (*Z. mays*), where its expression was shown to be important for maintaining ATP formation during hypoxic conditions (Sowa et al. 1998). Consistent with this, transgenic *A. thaliana* plants with overexpression of *Glb1* (Hunt et al. 2002, Hebelstrup et al. 2006) or *Glb2* (Hebelstrup et al. 2006) had an enhanced survival during severe hypoxic stress, whereas plants with silencing of *Glb1* had a decreased survival. Mutant *glb2* plants with silencing of *Glb2* had the same survival rate as wild-type (WT) plants (Hebelstrup et al. 2006). Studies on the mechanism behind this increased resistance to hypoxic stress, demonstrated in transgenic alfalfa (*Medicago sativa*) and liquid maize cell culture that the NO concentration was inversely correlated to the hemoglobin levels (Dordas et al. 2003a, 2003b, 2004). Transgenic silencing of hemoglobin in alfalfa roots also resulted in enhanced ratio of NADH to NAD^+ and ratio of NADPH to $NADP^+$ during hypoxia, whereas hemoglobin overexpression reduced these ratios. In vitro studies with fractionated protein extracts from transgenic alfalfa overexpressing barley hemoglobin showed that an enzymatic NAD(P)H-dependent oxidation of NO was located in a purified protein fraction containing hemoglobin (Igamberdiev et al. 2004). The reaction was later found to be dependent on the presence of the enzyme monodehydroascorbate reductase (MDHAR), which was demonstrated to be able to reduce plant hemoglobin from its Hb(Fe^{3+}) to its Hb(Fe^{2+}) form (Igamberdiev et al. 2006a). Altogether this mechanism, described as the Hb/NO cycle contributes to metabolism and modulation of endogenous NO as described in Igamberdiev et al. (2005) and schematically presented in Fig. 3. The Hb/NO cycle plays an important role in N-metabolism during hypoxia, where the hemoglobin-dependent scavenging of NO prevents a significant loss of nitrogen from NO emission (Hebelstrup et al. 2012).

There are indications that plant hemoglobins may modulate NO in both biotic and abiotic stress responses. In line with the effect of NO on stomatal closure (Neill et al. 2002), tobacco plants with overexpression of hemoglobin had an increased stomatal aperture

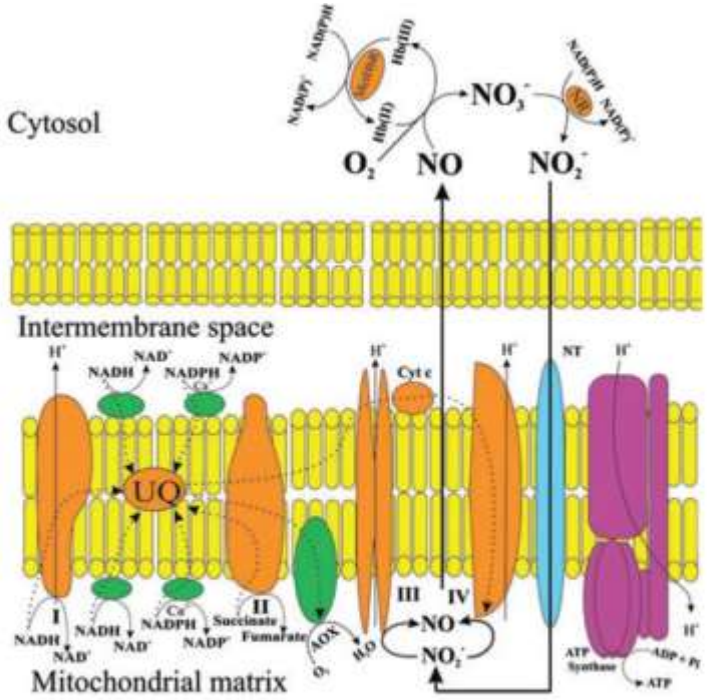

Fig. 3. Formation and scavenging of NO (Hb/NO cycle) under low oxygen conditions. Mitochondrial reduction of nitrite to NO takes place at the sites of cytochrome oxidase (complex IV) and complex III. Accumulating NO diffuses to the cytosol where it is converted to nitrate (NO_3^-) by the non-symbiotic hemoglobin in its ferrous form [Hb(II)]. High affinity of Hb to O_2 keeps it in the oxygenated state even at nanomolar O_2 concentration. Hb is oxidized to methemoglobin [Hb(III)], which is then reduced by methemoglobin reductase (MetHbR). Nitrate generated in the Hb reaction is reduced by NR to nitrite, which is transported to mitochondria by a putative nitrite transporter (NT). NADH and NADPH generated in the cytosol are oxidized by the externally facing calcium-dependent mitochondrial dehydrogenases. The cycle may contribute to proton pumping at sites of complex III and IV and to a limited ATP synthesis. (Modified from Møller 2001.)

under waterlogging stress compared with control plants (Shi et al. 2012). Hemoglobin gene silencing stimulates flooding-associated leaf tropism known as the hyponastic response. This most likely happens through an effect of increased NO either directly enhancing the hyponastic response or through NO stimulating the response to ethylene (Hebelstrup et al. 2012). In line with the role of NO acting broadly in response to both necrotrophic and biotrophic plant pathogens, hemoglobin gene expression was recently found to effectively modulate susceptibility toward both the hemibiotroph *Pseudomonas syringae* and the necrotroph *Botrytis cinerea* (Mur et al. 2012).

The modulation of NO under hypoxic condition via participation of class 1 hemoglobins has an important consequence for morphogenetic phenomena observed under low oxygen. Among them (e.g. suppression of growth which can also be linked to NO and hemoglobin), the most important is aerenchyma formation. Aerenchyma is a tissue formed by dead cells that facilitates transfer of oxygen to submerged parts of plant. It has been hypothesized that the differential expression of class 1 hemoglobin with high expression in the cells that remain alive in hypoxia and underexpression in the cells undergoing apoptosis that leads to aerenchyma formation in NO accumulating cells results in the morphological structure that facilitates oxygen delivery to roots and other submerged tissues (Igamberdiev et al. 2013).

Additional mechanisms of hemoglobin other than NO scavenging may exist in plants. Upregulation of class 2 hemoglobin expression in developing seeds of

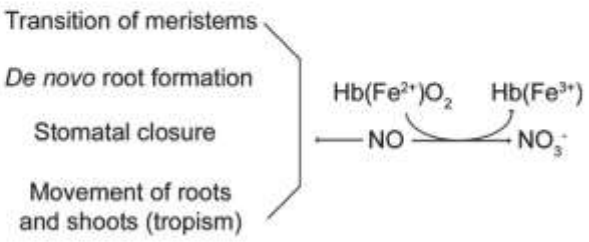

Fig. 4. Localized expression of hemoglobin controls a number of morphogenetic processes in plants.

A. thaliana has a significant effect on lipid composition and lipid content in seeds. Transgenic seeds contained more polyunsaturated fatty acids and total oil content was higher (Vigeolas et al. 2011). No direct link to NO modulation was demonstrated in this study, and the effect could be the result of a biochemical or enzymatic mechanism other than NO scavenging.

In summary, the functional studies on hemoglobins in plant development have shown that hemoglobin gene expression is predominantly observed in meristematic tissues. Meristematic tissues are often depleted in oxygen due to its high O_2 consumption and compact packing of cells. Hemoglobin gene expression in these tissues is involved in controlling development and morphogenesis. This effect is correlated to modulation of the endogenous level of NO. The mechanism behind plant hemoglobin function is based on its ability to directly control NO by oxidation in a biochemical cycle described as the Hb/NO cycle.

Conclusion

NO is a central signaling molecule with several effects on control of plant growth and development, such as shoot and root architecture. The effect of NO is present throughout the life of a plant starting from seed germination and finishing at plant senescence. NO is produced in plants mainly via reduction of nitrite and scavenged via the action of non-symbiotic hemoglobin. We suggest that the effect of NO is expressed via its effects on nitrosylation of proteins and on the maintenance of redox and energy levels, in particular on glutathione potentials. Removal of cellular NO is carried out by non-symbiotic hemoglobin, which modulates plant morphogenesis through the localized control of the NO level in specific tissues (Fig. 4). This is supported by comparison of experiments demonstrating the effect of NO and/or hemoglobin on different morphogenetic events; e.g. those presented in Table 1. The effect of hemoglobin gene expression in plants should therefore always be considered in processes controlled directly or indirectly by NO.

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REVIEW

Anoxic nitric oxide cycling in plants: participating reactions and possible mechanisms

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At sufficiently low oxygen concentrations, hemeproteins are deoxygenated and become capable of reducing nitrite to nitric oxide (NO), in a reversal of the reaction in which NO is converted to nitrate or nitrite by oxygenated hemeproteins. The maximum rates of NO production depend on the oxygen avidity. The hemeproteins with the highest avidity, such as hexacoordinate hemoglobins, retain oxygen even under anoxic conditions resulting in their being extremely effective NO scavengers but essentially incapable of producing NO. Deoxyhemeprotein-related NO production can be observed in mitochondria (at the levels of cytochrome *c* oxidase, cytochrome *c*, complex III and possibly other sites), in plasma membrane, cytosol, endoplasmic reticulum and peroxisomes. In mitochondria, the use of nitrite as an alternative electron acceptor can contribute to a limited rate of ATP synthesis. Non-heme metal-containing proteins such as nitrate reductase and xanthine oxidase can also be involved in NO production. This will result in a strong anoxic redox flux of nitrogen through the hemoglobin-NO cycle involving nitrate reductase, nitrite: NO reductase, and NO dioxygenase. In normoxic conditions, NO is produced in very low quantities, mainly for signaling purposes and this nitrogen cycling is inoperative.

Nitrite reduction to nitric oxide catalyzed by hemeproteins

Nitric oxide (NO) production from nitrite (nitrous acid) is a common chemical phenomenon occurring non-enzymatically at low pH. This non-enzymatic reaction may take place in low pH compartments such as aleurone layers during seed germination, with reducing agents such as ascorbate and phenolics involved in its facilitation (Bethke et al. 2004). However, in most tissues even under anoxia, the calculated rates of non-enzymatic NO synthesis are too low to account for the tissue levels of NO that are produced. Much higher rates can be achieved by the involvement of deoxyhemeproteins which are able to reduce nitrite (Doyle et al. 1981, Nagababu et al. 2003). In general, hemeproteins can serve both as NO scavengers and NO producers. If oxygen concentration is low and the hemeprotein molecule is deoxygenated, NO can be formed from nitrite, while at oxygen concentrations at which hemeproteins are oxygenated, NO conversion to nitrate or to nitrite occurs (Gladwin and Kim-Shapiro 2008). If the affinity of a hemeprotein to oxygen is extremely high, as in the case of hexacoordinated hemoglobins induced in plants under anoxia (Dordas et al. 2003, 2004), it is unlikely that the hemoglobin will function to produce NO as the oxygen dissociation constant is of the order of 2 nM (Duff et al. 1997).

The ability of deoxygenated hemoglobin to reduce nitrite to NO was first described by Brooks (1937). It

Abbreviations – AOX, alternative oxidase; COX, cytochrome *c* oxidase; NO, nitric oxide.

has been proposed that nitrite represents a biologic reservoir of NO that can regulate major physiological processes (Gladwin et al. 2009). Hemeproteins were found to perform a process by which the NO formed in the reaction of a deoxyhemeprotein with nitrite, is released because of heme oxidation to its ferric form (Huang et al. 2005). Maximal NO production is observed at approximately 50% hemeprotein oxygen saturation (Grubina et al. 2007, Gladwin et al. 2009). Equation (1) describes the reaction:

$$NO_2^- + Fe^{2+} + H^+ \rightarrow NO + Fe^{3+} + OH^- \quad (1)$$

The opposite reaction of NO scavenging is described by the following equation:

$$NO + Fe^{2+} + O_2 \rightarrow NO_3^- + Fe^{3+} \quad (2)$$

The mechanism of NO scavenging by hemoglobin has been investigated in plants exposed to the anoxic conditions and shown to have an important physiological role in the maintenance of redox and energy balance (Igamberdiev et al. 2004a, 2006, Stoimenova et al. 2007). The opposite process leading to NO formation during anoxia has been demonstrated in plants (Dordas et al. 2003, Planchet et al. 2005), although the mechanism by which this occurred remained unknown. Recent advances in understanding the mechanism of NO formation in blood by hemoglobin (Gladwin and Kim-Shapiro 2008) can also shed light on the mechanisms of this process in plants. The chemistry of nitrite-dependent NO production by hemeproteins relies on their ligand binding properties being regulated by pH, which in turn is dependent on CO_2 concentration and activity of carbonic anhydrase (Gladwin et al. 2009). As a result of the high oxygen affinity of plant hemoglobins, a nitrite-deoxyhemoglobin interaction (Angelo et al. 2006) is unlikely in plants, but other hemeproteins (e.g. cytochromes) may participate in this reaction.

Hemeproteins of the mitochondrial electron transport chain, including those of cytochrome *c* oxidase (COX) (Castello et al. 2006, 2008), the bc_1 complex (Kozlov et al. 1999), and cytochrome *c* itself (Basu et al. 2008), can participate in NO formation from nitrite. The reaction is not limited to hemeproteins. Molybdocofactors of nitrate reductase (Dean and Harper 1988, Yamasaki and Sakihama 2000), xanthine oxidase (Li et al. 2008) and iron–sulfur clusters (Li et al. 2004, 2008) have also been shown to be capable of producing NO.

In this paper we critically analyze the sources of NO production in plants under conditions of low oxygen and discuss the importance of NO turnover in plant anoxic metabolism.

Nitrite as an alternative electron acceptor of COX

Aerobically, NO can be converted by COX to nitrite (Cooper 2002, Pearce et al. 2002). Under very low oxygen tensions, the reaction can be reversed (Walters and Taylor 1965) and nitrite may become a source of NO (Castello et al. 2006). COX contains three redox-active metal sites: copper A, heme *a*, and heme a_3/copper B ($Fe_{a3}Cu_B$) binuclear center (Cooper 2002). Copper A and heme *a* catalyze electron transfer from the substrate (cytochrome *c*) to the catalytic site, whereas the heme a_3/copper B binuclear center catalyzes the reduction of oxygen to water. In explanation of the mechanism of nitrite/NO interconversion, the $Fe_{a3}Cu_B$ binuclear center is considered to be the major factor in the reaction (Wilson and Torres 2004). The mechanism involves nitrite binding to different species of partially reduced $Fe_{a3}Cu_B$ center (Fe oxidized, Cu reduced) which exists in deoxyferryl (no oxygen bound) or oxyferryl (oxygen atom bound) forms. The latter form appears upon binding of oxygen to the fully reduced center (Fig. 1). This binding, in turn, is competitively inhibited by NO (Cooper 2003, Mason et al. 2006).

Oxygen concentrations, at which the half-maximal reduction of the heme iron is observed, vary with the energy state and the respiratory rate. There is a two-fold difference between the state 3 and state 4 K_m (O_2) of the heme iron, while the half-reduction of copper is independent of both the energy state and the respiratory rate (Hoshi et al. 1993). Depending on the physiological conditions, therefore, there are oxygen concentrations in which iron is oxidized while copper is still reduced. These conditions are favorable for nitrite reduction to NO:

$$Fe_{a3}{}^{3+}Cu_B^+ + NO_2^- + H^+ \rightarrow Fe_{a3}{}^{3+}Cu_B^{2+} + NO + OH^- \quad (3)$$

Here, in contrast to equation (1), the electron for nitrite reduction is derived from reduced copper, not iron. The low nitrite affinity initially reported for COX (Paitian et al. 1985, Walters and Taylor 1965) likely did not take into consideration that, when the binuclear center iron is oxidized and copper is reduced, nitrite affinity increases many fold (Brunori et al. 2006). Catalysis of nitrite reduction to NO by COX has been confirmed to be operative in mammalian and yeast (Castello et al. 2006), algal (Tischner et al. 2004) and higher plant (Gupta et al. 2005, Planchet et al. 2005, Stoimenova et al. 2007) mitochondria. The rate of the reductase reaction increases with decreasing pH and increasing ADP concentration (Castello et al. 2006, Sarti et al. 2000), i.e. in the conditions that exist during anoxia.

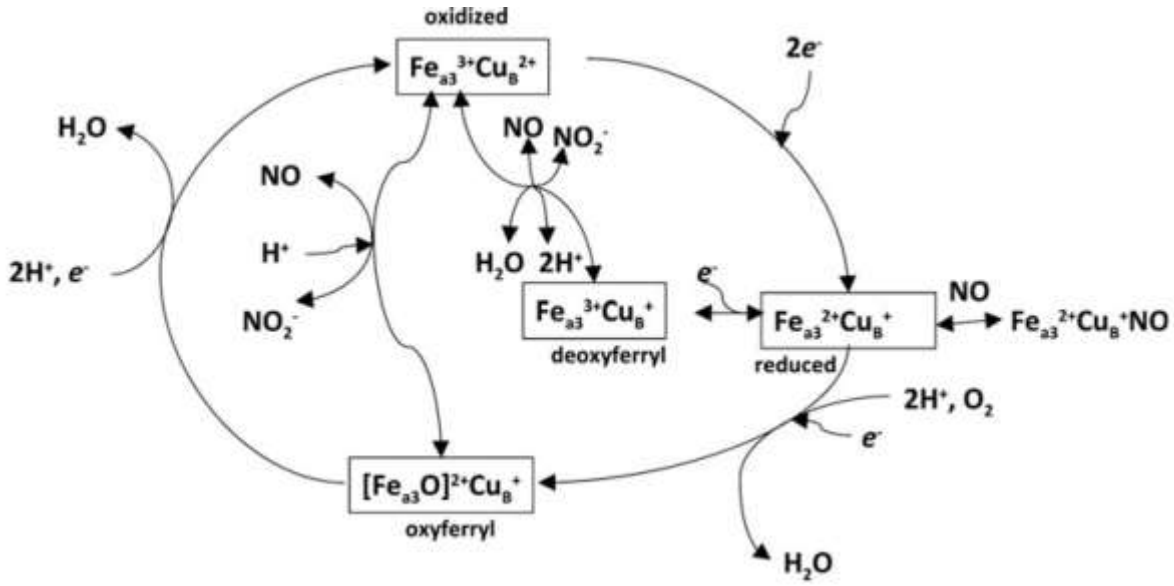

Fig. 1. Transformations of redox states of the COX center containing heme a3 and copper B ($Fe_{a3}Cu_B$) in relation to reduction of oxygen and conversions between nitrite and NO. While in the fully reduced state with Fe^{2+} and Cu^+, NO binds competitively and prevents binding and reducing oxygen, the partially reduced center (Fe-oxidized Cu-reduced) can bind nitrite and reduce it to NO. This partially reduced center can exist in deoxyferryl and oxyferryl states. Oxygen can participate in nitrite reduction when the latter binds to the oxyferryl state.

Nitrite itself also inhibits the COX reaction with NO (Wilson and Torres 2004) and thus facilitates its own reduction. Nitrite can be transported to mitochondria by a specific protein similarly to that recently identified for chloroplasts (Sugiura et al. 2007). It is likely that NO_2^- transport into the mitochondrial matrix occurs via the plant mitochondria inner membrane anion channel. The channel has been shown to be active under conditions of low ATP and membrane depolarization, i.e. under stress (Laus et al. 2008), and would be important, in particular, for NO_2^- reduction at the matrix side of Complex III.

Nitrite reduction becomes detectable in rat liver and yeast mitochondria at oxygen concentrations below 20 μ*M* and nitrite concentrations of 15–25 μ*M* (Castello et al. 2006). Castello et al. (2008) have demonstrated that in yeasts, during NO formation, the role for various COX subunits may be different. When the O_2 concentration drops below a threshold of 0.5 μ*M* O_2, *COX5a* gene is switched off and *COX5b* is switched on. It appears that the induction of *COX5b* constitutes a positive feedback mechanism in which hypoxia induces the expression of a gene whose product is incorporated into the cytochrome oxidase to make it better able to produce NO. From this point of view, it is worth examining the role of plant-specific subunits of complex IV (Millar et al. 2004) in relation to nitrite reduction.

Oxygen can be reduced by COX only when both iron and copper are reduced, with the reaction being inhibited by NO competing with oxygen at the binuclear center:

$$Fe_{a3}^{2+}Cu_B^+ + NO \leftrightarrow Fe_{a3}^{2+}Cu_B^+NO \qquad (4)$$

The affinity of NO for the oxygen-binding ferrous heme site is quite high (0.2 n*M*) (Mason et al. 2006). Only the non-competitive interaction described in equation (3) results in oxidation of NO to nitrite and behaves kinetically as if it had an apparent affinity of 28 n*M* (Mason et al. 2006).

There is evidence that nitrite reduction by COX is linked to membrane proton translocation (Jancura et al. 2006, Ruitenberg et al. 2002). Nitrite reduction can precede the proton pumping process when COX copper is reduced by cytochrome *c* but can also contribute to proton pumping when COX copper is oxidized in the course of NO formation (Wilson and Torres 2004) (Fig. 1). This may explain the observed ATP synthesis when anaerobic plant mitochondria are supplied with nitrite (Stoimenova et al. 2007).

Possible reduction of nitrite at the site of complex III

The suppression of oxygen reduction by COX in the presence of its inhibitors (including NO itself) triggers the formation of O_2^- at the level of complex III (Poderoso et al.

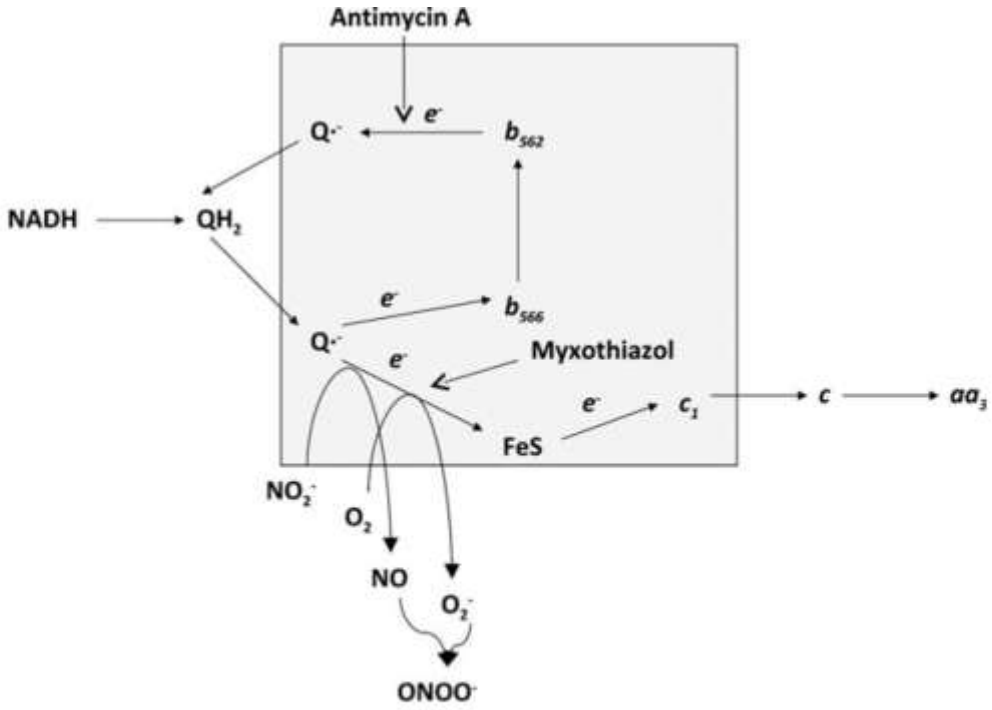

Fig. 2. Possible nitrite reduction at the site of complex III (bc_1 complex). Nitrite can be reduced at the site which transfers an electron from semiquinone to the FeS cluster. The leakage of this electron to nitrite reduces it to NO in the same manner as the leakage to oxygen forms superoxide (O_2^-). If both NO and superoxide are formed, they can bind together resulting in peroxynitrite formation ($ONOO^-$). Formation of NO (and O_2^-) is inhibited by myxothiazol and not inhibited by antimycin A.

1996). A similar electron leak at the level of complex III was postulated to participate in nitrite to NO reduction (Kozlov et al. 1999, Nohl et al. 2005). Although NO formation at Complex III is not essentially an anoxic process, under low oxygen the reduction of nitrite will become more dominant than the reduction of O_2. This reaction is inhibited by myxothiazol and insensitive to another complex III inhibitor, antimycin (Kozlov et al. 1999, Stoimenova et al. 2007), assuming a single electron leak from the respiratory chain, in which the redox cycling of semiquinones can supply single electrons that are used for nitrite or oxygen reduction (Fig. 2). This scheme is based on the inhibitory effects of antimycin A and myxothiazol (Kozlov et al. 1999, Planchet et al. 2005). It is unlikely that such a process can generate proton pumping and contribute to ATP synthesis. Simultaneous O_2^- and NO formation at the level of complex III can generate peroxynitrite (ONOO−), another reactive nitrogen species which may not be as toxic for plant cells as for animal cells (Halliwell et al. 1999) and may be scavenged by COX (Pearce et al. 2002). Incomplete inhibition of nitrite-dependent NAD(P)H oxidation, ATP synthesis and NO production in plants by myxothiazol suggests that a bypass of the myxothiazol-sensitive site is possible, so the rest of the electron flow can be used for nitrite reduction at the site of COX where it is inhibited completely by KCN (Planchet et al. 2005, Stoimenova et al. 2007, Tischner et al. 2004).

Other metal-containing proteins that can produce NO from nitrite

Other sites of nitrite: NO reduction include cytochrome *c* itself, cytochrome b_5 and P450 (Basu et al. 2008, Feelisch et al. 2008). Their contribution has to be quantified particularly in relation to hypoxic plant tissues. Proteins other than hemeproteins can be involved in the nitrite: NO reaction. The alternative oxidase (AOX) of plant mitochondria may also be involved based on the observed effect of its inhibitor, salicylhydroxamic acid, on NO evolution from mitochondria of Chlorella (Tischner et al. 2004) and higher plants (Gupta et al. 2005, Planchet et al. 2005). The AOX is a diiron carboxylate protein (Moore et al. 2008). Some proteins of a similar iron structure were found to be effective in reducing NO (Coufal et al. 1999, Kurtz 2007). If AOX is capable of nitrite: NO reduction, it may be

functional in anaerobic conditions through its contribution to this reaction. This conclusion should be taken carefully considering the inhibitor (salicylhydroxamic acid) is not specific and can affect different other iron-containing proteins including peroxidases. There is also no evidence to support an argument for involvement of AOX. The reaction of AOX with oxygen is not inhibited by NO (Millar and Day 1996) and its affinity to oxygen is in the micromolar range making it unlikely to provide sufficient oxygenic respiration under very low oxygen tensions. During hypoxia, the expression of this enzyme is usually decreased initially, followed by an increase under prolonged hypoxia (Klok et al. 2002). At high reduction levels, AOX together with non-proton pumping NAD(P)H dehydrogenases may take part in the regulation of NO levels by an indirect mechanism (Huang et al. 2002). By preventing electron leakage, these proteins can decrease NO, superoxide and subsequent $ONOO^-$ formation at the site of complex III (Wulff et al. 2009).

The involvement of xanthine oxidase in nitrite reduction to NO is a well-known phenomenon (Li et al. 2008). Xanthine oxidase is a non-heme protein having two flavin moieties, two molybdenum atoms, and eight iron atoms per enzymatic unit. The molybdenum atoms are involved as molybdopterin cofactors and are the active sites of the enzyme. The iron atoms are part of [2Fe–2S] ferredoxin iron–sulfur clusters and participate in electron transfer reactions. The formation of NO from nitrite by xanthine oxidase occurs under both anaerobic and aerobic conditions. Using nicotinamide adenine dinucleotide (NADH) as an electron donor, the aerobic rate can reach 70% of the anaerobic rate (Li et al. 2004). Aldehyde oxidase can also participate in nitrite reduction to NO (Li et al. 2008). It, too, has a molybdenum cofactor and iron–sulfur region. The hypoxic production of NO by proteins other than hemeproteins is, therefore, possible.

Initially, nitrate reductase was considered as the main enzyme producing NO from nitrite (Dean and Harper 1986, 1988, Harper 1981, Yamasaki and Sakihama 2000). Being one of many proteins possessing a molybdocofactor and heme, it can contribute to hypoxic NO formation but its relatively low abundance as compared with other NO sources may prevent it from being a dominant player in this field (Meyer et al. 2005) despite its higher expression and activation under hypoxia. NO synthase was also found to reduce nitrite to NO under anoxia (Vanin et al. 2007) but this enzyme may not exist in plants (Moreau et al. 2008).

When oxygen is available, hemeproteins become oxygenated and nitrite reduction to NO ceases. Only very low NO levels are reported for normoxic conditions in plants (Dordas et al. 2003). It is possible that NO may be produced from arginine by the oxygen-dependent NO synthase under these conditions. An Arabidopsis gene (*AtNOS1*) identified as a candidate for encoding NO synthase reveals no sequence similarity to any known animal NO synthase (Crawford 2006). Moreau et al. (2008) has demonstrated that AtNOS1 is unable to bind and oxidize arginine to NO and showed that this protein is a member of the circularly permuted GTPase family. This means that the arginine-dependent oxidative pathway of NO synthesis in plants is not unambiguously proven, and that NO signaling under normoxic conditions may also have a reductive nitrite origin (Zemojtel et al. 2006).

Scavenging mechanisms for NO

Hypoxically induced class 1 hemoglobins in plants cannot produce NO under physiological conditions. The deoxy form of class 1 hemoglobin exists at oxygen tensions several hundred times lower than necessary to saturate COX and is, therefore, not physiologically relevant to NO formation. These hemoglobins are, however, efficient NO scavengers (Hebelstrup et al. 2007, Igamberdiev et al. 2004a, 2006) and can be involved in turnover of NO formed by deoxyhemeproteins and other sources under anoxic conditions. The sequence of reactions involving hemeproteins participating in the production and scavenging of NO during anoxia, which are referred to as the hemoglobin-NO cycle have been previously described (Igamberdiev and Hill 2004, Igamberdiev et al. 2005, 2009). The operation of the cycle depends upon the differences in oxygen affinity amongst the various involved hemeproteins. It is this difference in oxygen affinity that allows for the nitrite- and NAD(P)H-dependent maintenance of redox and energy balance during plant hypoxia.

Oxygenated class 1 hemoglobins reacting with NO to produce nitrate is the main mechanism by which NO is oxidized in plants. While some hemoglobins have high dissociation constants for oxygen and can be used for oxygen transport or storage, class 1 hemoglobins induced in plants under hypoxia, bind strongly to oxygen with a K_d of approximately 2 n*M*. As a result they can effectively scavenge NO at oxygen levels far below saturation of COX. Hemoglobin ferrous ion is oxidized, forming methemoglobin, during NO scavenging. The extent of the reaction is, therefore, limited by the ability to reduce methemoglobin (Smagghe et al. 2008). Participation of cytosolic monodehydroascorbate reductase for reduction of methemoglobin in plants has been demonstrated (Igamberdiev et al. 2006) but may not be

the only reductase capable of reducing methemoglobin in plants. This mechanism described previously (Igamberdiev and Hill 2004, 2009, Igamberdiev et al. 2005) forms a non-toxic product (nitrate), is operative at extremely low oxygen concentrations and is a critical step in plant survival under hypoxia (Hunt et al. 2002). It has been claimed that class 1 hemoglobins scavenge NO through formation of nitrosohemoglobin (Perazzolli et al. 2006). Considering the high affinity for oxygen of these hemoglobins, it is difficult to envisage how nitrosohemoglobins can be formed when the hemoglobin is oxygenated. It has been well established that NO reacts rapidly with oxyhemoglobin, with the reaction proceeding via oxygenation rather than nitrosylation (Gardner et al. 2006). Clearly this issue needs revisiting using more rigorous experimental conditions.

Other mechanisms of NO scavenging include its conversion to nitrite by COX (Pearce et al. 2002) or NADP-dependent cytochrome P450 reductase (Hallstrom et al. 2004), nitrosylation of thiols and other compounds (Vanin et al. 2004), participation of lipoamide dehydrogenase (Igamberdiev et al. 2004b), and interaction with superoxide to form peroxynitrite which in turn can be reduced to nitrite by COX (Pearce et al. 2002). However, only hemoglobins scavenge NO with a high fidelity at subnanomolar concentrations and at extremely low oxygen tensions, in the case of class 1 hemoglobins (Gardner et al. 2006). For example, the Km (NO) of dihydrolipoamide dehydrogenase is approximately 0.5 μM, reflecting a relatively low NO affinity (Igamberdiev et al. 2004b) with potential for leaving toxic NO levels within the cell.

Rates of NO production/scavenging during hypoxia

The hypoxic rates of NO production remain a matter of controversy. The observed rates of nitrite-dependent NADH oxidation by anoxic mitochondria were found to be in the range of 8–12 nmol mg^{-1} min^{-1} of total mitochondrial protein for barley and 15–17 nmol mg^{-1} min^{-1} for rice (Stoimenova et al. 2007). However, the NO emission rates were much slower and corresponded to a maximum of 0.1 nmol mg^{-1} min^{-1} protein (Planchet et al. 2005, Stoimenova et al. 2007). It is possible that immediate scavenging of NO occurred as a result of the presence of sulfhydryl and thiols group compounds which are usually abundant in the medium or by superoxide which can be formed even in highly hypoxic conditions when the electron transport chain components are highly reduced (De Oliveira et al. 2008). In leaves, the immediate NO scavenging masks up to 95% of formed NO (Vanin et al. 2004). There is also the possibility of N_2O_3 and N_2O formation (see below) that may represent a substantial unidentified amount of nitrogen released during nitrite reduction (Goshima et al. 1999, Morikawa et al. 2004, Basu et al. 2007).

The rate of NO turnover in plant cells may be very high. While the interaction with thiols results in undesirable protein nitrosylation, specific NO scavenging at low oxygen levels can be provided by hexacoordinated hemoglobins (Igamberdiev and Hill 2004). Hemoglobin itself is capable of metabolizing NO at an average level of approximately 10 nmol mg^{-1} min^{-1} in the presence of NADH (Bykova et al. 2006, Igamberdiev et al. 2006). Considering that there are low concentrations of hemoglobin, even in anoxic tissues (Hebelstrup et al. 2007), these rates are insufficient to account for the rates of plant NO formation during anoxia. The rate-limiting step determining the turnover rate may be the reduction of methemoglobin to regenerate hemoglobin for continuing the cycle (Igamberdiev et al. 2006, Smagghe et al. 2008). There are several candidates for methemoglobin reductase, in animals it is cytochrome b_5 and its reductase (Hashimoto et al. 2008). In plants one confirmed candidate is the cytosolic form of monodehydroascorbate reductase (Igamberdiev et al. 2006). In the presence of the latter, the rate increases by three orders of magnitude reaching 9–10 $\mu mol\, min^{-1}$ mg^{-1} min^{-1} calculated per reductase protein. The measured NO scavenging activity in plant extracts is quite high and comparable with the rates determined with monodehydroascorbate reductase (Igamberdiev et al. 2004a, 2006).

Concentrations of NO in tissues estimated for normoxia and hypoxia can be compared with the rates of NO production and scavenging. By using spin trap techniques, Dordas et al. (2003, 2004) showed that NO accumulation in alfalfa root cultures reached 120 nmol g^{-1} of fresh weight after 24 h incubation. It was 50% lower in the hemoglobin over-expressing line and 1.5 times higher in the hemoglobin down-regulating line. In aerobic conditions, NO accumulation was at least two orders of magnitude lower. Usually the physiological NO concentrations in animal tissues are nanomolar rising to submicromolar and micromolar range under stress conditions (Pearce et al. 2002).

Fig. 3 shows participation of mitochondria in NO formation and hemoglobin in NO scavenging. As shown in the figure, NO formed in mitochondria is scavenged in the cytosol in the reaction involving hexacoordinated hemoglobin.

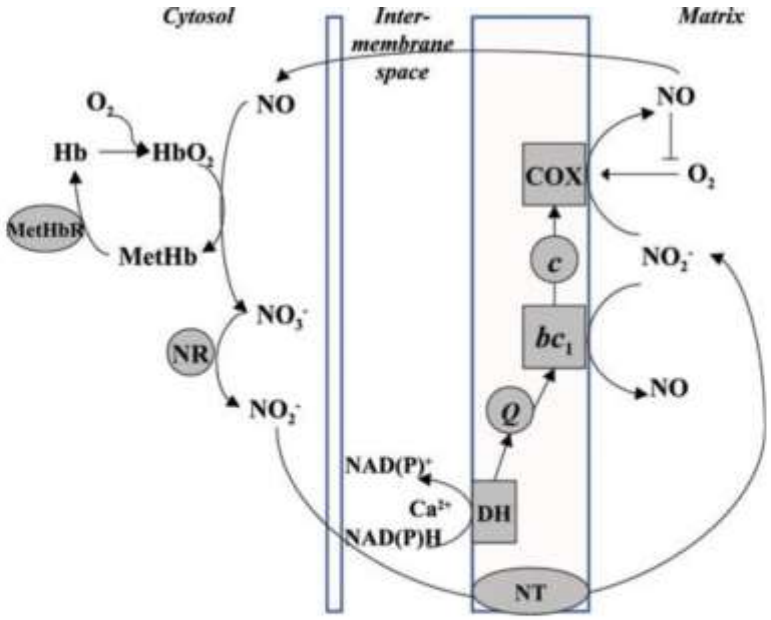

Fig. 3. Nitrogen turnover between mitochondria and cytosol. Under hypoxic conditions, COX and complex III (bc_1) can reduce nitrite (NO_2^-) to NO. The latter also competes with O_2 for binding at fully reduced $Fe_{a3}Cu_B$ center of COX increasing its $K_m(O_2)$. NO diffuses to the cytosol where it is oxygenated to nitrate (NO_3^-) by class 1 hemoglobin (Hb) in its oxygenated form (HbO_2). The latter forms methemoglobin (MetHb) in the reaction, which is then reduced by methemoglobin reductase (MetHbR). Nitrate is reduced by nitrate reductase to nitrite which is transported to mitochondria by a putative nitrite transporter similar to that found in chloroplasts. NADH formed in glycolysis and NADPH from lipid breakdown are oxidized by externally facing mitochondrial dehydrogenases. *Q*, ubiquinone; *c*, cytochrome *c*.

On the possibility of N_2O and N_2O_3 formation in plants

NO is not the only product of hypoxic nitrite reduction. Other products could be nitrous oxide (N_2O), nitrogen dioxide (NO_2), and dinitrogen trioxide (N_2O_3). The possibility of NO reduction to N_2O was shown by Zhao et al. (1995). The reaction occurs at low NO and oxygen levels and is inhibited by cyanide. In plants, N_2O emission is a well-known phenomenon but it is often attributed to the activity of denitrifying microorganisms (Pihlatie et al. 2005). In the sterile transgenic tobacco plant expressing antisense nitrite reductase mRNA, a significant emission of N_2O was detected, comprising more than 0.01% of the total nitrogen (Goshima et al. 1999). This rate may reflect a real capacity for plants under hypoxic conditions, when nitrite reduction to ammonium is limited.

The mechanism of N_2O formation can be linked to the same mechanism involved in NO formation and may be common to ferrous deoxyproteins. It has been suggested that the respiratory complexes originally evolved in an anoxygenic atmosphere when the main reaction was reduction of NO to N_2O and later adapted to the reduction of oxygen (Castresana and Sarasate 1995). This is supported by sequence similarities of COX and NO reductase (Ducluzeau et al. 2009). The eukaryotic COX catalyzes NO to N_2O reduction probably at a very slow rate (Cooper 2002), based on investigations of the bovine complex IV (Stubauer et al. 1998). An overlooked and important source for NO reduction is the reaction of ascorbate with NO in the presence of quinones (Alegria et al. 2004). The abundance of ascorbate and quinones in plant tissues and the activity of the ascorbate – glutathione cycle for recycling of formed dehydroascorbate make it plausible that a significant amount of NO formed in plants is released as N_2O. NO can also react with reduced glutathione (GSH) forming GSNO which can be further metabolized to form oxidized glutathione (GSSG) and NH_3 (Wilson et al. 2008).

N_2O_3 can be formed by hemeproteins in hypoxic conditions. Nitrite binding to methemoglobin produces an intermediate with $NO_2^{\cdot}$ radical properties. NO can then react with this intermediate at near radical – radical reaction rates that compete with NO scavenging reactions with vicinal ferrous hemes (Basu et al. 2007). Thus the production of N_2O_3 and also of small quantities of NO_2 and possibly N_2O_4 follows from the chemistry of nitrite reduction by hemeproteins. N_2O_3 is usually more stable than NO, it can easily cross lipid membranes and can break down to NO and $NO_2^{\cdot}$ in

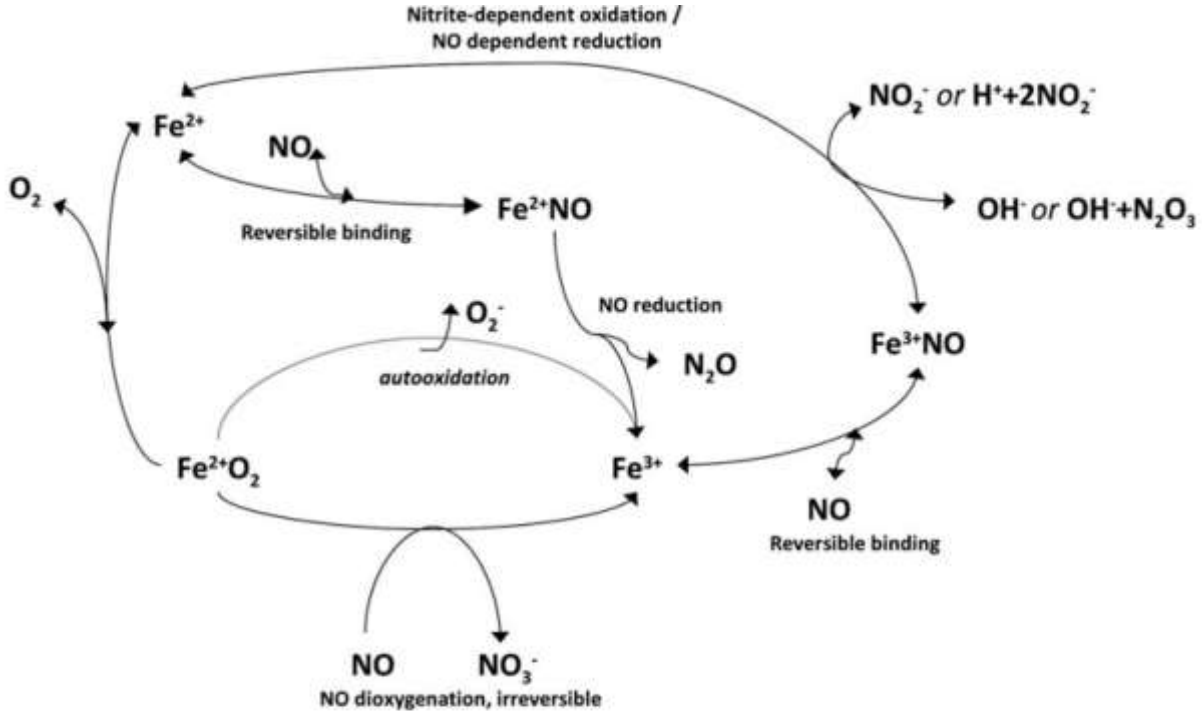

Fig. 4. Participation of hemeproteins in the turnover of nitrogen species. Ferrous (Fe^{2+}) heme can bind nitrite and convert it to NO with the possibility of also forming N_2O_3. In the reverse reaction, bound NO can reduce ferric (Fe^{3+}) heme to the ferrous state. Binding NO to ferrous heme can result in NO reduction to N_2O and conversion to ferric heme. Oxygen binding to ferrous hemoglobin can trigger irreversible conversion of NO to nitrate. Oxygenated heme slowly autooxidates to the ferric state with superoxide formation.

the cell (Gladwin et al. 2008). The importance of N_2O_3 formation accompanying NO formation has probably been overlooked and its significance may be higher than initially assumed. It is even possible that N_2O_3 constitutes a higher portion of nitrogen reduced from nitrite than NO (Gladwin et al. 2008).

Possible mechanisms of participation of hemeproteins in nitrogen turnover are presented in Fig. 4. This scheme summarizes in a simplified form the network of reactions individually described in several papers (Gladwin et al. 2009, Nagababu et al. 2003, Smagghe et al. 2009).

Conclusion

For many years, NO has been considered as a signaling molecule and even treated as a hormone, although its low stability and short-distance transport should prevent such classification. Recently, it has become more evident that NO (possibly together with other nitrous oxides such as N_2O_3 and N_2O) is generated at much higher quantities under hypoxic conditions because of nitrite reduction by deoxyhemeproteins, molybdocofactors and iron–sulfur clusters. These protein moieties, being exposed to a hypoxic medium, exhibit functions that they probably possessed in early Earth anoxygenic atmosphere where NO likely was a major oxidant (Ducluzeau et al. 2009). While in oxygenated tissues, NO is formed only in quantities sufficient for a signaling function, in hypoxic tissues it becomes a major metabolite playing a role in the bioenergetics of the hypoxic cell. Thus it is possible to conclude that, under low oxygen tensions, NO and other nitrous oxides serve as essential metabolites, the turnover of which contributes to the maintenance of redox and energy balance. The net effect is to keep NAD(P)H/$NAD(P)^+$ ratios low while maintaining energy charge and ATP/ADP ratio sufficiently high for short-term plant survival.

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