The Haemoglobin/Nitric Oxide Cycle: Involvement in Flooding Stress and Effects on Hormone Signalling

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INTRODUCTION

The accepted view of plant adaptation to low oxygen concentrations holds that below certain oxygen limits, plant cells cannot sustain respiration due to lack of an electron acceptor for the terminal oxidases (Geigenberger, 2003). They therefore require alternative pathways, such as fermentation, to maintain cell energy status. In addition to the glycolytic pathway to lactate and to ethanol, nitrate reduction could play a role in NADH turnover (Vartapetian et al., 2003). However, some authors (reviewed in Gibbs and Greenway, 2003) find it unlikely that nitrate serves as a terminal electron acceptor in hypoxia (as is the case in denitrifying bacteria) since there is no accumulation of alternative fermentation products in plants grown on nitrate. On the other hand, the abundant evidence of positive effects of supplied nitrate (Arnon, 1937; Reggiani et al., 1993a, b) and negative effects of inhibited nitrate reduction on plant flooding survival (Stoimenova et al., 2003) suggests that nitrate reduction might be involved as a part of a more general process regenerating NADH under hypoxia.

Class 1 haemoglobins (Hbs) may act in a pathway as an additional potential source for respiratory metabolism at oxygen levels below saturation of cytochrome c oxidase. Hb induction under hypoxic conditions has been studied from the point of view of its relevance to plant acclimation to low oxygen environments. Significant progress has been made towards understanding its function. One main role of Hb is to modulate the nitric oxide (NO) produced in the hypoxic stress response (Dordas et al., 2003a, b, 2004; Igamberdiev and Hill, 2004; Igamberdiev et al., 2004). NO is a molecule involved in many signal transduction pathways connected with stress responses, and modulation of NO levels can lead to significant changes in hormonal responses in plants (Wendehenne et al., 2004). There is a very efficient NAD(P)H- and Hb-dependent NO-scavenging system operative under hypoxic conditions (Igamberdiev et al., 2004).

In this hypoxia-induced respiration, nitrate is used as an intermediate electron acceptor, while oxygen remains as a terminal electron acceptor. In addition to mitochondrial respiration, Hb could act as one component of a soluble (probably cytosolic) oxygenase of NO, the operation of which contributes to the oxidation of NADH and, in an as yet to be determined way, is linked to improved cell energy status. In this review, we discuss both metabolic and regulatory (signalling) consequences of Hb expression in the framework of a possible strategy for plant adaptation to low oxygen environments.

MITOCHONDRIA UNDER HYPOXIA

Normal mitochondrial respiration functionally ceases when the available oxygen concentration declines below the half-saturation value of the terminal oxidase. Of the two mitochondrial terminal oxidases, cytochrome c oxidase and the alternative oxidase (AOX), the latter has an exceedingly low affinity for oxygen. The \( K_m \) for oxygen of the AOX is in the micromolar range (Millar et al., 1994) and according to recent data may be near 30 \( \mu \)M (Affourtit et al., 2001), eliminating it as a factor at concentrations below 10% of the oxygen in air-saturated water. This explains the fact that
the AOX is downregulated under hypoxia (Szal et al., 2003) and is important only in the post-anoxic period when reactive oxygen species (ROS) are intensively formed. Accumulation of transcripts of AOX under hypoxic conditions (Klok et al., 2002) could possibly be mediated by the elevation of NO (Huang et al., 2002). This may indicate that plants are preparing for post-anoxic injury, since the participation of AOX under high respiratory flux can prevent increased formation of ROS (Maxwell et al., 1999).

Cytochrome c oxidase has a $K_m$ value of 140 nM (Millar et al., 1994), making it effective at relatively low levels of tissue oxygen, in the absence of potential inhibitors of the oxidase. When the oxygen concentration falls below the level of saturation of cytochrome c oxidase, i.e. approx. 0.1–0.2 μM at the mitochondrial sites or higher in the case of its inhibition, conditions are considered fully anaerobic. One of the inhibitors of cytochrome c oxidase is NO. NO inhibition occurs at nanomolar concentrations and is competitive with oxygen, increasing the effective oxygen $K_m$ in vivo to 1 μM or higher (Brown, 1995). The physiological situation is more complex, however, as cytochrome c oxidase is capable of converting NO to nitrite (Pearce et al., 2002; Cooper, 2003). Cytochrome c oxidase also exhibits peroxynitrite reductase activity (Pearce et al., 2002), removing toxic peroxynitrite formed in the reaction of NO with superoxide. The evidence for cytochrome c oxidase interaction with NO is largely the result of work with animal tissues; however, the similarity of plant and animal enzymes (Siedow and Umbach, 1995) suggests that the mechanisms are likely to be the same. Oxygen concentration is a critical factor in any consideration of these reactions in the hypoxic stress response, which seriously complicates any analysis of the role of these reactions in the process.

There is abundant evidence that mitochondria may be functional under strict anoxic conditions. Exposure to anoxia results in some changes in enzyme composition in mitochondria (Couee et al., 1992), with the pattern possibly related to the transition to the partial tricarboxylic acid (TCA) cycle operating at higher reduction levels in the cell (Igamberdiev and Gardestrom, 2003). In this modification of the TCA cycle, citrate is exported from mitochondria (Couee et al., 1992), with the pattern possibly related to the transition to the partial TCA cycle. Mitochondria preserve their ultrastructure and function only when anaerobic plants are exposed to nitrate (Vartapetian et al., 2003). This was interpreted as an indication of the role of nitrate as a terminal electron acceptor under anoxia. However, it is plausible that nitrate is utilized as an intermediate electron acceptor by the mechanism discussed in this review.

**CLASS 1 HAEMOGLOBINS IN PLANTS AND HYPOXIA/FLOODING STRESS**

The properties and function of class 1 Hbs are dominated by their very strong avidity for oxygen in comparison with other Hbs. Thus, although the kinetics of oxygen association with barley (*Hordeum vulgare*) Hb are comparable with those of myoglobin and about an order of magnitude lower than those of leghaemoglobin (Duff et al., 1997), the rate of release of oxygen from barley oxyHb is extremely slow. The result is that the equilibrium dissociation constant of the complex is approx. 3 nM. This low dissociation constant, along with other characteristics, makes it unlikely that this type of Hb serves as an oxygen carrier, store or sensor (Hill, 1998).

Class 1 Hb genes are expressed in both aleurone (Taylor et al., 1994) and embryo (Guy et al., 2002) of germinating barley kernels, probably due to the hypoxic conditions existing in the grain prior to emergence of the radicle (Guy et al., 2002). In addition to hypoxia, the gene is induced by an increased sucrose supply (Hunt et al., 2001) and inhibition of mitochondrial oxidative phosphorylation (Nie and Hill, 1997).

We have proposed that this stress-induced Hb, with high affinity for oxygen, participates in a process regenerating NAD$^+$ through a reaction involving NO and HbO$_2$ (Dordas et al., 2003b). Class 1 Hbs have been shown to affect NO levels in several experimental systems (Dordas et al., 2003a, 2004; Perazzoli et al., 2004). Maize (*Zea mays*) recombinant HbO$_2$ reacted with NO to form NO$^-_2$ and methHb. Furthermore, using either NO traps or an NO electrode, it was shown that significant amounts of NO are formed in hypoxic maize cells and in alfalfa (*Medicago sativa*) root cultures during the first 24 h of hypoxic treatment (Dordas et al., 2003b, 2004; Igamberdiev et al., 2004). Transformed lines with reduced stress-induced Hb expression produced greater amounts of NO than wild-type or Hb-overexpressing lines, suggesting that Hb may be involved in turnover of the NO.

For transgenic maize cells and alfalfa root cultures, overexpressing class 1 Hb has been shown to improve plant energy status and growth during exposure to hypoxic stress (Sowa et al., 1998; Dordas et al., 2003a; Igamberdiev et al., 2004). Additionally, *Arabidopsis thaliana* plants overexpressing either *A. thaliana* (GLB1; high affinity) or *Parasponia andersonii* (GLB1S; medium affinity) class 1 Hbs have demonstrated enhanced tolerance and survival to hypoxic stress relative to non-transformed controls (Hunt et al., 2002). The gradient of hypoxic protection observed between *A. thaliana* plants expressing GLB1, GLB1S and mutated GLB1 (HE7L; low affinity) has led to the suggestion that the hypoxic protection provided by class 1 Hbs is intimately linked with their affinity for gaseous ligands such as O$_2$, NO and CO (Hunt et al., 2002). Extending the work with maize cell and alfalfa root cultures (Dordas et al., 2003a; Igamberdiev et al., 2004), regenerated transgenic alfalfa plants containing the barley Hb gene in either the sense or antisense orientation have been produced. In controlled environment studies, simulated flooding stress imposed upon such plants corroborates previous work where Hb overexpression enhances, and underexpression diminishes, the ability of these transgenic plants to tolerate flooding (hypoxic) stress relative to empty vector controls (Baron, 2005).

In microorganisms, NO is scavenged by a flavohaemoglobin possessing NAD(P)H-dependent NO-scavenging
enzymatic activity (Gardner et al., 1998, 2000). The reaction catalysed by this protein is described by the equation

\[2\text{NO} + 2\text{O}_2 + \text{NAD(P)H} \rightarrow 2\text{NO}_3^- + \text{NAD(P)}^+ + \text{H}^+\]

In animal tissues, dioxygen-dependent metabolism of NO is likely to be catalysed by both a haemoprotein and a flavoprotein (Gardner et al., 2001). The identity of the particular proteins involved, however, remains undetermined.

Plant scavenging of NO has been demonstrated in alfalfa root extracts expressing barley Hb (Igamberdiev et al., 2004). This scavenging is likely to occur in the cytoplasm as the Hb gene shows no evidence of being targeted to organelles or exported (Taylor et al., 1994). NO is actively scavenged in a reaction that is dependent on the presence of both NAD(P)H and barley Hb. Anoxic maize cells overexpressing class 1 Hb have lower alcohol dehydrogenase (ADH) activity compared with wild type and with lines downregulating Hb (Sowa et al., 1998). Higher Hb levels in the presence of nitrate would result in a greater turnover of NO and a corresponding increased NADH oxidation, replacing to some extent the requirement for ADH activity under hypoxic conditions. Lower NADH/NAD\(^+\) and NADPH/NADP\(^+\) ratios in plants overexpressing Hb are in agreement with this mechanism (Igamberdiev et al., 2004). These ratios are not affected significantly by hypoxia and suppression of the mitochondrial electron transport in Hb-overexpressing lines, whereas in Hb-downregulating lines, the ratios increase dramatically under low oxygen tension.

Strong hypoxic induction of the Hb gene, comparable with the induction of ADH, occurs in A. thaliana root cultures in concert with induction of enzymes of nitrogen metabolism, including two nitrate reductases and other nitrogen-related enzymes (Klok et al., 2002). Hb induction is observed in response to nitrate (Nie and Hill, 1997), nitrite and NO treatment (Ohwaki et al., 2005). It is known that in hypoxic conditions, nitrate reductase is usually induced and nitrite is accumulated (Morard et al., 2004). Nitrite may serve as an alternative substrate for nitrate reductase (Rockel et al., 2002) forming NO, or it can be a substrate for nitrite: NO reductase associated either with plasma membrane (Stöhr and Mäck, 2001) or with the mitochondrial electron transport chain (Kozlov et al., 1999). It was shown for mammalian mitochondria that nitrite can accept electrons from ubiquinone (Kozlov et al., 1999), forming NO. Similar activity, in which the mitochondrial electron transport is involved in NO production, has been reported for algae (Tischner et al., 2004) and plants (Planchet et al., 2005), indicating that a nitrite: NO reductase function may be associated with mitochondria.

Our own studies (A. U. Igamberdiev et al., submitted) with a mutant barley Hb, in which the only cysteine in the monomer has been modified to a serine (Cys\(^79\) to Ser), have demonstrated that the NO dioxygenase activity associated with the mutant Hb involves the participation of a component that is sensitive to sulphydryl reagents, indicating that Hb alone is incapable of sustaining the NO-degrading activity. Furthermore, we find that the sulphydryl group of the barley Hb monomer is involved in disulphide bond formation to form Hb dimers. Our results are in contrast to those of Perazzolli et al. (2004), who suggest that Arabidopsis class \(l\) Hb scavenges NO via S-nitrosylation and that the reaction is catalysed by Hb and NADPH, without the participation of another protein. We favour a mechanism in which synthesis and degradation of NO are the result of the joint operation of an NO-producing system (e.g. nitrate reductase and nitrite: NO reductase), Hb and an enzyme reducing ferric Hb to its ferrous form (metHb reductase). These components constitute the Hb/NO cycle (Fig. 1). In this cycle, metHb produced in the reaction of oxyHb with NO is reduced by a separate metHb reductase (Igamberdiev and Hill, 2004).

Hb gene induction is more directly related to cell ATP status than to oxygen levels (Nie and Hill, 1997). Expression of the gene during hypoxia probably affects cell survival as overexpression of Hb in hypoxic maize cell cultures resulted in the maintenance of cell energy status (Sowa et al., 1998). Several protein kinases have a similar profile of induction (Klok et al., 2002), indicating a possible link between decreasing ATP levels and Hb synthesis (Nie and Hill, 1997). A major question is how Hb induction during hypoxia is linked to ATP synthesis. It is unlikely that substrate level phosphorylation will contribute to the maintenance of ATP levels in Hb-overexpressing plants. maize cells overexpressing barley Hb demonstrate a decrease of glycolytic decarboxylation compared with control or Hb antisense lines (Sowa et al., 1998). In their study of the products of anaerobic nitrate and ammonium ion metabolism in rice coleoptiles, Fan et al. (1997) found an approx. 55 % higher production of glycolytic products in the presence of ammonium ion in comparison with nitrate under anaerobic conditions. This would favour an argument that
the observed effects on NO, ATP and NADH levels during hypoxia in plants overexpressing Hb are not the result of increases in glycolytic flux.

Evidence has been accumulating of a putative nitrate respiratory pathway in bacteria, capable of generating a proton motive force (Jormakka et al., 2003). An equivalent pathway in plants is complicated by the fact that the known ATPases present in plasma membrane fractions are not of a type that are capable of utilizing chemiosmotic gradients. Alternatively, the production of NO by mitochondria from nitrite (Kozlov et al., 1999; Tischner et al., 2004; Gupta et al., 2005) may be linked to the generation of a membrane potential at the site of complex I and, hence, to ATP synthesis. The presence of such a pathway in plants, potentially generating ATP more efficiently than glycolysis, might explain why Fan et al. (1997) observe less carbon flow with nitrate as a nitrogen source under anoxia as opposed to ammonium ion. There is clearly a need for further study to unravel the mechanism by which Hb overexpression results in enhanced energy status in anoxic plants.

HAEMOGLOBIN EXPRESSION AND HORMONE RELATIONSHIPS

From the mounting data of involvement of NO in various environmental and hormonal stimuli (Neill et al., 2002, 2003; Lamattina et al., 2003; Wendehenne et al., 2004) (Fig. 2), it is worthwhile to speculate that given the expression, localization and NO-related functions of class 1 plant Hbs, the presence of this molecule is likely to interfere with signal transduction pathways employing NO as a signal element. This is supported by the capacity for class 1 plant Hbs to modulate plant NO levels and bioactivity (Dordas et al., 2004; Perazzolli et al., 2004). Interference with environmental or hormonal signal transduction may be one possible reason why Hb gene expression is relatively limited until cells are faced with certain environmental/hormonal stimuli, most notably depressed cell energy status.

NO, by its activation of guanylate cyclase, has pronounced effects on cGMP-dependent signal transduction pathways (Wendehenne et al., 2004). NO binds to soluble guanylate cyclase haem, activating the enzyme and increasing the level of cGMP. In addition to cGMP, NO acts through cyclic ADP-ribose and Ca\textsuperscript{2+} mobilization (Wendehenne et al., 2004). Inhibitors of signal transduction pathways that have been linked to cGMP in mammalian cells also affect aerenchyma formation in maize roots. A relationship between NO and cGMP signalling pathway has been demonstrated in mosaic virus-infected tobacco (Nicotiana tabacum) (Durner et al., 1998). An involvement of another NO-dependent signalling molecule, cADP-ribose, was also detected (Durner et al., 1998). An NO effect on the functioning of plant peroxidases participating in cell wall lignification has also been shown (Ferrer and Ros-Barcelo, 1999).

NO signalling may explain the observation that plants overexpressing and downregulating Hb differ in morphology as evidenced by morphological alterations in the roots of GLB1-overexpressing A. thaliana plants (Hunt et al., 2002) and the divergent root and shoot phenotypes of alfalfa plants containing sense or antisense barley Hb transcripts (Baron, 2005). A number of these phenotypic characteristics show striking similarity to documented responses of alfalfa to exogenous applications of plant growth regulators and/or that of plants following treatment with NO donors and scavengers.

In plants, NO production occurs in response to hypoxic stress (Dordas et al., 2003a, 2004) in addition to treatment with the phytohormones auxin (IAA) (Pagnussat et al., 2003), cytokinin (Tun et al., 2001) and abscisic acid (ABA) (Neill et al., 2002; Guo et al., 2003). It has even been suggested that NO itself can be considered a hormone-like molecule (Wendehenne et al., 2004).

Considering the NO-related functions recently proposed for class 1 Hbs (Dordas et al., 2004), in addition to the diverse and versatile nature of NO as a signal molecule in plants (Wendehenne et al., 2004), it is possible that under specific conditions, and in specific cell types, NO-metabolizing class 1 Hbs might serve as negative regulators of NO levels or bioactivity. They might also serve to interfere with any one of a multitude of signal transduction pathways for which NO is known or proposed to function as a signal element. Furthermore, although class 2 and class 3 (truncated) Hbs demonstrate significantly different ligand-binding characteristics relative to class 1 Hbs, their localization and expression patterns (Hunt et al., 2001; Watts et al., 2001; Lee et al., 2004) invite speculation as to possible NO-related functions in plants in addition to those associated with oxygen.

The distinct root and shoot phenotype(s) of alfalfa plants containing sense and antisense transcripts of barley Hb strongly suggest class 1 Hbs to possess housekeeping functions associated with normoxic plant growth, fertility and

![Plant hormones and plausible ways of NO involvement in the signal transduction cascade.](image-url)
hormonal signalling. More recently, Dat et al. (2004) have entertained the involvement of NO and Hb in sensing and signalling during plant flooding. We will discuss below possible links between Hb expression and the function of particular hormones.

Cytokinins

Recent findings suggest that expression of class 1, class 2 and class 3 (truncated) plant Hbs may be positively regulated following cytokinin treatment (Hunt et al., 2001; Lee et al., 2004; Ross et al., 2004). Interestingly, NO generated via NO synthase, but not nitrate reductase, has been implicated alongside the cytokinin regulatory response protein, ARR5, as a secondary messenger in cytokinin signal transduction (Tun et al., 2001; Scherer, 2003). This raises the possibility that the cytokinin- or ARR1-mediated induction of NO-metabolizing class 1 Hbs (Ross et al., 2004) might serve as a negative regulator of cytokinin action, through metabolism of NO, in specific cell types.

Auxins

NO has been shown to participate in various auxin-mediated rooting processes, including adventitious rooting (Pagnussat et al., 2003), initiation of lateral roots and elongation of primary roots (Correa-Aragunde et al., 2004). Furthermore, in Arabidopsis (Hunt et al., 2002) and alfalfa plants (Baron, 2005), expressing varying levels of class 1 Hb results in dramatic alterations in root morphology. These observations have been interpreted as Hbs modulating endogenous plant NO levels and/or bioactivity, with the aforementioned NO-related rooting processes being positively or negatively influenced depending upon enhanced or suppressed Hb expression.

Furthermore, an obvious spatial relationship exists between NO synthase activity (Ribeiro et al., 1999; Guo et al., 2003) and class 1 Hb expression (Andersson et al., 1997; Hunt et al., 2001; Ross et al., 2004) in the root tips of both native and transgenic plants. Such observations support the potential for NO and Hb to interact with one another in both normoxic and hypoxic root environments.

Mitogen-activated protein kinases, abscisic acid and nitric oxide

The NO-mediated activation of a mitogen-activated protein kinase (MAP kinase) during adventitious root development (Pagnussat et al., 2004) begs the question as to how copious amounts of NO evolved from hypoxically stressed plant tissues participate in flood-induced adventitious rooting (Visser et al., 1996). Interestingly, both NO and H2O2 have recently been found to function as localized and long-range root-derived signals capable of rapidly communicating the redox status and indirectly activating MAP kinase-like activity in the shoots of A. thaliana (Capone et al., 2004).

For flooding research, it will be interesting to determine whether observed increases in NO evolution from flooded roots or soils can contribute as a positive message in root-to-shoot communication (reviewed in Jackson, 2002).

The distinct phenotype(s) of transgenic plants expressing sense and antisense barley transcripts strongly suggest that hormonal pathways are modified under both non-stressed and flooded conditions.

Nitrate reductase and NO synthase-mediated NO synthesis has implications for ABA signalling (Desikan et al., 2002; Guo et al., 2003). Also, a non-enzymatic reduction of nitrite to NO occurring in the apoplastic space of barley aleurone layers is observed in response to ABA and gibberellin (Bethke et al., 2004). It is important to examine how NO-related processes including MAP kinase activity (Capone et al., 2004) and ABA-induced stomatal closure (Guo et al., 2003; Neill et al., 2003) are modified as a consequence of Hb expression.

Ethylene and aerenchyma formation

Ethylene release is one of the most widespread responses to biotic or abiotic stresses in plants (Morgan and Drew, 1997), including the response to low oxygen (Jackson, 1985; Drew, 1997; Grichko and Glick, 2001). Ethylene biosynthesis is mediated by two key enzymes in higher plants. ACC synthase catalyses the formation of 1-aminocyclopentane-1-carboxylic acid (ACC) from S-adenosylmethionine. The second step in the hormone biosynthesis is the oxidation of ACC to ethylene by ACC oxidase. Both steps are considered rate limiting for ethylene biosynthesis. ACC synthase and ACC oxidase are both encoded by a multigene family which can be induced under various conditions. Stimulations of ACC synthase and ACC oxidase enzyme activities have been reported with repercussions on ethylene evolution in plant tissues (Grichko and Glick, 2001).

A few hours after flooding, the decrease in O2 concentration results in an increase in ACC in the roots where it accumulates. Limited O2 availability will prevent ACC oxidation by ACC oxidase, and ethylene will form only if ACC can diffuse towards aerial parts of the plant. ACC can travel to the shoot through the xylem and induce leaf epinasty, hypertextrophy of the stem or adventitious rooting (Morgan and Drew, 1997). In the case of submerged shoots such as in rice, ethylene, in concert with elevated CO2 concentrations and low O2 levels, can promote fast coleoptile elongation (Jackson, 1985; Almeida et al., 2003). In wetland species, ethylene-induced formation of aerenchyma in stems and roots is often constitutive, but it can also be induced by hypoxia. Aerenchyma is formed by cell separation or selective cell death and disintegration. The resulting empty spaces in the stem and roots facilitate gas diffusion and transfer throughout the plant (Jackson, 1985; Drew, 1997). Ethylene has been shown to be associated with aerenchyma formation in hypoxic maize roots (Drew et al., 2000). NO is an attractive candidate for involvement in aerenchyma formation. Aerenchyma cannot develop under complete anoxia. Low concentrations of ethylene (0.1–1 mL L−1 air) can trigger cell death in the cortex of maize roots under normoxic conditions (Drew, 1997). The diffusion of gas in water is 10 000 times slower than in air; therefore, the ethylene formed shortly after the onset of hypoxia is trapped and accumulates, reaching levels
that are sufficient to promote apoptotic aerenchyma formation (Evans, 2003). Even though the positive effect of hypoxia on the activation of ACC synthase and ACC oxidase enzymes has been well documented (English et al., 1995; Banga et al., 1996), it is still not known how low oxygen stress is sensed and leads to activation of ethylene biosynthesis (Drew, 1997; Drew et al., 2000; Evans, 2003). The action of ethylene is mediated by a signalling pathway involving a unique MAP kinase cascade (Guo and Ecker, 2004). MAP kinases have been implicated in NO-mediated auxin signalling (Pagnussat et al., 2004), which raises the possibility of similar NO-mediated events in the ethylene signal transduction pathway. Both promotive and inhibitory effects of NO upon ethylene generation in plants have been demonstrated. The majority of this work, however, has been conducted on climacteric species (Leshem et al., 1998; Leshem and Pinchasov, 2000; Sozzi et al., 2003).

Alfalfa roots overexpressing class 1 barley Hb exposed to hypoxic conditions for several hours show no sign of aerenchyma development, whereas control roots and roots underexpressing Hb showed evidence of cell disruption (Dordas et al., 2003). Suppressing Hb expression in maize cell suspensions resulted in elevated ethylene formation (Manac’h-Little et al., 2005). This indicates a potential role for Hb in regulating ethylene levels in the cell. In maize cell suspensions, both ethylene and NO levels increase when Hb expression is impaired. In this case, NO appears to have a promoting effect on ethylene biosynthesis. Decreasing the levels of NO in the cell by overexpressing Hb (Dordas et al., 2003a, 2004; Igamberdiev et al., 2004) may directly or indirectly turn off the signal triggering the activation of ethylene biosynthesis. This might indicate a participation of Hb in an acclimation response to low oxygen stress, regulating the levels of NO and ethylene, delaying apoptosis and aerenchyma formation. There is also the possibility that a selective expression of Hb in root cells may regulate the process of aerenchyma formation via direct effects of NO on programmed cell death.

**SUMMARY**

Plant tissues that express sufficient Hb soon after exposure to hypoxic stress may modulate levels of NO, produced as a result of the stress, through reaction of the NO with oxyHb. We hypothesize that this modulation would lead to maintenance of the energy and redox status of the cell and the prevention of cell death. In primary roots, this may provide sufficient time for the plant to develop adventitious roots, needed for prolonged survival under hypoxia. In roots developing aerenchyma, Hb expression or action may be cell selective, resulting in (programmed) death of some cells that underexpress the gene.

Plant adaptation to hypoxia, besides the well-known glycolytic fermentation pathway, may involve an alternative respiratory pathway in which nitrate reduction serves as an intermediate step, providing NO. NO is oxidized back to nitrate in a reaction involving non-symbiotic Hb induced under hypoxia. This sequence of reactions constitutes the Hb/NO cycle in which NADH accumulating under hypoxia due to the lack of electron acceptors is oxidized. The stoichiometry of the cycle is 2:5 NADH mol⁻¹ of nitrate (or NO). Indirect data show that the cycle is linked to ATP synthesis.

A generalized scheme of potential events associated with Hb and NO is presented in Fig. 3. The onset of hypoxia leads to a decline in mitochondrial respiration, triggering an increase in NADH and a drop in ATP levels. Hypoxia induces Hb gene expression and accumulation of nitrite, the latter being converted to NO. A reaction between the NO and the oxygenated form of newly synthesized class 1 Hb leads to the oxidation and oxygenation of NO, resulting in restoration of the cell redox and energy status.

![Diagram of the Haemoglobin/Nitric Oxide Cycle](https://academic.oup.com/aob/article-abstract/96/4/557/332557/fig3)

**Fig. 3.** Generalized scheme of potential acclimation events associated with Hb and NO in a low oxygen environment. The onset of hypoxia leads to a decline in mitochondrial respiration, triggering an increase in NADH and a drop in ATP levels. Hypoxia induces Hb gene expression and accumulation of nitrite, the latter being converted to NO. A reaction between the NO and the oxygenated form of newly synthesized class 1 Hb leads to the oxidation and oxygenation of NO, resulting in restoration of the cell redox and energy status.
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