

REVIEW

Plant Haemoglobins, Nitric Oxide and Hypoxic Stress

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It is now known that there are several classes of haemoglobins in plants. A specialized class of haemoglobins, symbiotic haemoglobins, were discovered 62 years ago and are found only in nodules of plants capable of symbiotic nitrogen fixation. Plant haemoglobins, with properties distinct from symbiotic haemoglobins were discovered 18 years ago and are now believed to exist throughout the plant kingdom. They are expressed in different organs and tissues of both dicot and monocot plants. They are induced by hypoxic stress and by oversupply of certain nutrients. Most recently, truncated haemoglobins have been shown to also exist in plants. While hypoxic stress-induced haemoglobins are widespread in the plant kingdom, their function has not been elucidated. This review discusses the recent findings regarding the function of these haemoglobins in relation to adaptation to hypoxia in plants. We propose that nitric oxide is an important metabolite in hypoxic plant cells and that at least one of the functions of hypoxic stress-induced haemoglobins is to modulate nitric oxide levels in the cell.

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INTRODUCTION

Haemoglobins are found ubiquitously in eukaryotes and are also present in many bacteria (Wittenberg and Wittenberg, 1990). In plants there are at least three distinct types of haemoglobins that have been categorized as symbiotic, nonsymbiotic and truncated haemoglobins. Symbiotic haemoglobins, the most well known of the plant haemoglobins, are found mainly in nodules of plants capable of symbiotic nitrogen fixation and their function is to regulate oxygen supply to nitrogen-fixing bacteria (Appleby, 1992). Nonsymbiotic haemoglobins, as the name implies, are not involved in symbiotic nitrogen fixation. They are believed to exist throughout the plant kingdom, including plants capable of symbiosis, and are expressed in seed, root and stem tissue of both dicot and monocot plants (Hill, 1998). The most recently discovered of the plant haemoglobins, truncated haemoglobins, also appear to be ubiquitous and share some characteristics with nonsymbiotic haemoglobins (Watts *et al.*, 2001). The different origins, biochemistry and likely tertiary structure of the truncated plant haemoglobins suggest that these proteins may have separate cellular roles.

There are two classes of nonsymbiotic haemoglobins; one has similar oxygen-binding properties to symbiotic haemoglobins (class 2), the second with dramatically different oxygen-binding properties (class 1). Classic 1 haemoglobins are induced by hypoxic stress and oversupply of some nutrients. We shall refer to class 1 haemoglobin as stress-induced haemoglobin.

Their presence across the plant kingdom, together with the fact that they are likely evolutionary predecessors of symbiotic haemoglobins, suggests that stress-induced haemoglobins are likely to have an important role in the metabolism of plants. While the presence of stress-induced

haemoglobins is widespread in the plant kingdom, their function has not been elucidated. This review summarizes what is presently known of stress-induced haemoglobins in higher plants and their possible function in plant metabolism.

HISTORICAL OVERVIEW OF PLANT HAEMOGLOBINS

Kubo (1939) was the first to describe a plant haemoglobin in soybean root nodules. The isolation of haemoglobin from *Parasponia andersonii* (Appleby *et al.*, 1983), a non-leguminous plant, led to the hypothesis that the presence of haemoglobin may extend beyond legumes. This finding attracted great attention among researchers, not only because it was the first haemoglobin in the plant kingdom to be found in a non-leguminous plant, but also because it was expressed in both nodules and nonsymbiotic parts of the plant. The same group demonstrated haemoglobin expression in *Trema*, showing for the first time the expression of haemoglobin in a non-nodulating plant (Bogusz *et al.*, 1988). Attempts to find similar haemoglobin genes in other species were met with little success, probably because of poor cross-hybridization between the cDNA probes and nucleic acids isolated from nonsymbiotic plants. The amino acid sequence of stress-induced haemoglobins is sufficiently distinct from the symbiotic haemoglobins to account for the problems. The discovery of a barley haemoglobin and the subsequent demonstration that haemoglobin was present in a number of other cereals, such as maize, wheat, rye and triticale (Taylor *et al.*, 1994) was the first of a number of papers on stress-induced haemoglobins in other species. Two haemoglobins have been found in rice (Sasaki *et al.*, 1994) and a chloroplast haemoglobin in *Chlamydomonas eugametos* (Couture *et al.*, 1994). Recently, another plant

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TABLE 1. Species known to express haemoglobins

Species	Reference
<i>Parasponia</i>	Landsmann <i>et al.</i> (1986)
<i>Trema</i>	Bogusz <i>et al.</i> (1990)
<i>Casuarina</i>	Christensen <i>et al.</i> (1991)
<i>Hordeum, Triticum, Secale</i>	Taylor <i>et al.</i> (1994)
<i>Oryza</i>	Sasaki <i>et al.</i> (1994)
<i>Chlamydomonas</i> (chloroplast)	Couture <i>et al.</i> (1994)
<i>Glycine, Pisum, Medicago, Trifolium</i>	Andersson <i>et al.</i> (1996)
<i>Chichorium</i>	Hendriks <i>et al.</i> (1998)
<i>Medicago</i>	Seregelyes <i>et al.</i> (2000)
<i>Arabidopsis</i>	Trevaskis <i>et al.</i> (1997), Watts <i>et al.</i> (2001)
<i>Zea</i>	Guy and Hill (2000), Arechaga-Ocampo <i>et al.</i> (2001)
<i>Echinochloa, Avena</i>	Hill RD <i>et al.</i> (unpublished data)
<i>Beta, Brassica, Citrus, Gossypium, Solanum</i>	Hunt PW <i>et al.</i> (2001)

haemoglobin was found in *Arabidopsis* which is similar to the truncated haemoglobins found in bacteria, protozoa and algae (Watts *et al.*, 2001). More than 33 plant haemoglobins have been found in more than 20 species (Table 1).

EVOLUTION

It is believed that plant and animal haemoglobins originated from the same ancestral globin gene about 1500 million years ago and have been shaped by vertical evolution (Zhu and Riggs, 1992). The position and number of introns in the haemoglobin genes vary considerably amongst the species. Symbiotic haemoglobins and stress-induced haemoglobins have three introns and four exons (Arredondo-Peter *et al.*, 1998). The introns in stress-induced haemoglobins are in exactly the same position as those found in the symbiotic haemoglobins of soybean and *Parasponia*.

Dicot species differentially express at least three genes that code for haemoglobins with potentially different biochemical properties. In *Arabidopsis*, for example, AHb1 has sequence and oxygen-binding characteristics typical of stress-induced haemoglobins, whereas AHb2 has greater similarity to symbiotic haemoglobins in sequence and O₂ binding characteristics (Arredondo-Peter *et al.*, 1998). Furthermore, the pattern of expression of the three genes is different. AHb1 is induced by hypoxia and nitrate, AHb2 is induced by cold stress, while the AHb3 is expressed in both roots and shoots and is down regulated by hypoxia (Trevaskis *et al.*, 1997; Wang *et al.*, 2000; Watts *et al.*, 2001). It has been suggested (Trevaskis *et al.*, 1997) that there may be two classes of nonsymbiotic haemoglobins in plants and that it is likely that all dicots have both classes of haemoglobins, whereas in monocots only genes coding for stress-induced haemoglobins have been detected (Hunt *et al.*, 2001).

CHEMISTRY

Haemoglobins reversibly bind oxygen. The rate at which they bind and release oxygen varies, depending on the type of haemoglobin and is a defining characteristic in their

cellular function. The kinetics of oxygen association with barley haemoglobin is comparable with myoglobin and about an order of magnitude lower than that of leghaemoglobin (Duff *et al.*, 1997) (Table 2). The rate of release of oxygen from barley haemoglobin is extremely slow, however, with the result that the equilibrium dissociation constant of the complex is about 3 nM. This low dissociation constant, along with other characteristics, make it unlikely that this type of haemoglobin serves as an oxygen carrier, store or sensor (Hill, 1998).

Spectra of barley haemoglobin have a typical Soret band in the 400 nm region and two additional bands (α and β bands) in the 500–600 nm region (Duff *et al.*, 1997). The characteristics of the spectrum in the α and β region suggest that the preferred orientation of the ferrous and ferric forms is low spin 6-coordinate. An attacking ligand, such as oxygen or nitric oxide (NO), would be expected to react with the 5-coordinate species, suggesting that the rate of formation of the 5-coordinate form may be rate-limiting. The kinetics of the reaction with oxygen bear out this hypothesis (Duff *et al.*, 1997).

FACTORS AFFECTING STRESS-INDUCED HAEMOGLOBIN EXPRESSION IN PLANTS

Stress-induced haemoglobins are expressed in plant tissue in response to specific metabolic stresses. Thus, haemoglobin is induced by hypoxia in barley aleurone layers, maize roots and embryos (Taylor *et al.*, 1994). Respiratory chain inhibitors (e.g. cyanide, dinitrophenol and oligomycin) that inhibit ATP production also induce haemoglobin expression, suggesting that expression is not directly influenced by the levels of O₂, but is influenced by the levels of ATP or some consequence of ATP action (Nie and Hill, 1997). Stress-induced haemoglobin is also induced by nitrate ions in aleurone layers and in *Arabidopsis* roots (Nie and Hill, 1997; Wang *et al.*, 2000).

Stress-induced haemoglobins are expressed in callus, cell suspension, seed, root and stem tissue of both dicot and monocot plants (Hill, 1998; Dordas *et al.*, 2002). They are generally found at low concentrations (1–20 μ mol per kg

TABLE 2. Oxygen binding kinetics of various haemoglobins

Protein	k_{on} ($\times 10^{-6}$ mol l ⁻¹ s ⁻¹)	k_{off} (s ⁻¹)	K_d ($k : k'$) (nmol l ⁻¹)
Barley ^a	9.5	0.0272	2.86
Rice ^b	68	0.038	0.5
Arabidopsis Hb1 ^c	75	0.12	1.6
Arabidopsis Hb2 ^c	86	0.14	1.6
Arabidopsis Hb3 ^c	0.2	0.3	1500
Soybean leghaemoglobin ^d	120	5.6	48
Parasponia haemoglobin ^d	165	15	89
Sperm whale myoglobin ^e	14	12	857
Ascaris haemoglobin ^f	1.5	0.0041	2.7
Trematode haemoglobin (Gastrothylax crum.) ^g	205	0.4	1.95

References: ^aDuff *et al.* (1997); ^bArredondo-Peter *et al.* (1997), Trevaskis *et al.* (1997); ^cTrevaskis *et al.* (1997), Watts *et al.* (2001); ^dGibson *et al.* (1989); ^eSpringer *et al.* (1989); ^fGibson and Smith (1965); ^gKiger *et al.* (1998).

fresh weight) in plant organs. In addition to their induction by hypoxic stress (Taylor *et al.*, 1994), they are also found in rapidly growing tissues such as root tips of germinating seeds (Hill, 1998). There is reason to believe that their appearance in rapidly growing tissues may be due to hypoxic stress as well (Guy *et al.*, 2002). Elevated sucrose levels have also been shown to increase stress-induced haemoglobin gene expression in *Arabidopsis* (Trevaskis *et al.*, 1997). The class 2 haemoglobin gene is expressed in roots, leaves and inflorescence and can be induced in young plants by cytokinin treatment (Hunt *et al.*, 2001). Bogusz *et al.* (1990), using a promoter of *Parasponia* and *Trema* with a GUS reporter gene, showed that the promoter regions of nonsymbiotic haemoglobin genes directed expression mainly to the root tip and the vascular cylinder of transformed tobacco and lotus roots. In alfalfa cell suspensions, a nonsymbiotic haemoglobin was concentrated in the nucleus, as well as the cytoplasm of the cells and was induced during the G2/M transition of the cell cycle (Serégelyes *et al.*, 2000).

HAEMOGLOBINS, NITRIC OXIDE AND HYPOXIC STRESS

Stress-induced haemoglobins have been shown to affect plant metabolism and growth under low oxygen tensions. Constitutive expression of barley haemoglobin in wild-type and transformed maize cells lines maintained cell adenine nucleotide levels and energy charge under hypoxic conditions, whereas wild-type cells and cells in which haemoglobin expression is suppressed had lowered adenine nucleotide levels and energy charge (Sowa *et al.*, 1998). In similarly transformed alfalfa root cultures, lines constitutively expressing barley haemoglobin maintained root growth during hypoxic treatment, whereas wild-type and lines with suppressed stress-induced haemoglobin expression had slower root growth (Dordas *et al.*, 2002). It is unlikely that the observed effects were due to haemoglobin behaving as a signal molecule to trigger the activation of other genes because of the low dissociation constant of the oxyhaemoglobin complex (Duff *et al.*, 1997) and the observation that haemoglobin induction is regulated, not by oxygen but by ATP or some consequence of ATP action

(Nie and Hill, 1997). While the low oxygen dissociation constant may preclude functions for stress-induced haemoglobins as oxygen stores, carriers or signal molecules, the molecule is a highly efficient scavenger of oxygen at low oxygen tensions. There is, thus, the possibility that it may act in a metabolic reaction involving oxygen, where it could potentially interact with other enzyme proteins or molecules in an oxygen-consuming reaction in low oxygen environments.

Most haemproteins ligate NO as well as oxygen. Many haemoglobins are known to have a function involving NO. Red blood cell haemoglobin forms NO complexes in the lung, which is transported to distant capillaries where, through activation of guanylate cyclase, the NO causes expansion of the capillaries, facilitating oxygen transfer (Furchgott, 1990; Ignarro, 1992; McDonald and Murad, 1996). It has been proposed that in the parasitic intestinal nematode, *Ascaris*, haemoglobin functions as a 'deoxygenase', using NO to detoxify oxygen in the perienteric fluid of the anaerobically metabolizing worm (Minning *et al.*, 1999). In bacteria and yeast, the flavohaemoglobins are believed to act as dioxygenases, breaking down toxic NO to protect the organism (Gardner *et al.*, 1998). These observations led us to examine the possibility of the involvement of NO and haemoglobin in hypoxic stress adaptation.

We have detected the presence of NO/haem complexes in both hypoxic maize cell cultures and alfalfa root cultures using electron paramagnetic resonance (EPR) spectroscopy (unpublished data). The characteristic signal for the complex is not evident in aerobic systems, even though haemoglobin is present. Furthermore, using NO traps, we have shown that significant amounts of NO are formed in hypoxic maize cells during the first 24 h of hypoxic treatment. Transformed lines with reduced stress-induced haemoglobin expression produced greater amounts of NO than wild-type or overexpressing-haemoglobin lines, suggesting that haemoglobin may be involved in turnover of the NO.

The results suggest that stress-induced haemoglobins may function as dioxygenases, detoxifying NO produced during hypoxia. This raises the question as to why NO is produced during plant hypoxia. There is the possibility that NO may be activating guanylate cyclase as it is reputed to

do in defence gene induction (Durner *et al.*, 1998). It is also possible that it might be involved as a product in a series of reactions to assist in the regeneration of NAD^+ to maintain glycolysis under hypoxic conditions, as an alternative to the use of alcohol dehydrogenase. Nitrate ion has been shown to act as a terminal electron acceptor in the denitrification process in flooded soils and has been suggested to play a similar role in plants (Crawford, 1978). Stress-induced haemoglobins have also been implicated in regeneration of NAD^+ during hypoxia (Hill, 1998) based on the observations that alcohol dehydrogenase activity and CO_2 production is reduced under hypoxia in maize cells constitutively expressing barley haemoglobin (Sowa *et al.*, 1998). The induction of stress-induced haemoglobin in *Arabidopsis* in the presence of elevated nitrate (Wang *et al.*, 2000) may also relate to a requirement to modulate NO levels.

Figure 1 outlines a metabolic scheme showing how reactions involving both NO and haemoglobin could function in plant cells during hypoxia. There are two known routes, NO synthase (Cueto *et al.*, 1996; Barroso *et al.*, 1999; Ribeiro *et al.*, 1999) and nitrate reductase (Yamasaki and Sakihama, 2000; Stöhr *et al.*, 2001), for formation of NO in plants. While both pathways consume reduced pyridine nucleotide in the reactions, formation via nitrate reductase appears to be the more favoured route for the following reasons. Nitric oxide synthase consumes 1.5 moles of NADPH per mole of NO produced, but it also consumes 2 moles of oxygen in the process. Nitrate reductase consumes 2 moles of NADH, without oxygen consumption, per mole of NO. Furthermore, nitrate reductase is activated upon exposure of plant roots to hypoxia (Botrel and Kaiser, 1997) and nitrate assimilation during anoxia is blocked at the nitrite reductase step (Ferrari and Varner, 1971).

The reactions shown for metabolism of the NO are based on the following considerations. NO is highly reactive and toxic to cells. The reaction of NO with haemoglobin is considered to be a major route for detoxification (Wennmalm *et al.*, 1992). NO reacts rapidly with oxyhaemoglobin forming nitrate and methaemoglobin [$\text{Hb}(\text{Fe}^{+3})$] (Doyle and Hoekstra, 1981). This route for metabolism of NO, with nitrate being recycled, would be advantageous to the hypoxic plant cell, exposed to conditions of prolonged soil waterlogging where nitrates would be severely depleted (Tiedje *et al.*, 1982). Haemoglobins (Fe^{+3}) can be reduced to haemoglobins (Fe^{+2}) via NADH-dependent reductases (Jakob *et al.*, 1992; Poole, 1994). An NADH-dependent reductase in hypoxic plant cells would provide an additional NAD^+ for glycolysis. Deoxyhaemoglobins form nitrosylhaemoglobins with NO (Antonini *et al.*, 1966) and the rate of formation of the complex has comparable kinetics to that of the oxyhaemoglobin complex (Cassoly and Gibson, 1975; Eich *et al.*, 1996). It is, therefore, evident that nitrosylhaemoglobins will represent a significant proportion of the population of liganded haemoglobin molecules when NO is present and the oxygen tension is low. Other than detoxifying NO as a consequence of the ligand formation, there are few references to metabolic reactions in which the complex might participate. Bacterial flavohaemoglobins oxidize NO to nitrate aerobically and reduce it to nitrous

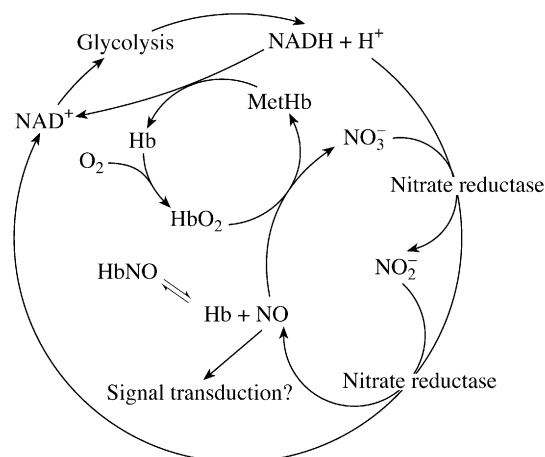


FIG. 1. Suggested pathways by which nitric oxide formation and its interaction with haemoglobin may be involved in adaptation to hypoxic stress.

oxide anaerobically (Poole and Hughes, 2000). Flavohaemoglobins, unlike haemoglobins, contain a ferredoxin-NADP⁺ reductase-like domain in addition to a haemprotein domain. Thus, any similar reactions of nitrosylated haemoglobins would require interaction with some other protein in the cell. No such interaction has yet been shown.

NITRIC OXIDE AND AERENCHYMA FORMATION

The subject of NO in relation to plant biology is rapidly expanding and there are other areas relative to its involvement in plant anaerobiosis that warrant further investigation, in addition to its interactions with haemoglobins. One of these is the question of aerenchyma formation. Ethylene has been shown to be associated with aerenchyma formation in hypoxic maize roots (Drew *et al.*, 1989, 1994; He *et al.*, 1992). This relationship has remained tenuous because the observation has been restricted to maize (Jackson, 1985) and some of the data are conflicting (Drew, 1997). NO is an attractive candidate for involvement in aerenchyma formation. It has been suggested that NO may interact with reactive oxygen species to produce peroxynitrite and directly kill plant pathogens (Durner and Klessig, 1999). There is an abundance of literature on NO and programmed cell death in many mammalian tissues. Depending on NO concentration and other factors, NO may either accelerate or inhibit apoptosis (Kim *et al.*, 2001). The effect may be either direct, through cell necrosis, or through regulatory pathways and it may also be selective in relation to the cells that do respond. A similar type of reaction could be responsible for selected cell death during aerenchyma formation in roots exposed to waterlogging (Drew, 1997). NO, by its activation of guanylate cyclase, has pronounced effects on signal transduction pathways. Inhibitors of signal transduction pathways that have been linked to cGMP in mammalian cells (McDonald and Murad, 1996) also effect aerenchyma formation in maize roots (Drew *et al.*, 2000). Similarly, NO

has been implicated in programmed cell death of *Arabidopsis* cell suspension cultures through its action on signal transduction pathways involving guanylate cyclase (Clarke *et al.*, 2000). It will be interesting to see if these observations on the possible involvement of NO in aerenchyma formation are borne out experimentally.

Haemoglobin may be pivotal in the short-term survival of plant root cells by regulating the levels of NO. Plant roots that express sufficient haemoglobin soon after exposure to hypoxic stress may modulate levels of NO, produced as a result of the stress, either through reaction of the NO with oxyhaemoglobin or through formation of nitrosylhaemoglobin. This would prevent the onset of cell death, maintaining ATP levels and energy charge, as has been observed in hypoxic maize cells overexpressing haemoglobin (Sowa *et al.*, 1998). 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, haemoglobin expression or action may be cell selective, resulting in (programmed) death of some cells that underexpress the gene.

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