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Review

Nonsymbiotic haemoglobins in plants

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General aspects regarding the presence of nonsymbiotic haemoglobin in plants are presented with the emphasis on those related to its function. As it becomes apparent that the nonsymbiotic haemoglobins are widespread across the plant kingdom and that they represent a more primitive and evolutionary older form of the plant globin genes, the question of their function becomes more attractive. While the physiological functions of the symbiotic haemoglobins in plants are well understood, almost nothing is known about their nonsymbiotic predecessors. Therefore, the known and hypothetical functions of haemoglobins in various systems are described along with information concerning properties and the regulation of expression of the nonsymbiotic haemoglobins.

Interestingly, a number of nonsymbiotic haemoglobins have been shown to be hypoxia-inducible. The spatial and temporal pattern of this induction in barley may suggest that it is an integral part of the plants response to limiting oxygen stress.

Haemoglobins are wide spread throughout the biosphere. They are found in a broad range of organisms from bacteria, through unicellular eukaryotes, to plants and animals (Hardison, 1996) suggesting that they predate divergence of life into plant and animal forms. Plant haemoglobins have been classified into symbiotic and nonsymbiotic types. Symbiotic haemoglobins are found in plants that are capable of participating in microbial symbioses, where they function in regulating oxygen supply to nitrogen fixing bacteria. Nonsymbiotic haemoglobins have only recently been discovered and are thought to be evolutionary predecessors of the more specialized symbiotic leghaemoglobins (Andersson *et al.*, 1996). The ubiquitous nature of nonsymbiotic haemoglobins is evidenced by their broad presence across the plant kingdom. The widespread presence and long evolutionary history of plant haemoglobins suggest a major role for them in the life of plants.

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Very little, however, is known about their function, although it has been proposed that nonsymbiotic haemoglobins may act either as oxygen carriers to facilitate oxygen diffusion, or oxygen sensors to regulate expression of anaerobic proteins during periods of low oxygen supply. Recent findings of high oxygen avidity of the nonsymbiotic haemoglobins (Duff *et al.*, 1997; Arredondo-Peter *et al.*, 1997) may, however, seriously question validity of these hypotheses. Experiments with barley haemoglobin show the involvement of ATP in regulation of haemoglobin gene expression (Nie & Hill, 1997). It has also been shown that barley haemoglobin improves energy status of maize cells under low oxygen stress (Sowa *et al.*, 1998). These findings support a hypothesis that haemoglobin may play a role in energy metabolism in plants (Hill, 1998).

WHAT ARE HAEMOGLOBINS?

Haemoglobins are characterised by their ability to reversibly bind oxygen. A functional haemoglobin consists of a globin polypeptide and the prosthetic group, haem, that is non-covalently bound to the globin. Haem is a tetra pyrrole ring, protoporphyrin IX, with a chelated iron atom and represents the oxygen binding site. Haemoglobins are relatively small, globular molecules with relative molecular mass clustered around 18000 (Wittenberg & Wittenberg, 1990). In nature, haemoglobins exist in monomeric, dimeric, or tetrameric forms. The red blood cell haemoglobins of vertebrates, for example, are heterotetramers built with two α and two β subunits. Plant haemoglobins are either homodimers without haem–haem interaction or monomers (Appleby, 1992; Duff *et al.*, 1997). The three-dimensional structure of haemoglobins or their subunits is best represented by myoglobin, a monomeric haemoglobin of vertebrate muscle cells.

Oxygen binding can occur only if the haem iron is in the Fe^{2+} (ferrous) valence state and the hydrophobic environment in the protein cleft, provided by valine and phenylalanine residues, minimises the oxidation rate of Fe^{2+} (Appleby, 1992). The interaction between the polypeptide and the haem affects the kinetics of oxygen binding by haemoglobins, therefore, haemoglobins with different amino-acid sequences differ in their affinity for oxygen. Haemoglobins have evolved to perform various functions, reflected by their different ligand binding kinetics (Table 1).

Oxygen affinity of haemoglobins can be greatly affected by environmental factors, such as pH, CO_2 and organic phosphate. Concentration of haemoglobin itself can also be a factor affecting its ligand binding. Certain small molecules, such as CO, NO and H_2S, bind to the sixth liganding position of the Fe(II) in haemoglobin with much higher affinity than does O_2. This, as well as their similar binding to the haem of cytochromes, accounts for their highly toxic properties.

In haemoglobins that are built from more than one subunit, such as tetrameric haemoglobins of vertebrates, haem–haem interactions affect oxygen binding. This interactive binding phenomenon is called cooperativity. Binding of one oxygen molecule to one haem facilitates binding of oxygen to the remaining haems within the same haemoglobin molecule.

A consequence of reversible combination with oxygen are two different forms of haemoglobin: oxygenated and deoxygenated. Binding of oxygen, or any other ligand, changes conformation of the protein. The functional implications of this change will be discussed later in this review.

A distinct characteristic of haemoglobin, as well as other haemoproteins, is its red colour brought about by the presence of haem. The oxy-haemoglobin shows two absorbance peaks in the visual spectrum, at about 540 and 574 nm, whereas the deoxy form peaks at

Table 1. Kinetics and equilibrium constants for the reactions of various haemoglobins with oxygen

Protein	k'_{on} $\mu M^{-1}s^{-1}$	k_{off} s^{-1}	K_D (=k:k') nM	Reference
Ascaris Hb	1.5	0.004	2.7	Gibson & Smith, 1965
Barley Hb	9.5	0.0272	2.86	Duff *et al.*, 1997
Rice Hb1	68	0.038	0.5	Arredondo-Peter *et al.*, 1997
Arabidopsis Hb1	75	0.12	1.6	Trevaskis *et al.*, 1997
Arabidopsis Hb2	1.1	0.14	130	Trevaskis *et al.*, 1997
Parasponia Hb1	165	5	89	Gibson *et al.*, 1989
Casuarina symHb1	41	6	135	Gibson *et al.*, 1989
Soybean Lb	120	5.6	48	Gibson *et al.*, 1989
Vitreoscilla Hb			6000	Dikshit & Webster, 1988

The equilibrium dissociation constant (K_D) represents the dissolved oxygen concentration at which the protein is half combined with ligand. Hb, haemoglobin; Lb, leghaemoglobin; Mb, myoglobin; symHb, symbiotic haemoglobin.

about 557 nm (Appleby, 1992). A Soret band at about 412 nm is characteristic of the optical spectrum for all haemoproteins regardless of their oxygenation state.

OCCURRENCE OF HAEMOGLOBIN IN THE BIOSPHERE

While the existence of haemoglobin in the animal kingdom has long been a common knowledge, the findings of its presence in non animal sources are relatively recent. The evidence accumulated already supports the wide spread occurrence of haemoglobin across the biosphere. Haemoglobins have been found in all major groups of organisms including eubacteria, unicellular eukaryotes, plants and animals (Hardison, 1996).

Two types of haemoglobin are known to exist in vertebrates. Myoglobin an oxygen storage and delivery protein, and oxygen carrying erythrocyte haemoglobin. Apart from vertebrates, haemoglobins have been found in a range of invertebrates from nematodes (Dixon *et al.*, 1992) to annelids and arthropods (Riggs, 1991; Sherman *et al.*, 1992).

Haemoglobins isolated from plants fall into two general categories. Symbiotic haemoglobins are found in plants entering symbiosis with microorganisms. They are best represented by leghaemoglobins that are produced in the nodules of legume plants (Kubo, 1939) where they function in controlling oxygen supply to nitrogen fixing *Rhizobia* (Appleby, 1992). The nonsymbiotic haemoglobins, also called plant haemoglobins, were first found in dicotyledonous *Trema tomentosa* (Bogusz *et al.*, 1988), but the wide spread existence of haemoglobin in the plant kingdom was truly proven by its discovery in monocotyledonous barley (*Hordeum vulgare*) (Taylor *et al.*, 1994), rice (*Oryza sativa*) (Sasaki *et al.*, 1994) and algae, *Chlamydomonas eugametos* (Couture *et al.*, 1994). Although the differences in the primary sequence between haemoglobins from plant and animal sources are significant (Riggs, 1991), structural analysis of soybean leghaemoglobin suggests that they all fold into virtually identical globin structures (Vanhstein *et al.*, 1975). Haemoglobins have also been found in far less advanced species representing protozoa, fungi and bacteria (Hardison, 1996).

HAEMOGLOBINS IN PLANTS

Symbiotic haemoglobins

Nodulating plants are able to participate in free nitrogen fixing symbioses with bacterial micro symbionts. An obvious benefit of such symbioses to plants is the additional source of nitrogen. *Rhizobium* and *Bradyrhizobium* of the family *Rhizobaceae,* and *Frankia* a member of Actinomycetes are the only known micro symbionts. The best characterized are the symbioses of *Rhizobium* and *Bradyrhizobium* bacteria with leguminous plants. The only nonleguminous plant known to be nodulated by *Rhizobium* or related members of *Rhizobaceae* is *Parasponia,* a member of *Ulmaceae* (Appleby *et al.,* 1983). *Frankia* nodulate a variety of nonleguminous dicotyledonous plants forming actinorhizal symbioses. Reduction of free nitrogen in all of these symbiotic systems depends on the activity of bacteria encoded nitrogenase.

The nitrogenase reaction requires substantial amounts of energy in the form of ATP. These energy needs have to be met by bacteroid respiration. Paradoxically, the oxygen that is necessary for respiration readily inhibits the activity of nitrogenase (Appleby, 1992). It has been well documented that haemoglobin found in symbiotic nodules plays a crucial role in the control of oxygen supply within nodules. It allows the system to meet the oxygen requirement for bacteroid respiration while keeping oxygen tensions low enough to sustain the function of nitrogenase (Appleby, 1992). Other than haemoglobin factors, such as mechanical barriers, have also been implemented in the control of nodule oxygen supply (Hunt & Layzell, 1993).

Isolation of haemoglobin from *Parasponia anderssoni,* the host of *Rhizobium* endophyte (Appleby *et al.,* 1983), attracted great attention among researchers. Not only was it the first haemoglobin in the plant kingdom to be found in other than leguminous plant source, but also it was expressed in both nodules and nonsymbiotic parts of the plant. This implicated a symbiotic as well as nonsymbiotic function for this protein and led to the hypothesis that the presence of haemoglobin may extend beyond nodulating plants.

Nonsymbiotic haemoglobins

The *Parasponia* haemoglobin cDNA was used as a probe to detect a haemoglobin gene in *Trema tomentosa* (Bogusz *et al.,* 1988). Although *Trema* belongs to the same family as *Parasponia* (*Ulmaceae*) it has no known symbiotic association. This finding made it apparent that plant haemoglobins exist more widely than previously thought and may have other than symbiotic functions.

The search for other nonsymbiotic plant haemoglobins that followed the *Trema* haemoglobin discovery, was for many years unsuccessful, due to poor cross-hybridization between symbiotic haemoglobin cDNA probes and nucleic acids isolated from nonsymbiotic plants. The breakthrough came with the serendipitous discovery of haemoglobin in barley (Taylor *et al.,* 1994). Using the barley haemoglobin cDNA probe the same authors detected related sequences in maize, wheat, rye and triticale (Taylor *et al.,* 1994). This work made it clear that haemoglobins are indeed wide spread in plants. The same year brought reports of the presence of two nonsymbiotic haemoglobins in rice (Sasaki *et al.,* 1994) and in chloroplasts of the algae, *Chlamydomonas eugametos* (Couture *et al.,* 1994). To date nonsymbiotic haemoglobins have been reported in a variety of plants, including the symbiotic soybean (Andersson *et al.,* 1996) and actinorhizal *Casuarina* (Christensen *et al.,* 1991; Jacobsen-Lyon *et al.,* 1995).

The plant haemoglobin protein similarity tree (Fig. 1) (Andersson *et al.,* 1996), shows clearly that symbiotic and nonsymbiotic haemoglobins, regardless of the source species, represent two distinct and coherent subdivisions within the haemoglobin family. Analys-

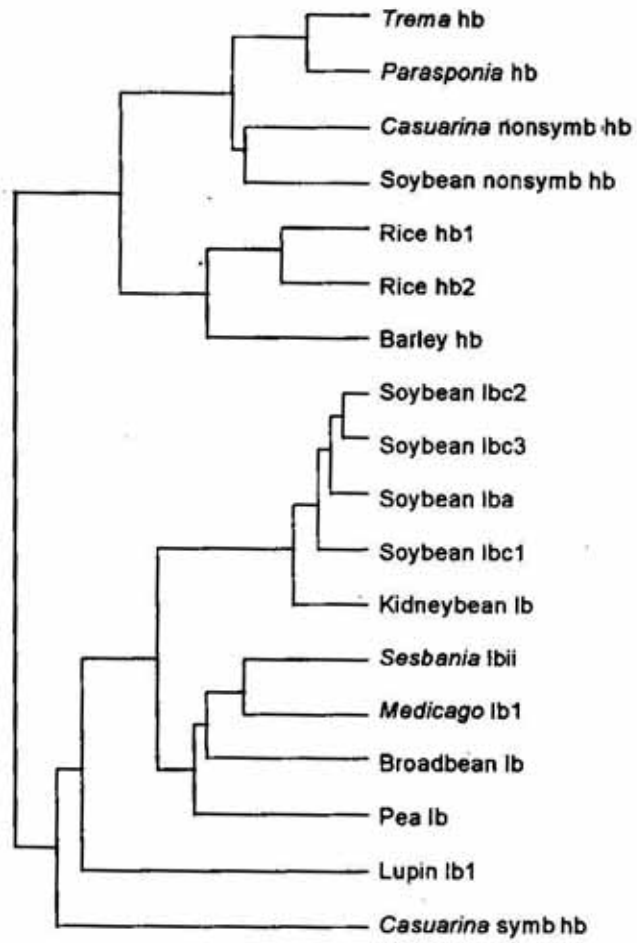

Figure 1. Plant haemoglobin protein similarity tree (Andersson *et al.*, 1996).

ing oxygen binding characteristics of plant haemoglobins, one can observe another distinction between these two haemoglobin types. The nonsymbiotic proteins (Duff *et al.*, 1997; Arredondo-Peter *et al.*, 1997) are much more oxygen avid than their symbiotic counterparts (Gibson *et al.*, 1989).

A new insight into the nonsymbiotic haemoglobins have been recently brought by the isolation of two nonsymbiotic *Hb* genes and their products in *Arabidopsis thaliana* (Trevaskis *et al.*, 1997). *AHb1* gene product represents, in sequence and oxygen affinity, a classical nonsymbiotic haemoglobin, whereas the product of *AHb2* gene shows higher similarity in both sequence and oxygen binding to the symbiotic haemoglobins. The subsequential identification of a AHb2 protein homologue from *Brassica napus* (unpublished) has led these authors to propose a further division within the nonsymbiotic haemoglobins, namely they suggest that classical nonsymbiotic haemoglobins should be called "class 1" nonsymbiotic haemoglobins and the *AHB2* like genes product "class 2" nonsymbiotic haemoglobins. The two nonsymbiotic haemoglobin genes isolated from rice (Sasaki *et al.*, 1994) were not included in this classification of genes. It would be valuable, from the evolutionary and functional point of view, to know whether the class 2 haemoglobins extend into monocotyledonous plants.

ORIGIN AND EVOLUTION OF PLANT HAEMOGLOBINS

Since the discovery of leghaemoglobins almost sixty years ago (Kubo, 1939), questions have been raised regarding the origin and

evolution of haemoglobin. For almost fifty years the apparent lack of haemoglobin presence in other than legume plant sources, supported a notion that haemoglobins have been introduced to plants by horizontal transfer from the animal kingdom, possibly through an insect or nematode vector (Appleby *et al.*, 1983). Arguments against this hypothesis came with relatively recent identification of haemoglobins in nonlegume and nonsymbiotic plant sources as well as findings of haemoglobins in protozoa, fungi and bacteria.

It is now widely accepted that plant and animal haemoglobins originate from the same ancestral globin gene, about 1500 million years ago, and have been shaped through vertical evolution. Moreover, finding haemoglobins in bacteria shows that haemoglobin is a truly ancient gene, preceding the divergence of prokaryotes and eukaryotes, about 1800 million years ago (Appleby, 1992; Hardison, 1996). Zhu & Riggs (1992) analysed the phylogenesis of haemoglobins and proposed the phylogenetic tree shown in Fig. 2.

Gene structure analyses are usually very helpful in tracking the steps of a gene's evolution. Bacterial haemoglobin gene, just like all bacterial genes, does not have any introns. Nor does the fungal haemoglobin gene (Zhu & Riggs, 1992). Symbiotic plant haemoglobin genes as well as the nonsymbiotic plant *Hb* genes have a three intron and four exon structure (Guy *et al.*, 1997). Interestingly, animal haemoglobin genes have either three (nematode), two (most arthropods, vertebrate and annelid) or no (insect) introns (Hardison, 1996). Close analysis shows that the two introns of the vertebrate, arthropod and annelid *Hb* genes are in the same positions as the first and third introns of the plant genes. It would appear that these animal genes lost the middle intron during the course of evolution. The third, middle intron found in the nematodes *Ascaris* and *Pseudoterranova* (Dixon *et al.*, 1992; Goldberg, 1995) might represent the link between plant and animal genes. Moreover, the high oxygen affinity of nematode haemoglobin (Goldberg, 1995) is within the range of nonsymbiotic plant haemoglobins. The placement of this middle intron in nematode genes is close, but not identical to its position in plant *Hb* genes. This may suggest either an intron sliding or independent insertion. Interestingly, in *Chlamydomo-*

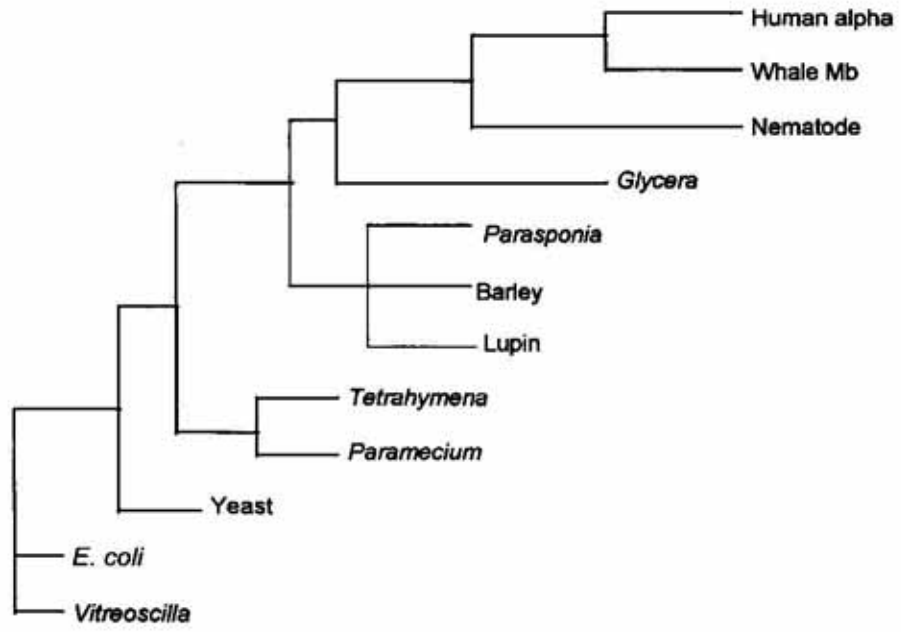

Figure 2. Phylogenetic analysis of the relationship between various haemoglobins.

(Zhu & Riggs, 1992; Barley Hb was not included in the original tree.

nas the middle intron is in the same position as in plant *Hb*, however the two remaining introns come in different positions than in plant and animal genes (Couture *et al.*, 1994). These phenomena of sliding or independently inserted introns may be difficult to explain, but the overwhelming evidence supports vertical, and at least 1800 million years long, evolution of haemoglobin genes (Hardison, 1996).

The very existence of two types of haemoglobins in plants raises a question: which one was the first? Symbiotic *Hb* genes are restricted only to plants capable of participation in N_2 fixing symbioses, whereas the nonsymbiotic genes are far more widespread. The finding of nonsymbiotic haemoglobins in symbiotic plants (Andersson *et al.*, 1996; Christensen *et al.*, 1991) favour the hypothesis that symbiotic haemoglobins arose through duplication of nonsymbiotic genes followed by speciation to fit the needs of symbiosis. Further evidence comes from the identification of noduline motifs 5′-AAAGAT-3′ and 5′-CTCTT-3′ in symbiotic genes of legumes and actinorhizal plants (Christensen *et al.*, 1991). These motifs are absent from the nonsymbiotic haemoglobin gene of *Casuarina* (Jacobsen-Lyon *et al.*, 1995), which may suggest that symbiotic haemoglobins evolved from the nonsymbiotic ones in response to the needs of symbiosis. In *Parasponia* one haemoglobin gene, whose sequence shows more homology with the nonsymbiotic than with symbiotic genes, the haemoglobin has both symbiotic and nonsymbiotic functions (Landsmann *et al.*, 1986). Although the *Parasponia* situation supports the idea of a nonsymbiotic gene duplication leading to the symbiotic genes, an alternative explanation may be suggested by the most recent finding of class 2 nonsymbiotic genes in *Arabidopsis* and *Brassica* (Trevaskis *et al.*, 1997). The class 2 nonsymbiotic haemoglobin shares more similarities with symbiotic than with nonsymbiotic genes and could be used to argue that the symbiotic haemoglobin genes gave rise to nonsymbiotic ones. Nevertheless, the stronger evidence is in support of the earlier hypothesis and it is generally accepted that the nonsymbiotic haemoglobins were evolutionary predecessors of the more specialized symbiotic haemoglobins (Trevaskis *et al.*, 1997; Andersson *et al.*, 1996).

Since haemoglobins are not the only haem binding proteins, valid questions have been raised concerning the possible common origin of haemoglobins and other haemoproteins. It has been proposed, based on similarities between amino-acid sequences, that globins evolved from cytochrome b_5 (Runnegar, 1984; Hardison, 1996).

Little is known about the functional evolution of haemoglobin. Hardison (1996) hypothesizes that metal-bound porphyrin rings were present when photosynthesis evolved. Such a ring chelating Mg^{2+} is a basic structure of chlorophyll. Haemoproteins could have been used at that time to protect the cell from oxygen produced by photosynthetic processes. From there, evolution would take them to electron transfer agents, such as cytochromes, and oxygen transport proteins. Today haemoglobins have various functions in different organisms, from accepting electrons in bacteria through buffering oxygen tensions in muscles of vertebrates and plant nodules to transporting oxygen in animals.

TOWARDS THE FUNCTION OF NONSYMBIOTIC HAEMOGLOBIN IN PLANTS

Haemoglobin gene expression and regulation

Symbiotic haemoglobin genes are expressed only in nodule tissue (Appleby, 1992). Noduline motifs present in promoters of leghaemoglobins and many other noduline genes direct their expression specifically to the nodules. The 5′-AAAGAT-3′ and 5′-CTCTT-3′ *cis*-elements, known as noduline motifs, are sepa-

rated in leghaemoglobin gene only by 6 to 7 nucleotides. It appears that the latter motif is of greater importance for the expression of nodule specific genes (Szczyglowski *et al.*, 1994). The same element is found in promoters of symbiotic haemoglobin genes from the actinorhizal plant *Casuarina* (Jacobsen-Lyon *et al.*, 1995). Functioning as oxygen buffers symbiotic haemoglobin genes are expressed at a very high level. Leghaemoglobin in soybean nodules is found at concentrations of 700 μM (Gibson *et al.*, 1989).

The nonsymbiotic haemoglobins, or their respective mRNAs, are found in metabolically active tissues such as roots (Taylor *et al.*, 1994; Arredondo-Peter *et al.*, 1997; Trevaskis *et al.*, 1997; Andersson *et al.*, 1996), aleurone (Taylor *et al.*, 1994) and vascular tissues of leaves, stems and seedling cotyledons (Andersson *et al.*, 1996). The expression of these proteins are relatively low in the order of 20–30 μM (Duff *et al.*, 1997).

There is no published information on the promoter sequences of haemoglobin genes from monocotyledonous sources. The sequences of nonsymbiotic haemoglobin genes from *Casuarina*, *Parasponia*, *Trema* and soybean does not show noduline motifs found in symbiotic genes (Andersson *et al.*, 1997; Jacobsen-Lyon *et al.*, 1995).

Haemoglobins in barley, maize (Taylor *et al.*, 1994), and *Arabidopsis* (Trevaskis *et al.*, 1997) are stress inducible, suggesting that at least some nonsymbiotic haemoglobins are involved in stress response in plants. Low oxygen tensions induce expression of barley, maize and *Arabidopsis AHB1* (class 1) *Hb* genes. Barley haemoglobin is induced at atmospheric oxygen levels of about 5%. The induction is rapid, with increased transcription becoming evident within 30 min and maximum expression occurring about 12 h after placing the tissue under a nitrogen atmosphere (Taylor *et al.*, 1994). AHB1, in addition, is also induced by 1% sucrose. *AHB2* (class 2) gene of *Arabidopsis* is induced by chilling (Trevaskis *et al.*, 1997).

Nie & Hill (1997) studied signal transduction leading to the induction of *Hb* expression in barley aleurone tissue. Their work shows that blocking of mitochondrial electron transport chain, as well as blocking of ATP synthase activity, in the presence of oxygen, results in haemoglobin induction at the levels similar to those observed under oxygen stress. An obvious conclusion, drawn by these authors, is that haemoglobin induction is triggered not by the lack of oxygen *per se*, but by the resulting reduction of ATP synthesis.

Properties of nonsymbiotic haemoglobins

Recent isolations of nonsymbiotic haemoglobins from barley, rice, soybean and *Arabidopsis* enabled studies of the chemical properties of these proteins. The first to be characterized was haemoglobin from barley (Duff *et al.*, 1997), which was followed by less detailed studies on haemoglobins from rice (Arredondo-Peter *et al.*, 1997) and *Arabidopsis thaliana* (Trevaskis *et al.*, 1997). In all cases recombinant proteins were produced in bacteria, then purified and used to determine subunit structure, spectral characteristics and, most importantly, ligand binding kinetics. In the case of barley haemoglobin the results obtained with recombinant protein were confirmed with the native one (Duff *et al.*, 1997). Barley haemoglobin is a homodimer with a subunit molecular mass of 18.5 kDa. Each subunit possesses a haem chromophore giving spectral characteristics of a Soret band at 412 nm with visible bands at 540 and 576 nm. The optical spectra of barley haemoglobin, in both ferrous and ferric state, are reminiscent of many low spin 6-C haemoproteins (Duff *et al.*, 1997).

A characteristic of all studied nonsymbiotic haemoglobins is a very slow ligand dissociation reaction, resulting in extremely high ligand affinities of these proteins (Table 1). The exceptions are the bifunctional haemoglobin from *Parasponia* and the class 2 haemoglobin from *Arabidopsis*. Both of these pro-

teins function either in symbiotic associations or share extensive sequence homology with symbiotic haemoglobins. These ligand binding kinetics, apart from sequence differences, make a clear functional division between the symbiotic and nonsymbiotic plant haemoglobins.

Functions of haemoglobins

Haemoglobin as an oxygen diffusion facilitator and in oxygen storage

In order to act as an oxygen carrier, or to facilitate oxygen diffusion within the cell, haemoglobin must fulfil two ground conditions. First, it must be present in sufficient quantities to make it effective in delivering oxygen in quantities that maintain oxygen demands of the cell. To be effective in facilitating diffusion of oxygen, its concentration must be high enough to create a gradient that supplies oxygen faster than the rate of diffusion of free oxygen. Second, it has to exist in a state of partial oxygenation, that is, a certain percentage of haemoglobin molecules must be in an oxygenated state and the equilibrium between the oxygenated and deoxygenated haemoglobin ought to be maintained (Appleby, 1992). In fact, some haemoglobins, including myoglobin, are found to function in partial oxygenation state in various tissues (Wittenberg & Wittenberg, 1990). In myoglobin dependent systems, such as vertebrate heart and muscle, free oxygen diffusion within the cell is extremely slow due to the low oxygen solubility in thick cytoplasm. Free oxygen pressure in mitochondria is at very low levels, about 20- to 25-fold lower than in erythrocyte. This creates a gradient of oxygen pressure, in which the diffusion of oxygen bound haemoglobin generates a flux of oxygen (Wittenberg & Wittenberg, 1990). It has been well documented that leghaemoglobin plays a key role in regulation of oxygen supply to the respiring bacteroids. A membrane separates the bacteroid from the host plant cell cytoplasm where the concentration of leghaemoglobin may reach nearly 4 mM (Appleby, 1984). With the estimated 20% oxygenation of leghaemoglobin, the steady state concentration of free dissolved oxygen at the bacteroid surface would be in the neighbourhood of 10 nM (Appleby, 1984). The leghaemoglobin bound oxygen is, then, at a concentration of about 0.75 mM. That is 75000 times greater than that of free oxygen, indicating that the entire flux of oxygen to the bacteroid is leghaemoglobin mediated (Appleby, 1992).

Another significant factor, in haemoglobin facilitated oxygen transport or diffusion, are the kinetics of oxygen binding. At a given concentration of free, dissolved oxygen, haemoglobin should be able to efficiently bind O_2 and release it at a non limiting rate. Both leghaemoglobin and vertebrate myoglobin have the oxygen binding kinetics that make them fit to perform their respective functions (Table 1). Although the oxygen affinity (dissociation constant, K_D) of symbiotic haemoglobins can be tenfold higher than that of myoglobin (Table 1), the oxygen dissociation reactions are almost equally fast (Appleby, 1992). This makes them a model for facilitated oxygen diffusion.

Haemoglobins as oxygen sensors

Changing conditions in a natural environment may often be life threatening to living organisms. Environmental stresses such as cold, heat, drought and flooding are especially dangerous to plants since they can not simply run away from them. Several defence mechanisms, often involving metabolic alterations, have evolved to increase stress survival chances. The first step in signal transduction pathways that activate these mechanisms must be the detection of the changing conditions. During inadequate oxygen supply, anaerobic metabolism is often turned on as an alternative pathway of energy production. A molecule that senses oxygen levels has been

proposed in many species from bacteria to mammals. Haemoprotein would be a natural candidate for an oxygen sensor, and in fact there is substantial evidence to support haemoprotein based oxygen sensing (Gilles Gonzalez *et al.*, 1994; Poole *et al.*, 1994a; Goldberg *et al.*, 1988).

In mammals, erythropoietin, a hormone, is involved in the regulation of haemoglobin synthesis. The synthesis of erythropoietin is, in turn, stimulated under hypoxia (Goldberg *et al.*, 1988). This hypoxic induction of erythropoietin gene, in human hepatoma cells, is blocked by inhibitors of haem synthesis and by carbon monoxide, which locks haemoproteins in their oxy-conformation, indicating that haemoprotein is involved in oxygen sensing (Goldberg *et al.*, 1988).

In bacteria, two proteins, Fix L (Gilles Gonzalez *et al.*, 1994) and Hmp (Poole *et al.*, 1994a) have been proposed as oxygen sensors. Both have a haem domain but, in addition, FixL possesses a protein kinase domain while Hmp has a flavoprotein domain with NADH oxidase activity. In both cases the occupancy of haem by oxygen affects the activity of the second domain. In oxygenated Hmp flavin is predominantly oxidized, whereas in the absence of oxygen flavin is reduced allowing Hmp to act as a reductase. FixL, found in *Rhizobium meliloti*, is membrane-bound. Upon deoxygenation, its kinase domain is activated, phosphorylating a transcriptional factor that activates a cascade reaction leading to the expression of critical nitrogen fixation genes (Gilles Gonzales *et al.*, 1994).

It has been proposed, that the nonsymbiotic plant haemoglobin may act as an oxygen sensor (Appleby *et al.*, 1992). A conformation change, in deoxygenated haemoglobin, would trigger the activation of anaerobic metabolism. The low level of expression of nonsymbiotic haemoglobins may not support the oxygen transport or storage, but the concentration present would be enough to support the sensing function.

Haemoglobin as a terminal oxidase

The presence of a haem domain in cytochromes as well as the possible common origin of cytochromes and globins invites a theory of possible functional similarity between the two groups. Haemoglobins of yeast and bacteria have been proposed to function as terminal oxidases, the sites of oxygen reduction (Wittenberg & Wittenberg, 1990). Both yeast and *Escherichia coli* haemoglobins have two prosthetic groups, haem and a flavin (Zhu & Riggs, 1992). The structure of yeast haemoglobin resembles that of flavocytochrome b_2 (Xia *et al.*, 1987). The flavin domain of yeast haemoglobin is homologous with members of a flavoprotein family that includes ferredoxin reductase, nitric oxide synthase, and cytochrome P-450 reductase (Zhu & Riggs, 1992). Binding of oxygen by the haem domain may be accompanied by conformation change which acts as a switching mechanism to control the activity of the second domain.

Vitreoscilla is an obligate aerobic bacterium that often lives in oxygen poor environments. Its haemoglobin is a dimer of identical subunits that demonstrate a significant amino-acid sequence similarity with eukaryotic globins (Wakabayashi *et al.*, 1986). Expression of *Vitreoscilla* haemoglobin gene increases greatly under limiting oxygen pressure. Intracellular expression of *Vitreoscilla* haemoglobin (VHb) gene in *E. coli* significantly improves ATP production and culture growth under oxygen limited conditions (Khosla & Bailey, 1988) by maintaining energy efficient, aerobic respiration. When expressed in baker's yeast, VHb alters the aerobic metabolism of the yeast, probably by affecting the mitochondrial electron transport pathway (Chen *et al.*, 1994). Moreover, expression of VHb in *E. coli* that lack terminal oxidases, cytochrome *o* and *d*, restores culture growth in the presence of aerobic substrates, such as succinate and lactate, and restores aerobic respiration (Dikshit *et al.*, 1992).

FUNCTION OF NONSYMBIOTIC HAEMOGLOBIN IN PLANTS

The available evidence suggests that nonsymbiotic haemoglobins are present widely across the plant kingdom. Based on protein sequence comparisons and the common occurrence of the nonsymbiotic haemoglobins in plants, it has been proposed that the nonsymbiotic haemoglobins appeared early in the evolution of plants (Andersson *et al.*, 1996). Nonsymbiotic haemoglobins are expressed in a number of plant tissues such as roots (Taylor *et al.*, 1994; Arredondo-Peter *et al.*, 1997; Trevaskis *et al.*, 1997; Andersson *et al.*, 1996), aleurone (Taylor *et al.*, 1994) and vascular tissues of leaves, stems and seedling cotyledons (Andersson *et al.*, 1996). Recently the expression of a nonsymbiotic haemoglobin was observed in germinating seeds and growing coleoptiles, and hypoxia-stressed leaves of barley (Nie & Hill, unpublished). Sowa *et al.* (1998) show expression of haemoglobin in stressed, suspension cultured, undifferentiated cells of maize. It would appear that the expression of nonsymbiotic haemoglobin is specific not to the tissue but to the conditions of growth and metabolic activity of the cell. Haemoglobin is likely expressed in either rapidly growing and metabolically active tissues that are under high demand for energy, or in cells under oxygen stress which limits the production of energy. All the above reasons may suggest a fundamental role for nonsymbiotic haemoglobins in a process common to all plants and cells.

It has been proposed that nonsymbiotic haemoglobins of plants may function as an oxygen sensor to monitor the oxygen level in tissues and, when necessary, to switch the metabolism from an aerobic to an anaerobic pathway (Appleby *et al.*, 1992). According to this hypothesis, a transition from the oxygenated to the deoxygenated state under lowered oxygen concentration results in a changed conformation of haemoglobin, that in turn triggers the anaerobic response. The bacterial haemoprotein oxygen sensors, FixL and Hmp have oxygen dissociation constants of 50 μM and 2 μM, respectively (Poole *et al.*, 1994b; Gilles Gonzalez *et al.*, 1994), whereas the constants of plant nonsymbiotic haemoglobins range from 0.5 nM to 2.7 nM (Table 1), are three orders of magnitude lower. With so low oxygen dissociation constants plant haemoglobins will remain oxygenated at oxygen concentrations far below those at which anaerobic processes are activated (Hill, 1998). Furthermore blocking the expression of haemoglobin in maize cells did not result in their decreased anaerobic response, rather the opposite, while increased expression of haemoglobin resulted in lower activity of the fermentative pathway in transgenic maize cells under hypoxic stress (Sowa *et al.*, 1998). Also the hypoxic induction of haemoglobin in plant tissues suggests that haemoglobin is a part of the anaerobic response rather than a sensor that switches it on. Finally, it has been demonstrated in barley aleurone tissue that the induction of haemoglobin expression is triggered not directly by the lack of oxygen but by the resulting impaired ATP synthesis (Nie & Hill, 1997).

The low oxygen dissociation constant of the nonsymbiotic haemoglobins also challenges another persisting theory that views haemoglobins as facilitators of oxygen diffusion or oxygen stores supporting mitochondrial respiration under limited oxygen tensions. The K_m for oxygen of cytochrome *c* oxidase is approximately 100 nM (Appleby, 1992). Having an oxygen dissociation constant of about 2 nM (Table 1), nonsymbiotic haemoglobins would be completely ineffective in providing oxygen to the mitochondrial respiratory process. No other known oxidases have a K_m for O_2 sufficiently low to make use of this oxygen. Barley haemoglobin shows an extraordinarily slow oxygen dissociation ($t_{1/2}$ = 27 s). This would appear to preclude this haemoglobin from oxygen transport or facilitated diffusion involving reversible oxygen dissociation (Duff *et al.*, 1997; Hill, 1998).

For all the above reasons, unless the high oxygen avidity of plant nonsymbiotic haemoglobins is altered by an association with another molecule its function as an oxygen sensor or facilitator of oxygen diffusion would seem to be impossible.

Using barley haemoglobin gene Sowa *et al.* (1998) prepared a system of transgenic maize cells, expressing sense and antisense haemoglobin constructs, with increased and decreased expression of haemoglobin. Experiments in this system showed that the presence of haemoglobin helped maintaining the energy status of maize cells under low oxygen stress.

Similar to the nonsymbiotic haemoglobins of barley, maize, rice and *Arabidopsis* (Taylor *et al.*, 1994; Arredondo-Peter *et al.*, 1997; Trevaskis *et al.*, 1997) the haemoglobin of the bacterium *Vitreoscilla* (VHb) is induced under conditions of limiting oxygen (Wakabayashi *et al.*, 1986; Dikshit *et al.*, 1992). This bacterial haemoglobin enhances growth of transgenic microorganisms such as *E. coli* and *Saccharomyces cerevisiae* (Khosla & Bailey, 1988; Chen *et al.*, 1994), and plants such as *Nicotiana tabaccum* and *Datura* (Holmberg *et al.*, 1997). This invites a comparison with barley haemoglobin. In transformed yeast VHb appears to interfere with the mitochondrial electron transport chain (Chen *et al.*, 1994). In fact about 40% of the VHb expressed in yeast was found in mitochondria. Contrary to that, the nonsymbiotic haemoglobin of barley was found only in the cytoplasm and not in mitochondria (Nie & Hill, unpublished). Furthermore, it has been shown that the interaction of barley haemoglobin with mitochondrial respiration is unlikely (Sowa *et al.*, 1998).

The available data, taken together, support a hypothesis that nonsymbiotic haemoglobins may function as a terminal oxidase or oxygenase in cells exposed to low oxygen tensions (Hill, 1998), although other hypotheses can not be disregarded. The very low dissociation constant of barley oxyhaemoglobin make it an ideal candidate for sequestering oxygen in low oxygen environments. Interaction with another compound, perhaps a flavoprotein, could create a complex capable of oxidizing NADH, in a manner analogous to the Hmp protein of *E. coli* (Poole *et al.*, 1994b; Hill, 1998). This would provide an efficient means of oxidatively regenerating NAD to support glycolysis, bypassing the fermentative route to ethanol.

The effects of expression of sense and antisense haemoglobin on energy charge (Sowa *et al.*, 1998) are reminiscent of hypoxic acclimation of plant tissues. Maize root tips develop a tolerance to short term anoxia if they have been acclimated by exposure to hypoxic conditions (Johnson *et al.*, 1989). Acclimation is accompanied by increased energy charge resulting from a sustained glycolytic rate compared to non-acclimated root tips (Xia & Saglio, 1992). Similarly, winter cereals show increased survival to hypoxia caused by ice encasement if they have been acclimated by exposure to hypoxic conditions. Acclimated plants maintain higher levels of adenylates and ATP during ice encasement as a result of accelerated rates of glycolysis compared to non-acclimated plants (Andrews & Pomeroy, 1989). Maximum induction of barley haemoglobin message occurs within 12 h exposure to hypoxic conditions (Taylor *et al.*, 1994), which is well within the time interval used for acclimation in the above conditions. Furthermore, it has been shown that the expression of haemoglobin is not directly influenced by oxygen usage or availability but is influenced by the availability of ATP in the tissue (Nie & Hill, 1997). It may be, therefore, that the increased survival of plants to anoxia as a result of hypoxic acclimation, is a consequence of haemoglobin gene induction during the acclimation process as a result of declining ATP levels during the acclimation process.

From an evolutionary standpoint it has been suggested that nonsymbiotic haemoglobins represent one of the more ancient forms of plant haemoglobins (Andersson *et al.*, 1996). Since early life on earth existed in oxygen-

poor environments, the presence of a haemoglobin capable of utilizing oxygen at low oxygen tensions would have provided an evolutionary advantage to an organism. Oxygen produced during photosynthesis and retained as oxyhaemoglobin would provide a source of oxygen to oxidize NADH, maintaining a high glycolytic flux during darkness to provide ATP for cell growth and development.

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