A role for haemoglobin in all plant roots?

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Abstract. We have found haemoglobin in plant roots whereas previously it has been recorded only in nitrogen fixing nodules of plants. Haemoglobin occurs not only in the roots of those plants that are capable of nodulation but also in the roots of species that are not known to nodulate. We suggest that a haemoglobin gene may be a component of the genome of all plants. The gene structure and sequence in two unrelated families of plants suggests that the plant haemoglobins have had a single origin and that this origin relates to the haemoglobin gene of the animal kingdom. At present we cannot completely rule out the possibility of a horizontal transfer of the gene from the animal kingdom to a progenitor of the dicotyledonous angiosperms but we favour a single origin of the gene from a progenitor organism to both the plant and animal kingdoms. We speculate about the possible functions of haemoglobin in plant roots and put the case that it is unlikely to have a function in facilitating oxygen diffusion. We suggest that haemoglobin may act as a signal molecule indicating oxygen deficit and the consequent need to shift plant metabolism from an oxidative to a fermentative pathway of energy generation.

Key-words: plant haemoglobin evolution; symbiotic nitrogen fixation; anaerobic response; gene regulation; oxygen transport.

Introduction

Until recently it was thought that haemoglobin was present in plants only in the nodule tissue of those species that are able to fix nitrogen by virtue of a symbiotic association between a microorganism and root tissue. That observation, first made for a large number of legume plants, has been extended to non-legumes and in particular to *Parasponia* in the family Ulmaceae, and subsequently to *Casuarina* (Casuarinaceae) (Appleby, 1984). Other non-leguminous plants, in widely divergent families, have also been reported to have haemoglobin associated with nitrogen fixing nodules (Tjepkema, 1983).

Recently we have determined that haemoglobin can be found in root tissue of plants which are not involved in any symbiotic association with bacteria

for nitrogen fixation. We have also shown that haemoglobin occurs in root tissue (Bogusz *et al.*, 1988) of plants which are capable of nodule formation, when they are grown under sterile conditions so that no nodules form and no nitrogen fixation occurs.

So far we have been limited in the plant species we have been able to examine, but, on the basis of our data, we suggest it is possible that *all* plants have functional haemoglobin protein, at least in their roots and possibly in other tissues. These observations have important implications in three areas. First, the hitherto unsuspected presence of haemoglobin in plant roots raises the question of the function of this root haemoglobin. Second, these observations have a bearing on the origin of the haemoglobin gene in plants; initially the gene was hypothesized to have been introduced to a primitive legume by horizontal transfer from an animal. Since we now think haemoglobin is likely to be present in all plants it may be unnecessary to postulate any special or unusual features of gene evolution. Third, the observations bear on the evolution of symbiotic associations between plants and bacteria for nitrogen fixation.

In order to examine these three areas we will review briefly some of the key observations concerning plant haemoglobins.
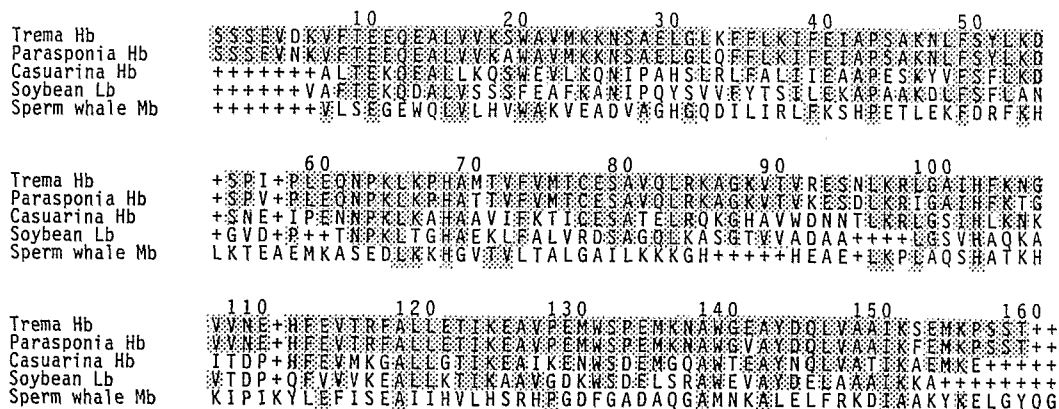
Haemoglobin and its gene in nodulating plants

Haemoglobins isolated from the nodules of legumes (leghaemoglobins) have many properties similar to those of animal haemoglobins (Appleby, 1974). The key property of both animal and legume haemoglobins is the capacity for reversible oxygen binding. Some kinetic properties of the oxygenation of leghaemoglobins differ from those of animal haemoglobins and these will be taken up in detail later in the review. An early finding of significance was that the critical amino acid residues of animal haemoglobins, concerned with haem binding and oxygen carrying, were also present in the soybean haemoglobin molecule. Other regions of amino acid homology were observed throughout the plant and animal haemoglobin molecules (Fig. 1; Ellfolk, 1972). Since haemoglobin was thought to be present in the plant kingdom only in legumes, the similarities between animal and legume haemoglobins suggested that the haemoglobin gene was introduced into a progenitor of legumes by horizontal gene transfer from the animal world (Jeffreys, 1982). When the haemoglobin gene from legumes was

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                    10        20        30        40        50
Trema Hb        SSSEVDKVFTEEQEALVVKSWAVMKKNSAELGLKEFLKIFEIAPSAKNLFSYLKD
Parasponia Hb   SSSEVNKVFTEEQEALVVKAWAVMKKNSAELGLQFFLKIFEIAPSAKNLFSYLKD
Casuarina Hb    +++++++ALTEKQEALLKQSWEVLKQNIPAHSLRLFALIIEAAPESKYVESFLKD
Soybean Lb      ++++++VAFTEKQDALVSSSFEAFKANIPQYSVVFYTSILEKAPAAKDLFSFLAN
Sperm whale Mb  +++++++VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFDRFKH

                      60        70        80        90       100
Trema Hb        +SPI+PLEQNPKLKPHAMTVFVMTCESAVQLRKAGKVTVRESNLKRLGAIHFKNG
Parasponia Hb   +SPV+PLEQNPKLKPHATTVFVMTCESAVQLRKAGKVTVKESDLKRIGAIHFKTG
Casuarina Hb    +SNE+IPENNPKLKAHAAVIEKTIGESATELRQKGHAVWDNNTLKRLGSIHLKNK
Soybean Lb      +GVD+P++TNPKLTGHAEKLFALVRDSAGQLKASGTVVADAA++++LGSVHAQKA
Sperm whale Mb  LKTEAEMKASEDLKKHGVTVLTALGAILKKKGH+++++HEAE+LKPLAQSHATKH

                     110       120       130       140       150      160
Trema Hb        VVNE+HFEVTRFALLETIKEAVPEMWSPEMKNAWGEAYDQLVAAIKSEMKPSST++
Parasponia Hb   VVNE+HFEVTRFALLETIKEAVPEMWSPEMKNAWGVAYDQLVAAIKFEMKPSST++
Casuarina Hb    ITDP+HFEVMKGALLGTIKEAIKENWSDEMGQANTEAYNGLVATIKAEMKE+++++
Soybean Lb      VTDP+QFVVVKEALLKTIKAAVGDKWSDELSRAWEVAYDELAAAIKKA++++++++
Sperm whale Mb  KIPIKYLEFISEAIIHVLHSRHPGDFGADAQGAMNKALELFRKDIAAKYKELGYQG
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Figure 1. Amino acid sequence alignments of plant and animal haemoglobins. The sequences 'Trema Hb' representing the monomeric haemoglobin present in roots of the non-nodulating plant *Trema tomentosa* (Bogusz *et al.*, 1988) and 'Parasponia Hb' representing the dimeric haemoglobin I from *Parasponia andersonii* root nodules (Appleby, Tjebkema & Trinick, 1983; Landsmann *et al.*, 1986) were deduced from gene sequences. The sequence 'Casuarina Hb' represents the monomeric haemoglobin I isolated from *Casuarina glauca* nodules (Fleming *et al.*, 1987) and was determined directly (Kortt *et al.*, 1988). The sequence 'Soybean Lb' was deduced from the gene sequence of the monomeric soybean leghaemoglobin (Hyldig-Nielsen *et al.*, 1982) and the sequence 'Sperm whale Mb' was determined directly from the monomeric sperm whale myoglobin (Dickerson & Geis, 1983). In this figure the sequences are arranged in order of decreasing homology with respect to *Trema* Hb, namely 93%, *Trema:Parasponia*; 50%, *Trema:Casuarina*; 39%, *Trema*:Soybean; 17% *Trema*:Whale myoglobin. Note that some residues of critical importance for stabilization of the haem-protein-oxygen complex (cf. Dickerson & Geis, 1983), those numbered 44 (proline C2 in the myoglobin helical structure), 50 (phenylalanine CD1), 69 (distal histidine) and 104 (proximal histidine) are present in all of these haemoglobins and in almost every other functional haemoglobin.

isolated it was found to contain three introns, two of which were in locations identical to the two introns commonly found in animal haemoglobins (Fig. 2; Hyldig-Nielsen *et al.*, 1982). Furthermore, the third intron was in a position which, from an analysis of animal haemoglobins had been predicted as a potential intron position, separating two structural domains (Go, 1981). This similarity in gene structure emphasized the evolutionary homologies between the legume and animal haemoglobins and was consistent with the suggested horizontal transfer from animals to plants.

The next finding of significance was that haemoglobin also occurred in nodules of non-legume plants.

For some time it has been thought that plants like *Parasponia*, a non-legume that both nodulated and fixed nitrogen in association with its symbiont *Rhizobium*, did not contain haemoglobin (*cf.* Appleby, 1984). Better techniques of protein isolation showed that haemoglobin was present in *Parasponia* nodules and that it had the classical protein structure and properties of animal and legume haemoglobins (Appleby, Tjepkema & Trinick, 1983). Another similar haemoglobin was isolated from the nitrogen fixing nodules of *Casuarina*, a member of a family unrelated to either the legumes or to *Parasponia* and nodulated by *Frankia*, an actinomycete (Fleming *et al.*, 1987). We isolated the gene coding for *Parasponia* haemog-
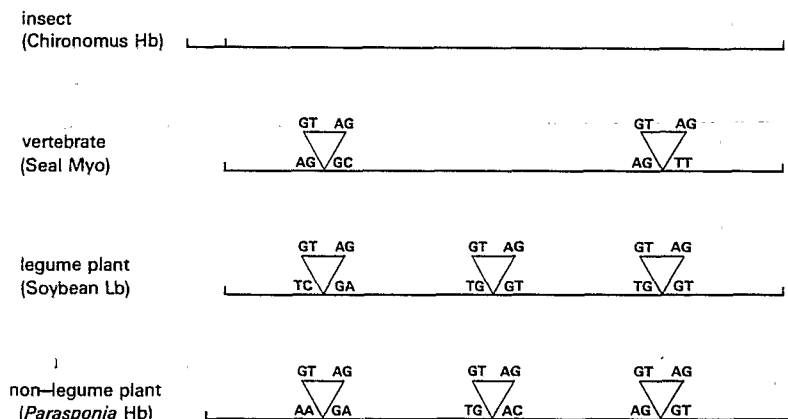
Figure 2. Diagram showing position of introns in haemoglobin genes. The insect (*Chironomus*) haemoglobin gene contains no introns; this is relevant since insects have been proposed as a vector for horizontal gene transfer. In the vertebrate (seal myoglobin) and plant haemoglobins (soybean and *Parasponia*) two of the introns are in identical positions, precisely to the nucleotide, in the genes. The third intron is in an identical position in legume and non-legume genes. The length of the line indicates the length of the coding region of the gene. Nucleotides at the intron/exon boundaries are indicated.

lobin and showed that its intron/exon structure was identical to that of the haemoglobin genes of legumes (Fig. 2; Landsmann *et al.*, 1986). It is clear that the haemoglobin genes of legumes and of *Parasponia* have had a common evolutionary origin.

Functional haemoglobin genes in non-nodulating plant species

The observations we made in *Parasponia* and *Casuarina* led us to doubt the horizontal transfer hypothesis and to suggest that there had been a normal vertical evolution of the haemoglobin gene in plants. In other words the haemoglobin gene could be regarded as a general component of plant genomes. If this were the case, and if haemoglobin occurred only in nodules as protein analyses seemed to indicate, then it could be expected that the haemoglobin gene would have been deleted or at least rendered non-functional in many taxa during the evolutionary period of some 1500 million years which elapsed after plants and animals separated, and before the first nodulated plant species appeared. On the other hand, if haemoglobin had functions in plant tissues other than nodules, the gene would have survived and could be expected to occur in the genome of most present day plant species.

Recently, we asked if functional haemoglobin genes do occur in the genomes of plants which are not known to nodulate. We were restricted in our choice of species for analysis because of the limitations set by nucleic acid cross-hybridization and by antibody cross-reactivity. We knew that we were unable to detect either the haemoglobin gene or protein in *Parasponia* using a soybean cDNA (Landsmann *et al.*, 1986) or antibody probe (Fleming *et al.*, 1987) although haemoglobin could be isolated from *Parasponia*. We therefore first examined species in the genus *Trema*, which is closely related to *Parasponia*, and had been shown not to be involved in any symbiotic association with known nitrogen fixing bacteria. Using a *Parasponia* cDNA probe we were able to detect haemoglobin gene sequences in *Trema* DNA. We then isolated and sequenced the *Trema* gene and showed it to have all the characteristics of a functional gene (Bogusz *et al.*, 1988). When we examined *Trema* root tissue for the products of this gene, for haemoglobin mRNA and haemoglobin protein, we detected both. The gene appeared to be expressed in root tissue but not in leaf tissue. Continuing to use the *Parasponia* cDNA probe we then looked beyond the *Trema* species at other genera in the same family, the Ulmaceae, and again found hybridization to haemoglobin gene sequences in the genus *Celtis* and possibly in *Ulmus* (Fig. 3; Bogusz *et al.*, 1988).

Tissue specific expression of haemoglobin genes

The observation that the haemoglobin gene in *Trema* was expressed in roots and not in leaves, is a possible example of organ specific gene regulation. If this were
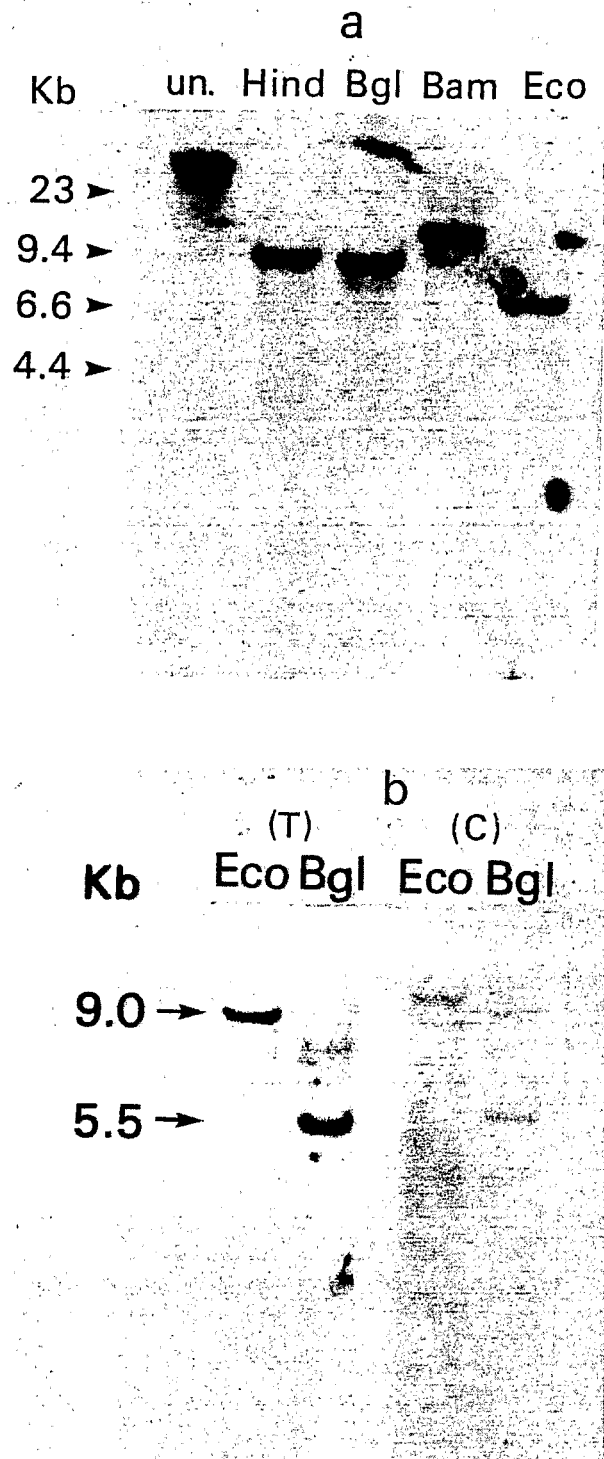

Figure 3. Southern blot hybridization of genomic DNA isolated from (a) *Parasponia* and (b) *Trema* (T) and *Celtis* (C). DNA was digested with the restriction enzymes indicated and probed with radioactively labelled cDNA (pL209, Landsmann *et al.*, 1986) of *Parasponia* haemoglobin. The simple patterns of labelled bands suggest a single haemoglobin gene in *Parasponia* and *Trema* and probably a single gene in *Celtis*.

the case it would strengthen our assumption that haemoglobin has survived in plant species which do not undergo nodulation because of specific and essential functions in particular plant tissues. We therefore asked, in *Parasponia*, whether we could detect haemoglobin in non-nodule as well as in nodule tissue and if so, was it found only in cells of roots? The answer to both questions was in the affirmative. We detected haemoglobin of the same molecular weight as the nodule haemoglobin in the roots of aseptically grown *Parasponia* plants (Bogusz *et al.*, 1988). We also detected haemoglobin mRNA in the roots but not in leaf tissue. It appears that the single *Parasponia* haemoglobin gene is expressed at high level in nodule tissue, is not expressed in leaves and is expressed at a low level in root tissues; thus the *Parasponia* gene has two modes of regulated expression.
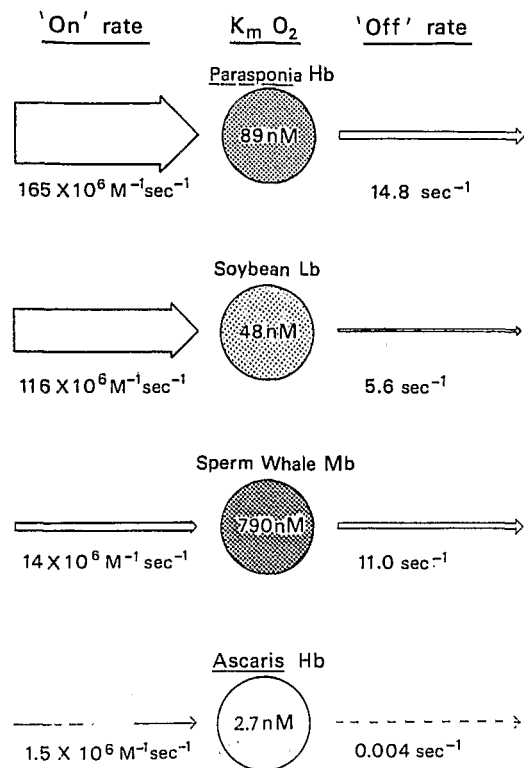
Stougaard, Petersen & Marcker (1987) demonstrated nodule specific expression of a soybean haemoglobin gene in transgenic *Lotus*, another genus of the legume family. We asked a similar question about the root expression of the *Parasponia* gene by introducing it into *Nicotiana tabacum*, choosing *Nicotiana* only because of its facility as a host in *Agrobacterium tumefaciens*-mediated gene transfer. Both the complete *Parasponia* gene and a chimaeric gene which had the upstream region (the promoter) of the *Parasponia* haemoglobin gene linked to a bacterial chloramphenicol acetyl transferase reporter gene, were expressed in roots of the transgenic *Nicotiana* plants (Landsmann *et al.*, 1988). In some transformants a low level of expression was also detected in leaf tissue. Since any expression of a gene depends both on the promoter DNA sequence and regulatory factors, generally proteins, which bind to the DNA sequences, we conclude that this experiment has shown that tobacco has regulatory proteins which interact with the *Parasponia* haemoglobin gene promoter to give an organ specific pattern of expression. It is therefore possible that *Nicotiana* has its own haemoglobin gene functioning in its root cells, but this needs direct confirmation by observations of mRNA or protein.

In *Casuarina*, one of the other plant species which does nodulate and is known to have haemoglobin in its nodules, we found that we could also detect haemoglobin in roots which had been grown in sterile culture, without nodule formation. In this case the molecular weight of the haemoglobin in the root tissue differed from that of the haemoglobin in nodule tissue (C. A. Appleby *et al.*, unpublished observations), suggesting that different genes could be encoding these haemoglobins. So, in *Casuarina* there may well be different haemoglobin genes involved in the production of haemoglobin in normal root tissue and in nodule tissue, each gene having a tissue-specific pattern of expression. In *Parasponia*, on the other hand, where there is a single gene, there appear to be two patterns of expression possible for that gene, one for roots and one for nodules.

Properties and functions of nodule haemoglobin

As a prerequisite to speculation about root haemoglobin function we summarize what is known about nodule haemoglobin with respect to its oxygen binding properties and functions. Contrary to a persistent view that haemoglobin in nodules serves as a barrier to prevent oxygen from reaching an oxygen-sensitive nitrogenase enzyme (Dickerson & Geis, 1983), it is now clear that this protein functions to provide an adequate supply of oxygen to the terminal oxidases of the symbiotic bacteria (Appleby, 1984). This occurs at a stabilized free oxygen level sufficient for those oxidases to function, but low enough to prevent inactivation of the nitrogenase enzymes also located in the bacteroids. The key features of nodule haemoglobin which suit it to this function are high oxygen affinity and fast oxygen turnover rates, with $K_m O_2$ (see Fig. 4) values lying between 27–135 μmol m^{-3} (Q. H. Gibson *et al.*, manuscript in preparation). The rate limiting step for oxygen turnover in haemoglobins is generally the oxygen 'off' rate as shown diagramatically in Fig. 4. The high oxygen affinities of non-legume (*Parasponia*) and legume (soybean) nodule haemoglobins are shown to be due to extremely fast oxygen 'on' rates and moderately fast 'off' rates. Of course, a high oxygen affinity does not guarantee the efficient facilitation of oxygen diffusion. Fig. 4 shows that for *Ascaris* perienteric haemoglobin ($K_m O_2 = 2.7$ μmol m^{-3}) the oxygen 'off' rate is only 0.004 s^{-1}. This resulted in an oxygen turnover rate, when the haemoglobin was used in a model system, too slow for efficient bacteroid respiration (Wittenberg *et al.*, 1974). On the other hand, although sperm whale myoglobin has a relatively fast oxygen 'off' rate, its low overall oxygen affinity means that it functions best, in model experiments, at a stabilized oxygen concentration which is high enough to limit nitrogenase activity (Bergersen, 1980, 1982). These comparisons indicate that the various haemoglobins have evolved different kinetic properties appropriate to the specialized conditions in which each functions.

Since spectrophotometric examination of leghaemoglobin in intact or minimally damaged legume nodules exposed to air show it to be only 5–20% oxygenated (Klucas *et al.*, 1985), it can be calculated that the free oxygen concentration in symbiotic tissue of legume nodules might be only 1–10 μmol m^{-3}, whereas leghaemoglobin-bound oxygen could be at least 10–100 mmol m^{-3} (10,000–100,000 times greater), depending on leghaemoglobin concentration (Bergersen, 1982; Appleby, 1985). Model experiments in which nitrogen fixing bacteroids isolated from soybean or other legume nodules were mixed with purified partially-oxygenated leghaemoglobin, showed that rapid transfer of oxygen occurs by a process of facilitated diffusion, enabling the bacteroids to respire vigorously and make the ATP necessary for nitrogen fixation (Wittenberg *et al.*, 1974; Bergersen, 1982). At the same time the buffered free

'On' rate K_m O_2 'Off' rate

Parasponia Hb

89 nM

165 $\times 10^6$ M^{-1}sec^{-1} 14.8 sec^{-1}

Soybean Lb

48 nM

116 $\times 10^6$ M^{-1}sec^{-1} 5.6 sec^{-1}

Sperm Whale Mb

790 nM

14 $\times 10^6$ M^{-1} sec^{-1} 11.0 sec^{-1}

Ascaris Hb

2.7 nM

1.5 $\times 10^6$ M^{-1}sec^{-1} 0.004 sec^{-1}

Figure 4. The oxygenation reactions of some plant and animal haemoglobins. The 'on' rates represent the oxygen combination rate constant k', and the 'off' rates represent the oxygen dissociation rate constant k. As shown elsewhere (Appleby, 1974), k/k' = K', the equilibrium dissociation constant, the measure of oxygen affinity. The value of K' is numerically equal to the Michaelis constant K_m O_2, the term used here and in the text; this is the free dissolved oxygen concentration at which the haemoglobin will be half combined with oxygen. When used in relation to oxidase function, K_m O_2 indicates the oxygen concentration at which oxidase turnover rate is half maximal. In this figure the widths of arrows approximately indicates the quantitative relationships among 'on' rates and 'off' rates respectively. Note that for the circles representing K_m O_2, heavy stippling indicates a high numerical value (low oxygen affinity), and light stippling a low numerical value (high affinity). For 'Parasponia Hb' rates see Wittenberg et al., (1986), for 'Soybean Lb' see Appleby et al., (1983), for 'Sperm whale Mb' and 'Ascaris Hb' rates see Wittenberg et al., (1974).

oxygen concentration, 1–10 μmol m^{-3} in the host cell cytoplasm where leghaemoglobin is located, is apparently too low to affect the activity of the oxygen-intolerant nitrogenase enzyme complex located inside the bacteroids. It may be helpful to emphasize that in similar model experiments in the absence of leghaemoglobin, where attempts are made to keep the concentration of dissolved oxygen concentration low (< 10 μmol m^{-3}), the flux of oxygen is far too low to sustain efficient bacteroid respiration. Only when partially-oxygenated leghaemoglobin, or another high affinity carrier protein, is present, providing a far greater total oxygen concentration than is available as free dissolved oxygen does facilitated oxygen diffusion occur.

Predicted properties and functions of root haemoglobin

No root haemoglobin has yet been purified in an amount sufficient for analysis of its oxygen-binding properties, but since there appears to be only one haemoglobin gene in Parasponia (Fig. 3) and since Parasponia root and nodule haemoglobins have the same molecular weight, we assume that this single gene codes for both proteins, and that they therefore have the same oxygen affinity. Furthermore, since Trema root haemoglobin has an amino acid sequence very similar to that of Parasponia nodule haemoglobin (see Fig. 1) we assume that it too has the same high oxygen affinity.

On the basis of these assumptions, together with a few additional facts and some reasonable analogies, we have attempted to make a comparison of oxygen relationships and of haemoglobin in Parasponia nodules and in aerobic roots (Table 1). Inspection of this table immediately raises doubt that the function of haemoglobin in roots could be to facilitate the flux of oxygen necessary for root mitochondrial respiration. We note that Parasponia nodules have the relatively high average concentration of haemoglobin necessary for facilitation of oxygen flow to the vigorously respiring Rhizobium bacteroids. On the other hand the volume-averaged concentration of haemoglobin in Parasponia aerobic roots is much lower and is probably less than the concentration of free dissolved oxygen. We therefore doubt that haemoglobin could have a role in overall oxygen flow or oxygen storage within the root tissue. It is possible that the haemoglobin could be concentrated in a small number of cells, perhaps in rapidly respiring cells near the root cap or in the zone of elongation, where it could be present at a concentration sufficient to facilitate oxygen flow. This is an obvious area for further experiment.

In animals, muscle myoglobin functions as an oxygen carrier at an oxygen concentration corresponding to the lower end of its oxygen dissociation range, where this protein would be substantially deoxygenated; in skeletal muscle the K_m O_2 for the mitochondrial cytochrome oxidase (Cole et al., 1982) is much lower than the K_m O_2 for muscle myoglobin (cf. Fig. 4). Similarly, leghaemoglobin functions at the lower end of its oxygen dissociation range (1–10 μmol m^{-3}) during Rhizobium bacteroid respiration in legume nodules (Bergersen & Turner, 1988), and we have recently found this to be true for bacteroids from Parasponia nodules (Bergersen, Turner & Appleby, manuscript in preparation). In contrast, plant haemoglobin would have to function at the upper end of its oxygenation range if it were to supply oxygen to root mitochondrial oxidases (K_m O_2 of approximately 100 μmol m^{-3}; Rawsthorne & La Rue, 1986) in non-nodule tissue; it is likely that in Parasponia roots the oxidases of mitochondria respire at oxygen concentrations one to two orders of magnitude greater than the oxidases of the nodule bacteroids. In itself this is not a

convincing reason for rejecting a facilitated oxygen diffusion role for root haemoglobin but it deepens our unease and has led us to think of other possible functions.

Given the oxygen binding properties of root haemoglobin and the probable oxygen concentrations in roots, what might we deduce about haemoglobin function in normal root cells? Plant roots undergo a major change in metabolism when starved of oxygen (Davies, 1980; Roberts *et al.*, 1984a,b). This anaerobic response is widespread in the plant kingdom and features a changeover in the mechanism of energy generation, from oxidative phosphorylation associated with the tricarboxylic acid cycle, to alternative fermentative pathways involving both lactate and ethanol as terminal electron acceptors. In this metabolic changeover, important for survival of plants under anoxic conditions such as occur during flooding, normal protein synthesis stops and a specific set of genes is induced (Sachs, Freeling & Okimoto, 1980).[3] These genes code for many of the key enzymes of the fermentative pathways (Lazlo & St. Lawrence, 1983; Kelley & Freeling, 1984; Springer *et al.*, 1986) and their mRNAs are selectively translated under the hypoxic conditions (Gerlach *et al.*, 1982) so that polypeptide synthesis is mostly concerned with producing the enzymes necessary for energy generation by fermentation. The necessity for fermentative metabolism has been demonstrated for the final step in the ethanol pathway. Maize seedlings lacking the alcohol dehydrogenase I enzyme do not survive an anaerobic treatment (Schwartz, 1969).

Very little is known of the way in which plant root cells sense oxygen levels and are able to bring about changes in patterns of transcription and translation so that the fermentative metabolism is operative under conditions of low oxygen tension. Could haemoglobin be involved in the sensing pathway? From Table 1 we can predict that in normally aerated roots the haemoglobin would be substantially oxygenated, in contrast to the low level of oxygenation of haemoglobin in nodule tissue. Below the level of oxygen concentration which would lead to the shut-down of mitochondrial oxidative phosphorylation (say 5–$10 \ \mu\text{mol m}^{-3}$ free dissolved oxygen) the root haemoglobin would become substantially deoxygenated. Perhaps the change in concentration of deoxyhaemoglobin is the beginning of a cascade path for the initiation of the anaerobic response mechanism. This implies that haemoglobin interacts with another molecule which can detect its state of oxygenation, perhaps through a change in its conformation.

Evolutionary path of the plant haemoglobin gene

The haemoglobin genes of legumes, and of *Parasponia* and *Trema* in the Ulmaceae, have identical exon/intron structures (Landsmann *et al.*, 1986; Bogusz *et*

[3] See article by Bailey-Serres, J. in this issue of *Plant, Cell and Environment.*

Table 1. Haemoglobin and oxygen status in Parasponia

	Nodules	Aerobic roots
Haemoglobin[1] conc.	100 μM	$\sim$100 nM
Oxyhaemoglobin[2] conc.	5-20 μM	($\sim$100 nM)
Free O_2 conc	(1-10 nM)[3]	($<$1.4 μM)[4]
Principal Oxidase $K_m O_2$	(5-10 nM)[5]	(100 nM)[6]
Haemoglobin $K_m O_2$	89nM[7]	(89 nM)

[1] From C.A. Appleby, unpublished observations. These are volume averaged concentrations; in nodules the concentrations in symbiotic tissue may be ten times greater, in roots the distributon is unknown.

[2] See text.

[3] All values in parenthesis are guesses based on observations made on other nodules or root tissues.

[4] cf. Armstrong & Gaynard (1976): values for roots of rice and cotton grass.

[5] cf. Bergersen & Turner (1988); values for the principal bacteroid oxidase of soybean nodules.

[6] cf. Rawsthorne & La Rue (1986); value for the mitochondrial oxidase of cowpea hypocotyls.

[7] Wittenberg *et al.* (1986).

al., 1988); this fact and the sequence relatedness of the genes (*Parasponia/Trema*, 93% in the coding region; *Trema*/soybean, 49% in the coding region) point to a single origin of these genes in a plant ancestral to these widely separated angiosperm families. Amino acid sequence comparisons show that the haemoglobin in these plants is related to that in *Casuarina* (Fig. 1) and it seems likely, from known amino acid sequence data, that the sequences of other plant haemoglobins will fit in with the assumption of a single evolutionary origin of the plant and animal haemoglobin genes. The gene sequence and amino acid homology data make it highly improbable that there were separate horizontal gene transfers into the progenitors of the Leguminaceae, Ulmaceae and Casuarinaceae. However, logically it is still possible, despite the lack of knowledge of any animal group containing a three intron haemoglobin gene, to entertain the possibility that there was a horizontal transfer of the gene from the animal kingdom into a precursor of the flowering plant families (cf. Runnegar, 1984). We consider this less probable than a common origin of the haemoglobin gene in both animal and plant kingdoms from a precursor organism which contained the gene.

Horizontal transfer to the angiosperm stock must remain a possibility, because at present haemoglobin and haemoglobin genes are known only in scattered families within the dicotyledonous angiosperms. If haemoglobin could be detected in cereal plants (monocotyledonous angiosperms) and in other plant groups such as the gymnosperms (pines and cycads), pteridophytes (ferns) and bryophytes (mosses and liverworts) then the alternative possibility of a single origin of the haemoglobin gene from a progenitor

common to both animals and plants would be very much preferred. Haemoglobin-like molecules have been reported in yeast (Oshino *et al.*, 1973) and in a bacteria (Appleby, 1969; Wakabayashi, Matsubara & Webster, 1986) but the available biochemical data indicate they are not closely related to the plant and animal haemoglobins.

The availability of haemoglobin gene probes and antibodies from both Leguminosae and Ulmaceae haemoglobins have increased the chances of detection of the gene or protein in other plant groups, but the limitations set by nucleotide divergence and by amino acid substitution may still prevent the ready detection of haemoglobins, or their genes, over a wide range of plant groups. There have been claims of leghaemoglobin-like sequences in the DNAs of several plants, both monocots and dicots, on the basis of Southern hybridization experiments (Roberts, Jafar & Mullin, 1985a,b). In their experiments all plant species showed leghaemoglobin related fragments of similar sizes. Because of the size identity and because the intensity of hybridization was equal in the heterologous species, *Alnus* and *Casuarina*, to that of the homologous (relative to the probe) soybean, we are reluctant to accept their conclusions. Cloning and sequencing of the fragments is needed to substantiate the claims.

Evolution of nodulation and nodule haemoglobins

Nodulation results from a symbiotic association between plant roots and a bacterium capable of fixing atmospheric nitrogen into forms suitable for uptake by the plant as a nutrient (Bergersen, 1982). Our observation of haemoglobin in normal plant roots (Bogusz *et al.*, 1988) suggests that it is widespread in plants, that it has a function in normal roots and that the specialized association of haemoglobin with nitrogen fixing nodules in the relatively few plants which have evolved nodulation is a secondary development. Our observations suggest that the haemoglobin gene has been one of the several genes in plants which were able to be recruited for particular service in the specialized structure and environment of the nitrogen fixing nodule. The kinetic parameters of reversible oxygen binding of plant haemoglobin are ideally suited to the provision of a steady, low-concentration flow of oxygen to the bacterial oxidases for energy geneation in the nitrogen fixing process (Bergersen, 1982; Appleby, 1984, 1985).

In *Parasponia* there seems to be only one haemoglobin gene sequence (Landsmann *et al.*, 1986), so the low level expression of haemoglobin in normal root tissue (Bogusz *et al.*, 1988) must be under a regulatory control different from the high level expression that occurs in nodule tissue. it is known that in other genes a single coding sequence may have two alternative sets of upstream controls (Benyajati *et al.*, 1983). It will be of interest to us to compare the promoter regions of the *Parasponia* and *Trema* genes to determine if there are controls being used specifically for nodule tissue

expression in *Parasponia*. We do know that the haemoglobin mRNA transcription start is identical in *Parasponia* nodules, *Trema* roots or roots of transgenic tobacco plants carrying the *Parasponia* haemoglobin gene, suggesting that the same TATA box is being used. The presence of upstream elements in the genes which are specific for either nodule or root expression remains a likely possibility. On the other hand the level of expression of haemoglobin may be sensitive to the concentration of haem. Haem is made by bacteria, and following bacterial infection, high levels of haem could induce high levels of expression of the haemoglobin gene in nodules (Appleby, 1984), much higher than would occur in the normal root cells. However in soybeans nodulated with a mutant of *Bradyrhizobium japonicum* apparently deficient in haem synthesis, apoleghaemoglobin is synthesized by the plant indicating that its regulation is not exclusively under the control of haem (O'Brian, Kirshbom & Maier, 1987).

The recruitment of the haemoglobin gene for function in nodule tissue in *Casuarina* appears to have evolved by a different path. We have not yet examined the gene sequences in *Casuarina* but we know that the nodule and root tissues have haemoglobins of different molecular weights so there are likely to be separate genes encoding these proteins. Differential expression of genes frequently occurs following a gene duplication event, with one gene copy becoming specialized for a function in a particular tissue different from the tissue in which the other gene copy is expressed. This is a common evolutionary strategy.

In the case of legumes there are multiple copies of the nodule haemoglobin genes and these are thought to have arisen by gene duplication (Bojsen *et al.*, 1983; Lee *et al.*, 1983). We, and Stougaard *et al.*, (1987) have not been able to detect expression of any of these haemoglobin genes in the normal root cells of legumes. We suggest that there may be another haemoglobin gene or genes encoding the legume root haemoglobin and that we have not yet been able to detect the gene sequence by hybridization because of the nucleotide divergence that has occurred since the gene duplication event.

We are suggesting that nodulation has evolved independently in many of the plant families in which it is now observed. In families which are not closely related to each other, the structural features of nodules differ markedly and the symbiont may differ. In legumes and *Parasponia*, *Rhizobium* species are the symbiotic bacteria, whereas in *Casuarina*, *Alnus*, *Myrica* and some other plant species the actinomycete *Frankia* is the symbiotic organism (Tjepkema, 1983; Appleby, 1984). In the legumes, in *Parasponia*, and in *Casuarina*, there has been a supervention of haemoglobin for specialized function in the nodule. However, it seems likely that some nodule structures could be such that recruitment of the haemoglobin gene may not have been necessary. This may be the case with the actinomycetous nodules in *Datisca glomerata* (Tjepkema & Asa, 1987) and in nodules formed by symbio-

sis between *Cyanobacteria* and the roots of cycads (Grilli Caiola, 1980) where haemoglobins have not been found. It appears that in these nodules other physiological or anatomical structures have evolved to regulate the supply of a low concentration of oxygen (Tjepkema, Schwintzer & Benson, 1986).

Concluding remarks

Our recent data have shown that, in plants, haemoglobin occurs in normal root tissue as well as in nodule tissue. Previously, haemoglobin had been demonstrated only in nodule tissue in legumes and in some non-legume plants, but we now know that the haemoglobin gene is expressed in non-nodule tissue of the roots of *Parasponia* and *Casuarina* and we expect that this is the case in the legumes as well. We have not examined other organs or other stages of development for the expression of haemoglobin. It is possible that its function may be important in other organs or tissues where oxygen deficits arise, e.g. in the developing endosperm and embryo.

We have also shown that the gene occurs and is expressed in plant species that are related to *Parasponia* but which do not have any symbiotic relationship with a bacterium to form nodules. The demonstration of the gene and its products in these non-nodulating species has so far been limited to species which are taxonomically close to *Parasponia*; a *Parasponia* antibody and *Parasponia* gene probes have given positive results in the related species. It may be possible, either with protein or gene probes, gradually to extend the range of plants in which haemoglobin or its gene can be demonstrated. It seems to be a reasonable conclusion, given the wide phyletic distribution of haemoglobin in the dicotyledonous angiosperms, that the gene is present in all such plants.

If haemoglobin is present only in the dicotyledonous flowering plants it is possible that there has been a horizontal gene transfer from the animal kingdom (Runnegar, 1984), but if haemoglobin or haemoglobin genes can be demonstrated in other plant groups, especially the evolutionarily more primitive ones such as gymnosperms, pteridophytes or bryophytes, then it becomes much more probable that the haemoglobin gene of plants, which is certainly related to the haemoglobin gene of animals, has had a linear evolutionary path from an organism ancestral to both animals and plants.

A corollary of this postulate is that haemoglobin would be functional in both the animal and plant lineages. There has been molecular evolution within the known animal haemoglobins to give characteristics suitable for their specialized environments, e.g. monomeric myoglobin in muscle or tetrameric haemoglobin in erythrocytes (Dickerson & Geis, 1983) so we must not automatically suppose that haemoglobin in plants will necessarily have the same oxygen affinity and intracellular function in all extant species. Nevertheless in those species, in unrelated families, where

the nodule haemoglobin has been examined, the characteristics of the molecule are essentially similar; it is a haemoglobin with high oxygen affinity and fast turnover rate suited to a role in facilitating oxygen diffusion at low partial pressures of oxygen.

Parasponia is a key species in thinking about the function of haemoglobin in root cells because our Southern hybridization data (Fig. 3) suggest that only one haemoglobin gene sequence is present in this species (Landsmann *et al.*, 1986). Presumably the haemoglobins have the same kinetic characteristics with respect to oxygen binding (Table 1). On the basis of the characteristics of the *Parasponia* haemoglobin we have considered what the function in root cells might be. This is limited to some extent because we, as yet, know nothing of its cellular or intracellular distribution in the root. Given a low intracellular concentration, similar to or lower than the free dissolved oxygen concentration, it is unlikely that haemoglobin functions to facilitate oxygen supply to the mitochondrial oxidases which are presumably the key oxygen consuming molecules in these cells. We have no data directly suggesting an alternative function, but even at low concentration the molecule could be important in the recognition of oxygen tension in root cells and in the subsequent deployment of oxidative or non-oxidative pathways of energy generation.

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