Plant Molecular Biology **24**: 853–862, 1994.

A cereal haemoglobin gene is expressed in seed and root tissues under anaerobic conditions

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Received 25 October 1993; accepted in revised form 10 December 1993

Key words: aleurone, anaerobiosis, barley, haemoglobin, RNA

Abstract

Legumes, and a very few non-legume plant species, are known to possess functioning haemoglobin genes. We describe here the characterization of a haemoglobin cDNA isolated from barley. The deduced amino acid sequence shows 71% amino acid identity with a non-legume haemoglobin gene, a further 16% of the residues being conservative replacements. The barley cDNA also hybridizes to genomic sequences in rye, maize and wheat. The demonstration of a gene from a monocotyledon with close sequence homology to the known non-legume plant haemoglobins fills a major gap in the known distribution of haemoglobin genes in the plant kingdom. The expression of the gene is induced in isolated barley aleurone layers exposed to anaerobic conditions, and the roots of flooding-stressed barley plants. The expression of the RNA under anoxic conditions is similar to that of a known anaerobic response gene, alcohol dehydrogenase. Our results suggest that the increased expression of haemoglobin RNA is an integral part of the normal anaerobic response in barley. The findings are discussed in the light of current theories of haemoglobin function and evolution.

Introduction

Plant haemoglobins (Hb) in the form of leghaemoglobins (Lb) have, for many years, been known to exist in the root nodules of legumes [27, 30]. Haemoglobins have more recently been positively identified in two closely related members of the Ulmaceae *Parasponia andersonii* (nodulating) and *Trema tomentosa* (non-nodulating), and in one species *Casuarina glauca* of the Casuarinaceae [8, 29]. There is considerable support [8, 10, 14, 31] for the hypothesis that plant and animal Hbs have a common evolutionary origin. However, the inability to demonstrate the presence of an Hb gene in monocotyledons has left open the possibility of inter-kingdom horizontal transfer to the dicots [8, 31]. There have been no reports which unequivocally demonstrate the presence of an Hb gene in a monocotyledon [3, 6], although claims of Lb-like sequences in the genomes of a number of non-legumes have appeared [36, 37]. These, however, were based only on Southern blotting

The nucleotide sequence data reported will appear in the GenBank Nucleotide Sequence Database under the accession number U01228.

experiments and the results have been questioned [3]. Several authors [8, 31] have noted the limitations which have frustrated attempts at cross species screening using available antibody and DNA probes.

The role of Lb in legume symbioses has been well-documented [2]. It is believed to act as an oxygen carrier in the symbiosomes of root nodules, supplying oxygen to the bacterial respiratory chain whilst preserving a low free-oxygen concentration [2]. The sequences of other plant Hb proteins are quite distinct from those of the Lb type and have been reviewed in some detail by Arredondo-Peter and Escamilla [6]. Current knowledge of the properties of non-legume plant Hb is based largely upon studies of the *Parasponia* protein. This protein has a very high oxygen affinity (Km 89 nM), and shows rapid oxygen rebinding (geminate reaction) [17, 43]. Transgenic studies have shown that the *Parasponia* Hb promoter is activated in the root meristem and vascular bundle in tobacco, and additionally expressed in root nodules of *Lotus* [9, 31].

The main body of work on the response of plants to anaerobiosis has dealt with glycolytic metabolism. Seed and root tissues that are tolerant of hypoxic conditions respond to oxygen deficit by increased utilization of glycolytic pathways [28]. In barley aleurone tissue, for example, low oxygen stress results in increased expression of alcohol dehydrogenase and lactate dehydrogenase RNA and protein [20, 22, 26]. Whilst changes in glycolysis are clearly major factors in plant adaptation to hypoxia, there are probably other factors involved. It is likely that under otherwise anoxic conditions roots can receive some oxygen input by axial movement from the aerial parts of the plant [4, 5, 11]. It has also been suggested that facilitated diffusion of oxygen and the accompanying buffering of oxygen tensions may be significant factors for cellular metabolism [40].

We have isolated a barley cDNA clone which has strong sequence homology to other non-legume Hbs. We have examined the Hb transcript levels, under varying exposures to oxygen stress, in comparison to the response of alcohol and lactate dehyrodegenase, two glycolytic enzymes that have been studied extensively in the normal anaerobic response of aleurone tissues. We also examined the behaviour of the gene in root tissues under flooding (hypoxic) conditions. On the basis of our results, we suggest that all monocots, and probably all plants, may in fact possess an Hb gene. Our evidence also indicates that the expression of the barley Hb gene is highly induced in response to anaerobic stress, and that this property of the gene is not limited to barley. The implications of these findings are discussed.

Materials and methods

Materials

Wheat (cv. Biggar), rye (cv. Musketeer) and maize were available locally. Barley seeds (cv. Harrington) were provided by the Canadian Grain Commission, Winnipeg, Manitoba. The Adh 1 probe was a gift from E. S. Dennis, and the barley Ldh probe was a gift from A. D. Hanson.

Library screening and sequence analysis

Clones were isolated from a λgt22 cDNA library of RNA from aleurone layers of Harrington barley half seeds, incubated with 1 μM gibberellic acid for 5 days [11, 34]. The initial Hb cDNA was isolated serendipitously in an immunological screen for other proteins. This cDNA was then used as a probe to identify full-length clones. The library was screened with the radiolabelled Hb cDNA probe according to Maniatis *et al.* [32]. cDNA inserts were subcloned into bluescript SK plasmid, and sequenced using a Gibco/BRL cycle sequencing kit. Sequence analysis and manipulation were carried out using the FASTA and FSAP packages and other software [15, 16, 35].

Southern blotting

Genomic DNA samples were prepared from seedling leaves by a modification of the CTAB

method [38] and aliquots of 10 or 20 μg DNA were digested with appropriate restriction enzymes before size fractionation on agarose gels in Tris-acetate buffer. DNA was blotted onto Hybond N^+ (Amersham) membrane in 20 × SSC overnight and fixed by alkali and UV treatment. Hybridisations were carried out overnight in a mixture containing 6 × SSC, 5 × Denhardt's solution, 1% SDS, 100 μg/μl herring testis DNA, at 65 °C. Washes were carried out at 65 °C in 0.5% SDS plus varying concentrations of SSC. Washes were initially performed in 2 × SSC, then in 1 × SSC, 0.5 × SSC, or finally 0.2 × SSC as appropriate. Filters were exposed to Kodak XAR-5 film with screens at −75 °C. Fragment sizes were estimated relative to Gibco/BRL size standards.

Northern blotting

Total RNA was prepared from frozen seedling leaves, seedling roots, coleoptiles of 10 day dark germinated seeds, and aleurone layers (usually batches of 75 aleurone layers per sample) according to the procedure described by Mohapatra *et al.* [33]. RNA was quantitated by UV absorption spectroscopy [32] and size-fractionated on agarose/formaldehyde gels [32]. Loading equivalence and RNA integrity were confirmed by ethidium staining of gels prior to transfer. RNA was blotted to GeneScreen (NEN) or Hybond N^+ (Amersham) membranes according to the manufacturer's instructions, and fixed with UV. Hybridisation, washing, and exposure to film, were carried out as described for southern blotting. Transcript sizes were estimated relative to Gibco/BRL RNA size standards, which were visualised by methylene blue staining [32]. Blots were re-probed in sequence with Hb probe, Ldh probe and Adh probe.

Preparation and use of probes

Purified plasmid inserts were radioactively labelled according to standard procedures [32]. Labelling was performed using ^{32}P-dCTP (ICN), unincorporated label was removed by spinning through Sephadex G50 (Pharmacia). Probes were added at concentrations of $1-5 \times 10^6$ cpm per ml of hybridisation fluid. Higher probe concentrations were used for cross-species Southern blots where signal strengths were extremely low.

Preparation of aleurone layers

Aleurone layers from barley (*Hordeum vulgare* cv. Harrington) were isolated by normal procedures [11, 34]. Briefly, embryo-free barley half seeds were sterilised in 0.25% bleach for 30 min, rinsed four times for 5 to 10 min in sterile water. After 3 days imbibition at 22 °C, aleurone layers were separated. Twenty or 25 layers were placed in sterile 50 ml conical flask containing 3 ml H_2O, 1 μM GA, 10 μg/ml chloramphenicol, 10 mM $CaCl_2$, and incubated on a rotary shaker running at 65 cycles/minute, at ca. 24 °C.

Oxygen stress treatments

Isolated aleurone layers were incubated under controlled oxygen tensions essentially as described by Hanson and Jacobsen [22]. For time course incubation under N_2, each flask was flushed with high purity N_2 for 2 min, then flasks were placed in 3 litre jars, which were in turn purged with N_2 for 1 h. The jars were reflushed with N_2 for 30 min every 6 h thereafter. For varied oxygen tensions, aleurone flasks were placed in 1-litre jars; these were sealed and flushed with the appropriate mixture of O_2 and N_2 gases for 15 min. Jars were re-purged with the same gas mixture every 3 to 6 h. Samples were harvested at appropriate time points, frozen in liquid nitrogen, and stored at −75 °C.

Flooding experiments

Batches of 10 barley or maize seeds were planted in a mixture of peat, sand, and soil and allowed

to germinate normally up to the two leaf stage. Individual pots were used for each treatment. At the start of the experimental treatment, an appropriate number of pots were placed in a deep tray of water, so as to completely submerge the root system up to soil level. For sampling, the contents of whole pots were removed and washed to clear the roots of soil and debris. Root tissue was frozen in liquid nitrogen and used to prepare total RNA as described above.

Extraction and assay of ADH and LDH

Batches of 6 aleurone layers were homogenised using a blender (Polytron, Brinkman) at top speed for 30 s in a 10 ml plastic tube containing 2 ml ice-cold isolation buffer (0.5 M Tris-HCl pH 8.0, 10 mM DTT). The homogenate was clarified by centrifugation at 10000 rpm (12000 × *g*) in a Sorvall SS34 rotor for 20 min. The supernatants were transferred to 1.5 ml microfuge tubes and respun at 10000 rpm for 20 min. The supernatants were used for assays. Lactate dehydrogenase (EC 1.1.1.1) and alcohol dehydrogenase (EC 1.1.1.27) assays were performed exactly as described by Hanson and Jacobsen [22]. Total protein concentrations were standardised using a Biorad protein assay kit with γ-globulin as a standard. Enzyme activity was expressed in IU (μmol of NADH oxidised or reduced per minute). Each time point represents the results of three separate measurements.

Results

Cloning and sequencing

During the course of immunologically screening an aleurone cDNA library with antiserum for the 1,6-α-glucosidase, limit dextrinase, a cDNA was isolated which encoded a protein with strong sequence homology to *Parasponia andersonii* and *Trema tomentosa* Hbs [8, 31]. It is possible that this is due to some contaminant in the protein used to raise antiserum, but the serum does not detect an Hb-sized protein on western blots. The initial cDNA isolate represents bases 354 to 706 of the sequence presented here (Fig. 1). This fragment was used as a probe to re-screen the library yielding a number of clones of varying lengths, the sequence of the longest clone is the basis for the data presented. Partial sequencing of a number of independent clones indicated no rearrangement of the cDNA sequence during cloning. It also revealed no variations in nucleotide sequence between different clones.

The complete open reading frame encodes a predicted translation product 162 amino acids long (17.8 kDa). The predicted protein sequence shows 71% amino acid identity with *Parasponia* Hb, with a further 16% of residues being conservative replacements. The sequence is clearly distinct from the Lb class of proteins, relative to which it shows ca. 40% sequence identity and a further 40% of conserved changes. Residues conserved between known dicotyledons and animal Hb [6] are also conserved in the barley Hb. These residues are underlined in Fig. 1. The nucleotide sequence is likewise highly conserved relative to other non-legume Hbs, being 70% homologous to *Parasponia*. Sequence comparisons of different plant Hbs have revealed a consensus for nine specific residues amongst those which show rapid oxygen rebinding (geminate plus) [17], six of these are also conserved in the barley and *Parasponia* sequences. Two of the changed positions (Thr-92 and Gln-149) are identical in the barley and *Parasponia* sequences (see Fig. 1). The barley protein also shares the cysteine residue (residue 79 in the barley protein) seen in all the sequenced non legume plant Hbs and in none of the sequenced Lbs [6]

The haemoglobin DNA sequence is present in the genomic DNA of barley and other cereals

Southern blotting (Fig. 2a) confirmed that the cDNA sequence was present in the barley genome. Hybridising fragments were ca. 5.0 kb with *Bam* HI, 4.9 kb with *Bgl* II, 7.5 kb with *Hin*d III, and 4.0 kb with *Bam* HI and *Hin*d III combined.

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        CTGGCCAAACCATTCAGTCGTCGTGCCCACTTACGAGAACGAAATTAAGCGGGAAGGAAGCCATGTCTGCCGCGGAGGGGGCGCGTCGTCTTCAGC  95
blyhb                                                                   M  S  A  A  E  G  A  V  V  F  S   11
parhb                                                                      +  S  S  +  V  N  K  +  +  T   10

        GAGGAGAAGGAGGCGCTGGTGCTCAAGTCATGGGCCATCATGAAGAAGGATTCCGCCAACCTTGGGCTCCGCTTCTTCGTCAAGATCTTCGAGATC 191
blyhb   E  E  K  E  A  L  V  L  K  S  W  A  I  M  K  K  D  S  A  N  L  G  L  R  F  F  L  K  I  F  E  I   43
parhb   +  +  Q  +  +  +  +  V  +  A  +  +  V  +  +  +  N  +  +  E  +  +  +  Q  +  +  +  +  +  +  +   42

        GCGCCGTCGGCGAGGCAGATGTTCCGCGTTCGCTGCGCGACTCCGACGTGCGCGCTGGAGACCAACCGCCAAGCTCAAGACCCACGGCCGTGTCCGTCTTC 287
blyhb   A  P  S  A  R  Q  M  F  P  F  L  R  D  S  D  V  P  L  E  T  N  P  K  L  K  T  H  A  V  S  V  F   75
parhb   +  +  +  +  K  N  L  +  S  Y  +  K  +  +  P  +  +  +  +  Q  +  +  +  +  +  P  +  +  -  T  T  +   73

        GTCATGACCTGCGAGGCGGCTGCGCAGTTGCGGAAAGCCGGCAAGATCACCGTCAGGGAGACCACCCTGAAGAGGCTGGGCGGCACGCACTTGAAA 383
blyhb   V  M  T  C  E  A  A  A  Q  L  R  K  A  G  K  I  T  V  R  E  T  T  L  K  R  L  G  G  T  H  L  K  107
parhb   +  +  +  +  +  S  +  V  +  +  +  +  +  +  V  +  +  K  +  S  D  +  +  +  I  +  A  I  +  F  +  105

        TACGGCGTGGCAGATGGCCACTTCGAGGTGACGCGGTTCGCTCTGCTCGAGACGATCAAGGAGGCGCTTCCGGCTGACATGTGGGGGCCCGAGATG 479
blyhb   Y  G  V  A  D  G  H  F  E  V  T  R  F  A  L  L  E  T  I  K  E  A  L  P  A  D  M  W  G  P  E  M  139
parhb   T  +  +  V  N  E  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  V  +  -  E  +  +  S  +  +  +  136

        AGGAACGCGTGGGGCGAGGCATACGATCAACTGGTCGCGGCCATCAAGCAAGAGATGAAGCCAGCTGAGTAGCTCCACCGCACTCATATACCACGC 576
blyhb   R  N  A  W  G  E  A  Y  D  Q  L  V  A  A  I  K  Q  E  M  K  P  A  E                              162
parhb   K  +  +  +  +  V  +  +  +  +  +  +  +  +  +  +  F  +  +  +  +  S  S  T                        160

        CATTTTCGCCGATTGTCCGTTCAACCTTCGTTGCTTCACCAATTCACTCATTTCACCGTTGTGTTTGTATTGTGTGTTTATGTGCACTAAAGTGTA 672
        TTGTAACACTCAATCAAAAGTACAAATTATGCACGATATTCACCCCGCTCTACTTTACAGTTTTTTTTTTCTGTTAGAGGAACTCTGATTTAGAGG 768
        TTATGCCTCGTACTAGTACATTGCAATACGCTTAGAAAAGATGCTTAAATAAATAAATCAATGTTTTTTAGGCACGCGTGCTTATATGTATCGAGT 864
        AAACGTTTAGCCTGTATAAATGAACACCCGTGCTTCAGAAAAACGGGTTTATTTTTTA$_n$                                      924
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Fig. 1. Nucleotide sequence of a barley Hb cDNA clone with predicted translation product (blyhb), showing similarity to *Parasponia* Hb [8] (parhb). + indicates identical residue, – indicates gap inserted to optimise similarity. Amino acid residues conserved between animal and dicot Hbs [6] are underlined. Positions of the nine residues conserved amongst the geminate plus (fast oxygen rebinding) Hbs are shaded. Thr-92 and Gln-149 replace Leu and Val, respectively, in the geminate plus consensus (double-underlined) [17]. The conserved cysteine (Cys-79) is also indicated (double-underlined). Nucleotide and protein sequences are numbered.

The results suggest that in barley, like in *Parasponia* [8], the gene is present at very low copy number. Lower-stringency washes did not indicate the presence of multiple, related sequences. None of the blotting results suggest the presence of a multigene family as seen with the Lbs.

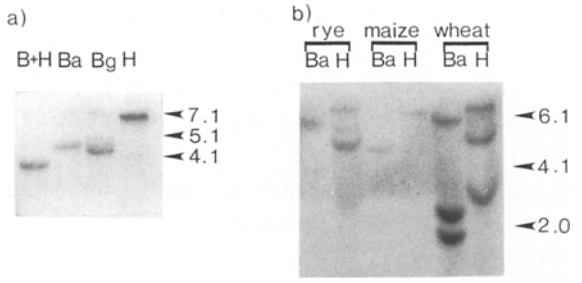

Fig. 2. Southern blots probed with barley Hb cDNA. a. Barley DNA digested with, *Bam* HI and *Hind* III (B + H), *Bam* HI (Ba), *Bgl* II (Bg), *Hind* III (H). b. Rye, maize and wheat DNA, digested with *Bam* HI (Ba) or *Hind* III (H). Samples were run on a 1% agarose gel and blotted as described in Materials and methods. Positions of size markers are indicated in kb. Unhybridised probe was removed by washing in 2× SSC and then 0.5× SSC/0.5%; SDS (rye, maize, wheat) or 0.2× SSC/0.5%; SDS (barley), at 65 °C. Blots were exposed to film at −75 °C.

The barley probe also hybridised to genomic sequences in a number of other cereals (Fig. 2b), even at quite high stringency. In rye, *Bam* HI gave a 4.8 kb hybridising band, and *Hind* III, 7.5 kb and 5.0 kb bands. In maize, *Bam* HI gave a fragment of 4.8 kb and *Hind* III 7.5 kb. Within the limits of cross-species probing, it seems that in both of these cases, as in barley and *Parasponia*, the gene is present at low copy number. Again, even at low stringency no evidence was seen for a multigene family as observed amongst the Lbs. In hexaploid wheat, there are three hybridising fragments with each enzyme, *Bam* HI giving bands at 6.3, 2.4 and 1.8 kb, and *Hind* III giving bands of 7.6, 5.0 and 3.8 kb. It is possible that each of the three bands represents one chromosome complement. We cannot rule out the possibility, however, that these three bands represent a gene family.

The haemoglobin RNA is expressed in roots and aleurone layers

Northern blot analysis of barley RNA indicated a transcript of ca. 900 nucleotides, which was present in both total and poly(A)$^+$ RNA (Fig. 3a and b). A transcript was easily detectable in total RNA isolated from the roots of seedlings at the one- and three-leaf stages. There was no detectable expression of the transcript in leaf tissue of the same plants. This is consistent with the expression pattern seen in the non-legume dicot species which have been examined [3, 8].

In addition to its expression in root tissue, the Hb transcript can be detected at very low levels in coleoptile tissue (Fig. 3a) and in oxygen-stressed aleurone layers (Fig. 3b) of imbibed half seeds.

Effect of O_2 concentration on the expression of haemoglobin, alcohol dehydrogenase and lactate dehydrogenase mRNA expression

Since the expression of Hb message increased in response to O_2 deprivation, a series of experiments were conducted in which aleurone layers were incubated under imposed oxygen concentrations ranging from 20% to 0% O_2 for a period of 24 h. Hb mRNA was not detectable at 20% and 10% O_2 was barely visible at 5% O_2 and became easily detectable at 2% and 0% O_2 (Fig. 4a). The same blots used to examine the Hb message were re-probed for Adh and Ldh transcripts. Adh transcript levels increased steadily as the O_2 concentration was decreased from 10% to 0%. Ldh message was noticeably elevated between 2% and 10% O_2 with a lower but easily detectable basal level of expression in 0% and 20% O_2.

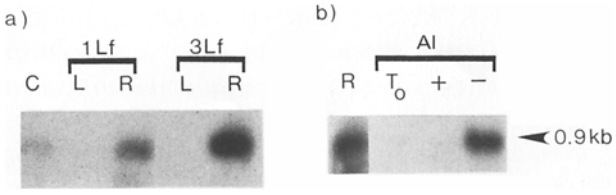

Fig. 3. Northern blots of barley RNA probed with barley Hb cDNA. a. 30 μg total RNA from, coleoptiles (C), leaves (L), and roots (R). Growth stages of 1 leaf and 3 leaf are indicated. b. 0.5 μg poly A$^+$ RNA from roots of 3-leaf stage seedlings (R). 20 μg total RNA from aleurone layers (Al) at a zero time point (T_0) and after 24 h in normal atmospheric conditions (+), or oxygen deficient conditions (−). Approximate transcript size is shown in kb. Gels were run and blotted as described in Materials and methods. Blots were washed in 0.2× SSC at 65 °C,

Temporal expression of haemoglobin, alcohol dehydrogenase and lactate dehydrogenase RNA in aleurone layers under anoxic conditions

The expression of Hb, Ldh and Adh messages was examined in aleurone layers under sustained anoxia (Fig. 4b). Increased levels of Hb message were first observable after 1 h of incubation under N_2, and continued to increase up to 12 h, the last sampling point in this experiment. No significant changes in Hb message level were observed beyond 12 h (results not shown). Increases in Adh and Ldh transcript levels also became apparent after the first hour of anoxia. After the first two hours of treatment changes became less pronounced. High basal levels of Ldh message were apparent in the 0 h and 12 h control (incubated in normal air) samples.

Changes in enzyme activity of ADH and LDH under anoxia and hypoxia

As a confirmation that the aleurone preparations were behaving normally under oxygen stress, activity assays for LDH and ADH enzymes were carried out on aleurone layers exposed to a range of imposed O_2 concentrations, and under sustained anoxia. When aleurones were incubated under O_2 concentrations ranging from 20% to 0% (Fig. 4a, top), the results indicated that ADH activity remained low until the O_2 concentration dropped below 10%, activity then increased steadily up to a maximum in 0% O_2 LDH activity rose as the O_2 concentration was decreased to

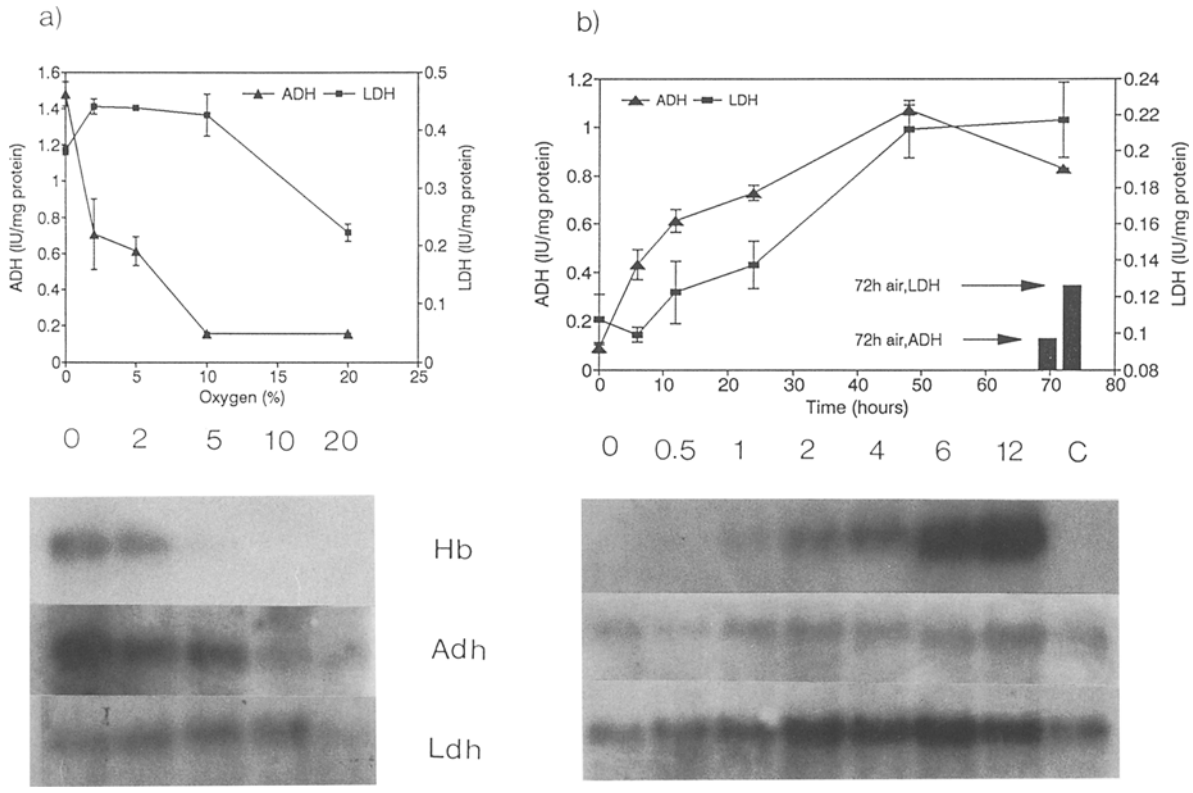

Fig. 4. Response of aleurone layers to (a) different oxygen concentrations, and (b) sustained anoxia. Northern blots were probed in sequence with Hb probe, Ldh probe, and Adh probe. Each lane carries 15 μg total RNA, blots were washed at 0.2 × SSC 65 °C. In a, numbers represent percentage oxygen, from 0 to 20%. In b, numbers represent time in hours after beginning the experiment, from 0 to 12 hours. Graphs show the corresponding changes in ADH and LDH enzyme activity observed in similar experiments. The standard deviation for each measurement is shown. The time-course of enzyme activities shown in b is over 72 h to allow direct comparison with the results of Hanson *et al.* [22]. Gels were run and blotted as described in Materials and methods. Blots were washed in 0.2 × SSC at 65 °C.

10%, remained relatively constant over the range of 10% to 2% O_2, then declined slightly at 0% O_2.

Under sustained anoxia (Fig. 4b, top) ADH activity increased rapidly during the first 12 h, and continued to increase up to 48 h. Following a slight decline in the first 6 h, LDH activity increased steadily until 48 h. A comparison of anoxic and oxygenated aleurone layers after 72 h indicated about an 8-fold increase in ADH activity and less than a 2-fold increase in LDH activity.

These results are very similar to those of Hanson and Jacobsen [22]. They confirm that the aleurone preparations behaved normally in response to low oxygen stress. They place results regarding Hb transcription in the context of a normal anaerobic response.

Levels of the haemoglobin transcript are increased in flooded roots of barley and maize

In view of the increase in Hb transcript levels in oxygen-stressed aleurone layers it was important to know if this finding was also relevant to the gene in its major site of expression, root tissue. Barley and maize roots were examined for Hb RNA expression under flooding stress. Figure 5 shows RNA samples from the flooded and unflooded plants, probed with the Hb cDNA. After a 12 h period of flooding stress, both maize and

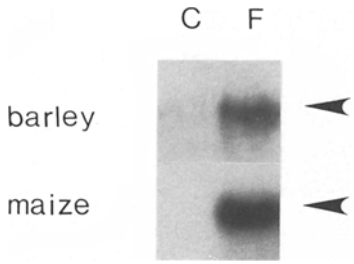

Fig. 5. Hb transcript expression in RNA from the roots of barley and maize plants. Lanes are (C) control roots, and (F) flooded root systems, completely submerged in water for 12 h. Each lane carries 15 μg of total RNA. Gel was run and blotted as described in Materials and methods. Blot was washed in 0.2× SSC at 65 °C.

barley expressed greatly increased levels of Hb RNA. In this exposure the Hb transcript is barely visible in control roots, but is clearly visible in the flooding-stressed samples.

Discussion

It is now generally accepted that the known animal and dicot plant Hbs evolved from a common ancestor about 1400 million years ago [10, 14, 31]. However, in the absence of a monocot Hb gene, the possibility that the dicot gene is a result of interkingdom horizontal transfer has remained troubling [3]. The demonstration of a cereal Hb gene confirms that it is present in two of the major divisions of the plant kingdom. This, in turn, makes it all the more likely that an Hb gene is indeed present in all plants [3, 6, 14], and perhaps serves a common function there [3]. The conservation of kinetically important residues between the barley and *Parasponia* Hbs (Fig. 1) suggests that the barley protein is functional and that it may share kinetic properties with the *Parasponia* protein. Some authors have suggested that the identification of a monocot Hb gene may circumvent one conceptual obstacle in the generation of artificial nitrogen-fixing symbioses involving monocot plants [3].

Based on the available information concerning the expression pattern and kinetic properties of non-legume plant Hb, two major hypotheses have been put forward to account for the expression of the protein in non-nodulating plants. One involves Hb-facilitated diffusion of oxygen within the root tissues [3, 9]. Another proposes a role as an oxygen sensor, detecting changing levels of environmental oxygen and causing the switch from aerobic to anaerobic metabolism [3, 9]. There is already evidence from work on mammalian and rhizobial systems [18, 19] that at least some oxygen sensors are haem proteins. However, work on the response of *E. coli* to low oxygen stress suggests that the switch mechanism need not rely on monitoring oxygen levels at all [39]. Work on a range of experimental systems has suggested a number of other functions for proteins of the Hb family, including reduction of metal ions [7], and a role as a terminal oxidase [12, 13]. These and many other possibilities have been extensively reviewed by Wittenberg and Wittenberg [42].

Whether the major role of plant Hb is as an oxygen sensor, or as a facilitator of oxygen diffusion, the expression of the gene would be expected in organs such as roots and seed tissues (the aleurone and embryo itself) which might be expected to experience oxygen stress [3, 22]. Aside from one unconfirmed report of an Hb protein in seed tissues [25], there have been no previous reports of Hb expression other than in root and root nodule tissues.

If the Hb protein has some functional role in the anaerobic response (as a facilitator of oxygen diffusion for example), one would expect it's RNA levels to be up-regulated under low oxygen stress. That is, it should behave in much the same way as other genes involved in the anaerobic response [1, 22, 24, 41]. Experimental analysis showed that if barley aleurone layers are subjected to a low oxygen regime (Fig. 4), the level of the Hb RNA does indeed increase dramatically. The increase in transcript levels in response to low oxygen levels is apparent at lower O_2 concentrations for Hb than it is for Adh or Ldh. The timing of the Hb response to anoxia is similar to that of the Adh and Ldh genes, the increase in Hb transcript levels seems to be relatively large. Taken together with the changes in ADH and LDH enzyme activity, these results indicate that the induction of

Hb RNA expression is occurring within the framework of a normal anaerobic response [20, 22, 23]. The Hb RNA induction parallels rather than anticipates the expression of the Adh and Ldh messages. This would not be expected if the only function of the protein were as an oxygen sensor triggering the anaerobic response and thus the increased expression of these two transcripts.

An examination of the expression of the Hb RNA in roots of both barley and maize gives a similar picture, the gene is again very responsive to low oxygen stress. The results demonstrate that this responsiveness to O_2 levels is not limited to the aleurone system. It is also important to note that the Hb gene shows same O_2 responsiveness in at least two different monocot species.

The expression of an Hb gene in roots and aseptically cultured aleurone layers of a monocot with no known symbiotic partners strengthens the proposition that plant Hbs do have a more universal function than their role in symbioses [3, 31, 42]. If this is true, together with the high level of sequence conservation between the barley and dicot non-legume Hbs, it provides some circumstantial support for the proposal that even legumes may still possess a copy of the 'non-Lb' gene, from which the Lbs themselves have arisen for specialist purposes by gene duplication and subsequent evolution [3, 31]. Such a conserved locus could easily have been overlooked due to the well documented problems inherent in cross species screening with available Hb probes [3, 31]. In our own work, we have observed that Lb probes and barley Hb probes do not cross hybridise even at very low stringency and in the presence of a large excess of the target sequence and the probe (unpublished data).

Hb transcription in a well characterised cell system, the barley aleurone layer, provides a sufficiently defined system for transfection experiments to directly address the question of Hb function and its possible role in the anaerobic response [21, 22]. A clearer picture of this response is crucial to a better understanding of the factors which lead to flooding tolerance in some cereals and not in others [24]. We are currently pursuing experiments designed to directly assess the physiological significance of the Hb protein.

Acknowledgements

This work was funded by A Research Grant OGP4689 from the Natural Sciences and Engineering Research Council of Canada. We thank Doug Durnin for technical assistance and Ma Luo for the aleurone cDNA library. X.Z.N and E.R.T contributed equally to this work.

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