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Molecular Characterization and Expression Analysis of Hemoglobin cDNA from Small Radish (*Raphanus sativus* L. var. *sativus*)

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We have characterized *RSHb*, a hemoglobin cDNA clone isolated from a seedling cDNA library of the small radish (*Raphanus sativus* L. var. *sativus*). Its 480-bp ORF encodes a protein of 160 amino acids, with a predicted molecular weight of 17.98 kDa and an estimated pI of 8.77. Sequence analysis and multiple alignment revealed that the *RSHb* shares 95% nucleotide sequence and 93% amino acid sequence identities with those of *AHB 1* from *Arabidopsis thaliana*, a Class I non-symbiotic hemoglobin. Our Southern blot analysis showed that *RSHb* is a single-copy gene in the small radish genome. The northern blot analysis demonstrated that this gene is expressed in all organs, including the roots, hypocotyls, and leaves, with transcription levels increasing under flooding and high-sucrose conditions. However, cold treatment does not affect its expression pattern. We conclude that *RSHb* is a Class I non-symbiotic hemoglobin gene.

Keywords: cold, dehydration, flooding, non-symbiotic hemoglobin, small radish

Hemoglobins are present in a broad range of organisms, from bacteria to unicellular eukaryotes, plants, and animals (Sowa et al., 1998). They function by binding and transporting oxygen and other gaseous ligands. In higher plants, two families of hemoglobin are distinguished – symbiotic and non-symbiotic. Genes of the first family are expressed at high concentrations in the nodules, where their products facilitate oxygen diffusion to the respiring nitrogen-fixing symbionts (Appleby, 1984). Those of the second family have been identified both in nitrogen-fixing plants, such as *Parasponia* (Appleby et al., 1983) and soybean (*Glycine max*; Andersson et al., 1996), and in non-nitrogen-fixing species, including *Trema tomentosa* (Bogusz et al., 1998), *Arabidopsis* (Trevaskis et al., 1997), barley (*Hordeum vulgare*; Taylor et al., 1994), and rice (*Oryza sativa*; Arredondo-Peter et al., 1997). Based on those discoveries, Appleby (1992) has proposed that hemoglobin may be present in all plant species.

Two dissimilar non-symbiotic hemoglobin genes have now been described from *Arabidopsis thaliana*: 1) *AHB 1*, which is similar to the classical non-symbiotic hemoglobin in a number of plant species; and 2) *AHB 2*, more closely related to the symbiotic hemoglobins from legumes and *Casuarina* (Trevaskis et al., 1997). Non-symbiotic hemoglobins are expressed in various plant organs, depending on species (Bogusz et al., 1990), and are commonly over-expressed either under hypoxic conditions (Taylor et al., 1994; Trevaskis et al., 1997; Arechaga-Ocampo et al., 2001) or when ATP synthesis is blocked (Seregelyes et al., 2000). In *Arabidopsis*, increased transcription of two non-symbiotic hemoglobin genes occurs in response to different environmental stimuli. Like other Class I hemoglobins, the *AHB1* gene can be induced by adding sucrose to the growth medium, decreasing the oxygen concentration, or placing the plants under hypoxia (Jacobsen et al., 1995; Nie et al., 1997; Trevaskis et al., 1997). However, *AHB2*, a Class II gene, responds not to those stimuli, but to treatment with low temperatures (Trevaskis et al., 1997). These contrasting response patterns suggest different functions for the Class I and Class II proteins (Hunt et al., 2001).

Sowa et al. (1998) and Durner et al. (1999) have proposed that non-symbiotic hemoglobins may serve as O_2 carriers or scavengers, or as substrate for nitric oxide synthase. Because their functional role is still not clear, the objective of the current study was to isolate and characterize a hemoglobin clone from small radish, and to analyze its expression levels in different organs and in response to stress treatments.

MATERIALS AND METHODS

Plant Growth

Seeds of the small radish (*Raphanus sativus* L. var.

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sativus) were surface-sterilized in 70% ethanol for 30 s, then in a 1% sodium hypochlorite solution for 5 min. After being washed three times in deionized water (dH_2O), they were soaked in dH_2O for 24 h and germinated for 3 or 4 d on a wet paper towel in the dark at 28°C. The seedlings were then held in a growth chamber (28°C, 12-h photoperiod) for another 3 d. Afterward, they were either subjected to the stress treatments or transferred to soil and returned to the growth chamber for another three weeks before their organs were sampled.

PCR Reaction for Probe Preparation

Ten μL of a cDNA library (Kwon, 2000) was placed in boiled water for 10 min, then centrifuged. The supernatant was used as a template for the PCR reaction. Degenerate primers were designed from the conserved regions of plant non-symbiotic hemoglobins (Andersson et al., 1996; Trevaskis et al., 1997) as follows: nonsym-F (forward primer) 5'AT(A/C)TT(C/T)GAGAT(C/T)GC(A/G)CC(A/G)(A/Y)C3' and nonsym-R (reverse primer) 5'AC(C/T)GC(C/T)TC(C/T)TT(G/T)AT(G/C)GTCTC3'. The PCR program involved denaturation at 94°C for 5 min, followed by 30 cycles of annealing at 42°C for 1 min and polymerization at 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR fragment was then cloned into a pGEM T-Easy cloning vector (Promega).

Screening of cDNA Library for *RSHb*

Approximately 100,000 pfu of the cDNA library were placed with XL1-blue cells on six plates, and the phage DNA was transferred onto a Hybond N nylon membrane (Amersham). The filters were denatured in 1.5 M NaCl and 0.5 N NaOH for 3 min; neutralized in 0.5 M Tris-HCl (pH 7.5) and 1.5 M NaCl for 5 min; and rinsed in 2×SSC [0.15 M NaCl/0.015 M sodium citrate (pH 7.6)] for 30 s. Afterward, they were dried briefly on Whatman 3 MM paper, and baked at 80°C for 2 h. The library was screened with the PCR fragment labeled with [α-^{32}P]dCTP, using a random primer labeling kit (Promega) as a probe. Hybridization was carried out according to the method of Sambrook et al. (1989).

Sequencing and Analysis of *RSHb*

Nucleotide sequencing of the PCR fragment clone and a positive cDNA clone (*RSHb*) was carried out as described by Sanger et al. (1977). The Sequenase Version 2.0 DNA sequencing Kit was used with [α-^{35}S] dATP. Sequence analyses were carried out by the Blast Search program, the ExPASy system (Molecular Biology Center, Geneva, Switzerland), and the Clustal W program.

DNA Isolation and Southern Hybridization

Genomic DNA was purified from seedlings of small radish according to the method of Ausubel et al. (1987). Briefly, 10 μL was digested with BamHI, EcoRI, PstI, XhoI, or SmaI, and separated on a 0.7% agarose gel. The DNA was then transferred to a Hybond N nylon membrane and hybridized at 61°C for 20 h with the *RSHb* insert, which was labeled with [α-^{32}P]dCTP in a hybridization buffer (1 mM EDTA, 0.25 M sodium phosphate, 1% BSA, and 7% SDS). Afterward, the filters were washed with 2×SSC at 61°C for 1 h, and 1×SSC/0.1% SDS at 61°C for 30 min, followed by visualization via autoradiography at –80°C.

RNA Isolation and Northern Hybridization

Total RNA from the samples of different organ types was isolated with guanidine-isothiocyanate buffer (Chomczynski and Sacchi, 1987). After being dissolved in diethylpyrocarbonate (DEPC)-treated distilled water and stored at –80°C, it was separated on a 1% formaldehyde-agarose gel in MOPS (3-[N-morpholino] propanesulfonic acid) buffer. The gel was blotted onto a Hybond-NX membrane (Amersham), baked at 80°C for 2 h, and hybridized with the [α-^{32}P]dCTP-labeled inserts from *RSHb*. Hybridization and washing conditions were the same as for the Southern analysis.

Stress Treatments

For the flooding treatment, 30 seedlings were completely submerged in a deep tray of water. Treatment with sucrose involved placing the seedlings in a 5% solution (Logemann et al., 1987). Seedlings from both these experiments were then incubated in a growth chamber for 24 h. For the dehydration treatment, we incubated the seedlings on a shaker (50 rpm) in the dark for 24 h at 28°C in a 0.6 M mannitol solution (Dolferus et al., 1994). This procedure proved to be faster and more reproducible than the air-drying method described by Lang and Palva (1992). Samples were then frozen in liquid nitrogen and used to prepare total RNA, as described above.

RESULTS AND DISCUSSION

A Non-Symbiotic Hemoglobin cDNA from Small Radish

Using degenerate primers, we amplified a 270-bp fragment of a hemoglobin gene from the cDNA of small radish. The partial sequence showed 91% homology with an *Arabidopsis* non-symbiotic hemoglobin, *AHB 1* (data not shown). To isolate the full-length cDNA clone, we sub-cloned the PCR fragment into a pGEM T-Easy cloning vector and used it as a probe. After the primary and secondary library screenings of approximately 100,000 pfu, a 683-bp-long hemoglobin cDNA clone, encoding 160 amino acids, was isolated. Its nucleotide sequence (GenBank Accession No. AY286331) showed that *RSHb* has a 5' untranslated region of 50 bp, a 480-bp coding region, and a 153-bp 3' untranslated region. The deduced amino acid sequence shared strong homology (about 93%) with those from other non-symbiotic hemoglobins. The predicted ORF also contained amino acid residues (Fig. 1) that are highly conserved in plant hemoglobins: both proximal (H104) and distal (H69) histidines, F50 and P43 (Ota et al., 1997). RSHb also possessed the Cys residue, C78, which is conserved in non-symbiotic hemoglobins (Arredondo-Peter et al., 1997). Therefore, these results demonstrate that

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Trema          -------MSSS EVDKVFTEEQ EALVVKSWAV MKKNSAELGL KFFLKIFEIA PSAKNLFSYL  54
Parasponia     -------MSSS EVNKVFTEEQ EALVVKAWAV MKKNSAELGL QFFLKIFEIA PSAKNLFSYL  54
Glycine        --------MTT TLERGFSEEQ EALVVKSWNV MKKNSGELGL KFFLKIFEIA PSAQKLFSFL  53
Arabidopsis    --------MES EGKIVFTEEQ EALVVKSWSV MKKNSAELGL KLFIKIFEIA PTTKKMFSFL  53
Raphanus       --------MES EGKIVFTEEQ EALVVKSWSV MKKNSADLGL KLFIKIFEIA PTAKKLFSFL  53
Oryza1         MALVEDNNAV- --AVSFSEEQ EALVLKSWAI LKKDSANIAL RFFLKIFEVA PSASQMFSFL  58
Oryza2         MALVEGNNGVS GGAVSFSEEQ EALVLKSWAI MKKDSANIGL RFFLKIFEVA PSASQMFSFL  61
                                 * *** **** ***   ***    **  **** ** * ***   ** *

Trema          KDSPIPLEQN PKLKPHAMTV FVMTCESAVQ LRKAGKVTVR ESNLKRLGAI HFKNGVVNEH  114
Parasponia     KDSPVPLEQN PKLKPHATTV FVMTCESAVQ LRKAGKVTVK ESDLKRIGAI HFKTGVVNEH  114
Glycine        RDSTVPLEQN PKLKPHAVSV FVMTCDSAVQ LRKAGKVTVR ESNLKKLGAT HFRTGVANEH  113
Arabidopsis    RDSPIPAEQN PKLKPHAMSV FVMCCESAVQ LRKTGKVTVR ETTLKRLGAS HSKYGVVDEH  113
Raphanus       RDSPIPAEQN PKLKPHAMSV FVMCCESAAQ LRKTGKVTVK ETTLKRLGAN HSKYGVVDEH  113
Oryza1         RNSDVPLEKN PKLKTHAMSV FVMTCEAAAQ LRKAGKVTVR DTTLKRLGAT HLKYGVGDAH  118
Oryza2         RNSDVPLEKN PKLKTHAMSV FVMTCEAAAQ LRKAGKVTVR DTTLKRLGAT HFKYGVGDAH  121
               *  *** * *** ***  * ****** * * **********   ** ***  *   **   *

Trema          FETR-FALLE TIKEAVP-EM WSPEMKNAWG EAYDQLVAAI KSEMKPSST  161
Parasponia     FEVTRFALLE TIKEAVP-EM WSPEMKNAWG VAYDQLVAAI KFEMKPS--  160
Glycine        FEVTKFALLE TIKEAVP-EM WSPAMKNAWG EAYDQLVDAI KSEMKPPSS  161
Arabidopsis    FEVAKYALLE TIKEAVP-EM WSPEMKVAWG QAYDHLVAAI KAEMNLSN-  160
Raphanus       FEVTKYALLE TIKEAVP-EM WSPEMKSAWG QAYDHLVAAI KAEMKPSH-  160
Oryza1         FEVVKFALLD TIKEEVPADM WSPAMKSAWS EAYDHLVAAI KQEMKPAE-  166
Oryza2         FEVTRFALLE TIKEAVPVDM WSPAMKSAWS EAYNQLVAAI KQEMKPAE-  169
               ***   **** ***** *  * *** ** **   **  ***** * *****
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Figure 1. Amino acid sequence alignment of RSHb and selected plant non-symbiotic hemoglobins. Conserved residues of heme and ligand bindings are shown in bold, and Cys residues are underlined. Asterisks indicate the most conserved residues. Trema, *T. tomentosa*; Parasponia, *P. andersonii*; Glycine, *G. max*; Arabidopsis, *A. thaliana*; Raphanus, *R. sativus*; Oryza, *O. sativa*.

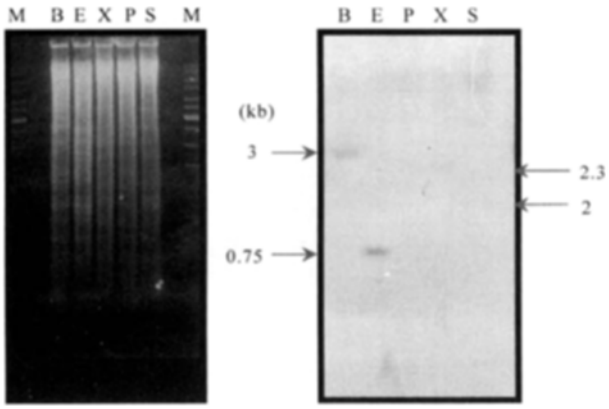

Figure 2. Genomic DNA Southern analysis. Ten μg genomic DNAs digested with BamHI, EcoRI, PstI, XhoI, and SmaI (B, E, P, X, and S, respectively), and hybridized with *RSHb* probe. M: 1-kb ladder DNA size marker.

RSHb encodes a plant non-symbiotic hemoglobin.

Genomic Blot Analysis of *RSHb*

To determine the copy number of *RSHb*, we performed DNA-DNA hybridization of the genomic DNA. Single bands were detected from the digests of BamHI (3 kb), EcoRI (0.75 kb), XhoI (2.3 kb), and SmaI (2 kb), indicating that the *RSHb* gene is present in single copy (Fig. 2). In *Arabidopsis*, *AHB 1* is also present as a single or low-numbered copy, whereas *AHB 2* is multicopied (Arredondo-Peter et al., 1997; Trevaskis et al., 1997). Generally, plant non-symbiotic hemoglobin genes exist as singles or low copies (Taylor et al., 1994; Jacobsen et al., 1995; Andersson et al., 1996; Seregelyes et al., 2000).

Expression Pattern of *RSHb* in Different Organs

Based on our northern hybridization, expression of *RSHb* was detectable, albeit at low levels, in all organ types, including the roots, hypocotyls, and leaves (Fig. 3). In contrast to symbiotic hemoglobin genes, which are expressed only in the nodules of N_2-fixing plants, non-symbiotic hemoglobin transcripts have been reported in many tissues, e.g., the root meristems of *T. tomentosa* (Bogusz et al., 1998); the root vascular bundles of transgenic tobacco (Bogusz et al., 1990); the seed aleurone and roots of barley grown under microaerobic conditions (Taylor et al., 1994); diverse organs of the soybean (Andersson et al., 1996), *Arabidopsis* (Trevaskis et al., 1997), and rice (Arredondo-Peter et al., 1997); the roots of alfalfa (*Medicago sativa*; Seregelyes et al., 2000); and the coleoptiles, seminal roots, and embryos of maize (*Zea mays*; Arechaga-Ocampo et al., 2001).

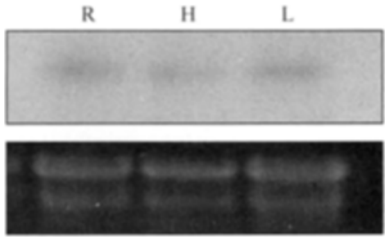

Figure 3. Northern blot analysis, in which 20 μg of total RNA was extracted from roots (R), hypocotyls (H), and leaves (L) and loaded. Blot was hybridized with *RSHb* cDNA probe.

Expression of *RSHb* under Stress Conditions

Northern blot analysis was conducted to determine whether activity of the *RSHb* gene was regulated by stress stimuli, including hypoxia, high sucrose concentrations, and dehydration. Here, all those treatments induced its expression (Fig. 4). For example, under hypoxic conditions, transcript levels reached their maximum 12 to 15 h after flooding, then rapidly declined (Fig. 4A). Likewise, time-course monitoring of the response to 5% sucrose showed a gradual increase in expression until 18 h, with a maximum recorded at 12 to 18 h, followed by a gradual decrease (Fig. 4B). Transcript levels also increased significantly after 12 h of dehydration (Fig. 4C). In contrast, the low, basal

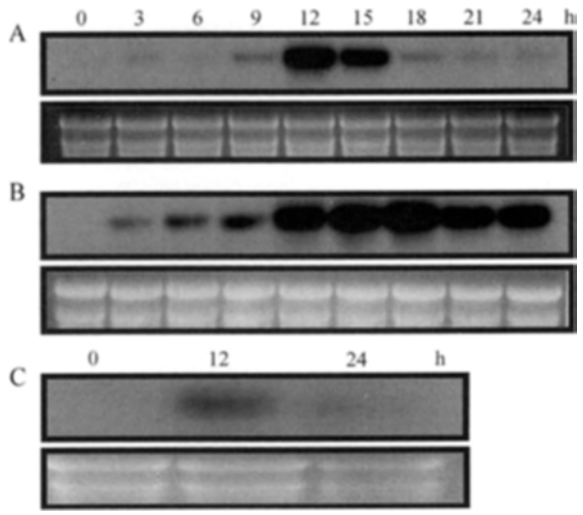

Figure 4. Time-course analysis of *RSHb* gene induction under treatment with flooding (A), 5% sucrose (B), and 0.6% mannitol (C). Twenty μg RNA was loaded and the blot hybridized with *RSHb* cDNA probe.

levels of *RSHb* transcript were not influenced by cold stress (data not shown).

In general, plant non-symbiotic hemoglobins genes are highly expressed under hypoxic conditions as well as in metabolically activated tissues. This occurs to maintain the energy status of cells in low-oxygen environments (Sowa et al., 1998). Expression of Class I non-symbiotic hemoglobin genes is also increased by hypoxia and sucrose treatments (Jacobsen et al., 1995; Nie et al., 1997; Trevaskis et al., 1997), but not by low temperatures (Trevaskis et al., 1997). Therefore, our results further demonstrate that *RSHb* is a member of that Class I gene group.

We are currently analyzing the promoter region of the *RSHb* genomic clone and measuring the kinetics and binding affinity of recombinant RsHb and its mutants for ligands. Our efforts should help in better understanding the function and regulation of non-symbiotic hemoglobin genes in plants.

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LITERATURE CITED

Andersson CR, Jensen EO, Llewellyn DJ, Dennis ES, Peacock WJ (1996) A new hemoglobin gene from soybean: A role for hemoglobin in all plants. Pro Natl Acad Sci USA 93: 5682-5687

Appleby CA (1984) Leghemoglobin and *Rhizobium* respiration. Annu Rev Plant Physiol 35: 443-478

Appleby CA (1992) The origin and functions of haemoglobin in plants. Sci Prog 76: 365-398

Appleby CA, Tjepkema JD, Trinick MJ (1983) Hemoglobin in a non-leguminous plant *Parasponia*: Possible genetic origin and function in nitrogen fixation. Science 220: 951-953

Arechaga-Ocampo E, Saenz-Rivera J, Sarath G, Klucas RV, Arredondo-Peter R (2001) Cloning and expression analysis of hemoglobin genes from maize (*Zea mays* ssp. *mays*) and teosinte (*Zea mays* ssp. *Parviglumis*). Biochim Biophys Acta 1552: 1-8

Arredondo-Peter R, Hargrove MS, Sarath G, Moran JF, Lohrman J, Olson JS, Klucas RV (1997) Rice hemoglobins: Gene cloning, analysis, and O_2-binding kinetics of a recombinant protein synthesized in *Escherichia coli*. Plant Physiol 115: 1259-1266

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1987) Current Protocols in Molecular Biology. John Wiley & Sons, NY, pp 4.5.1-4.5.3

Bogusz D, Appleby CA, Landmann J, Dennis ES, Trinick MJ, Peacock WJ (1998) Functioning of haemoglobin genes in a non-nodulating plant. Nature 331: 178-180

Bogusz D, Llewellyn DJ, Craig S, Dennis ES, Appleby CA, Peacock WJ (1990) Nonlegume hemoglobin genes retain organ-specific expression in heterologous transgenic plants. Plant Cell 2: 633-641

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Analytic Biochem 162: 156-159

Dolferus R, Hacobs M, Peacock WJ, Dennis ES (1994) Differential interactions of promoter elements in stress response of the *Arabidopsis Adh* gene. Plant Physiol 105: 1075-1087

Durner J, Gow AJ, Sramler JS, Glazebrook J (1999) Ancient origins of nitric oxide signaling in biological systems. Proc Natl Acad Sci USA 196: 14206-14207

Hunt PW, Watts RA, Trevaskis B, Llewellyn DJ, Burnell J, Dennis ES, Peacock WJ (2001) Expression and evolution of functionally distinct haemoglobin genes in plants. Plant Mol Biol 47: 677-692

Jacobsen LK, Jensen EO, Jorgensen JE, Marker KA, Peacock WJ, Dennis ES (1995) Symbiotic and nonsymbiotic hemoglobin genes of *Casuarina glauca*. Plant Cell 7: 213-223

Kwon SI (2000) Structure and expression patterns of genes encoding catalase and superoxide dismutase from small radish seedlings. Ph.D. thesis. Seoul National University, Korea, pp 19-20

Lang V, Palva ET (1992) The expression of a *rab*-related gene, *ran18*, is induced by abscisic acid during the cold acclimation process of *Arabidopsis thaliana* (L.) Heynh. Plant Mol Biol 20: 951-962

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem 163: 16-20

Nie X, Hill RD, Nie XZ (1997) Mitochondrial respiration and hemoglobin gene expression in barley aleurone tissue. Plant Physiol 114: 835-840

Ota M, Isogai Y, Nishikawa K (1997) Structural requirement of highly conserved residues in globins. FEBS Letters 415: 129-133

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, NY

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463-5467

Seregelyes C, Mustardy L, Ayaydin F, Sass L, Kovacs L, Endre G, Lukacs N, Kovacs I, Vass I, Kiss IV, Kiss GB, Horvath GV, Dudits D (2000) Nuclear localization of a hypoxia-inducible novel non-symbiotic hemoglobin in cultured alfalfa cells. FEBS Letters 482: 125-130

Sowa AW, Duff SM, Guy PA, Hill RD (1998) Altering hemoglobin levels changes energy status in maize cells

under hypoxia. Proc Natl Acad Sci USA 95: 10317-10321

Taylor ER, Nie XZ, MacGregor AW, Hill RD (1994) A cereal haemoglobin gene is expressed in seed and root tissues under anaerobic conditions. Plant Mol Biol 24: 853-862

Trevaskis B, Watts RA, Andersson SR, Llewellyn DJ, Hargrove MS, Olson JS, Dennis ES, Peacock WJ (1997) Two hemoglobin genes in *Arabidopsis thaliana*: The evolutionary origins of leghemoglobins. Proc Natl Acad Sci USA 94: 12230-12234