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Rice Hemoglobins[1]

Gene Cloning, Analysis, and O_2-Binding Kinetics of a Recombinant Protein Synthesized in *Escherichia coli*

Raúl Arredondo-Peter*, Mark S. Hargrove, Gautam Sarath, Jose F. Moran, Joseph Lohrman, John S. Olson, and Robert V. Klucas

Department of Biochemistry, University of Nebraska, The Beadle Center, P.O. Box 880664, Lincoln, Nebraska 68588–0664 (R.A.-P., G.S., J.F.M., J.L., R.V.K.); and Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77005–1892 (M.S.H., J.S.O.)

Although nonsymbiotic hemoglobins (Hbs) are found in different tissues of dicots and monocots, very little is known about *hb* genes in monocots and the function of Hbs in nonsymbiotic tissues. We report the cloning and analysis of two rice (*Oryza sativa* L.) *hb* genes, *hb1* and *hb2*, that code for plant Hbs. Rice *hb1* and *hb2* genes contain four exons and three introns, as with all of the known plant *hb* genes. At least three copies of the *hb* gene were detected in rice DNA, and analysis of gene expression shows that *hb1* and *hb2* are expressed in leaves but only *hb1* is expressed in roots. A cDNA for rice Hb1 was expressed in *Escherichia coli*, and the recombinant Hb (rHb1) shows an unusually high affinity for O_2 because of a very low dissociation constant. The absorbance spectra of the ferric and deoxyferrous rHb1 indicate that, in contrast to symbiotic Hbs, a distal ligand is coordinated to the ligand-binding site. Mutation of the distal His demonstrates that this residue coordinates the heme Fe of ferric and deoxyferrous rHb1 and stabilizes O_2 in oxy-rHb1. The biochemical properties of rice rHb1 suggest that this protein probably does not function to facilitate the diffusion of O_2.

Hbs are widely distributed throughout higher plants, including both dicots and monocots. Comparison of protein sequences and analysis of gene expression suggest that two families of Hbs, the symbiotic and nonsymbiotic, exist in higher plants (Appleby, 1992; Andersson et al., 1996). Symbiotic Hbs are detected only in N_2-fixing nodules, but not in other plant organs or in non-N_2-fixing plants. The function of Hbs in legume nodules is to facilitate the diffusion of O_2 to the N_2-fixing bacteroids (Appleby, 1984, 1992). Nonsymbiotic Hbs are found in both dicot and monocot plants, and thus appear to be more widely distributed in higher plants than symbiotic Hbs (Appleby, 1992; Andersson et al., 1996).

A Hb that exhibits characteristics of both symbiotic and nonsymbiotic Hbs was isolated from nodules of *Parasponia andersonii*, a nonlegume infected by rhizobia (Appleby et al., 1983). The *P. andersonii hb* gene is expressed in both root nodules and noninfected organs (Bogusz et al., 1988; Appleby, 1992), and codes for a protein that has O_2-binding kinetics similar to symbiotic Hbs, which suggests that both Hbs have a similar function in nodules (Gibson et al., 1989). The *P. andersonii hb* gene contains three introns located identically to the symbiotic *hb* genes (Landsman et al., 1986). A *hb* gene has also been cloned from *Trema tomentosa*, a nonnodulating relative of *P. andersonii* (Bogusz et al., 1988) that is expressed in roots but not in leaves (Bogusz et al., 1988). Nonsymbiotic *hb* genes have been cloned from other dicots such as soybean (*Glycine max* L.) (Andersson et al., 1996) and Arabidopsis (Trevaskis et al., 1997). The soybean *hb* gene is similar to nonsymbiotic *hb*s and it is expressed in diverse organs, with the highest level of expression detected in stems (Andersson et al., 1996).

A Hb transcript from a monocot was cloned by Taylor et al. (1994) using an aleurone cDNA library from barley (*Hordeum vulgare* L.), and its corresponding gene sequence was recently deposited in the GenBank database (accession no. U94968). The amino acid sequence of the predicted barley Hb is similar to nonsymbiotic Hbs. A single copy of the *hb* gene apparently exists in barley. It is expressed in roots of plants grown under normal conditions, but it is expressed at higher levels when the plants are grown under microaerobiosis, suggesting that the expression of the *hb* gene may be associated with the anaerobic response.

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* Corresponding author; e-mail ra@unlinfo.unl.edu; fax 1–402–472–7842.

Abbreviations: DDBJ, DNA Data Bank of Japan; EST, expressed sequence tags; Hb, hemoglobin; Mb, myoglobin; RGRP, Rice Genome Research Program (Japan); rHb, recombinant hemoglobin.

Incomplete sequences of cDNAs coding for rice Hbs have been deposited in the DDBJ database, and the sequences for the predicted proteins have been published by Andersson et al. (1996). Very little information is available on the *hb* genes in monocots and the biochemical properties of nonsymbiotic Hbs, and nothing is known about the function of these proteins in plants. A nonsymbiotic Hb has been isolated from barley seeds (Duff et al., 1997), and recombinant nonsymbiotic Hbs have been studied (Duff et al., 1997; Trevaskis et al., 1997). These proteins exhibit unusual spectra when reduced and unligated and possess a very high affinity for O_2. In this work we report the cloning and analysis of two *hb* genes, *hb1* and *hb2*, from rice.

A cDNA coding for Hb1 was expressed in *Escherichia coli*, and the recombinant wild-type protein (rHb1) and a mutant in which the distal His was replaced by a Leu were spectroscopically and kinetically characterized. Our results show that rice *hb* genes are similar to other nonsymbiotic *hbs*, and are differentially expressed in roots and leaves of rice plants grown under normal conditions. rHb1 shows spectral characteristics similar to other Hbs, but the spectra for the ferric and deoxyferrous forms are unusual. Furthermore, despite the very high affinity for O_2, rHb1 appears to function through a unique mechanism in which the distal His binds to the heme Fe in the deoxyferrous state, but repositions to stabilize bound O_2, resulting in an extremely low dissociation rate.

MATERIALS AND METHODS

Plant Growth and Total DNA and RNA Isolation

Rice (*Oryza sativa* var. Jackson) seeds were germinated for 5 d and then planted in pots containing vermiculite. Rice plants were grown in a greenhouse at 22°C with light/dark periods of 16 h/8 h and watered with tap water every 3 d and with nutrient solution (Becana et al., 1991) every 6 d. Plants were grown for 5 weeks and then the roots and leaves were collected, washed, and immediately frozen. Total DNA was isolated from roots or leaves using a modification of the cetyltrimethylammonium bromide method (Doyle and Doyle, 1990). Poly(A^+) RNA was isolated from rice roots or leaves using a QuickPrep mRNA purification kit (Pharmacia) and quantitated by spectrophotometry, assuming $1A_{260}$ = 40 μg/mL (Ausubel et al., 1995).

Sequencing of Two cDNAs Coding for Rice Hb1 or Hb2

Rice cDNA clones with sequences similar to plant Hbs were generated by the RGRP (Sasaki et al., 1994) and deposited in the DDBJ database as EST sequences. We obtained the clones C741 and C2576 (DDBJ accession nos. D15507 and D38931, respectively), from the RGRP, which were fully sequenced. Clones C741 and C2576 were named rice Hb1 and Hb2, respectively.

Oligonucleotides and PCR Amplification

Primers were designed for PCR to amplify the *hb1* or *hb2* genes using the sequences that are immediately upstream and downstream from the start and stop codons of the rice Hb1 or Hb2 cDNAs. The oligonucleotide sequences were: Hb1/5′ (sense), 5′-TAAACCAGCTGTCAGGAAGCA-3′; Hb1/3′ (antisense), 5′-AGCAGCT-AGCATGCCTGTCGA-3′; Hb2/5′ (sense), 5′-AGGAATCAAATCGAAGCAGCC-3′; and Hb2/3′ (antisense), 5′-GGAGGTGGAGCAGT-ATATATA-3′. Total rice DNA (approximately 0.5 μg) was used as the template for PCR amplification. PCR components and concentrations were: 0.5 μM of each sense and antisense primer, 200 μM of each dNTP, and 0.4 unit of *Taq* DNA polymerase (Gibco-BRL) in 1× PCR buffer. PCR was done in a final volume of 10 μL using a rapid-cycling apparatus (Idaho Technology, Idaho Falls, ID). Amplification was carried out for 35 cycles at 65°C (for *hb1*) or 60°C (for *hb2*)/30 s for annealing. PCR products were isolated from the agarose gel using the GeneClean kit (Bio 101, Bio-Rad) and then cloned into the vector pCRII (Invitrogen, San Diego, CA) following standard procedures (Sambrook et al., 1989). Cloned fragments were sequenced and DNA sequences were compared with sequences deposited in the GenBank database using the BLAST program (Altschul et al., 1990). Additional computer analyses were done using the GCG package (Genetics Computer Group, Madison, WI).

Southern-Blot Analysis

The clone with the rice *hb1* gene was labeled with digoxigenin-11-dUTP (Boehringer Mannheim) by PCR as described by Lu et al. (1993). Rice total DNA was digested with restriction enzymes, and the samples were Southern-blotted using standard procedures (Sambrook et al., 1989). Membranes were hybridized at 55°C overnight with the rice *hb1* probe, washed at high stringency (60°C) in 2× SSC/0.1% (w/v) SDS twice for 5 min each, and in 0.5× SSC/0.1% (w/v) SDS twice for 15 min each, and then incubated in the NBT/x-Phosphate mix of the Genius kit (Boehringer Mannheim) to develop color.

Expression of *hb* Genes in Rice Organs

Expression of the rice *hb* gene was examined in roots and leaves of 5-week-old plants by RNA-PCR (Wang et al., 1989) using a kit (Cetus). Reverse transcription was done using 30 ng of poly(A^+) RNA as the template and oligo $d(T)_{16}$ as the primer following the manufacturer's protocol. PCR amplification was performed using the specific primers for the amplification of rice Hb1 or Hb2 cDNAs and the same conditions used for the isolation of the rice *hb1* and *hb2* genes (above), but for 40 cycles. For a positive control, ubiquitin transcripts were amplified using primers that were designed from sequences located at positions 109 to 130 (sense: 5′-ATGCAGATCTTCGTGAAGACCC-3′) and 316 to 336 (antisense: 5′-ACCTCCACGAAGGCGCAG-GAC-3′) of a rice ubiquitin cDNA (Nishi et al., 1993). PCR

products were detected in 2% (w/v) agarose gels after staining with EtBr.

Expression of the Rice Hb1 cDNA in *Escherichia coli* and Spectroscopic and Kinetic Characterization of the Recombinant Wild-Type and Mutant Proteins

A cDNA coding for rice Hb1 was amplified by PCR using the primers 5′-*CCATGG*CTCTCGTGGAGGATAAC-3′ (sense) and 5′-*GAATTC*TCACTCCGCGGGCTTCATCTC-3′ (antisense) that are located at the start and stop codons of the *hb1* gene, respectively, and were degenerated with restriction sequences for *Nco*I or *Eco*RI (underlined). PCR conditions were the same as above. The PCR fragment for Hb1 was subcloned into the expression vector pET28a (Novagen, Madison, WI) and transformed in *E. coli* as described by Hargrove et al. (1997) and Arredondo-Peter et al. (1997). The Kunkel method (Kunkel, 1985) of mutagenesis was used to generate an H74L (His→Leu) mutant starting from the cloned cDNA for Hb1. Recombinant rice Hbs (wild type and mutant) were purified by $(NH_4)_2SO_4$ precipitation and chromatography on DEAE-cellulose, and then partially sequenced from their N terminus using standard procedures (Jun et al., 1994a, 1994b). Pure recombinant Hbs were spectrophotometrically characterized as described by Arredondo-Peter et al. (1997), and O_2- and CO-binding affinities were determined as described by Hargrove et al. (1997).

RESULTS AND DISCUSSION

Sequencing of Two Rice cDNAs That Code for Hb1 and Hb2 and Analysis of the Predicted Proteins

Partial sequences (approximately 300 bp) of two cDNAs, corresponding to the clones C741 and C2576, with high similarity to nonsymbiotic *hb* genes, have been generated by the RGRP program and deposited in the DDBJ database (Sasaki et al., 1994). We fully sequenced the clones C741 and C2576 in both directions, and the sequences were compared with sequences deposited in the GenBank database. Clones C741 and C2576 were found to be highly similar to plant Hbs, so they were designated Hb1 and Hb2, respectively. The rice Hb1 and Hb2 clones are 812 and 786 bp in length, contain putative polyadenylation signals at positions 772 and 747, and code for predicted proteins of 166 and 169 amino acid residues, respectively (Fig. 1).

Sequence comparisons show that the predicted Hb1 and Hb2 proteins are 93% similar to each other, and that the rice Hbs are 68 to 82% similar to nonsymbiotic Hbs and about 50% similar to symbiotic Hbs. Rice Hbs contain distal (H77) and proximal (H112) His residues, as well as the P52, F58, F82, and F122 that are conserved in plant Hbs (Fig. 2) (Arredondo-Peter and Escamilla, 1991). Rice Hb1 and Hb2 also contain a single Cys residue, C86, that is highly conserved in nonlegume Hbs (Arredondo-Peter and Escamilla, 1991; Taylor et al., 1994; Andersson et al., 1996). Andersson et al. (1996) reported a second Cys at position 92 (numbering as in Fig. 2) from partial sequences of rice Hb1 and Hb2. However, after sequencing many clones of rice Hbs we did

A

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CCACGCGTCCGGTTGTTTTCAGAGCCCAGCTAGCTCTCGATCATTTGTTA   50
CAGAGAAATTGATCAAAGCAGGAAATTAAACCAGCTGTCAGGAAGCAATG  100
                                                 M    1

GCTCTCGTGGAGGATAACAATGCCGTAGCGGTGAGCTTCAGCGAGGAGCA  150
 A  L  V  E  D  N  N  A  V  A  V  S  F  S  E  E  Q   18

GGAGGCGCTGGTGCTCAAGTCATGGGCGATCTTGAAGAAGGATTCCGCCA  200
  E  A  L  V  L  K  S  W  A  I  L  K  K  D  S  A    34

ATATTGCCCTCCGCTTCTTCTTGAAGATCTTCGAGGTCGCGCCGTCGGCG  250
N  I  A  L  R  F  F  L  K  I  F  E  V  A  P  S  A   51

AGCCAGATGTTCTCGTTCCTGCGAAACTCCGACGTGCCGCTCGAGAAGAA  300
 S  Q  M  F  S  F  L  R  N  S  D  V  P  L  E  K  N   68

CCCCAAGCTCAAGACCCACGCCATGTCCGTCTTCGTCATGACATGCGAGG  350
  P  K  L  K  T  H  A  M  S  V  F  V  M  T  C  E    84

CCGCCGCGCAGCTGCGGAAAGCCGGGAAGGTCACCGTGAGAGACACCACC  400
A  A  A  Q  L  R  K  A  G  K  V  T  V  R  D  T  T   101

CTCAAGAGGCTCGGCGCCACGCACCTCAAGTACGGCGTCGGAGACGCCCA  450
 L  K  R  L  G  A  T  H  L  K  Y  G  V  G  D  A  H   118

CTTCGAGGTGGTGAAGTTCGCGCTGCTTGACACGATCAAGGAGGAGGTTC  500
  F  E  V  V  K  F  A  L  L  D  T  I  K  E  E  V    134

CGGCGGACATGTGGAGCCCGGCGATGAAGAGCGCGTGGAGCGAAGCCTAC  550
P  A  D  M  W  S  P  A  M  K  S  A  W  S  E  A  Y   151

GACCACCTGGTCGCTGCCATCAAGCAGGAGATGAAGCCCGCGGAGTGATC  600
 D  H  L  V  A  A  I  K  Q  E  M  K  P  A  E  *      166

GACAGGCATGCTAGCTGCTCCACCTCCATGATCCTCGCCTCGCGAGTCCG  650
ATTAGCTTTTGTTGCTTTCAAATGCTCGTTTCATATTCATCGTGTCCCAC  700
AAAAAAAGGAGTGTGTATGTGGTGTACGATTGGTGCACGCTCCTGCTGTT  750
TTCTTCTCGTGATAAGACATAAATAAAGATGGTTTTCTACGCTAAAAAAA  800
AAAAAAAAAAAG                                        812
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B

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CCACGCGTCCGGTTGAGTTGAATTGAGCTCGAATTGTACTCGATTCACCA   50
CACAGAGGAATCAAATCGAAGCAGCCATGGCTCTCGTGGAGGGAAACAAC  100
                           M  A  L  V  E  G  N  N     8

GGCGTGTCGGGGGGAGCGGTCAGCTTCAGCGAGGAGCAGGAGGCGCTTGT  150
 G  V  S  G  G  A  V  S  F  S  E  E  Q  E  A  L  V   25

GCTCAAGTCGTGGGCCATCATGAAGAAGGATTCCGCCAACATTGGACTCC  200
  L  K  S  W  A  I  M  K  K  D  S  A  N  I  G  L    41

GCTTCTTCTTGAAGATCTTCGAGGTCGCGCCGTCGGCGAGCCAGATGTTC  250
R  F  F  L  K  I  F  E  V  A  P  S  A  S  Q  M  F   58

TCGTTCCTGCGCAACTCCGACGTGCCGCTCGAGAAGAACCCCAAGCTCAA  300
 S  F  L  R  N  S  D  V  P  L  E  K  N  P  K  L  K   75

GACCCACGCCATGTCCGTCTTCGTCATGACATGTGAGGCCGCCGCGCAGC  350
  T  H  A  M  S  V  F  V  M  T  C  E  A  A  A  Q    91

TGCGGAAAGCCGGGAAGGTCACCGTGAGAGACACCACCCTGAAGAGGCTC  400
L  R  K  A  G  K  V  T  V  R  D  T  T  L  K  R  L   108

GGCGCCACGCACTTCAAGTACGGCGTCGGAGACGCCCACTTTGAGGTGAC  450
 G  A  T  H  F  K  Y  G  V  G  D  A  H  F  E  V  T   125

GAGGTTCGCGCTGCTTGAGACGATCAAGGAGGCGGTTCCGGTGGACATGT  500
  R  F  A  L  L  E  T  I  K  E  A  V  P  V  D  M    141

GGAGCCCCGCGATGAAGAGCGCGTGGAGCGAAGCCTACAACCAACTGGTC  550
W  S  P  A  M  K  S  A  W  S  E  A  Y  N  Q  L  V   158

GCGGCCATCAAGCAGGAGATGAAGCCTGCTGAGTGATATATATACTGCTC  600
 A  A  I  K  Q  E  M  K  P  A  E  *                  169

CACCTCCATGATCCTCGCTGATCAACTTTGTTGCATTGTGCTCGTTCAAT  650
ATTCCTCGCCCCACAAAAGGGACTTTTGTCGGTGTGTGTATGTCCAAATG  700
ATTAAATCAACTGCTGTTTTGTTCTATGTAAGATACATAACTCATAAATA  750
AAGATGGTTTTCTACATGCAAAAAAAAAAAAAAAAG                786
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Figure 1. Nucleotide and deduced protein sequences of clones C741 (A) and C2576 (B) that code for rice Hb1 and Hb2, respectively. Sequences used to design the sense and antisense primers for the specific amplification of Hb1 and Hb2 are underlined. Putative polyadenylation signals are double-underlined.

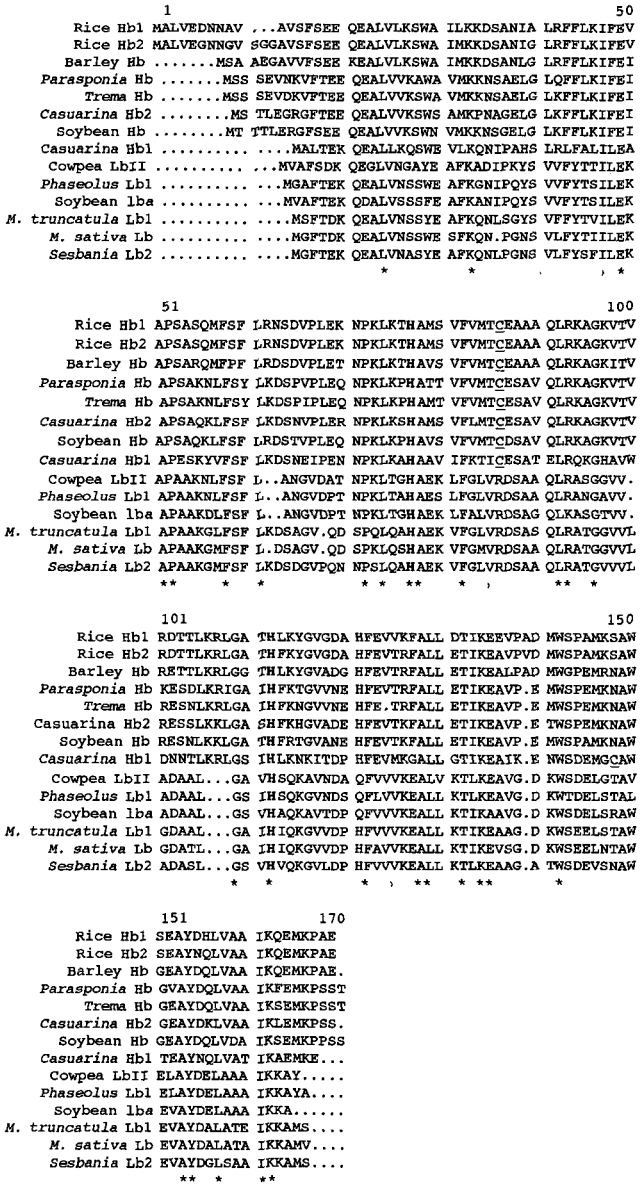

Figure 2. Sequence alignment of nonsymbiotic and selected symbiotic Hbs. Distal (H77) and proximal (H112) His residues are shown in bold type, and Cys residues are underlined. Intron I is at position 47, intron II is at position 85, and intron III is at position 125; asterisks show the most conserved residues. Amino acid sequences were obtained from the GenBank database using the following accession numbers: U01228 (barley Hb), M36509 (*Parasponia andersonii* Hb), Y00296 (*Trema tomentosa* Hb), X53950 (*Casuarina glauca* Hb2), U47143 (soybean Hb), L28826 (*C. glauca* Hb1), U33207 (cowpea LbII), K03152 (*Phaseolus vulgaris* Lb1), V00453 (soybean Lba), X57732 (*Medicago truncatula* Lb1), M32883 (*M. sativa* Lb), and X13815 (*Sesbania rostrata* Lb2). Alignment of sequences was done using the PileUp routine of the GCG program.

not detect a Cys C92, but rather a Leu L92, which is highly conserved in plant Hbs (Fig. 2). Thus, our results support the conclusion that rice Hbs contain only one Cys residue, which is located at position 86.

Cloning and Analysis of Rice *hb* Genes

To clone the gene for rice Hb, we used total rice DNA as the template with specific primers for rice Hb1 or Hb2, which generated PCR products of approximately 900 bp. The PCR products were purified, cloned, and sequenced (Fig. 3), and the coding sequences of the PCR products were identical to cDNA sequences for Hb1 and Hb2; therefore, they were the rice *hb1* and *hb2* genes.

The *hb* gene and cDNA sequences were compared to identify the exon and intron (IVS) sequences of the rice *hb* genes. Rice *hb* genes have four exons and three introns, with the introns located at the same position as all of the known plant *hb* genes. The exon/intron boundaries of the rice *hb* genes are identical to the boundaries of the *Parasponia andersonii hb* gene (Appleby et al., 1988) (Fig. 3). Homologous introns of rice *hb1* and *hb2* were very conserved, with 92% similarity for IVS-II, 84% similarity for IVS-III, and 72% similarity for IVS-I. The highly conserved location of the introns and sequences of the exon/intron boundaries in monocot and dicot *hb* genes suggest that the ancestral *hb* gene of flowering plants had three introns in an identical location.

When rice DNA was digested with restriction enzymes and then subjected to Southern blotting with the rice *hb1* probe at high stringency, between one and three hybridizing fragments were detected (Fig. 4). Rice *hb* genes have no restriction sites for the enzymes used to cut the DNA, so at least three copies of the *hb* gene exist in rice. The existence of cDNAs encoding for Hb1 and Hb2 (Fig. 1) indicates that *hb1* and *hb2* are functional genes in rice.

Expression of *hb* Genes in Rice Organs

In contrast to symbiotic *hb* genes, which are expressed only in nodules of N_2-fixing plants, nonsymbiotic Hb transcripts have been reported to exist in many tissues, including: (a) root meristems of *Trema tomentosa* (Bogusz et al., 1988), (b) root vascular bundles of transgenic tobacco (*Nicotiana tabacum*) (Bogusz et al., 1990), (c) seed aleurone and roots grown under microaerobiosis of barley (*Hordeum vulgare*) (Taylor et al., 1994), and (d) diverse organs of soybean (*Glycine max*) (Andersson et al., 1996) and Arabidopsis (Trevaskis et al., 1997).

To determine the pattern of *hb* gene expression in rice, we isolated poly(A^+) RNA from roots and leaves, and then subjected it to PCR using specific primers for rice Hb1 or Hb2. Amplification products were detected for Hb1 in roots, and for Hb1 and Hb2 in leaves (Fig. 5). Hb transcripts of approximately 550 bp were cloned and sequenced, and the resulting sequences were identical to those of the rice Hb1 and Hb2 cDNAs, indicating that *hb* genes are detectable and functional in rice roots and leaves. We did not detect any Hb2 transcripts in rice roots using our protocols, suggesting that the *hb2* gene is probably not expressed in the roots of rice grown under normal growth conditions. The pattern of expression of rice *hb* genes is similar to the expression of *hb* genes in Arabidopsis reported by Trevaskis et al. (1997). The differential expression of *hb1* and *hb2* genes in the rice plant indicates that these genes

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hb1    1 ATGGCTCTCGTGGAGGATAACAATGCCGT.....AGCGGTGAGCTT 41
         |||||||||||||||  |||||  | |||       ||||||  |||||
hb2    1 ATGGCTCTCGTGGAGGGAAACAACGGCGTGTCGGGGGGAGCGGTCAGCTT 50
         .    .    .    .    .    .    .    .    .    .
      42 CAGCGAGGAGCAGGAGGCGCTGGTGCTCAAGTCATGGGCGATCTTGAAGA 91
         ||||||||||||||||||||| ||||||||||| ||||| ||| ||||||
      51 CAGCGAGGAGCAGGAGGCGCTTGTGCTCAAGTCGTGGGCCATCATGAAGA 100
         .    .    .    .    .    .    .    .    .    .
      92 AGGATTCCGCCAATATTGCCCTCCGCTTCTTCTTGAAgtatgtac..atg 139
         |||||||||||| ||||  |||||||||||||||||||||||||   |
     101 AGGATTCCGCCAACATTGGACTCCGCTTCTTCTTGAAgtatgtactacta 150
         .    .    .    .    .    .    .    .    .    .
     140 cgtgttacta.....ccattt.....ctctttttgcggaatc 171
         | || |||||          ||||||          || ||| ||| | |
     151 catgctactactcactagtgccatttttcgcagagtcagtttacggcacc 200
         .    .    .    .    .    .    .    .    .    .
     172 agagattgggtt.tgtgaagcat..taaattgagcaatgcat..ttcgct 216
         |  |||| || |||  ||||  || |||  |  ||| |  || | |
     201 aaccattggtttctgtagtgcatcatacattttggtgtgcgtgatttggt 250
         .    .    .    .    .    .    .    .    .    .
     217 gatacatgtgtgtctgattgtgttgtagGATCTTCGAGGTCGCGCCGTCG 266
         | |||       | |  || | ||  |||||||||||||||||||||||
     251 gctac.......tatatttttattccagGATCTTCGAGGTCGCGCCGTCG 293
         .    .    .    .    .    .    .    .    .    .
     267 GCGAGCCAGATGTTCTCGTTCCTGCGAAACTCCGACGTGCCGCTCGAGAA 316
         ||||||||||||||||||||||||| |||||||||||||||||||||||
     294 GCGAGCCAGATGTTCTCGTTCCTGCGCAACTCCGACGTGCCGCTCGAGAA 343
         .    .    .    .    .    .    .    .    .    .
     317 GAACCCCAAGCTCAAGACCCACGCCATGTCCGTCTTCGTCATGgtaatac 366
         |||||||||||||||||||||||||||||||||||||||||||||||||
     344 GAACCCCAAGCTCAAGACCCACGCCATGTCCGTCTTCGTCATGgtaatac 393
         .    .    .    .    .    .    .    .    .    .
     367 taccatcattat...ttcaggca..agtaaatttgttgtggtagtagaca 411
         ||||||||  |||   | |||  ||  ||||||||||||| |||||||  ||
     394 taccatcagtattgctgcagacaagagtaaatttgttgtagtagtaggca 443
         .    .    .    .    .    .    .    .    .    .
     412 ctgacagaatgtgtgcgtgcgtcgcgatcaatcgatattgcagACATGCG 461
         |||||||||||||||||| |||| |||||||||| |||||||||| |
     444 ctgacagaatgtgtgcgtgtgtcgtgatcaatcgatgttgcagACATGTG 493
         .    .    .    .    .    .    .    .    .    .
     462 AGGCCGCCGCGCAGCTGCGGAAAGCCGGGAAGGTCACCGTGAGAGACACC 511
         ||||||||||||||||||||||||||||||||||||||||||||||||||
     494 AGGCCGCCGCGCAGCTGCGGAAAGCCGGGAAGGTCACCGTGAGAGACACC 543
         .    .    .    .    .    .    .    .    .    .
     512 ACCCTCAAGAGGCTCGGCGCCACGCACCTCAAGTACGGCGTCGGAGACGC 561
         |||||  ||||||||||||||||||||  |||||||||||||||||||||
     544 ACCCTGAAGAGGCTCGGCGCCACGCACTTCAAGTACGGCGTCGGAGACGC 593
         .    .    .    .    .    .    .    .    .    .
     562 CCACTTCGAGgtacagtgatccccaatggctgcctgcgctccattcgatc 611
         ||||||  ||||||  ||||  | | |     ||||||||||||||
     594 CCACTTTGAGgtagagtgatatccagttgtt......gctccattcgatc 637
         .    .    .    .    .    .    .    .    .    .
     612 gacatgaaac....ttgatcgttttctgatcgtgtctttgtcgaacaacg 657
         ||||||  |||    | ||||||  |||||||  |||  | |||||||
     638 gacatggaactctatcgatcgtgttctgattgtggccttgtcgaa..... 682
         .    .    .    .    .    .    .    .    .    .
     658 tacatgcgatcgwtcgatcgtgtawacagGTGGTGAAGTTCGCGCTGCTT 707
                ||:||      |||:|||||||  ||  ||||||||||||||
     683 .....cgttc......gtaaacagGTGACGAGGTTCGCGCTGCTT 716
         .    .    .    .    .    .    .    .    .    .
     708 GACACGATCAAGGAGGAGGTTCCGGCGGACATGTGGAGCCCGGCGATGAA 757
         || ||||||||||||| ||||||||| |||||||||||||||| ||||||||
     717 GAGACGATCAAGGAGGCGGTTCCGGTGGACATGTGGAGCCCCGCGATGAA 766
         .    .    .    .    .    .    .    .    .    .
     758 GAGCGCGTGGAGCGAAGCCTACGACCACCTGGTCGCTGCCATCAAGCAGG 807
         |||||||||||||||||||||  |||| ||||||||  ||||||||||||||
     767 GAGCGCGTGGAGCGAAGCCTACAACCAACTGGTCGCGGCCATCAAGCAGG 816
         .    .    .    .    .    .    .    .    .    .
     808 AGATGAAGCCCGCGGAGTGA 827
         ||||||||||  ||  ||||||
     817 AGATGAAGCCTGCTGAGTGA 836
```

Figure 3. Sequence alignment of rice *hb1* and *hb2* genes. Coding and noncoding sequences are shown as upper or lowercase, respectively. Sequences flanking the exon/intron boundaries that are conserved in rice and *P. andersonii* sp. (Appleby et al., 1988) *hb* genes are underlined.

are not linked to each other, and that each *hb* gene is probably regulated by different promoter sequences and *trans*-acting factors.

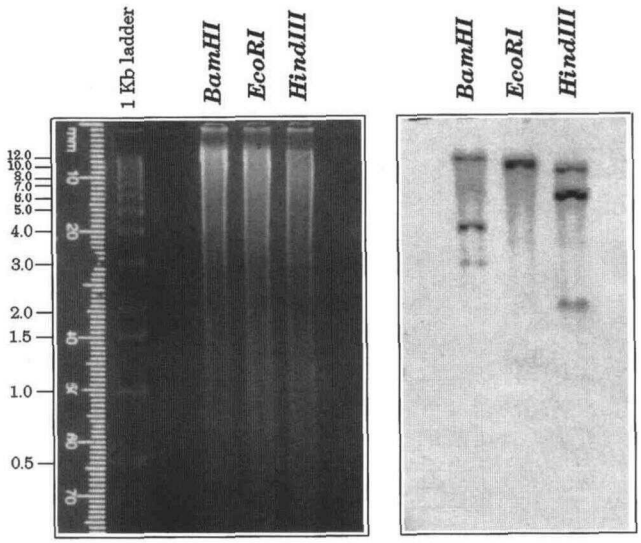

Figure 4. Southern-blot analysis of total rice DNA using the rice *hb1* gene as a probe. Signals were detected by colorimetry using the chromogenic mix NBT/x-phosphate. Molecular size markers are shown in kilobars.

Expression of the Rice Hb1 cDNA in *E. coli* and Spectroscopic and Kinetic Characterization of the Recombinant Wild-Type and Mutant Protein

Nonsymbiotic Hbs are of low abundance in plant tissues and thus are difficult to isolate and purify from plants. The synthesis of recombinant proteins provides a useful method of producing large amounts of protein for bio-

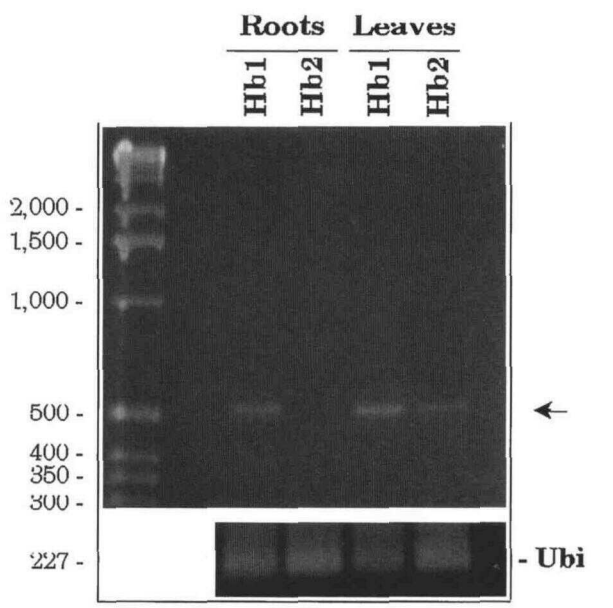

Figure 5. Agarose gel electrophoresis of the RNA-PCR products that were obtained using as a template poly(A^+) RNA isolated from rice roots or leaves and specific primers for rice Hb1 or Hb2. Arrow shows the approximately 550-bp fragments that were cloned and sequenced. Rice ubiquitin (Ubi) was used as positive control. Molecular size markers are shown in base pairs.

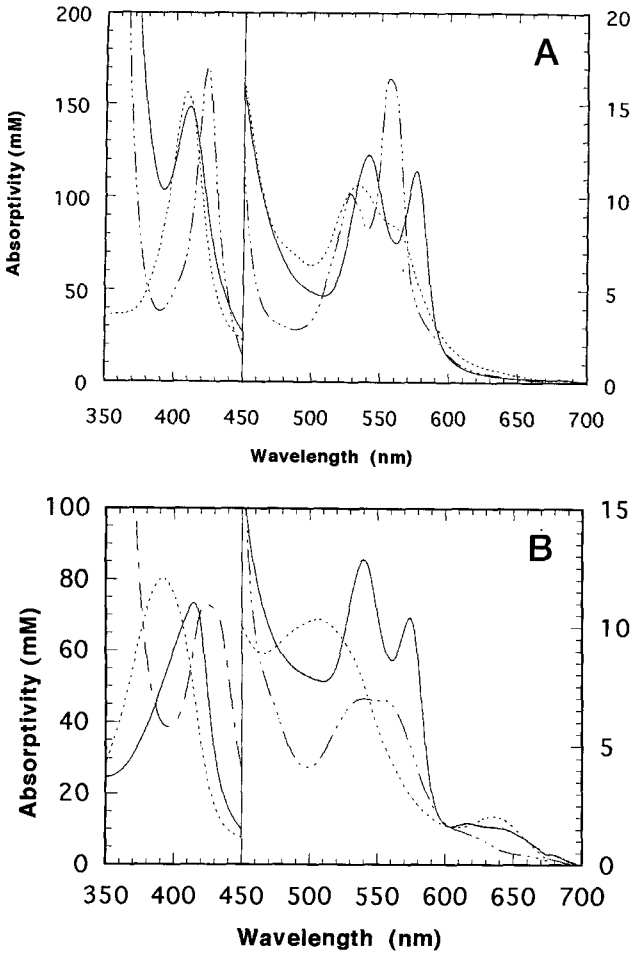

Figure 6. Absorption spectra of rice wild-type rHb1 (A) and the H77L mutant of rHb1 (B). Dashed lines, Ferric; solid lines, oxygenated; and combination dashed/solid lines, ferrous forms of Hb.

chemical studies. We prepared a recombinant Hb by subcloning a cDNA for rice Hb1 into the vector pET28a and then expressing it in *E. coli*. The N terminus of the rHb1 was determined to be ALVEDNNAVAV, which is identical to the predicted sequence of the Hb1 cDNA (Fig. 1A), indicating that the correct recombinant protein had been synthesized.

Analysis of the rHb1 shows that it exhibits spectral characteristics that are similar to Hbs (Fig. 6A), including reversible binding of O_2. However, rHb1 exhibits some distinctive absorption bands both in the ferric and the deoxyferrous forms. Differences in the globin-heme linkage were apparent from the deoxyferrous spectrum because, at pH 7.0, the unligated ferrous state exhibits two peaks at 526 and 556 nm, which is similar to the absorption spectra of Cyt *b*, where the heme is hexacoordinate and the Fe is principally in the low-spin state (Smith, 1978; Weiss and Ziganke, 1978). These spectra are in marked contrast to the symbiotic plant Hbs and animal Mbs and Hbs, in which the Fe is pentacoordinate and displays a broad peak centered at 556 nm in their deoxyferrous form (Appleby, 1974, 1992). Thus, it is apparent that the ferric and deoxyferrous forms of rHb1 contain a distal ligand that was identified as His74. Absorbance spectra of the ferric, oxy-, and deoxyferrous forms of an H74L mutant of rHb1 shows no evidence of His coordination (Fig. 6B), and the ferric peak at 405 nm suggests that the ligand-binding site is partially occupied by a water molecule. Furthermore, the addition of exogenous imidazole to ferric or deoxyferrous H74L mutant results in a spectrum identical to the corresponding form of the wild-type rHb1.

Kinetic analysis of ligand binding shows that the rHb1 has an unusually high affinity for O_2 (Table I). The O_2-association constant of rHb1 is similar to other O_2 storage and transport proteins, such as soybean Lb*a*, however, the high affinity of rHb1 for O_2 results from a very low dissociation constant. Similar values for the dissociation constant have been reported for barley (Duff et al., 1997) and Arabidopsis (Trevaskis et al., 1997) recombinant Hbs, and suggest that these proteins are not involved in O_2 transport through facilitated diffusion. Rate constants of rice rHb1 for the reaction with CO are similar to O_2-transport proteins, which indicates that the unique O_2 reactivity is a result of specific interactions between O_2 and the protein.

Removal of His74 had a profound effect on the rate constants for O_2 binding. The association constant increases approximately 9-fold as a result of the H74L mutation, and the dissociation constant increases nearly 1000 times. This is similar to the effect of removal of the distal His in Mb (Springer and Sligar, 1987), but is different from the smaller effect seen with the same mutation in soybean Lb*a* (Hargrove et al., 1997) (Table I). These results suggest that His74 forms a strong H^+ bond with bound O_2, which contributes to the slow dissociation constant. However, the rate constant for the mutant, which is similar to soybean Lb, combined with the extremely low value for the wild-

Table I. *Rate and equilibrium constants for the reaction of O_2 and CO with rice wild-type rHb1 and the H77L mutant of rHb1*

Protein	k'_{O_2}[a]	k_{O_2}[b]	K_{O_2}[c]	k'_{CO}[a]	k_{CO}[b]	K_{CO}[c]
	$\mu M^{-1}\ s^{-1}$	s^{-1}	μM^{-1}	$\mu M^{-1}\ s^{-1}$	s^{-1}	μM^{-1}
Rice rHb1	68	0.038	1800	7.2	0.001	7,200
rHb1-H77L	620	51	12	150	0.002	75,000
Soybean Lba[d]	130	5.6	23	16	0.0084	1,900
Lba-H61L[d]	400	24	16	170	0.0024	71,000

[a] Association constant. [b] Dissociation constant. [c] Ligand affinity. [d] Values from Hargrove et al. (1997).

type rHb1, suggests that there might be an additional mechanism limiting O_2 dissociation.

Nonsymbiotic Hb from rice, and presumably all Hbs of this class, appear to operate through a unique mechanism. His H74 coordinates the heme Fe in the deoxy-protein, but when O_2 binds the side chain moves to a position from which it can form a stabilizing interaction with bound O_2. This is very different from the behavior of Mb mutants, in which a His coordinates the heme Fe. These proteins are very unstable and do not react with O_2 due to an extraordinarily high autooxidation rate (Duo et al., 1995).

Function of Nonsymbiotic Hbs

The function of plant Hbs in nonsymbiotic tissues is not known; however, it has been hypothesized that the role of nonsymbiotic Hbs is probably not to facilitate the diffusion of O_2 in roots, but rather to sense levels of O_2 (Appleby et al., 1988). Appleby et al. (1988) suggested that under normal aerobic conditions Hb would be oxygenated, and that under O_2-limiting conditions, deoxyferrous Hb levels would increase and trigger an anaerobic response. Although O_2-sensor heme proteins have been described in other systems (Gilles-Gonzalez et al., 1991, 1994, 1995; Gilles-Gonzalez and Gonzalez, 1993), the above hypothesis was questioned recently by Andersson et al. (1996), who suggest that Hbs may function as O_2 carriers in metabolically active tissues.

Our results with rice plants, including the expression profiles in aerobic tissues (Fig. 5), expected levels of Hbs in tissues, and kinetic parameters of Hb1 (Table I), suggest that these proteins are unlikely to facilitate the diffusion of O_2. Also, because of the extremely low dissociation constant exhibited by rHb1, it is unlikely that rice Hbs function as an effective O_2 donor to other proteins, such as mitochondrial oxidases. However, under specific circumstances, such as in barley roots grown under microaerobic conditions, in which high levels of Hb transcripts have been detected (Taylor et al., 1994), nonsymbiotic Hbs may have other functions, including participation in the anaerobic response and possibly in specific metabolic aspects of dedifferentiated tissues. Recently, a multitude of functions have been suggested for nonplant Hbs, which includes the transport of ligands other than O_2 (such as NO or CO), interaction with small organic molecules, O_2 scavenging, or formation of complexes with regulatory proteins (Götz et al., 1994; Giardina et al., 1995; Goldberg, 1995; Jia et al., 1996). The pattern of expression of nonsymbiotic *hb* genes in plants and the biochemical properties of the recombinant Hbs suggest that these proteins have other functions besides O_2 transport, which are yet to be determined.

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