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Cloning and expression analysis of hemoglobin genes from maize (*Zea mays* ssp. *mays*) and teosinte (*Zea mays* ssp. *parviglumis*)

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Abstract

With the exception of barley and rice, little is known about the existence of hemoglobins (Hbs) in cereals. This work reports the cloning and analysis of *hb* genes from maize (*Zea mays* ssp. *mays*) and teosinte (*Zea mays* ssp. *parviglumis*). Coding sequences of maize and teosinte *hb* genes (*hbm* and *hbt*, respectively) are highly similar to each other and are interrupted by three introns located at identical positions as other plant *hb* genes. Sequences of predicted Hbm and Hbt proteins are identical. The hydropathic profile of Hbm and Hbt is highly similar to that of rice Hb1, suggesting that Hbm, Hbt and Hb1 have the same tertiary structure and biochemical properties. Expression analysis showed that low levels of Hb transcripts, but considerable levels of Hb proteins exist in maize embryonic organs. No Hb transcripts and proteins were detected in teosinte embryonic organs. Low levels of Hb proteins, but no Hb transcripts, were detected in maize and teosinte vegetative organs. These observations suggest that the regulation of *hb* genes is different in maize and teosinte embryonic organs, and that the expression of *hb* genes is down- or up-regulated in maize and teosinte, respectively, from germination to vegetative growing.

Keywords: Gene expression, Hemoglobin, Maize, Non-symbiotic, Teosinte, *Zea*

Abbreviations: Hb, hemoglobin; Hbm, maize Hb; Hbt, teosinte Hb

1. Introduction

Hemoglobins (Hbs) are heme proteins that have been detected in all phyla, and function by binding and transporting oxygen and other gaseous ligands [1–3]. In plants, non-symbiotic Hbs have been detected in organs of vascular and non-vascular species; however, the function of this group of proteins is still not known [4, 5]. Among flowering plants, genes coding for non-symbiotic Hbs were cloned from dicot and monocot species. In dicots, non-symbiotic Hbs were first detected in root nodules and roots of *Parasponia* [6], and roots of *Trema tomentosa* [7], and more recently non-symbiotic *hb* genes were cloned from a number of dicot plants, including soybean [8] and *Arabidopsis* [9]. Apparently, the expression of non-symbiotic *hb* genes varies significantly, and the highest levels of expression were detected in metabolically active or stressed organs. For example, high-est levels of non-symbiotic Hb transcripts were detected in soybean stems [8], which are metabolically active organs. In *Arabidopsis*, two *hb* genes were cloned and analyzed: *ahb1* gene is expressed in roots and rosette leaves but overexpressed under hypoxic conditions, whereas *ahb2* gene is expressed at low levels in rosette leaves but overexpressed at low temperatures [9].

In monocots, genes coding for non-symbiotic Hbs were first cloned from barley [10] and subsequently from rice [11], but Southern blot analysis using a heterologous *hb* probe from barley showed that *hb* genes also exist in a number of cereals, such as maize, rye and wheat [10]. In barley, a single copy of the non-symbiotic *hb* gene exists, which is expressed in plant roots, and overexpressed in roots from plants growing in microaerobiosis. In rice four copies of the *hb* gene exist, and different promoters were identified suggesting that rice *hb* genes are regulated by different *trans*-acting factors

(Lira-Ruan et al., submitted; [11]).

A cDNA with sequence similarity to non-symbiotic Hbs was cloned from stressed maize organs, and the full sequence was subsequently deposited in the Gen-Bank database (accession No. AF236080). Despite the above entry, no work has been reported on maize Hbs. Maize is an important crop and a good model to analyze the role of non-symbiotic Hbs in plants because much information has been published concerning maize physiology, biochemistry and genetics. Moreover, the origin of maize is well documented and it has been postulated that the contemporary maize evolved from the domestication of teosinte [12–16]. Thus, the evolutive relationship between maize and teosinte is an interesting model to perform analyses of plant Hbs. In this work we report the cloning and analysis of *hb* genes from maize and teosinte.

2. Materials and methods

2.1. Seed germination and plant growing

Maize (*Zea mays* ssp. *mays*) seeds were obtained commercially, and teosinte (*Zea mays* ssp. *parviglumis*) seeds were either provided by Dr. Mark J. Millard (Iowa State University, USA) or collected at Teloloapan (Guerrero State) in the Balsas Region, México. Maize and teosinte seeds were germinated for 5–7 days in paper towels imbibed in water. Embryonic organs, such as coleoptiles, seminal roots and embryos, were collected from germinated seeds. To avoid any contamination from the endosperm, embryos were carefully detached from the cotyledon using a microspatula and, if necessary, endosperm remains were removed using a razor blade. Organs were immediately frozen in liquid nitrogen and stored at −80°C until used. Germinated seeds were also planted in pots containing vermiculite and then grown in a greenhouse at 22°C with light/dark periods of 12 h/12 h. Plants were watered with tap water every third day and with nutrient solution [17] every sixth day. Young and mature plants were grown for 2 or 16 weeks after germination, respectively, and roots and leaves were collected as above and stored at −80°C until used.

2.2. Total DNA and RNA isolation

Total DNA was isolated from 1–3 g of roots or leaves using a modification of the hexadecyltrimethylammonium bromide method [18]. Total RNA was isolated from 100 mg of frozen tissues using the Tryzol reagent (Gibco BRL), and poly(A)$^+$ RNA was isolated using a Quick-Prep mRNA purification kit (Pharmacia-Amersham) following the instructions of the manufacturers. DNA and total RNA and poly(A)$^+$ RNA were quantitated by spec-trophotometry assuming A_{260} = 50 or 40 µg/ml for DNA and RNA, respectively [19].

2.3. Primers and PCR amplification

PCR primers were designed to amplify the maize and teosinte *hb* genes (*hbm* and *hbt*, respectively) using sequences from the Start and Stop codons of the maize Hb cDNA (GenBank accession No. AF236080). Oligo-nucleotide sequences were 5′-ATGGCACTCGCGGAG-GCCGAC-3′ and 5′-CTAGGCATCGGGCTTCAT-3′ for the sense (Maize Hb/ATG) and antisense (Maize Hb/TAG) primers, respectively. Total plant DNA (~0.5 µg) was used as template for PCR amplification. PCR components and concentrations were: 0.5 µM of each sense and antisense primer, 200 µM of each dNTP and 0.5 U of *Taq* DNA polymerase (Gibco BRL) in 1×PCR buffer containing 1.5 mM MgCl$_2$. PCR was done in a final volume of 25 µl using a thermal cycler (Progene). Amplification was carried out for 35 cycles at 60°C for annealing, and PCR products were detected in a 1.4% (w/v) agarose gel after staining with ethidium bromide.

2.4. Cloning of hbm and hbt genes

PCR products were isolated from the agarose gel using the Genclean kit (Bio 101) and then cloned into the vector pCR2.1 (Invitrogen) following standard protocols [19]. Cloned fragments were fully sequenced in both directions at the DNA Sequencing Facility of the University of Nebraska-Lincoln, USA, and DNA sequences were compared with sequences deposited in the GenBank database using the BLAST program [20]. Additional computer analyses were performed using the GCG package (Genetics Computer Group).

2.5. Detection of Hbm and Hbt transcripts in plant organs

Detection of Hb transcripts in plant organs was performed by RT-PCR [21] using a RNA-PCR kit (Cetus or Gibco BRL). Reverse transcription was done using 1 µg of total RNA or 45 ng of poly(A)$^+$ RNA as the template for embryonic and vegetative organs, respectively, and oligo d(T)$_{16}$ as primer following the manufacturer's protocol. PCR amplification was performed with the primers Maize Hb/ATG and Maize Hb/TAG that had been used for the amplification of *hbm* and *hbt* and the same conditions described above, but for 40 cycles. For a positive control, ubiquitin transcripts were amplified using the primers Ubi sense (5′-GTTCTCCGCCT-CAGGGGAGGC-3′) and Ubi antisense (5′-GCTTA-AATGAACCATGGCTTA-3′) from a maize ubiquitin cDNA (GenBank accession No. U29158). PCR products were detected in a 1.4% (w/v) agarose gel after staining with ethidium bromide.

2.6. Purification of recombinant rice Hb1 and generation of anti-rice Hb1 antibodies

Purification of recombinant rice Hb1 from transgenic *Escherichia coli* pEMBL18$^+$/rHb1 [11] was performed essentially as described by Arredondo-Peter et al. [22]. Pure rice Hb1 was used to generate polyclonal anti-rice Hb1 antibodies in rabbits by the Monoclonal Antibody Core Facility of the University of Nebraska–Lincoln, USA.

2.7. Western blot analysis of maize and teosinte organs

Plant organs (0.5–1 g) were homogenized with liquid nitrogen, and the homogenate was resuspended in 2 vols. of extraction buffer (20 mM Tris–HCl, pH 8, containing 1 mM phenylmethylsulfoxide fluoride). The resulting solution was centrifuged at 13 000×g at 4°C, and the supernatant was used for determination of total proteins by a dye-binding assay (Sigma-Aldrich) using bovine serum albumin as standard. To adjust to a protein concentration of 1–2 µg/µl, samples were concentrated by centrifugation using centrifugal concentrators (Centricon). Samples consisting of fresh extracts (40 µg of total soluble proteins/lane) were subjected to electrophoresis in a 15% (w/v) sodium dodecyl sulfate–polyacrylamide gel, and then transferred in transfer buffer (25 mM Tris–HCl, pH 8.0, 192 mM glycine, 20% (v/v) methanol) to a Hybond C-extra nitrocellulose membrane (Pharmacia-Amersham) at 4°C overnight.

Western blotting was performed at least in triplicate following standard protocols [23]. Briefly, membranes were blocked by incubation with 3% (w/v) non-fat dry milk in TBS buffer (100 mM Tris–HCl, pH 8.0, 150 mM NaCl) containing 0.05% (v/v) Tween 20. Protein blots were incubated with a 1/5000 dilution of the anti-rice Hb1 antibodies for 1 h at room temperature. Blots were washed with TBS-Tween and then incubated with a 1/3000 dilution of the anti-rabbit IgG conjugated with alkaline phosphatase (Roche) for 1 h at room temperature. After washing with TBS-Tween, blots were developed using the chromogenic mix nitroblue tetrazolium/5-bromo-4-chloroindolyl phosphate (Roche).

3. Results and discussion

3.1. Cloning and characterization of hbm and hbt genes

When total DNA from maize and teosinte was used as the template with primers designed for the maize Hb cDNA, two PCR products of approx. 650 and 880 bp were obtained (Figure 1). These PCR products were purified, cloned and sequenced, and the sequences were compared with sequences deposited in the GenBank database. The sequence of the smaller fragment (650 bp) showed no similarity with Hbs and was a non-specific amplification. The sequence of the larger fragment (880 bp) from maize (Figure 1A) and teosinte (Figure 1B)
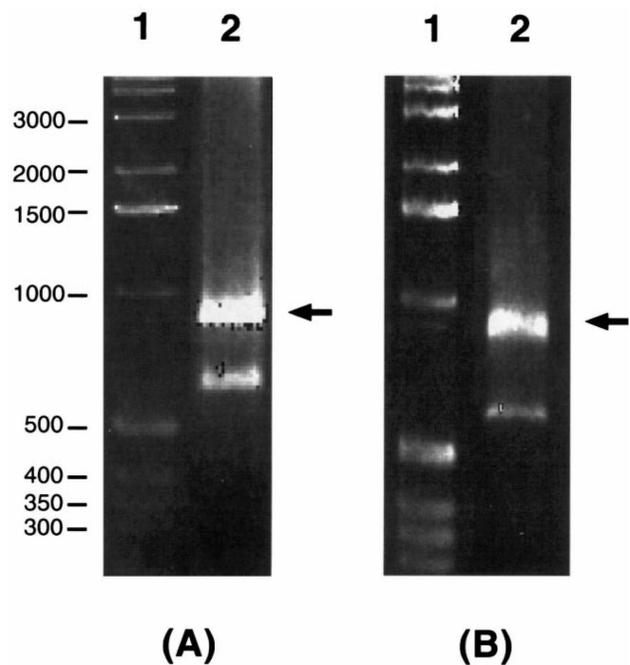

Figure 1. Amplification of maize (A) and teosinte (B) *hb* genes by PCR using the primers Maize Hb/ATG and Maize Hb/TAG. Samples were subjected to electrophoresis in a 1.4% (w/v) agarose gel and stained with ethidium bromide. Lane 1, 1 kb ladder; lane 2, total PCR reaction. Arrows show the 880 bp fragment corresponding to maize and teosinte *hb* genes. Molecular size markers are shown in bp.

was identical or very similar to the maize Hb cDNA sequence, respectively, indicating that these fragments corresponded to the *hbm* and *hbt* genes.

The *hbm* and *hbt* sequences were compared to the maize Hb cDNA to identify exon and intron (IVS) sequences. Both *hbm* and *hbt* genes have four exons and three introns (Figure 2) located at identical positions as all of the known plant *hb* genes [11, 24, 25]. However, the nucleotide sequence at the 3′ splice site of the first intron and 5′ splice site of the third intron was unusual in *hbm* and *hbt* (agGG and AGgc, respectively) (Figure 2) compared to those sequences from *Parasponia* [25] and rice [11] *hb* genes (agGA and AGgt, respectively). Sequence comparison between *hbm* and *hbt* showed that exons were highly conserved (~99% similar), but intron sequences were rather variable (similarity values were 68, 95 and 83% for IVS-I, IVS-II and IVS-III, respectively).

Maize and teosinte *hb* genes code for predicted proteins of 164 amino acid residues in length (Figure 3), with a calculated molecular mass of 18.3 kDa. Sequence comparison showed that the predicted Hbm and Hbt proteins are identical to each other. Hbm and Hbt contain distal (H76) and proximal (H111) His residues, as well as the P51, F57, F81, and F121 that are highly conserved in plant Hbs [24]. Also, Hbm and Hbt contain a single Cys residue, C85, that is conserved in non-symbiotic Hbs (Figure 3) [11].

```
   Maize hb   1 ATGGCACTCGCGGAGGCCGACGACGGCGCGGTGGTCTTCGGCGAGGAGCA  50
               ||||||||||||||||||||||||||||||||||||||||||||||||||
Teosinte hb   1 ATGGCACTCGCGGAGGCCGACGACGGCGCGGTGGTCTTCGGCGAGGAGCA  50

             51 GGAGGCGCTGGTGCTCAAGTCGTGGGCCGTCATGAAGAAGGACGCCGCNA 100
                ||||||||||||||||||||||||||||||||||||||||||||||||| |
             51 GGAGGCGCTGGTGCTCAAGTCGTGGGCCGTCATGAAGAAGGACGCCGCCA 100

            101 ACCTGGGCCTCCGCTTCTTCCTCAAgtacgtgcgtttccgtgc....cgc 146
                |||||||||||||||||||||||||||  ||  ||||||||||||    | |
            101 ACCTGGGCCTCCGCTTCTTCCTCAAgtaagtacgtttccgtgctacacac 150

            147 tgcctggtgctggtgcctgcgtttgggt......tgcaccggcggccatc 190
                ||||||       |   |  ||||  ||||||       |||||||||||||||
            151 tgcctg.....cgcacgtgcgcttgggttgcacctgcaccggcggccatc 195

            191 gtcgcaattcgcacctagtagctgctccttgactaacgggcgccg.ccgc 239
                              |   |||||||||||||||||||||||||||  ||||
            196 ................gagcctgctccttgactaacgggcgccgcccgc 228

            240 cgtccgcttctgccg.cagGGTCTTCGAGATCGCGCCGTCGGCGAAGCAG 287
                |||||||  ||||||  ||||||||||||||||||||||||||||||||||||
            229 cgtccgctcctgccgtcagGGTCTTCGAGATCGCGCCGTCGGCGAAGCAG 278

            288 ATGTTCTCGTTCCTGCGCGACTCCGACGTGCCGCTAGAGAAGAACCCCAA 337
                |||||||||||||||||||||||||||||||||||||   |||||||||||
            279 ATGTTCTCGTTCCTGCGCGACTCCGACGTGCCGCTGGAGAAGAACCCCAA 328

            338 GCTCAAGACGCACGCCATGTCCGTCTTCGTCATGgtacgtacgttctcat 387
                |||||||||||||||||||||||||||||||||||||||||||||||||||
            329 GCTCAAGACGCACGCCATGTCCGTCTTCGTCATGgtacgtacgttctcat 378

            388 gctagctcccaagctagctagcatcgatcgatggaaattccacgcgaccc 437
                |||||||||| ||||||||||||||| ||  |||||||||||||||||||
            379 gctagctcccacgctagctagcatcgaccgatggaaattccacgcgaccc 428

            438 atcgagcggcaaaaag....gactgacaggactgtgtgtctctcgctgtc 483
                |||||||||||||||       ||||||||||||||||||||||||||||
            429 atcgagcggcaaaaaggactgactgacaggactgtgtgtctctcgctgtc 478

            484 attgtcaatgctgctgcagACCTGCGAGGCGGCGGCGCAGCTTCGCAAGG 533
                |||||||||||||||||||||||||||||||||||||||||||||||||||
            479 attgtcaatgctgctgcagACCTGCGAGGCGGCGGCGCAGCTTCGCAAGG 528

            534 CCGGGAAGGTCACCGTGAGGGAGACCACGCTCAAGAGGCTGGGCGCCACG 583
                |||||||||| ||||||||||||||||||||||||||||||||||||||||
            529 CCGGGAAGGTCACGGTGAGGGAGACCACGCTCAAGAGGCTGGGCGCCACG 578

            584 CACTTGAGGTACGGCGTCGCAGATGGACACTTCGAGgcaagtcaaaaccg 633
                || |||||||||||||||||||||||||||||||||||||||||||||||
            579 CATTTGAGGTACGGCGTCGCAGATGGACACTTCGAGgcaagtcaaaaccg 628

            634 cccgcccaacatgtc....aattcacctct.attcctgactctggcttgg 678
                ||||||||||||| ||       |||||||| ||||||||||||||  ||||
            629 cccgcccaacatttcaattaattcacctctaattcctgactctgccttgg 678

            679 aaaggcagcaagaacgaaagc.............attgagattgaaacac 715
                ||||     ||||||||||||||              |||||||||||||||
            679 aaag...gcaagaacgaaagcgtccgcctgctagattgagattgaaaca. 724

            716 gcgcttgaatacatacagGTGACGGGGTTCGCGCTGCTTGAGACGATCAA 765
                ||||||||||||||||||||||||||||||||||||||||||||||||||
            725 .cgcttgaatacatacagGTGACGGGGTTCGCGCTGCTTGAGACGATCAA 773

            766 GGAGGCGCTCCCCGCTGACATGTGGAGCCTCGAGATGAAGAAAGCCTGGG 815
                ||||||||||||||||||||||||||||||||||||||||||||||||||
            774 GGAGGCGCTCCCCGCTGACATGTGGAGCCTCGAGATGAAGAAAGCCTGGG 823

            816 CCGAGGCCTACAGCCAGCTGGTGGCGGCCATCAAGCGGGAGATGAAGCCC 865
                |||||||||||||||||||||||||||||||||||||| |||||||||||||
            824 CCGAGGCCTACAGCCAGCTGGTGGCGGCCATCAAGAGGGAGATGAAGCCC 873

            866 GATGCCTAG 874
                |||||||||
            874 GATGCCTAG 882
```

Figure 2. Sequence alignment of maize and teosinte *hb* genes. Coding (exon) and non-coding (intron) sequences are shown in upper and lowercase letters, respectively. Vertical bars show identical bases in both sequences. Dots correspond to indels (insertion/deletion). Sequences flanking the exon/intron boundaries are underlined. N at position 99 of maize *hb* gene corresponds to C or A. Sequences were aligned using the PileUp routine of the GCG program. Maize and teosinte *hb* gene sequences were deposited in the GenBank database under accession Nos. AY005818 and AF291052, respectively.
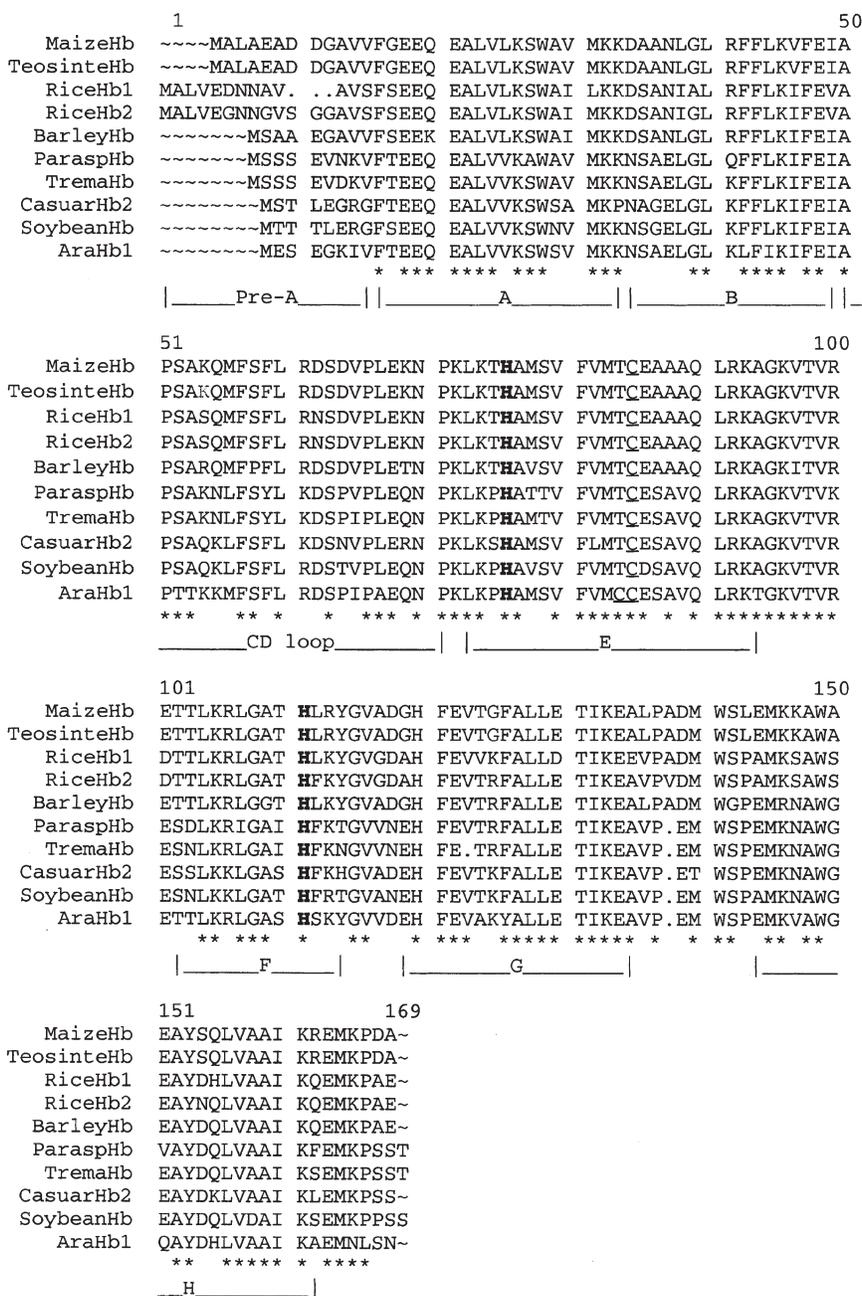
```
             1                                                        50
    MaizeHb  ~~~~MALAEAD DGAVVFGEEQ EALVLKSWAV MKKDAANLGL RFFLKVFEIA
 TeosinteHb  ~~~~MALAEAD DGAVVFGEEQ EALVLKSWAV MKKDAANLGL RFFLKVFEIA
    RiceHb1  MALVEDNNAV. ..AVSFSEEQ EALVLKSWAI LKKDSANIAL RFFLKIFEVA
    RiceHb2  MALVEGNNGVS GGAVSFSEEQ EALVLKSWAI MKKDSANIGL RFFLKIFEVA
   BarleyHb  ~~~~~~~MSAA EGAVVFSEEK EALVLKSWAI MKKDSANLGL RFFLKIFEIA
   ParaspHb  ~~~~~~~MSSS EVNKVFTEEQ EALVVKAWAV MKKNSAELGL QFFLKIFEIA
    TremaHb  ~~~~~~~MSSS EVDKVFTEEQ EALVVKAWAV MKKNSAELGL KFFLKIFEIA
  CasuarHb2  ~~~~~~~~MST LEGRGFTEEQ EALVVKSWSA MKPNAGELGL KFFLKIFEIA
  SoybeanHb  ~~~~~~~~MTT TLERGFSEEQ EALVVKSWNV MKKNSGELGL KFFLKIFEIA
      AraHb1 ~~~~~~~~MES EGKIVFTEEQ EALVVKSWSV MKKNSAELGL KLFIKIFEIA
                        * *** **** ***    ***      **  **** ** *

             |_____Pre-A_____||_____A_____||_____B_____||_
```

```
             51                                                      100
    MaizeHb  PSAKQMFSFL RDSDVPLEKN PKLKTHAMSV FVMTCEAAAQ LRKAGKVTVR
 TeosinteHb  PSAKQMFSFL RDSDVPLEKN PKLKTHAMSV FVMTCEAAAQ LRKAGKVTVR
    RiceHb1  PSASQMFSFL RNSDVPLEKN PKLKTHAMSV FVMTCEAAAQ LRKAGKVTVR
    RiceHb2  PSASQMFSFL RNSDVPLEKN PKLKTHAMSV FVMTCEAAAQ LRKAGKVTVR
   BarleyHb  PSARQMFPFL RDSDVPLETN PKLKTHAVSV FVMTCEAAAQ LRKAGKITVR
   ParaspHb  PSAKNLFSYL KDSPVPLEQN PKLKPHATTV FVMTCESAVQ LRKAGKVTVK
    TremaHb  PSAKNLFSYL KDSPIPLEQN PKLKPHAMTV FVMTCESAVQ LRKAGKVTVR
  CasuarHb2  PSAQKLFSFL KDSNVPLERN PKLKSHAMSV FLMTCESAVQ LRKAGKVTVR
  SoybeanHb  PSAQKLFSFL RDSTVPLEQN PKLKPHAVSV FVMTCDSAVQ LRKAGKVTVR
      AraHb1 PTTKKMFSFL RDSPIPAEQN PKLKPHAMSV FVMCCESAVQ LRKTGKVTVR
             ***    ** *    *  *** * **** **  * ****** * * **********

             _____CD loop_____|  |_____E_____|
```

```
             101                                                     150
    MaizeHb  ETTLKRLGAT HLRYGVADGH FEVTGFALLE TIKEALPADM WSLEMKKAWA
 TeosinteHb  ETTLKRLGAT HLRYGVADGH FEVTGFALLE TIKEALPADM WSLEMKKAWA
    RiceHb1  DTTLKRLGAT HLKYGVGDAH FEVVKFALLD TIKEEVPADM WSPAMKSAWS
    RiceHb2  DTTLKRLGAT HFKYGVGDAH FEVTRFALLE TIKEAVPVDM WSPAMKSAWS
   BarleyHb  ETTLKRLGGT HLKYGVADGH FEVTRFALLE TIKEALPADM WGPEMRNAWG
   ParaspHb  ESDLKRIGAI HFKTGVVNEH FEVTRFALLE TIKEAVP.EM WSPEMKNAWG
    TremaHb  ESNLKRLGAI HFKNGVVNEH FE.TRFALLE TIKEAVP.EM WSPEMKNAWG
  CasuarHb2  ESSLKKLGAS HFKHGVADEH FEVTKFALLE TIKEAVP.ET WSPEMKNAWG
  SoybeanHb  ESNLKKLGAT HFRTGVANEH FEVTKFALLE TIKEAVP.EM WSPAMKNAWG
      AraHb1 ETTLKRLGAS HSKYGVVDEH FEVAKYALLE TIKEAVP.EM WSPEMKVAWG
               ** ***  *    **    * ***  ***** ***** *   *  **   ** **

             |_____F_____|    |_____G_____|          |_____
```

```
             151           169
    MaizeHb  EAYSQLVAAI KREMKPDA~
 TeosinteHb  EAYSQLVAAI KREMKPDA~
    RiceHb1  EAYDHLVAAI KQEMKPAE~
    RiceHb2  EAYNQLVAAI KQEMKPAE~
   BarleyHb  EAYDQLVAAI KQEMKPAE~
   ParaspHb  VAYDQLVAAI KFEMKPSST
    TremaHb  EAYDQLVAAI KSEMKPSST
  CasuarHb2  EAYDKLVAAI KLEMKPSS~
  SoybeanHb  EAYDQLVDAI KSEMKPPSS
      AraHb1 QAYDHLVAAI KAEMNLSN~
             **   ***** * ****

             __H_____|
```

Figure 3. Sequence alignment of cereal and selected non-symbiotic Hbs. Distal (H76) and proximal (H111) His residues are shown in bold type, and Cys residues are underlined. Asterisks show the most conserved residues. Helix regions are calculated from the structure of rice Hb1 [26]. Amino acid sequences were obtained from the GenBank database using the following accession Nos.: U76030 (rice Hb1), U76031 (rice Hb2), U01228 (barley Hb), M36509 (*Parasponia andersonii* Hb), Y00296 (*Trema tomentosa* Hb), X53950 (*Casuarina glauca* Hb2), U47143 (soybean Hb) and U94998 (*Arabidopsis thaliana* Hb1). Alignment of sequences was done using the PileUp routine of the GCG program.

Computer analysis showed that the hydropathy profile of Hbm and Hbt is highly similar to that of recombinant rice Hb1 (Figure 4). The tertiary structure of rice Hb1 was elucidated recently [26], and it was shown that rice Hb1 is a dimer when the concentration is approx. 0.8 mM [27]. Thus, the above observation suggests that the tertiary structure of Hbm and Hbt is very similar to that of rice Hb1. Moreover, it has been proposed that residues I/V49, S52, E122, V123 and F126 form the dimer interface of rice Hb1 and other non-symbiotic Hbs [26]. These residues are also conserved in Hbm and Hbt (Figure 3), which strongly suggests that Hbm and Hbt can also form dimers. Thus, we predict that the biochemical properties of Hbm and Hbt are the same to those reported for the rice Hb1 [11, 26, 27], i.e. Hbm and Hbt have a very high affinity for oxygen.
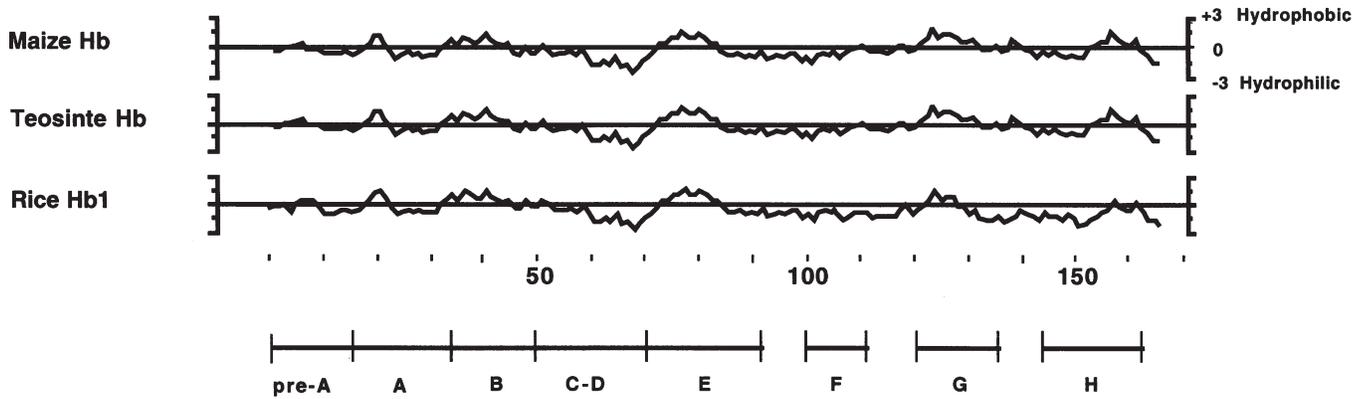
Figure 4. Hydropathy profiles of Hbm, Hbt and rice Hb1. Helix regions are calculated from the structure of rice Hb1 [26]. Hydropathy analysis was performed with the Peplot routine of the GCG program.

3.2. Expression analysis of hbm and hbt genes in plant organs

Non-symbiotic Hbs have been detected in plant organs by Northern blot [8], RT-PCR [11] and Western blot [28, 29]. In this work we analyzed the expression of *hbm* and *hbt* genes in plant organs by evaluating the levels of Hb transcripts and proteins by RT-PCR and Western blot, respectively.

To determine the levels of Hb transcripts in maize and teosinte organs, total and poly(A)$^+$ RNA were isolated from embryonic (coleoptiles, seminal roots and embryos) and vegetative (young and mature leaves and roots) organs, and then subjected to PCR using primers for Hbm cDNA (above). Low levels of amplification products of the expected size (498 bp) were detected in maize coleoptiles, seminal roots and embryos (Figure 5A), indicating that *hbm* expresses in maize embryonic organs. However, no amplification products were detected in teosinte embryonic (Figure 5B) and maize and teosinte vegetative (Figure 6) organs. Thus, to explore the possibility that the amount of Hb transcripts was below the detection limit in the above organs an (10 μl) aliquot of the RT-PCR reaction was subjected to a re-PCR for 40 additional cycles; however, no Hb transcripts were detected after re-PCR.

Maize and teosinte *hb* genes code for proteins with a predicted molecular mass of 18.3 kDa, which is nearly identical to the molecular mass of recombinant rice Hb1 (18.4 kDa) [11]. Our previous analysis by Western blot showed that soluble extracts of maize and teosinte organs contain proteins that comigrate with rice Hb1, and that specifically cross-react with anti-rice Hb1 antibodies (not shown), suggesting that these proteins were maize and teosinte Hbs. To determine the level of Hb proteins in maize and teosinte organs the same amount of fresh plant soluble extracts were Western blotted with anti-rice Hb1 antibodies. Results showed that high levels of Hbm protein exist in maize embryonic organs (Figure 5A), and that the highest levels of Hbm were detected in seminal roots and embryos, and the lowest in

coleoptiles. Hbm proteins were also detected in maize vegetative organs (young and mature leaves and roots) (Figure 6A), but the Hb levels were considerably lower than those detected in embryonic organs. No differences were observed between Hbm levels from leaves and roots, or young and mature organs. In contrast to maize, Hbt proteins were not detected in teosinte embryonic organs (Figure 5B), which is consistent with the
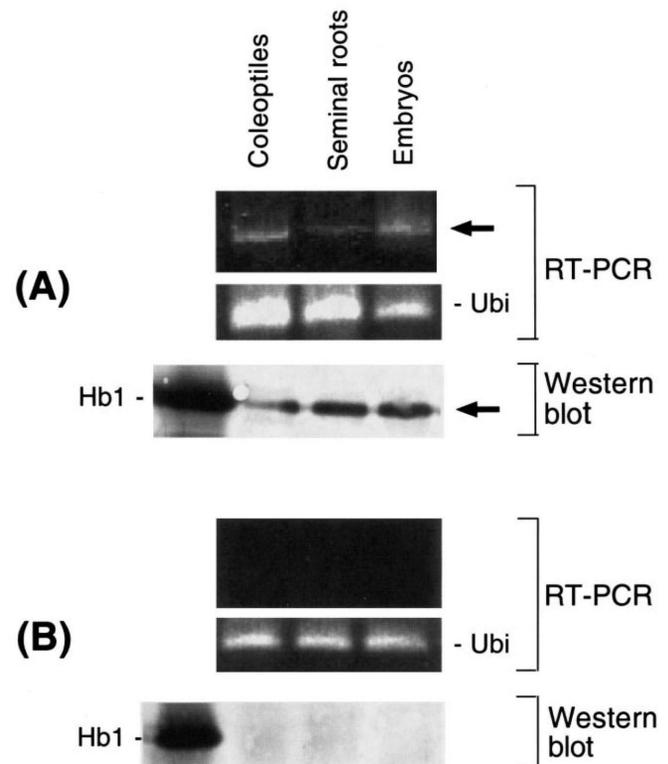

Figure 5. Expression of *hb* genes in embryonic organs from maize (A) and teosinte (B). Arrows show the Hb transcripts (498 bp) and proteins (18.3 kDa) detected by RT-PCR and Western blot, respectively. Maize ubiquitin (Ubi) (500 bp) and rice Hb1 (Hb1) (18.4 kDa) were used as controls for RT-PCR and Western blot, respectively. These results are representative of at least three replicates.
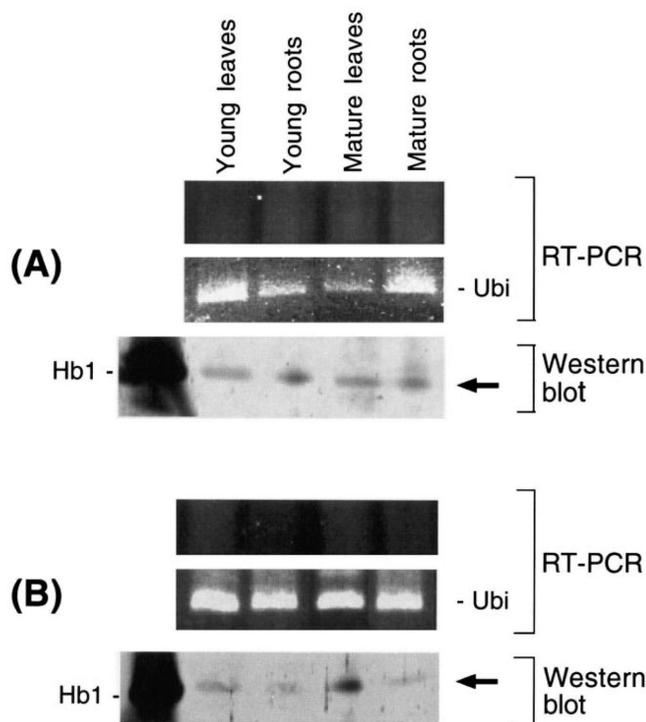
Figure 6. Expression of *hb* genes in vegetative organs from maize (A) and teosinte (B). Arrows show the Hb proteins (18.3 kDa) detected by Western blot. Maize ubiquitin (Ubi) (500 bp) and rice Hb1 (Hb1) (18.4 kDa) were used as controls for RT-PCR and Western blot, respectively. These results are representative of at least three replicates.

inability to detect Hbt transcripts by RT-PCR and re-PCR (above). However, low levels of Hbt proteins were detected in teosinte young and mature leaves and roots (Figure 6B); apparently, Hbt levels were higher in leaves than in roots from young and mature plants.

The above observations suggest that *hb* genes are differently expressed in maize and teosinte organs. In maize embryonic organs *hb* genes express and transcripts are translated at considerable levels of Hbm protein; in contrast, in teosinte embryonic organs apparently *hb* genes are shut down as no Hbt transcripts and proteins were detected. These results were surprising given the relatedness of teosinte to maize, and the existence of Hbs in seeds of other cereals [10, 29]. However, it is still possible that Hbt could be below detection limits, or that *hbt* gene has a different type of regulation. In vegetative organs from maize and teosinte no Hb transcripts were detected, but low levels of Hb proteins were detected. This observation suggests that in vegetative organs *hbm* and *hbt* express at very low levels (below the detection limit) resulting in low abundance of Hbm and Hbt transcripts, which are translated in detectable levels of proteins. Therefore, the results obtained in this work suggest that the expression of *hb* genes is down- or up-regulated in maize and teosinte, respectively, from germination to vegetative growing.

4. Conclusions

The results presented above show that *hb* genes from maize and teosinte are highly conserved and code for identical Hb proteins. This suggests that the domestication of teosinte to maize did not substantially affect the evolution of Hbs in these plants. The function of non-symbiotic Hbs in plants is not known [4, 30], but the existence of Hbs in specific tissues of plant organs suggests that non-symbiotic Hbs play specialized rather than housekeeping functions (Ross et al., submitted). In this work we showed that the *hb* genes are expressed and Hb proteins are synthesized in maize and teosinte organs. However, apparently the regulation of *hb* genes is different in maize and teosinte seeds suggesting that Hbm and Hbt play different roles in the plant embryonic organs. Taylor et al. [10] and Lira-Ruan et al. [29] detected Hbs in germinating seeds from barley and rice, respectively, and it was postulated that Hbs play a role in germination, i.e. by providing O_2 to sustain an active metabolism. However, no Hbs were detected in germinating seeds from teosinte suggesting that these proteins are not essential for germination in this plant. Also, as judged by the level of sensitivity of the procedures used in this work, the expression of *hb* genes in maize and teosinte vegetative organs is extremely low but high enough for the synthesis of detectable Hb proteins. Level of Hb proteins in maize and teosinte vegetative organs were comparable, thus it is likely that Hbs have a similar function in leaves and roots from maize and teosinte.

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