6-1-2001

Nonsymbiotic hemoglobins in rice are synthesized during germination and in differentiating cell types

E. J. H. Ross
University of Nebraska - Lincoln

L. Shearman
University of Nebraska - Lincoln

M. Mathiesen
University of Nebraska - Lincoln

Y.J. Zhou
University of Nebraska - Lincoln

R. Arredondo-Peter
Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca, Morelos

See next page for additional authors

Published in *Protoplasma* 218 (2001), pp. 125-133. http://www.springerlink.com/content/107724/

Submitted March 29, 2001; accepted June 12, 2001.

Nonsymbiotic hemoglobins in rice are synthesized during germination and in differentiating cell types

E. J. H. Ross[1], L. Shearman[1], M. Mathiesen[2], Y. J. Zhou[2], R. Arredondo-Peter[3], G. Sarath[1,2,*], and R. V. Klucas[1]

[1] Department of Biochemistry and [2] Center for Biotechnology, George W. Beadle Center, University of Nebraska–Lincoln, Lincoln and [3] Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca, Morelos

* Corresponding author: Department of Biochemistry, George W. Beadle Center, University of Nebraska–Lincoln, Lincoln, NE 68588-0664, U.S.A.

Abstract: Nonsymbiotic hemoglobins (ns-Hbs) previously have been found in monocots and dicots; however, very little is known about the tissue and cell type localization as well as the physiological function(s) of these oxygen-binding proteins. We report the immunodetection and immunolocalization of ns-Hbs in rice *(Oryza sativa* L.) by Western blotting and in situ confocal laser scanning techniques. Ns-Hbs were detected in soluble extracts of different tissues from the developing rice seedling by immunoblotting. Levels of ns-Hbs increased in the germinating seed for the first six days following imbibition and remained relatively constant thereafter. In contrast, ns-Hb levels decreased during leaf maturation. Roots and mesocotyls contained detectable, but low levels of ns-Hbs. Split-seed experiments revealed that ns-Hbs are synthesized de novo during seed germination and are expressed in the absence of any signal originating from the embryo. Immunolocalization of ns-Hbs by con- focal microscopy indicated the presence of ns-Hbs primarily in differentiated and differentiating cell types of the developing seedling, such as the aleurone, scutellum, root cap cells, sclerenchyma, and tracheary elements. To our knowledge, this is the first report of the specific cellular localization of these proteins during seedling development.

Keywords: Nonsymbiotic hemoglobin, Plant development, Immunodetection, Confocal microscopy, Seed germination, *Oryza sativa L.*

Abbreviations: Hbs hemoglobins; Ns nonsymbiotic.

Introduction

Hemoglobins (Hbs) are ubiquitous proteins found in bacteria, fungi, plants, protozoa, and animals (Vinogradov et al. 1993, Bolognesi et al. 1997). Plants contain two types of Hbs: the symbiotic and nonsymbiotic (ns) Hbs (Appleby 1992, Andersson et at. 1996). Symbiotic Hbs are found only in the nitrogen-fixing, nodulated dicot species and their function is to facilitate the diffusion of oxygen to the nitrogen-fixing bacteroids in the nodule (Appleby 1984, 1992). In contrast, ns-Hbs are more widely distributed and have been characterized in both monocot and dicot species (Appleby et al. 1983, Appleby 1992, Taylor et al. 1994, Andersson et al. 1996, Arredondo-Peter et al. 1997). Specific functions have not been defined for ns-Hbs.

Expression of ns-Hbs in plants appears to be primarily in metabolically active or stressed tissues. The activity of *hb* promoters of *Parasponia andersonii* and *Trema tomentosa* was localized to the root meristem and vascular cylinder of transgenic tobacco, using GUS as a reporter (Bogusz et al. 1990). Jacobsen-Lyon et al. (1995) reported that when the *Casuarina glauca hb* gene was fused to GUS and transformed into *Lotus corniculatus,* GUS expression was found in the meristem of the root tips, the vascular stele of roots, and the parenchyma internal to the endodermis. Using Northern blot analysis, Andersson et al. (1996) detected the highest level of Hb transcripts in stems of soybeans.

Other studies also suggest that ns-Hbs are stress-related proteins. For example, under hypoxic conditions, the *ns-hb* gene is expressed in roots and rosette leaves of *Arabidopsis thaliana (arahb1)* (Trevaskis et al. 1997) and the seeds of germinating barley (Taylor et al. 1994, Hill 1998). Nie and Hill (1997) found induction of the

barley *hb* gene under low oxygen tension and high levels of CO in aleurone tissue, as well as in the presence 10 mM nitrate. Arredondo-Peter et al. (1997) showed that under normal growth conditions, *hb1* and *hb2* from rice are expressed in leaves but that only *hb1* is expressed in roots, suggesting differential regulation of this small gene family during seedling development.

In monocots, the earliest expression of *ns-hb* genes is observed in germinating seeds. In barley, ns-Hb transcripts and protein are present predominantly in the aleurone cells (Taylor et al. 1994). When subjected to abiotic stress, such as low oxygen tension and high levels of CO, the levels of ns-Hb transcripts are markedly enhanced in isolated barley aleurone. Using immunoblotting methods, Duff et al. (1998) have reported the presence of ns-Hbs in excised barley embryos, embryo-containing and embryoless half seeds, and aleurone tissue.

These collective data indicate that ns-Hbs have a role in unstressed and stressed plant tissues, although the biochemical function(s) of ns-Hbs is still unresolved (Arredondo-Peter et al. 1998, Hill 1998). Appleby et al. (1988) suggested that ns-Hbs may not be involved in the facilitated diffusion of oxygen but rather may function as an oxygen sensor. In contrast, Andersson et al. (1996) suggested that ns-Hbs might function as oxygen carriers in metabolically active tissues, such as stems of soybean. However, no information on the quantity of ns-Hb protein or the affinity of soybean ns-Hbs to gaseous ligands was reported. Ns-Hbs are now known to possess the highest reported oxygen affinities among plant Hbs (Arredondo-Peter et al. 1997), which leads to intriguing questions about their function(s) in plant tissues. The high oxygen binding affinity of recombinant ns-Hbs (r-ns-Hbs) from several species is brought about by a moderate association constant coupled to an extremely low dissociation constant (Duff et al. 1997, Trevaskis et al. 1997, Arredondo-Peter et al. 1998, Hargrove 2000). The very tight binding of oxygen by r-ns-Hbs does not readily support a role in oxygen transport for these proteins. Instead, these proteins may function in binding of ligands such as CO or NO and/or may interact with other cellular molecules (Arredondo-Peter et al. 1998, Goodman and Hargrove 2001).

In all previous work on ns-Hbs in plants, mRNA transcripts and in some instances protein content have been determined in tissue extracts. However, to understand the function(s) of these proteins in plants, specific cell type localization is needed to define the role of ns-Hbs.

We have studied the temporal and spatial distribution of ns-Hbs in rice tissues by immunoblotting and confocal laser scanning microscopy. These proteins were immunodetected in all tissue types examined during the first two weeks following imbibition and were immunolocalized in a number of cell types during germination, with specificity to the aleurone, scutellum, root cap, and in differentiating tracheary elements. We have also determined that up-regulation of ns-Hbs during rice seed germination does not require an embryonic signal.

Material and methods

Plant growth

Rice *(Oryza sativa* cv. Jackson) seeds were germinated on paper towels, in a greenhouse at 22 °C with light and dark periods of 16 h and 8 h at 950 microeinstein/m^2 • s. Plants were watered daily with tap water.

For half-seed experiments, dry, ungerminated rice seeds were sliced with a razor blade into embryo-containing and embryoless halves, incubated separately in petri dishes on moist paper towels in the dark, and wet daily with either distilled water or 5 μM cycloheximide (Jiao et al. 1991).

Antibodies to rice r-ns-Hb1 and immunoblotting of native rice Hbs

Recombinant rice ns-Hb1 was purified according to Arredondo-Peter et al. (1997). Polyclonal antibodies were raised against this r-ns-Hb1 in rabbits by the Antibody Core Facility of the University of Nebraska–Lincoln. Serum titers were analyzed by Western blotting techniques (Penheiter et al. 1997).

Rice seeds were germinated on filter paper soaked with distilled water. Germinating seeds and young seedlings were harvested 2, 4, 6, 8, 10, 12, and 14 days post imbibition. When possible (>4 days), seedlings were separated into root, seed, mesocotyl, and leaf tissues and were immediately frozen in liquid N_2. Frozen plant tissue was either processed immediately or kept at –80 °C until used. Plants were ground in liquid N_2 and resuspended in 20 mM Tris-HCl, pH 8.0, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was squeezed through one layer of cheesecloth and centrifuged at 16,000 *g* for 10 min. The resulting supernatant fraction was analyzed for protein content by the BCA protein assay (Pierce Chemical Co.). 30 μg of total soluble protein was precipitated using 5% trichloroacetic acid and the pellet was washed three times with cold 100% ethanol. Pellets were resuspended in Laemmli sample buffer and heated at 95 °C for 3 min (Laemmli 1970) before separation on 13% sodium dodecyl sulfate-polyacrylamide gels. The resolved polypeptides were electroblotted onto a nitrocellulose membrane and blocked with Tris-buffered saline with 0.05% (v/v) Tween 20 supplemented with 3 % (w/v) nonfat dry milk. Western blotting was performed with primary antibody raised against recombinant rice ns-Hb1 (0.8 ng/ml) and secondary antibody of goat anti-rabbit immunoglobulin G peroxidase (1.6 ng/ml; Sigma). Blots were developed with CL-HRP substrate system (Pierce Chemical Co.) and detected by using Biomax ML imaging film (Kodak).

Localization of ns-Hbs in rice tissues

Rice tissues were collected at various developmental stages and were frozen in OCT compound (Tissue-Tek) at –20 °C. Frozen sections (ca. 8 μm thick) were cut on a cryostat microtome (Leica, CM-19-00) and collected on poly-prep slides (Sigma). Slides were first treated in 100% methanol for 5 min at –20 °C and washed for 2 min in PBS (phosphate-buffered saline: 137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2, at 25 °C They were then incubated in 3% (w/v) bovine serum albumin (BSA) and PBS–0.05 % (v/v) Tween 20 (PBST) for 45 min at 25 °C Primary antibody against anti-recombinant ns-Hb1 was diluted to 8 ng/ml in PBST containing 1% BSA; the negative control used was pre-immune serum. Slides were incubated at room temperature for 2 h, washed thrice in PBST, and then in secondary antibody consisting of Cy-2-conjugated goat anti-rabbit immunoglobulin G (Jackson Immuno-Research) (300 ng/ml in PBST containing 1% BSA), and the slides were incubated in the dark for 1 h. Samples were then washed twice in PBST and once in PBS, mounted, and examined with a confocal laser scanning microscope (Bio-Rad MRC-1024ES). Images were collected and analyzed by Bio-Rad LaserSharp (V3.3) software.

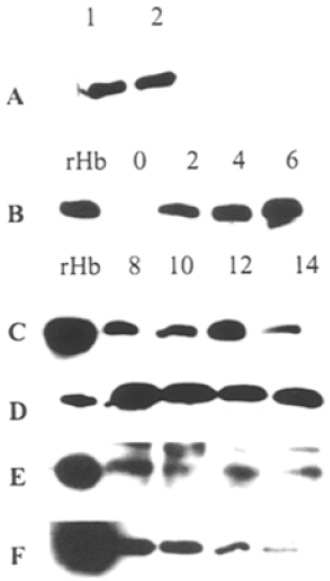

Fig. 1, A-E: Protein immunoblot analysis of rice ns-Hb expression during seed germination. **A:** 50 ng of rHb1 (1) and rHb2 (2) were analyzed by Western blot analysis using rHb1 antibody (0.8 ng/ml). **B-F:** 30 μg of total soluble protein from various rice extracts were separated by 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with rHb1 antibody (0.8 ng/ml). The rHb standard was 50 ng of rHb2. **B:** Extracts prepared from 0-, 2-, 4-, and 6-day-old whole plant seedlings. **C-F:** Extracts prepared from 8-, 10-, 12-, and 14-day-old root, seed, mesocotyl, and leaf tissues, respectively.

Results

Detection of ns-Hbs in cell extracts

Immunodetection of ns-Hbs in germinating rice seedling tissue extracts was performed with antibodies raised against rice r-ns-Hb1 at 2, 4, 6, 8, 10, 12, and 14 days post imbibition. These antibodies reacted equally to r-ns-Hb1 and r-ns-Hb2 (Fig. 1A), indicating their specificity for rice ns-Hbs. The antibodies also did not cross-react to proteins other than ns-Hbs in rice extracts, indicating their utility for tissue localization experiments. To analyze the relative amounts of ns-Hbs in soluble tissue extracts from germinating rice seedlings (Fig. 1 B-F) signals attributable to ns-Hbs in tissue extracts were compared to the signal observed for 50 ng of r-ns-Hb2 which was included on each blot as a standard. In dry seeds, ns-Hbs were barely detectable (Fig. 1B). By 2 days after imbibition, ns-Hbs were detectable, and increased in abundance over the next 4 days. These data indicate that ns-Hb synthesis is apparently up-regulated during rice seed germination (Fig. 1B). Under our conditions of growth, the seminal root and the coleoptile had emerged from the seed at 4 days after imbibition. By 6 to 8 days, the primary leaf was present and the embryo contained the organs associated with a normally developing rice seedling, including the scutellum, coleoptile, coleorhiza, radicle, and vascular procambium (T. Jones and Rost 1989). Eight days after imbibition, the rice seedlings were large enough to separate into root, seed fraction (seed coat, cotyledon, and coleorhiza), mesocotyl, and leaf, As shown in Fig. 1 C, ns-Hb levels in root tissue were low, and blots had to be overdeveloped to detect a signal. In the seed fraction, immunoblotting detected relatively greater amounts of ns-Hbs between 8 and 14 days as compared to the earlier stages (Fig. 1D). Ns-Hbs were also present in 8- to 14-days-old mesocotyl tissue, but at levels lower than in other tissues except leaves (Fig. 1E). The amounts of ns-Hbs in leaf extracts were apparently much lower than thee 50 ng r-ns-Hb2 standard and decreased with increasing age of the leaf tissue (Fig. 1F). Blots of the different tissue extracts were developed for varying lengths of time to obtain signals corresponding to the ns-Hbs. Thus the intensity of the signal arising from the 50 ng r-ns-Hb2 standard included in each blot was important for determining the relative abundance of ns-Hbs in tissue extracts. Our data indicate that ns-Hbs are present in varying amounts in the different tissues during normal rice seed germination, and suggest that there may be age-dependent regulation of Hb levels in maturing tissues such as the leaf.

Immunolocalization of rice ns-Hbs by confocal microscopy

Ns-Hbs in specific rice tissues

To determine the specific cellular distribution of ns-Hbs in rice seedling tissues, immunolocalization of ns-Hbs was performed on cryofixed fresh tissue sections. However, the starchy nature of the rice seed made it difficult to obtain unfragmented sections from germinating seeds prior to 3 days post imbibition. Although we analyzed many sections obtained from varying tissues at different times during the germination process, we have selected micrographs that are representative of ns-Hb localization in the different rice tissues. Paired confocal images of specific regions of tissues were collected from adjacent sections as shown in Fig. 2. Ns-Hbs were found in specific cell types throughout the rice seedling during the first two weeks of germination. For example, a section of a 4-day-old seedling through its radicle shows ns-Hb protein is present primarily in the root cap cells (Fig. 2A–C). As seen in these images of the root cap, the ns-Hbs are apparently in the cytoplasm, as determined by the position of the nucleus, observed by an elliptical shadow within each cell. The lack of a signal peptide on rice Hb1 and Hb2 (Arredondo-Peter et al. 1997) also supports this conclusion. The aleurone and scutellum,

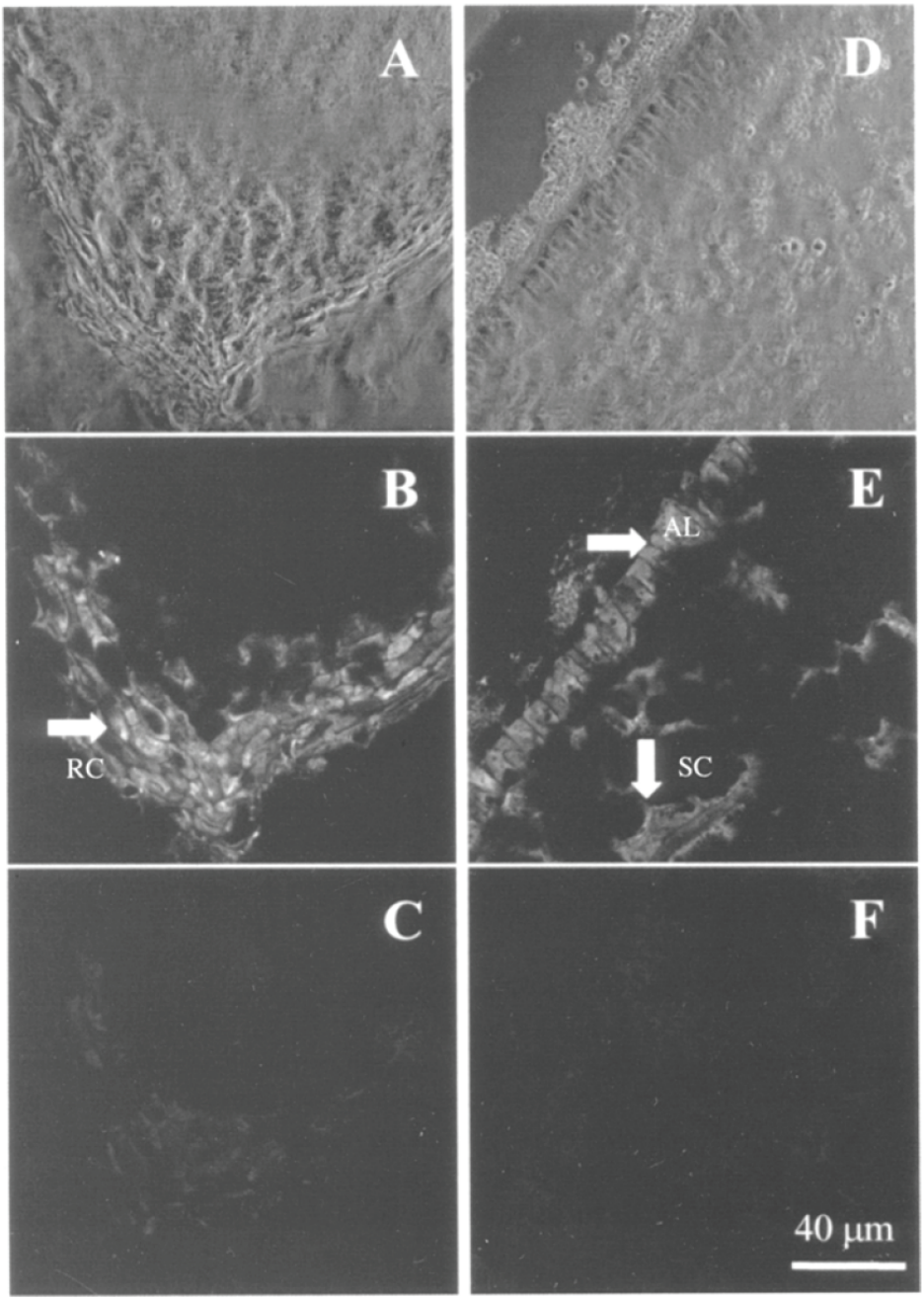

Fig. 2, A-F: In situ immunolocalization of ns-Hbs in rice tissues by confocal laser scanning microscopy. Rice tissues were sectioned on a cryostat, permeablized with methanol, and incubated with primary and secondary antibodies. Concentrations of the primary antibody were 0.8 ng/ml for anti-rHb1 (positive) and preimmune (negative) and for the secondary, 300 ng/ml goat anti-rabbit immunoglobulin G conjugate Cy-2. **A-C:** 4-day-old rice root cap. **A:** Phase image; **B:** positive test; **C:** negative control. **D-F:** 6-day-old aleurone and scutellum of rice. **D:** Phase image; **E:** positive test; **F:** negative control. Ns-Hbs are localized as indicated by the arrows. *RC* Root cap cells, *AL* aleurone, *SC* scutellum

which play a key role in signaling and hydrolytic enzyme secretion during the onset of germination, showed the presence of ns-Hbs, as seen in Fig. 2D-F. The aleurone cells are characterized by their columnar appearance and the scutellum is the adjoining tissue. Distribution of ns-Hbs in this region is shown in a representative micrograph of an oblique section from a 6-day-old germinating seedling. In addition, ns-Hbs were found in a number of tissues of the leaf, including the sclerenchyma (Fig. 3 G-I).

Ns-Hbs in the vasculature of rice seedlings

Immunolocalization of ns-Hbs by confocal laser scanning microscopy led to the detection of ns-Hb expression throughout the vasculature of rice seedlings (Fig. 3). Ns-Hbs were detectable in vascular tissues as early as 4 days after imbibition and were found in all developing vascular tissues examined by this technique. Specifically, ns-Hbs were localized in differentiating xylem cells, as shown in sections of the root and mesocotyl from 10-day-old seedlings (Fig. 3A-C, D-F). The ns-Hb proteins appear to be localized primarily in the xylem. Additionally, ns-Hbs were detected in the vasculature of the leaf and in particular the vasculature next to the sclerenchyma (Fig. 3G-I).

The recurrent immunolocalization of ns-Hbs in the vasculature of root, mesocotyl, and leaf tissues raises questions about the function of these proteins. The fact

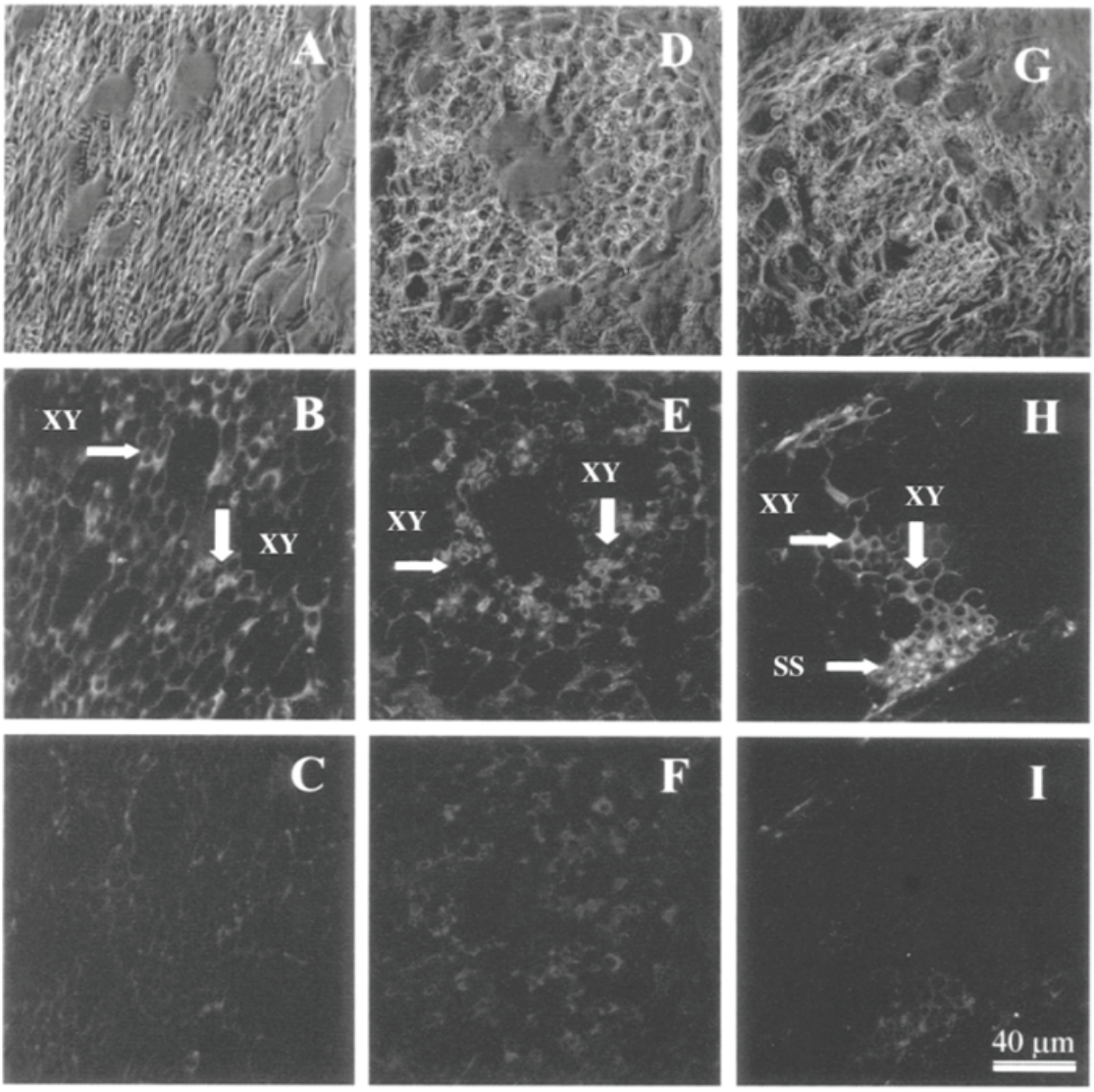

Fig. 3, A-I. In situ immunolocalization of ns-Hbs in vascular bundles of rice tissues. Rice tissues were treated as described in the legend to Fig. 2. **A-C:** 10-day-old root vasculature. **A:** Phase image of an oblique section; **B:** positive test; **C:** negative control. Ns-Hbs are immunolocalized to the xylem as indicated by the arrows. **D-F:** 10-day-old mesocotyl vasculature. **D:** Phase image; **E:** positive test; **F:** negative control. **G-I:** 10-day-old leaf vasculature. **G:** Phase image; **H:** positive test; **I:** negative control. Ns-Hbs are immunolocalized to the xylem as indicated and in the sclerenchyma sheath as indicated by the arrows. *XY* Xylem, *SS* sclerenchyma sheath

that ns-Hbs were found specifically in the xylem indicates that these proteins may have a particular role in the metabolism accompanying the differentiation of the xylem.

Ns-Hbs in differentiating tracheary elements

Differentiating xylem cells can be distinguished morphologically from nondifferentiating cells by the radial thickening of the cell walls (Fig. 4). To determine in which xylem cells the ns-Hb proteins accumulated, the immunolocalization micrographs were overlaid on the phase micrograph of the same image (Fig. 4). As seen in the representative micrographs of 12- (Fig. 4 A) and 6-day-old mesocotyl (Fig. 4B), ns-Hbs were predominantly localized in cells that contain radial thickening of the cell walls. Ns-Hbs were also observed in parenchyma cells adjacent to the cells containing radial cell wall thickenings. We are uncertain if these parenchyma cells were in an early stage of differentiation into tracheary elements. However, proteins were not detected in fully mature xylem cells, indicating that ns-Hbs are present at distinct stages of the xylem differentiation process. While the exact stage of xylogenesis of the cells shown in Fig. 4 is unknown, the localization of ns-Hbs is a first indicator that these proteins are involved in the xylogenesis process.

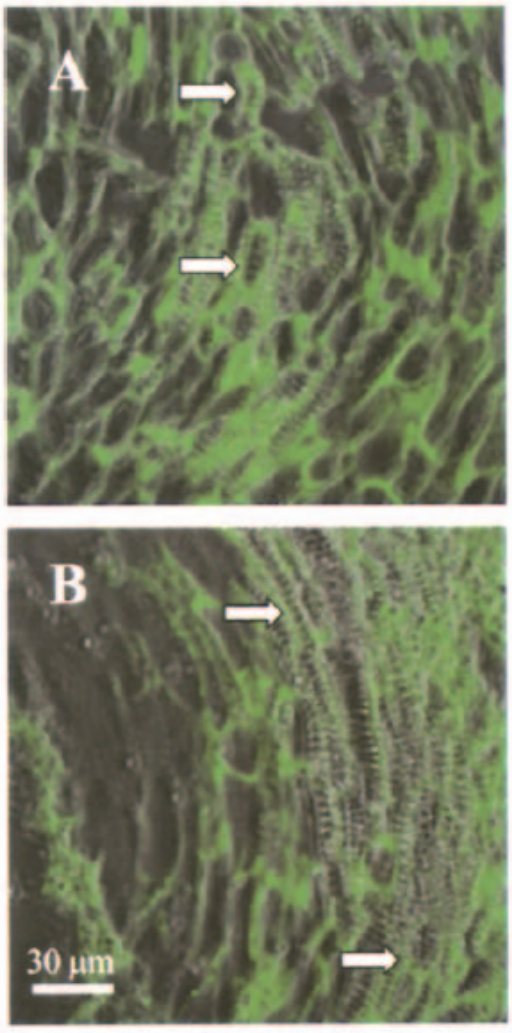

Fig. 4, A, B. In situ immunolocalization of ns-Hbs in differentiating xylem cells. Immunolocalization techniques were described in the legend to Fig. 2. Phase micrograph and positive micrograph were overlaid by Adobe Photoshop 5.5. **A:** Positive and phase overlay of 12-day-old mesocotyl tissue. **B:** Positive and phase overlay of 6-day-old mesocotyl tissue. Ns-Hbs are immunolocalized in differentiating xylem ceils, as distinguished by the radial cell wall thickenings (see arrows)

Synthesis of ns-Hbs does not require the embryo upon imbibition

On the basis of the finding that ns-Hbs were synthesized in the aleurone cells of barley (Taylor et al. 1994, Duff et al. 1998) and rice (this work), we were interested in understanding if the embryo was required for expression of ns-Hbs in these cells. Dry rice seeds were separated into embryo-containing and embryoless halves, imbibed in the presence or absence of cycloheximide, and probed for ns-Hb levels by immunoblotting. By 3 days of imbibition, both halves showed the presence of ns-Hbs, but with more protein in the embryoless half (Fig. 5, lanes 3 and 4). This indicated that the embryo was not required for ns-Hb synthesis. When the embryoless and embryo-containing halves were incubated in the presence of 5 µM cycloheximide, a eukaryotic cytoplasmic protein synthesis inhibitor, immunoblots showed less protein in both seed halves after 3 days of incubation (Fig. 5, lanes 5 and 6). However, considerably more ns-Hbs were now found in the embryo-containing half as compared to the embryoless half.

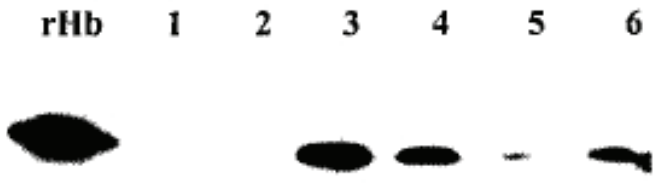

Fig. 5. Protein immunoblot analysis of rice seeds grown in the absence and presence of cycloheximide. Rice seeds were sliced into embryo-containing and embryoless halves and germinated in the absence or presence of 5 µM cycloheximide. 30 µg of total soluble protein was separated by 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and analysis of ns-Hbs was as described in the legend to Fig. 1. **1:** Dry, embryoless; **2:** dry, embryo-containing; **3:** 3 days imbibed without cycloheximide, embryoless; **4:** 3 days imbibed without cycloheximide, embryo-containing; **5:** 3 days treated with cycloheximide, embryoless; 6 3 days treated with cycloheximide, embryo-containing. The r-Hb standard was 50 ng of rHb2.

Discussion

Using immunolocalization, ns-Hbs were identified in primarily differentiated and differentiating cell types, including the aleurone, scutellum, root cap cells, sclerenchyma, and tracheary elements. Dry, ungerminated rice seeds contained very low amounts of ns-Hbs and levels increased during germination as observed by immunochemical analyses of soluble proteins from various tissues (Fig. 1B-F). The increase in ns-Hb protein during seed germination appears to be due in part to de novo synthesis, as it is inhibited by the addition of cycloheximide upon seed imbibition (Fig. 5). However, signals originating from the embryo do not apparently control the production of ns-Hbs. These data corroborate and significantly extend earlier studies performed with barley (Taylor et al. 1994, Nie and Hill 1997, Duff et al. 1998). During seed germination and early seedling development, ns-Hb proteins were present in roots, seeds, mesocotyl, and leaves. However, there appeared to be a down-regulation of ns-Hb synthesis or up-regulation of protein turnover in maturing leaves.

Utilizing confocal laser scanning microscopy, ns-Hbs were immunolocalized in specific cell types; namely the aleurone, scutellum, sclerenchyma, root cap, and developing xylem (Fig. 2-4). Seed germination and early seedling development are processes accompanied by the programmed depletion of stored seed reserves. Typically, during germination of cereal grains such as rice, the aleurone and cells of the scutellum play a central role in mobilizing reserves (Radi and Maeda 1987). These two tissues are the principal sites for the conversion of starch into sucrose, which is then transported into the embryonic axis for subsequent metabolism (Nomura et al. 1969, Briggs 1972). With the onset of germination, new cell types are formed which take on specific roles for the growth and maintenance of the plant. These include gravi-sensing root cap cells, which differentiate from peripheral root cap cells into border cells and eventually slough off (Woo and Hawes 1997), and the cells of the vasculature and tissue such as sclerenchyma.

Analyses of different plant species transformed with a variety of *hb-promoter-gus* fusions have indicated that these promoters will drive *gus* expression in different tissues. Root-nodule-specific promoters appear to maintain this specificity in transformed plants (Andersson et al. 1997, Franche et al. 1998, Strózycki et al. 2000). In nonnodulating transgenic plants or plants transformed with *ns-hb-promoter-gus* fusions, GUS activity was principally detected in the vascular bundles, roots and root cap cells (Bogusz et al. 1990, Jacobsen-Lyon et al. 1995, Franche et al. 1998, Strózycki et al. 2000). Our observations of ns-Hb proteins in root cap cells and the vasculature are consistent with these earlier studies, and have for the first time demonstrated that native Hb proteins are indeed present in these tissues.

Localization of ns-Hbs in differentiating xylem is particularly interesting. Our studies strongly suggest that ns-Hbs are synthesized during an early stage of tracheary-element differentiation. However, ns-Hb signals were also detected in cells at a more advanced stage of xylogenesis, possibly due to sequestration of proteins within a cellular compartment (for example, cell walls) which delays their eventual degradation by proteases released during the differentiation process. Xylogenesis is a complex cytodifferentiation process in which a distinct metabolism has been documented for specific stages (Fukuda 1996, A. Jones 2000). It is unknown at what stage(s) of xylogenesis *ns-hb* genes are expressed. The availability of elegant *in vitro* and *in vivo* systems to study xylogenesis (Fukuda and Komamine 1980, Fukuda 1996, Jones 2000, Roberts and McCann 2000) will be an asset to the future determination of the role of ns-Hbs during this process.

Previously, ns-Hbs have been detected in diverse tissues under stressed and unstressed conditions (Andersson et al. 1996, Arredondo-Peter et al. 1997, Trevaskis et al. 1997, Hill 1998). For example, in 6-week-old plants, the *ns-hb1* and *ns-hb2* genes are differentially expressed (Arredondo-Peter et al. 1997), indicating tissue-specific regulation. It is conceivable that ns-Hbs have multiple roles in the plant, which are determined by the specific gene product, the levels of functional protein, and the specific sites of protein accumulation. Seregélyes et al. (2000) have shown that cultured alfalfa cells synthesize an ns-Hb under hypoxia that is apparently nuclearly localized. This is interesting, since all plant ns-Hbs reported to date lack a nuclear-localization signal and appear to be cytoplasmic. However, ultrastructural procedures may unmask sites of protein localization not evident at lower levels of resolution.

Ns-Hbs may be involved as oxygen scavengers or in oxygen signaling (Appleby 1992, Goodman and Hargrove 2001). However, compared to symbiotic Hbs, which are found in concentrations that range from 1 to 3 mM (Appleby 1984), ns-Hbs are found in much lower concentrations (Duff et al. 1998, Hill 1998; this work). In addition, given the *in vivo* localization of ns-Hbs in metabolically active tissues and the very high affinity for oxygen binding of several r-ns-Hbs, it is unlikely

that they function as oxygen scavengers (Arredondo-Peter et al. 1998, Hill 1998). Both Arredondo-Peter et al. (1998) and Hill (1998) have suggested that plant ns-Hbs could act as NO scavengers, in a similar manner to those of the bacterial flavohemoglobins, which have been documented to possess nitric oxide dioxygenase properties (R Gardner et al. 1998, A. Gardner 2000). However, ns-Hbs lack a flavin domain, which would be required for functioning as an NO dioxygenase. It has also been suggested that there is a general function for ns-Hbs as an oxygenase by oxidizing NADH in conjunction with a flavoprotein in order to maintain the energy demands of the cell (Arredondo-Peter et al. 1998, Hill 1998). Conceivably, such interactions could occur at the dimer interface of differently ligated ns-Hbs (Goodman and Hargrove 2001).

On the basis of the nature of ns-Hb expression in specific rice cell types we propose that these proteins likely play a key role in a particular metabolic function(s). We have observed ns-Hbs so far only in terminally differentiated or terminally differentiating tissues such as the aleurone, root cap cells, or differentiating xylem. All of these tissues have inherently different cell-specific metabolism but need to maintain this metabolism until no longer required by the plant (for instance, the aleurone) or have become terminally differentiated (xylem). It is possible that ns-Hbs prevent an early demise of these cells or are part of a pathway that permits the orderly progression of cell development. For instance, if they were found in all rice tissue and cell types it would be more likely that they played a general "housekeeping" role; however, the nature of our findings indicates that, indeed, there may be a specific developmental function(s) of the ns-Hbs in vivo.

Acknowledgments

This work was supported in part by the University of Nebraska Center for Biotechnology funded through the Nebraska Research Initiative. Partial funding for R.A.-R was from DGAPA/PAPIIT- UNAM (project number IN202399) and CONACYT (project number 25229N). This work is published as journal series number 13123 from the Agricultural Research Division, University of Nebraska–Lincoln.

References

Andersson CR, Jensen EO, Llewellyn D J, Dennis ES, Peacock WJ (1996) A new hemoglobin gene from soybean: a role for hemoglobin in all plants. Proc Natl Acad Sci USA 93:5682-5687, Llewellyn DJ, Peacock WJ, Dennis ES (1997) Cell-specific expression of the promoters of two nonlegume hemoglobin genes in transgenic legume, *Lotus corniculatus.* Plant Physiol 113: 45- 57

Appleby CA (1984) Leghemoglobins and *Rhizobium* respiration. Annu Rev Plant Physiol 35:443-478, (1992) The origin and functions of haemoglobin in plants. Sci Prog 76:365-398 , Tjepkema JD, Trinick MJ (1983) Hemoglobin in a nonleguminous plant *Parasponia:* possible genetic origin and function in nitrogen fixation. Science 220:951-953 , Bogusz V, Dennis ES, Peacock WJ (1988) A role for hemoglobin in all plant roots? Plant Cell Environ 11:359-367

Arredondo-Peter R, Hargrove MS, Sarath G, Moran JF, Lohrman J, Olson JS, Klucas RV (1997) Rice hemoglobins: gene cloning, analysis and oxygen-binding kinetics of a recombinant protein synthesized in *Escherichia coli.* Plant Physiol 115:1259-1266, Moran JF, Sarath G, Klucas RV (1998) Plant hemoglobins. Plant Physiol 118:1121-1125

Bogusz D, Llewellyn D J, 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

Bolognesi M, Bordo D, Rizzi M, Tarricone C, Ascenzi P (1997) Non- vertebrate hemoglobins: structural bases for reactivity. Prog Biophys Mol Biol 68:29-68

Briggs DE (1972) Enzyme formation, cellular breakdown and the distribution of gibberellins in the endosperm of barley. Planta 108: 351-358

Duff SMG, Wittenberg JB, Hill RD (1997) Expression, purification and properties of recombinant barley *(Hordeum* spp.) hemoglobin: optical spectra and reactions with gaseous ligands. J Biol Chem 272:16746-16752, Guy PA, Nie X, Durnin DC, Hill RD (1998) Haemoglobin expression in germinating barley. Seed Sci Res 8:431-436

Franche C, Diouf D, Laplaze L, Auguy F, Frutz T, Rio M, Duhoux E, Bogusz D (1998) Soybean *(lbc3), Parasponia* and *Trema* hemoglobin gene promoters retain symbiotic and nonsymbiotic specificity in transgenic casuarinaceae: implications for hemoglobin gene evolution and root nodule symbioses. Mol Plant Microbe Interact 11:887-894

Fukuda H (1996) Xylogenesis: initiation, progression, and cell death. Annu Rev Plant Physiol Plant Mol Biol 47:299-325, Komamine A (1980) Direct evidence for cytodifferentiation to tracheary elements without intervening mitosis in a culture of single cells isolated from the mesophyll of *Zinnia elegans*. Plant Physiol 65:61-64

Gardner AM, Martin LA, Gardner PR, Dou Y, Olson JS (2000) Steady state and transient kinetics of *Escherichia coli* nitric-oxide dioxygenase (flavohemoglobin). J Biol Chem 275:12581-12589

Gardner PR, Gardner AM, Martin LA, Salzman AL (1998) Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. Proc Natl Acad Sci USA 95:10378-10383

Goodman MD, Hargrove MS (2001) Quaternary structure of rice nonsymbiotic hemoglobin. J Biol Chem 276:6834-6839

Hargrove MS (2000) A flash photolysis method to characterize hexacoordinate hemoglobin kinetics. Biophys J 79:2733-2738

Hill RD (1998) What are hemoglobins doing in plants? Can J Bot 76:707-712

Jacobsen-Lyon K, Jensen EO, Jorgensen J, Marcker KA, Peacock WJ, Dennis ES (1995) Symbiotic and nonsymbiotic hemoglobin genes of *Casuarina glauca.* Plant Cell 7:213-233

Jiao J, Echevarria C, Vidal J, Chollet R (1991) Protein turnover as a component in the light/dark regulation of *phosphoenolpyruvate* carboxylase protein-serine kinase activity in C4 plants. Proc Natl Acad Sci USA 88:2712-2715

Jones A (2000) Does the plant mitochondrion integrate cellular stress and regulate programmed cell death? Trends Plant Sci 5:225-230

Jones TJ, Rost TL (1989) The developmental anatomy and ultrastructure of somatic embryos from rice *(Oryza sativa* L) scutellum epithelium cells. Bot Gaz 150:4149

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685

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

Nomura T, Kono Y, Akazawa T (1969) Enzymic mechanism of starch breakdown in germinating rice seeds II: scutellum as the site of sucrose synthesis. Plant Physiol 44:765-769

Penheiter A, Duff SMG, Sarath G (1997) Soybean root nodule acid phosphatase. Plant Physiol 114:597-604

Radi SH, Maeda E (1987) Ultrastructures of rice scutellum cultured with attached root using two separate media as compared to the intact seedling. Jpn J Crop Sci 56:73-84

Roberts K, McCann MC (2000) Xylogenesis: the birth of a corpse. Curr Opin Plant Biol 3:517-522

Seregélyes C, Mustárdy L, Ayaydin F, Sass L, Kovács L, Endre G, Lukács N, Vass I, Kiss GB, Horváth GV, Dudits D (2000) Nuclear localization of a hypoxia-inducible novel non-symbiotic hemoglobin in cultured alfalfa cells. FEBS Lett 482:125-130

Strózycki PM, Karlowski WM, Dessaux Y, Petit A, Legocki AB (2000) Lupine *leghemoglobin I:* expression in transgenic *Lotus* and tobacco tissues. Mol Gen Genet 263:173-182

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 CR, Llewellyn D J, 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

Vinogradov SN, Walz DA, Pohajdak B, Moens L, Kapp OH, Suzuki T, Trotman CN (1993) Adventitious variability? The amino acid sequences of nonvertebrate globins. Comp Biochem Physiol (b) 106:1-26

Woo H, Hawes MC (1997) Cloning of genes whose expression is correlated with mitosis and localized in dividing cells in root caps of *Pisurn sativum* L. Plant Mol Biol 35:1045-1051