Nuclear localization of a hypoxia-inducible novel non-symbiotic hemoglobin in cultured alfalfa cells[1]

Csaba Seregélyes[a], László Mustárdy[a], Ferhan Ayaydin[a], László Sass[a], László Kovács[a], Gabriella Endre[b], Noémi Lukács[a], Izabella Kovács[a], Imre Vass[a], György B. Kiss[b], Gábor V. Horváth[a,c], Dénes Dudits[a,c,*]

[a] *Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, P.O. Box 521, 6701 Szeged, Hungary*
[b] *Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, P.O. Box 521, 6701 Szeged, Hungary*
[c] *Biotechnology Institute, Bay Zoltán Applied Research Foundation, Derkovits fasor 2, 6726 Szeged, Hungary*

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Abstract We have isolated a 483-bp-long full-length cDNA clone encoding a non-symbiotic hemoglobin called *Mhb1*, the first one found in alfalfa. This non-symbiotic hemoglobin is a single copy gene localized in linkage group 4 in diploid *Medicago* genome. The *Mhb1* mRNA was found only in the roots of alfalfa plants. The *Mhb1* gene was inducible by hypoxia and showed no induction by cold stress treatment. The *Mhb1* transcript level increased at the G2/M boundary in a synchronized alfalfa cell suspension culture. The majority of Mhb1 protein was shown to be localized in the nucleus and smaller amounts were detected in the cytoplasm. A potential link to the nitric oxide signalling pathway is also discussed. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Non-symbiotic hemoglobin; Immunolocalization; Hypoxia; Cell division cycle

1. Introduction

Plant hemoglobins are usually divided into two major groups on the basis of their common features. The first group is formed by symbiotic hemoglobins. They can be found in leguminous plants and non-legumes living in symbiosis with nitrogen fixing organisms. Their role is to provide oxygen to symbionts in tissues actively fixing nitrogen [1].

The second major group contains non-symbiotic hemoglobins. They are more ancestral [2] and more widespread than symbiotic hemoglobins since they are not only present in plants containing symbiotic hemoglobins ([1–5], this study), but also in other plant species, e.g. *Arabidopsis* [6], barley [7], rice [8] and *Trema tomentosa* [9]. Non-symbiotic hemoglobins possess a set of characteristic features. They have a much higher affinity to oxygen than symbiotic hemoglobins [10] and they are inducible by hypoxia ([6,7], this study). Barley hemoglobin was shown to be involved in ATP metabolism under hypoxia [11]. A hemoglobin gene (*AHB2*) from *Arabidopsis thaliana* with close homology to non-symbiotic hemoglobins is inducible by cold stress but not by hypoxia [6]. High mRNA levels of non-symbiotic hypoxia-inducible hemoglobin were observed in the roots and rosette leaves of barley [7], young leaves, stems and roots of soybean [2], in rice leaves and roots [8] and in *A. thaliana* roots [6]. 2,4-Dinitrophenol (2,4-D), a respiratory chain uncoupler, was shown to increase both oxygen consumption and barley hemoglobin expression in barley aleurone tissue. This indicated that the expression of barley hemoglobin is influenced by the availability of ATP in the tissue [12]. Accumulation of non-symbiotic hemoglobin mRNA under non-hypoxic conditions is supposed to occur because of the high metabolic activity of the above mentioned tissues [2].

Here we describe that *Mhb1* gene transcripts are accumulated under hypoxia and this protein is primarily localized in the nuclei of alfalfa suspension cells grown under normal conditions. Furthermore, we show that the induction of the *Mhb1* gene is increased in synchronized G2/M cells.

2. Materials and methods

2.1. Plant material

A highly homogeneous, fast-growing cell suspension culture of *Medicago sativa* ssp. *varia* (genotype A2, tetraploid) was established and maintained in Murashige and Skoog medium [13] in the presence of 1 mg/l 2,4-D and 0.2 mg/l kinetin [14].

2.2. Construction and random sequencing of an alfalfa cDNA library

An alfalfa cell suspension cDNA library was constructed with a cDNA Library Construction Kit purchased from Clontech (Palo Alto, CA, USA) according to the instructions of the manufacturer. On random clones from this cDNA library automated sequencing was performed according to company specifications (Applied Biosystem, Foster City, CA, USA).

2.3. Genomic hybridization

Genomic hybridization of the *Mhb1* gene was carried out on filters containing *Dra*I-digested genomic DNA of diploid *M. sativa* plants. These individuals belong to a segregating F2 population of self-mated progeny of F1/1 which originates from the cross between *M. sativa* ssp. *coerulea* (male parent) and *M. sativa* ssp. *quasifalcata* (female parent) used earlier for genetic mapping by [15]. Conditions for hybridization were the same as described by [15].

2.4. Synchronization of the cell cycle and flow cytometric analysis

Alfalfa suspension cells were treated with hydroxyurea (Sigma, St. Louis, MO, USA) at a concentration of 10 mM for 36 h to synchronize cells. Then the cells were washed three times with fresh Murashige and Skoog medium [13], and cultured further for synchronous growth [16]. The isolation of nuclei and the flow cytometric analysis were performed according to [17].

2.5. Stress treatments

Hypoxia treatment: gaseous N_2 was bubbled for 90 s through 50 ml of alfalfa cell suspension culture in a 100-ml culture flask which was

*Corresponding author. Fax: (36)-62-433 434.
E-mail: dudits@nucleus.szbk.u-szeged.hu

[1] GenBank accession number: AF172172.

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MHB1   MGTLDTKGFTEEQEALVVKSWNAMKKNSAELGLKLFLKIFEIAPSAQKLFSFLKDSKVPL 60
CASNON .S..EGR.............S...P.AG.....F.....................N... 60
SOYNON .T.TLER..S.............V.....G.....F.................R..T... 60
TREMA        .V.............AV...........F...........KN...Y....PI.. 54
PARA         .V............A.AV.........QF...........KN...Y....P... 54
AHB1           .............SV............I.......TTK.M....R..PI.A 52
RICE2          .S.......L...AI...D..NI..RF......V....SQM....RN.D... 52
BARLEY         .S..K....L...AI...D..N...RF..........RQM.P..R..D... 52
RICE1          .S.......L...AIL..D..NIA.RF......V....SQM....RN.D... 52
AHB2          ....K.....KE..EIL.QDIPKYS.HF.SQ.L....A.KG.....R..DEVP 53
CASSYM         ..K....LKQ..EVL.Q.IPAHS.R..AL.I.A..ESKYV.......NEIP 51
MLB1           ..DK.....NS..E.F.Q.LPRYSVFFYTVVL.K..A.KG......N.-AEV 51
PEALB         ....K.....NS..ELF.Q.PS-YSVLFYTI.LKK..A.KGM.......AEVV 52

MHB1   EQNTKLKPHAMSVFLMTCESAVQLRKSGKVTVRESSLKKLGANHFKYGVVDEHFEVTKFA 120
CASNON .R.P...S.....................A..............S...H..A......... 120
SOYNON ...P......V...V...D.......A........N......T..RT..AN......... 120
TREMA  ...P.......T..V...........A........N..R...I...N...N......R.. 114
PARA   ...P......TT..V...........A.....K..D..RI..I...T...N......R.. 114
AHB1   ...P..........V.C.........T.......TT..R...S.S..........A.Y. 112
RICE2  .K.P...T......V....A.A....A......DTT..R...T......G.A.....R.. 112
BARLEY .T.P...T..V...V....A.A....A..I....TT..R..GT.L....A.G.....R.. 112
RICE1  .K.P...T......V....A.A....A......DTT..R...T.L....G.A....V... 112
AHB2   HN.P...A..VK..K....T.I...EE...V.ADTT.QY..SI.L.S..I.P....V.E. 113
CASSYM .N.P...A..AVI.KTI....TE..QK.HAVWDNNT..R..SI.L.NKIT.P....M.G. 111
MLB1   QDSPQ.QA..EK..GLVRD......AT.G.VLGDAT.---..I.VRK....P..V.V.E. 108
PEALB  D-SP..QA..EK..G.VHD..I...A..E.VLGDVT.---..I.IQK..I.P..V.V.E. 108

MHB1   LLETIKEAVP-EMWSPAMKNAWGEAYDQLVNAIKSEMKPSS 160
CASNON ..........-.T...E..........K..A...L..... 160
SOYNON ..........-.....................D.......P. 160
TREMA  ..........-.....E.............A.......... 154
PARA   ..........-.....E......V......A...F..... 154
AHB1   ..........-.....E..V...Q...H..A...A..NL.N 152
RICE2  ..........VD.......S..S...N...A...Q....A 153
BARLEY .........L.AD..G.E.R..........A...Q....A 153
RICE1  ..D....E..AD.......S..S....H..A...Q....A 153
AHB2   ..R.L..GLG-.KYNEEVEG..SQ...H.AL...T...   150
CASSYM ..G.....IK-.N..DE.GQ..T...N...AT..A...   148
MLB1   ..K.....AG-DK..EELNT..EV...A.AT...KA.    144
PEALB  ..D.....SG-.K..EELST..EI..EG.AS...KA.    144
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Fig. 1. Alignment of some plant hemoglobin protein sequences using the BLASTP program. The highly conserved residues of heme and ligand binding (the distal and proximal histidines, phenylalanine CD1 and proline CD2) are in bold [21]. Plant globin motif characteristic of plant hemoglobins is shown underlined. Dots mean identical amino acids. Hemoglobins (with GenBank accession numbers or with reference numbers): MHB1: this study (AF172172), CASNON: *Casuarina glauca* non-symbiotic hemoglobin (X53950, 86% amino acid identity to Mhb1 protein), SOYNON: soybean non-symbiotic hemoglobin (U47143, 85% identity), TREMA: *T. tomentosa* non-symbiotic hemoglobin (Y00296, 85% identity), PARA: *Parasponia andersonii* hemoglobin (U27194, 82% identity), AHB1: *A. thaliana* class 1 non-symbiotic hemoglobin (U94998, 79% identity), RICE2: rice class 2 non-symbiotic hemoglobin (U76028, 74% identity), BARLEY: barley non-symbiotic hemoglobin (U01228, 72% identity), RICE1: rice class 1 non-symbiotic hemoglobin (U76029, 71% identity), AHB2: *A. thaliana* class 2 non-symbiotic hemoglobin (U94999, 52% identity), CASSYM: *C. glauca* symbiotic hemoglobin ([22], 51% identity), MLB1: *M. sativa* class 1 leghemoglobin (X13375, 45% identity), PEALB: pea leghemoglobin (AB015720, 46% identity).

then closed in an airtight manner and was left unshaken for 24 h. Samples were taken at 2, 6, 12 and 24 h. N_2 bubbling for 90 s through the sample was repeated after each sample taking step. The samples were snap-frozen in liquid nitrogen and stored at −70°C.

Cold stress treatment [6]: a culture flask containing alfalfa cell suspension was placed in a 4°C atmosphere and was shaken for 24 h. Samples were taken at 0 and 24 h, frozen in liquid nitrogen and stored at −70°C.

2.6. RNA isolation and Northern analysis

Total RNA was prepared from frozen alfalfa cells harvested at the indicated timepoints according to the procedure described by [18] with a slight modification to scale the method to the volume of Eppendorf tubes. Total RNA was quantified by optical density at 260 nm [18]. Twenty μg total RNA was loaded on 1% formaldehyde gel containing 0.01% ethidium bromide. Transfer of the RNAs to an Hybond N^+ filter (Amersham, UK) was performed with the capillary action technique, and the filter was examined under UV light to verify the efficiency of transfer and to test the quality and quantity of loaded RNA samples. Hybridization of the filter was performed in hybridization buffer containing 5 mM EDTA, 50 mM pH 7 mixture of Na_2HPO_4 and NaH_2PO_4 [19], 900 mM NaCl, 100 μg/ml tRNA, 1× Denhardt's reagent [19] and 0.1% sodium dodecyl sulfate (SDS) at 65°C. Radiolabeled probes were generated by random-primed ^{32}P-labelling of the coding regions of *CycIIIMs* [17] and *Mhb1* and cDNA fragments

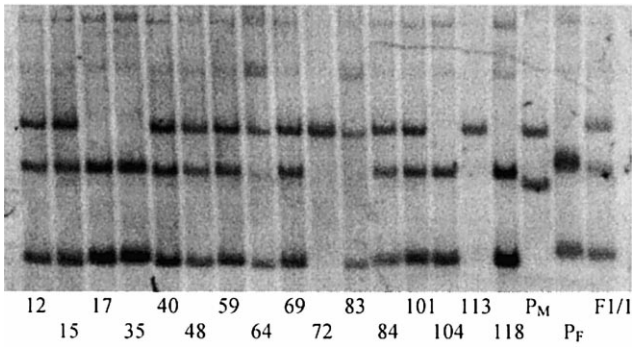

Fig. 2. DNA–DNA hybridization analysis and genetic mapping of the alfalfa non-symbiotic hemoglobin gene *Mhb1*. P_M and P_F are male and female parental plants, respectively. Numbers identify the individuals of self-mated progeny of the F1/1 plant.

encoding the 3′ non-translated regions of the actin gene from *Medicago truncatula* and *Mhb1*.

2.7. Protein extraction, antibody raising, immunoblotting

Proteins were extracted by grinding frozen alfalfa cells harvested at the indicated timepoints with quartz sand in homogenization buffer containing 25 mM Tris–HCl pH 7.7, 75 mM NaCl, 15 mM $MgCl_2$, 15 mM EGTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and leupeptin and chymostatin 10 μg/ml each.

The *Mhb1* hemoglobin gene was first cloned into a pTRC HisB expression vector (Invitrogen, Carlsbad, USA). Polyhistidine-tagged Mhb1 recombinant protein was expressed in *Escherichia coli* XL-I Blue cells (Stratagene, USA) and purified with the help of Xpress System according to the instructions of the manufacturer (Invitrogen, Carlsbad, USA). Polyclonal antibody against recombinant Mhb1 protein was raised in mice.

For immunoblotting, the SDS–polyacrylamide gels loaded with equal amounts (50 μg) of protein were transferred onto polyvinylidene difluoride (Millipore, Bedford, MA, USA) membranes in 50 mM Tris-base–50 mM boric acid buffer at a constant 10 V overnight. The filters were blocked in 5% milk powder–0.02% Tween 20 in TBS (25 mM Tris–Cl pH 7.4, 150 mM NaCl) buffer for 2 h at room temperature, reacted with the first antibody in the blocking buffer for 2 h at room temperature, washed three times with TBST (0.02% Tween 20 in TBS pH 7.4) and reacted with horse radish peroxidase-conjugated goat anti-rabbit IgG using the dilution recommended by the manufacturer (Sigma, St. Louis, MO, USA). After washing the membrane three times with TBST, signals were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and detected on X-ray films.

2.8. Immunogold labelling

Alfalfa suspension cells were pelleted by centrifugation and fixed with 1% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 1 h. After rinsing in the same buffer, the samples were dehydrated with ethanol and embedded in Lowicryl K4M resin (TAAB Laboratories Equipment, Berkshire, UK). Immunocytochemical localization of the Mhb1 protein was carried out according to Mustárdy et al. [20].

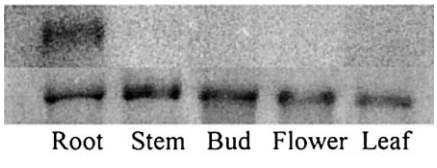

Fig. 3. Transcription pattern of the *Mhb1* gene in various plant tissues. The blot was probed with an actin clone from *M. truncatula* to verify the equal loading of RNA samples (lower lane).

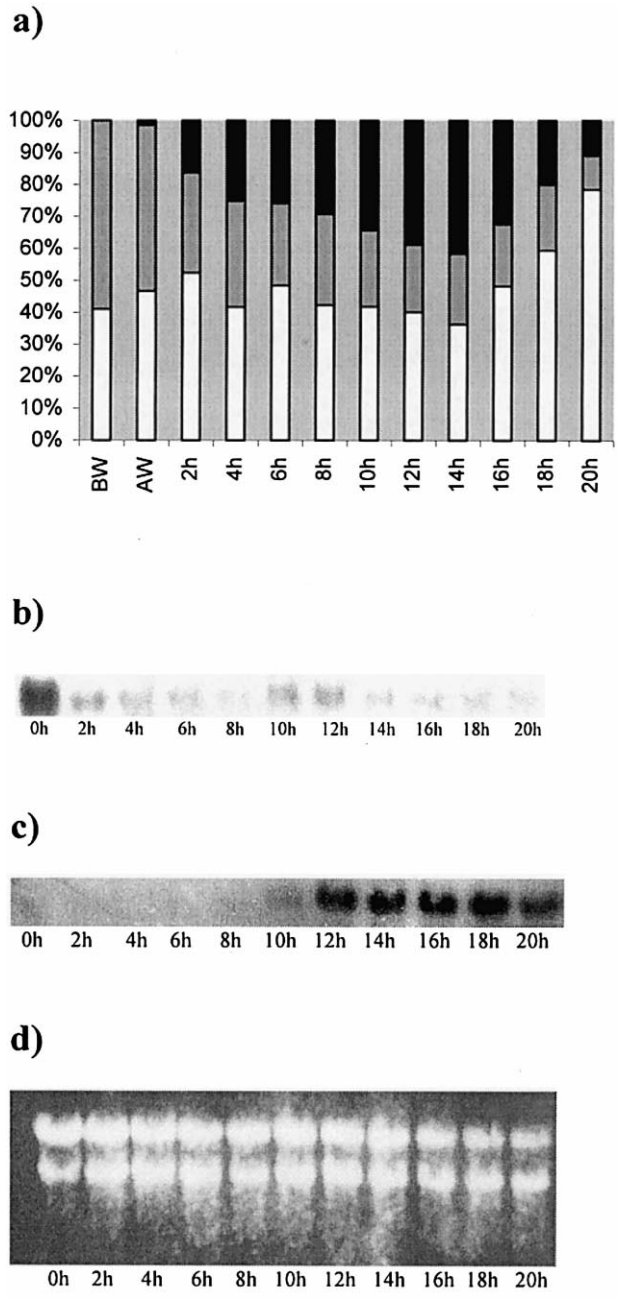

Fig. 4. a: Data from flow cytometric analysis show the proportion of cells found in different phases (G2, S, G1, black, gray and white bars, respectively) of the cell division cycle at the examined points of time. 'BW' and 'AW' stand for 'before wash' and 'after wash', respectively. b: Time course analysis of changes in *Mhb1* gene induction during cell division cycle of a synchronized alfalfa cell suspension culture. c: Changes in the mRNA levels of the mitotic cyclin *CycIIIMs* [18] at the examined timepoints of the experiment show that mitosis begins approximately 12–14 h and ends approximately 20 h after removing hydroxyurea. d: Photograph of the membrane used in the above hybridizations shows the RNA levels relative to each other.

3. Results and discussion

3.1. A non-symbiotic hemoglobin gene from alfalfa

In sequenced alfalfa cDNAs we have found a 483-bp-long clone that we identified with the help of the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) as *Mhb1*, a 160-amino acid-long (18 kDa) non-symbiotic non-leghemoglobin. It turns out from sequence alignments (Fig. 1) that *Mhb1* is a full-length clone and has all the features that are characteristic of plant hemoglobins. These features include a strong homology to other non-symbiotic hemoglobins and conserved amino acid residues: proximal (F8) and distal (E7) histidines, phenylalanine CD1 and proline C2 [21]. Plant globin motif is also present in the amino acid sequence of Mhb1 protein (PROSITE search, http://genomic.sanger.ac.uk/pss/pss.shtml), but not exactly in the [SN]-P-x-L-x(2)-H-A-x(3)-F form because in the case of Mhb1 protein the proline in the motif is exchanged by threonine. The PROSITE search has also revealed a cAMP- and cGMP-dependent protein kinase phosphorylation site ([RK]-x(2)-[ST], amino acids from position 26 to 29) that can be of functional relevance.

3.2. Genomic analysis and mapping of the Mhb1 gene

To determine *Mhb1* gene copy number and to investigate if there are any cross-hybridizations to leghemoglobins or to other types of non-symbiotic hemoglobins, DNA–DNA hybridization was performed to genomic DNA isolated from diploid *M. sativa* plants. The hybridization resulted in three strong bands on the *Dra*I-digested genomic DNA of diploid alfalfa plants (Fig. 2) which could mean the presence of the *Mhb1* gene either in single copy or low copy number. Since the DNA was isolated from individuals of the diploid *M. sativa* F2 segregating population, it was possible to determine by following their inheritance that all hybridization bands

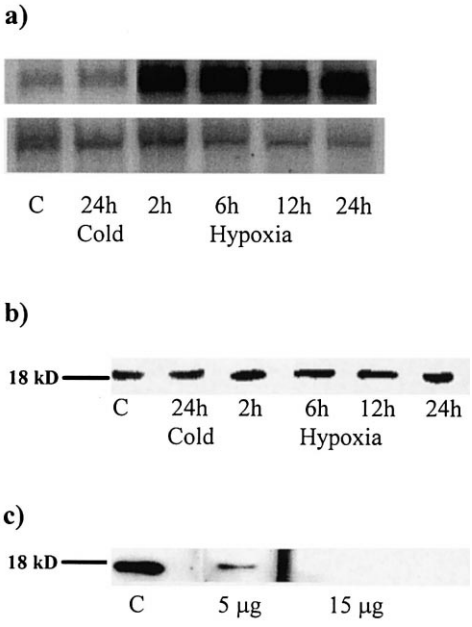

Fig. 5. a: Time course analysis of *Mhb1* gene induction under cold stress and hypoxia in alfalfa suspension cells. An increase in *Mhb1* mRNA level can be observed under hypoxic conditions only. 'C' stands for control. The blot was probed with an actin clone from *M. truncatula* to verify the equal loading of RNA samples (lower lane). b: Time course analysis of Mhb1 protein accumulation using polyclonal antibody raised against recombinant Mhb1 protein. No change is observable in comparison with control after cold stress or hypoxic treatment. 'C' stands for control. c: Preincubation of anti-Mhb1 antiserum aliquots with increasing amounts of recombinant Mhb1 protein (5 and 15 μg, respectively) results in the weakening and disappearance of the Mhb1 bands.

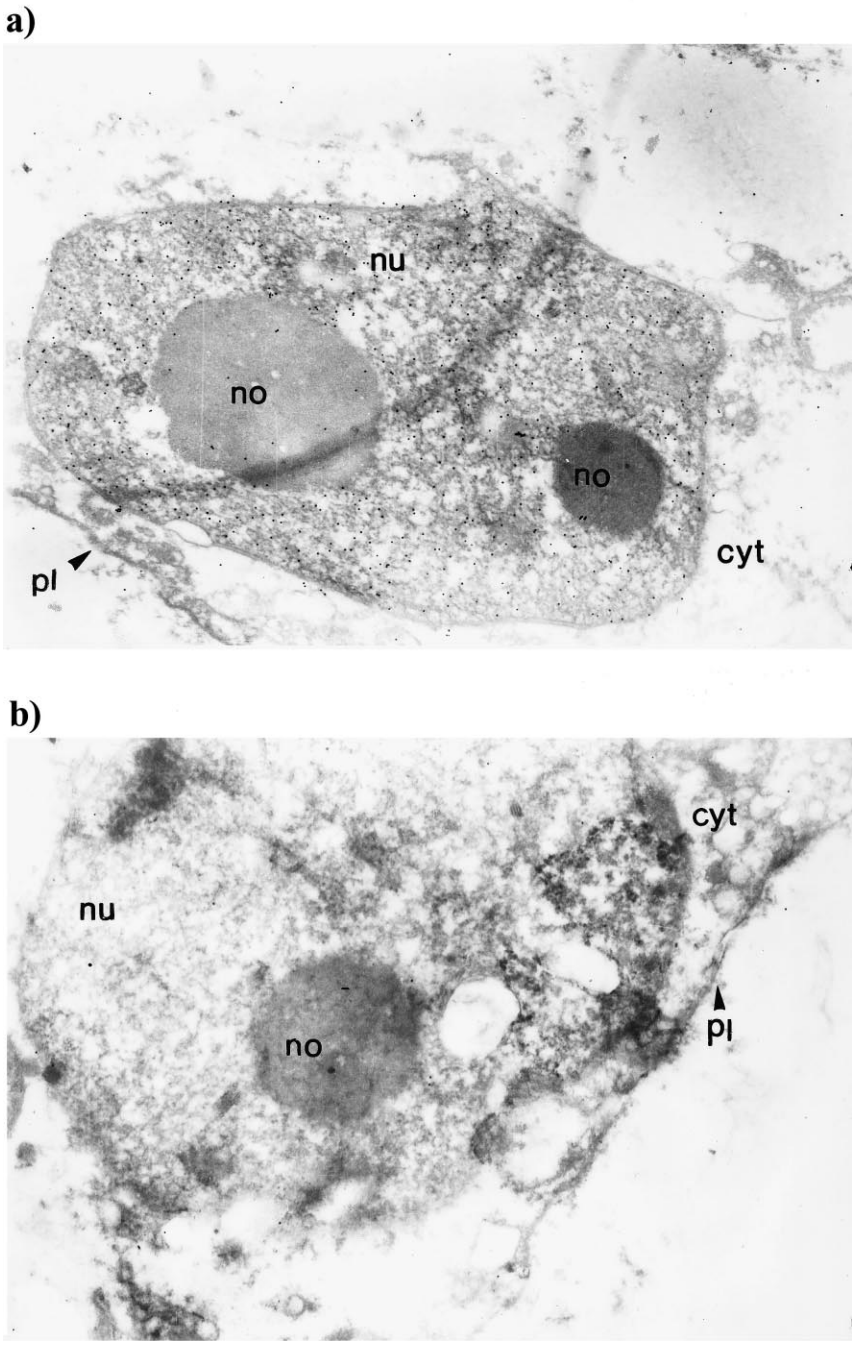

Fig. 6. Immunogold localization of Mhb1 using anti-Mhb1 antibodies in the alfalfa suspension cells. a: Cross-section of an alfalfa cell suspension cell showing mainly nuclear localization of the Mhb1 protein. Magnification: 11 520×. Abbreviations for (a) and (b): nu = nucleus, cyt = cytoplasm, no = nucleolus, pl = plasmalemma. b: Pre-immune control for (a), replacement of anti-Mhb1 with pre-immune mouse serum. Magnification: 11 520×.

represent the alleles of a single gene. The same population was used earlier to construct the detailed genetic map of alfalfa [15], so with the help of the already existing genotype data the genetic mapping *Mhb1* was also carried out. This gene is located on the 40–47 cM region of linkage group 4 between two codominant RAPD markers, OPA12A and OPA17A. We compared the *Mhb1* hybridization pattern to that of previously used leghemoglobin gene probes, but no cross-hybridization between leghemoglobins and the coding region of *Mhb1* gene could be detected. However, as can be seen in Fig. 2, besides the strong hybridization signals of the alleles of the single *Mhb1* gene, weakly cross-hybridizing bands can also be observed. These weak signals could possibly appear as a consequence of the *Mhb1* coding region hybridizing to other, still unidentified, types of non-symbiotic hemoglobins in alfalfa.

3.3. Expression of Mhb1 in various alfalfa organs

Northern analyses of total RNA samples obtained from various plant tissues grown under normal conditions have revealed an observable *Mhb1* RNA level only in the roots of alfalfa (Fig. 3). Hybridization of the same filter with actin

(control cDNA) gene indicated the equal RNA quantity in each lane (Fig. 3).

In contrast to the *Mhb1* gene, other non-symbiotic hemoglobin gene transcripts were detected also in tissues other than root [2,7,8]. The specificity of *Mhb1* gene induction can be explained by the relative hypoxia of the roots in the soil.

3.4. Mhb1 gene is activated during the cell cycle

To test if there is a cell cycle dependence of *Mhb1* gene transcription, we synchronized alfalfa cell suspension culture with hydroxyurea. Flow cytometric analysis (Fig. 4a) shows that the majority of cells are synchronized in the S phase at the beginning of the experiment. Fig. 4b shows changes in the *Mhb1* RNA level during cell division. Although the initial high RNA levels of the *Mhb1* gene could well have been caused by the 36 h long hydroxyurea treatment, the later RNA level changes at 10–12 h show the induction of the *Mhb1* gene before mitosis. A Northern hybridization was carried out with the mitotic cyclin *CycIIIMs* [18] in order to follow the procedure of cells through mitosis. This shows that G2/M begins at about 12–14 h (Fig. 4c). The relative amount of RNA in the lanes on the membrane can be seen in Fig. 4d.

The observed activation of the *Mhb1* gene at G2/M transition during the cell division cycle in a synchronized alfalfa cell suspension culture may reflect an increased metabolic activity and oxygen consumption. A similar process is also possible during cell division in the root tip meristems and in growing nodules as a result of high metabolic activity.

3.5. Induction of Mhb1 gene by hypoxia in cultured alfalfa cells

We used Northern blot experiments to study the *Mhb1* gene expression after exposing cultured alfalfa cells to hypoxia and cold stress. In undifferentiated alfalfa callus cells grown in the presence of 2,4-D and cytokinin the *Mhb1* gene showed a low, basal level of transcription that was not influenced by cold stress treatment (Fig. 5a). In the same culture a significant change in the amount of *Mhb1* RNA was detected as a result of the hypoxic treatment. It is clearly visible that the increase in the *Mhb1* transcript level appears as early as 2 h after the start of hypoxia, and this high level is maintained throughout the experiment (Fig. 5a). We hybridized the same RNA filter with actin (control cDNA) gene to show the equal RNA quantity in each lane.

After exposing cultured cells to cold stress and hypoxia, respectively, the Mhb1 protein accumulation was the same as in cells grown under normal conditions (Fig. 5b). The induction upon hypoxia is clearly detected on an RNA level, and the constant level of protein accumulation may indicate that the *Mhb1* mRNA is unstable or that a higher protein turnover occurs. The presence of Mhb1 protein in constantly dividing alfalfa suspension cells grown without stress treatment may also serve as evidence for the results mentioned in Section 3.4. Furthermore, the *Mhb1* gene encodes a member of the class 1 non-symbiotic hemoglobin group according to the classification of non-symbiotic hemoglobins of *A. thaliana* by Trevaskis et al. [6] in the sense that it can be induced by hypoxia but not by cold stress.

Before completing the above immunoblot, the anti-Mhb1 antiserum had been checked on an A2 cell suspension total protein extract. The antiserum recognized one clear band of the right size of 18 kDa. To test the specificity of this polyclonal antibody, its aliquots were incubated on ice for 1 h with increasing amounts (5 and 15 μg) of purified recombinant polyhistidine-tagged Mhb1 hemoglobin. This resulted in the gradual disappearance of the signal (Fig. 5c).

3.6. Immunogold localization of Mhb1

In order to determine the intracellular distribution of Mhb1 protein in alfalfa cells, immunoelectron microscopy was carried out. Fig. 6a shows the results obtained from immunolabelling using polyclonal antibody raised against recombinant Mhb1 protein. The vast majority of the labelling can be seen within the nucleus, with nucleoli virtually unlabelled. However, to a lower extent, cytoplasmic labelling is also observable. In the control picture, where pre-immune mouse serum was used instead of anti-Mhb1 antibody, no immunogold labelling is visible (Fig. 6b).

The result obtained by electron-microscopic immunolocalization studies can be particularly important if we consider that heme can serve as a substrate of nitric oxide synthase [23], on the one hand. On the other hand, in maize roots a nitric oxide synthase was localized in the nuclei of elongation zone cells [24]. Furthermore, the interaction between nitric oxide and hemoglobins in animal cells can contribute to oxygen delivery [25]. This and the the presence of both of the above mentioned proteins in the nucleus may have functional significance in the nitric oxide signalling pathway that is suggested to be the subject of further investigations.

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