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Cloning of a full-length symbiotic hemoglobin cDNA and in situ localization of the corresponding mRNA in *Casuarina glauca* root nodule

Hassen Gherbi, Emile Duhoux, Claudine Franche, Katharina Pawlowski, Atef Nassar, Alison M. Berry and Didier Bogusz

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We have characterized a full-length cDNA (*hb*-Cg1F) that represents symbiotic mRNA hemoglobin (*hb*) from *Casuarina glauca* root nodules. In situ hybridization was used to examine the correlation between *hb*-Cg1F mRNA and the state of the *Frankia* infection process. The efficiency of in situ hybridization using DIG-labeled vs [^{35}S]-labeled probes was compared. The expression of *hb*-Cg1F gene is induced in young infected host cells prior to the detection of *Frankia nif*H mRNA. Since *Frankia* does not form vesicles in *C. glauca* nodules, it is proposed that Hb is necessary to reduce the O_2 concentration in the cytoplasm of the host cells before the *nif* genes are expressed.

Key words – Actinorhizal symbiosis, *Casuarina glauca*, in situ hybridization, nitrogen-fixing nodules, plant hemoglobins.

D. Bogusz (corresponding author, e-mail bogusz@orstom.rio.net), H. Gherbi, E. Duhoux and C. Franche, ORSTOM-GeneTrop, BP5045, F-34032 Montpellier, France; K. Pawlowski, Dept of Molecular Biology, Agricultural Univ. Dreijenlaan 3, 6703 HA Wageningen, The Netherlands; A. Nassar (present address: INRA, Pathologie végétale, F-78026 Versailles Cedex) and A. Berry, Dept of Environmental Horticulture, Univ. of California, Davis, CA 95616, USA.

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Introduction

Hemoglobins (Hbs) occur in all legume root nodules that fix nitrogen (Appleby 1984) and in nodules formed on roots of the nonlegume *Parasponia* in symbiosis with *Rhizobium* (Wittenberg et al. 1986). More recently, Hbs have also been found in *Casuarina* one of the genera of the taxonomically highly diverse actinorhizal plants whose species are capable of forming root nodules with the actinomycete *Frankia* as endosymbiont (Tjepkema and Torrey 1979). The role of Hb in symbiosis is to supply oxygen to the bacterial respiration chain while preserving the activity of the oxygen-intolerant nitrogenase enzyme complex (Appleby 1984).

Frankia strains isolated from *Casuarina* nodules can fix nitrogen when they are grown in pure culture at atmospheric pO_2 because they are able to form vesicles in the free-living state. Vesicles are a barrier to oxygen diffusion (Meesters 1987). *Casuarina* nodules differ from other actinorhizal nodules in that *Frankia* does not form vesicles within the infected cells of the nodules (Berg 1983). Thus in the nodule, host cellular mechanisms exist for regulating the distribution of oxygen. Three major Hb components have been isolated from the nodules

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of *C. glauca* (Fleming et al. 1987). The amino acid sequence and the physiological properties of the *C. glauca* Hb component HbI have established the relationship of *Casuarina* Hbs to other plant symbiotic Hbs (Kortt et al. 1988). Furthermore, a *C. glauca hb* gene, *hb*II, has been cloned and characterized (Christensen et al. 1991). The comparison of the amino acid sequence of HbI and the deduced amino acid sequence of HbII with the amino acid sequence of other plant Hbs showed that *C. glauca* contains at least two different *hb* genes. *hb*I is closely related to symbiotic *lb* from legume whereas *hb*II shows extensive homology to the *Trema* nonsymbiotic *hb* gene and to the *hb* gene found in *Parasponia* (Christensen et al. 1991). Recently, symbiotic and nonsymbiotic *C. glauca hb* genes have been isolated and the corresponding promoters analysed. In the transgenic legume *Lotus corniculatus*, the *hb* promoters retained the symbiosis-specific pattern of expression observed in *Casuarina* (Jacobsen-Lyon et al. 1995).

The root nodules of *Casuarina* sensu stricto are composed of closely packed nodule lobes with uninfected roots emerging from the tips of each lobe (Torrey and Callaham 1978). The endophyte is intracellular and, as in legume symbioses, is separated from the plant cytoplasm by the host plant plasma membrane. However, in contrast to legume-*Rhizobium* symbioses, *Frankia* is not released from the infection thread. Each nodule lobe arises as a lateral root from the pericycle, and contains a central vascular cylinder (Torrey 1976).

In the present study, we describe the isolation and the characterization of a full-length Hb cDNA isolated from a *C. glauca* nodule cDNA library. The localization of *hb* mRNA in nodules of *C. glauca* was also studied by in situ hybridization. We found that *hb* transcripts were first detectable in cells adjacent to the apical meristem, where the endophyte began to infect cortical cells. The largest amont of *hb* mRNA was present in cells completely filled by the microsymbiont *Frankia*. By contrast, expression of *Frankia nif*H was only detectable in cells completely filled by *Frankia*.

Abbreviations – CTAB, cethyl ethyl ammonium bromide; DDC, diethyldithiocarbamate; DIG, digoxigenine; Hb, hemoglobin; PCR, polymerisation chain reaction.

Materials and methods

Plant material

Casuarina glauca seeds were planted and grown in a sand/vermiculite substrate (1:1, v/v) in a glasshouse under natural light at temperature between 25 and 30°C.

Well-developed seedlings were transferred to one-fourth strength Hoagland's solution (Hoagland and Arnon 1950) in containers (Torrey 1990) and inoculated with *Frankia* strain Thr (Girgis et al. 1990). Root nodules (3–6 lobes, 0.5 cm in diameter) were harvested into liquid nitrogen at 3–4 weeks post inoculation and stored at –70°C.

RNA preparation

Total RNA was extracted from root nodules and uninfected root tips using a modification of the method of Manning (1991) essentially as described by Phelep (M. Phelep 1992. Thesis, Ecole Nationale du Génie Rural, des Eaux et Forêts, Paris, France). Nodules and roots were frozen in liquid nitrogen, and homogenized in a blender with homogenization buffer which contained 10 m*M* Tris-HCl (pH 7.5), 100 m*M* NaCl, 1 m*M* disodium salt ethylenediaminetetraacetic acid (Na_2EDTA) (pH 8.0), 0.5% sodium dodecyl sulfate (SDS), 100 m*M* diethyldithiocarbamate (DDC), 20 mg ml^{-1} sodium metabisulfite and phenol (2:2:3, w/v/v). After centrifugation (20 000 *g*, 10 min) the aqueous phase was transferred and nucleic acids were precipitated in 2-butoxyethanol; RNA was separated from DNA by precipitation with 3 *M* LiCl (Manning 1991). Poly(A^+) RNA was selected by chromatography on oligo(dT) cellulose (Pharmacia, Piscataway, NJ, USA).

cDNA synthesis and cloning

cDNA was synthesized from 4 μg poly(A^+) mRNA from root nodules with a Time Saver cDNA synthesis kit from Pharmacia, used as recommended by the manufacturer. *Eco*RI/*Not*I adapters (Pharmacia) were ligated to the ends of the blunt-ended double-stranded cDNAs. The *Eco*RI-terminated cDNAs were ligated into the λgt10 vector (Stratagene, La Jolla, CA, USA). The ligated products were packaged into phage particles in vitro using a GigapackII Lambda DNA packaging system (Stratagene) and transformed into a host *Escherichia coli* strain NM514 (Stratagene). The resulting cDNA library consisted of approximately 2×10^6 bacteriophage particles, containing 60% recombinants.

Differential screening

Samples of phage plaques to be amplified were cut out with a sterile pipette tip and agitated in TM buffer (Sambrook et al. 1989) consisting of 50 m*M* Tris-HCl, pH 7.5, 10 m*M* $MgSO_4$. The released DNA was used as template for a polymerase chain reaction (PCR). The primers used for PCR were λgt10 21- and 24-mer universal primers (New England Biolabs, MA, USA) flanking the *Eco*RI site. The conditions used for PCR were as follows: 1× PCR buffer (Appligene, Illkirch, France), 20 μ*M* dATP, dCTP, dGTP and dTTP, 10 μ*M* for each oligonucleotide primer, and 0.25 unit of *Taq* polymerase (Appligene). PCR was performed for 35 cycles in a Perkin-Elmer Cetus thermal cycler (programmed for 1 min at 94°C, 1 min at 55°C and 2 min at 72°C per cycle).

Equal amounts of each of the PCR products were run on two duplicate 1% agarose gels that were then transferred onto two nylon membranes (Tropix, Bedford, MA, USA) by the Southern method (Southern 1975) and cross-linked by UV treatment.

The probes were synthesized by amplification of non-infected root and nodule cDNAs prepared as described above in cDNA synthesis protocol. Using 0.1 μ*M* of PCR primers complementary to the *Eco*RI/*Not*I adapter sequence (5'-CCGGAATTCGCGGCCGCT-3') cDNAs were labeled by including biotinylated-11-dUTP (Clontech, CA, USA) during amplification (Dennis et al. 1990). The reaction mixtures contained 1 unit *Taq* polymerase, 0.1 μ*M* bio-11-dUTP, 20 μ*M* of each dNTP in *Taq* reaction buffer (Appligene). The temperature programme used for the PCR was set up as described above for the phage plaques screening.

Hybridization and chemiluminescence detection were performed essentially following the manufacturer's protocol for the Southern-Light DNA detection system (Tropix). Filters were exposed to X-Omat film (Eastman Kodak, Rochester, NY, USA) for 20–90 min. Clones hybridizing specifically with the nodule cDNA were isolated by rescreening and plaque purification.

Hb cDNA clones were isolated by PCR using degenerated primers corresponding to a nucleotide sequence specifying a conserved region in plant Hb proteins (5'-ACWGARAARCARGARGCTTT-3' and 5'-AGYTTRG GRTTRTTYTCHGG-3') (M. Phelep 1992. Thesis, Ecole Nationale du Génie Rural, des Eaux et Forêts, Paris, France). PCR was performed for 35 cycles (1 min at 94°C, 1 min at 40°C and 3 min at 72°C per cycle).

DNA sequencing and analysis

cDNA inserts from purified plaques were amplified using the λgt10 21- and 24-mer primers, and sequencing of PCR products was performed using the Applied Biosystems 373A (Foster City, CA, USA) automatic sequencing system. The insert DNAs were partially sequenced. One clone, *hb*-Cg1F, was completely sequenced on both strands. The DNA sequences and deduced peptide sequences were analyzed using the Lasergene software (DNASTAR, Madison, WI, USA).

Southern blot analysis

Genomic DNA was prepared from *C. glauca* seedlings (2–3 cm long) by a modification of the cethyl ethyl ammonium bromide (CTAB) method (Rogers and Bendich 1985). The tissue was frozen in liquid nitrogen, pulverized with mortar and pestle, homogenized in 10 ml of nucleic acid extraction buffer (100 m*M* Tris-HCl, pH 7.5, 0.7 *M* NaCl, 20 m*M* Na_2EDTA, pH 8.0, 1% CTAB, 0.1 *M* DDC) and 5 m*M* dithiothreitol (DTT) at 65°C; after 10 min at room temperature, samples were extracted twice with chloroform and the aqueous phase was precipitated with two volumes of ethanol. The DNA pellet was resuspended in 5 ml of sterile distilled water and purified on two successive cesium chloride gradients. Ten micrograms of *C. glauca* genomic DNA were digested with *Eco*RI or *Bgl*II, subjected to agarose gel electrophoresis and blotted on nylon transfer membrane (Tropix). Hybridization was performed overnight at 42°C in hybridization buffer containing 50% formamide (Fluka-Puriss), 7% SDS, 0.25 *M* Na_2HPO_4, 2 m*M* Na_2EDTA, 100 mg ml^{-1} heparine, and 100 mg ml^{-1} salmon sperm sonicated DNA (Sigma Chemical Co.). The insert of *hb*-Cg1F was labeled with [^{32}P]-dCTP using an oligolabeling kit (Pharmacia). After hybridization the filters were washed twice with 0.2× saline-sodium citrate buffer (SSC), 1% SDS, 0.1% Na-pyrophosphate at room temperature and three times in the same solution at 65°C for 20 min (Church and Gilbert 1984). The filters were finally exposed to X-ray film at –70°C with two intensifying screens during 3–10 days for autoradiography.

Northern blot analysis

Total RNA was isolated from *C. glauca* roots and nodules as described for RNA preparation, while for RNA from stems and leaves LiCl precipitation was replaced by a centrifugation (200 000 *g*, 16 h) on a layer of 5.7 *M* CsCl (M. Phelep 1992. Thesis, Ecole Nationale du Génie Rural, des Eaux et Forêts, Paris, France). RNA samples (about 10 μg each) were separated on an agarose-formaldehyde gel (Sambrook et al. 1989), along with a size marker (0.24–9.5 kb RNA ladder; BRL, MD, USA), transferred to a nylon membrane, and hybridized with the biotinylated-11-dUTP-labeled *hb*-Cg1F (1 083 bp). The filter was washed and hybridization was detected as described for the differential screening.

In situ hybridization

In situ hybridization was performed essentially as described by Scheres et al. (1990) and van de Wiel et al. (1990).

For generation of the RNA probe, a λgt10 cDNA clone containing a fragment of the *hb*-Cg1F cDNA was amplified with two primers homologous to the coding region (5'-AACAATCATGGGAGGTAC-3' and 5'-TGG TTGTAGGCTTCAGTC-3'). The 390-bp PCR product was cloned into the *Sma*I site of a Bluescript II KS$^+$ vector (Stratagene). This plasmid was linearized using *Bam*HI and transcribed with T3 RNA polymerase (sense) or linearized with *Eco*RI and transcribed with T7 RNA polymerase (antisense), respectively. For the *Frankia nif*H probe, pFnifH1 (Ribeiro et al. 1995) was linearized with *Eco*RI and in vitro transcribed using RNA polymerase T7 for production of antisense RNA.

Radioactive and nonradioactive probes were used. The radioactive [^{35}S]-rUTP-labeled sense and antisense RNA were obtained according to Scheres et al. (1990). Probes were partially degraded to an average length of 150 nucleotides by heating for 30 min at 60°C in a buffer containing 0.2 *M* Na_2CO_3 and 0.2 *M* $NaHCO_3$. The transcription with DIG-rUTP was identical to [^{35}S]-rUTP labeled, except that probes were not degraded.

Nodules harvested at 6–8 weeks after *C. glauca* inoculation were fixed for 5 h in 4% paraformaldehyde,

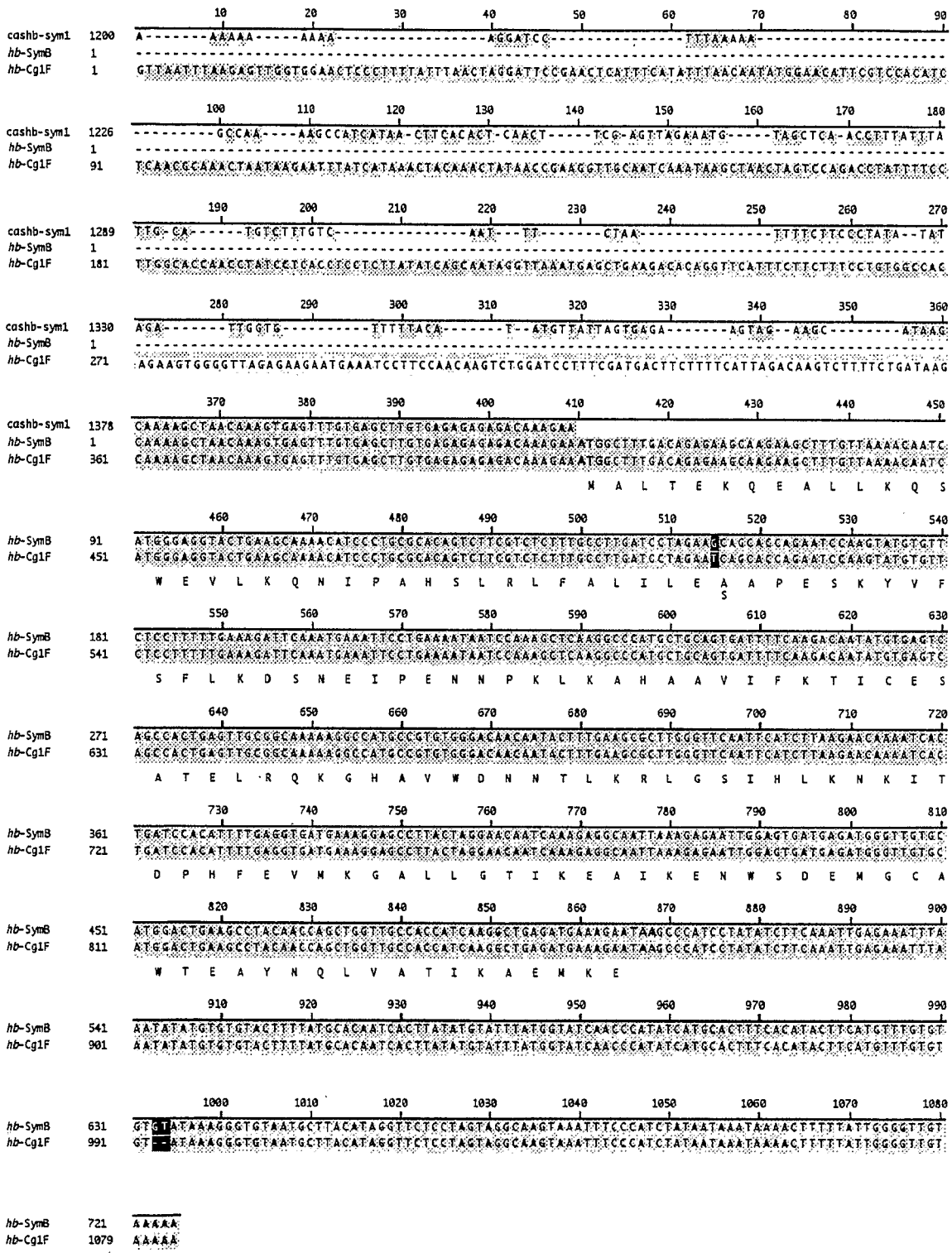

Fig. 1. Nucleotide sequence alignment of *hb*-Cg1F, *hb*-Symb (Jacobsen-Lyon et al. 1995) and the genomic sequence of ATG upstream region (nucleotides 1 200 to 1 427) of the cash-sym1 gene (Jacobsen-Lyon et al. 1995). The deduced amino acid sequences of the two cDNAs (*hb*-Cg1F and *hb*-SymB) are compared; differences between the two clones in the coding region are labeled in black. The start and the stop codon are given in bold print.

0.25% glutaraldehyde, 10% dimethyl sulfoxide (DMSO), 70 m*M* ethyleneglycol-bis(β-aminoethyl-ether)-N,N'-tetraacetic acid (EGTA) and 100 m*M* phosphate buffer, pH 7.2, and embedded in melted paraffin. Seven μm thick sections were cut with a steel knife on a microtome and stretched on poly-L-lysine coated glass slides at 42°C overnight. Hybridization conditions were as described by van de Wiel et al. (1990), 2×10^6 cpm of [^{35}S]-rUTP label were used per slide. After exposure for 4 weeks at 4°C, sections were stained with 0.025% toluidine blue and photographed with a Nikon microscope. Hybridization of digoxigenin-labeled probes was similar to the radioactive procedure except that the hybridization buffer did not contain DTT and detection was performed essentially as recommended by the manufacturer's protocol DIG Nucleic Acid Detection Kit (Boehringer Mannheim, GmbH, Germany). The alkaline phosphate-conjugated antidigoxigenin Fab fragment (Boehringer Mannheim) was diluted 1/5000 in buffer (100 m*M* Tris, pH 7.5, 150 m*M* NaCl) and applied to the slides for 60 min at room temperature. The 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium (BCIP-NBT) development was done according to the instructions supplied by the manufacturer (Boehringer Mannheim).

Results

Isolation and structure of *hb*-Cg1F cDNA

A cDNA library was constructed from 3- to 4-week-old *C. glauca* nodules using λgt10 as a vector. The library was screened for the presence of nodule-specific cDNAs by differential hybridization of randomly picked clones with biotin-labeled root or nodule cDNAs, respectively. Of a total of 160 clones, 28 hybridized with the nodule probe only. PCR reactions were performed on all 28 nodule specific clones with degenerated primers corresponding to the nucleotide sequence of a conserved region in plant Hb proteins (M. Phelep 1992. Thesis, Ecole Nationale du Génie Rural, des Eaux et Forêts, Paris, France). All nodule specific cDNAs could be amplified with *hb* primers. The size of these cDNAs ranged between 0.5 kb and 1.0 kb. The largest one, *hb*-Cg1F, was isolated and completely sequenced.

The cDNA was 1083 bp in length and contained an open-reading-frame of 459 bp; the 5'- and 3'-untranslated regions were 409 and 215 bp long, respectively. Sequence analysis revealed that *hb*-Cg1F showed homology to a *C. glauca* Hb cDNA clone (*hb*-SymB) recently described by Jacobsen-Lyon et al. (1995), differing only by one nucleotide in the coding region at position 515 and two gaps in the 3'-noncoding region at position 993 (Fig. 1). Forty-eight bp of the 5'-untranslated region of *hb*-SymB were identical to the corresponding sequence upstream of the ATG initiation codon of *hb*-Cg1F; however, in the 5'-end of this region (nucleotides 1 to 360), no significant homology could be found between the sequences (Fig. 1). The deduced amino acid sequence of *hb*-Cg1F is 98.7% identical to the *hb*-SymB deduced amino acid sequence and 97.4% identical to the HbI protein sequence reported by Kortt et al. (1988).

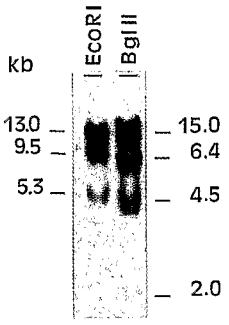

Fig. 2. Southern blot hybridization analysis of *hb*-Cg1F in the *C. glauca* genome. Total DNA from *C. glauca* was digested with *Eco*RI and *Bgl*II, respectively, and separated by agarose gel electrophoresis. The Southern blot was hybridized with the cDNA *hb*-Cg1F.

Genomic blot hybridization

To determine the number of genes coding for Hb in *C. glauca*, a Southern blot containing genomic DNA digested with various restriction enzymes was hybridized with the 1083-bp insert of *hb*-Cg1F as a probe. This insert hybridized strongly with several *Bgl*II (2.0, 4.5, 6.4, 15.0 kb) and *Eco*RI (5.3, 9.5, 13.0 kb) fragments suggesting that there are three to four *hb*-Cg1F homologs in the nuclear genome (Fig. 2). Similar results were observed by Jacobsen-Lyon et al. (1995) using a different *C. glauca* Hb cDNA as a probe.

Northern blot hybridization

A northern blot containing total RNA from roots, nodules and leaves/stems from *C. glauca* was hybridized with the insert of *hb*-Cg1F. The probe hybridized strongly with a 1.0-kb transcript present in root nodule RNA (Fig. 3). No *hb*-Cg1F gene expression was detectable in roots and leaves/stems. This is in agreement with the expression pattern seen in *C. glauca* by Jacobsen-Lyon et al. (1995) and with other studies that have exam-

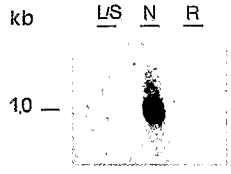

Fig. 3. Nodule-dependent accumulation of *hb* mRNA. Northern blots containing 10 μg slot^{-1} of *C. glauca* RNA were hybridized with the *C. glauca hb*-Cg1F cDNA. Total RNA had been isolated from non-nodulated roots (R), young leaves and stems (L/S) and nodules (N).

ined symbiotic *lb* (leghemoglobin) gene expression in legumes (Marcker et al. 1984, Jensen et al. 1986, Stougaard et al. 1987). The size of the hybridizing RNA (1.0 kb) was consistent with that of the cDNA insert (1 083 bp), suggesting that the *hb*-Cg1F clone contains a full-length cDNA.

Localization of *hb* transcripts in *C. glauca*

We examined longitudinal sections of nodule lobes to determine the location of *Frankia* infected cells. As shown in Fig. 4a, longitudinal or oblique longitudinal sections of a mature *C. glauca* nodule lobe confirmed

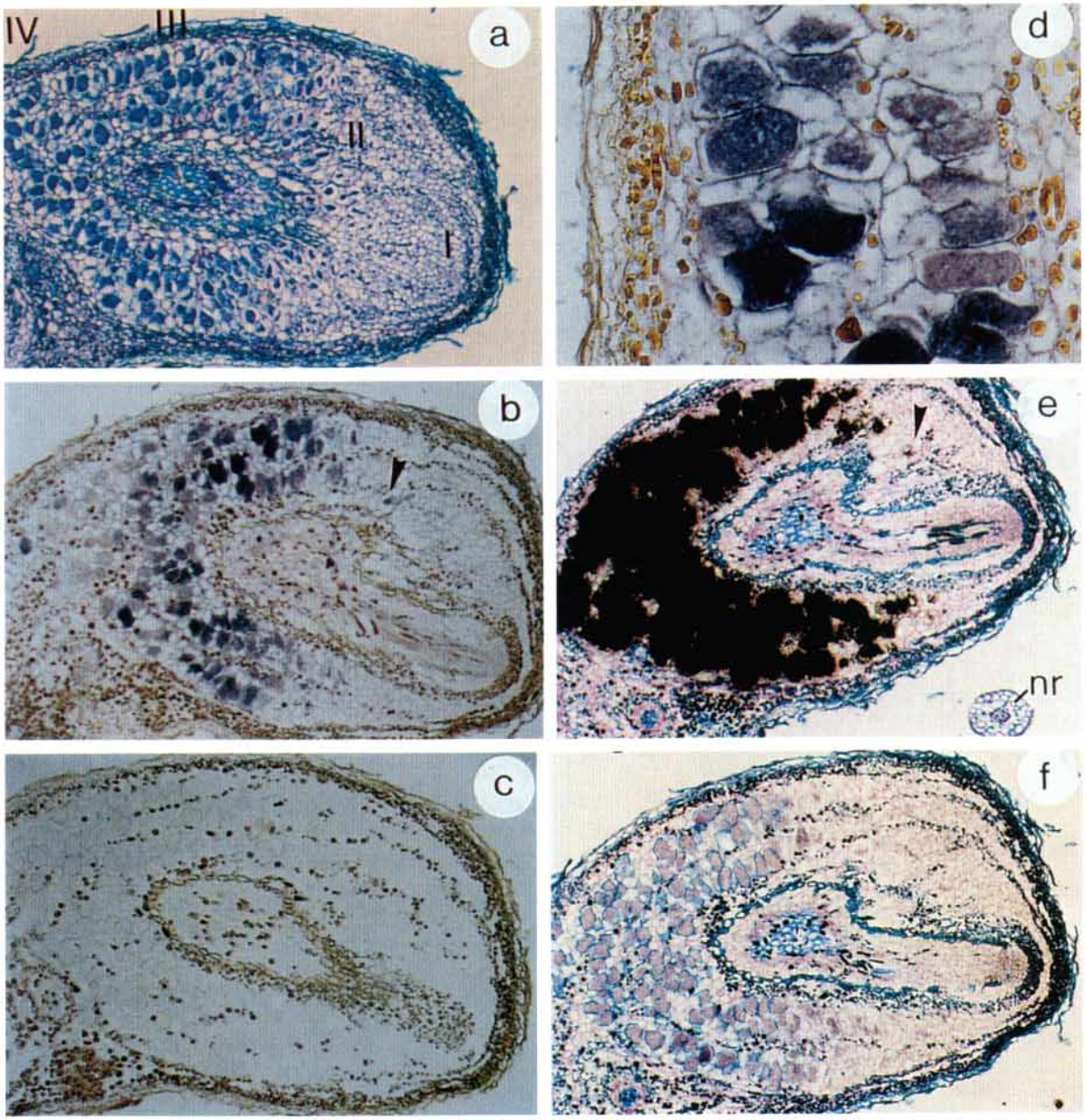

Fig. 4. Localization of *hb* transcripts in *C. glauca* nodule lobe. The sections in panels (b), (c) and (d) were hybridized with DIG-labeled probes, in panels (e) and (f) with [^{35}S]-labeled probes. For DIG-labeled probe, hybridization signal is seen as purple precipitate in panels (b) and (d); for [^{35}S]-labeled probe, silver grains denoting hybridization are visible as black dots in panels (e) and (f). Sections in panels (e) and (f) were stained with ruthenium red and toluidine blue. (a), Pseudo-longitudinal nodule lobe section of *C. glauca* stained with toluidine blue. Zone I, apical meristem; zone II, young infected and uninfected cells; zone III, mature infected cells; zone IV, senescent zone. (b, e), *hb* antisense RNA probes. *hb* transcripts were found mostly in the mature infected cells (zone III); low levels (arrow) of *hb* transcripts were observed in zone II; nr, nodule root. (c, f), *hb* sense RNA probes. No hybridization signal could be observed. (d), Magnification of Fig. 4b. Small infected cells were observed adjacent to cells fully infected with *Frankia*.

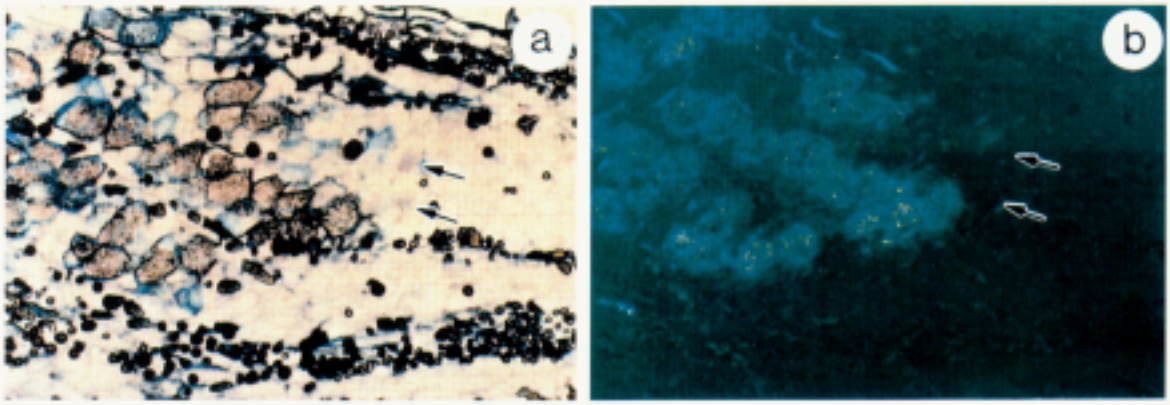

Fig. 5. Localization of *Frankia nif*H mRNA in a *C. glauca* nodule lobe. a, Bright-field micrographs of a longitudinal nodule lobe section of *C. glauca* hybridized with [^{35}S]-labeled *Frankia nif*H antisense RNA and stained with toluidine blue. Silver grains denoting hybridization are visible as black dots. Young infected cells of zone II (arrows) do not show *Frankia nif*H expression. b, In the dark-field micrograph taken under epipolarized light, silver grains denoting hybridization are visible as yellow dots. No signal is found in young infected cells of zone II (arrows).

that *Frankia* was restricted to the cortical tissues of the lateral root axis. In a given lobe, *Frankia* invaded cortical parenchyma cells growing acropetally towards the nodule lobe meristem so that a developmental gradient consisting of four distinct zones was formed, the apical meristematic zone (zone I), the young infected cells (zone II), the mature infected cells (zone III) and the senescent zone (zone IV). The senescent zone was observed in older nodules. As in most actinorhizal nodules, invasion of *Frankia* was correlated structurally with expansion and hypertrophy of the host cells.

To localize mRNA by in situ hybridization, longitudinal sections of nodules were hybridized to antisense (Fig. 4b,e) and sense RNA probes (Fig. 4c,f). Because the tannin-filled and suberized cell layers in the nodule periderm (Fig.4a) were expected to interfere with hybridization techniques, two different ways of probe labeling were used to enhance the possibility of the transcript detection, radioactive labeling with ^{35}S, detected by autoradiography and hapten (DIG) nonradioactive labeling which was detected by immunocytochemistry.

In situ hybridization of longitudinal sections with *hb*-Cg1F antisense RNA revealed that the transcripts were concentrated in the mature infected cells (zone III). Hybridization was present but weak in infected cells of zone II (Fig. 4b,e, arrows). No hybridization was observed in the uninfected cells of zones II and III, in the meristematic apical zone (zone I) or in the vascular bundle and in nodule roots (Fig. 4b,e). Infected cells are distinguishable from uninfected cells in zones II and III because they enlarge more (Fig. 4a,d; Berry and Sunnell 1990). In zone II, where the infection of cortical cells by *Frankia* hyphae had begun, the infected cells were already larger than uninfected cells (Fig. 4a). In the control experiments using [^{35}S]- and DIG-labeled RNA sense probes no hybridization was detected (Fig. 4c,f).

Hybridizations with antisense RNA of the *Frankia* nitrogenase structural gene *nif*H were also performed on longitudinal sections of nodule lobes. *nif*H mRNA was detected in the mature infected cells (zone III), while no transcripts were detectable in the young infected cells of zone II (Fig. 5).

Discussion

In order to identify *C. glauca* genes involved in nodule formation and function, we analyzed cDNA clones corresponding to nodule-specific genes. We report the sequence of a full-length Hb cDNA clone (*hb*-Cg1F) (Fig. 1). The *hb*-Cg1F cDNA encodes a protein highly homologous to *C. glauca* HbI (Kortt et al. 1988) and deduced *hb*-SymB protein (Jacobsen-Lyon et al. 1995). Furthermore, Jacobsen-Lyon et al. (1995) isolated a genomic clone cashb-sym1 corresponding to the cDNA clone *hb*-SymB. As concluded by these authors, the deduced ami no acid composition of the protein encoded by *hb*-SymB is identical to that of cashb-sym1 and is 98% identical to the protein sequence reported by Kortt et al. (1988). However, the 5'-untranslated sequence of *hb*-Cg1F and cashb-sym1 are dissimilar, except for 48 nucleotides close to the translation start (Fig. 1). These data suggest that *hb*-Cg1F and *hb*-SymB represent two different genes in the *C. glauca* genome, as there appear to exist three to four copies of *hb* genes in *C. glauca* (see Fig. 2). The 5'-untranslated region of *hb*-Cg1F consists of 409 nucleotides, which is unusually long since 75% of the 5'-untranslated region in eucaryotic mRNAs are in the range of 20–100 bases and 25% in the range of 100–300 nucleotides. However, 5'-noncoding regions as long as 1000 nucleotides have been reported (Kozak 1988). It has been suggested that the 5'-untranslated leader sequence can be important for translation efficiency (Kozak 1989). This might mean that the 48-nucleotide conserved motif upstream from the ATG could be involved in regulation of translation. Besides, in view of the dissimilarity between the 5' region of *hb*-Cg1F and *hb*-Symb, we examined the

possibility that *hb*-Cg1F was chimeric. To test this hypothesis, RNA from *C.glauca* root nodule was amplified with two primers homologous to the *hb*-Cg1F sequence. We employed a reverse complement primer (5'-TGGT TGTAGGCTTCAGTC-3') containing bases 814 to 831, and the primer (5'-TCGTCCACATCTCAACGC-3') containing bases homologous to those (80 to 97). The results showed the amplification of a DNA fragment of expected size (754 bp) which hybridized with *hb*-Cg1F probe (data not shown). This demonstrated that *hb*-Cg1F cDNA clone was not chimeric.

We have performed in situ hybridization with [^{35}S]- and DIG-labeled *hb* probes in sections of nodule lobes of *C. glauca*. In this work, the sensitivity of DIG immunodetection has been found to be comparable to that obtained by radiolabeling. Moreover, when a single cell resolution was required (Fig. 4d), DIG labeling was the method of choice. Comparably low levels of transcripts were observed in the first infected cells of zone II with the two labeling methods.

In situ hybridizations carried out on nodule sections demonstrated a developmental gradient along the lobe axis from the distal region (lobe meristem, zone I) to the senescence zone IV (Fig. 4a). *hb* transcripts were first detectable in the youngest infected cells (zone II) reaching a maximum concentration in zone III where the induction of *nif*H of *Frankia* occurred. Although *Casuarina* and legume nodules differ in development and morphology (reviewed by Newcomb and Wood 1987), the expression of *C. glauca hb* gene shows basic similarities to the pattern described for the indeterminate nodule of pea (Kardailsky et al. 1993) and alfalfa (de Billy et al. 1991). Also on legume nodules, plant *lb* gene expression in infected cells has been shown to precede bacterial *nif* gene expression (Yang et al. 1991). Soupène et al. (1995) have even found evidence that the O_2 concentration is a crucial factor in the induction of bacterial *nif* gene expression in nodules. Although the link between *lb* and *nif* gene expression has not yet been firmly estabilished, it is possible that the expression of *lb* in legume nodule contributes to the reduction of oxygen tension which in turn induces *nif* gene expression. Nodules of *Casuarina* differ from those of other actinorhizal species in that *Frankia* vesicles are absent (Berg and McDowell 1987). The regulation of oxygen tension at the sites of nitrogen fixation appears to be more strongly under host control: *Casuarina* nodules are distinctive in that the cell walls of the infected and adjacent uninfected cortical cells are lignified (Berg and McDowell 1988) and Hb is present at high concentration, unlike in other actinorhizal species with the exception of *Myrica* (Silvester et al. 1990). Thus, O_2 protection of bacterial nitrogen fixation appears to be similar in *Casuarina* and legume nodules, i.e. mediated by host-derived O_2 diffusion barrier and an O_2 transport protein. It is reasonable to suggest that the same mechanism of bacterial *nif* gene induction exists in both systems, too.

Several studies revealed that gas diffusion from the nodule surface to the site of N_2 fixation differs among actinorhizal species (Silvester et al. 1990). The present results establish the pattern of transcription of *hb* genes in *C. glauca* nodules where *Frankia* vesicles are absent. An important question is to localize *hb* mRNA in nodules where *Frankia* vesicles are present. Such studies could be done in nodules of *Myrica gale* from which Pathirana and Tjepkema (1995) recently reported the purification of Hb.

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