A novel nonsymbiotic hemoglobin from oak: cellular and tissue specificity of gene expression

Claire Parent¹, Audrey Berger², Hélène Folzer³, James Dat¹, Michèle Crevècoeur², Pierre-Marie Badot¹ and Nicolas Capelli¹

¹Laboratoire de Biologie Environnementale (EA 3184 MR usc INRA), Université de Franche-Comté, Place Leclerc, F-25030 Besançon cedex, France; ²Département de Botanique et Biologie Végétale, Université de Genève, 30 Quai Ernest Ansermet, CH-1211 Genève 4, Switzerland; ³Institut Méditerranéen d’Ecologie et de Paléoécologie (UMR CNRS 6116), Université Paul Cézanne, Avenue Escadrille Normandie-Niemen, F-13397 Marseille cedex 20, France

Summary

• This study presents the isolation and characterization of a novel nonsymbiotic Hb gene from sessile oak (Quercus petraea) seedlings, herein designated QpHb1.
• The cellular and tissue expression of QpHb1 was analysed by Northern blotting and in situ hybridization.
• The encoded protein was predicted to consist of 161 amino acid residues, and shares 71 and 51% amino acid sequence identity with the Arabidopsis class 1 and 2 nonsymbiotic Hb, respectively. Northern blot analysis revealed that QpHb1 was strongly expressed in roots. Spatial expression analysis of QpHb1 in the root apical region of sessile oak by in situ hybridization indicated that transcripts were mostly abundant in protoxylem cell initials, some cortical cells and the protoderm. In addition, when comparing the expression profile of QpHb1 in sessile and pedunculate oak (Quercus robur), two species with contrasted hypoxia tolerance, the transcript level of QpHb1 rose early in the most flood-tolerant species, pedunculate oak, during root submergence.
• The spatial–temporal expression of QpHb1 suggests that this gene could participate in perception and signalling during hypoxia.

Key words: gene expression, hypoxia, in situ hybridization, nonsymbiotic hemoglobin, oak (Quercus petraea, Quercus robur).

Introduction

Soil waterlogging has become a major factor affecting the growth, development and survival of many plant species, not only in natural ecosystems, but also in agricultural and horticultural systems (Dat et al., 2006). Transient flooding periods are frequently observed as a result of over-irrigation, inadequate drainage, the removal of vegetation cover and/or global warming. In addition, climate predictions suggest that the occurrence of this event will increase in frequency in the near future. Some of the best characterized plant adaptations to hypoxia include a switch in biochemical and metabolic processes commonly observed when O₂ availability becomes limiting (Dat et al., 2004). Most plant species synthesize a set of c. 20 anaerobic proteins (ANP) that enable an oxygen-independent energy-generating metabolism to proceed when conditions become unfavourable for aerobic energy production (Subbaiah & Sachs, 2003). Other observed adaptations include the formation of hypertrophied lenticels, the development of aerenchyma, root cortical air spaces that enhance the efficiency of gas transfer between aerial and

The nucleotide sequence data reported in this paper will appear in the EMBL/GenBank/DDBJ databases with accession number EF186909 (QpHb1).
submerged organs, as well as the promotion of adventitious roots (Vartapetian & Jackson, 1997; Jackson & Colmer, 2005; Folzer et al., 2006).

In contrast to the wealth of data available concerning the molecular and cellular mechanisms of hypoxia sensing and signalling in animals, such mechanisms have been more rarely described in plants, even less in woody species. In mammals, the hypoxia-inducible heterodimeric transcription factor (HIF) is a key regulatory element in the response to hypoxia (Giaccia et al., 2004; Semenza, 2004). However, to date no such sensor has been identified in plants (Baiyer-Serres & Chang, 2005; Agarwal & Grover, 2006). Recent broad-range approaches (DNA chip technology or proteome analysis) have helped to identify novel genes and proteins involved in plant responses to soil waterlogging and anaerobiosis (Chang et al., 2000; Klok et al., 2002; Agarwal & Grover, 2005; Branco-Price et al., 2005; Liu et al., 2005; Loreti et al., 2005). However, novel components of the signal transduction pathway leading to hypoxia-induced gene expression have been documented exclusively in crop plants (Lasanthi-Kudahettige et al., 2007) and model organisms such as Arabidopsis thaliana (Miyashita et al., 2007). These components include rapid changes in cytosolic Ca$^{2+}$ levels (Snedden & Fornom, 1998; Subbaiah et al., 1998, 2000; Luan et al., 2002) and Ca$^{2+}$-binding proteins (Folzer et al., 2005), the induction of ethylene biosynthesis (Drew et al., 2000; Nie et al., 2002), Rop (RHO-related GTPase of plants) G-protein signalling (Baxter-Burrell et al., 2002), as well as a large number of transcription factor families (AtMYB2, ZAT 12, WRKY factors; Bailey-Serres & Chang, 2005). In contrast to the amount of data available for herbaceous species, very little is known about the molecular mechanisms that underlie the sensing and signalling to hypoxia in woody species. This is especially apparent with forest tree species, which are not only of primary interest to the wood industry, but are also critical for the preservation and/or conservation of forest biodiversity.

Recently, additional insight into the response of plants to hypoxia has been provided by the discovery of stress-induced genes that affect plant metabolism and growth under low oxygen tensions (Dordas et al., 2003a). Among these, hemoglobin (Hbs) are ubiquitous molecules that have been found in various species from most of the taxonomic kingdoms, including bacteria, yeasts, protists, plants and animals (Wittenberg & Wittenberg, 1990; Hardison, 1996; Suzuki & Imai, 1998). All Hbs contain a heme group carrying an iron ion, which is responsible for the reversible binding to gaseous ligands such as oxygen (O$_2$) and carbon monoxide (CO) (Weber & Vinogradov, 2001). In plants, at least three different Hb families have been identified: symbiotic, nonsymbiotic and truncated Hbs (Ross et al., 2002). Symbiotic Hbs, or leghemoglobins, are specifically synthesized in nitrogen-fixing legume root nodules, and their main function is to facilitate oxygen transport and scavenging to protect Rhizobium nitrogenase from inactivation (Appleby, 1984). Plant truncated Hbs are short versions of the classical globin fold. The function of these proteins, recently detected in organs of angiosperm species such as Arabidopsis (Watts et al., 2001) and wheat (Larsen, 2003), is still unknown. Finally, nonsymbiotic Hbs occur at much lower abundance, but appear ubiquitous in all plant species examined (Dordas et al., 2003a). In vascular plants, two classes occur. Class 2 nonsymbiotic Hbs present similar O$_2$-binding properties to those of symbiotic Hbs and are inducible by cold stress (Trevaskis et al., 1997) or cytokinin treatment (Hunt et al., 2001). In contrast, class 1 nonsymbiotic Hbs have high O$_2$ affinity and are induced under hypoxic conditions (Duff et al., 1997; Trevaskis et al., 1997). Because of an extremely low O$_2$-dissociation constant, class 1 nonsymbiotic Hbs might not function as O$_2$ carriers, as originally thought. In fact, recent studies suggest that their presence could regulate cellular nitric oxide (NO) levels, thus improving the redox and/or energy status of the plant cell during hypoxia (Dordas et al., 2003b; Perazzolli et al., 2004).

In an attempt to gain further understanding of the difference in the molecular responses of tree species to hypoxia, the cloning and characterization of a nonsymbiotic Hb gene from sessile oak was undertaken. The analysis was further complemented by comparing the expression profile of the gene in two oak species with a contrasted response to flooding. The genus Quercus (oaks), which includes over 300 woody species, is widespread in the northern hemisphere, where it represents the dominant vegetation of temperate forests (Nixon, 1993). We decided to focus on the two predominant European oak species, pedunculate and sessile oak, to investigate the spatial and temporal expression patterns of Hb during the early response to hypoxia. The two species generally cohabit in forest ecosystems; however, sessile oak is found more frequently on well drained soils, whereas pedunculate oak can populate poorly drained sites where temporary waterlogging occurs (Lévy et al., 1992).

Materials and Methods

Plant material and growth conditions

Sessile (Quercus petraea (Matt.) Liebl.) and pedunculate (Quercus robur L.) oak acorns, harvested in north-eastern France, were provided by the Office National des Forêts (ONF; Preney et al., 1997) and stored in moist vermiculite at 4°C until use. The acorns were sterilized by dipping in a 1% bleach solution and oxygenated overnight in running water to favour germination. Individual acorns were then grown in 1.8-l plastic pots containing river sand (Dekoline Carat 4, Aquatic Nature, Belgium). This substrate was chosen to enable harvesting of quality roots for histological and molecular studies. The plants were grown for 5 wk in a controlled growth chamber with environmental conditions set as follows: a 16 h photoperiod, a photosynthetically active radiation (PAR) of 170 µmol m$^{-2}$ s$^{-1}$ at canopy level.
(provided by high-pressure sodium lamps), an average temperature of 20 ± 1.5°C and a relative humidity of 70%. Plants were irrigated four times daily with a fertilizer solution (0.5 ml l⁻¹, NPK 6/5/6; SEM, Compo France, Roche-Lez-Beaupré, France, Germany) using an automated ebb-and-flow system. After 5 wk, which corresponded to the establishment of the first true leaves, each seedling was checked for leaf morphology and growth characteristics to make sure the seedlings grown from acorns provided by the ONF were not misidentified. A clear difference in leaf morphology and growth characteristics could be observed between both species. However, when in doubt, the seedlings were discarded. For molecular analysis, the root system of plants used for shoot water potential measurements were immediately harvested, frozen in liquid nitrogen, and stored at −80°C until use. Other plant samples (shoots and leaves) were treated similarly.

**Hypoxia treatment**

Low oxygen stress was imposed by immersing 5-wk-old seedlings up to the root collar in the irrigation solution for the desired period (1–48 h). Control plants (not submerged) were harvested in parallel with flooded plants at each time point. In addition, the O₂ level of the solution surrounding the roots was monitored for each stress period by measuring O₂ concentration with a portable O₂ electrode (CellOx325, WTW, Weilheim, Germany) in the rhizospheric solution (see Fig. 7a). The measurements were undertaken on nine individual pots (each containing one plant) from three independent experiments.

**Shoot water potential**

Shoot water potential measurements were made with a Scholander-type pressure chamber (DPI 700, GE Druck, New Fairfield, CT, USA) on whole shoots of flooded and control 5-wk-old sessile and pedunculate oak seedlings, as described by Folzer et al. (2006). The average values for shoot water potential of control and flooded plants were calculated at each time point from 12 seedlings for each species, obtained from four independent experiments. The shoot water potential was then expressed as the difference between the flooded and control values calculated for each species.

**5′ and 3′ rapid amplification of cDNA ends (RACE)**

In order to isolate sessile oak nonsymbiotic hemoglobin (Hb) cDNA clones, total RNA from pooled samples of roots of plants exposed to hypoxia for either 1 or 3 h were prepared using the RNeasy Plant Mini Kit (Qiagen S.A., Courtaboeuf, France). cDNA synthesis was carried out using the SMART RACE cDNA amplification kit according to the manufacturer's instructions (Clontech, Palo Alto, CA, USA). After treatment with RNase-free DNase, the first-strand cDNA was synthesized by reverse transcription of 1 µg total RNA using the 5′-CDS primer A and the SMART II A oligo (both provided in the kit). The degenerated reverse primer Hb (5′-G[C/T]T[C/T]T[A/G/T]AT[A/T/C/G]AT[C/T/T]C/ T][A/TC/G][A/T/C/G]AG[A/T/C/G]G-3′) for 5′-RACE was designated and synthesized based on the conserved region identified among several plant Hbs (A. thaliana, barley, soybean, Casuarina glauca, rice) deposited in the GenBank database (http://www.ncbi.nlm.nih.gov).

Amplification conditions were as follows: preamplification denaturation at 94°C for 2 min, 41 cycles of denaturation at 94°C for 15 s, primer annealing at 64°C for 25 s and primer extension at 72°C for 2 min, and a final polymerization and extension step of PCR products at 72°C for 10 min. The resulting 500–600-bp RT-PCR products were purified using the MinElute gel extraction kit (Qiagen), cloned individually using the pGEM-T Easy Vector System (Promega, Madison, WI, USA), and fully sequenced (MlleGen, Biostep, Labège, France). Based on the sequence of the 5′-RACE fragment, a gene-specific sense primer Hb2 (5′-CGGGTTGTGT-TCACCTTCAAAATGTTGGGTCG-3′) was designated and synthesized to amplify the remaining part of the corresponding Hb gene. RT-PCR was used to isolate the full-length cDNA of *Q. petraea* Hb (designated *QpHb1*).

**Northern blot analysis**

To obtain a gene-specific probe for *QpHb1*, a 349-bp DNA fragment was amplified by PCR from both the 5′-UTR and coding sequence of the cDNA clone. Primers used were *QpHbp* (5′-TTTCCAAAATCCTCTAACTAATTCTTGACC-3′) and *QpHbrp* (5′-AAGTTGCCACCGGTGATTTCAACAAGTTCG-3′). Bacteriophage promoters T7 and SP6 were added to primers *QpHbp* and *QpHbrp*, respectively, generating a PCR product that could be used for *in vitro* transcription (Stofflet et al., 1988). Total RNA (5 µg) from each tissue was mixed with sample buffer, separated by formaldehyde-denaturing agarose gel electrophoresis (1.2% w/v) and capillary-blotted with 10× saline sodium citrate (SSC) onto positively charged nylon membranes (Roche Diagnostics SAS, Meylan, France, Germany). After RNA fixation by baking for 2 h at 80°C, prehybridization (1 h) and hybridization (overnight) were performed in Dig Easy Hyb buffer (Roche Diagnostics) at 68°C. Membranes were washed for 15 min twice with 2× SSC, 0.1% SDS at room temperature, and for 30 min twice with 0.1× SSC, 0.1% SDS at 60°C. The hybridized antisense RNA probe was immunodetected with an alkaline phosphatase-conjugated antidiogenibogen antibody and visualized with a chemiluminescence system (Roche Diagnostics) as recommended by the manufacturer. The Northern analysis was replicated three times with RNA from three independent experiments and a pool of at least six plants per experiment. Densitometric measurements were performed and a one-way ANOVA was undertaken on the results to identify main effects.
Bio-informatics and phylogenetic analysis

Multiple-sequence alignments were carried out using CLUSTAL W algorithms from EMBL-EBI (http://www.ebi.ac.uk/clustalw). Cluster analysis was performed by the neighbor-joining method (Saitou & Nei, 1987). The GenBank accession numbers for all 56 proteins are given in the legends of Figs 1, 2.

In situ hybridization

Root segments from *Q. petraea* (7 mm long) were fixed in 0.25% glutaraldehyde (w/v) and 4% formaldehyde (w/v) in 0.1 M phosphate buffer pH 7.2 overnight at 4°C. They were then washed thoroughly in phosphate buffer, dehydrated in a graded-alcohol series and embedded in paraffin. Thin

Fig. 1  Comparative alignment of the predicted amino acid sequences of hemoglobin (Hb) from different plant species. The alignment was generated using the CLUSTALW program (http://www.ebi.ac.uk/clustalw). Conserved residues of heme- and ligand-binding (distal (E7) and proximal (F8)) His residues are in bold type; Cys residues are underlined (Ota *et al.*., 1997). The plant globin motif characteristic of plant hemoglobins is indicated in thick gray. Protein sequences were obtained from the GenBank database using the following accession numbers: EF186909 (*Quercus petraea* QpHb1 nonsymbiotic Hb, this study); AB221344 (*Alnus firma* nonsymbiotic Hb, 85% aa identity to QpHb1 protein); U47143 (soybean nonsymbiotic Hb, 80% identity); AF172172 (alfalfa MHB1 nonsymbiotic Hb, 81% identity); AY899302 (cotton GHB1 nonsymbiotic Hb, 78% identity); Y00296 (*Trema tomentosa* Hb, 82% identity); U27194 (*Parasponia andersonii* Hb, 80% identity); AY221433 (*Ceratodon purpureus* GLb1 nonsymbiotic Hb, 70% identity); AF309562 (*Physcomitrella patens* nonsymbiotic Hb, 44% identity); X77694 (*Casuarina glauca* symA symbiotic Hb, 50% identity); AB238218 (*Lotus japonicusc* LjLb2 legHb, 43% identity); X14311 (alfalfa legHb, 46% identity); AB015720 (pea legHb, 47% identity); V00453 (soybean Lba legHb, 43% identity); U33206 (*Vigna unguiculata* LbI legHb, 40% identity).
sections (7 µm) were attached on poly-L-lysine coated slides (Polysine™*) from Menzel-Glaser (Menzel Gmbh & Co KG, Braunschweig, Germany) in the presence of DEPC-treated water. The sections were deparaffinized, rehydrated through a graded ethanol series and rinsed in DEPC-treated water. They were then incubated for 30 min at 37°C with 1 µg ml⁻¹ proteinase K (Sigma, Buchs, Switzerland) in 100 mM Tris-HCl pH 7.5 containing 5 mM EDTA and washed in 0.2% glycine in PBS. After acetylation with 100 mM triethanolamine pH 8.0 containing 0.25% acetic anhydride, the sections were rinsed successively in 2× SSC and in DEPC-treated water. Slides were then dehydrated in ethanol and dried in a desiccator, and prehybridization was carried out at 50°C for 2 h in the prehybridization solution containing 2× SSC, 50% formamide, 1× Denhardt's solution, 5% dextran sulfate, 1 µg ml⁻¹ salmon-sperm DNA and 0.25 µg ml⁻¹ yeast tRNA in DEPC-treated water. Slides were then hybridized overnight at 50°C in the prehybridization solution with 4 ng ml⁻¹ sense or antisense Fig. 2 Phylogenetic relationship of *Quercus petraea* QpHb1 with other hemoglobins (Hb) from various organisms. Complete protein sequences of Hb-encoding genes were aligned and the tree was constructed using the CLUSTALW method (Higgins *et al.*, 1994). The bar indicates a distance of 0.05 substitutions per site. Nonsymbiotic Hbs from leguminous and nonleguminous plants are shown in blue and black, respectively. Symbiotic Hbs from leguminous plants are shown in red; symbiotic Hbs from nonleguminous plants in green. Two dicotyledon plant nonsymbiotic class 1 Hb clusters (groups Ia, Ib; IV), one monocotyledon plant nonsymbiotic class 1 Hb cluster (group III) and a dicotyledon plant symbiotic Hb and class 2 nonsymbiotic Hb cluster (group II) are indicated on the right. Hbs from *Paraparona* and *Myrica gale* were classified as group I, however they are intermediate because they retain both symbiotic and nonsymbiotic specificity. Sequences are as in Fig. 1, with the following additions: *Trema orientalis* Hb (Z99635), *Trema virgata* Hb (AJ131349), *Paraparona rigida* Hb (P68169), *Astragalus sinicus* legHb (DQ199647), *Sesbania rostrata* Srglb2 legHb (X13815), *Sesbania rostrata* Srglb3 legHb (X13814), alfalfa legHb1 (M32883), barley medicinal MtHb2 legHb (X57733), *Vicia faba* legHb 49 (Z54195), *Psophocarpus tetragonolobus* legHb (X65874), *Vigna unguiculata* LbbHb legHb (U33207), *Phaseolus vulgaris* legHb (P02234), *Canavalia lineata* legHb (U09671), yellow lupin legHb1 (Y00401), *Brassica napus* GLB2 nonsymbiotic Hb class 2 (AY026337), cotton GLB2 nonsymbiotic Hb class 2 (AY026339), *Lycopersicon esculentum* GLb2 nonsymbiotic Hb class 2 (AY026344), *Cichorium intybus × Cichorium endivia* nonsymbiotic Hb (A997507, A127797), *Cassuariar glauca* symbiotic Hb (X77695), rice Hb (U76031, AF335504), teosinte Hb (AF291052), maize Hb (AF236080), *Raphanus sativus* nonsymbiotic Hb (AY286331), *Lotus japonicus* LiHb1 nonsymbiotic Hb (AB328202), *Nicotiana tabacum* Hb (8Q842804), cotton GLB1 nonsymbiotic Hb class 1 (AF329368), *C. glauca* HbII (X53950), *M. gale* Hb (EF405885).
RNA probe. After hybridization, the slides were washed at 50°C in decreasing SSC solutions (2×, 1× and 0.5×) and treated with 0.5 g μl–1 RNase A (Roche Diagnostics) in NTE buffer (10 mM Tris–HCl pH 7.5, 500 mM NaCl, 1 mM EDTA) at 37°C for 30 min. The RNase treatment was followed by 2× 5-min rinse in NTE buffer, 45 min at 50°C in 0.5× SSC and finally in 100 mM Tris–HCl pH 7.5 containing 150 mM NaCl. The immunodetection of probes was performed as described by Carpin et al. (1999) and according to the instructions of the manufacturer (Roche Diagnostics). The sections were mounted in PBS-glycerine.

**Histological staining**

Deparaffinized sections were stained with 1 μg ml–1 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) in water for 20 min. The sections were rinsed in water and mounted in PBS containing 0.1% p-phenylenediamine as antifading reactive and 61% glycerol. For safranine-fast green staining, sections were deparaffinized and rehydrated from alcohol 100 to 70%. They were dipped for 24 h in a solution of 1% safranine T in 50% ethanol, washed in water, stained for a few seconds in 0.2% fast green in 92% ethanol, washed and mounted in Assistent-Histokitt (Assistent–Sondheim/Rhön, Germany).

**Photographs**

All sections were observed and photographed with a Leica DM IRE 2 microscope equipped with a Leica DC300F CCD color camera. DAPI-stained sections were examined by epifluorescence with the same microscope, with an A filter block (Leica, filters BP 340–380 nm for excitation, LP 425 for emission).

**Statistical analysis**

For water potential and densitometric data analysis, a one-way ANOVA with the general linear model (GLM) procedure of SAS 6.0 (SAS Institute Inc., Cary, NC, USA) for main effects, species and hypoxia treatment was used. Individual means were compared using Duncan’s test.

**Results**

**Molecular characteristics of the QpHb1 gene**

The QpHb1 clone (GenBank accession number EF186909) consists of 729 nucleotides, excluding the poly(A)+ tail, with an open reading frame of 486 bp, flanked by a 93-bp 5'-UTR and a 150-bp 3'-UTR. The cDNA sequence encodes a 161-aa predicted polypeptide with a calculated molecular mass of 17 913 Da and an isoelectric point of 8.57, revealed by an analysis with the ExPASy Molecular Biology Server (http://us.expasy.org). Alignment of the predicted QpHb1 amino acid sequence with selected plant Hb sequences, using the CLUSTALW method (Higgins et al., 1994) is presented in Fig. 1. The QpHb1 deduced protein contained all conserved amino acid and characteristic peptide motifs of plant Hbs.

In an effort to analyse the molecular evolution of the Q. petraea nonsymbiotic Hb, 55 full Hb amino acid sequences (incomplete sequences were not included in this study) from other plant species were retrieved from the GenBank database and compared using the CLUSTALW software. Subsequently, a phylogenetic tree was constructed by the neighbor-joining method (Saitou & Nei, 1987). The phenogram (Fig. 2) revealed that class 1 nonsymbiotic Hbs diverge into three separate major clusters (groups I, III and IV), while leghemoglobins converge into a single major cluster (group II) with homology to a group of class 2 nonsymbiotic Hbs and symbiotic Hbs from the actinorhizal shrub C. glauca. As expected, the monocot sequences form a distinct clade within the class 1 nonsymbiotic Hbs (group III) and QpHb1 is in group I with other tree species. In addition, the phylogenetic analysis shows that Hbs from the bryophytes Physcomitrella patens and Ceratodon purpureus constitute a separate clade, which could indicate that Hbs are widespread in land plants.

**QpHb1 transcript levels in various organs of sessile oak**

The QpHb1 antisense riboprobe hybridizes with a single specific transcript of approx. 0.75 kb in all vegetative organs examined (root, stem, leaf) in sessile oak seedlings grown under control conditions (Fig. 3a). The mRNA encoding QpHb1 was detected primarily in root tissues. In contrast, only a very faint signal representing a low level of QpHb1 mRNA accumulation could be observed in the leaves. The dendrometric analysis of QpHb1 expression clearly supports the observation that its expression level is generally far superior in roots than in other tissues (Fig. 3c).

**In situ expression of QpHb1 in sessile oak roots**

Figure 4 shows cross sections made at three different levels in the primary root tip of Q. petraea, in the region where in situ hybridization was performed. To identify the different tissues or cell layers in each section, cross serial sections were made from the tip of the root cap (Rc). The position of each section was expressed in micrometres estimated from the extremity of the Rc. The sections allowed us to identify the distance from the Rc at which the first provascular elements can be observed (arrows in Fig. 4c).

Figure 5 shows the in situ QpHb1 expression in cross sections from different regions of a primary root tip. In sections made at 200 μm from the tip of the Rc (Fig. 5a), QpHb1 expression was associated with the central region of the Rc but was not detected in the outer cell layers. At approx. 400 μm from the tip of the Rc (Fig. 5b), QpHb1 was expressed mainly...
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in the cell layers corresponding to the outer ground meristem. A lower expression was detected in the inner part of the cortex. The outer cell layers, corresponding to the Rc, as well as the central part of the section (future vascular cylinder) did not show any QpHb1 expression. About 600 µm from the tip of the Rc (Fig. 5c), patterns of QpHb1 gene expression were as follows: no expression in the outer cell layers corresponding to the Rc, a strong expression in the cell layers located in the outer ground meristem, and finally a lower expression in the cortical parenchyma. QpHb1 mRNA accumulation was associated with the central region of the vascular cylinder in which the xylem will differentiate. No QpHb1 expression was detected in the outer region of the vascular cylinder in which protophloem elements are found. Control sections incubated with the sense probe did not show any staining (Fig. 5d). A very similar expression pattern was observed at c. 800 and 1000 µm (Fig. 5f,g). At these levels, QpHb1 mRNA transcripts were found in cells of the endodermis (Fig. 5g) as well as the epidermis (Fig. 5f,g). DAPI staining of a section at 800 µm from the tip of the Rc (Fig. 5c) confirms the localization of the epidermal cell layer (densely stained nuclei in the outer region of the section).

Effect of a short hypoxia stress on shoot water potential and QpHb1 expression level in sessile and pedunculate oaks

We decided to monitor the plant water potential and the expression pattern of QpHb1 in sessile oak and in another species considered more tolerant to soil waterlogging, pedunculate oak. As the expression pattern of a gene coupled to the plant physiological status may help understand the difference in tolerance between species, we investigated the effect of imposing hypoxia on the roots for 48 h (Figs 6, 7b). The monitoring of O2 evolution in the rhizosphere of flooded oaks (Fig. 7a) shows that O2 concentration rapidly decreased within 12 h of the start of treatment. During the next 36 h, the O2 concentration remained low. The differences in shoot water potential monitored during the same time period in the two oak species indicated a rapid decrease in shoot water potential in both species as early as 3 h after the start of root submergence (Fig. 6). However, the decline was much more pronounced in sessile than in pedunculate oak. An ANOVA of the expression of QpHb1 during the same stress period in sessile and pedunculate oaks indicated a significant negative effect of hypoxia after 24 and 48 h for both species (Table 1). However, there was also a significant species effect on QpHb1 expression. Furthermore, the monitoring of QpHb1 indicated a constant and gradual decline in QpHb1 expression in sessile oak, whereas there was a significant transient increase in QpHb1 in pedunculate oak during the first hour of stress (Fig. 7b). This initial rise was followed by a significant drop within 3 h, then the level of expression increased to 60% of its initial level and remained low thereafter, being not significantly different from sessile oak after 24 h of hypoxia.

Discussion

Cloning and characterization of the QpHb1 gene

The QpHb1 gene is the first nonsymbiotic Hb gene that has been isolated from oak roots and characterized. The QpHb1 deduced protein shows characteristic features of other plant...
Hbs (Fig. 1). These include the CD1 phenylalanine, C2 proline and F8 proximal histidine residues needed for heme binding, and the E7 distal histidine which is involved in ligand binding (Ota et al., 1997). A cysteine residue found in most known plant nonsymbiotic Hbs is also present in QpHb1. Sequence comparison also confirmed the presence of the plant Hbs signature (SN)-P-x-(LV)-x(2)-H-A-x(3)-F (Dickerson & Geis, 1983). Based on these structural features, QpHb1 can be categorized as a class 1 nonsymbiotic Hb. Furthermore, the phylogenetic analysis of QpHb1 with other known Hb sequences available in public resources highlights the fact that QpHb1 forms a cluster together with other woody species (*Trema tomentosa*, *Trema orientalis*, *Parasponia andersonii*, *Alnus firma*, *Malus domestica*), indicating its close primary structural relationship with other lignified plants (Fig. 2). However, as seen in the evolutionary tree constructed with polypeptide sequences, QpHb1 clearly belongs to subgroup Ib, clustering with *A. firma*, *pear* and *apple* Hbs, but distant from the *Trema* nonnodulating (Ulmacean) plant Hbs, which are closely related to the nitrogen-fixing tree *P. andersonii* Hb (subgroup Ia).

In plants, three distinct types of Hb have been isolated: symbiotic, nonsymbiotic and truncated. The nonsymbiotic Hbs are widespread in the plant kingdom, suggesting multiple or essential functions (Hebelstrup et al., 2007). They are characterized by a relatively low abundance in plant tissues and are divided into two classes (Dordas et al., 2003a). Class 1 nonsymbiotic Hbs have a high affinity for oxygen (Hill, 1998), are induced during flooding (Taylor et al., 1994; Hunt et al., 2002; Dordas et al., 2003b), and their induction is generally related to cellular ATP levels (Nie & Hill, 1997). In contrast, class 2 nonsymbiotic Hbs have a lower affinity for oxygen and are induced by low temperatures (Trevaskis et al., 1997) and cytokinin treatment (Hunt et al., 2001, 2002). However, the molecular mechanisms behind the distinct induction characteristics of both classes of nonsymbiotic Hbs have yet to be elucidated.

All plant species studied to date possess nonsymbiotic Hb gene(s), the expression patterns of which vary in different plant tissues and in response to different stress conditions (Hunt et al., 2001). The analysis of the *QpHb1* expression pattern in the various plant organs shows that the gene is strongly expressed in roots as compared with the other plant vegetative tissues, suggesting a more prominent role for *QpHb1* in roots. In most studies in which nonsymbiotic Hb expression has been analysed in the different organs of the same plant, high levels of expression have been found in root tissues (Jacobsen-Lyon et al., 1995; Andersson et al., 1996; Seregélyes et al., 2000; Larsen, 2003; Silva-Cardenas et al., 2003; Qu et al., 2005).

At the tissue level, there is indication that the expression of nonsymbiotic Hbs varies greatly, with generally high levels reported in metabolically active or stressed tissues (Hill, 1998). Using nonradioactive in situ hybridization, we describe the distribution under normal conditions of *QpHb1*, in the first millimetre of the primary root tip, in the region including the meristem and the Rc. This is the first report of in situ localization of a nonsymbiotic Hb gene in the primary root of a woody plant. In the root meristem, *QpHb1* was mainly expressed in the outer ground meristem and in the differentiation region of the xylem. Differential expression of nonsymbiotic Hb was also reported in soybean, and Northern blot analysis indicated a higher expression in the elongating region than in the root tip (Andersson et al., 1996). Using GUS reporter fusions in *L. corniculatus*, the *C. glauca* nonsymbiotic Hb promoter was detected primarily in meristematic regions of the root tip, the vascular stele and the pith parenchyma (Jacobsen-Lyon et al., 1995). Furthermore, the activity of Hb promoters of *P. andersonii* and *T. tomentosa*...
Fig. 5 in situ QpHb1 expression in cross sections at different distances from the tip of the root cap: (a) 200; (b) 400; (c–d) 600; (e–f) 800; (g) 1000 µm. Negative control with sense probe in (d). DAPI-stained section in (e). Rc, root cap; ep, epidermis; en, endodermis. Bar, 100 µm.
was detected in root meristems and in the vascular cylinder of transgenic tobacco (Bogusz et al., 1990). In monocots, nonsymbiotic Hbs were also immunologically localized in differentiating tissues, mainly the vascular and Rc cells (Arechaga-Ocampo et al., 2001; Lira-Ruan et al., 2001; Ross et al., 2001). In addition to these data, immunolocalization of nonsymbiotic Hb1 in rice has been described in the cytoplasm in primary differentiated and differentiating cell types, including the Rc and the differentiating xylem (Ross et al., 2001). These results suggest that nonsymbiotic Hb proteins are synthesized early during the differentiation of conductive elements, as well as in cells in more advanced stages of xylogenesis. We show here that the expression of QpHb1 is detected at a very early stage of xylem differentiation, but not in the protophloem, the earliest vascular tissue to differentiate in Quercus and commonly in angiosperm roots. QpHb1 expression was also found in the Rc cells. A similar localization was reported for a nonsymbiotic Hb protein in 4-d-old rice seedlings, and the authors relate this localization to the formation of new cell types essential for growth of the root (e.g. gravisensing cells; Ross et al., 2001).

However, more recent data on the role of nonsymbiotic Hb in regulating the level of NO in plants may also provide some clues as to the spatial root distribution and function of

![Fig. 6](image)

Fig. 6 Changes in the difference in shoot water potential between stressed and control sessile (Quercus petraea; open bars) and pedunculate (Quercus robur; closed bars) oak seedlings exposed to a short hypoxia treatment. Vertical bars, ±SEM, n = 6.

![Fig. 7](image)

Fig. 7 (a) Evolution in oxygen concentration in the rhizospheric solution during the hypoxia treatment. Vertical bars, ±SEM, n = 3. (b) Time-course analysis of QpHb1 gene induction under hypoxia in sessile (Quercus petraea) and pedunculate (Quercus robur) oak. Five-wk-old oak seedlings were submerged for 0, 1, 3, 6, 12, 24 and 48 h. For each treatment, 5 µg total RNA was used to determine the transcript level by Northern hybridization. Densitometric scanning was used to quantify the signal as indicated in Fig. 3; the ANOVA for main effects is shown in Table 1.
QpHb1 (Ross et al., 2002; Igamberdiev & Hill, 2004; Sakamoto et al., 2004). NO is a stress-related metabolite known to participate in the signalling pathway during plant stress responses, produced in response to a variety of environmental cues (Durner & Klessig, 1999). Interestingly, NO has also been associated with many physiological and developmental processes, including the differentiation of xylem cells (Gabaldon et al., 2005). Thus the spatial expression pattern of QpHb1 in the protoxylem cells is also in agreement with a role of Hb in controlling NO accumulation in these cells, further supporting a close relationship between both molecules in controlling developmental processes, as proposed previously in Arabidopsis (Hebelstrup et al., 2006).

On the other hand, the spatial distribution of QpHb1 in the protoderm cells, the first layer of living cells in the root in contact with the outside environment, strongly suggests that QpHb1 could play an important role during the perception of changes in the rhizosphere, such as oxygen depletion. In order to address this possibility, we compared the expression of QpHb1 during a short hypoxia stress in two sympatric oak species: sessile and pedunculate oak.

Possible role of QpHb1 in the response to hypoxia

Monitoring shoot water potential during hypoxia in both pedunculate and sessile oak seedlings indicated a contrasting response for both species (Fig. 6). The difference between stressed and control plants shows that pedunculate oak is physiologically less affected than sessile oak early during hypoxia. These results suggest an enhanced tolerance to short hypoxia in pedunculate oak. Parelle et al. (2006, 2007) have previously shown that pedunculate oak is more tolerant than sessile oak to long-term hypoxia, and the enhanced tolerance could be attributed to a higher capacity to develop aerenchyma tissue as well as adventitious roots. However, no data are available on differences in the early response to hypoxia in both species. The expression analysis of QpHb1 during hypoxia in both oak species showed that the gene is induced transiently in pedunculate oak (Fig. 7b). However, the decreased expression in sessile oak was more unexpected.

Class 1 Hb genes have been shown to affect NO levels in several experimental systems, and their main function may be in the removal of NO during oxygen deficiency in plants (Dordas et al., 2003a, 2004; Perazzolli et al., 2004). Hypoxic root cultures of Hb-deficient alfalfa and maize mutants accumulated high levels of NO, 2–3 cm behind the root tip, during the first 24 h of hypoxia (Dordas et al., 2003b, 2004; Igamberdiev & Hill, 2004). NO and Hb are intimately linked during the response to hypoxia in many biological systems (Durner & Klessig, 1999; Dordas et al., 2003b; Wendehenne et al., 2004). Both NO and Hb levels increase in tissues with a similar temporal sequence after exposure to hypoxia or anoxia (Dordas et al., 2004). It was thus concluded by the authors that Hb modulation of NO is closely linked to short-term survival under hypoxic or anoxic stress. The data presented here could support the role of nonsymbiotic Hb1 in the spatial and temporal control of NO accumulation.

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References


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