Endogenous PttHb1 and PttTrHb, and heterologous Vitreoscilla vhb haemoglobin gene expression in hybrid aspen roots with ectomycorrhizal interaction

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Abstract

Present knowledge on plant non-symbiotic class-1 (Hb1) and truncated (TrHb) haemoglobin genes is almost entirely based on herbaceous species while the corresponding tree haemoglobin genes are not well known. The function of these genes has recently been linked with endosymbioses between plants and microbes. In this work, the coding sequences of hybrid aspen (Populus tremula×tremuloides) PttHb1 and PttTrHb were characterized, indicating that the key residues of haem and ligand binding of both genes were conserved in the deduced amino acid sequences. The expression of PttHb1 and PttTrHb was examined in parallel with that of the heterologous Vitreoscilla haemoglobin gene (vhb) during ectomycorrhiza/ectomycorrhizal (ECM) interaction. Both ECM fungi studied, Leccinum populinum and Xerocomus subtomentosus, enhanced root formation and subsequent growth of roots of all hybrid aspen lines, but only L. populinum was able to form mycorrhizas. Real-time PCR results show that the dual culture with the ECM fungus, with or without emergence of symbiotic structures, increased the expression of both PttHb1 and PttTrHb in the roots of non-transgenic hybrid aspens. PttHb1 and PttTrHb had expression peaks 5 h and 2 d after inoculation, respectively, pointing to different functions for these genes during interaction with root growth-improving fungi. In contrast, ECM fungi were not able to enhance the expression of hybrid aspen endogenous haemoglobin genes in the VHb lines, which may be a consequence of the compensating action of heterologous haemoglobin.

Key words: Ectomycorrhizas, non-symbiotic haemoglobin, Populus tremula×tremuloides, real-time PCR, truncated haemoglobin, Vitreoscilla haemoglobin (VHb).

Introduction

Plant haemoglobins (Hbs) currently comprise three major groups of oxygen-binding proteins: symbiotic, non-symbiotic, and truncated Hbs (reviewed by Dordas et al., 2003; Perazzolli et al., 2006). For over two decades, the symbiotic Hbs have been known to facilitate oxygen diffusion to nitrogen-fixing bacteria in the nodules of plant roots (Appleby, 1984). In contrast, the roles of non-symbiotic and truncated Hbs have remained more obscure. Originally, the non-symbiotic plant Hbs were divided into two distinct classes based on phylogenetic analyses, different expression patterns, and oxygen-binding properties (Trevaskis et al., 1997; Hunt et al., 2001). Class-1 Hbs have an extremely high affinity for oxygen, and their expression has been shown to be inducible by hypoxic stress (Trevaskis et al., 1997; Lira-Ruan et al., 2001), oversupply of nitrate, nitrite, and nitric oxide (NO) (Wang et al., 2000; Sakamoto et al., 2004; Ohwaki et al., 2005;
TrHb workers (2005) observed the up-regulation of certain phytohormones (Watts et al., 2004; Hebelstrup et al., 2006), thus suggesting that the classification of non-symbiotic Hbs might need to be reconsidered when more information is available (Ross et al., 2004).

The most recently identified group of plant globin proteins are truncated Hbs (TrHbs), which share strong similarity with a subset of the bacterial 2-on-2 Hbs (Watts et al., 2001). TrHbs in both plants and bacteria differ from other Hbs by having a 2-on-2 arrangement of α-helices instead of 3-on-3 arrangement of the standard globin fold which possibly leads to different ligand binding kinetics of TrHbs relative to the superfamily of Hbs (Hoy and Hargrove, 2008). Because of the recent discovery of plant TrHbs, the number of studies analysing their functional roles is still very limited. Their expression was shown to remain uninduced under hypoxia or upon treatment with phytohormones (Watts et al., 2001), but Vieweg and co-workers (2005) observed the up-regulation of certain TrHb genes of Medicago truncatula Gaernt. during symbiotic association in root nodules and in roots colonized by arbuscular mycorrhizal fungi.

For the past decade, different industrially important microbial and plant species have been metabolically engineered to express the Hb protein VHb of the Gram-negative bacterium Vitreoscilla sp. VHb is produced in its native host under oxygen-limited conditions, but its physiological and biochemical properties have mainly been analysed in Escherichia coli. VHb-expressing E. coli cells have higher demand for oxygen, and an enhanced amount and activity of energetically more favourable bo3 complexes (Kallio et al., 1994; Tsai et al., 1996). As a consequence, these characteristics are able to enhance the efficiency of energy production by generation of a higher proton flux per reduced O2 molecule and leading to a 65% higher ATP turnover rate and a 30% increase in ATP synthase activity (summarized in Frey and Kallio, 2003). Recently, it has been shown that microbial globins possess NO dioxygenase activity and, therefore, are able to catalyse O2-dependent cellular NO metabolism and protect cells against the toxic effects of NO (Frey et al., 2002; Gardner, 2005).

Holmberg et al. (1997) characterized VHb-expressing tobacco plants that exhibited faster germination rates, gave higher yield of plant material, had enhanced chlorophyll content, and had a shift in secondary metabolite production towards nicotine relative to controls. Similar positive effects were also observed when VHb was expressed in suspension-cultured tobacco cells (Farrés and Kallio, 2002), rice plants (Cao et al., 2004), and Hyoscyamus muticus L. hairy root cultures (Wilhelmson et al., 2005). Contrary to the results of Holmberg et al. (1997), Frey and co-workers (2004) could only detect the growth-promoting effect of VHb on tobacco cell cultures under nitrosative stress, i.e. under conditions where VHb is able to protect the enzyme activity of cell extracts against the deleterious effects of NO. In a previous study with VHb-expressing heterologous hybrid aspen (Populus tremula L. × tremuloides Michx.) lines, enhanced starch accumulation was observed, which points to changes in cellular energy metabolism and to extra energy resources for secondary metabolite production, but no general improvement of elongation growth (Håggman et al., 2003). Similarly, Zelasco et al. (2006) did not observe general growth improvements or enhanced survival rate under either submergence, oxidative, or nitrosative conditions in white poplar (P. alba L.) expressing VHb. Although the heterologous expression of VHb can be used to modify plant metabolism, its molecular mechanism has not been fully characterized.

Most economically important forest trees, including aspen, live in symbiosis with root-colonizing ectomycorrhizal (ECM) fungi. In ECMs of aspens as well as of other angiosperms, the fungal hyphae cover feeder roots as a mantle and penetrate between radially elongated epidermal cells of the roots, forming a highly differentiated structure called a Hartig net (Godbout and Fortin, 1985; Neville et al., 2002). ECM fungi are known to enhance the growth of the plant by increasing both water and nutrient acquisition, and by releasing different plant growth regulators. As a result, mycorrhizal plants are often more competitive and tolerate biotic and abiotic stresses better than non-mycorrhizal plants (Smith and Read, 1997). Specific ECM fungi have also shown potential for improving the growth of vegetative propagated plants. Both in vitro and ex vitro studies have shown positive effects of inoculation on adventitious root formation and subsequent growth of the roots, as well as acclimatization (Niemi et al., 2004).

Recent studies have connected plant TrHb genes to arbuscular mycorrhizas (Vieweg et al., 2005) and Hb1 genes to rhizobial (Shimoda et al., 2005) and actinorhizal (Sasakura et al., 2006) nodule symbioses. In the present work, a study was carried out to determine whether plant Hb genes have a more general role in symbiotic associations. The effects of two ECM fungi on the root formation and root growth of non-transgenic hybrid aspen lines and lines expressing the vhb gene were examined, and changes in expression of endogenous and heterologous Hb genes during ECM interaction were analysed.

Materials and methods

Plant and fungal material

The plant material was originally derived from suckers of selected hybrid aspen (P. tremula L. × tremuloides Michx.) plus trees growing in Southern Finland (61°48′N, 28°22′E). Multiplication
of their bud material was performed according to Ryynänen (1991). Two non-transgenic hybrid aspen lines, V613 and V617, as well as two transgenic lines, V613/3 and V617/45, were originally produced by Häggman et al. (2003). The transgenic lines were genetically modified by Agrobacterium-mediated gene transfer and selected for the present work due to their constitutive VhB expression (Häggman et al., 2003). The transgenic lines include the vhb gene driven by the 35S CaMV (cauliflower mosaic virus) promoter and the selectable marker gene neomycin phosphotransferase II (nptII) under the control of the nos promoter (Farrés and Kallio, 2002). Prior to dual culture with ECM fungi, hybrid aspen in vitro shoots were multiplied in RITA® temporary immersion containers (Vitropic, Saint-Mathieu-de-Tréviers, France) on liquid MS medium [full strength of C10H12FeN2NaO6, half strength of other micro- and macronutrients; 2.22 μM benzyladenine (BA) and 2.85 μM indole acetic acid (IAA); Murashige and Skoog, (1962)] under a 16 h/8 h light/dark photoperiod (110–130 μmol m−2 s−1) at 22 °C.

The ECM fungi, Leccinum populinum M. Korhonen and Xerocomus subtomentosus (L.: Fr.) Quél., were isolated from fruiting bodies growing under hybrid aspen and European aspen (P. tremula) stands, respectively, in Southern Finland. The fruiting bodies were halved and a piece of sterile mycelium was taken from the point of contact between the cap and the stipe, and transferred to Hagem’s agar medium (Modess, 1941) supplemented with 35 μg ml−1 streptomycin. Four weeks later, the fungal mycelium was transferred to the fresh medium. After a further 4 weeks on streptomycin-containing medium, the fungal mycelium was transferred to Hagem’s medium without streptomycin and subcultured in the dark at 21 °C. For inoculations, the mycelia of L. populinum and X. subtomentosus on the root formation, root growth, and mycorrhiza formation of hybrid aspen shoots were examined after 3 weeks in dual culture. The expression of endogenous Hb genes PttHb1 (P. tremula×trumuloides class-1 Hb gene) and PttTrHb (P. tremula×tremuloides truncated Hb gene), as well as the heterologous Hb gene vhb from Vitreoscilla in the roots was also analysed. In the time-course experiment, the expression of PttHb1, PttTrHb, and vhb in the hybrid aspen roots inoculated with L. populinum was analysed in a time-dependent manner 5 h, 2 d, 7 d, and 21 d after inoculation.

In the rooting experiment, the effects of L. populinum and X. subtomentosus on the root formation, root growth, and mycorrhiza formation of hybrid aspen shoots were examined after 3 weeks in dual culture. The expression of endogenous Hb genes PttHb1 (P. tremula×tremuloides class-1 Hb gene) and PttTrHb (P. tremula×tremuloides truncated Hb gene), as well as the heterologous Hb gene vhb from Vitreoscilla in the roots was also analysed. In the time-course experiment, the expression of PttHb1, PttTrHb, and vhb in the hybrid aspen roots inoculated with L. populinum was analysed in a time-dependent manner 5 h, 2 d, 7 d, and 21 d after inoculation.

Identification and cloning hybrid aspen endogenous haemoglobin genes
Using previously identified plant Hb genes, non-symbiotic Hb class-1 (Hb1) and class-2 (Hb2), as well as truncated Hb (TrHb) gene homology searches were performed against the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) and the genome assembly of P. trichocarpa that is available at the Joint Genome Institute (JGI, http://genome.jgi-psf.org/Porp1/Porp1.home.html). Furthermore, because, for example, vine (Vitis) and citrus (Citrus) genera possess rather large expressed sequence tag (EST) collections, Hb2 homology searches were also performed with seven different herbaceous Hb2 genes (Arabidopsis thaliana, Beta vulgaris, Brassica napus, Cichorium intybus×endivia, Gossypium hirsutum, Solanum lycopersicum, and Orzya sativa; NCBI accession nos NM_111887, BE590299, AY026337, AJ007507, AY026340, AY026344, and U76031, respectively) against vine and citrus NCBI EST databases. PCR primers to amplify the open reading frames (ORFs) of hybrid aspen PttHb1 and PttTrHb genes were designed based on the P. trichocarpa LG15 sequence deposited in the JGI database and the P.×canadensis NCBI EST sequence CX187036, respectively.

For sequencing reactions, total RNA was extracted using the method of Chang et al. (1993), with modifications according to Jaakola et al. (2001), and reverse transcribed by SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) from an anchored oligo(dT) primer according to the manufacturer’s instructions. Hybrid aspen genomic DNA was isolated by the method of Doyle and Doyle (1990) with minor modifications according to Areen and Häggman (1995). The coding region and genomic PttHb1 and PttTrHb sequences were amplified using a standard PCR method, and the amplification products were separated by 1% agarose gel electrophoresis. The amplicons were purified from the agarose gel with the Montage DNA Gel Extraction Kit (Millipore, Bedford, MA, USA) and cloned with a TOPO TA cloning Kit (Invitrogen). The cloned fragments were sequenced on both strands using an automated sequencer (ABI 3730, PE Applied Biosystems, Foster City, CA, USA) and dye terminator sequencing reagents (PE Applied Biosystems). Based on the obtained sequences, the exon and intron boundaries of hybrid aspen PttHb1 and PttTrHb were defined with the ClustalW program.

Haemoglobin expression analyses of the rooting experiment
At the end of the rooting experiment, the whole root systems of three individual plantlets per treatment were harvested into liquid nitrogen and stored at −80 °C. Total RNA was isolated from the
samples according to Vuosku et al. (2004) using the KingFisher™ mL Magnetic Particle Processor (Thermo Electron Corporation, Vantaa, Finland) with the MagExtractor-RNA-Nucleic Acid Purification Kit (TOYOBO, Osaka, Japan), and transcribed to cDNA with a Transcriptor First Strand cDNA Synthesis Kit (Roche, Penzberg, Germany) from an anchored oligo(dT) primer according to the manufacturer’s instructions.

The relative expression of the target genes PttHb1, PttTrHb, and vhb was analysed by real-time quantitative PCR using FBN (TUA) as the non-regulated reference gene. The expression patterns were also confirmed by normalization of data with the actin (ACT) housekeeping gene. Both ACT and TUA have been commonly applied as internal control genes of Populus species (Brunner et al., 2004). The gene fragments ranging from 149 bp to 210 bp were amplified using the following primers: PttHb1: 5'-CTGCTTGTGTTGAATGGAGG-3' (forward) and 5'-GCTTGTTGACATCAACGAGGACC-3' (reverse); PttTrHb: 5'-GCTGCTGATGTTGAGGTCG-3' (forward) and 5'-GCTTGATGTTGAGGTCG-3' (reverse); TUA: 5'-GCTGCTGATGTTGAGGTCG-3' (forward) and 5'-GCTGCTGATGTTGAGGTCG-3' (reverse); ACT 5'-GGGATTCCGCGCGGGTGGTCG-3' (forward) and 5'-CCCATTGCTAAGCGATACGC-3' (reverse). Both PttHb1 and PttTrHb primers were designed to contain an intron in the sequence between the primers to reveal any possible genomic DNA contamination.

The real-time quantitative PCR was performed in a LightCycler 2.0 instrument (Roche) in a 20 μl reaction mixture consisting of 2 μl of LightCycler FastStart DNA Master SYBR Green I (Roche), 3 mM MgCl₂, forward and reverse primers (0.5 μM each), cDNA template, and nuclelease-free water. PCR amplification was initiated by incubation at 95 °C for 10 min and followed by 45 cycles: 10 s at 95 °C, 5 s at 60 °C, and 10 s at 72 °C. The normalization of target gene expression was performed with LightCycler software version 4.05 (Roche) using the calibrator-normalized PCR efficiency-corrected method (Technical Note No. LC 13/2001, Roche Applied Science). After amplification, the specificities of PCR products were verified by melting curve analysis.

Time-course experiment of haemoglobin expression
To analyse Hb expression in a time-dependent manner during ECM fungus interaction, rooted in vitro plantlets of non-transgenic line V617 and VHB line V617/45 were dual cultured with the mycelia of Leccinum populinum and Xerocomus subtomentosus from all the cuttings within the line and treatment, of non-inoculated plants and VHb lines (Fig. 1C).

For the RNA extractions, the root systems of two plantlets growing on each Petri dish were pooled for one biological sample, and four replicates per treatment and sampling time were harvested and four replicates per treatment and sampling time were harvested into liquid nitrogen and stored at –80 °C. The expression analyses of the samples were carried out as described above with minor modifications. Briefly, total RNA was transcribed to cDNA with SuperScript II reverse transcriptase (Invitrogen) from an anchored oligo(dT) primer according to the manufacturer’s instructions. The real-time PCR was performed in a LightCycler 480 plate instrument (Roche, Penzberg, Germany) in a 20 μl reaction mixture consisting of 10 μl of LightCycler 480 SYBR Green I Master Mix (Roche), forward and reverse primers (0.5 μM each), 30 ng of cDNA template, and nuclelease-free water. The real-time quantitative PCR was carried out by incubation at 95 °C for 5 min followed by 45 cycles: 10 s at 95 °C, 10 s at 60 °C, and 20 s at 72 °C.

Statistical analyses
Rooting percentages and the percentage of mycorrhizal plants were analysed by the χ²-test with Bonferroni correction (Zar, 1984; Altman, 1991). Statistical comparisons in the number of lateral roots were made using analysis of variance (ANOVA) combined with Tukey’s HSD test. Differences in the number of adventitious roots and the root fresh weight were compared using a non-parametric Kruskal–Wallis test combined with the Mann–Whitney U-test with Bonferroni correction (Zar, 1984; Altman, 1991). Among the expression data of the rooting experiment, statistical comparisons between the relative mean expressions of the studied genes were performed using either ANOVA or Kruskal–Wallis test. The relative mean expressions of the time-course experiment were compared with either t-test or Mann–Whitney U-test. All statistical analyses were conducted with SPSS/PC software.

Results
The effect of dual culture with the ECM fungi Leccinum populinum and Xerocomus subtomentosus on rooting percentage, i.e. percentage of cuttings with adventitious roots from all the cuttings within the line and treatment, of non-transgenic hybrid aspen lines V613 and V617, and the VHb-expressing lines V613/3 and V617/45

| Hybrid aspen line | Rooting percentage |
|-------------------|---------------------|
|                   | Non-inoculated   | L. populinum inoculated | X. subtomentosus inoculated |
| V613              | 75.0              | 85.7                    | 85.7                        |
| V613/3            | 83.3              | 84.6                    | 92.9                        |
| V617              | 100.0 a           | 89.3 b                  | 96.4 a,b                    |
| V617/45           | 91.7              | 96.0                    | 92.6                        |

Table 1. The effect of dual culture with the ECM fungi Leccinum populinum and Xerocomus subtomentosus on rooting percentage, i.e. percentage of cuttings with adventitious roots from all the cuttings within the line and treatment, of non-transgenic hybrid aspen lines V613 and V617, and the VHb-expressing lines V613/3 and V617/45

Different letters after the values denote a significant difference (P < 0.05) within the line V617 (n=24 for non-inoculated shoots and n=26–28 for inoculated shoots).
Only *L. populinum* was able to form ECMs with the hybrid aspen lines studied during the 3 week rooting experiment. At the end of the experiment, 37.0% of the plantlets representing the transgenic line V613/3 had lateral roots covered with the hyphae of *L. populinum*, whereas in the control line V613 the comparable percentage was only 4.2% and significantly (*P* < 0.01) lower than in the transgenic line V613/3. In contrast, the transgenic line V617/45, with 36% fungal coverage, and its control line V617, with 33.3% coverage, did not differ markedly from each other in mycorrhiza formation. The anatomical examinations revealed that *L. populinum* formed a thick mantle around the lateral roots and Hartig net that enveloped epidermal cells. Typically for angiosperms, the epidermal root cells elongated radially during Hartig net formation (Fig. 2A–D).

**Endogenous haemoglobin genes in hybrid aspen**

The coding regions and corresponding genomic sequences of hybrid aspen non-symbiotic class-1 *PttHb1* and truncated *PttTrHb* genes were cloned and characterized by DNA sequencing. The coding regions of *PttHb1* (NCBI accession no. EF180083) and *PttTrHb* (EF180084) were 483 bp and 498 bp, respectively, and they consisted of four exons. Both *PttHb1* (73–82%) and *PttTrHb* (74–79%) showed a high degree of nucleic acid sequence similarity to orthologous Hb genes isolated from other plant species. When hybrid aspen *PttHb1* and *PttTrHb* were compared with the genomic sequence of *P. trichocarpa* and the EST sequence of *P. 3 canadensis* that were originally used to design the primers for ORFs, the nucleotide identities were 98.3% and 98.4%, respectively. The key residues of haem and ligand binding, ProC2, PheCD1, distal HisE7 and proximal HisF8 of Hb1, as well as the HisF8 and Phe–Tyr pair at the sites B9–B10 of TrHb appeared to be conserved in the deduced amino acid sequences (Fig. 3).

A database search with seven different herbaceous *Hb2* genes (*A. thaliana*, *B. vulgaris*, *B. napus*, *C. intybus* × *endivia*, *G. hirsutum*, *S. lycopersicum*, and *O. sativa*) did not reveal any sequence specificity for class-2 non-symbiotic Hbs in the *Populus* genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html). Besides *Populus*, vine and citrus are other genera of woody angiosperms, for which rather large EST collections are available. Therefore, *Hb2* homology searches were also performed against vine and citrus EST GenBank databases, but no DNA sequences showing similarities to *Hb2* were found.

**Haemoglobin gene expression in the roots of hybrid aspen**

At the end of the rooting experiment, the relative mean expression levels of *PttHb1* and *PttTrHb* were increased in the roots of non-transgenic lines V613 and V617 inoculated with *X. subtomentosus* or *L. populinum*, while the increase was absent in the lines expressing the heterologous *vhb* gene (Fig. 4A, B). When comparing the effects of ECM fungi on an average fold change level, the dual culture with *X. subtomentosus* caused the strongest changes. The relative mean expression of *PttHb1* and
*PttTrHb* genes was up-regulated by 2.9- and 3.3-fold, respectively, when expression was normalized with the TUA housekeeping gene. However, only the 1.7-fold up-regulation of *PttHb1* in the roots of line V613 as a result of the inoculation with *X. subtomentosus* was statistically significant (*P* < 0.05). The expression patterns were also validated by normalization with the ACT reference gene which produced similar expression patterns to TUA (data not shown).

When the function of hybrid aspen Hb genes was analysed in a time-dependent manner, 5 h, 2 d, 7 d, and 21 d after inoculation, the relative mean expression of both *PttHb1* and *PttTrHb* was higher in the roots of the non-transgenic line V617 inoculated with *L. populinum* than in non-inoculated control roots at all sampling times (Fig. 5A, C). From the studied time points, the peaks, i.e. a 2.1- and 2.9-fold increase in the expression of *PttHb1* and *PttTrHb*, were observed 5 h and 2 d after inoculation, respectively, showing a significant (*P* < 0.05) difference compared with control roots. As in the rooting experiment, no up-regulation of *PttHb1* and *PttTrHb* was observed in the roots of the VHB-expressing line V617/45 due to the dual culture with ECM fungus *L. populinum* (Fig. 5B, D).

**Discussion**

Generally, ECM fungal inoculation has been found to improve root formation and subsequently to affect the growth characteristics of the plants positively, including higher fresh weight, increased number of both adventitious and lateral roots, and increased root length (reviewed by Niemi et al., 2004). In the present *in vitro* study, both ECM fungi *L. populinum* and *X. subtomentosus* enhanced root formation and growth of the roots of both non-transgenic and transgenic hybrid aspen lines, but only *L. populinum* was able to form mycorrhizas, which supports our earlier observations that mycorrhiza formation is not a prerequisite for root induction by the fungus (Niemi et al., 2000, 2002).
To date, the plant non-symbiotic and truncated Hbs have been studied almost entirely in herbaceous species (reviewed by Dordas et al., 2003; Perazolli et al., 2006) while the corresponding tree Hbs were poorly characterized. In the present study, the coding sequences of hybrid aspen PttHb1 and PttTrHb genes were identified and cloned. To our knowledge, the latter is the first coding region of tree truncated Hb that has been characterized.

The TrHb proteins are typically 20–40 residues shorter than normal Hb proteins in bacteria (Frey and Kallio, 2003, 2005). However, the known plant truncated Hb genes are in general longer than 3-on-3 Hb genes (Vinogradov et al., 2006). Consistently, the coding regions of hybrid aspen PttHb1 and PttTrHb reported herein were found to be 483 bp and 498 bp long, respectively. Both hybrid aspen endogenous Hb genes showed high sequence similarity to other plant Hb genes isolated so far.

The recent phylogenetic analyses support the hypothesis that two distinct classes of non-symbiotic Hbs, class-1 and class-2, arose through a gene duplication event that occurred before the divergence between monocots and eudicots (Hunt et al., 2001; Gouldner et al., 2004). At the moment, however, the existence of class-2 Hb genes in monocots has not been verified. The present database searches did not reveal any homologous DNA sequence of a class-2 non-symbiotic Hb gene in the Populus genome, or in vine and citrus EST collections. This suggests that the Hb2 class might be more limited to specific plant species or genera than originally hypothesized (Trevaskis et al., 1997).

In the present study, inoculation with specific ECM fungi enhanced the expression of PttHb1 and PttTrHb in the roots of non-transgenic hybrid aspens. When the expression of the genes was compared in a time-dependent manner in the roots inoculated with L. populinum, PttHb1 had an expression peak at 5 h from the studied sampling times. This is in accordance with the work of Shimoda and co-workers (2005) in which the endosymbiotic rhizobium Mesorhizobium loti caused transient

Fig. 3. The deduced amino acid sequences of hybrid aspen endogenous haemoglobins aligned with other plant and Vitreoscilla (NCBI accession nos. L21670) haemoglobins. (A) Hybrid aspen Hb1 aligned with class 1 haemoglobins from Alnus firma, Arabidopsis thaliana, Glycine max, and Trema tomentosa (accession nos AB221344, NM_127165, U47143, and Y00296, respectively) and VHb. (B) Hybrid aspen TrHb aligned with truncated haemoglobins from A. thaliana, Datisca glomerata, G. max, and Hordeum vulgare (accession nos NM_119421, AJ489324, AY547292, and AF376063, respectively) and VHb. The shading shows the degree of conservation in each column. The positions of key residues are shown with upper case letters above the alignment.
up-regulation of *LjHb1* in *Lotus japonicus* 4 h after inoculation. In contrast, the peak in the expression of *PttTrHb* was observed 2 d after inoculation, suggesting that in hybrid aspen the function of the truncated Hb gene differs from that of class-1 during the interaction with the ECM fungus. The most pronounced enhancements of *PttHb1* and *PttTrHb* gene expression caused by *L. populinum* were 2.1- and 2.9-fold, respectively. Similar expression levels were obtained by Vieweg and co-workers (2005) who reported that the interaction with the arbuscular mycorrhizal fungus *Glomus intraradices* increased the expression of the truncated Hb gene *MtTrHb2* by 2.6-fold in the roots of *M. truncatula*. In the same study, *MtTrHb2* and *MtTrHb1* were also found to be up-regulated in response to nitrogen-fixing *Rhizobium* bacteria. In the present experiments, the relative mean expression of *PttHb1* and *PttTrHb* was also raised due to the inoculation with *X. subtomentosus*, which significantly increased the number of lateral roots and inhibited root hair proliferation, characteristics typical of ECM symbiosis (Béguiristain and Lapeyrie, 1997; Karabaghi-Dergon et al., 1998; Tranvan et al., 2000), but was unable to form a hyphal mantle and Hartig net during the experiment. These findings together show that a wide range of microorganisms generally enhancing host plant growth are able to increase the expression of plant non-symbiotic and truncated Hb genes.

The induced expression of *PttHb1* and *PttTrHb* in dual culture of non-transgenic hybrid aspen with specific ECM fungi raises the question of the biological function of Hbs in plant–mycorrhizal fungus interaction. Generally, bacterial Hbs and flavohaemoglobins as well as bacterial truncated and plant Hb1 proteins have been found to be capable of detoxifying and regulating the levels of the highly reactive signalling molecule NO (Ouellet et al., 2002; Frey and Kallio, 2003, 2005; Gardner, 2005). In plants, NO has been reported to be involved in pathogen resistance responses (Delledonne, 2005; Mur et al., 2006), but it has also been shown to increase transiently after inoculation with symbiotic bacteria (Shimoda et al., 2005) and it has been speculated that NO acts as a messenger molecule in symbiotic interactions (Perazzolli et al., 2005; Shimoda et al., 2005; Vieweg et al., 2005).

On the other hand, NO has also been shown to be involved in adventitious (Pagnussat et al., 2002) and lateral root development (Correa-Aragunde et al., 2004), and overexpression or down-regulation of endogenous Hb1 has been shown to result in altered root morphology (Hunt et al., 2002; Igamberdiev et al., 2005). In the present study, the increased expression of *PttHb1* and *PttTrHb* genes in the presence of the ECM fungi was accompanied by improved adventitious and lateral root formation in non-transgenic lines, which may indicate that the native non-symbiotic class-1 and truncated Hbs of hybrid aspen separately or in concert modulated NO levels in early reactions involved in root growth. Moreover, inoculation with ECM fungi improved the growth of the lines expressing *Vitreoscilla* VHb without up-regulating the expression of endogenous Hb genes, which indicates possible substitution of the function of the endogenous Hbs by VHb. A similar hypothesis was presented by Frey et al. (2004), even though in their work the expression of endosymbiotic plant Hb genes was not analysed.

**Fig. 4.** The expression data of the rooting experiment. The relative mean expressions ±SE of hybrid aspen endogenous haemoglobin genes *PttHb1* (A) and *PttTrHb* (B), and the heterologous haemoglobin gene *vhb* (C) in the roots of non-transgenic control lines (V613 and V617) and transgenic VHb lines (V613/3 and V617/45) 3 weeks after inoculation with the ECM fungi *Leccinum populinum* and *Xerocomus subtomentosus*. An asterisk represents a statistically significant (*P < 0.05*) difference in contrast to control treatment within the hybrid aspen line. The results were normalized using *TUA* as the reference gene.
To conclude, in the present study, two new coding sequences, hybrid aspen *PttHb1* and *PttTrHb*, were characterized and their expression was studied together with that of the bacterial Hb gene *vhb* during the interaction with specific ECM fungi. This is the first report in which the expression of representatives of these two major groups of plant Hb genes has been studied in parallel in a time-course experiment. The results show that the dual culture with the ECM fungus, with or without emergence of symbiotic structures, increased the expression of both *PttHb1* and *PttTrHb* in the roots of non-transgenic hybrid aspens. The up-regulation of *PttHb1* and *PttTrHb* genes was found to be separated in time; the peak in the expression of *PttHb1* was earlier whereas the expression *PttTrHb* was enhanced more strongly. In contrast, the ECM fungi were not able to up-regulate the hybrid aspen endogenous Hb genes in the lines expressing the heterologous *vhb* gene. Therefore, it is hypothesized that endogenous Hbs may relate to early growth responses caused by specific ECM fungi and that VHb may compensate the function of endogenous Hbs.

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