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RESEARCH PAPER

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

Soile Jokipii[1,*], Hely Häggman[1], Günter Brader[2], Pauli T. Kallio[3] and Karoliina Niemi[4]

[1] *Department of Biology, PO Box 3000, 90014 University of Oulu, Finland*

[2] *Department of Biological and Environmental Sciences, PO Box 56, 00014 University of Helsinki, Finland*

[3] *Institute of Microbiology, ETH-Zürich, CH-8093 Zürich, Switzerland*

[4] *Department of Applied Biology, PO Box 27, 00014 University of Helsinki, Finland*

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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;

* To whom correspondence should be addressed. E-mail: Soile.Jokipii@oulu.fi
The nucleotide sequences of hybrid aspen *PttHb1* and *PttTrHb* coding regions that were reported in this paper have been submitted to the NCBI database under accession numbers EF180083 and EF180084, respectively.
Abbreviations: BA, 6-benzylaminopurine; ECM, ectomycorrhizal; EST, expressed sequence tag; IAA, indole-3-acetic acid; MS, Murashige and Skoog; NO, nitric oxide; ORF, open reading frame; RNAi, RNA interference.

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Sasakura *et al*., 2006), as well as deficiency of phosphorus, potassium, and iron (Wang *et al*., 2003). Depending on the pH, class-2 Hbs, in turn, have more moderate affinity for oxygen, and their expression is up-regulated by cold (Trevaskis *et al*., 1997) or cytokinins (Hunt *et al*., 2001). Recent studies indicate that the activation patterns and functions of *Hb1* and *Hb2* classes may overlap (Ross *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* Gaertn. 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 bo_3 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 O_2 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 O_2-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 (Häggman *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 importat 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 multiplicated in RITA® temporary immersion containers (Vitropic, Saint-Mathieu-de-Tréviers, France) on liquid MS medium [full strength of $C_{10}H_{12}FeN_2NaO_8$; 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* were cultivated for 4 weeks and the mycelia of *X. subtomentus* for 5 weeks on a semi-circular cellophane membrane (8 cm diameter, P 400, Visella Oy, Valkeakoski, Finland) lying on modified Hagem's medium.

Rooting and time-course experiments

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.

Dual culture of hybrid aspen shoots and fungal mycelia

The dual culture of hybrid aspen *in vitro* shoots and fungal mycelium was carried out using the method of Niemi *et al*. (2002) with slight modifications. Two pieces of the cellophane membranes covered with actively growing mycelia were transferred to Petri dishes (14 cm in diameter) containing modified Melin–Norkrans (MMN) agar medium [3.7 mM KH_2PO_4, 1.9 mM $(NH_4)_2HPO_4$, 0.45 mM $CaCl_2$, 0.43 mM NaCl, 0.61 mM $MgSO_4 \cdot 7\ H_2O$, 0.2 μM thiamine-HCl, 18.4 μM $FeCl_3 \cdot 6\ H_2O$, and 4.1 mM glucose, pH 5.8; Marx, 1969]. A 3-week-old hybrid aspen shoot was laid horizontally on both membranes so that the base of the shoot was in contact with the mycelium. The mycelia and the bases of shoots were covered by moist semi-circular filter papers. In non-inoculated cultures, a mycelium-covered cellophane membrane was substituted by a membrane moistened with sterile water. A semi-circular piece of brown paper was placed on the lower part of the lid of the Petri dish to protect the fungus and the developing roots from direct illumination while leaving the shoots unshaded. The Petri dishes were slanted at a 70 ° angle and incubated under a 16 h/8 h light/dark photoperiod (140–150 μmol m^{-2} s^{-1}) at 24 °C.

Growth measurements and microscopic examination

After 3 weeks of dual culture, the rooting percentage, i.e. the pecentage of cuttings with adventitious roots from all the cuttings within the line and treatment, the fresh mass of the roots, and the number of adventitious and lateral roots of the plants were determined. The number of root systems with ECMs was evaluated using a dissecting microscope. The root tip samples for examination of ECM structures by light microscopy were prepared according to the method described by Niemi and Häggman (2002). After fixation, the root tips were infiltrated and embedded in Spurr resin (Agar Scientific Ltd, Stansted, UK). The microscopy sections were cut in an LKB IV Ultratome (LKB, Bromma, Sweden) and stained with toluidine blue.

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/Poptr1/Poptr1.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 *Oryza 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* LG_IX 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 Aronen 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 definied 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 α-tubulin (*TUA*) as the non-regulated reference gene. The expression patterns were also verified 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′-GCTCTTGTTGTGAAGTCATGGAG-3′ (forward) and 5′-GCTTGAGTTTTGGATTCTGCTCG-3′ (reverse); *PttTrHb*: 5′-CTGACGCGTTTGCTATTGACAAC-3′ (forward) and 5′-TCCGTTGAGAATACAAAGGAGCG-3′ (reverse); *vhb*: 5′-GTCCTTTGTTTGATATGGGTCGCC-3′ (forward) and 5′-GCCTGCTTGACAATGTTTGACTGC-3′ (reverse); *TUA*: 5′-GCTAGAGGACACTATACAGTTGG-3′ (forward) and 5′-GGAGAAGGGTAAATGGTGAATCC-3′ (reverse); *ACT* 5′-GGATATTCAGCCTCTTGTGTGCG-3′ (forward) and 5′-CCCAGTTGCTAACGATACCATGC-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 $MgCl_2$, forward and reverse primers (0.5 µM each), cDNA template, and nuclease-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-depent 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 *L. populinum*. To induce root formation before the dual culture, *in vitro* shoots were grown for 2 weeks on semi-solid MS medium (full strength of $C_{10}H_{12}FeN_2NaO_8$; half stregth of other micro- and macronutrients; no hormones) under a 16 h/8 h light/dark photoperiod (140–150 µmol m^{-2} s^{-1}) at 24 °C. The dual culture of the plantlets and mycelia was performed as described above.

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 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 nuclease-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 χ^2-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–Withney U-test. All statistical analyses were conducted with SPSS/PC software.

Results

Root growth and mycorrhiza formation

The rooting percentages of the hybrid aspen lines ranged from 75% to 100% after 3 weeks of dual culture (Table 1). There was no significant difference in rooting percentages between the non-transgenic and transgenic lines. Inoculation with *L. populinum* decreased the rooting percentage significantly ($P < 0.05$) in the line V617. In other cases, the fungi had no significant effect on the rooting percentage, but they decreased the variation in rooting percentage between hybrid aspen lines. Inoculation with both *L. populinum* and *X. subtomentosus* significantly increased ($P < 0.05$) the number of adventitious roots of lines V613 and V613/3 (Fig. 1A). The number of lateral roots was significantly ($P < 0.05$) higher in inoculated than in non-inoculated plants in all the studied lines (Fig. 1B). Moreover, the induced root growth in the presence of the fungi resulted in significantly ($P < 0.05$) increased root fresh weights in both non-transgenic control lines and VHb lines (Fig. 1C).

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).

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

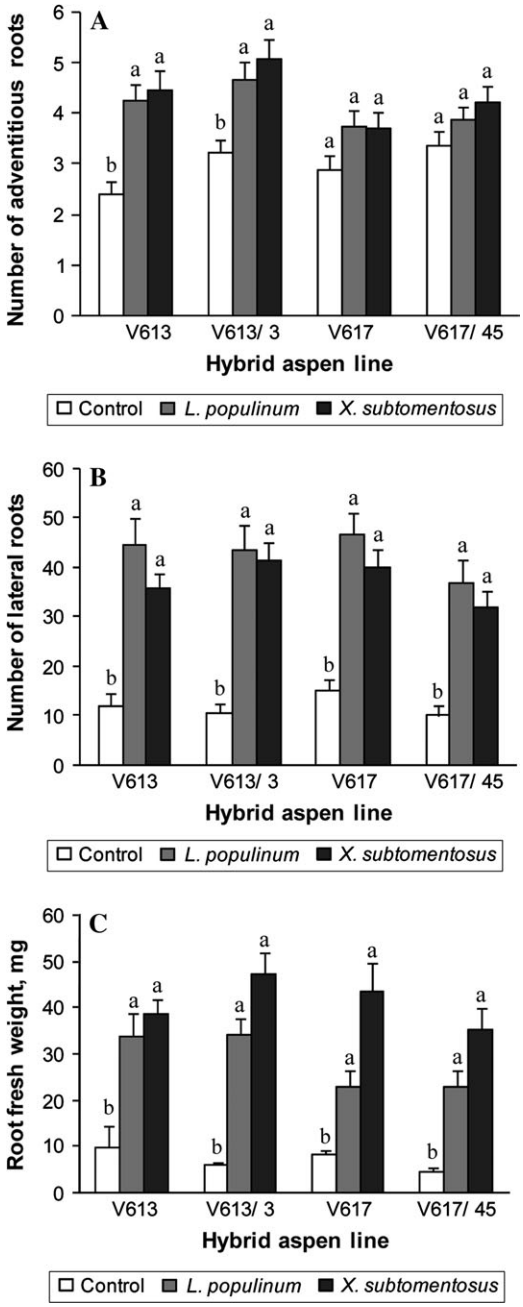

Fig. 1. Effects of the ECM fungi *Leccinum populinum* and *Xerocomus subtomentosus* on the root development and subsequent root growth of the non-transgenic hybrid aspen lines V613 and V617, and lines expressing the heterologous *vhb* gene (V613/3 and V617/45) after 3 weeks in dual culture *in vitro*. (A) Number of adventitious roots. (B) Number of lateral roots. (C) Root fresh weight. Values are means ±SE. Different letters above the columns denote a statistically significant ($P < 0.05$) difference between the means within the hybrid aspen line.

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.×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

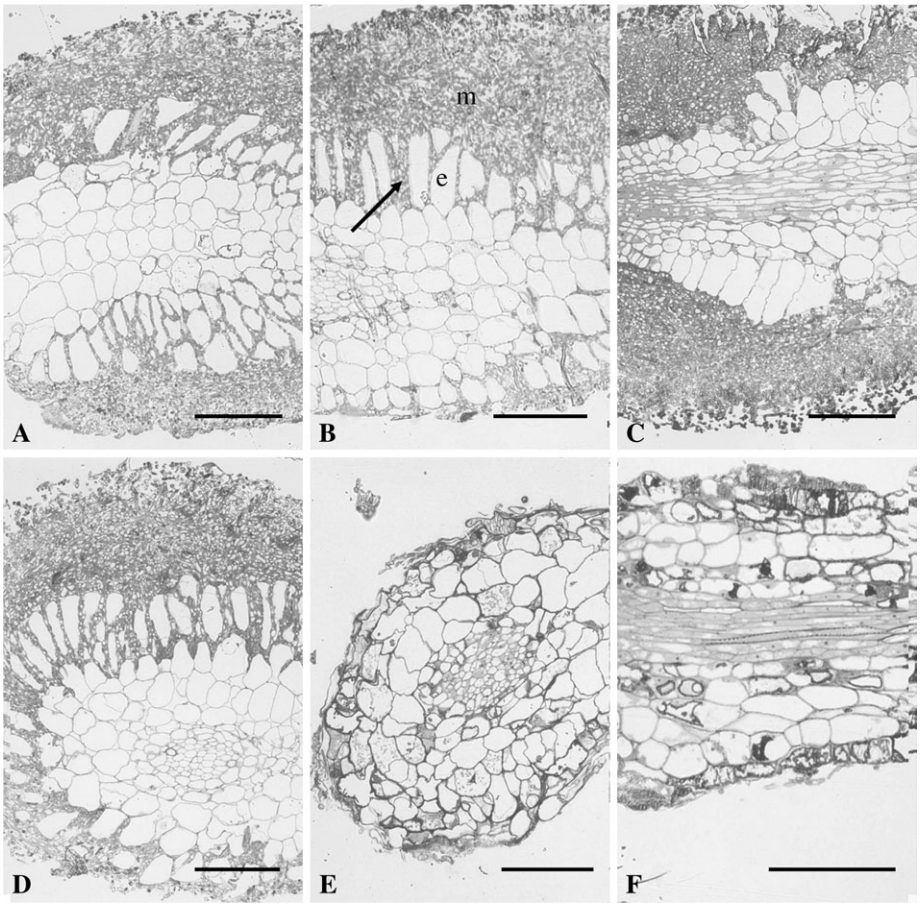

Fig. 2. Hybrid aspen roots after 3 weeks in dual culture with the ECM fungi *Leccinum populinum* (A, B, C, D) and *Xerocomus subtomentosus* (E, F). (A, E) Non-transgenic hybrid aspen line V613. (B) Non-transgenic line V617. (C, F) VHb line V613/3. (D) VHb line V617/45. The hyphae of *L. populinum* cover a lateral root as a thick mantle and have fully enveloped the radially elongated epidermal cells as a Hartig net. *Xerocomus subtomentosus* did not form mycorrhizal structures with any of the hybrid aspen lines. E, epidermal cell; m, mantle. The arrow indicates the Hartig net. The scale bar represents 100 μm.

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).

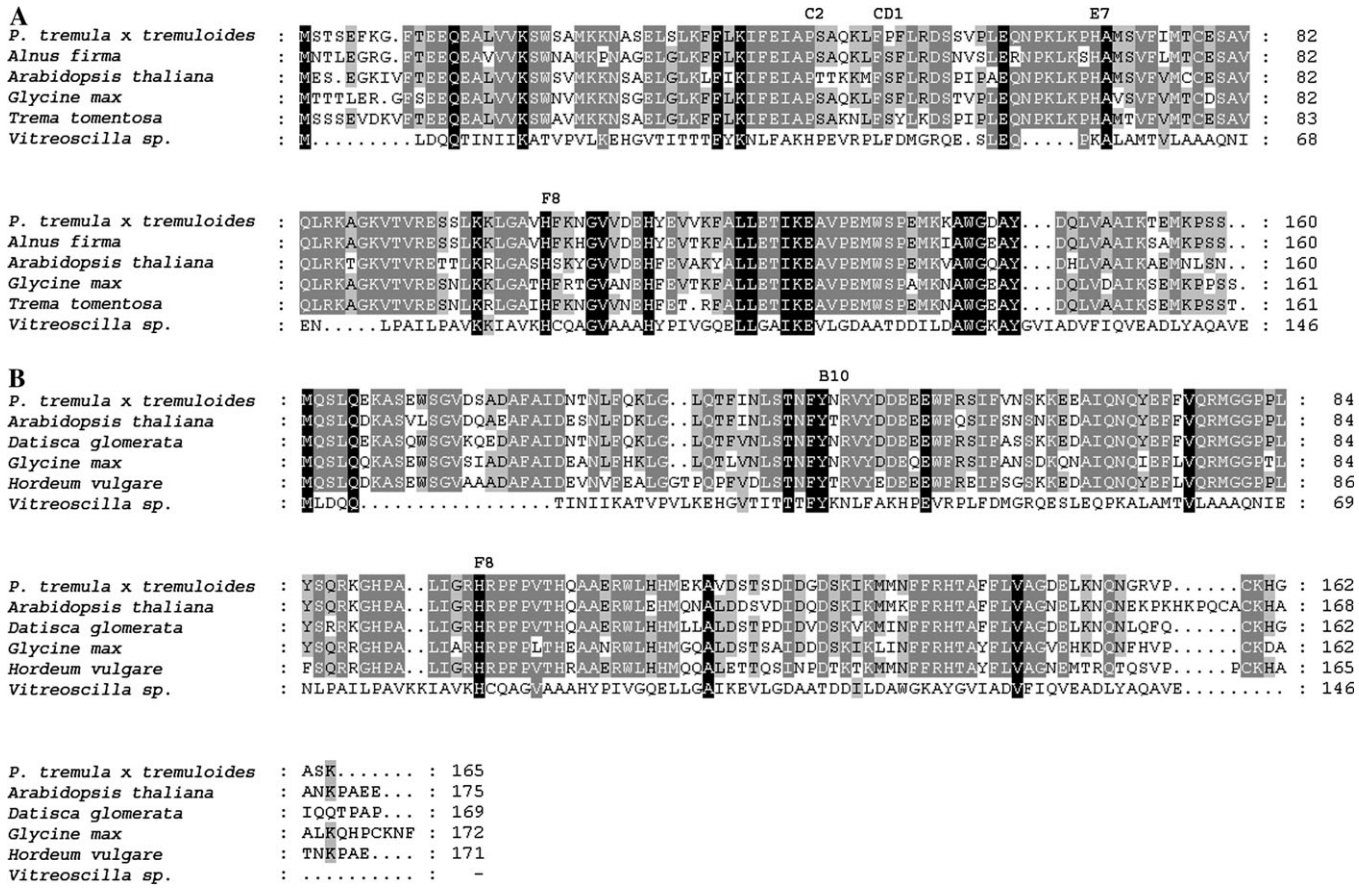

Fig. 3. The deduced amino acid sequences of hybrid aspen endogenous haemoglobins aligned with other plant and *Vitreoscilla* (NCBI accession no. L21670) haemoglobins. (A) Hybrid aspen Hb1 aligned with class 1 haemoglobins from *Alnus firma*, *Arabidopsis thaliana*, *Glycine max*, and *Trema tomentosa* (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.

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; Guldner *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

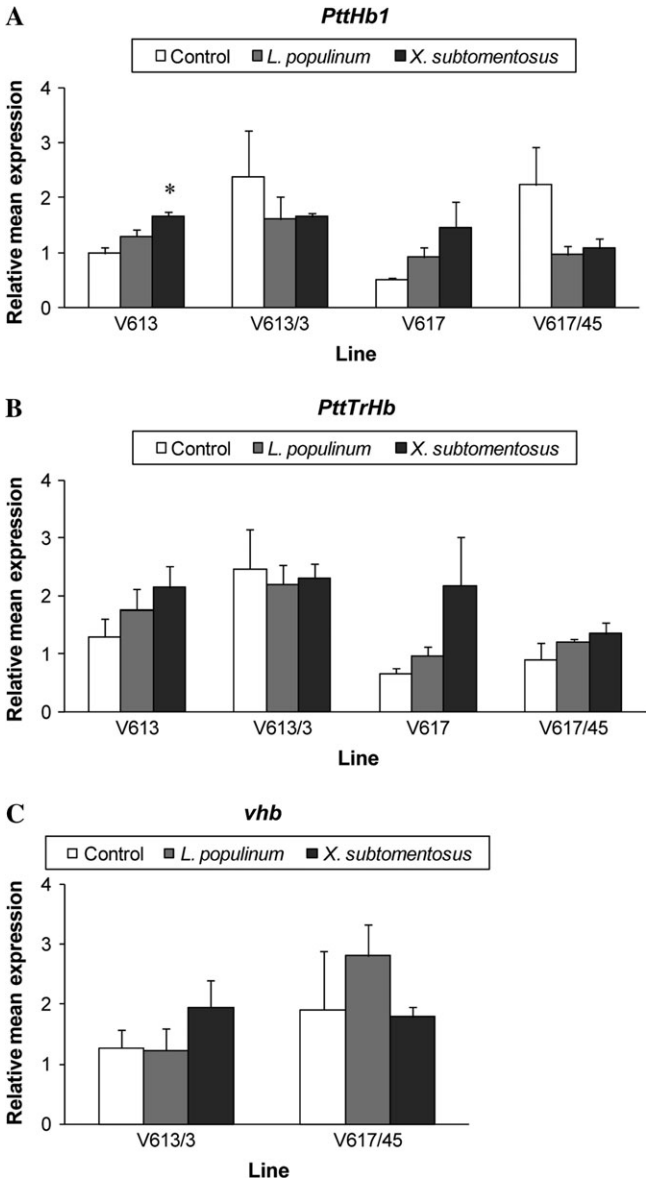

Fig. 4. The expression data of the rooting experiment. The relative mean expressions ±SE of hybrid aspen endogenous haemoglobin genes *PttHb1* (A) and *PttTrHb* (B), and the heterologous haemoglobin gene *vhb* (C) in the roots of non-transgenic control lines (V613 and V617) and transgenic VHb lines (V613/3 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.

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; Karabaghli-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 messager 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.

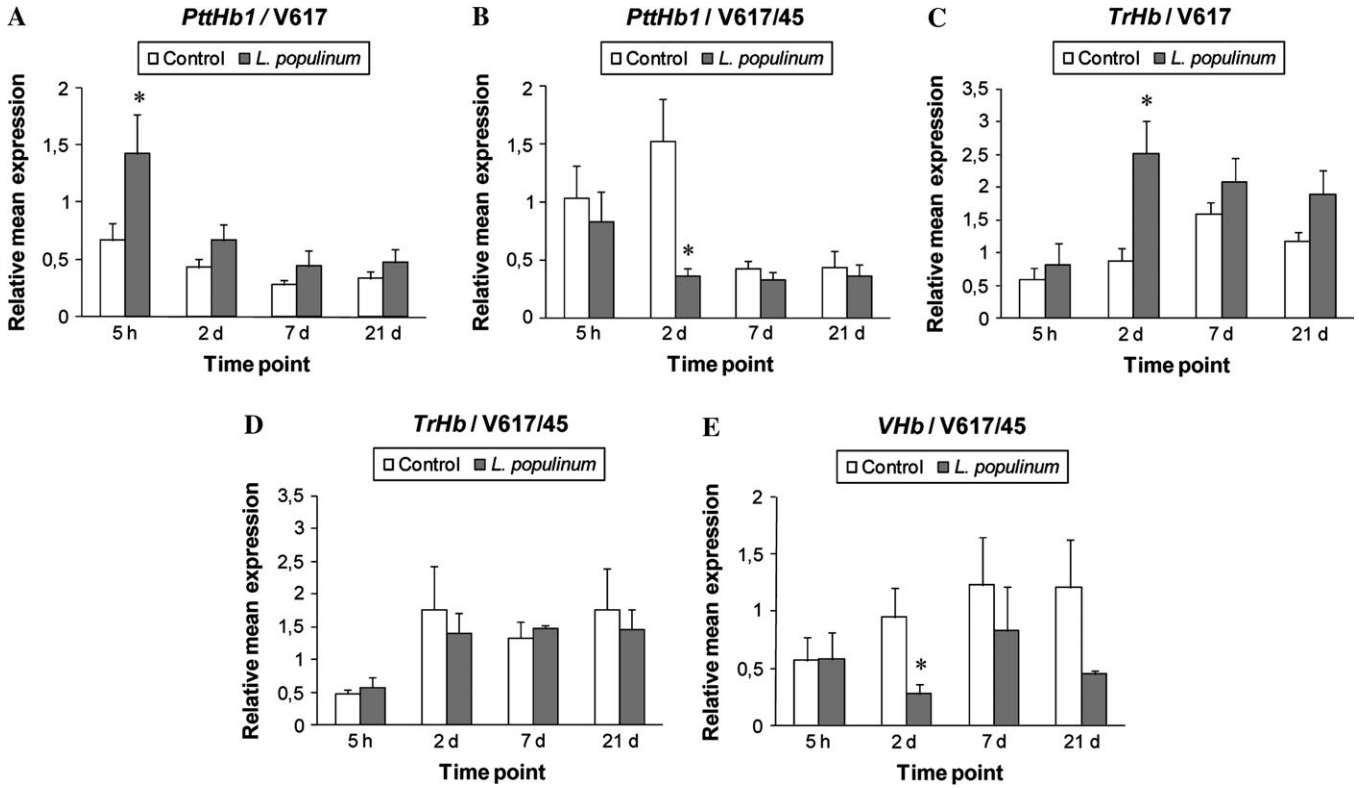

Fig. 5. The expression data of the time-course experiment, analysed 5 h, 2 d, 7 d, and 21 d after inoculation. The relative mean expression ±SE of hybrid aspen endogenous haemoglobin genes *PttHb1* (A, B) and *PttTrHb* (C, D), and the heterologous haemoglobin gene *vhb* (E) in the roots of the non-transgenic control line V617 (A, C) and the transgenic VHb line V617/45 (B, D, E) during dual culture with the ECM fungus *Leccinum populinum*. Asterisks represent statistically significant ($P < 0.05$) differences between treatments within each time point and 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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