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Soile Jokipii-Lukkari

ENDOGENOUS HAEMOGLOBINS AND HETEROLOGOUS *VITREOSCILLA* HAEMOGLOBIN IN HYBRID ASPEN

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SOILE JOKIPII-LUKKARI

ENDOGENOUS HAEMOGLOBINS AND HETEROLOGOUS *VITREOSCILLA* HAEMOGLOBIN IN HYBRID ASPEN

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Abstract

In plants, there are at least three types of haemoglobins (Hbs): symbiotic, non-symbiotic and truncated. Symbiotic Hbs are known to participate in nitrogen fixation, but the roles of the latter two groups are more obscure. Previous reports have connected both plant non-symbiotic and truncated Hbs to the scavenging of an important signal molecule, nitric oxide (NO). The aim of the present thesis was to study the effects of a bacterial Hb of *Vitreoscilla* sp. (VHb) on a woody model organism, hybrid aspen (*Populus tremula* x *tremuloides*), and the role of endogenous hybrid aspen Hbs. To store the produced hybrid aspen lines, the suitability of different cryopreservation methods was also tested.

VHb-expressing hybrid aspens were generated by *Agrobacterium*-mediated gene transfer. The effects of VHb expression were examined in standard greenhouse conditions, under elevated UV-B light as well as during culture with ectomycorrhizal fungi. Both slow cooling and vitrification methods were applied in cryostoring samples of the different genetic backgrounds. Hybrid aspen non-symbiotic and truncated *Hb* genes *PttHb1* and *PttTrHb*, respectively, were also isolated. The function of the genes and corresponding proteins PttHb1 and PttTrHb were studied using non-transgenic and VHb hybrid aspen lines as well as a mutant yeast *(Saccharomyces cerevisiae)* defective in NO resistance.

VHb expression did not improve the general growth of hybrid aspen but resulted in enhanced starch accumulation in chloroplasts, pointing to changes in energy metabolism. Of the studied cryopreservation protocols, the slow cooling of dormant *in vivo* buds proved to be the most feasible way of cryostoring hybrid aspen lines. The culture with the ectomycorrhizal fungus was shown to increase the expression of both *PttHb1* and *PttTrHb* in the roots of non-transgenic lines. However, the fungi did not up-regulate the hybrid aspen *Hb* genes in the VHb lines. Therefore, it is hypothesized that endogenous Hbs may contribute to the growth of roots and that VHb may compensate this function. When expressed alone in the mutant yeast, the recombinant PttHb1 and PttTrHb did not protect cells against the toxicity of NO. Subsequently, a novel mRNA transcript of the heterotrophic ferredoxin $NADP^+$ oxidoreductase gene *PtthFNR* was found. The absence of a plastid presequence in the transcript suggests targeting of the encoded protein into cytosol. The coexpression of PttHb1 and cytosolic PtthFNR partially rescued the mutant yeast during NO treatment, demonstrating for the first time that plant Hb1 with an applicable reductase scavenges NO *in vivo* at a physiologically relevant rate.

This thesis extends current knowledge about plant Hbs and the effects of VHb on a phenotype of a tree. It also provides new information about plant ferredoxin reductase genes.

Keywords: cryopreservation, ectomycorrhizal fungi, heterologous expression, hybrid aspen, non-symbiotic haemoglobin, Populus tremula x tremuloides, truncated haemoglobin, Vitreoscilla haemoglobin VHb

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Tiivistelmä

Kasvihemoglobiinit jaetaan symbioottisiin, ei-symbioottisiin sekä ns. katkaistuihin eli truncated-hemoglobiineihin. Symbioottisten hemoglobiinien tiedetään osallistuvan typen sitomiseen, kun taas kahden muun ryhmän toiminta tunnetaan heikosti. Aiemmissa tutkimuksissa ei-symbioottiset ja truncated-hemoglobiinit on yhdistetty tärkeän signaalimolekyylin, typpimonoksidin (NO), haitallisuuden vähentämiseen. Tämän työn tarkoituksena oli tutkia *Vitreoscilla* sp. -bakteerin VHb-hemoglobiinin vaikutuksia puuvartiseen mallikasviin, hybridihaapaan (*Populus tremula* x *tremuloides*), sekä hybridihaavan omien hemoglobiinien merkitystä. Lisäksi työssä kokeiltiin eri nestetyppisäilytysmenetelmiä hybridihaapalinjojen varastoimiseksi.

VHb:tä ilmentävät hybridihaavat tuotettiin *Agrobacterium*-välitteisellä geeninsiirrolla. VHb:n vaikutuksia tutkittiin kasvihuoneolosuhteissa, kohotetussa UV-B-säteilyssä sekä sienijuurikasvatuksen aikana. Eri linjojen nestetyppisäilytykseen sovellettiin sekä hidasta että nopeaa jäähdytystä. Tutkimuksissa myös eristettiin hybridihaavan *PttHb1*- ja *PttTrHb*-hemoglobiinigeenit. Näiden geenien sekä niiden koodaamien PttHb1- ja PttTrHb-proteiinien toimintaa tutkittiin siirtogeenittömien ja VHb-hybridihaapalinjojen sekä NO-herkän hiivamutantin (*Saccharomyces cerevisiae*) avulla.

VHb-ilmennys ei parantanut hybridihaavan kasvua, mutta lisäsi tärkkelyksen kertymistä viherhiukkasiin, mikä viittaa muutoksiin energia-aineenvaihdunnassa. Tutkituista menetelmistä parhaiten hybridihaapalinjojen nestetyppisäilytykseen soveltui lepovaiheessa olevien *in vivo* -silmujen hidas jäähdytys. Toisin kuin VHb-kasveissa, sienijuurikasvatus voimisti sekä *PttHb1*:n että *PttTrHb*:n ilmenemistä siirtogeenittömien hybridihaapojen juurissa. Tästä johtuen esitettiin, että hybridihaavan hemoglobiinit voivat vaikuttaa juurien kasvuun ja VHb pystyy korvaamaan tämän toiminnon. Kun PttHb1 ja PttTrHb ilmennettiin yksin mutanttihiivassa, proteiinit eivät suojanneet soluja NO:n myrkyllisyydeltä. Tämän jälkeen työssä kuvattiin heterotrofisen ferredoksiini-$NADP^+$-oksidoreduktaasi-geenin *PtthFNR*:n uusi mRNA-muoto. Plastidiin ohjaavan sekvenssin puuttuminen geenituotteesta viittaa siihen, että mRNA:n koodaama proteiini sijoittuu solulimaan. PttHb1:n ja PtthFNR:n yhtäaikainen ilmentäminen mutanttihiivassa paransi solujen elossa säilymistä NO-käsittelyn aikana, mikä osoittaa ensimmäistä kertaa, että kasvin Hb1 pystyy sopivan reduktaasin kanssa vähentämään NO-pitoisuutta elävässä eliössä.

Tämä työ laajentaa tietämystä kasvihemoglobiineista sekä VHb:n vaikutuksesta puiden ilmiasuun. Työ myös lisää tietoa kasvien ferredoksiinireduktaasi-geeneistä.

Asiasanat: ei-symbioottinen hemoglobiini, heterologinen ilmentäminen, hybridihaapa, katkaistu hemoglobiini, nestetyppisäilytys, Populus tremula x tremuloides, sienijuuri, Vitreoscilla-hemoglobiini VHb

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Oulu, February 2011 Soile Jokipii-Lukkari

Abbreviations

AFLP	amplified fragment length polymorphism
ATP	adenosine triphosphate
DIG	digoxigenin
ECM	ectomycorrhizal
Hb	haemoglobin
Hb1	class-1 non-symbiotic haemoglobin
Hb2	class-2 non-symbiotic haemoglobin
HPLC	high-performance liquid chromatography
MS	Murashige & Skoog
NAD(P)H	nicotiamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
nptII	neomycin phosphotransferase II gene
ORF	open reading frame
PEG	polyethylene glycol
PGD	mixture of polyethylene glycol, glucose and DMSO in water
BiFC	bimolecular fluorescence complementation
PttHb1	*Populus tremula* x *tremuloides* class-1 non-symbiotic haemoglobin
PttTrHb	*Populus tremula* x *tremuloides* truncated haemoglobin
PtthFNR	*Populus tremula* x *tremuloides* heterotrophic ferredoxin $NADP^+$ oxidoreductase
PVS2	Plant vitrification solution 2
RAPD	random amplified polymorphic DNA
ROS	reactive oxygen species
SNP	sodium nitroprusside
SSP	small secreted protein
VHb	*Vitreoscilla* haemoglobin
YFP	yellow fluorescent protein
Yhb1p	yeast native flavohaemoglobin

List of original publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I Häggman H, Frey AD, Ryynänen L, Aronen T, Julkunen-Tiitto R, Tiimonen H, Pihakaski-Maunsbach K, Jokipii S, Chen X & Kallio PT (2003) Expression of *Vitreoscilla* haemoglobin in hybrid aspen (*Populus tremula* x *tremuloides*). Plant Biotechnology Journal 1: 287–300.

II Jokipii S, Ryynänen L, Kallio PT, Aronen T & Häggman H (2004) A cryopreservation method maintaining the genetic fidelity of a model forest tree, *Populus tremula* L. x *Populus tremuloides* Michx. Plant Science 166: 799–806.

III Jokipii S, Häggman H, Brader G, Kallio PT & Niemi K (2008) Endogenous *PttHb1* and *PttTrHb*, and heterologous *Vitreoscilla vhb* haemoglobin gene expression in hybrid aspen roots with ectomycorrhizal interaction. Journal of Experimental Botany 59: 2449–2459.

IV Jokipii-Lukkari S, Frey AD, Kallio PT & Häggman H (2009) Intrinsic non-symbiotic and truncated haemoglobins and heterologous *Vitreoscilla* haemoglobin expression in plants. Journal of Experimental Botany 60: 409–422.

V Jokipii-Lukkari S, Kastaniotis AJ, Nymalm Y, Sundström R, Blokhina O, Kukkola E, Fagerstedt KV, Salminen TA, Läärä E, Ohlmeier S, Hiltunen JK, Kallio PT & Häggman H (2011) Coexpression of hybrid aspen haemoglobin PttHb1 and heterotrophic ferredoxin $NADP^+$ oxidoreductase PtthFNR alleviates nitric oxide sensivity *in vivo*. Manuscript.

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1 Introduction

1.1 Hybrid aspen

Poplars and aspens are dioecious trees that belong to the genus *Populus* in the family Salicaceae. *Populus* species are widely distributed across the Northern Hemisphere, European aspen (*Populus tremula* L.) being presumably the most widespread tree species in the world (Worrell 1995). In Finland, aspen grows mostly in mixed stands, and the occurrence of aspen-dominated forests is only 0.3% of the total forest area (Sevola 2001).

The best known hybrid aspen (*P. tremula* L. x *tremuloides* Michx., also known as *P.* x *wettsteinii*) is an artificially produced F_1 hybrid of European and Quaking aspen (*P. tremuloides* Michx.). In recent years, the paper industry has renewed its interest in this fast-growing cross due to its superior growth characteristics. The annual height increment of hybrid aspen can be up to one meter per year during the first 25 years, which is approximately 50% more than that of European aspen (Yu 2001). Due to a relatively low lignin and a high carbohydrate content, aspen wood has been found favourable for both chemical and mechanical pulping. Furthermore, aspen fibres have a small diameter and thin walls which are ideal for producing high-density paper sheets with good optical properties (Ranua 1996, Mansfield & Weineisen 2007).

Populus species and hybrids are also regarded as the main model systems of tree and woody perennial plant biology. This is because of their relatively small genome size (480 Mb); publicly accessible molecular markers and functional genomic tools, such as DNA microarrays; high-troughput transformation and regeneration capabilities; and the availability of gene sequence data (Taylor 2002, Bhalerao *et al*. 2003, Jansson & Douglas 2007). At present, the large-scale *Populus* expressed sequence tag (EST) collections represent sequences from multible species or hybrids, tissues and treatments (e.g. Sterky *et al*. 1998, Nanjo *et al*. 2004, Brosche *et al*. 2005), and, furthermore, the draft sequence of the whole genome of *P. trichocarpa* has been released (Tuskan *et al*. 2006).

1.2 *Populus* ectomycorrhizal symbiosis

Mycorrhizal symbiosis refers to interaction between feeder roots of a vascular plant and specific soil fungi. In mycorrhizal association, a fungal partner benefits

from the carbohydrates synthezised by a host plant while the large surface area of fungal mycelium increases the acquisition of water and mineral nutrients by the plant. The fungus also improves the growth of the plant by releasing different plant growth regulators. As a result, mycorrhizal plants are often more competitive and tolerate diseases, pests and unfavorable growth conditions better than plants without mycorrhizas (Smith & Read 2008).

Populus species form mycorrhizal symbiosis predominantly with ectomycorrhizal (ECM) fungi (Godbout & Fortin 1985). In ECM symbiosis the fungus colonizes the plant tissues by covering the root as a mantle and growing between the elongated epidermal cells as a Hartig net. From the mantle, fungal hyphae extend outwards into the surrounding soil as extraradical mycelium. The interaction between the ECM fungus and the host root induces a cascade of changes in gene expression in both fungus and plant. Because the amount of ectomycorrhiza-specific genes is very low, translational control presumably has an important role in gene regulation during symbiosis development (Martin & Nehls 2009). The transcripts showing different expression profile in the fungal partner encode proteins involved in, for example, cell wall modification, glycolysis, tricabocylic acid (TCA) cycle and mitochondrial respiration, whereas genes related to defence, water permeability, water stress tolerance and hormone metabolism are differentially expressed in plant roots (Johansson *et al*. 2004, Duplessis *et al*. 2005, Le Quéré *et al*. 2005, Heller *et al*. 2008).

Sequencing the complete genomes of one of the symbiotic associates of *Populus*, basidiomycete *Laccaria bicolor* (Martin *et al*. 2008), and ascomycete *Tuber melanosporum* (Martin *et al*. 2010) has also provided insights into the molecular bases of ECM symbiosis. The *L. bicolor* genome appears to contain a large number of multigene families and genes that code for effector-type small-secreted proteins (SSP), which have been proposed to serve as signaling factors in the rhizosphere and in the functioning of mycorrhizas (Martin *et al*. 2008, Podila *et al*. 2009). By contrast, the compact genome of *T. melanosporum* with very few multigene families lacks SSPs entirely (Martin *et al*. 2010). The significant differences between the proteomes of *L. bicolor* and *T. melanosporum* suggest that ECM symbiosis has evolved more than once during the course of Mycota evolution.

1.3 Plant endogenous haemoglobins and VHb haemoglobin of *Vitreoscilla* bacterium

1.3.1 Plant haemoglobins

Haemoglobins (Hbs) are a versatile set of globular proteins that reversibly bind oxygen to the iron ion of their prosthetic haem group. The first Hb was found in the blood of earthworm Lumbricus in 1839 by Hünefeld (Mann 1906), and today, Hbs are known to exist in all kingdoms of organisms (Vinogradov *et al*. 2006). In plants, there are at least three types of Hbs, including symbiotic, non-symbiotic and truncated Hbs (reviewed by Hoy & Hargrove 2008, IV). Symbiotic Hbs or legHbs are monomeric proteins of nodulating plants that participate in nitrogen fixation. During symbiotic nitrogen fixation, atmospheric dinitrogen (N_2) is converted into ammonia (NH_3) in the plant root nodules by nitrogenase enzyme of rhizobia. Nitrogenase, however, is sensitive to oxygen that causes rapid, irreversible oxidation of metal-S centers and represses the synthesis of the protein. It has been confirmed that the function of symbiotic Hbs is to facilitate the flux of oxygen to respiring N_2 fixing bacteria while maintaining a low, nontoxic free O_2 concentration (Appleby 1984).

The roles of non-symbiotic and truncated plant Hbs are less well known. Non-symbiotic plant Hbs are generally further divided into two distinct classes, class-1 (Hb1, also called Glb1) and class-2 (Hb2, also called Glb2), based on phylogenetic analyses, oxygen-binding properties and partially different expression patterns (Trevaskis *et al*. 1997, Hunt *et al*. 2001, Ross *et al*. 2004). Hb1 proteins have an unusually high affinity for oxygen whereas Hb2s appear to exhibit more moderate P_{50} values (Trevaskis *et al*. 1997, Hoy *et al*. 2007, Smagghe *et al*. 2009). Smagghe *et al*. (2009) have suggested that differences detected in the oxygen-binding kinetics of class-1 and class-2 Hbs arise from the strong hexacoordination of Hb2s and the very strong electrostatic stabilization of oxygen by hydrogen bond from $His^{(E7)}$ in Hb1s. The former characteristic results in the low oxygen association rate constant of Hb2s, and the latter causes the very small rate of oxygen dissociation of Hb1s.

Mutant studies on *Arabidopsis thaliana* have emphasized the central role of non-symbiotic Hbs during plant development by showing that at least one functional non-symbiotic *Hb* gene is necessary for the survival of a seedling (Hebestrup *et al*. 2006). To examine the function of *Hb1* genes in detail, numerous studies on their expression have been conducted. *Hb1* genes have been

reported to be up-regulated by hypoxic, cold, osmotic and high-salt stress (Trevaskis *et al*. 1997, Lira-Ruan *et al*. 2001, Shimoda *et al*. 2005, Zhao *et al*. 2008, Bustos-Sanmamed *et al*. 2011), treatments with various nitrogen compounds, plant hormones, sucrose and H_2O_2 (Trevaskis *et al*. 1997, Wang *et al*. 2000, Sakamoto *et al*. 2004, Ohwaki *et al*. 2005, Shimoda *et al*. 2005, Sasakura *et al*. 2006, Qu *et al*. 2006, Bustos-Sanmamed *et al*. 2011), deficiency of phosphorus, potassium and iron (Wang *et al*. 2003) as well as inoculation with pathogenic fungus (Qu *et al*. 2005), symbiotic rhizobia (Shimoda *et al*. 2005, Nagata *et al*. 2008) and ECM fungi (III). By contrast, the expression of *Hb2* genes is known to increase on the basis of cold (Trevaskis *et al*. 1997), hormone (Hunt *et al*. 2001, Bustos-Sanmamed *et al*. 2011) and sucrose (Shimoda *et al*. 2005) treatments.

The most recently identified truncated Hbs (also known as Glb3s or 2/2 Hbs) have sequence homology to bacterial Hbs that have shorter amino acid sequences than other Hbs and a 2-on-2 arrangement of α-helices in a three-dimensional structure instead of the 3-on-3 arrangement of the standard globin fold (Watts *et al*. 2001). The characterized crystal structures of truncated Hbs typically show a shortened A helix, a decreased CE inter-helical region and most of the F helix occurring as a loop (Lecomte *et al*. 2005). However, plant truncated Hbs have longer polypeptides than nonsymbiotic Hbs, and it is unclear if the 2-on-2 structure applies to them because no crystal or NMR structures of truncated Hbs of higher plants have been determined so far (Smagghe *et al*. 2009). The expression of genes encoding truncated Hbs (*TrHb*s) has been found to increase during root nodule and arbuscular mycorrhiza symbioses (Vieweg *et al*. 2005), dual culture with ECM fungi (III) and treatments with 1-aminocyclopropane-1-carboxylic acid and polyamines (Bustos-Sanmamed *et al*. 2011).

1.3.2 Vitreoscilla haemoglobin VHb

Vitreoscilla sp. is a gram negative, strictly aerobic filamentous bacterium (Strohl 2005). In oxygen-limited conditions, *Vitreoscilla* increases the synthesis of soluble homodimeric protein VHb, which is the best characterized bacterial Hb at the moment (Wu *et al*. 2003). Although the mechanism behind the action of the VHb protein has not been fully characterized, the heterologous expression of *vhb* has been intensively utilized to engineer the metabolism of diverse organisms (reviewed by Frey & Kallio 2003, Frey *et al*. in press). The genetically modified plant species expressing VHb represent both monocots and dicots as well as herbaceous and tree species. The reported positive effects of VHb on plant growth

and productivity include, for example, faster germination, increased dry weight and height, a shift in secondary metabolite production and enhanced submergence tolerance (reviewed by Zhang *et al*. 2007, IV).

Originally called cytochrome *o*, VHb was believed to be a terminal oxidase (Webster & Hackett 1966), but sequence and structural information later proved the Hb character of the protein (Wakabayashi *et al*. 1986, Tarricone *et al*. 1997). Further experiments indicated that VHb expression improves cellular respiration and the efficiency of energy production. Compared with a VHb-negative control, VHb-expressing *Escherichia coli* cells were able to generate a 50% higher proton flux per oxygen atom consumed (Kallio *et al*. 1994). This was shown to lead to higher ATP synthase activity and a higher net ATP accumulation rate (Chen & Bailey 1994, Kallio *et al*. 1994). Under near anoxic conditions, the expression of VHb also reduced the cellular NAD(P)H level by 1.8-fold without significantly affecting the size of the NAD(P)H/NAD(P)$^+$ pool (Tsai *et al*. 1995a). Based on the gathered results, Kallio *et al*. (1994) proposed the hypothesis that VHb increases the effective concentration of intracellular oxygen, i.e. the sum of haemoglobin-bound and -free oxygen and, thus, shifts the cellular physiology to an energetically more favourable state. Subsequent microarray analyses have indicated that the expression of genes associated with electron transport and oxidative phosphorylation are up-regulated due to VHb expression (Roos *et al*. 2004). Furthermore, VHb has been shown to interact with subunit I of cytochrome *bo* complex of the electron transport chain, suggesting that VHb provides oxygen directly to the terminal oxidase (Park *et al*. 2002).

A high oxygen concentration in a cell is known to cause generation of reactive oxygen species (ROS) that may harm lipids, proteins and DNA (reviewed by Apel & Hirt 2004). On the other hand, VHb also enhances antioxidant metabolism of certain hosts and protects cells from oxidative stress (Wang *et al*. 2009, Anand *et al*. 2010). The *vhb* gene has an oxygen-responsive promoter including binding sites for the redox-sensitive transcriptional regulators Fnr and OxyR of *E. coli* (Tsai *et al*. 1995b, Anand *et al*. 2010). Under hypoxic conditions, *vhb* is up-regulated by Fnr (Tsai *et al*. 1995b) whereas during oxidative stress, VHb has been suggested to convert OxyR into its oxidized form (Anand *et al*. 2010). Oxidized OxyR, in turn, up-regulates antioxidant genes and down-regulates *vhb* expression, resulting thus in a balance between oxygen and ROS concentrations.

1.3.3 Plant haemoglobins, VHb and nitric oxide (NO)

Both plant Hb1 (Dordas *et al*. 2003, Igamberdiev *et al*. 2004) and truncated Hb (Vieweg *et al*. 2005) as well as VHb expressed in tobacco (Frey *et al*. 2004) have been proposed to be capable of detoxifying a highly reactive signaling molecule, nitric oxide (NO). NO is a small gaseous free radical that functions in the control of many important processes in plants, e.g. germination (Beligni & Lamattina 2000), defence responses (Delledonne *et al*. 1998), flowering (He *et al*. 2004) and stomatal closure (García-Mata & Lamattina 2001). However, NO is also cytotoxic as it inhibits enzymes and produces detrimental secondary reactive nitrogen species such as peroxynitrite ($ONOO^-$; Pacher *et al*. 2007).

Sowa *et al*. (1998) pioneered in the studies that elucidated the connection between Hb1s and NO by observing that hypoxic maize cells expressing barley Hb maintained their energy status more effectively than wild type cells or a Hb silenced mutant line. Dordas *et al*. (2003, 2004) discovered later that plants respond to low oxygen tensions with the production of NO and that Hb1 expression has an inverse relationship to NO concentration in hypoxic plants. Based on these results, the authors suggested that Hb1 functions by modulating the levels of NO in cells (Dordas *et al*. 2003, 2004). Igamberdiev & Hill (2004, 2010) modified the hypothesis further and proposed that in oxygen limited conditions, Hb1 dioxygenates NO to nitrate ion (NO_3^-), which is then used as an alternative electron acceptor in a respiratory pathway. The suggested overall reaction consumes NAD(P)H and maintains cellular ATP levels sufficiently high for short-term plant survival (Figure 1). A recent study by Hebelstrup & Jensen (2008) has shown that *A. thaliana GLB2* overexpression also results in reduced NO emission of transgenic plants compared with control plants. However, the *GLB2* gene seems to play only a secondary role to *GLB1*, which is able to complement the function of *GLB2*. *GLB2* knockout plants do not show a mutant phenotype, whereas *GLB1* silenced plants have abnormal leaf hydathodes, flowers and floral buds (Hebelstrup *et al*. 2006).

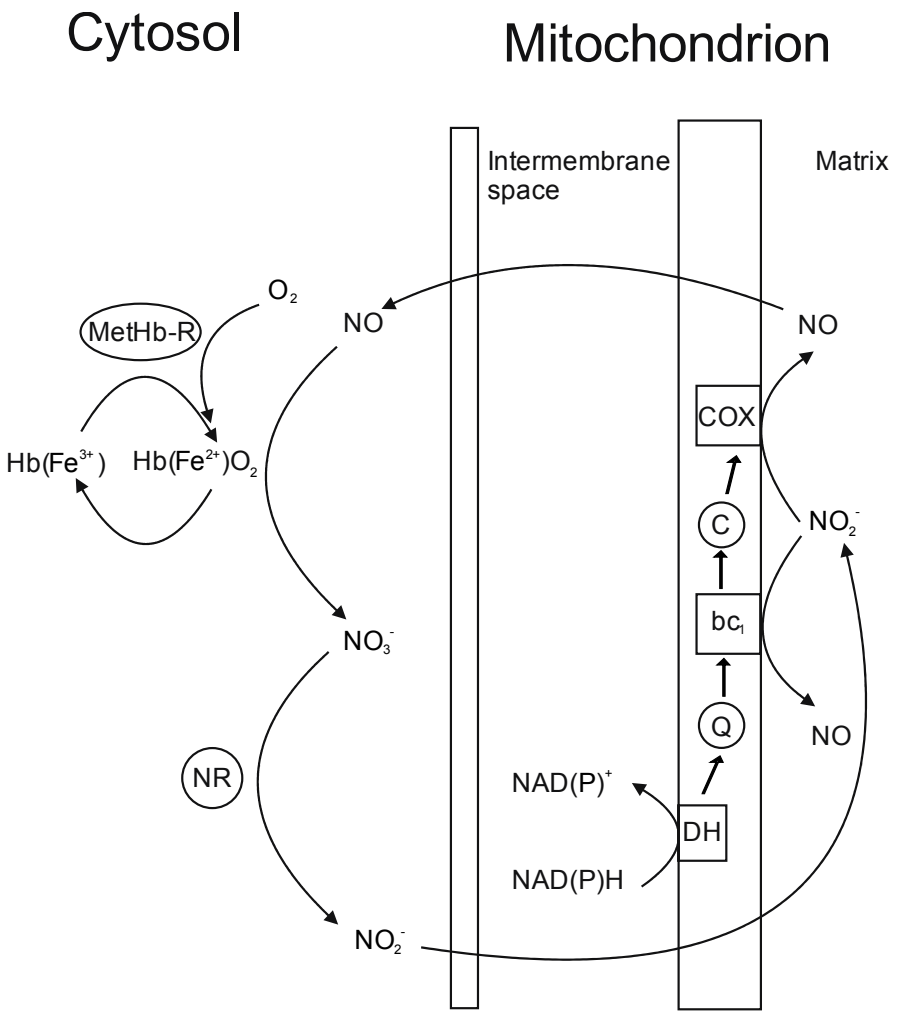

Fig. 1. A suggested schema for NO detoxification in plants. Under hypoxia, cytochrome *c* oxidase (COX) and complex III (bc_1) can reduce nitrite (NO_2^-) to NO. From a mitochondrion NO diffuses to cytosol, where it is dioxygenated to nitrate (NO_3^-) by Hb1 in its oxygenated form ($Hb(Fe^{2+})O_2$). OxyHb1 turns to metHb ($Hb(Fe^{3+})$) in the reaction. Because only Fe^{2+} can bind O_2, metHb1 has to be reduced by methaemoglobin reductase (MetHb-R) to maintain the reaction cycle. Nitrate is reduced by nitrate reductase (NR) to nitrite which is then transported to the mitochondrion. NADH formed in glycolysis and NAD(P)H from lipid breakdown are oxidized by mitochondrial dehydrogenases (DH) to provide electrons to the respiratory chain. Q, ubiquinone; c, cytochrome *c* (modified from Igamberdiev *et al*. 2010).

1.4 Cryopreservation as a means to preserve genetically modified plant material

The production of multiple genetically modified plant lines creates demand for the development of feasible non-genotype-specific methods for the long-term maintenance of living plant material (Tsai & Hubscher 2004). One such method may be cryopreservation, i.e. the storage of viable cells, tissues, organs or organisms at a very low temperature, usually in liquid nitrogen or in its vapour phase at ca. -196 ºC to -140 ºC (Benson 2008a). At ultra-low temperatures cellular metabolic activities slow down or arrest and, therefore, the risks of genetic changes are minimized. Theoretically, cryopreserved materials can be stored for an unlimited time. In the case of microalgae, Müller *et al*. (2007) have reported on the viability of *Coccomyxa arvernensis* that was cryostored for 27 years.

In comparison with maintenance of plant material as traditional plant collections or by tissue culture *in vitro*, the advantages of cryopreservation are its minimal requirements for space and labour as well as the avoidance of herbivores, plant pathogens, microbial contaminations and somaclonal mutations. The liquid nitrogen storage may also be used for cryotherapy, i.e. for eliminating plant pathogens such as viruses, phytoplasmas and other bacteria from infected shoot tips (Wang *et al*. 2009).

Currently, cryogenic storage techniques exist for over 100 plant species (Harding 2010). The techniques can be divided into traditional methods which exploit controlled rate cooling, and vitrification-based methods, in which cellular water solidifies into amorphous form without crystallization (Benson 2008b). Both of these approaches have been applied to *Populus* species and hybrids. To date, *in vivo* twigs or buds of *P. sieboldi* (Sakai *et al*. 1960) and *P. tremula* x *tremuloides* (II); calli of *P. euramericana* (Sakai & Sukawara 1973) and *P. glandulosa* (Son *et al*. 1997); and *in vitro* shoot tips of *P. tremula* x *alba* (Accart *et al*. 1995), *P. alba* and *P. tremula* x *tremuloides* (Lambardi *et al*. 2000, Tsvetkov *et al*. 2009) have been cryopreserved successfully. In the case of *P. tremula* x *alba* and *in vivo* buds of *P. tremula* x *tremuloides*, the protocols were shown to be suitable for preserving both non-transgenic as well as genetically modified plant material (Accart *et al*. 1995, II).

1.5 Aim of the thesis

The main objective of this thesis was to investigate the function of plant Hb1s and truncated Hbs by using both non-transgenic and VHb-expressing hybrid aspens (*P. tremula* L. x *tremuloides* Michx.). The specific aims of the studies were

1. to produce and analyse VHb-expressing hybrid aspen lines (I);
2. to develop an effective cryopreservation method for non-transformed and genetically modified hybrid aspens (II);
3. to isolate and characterize endogenous hybrid aspen *Hb* genes and study their expression in roots of non-transgenic and VHb-expressing hybrid aspens during ECM interaction (III);
4. to examine the effect of NO on the expression of hybrid aspen *Hb* genes *PttHb1* and *PttTrHb*. The objective of the study was also to examine whether recombinant PttHb1 and PttTrHb proteins are able to scavenge NO *in vivo* in yeast (*Saccharomyces cerevisiae*) cells (V); and
5. to review the state of knowledge of plant non-symbiotic and truncated Hbs as well as heterologous expression of VHb in plants in year 2009 (IV).

2 Materials and methods

2.1 Plant and fungi material

The hybrid aspen (*Populus tremula* L. x *tremuloides* Michx.) clones were originally derived from suckers of selected plus trees growing in Eastern Finland (61°48′N, 28°22′E). Greenhouse-grown *in vivo* (I, II) and migropropagated *in vitro* plants (I, II, III, V) were used as study materials. *In vitro* plants were cultured on semisolid (I, II, III, V) or liquid MS medium (Murashige & Skoog 1962; III, V) containing 2.22 µM benzyladenine (BA) and 2.85 µM indole-3-acetic acid (IAA). To induce root formation prior to dual culture with specific ECM fungi (III) and NO treatment (V), *in vitro* shoots were transferred on MS medium containing half-strength of sucrose content and no growth regulators. Protoplasts isolated for BiFC (bimolecular fluorescence complementation) analyses were derived from the leaves of three- to four-week-old *A. thaliana* (Landsberg ecotype) plants (V).

The two ECM fungus strains used in the studies, *Leccinum populinum* M. Korhonen and *Xerocomus subtomentosus* (L.: Fr.) Quél, were isolated from fruiting bodies growing in hybrid aspen (60º11′N, 23º57′E) and European aspen (*P. tremula*; 60º9′N, 25º3′E) stands in Southern Finland, respectively. The fungi were subcultured on Hagem's agar medium (Modess 1941) and dual cultured with hybrid aspen on modified Melin-Norkrans (MMN) agar medium (Marx 1969).

The capability of hybrid aspen recombinant Hbs PttHb1 and PttTrHb to detoxify NO was studied with yeast (*Saccharomyces cerevisiae*) *Δyhb1* mutant BY4741 (*MAT***a**; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) *YGR234w::kanMX4* (EUROSCARF, Frankfurt, Germany) lacking the native flavohaemoglobin Yhb1p (V). Depending on the genotype, yeast strains were maintained on synthetic complete (SC) selection media (SIGMA-Aldrich, St. Louis, MO, USA) lacking either uracil or both uracil and leucin for plasmid maintenance. Growth inhibition assays of yeast strains expressing recombinant Hbs were performed on rich YPD medium.

2.2 Genetic transformations

The *vhb* gene was introduced to hybrid aspen via *Agrobacterium tumefaciens* mediated transformation system (I). The wounded shoot and leaf pieces were

cocultivated with disarmed *A. tumefaciens* strain LBA 4404, carrying a VHb-expression plasmid pBVHb (Farrés & Kallio 2002) which includes the *vhb* gene driven by the 35S CaMV promoter and the neomycin phosphotransferase (*nptII*) selectable marker gene under the control of the *nos* promoter. The non-transgenic control lines were produced by regenerating individual shoots from non-transformed callus pieces.

In order to express hybrid aspen Hbs and ferredoxin $NADP^+$ Oxidoreductase PtthFNR in *YHB1*-defective yeast, the plasmids carrying the *Hb* and *PtthFNR* ORFs were heat shock transformed to the mutant yeast as described by Chen *et al.* (1992; V). Δ*yhb1* strains transformed with the corresponding empty vectors, YEp195 alone or together with YEp181, represented the negative controls in the assays. For positive controls, a so-called "wild type", YEp195*YHB1* alone or with empty YEp181, was cloned to Δ*yhb1*.

A. thaliana protoplasts were transformed with yellow fluorescent protein (YFP) PttHb1 and PtthFNR fusion proteins by polyethylene glycol (PEG)-mediated transformation (V). Protoplasts transformed with empty vectors or an empty vector with a fusion protein were used as negative controls.

2.3 Greenhouse experiments (I)

The phenotypes of VHb-expressing hybrid aspen lines were characterized under different greenhouse conditions and under elevated UV-B illumination. The first greenhouse experiment was conducted for circa six weeks in October and November. Altogether, 15 VHb-expressing and 5 non-transgenic lines from clones V613, V617 and V619 were compared for their morphology and growth characteristics. Furthermore, stable integration of the *vhb* gene was verified by Southern and Western blot analyses.

The second greenhouse experiment was performed within eight weeks in August and October to follow VHb expression and secondary metabolism during growing season. The anatomy and growth characteristics of the hybrid aspen lines were also studied. The eight VHb-expressing lines from clones V613 and V617 were selected for the experiment based on the best growth rates obtained during the first greenhouse experiment. Three non-transgenic lines represented controls. The greenhouse conditions were optimized with pre-fertilized peat, HPI-T multimetal lamps and increased temperature during light period in contrast with the first experiment.

One-year-old plants from the second greenhouse experiment were illuminated with UV-B radiation to study the effects of VHb expression on environmental stress responses. The plants were at first acclimatized for one week and then treated with ambient or elevated UV-B illumination for three weeks. The hybrid aspen lines were compared for their growth rates, phenolic compounds and VHb expression.

2.4 Dual culture with ECM fungi (III)

In the rooting experiment, the effects of specific ECM fungi on the root formation, root growth and mycorrhiza formation of VHb-expressing and non-transgenic hybrid aspen *in vitro* shoots were examined after three weeks in dual culture. Additionally, the expression of endogenous *Hb* and heterologous *vhb* genes in the roots were analysed by real-time quantitative PCR. The study material consisted of two non-transgenic and two VHb hybrid aspen lines as well as ECM fungi *L. populinum* and *X. subtomentosus*.

Subsequently, the function of hybrid aspen *Hb* genes in the roots with ECM interaction was examined in a time-dependent manner 5 h, 2 d, 7 d and 21 d after inoculation. As *X. subtomentosus* was unable to form mycorrhizal structures in the previous experiment, in the time-course experiment one non-transgenic and one VHb hybrid aspen line were inoculated only with *L. populinum*.

2.5 Cryopreservation (II)

Both controlled rate cooling and vitrification-based cryopreservation methods were tested for the long-term storage of different hybrid aspen lines. The slow cooling of PGD (10% (w/v) polyethylene glycol 6000, 10% glucose (w/v) and 10% DMSO (v/v) in water) treated *in vitro* apical segments was carried out by modifying a method described by Ryynänen (1996a). The effects of both the duration from the previous subculturing and the duration of cold acclimation were studied. The apical segments were frozen slowly in a programmable cooling chamber (Lauda Ultra-Kryomat RUK 60, Königshofen, Germany). The cryostored material consisted of two transgenic lines.

The vitrification of PVS2 (Plant vitrification solution 2; 30% glycerol (v/v), 15% (w/v) ethylene glycol, 15% (v/v) DMSO and 0.4 M sucrose in hormone-free MS medium; Sakai *et al*. 1990) treated *in vitro* buds was performed slightly modifying a method described by Lambardi *et al*. (2000). The vitrificated *in vitro*

buds were derived from three non-transgenic clones and two genetically modified lines.

The dormant *in vivo* axillary and apical buds were cryopreserved with a slow cooling method using a programmable cooling chamber according to Ryynänen (1996b). The effects of the bud type and the position of the bud on the seedling were studied. The *in vivo* buds were collected from the greenhouse-grown hybrid aspen plants representing three non-transgenic clones and two genetically modified lines.

2.6 DNA analyses

The hybrid aspen genomic DNA was isolated using a method as described by Doyle & Doyle (1990), with minor modifications made according to Aronen & Häggman (1995; I, III), or as described by Lodhi *et al*. (1994) and further modified by Valjakka *et al*. (2000; I, II). The polymerase chain reaction (PCR) was used for preliminary screening of possible VHb lines (I); to produce labelled probes for molecular studies (I, II, V); to amplify the coding regions and genomic sequences of the isolated genes *PttHb1*, *PttTrHb* and *PtthFNR* (III, V); and to introduce restriction sites to the DNA fragments to be cloned (V). PCR primers to amplify hybrid aspen ORFs were designed based on gene homology searches against the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) and the genome assembly of *P. trichocarpa* at Phytozome database (http://www.phytozome.net/poplar.php).

The PCR products were inserted to cloning vectors either in a ligation reaction (V) or with a TOPO TA cloning Kit (Invitrogen, Carlsbad, CA, USA; III, V) and sequenced using an automated sequencer (ABI 3730, PE Applied Biosystems, CA, USA). The genetic fidelities of the control and cryopreserved materials were tested by comparing their random amplified polymorphic DNA (RAPD) profiles (II). The stable integration and copy number of the *vhb* (I) and *nptII* (II) genes were examined by Southern blotting with digoxigenin (DIG) - labelled DNA probes.

2.7 RNA analyses

Total hybrid aspen RNA was isolated either manually (Jaakola *et al*. 2001) or with automated extraction procedure using a KingFisher^{TM} mL Magnetic Particle Processor (Thermo Electron Corporation, Vantaa, Finland; III, V). Subsequently,

the RNA samples were transcribed to cDNA with a Transcriptor First Strand cDNA Synthesis Kit (Roche, Penzberg, Germany) or with a SuperScript II reverse transcriptase (Invitrogen) from an anchored-oligo(dT) primer. The relative expressions of the target genes *PttHb1*, *PttTrHb* and *vhb* were analysed by real-time quantitative PCR using α-tubulin (*TUA*) or actin (*ACT*) as the non-regulated reference gene in LightCycler 2.0 (Roche) or in a LightCycler 480 plate instrument (Roche). The normalization of the target gene expression was performed with the calibrator-normalized PCR efficiency-corrected method.

In order to search for alternative *PtthFNR* transcripts, 5' ends of *PtthFNR* mRNAs were amplified with 5'RACE method using a 5'/3' RACE Kit (2nd Generation, Roche; V). *PtthFNR* mRNAs were localized with *in situ* hybridization using full-length DIG-labelled RNA probes.

2.8 Protein analyses

Total soluble proteins from hybrid aspen samples were isolated as described in I, and the protein extraction of yeast cells was carried out according to Ruohonen *et al*. (2006; V). The concentrations of the protein samples were determined using a method described by Bradford (1976). The expression level of VHb in transgenic hybrid aspen lines was studied by Western blot analysis using chemiluminescent reactions (I). The biological activities of the recombinant Hbs produced in yeast were assayed by recording CO difference spectra (CO-ligated minus reduced) of the protein samples (Tsai *et al*. 1996). Interaction between the PttHb1 and PtthFNR proteins in plant cells was verified by BiFC in *A*. *thaliana* protoplasts (http://genetics.mgh.harvard.edu/sheenweb/; V).

To examine the possible mode of ligand binding of PttHb1, three-dimensional PttHb1 dimer structures with either penta- or hexacoordinated haem group were made with MODELLER (Sali & Blundell 1993) and visualized with a Bodil visualization and modeling package (Lehtonen *et al*. 2004; V). The cavities in the PttHb1 models were calculated with SURFNET (Laskowski 1995) and created with PYMOL Molecular Graphics System (DeLano Scientific).

2.9 Metabolite analyses

The leaf phenolics of greenhouse-grown hybrid aspen lines were extracted as described in I and analyzed with the DAD-HPLC (high-performance liquid chromatography with photodiode array detection) method. Retention time, UV-

spectrum and HPLC/API-ES (atmospheric pressure electrospray ionisation; Julkunen-Tiitto & Sorsa 2001) were used to identify the compounds. The chlorophylls were extracted as described in I. The concentrations of chlorophylls *a* and *b* were calculated according to Arnon (1949).

2.10 Microscopy

The leaf anatomy of VHb and control hybrid aspen lines was studied with light and transmission electron microscopy (I), whereas the number and structure of ECMs in the studied lines were examined using a dissecting and light microscope, respectively (III). The transient expression of YFP-fusion proteins of BiFC analyses was monitored by confocal microscopy (V).

2.11 NO treatments of hybrid aspen roots and Hb expressing yeast cultures

To study the effects of nitrosative stress on the expression of *PttHb1* and *PttTrHb*, the hybrid aspen *in vitro* roots were incubated in liquid culture medium supplemented with the NO-releasing compound sodium nitroprusside (SNP) for 5 h (V). The *in vivo* NO detoxification efficiency of recombinant PttHb1 and PttTrHb was examined with the complementation analyses in a yeast. The *Δyhb1* yeast strains expressing Hbs with or without a reductase PtthFNR were treated with various concentrations of a NO-donor DETA NONOate ((Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; Cayman Chemicals, Ann Arbor, MI, USA). The growth of yeast cells was monitored by OD_{600} readings during 10 h assays.

2.12 Statistical analyses

Statistical comparisons among data were performed using SPSS/PC software or the R enviroment (R Development Core Team, 2010). The analyses are described in detail in the original publications I, II, III and V.

3 Results

3.1 Phenotypic comparisons between VHb and control hybrid aspens grown in greenhouse (I)

According to the PCR findings, *Agrobacterium*-mediated gene transfer resulted in a total of 40 independent VHb-expressing hybrid aspen lines. The lines selected for subsequent studies showed stable but genotype-dependent VHb expression. During the first, non-optimized greenhouse experiment, the VHb lines of clone V613 had significantly ($p<0.05$) higher dry and fresh weight values than the corresponding control line. No differences were found in chlorophyll or soluble protein concentrations nor in the number of leaves or side branches between VHb and non-transgenic plants. When leaf areas were compared, only the VHb line V613/3 had larger leaves than the controls ($p<0.05$).

No positive effects of VHb expression on the fresh and dry weights could be detected during the second, optimized greenhouse experiment. By contrast, three specific VHb lines had significantly ($p<0.05$) lower whole plant dry weights than the control lines at the end of the experiment. Microscopic examinations revealed that the total number of palisade parenchyma cells and the volume of starch in the chloroplasts were higher in the VHb plants than in the control plants. Over the course of the experiment, there were not any consistent differences in secondary metabolite composition due to VHb expression.

Under elevated UV-B illumination, the VHb-expressing line V617/45 grew in height significantly ($p<0.05$) better than the control line. The UV-B treatment did not cause changes in the secondary metabolite profiles of the control lines, but the VHb lines responded to the treatment individually relative to the controls. The VHb lines V617/45 and V617/60 had more quercetin-, kaempfereol- and myricetin-derivatives and total flavonoids than either or both corresponding control lines.

3.2 Root growth and mycorrhiza formation of VHb and control hybrid aspens during ECM fungus interaction (III)

After three weeks of culture, the rooting percentages of the non-inoculated and ECM fungus inoculated hybrid aspens ranged between 75–100% and between 85–96%, respectively. No difference in the rooting ability of the VHb and control

lines was found. Inoculation with both *L. populinum* and *X. subtomentosus* significantly ($p<0.05$) increased the number of adventitious roots in one control and one VHb line. The number of lateral roots was also higher ($p<0.05$) in inoculated than non-inoculated plants in all four studied lines. Fungus-induced root growth resulted in increased ($p<0.05$) root fresh weights in both non-transgenic control lines and VHb lines.

Only *L. populinum* was able to form ECMs during the three-week rooting experiment. At the end of the experiment, 37.0% of the plants representing the VHb line V613/3 had lateral roots covered with the hyphae of *L. populinum*, whereas in the control line V613 the comparable percentage was significantly ($p< 0.01$) lower: 4.2%. By contrast, lines V617 and V617/45 did not differ from each other in mycorrhiza formation.

3.3 Non-genotype-specific cryopreservation of hybrid aspen (II)

The slow cooling of PGD-treated *in vitro* apical segments was not an applicable cryopreservation method for genetically modified and non-transgenic hybrid aspen lines. Altogether, only 4 cryostored samples out of 360 survived. The vitrification of PVS2-treated *in vitro* buds was more successful: all the studied genotypes resumed growth after cryostorage. However, the regeneration of the buds varied significantly ($p<0.001$) among genotypes and was lower ($p<0.001$) than those of the controls. The regrowth rates of cryopreserved *in vitro* buds ranged between 3–75% after six weeks of culture, while 65–100% of the treatment controls and all non-treated controls proliferated.

The best recovery rates were achieved when dormant *in vivo* buds were cryopreserved using the slow cooling method. Regeneration percentages of the thawed buds varied between 72–96% after four weeks of culture, while 90–100% of the control buds showed regrowth. The difference between the regeneration of genotypes ($p<0.05$) and between cryopreserved and control buds ($p<0.01$) was statistically significant. The bud type or its position on the seedling did not affect regrowth.

The regenerated plants appeared to have normal phenotypes during both tissue culture and greenhouse cultivation, and no genetic changes in their RAPD profiles that would have been caused by the cryopreservation treatments were observed. According to Southern blot analyses, the slow cooling and vitrification methods used did not affect the copy number of the *nptII* transgene.

3.4 Hybrid aspen haemoglobin genes and their expression in roots with ECM interaction and during NO stress (III, V)

The coding regions and genomic sequences of hybrid aspen non-symbiotic class-1 *PttHb1* and truncated *PttTrHb* haemoglobin genes were cloned and characterized (III). The coding regions of *PttHb1* (NCBI accession number EF180083) and *PttTrHb* (EF180084) were 483 bp and 498 bp long, respectively, and they were composed of four exons. Both *PttHb1* (73–82%) and *PttTrHb* (74–79%) showed high degrees of nucleic acid sequence similarity to orthologous *Hb* genes of other plant species. The key residues of heme and ligand binding appeared to be conserved in deduced amino acid sequences of PttHb1 and PttTrHb. No *Hb2* sequences were found in the fully sequenced *Populus* genome.

At the end of the rooting experiment, the relative mean expression levels of both *PttHb1* and *PttTrHb* had increased in the roots of non-transgenic lines inoculated with ECM fungi, but no the up-regulation could be detected in the VHb lines. The dual culture with *X. subtomentosus* unable to form mycorrhizal structures caused the strongest changes: the expressions of *PttHb1* and *PttTrHb* were enhanced by 2.9- and 3.3-fold, respectively.

When the function of hybrid aspen haemoglobin genes in the roots was analysed in a time-dependent manner, the expression peaks of *PttHb1* and *PttTrHb* were observed at 5 h and 2 d, respectively, after inoculation with *L. populinum*. The former gene was up-regulated by 2.1- and the latter by 2.9-fold, both showing a significant ($p<0.05$) difference in comparison with the control roots. Similar to the rooting experiment, no increase in the expressions of *PttHb1* and *PttTrHb* genes was found in the ECM inoculated roots of the VHb line.

To examine the effects of nitrosative stress on the expression of hybrid aspen *Hb* genes, the roots of *in vitro* grown hybrid aspen plants were treated with SNP (V). After 5 h of incubation, the relative mean expressions of *PttHb1* and *PttTrHb* increased in the roots treated with 200 μM SNP by 2.3- and 2.7-fold, respectively, in comparison with the control plants.

3.5 Functional expression of *PttHb1* or *PttTrHb* in a yeast mutant lacking the native flavohaemoglobin (V)

The capability of the yeast native flavohaemoglobin gene *YHB1* to alleviate the deleterious effects of NO was clearly observed in the cell cultures that were treated with DETA NONOate. However, neither the cytosolic nor the

mitochondrially targeted hybrid aspen Hbs were able to rescue the NO resistance of the mutant yeast under the studied DETA NONOate concentrations. According to the CO-binding assays, all of the transformed yeast strains produced the recombinant Hbs in their active form.

3.6 Hybrid aspen ferredoxin $NADP^+$ oxidoreductase gene *PtthFNR*: coding sequence, location of transcripts and discovery of alternative mRNA (V)

The coding region of the hybrid aspen ferredoxin $NADP^+$ oxidoreductase gene (*FNR*) was cloned and sequenced, and it proved to be 1137 bp long. As it showed high nucleic acid sequence similarity (75–78%) to plant heterotrophic *FNR*s, the gene was designated as *PtthFNR* (NCBI code GU124691). *PtthFNR* transcripts were detected abundantly in the epidermis and in the vascular tissues of hybrid aspen *in vitro* leaves.

Analyses of the putative amino acid sequence of PtthFNR with TargetP (Emanuelsson *et al*. 2000) program predicted the cleavage site for its transit peptide to appear at Val60 followed by a Methionine at amino acid place 62. The gel electrophoresis of *PtthFNR* 5'RACE products revealed two DNA fragments of different sizes and a smear between them. The sequencing of the products showed that the largest fragment represented the *PtthFNR* transcript containing the plastid targeting sequence and that the smallest fragment represented the sequence starting at the ATG corresponding to Met62. The smear contained PCR products of various lengths.

3.7 Coexpression of PttHb1 and PttTrHb with PtthFNR (V)

PttHb1 together with the mature cytosolic PtthFNR was able to partially complement the NO resistance defect of the mutant yeast phenotype. After 10 h of culture, the average cell densities of the strains coexpressing PttHb1 and PtthFNR were 52% and 40% higher than that of the negative control in 0.5 mM and 1.0 mM DETA NONOate, respectively. When PtthFNR was expressed alone in the Δ*yhb1* strain, there was no evidence of improvement in growth rate. In contrast to PttHb1, the expression of PttTrHb together with PtthFNR did not significantly affect the growth inhibition caused by DETA NONOate. According to the CO difference spectra, PttTrHb was also expressed as a functional protein during the coexpression analyses.

The expression of YFP fusion proteins in *Arabidopsis* protoplasts confirmed the interaction between PttHb1 and PtthFNR also in plant cells. YFP signal was detected using a pair of constructs in which PttHb1 was fused to a C-terminal fragment of YFP (PttHb1-YFP^C) and PtthFNR to an N-terminal fragment (PtthFNR-YFP^N). With a reversed construct pair, PtthFNR-YFP^C and PttHb1-YFP^N, or when protoplasts were transformed with empty vector and either PttHb1-YFP^C or PtthFNR-YFP^N, no YFP fluorescence was detected. In the case of co-transformation with both empty vectors, a non-specific signal near the plasma membrane was occasionally observed, which was possibly caused by a non-specific YFP folding.

3.8 Structural models of PttHb1 (V)

To visualize the possible movement of amino acids during NO detoxification, the structural *in silico* models of unliganded, hexacoordinated and liganded, pentacoordinated PttHb1 were constructed. In the unliganded form, the haem group of PttHb1 is coordinated by the distal histidine (His69), whereas upon ligand binding it is shifted away due to movement of the E-helix. The movement of the E-helix enables the formation of a channel that would seem to fit small molecules, such as NO. The channel connects the haem group with the conserved cysteine (Cys78), which is possibly S-nitrosylated. In both constructed models Cys78 is surrounded by acidic and basic amino acids, which may function as catalysts in S-nitrosylation (Stamler *et al*. 1997).

4 Discussion

4.1 Growth characteristics of VHb-expressing hybrid aspens

Genetically modified organisms can be exploited to study the function of specific genes or to engineer organism traits to meet human needs. To date, the heterologous expression of VHb has led to enhanced growth and altered metabolism in several microbial species (reviewed by Frey & Kallio 2003, Frey *et al*. in press), but the reported results concerning plants have been more contradictory (IV). In the present thesis, no general growth improvement due to VHb expression could be detected (I, III). However, the volume of starch in the chloroplasts was higher in the two studied VHb hybrid aspen lines than in the control plants, indicating changes in energy metabolism (I). Furthermore, significantly more plants of one VHb line formed symbiotic structures during dual culture with the specific ECM fungus than the non-transgenic line (III). The overall growth characteristics of VHb hybrid aspens are in accordance with the findings of Zelasco *et al*. (2006) who found that only one out of six greenhouse-grown *P. alba* VHb lines had significantly higher values for height, stem and root biomass as well as stem diameter than wild type plants. On the other hand, Holmberg and co-workers (1997) have reported faster germination; higher dry weight; and higher chlorophyll and nicotine content in VHb-expressing tobacco plants than in wild type plants. In their repetition experiments, Frey *et al*. (2004) were, however, unable to discern significant growth differences between transgenic and control tobacco lines under standard growth conditions.

When expressed in microorganisms, the VHb protein has been suggested to affect aerobic metabolism and growth by increasing the effective concentration of intracellular oxygen (Kallio *et al*. 1994) or by protecting cells against nitrosative stress (Frey *et al*. 2002, Kaur *et al*. 2002). As it is commonly assumed that normal atmospheric oxygen content is not a limiting factor for plant growth, the potential beneficial effects of VHb expression might be more clearly seen in oxygen-limited or other stress conditions in specific plant species. Indeed, VHb-expressing plants have shown increased tolerance against hypoxic, nitrosative and photo-oxidative stress (Frey *et al*. 2004, Li *et al*. 2005, Wang *et al*. 2009). Moreover, two VHb lines in the present study had higher levels of certain flavonoids than the controls under elevated UV-B illumination, which may reflect

the availability of extra energy resources for protective secondary metabolite production (I).

The increase in human population and the climate change create a need for the "sustainable intensification" of plant production in which yields are increased without adverse environmental impact and without the cultivation of more land (The Royal Society 2009). Plant breeding by genetic modification can potentially contribute to achieving this objective. In order to target the heterologous expression of VHb in plants more efficiently, it is vital to study in detail how the protein functions at molecular level. In the future, it is also highly important to examine the metabolic pathways of VHb plants showing positive growth characteristics at global genome, proteome and metabolome levels.

4.2 Successful cryopreservation of genetically modified and non-transgenic hybrid aspens

In order to maintain multiple genetically modified and non-transgenic lines, various methods were tested for cryostoring hybrid aspen (II). The slow cooling of dormant *in vivo* buds proved to be the most efficient method, resulting in recovery rates as high as 72–96%, which are similar with the values obtained with PVS2 vitrificated hybrid aspen (78%; Tsvetkov *et al*. 2009) and *P. alba* shoot tips (90%; Lambardi *et al*. 2000). In comparison with the vitrification protocols, the benefit of the applied slow cooling method is the avoidance of pretreatments and potentially mutagenic cryoprotectants. It should be noted, however, that the preservation of dormant buds requires specific freezing equipment and larger storage space for plants.

The cryopreservation process exposes plant tissues to physical, chemical and physiological stress, which may potentially lead to genetic alterations (Harding 2010). Therefore, it is important to assess whether plant germplasm recovered from cyostorage is genetically identical with the material used prior to the storage. In the present thesis, the genetic integrity of the cryopreserved hybrid aspen lines were studied with RAPD and Southern blot analyses. No genetic differences were detected in the RAPD profiles or in the copy number of the transgene between non-cryopreserved controls and *in vivo* plants regenerated from cryopreserved materials. Additionally, AFLP (amplified fragment length polymorphism), AMP (amplified DNA methylation polymorphism), MSAP (methylation-sensitive amplified polymorphism), RFLP (restriction fragment length polymorphism), RAF (randomly amplified DNA fingerprinting), SSR (simple sequence repeat)

and ISSR (inter-simple sequence repeat) molecular marker techniques have been used to examine the genetic stability of cryopreserved plant materials (Harding 2004, Kaity *et al*. 2008, Liu *et al*. 2008). Most of the studies do not provide any evidence of genetic alterations occurring during cryopreservation, but there are also conflicting reports. After cryopreservation, changes have been observed in the genomic DNA sequence or the methylation status of, for example, *in vitro* grown *Prunus* (Helliot *et al*. 2002), *Carica papaya* (Kaity *et al*. 2008) and *Rubus* (Castillo *et al*. 2010) plants.

Modifications in DNA methylation have been argued to be the most likely explanation for genetic differences detected with restriction enzyme-based analysis or HPLC methods in cryopreserved materials (Johston *et al*. 2009, Castillo *et al*. 2010). As a reversible regulator of gene expression, DNA methylation may be a necessary adaptive response to enable plant tissues to tolerate high osmotic stress that is associated with cryopreservation (Johnston *et al*. 2009). Castillo *et al*. (2010) have recently shown that AFLP found in *Rubus* genotypes during subculture after cryostorage was no longer observed in field-grown plants. Thus, the transitory nature of these polymorphisms should be carefully considered when evaluating the feasibility of the cryopreservation protocol.

It is also important to note that molecular marker techniques screen only a small portion of a genome. For example, Harding (2004) has evaluated that molecular analysis with a mean number of 10 fragments and an average fragment size of 1000 bp results in 0.001% coverage of the potato genome (10^9 bp). Furthermore, detected variations can be located in non-coding regions and, thus, cannot affect phenotypic characteristics. Preferably, molecular markers should be used together with other approaches, such as morphological and cytological observations, to assay the genetic fidelity of the cryostored material, and, more importantly, the cryopreservation methods should be optimized to be least stressful as possible for plant tissues.

4.3 The role of endogenous haemoglobins of hybrid aspen

To date, the overall majority of plant endogenous Hb research has focused on herbaceous species although the localization of non-symbiotic Hbs in differentiating xylem cells has suggested that these proteins may function in wood formation (Ross *et al*. 2001). In the present thesis, the coding sequences of the *PttHb1* and *PttTrHb* genes of a model woody organism, hybrid aspen, were

characterized and their expressions were studied using both non-transgenic and VHb-expressing lines (III, V). By contrast, no evidence for the existence of the *Hb2* gene in *Populus* genome could be found (III), indicating that this Hb subgroup is more limited than originally proposed (Trevaskis *et al*. 1997).

The close connection between nitrogen-fixing bacteria and symbiotic Hbs has prompted studies concerning microsymbionts and the expression of non-symbiotic and truncated Hbs. The *Hb1*, *Hb2* as well as *TrHb* genes have been shown to express strongly in root nodules colonized by symbiotic bacteria (Uchiumi *et al*. 2002, Shimoda *et al*. 2005, Vieweg *et al*. 2005, Sasakura *et al*. 2006, Pawlowski *et al*. 2007, Bustos-Sanmamed *et al*. 2011), whereas the arbuscular mycorrhizal fungus *Glomus* sp. enhanced the expression of *MtTrHb2* in *Medicago truncatula* (Vieweg *et al*. 2005) but repressed the expression of the *LjNSG1* (*Hb1*) gene in *Lotus japonicus* (Uchiumi *et al*. 2002). ECM fungi differ from the previous endosymbionts because they do not penetrate inside the root cells. In order to examine whether *Hb* genes have a more general role in symbiotic associations, the expressions of *PttHb1* and *PttTrHb* were analysed from hybrid aspen roots inoculated with specific ECM fungi (III). Regardless of the emergence of symbiotic structures, i.e. a hyphal mantle and a Hartig net, the dual culture with the ECM fungus up-regulated both *PttHb1* and *PttTrHb* in the roots of non-transgenic hybrid aspens. Together, the results indicate that a wide range of symbionts generally enhancing plant growth affect the expression of *Hb* genes. *PttHb1* and *PttTrHb* had, however, their expression peaks at different points in time after inoculation, which suggests distinct functions for these genes.

Interestingly, the ECM fungi did not enhance the expression of the hybrid aspen *Hb* genes in the lines expressing VHb (III). Similarly, Wilhelmson *et al*. (2007) have found lower levels of endogenous Hb in the roots of greenhouse-grown VHb-expressing barley plants in contrast with their controls. As the *vhb* sequence shares very low similarity with *PttHb1* or *PttTrHb*, it is unlikely that the silencing of endogenous *Hb* genes by *vhb* mRNA would explain the observed phenomenon. On the other hand, VHb relieves NO stress when expressed in bacteria (Frey *et al*. 2002, Kaur *et al*. 2002), pancreatic cells (Frey *et al*. in press) or plants (Frey *et al*. 2004, Wang *et al*. 2009), and NO is known to induce adventitious root (Pagnussat *et al*. 2002) and lateral root primordia (Correa-Aragunde *et al*. 2006) formation. Moreover, the up-regulation of *Hb1* genes during the nodulation process is accompanied by NO production (Shimoda *et al*. 2005, Nagata *et al*. 2008), and the overexpression of *Hb1* genes reduces NO emission from root nodules (Shimoda *et al*. 2009). Therefore, it can be

hypothesized that PttHb1 and PttTrHb, separately or together, modulate the NO concentration during early root growth responses caused by ECM fungi and that VHb may substitute the function of endogenous Hbs (III, IV).

In the subsequent experiment, the NO-releasing compound SNP was, indeed, found to increase the relative mean expressions of both the *PttHb1* and *PttTrHb* genes, indicating the role of Hb proteins in NO metabolism (V). However, when the recombinant PttHb1 and PttTrHb were expressed alone as cytosolic or mitochondrially targeted variants in the yeast strain defective in NO resistance, no complementation of the mutant phenotype was observed. In accordance with these results, Smagghe *et al*. (2008) did not achieve complementation, either, when they expressed rice Hb1 in a flavohaemoglobin knockout line of *E. coli*. The gathered data indicate that Hb1 or truncated Hb alone is not able to detoxify NO efficiently *in vivo*.

Microbial flavohaemoglobins that catalyse oxygenation of NO contain a haem domain and a ferredoxin-$NADP^+$ reductase (FNR) -like domain that converts the globin into an NAD(P)H-oxidizing protein with reductase activities (Poole & Hughes 2000). Because there is no evidence of a flavin-binding domain in plant Hbs, an analogous system in plants would require Hb to react with another protein to maintain the turnover of NO oxygenation reaction via haem iron reduction (Igamberdiev & Hill 2004). The hybrid aspen heterotrophic *FNR* gene (*PtthFNR*) was identified as a candidate gene encoding metHb-reductase (V) based on the *A. thaliana FNR* sequences that had shown a similar expression pattern with *AHB1* in response to nitrate treatment (Wang *et al*. 2000). When *PtthFNR* mRNAs were analysed by *in situ* hybridization, the transcripts were found abundantly in the epidermis and vascular bundles, i.e. in the same tissues in which *Hb1* mRNAs (Parent *et al*. 2008, Bustos-Sanmamed *et al*. 2011) and Hb1 proteins (Ross *et al*. 2001, Hebelstrup *et al*. 2007) had previously been detected.

However, the same subcellular location is also a prerequisite for protein interaction. To date, plant Hb1 proteins have been localized mainly to cytosol (Hebelstrup *et al*. 2007), whereas hFNRs are considered to function only in plastids (Hanke *et al*. 2005). Proteins that are targeted to mitochondria and chloroplasts or plastids are mostly post-translationally translocated to these organelles by specific targeting sequences (Silva-Filho 2003). The analysis of the deduced amino acid sequence of PtthFNR and the 5' ends of PtthFNR transcripts resulted in the discovery of a novel *PtthFNR* mRNA (V). The absence of the plastid presequence in the characterized *PtthFNR* transcript indicates that the protein encoded by the mRNA is targeted into cytosol. The subsequent

coexpression of the recombinant PttHb1 and cytosolic PtthFNR was shown to alleviate the NO sensivity of the yeast strain lacking the native flavohaemoglobin (V). The data show, for the first time, that plant Hb1 with an applicable reductase scavenges NO *in vivo* with a rate that has physiological relevance. The transient expression of YFP fusion proteins verified the interaction between PttHb1 and PtthFNR also in plant cells. By contrast, PttTrHb coexpressed together with PtthFNR failed to rescue the mutant yeast cells during NO treatment, pointing to different functions for the PttHb1 and PttTrHb proteins.

At the moment, no cytosolic plant ferredoxins are known, but Wang *et al*. (2007) have identified a NADPH-dependent ferredoxin reductase of *Pseudomonas aeruginosa* that transfers electrons to heme oxygenase without the need of a ferredoxin. Thefore, the yet uncharacterized plant cytosolic ferredoxin or FNR that relies on another reductant than ferredoxin could explain the production of cytosolic *PtthFNR* transcript. The cytosolic *PtthFNR* mRNA appears to be rare compared with the plastid form (V), and thus, the amount of cytosolic PtthFNR protein can be expected to be low, as well. As it has been suggested that a protein could change its intracellular localization in response to external signals (Silva-Filho 2003), analyzing the proteome of roots treated with various nitrogen compounds may facilitate in confirming the existence of cytosolic PtthFNR protein in the future.

NO can react with sulfhydryl groups of proteins, leading to the formation of S-nitrosothiols and to changes in protein functions or activities (Grennan 2007). To visualize the possible course of NO detoxification, the computational models of hexa- and pentacoordinated tertiary structures of PttHb1 were constructed in the current thesis (V). Interestingly, the vicinity of basic and acidic residues and the location at the end of the hydrophobic channel (Lindermayr & Durner 2009) could promote S-nitrosylation of the conserved Cys78 in the liganded PttHb1. In accordance with this finding, Perazolli *et al*. (2004) have observed S-nitrosylation in *A*. *thaliana* AHb1. The authors postulated that AHb1 scavenges NO through production of S-nitrosohaemoglobin, but Igamberdiev *et al*. (2006) later showed that S-nitrosylation does not affect the NO turnover of the recombinant barley Hb1. As S-nitrosylation is rapidly reversible, it has been suggested to contribute to signal transduction (Grennan 2007). Therefore, instead of having a direct effect on the NO detoxification rate, the possible S-nitrosylation of Cys78 of PttHb1 could rather have significance in cell signalling. Whether Cys78 is involved in NO signalling or not will be the subject of forthcoming studies.

5 Conclusions and future prospects

This thesis and the original papers deal with endogenous Hbs and a bacterial Hb of *Vitreoscilla* sp. (VHb) in hybrid aspen. The thesis describes the production of VHb-expressing hybrid aspens and the effects of the heterologous expression on the phenotype of the genetically modified lines. Together with non-transgenic control hybrid aspens and yeast cells, the VHb lines were used to explore the role of hybrid aspen endogenous non-symbiotic and truncated Hbs, PttHb1 and PttTrHb, respectively. To store the produced hybrid aspen lines, the suitability of different cryopreservation methods was also tested.

VHb expression did not improve the general growth of hybrid aspens, but the volume of starch in the chloroplasts was higher in the VHb plants than in the controls, pointing to changes in energy metabolism. Under elevated UV-B illumination, two VHb lines had higher levels of certain flavonoids than the control lines, which may also reflect the availability of extra energy resources for secondary metabolite production. However, in order to exploit VHb expression in plant breeding in the future, it will be important to examine in detail the mode of action of the VHb protein at global genome, proteome and metabolome levels.

Of the studied cryopreservation protocols, the slow cooling of dormant *in vivo* buds proved to be the most feasible way of cryostoring multiple hybrid aspen lines, resulting in a regrowth rate as high as 72–96%. The plants that regenerated after cryopreservation had normal phenotypes, and no genetic changes that would have been caused by the cryopreservation treatments were observed. The adequacy of the method for several hybrid aspen lines also indicates the possible usage of the protocol for other plant species going through dormancy.

To study endogenous *Hb* genes of a woody model organism, two new coding sequences, hybrid aspen *PttHb1* and *PttTrHb*, were cloned and characterized. The dual culture with the ECM fungus, with or without the emergence of symbiotic structures, was shown to increase the expressions of both *PttHb1* and *PttTrHb* in the roots of non-transgenic hybrid aspens. The expression peaks of the *PttHb1* and *PttTrHb* genes emerged at different points in time during the dual culture, suggesting diverse functions for the genes. By contrast, the ECM fungi were not able to up-regulate the hybrid aspen *Hb* genes in the lines expressing the heterologous *vhb* gene. Therefore, it is hypothesized that endogenous Hbs may contribute to early root growth responses caused by specific ECM fungi and that VHb may compensate the function of endogenous Hbs. Currently, studies testing

this hypothesis by silencing the endogenous *Hb* genes in the VHb-expressing lines during ECM interaction are under way.

Previous reports have connected both plant non-symbiotic and truncated Hbs to the scavenging of NO. In the present thesis, NO treatment increased the relative mean expressions of both *PttHb1* and *PttTrHb*, suggesting that they play a role in NO metabolism. The results of the yeast complementation analyses showed, however, that PttHb1 and PttTrHb did not protect yeast cells efficiently against the toxicity of NO when expressed alone in the yeast strain defective in NO resistance. Subsequently, a novel mRNA transcript of *PtthFNR* containing no plastid presequence was found, which indicates the existence of cytosolic hFNR in plants. *PtthFNR* mRNAs were observed abundantly in the epidermis and vascular bundles, i.e. in the same tissues in which *Hb1* mRNAs and Hb1 proteins had previously been detected. The coexpression of PttHb1 and cytosolic PtthFNR partially complemented the NO resistance defect of the mutant yeast strain demonstrating, for the first time, that plant Hb1 with an applicable reductase scavenges NO with a physiologically relevant level *in vivo*. The transient expression of YFP fusion proteins confirmed the interaction between PttHb1 and PtthFNR in the plant cells. However, the presence of the cytosolic PtthFNR protein in hybrid aspen cells remains to be verified in forthcoming studies.

By contrast, PttTrHb coexpressed together with PtthFNR did not alleviate the NO sensitivity of the mutant yeast, suggesting different functions also for the PttHb1 and PttTrHb proteins. In the future, the crystallizing truncated Hb of a higher plant species and studying the global gene expression of *TrHb* RNAi or knockout lines will provide interesting tools for revealing the role of TrHbs in plants.

References

Accart F, Monod V, Poissonnier M, Dereuddre J & Paques M (1995) Cryoconservation d'apex de *Populus tremula* x *alba* cultives *in vitro* transformes genetiquement ou non. Annales de Recherches Sylvicoles 1993/1994: 23–39.

Anand A, Duk BT, Singh A, Akbas MY, Webster DA, Stark BC & Dikshit KL (2010) Redox-mediated interactions of VHb (*Vitreoscilla* haemoglobin) with OxyR: novel regulation of VHb biosynthesis under oxidative stress. Biochem J 426: 271–280.

Apel K & Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55: 373–399.

Appleby CA (1984) Leghemoglobin and *Rhizobium* respiration. Annu Rev Plant Physiol 35: 443–478.

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15.

Aronen T & Häggman H (1995) Differences in *Agrobacterium* infections in silver birch and Scots pine. Eur J For Path 25: 197–213.

Beligni MV & Lamattina L (2000) Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible reponses in plants. Planta 210: 215–221.

Benson EE (2008a) Cryopreservation of phytodiversity: a critical appraisal of theory and practice. Crit Rev Plant Sci 27: 141–219.

Benson EE (2008b) Cryopreservation theory. In: Reed BM (ed) Plant Cryopreservation. A practical guide. New York, Springer Science+Business Media: 15–32.

Bhalerao R, Nilsson O & Sandberg G (2003) Out of the woods: forest biotechnology enters the genomic era. Curr Opin Biotech 14: 206–213.

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Brosché M, Vinocur B, Alatalo ER, Lamminmäki A, Teichmann T, Ottow EA, Djilianov D, Afif D, Bogeat-Triboulot M-B, Altman A, Polle A, Dreyer E, Rudd S, Paulin L, Auvinen P & Kangasjärvi J (2005) Gene expression and metabolite profiling of *Populus euphratica* growing in the Negev desert. Genome Biol 6: R101.

Bustos-Sanmamed P, Tovar-Méndez A, Crespi M, Sato S, Tabata S & Becana M (2011) Regulation of nonsymbiotic and truncated hemoglobin genes of *Lotus japonicus* in plant organs and in response to nitric oxide and hormones. New Phytol 189: 765–776.

Castillo NRF, Bassil NV, Wada S & Reed BM (2010) Genetic stability of cryopreserved shoot tips of *Rubus* germplasm. In Vitro Cell Dev Biol–Plant 46: 246–256.

Chen D-C, Yang B-C & Kuo T-T (1992) One step transformation of yeast in stationary phase. Curr Genet 21: 83–84.

Chen R & Bailey JE (1994) Energetic effect of *Vitreoscilla* hemoglobin expression in *Escherichia coli*: an on-line ^{31}P NMR and saturation transfer study. Biotechnol Prog 10: 360–364.

Correa-Aragunde N, Graziano M, Chevalier C & Lamattina L (2006) Nitric oxide modulates the expression of cell cycle regulatory genes during lateral root formation in tomato. J Exp Bot 57: 581–588.

Delledonne M, Xia Y, Dixon RA & Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. Nature 394: 585–588.

Dordas C, Hasinoff BB, Igamberdiev AU, Manac'h N, Rivoal J & Hill RD (2003) Expression of a stress-induced hemoglobin affects NO levels produced by alfalfa root cultures under hypoxic stress. Plant J 35: 763–770.

Dordas C, Hasinoff BB, Rivoal J & Hill RD (2004) Class-1 hemoglobins, nitrate and NO levels in anoxic maize cell-suspension cultures. Planta 219: 66–72.

Doyle JJ & Doyle JL (1990) Isolation of plant DNA from fresh tissue. BRL Focus 12: 13–15.

Duplessis S, Courty P-E, Tagu D & Martin F (2005) Transcript patterns associated with ectomycorrhiza development in *Eucalyptus globulus* and *Pisolithus microcarpus*. New Phytol 165: 599–611.

Emanuelsson O, Nielsen H, Brunak S & von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol 300: 1005–1016.

Farrés J & Kallio PT (2002) Improved cell growth in tobacco suspension cultures expressing *Vitreoscilla* hemoglobin. Biotechnol Prog 18: 229–233.

Frey AD, Farrés J, Bollinger CJT & Kallio PT (2002) Bacterial hemoglobins and flavohemoglobins for alleviation of nitrosative stress in *Escherichia coli*. Appl Environ Microbiol 68: 4835–4840.

Frey AD & Kallio PT (2003) Bacterial hemoglobins and flavohemoglobins: versatile proteins and their impact on microbiology and biotechnology. FEMS Microbiol Rev 789: 1–21.

Frey AD, Oberle BT, Farrés J & Kallio PT (2004) Expression of *Vitreoscilla* haemoglobin in tobacco cell cultures relieves nitrosative stress *in vivo* and protects from NO *in vitro*. Plant Biotechnol J 2: 221–231.

Frey AD, Shepherd M, Jokipii-Lukkari S, Häggman H & Kallio PT (in press) The single-domain globin of *Vitreoscilla*: augmentation of aerobic metabolism for biotechnological applications. Adv Microb Physiol.

García-Mata C & Lamattina L (2001) Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. Plant Physiol 126: 1196–1204.

Godbout C & Fortin JA (1985) Synthesized ectomycorrhizae of aspen: fungal genus level of structural characterization. Can J Bot 63: 252–262.

Grennan AK (2007) Protein S-nitrosylation: potential targets and roles in signal transduction. Plant Physiol 144: 1237–1239.

Hanke GT, Okutani S, Satomi Y, Takao T, Suzuki A & Hase T (2005) Multiple iso-proteins of FNR in *Arabidopsis*: evidence for different contributions to chloroplast function and nitrogen assimilation. Plant Cell Environ 28: 1146–1157.

Harding K (2004) Genetic integrity of cryopreserved plant cells: a review. CryoLetters 25: 3–22.

Harding K (2010) Plant and algal cryopreservation: issues in genetic integrity, concepts in cryobionomics and current applications in cryobiology. AsPac J Mol Biol Biotechnol 18: 151–154.

He Y, Tang R-H, Hao Y, Stevens RD, Cook CW, Ahn SM, Jing L, Yang Z, Chen L, Guo F, Fiorani F, Jackson RB, Crawford NM & Pei Z-M (2004) Nitric oxide represses the *Arabidopsis* floral transition. Science 305: 1968–1971.

Hebelstrup KH, Hunt P, Dennis E, Bjerregaard Jensen S & Østergaard Jensen E (2006) Hemoglobin is essential for normal growth of Arabidopsis organs. Physiol Plantarum 127: 157–166.

Hebelstrup KH, Igamberdiev AU & Hill RD (2007) Metabolic effects of hemoglobin gene expression in plants. Gene 398: 86–93.

Heller G, Adomas A, Guosheng L, Osborne J, van Zyl L, Sederoff R, Finlay RD, Stenlid J & Asiegbu FO (2008) BMC Plant Biol 8: 19.

Helliot B, Madur D, Dirlewanger E & de Boucaud MT (2002) Evaluation of genetic stability in cryopreserved *Prunus*. In Vitro Cell Dev Biol–Plant 38: 493–500.

Holmberg N, Lilius G, Bailey JE & Bülow L (1997) Transgenic tobacco expressing *Vitreoscilla* hemoglobin exhibits enhanced growth and altered metabolite production. Nat Biotechnol 15: 244–247.

Hoy JA & Hargrove MS (2008) The structure and function of plant hemoglobins. Plant Physiol Bioch 46: 371–379.

Hoy JA, Robinson H, Trent III JT, Kakar S, Smagghe BJ & Hargrove MS (2007) Plant hemoglobins: a molecular fossil record for the evolution of oxygen transport. J Mol Biol 371: 168–179.

Hunt PW, Watts RA, Trevaskis B, Llewelyn DJ, Burnell J, Dennis ES, & Peacock WJ (2001) Expression and evolution of functionally distinct haemoglobin genes in plants. Plant Mol Biol 47: 677–692.

Igamberdiev AU, Bykova NV, Shah JK & Hill RD (2010) Anoxic nitric oxide cycling in plants: participating reactions and possible mechanisms. Physiol Plantarum 138: 393–404.

Igamberdiev AU & Hill RD (2004) Nitrate, NO and haemoglobin in plant adaptation to hypoxia: an alternative to classic fermentation pathways. J Exp Bot 55: 2473–2482.

Igamberdiev AU, Seregélyes C, Manac'h N & Hill RD (2004) NADH-dependent metabolism of nitric oxide in alfalfa root cultures expressing barley haemoglobin. Planta 219: 95–102.

Jaakola L, Pirttilä AM, Halonen M & Hohtola A (2001) Isolation of high quality RNA from bilberry (*Vaccinium myrtillus* L.) fruit. Mol Biotechnol 19: 201–203.

Jansson S & Douglas CJ (2007) *Populus*: a model system for plant biology. Annu Rev Plant Biol 58: 435–458.

Johansson T, Le Quéré A, Ahren D, Söderström B, Erlandsson R, Lundeberg J, Uhlén M & Tunlid A (2004) Transcriptional responses of *Paxillus involutus* and *Betula pendula* during formation of ectomycorrhizal root tissue. Mol Plant-Microbe Interact 17: 202–215.

Johnston JW, Benson EE & Harding K (2009) Cryopreservation induces temporal DNA methylation epigenetic changes and differential transcriptional activity in *Ribes* germplasm. Plant Physiol Bioch 47: 123–131.

Julkunen-Tiitto R & Sorsa S (2001) Testing the effects of drying methods on willow flavonoids, tannins, and salicylates. J Chem Ecol 27: 779–798.

Kaity A, Ashmore SE, Drew RA & Dulloo ME (2008) Assessment of genetic and epigenetic changes following cryopreservation in papaya. Plant Cell Rep 27: 1529–1539.

Kallio PT, Kim DJ, Tsai PS & Bailey JE (1994) Intracellular expression of *Vitreoscilla* hemoglobin alters *Escherichia coli* energy metabolism under oxygen-limited conditions. Eur J Biochem 219: 201–208.

Kaur R, Pathania R, Sharma V, Mande SC & Dikshit KL (2002) Chimeric *Vitreoscilla* hemoglobin (VHb) carrying a flavoreductase domain relieves nitrosative stress in *Escherichia coli*: new insight into the functional role of VHb. Appl Environ Microbiol 68: 152–160.

Lambardi M, Fabbri A & Caccavale A (2000) Cryopreservation of white poplar (*Populus alba* L.) by vitrification of in vitro-grown shoot tips. Plant Cell Rep 19: 213–218.

Laskowski RA (1995) SURFNET: a program for visualizing molecular surfaces, cavities, and intermolecular interactions. J Mol Graph 13: 323–330, 307–328.

Lecomte JTJ, Vuletich DA & Lesk AM (2005) Structural divergence and distant relationships in proteins: evolution of the globins. Curr Opin Struct Biol 15: 290–301.

Le Quéré A, Wright DP, Söderström B, Tunlid A & Johansson T (2005) Global patterns of gene regulation associated with the development of ectomycorrhiza between birch (*Betula pendula* Roth.) and *Paxillus involutus* (Batsch) Fr. Mol Plant-Microbe Interact 18: 659–673.

Lehtonen JV, Still D-J, Rantanen V-V, Ekholm J, Björklund D, Iftikhar Z, Huhtala M, Repo S, Jussila A, Jaakkola J, Pentikäinen O, Nyrönen T, Salminen T, Gyllenberg M & Johnson MS (2004) BODIL: a molecular modeling environment for structure-function analysis and drug design. J Comput Aided Mol Des 18: 401–419.

Li X, Peng R-H, Fan H-Q, Xiong A-S, Yao Q-H, Cheng Z-M & Li Y (2005) *Vitreoscilla* hemoglobin overexpression increases submergence tolerance in cabbage. Plant Cell Rep 23: 710–715.

Lindermayr C & Durner J (2009) S-nitrosylation in plants: pattern and function. J Proteomics 73: 1–9.

Lira-Ruan V, Sarath G, Klucas RV & Arredondo-Peter R (2001) Synthesis of hemoglobins in rice (*Oryza sativa* var. Jackson) plants growing in normal and stress conditions. Plant Sci 161: 279–287.

Liu YG, Liu LX, Wang L & Gao AY (2008) Determination of genetic stability in surviving apple shoots following cryopreservation by vitrification. Cryo Lett 29: 7–14.

Lodhi MA, Ye G-N, Weeden NF & Reisch BI (1994) A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. Plant Mol Biol Rep 12: 6–13.

Mann G (1906) Chemistry of the proteids. London, MacMillan and Co.

Mansfield SD & Weineisen H (2007) Wood fiber quality and kraft pulping efficiencies of Trembling Aspen (*Populus tremuloides* Michx) clones. J Wood Chem Technol 27: 135–151.

Martin F, Aerts A, Ahrén D, Brun A, Danchin EGJ, Duchaussoy F, Gibon J, Kohler A, Lindquist E, Pereda V, Salamov A, Shapiro HJ, Wuyts J, Blaudez D, Buée M, Brokstein P, Canbäck B, Cohen D, Courty PE, Coutinho PM, Delaruelle C, Detter JC, Deveau A, DiFazio S, Duplessis S, Fraissinet-Tachet L, Lucic E, Frey-Klett P, Fourrey C, Feussner I, Gay G, Grimwood J, Hoegger PJ, Jain P, Kilaru S, Labbé J, Lin YC, Legué V, Le Tacon F, Marmeisse R, Melayah D, Montanini B, Muratet M, Nehls U, Niculita-Hirzel H, Oudot-Le Secq MP, Peter M, Quesneville H, Rajashekar B, Reich M, Rouhier N, Schmutz J, Yin T, Chalot M, Henrissat B, Kües U, Lucas S, Van de Peer Y, Podila GK, Polle A, Pukkila PJ, Richardson PM, Rouzé P, Sanders IR, Stajich JE, Tunlid A, Tuskan G & Grigoriev IV (2008) The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. Nature 452: 88–92.

Martin F, Kohler A, Murat C, Balestrini R, Coutinho PM, Jaillon O, Montanini B, Morin E, Noel B, Percudani R, Porcel B, Rubini A, Amicucci A, Amselem J, Anthouard V, Arcioni S, Artiguenave F, Aury J-M, Ballario P, Bolchi A, Brenna A, Brun A, Buée M, Cantarel B, Chevalier G, Couloux A, Da Silva C, Denoeud F, Duplessis S, Ghignone S, Hilselberger B, Iotti M, Marçais B, Mello A, Miranda M, Pacioni G, Quesneville H, Riccioni C, Ruotolo R, Splivallo R, Stocchi V, Tisserant E, Viscomi AR, Zambonelli A, Zampieri E, Henrissat B, Lebrun M-H, Paolocci F, Bonfante P, Ottonello S & Wincker P (2010) Périgord black truffle genome uncovers evolutionary origins and mechanisms of symbiosis. Nature 464: 1033–1038.

Martin F & Nehls U (2009) Harnessing ectomycorrhizal genomics for ecological insights. Curr Opin Plant Biol 12: 508–515.

Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59: 153–163.

Modess O (1941) Zur Kentniss der Mycorrhizabildner von Kiefer und Fichte. Symbolae Botanicae Upsaliensis 5: 1–147.

Murashige T & Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plantarum 15: 473–497.

Müller J, Day JG, Harding K, Hepperle D, Lorenz M & Friedl T (2007) Assessing genetic stability of a range of terrestial microalgae after cryopreservation using amplified fragment length polymorphism (AFLP). Am J Bot 94: 799–808.

Nagata M, Murakami E, Shimoda Y, Shimoda-Sasakura F, Kucho K, Suzuki A, Abe M, Higashi S & Uchiumi T (2008) Expression of a class 1 hemoglobin gene and production of nitric oxide in response to symbiotic and pathogenic bacteria in *Lotus japonicus*. Mol Plant-Microbe Interact 21: 1175–1183.

Nanjo T, Futamura N, Nishiguchi M, Igasaki T, Shinozaki K & Shinohara K (2004) Characterization of full-length enriched expressed sequence tags of stress-treated poplar leaves. Plant Cell Physiol 45: 1738–1748.

Ohwaki Y, Kawagishi-Kobayashi M, Wakasa K, Fujihara S & Yoneyama T (2005) Induction of class-1 non-symbiotic hemoglobin genes by nitrate, nitrite and nitric oxide in cultured rice cells. Plant Cell Physiol 46: 324–331.

Pacher P, Beckman JS & Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87: 315–424.

Pagnussat GC, Simontacchi M, Puntarulo S & Lamattina L (2002) Nitric oxide is required for root organogenesis. Plant Physiol 129: 954–956.

Parent C, Berger A, Folzer H, Dat J, Crevècoeur M, Badot P-M & Capelli N (2008) A novel nonsymbiotic hemoglobin from oak: cellular and tissue specificity of gene expression. New Phytol 177: 142–154.

Park K-W, Kim K-J, Howard AJ, Stark BC & Webster DA (2002) *Vitreoscilla* hemoglobin binds to subunit I of cytochrome *bo* ubiquinol oxidases. J Biol Chem 277: 33334–33337.

Pawlowski K, Jacobsen KR, Alloisio N, Denison RF, Klein M, Tjepkema JD, Winzer T, Sirrenberg A, Guan C & Berry AM (2007) Truncated hemoglobins in actinorhizal nodules of *Datisca glomerata*. Plant Biol 9: 776–785.

Perazzolli M, Dominici P, Romero-Puertas MC, Zago E, Zeier J, Sonoda M, Lamb C & Delledonne M (2004) Arabidopsis nonsymbiotic hemoglobin AHb1 modulates nitric oxide bioactivity. Plant Cell 16: 2785–2794.

Podila GK, Sreedasyam A & Muratet MA (2009) *Populus* rhizosphere and the ectomycorrhizal interactome. Crit Rev Plant Sci 28: 359–367.

Poole RK & Hughes MN (2000) New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. Mol Microbiol 36: 775–783.

Qu Z-L, Wang H-Y & Xia G-X (2005) *GhHb1*: A nonsymbiotic hemoglobin gene of cotton responsive to infection by *Verticillium dahliae*. Biochim Biophys Acta 1730: 103–113.

Qu Z-L, Zhong N-Q, Wang H-Y, Chen A-P, Jian G-L & Xia G-X (2006) Ecotopic expression of the cotton nonsymbiotic hemoglobin gene *GhHb1* triggers defence responses and increases disease tolerance in Arabidopsis. Plant Cell Physiol 47: 1058–1068.

R Development Core Team (2010) R: A language and environment for statistical computing. Vienna, R Foundation for Statistical Computing.

Ranua J (1996) Why is the Finnish forest industry interested in Aspen? Sorbifolia 27:178–180.

Roos V, Andersson CIJ & Bülow L (2004) Gene expression profiling of *Escherichia coli* expressing double *Vitreoscilla* haemoglobin. J Biotechnol 114: 107–120.

Ross EJH, Shearman L, Mathiesen M, Zhou YJ, Arredondo-Peter R, Sarath G & Klucas RV (2001) Nonsymbiotic hemoglobins in rice are synthesized during germination and in differentiating cell types. Protoplasma 218: 125–133.

Ross EJH, Stone JM, Elowsky CG, Arredondo-Peter R, Klucas RV & Sarath G (2004) Activation of the *Oryza sativa* non-symbiotic haemoglobin-2 promoter by the cytokinin-regulated transcription factor, ARR1. J Exp Bot 55: 1721–1731.

Ruohonen L, Aristidou A, Frey AD, Penttilä M & Kallio PT (2006) Expression of *Vitreoscilla* hemoglobin improves the metabolism of xylose in recombinant yeast *Saccharomyces cerevisiae* under low oxygen conditions. Enzyme Microb Technol 39: 6–14.

Ryynänen L (1996a) Cold hardening and slow cooling: tools for successful cryopreservation and recovery of in vitro shoot tips of silver birch. Can J For Res 26: 2015–2022.

Ryynänen L (1996b) Survival and regeneration of dormant silver birch buds stored at super-low temperatures. Can J For Res 26: 617–623.

Sakai A (1960) Survival of the twig of woody plants at -196º C. Nature 185: 393–394.

Sakai A, Kobayashi S & Oiyama I (1990) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. Plant Cell Rep 9: 30–33.

Sakai A & Sugawara Y (1973) Survival of poplar callus at super-low temperatures after cold acclimation. Plant Cell Physiol 14: 1201–1204.

Sakamoto A, Sakurao S, Fukunaga K, Matsubara T, Ueda-Hashimoto M, Tsukamoto S, Takahashi M & Morikawa H (2004) Three distinct *Arabidopsis* hemoglobins exhibit peroxidase-like activity and differentially mediate nitrite-dependent protein nitration. FEBS Lett 572: 27–32.

Sali A & Blundell TL (1993) Comparative protein modelling by satisfaction of spatial restraints. J Mol Biol 234: 779–815.

Sasakura F, Uchiumi T, Shimoda Y, Suzuki A, Takenouchi K, Higashi S & Abe M (2006) A class 1 hemoglobin gene from *Alnus firma* functions in symbiotic and nonsymbiotic tissues to detoxify nitric oxide. Mol Plant-Microbe Interact 19: 441–450

Sevola Y (2001) Metsävarat. In: Peltola A (ed) Finnish Statistical Yearbook of Forestry. Vammala, Vammalan Kirjapaino Oy: 31–74.

Shimoda Y, Nagata M, Suzuki A, Abe M, Sato S, Kato T, Tabata S, Higashi S & Uchiumi T (2005) Symbiotic Rhizobium and nitric oxide induce gene expression of non-symbiotic hemoglobin in *Lotus japonicus*. Plant Cell Physiol 46: 99–107.

Shimoda Y, Shimoda-Sasakura F, Kucho K, Kanamori N, Nagata M, Suzuki A, Abe M, Higashi S & Uchiumi T (2009) Overexpression of class 1 plant hemoglobin genes enhances symbiotic nitrogen fixation activity between *Mesorhizobium loti* and *Lotus japonicus*. Plant J 57: 254–263.

Silva-Filho MC (2003) One ticket for multiple destinations: dual targeting of proteins to distinct subcellular locations. Curr Opin Plant Biol 6: 589–595.

Smagghe BJ, Hoy JA, Percifield R, Kundu S, Hargrove MS, Sarath G, Hilbert J-L, Watts RA, Dennis ES, Peacock WJ, Dewilde S, Moens L, Blouin GC, Olson JS & Appleby CA (2009) Correlations between oxygen affinity and sequence classifications of plant hemoglobins. Biopolymers 91: 1083–1096.

Smagghe BJ, Trent JT III & Hargrove MS (2008) NO dioxygenase activity in hemoglobins is ubiquitous *in vitro*, but limited by reduction *in vivo*. PLoS ONE 3: e2039.

Smith SE & Read DJ (2008) Mycorrhizal symbiosis. London, Academic Press.

Son SH, Park YG, Chun YW & Hall RB (1997) Germplasm preservation of *Populus* through *in vitro* culture systems. In: Klopfenstein NB, Chun YW, Kim M-S & Ahuja MR (eds) Micropropagation, genetic engineering, and molecular biology of *Populus*. General Technical Report RM-GTR-297. Fort Collins, Department of Agriculture, Forest Service, Rocky Mountain Research Station: 44–49.

Sowa AW, Duff SMG, Guy PA & Hill RD (1998) Altering hemoglobin levels changes energy status in maize cells under hypoxia. Proc Natl Acad Sci USA 95: 10317–10321.

Stamler JS, Toone EJ, Lipton SA & Sucher NJ (1997) (S)NO signals: translocation, regulation, and a consensus motif. Neuron 18: 691–696.

Sterky F, Regan S, Karlsson J, Hertzberg M, Rohde A, Holmberg A, Amini B, Bhalerao R, Larsson M, Villarroel R, Van Montagu M, Sandberg G, Olsson O, Teeri TT, Boerjan W, Gustafsson P, Uhlén M, Sundberg B & Lundeberg J (1998) Gene discovery in the wood-forming tissues of poplar: analysis of 5,692 expressed sequence tags. Proc Natl Acad Sci USA 95: 13330–13335.

Strohl WR (2005) Genus XII. *Vitreoscilla* Pringsheim 1949c, 70^{AL}. In: Garrity GM (ed) Bergey's manual of systematic bacteriology. New York, Springer: 851–858.

Tarricone C, Calogero S, Galizzi A, Coda A, Ascenzi P & Bolognesi M (1997) Expression, purification, crystallization, and preliminary X-ray diffraction analysis of the homodimeric bacterial hemoglobin from *Vitreoscilla stercoraria*. Protein Struct Funct Genet 27: 154–156.

Taylor G (2002) *Populus*: Arabidopsis for forestry. Do we need a model tree? Ann Bot 90: 681–689.

The Royal Society (2009) Reaping the benefits: science and the sustainable intensification of global agriculture. London, The Royal Society.

Trevaskis B, Watts RA, Andersson CR, Llewellyn DJ, Hargrove MS, Olson JS, Dennis ES & Peacock WJ (1997) Two haemoglobin genes in *Arabidopsis thaliana*: The evolutionary origins of leghemoglobins. Proc Natl Acad Sci USA 94: 12230–12234.

Tsai C-J & Hubscher SL (2004) Cryopreservation in *Populus* functional genomics. New Phytol 164: 73–81.

Tsai PS, Nägeli M & Bailey JE (1996) Intracellular expression of *Vitreoscilla* hemoglobin modifies microaerobic *Escherichia coli* metabolism through elevated concentration and specific activity of cytochrome *o*. Biotechnol Bioeng 49: 151–160.

Tsai PS, Rao G & Bailey JE (1995a) Improvement of *Escherichia coli* microaerobic oxygen metabolism by *Vitreoscilla* hemoglobin: new insights from NAD(P)H fluorescence and culture redox potential. Biotechnol Bioeng 47: 347–354.

Tsai PS, Kallio PT & Bailey JE (1995b) Fnr, a global transcriptional regulator of *Escherichia coli*, activates the *Vitreoscilla* hemoglobin (VHb) promoter and intracellular VHb expression increases cytochrome *d* promoter activity. Biotechnol Prog 11: 288–293.

Tsvetkov I, Benelli C, Capuana M, De Carlo A & Lambardi M (2009) Application of vitrification-derived cryotechniques for long-term storage of poplar and aspen (*Populus* spp.) germplasm. Agr Food Sci 18: 160–166.

Tuskan GA, DiFazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A, Schein J, Sterck L, Aerts A, Bhalerao RR, Bhalerao RP, Blaudez D, Boerjan W, Brun A, Brunner A, Busov V, Campbell M, Carlson J, Chalot M, Chapman J, Chen G-L, Cooper D, Coutinho PM, Couturier J, Covert S, Cronk Q, Cunningham R, Davis J, Degroeve S, Déjardin A, dePamphilis C, Detter J, Dirks B, Dubchak I, Duplessis S, Ehlting J, Ellis B, Gendler K, Goodstein D, Gribskov M, Grimwood J, Groover A, Gunter L, Hamberger B, Heinze B, Helariutta Y, Henrissat B, Holligan D, Holt R, Huang W, Islam-Faridi N, Jones S, Jones-Rhoades M, Jorgensen R, Joshi C, Kangasjärvi J, Karlsson J, Kelleher C, Kirkpatrick R, Kirst M, Kohler A, Kalluri U, Larimer F, Leebens-Mack J, Leplé J-C, Locascio P, Lou Y, Lucas S, Martin F, Montanini B, Napoli C, Nelson DR, Nelson C, Nieminen K, Nilsson O, Pereda V, Peter G, Philippe R, Pilate G, Poliakov A, Razumovskaya J, Richardson P, Rinaldi C, Ritland K, Rouzé P, Ryaboy D, Schmutz J, Schrader J, Segerman B, Shin H, Siddiqui A, Sterky F, Terry A, Tsai C-J, Uberbacher E, Unneberg P, Vahala J, Wall K, Wessler S, Yang G, Yin T, Douglas C, Marra M, Sandberg G, Van de Peer Y & Rokhsar D (2006) The genome of Black Cottonwood *Populus trichocarpa* (Torr. & Gray). Science 313: 1596–1604.

Uchiumi T, Shimoda Y, Tsuruta T, Mukoyoshi Y, Suzuki A, Senoo K, Sato S, Kato T, Tabata S, Higashi S & Abe M (2002) Expression of symbiotic and nonsymbiotic globin genes responding to microsymbionts on *Lotus japonicus*. Plant Cell Physiol 43: 1351–1358.

Valjakka M, Aronen T, Kangasjärvi J, Vapaavuori E & Häggman H (2000) Genetic transformation of silver birch (*Betula pendula*) by particle bombardment. Tree Physiol 20: 607–613.

Vieweg MF, Hohnjec N & Küster H (2005) Two genes encoding different truncated hemoglobins are regulated during root nodule and arbuscular mycorrhiza symbioses of *Medicago truncatula*. Planta 220: 757–766.

Vinogradov SN, Hoogewijs D, Bailly X, Arredondo-Peter R, Gough J, Dewilde S, Moens L & Vanfleteren JR (2006) A phylogenomic profile of globins. BMC Evol Biol 6: 31.

Wakabayashi S, Matsubara H & Webster DA (1986) Primary sequence of a dimeric bacterial haemoglobin from *Vitreoscilla*. Nature 322: 481–483.

Wang A, Zeng Y, Han H, Weeratunga S, Morgan BN, Moënne-Loccoz P, Schönbrunn E & Rivera M (2007) Biochemical and structural characterization of *Pseudomonas aeruginosa* Bfd and FPR: ferredoxin $NADP^+$ reductase and not ferredoxin is the redox partner of heme oxygenase under iron-starvation conditions. Biochemistry 46: 12198–12211.

Wang QC, Panis B, Engelmann F, Lambardi M & Valkonen JPT (2009) Cryotherapy of shoot tips: a technique for pathogen eradication to produce healthy planting materials and prepare healthy plant genetic resources for cryopreservation. Ann Appl Biol 154: 351–363.

Wang R, Guegler K, LaBrie ST & Crawford NM (2000) Genomic analysis of a nutrient response in Arabidopsis reveals diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate. Plant Cell 12: 1491–1509.

Wang Y-H, Kochian LV, Doyle JJ & Garvin DF (2003) Two tomato non-symbiotic haemoglobin genes are differentially expressed in response to diverse changes in mineral nutrient status. Plant Cell Environ 26: 673–680.

Wang Z, Xiao Y, Chen W, Tang K & Zhang L (2009) Functional expression of *Vitreoscilla* hemoglobin (VHb) in *Arabidopsis* relieves submergence, nitrosative, photo-oxidative stress and enhances antioxidants metabolism. Plant Science 176: 66–77.

Watts RA, Hunt PW, Hvitved AN, Hargrove MS, Peacock WJ & Dennis ES (2001) A hemoglobin from plants homologous to truncated hemoglobins of microorganisms. Proc Natl Acad Sci USA 98: 10119–10124.

Webster DA & Hackett DP (1966) The purification and properties of cytochrome *o* from *Vitreoscilla*. J Biol Chem 241: 3308–3315.

Wilhelmson A, Kallio PT, Oksman-Caldentey K-M & Nuutila AM (2007) Heterologous expression of *Vitreoscilla* haemoglobin in barley (*Hordeum vulgare*). Plant Cell Rep 26: 1773–1783.

Worrell R (1995) European aspen (*Populus tremula* L.): a review with particular reference to Scotland I. Distribution, ecology and genetic variation. Forestry 68: 231–244.

Wu G, Wainwright LM & Poole RK (2003) Microbial globins. Adv Microb Physiol 47: 255–308.

Yu Q (2001) Selection and propagation of hybrid aspen clones for growth and fibre quality. PhD thesis. Univ Helsinki, Dept Appl Biol.

Zelasco S, Reggi S, Calligari P, Balestrazzi A, Bongiorni C, Quattrini E, Delia G, Bisoffi S, Fogher C & Confalonieri M (2006) Expression of the *Vitreoscilla* hemoglobin (VHb)-encoding gene in transgenic white poplar: plant growth and biomass production, biochemical characterization and cell survival under submergence, oxidative and nitrosative stress conditions. Mol Breeding 17: 201–216.

Zhang L, Li Y, Wang Z, Xia Y, Chen W & Tang K (2007) Recent developments and future prospects of *Vitreoscilla* hemoglobin application in metabolic engineering. Biotechnol Adv 25: 123–136.

Zhao L, Gu R, Gao P & Wang G (2008) A nonsymbiotic hemoglobin gene from maize, *ZmHb*, is involved in response to submergence, high-salt and osmotic stresses. Plant Cell Tiss Organ Cult 95: 227–237.

Original publications

I Häggman H, Frey AD, Ryynänen L, Aronen T, Julkunen-Tiitto R, Tiimonen H, Pihakaski-Maunsbach K, Jokipii S, Chen X & Kallio PT (2003) Expression of *Vitreoscilla* haemoglobin in hybrid aspen (*Populus tremula* x *tremuloides*). Plant Biotechnology Journal 1: 287–300.

II Jokipii S, Ryynänen L, Kallio PT, Aronen T & Häggman H (2004) A cryopreservation method maintaining the genetic fidelity of a model forest tree, *Populus tremula* L. x *Populus tremuloides* Michx. Plant Science 166: 799–806.

III Jokipii S, Häggman H, Brader G, Kallio PT & Niemi K (2008) Endogenous *PttHb1* and *PttTrHb*, and heterologous *Vitreoscilla vhb* haemoglobin gene expression in hybrid aspen roots with ectomycorrhizal interaction. Journal of Experimental Botany 59: 2449–2459.

IV Jokipii-Lukkari S, Frey AD, Kallio PT & Häggman H (2009) Intrinsic non-symbiotic and truncated haemoglobins and heterologous *Vitreoscilla* haemoglobin expression in plants. Journal of Experimental Botany 60: 409–422.

V Jokipii-Lukkari S, Kastaniotis AJ, Nymalm Y, Sundström R, Blokhina O, Kukkola E, Fagerstedt KV, Salminen TA, Läärä E, Ohlmeier S, Hiltunen JK, Kallio PT & Häggman H (2011) Coexpression of hybrid aspen haemoglobin PttHb1 and heterotrophic ferredoxin $NADP^+$ oxidoreductase PtthFNR alleviates nitric oxide sensivity *in vivo*. Manuscript.

Original publications are not included in the electronic version of the dissertation.

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