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REVIEW PAPER

Intrinsic non-symbiotic and truncated haemoglobins and heterologous *Vitreoscilla* haemoglobin expression in plants

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Abstract

To date, haemoglobins (Hbs) have been shown to exist in all kingdoms of life. The least studied and understood groups are plant non-symbiotic haemoglobins (nsHbs) and the recently found plant truncated Hbs (trHbs). From a biotechnological point of view, the best characterized and almost exclusively applied Hb is the bacterial *Vitreoscilla* haemoglobin (VHb). In this review, the present state of knowledge of structural features and ligand binding kinetics of plant nsHbs and trHbs and their proposed roles as oxygen carriers, oxygen sensors, and for oxygen storage, in nitric oxide (NO) detoxification, and in peroxidase activity are described. Furthermore, in order to predict the functioning of plant Hbs, their characteristics will be compared with those of the better known bacterial globins. In this context, the effects of heterologous applications of VHb on plants are reviewed. Finally, the challenging future of plant Hb research is discussed.

Key words: Bacterial globins, non-symbiotic, plant haemoglobin, truncated, *Vitreoscilla* haemoglobin.

Introduction

At present, ubiquitous haemoglobins (Hbs) have been shown to exist in all kingdoms of life (Vinogradov *et al.*, 2006). Putative globins, also including the group of globin-containing sensor proteins, have been identified in 426 bacterial, 32 Archaeal, and 67 eukaryote genomes (Vinogradov *et al.*, 2007). However, ''true'' globins have been characterized only in 264 bacterial, eight Archaeal and 54 eukaryote genomes (Vinogradov *et al.*, 2007). The bacterial globins are generally divided into three types of Hb proteins: truncated haemoglobins (trHbs), haemoglobins (Hbs), and flavohaemoglobins (flavoHbs). The best characterized and almost exclusively applied Hb in microbe and plant biotechnology is *Vitreoscilla* haemoglobin (VHb) (Frey and Kallio, 2003, 2005; Kallio *et al.*, 2008).

In plants, upon binding of CO and O_2, haemoproteins with absorption spectra typical of Hbs had already been discovered in soybean root nodules by Kubo in 1939. These globins are today termed as symbiotic or leghaemoglobins (sHbs), and their role in facilitating oxygen diffusion to nitrogen-fixing bacteria in the nodules of plants capable of symbiotic nitrogen fixation is also well described (Appleby, 1984; Appleby *et al.*, 1988). Plant non-symbiotic haemoglobins (nsHbs) were first found in the roots of the tropical trees *Parasponia andersonii* and *Trema tomentosa* (Bogusz *et al.*, 1988). Since these findings, expression of nsHbs has been reported in several plant species, especially in crop plants, as recently reviewed by Hoy and Hargrove (2008). The most recently found and still poorly characterized group of plant Hbs are trHbs. Plant *TrHb* genes were first discovered by Watts *et al.* (2001), and even today the number of trHbs characterized in plants is low and their role remains obscure.

The aim of this review is to introduce the present state of knowledge of nsHbs and trHbs in plants. In order to predict the functioning of plant Hbs, their characteristics are compared with those of the better known bacterial globins. In this context, the effects of heterologous applications of

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Abbreviations: ADH, alcohol dehydrogenase; Hb, haemoglobin; HR, hypersensitive response; NO, nitric oxide; NOD, nitric oxide dioxygenase; NOS, nitric oxide synthase; nsHb, non-symbiotic haemoglobin; RNS, reactive nitrogen species; sHb, symbiotic haemoglobin; trHb, truncated haemoglobin.

VHb in plants are reviewed. Finally, the future challenges of plant Hb research are also discussed.

Structural comparison of plant non-symbiotic and truncated haemoglobins and their bacterial counterparts

Plant non-symbiotic haemoglobins

In plants, the nsHbs were originally divided into class-1 (Hb1) and class-2 (Hb2) subgroups (Trevaskis *et al.*, 1997; Hunt *et al.*, 2001), the differentiation of which is based on phylogenetic analyses, gene expression patterns, and oxygen-binding properties of the encoded proteins. The first crystal structure of monocot class-1 nsHb was that of rice (riceHb1) (Hargrove *et al.*, 2000). The crystal structure of riceHb1 displays a typical hexacoordination, with the distal histidine linked to the haem iron, a bend in the E-helix, absence of the D-helix, disorder in the CD-region and a conserved phenylalanine (Phe-B10) in the B-helix that interacts with the distal histidine (Hargrove *et al.*, 2000; Smagghe *et al.*, 2006). However, there are also species-dependent differences within the monocots. For instance, the tertiary stucutures of riceHb1 and barley Hb (barHb1) are different (Bykova *et al.*, 2006; Hoy and Hargrove, 2008). In corn, the computational modelling predicted a structure similar to riceHb1 (Saenz-Rivera *et al.*, 2004), but cornHb1 has more interhelical bonds than riceHb1, which suggests reduced flexibility of corn Hb1 and putative kinetic differences in ligand binding (Smagghe *et al.*, 2006).

The structures of nsHbs in dicots are not well known. In tomato, nsHb1 reveals hexacoordination in the ferric form and a mixture of pentacoordination and hexacoordination in the ferrous form (Ioanitescu *et al.*, 2005). In *Arabidopsis*, AtHb1 (class-1 Hb, also referred to as AtGLB1 or AHb1 in the literature) is present at a 40% fraction in a pentacoordinated state and the distal haem cavity is suggested to be connected to the exterior by a relatively open channel (Bruno *et al.*, 2007). The structure and functioning of class-2 nsHbs have been more precisely described only in the case of *Arabidopsis* AtHb2 (also referred to as AtGLB2 or AHb2) (Trevaskis *et al.*, 1997; Bruno *et al.*, 2007). In AtHb2, unlike in AtHb1, protein dynamics have a major role in the ligand migration from the distal cavity to the solvent (Bruno *et al.*, 2007).

Bacterial haemoglobins and flavohaemoglobins versus plant non-symbiotic haemoglobins

In bacteria, the Hbs and flavoHbs exhibit a classical 3-on-3 α-helical globin fold, and share a high degree of sequence homology and structural similarity in their globin domain, the size of which is ~150 residues. FlavoHbs, which have not been identified in plants, are chimeric proteins (~400 residues). In addition to the N-terminal oxygen-binding Hb domain, they possess a flavin-carrying reductase domain of the ferredoxin-$NADP^+$ reductase family, which is able to transfer electrons from NADPH via FAD to the haem group (Karplus *et al.*, 1991; Frey and Kallio, 2003, 2005; Gardner, 2005).

VHb is a homodimeric pentacoordinate Hb, adopting the classical Hb fold, where the haem is embedded in a hydrophobic crevice formed by helices B, C, E, F, G, and H (Tarricone *et al.*, 1997). No protein–haem interactions are detected at the distal site. The proximal site residues His-F8, Tyr-G5, and Glu-H23 form a hydrogen bond network which modulates the redox properties of the haem in a similar fashion to that observed for cytochrome *c* peroxidase (Goodin and McRee, 1993; Mukai *et al.*, 2001). These residues are also present in flavoHbs (Ermler *et al.*, 1995; Ilari *et al.*, 2002), which possess a well established function in nitric oxide (NO) detoxification. This suggests a role different from oxygen binding and storage of bacterial globin proteins. Notably, Lys-F7, which has been implicated in the transfer of electrons from FAD to the haem iron in flavoHbs, is present in VHb but absent from other bacterial Hb proteins (Ermler *et al.*, 1995; Tarricone *et al.*, 1997; Ilari *et al.*, 2002).

Similar to bacterial Hbs and flavoHbs, plant nsHbs also exhibit a classical 3-on-3 α-helical globin fold. In contrast to their pentacoordinated bacterial homologues and plant symbiotic Hbs, plant nsHbs are mostly hexacoordinated, as also observed for neuroglobin in humans (Trent *et al.*, 2001) and cyanoglobin in cyanobacteria (Hvitved *et al.*, 2001).

Sequence comparison of bacterial globins, represented by VHb and *Escherichia coli* flavoHb (Hmp), and of plant nsHb reveals that the residues Tyr-G5 and Glu-H23 implicated in the activation of the haem-bound ligand are missing (Fig. 1). Three similarly interesting observations have been made. First, Tyr-B10, which has been suggested to aid in the stabilization of the haem ligand in bacterial globin proteins, is only present in trHb and symbiotic leghaemoglobin but exchanged for phenylalanine in plant nsHbs (Smagghe *et al.*, 2006). Secondly, the distal His-E7 present in hexacoordinate Hbs is replaced by glutamine in bacterial globins. The replacement of Tyr-B10 by phenylalanine strongly increased the O_2 dissociation constant of the Hmp of *E. coli* (Gardner *et al.*, 2000). Thirdly, Lys-F7, which is involved in the transfer of electrons from the FADH present in the reductase domain of flavoHb or from a non-linked reductase in the case of VHb, is also potentially missing. This might indicate that the mechanism for reducing the oxidized haem iron to the ferrous form involves a protein which is not structurally related to ferredoxin-$NADP^+$ type reductase (Karplus *et al.*, 1991). The importance of the reductase domain for detoxification of NO has been nicely demonstrated for VHb and very recently also for hexacoordinated Hbs (Frey *et al.*, 2002; Smagghe *et al.*, 2008).

Truncated haemoglobins in plants and bacteria

TrHb is a rather new class of small myoglobin-like (2-on-2) proteins, which are widely distributed and can be identified in bacteria, unicellular eukaryotes, and plants. In bacteria, their primary structure is usually ~20–40 residues shorter

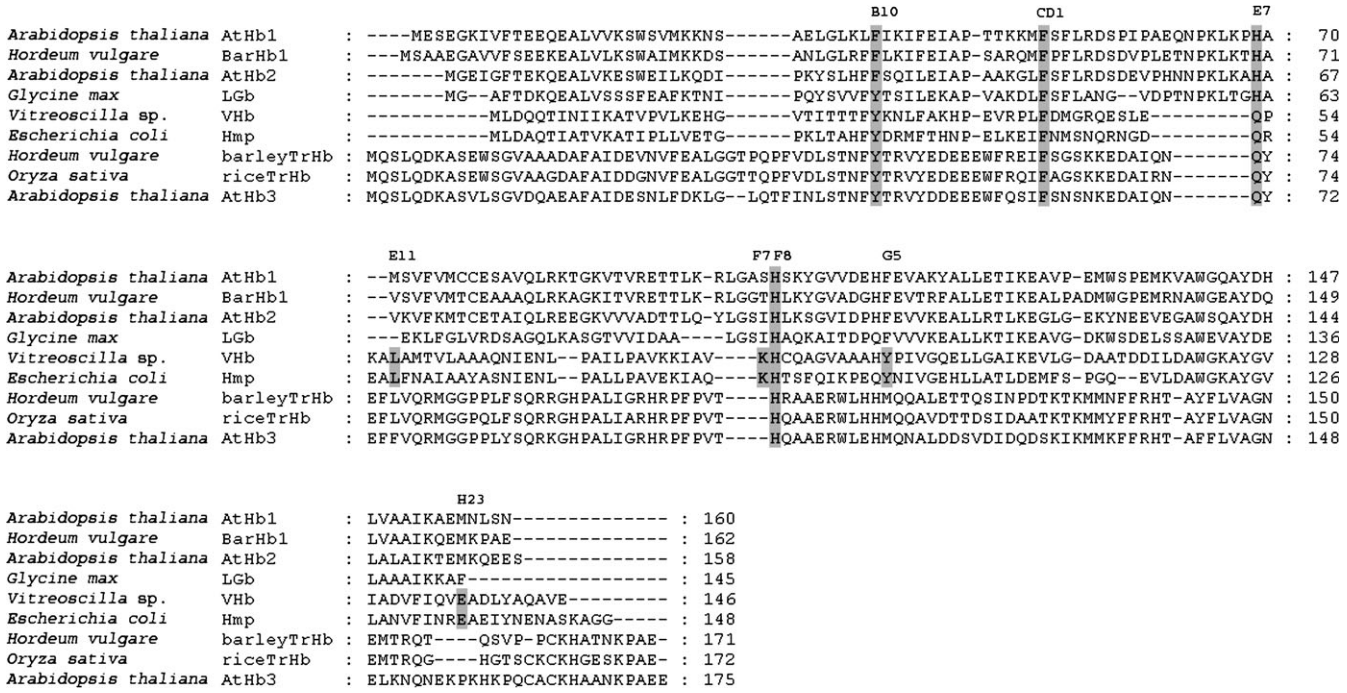

Fig. 1. The deduced amino acid sequences of specific plant symbiotic, non-symbiotic, and truncated Hbs aligned with the *Vitreoscilla* Hb (VHb) and *Escherichia coli* flavoHb (Hmp) globin domain. The topological positions for key residues are shown with upper case letters above the alignment.

than the classical (non-)vertebrate Hb proteins and myoglobin, but the length can vary significantly, as seen in mycobacteria (121–215 residues) (Ascenzi *et al.*, 2007). In contrast, the identified plant *TrHb* genes are longer than genes encoding 3-on-3 Hbs (Vinogradov *et al.*, 2006; Jokipii *et al.*, 2008). This new globin family can be divided, based on sequence clustering, into three groups in bacteria: I (trHbN), II (trHbO), and III (trHbP) (Wittenberg *et al.*, 2002; Vinogradov *et al.*, 2007). The crystallographic analyses have shown that all three trHb groups adopt a 2-on-2 α-helical fold in which helices B, E, G, and H surround the haem (Nardini *et al.*, 2007). The phylogenetic analysis conducted by Vuletich and Lecomte (2006) suggests that trHbO is the ancestral gene and groups I and III are the result of various replacement events. Structural analyses of plant trHbs are still scarce. The group I trHb of *Chlamydomonas eugametos* (CeTrHb) has an apolar tunnel that connects the protein surface to the distal haem pocket with xenon-binding sites and modulation of carbonmonoxy derivatives along the tunnel, which indicate putative ligand binding in the tunnel (Samuni *et al.*, 2003; Milani *et al.*, 2004*a*, *b*; Hoy and Hargrove, 2008). In *Arabidopsis*, the group II trHb (AtTrHb and also called AtGLB3 or AHb3) adopts a pentacoordinate state with a transient hexacoordinate state (Watts *et al.*, 2001).

Ligand binding kinetics

Similar to other globin proteins, Hbs can bind a number of different ligands, including O_2, CO, NO, and CN. Ligand binding kinetics that cover examples of plant Hbs, bacterial Hbs, and flavoHbs are summarized in Table 1. Intriguingly, bacterial flavoHbs possess a high autoxidation rate under aerobic conditions, with a rapid consumption of NADH (Farrés *et al.*, 2005). Despite their high sequence similarity, the ligand-binding properties of flavoHbs and Hbs differ considerably, but the ligand-binding constants of a specific globin are independent of the presence of a reductase (Farrés *et al.*, 2007). O_2 and CO affinity constants and rates of NO binding to the ferric form of bacterial Hbs are roughly one order of magnitude higher than the respective values from flavoHbs and plant nsHbs, resembling more the oxygen affinities of sHbs. The observed biphasic behaviour for ligand binding of bacterial pentacoordinate globins can be attributed to the displacement of a lipid ligand from the haem pocket (Bonamore *et al.*, 2003). NsHbs possess similar k_{on} values for O_2 relative to bacterial globins but generally lower k_{off}, yielding higher affinity constants (Table 1). NO binding kinetics for ferrous plant nsHbs have not been determined; k_{on} of ferric riceHb is five orders of magnitude lower than the corresponding values for Hbs and flavoHbs (Gardner *et al.*, 2000; Farrés *et al.*, 2005, 2007; Smagghe *et al.*, 2008).

Diverse functions of plant non-symbiotic and truncated haemoglobins compared with bacterial haemoglobins and flavoglobins

Physiological functions of plant non-symbiotic haemoglobins

Since their discovery, several important biochemical and physiological roles have been suggested for plant Hbs,

Table 1. Kinetic parameters for ligand binding of plant haemoglobins in comparison with their bacterial homologues

	k_{on} (μM^{-1} s^{-1}) O_2	k_{off} (s^{-1}) O_2	k_{on} (μM^{-1} s^{-1}) CO_2	k_{off} (s^{-1}) CO_2	k_{on} (μM^{-1} s^{-1}) NO, ferrous	k_{off} (s^{-1}) NO, ferrous	k_{on} (μM^{-1} s^{-1}) NO, ferric	k_{off} (s^{-1}) NO, ferric	Reference
Myoglobin	13	11	0.5	15	21	1.1	0.044	ND	Farrés *et al.* (2005)
				Symbiotic					
Soybean leghaemoglobin	120	5.6	13	0.0078	170	2×10^{-5}	0.14*	3*	Gibson *et al.* (1989), Hargrove *et al.* (1997), *Herold and Puppo (2005)
				Non-symbiotic					
Class 1 (Hb1)									
Arabidopsis AtHb1	75	0.12	0.55	0.0012					Trevaskis *et al.* (1997)
Tomato	30	0.5	1	0.02					Ioanitescu *et al.* (2005)
Rice	68	0.038	7.2	0.001			0.0035*		Goodman and Hargrove (2001), *Smagghe *et al.* (2008)
Barley	9.5	0.027	0.57	0.0011					Duff *et al.* (1997)
Class 2 (Hb2)									
Arabidopsis AtHb2	86	0.14	22	0.0013					Trevaskis *et al.* (1997)
Truncated (Hb3)									
Arabidopsis	0.2	0.3	0.014	0.001					Watts *et al.* (2001)
				Bacterial globins					
VHb	200* (0.15)	4.2*	ND	29			263		Farrés *et al.* (2007), *Giangiacomo *et al.* (2001)
E. coli HMP	38	0.44	22	5.7	26	2	44	4000	Gardner *et al.* (2000)
B. subtilis trHB	14	0.0021	0.22	0.00046					Giangiacomo *et al.* (2005)
M. tuberculosis trHbO	0.11	0.0014							Ouellet *et al.* (2003)

As prototype for an oxygen-binding protein, sperm whale myoglobin has been included.

including functioning as oxygen carriers, in oxygen storage, and as oxygen sensors, ligand transport, scavenging, sensing, detoxification, and electron transfer (Arredondo-Peter *et al.*, 1998). The recent silencing and knockout studies on *Arabidopsis thaliana* have emphasized the vital role of nsHbs during plant development by showing that at least one functional *nsHb* gene is essential for survival of young seedlings (Hebelstrup *et al.*, 2006).

To date, *nsHb1* genes have been reported to be up-regulated by hypoxic, osmotic, and high salt stresses (Trevaskis *et al.*, 1997; Lira-Ruan *et al.*, 2001; Zhao *et al.*, 2008), treatments with nitrate, nitrite, NO, salicylic acid, methyl jasmonic acid, ethylene, and H_2O_2 (Wang *et al.*, 2000; Sakamoto *et al.*, 2004; Ohwaki *et al.*, 2005; Sasakura *et al.*, 2006; Qu *et al.*, 2006), deficiency of phosphorus, potassium, and iron (Wang *et al.*, 2003), as well as dual culture with specific microsymbionts (Shimoda *et al.*, 2005; Jokipii *et al.*, 2008), whereas *nsHb2*s are known to be inducible by cold (Trevaskis *et al.*, 1997) or cytokinins (Hunt *et al.*, 2001). The characterization of *Oryza sativa* transcription factors regulating the expression of *nsHb* genes has indicated that cytokinin-induced activation of *OsNSHb2* may be mediated by ARR1 (Arapidopsis Response Regulator 1) containing a Myb-like DNA-binding domain (Ross *et al.*, 2004), while *nsHb1* expression in tissues under hypoxic stress may depend on an incomplete transcription factor, Mybleu, unable to bind DNA (Mattana *et al.*, 2007). Despite differential regulation, recent studies suggest that the functions of nsHb1s and nsHb2s also overlap (Ross *et al.*, 2004; Hebelstrup *et al.*, 2006). Moreover, recent database searches suggest that nsHb2 might be limited to specific plant species or genera (Jokipii *et al.*, 2008).

Carriage, storage, or sensing oxygen

Historically, bacterial Hb proteins were considered to function as oxygen scavenging and delivering proteins, but their exact physiological function is still not known. After finding Hbs from non-nodulating plants (Landsmann *et al.*, 1986), comparisons with globins of other organisms led to a hypothesis that plant nsHbs could possibly also function as general oxygen carriers, oxygen sensors, and for oxygen storage. However, nsHbs, especially Hb1s, have unusually high affinity for oxygen and they release oxygen slowly (Table 1), which are characteristics that later excluded these speculations on the function of nsHbs in oxygen delivery (Hill, 1998).

Peroxidase activity

Recently, VHb has been shown to possess peroxidase activity *in vitro*, reaching similar activity to horseradish peroxidase for 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) and ferrocene carboxylic acid, but acting poorly on phenolic compounds such as dopamine (Kvist *et al.*, 2007). Interestingly, *E. coli* Hmp has alkylhydroperoxide reductase activity.

Since the X-ray structures revealed the presence of lipids in the neighbourhood of the active site, there is speculation that this activity could help in the repair of oxidized membranes (Bonamore *et al.*, 2003). The *in vivo* significance of this activity is not yet known. In plants, peroxidase activity has so far been shown in all three *Arabidopsis* Hbs, AtHb1, AtHb2, and AtHB3, representing nsHbs of class-1 and class-2 as well as trHb, respectively (Sakamoto *et al.*, 2004). All three purified AtHb proteins, resolved by native PAGE, co-localized with peroxidase activity, but the signal generated by AtHb3 was considerably weaker relative to that of AtHb1 or AtHb2. In the activity staining, the oxidation of the chromogenic reagent was dependent on the presence of both H_2O_2 and the recombinant protein, and it was inhibited by KCN, thus revealing intrinsic peroxidase-like activity (Sakamoto *et al.*, 2004).

NO detoxification

One of the biochemical properties suggested for VHb and more generally for bacterial globins as well as for plant Hb1 proteins is the neutralization of reactive nitrogen species (RNS) (Frey *et al.*, 2002; Ouellet *et al.*, 2002; Frey and Kallio, 2003, 2005; Igamberdiev and Hill, 2004; Gardner, 2005). FlavoHbs detoxify NO into nitrate under aerobic conditions and under consumption of NADH in a catalytic reaction called the nitric oxide dioxygenase (NOD) reaction (Gardner *et al.*, 2000). Under physiological conditions with a limiting O_2 concentration, flavoHb-bound NO reacts with O_2 in a reaction termed the O_2 denitrosylase reaction, which is consistent with a peroxidase-like function (Hausladen *et al.*, 2001). In contrast to cells, cell-free extracts containing VHb were not able to degrade NO. This suggests that *in vivo* an endogenous reductase system has the capability to reduce the ferric VHb to the biochemically active ferrous form. However, upon preparation of cell-free extracts this reducing activity is lost (Frey *et al.*, 2002). The enzymatic NOD activities of *E. coli* Hmp and *Ralstonia eutropha* flavoHb (FHP) are 94 s^{-1} (at 20 °C) and 7.4 s^{-1} (at 20 °C), respectively. It has been shown that a group I trHb in *Mycobacterium bovis* possesses efficient NO neutralizing activity and that *M. tuberculosis* oxygenated trHb is also able to oxidize NO into nitrate in stoichiometric amounts with a second-order rate constant of 745μM^{-1} s^{-1} (at 23°C) (Ouellet *et al.*, 2002; Pathania *et al.*, 2002).

The interplay between NO and Hb1s has usually been studied by treatments of plants with exogenous NO donors, e.g. sodium nitroprusside, or using NO-generating growth conditions. One of these conditions is hypoxic stress, which is known to increase the NO emission of plant cells (Dordas *et al.*, 2003). This discovery has prompted intensive research on the possible functions of nsHbs under low oxygen tension. Sowa and co-workers (1998) were the first to observe that maize cells overexpressing barley Hb1 maintained ATP levels under hypoxic conditions more effectively than wild-type and Hb-negative cells. The NO concentration of the heterologous maize cells was later shown to have an inverse relationship with the level of Hb expression (Dordas *et al.*, 2004). Moreover, partially purified recombinant alfalfa Hb1 was found to exhibit NO degradation activity and to rely on the use of NAD(P)H as a cofactor *in vitro*, but to lose this activity after the size exclusion chromatography step, indicating that Hb1 alone is not able to scavenge NO (Seregélyes *et al.*, 2004). Based on the information gathered, Igamberdiev and Hill (2004) postulated that NO is produced during hypoxia through nitrate reduction in order to maintain the energy status of the cells. According to the authors, barley Hb1 prevents the accumulation of NO by acting as a dioxygenase, and turnover of this reaction is maintained by a methaemoglobin (metHb) reductase. Indeed, Igamberdiev *et al.* (2006) have recently identified a monodehydroascorbate reductase that is able to facilitate NO-scavenging activity of barley metHb in the presence of ascorbate and NADH. Furthermore, Smagghe *et al.* (2008) have recently compared, with various methods, NO reactions of rice, human (neuroglobin and cytoglobin), and *Synechocystis* haemoglobin with those of horse heart myoglobin. *In vitro*, all hexacoordinated oxygenated Hbs were able to destroy NO rapidly at a rate equal to the re-reduction of the ferric Hb. However, only *Synechocystis* Hb was able to replace the NO detoxification capability of *E. coli* flavoHb *in vivo*. Thus, Smagghe *et al.* (2008) suggested that future Hb research could be focused on the identification of possible reductases co-operating with Hbs in their natural environments. In early studies on VHb purifiction from its native host, a metHb reductase was co-purified (Webster and Liu, 1974; Gonzales-Prevatt and Webster, 1980). However, the elucidation of its function has not been pursued in the light of NO chemistry. Additionally, an alternative way for modulation of the NO level by Hbs has also been demonstrated by Perazzolli *et al.* (2004). They showed that *A. thaliana* AtHb1 protein scavanged NO through formation of *S*-nitrosohaemoglobin under hypoxic stress.

NO, a double-edged sword

NO has emerged as a key signalling molecule based on its chemical properties. NO can freely penetrate the lipid bilayer and hence be transported within the cell. NO is quickly produced on demand via inducible enzymatic or non-enzymatic routes. Due to its free radical nature (one unpaired electron), NO has a short half-life (in the order of seconds) and can be removed easily when no longer needed (Lamattina *et al.*, 2003; Neill *et al.*, 2003). In plants, NO has been shown to be involved in multiple processes, such as the expression of defence genes, programmed cell death, stomatal closure, seed germination, and senescense (reviewed by Neill *et al.*, 2003).

NO is a double-edged sword for plants: on the one hand, it is toxic to cells but, on the other hand, together with ROS, it contributes to triggering a hypersensitive response (HR) to prevent the propagation of biotrophic pathogens (Delledonne *et al.*, 1998). Despite the clear effects of Hb1 proteins on the abundance of NO under oxygen-limited conditions, studies with Hb1-overexpressing or -underexpressing lines treated with pathogens have resulted in controversial

findings. Seregélyes and co-workers (2003) showed that transgenic tobacco plants overexpressing an alfalfa *Mhb1* gene had reduced necrosis after pathogen infection, with either *Pseudomonas syringae* or tobacco necrosis virus. Additionally, Qu *et al.* (2006) reported enhanced resistance against *P. syringae* in *A. thaliana* plants that were genetically modified with *GhHb1* of cotton. On the other hand, Perazzolli *et al.* (2004) did not observe any changes in NO accumulation or HR in AtHb1-overexpressing *Arabidopsis* plants inoculated with *P. syringae* relative to wild-type plants. In order to defend themselves against invading pathogens, plants have to have mechanisms for recognizing the advantageous and the harmful NO. In mammals, this differentiation is based on NO concentration. Whereas constitutively expressed nitric oxide synthase (NOS) produces NO in response to physiological stimuli, the inducible form of NOS produces NO as a defence molecule in high amounts (Coleman, 2001). How this discrimination between these two functions is achieved in plant systems remains currently largely unknown.

Functioning of truncated haemoglobins in plants

Because trHbs have only recently been discovered in higher plants (Watts *et al.*, 2001), the number of studies analysing their functional roles is very limited and no body of information similar to that for some bacterial globins is yet available for trHbs. The expression of plant *TrHb* genes has been shown to remain uninduced under hypoxia or upon treatment with phytohormones (Watts *et al.*, 2001), but Vieweg and co-workers (2005) observed the up-regulation of certain *TrHb* genes of *Medicago truncatula* Gaertn. during symbiotic association in root nodules and in roots colonized by arbuscular mycorrhizal fungi. Moreover, Pawlowski *et al.* (2007) showed that expression of the *TrHb* gene of *Datisca glomerata* is induced prior to the onset of bacterial nitrogen fixation in actinorhizal nodules. It was found later that the up-regulation of *TrHb* genes is not restricted to endosymbionts but it was observed that the ectomycorrhizal fungi *Leccinum populinum* and *Xerocomus subtomentosus*, with or without emergence of symbiotic structures, also increased the expression of both *PttHb1* (encoding class-1 nsHb) and *PttTrHb* (encoding trHb) genes in *Populus tremula*×*tremuloides* (Jokipii *et al.*, 2008). *PttHb1* and *PttTrHb* had expression peaks 5 h and 2 d after inoculation, respectively, which suggests different functions for these genes during interaction with ectomycorrhizal fungi.

Biological background and biotechnological applications of VHb in plants

One of the main concerns in microbial biotechnology was, and still is, to find an efficient and cheap way to deliver oxygen to aerobic microorganisms during large-scale production processes. Historically, due to its extraordinarily high k_{off} rate for oxygen release and its inducibility by O_2 deprivation, VHb was considered for a long period of time an oxygen-binding and delivering protein (Orii and Webster, 1986). The synthesis of VHb seemed an obvious natural genetic strategy to combat oxygen limitation and improve phenotypic properties of *Vitreoscilla*. Therefore, it was hypothesized that heterologous expression of VHb could be used to alleviate oxygen limitation, and enhance oxygen delivery and the performance of industrially important bioprocesses. Motivated by this hypothesis, the VHb was first expressed in *E. coli* and the results showed that VHb enabled cells to reach higher cell densities under poorly oxygenated conditions (Khosla and Bailey, 1988). These successful experiments paved the way for future applications, and VHb has been expressed in numerous biotechnologically relevant microorganisms, and also later in eukaryotic cells and plants. In all these cases, positive effects on either cellular physiology and metabolism, cell growth, or production of industrially important compounds have been reported (Kallio *et al.*, 2001, 2008; Frey and Kallio, 2003, 2005). Re-examination of the kinetic constants for O_2 binding and release revealed a k_{off} for O_2 which is similar to that of other bacterial Hb proteins (Giangiacommo *et al.*, 2001; Farrés *et al.*, 2007). However, experiments directly revealing the biochemical function of VHb under these microaerobic conditions are still missing.

In bacteria, the expression of VHb has usually been obtained using the original *vhb* promoter (Khosla and Bailey, 1988; Frey and Kallio, 2003, 2005; Kallio *et al.*, 2008). The *vhb* promoter is not induced by nitrosative or oxidative stress, although the VHb is able to neutralize NO in *E. coli* (Frey *et al.*, 2003). However, expression of *vhb* is induced by oxygen deprivation. Its function has been thoroughly studied under microaerobic conditions, where it has been shown to change the gene expression pattern, increase the carbon flux through glycolysis, and enhance respiratory activity, leading to improved ATP synthesis and lowering the steady-state $NAD(P)^+$/NADPH ratio in *E. coli* (Kallio *et al.*, 1994; Tsai *et al.*, 1995, 1996; Frey *et al.*, 2001, 2007) Although these effects were formerly attributed to the oxygen delivering role, it is now believed that VHb enhances respiratory activity by shielding NO-sensitive cytochromes (Kaur *et al.*, 2002). In contrast to the expression of VHb in *E. coli*, the native *hmp* gene is induced by nitrosative stress conditions and its function is clearly implicated in protection from nitrosative stress (Poole *et al.*, 1996; Anjum *et al.*, 1998; Cruz-Ramos *et al.*, 2002).

It has also been hypothesized that the biochemical properties of VHb are not always optimal for foreign host cells. Therefore, VHb mutants showing highly improved growth properties in *E. coli* were generated using error-prone PCR (Andersson *et al.*, 2000; Kallio *et al.*, 2001). Unfortunately, the consequences on the kinetc properties for O_2 and NO binding have not been elucidated. In addition, the expression of an engineered VHb fusion protein, 'FlavoVHb', carrying the C-terminal reductase domain of the *R. eutropha hmp* gene, was able to enhance growth and improve energetic characters of *E. coli* significantly (Frey *et al.*, 2000, 2001). In the case of the chimeric VHb reductase fusion, the improvements in growth under

microaerobic conditions correlate with the potentiating of its NO detoxification activity in comparison with VHb (Frey *et al.*, 2002) The overwhelming genomic information has also allowed the cloning of novel bacterial Hb genes, yielding *E. coli* strains with improved phenotypical characters upon their expression (Bollinger *et al.*, 2001; Kallio *et al.*, 2007). This clearly shows that there is room for new developments, and other Hbs, natural or engineered, should also be tested for their efficacy in prokaryotic and eukaryotic cells and in plants.

Heterologous expression of Vitreoscilla *haemoglobin in plants*

As stated above, VHb and its engineered derivatives have been expressed in various heterologous hosts and have been shown to improve growth, cellular metabolism, and productivity of interesting products, e.g. antibiotics and amino acids in microorganisms, as summarized in Frey and Kallio (2003, 2005) and Kallio *et al.* (2008).

Due to the numerous successful achievements obtained in microorganisms, it is not surprising that Hb technology has also been tested in plants, and VHb has been the prime choice to be expressed *in planta* (Table 2). The pioneering work of Holmberg *et al.* (1997) with VHb revealed important phenotypic and metabolic changes, e.g. faster germination, enhanced growth, and changes in activities of metabolic pathways in *Nicotiana tabacum*, attributing the effects to increased intracellular oxygen levels. The follow-up study conducted using tobacco plant cell cultures also confirmed that VHb expression could be used to enhance growth and to generate more plant material relative to

Table 2. Effects of *Vitreoscilla* haemoglobin (VHb) expression in plants or during symbiosis

Plant species	VHb effects	Reference
Arabidopsis thaliana	Improved germination rate and increased tolerance against submergence, nitrosative, and photo-oxidative stresses. Up-regulation of endogenous genes involved in oxygen metabolism and antioxidant biosynthesis. Quantitative differences in secondary metabolites	Wang *et al.* (2008)
Brassica oleracea var. *Cabitata*	Faster germination of F_1 seeds, not uniform growth enhancement effect observed, improved tolerance to a prolonged submergence	Li *et al.* (2005)
Hordeum vulgare	Negligible alcohol dehydrogenase activity in the *vhb*-expressing roots, no improvement in the germination rate of barley kernels or plant growth, slight negative effect on root growth, showing shorter and/or fewer roots. In addition, the ratio of total root length to stem length was smaller	Wilhelmson *et al.* (2007)
Hyoscyamus muticus	On average 18% higher dry weight of hairy root cultures, no significant increase in hyoscyamine production, changes in alkaloid profile	Wilhelmson *et al.* (2005, 2006)
Nicotiana tabaccum SR1	Faster germination of tobacco F_1 seeds, enhanced growth, improved production of dry weight (on average 80–100%), 30–40% higher chlorophyll content, and 34% more nicotine, and altered distribution of secondary metabolites (anabasine)	Holmberg *et al.* (1997)
N. tabaccum SR1	No lag-phase in the growth of suspension cultures, ~20% higher final dry weight value	Farrés and Kallio (2002)
N. tabaccum SR1	No significant changes in growth and other phenotypical characteristics of suspension cultures were observed, improved growth under nitrosative stress, protection of NO-sensitive enzymes (40–80% higher acotinase activity), no protection under oxidative stress	Frey *et al.* (2004)
Ozyza sativa L. (cultivars Xiushui-11, Quifeng, Youfeng and Hanfeng)	Statistically significant increases were obtained in rice plant height, panicle length, and the total amount of grains per panicle and filled grains per panicle	Cao *et al.* (2004)
Populus alba L.	One out of six poplar lines showed improved growth and stem biomass and enhanced root biomass production. No differences in chlorophyll, total carotenoid. and protein content. No differences under submergence, or oxidative and nitrosative stresses.	Zelasco *et al.* (2006)
Petunia hybrida Vilm	Improved growth in hydroponic culture, enhanced survival rate, and improved growth in hypoxic conditions, better tolerance to water-logging	Mao *et al.* (2003)
Potato	Higher tolerance against low oxygen stress	Zhou *et al.* (2004)
P. tremula×*tremuloides*	No general growth improvement, no changes in chlorophyll content, ~75% and 30% higher starch volume in chloroplasts and enhanced relative volume of mitochondria, respectively, and changes in secondary metabolite production under UV-B illumination	Häggman *et al.* (2003)
Symbiosis		
Phaseolus vulgaris cv Negro Jamapa/*Rhizobium etli* (VHb^+)	Bean plants exhibited 68% higher nitrogenase content and 14–53% enhanced total nitrogen content, 10–20% higher foliage dry weight, earlier flowering when inoculated with VHb-expressing *R. etli*	Ramirez *et al.* (1999)
P. tremula×*tremuloides* (VHb^+)/*L. populinum* and *X. subtomentosus*	Ectomycorrhizal inoculation increased the expression of endogenous *PttHb1* and *PttTrHb* genes in the roots of wild-type hybrid aspens while the up-regulation was not observed in VHb-expressing hybrid aspen lines	Jokipii *et al.* (2008)

controls (Farrés and Kallio, 2002). Frey *et al.* (2004) were not able to repeat the experimental results of Holmberg *et al.* (1997) in tobacco, discerning no effect of VHb expression on growth. However, they were able to show that suspension cultures derived from VHb-expressing plants were more resistant against nitrosative stress. Furthermore, an enzyme assay for the NO-sensitive tricarboxylic acid cycle enzyme aconitase revealed that VHb was able to protect the NO-labile Fe–S cluster of aconitase against RNS inactivation (Frey *et al.*, 2004).

VHb has also been expressed in rice (Cao *et al.*, 2004), potato (Zhou *et al.*, 2004), and cabbage (Li *et al.*, 2005). In cabbage, VHb-positive seeds exhibited a faster early germination rate than the wild-type controls, and the transgenic plants also showed improved tolerance to prolonged submergence treatment (Li *et al.*, 2005). In rice, *vhb* was co-transformed and co-expressed with the *Agrobacterium tumefaciens tzs* gene encoding zeatin-type cytokinins and the mutated *aroA* gene from *Salmonella typhimurium* that renders recombinant plants resistant to glyphosate treatment (Cao *et al.*, 2004). When the rice plants expressed both *vhb* and *tzs* genes, the plants were able to display a significant increase in plant height and panicle length relative to the non-transformed controls. Zhou *et al.* (2004) transformed potato plants by using expression vectors that carry a *cry3A–vhb* expression cassette. The *cry3A* gene encodes the coleopteran-specific endotoxin of *Bacillus thuringiensis* (Donovan *et al.*, 1988). Interestingly, the water logging tests demonstrated that *cry3A–vhb*-positive potato plants were able to exhibit higher tolerance against low oxygen stress (Zhou *et al.*, 2004). However, the studies on *vhb* gene expression alone without co-transformation with herbicide and cytokinin genes or herbivore resistance genes might have improved the possibilities to evaluate VHb effects in rice and potato, respectively.

In VHb-expressing hybrid aspen (*P. tremula*× *tremuloides*), no general growth-promoting effects were obtained (Häggman *et al.*, 2003). VHb-expressing lines had higher relative volumes of mitochondria and showed significantly enhanced starch accumulation in chloroplasts. Under elevated UV-B illumination, some specific VHb lines had elevated levels of total flavonoids, and individual quercetin, kaempferol, and myricetin derivatives. In the experiments conducted using VHb-producing *Populus alba* L. lines, Zelasco *et al.* (2006) could reveal positive effects on growth in only one out of six transgenic lines. In contrast to the findings of Frey *et al.* (2004), no positive effects of VHb on the growth of suspension cultures under nitrosative stress conditions relative to wild-type controls were detected. VHb has also been expressed in barley and in a medicinal plant, *Hyoscyamus muticus* (Egyptian henbane), that produces a commercially important tropane alkaloid scopolamine (see Table 2 for references and a summary of key findings).

While VHb expression may have erratic effects on plant growth and tolerance against various stresses, the studies evaluating the global gene expression and secondary metabolism of genetically modified lines could lead to a deeper understanding of functioning of heterologous VHb protein. Wang and co-workers (2008) have recently studied in detail VHb-expressing *A. thaliana* lines that exhibited an improved germination rate and tolerance against waterlogging, as well as nitrosative and photo-oxidative stresses. It was revealed that VHb expression significantly increased the abundance of vitamin C and phytosterol in leaf samples. When the expression of *Arabidopsis* endogenous genes involved in oxygen metabolism and antioxidant biosynthesis was analysed, the genes encoding glutathione reductase, glutathione synthase, ascorbate peroxidase, GDP-mannose pyrophosphorylase, and GDP-mannose showed up-regulation in comparison with control plants.

The improved starch production and enhanced growth could indicate that VHb-expressing lines have an improved energy household (Holmberg *et al.*, 1997; Farrés and Kallio, 2002; Häggman *et al.*, 2003). Such positive changes in energy status have also been reported when barley Hb (barHb) has been expressed constitutively in maize cells under limited oxygen availability. Under such conditions, the ATP levels were not affected in plants overexpressing barHb but were reduced by 27% in wild-type and 61% in Hb-negative cells, respectively (Sowa *et al.*, 1998). Therefore, it has been hypothesized that nsHbs are needed in order to maintain the energy status, possibly by enhancing the substrate-level phosphorylation and concomitant NAD(P)H oxidation under diminished oxygen concentrations. This assumption is also supported by the findings of Igamberdiev *et al.* (2004), who reported lower NAD(P)H/NAD(P)$^+$ ratios in alfalfa root cultures expressing barHb. The VHb-positive barley plants did not react to oxygen deficiency by increasing the alcohol dehydrogenase (ADH) activity in the roots relative to the control plants (Wilhelmson *et al.*, 2007). Such an observation could mirror the ability of VHb to substitute ADH for recycling of NADH and to maintain glycolysis, and would require a high autoxidation rate of the globin protein and an efficient reductase system. Indeed, VHb and other bacterial globin proteins display a high autooxidation rate (Farrés *et al.*, 2005, 2007). However, the endogenous plant reductase system capable of reducing ferric VHb remains elusive. Overall, these results parallel the early findings of the effects of VHb expression on microaerobic metabolism in *E. coli* (Kallio *et al.*, 1994; Tsai *et al.*, 1995).

Although, heterologous expression of VHb has been shown to generate important metabolic effects, it is still the only bacterial Hb which has been expressed in plants. Therefore, it is surprising that neither engineered nor new Hbs, showing improved properties in bacteria, have been tested in plants yet.

Interplay betgween endogenous haemoglobins and VHb

Due to the similarities between the suggested roles of VHb and plant endogenous Hbs in the NO reactions (Dordas *et al.*, 2003, 2004; Frey *et al.*, 2004), the expression of the genes has also been examined simultaneously during root growth (Wilhelmson *et al.*, 2007; Jokipii *et al.*, 2008), the

developmental stage that is known to be regulated by NO (Pagnussat *et al.*, 2002; Correa-Aragunde *et al.*, 2004). The western analyses in barley indicated that the abundance of Hb1 was slightly lower in normoxic roots of VHb-expressing lines than in control plants, but the difference was not observed in anoxic root tissues (Wilhelmson *et al.*, 2007). In hybrid aspen, the expression of endogenous *PttHb1* and *PttTrHb* genes was studied by real-time RT-PCR in the roots of control and VHb-expressing plants that were cultivated with and without root growth-increasing ectomycorrhizal fungi (Jokipii *et al.*, 2008). The results indicated that *PttHb1* and *PttTrHb* genes were up-regulated in the roots of non-transgenic control lines during the dual culture with ectomycorrhizal fungi. Surprisingly, the fungi were not able to enhance the expression of endogenous *Hb* genes in the VHb lines, which suggests that endogenous Hbs may relate to early growth responses caused by fungi and that VHb may compensate the function of endogenous Hbs (Jokipii *et al.*, 2008).

Future challenges of plant haemoglobin research

The fascinating world of haemoproteins covers all kingdoms of life and interests of researchers in different disciplines, from medicine to plant sciences. Research on plant hexacoordinated nsHbs, and especially trHbs, is still in its infancy, although a lot of information has been accumulated during recent years. The new sequencing techniques will foster the sequencing of plant genomes and widen the repertoire of Hb sequences from the present model plants (such as *A. thaliana*, *M. truncatula*, *O. sativa, Zea mays*, and *Hordeum vulgare*) to more genera and species. This will certainly provide new information about the structure and biochemical properties of Hbs but will also verify the validity of the present grouping into class-1 and class-2 nsHbs. Such a clarification would be important because recent database searches already suggest that class-2 Hbs might be limited to specific plant species or genera (Jokipii *et al.*, 2008). Additionally, it will be interesting to know if corresponding groups of trHbs that have been encountered in bacteria can also be identified in plants.

The hexacoordinated Hbs are, generally, known for their high cross-species sequence identity, indicating proteins with important physiological roles. However, although there are several hypotheses on their roles, their physiological functions still remain obscure. Thus, more biochemical, biophysical, structural, and protein-level research is needed to enable their physiological importance to be understood. For instance, the recent silencing and knockout studies on *A. thaliana* have emphasized the important role of nsHbs during plant development (Hebelstrup *et al.*, 2006), but their physiological role is still elusive.

So far, very few scientific data have been gathered from plant trHbs and, for instance, structural and other physiological features have to be characterized thoroughly. Although there is convincing evidence that plant class-1 nsHbs are able to detoxify NO, some essential questions about their functioning and properties still need to be resolved. In plant cells, NO has been localized to mitochondria, cytosol, peroxisomes, and apoplasts (Blokhina and Fagerstedt, 2006), whereas nsHb1 proteins are apparently distributed throughout the nucleus and cytosol (Seregélyes *et al.*, 2000; Hebelstrup *et al.*, 2008) but are absent from mitochondria and peroxisomes (Hebelstrup *et al.*, 2007). Inevitably, this raises a question about the role of nuclear Hb1, which in alfalfa cells represents the majority of the Hb1 proteins (Seregélyes *et al.*, 2000). Moreover, it has recently been reported that NO dioxygenase activity in hexacoordinated haemoglobins is ubiquitous *in vitro* but limited by reduction *in vivo*. This clearly indicates that cognate reductases for each Hb within their natural environments have to be identified and analysed (Smagghe *et al.*, 2008).

Heterologous VHb expression has been shown to improve growth, cellular metabolism, and productivity of interesting products (Table 2). The achieved results in plants parallel well the early findings of the effects of heterologous VHb expression on microaerobic metabolism in bacteria (Frey and Kallio, 2003). The beneficial role of VHb in plant biotechnology has thus been emphasized, although some controversial results have also been reported (Table 2). It seems obvious that heterologous VHb expression, not excluding other potentially interesting bacterial Hbs or engineered variants (Andersson *et al.*, 2000; Frey *et al.*, 2000; Bollinger *et al.*, 2001; Kallio *et al.*, 2001, 2007, 2008), should be analysed on a case-by-case basis. Such experiments may reveal if VHb can be used to improve the energy status of specific plant species, potentially leading to economically important applications in the bioenergy field. On the other hand, starch accumulation can potentially lead to improved growth or carbon sequestration to secondary metabolites under suboptimal environmental conditions (e.g. under flooding in nature). New and fast screening methods and reliable high-throughput cultivation techniques are also needed to evaluate the biotechnologically important characters of Hbs originating from microorganisms and plants.

To conclude, many questions still remain open in the fascinating world of haemoproteins, especially in the case of plant nsHbs and trHbs. We will certainly learn a lot from the bacterial counterparts that are at the moment better characterized and known, but a great deal of research needs to be carried out concerning plants. Future research on plant nsHbs and trHbs will be challenging.

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References

Andersson CIJ, Holmberg N, Farrés J, Bailey JE, Bülow L, Kallio PT. 2000. Error-prone PCR of *Vitreoscilla* hemoglobin (VHb) to

support the growth of microaerobic *Escherichia coli*. *Biotechnology and Bioengineering* **70,** 446–455.

Anjum MF, Ioannidis N, Poole RK. 1998. Response of the NAD(P)H-oxidising flavohaemoglobin (Hmp) to prolonged oxidative stress and implications for its physiological role in *Escherichia coli*. *FEMS Microbiology Letters* **166,** 219–223.

Antonini E. 1965. Interrelationship between structure and function in hemoglobin and myoglobin. *Physiological Reviews* **45,** 123–170.

Appleby CA. 1984. Leghemoglobin and *Rhizobium* respiration. *Annual Review of Plant Physiology* **35,** 443–478.

Appleby CA, Bogusz D, Dennis ES, Peacock WJ. 1988. A role for haemoglobin in all plant roots? *Plant, Cell and Environment* **11,** 359–367.

Arredondo-Peter R, Hargrove MS, Moran JF, Sarath G, Klucas RV. 1998. Plant hemoglobins. *Plant Physiology* **118,** 1121–1125.

Ascenzi P, Bolognesi M, Milani M, Guertin M, Visca P. 2007. Mycobacterial truncated hemoglobins: from genes to functions. *Gene* **398,** 42–51.

Blokhina OB, Fagerstedt KV. 2006. Oxidative stress and antioxidant defenses in plants. In: Singh KK, ed. *Oxidative stress, disease and cancer*. London: Imperial College Press, 151–199.

Bogusz D, Appleby CA, Landsmann J, Dennis ES, Trinick MJ, Peacock WJ. 1988. Functioning haemoglobin genes in non-nodulating plants. *Nature* **331,** 178–180.

Bollinger CJT, Bailey JE, Kallio PT. 2001. Novel hemoglobins to enhance microaerobic growth and substrate utilization in *Escherichia coli*. *Biotechnology Progress* **17,** 798–808.

Bonamore A, Gentili P, Ilari A, Schinina ME, Boffi A. 2003. *Escherichia coli* flavohemoglobin is an efficient alkylhydroperoxide reductase. *Journal of Biological Chemistry* **278,** 22272–22277.

Bruno S, Faggiano S, Spyrakis F, *et al*. 2007. The reactivity with CO of AHb1 and AHb2 from *Arabidopsis thaliana* is controlled by the distal HisE7 and internal hydrophobic cavities. *Journal of the American Chemical Society* **129,** 2880–2889.

Bykova NV, Igamberdiev AU, Ens W, Hill RD. 2006. Identification of an intermolecular disulfide bond in barley hemoglobin. *Biochemical and Biophysical Research Communication* **347,** 301–309.

Cao MX, Huang JQ, Wei ZM, Yao QH, Wan CZ, Lu JA. 2004. Engineering higher yield and herbicide resistance in rice by *Agrobacterium*-mediated multiple gene transformation. *Crop Science* **44,** 2206–2213.

Coleman JW. 2001. Nitric oxide in immunity and inflammation. *International Immunopharmacology* **1,** 1397–1406.

Correa-Aragunde N, Graziano M, Lamattina L. 2004. Nitric oxide plays a central role in determining lateral root development in tomato. *Planta* **218,** 900–905.

Cruz-Ramos H, Crack J, Wu G, Hughes MN, Scott C, Thomson AJ, Green J, Poole RK. 2002. NO sensing by FNR: regulation of the *Escherichia coli* NO-detoxifying flavohaemoglobin, Hmp. *EMBO Journal* **21,** 3235–3244.

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

Donovan WP, Dankocsik CC, Gilbert MP. 1988. Molecular characterization of a gene encoding a 72-kilodalton mosquito-toxic crystal protein from *Bacillus thuringiensis* subsp. *israelensis*. *Journal of Bacteriology* **170,** 4732–4738.

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.

Dordas C, Rivoal J, Hill RD. 2003. Plant haemoglobins, nitric oxide and hypoxic stress. *Annals of Botany* **91,** 173–178.

Duff SM, Wittenberg JB, Hill RD. 1997. Expression, purification, and properties of recombinant barley (*Hordeum* sp.) hemoglobin. Optical spectra and reactions with gaseous ligands. *Journal of Biological Chemistry* **272,** 16746–16752.

Ermler U, Siddiqui RA, Cramm R, Friedrich B. 1995. Crystal structure of the flavohemoglobin from *Alcaligenes eutrophus* at 1.75 Å resolution. *EMBO Journal* **14,** 6067–6077.

Farrés J, Burckhardt-Herold S, Scherrer J, Frey AD, Kallio PT. 2007. Analysis of the contribution of the globin and reductase domains to the ligand-binding properties of bacterial haemoglobins. *Biochemical Journal* **407,** 15–22.

Farrés J, Kallio PT. 2002. Improved cell growth in tobacco suspension cultures expressing *Vitreoscilla* hemoglobin. *Biotechnology Progress* **18,** 229–233.

Farrés J, Rechsteiner MP, Herold S, Frey AD, Kallio PT. 2005. Ligand binding properties of bacterial hemoglobins and flavohemoglobins. *Biochemistry* **44,** 4125–4134.

Frey AD, Andersson CIJ, Schmid VH, Bülow L, Kallio PT. 2007. Globin-expression postpones onset of stationary phase specific gene expression in *Escherichia coli*. *Journal of Biotechnology* **129,** 461–471.

Frey AD, Bailey JE, Kallio PT. 2000. Expression of *Alcaligenes eutrophus* flavohemoprotein and engineered *Vitreoscilla* hemoglobin–reductase fusion protein for improved hypoxic growth of *Escherichia coli*. *Applied and Environmental Microbiology* **66,** 98–104.

Frey AD, Farrés J, Bollinger CJT, Kallio PT. 2002. Bacterial hemoglobins and flavohemoglobins for alleviation of nitrosative stress in *Escherichia coli*. *Applied and Environmental Microbiology* **68,** 4835–4840.

Frey AD, Fiaux J, Szyperski T, Wüthrich K, Bailey JE, Kallio PT. 2001. Dissection of central carbon metabolism of hemoglobin-expressing *Escherichia coli* by ^{13}C nuclear magnetic resonance flux distribution analysis in microaerobic bioprocesses. *Applied and Environmental Microbiology* **67,** 680–687.

Frey AD, Kallio PT. 2003. Bacterial hemoglobins and flavohemoglobins: versatile proteins and their impact on microbiology and biotechnology. *FEMS Microbiology Reviews* **27,** 525–545.

Frey AD, Kallio PT. 2005. Nitric oxide detoxification—a new era for bacterial globins in biotechnology? *Trends in Biotechnology* **23,** 69–73.

Frey AD, Koskenkorva T, Kallio PT. 2003. *Vitreoscilla* hemoglobin promoter is not responsive to nitrosative and oxidative stress in *Escherichia coli*. *FEMS Microbiology Letters* **224,** 127–132.

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 Biotechnology Journal* **2,** 221–231.

Gardner PR. 2005. Nitric oxide dioxygenase function and mechanism of flavohemoglobin, hemoglobin, myoglobin and their associated reductases. *Journal of Inorganic Biochemistry* **99,** 247–266.

Gardner AM, Martin LA, Gardner PR, Dou Y, Olson JS. 2000. Steady-state and transient kinetics of *Escherichia coli* nitric-oxide dioxygenase (flavohemoglobin)—the B10 tyrosine hydroxyl is essential for dioxygen binding and catalysis. *Journal of Biological Chemistry* **275,** 12581–12589.

Giangiacomo L, Ilari A, Boffi A, Morea V, Chiancone E. 2005. The truncated oxygen-avid hemoglobin from *Bacillus subtilis*: X-ray structure and ligand binding properties. *Journal of Biological Chemistry* **280,** 9192–9202.

Giangiacomo L, Mattu M, Arcovito A, Bellenchi G, Bolognesi M, Ascenzi P, Boffi A. 2001. Monomer–dimer equilibrium and oxygen binding properties of ferrous *Vitreoscilla* hemoglobin. *Biochemistry* **40,** 9311–9316.

Gibson QH, Wittenberg JB, Wittenberg BA, Bogusz D, Appleby CA. 1989. The kinetics of ligand binding to plant hemoglobins. Structural implications. *Journal of Biological Chemistry* **264,** 100–107.

Gonzales-Prevatt V, Webster DA. 1980. Purification and properties of NADH-cytochrome *o* reductase from *Vitreoscilla*. *Journal of Biological Chemistry* **255,** 1478–1482.

Goodin DB, Mcree DE. 1993. The Asp–His–Fe triad of cytochrome *c* peroxidase controls the reduction potential, electronic structure, and coupling of the tryptophan free radical to the heme. *Biochemistry* **32,** 3313–3324.

Goodman MD, Hargrove MS. 2001. Quaternary structure of rice nonsymbiotic hemoglobin. *Journal of Biological Chemistry* **276,** 6834–6839.

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

Hargrove MS, Barry JK, Brucker EA, Berry MB, Phillips GN Jr, Olson JS, Arredondo-Peter R, Dean JM, Klucas RV, Sarath G. 1997. Characterization of recombinant soybean leghemoglobin a and apolar distal histidine mutants. *Journal of Molecular Biology* **266,** 1032–1042.

Hargrove MS, Brucker EA, Stec B, Sarath G, Arredondo-Peter R, Klucas RV, Olson JS, Philips GN Jr. 2000. Crystal structure of a nonsymbiotic plant hemoglobin. *Structure with Folding and Design* **8,** 1005–1014.

Hausladen A, Gow A, Stamler JS. 2001. Flavohemoglobin denitrosylase catalyzes the reaction of a nitroxyl equivalent with molecular oxygen. *Proceedings of the National Academy of Sciences, USA* **98,** 10108–10112.

Hebelstrup KH, Hunt P, Dennis E, Bjerregaard Jensen S, Østergaard Jensen E. 2006. Hemoglobin is essential for normal growth of *Arabidopsis* organs. *Physiologia Plantarum* **127,** 157–166.

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

Hebelstrup KH, Østergaard-Jensen E, Hill RD. 2008. Bioimaging techniques for subcellular localization of plant hemoglobins and measurement of hemoglobin-dependent nitric oxide scavenging *in planta*. *Methods in Enzymology* **437,** 595–604.

Herold S, Puppo A. 2005. Kinetics and mechanistic studies of the reactions of metleghemoglobin, ferrylleghemoglobin, and nitrosylleghemoglobin with reactive nitrogen species. *Journal of Biological Inorganic Chemistry* **10,** 946–957.

Hill RD. 1998. What are hemoglobins doing in plants? *Canadian Journal of Botany* **76,** 707–712.

Holmberg N, Lilius G, Bailey JE, Bülow L. 1997. Transgenic tobacco expressing *Vitreoscilla* hemoglobin exhibits enhanced growth and altered metabolite production. *Nature Biotechnology* **15,** 244–247.

Hoy JA, Hargrove MS. 2008. The structure and function of plant hemoglobins. *Plant Physiology and Biochemistry* **46,** 371–379.

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 Molecular Biology* **47,** 677–692.

Hvitved AN, Trent JT III, Premer SA, Hargrove MS. 2001. Ligand binding and hexacoordination in *Synechocystis* hemoglobin. *Journal of Biological Chemistry* **276,** 34714–34721.

Igamberdiev AU, Bykova NV, Hill RD. 2006. Nitric oxide scavenging by barley hemoglobin is facilitated by a monodehydroascorbate reductase-mediated ascorbate reduction of methemoglobin. *Planta* **223,** 1033–1040.

Igamberdiev AU, Hill RD. 2004. Nitrate, NO and haemoglobin in plant adaptation to hypoxia: an alternative to classic fermentation pathways. *Journal of Experimental Botany* **55,** 2473–82.

Igamberdiev AU, Seregelyes C, Manac'h N, Hill RD. 2004. NADH-dependent metabolism of nitric oxide in alfalfa root cultures expressing barley hemoglobin. *Planta* **219,** 95–102.

Ilari A, Bonamore A, Farina A, Johnson KA, Boffi A. 2002. The X-ray structure of ferric *Escherichia coli* flavohemoglobin reveals an unexpected geometry of the distal heme pocket. *Journal of Biological Chemistry* **277,** 23725–23732.

Ioanitescu AI, Dewilde S, Kiger L, Marden MC, Moens L, Van Doorslaer S. 2005. Characterization of nonsymbiotic tomato hemoglobin. *Biophysical Journal* **89,** 2628–2639.

Jokipii S, Häggman H, Günter B, 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.

Kallio PT, Bollinger CJT, Koskenkorva T, Frey AD. 2008. Assessment of biotechnologically relevant characteristics of heterologous hemoglobins in *E.coli*. *Methods in Enzymology* **436,** 255–272.

Kallio PT, Frey AD, Bailey JE. 2001. From *Vitreoscilla* hemoglobin (VHb) to a novel class of growth stimulating hemoglobin proteins. In: Merten O-W, Mattanovich D, Lang C, Larsson G, Neubauer P, Porro D, Postma P, Teixeira de Mattos J, Cole JA, eds. *Recombinant protein production with prokaryotic and eukaryotic cells. A comparative view on host physiology*. Dordrecht, The Netherlands: Kluwer Academic Publishers, 75–87.

Kallio PT, Heidrich J, Koskenkorva T, Bollinger CJT, Farrés J, Frey AD. 2007. Analysis of novel hemoglobins during microaerobic growth of HMP-negative *Escherichia coli*. *Enzyme and Microbial Technology* **40,** 329–336.

Kallio PT, Kim D-J, Tsai PS, Bailey JE. 1994. Intracellular expression of *Vitreoscilla* hemoglobin alters *Escherichia coli* energy metabolism under oxygen-limited conditions. *European Journal of Biochemistry* **219,** 201–208.

Karplus PA, Daniels MJ, Herriott JR. 1991. Atomic-structure of ferredoxin-$NADP^+$ reductase—prototype for a structurally novel flavoenzyme family. *Science* **251,** 60–66.

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. *Applied and Environmental Microbiology* **68,** 152–160.

Khosla C, Bailey JE. 1988. Heterologous expression of a bacterial haemoglobin improves the growth properties of recombinant *Escherichia coli*. *Nature* **331,** 633–635.

Kubo H. 1939. Über hämoprotein aus den wurzelknöllchen von leguminosen. *Acta Phytochimica (Tokyo)* **11,** 195–200.

Kvist M, Ryabova ES, Norlander E, Bülow L. 2007. An investigation of the peroxidase activity of *Vitreoscilla* hemoglobin. *Journal of Biological Inorganic Chemistry* **12,** 324–334.

Lamattina L, García-Mata C, Graziano M, Pagnussat G. 2003. Nitric oxide: the versatility of an extensive signal molecule. *Annual Review of Plant Biology* **54,** 109–136.

Landsmann J, Dennis ES, Higgins TJV, Abbleby CA, Kortt AA, Peacock WJ. 1986. Common evolutionary origin of legume and non-legume plant hemoglobins. *Nature* **324,** 166–168.

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 Reports* **23,** 710–715.

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 Science* **161,** 279–287.

Mao ZC, Hu YL, Zhong J, Wang LX, Guo JY, Lin ZP. 2003. Improvement of the hydroponic growth and waterlogging tolerance of petunias by the introduction of *vhb* gene. *Acta Botanica Sinica* **45,** 205–210.

Mattana M, Vannini C, Espen L, *et al*. 2007. The rice Mybleu transcription factor increases tolerance to oxygen deprivation in Arabidopsis plants. *Physiologia Plantarum* **131,** 106–121.

Milani M, Pesce A, Quellet Y, Dewilde S, Friedman J, Ascenzi P, Guertin M, Bolognesi M. 2004*a*. Heme-ligand tunneling in group I truncated hemoglobins. *Journal of Biological Chemistry* **279,** 21520–21525.

Milani M, Quellet Y, Quellet H, Guertin M, Boffi A, Antonini G, Bocedi A, Mattu M, Bolognesi M, Ascenzi P. 2004*b*. Cyanide binding to truncated hemoglobins: a crystallographic and kinetic study. *Biochemistry* **43,** 5213–5221.

Mukai M, Mills CE, Poole RK, Yeh SR. 2001. Flavohemoglobin, a globin with a peroxidase-like catalytic site. *Journal of Biological Chemistry* **276,** 7272–7277.

Nardini M, Pesce A, Milani M, Bologesi M. 2007. Protein fold and structure in the truncated (2/2) globin family. *Gene* **398,** 2–11.

Neill SJ, Desikan R, Hancock JT. 2003. Nitric oxide signalling in plants. *New Phytologist* **159,** 11–35.

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 and Cell Physiology* **46,** 324–331.

Orii Y, Webster DA. 1986. Photodissociation of oxygenated cytochrome *o*(s) (*Vitreoscilla*) and kinetic studies of reassociation. *Journal of Biological Chemistry* **261,** 3544–3547.

Ouellet H, Juszczak L, Dantsker D, Samuni U, Ouellet YH, Savard PY, Wittenberg JB, Wittenberg BA, Friedman JM, Guertin M. 2003. Reactions of *Mycobacterium tuberculosis* truncated hemoglobin O with ligands reveal a novel ligand-inclusive hydrogen bond network. *Biochemistry* **42,** 5764–5774.

Ouellet H, Ouellet Y, Richard C, Labarre M, Wittenberg B, Wittenberg J, Guertin M. 2002. Truncated hemoglobin HbN protects *Mycobacterium bovis* from nitric oxide. *Proceedings of the National Academy of Sciences, USA* **99,** 5902–5907.

Pagnussat GC, Simontacchi M, Puntarulo S, Lamattina L. 2002. Nitric oxide is required for root organogenesis. *Plant Physiology* **129,** 954–956.

Pathania R, Navani NK, Gardner AM, Gardner PR, Dikshit KL. 2002. Nitric oxide scavenging and detoxification by the *Mycobacterium tuberculosis* haemoglobin HbN in *Escherichia coli*. *Molecular Microbiology* **45,** 1303–1314.

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 Biology* **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. *The Plant Cell* **16,** 2785–2794.

Poole RK, Anjum MF, Membrillo-Hernández J, Kim SO, Hughes MN, Stewart V. 1996. Nitric oxide, nitrite, and Fnr regulation of *hmp* (flavohemoglobin) gene expression in *Escherichia coli* K-12. *Journal of Bacteriology* **178,** 5487–5492.

Qu Z-L, Zhong N-Q, Wang H-Y, Chen A-P, Jian G-L, Xia G-X. 2006. Ectopic expression of the cotton non-symbiotic hemoglobin gene *GhHbd1* triggers defense responses and increases disease tolerance in Arabidopsis. *Plant and Cell Physiology* **47,** 1058–1068.

Ramírez M, Valderrama B, Arredondo-Peter R, Soberón M, Mora J, Hernández G. 1999. *Rhizobium etli* genetically engineered for the heterologous expression of *Vitreoscilla* sp hemoglobin: effects on free-living and symbiosis. *Molecular Plant-Microbe Interactions* **12,** 1008–1015.

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. *Journal of Experimental Botany* **55,** 1721–1731.

Saenz-Rivera J, Sarath G, Arredondo-Peter R. 2004. Modeling the tertiary structure of a maize (*Zea mays* ssp. *mays*) non-symbiotic hemoglobin. *Plant Physiology and Biochemistry* **42,** 891–897.

Sakamoto A, Sakurao S-H, 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 Letters* **572,** 27–32.

Samuni U, Dantsker D, Ray A, Wittenberg JB, Wittenberg BA, Dewilde S, Moens L, Quellet Y, Guertin M, Friedman JM. 2003. Kinetic modulation in carbonmonoxy derivatives of truncated hemoglobins: the role of distal heme pocket residues and extended apolar tunnel. *Journal of Biological Chemistry* **278,** 27241–27250.

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. *Molecular Plant-Microbe Interactions* **19,** 441–450.

Seregélyes C, Mustárdy L, Ayaydin F, *et al*. 2000. Nuclear localization of a hypoxia-inducible novel non-symbiotic hemoglobin in cultured alfalfa cells. *FEBS Letters* **482,** 125–130.

Seregélyes C, Barna B, Hennig J, Konopka D, Pasternak TP, Lukács N, Fehér A, Horváth GV, Dudits D. 2003. Phytoglobins can interfere with nitric oxide functions during plant growth and pathogenic responses: a transgenic approach. *Plant Science* **165,** 541–550.

Seregélyes C, Igamberdiev AU, Maassen A, Henning J, Dudits D, Hill RD. 2004. NO-degradation by alfalfa class 1 hemoglobin (Mhb1): a possible link to PR-1a gene expression in Mhb1-overproducing tobacco plants. *FEBS Letters* **571,** 61–66.

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 and Cell Physiology* **46,** 99–107.

Smagghe BJ, Kundu S, Hoy JA, Halder P, Weiland TR, Savage A, Venugopal A, Goodman M, Premer S, Hargrove MS. 2006. Role of phenylalanine B10 in plant nonsymbiotic hemoglobins. *Biochemistry* **45,** 9735–9745.

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

Sowa AW, Duff SMG, Guy PA, Hill RD. 1998. Altering hemoglobin levels changes energy status in maize cells under hypoxia. *Proceedings of the National Academy of Sciences, USA* **95,** 10317–10321.

Tarricone C, Galizzi A, Coda A, Ascenzi P, Bolognesi M. 1997. Unusual structure of the oxygen-binding site in the dimeric bacterial hemoglobin from *Vitreoscilla* sp. *Structure* **5,** 497–507.

Trent JT III, Watts RA, Hargrove MS. 2001. Human neuroglobin, a hexacoordinate hemoglobin that reversibly binds oxygen. *Journal of Biological Chemistry* **276,** 30106–30110.

Trevaskis B, Watts RA, Andersson CR, Llewellyn DJ, Hargrove MS, Olson JS, Dennis ES, Peacock WJ. 1997. Two hemoglobin genes in *Arabidopsis thaliana*: the evolutionary origins of leghemoglobins. *Proceedings of the National Academy of Sciences, USA* **94,** 12230–12234.

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*. *Biotechnology and Bioengineering* **49,** 151–160.

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

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, Arrendondo-Peter R, Gough J, Dewilde S, Moens L, Vanfleteren JR. 2006. A phylogenomic profile of globins. *BMC Evolutionary Biology* **6,** 31.

Vinogradov SN, Hoogewijs D, Bailly X, Mizuguchi K, Dewilde S, Moens L, Vanfleteren JR. 2007. Model of globin evolution. *Gene* **398,** 132–142.

Vuletich DA, Lecomte JT. 2006. A phylogenetic and structural analysis of truncated hemoglobins. *Journal of Molecular Evolution* **62,** 12622–12633.

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. *The 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 and Environment* **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 anti-oxidants metabolism. *Plant Science* **176,** 66–77. doi:10.1016/j.plantsci.2008.09.011.

Watts RA, Hunt PW, Hvitved AN, Hargrove MS, Peacock WJ, Dennis ES. 2001. A hemoglobin from plants homologous to truncated hemoglobins of microorganisms. *Proceedings of the National Academy of Sciences, USA* **98,** 10119–10124.

Webster DA, Liu CY. 1974. Reduced nicotinamide adenine dinucleotide cytochrome *o* reductase associated with cytochrome *o* purified from Vitreoscilla. Evidence for an intermediate oxygenated form of cytochrome *o*. *Journal of Biological Chemistry* **249,** 4257–4260.

Wilhelmson A, Häkkinen ST, Kallio PT, Oksman-Caldentey K-M, Nuutila AM. 2006. Heterologous expression of *Vitreoscilla* hemoglobin (VHb) and cultivation conditions affect the alkaloid profile of *Hyoscyamus muticus* hairy roots. *Biotechnology Progress* **22,** 350–358.

Wilhelmson A, Kallio PT, Oksman-Caldentey K-M, Nuutila AM. 2005. Expression of *Vitreoscilla* hemoglobin enhances growth of *Hyoscyamus muticus* hairy root cultures. *Planta Medica* **71,** 48–53.

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

Wittenberg JB, Bolognesi M, Wittenberg BA, Guertin M. 2002. Truncated hemoglobins: a new family of hemoglobins widely distributed in bacteria, unicellular eukaryotes, and plants. *Journal of Biological Chemistry* **277,** 871–874.

Zelasco S, Reggi S, Callihari 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. *Molecular Breeding* **17,** 201–216.

Zhao L, Gu R, Gao P, Wang G. 2008. A non-symbiotic hemoglobin gene from maize, *ZmHb*, is involved in response to submergence, high-salt and osmotic stresses. *Plant Cell, Tissue and Organ Culture* **95,** 227–237.

Zhou ZZ, Zhou YG, He CZ, Mang KQ, Tian YC. 2004. Expression of *cry3A* and *vhb* genes in transgenic potato plants. *Progress in Biochemistry and Biophysics* **31,** 741–745.