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Review

Plant hemoglobins: What we know six decades after their discovery ☆

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Abstract

This review describes contributions to the study of plant hemoglobins (Hbs) from a historical perspective with emphasis on non-symbiotic Hbs (nsHbs). Plant Hbs were first identified in soybean root nodules, are known as leghemoglobins (Lbs) and have been characterized in detail. It is widely accepted that a function of Lbs in nodules is to facilitate the diffusion of O_2 to bacteroids. For many years Hbs could not be identified in plants other than N_2-fixing legumes, however in the 1980s a Hb was isolated from the nodules of the non-legume dicot plant *Parasponia*, a *hb* gene was cloned from the non-nodulating *Trema*, and Hbs were detected in nodules of actinorhizal plants. Gene expression analysis showed that *Trema* Hb transcripts exist in non-symbiotic roots. In the 1990s nsHb sequences were also identified in monocot and primitive (bryophyte) plants. In addition to Lbs and nsHbs, Hb sequences that are similar to microbial truncated (2/2) Hbs were also detected in plants. Plant nsHbs have been characterized in detail. These proteins have very high O_2-affinities because of an extremely low O_2-dissociation constant. Analysis of rice Hb1 showed that distal His coordinates heme Fe and stabilizes bound O_2; this means that O_2 is not released easily from oxygenated nsHbs. Non-symbiotic *hb* genes are expressed in specific plant tissues, and overexpress in organs of stressed plants. These observations suggest that nsHbs have functions additional to O_2-transport, such as to modulate levels of ATP and NO.
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Keywords: History; Leghemoglobin; Non-symbiotic; Truncated

1. Introduction

Hemoglobins (Hbs) are hemeproteins that reversibly bind O_2. The most common function of Hbs is associated with the transport of O_2, however Hbs also bind other gaseous ligands, such as NO (Cooper, 1999; Poole and Hughes, 2000; Moller and Skibsted, 2002; Dordas et al., 2003b; Milani et al., 2003; Frey and Kalio, 2005), and organic molecules (Ollesch et al., 1999; Bonamore et al., 2003; D'Angelo et al., 2004; Rinaldi et al., 2006), which suggests that they are multifunctional proteins in living organisms. Phylogenomic analysis showed that Hbs are widespread in organisms, as they have been identified in genomes of archaeobacteria, eubacteria and eukaryotes, including plants (Vinogradov et al., 2005, 2006). In plants three types of Hbs have been identified: symbiotic, non-symbiotic (nsHb) and truncated (2/2) Hbs (tHbs) (Ross et al., 2002). This review describes contributions to the study of plant Hbs from a historical perspective with emphasis on nsHbs. The authors refer interested readers to reviews by Appleby (1992), Fuchsman (1992), Arredondo-Peter et al. (1998), Hill (1998), Ross et al. (2002) and Kundu et al. (2003) for detailed discussion and relevant references on the structure, function and molecular biology of plant Hbs.

2. The discovery of plant Hbs

Plant Hbs were first identified by Kubo (1939). This author analyzed the red pigment of soybean root nodules. Nodules form in legumes after root infection by soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Mesorhizobium* and *Sinorhizobium* (collectively know as rhizobia). Nodules

Abbreviations: Hb, hemoglobin; HGT, horizontal gene transfer; Lb, leghemoglobin; Mb, myoglobin; nsHb, non-symbiotic hemoglobin; NO, nitric oxide; tHb, truncated (2/2) hemoglobin.

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are red in color and are the plant organs where nitrogen fixation (by symbiotic rhizobia) occurs. Spectral examination of red pigment isolated by Kubo showed absorption bands that are characteristic of deoxy-ferrous, O_2-ferrous, CO-ferrous, and ferric Hbs. Kubo also crystallized hemin from this pigment and showed that crystals are identical to those of hemin from horse Hb. From this evidence Kubo concluded that the red pigment isolated from soybean root nodules was a hemeprotein.

To identify the physiological role of this hemeprotein in nodules, Kubo incubated nodule bacteria with it and observed stimulation of bacterial O_2-consumption. From this observation he concluded that the physiological role of the hemeprotein in nodules is to stimulate the assimilation and transport of O_2. The nodule hemeprotein discovered by Kubo has been characterized in detail by others. Biochemical, biophysical and molecular biology analyses have shown that Kubo's hemeprotein is a plant Hb with similar (*i.e.* structural) properties to animal Hbs (Fig. 1). Kubo's plant Hb was named as leghemoglobin (Lb) by Virtanen and Laine (1946) and is also known as plant symbiotic Hb.

3. The discovery of the function of Lb in root nodules

The role of Lb in N_2-fixing nodules was elucidated by Wittenberg, Bergersen, Appleby and Turner in 1974 (Wittenberg et al., 1974). These authors observed that the addition of Lb to suspensions of bacteroids enhanced the rate of O_2-uptake and nitrogenase activity (measured as the reduction of acetylene to ethylene), and promoted the formation of ethylene with increasing air O_2 concentrations. Ferric Lb, ferric horseradish peroxidase and ferric cytochrome *c* peroxidase were ineffective, however twelve O_2-binding proteins other than Lb also increased O_2-uptake and acetylene reduction by bacteroids. From these observations the authors concluded that oxyLb and other O_2-binding proteins facilitated the diffusion of O_2 to bacteroids in suspension, and that the function of Lb in nodules is to facilitate the diffusion of O_2 to bacteroids at an internal concentration too low to inhibit or destroy their O_2-sensitive nitrogenase. This concept of high O_2-flux at low free O_2 in the bacteroid vicinity is now generally accepted.

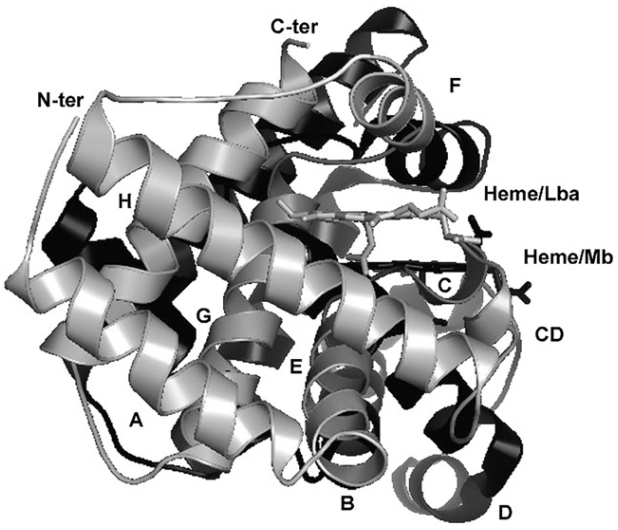

Fig. 1. Tertiary structure of Kubo's nodule hemeprotein (soybean leghemoglobin *a*) (gray) overlaid on the structure of spermwhale myoglobin (Mb) (black). Coordinates for the Lb*a* and Mb were obtained from the Brookhaven Protein Database using the identification numbers **1BIN** and **2MYC**, respectively, and structures were displayed using the PyMol program (http://www.pymol.org/funding.html). Helices (and CD loop in Lb*a*) are shown with the A–H letters.

4. The hypothesis for the origin of Lbs by horizontal gene transfer

For many years Hbs were identified in N_2-fixing legumes, but not elsewhere in the plant kingdom. Early attempts to detect Hbs in non-legumes using biochemical and molecular biology approaches were unsuccessful, so plant Hbs were apparently restricted to N_2-fixing legumes, and thus its origin was a mystery. In 1982 Jeffreys hypothesized that plant Hbs originated by a unique act of horizontal gene transfer (HGT) from a phytophagous insect to a primitive legume *via* a viral vector (Jeffreys, 1982). For this hypothesis to be correct the gene structure of insect *hb* and plant *lb* genes should be identical. Leghemoglobins are encoded by genes that are interrupted by three introns (Jensen et al., 1981; Hyldig-Nielsen et al., 1982). After Jeffreys' HGT hypothesis it was revealed that *hb* gene from the insect *Chironomus* contains no introns (in Appleby et al., 1988), thus the HGT hypothesis for the origin of plant Hbs was discarded, and the vertical evolution hypothesis of plant Hbs was favored. It is now known that other insect *hb* genes do contain introns, some in the exact positions found for legume *lb*s (Kloek et al., 1993; Hankeln et al., 2002; Hoogewijs et al., 2004); even so, other evidence in favor of vertical rather than horizontal evolution of plant *hb* genes soon emerged (see Section 5). An implication of the vertical evolution hypothesis is that Hbs might exist in all (legume and non-legume) land plants.

5. The vertical evolution of plant Hbs: discovery of plant non-symbiotic Hbs

A discovery that supported the vertical evolution hypothesis of plant Hbs was the identification of a Hb in the N_2-fixing root nodules of the non-legume dicot plant *Parasponia* (Appleby et al., 1983). *Parasponia* is a member of the Ulmaceae family, and the only non-legume known to be nodulated by *Rhizobium* strains. *Parasponia* Hb was extracted under anaerobic conditions and final purification was achieved by preparative isoelectric focusing, which produced a single major component of oxyHb (with pI of 6.28), and small amount of ferric Hb (with pI of 6.67). The absorption spectra of (deoxy-ferrous, O_2-ferrous and CO-ferrous) *Parasponia* Hb are similar to those of Lbs and animal Hbs and kinetic constants (O_2-affinity and O_2-association and -dissociation constants) are also similar in *Parasponia* Hb and soybean and other legume Lbs (Gibson et al., 1989). This evidence suggested that the function of Hb in *Parasponia* nodules is similar to function of Lb in legume nodules, *i.e.* to facilitate the diffusion of O_2 to bacteriods.

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During the 1980s a cDNA probe for *Parasponia* Hb was used to detect *hb* sequences in *Trema* and *Celtis*. *Trema* and *Celtis* are non-nodulating (ulmacean) plants closely related to *Parasponia*. Southern blot analysis revealed the existence of Hb-like sequences in *Trema* and (possibly) *Celtis* DNAs (Appleby et al., 1988; Boguz et al., 1988). DNA cloning and sequencing showed that *Trema* sequences have all the characteristics of a functional plant *hb* gene: the *Trema hb* gene is interrupted by three introns and exon sequences are 93% similar to *Parasponia hb*. Also, Northern blot analysis using the *Parasponia* Hb cDNA probe showed that Hb transcripts exist in *Trema* roots and in *Parasponia* roots and nodules, but not in *Trema* and *Parasponia* leaves. Western blot analysis using anti-*Parasponia* Hb antibodies showed that mRNA is translated into monomeric and dimeric Hbs in *Trema* roots and *Parasponia* roots and nodules, respectively (Boguz et al., 1988).

During the same decade Tjepkema detected high concentrations of Hb-like proteins in nodule extracts of actinorhizal plants (which are nodulated by the actinomycete *Frankia*), such as *Casuarina cunninghamiana* and *Myrica gale*, and low concentrations in nodules of *Comptonia peregrina*, *Alnus rubra* and *Eleagnus angustifolia* (Tjepkema, 1983). A few years latter after Tjepkema's report Hbs were purified and characterized from the actinorhizal *Casuarina glauca* (Fleming et al., 1987), *Alnus glutinosa* (Suharjo and Tjepkema, 1995), and *M. gale* (Pathirana and Tjepkema, 1995) root nodules. The spectral properties of actinorhizal Hbs are similar to those of Lbs and non-plant Hbs.

The detection of Hbs in *Parasponia*, *Trema* and actinorhizal plants suggested that plant Hbs are widespread in land plants and that plant *hb* genes vertically evolved from a common ancestor. Also, the detection of Hb transcripts in *Parasponia* and *Trema* roots showed that *hb* genes express and Hbs probably function in non-symbiotic plant organs.

cDNAs coding for monocot (barley and rice) nsHbs were cloned and sequenced in the 1990s. The first monocot Hb cDNA was cloned from barley (Taylor et al., 1994). The deduced amino acid sequence of barley Hb is 71% similar to *Parasponia* Hb. Southern blot analysis using the barley Hb cDNA probe showed that a single copy (or a very low copy number) of the *hb* gene exists in barley and that *hb*-like sequences exist in other monocot species, such as rye, maize and wheat. In 1997 Arredondo-Peter et al. reported the cloning and characterization of *hb1* and *2* genes from rice (Arredondo-Peter et al., 1997). Southern blot analysis showed that at least three copies of the *hb* gene exist in rice, however recent work showed that five copies of the *hb* gene exist in the rice genome (Lira-Ruan et al., 2002; Bustos-Rivera et al. manuscript in preparation). Sequence comparison showed that the predicted Hb1 and 2 proteins are 93% similar to each other, and that the rice Hbs are 68 to 82% similar to other non-symbiotic Hbs and about 50% similar to symbiotic Hbs (Arredondo-Peter et al., 1997).

Recently, Hb sequences were identified in very many evolved and primitive plants (Table 1), mostly because of numerous plant genome sequencing programs. For example, nsHb sequences have been identified in monocots such as maize and teosinte (Aréchaga-Ocampo et al., 2001) and wheat (Larsen, 2003), dicots such as soybean (Andersson et al., 1996), *Arabidopsis* (Trevaskis et al., 1997), chicory (Hendriks et al., 1998) and tomato (Wang et al., 2003), and bryophytes such as the moss *Physcomitrella patens* (Arredondo-Peter et al., 2000). The existence of nsHbs in the genome of primitive bryophytes suggests that the ancestor of land plant Hbs existed when the land was colonized by bryophytes, about 550 million years ago, and the existence of Hbs in primitive and evolved plants (Table 1) shows that Hbs are widespread in land plants. Moreover, *nshb* genes from the bryophytes *Marchantia polymorpha*, *Ceratodon purpureus* and *Physcomitrella patens* (GenBank accession numbers AY026341, EF028054 and EF028055, respectively) are interrupted by three introns in identical positions to introns of known plant *nshb* and *lb* genes. This evidence shows that *nshb* and *lb* genes evolved from an ancestral plant *hb* gene that was interrupted by three introns.

In addition to Lbs and nsHbs, Hb sequences that are similar to those of microbial truncated (2/2) Hbs (the reader is referred to reviews by Pesce et al. (2000) and Wittenberg et al. (2002) for a detailed description of the "truncated (2/2) folding" of globins) were detected in primitive and evolved plants (Table 1) (Watts et al., 2001). Sequence alignment showed that plant 2/2-like Hbs are highly conserved (not shown), and phylogenetic analysis showed that plant 2/2-like Hbs and Lbs and nsHbs evolved through different lineages (Fig. 2). Phylogenomic analysis by Vinogradov et al. (2006) revealed that plant 2/2-like Hbs are more similar to bacterial 2/2 Hbs than to plant (symbiotic and non-symbiotic) Hbs, which suggested that plant 2/2-like Hbs originated by a HGT event from a bacterial (*2/2 hb* gene) donor. Expression analysis showed that 2/2-like *hb* genes express in vegetative and embryonic organs and in organs from stressed plants (Hunt et al., 2001; Watts et al., 2001), however the function of 2/2-like Hbs in plant organs is not yet known, although kinetic properties of a recombinant *Arabidopsis* 2/2-like Hb suggest that these proteins may function as O_2-carriers (Watts et al., 2001).

6. Non-symbiotic (class 1) Hbs: hexacoordinate proteins with very high O_2-affinity

A distinctive characteristic of plant (class 1) nsHbs is that the affinity of these proteins for O_2 is very high (Duff et al., 1997; Arredondo-Peter et al., 1997; Trevaskis et al., 1997). For example, the O_2-affinity of rice Hb1 is 78-times higher than that from soybean Lb*a* (Arredondo-Peter et al., 1997). This high O_2-affinity of rice Hb1 is mainly due to a very low O_2-dissociation constant (k_{off}=0.038 s^{-1}), which suggests that O_2 is stabilized after binding to Hb1. Spectral analysis of deoxy-ferrous rice Hb1 showed that a distal ligand coordinates to Fe: the unliganded ferrous state of Hb1 exhibits peaks at 526 and 556 nm, which is similar to the absorption spectrum of (ferrous) cytochrome *b* where the heme Fe is hexacoordinate (Smith, 1978; Weiss and Ziganke, 1978). Histidine was identified as the distal ligand to heme Fe in wild type rice Hb1 by comparison with its mutant Hb1 H74L (in which distal His H74 was replaced by Leu). Absorbance spectra of mutant Hb1 showed no

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Table 1
Hemoglobin sequences identified in land plants

Organism	Hb[a] (accession number[b])
I. Seed plants	
a) Angiosperms	
Monocotyledonous	
Hordeum vulgare (barley)	nsHb-1 (U94968), tHb (AF376063)
Oryza sativa (rice)	nsHb1–1 (U76029), nsHb2–1 (U76031), nsHb3–1 (AF335504), nsHb4–1 (AF335504), tHb (BAD32857)
Saccharum spp. (sugarcane)	nsHb-1 (C0373680)
Triticum aestivum (wheat)	nsHb-1 (AY151390), tHb (AY151391)
Zea mays ssp. *mays* (maize)	nsHb-1 (AY005818), nsHb-2 (DQ171946)
Z. mays ssp. *parviglumis* (teosinte)	nsHb-1 (AF291052)
Dicotyledonous (legumes)	
Astragalus sinicus	symHb (DQ199647)
Canavalia lineata (bay bean)	symHb (UD9671)
Glycine max (soybean)	symHb (V00453), nsHb-1 (U47143), tHb (AA548191)
Lotus japonicus	symHb (AB238218), nsHb-1 (AB238220)
Lupinus luteus (yellow lupine)	symHb (Y00401)
Phaseolus vulgaris (kidney bean)	symHb (P02234)
Pisum sativum (pea)	symHb (P02233)
Psophocarpus tetragonolobus (winged bean)	symHb (X65874)
Medicago sativa (alfalfa)	symHb (M32883), nsHb-1 (AF172172)
M. trunculata (barrel medic)	symHb (X57733), tHb (ABE93362)
Sesbania rostrata	symHb (M23313)
Vicia faba (broad bean)	symHb (Z54159)
V. sativa (common vetch)	symHb (YO0229)
Vigna unguiculata (cowpea)	symHb I (U33206), symHb II (U33207)
Dicotyledonous (no-legumes)	
Alnus firma	nsHb-1 (AB221344)
Arabidopsis thaliana	nsHb-1 (NM_127165), nsHb-2 (NM_111887), tHb (NM_119421)
Beta vulgaris (sugar beet)	nsHb-2 (BE590299)
Brassica napus (canola)	nsHb-2 (AY026337)
Casuarina glauca (swamp oak)	nsHb-2 (X77694), nsHb-1(X53950)
Cichorium intybus × *C. endivia* (chicory)	nsHb1–2 (CAA07547), nsHb2–2 (AJ277797)
Citrus unshiu (satsuma orange)	nsHb-1 (AY026338)
Datisca glomerata (durango root)	tHb (CAD33536)
Euryale ferox (fox nut)	nsHb1–2 (AY281295), nsHb2–2 (AY281296)
Gossypium hirsutum (cotton)	nsHb-1 (AY899302), nsHb-2 (AY026340)
Lycopersicon esculentum (tomato)	nsHb-1 (AY026343), nsHb-2 (AY026344)
Malus domestica (apple)	nsHb-1 (AY224132)
Myrica gale	nsHb-1 (EF405885)
Nicotiana tabacum (tobacco)	nsHb-1 (BQ842804)

Table 1 (*continued*)

Organism	Hb[a] (accession number[b])
I. Seed plants	
a) Angiosperms	
Dicotyledonous (no-legumes)	
Parasponia andersonii	NsHb-1 (U27194)
P. rigida	nsHb-1 (P68169)
Pyrus communis (pear)	nsHb-1(AAY224133)
Raphanus sativus (horseradish)	nsHb-1 (AY286331)
Solanum tuberosum (potato)	nsHb2–1 (AY151389)
Trema orientalis	nsHb-1 (AF027215)
T. tomentosa	nsHb-1 (P07803)
T. virgata	nsHb-1 (AJ131349)
b) Gymnosperms	
Pinus tadea (pine)	tHb (BQ291271)
II. Bryophytes	
a) Mosses	
Physcomitrella patens	nsHb (AF218049)
Ceratodon purpureus	nsHb (AF309562)
b) Liverworts	
Marchantia polymorpha (liverwort)	nsHb (AY026341)

[a]nsHb, non-symbiotic Hb; nsHb-1, non-symbiotic Hb (class 1) with high O_2-affinity; nsHb-2, non-symbiotic Hb (class 2) with moderate O_2-affinity and/or sequence similarity to symbiotic Hbs; symHb, symbiotic Hb (including Lbs) with moderate O_2-affinity, which is specifically localized in nodules of N_2-fixing plants; tHb, 2/2-like plant Hb.
[b]Accession numbers in the GenBank database.

evidence of His coordination. Also, the substitution of H74 by L in Hb1 resulted in an increase of O_2-association and -dissociation constants. A conclusion from these observations was that distal His hexacoordinates to Fe and stabilizes bound O_2 in oxygenated Hb1 (Arredondo-Peter et al., 1997; Hargrove et al., 2000; Goodman and Hargrove, 2001).

7. Localization of nsHbs and expression of *nshb* genes in plant organs

Use of anti-rice Hb1 antibodies and Western blot showed that Hbs are localized in embryonic (coleoptiles, seminal roots and embryos) and (young and mature) vegetative (leaves and roots) rice organs. These observations showed that nsHbs are synthesized in different rice organs and during plant development (Lira-Ruan et al., 2001). However, analysis by confocal microscopy revealed that nsHbs are localized in the cytoplasm of differentiated and differentiating cell types of the developing rice seedling, such as the aleurone, scutellum, root cap cells, sclerenchyma, and tracheary elements. The immunolocalization of nsHbs in the vasculature of root, mesocotyl and leaf tissues, specifically in the xylem, suggests that nsHbs play roles in the metabolism accompanying the differentiation of the xylem (Ross et al., 2001).

Also, high levels of nsHb transcripts and proteins have been detected in organs and tissues of stressed plants. For example, *nshb* genes overexpress in barley aleurones subjected to microaerobiosis (<5% O_2) or sustained anoxia (up to 12 h) and in roots of flooded barley plants (Taylor et al., 1994). In

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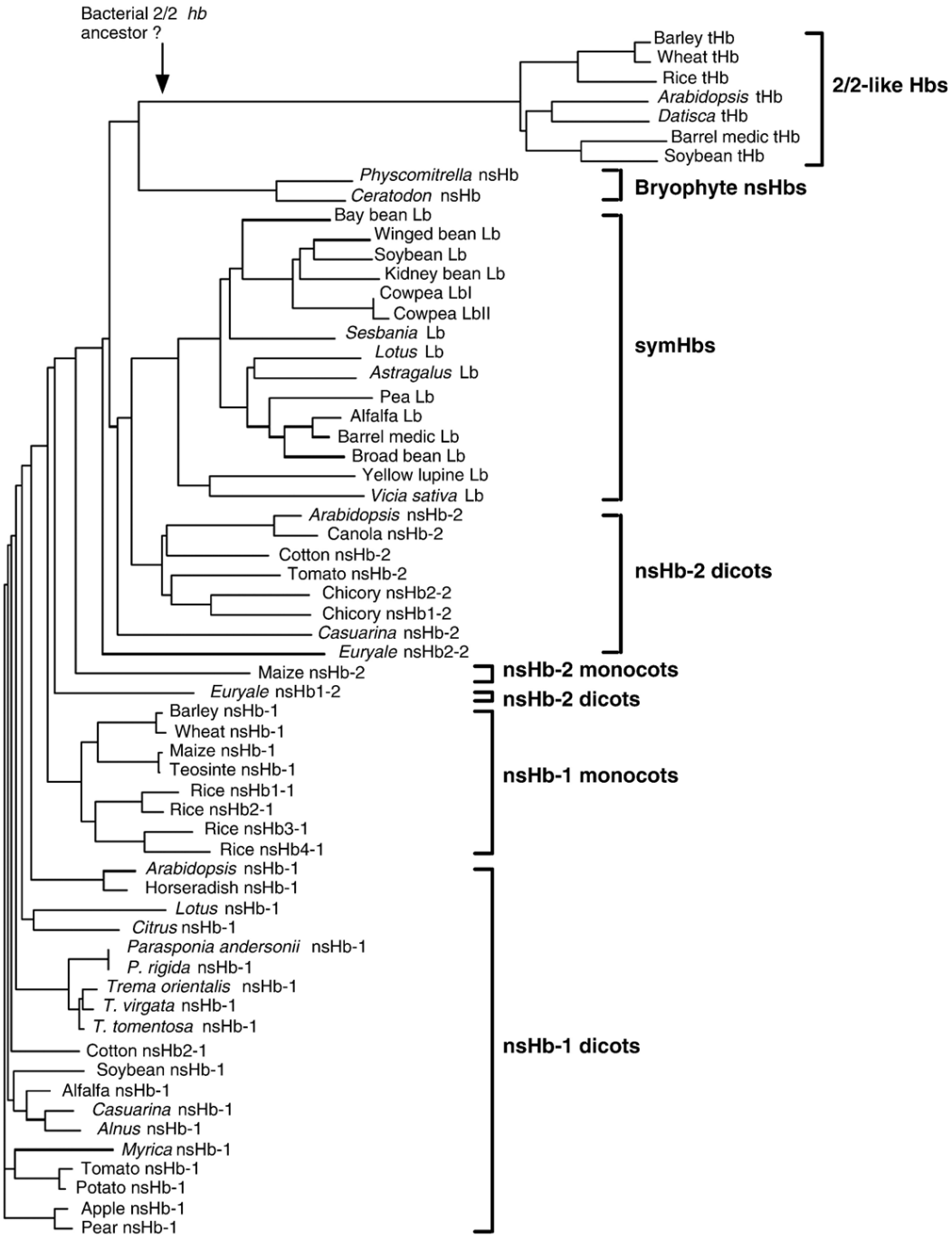

Fig. 2. Cluster (phenetic) analysis of selected plant Hbs. Full Hb sequences (partial or incomplete Hb sequences were not included in this analysis) were obtained from the GenBank database using the accession numbers shown in Table 1. Hb sequences were aligned and phenogram was constructed using the Clustal W program (Thompson et al., 1997). The possible origin of plant 2/2-like Hbs by HGT is indicated according to Vinogradov et al. (2006). Abbreviations are the same as in Table 1. *Parasponia* Hbs were classified as nsHb-1 because they cluster with high O_2-affinity (class 1) nsHbs, however *Parasponia* Hbs are intermediate because they are localized in nodules and non-symbiotic roots and their O_2-affinity is similar to that of symHbs (Lbs).

etiolated rice levels of Hbs increased 2.4-fold compared to control plants grown under a normal light/dark regimen (Lira-Ruan et al., 2001), and levels of nsHb transcripts increased in microaerobic (0.1% O_2) and osmotically (1% sucrose) stressed *Arabidopsis* roots (Trevaskis et al., 1997). These observations suggest that nsHbs take part of the plant response to some stress conditions.

8. Multiple functions of plant nsHbs?

The kinetic constants (see Section 6) and localization of nsHbs in metabolically active tissues as well as the over-expression of *nshb* genes in stressed plants (see Section 7) suggest that nsHbs have functions other than or additional to O_2 transport. In 1998 it was proposed that nsHbs may play three

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major roles in plant metabolism (Arredondo-Peter et al., 1998): 1) bind gaseous ligands, such as O_2, NO and CO, to modulate cell metabolism through interaction with other cell molecules, which could modify the rate constants for ligand binding; 2) bind small organic molecules, such as fatty acids, for transport and synthesis processes; and 3) scavenge O_2 based on the extremely tight binding of O_2 or interact with flavoproteins (for example with a ferric Hb reductase) for electron transfer.

Work aimed at the elucidation of nsHb function suggests that these proteins play roles in plant cells by maintaining levels of ATP and modulating levels of NO. Experiments with maize cells transformed with sense and antisense barley Hb cDNA (Hb^+ and Hb^- cells, respectively) showed that ATP levels decreased about 5, 40 and 65% when Hb^+, native (untransformed) and Hb^- maize cells were subjected to microaerobiosis, respectively (Sowa et al., 1998). Thus, it was concluded that a function of nsHbs is to maintain the energy *status* of cells under low O_2-concentrations. Also, work from the same laboratory showed that substantial amounts of NO were produced in alfalfa root cultures maintained under hypoxic (3% O_2) conditions, and that the amount of NO accumulated in alfalfa cells underexpressing Hb (Hb^- lines) was 2.5-fold higher than that in overexpressing barley Hb (Hb^+ lines) (Dordas et al., 2003a). It has been claimed that *Arabidopsis* nsHb1 scavenges NO and that it reduces levels of NO under hypoxic stress (Perazzolli et al., 2004). From these and others' observations it was concluded that a function of nsHbs is to modulate levels of NO (Dordas et al., 2003a; Perazzolli et al., 2004; Igamberdiev et al., 2005; Perazzolli et al., 2006) and, directly or indirectly, regulate a number of NO-dependent procceses in the plant cell.

9. Concluding remarks and future directions

Plant Hbs were described for the first time more than six decades ago. Since then substantial amounts of information about their structure, function and evolution have accumulated. However, there is still plenty of work to be done for a complete characterization and understanding of these plant proteins. For example, it is widely accepted that a function of Lb in nodules is to facilitate the diffusion of O_2 to the respiring bacteroids, however the mechanism for O_2-delivery from oxygenated Lb to the peribacteroid membrane is not yet known. It is well documented that Hbs interact with proteins (Low, 1986; Nagata et al., 1995; Park et al., 2002) and membrane lipids (Bonamore et al., 2003; Wakasugi et al., 2004; Rinaldi et al., 2006), thus it is of interest to evaluate whether or not Lb interacts with peribacteroidal membranes and if this interaction promotes O_2-delivery from oxygenated Lb. Also, primitive *nshb* genes could be selected within a particular plant family to help develop primitive symbiosis. Therefore, it is of interest to analyze primitive legumes to identify the ancestral *nshb*/*lb* that evolved into a functional symbiotic Hb.

Non-symbiotic Hbs have been characterized in detail. For example, *in vitro* analysis showed that O_2-affinity of (class 1) nsHbs is very high, mostly due to a very low O_2-dissociation constant. Thus, it is of interest to evaluate whether or not the O_2-affinity of nsHbs is also high *in vivo*, otherwise it is possible that nsHbs function as O_2-carriers or -sensors in plant tissues (Appleby et al., 1988). It is known that *nshb* genes (over)express in specific plant tissues (including organs from plants subjected to stress conditions), however details about the regulation of *nshb* gene expression are still not known. This information is essential to understand the role that nsHbs play in the physiology of a plant cell. A number of potential promoters have been identified upstream of the rice *hb1–4* genes (Lira-Ruan et al., 2002), thus it is likely that the regulation of *nshb* genes is complex. However, it has been shown that the expression of an *Arabidopsis nshb* gene is enhanced by the addition of the cytokinin 2iP (6-[γ-γ-dimethylallylamino]purine) (Hunt et al., 2001) and that a cytokinin-regulated element modulates the expression of rice *hb2* (Ross et al., 2004). This data shows that the expression of *nshb* genes is mediated by plant hormones, thus it is of interest to identify plant hormones that affect the synthesis of nsHbs and the role that these proteins play in the physiological/metabolic processes that are hormonally modulated.

Finally, compared to Lbs and nsHbs, little is known about the function of plant 2/2-like Hbs. However, it was postulated that these proteins originated (by HGT) from a bacterial tHb (Vinogradov et al., 2006), so it is likely that properties of plant 2/2-like Hbs and some bacterial tHbs are similar. Several lines of evidence suggest that a function of microbial tHbs is to detoxify NO (Ouellet et al., 2002; Milani et al., 2003; Giangiacomo et al., 2005; Sarma et al., 2005), thus it is of interest to evaluate whether or not plant 2/2-like Hbs participate in the metabolism of NO in plant cells. For example, (reduced) plant 2/2-like Hbs may bind O_2 and NO, dioxygenate NO and generate NO_3^- and ferric Hb. For this process, it is of interest to identify mechanisms that reduce ferric to ferrous plant 2/2-like Hb (which will subsequently bind O_2 for dioxygenation of NO), as has been documented for the reduction of ferric to ferrous Lbs (Lee and Klucas, 1984; Saari and Klucas, 1984, 1987; Klucas et al., 1988; Becana and Klucas, 1990; Ji et al., 1991).

In summary, the diversity of interactions of plant Hbs with molecules suggests that Hbs are multifunctional proteins that are essential for the functioning of plant cells. If this is true, Hbs have played an important role during the evolution and adaptation of plants to land environment.

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