Mini Review

Plant hemoglobins: Important players at the crossroads between oxygen and nitric oxide

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ABSTRACT

Plant hemoglobins constitute a diverse group of hemeproteins and evolutionarily belong to three different classes. Class 1 hemoglobins possess an extremely high affinity to oxygen and their main function consists in scavenging of nitric oxide (NO) at very low oxygen levels. Class 2 hemoglobins have a lower oxygen affinity and they facilitate oxygen supply to developing tissues. Symbiotic hemoglobins in nodules have mostly evolved from class 2 hemoglobins. Class 3 hemoglobins are truncated and represent a clade with a very low similarity to class 1 and 2 hemoglobins. They may regulate oxygen delivery at high O_2 concentrations. Depending on their physical properties, hemoglobins belong either to hexacoordinate non-symbiotic or pentacoordinate symbiotic groups. Plant hemoglobins are plausible targets for improving resistance to multiple stresses.

1. Introduction

When we think of hemoglobin we immediately remember oxygen transport within erythrocytes in the circulatory system of blood. It may be therefore surprising that non-blood containing plants also encode hemoglobins. The name hemoglobin comes from the globular structure associated with heme prosthetic group which binds oxygen. However, hemoglobins not only bind oxygen but also other ligands such as nitric oxide (NO), carbon monoxide (CO), hydrogen sulfide (H_2S) and even organic molecules such as certain membrane lipids [1,2]. The concentration of hemoglobin in erythrocytes is in the order of ten millimolar whilst that of myoglobin in the skeletal muscle is in the submillimolar range. In plants, hemoglobin was first studied within symbiotic nitrogen-fixing root nodules, where "leghemoglobin" (legHb) plays a key role transporting free oxygen away from the oxygen-sensitive-nitrogenase enzyme. The concentration of legHb can reach 0.7 mM in nodules rendering them with their characteristic red color. It is also the case than plants produce hemoglobins with a non-symbiotic role but concentrations are usually in the region of 5–20 μM upon induction [3], too low for plants to be red.

Plant hemoglobins constitute a diverse group of hemeproteins and evolutionary belong to three different classes. Class 1 hemoglobins possess an extremely high affinity to oxygen (K_m in the order of 2 nM [4,5]) and their main function seems to be related to scavenging NO [6]. This role appears to be essential as nitric oxide emerged as an important molecule that influences responses to abiotic and biotic stresses [7]. Further, NO controls many developmental processes such as seed dormancy, root development, and transition to flowering [8,9]. Plant acclimation and tolerance to cold is triggered by nitrate reductase-produced NO, which regulates the expression of genes involved in phosphatidic acid synthesis and sphingolipid phosphorylation. Class 1 hemoglobins modulate this response by scavenging NO [10].

Class 2 hemoglobins have a lower oxygen affinity (K_m of the order of 150 nM [11,12]) and their function is related to facilitating oxygen supply to developing tissues [12,13]. Symbiotic hemoglobins in nodules have mostly evolved from class 2 hemoglobins. As such they can be considered as a subclass of class 2 that have acquired specific structural properties to support symbiosis with nitrogen-fixing bacteria via buffering of oxygen concentration. It should be noted that a few symbiotic hemoglobins (sHb), such as Parasponia Hb, have originated from class 1, which are classically thought to have non-symbiotic roles (nsHb). Class 3 hemoglobins (truncated version; trHb) represent a group with a very low similarity to class 1 and 2 hemoglobins and having low affinity to O_2

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($K_m \sim 1500$ nM) [14]. Their function is obscure but may be related to regulation of oxygen delivery at high O_2 concentrations [14]. We will now further elaborate on the structure-functional and evolutionary basis of the plant hemoglobin classes.

2. Non-symbiotic plant hemoglobins are hexacoordinate while symbiotic are pentacoordinate

Biochemically, hemoglobins can be divided into two groups based on coordination of heme iron and this also corresponds in plants to the division between non-symbiotic and symbiotic hemoglobins. The heme prosthetic group contains an iron atom with four of the six coordination sites occupied by the heme pyrrole nitrogens. It is further attached to histidines of the globin moiety through coordination of either one or two histidine side chains [15] (Fig. 1). Those hemoglobins with one histidine coordinating the fifth site of the heme iron, leaving the sixth open for exogenous ligand binding, are designated "pentacoordinate" (examples include the erythrocyte hemoglobin and other oxygen transporters).

Those in which the iron coordinates with both the proximal and distal histidine, similarly to the heme active site of cytochrome b_5, are called "hexacoordinate hemoglobins". While the first (proximal) histidine is bound tightly to the heme iron, the coordination of the second (distal) histidine is reversible and differs significantly, allowing for binding of exogenous ligands such as oxygen, carbon monoxide, and nitric oxide. In most cases, hexacoordinate hemoglobins contain certain portion of molecules with the heme in a pentacoordinated state. Pentacoordinate hemoglobins are adapted to the function of oxygen buffering and delivery because the pentacoordinate state allows oxygen to bind reversibly giving optimal condition for storage and transportation functions.

3. Three classes of plant hemoglobins

While the classification of plant hemoglobins based on coordination of iron and on symbiotic/non-symbiotic functions is useful it does not fully reflect their evolutionary origin and structural properties. Since the discovery of non-symbiotic plant hemoglobins [16,17] which followed after the description of the unusual symbiotic hemoglobin from Parasponia by Appleby et al. [18], other globins genes were found in plants [19]. As a result all plant hemoglobins were classified into three distinct groups based on their evolutionary origin and physical behavior. Phylogenetic analyses readily differentiate plant hemoglobins into three different clades (Fig. 2) which correspond to class 1, class 2, and truncated (class 3) hemoglobins. In two of these clades (class 2 and to less extent class 1) the evolution was observed from nsHb hexacoordinate towards sHb pentacoordinate hemoglobins. The class 2 nsHb

has the highest hexacoordination level whereas the degree of coordination in class 1 nsHb is much lower.

Gene sequence analysis of class 1 and 2 plant hemoglobins with other hemoglobins show that they are distantly related to animal myo- and hemoglobin whereas the primary structure of the class 3 truncated type of (trHbs) suggests that this class is closer related to bacterial hemoglobins [14]. These proteins (class 3) share 40–45% sequence similarity with bacterial hemoglobins of the "2-on-2" structural motif, and likely appeared in plants due to a horizontal gene transfer from bacteria. Although they are characterized by "2-on-2" motif (the fold is based on a "2 on 2" α-helical sandwich) as compared to "3-on-3" sandwich of other plant hemoglobins (and therefore called truncated), in plants they actually have longer amino acid chain than the class 1 and 2 hemoglobins [4].

The relationship of class 1 and 2 plant hemoglobins with animal myo- and hemoglobins is confirmed by structural analysis. The molecular structure of barley or rice nsHb (class 1) and soybean sHb (class 2) shows a remarkably similarity with animal myo- and hemoglobin [20]. The relationship is also confirmed by the intron–exon structure of the genes. Animal myo- and hemoglobin genes have two conserved introns in common with nsHb and sHb. However nsHb and sHb have an additional third intron between the two conserved introns. Interestingly such a third central intron is also found in the genes of neuro- and cytoglobins of vertebrates (globins expressed in neural and brain tissue, most in marine mammals, and may offer protection against the hypoxia) and in hemoglobin genes from invertebrates [21,22]. This indicates that the three introns is a more ancient version of the gene, and that the third central intron was lost in the evolution of animal myo- and hemoglobins. Early comparisons of animal hemoglobin molecular structure with intron–exon predicted that each intron represents a protein sub-domain and that a third central intron had been lost during evolution. The relationship in molecular structure and DNA sequences of class I and II plant nsHbs with animal myo- and hemoglobin suggests that they are derived from a common primordial hemoglobin gene in a eukaryotic ancestor. Such a common unicellular ancestor of plants and animals is estimated to have lived 1500 million years ago [23].

4. Class 1 plant hemoglobins

4.1. Structural and functional properties of class 1 non-symbiotic hemoglobins

The common property of non-symbiotic class 1 hemoglobins that distinguishes them from other hemoglobins is a low value of the hexacoordination equilibrium constant (K_H), which is the bind-
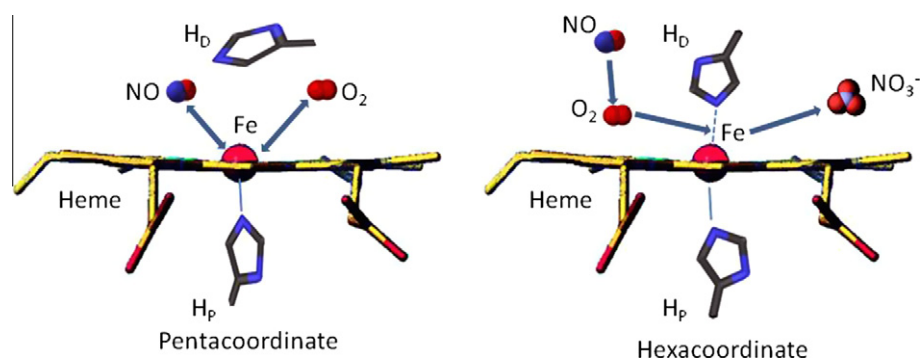

Fig. 1. Coordination of proximal (H_P) and distal (H_D) histidines in pentacoordinate and hexacoordinate heme. The pentacoordinate structure is open for reversible binding of ligands such as O_2 and NO, while the hexacoordinate structure facilitates tight binding of oxygen that can further accept an electron from iron and oxygenate NO resulting in formation of nitrate (NO_3^-).
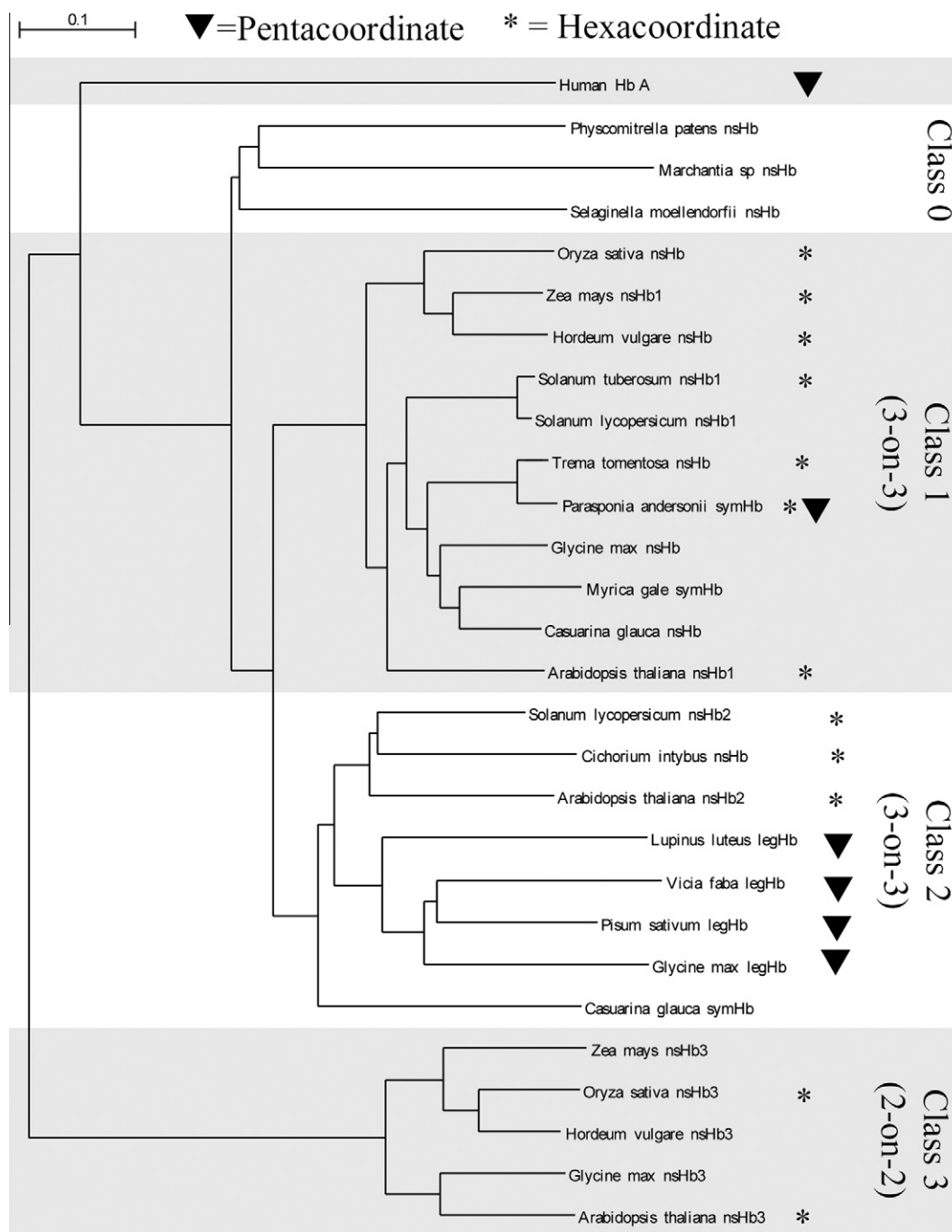
Fig. 2. The evolutionary tree of plant globins showing that they are classified into three "classes", each containing hexacoordinate members (marked by asterisks). Evolution to the symbiotic hemoglobins (which include leghemoglobins) corresponded to the structural transition to pentacoordinate state (marked by triangles). For non-marked globins the coordination state is not reported. Parasponia Hb has a transitional structure exhibiting hexacoordination in one subunit and pentacoordination in another.

ing constant of the distal histidine, allowing the equilibrium of pentacoordinated and hexacoordinated species and facilitating the binding of ligands. This weak hexacoordination causes an extremely high avidity to oxygen [5]. The dissociation constant of oxygen is in the nanomolar range and it follows from the rapid oxygen binding resulting in protein conformational changes that prevent fast dissociation from the heme site. Upon ligand binding the distal histidine moves away from the iron atom resulting in an overall more stable conformation [20]. This allows a very tight, slowly reversible binding of O_2, which is optimal for oxygen-dependent NO scavenging under near-anaerobic conditions – a well-described function for nsHb [24]. This function, however,

cannot be efficient without an associated reductase that converts the ferric form of Hb; arising from the reaction with NO, back to the ferrous form [25]. Following Hb binding of O_2, steric interactions encourage the reaction to form nitrate [26]. Structurally the non-symbiotic class 1 hemoglobins are protein dimers consisting of two identical subunits and they contain one or two cysteine residues per monomer.

Class 1 hemoglobins are characterized by an increased rate of Fe^{3+} reduction which is most probably mediated by a cysteine residue. This cysteine can form a reversible covalent bond between two monomers as shown by mass spectrometric analysis and, in addition to its structural role, also prevents the molecule from

autoxidation [27]. While the direct reduction of heme iron can be facilitated by ascorbate, the additional mechanism mediating this reduction and keeping the molecule in the ferrous state involves a cysteine residue and controlled by sulfhydryl reagents such as reduced glutathione [28].

The structural properties of class 1 hemoglobins allow them to serve as soluble electron transport proteins in the enzymatic system scavenging nitric oxide (NO) produced in low oxygen conditions primarily via reduction of nitrite in plants [29] (Fig. 3). During oxygenation of nitric oxide to nitrate, oxidized ferric hemoglobin is formed (methemoglobin), which can be reduced by an associated reductase. In line with the effectiveness of ascorbate as a reducing agent for ferric class 1 Hb, the cytosolic monodehydroascorbate reductase has been shown to act as an effective facilitator of the NO scavenging reaction [25]. Functionally, in terms of participation in electron transport, class 1 hemoglobins resemble cytochromes more than other classes hemoglobins, the main distinction of class 1 hemoglobins from cytochromes being their solubility, compared to cytochromes anchored to membranes.

NO is produced at high levels during the hypoxic conditions and class 1 nsHbs are able to scavenge it thus reducing the NO levels. By doing so class1 nHbs are involved in redox balance in hypoxic conditions and contributes to the flooding tolerance by increasing NADH recycling. By using spin trap techniques, Dordas et al. [29] showed that NO accumulation in alfalfa root cultures reached 120 nmol g^{-1} of fresh weight after 24 h incubation being at least 1.5 times higher in the hemoglobin down-regulating line, while in aerobic conditions, NO accumulation was at least two orders of magnitude lower. Class 1 hemoglobins are not only induced under hypoxia but also by high nitrite or nitrate concentrations that lead to the increased NO production [30,31]. In Arabidopsis seeds, Hb1 overexpression resulted in low levels of endogenous NO, in maintenance of a high energy status, and in higher fresh weight [32]. A coordinated spatiotemporal regulation of hemoglobin and nitrate reductase has been shown in maize roots in response to nitrate fertilization [31].

Oxygenated class 1 hemoglobins reacting with NO to produce nitrate represent the main mechanism by which NO is scavenged in plants [25]. While other hemoglobins have much higher dissoci-

Fig. 3. Scavenging of nitric oxide (NO) by class 1 hemoglobin. During this reaction, NO is converted to nitrate (NO_3^-) by the oxygenated ferrous (Fe^{2+}) hemoglobin (Hb), which turns to the MetHb (ferric, Fe^{3+}) form. The latter can be reduced by a corresponding reductase (MetHbR) and oxygenated again. Nitrate is converted to nitrite (NO_2^-) by nitrate reductase, while nitrite can form NO in reactions of hemeproteins and other redox systems possessing nitrite: NO reductase (NiNOR) activity.

ation constants for oxygen and can be used for oxygen transport or storage, class 1 hemoglobins induced in plants under hypoxia, bind oxygen very tightly ($K_d \sim 2$ nM). As a result they can effectively scavenge NO at oxygen levels far below the saturation of cytochrome *c* oxidase ($K_m \sim 150$ nM), while the reverse reaction of production of NO from nitrite by these hemoglobins can take place only at concentrations of oxygen below nanomolar [33], which likely cannot be reached even in oxygen-depleted tissues. The mechanism of NO scavenging involving class 1 hemoglobin results in a formation of a non-toxic product (nitrate), is operative at extremely low oxygen concentrations, and represents a critical step in plant survival under hypoxia and most likely other stress scenarios where high concentrations of NO are produced [34].

4.2. Evolution of class 1 hemoglobins towards symbiosis

The identification of an NO activated oxygen scavenging function of hemoglobin in the parasitic nematode *Ascaris lumbricoides* led to the suggestion that the oxygen transporting and storing function of animal hemoglobins evolved from a primordial NO detoxification function [35]. A similar evolutionary transition has happened independently in plants, where hemoglobins with a NO catalyzing function evolved into symbiotic hemoglobins with a primary function in oxygen binding and storage. In the class 1 group of hemoglobins examples of evolution toward symbiosis, although not as widespread as in the class 2 group, are also observed. These are recent emergences compared to legHbs at 200 million years ago and as a result legHbs share only ~40% sequence identity with non-symbiotic class 2 Hbs [36]. This evolutionary tendency is observed in the family of *Cannabaceae* in two genera, *Trema* and *Parasponia* [36,37]. In fact, the hemoglobin from *Trema tomentosa* was the first non-symbiotic hemoglobin discovered in plants in 1980s [16] with the class 1 hemoglobin from barley characterized only a few years later [17].

Trema hemoglobin is characterized by a high oxygen affinity and slow oxygen dissociation rate common for class 1 hemoglobins. The symbiotic hemoglobin from *Parasponia andersonii*, which can form rhizobial symbioses, shares 93% amino acid identity to Trema hemoglobin, indicating a recent divergence, but has characteristics common to oxygen transport hemoglobins in having a pentacoordinate ferrous heme iron, moderate oxygen affinity, and a relatively rapid oxygen dissociation rate constant [37]. Unlike the evolution of class 2 nsHbs to legHbs (both monomeric proteins), which related to an increase of O_2 affinity and an increase of O_2 dissociation constant, the emergence of Parasponia Hb resulted in lower O_2 affinity, faster O_2 dissociation, and tight dimeric structure [37]. This dimeric structure confers a 50 fold greater oxygen affinity than the Trema Hb. In the ferric state, Parasponia Hb acquires an unusual dissymmetric structure with a hexacoordinate heme in one subunit and pentacoordinate in another [37]. The evolution towards symbiosis is also observed in *Myrica gale*, the plant from the related but distant Myricaceae family that exhibits actinorhizal symbiosis. This plant expresses the hemoglobin evolved from the non-symbiotic class 1 precursor and interestingly is capable of playing both symbiotic and non-symbiotic roles [38].

The observed characteristics for *Trema* and *Parasponia* hemoglobins demonstrate distinct mechanisms for convergent evolution of oxygen transport in different phylogenetic classes of plant hemoglobins. This means that the evolution towards symbiosis occurred both in class 1 and 2 hemoglobins, and that in plants pentacoordinate hemoglobins evolved always from the hexacoordinate [39]. This occurred in opposite to the observed evolutionary trend in which hexacoordinate hemoglobins evolved from pentacoordinate. The cloning of a hemoglobin gene from the ancient legume *Chamaecrista fasciculata* similarly suggested that the transition
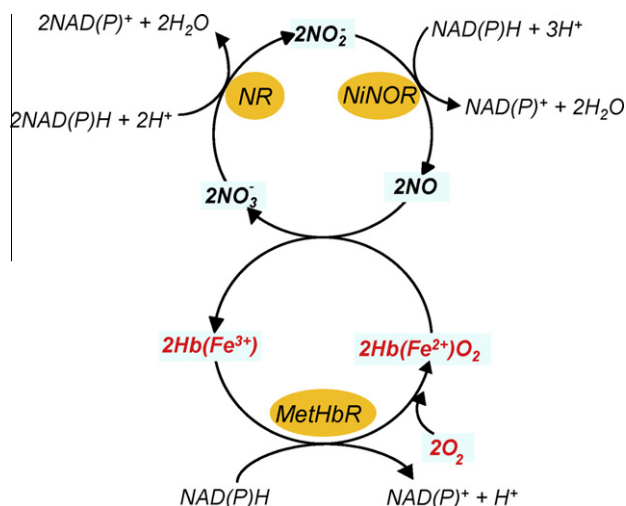
from nsHb to legHb involved transition from hexa- to pentacoordination and compaction of protein structure [40].

The cloning of a moss nsHbs from mosses *Ceratodon purpureus* and *Physcomitrella patens* suggests that class 1 and 2 nsHb evolved from a hexacoordinate nsHb with low O_2 dissociation rate [41]. The hemoglobins from mosses (bryophytes) and other lower higher plants (such as Selaginella) have a similarity to class 1 hemoglobins but they are more ancient and diverged early after separation of class 1 and 2 hemoglobins. They can be defined as class 0 hemoglobins. All this suggests that various plant hemoglobins are good 'molecular fossils' representing different steps in the evolution from primordial hexacoordinate hemoglobins with alternate functions (such as NO scavenging) to pentacoordinate symbiotic hemoglobins optimized for oxygen storage and transport.

5. Class 2 plant hemoglobins

5.1. Non-symbiotic class 2 hemoglobins

In contrast to class 1 nsHbs, the class 2 nsHbs are usually not induced under hypoxic conditions [42] but their overexpression can promote the survival under hypoxia [39]. Class 2 Hbs are characterized by a tighter hexacoordination than class 1 nsHbs and thus they have lower oxygen affinities (in the order of 100–200 nM). This makes them less efficient in NO scavenging but increases the possibility of functions related to sensing low levels of oxygen and to oxygen storage and diffusion [39]. The oxygen-binding characteristics of class 2 hemoglobins are comparable to leghemoglobin, and an affinity to oxygen resembles the K_m of cytochrome oxidase, which means that their role in facilitating oxygen diffusion is quite likely [12,13]. While class 1 Hbs possess two docking sites for small ligands permitting NO and O_2 to react, the only one site can be occupied for class 2 Hbs also making NO dioxygenase reaction for these Hbs less favorable [26].

Overexpression of class 2 hemoglobin in transgenic Arabidopsis (AtHb2) plants led to a 40% increase in the total fatty acid content of developing and mature seeds and to the elevated unsaturation/saturation index of total seed lipids [12]. The increase in fatty acid content was mainly due to a stimulation of the rate of triacylglycerol synthesis, which was attributable to a 3-fold higher energy state and a 2-fold higher sucrose content of the seeds. Under low external oxygen, AtHb2 overexpression maintained an up to 5-fold higher energy state and prevented fermentation. These results suggested that a specific function of class 2 hemoglobin is in seed oil production and in promoting the accumulation of polyunsaturated fatty acids by facilitating oxygen supply in developing seeds. A similar function related to a facilitated oxygen supply can be attributed not only to seeds but also to other plant tissues expressing class 2 hemoglobin.

5.2. Leghemoglobins

LegHb was the first plant hemoglobin discovered. It was described by Kubo [43] in 1939 and for a half of century remained the only known plant hemoglobin. It is present in root nodules of the nitrogen-fixing plants and plays an important role in symbiotic nitrogen fixation. LegHb has similar structures to mammalian myoglobins which are involved in oxygen storage. LegHb promotes oxygen diffusion from atmosphere to *Rhizobium* bacteria which are engaged in nitrogen fixation inside the nodules. Actual nitrogen fixation within nodules is catalyzed by nitrogenase ($N_2 + 8H^+ + 8e^- + 16\,ATP \rightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,Pi$) which must function at low oxygen tensions because oxygen represents an alternative electron acceptor. Therefore LegHb quenches the oxygen and delivers it to *Rhizobium* in low concentration and by doing so it fulfills a vital role in nitrogen fixation in leguminous plants and thus aids in improving soil fertility [44]. During symbiosis, the expression of non-symbiotic hemoglobins of all three classes is also enhanced pointing out complementary role of all types of hemoglobins and indicating that they are also required for symbiosis [45]. This may be related to fine adjustments of concentrations of oxygen and NO in symbiotic nodules to achieve high metabolic rates of nitrogen fixation.

6. Class 3 plant hemoglobins (truncated)

The class 3 plant Hb was originally described by Watts et al. [14]. They showed that this protein in *Arabidopsis thaliana* exhibits unusual concentration-independent binding of O_2 and CO. The protein, which is pentacoordinate in oxygenated state, forms a transient hexacoordinate structure after reduction and deoxygenation, which slowly converts to a five-coordinate structure. In *A. thaliana*, the truncated Hb is expressed throughout the plant but responds to none of the treatments that induce plant 3-on-3 (class 1 and 2) Hbs. It has been suggested that because of a lower O_2 affinity the truncated Hb in Arabidopsis might be an O_2 transport protein with a moderate O_2 affinity (50% saturation at 1500 nM, i.e., at 10 times higher O_2 level than for Hb2 and few hundred times higher than for Hb1 [14]). The deoxygenation of the truncated Hb leaves the protein in a hexacoordinate state with an endogenous (i.e., amino acid side-chain) or uncharacterized exogenous ligand (i.e., not O_2, CO, or NO) bound to the sixth bond of the heme iron molecule. This state is transitory, reverting to a five-coordinate form in ~20 min. This extremely slow conversion has not been observed with any other Hb.

The trHb group is maintained through evolutionary processes so presumably contributes towards plant fitness but their role remains obscure. In bacteria, downregulation of a truncated Hb results in a sensitivity to high solution oxygen pressure, to H_2O_2, and to a nitrosating agent [46]. The Arabidopsis trHb is expressed both in roots and shoots, but is suppressed rather than induced, as in other classes, when oxygen concentrations go down [14]. This corresponds to a low O_2 affinity of the trHbs and argues against their role in responses to hypoxia. Interestingly, in *Medicago truncatula* both trHbs (*MtTrHb1* and *MtTrHb2*) are induced in nodules and upon arbuscular mycorrhizal associations [47]. *MtTrHb1* expression in root nodules matched that of sHbs whilst *MtTrHb2* is active in root nodule base and vascular tissues and mycorrhizal roots. Based on these patterns it has been proposed that the function of these truncated Hbs involves the suppression of defence processes against symbioses and may be based on NO scavenging. The same role has been suggested for the truncated hemoglobin found in the Frankia-induced nodules of the actinorhizal plant *Datisca glomerata* [48]. Clearly, this function needs to be substantiated through (for example) mutational analysis as it is unclear why trHb rather than nsHb should be exploited in this role.

7. Conclusion

Plant hemoglobins are a wide group of proteins containing a heme structure. They are involved in the maintenance of redox balance and the energy state in cells. Their functions are achieved by different mechanisms, such as facilitation of oxygen delivery, transport and buffering of oxygen levels (legHbs evolved from class 2 nsHbs), nitric oxide scavenging (class 1 Hbs), and by other yet uncharacterized ways (trHbs). Evolutionary plant hemoglobins belong to three different clades (class 1, class 2 and truncated), while by their physical and functional properties they belong either to hexacoordinate non-symbiotic or pentacoordinate symbiotic groups. Since the main functions of plant hemoglobins are structurally determined by their affinities and ligand-binding properties

to O_2 and NO, they are important players on the crossroad between these two gases, and their functions are either more related to NO turnover (class 1) or to O_2 delivery and buffering (class 2). Yet these functions have species-specific and stress-related features that have to be established in each particular case, and there are significant gaps in understanding functional roles of plant Hbs, particularly this concerns the truncated (class 3) Hbs and class 2 nsHbs. Much more research is required on hemoglobin function but, given the current state of knowledge, one conclusion of this overview is that the manipulation of plant hemoglobins via genetic modification or through allele screens in plant germplasm population could improve plant resistance to multiple stresses and also increase sustainable plant productivity under changing environmental conditions.

Acknowledgements

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