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Biochemical and Physiological Characterization of Nonsymbiotic Plant Hemoglobins

Nélida Leiva Eriksson

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DOCTORAL DISSERTATION
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Abstract

Hemoglobins (Hb) are usually associated with blood in humans. However, these proteins are widely distributed among living organisms. In plants the most known group are the leghemoglobins. Still, other Hbs that not participate in symbiosis are also found. They are known as nonsymbiotic Hbs (nsHbs). NsHbs are divided into class-1 and class-2. In this thesis three nsHbs from sugar beet (BvHb1-1, BvHb1-2, and BvHb2) and one class-1 nsHb from poplar (PttHb1) have been studied. Additionally, the possibility of using nsHbs for biotechnological application is explored. A holistic expression study of the three *BvHbs* was done. Following the recombinant production of the three BvHbs, their kinetics and binding affinities to oxygen and CO were determined. Concerning PttHb1, its role as a NO scavenger was investigated by expressing it in roots and mutant yeast. We conclude that, even though differential roles have been given to both class-1 and class-2 nsHbs, a specific function can not be given to each group. On the contrary we found that for some nsHbs their roles may be complementary and overlapping. However, some class-1 nsHbs seem to have a clear NO dioxygenase function. In this study we demonstrate that PttHb1 alleviates NO toxicity in cells when expressed together with PtthFNR, a reductase. To finalize, given that nsHbs have a variety of potential roles, the possibility of being interesting biotechnological targets is studied. Early results regarding the expression of class-2 nsHbs in the model plant *A. thaliana* and in a potential crop *Lepidium campestre* are presented.

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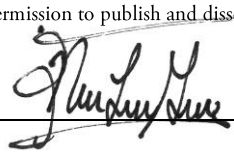
Signature  Date: September 3rd, 2014

Biochemical and Physiological Characterization of Nonsymbiotic Plant Hemoglobins

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Cover: Sugar beet plant (www.hancockseed.com), purified recombinant BvHbs (BvHb1-1, BvHb1-2, and BvHb2), and tertiary structure model of BvHb2.

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For you, who is always watching over us.

Para ti, que desde el cielo siempre nos cuidas.

Popular Summary

People normally associate hemoglobin with blood. It is true that the iron in the center of the hemoglobin molecule makes it excellent at carrying oxygen in the bloodstream from the lungs to every part of the body. However, seventy five years ago a hemoglobin was found in a plant. And plants do not have blood. So what is hemoglobin doing in plants? What are its functions in these sessile, oxygen-producing organisms? The first plant hemoglobin was found in the root nodules of soybean, a legume plant. Since legumes form symbiosis with soil-living bacteria, the hemoglobins found in these plant species became known as symbiotic hemoglobins. Their role is to deliver oxygen to bacteria to stimulate their growth. In return, the bacteria give nitrogen to the plant. About forty five years later, other types of hemoglobins were found in non-legume plants and hence they became known as nonsymbiotic hemoglobins. These new hemoglobins are present in all plants, including legumes. After analyzing their DNA sequence they were divided into three groups, class-1, class-2, and class-3. So, in total four different types of hemoglobins can be found in plants

Since plants neither have blood nor a system to transport oxygen in the same way as in humans, why do plants need all these different types of hemoglobin? Researchers have been trying to answer this question for many years and some advances have been done. For example, it is clear that all plant species have the class-3 nonsymbiotic hemoglobin, which is very similar to the hemoglobins found in bacteria. Also, it is well accepted that symbiotic hemoglobins evolved from the class-2 group of nonsymbiotic hemoglobin, and that class-2 evolved from the class-1 group. Regarding their role, it seems like nonsymbiotic hemoglobins do not transport oxygen. They hold on to it too strong and do not release it easily. Instead, it has been suggested that these hemoglobins bind nitric oxide, a signaling molecule produced by the plant; for instance, when it is under stress.

In this thesis we have mainly studied the two first classes of nonsymbiotic hemoglobins in sugar beet. We chose this plant because of its economical importance in Sweden and Europe. Also, because it belongs to a family where hemoglobins have not been studied at all. We started by identifying all the hemoglobin genes present in its genome. We found four different hemoglobins, two from class-1 and one from each of class-2 and class-3. Once identified, the gene expression of both class-1 and the class-2 hemoglobins in sugar beet plants was determined. Thanks to the information about their DNA sequence, the three hemoglobin proteins were

produced in the lab to be able to characterize them. Their capacity to bind both oxygen and carbon monoxide was analyzed. Additionally, a bacterial hemoglobin and two class-2 nonsymbiotic hemoglobins were expressed in seeds of *Arabidopsis thaliana* and *Lepidium campestre* in order to evaluate the effect on their oil content. Finally, a class-1 nonsymbiotic hemoglobin from aspen was characterized and its role as a NO scavenger determined.

Even though some preferential roles have been given to both class-1 and class-2 nonsymbiotic hemoglobins, we conclude that a specific function can not be given to them. A specific role can not be given to a specific class either, as their role can either complement or overlap depending on the plant species. Since the diversification of plant hemoglobins has followed the evolution of plants they need to be studied in a diverse variety of species. Only in this way it will be possible to determine their role in plants. On the other side, the variety of roles these proteins may have open up great research and biotechnological possibilities as they may play key roles within a plant cell. Hemoglobins are a clear example of successful protein adaptation which allowed them to keep on with the evolution of species all the way from bacterial ancestors to humans.

Abstract

Hemoglobins (Hb) are usually associated with blood in humans. However, these proteins are widely distributed among living organisms. In plants the most known group are the leghemoglobins. Still, other Hbs that not participate in symbiosis are also found. They are known as nonsymbiotic Hbs (nsHbs). NsHbs are divided into class-1 and class-2. In this thesis three nsHbs from sugar beet (BvHb1-1, BvHb1-2, and BvHb2) and one class-1 nsHb from poplar (PttHb1) have been studied. Additionally, the possibility of using nsHbs for biotechnological application is explored. A holistic expression study of the three *BvHbs* was done. Following the recombinant production of the three BvHbs, their kinetics and binding affinities to oxygen and CO were determined. Concerning PttHb1, its role as a NO scavenger was investigated by expressing it in roots and mutant yeast. We conclude that, even though differential roles have been given to both class-1 and class-2 nsHbs, a specific function can not be given to each group. On the contrary we found that for some nsHbs their roles may be complementary and overlapping. However, some class-1 nsHbs seem to have a clear NO dioxygenase function. In this study we demonstrate that PttHb1 alleviates NO toxicity in cells when expressed together with PtthFNR, a reductase. To finalize, given that nsHbs have a variety of potential roles, the possibility of being interesting biotechnological targets is studied. Early results regarding the expression of class-2 nsHbs in the model plant *A. thaliana* and in a potential crop *Lepidium campestre* are presented.

List of Papers

The thesis is based on the following publications, referred to in the text by Roman numerals. The publication and manuscripts are appended to the thesis.

I. **Differential Expression Patterns of Non-symbiotic Hemoglobins in Sugar Beet (*Beta vulgaris* ssp. *vulgaris*).** Nélida Leiva Eriksson, Pierre A. Pin, Thomas Kraft, Juliane C. Dohm, Andre E. Minoche, Heinz Himmelbauer, and Leif Bülow. 2014. *Plant Cell Physiology* (2014) 55 (4): 834-844. DOI: 10.1093/pcp/pcu027.

II. **Kinetic and Structural Properties of Recombinant Sugar beet (*Beta vulgaris* spp. *vulgaris*) Nonsymbiotic Hemoglobins** Leiva Eriksson N, Reeder B, Wilson M, and Bülow L. *Submitted* 2014.

III. **Direct electrochemistry and bioelectrocatalysis of a class II nonsymbiotic plant haemoglobin immobilised on screen-printed carbon electrodes.** Chekin F, Leiva N, Raoof JB, Gorton L, Bulow L. 2010. *Analytical and Bioanalytical Chemistry.* 398(4): 1643-1649.

IV. **Poplar Cytosolic Ferredoxin NADP$^+$ Oxidoreductase Interacts with Haemoglobin 1 to Alleviate Nitric Oxide Sensitivity.** Soile Jokipii-Lukkari, Alexander J. Kastaniotis, Robin Sundström, Vimal Parkash, Nélida Leiva Eriksson, Yvonne Nymalm, Olga Blokhina, Eija Kukkola, Kurt V. Fagerstedt, Tiina A. Salminen, Esa Läärä, Leif Bülow, Steffen Ohlmeier, J. Kalervo Hiltunen, Pauli T. Kallio and Hely Häggman. *Submitted* 2014.

V. **Utilizing Plant and Bacterial Hemoglobins to Alter the Fatty Acid Profile in Seeds of *Arabidopsis thaliana* and *Lepidium campestre*.** Leiva Eriksson, Nélida; Nelin Jonas; Eriksson Dennis; Zhu Li-Hua; Stymne Sten; and Bülow, Leif. *Manuscript.*

My contribution to the papers:

Paper I	I took a major part in planning, performed some of the *in silico* analysis and wrote the manuscript.
Paper II	I took a major part in planning, performed most of the experimental work, analyzed the results and wrote the manuscript.
Paper III	I took a major part in planning, expressed and purified the protein, and co-wrote the manuscript.
Paper IV	I performed the expression and purification of both Hemoglobin 1 (PttHb1) and Ferrodoxin $NADP^+$ oxidoreductasa (PtthFNR). Also performed the reduction experiments and co-wrote the manuscript.
Paper V	I took a major part in planning, performed the construction of the transformation cassettes, analyzed the data, and co-wrote the manuscript.

Abbreviations

Å	Ångström
ABA	Abscisic acid
BLAST	Basic Local Alignment Search
BvHb	*Beta vulgaris* hemoglobin
CO	Carbon monoxide
DNA	Deoxyribonucleic acid
EPR	Electron paramagnetic resonance
EST	Expressed sequence tag
Fe^{2+}	Ferrous oxidation state of iron
Fe^{3+}	Ferric oxidation state of iron
Fe^{4+}	Ferryl oxidation state
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
Hb	Hemoglobin
His	Histidine
hxHbs	Hexacoordinate hemoglobins
Lb	Leghemoglobins
LDs	Long days
Mb	Myoglobin
MetHb	Methemoglobin, ferric Hb
Mya	Millions of years ago
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
nm	Nanometers
NO	Nitric oxide
NOD	NO dioxygenase
nsHbs	Nonsymbiotic hemoglobins
O_2	Diatomic oxygen
PttHb1	Primula tremuloides nonsymbiotic hemoglobin class-1
PtthFNR	Primula tremuloides ferrodoxin NADH reductase
ROS	Reactive oxygen species
sHbs	Symbiotic hemoglobins
SDs	Short days
trHbs	Truncated hemoglobins

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Introduction and Aims

Hemoglobins (Hb) are widespread proteins belonging to the globin super family. They can be found in all the kingdoms of living organisms and are mainly recognized as oxygen transporters even though this function corresponds to a relatively recent adaptation in vertebrates (Vinogradov & Moens, 2008).

Hbs in plants were first discovered in root nodules of leguminous plants infected by soil bacteria (Kubo, 1939), collectively known as rhizobia (Garrocho-Villegas *et al.*, 2007). These plant Hbs were named Leghemoglobins (Lbs) (Virtanen & Laine, 1946) but are also known as symbiotic Hbs (sHbs). Later, the identification of a Hb in the root nodules of the non-legume plant *Parasponia* (Appleby *et al.*, 1983) led to the discovery of *Hb* sequences in *Trema* and *Celtis*, both non-nodulating plants (Bogusz *et al.*, 1988). Further, high concentrations of Hb-like proteins were detected in actinorhizal plants (Tjepkema, 1983). The detection of Hbs in *Parasponia*, *Trema* and actinorhizal plants suggested that not only symbiotic plants would have Hbs; with barley being the first crop in which an Hb cDNA was found (Taylor *et al.*, 1994). This finding was followed by the identification of other Hbs in rice (Arredondo-Peter *et al.*, 1997) and *Arabidopsis thaliana* (Trevaskis *et al.*, 1997). As these Hbs were present in nonsymbiotic plants, they are known as non-symbiotic Hbs (nsHbs) (Hunt *et al.*, 2001). Finally, thanks to the numerous plant genome sequencing programs a third group of Hbs in plants were discovered: truncated Hbs (trHbs) (Watts *et al.*, 2001). These trHbs are more similar to bacterial Hbs than to sHbs or nsHbs (Vinogradov *et al.*, 2006).

Hbs from plants have mainly been studied in monocot plants (rice, maize, and barley) and in some dicotyledonous plants from the rosid (tomato, chicory) and asterid (*Arabidopsis*) groups, leaving the caryophyllid apart (Smagghe *et al.*, 2009; Vazquez-Limon *et al.*, 2012). To date, only one nsHb from the caryophyllid group has been added to the study of Hbs evolution in plants (Vazquez-Limon *et al.*, 2012) and no other Hb has ever been studied as a gene or protein, except from the finding of an incomplete expressed sequence tag (EST) in sugar beet (*Beta vulgaris* spp. *vulgaris*) (Hunt *et al.*, 2001).

Most of what is known about plant Hbs comes from wide spread sequencing of genomes as well as biochemical and physical analysis of recombinant proteins. The extensive infrastructure for detailed biophysical research, established for decades to understand how the structures of oxygen transport Hbs work, has naturally welcomed all these newly discovered Hbs. As a result, a wealth of structures, spectroscopic

characterizations, and biochemical investigations have been obtained resulting in a high number of physical analysis that surpasses the amount of physiological studies. As discussed by Kakar *et al.* (2010), the lack of physiological studies is complicating the possibility of retrieving a confident interpretation of the results into clear physiological functions. This, added to the lack of diversity and representation of species, increases the risk of having misled functional hypothesis that deviate from biology.

The aim of this thesis was to investigate both the physiological and biochemical aspects of nsHbs in plants. The aim was accomplished by first identifying and characterizing the nsHb genes in sugar beet **(Papers I, II, and III)**. Sugar beet is a biennial crop whose transition from vegetative to reproductive growth relies on a period of vernalization in between. The expression of three nsHbs in sugar beet plants was analyzed **(Paper I)**. Then, the kinetic and structural features of the three proteins were determined by biophysical, electrochemical, and homology modeling analysis (**Papers II and III**). Finally, more hints about the role of nsHbs were obtained from studies *in vivo* by means of heterologous expression. For this, Hbs from other species such as *A. thaliana*, poplar, and *Vitreoscilla* were added to our studies (**Paper IV and V**).

We conclude that even though some specific roles have been given to both class-1 and class-2 nsHbs, they can not be clearly delineated. On the contrary, they suggest selective pressure for different physiological functions, but also for some role overlapping. Furthermore, the role of nsHbs cannot be precisely categorized in all plant species since overlaps in the sequence, ligand binding rates, and structures can be found. Hence, the classification of both nsHbs classes should be limited to its primary sequence to avoid misleading conclusions. The functional implications of our studies have been very much discussed in light of the different biological roles proposed for nsHbs. This study suggests that the evolution of nsHbs has been under high selective pressure which resulted in a wide variety of Hbs with different properties and capacities. This diversity opens up great research and biotechnological opportunities as plant Hbs may play key roles within a plant cell

I. Plant Hemoglobins

1.1. Classification

In plants, three types of Hbs have been identified: symbiotic (sHbs), nonsymbiotic (nsHbs) and truncated (Kubo, 1939; Taylor *et al.*, 1994; Watts *et al.*, 2001). NsHbs are further classified into class-1 (nsHb1) and class-2 (nsHb2) based on sequence similarity and O_2-affinity (Trevaskis *et al.*, 1997; Smagghe *et al.*, 2009) (Fig. 1).

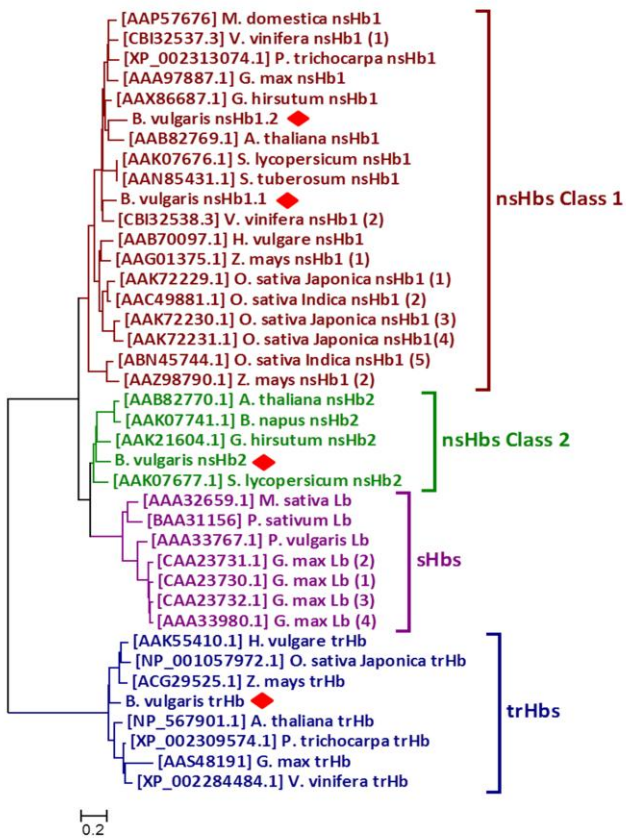

Figure 1. Phylogenetic tree of plant Hbs. All four Hbs in Beta vulgaris (BvHbs) are indicated by a red diamond. The GenBank accession numbers are at the left of each plant Hb (Leiva-Eriksson *et al.*, 2014)

SHbs were the first Hbs discovered in plants as they were easy to find due to their large quantities in root nodules (Fig. 2) (Kubo, 1939). At first, these proteins were found in legumes and that is why they were called Lbs; however, they were later also discovered in non-legumes such as the Ulmaceae *Parasponia andersonii* in symbiosis with *Rhizobium* (Appleby *et al.*, 1983), and dicotyledonous plants such as *Casuarina glauca* in symbiosis with the actinomycete *Frankia* (Tjepkema, 1983). Currently, a much broader term, symbiotic plant hemoglobins, is used when referring to these proteins.

In this plant-bacteria symbiosis, the plant provides the bacteria with sugars while the bacteria create ammonia from nitrogen in the air, which is used by the plant to form amino acids and nucleotides. The role of sHbs in this relationship is relatively well described. They carefully regulate the oxygen levels in the root nodule by facilitating its diffusion for nitrogen-fixation, and at the same time they keep a low free oxygen concentration to avoid the inhibition of the oxygen-sensitive-bacterial nitrogenase enzyme (Appleby *et al.*, 1988)

Figure 2. Root nodules in Medicago italica. They are formed when nitrogen fixing bacteria enter the cells of a host plant. The high amount of sHbs is clearly visible as judged by their bright red color. Figure adapted from www.wikipedia.org (Author: Ninjatacoshell, CC BY-SA 3.0)

Unlike sHbs, which are found in root nodules, nsHbs are well distributed in diverse organs of bryophytes and angiosperms along all their developmental stages (Hunt *et al.*, 2001; Garrocho-Villegas *et al.*, 2007; Vinogradov *et al.*, 2011a) (**Paper I**). As previously indicated, two types of nsHbs have been distinguished using phylogenetic analysis, class-1 (nsHb1) and class-2 (nsHb2) (Fig. 1) (Hunt *et al.*, 2001). Both classes have not been found in all plants. It appears that monocots lack nsHb2, but generally have one or more nsHb1. Dicots most often carry at least one

class-1 and one class-2 nsHb, unless the class-2 has evolved into a sHb (as in the case of legumes and some other nodulating dicots) (Hunt *et al.*, 2001; Garrocho-Villegas *et al.*, 2007; Smagghe *et al.*, 2009). These two types of nsHb mainly differ in their expression pattern which can be different depending on the organ and the developmental stage of the plant **(Paper I)**, and their oxygen binding properties (**Paper II**) (Trevaskis *et al.*, 1997; Hunt *et al.*, 2001). Multiple studies have been carried out to determine the functions of nsHbs, the most general proposal being that they would work as hormonal regulators via their dioxigenase activity. The role of these plant Hbs are more deeply discussed in Chapter III of this thesis.

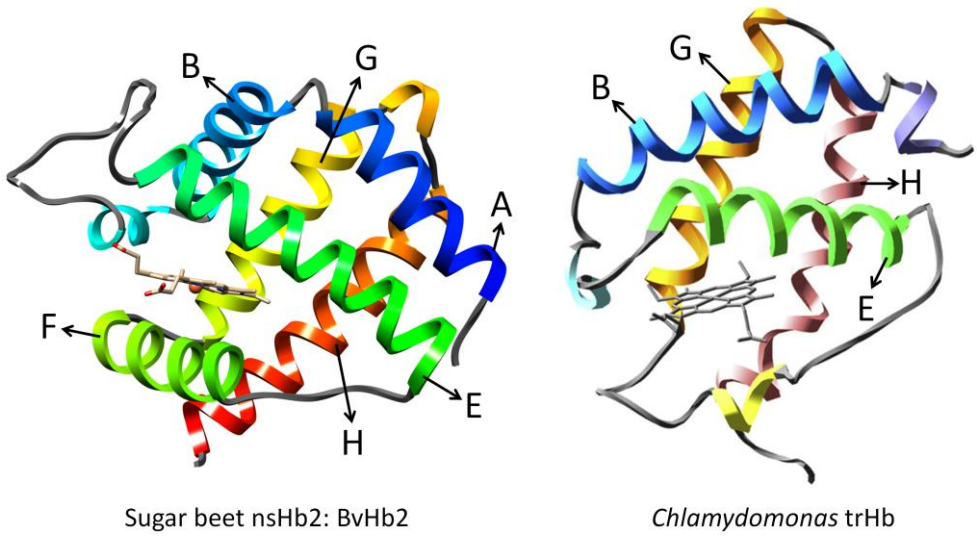

Figure 3. Tertiary structure of BvHb2 (3-on-3 Hb) versus *Chlamydomonas* trHb (2-on-2 Hb). Compared to BvHb2, the truncation of the globin fold in Chlamydomonas TrHb is evident. Successive helices are individually colored. NsHbs, as BvHb2, are described as having a 3-on-3 fold (helices A/E/F and B/G/H), while trHbs are described as having a 2-on-2 fold (helices B/E and G/H).

TrHbs owe their name to their truncated "2-on-2" fold (2/2) which differs from the typical "3-on-3" alpha-helical "sandwich fold" of most Hbs, including plant Hbs (Fig. 3) (Pesce *et al.*, 2000). Besides being found in eubacteria, cyanobacteria, and protozoa, they are nearly ubiquitous in the plant kingdom. TrHbs have been phylogenetically divided into three groups (Wittenberg *et al.*, 2002). Plant trHbs have been grouped in one of the four clusters of the second group and in contrast to nsHbs; studies on their functional roles are still scarce. The expression of *trHb*s has been found in symbiotic structures (Bustos-Sanmamed *et al.*, 2011) and after different abiotic stress treatments (Jokipii *et al.*, 2008). In wheat, the transcription levels of TatrHb increases in roots and leaves treated with NO-releasing compound sodium nitroprusside (SNP), indicating a potential role in NO scavenging or detoxification. Additionally, TatrHb was shown to interact with chloroplast proteins,

indicating a function involved in photosynthesis (Kim *et al.*, 2013). On the other hand, complementation analyses where PttTrHb was co-expressed with PtthFNR, a ferredoxin $NADP^+$ oxidoreductase, failed to rescue a NO resistance defective yeast mutant (**Paper IV**) even though immunolocalization studies showed that the occurrence of PttTrHb overlapped with areas of NO production (Dumont *et al.*, 2014). All together, these and many other studies indicate that trHbs in plants can have different roles depending on protein location and/or plant species.

1.2. Evolution

The current availability of numerous sequenced genomes has extended our knowledge about the diversity of Hbs in living organisms. Initially, when sHbs were discovered, it was believed that they originated in plants due to horizontal gene transfer from animals; however, gene structure and sequence analysis demonstrated that they came from a common ancestor to both plants and animals (Appleby *et al.*, 1988). In the case of trHbs, phylogenetic and three-dimensional analysis indicate that they have evolved vertically from a bacterial trHb (Dumont *et al.*, 2014).

3-on-3 plant Hbs have been found in almost all eukaryote groups and nowadays it is obvious that they share a common origin with bacterial globins (Vinogradov *et al.*, 2011b). Phylogenetic analyses have demonstrated that all Hbs are likely to have emerged from a bacterial single domain globin that had one or more enzymatic and oxygen sensing functions (Vinogradov & Moens, 2008; Vazquez-Limon *et al.*, 2012). This globin later evolved in multicellular eukaryotes with new properties, including reversible binding of diatomic ligands, such as oxygen (O_2), nitric oxide (NO), carbon monoxide (CO), and sulfide which enabled the evolution of transport and storage functions (Vinogradov & Moens, 2008).

In general, the evolution of land plant 3-on-3 Hbs has paralleled the major transitions in land plant evolution (Fig. 4) (Vazquez-Limon *et al.*, 2012). From phylogenetic analysis it was concluded that the Hbs of *Marchantia* (liverwort), *Physcomitrella patens* (moss), and the spike moss *Selaginella* (Lycopsid), the oldest living groups of land plants, are closest to the ancestral embryophyte Hbs. Since *Euryale ferox* (Nymphaeaceae) has class-1 and class-2 nsHbs, and because the Nymphaeales (water lilies) represent one of the earliest branching of the angiosperm lineages, it has been suggested that the origin of both classes occurred when the whole genome of the angiosperms ancestors duplicated (Jiao *et al.*, 2011). Finally, sHbs originated from an nsHb and spread among nodulating flowering plants, while Lbs evolved only within legumes.

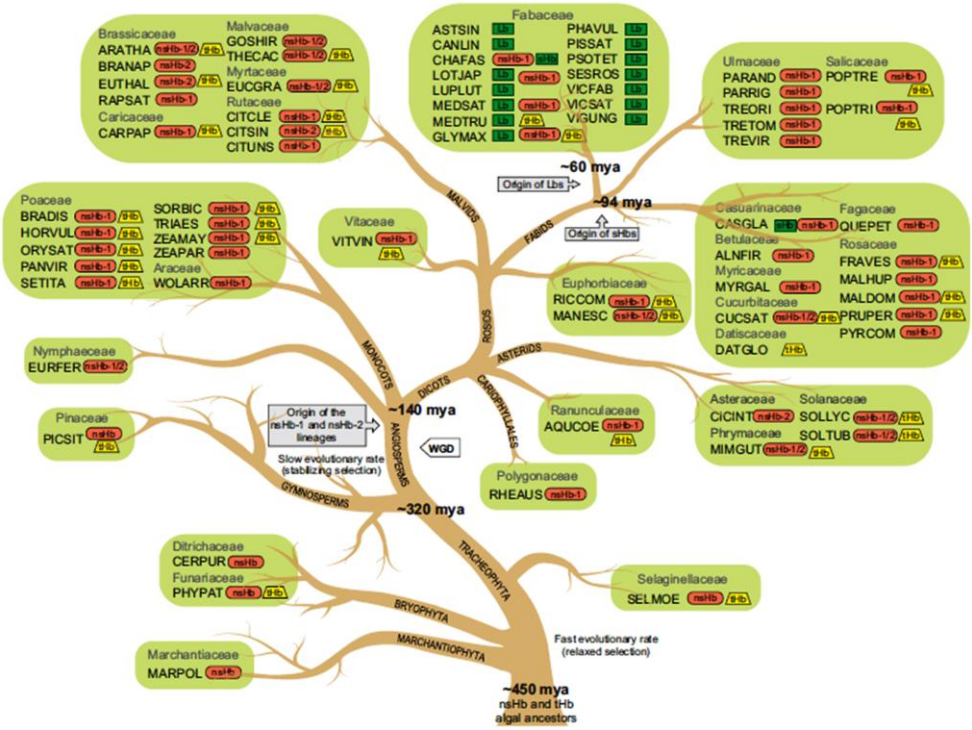

Figure 4. Evolution of land plant hemoglobins. The species are named following a binomial abbreviation. Lb: leghemoglobin; nsHb-1: nonsymbiotic hemoglobin class-1; nsHb-2: nonsymbiotic hemoglobin class-2; sHb: symbiotic hemoglobin; tHb: truncated hemoglobin; WGD: whole genome duplication. Figure adapted from Vazquez-Limon et al. (2012)

From the different studies carried out on plant Hbs, it is clear that the assigned functions have species-specific and stress-related features that have to be established in each particular case (Gupta *et al.*, 2011). Such a variation seems to be a direct consequence of the parallel evolution of plants and their Hbs. As an example of modification we have the variation of the N-termini region of these proteins. Thus, sequence alignment of primitive and evolved nsHbs together with sHbs, and Lbs revealed that the size of the polypeptide decreased over time at the N-terminal region (Fig. 5). This region, named pre-helix A, was suggested to function as a leader peptide in primitive nsHbs (Ross *et al.*, 2002), similarly to the T1 trHb from the green algae *Chlamydomonas*, which is translocated from the cell cytoplasm to the chloroplasts (Couture *et al.*, 1994). Since no transit sequences have been previously found at the N-termini of nsHbs in higher plants, it was generally believed that all nsHbs became cytoplasmic during the evolution of land plants and only the ancestors to land plant

nsHbs were translocated from cytoplasm to cellular organelles (Ross *et al.*, 2002; Vazquez-Limon *et al.*, 2012). However, in some species, this leader peptide is still conserved and some nsHbs still carry information to be translocated to chloroplasts. Protein alignment revealed that the class-1 nsHb from sugar beet, grape and spinach have a leading peptide at the N-terminus with information to be translocated into chloroplasts (**Paper I**). Even though experimental data is needed to confirm that these class-1 nsHbs are transported and located in chloroplasts, the results indicate that in these species, this leader peptide is still conserved and not all nsHbs became cytoplasmic during their evolution.

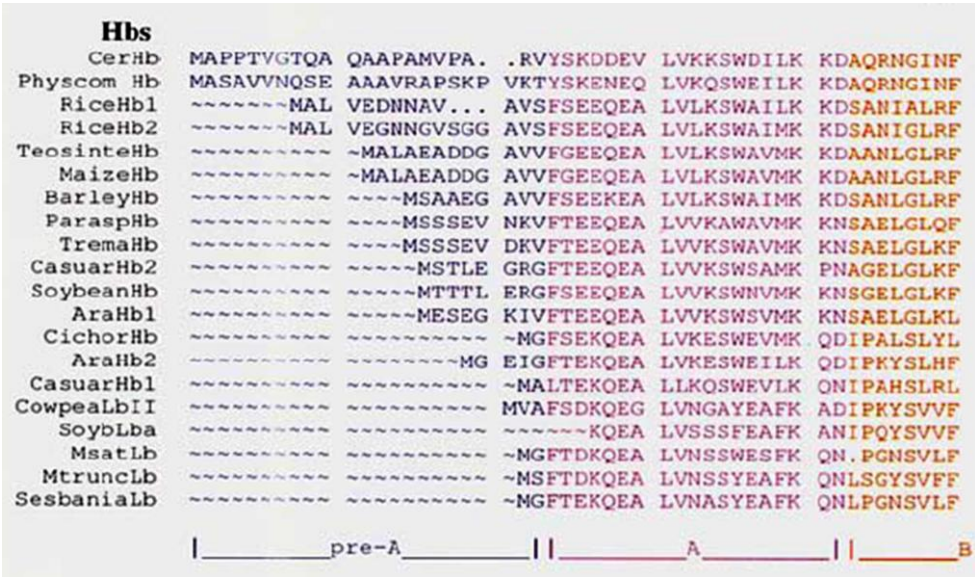

Figure 5. Sequence alignment of the amino-terminal region of plant Hbs. The size at the pre-A helix decreases from primitive (moss) to evolved Hbs (sHbs and Lbs). CerHb and PhyscomHb are nsHbs from mosses. Helices are indicated with different colors. Modified from Ross *et al.* (2002)

Undoubtedly, the era of genome sequencing is extending our understanding of the evolution of plants. Thanks to this technology it was possible to determine that the nsHbs of sugar beet and spinach, both amaranthaceae species (Caryophyllids), have arisen through a duplication event that took place in a common ancestor. It is probable that the ancestral nsHb1 gene had a long N-terminal region, but after duplication one of the new copies lost it over the time through deletion events (**Paper I**).

The identification of a leader peptide at the N-termini in Hbs of species coming from groups with complex taxonomy; caryophyllidae and vitaceae (Samuel F. Brockington *et al.*, 2009; The Angiosperm Phylogeny, 2009), make us realize that the understanding of the evolutionary history for the traits being investigated provides the framework for more thorough and varied analysis.

1.3. Protein structure

Plant Hbs display several characteristics that separate them into distinct clusters. However, detailed mechanism and functional relation to their structures remain to be elucidated (Mukhi *et al.*, 2013). To date, not so many three-dimensional structures of plant Hbs have been solved. In fact, no structure for a nsHb class-2 has been determined. Most of the structural details we know have been gathered from electron paramagnetic resonance, resonance raman, and UV/VIS spectroscopy, as well as from point mutation studies, mass spectrometry, electrochemistry, and computer modeling (Hoy & Hargrove, 2008).

1.3.1. The Heme group

In general, sHbs and nsHbs are globins with a heme *b* prosthetic group held within an α-helical secondary structure comprised of helices A-H, known as the myoglobin-fold (Mb-fold) or 3-on-3 structure. The heme contains an iron atom with four of the six coordination sites occupied by the heme pyrrole nitrogens (Hoy & Hargrove, 2008) (Fig 6). The heme group is attached to the globular protein by a covalent bond to a His side chain termed the ‘‘proximal His’’. The side of the heme opposite the proximal His is where the ligands bind. Nearby there is another His residue called the “distal His”. In the case of trHbs, the heme *b* group is within a 2-on-2 helical domain (Fig. 3) similar to that observed in bacterial trHbs (Reeder & Hough, 2014). It is important to note that although the above described globin fold is highly conserved, sequence homology among these proteins can be as low as 16% (Bashford *et al.*, 1987).

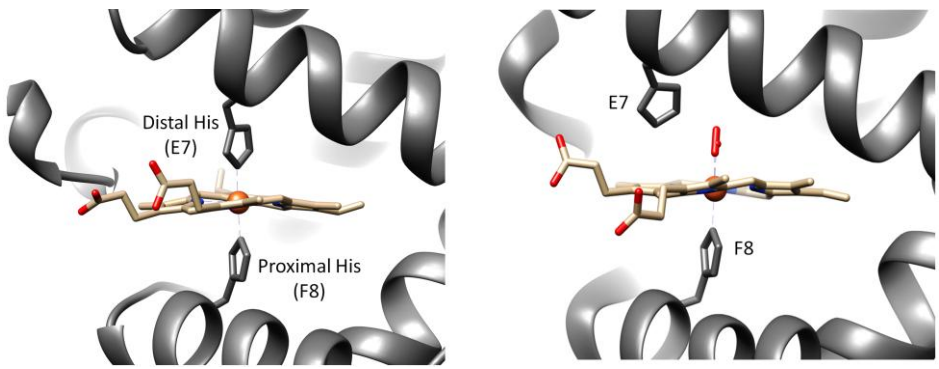

Figure 6. The heme *b* group in plant Hbs. The hexacoordinate structure to the left (AtHb1, 3ZHW) demonstrates distal His coordination to the sixth binding site of the heme iron (orange sphere). This protein structure has no ligand molecule bound to the heme group (brown). On the right, a pentacoordinate Hb (Lupin Lb, 2GDM) has oxygen (red) bound to the sixth binding site of the heme. The proximal and distal His are in the F- and E- helices, respectively.

1.3.2. Symbiotic hemoglobins

In sHbs, both the proximal and distal pockets have been analyzed in detail. This has been achieved through mutational and kinetic studies (Kundu *et al.*, 2002; Kundu & Hargrove, 2003) as well as by analyzing crystal structures in various oxidation states with many ligands (Harutyunyan *et al.*, 1995; Hargrove *et al.*, 1997). Those studies have concluded that sHbs share similar structures while using specific residues in the heme pocket to modulate the rates of ligand binding. This explains the kinetic homogeneity between sHbs from different species at the moment of binding oxygen for its transport.

Throughout the characterization of the structure and ligand binding mechanism of sHbs, comparison have usually been made with Mb, as both proteins are monomers and pentacoordinated in their ligand-free ferrous and ferric states. Different studies have determined that the mechanism of ligand binding regulation in sHbs is opposite to the one used by Mb. SHbs rely on the proximal pocket whereas Mb relies on the distal pocket. In Mb a "gating" mechanism due to steric hindrance from the distal His has been observed. Such a hindrance is because of a strong hydrogen bond with the distal His. In sHbs the replacement of this His with Leu has a minimal effect on oxygen affinity, suggesting a weak interaction (Hargrove *et al.*, 1997). Another differential feature between sHbs and Mb is the size of the distal binding pocket which is larger in sHbs allowing them to bind bulky ligands such as acetate and nicotinate (Ellis *et al.*, 1997). Also, the interaction between the Tyr at position 10 on the B-helix (TyrB10), which makes the hydrogen bond with the bound ligand longer, is not observed in Mb which has Leu at position B10. As a result, the distal bond of Mb with the ligand is much stronger (Kundu *et al.*, 2004).

1.3.3. Truncated hemoglobins

Unlike sHbs, whose tertiary structure has been very much studied, reports on plant trHbs are limited. The first crystal structure of this protein, also called class-3 nsHb, from *A. thaliana* has been recently reported (Reeder & Hough, 2014). This protein (AtHb3) forms a homodimer, with each monomer containing a 2-on-2 helical domain similar to that observed in bacterial trHbs (Fig. 3). The structure presents a novel N-terminal extension with two α-helices that play a major role in the dimer interface, occupying the periphery of the dimer–dimer face, surrounding an open central cavity. The heme cavity contains a proximal His but the distal pocket does not contain any His residue that could bind to either Fe or to a Fe-bound water or gas ligand. The open sixth Fe-coordination site instead has potential to form hydrogen bonds with a Tyr or a Trp residue. Unusually, the heme pocket of AtHb3 appears to be open to the external environment, with another cavity spanning the entrance of the two heme pockets. Reeder & Hough (2014) suggested that this open structure towards external solvent may be related to the unusual concentration-

independent binding kinetics for oxygen and CO following photodissociation (Watts *et al.*, 2001)

1.3.4. Nonsymbiotic hemoglobins

The significant kinetic variability in ligand binding and the potential for diverse functions displayed by nsHbs (Smagghe *et al.*, 2009) indicate broad variations in their three-dimensional structure. Initially, when the spectral properties of nsHbs were analyzed it was discovered that both their ferric and ferrous forms resembled those of cytochrome *b*5, indicating that the heme iron was six-coordinated in both oxidation states (Fig. 6) (Arredondo-Peter *et al.*, 1997; Duff *et al.*, 1997). The first solved structure from a class-1 nsHb, riceHb1, clearly confirmed that the iron atom coordinated both the proximal and distal His in both the deoxy and ferric forms (Hargrove *et al.*, 2000). Interestingly, such a hexacoordination does not hinder these proteins to bind exogenous ligands, as it happens with cytochrome *b5*.

When the barleyHb1 ligand-bound structure (Hoy *et al.*, 2007) was compared to the ligand-free structure of riceHb1, it was observed that structural changes prior to ligand binding were necessary. The distal His dissociation from the heme iron was accompanied by rotation and translation of the E-helix, through a "piston" movement along the helical axis. This movement is accompanied by stabilization of the CD loop region, including the formation of a D-helix, and development of several new trans-helical contacts in the EF turn. Those structural changes are clearly visualized in plots of root mean square (RMS) deviation between ligand-bound and ligand free riceHb1 (Hoy & Hargrove, 2008; Kakar *et al.*, 2010). Even though both class-1 nsHbs from the monocots rice and barley seem to bind ligands in a similar manner, the way these proteins form homodimers is different. BarleyHb1 contains a disulfide bridge between the monomers that helps to stabilize the protein (Hoy *et al.*, 2007), such a bridge does not exist in riceHb1.

Much less is known about the structure of nsHbs in dicotyledonous plants. So far, the tertiary structure of only two class-1 nsHbs has been solved, TremaHb from *Trema tomentosa* (Kakar *et al.*, 2011) and AtHb1 from *Arabidopsis thaliana* (Mukhi *et al.*, 2013). On the other hand, the structure of class-2 nsHbs remains unknown as no useful protein crystals have yet been generated.

The structure of TremaHb in its ferric state has been determined in order to be compared to the structure of ParaHb, a sHb from *Parasponia andersonii* (Kakar *et al.*, 2011). These two proteins are 93% identical in primary structures but differ in oxygen binding constants in accordance with their distinct physiological functions (Sturms *et al.*, 2010). The structure of TremaHb has been shown to be similar to the other class-1 nsHbs from monocot plants and its comparison to ParaHb has been mainly discussed in the context of evolution of oxygen transport in plants.

In order to get more information on the tertiary structure of nsHbs from dicot plants we took advantage of the high sequence identity among nsHbs. Thus, we could predict the structure of the three nsHbs of sugar beet by homology modeling **(Paper II)**. The X-ray structures of TremaHb from *T. tomentosa* (PDB ID: 3QQQ) and AtHb1 from *A. thaliana* (PDB ID: 3ZHW) were used as templates for the modeling of BvHb1-1 and BvHb1-2. For the modeling of BvHb2, templates with the highest sequence identity were used: AtHb1 and the legHb of yellow lupin (*Lupinus luteus*) (PDB ID: 1GDJ). Due to the high identity of the templates with the three BvHbs the obtained models are at the high end of the accuracy spectrum, except from some segments corresponding to the D loop as well as the EF-loop and the F-helix. This is mainly due to the high variability found in the amino acid sequence of these regions as observed when plants Hbs are aligned (Fig. 7). For instance, the D-loop of BvHb1-1 is similar to those of class-2 nsHbs with a nucleophilic amino acid surrounded by acidic residues. On the other hand, it has two additional amino acids (Ser and Asp) which are the same as class-1 nsHbs from rice and maize.

Another important source of variation is the number of the structurally critical Pro residues in the DE region of class-1 nsHbs of dicot plants. In this group it is possible to find from one (BvHb1-2) up to four Pro residues (AtHb1), the most common is to find only two in the other plant groups. On the other hand, the flexible Gly residues are absent in nsHbs except for BvHb1-2, which has one just before the distal HisE7 similar to LegHbs.

Both class-1 BvHbs were modeled as dimers and BvHb2 as a monomer. The formation of homodimers could result into cooperative ligand binding; however, no hint of this has been observed for any class-1 nsHb, including the two class-1 BvHbs. It has been suggested that the formation of dimers might not be physiologically relevant because of the very low intracellular concentrations of these proteins (Hargrove *et al.*, 2000). However, the apparently conserved dimer formation in several class-1 nsHbs may have some physiological significance (Mukhi *et al.*, 2013).

As reported for TremaHb (Kakar *et al.*, 2011) and AtHb1 (Mukhi *et al.*, 2013), there are three successive side chains at the beginning of the G helix that are directly involved in the subunit interface: AspG3, GluG4, and HisG5 (Fig. 8A). The electrostatic interaction between HisG5 and GluG4 was reported as rare both for Trema Hb and AtHb1 and it was suggested that it could influence the environment of the F helix and HisF8 affecting the heme coordination (Kakar *et al.*, 2011). However, judging by the sequence similarity, it may be very common within class-1 nsHbs of dicot plants. In both class-1 BvHbs, the position TyrG11 in AtHb1 has been replaced by a Phe, resulting in the interaction of the three side chains of LysH5, GluB15, and PheG11 (Fig. 8B). Given that these residues are highly conserved in most of the class-1 nsHbs from dicot plants, this interaction may be common (Fig. 7). Furthermore, an extended tunnel connecting the heme active site of both the subunits across the dimeric interface was also observed in both class-1 BvHbs **(Paper II)** and PttHb1 **(Paper IV)** as previously reported for AtHb1(Mukhi *et al.*, 2013).

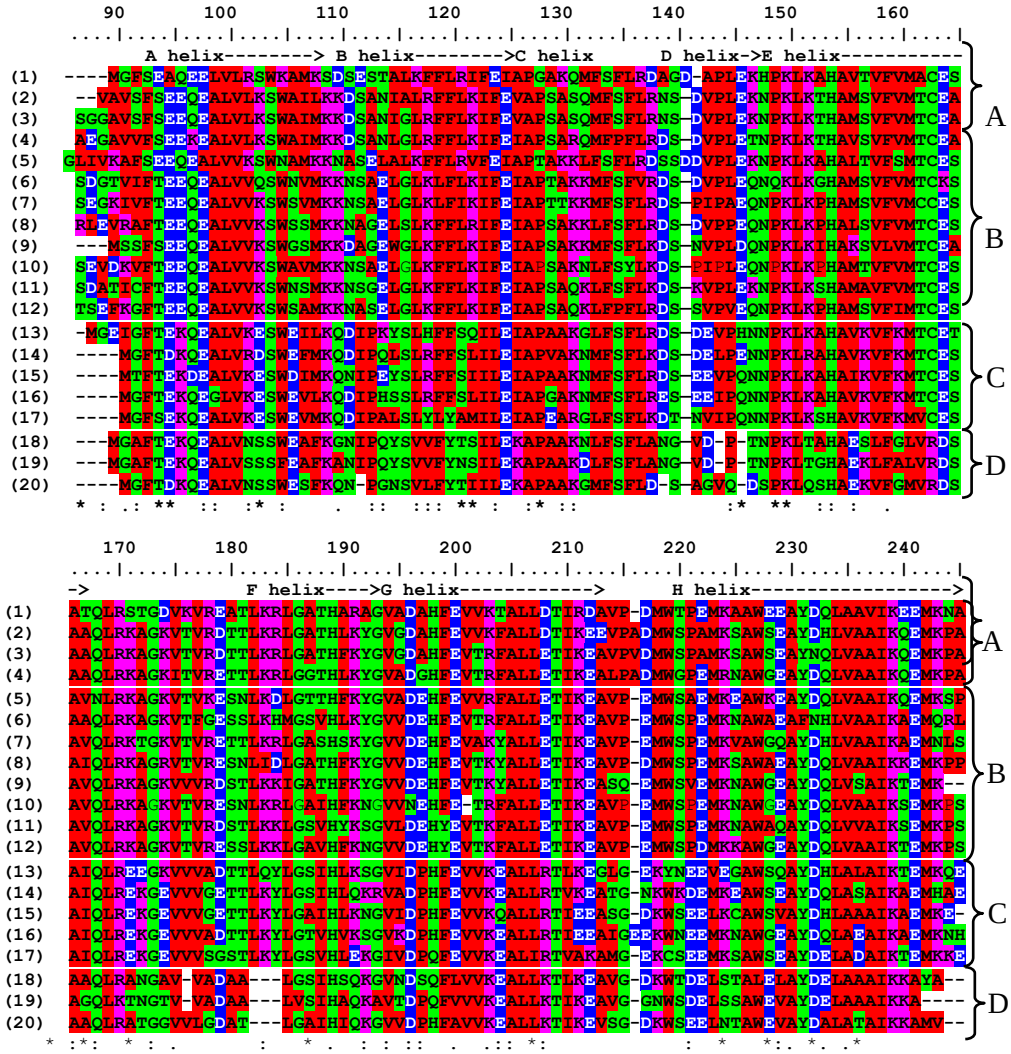

Figure 7. Sequence alignment of 3-on-3 plant Hbs. Conserved amino acids are marked with asterisks. The residues are colored according to their physicochemical properties. Small, hydrophobic residues including aromatic except Tyr are in red. Acidic residues are in blue. Basic residues except His are in magenta. Hydroxyl, sulfhydryl, amine and Gly residues are in green. A: Class-1 nsHbs from monocot plants, B: Class-1 nsHbs from dicot plants, C: Class-2 nsHbs, D: sHbs. (1) *Z. mays* nsHb1, (2) *Oryza sativa* nsHb1a, (3) *O. sativa* nsHb1b, (4) *Hordeum vulgare* nsHb1, (5) *Beta vulgaris* BvHb1-1, (6) *B. vulgaris* BvHb1-2, (7) *A. thaliana* AtHb1, (8) *Vitis vinifera* nsHb1b, (9) *Solanum lycopersicum* nsHb1, (10) *Trema tomentosa* nsHb1, (11) *V. vinifera* nsHb1a, (12) *Populus trichocarpa* nsHb1, (13) *A. thaliana* AtHb2, (14) *Solanum lycopersicum* nsHb2, (15) *B. vulgaris* BvHb2, (16) *Gossypium hirsutum* nsHb2, (17) *Cichorium intybus* Hb2a, (18) *Phaseolus vulgaris* Lb, (19) *Glycine max* Lb (1), (20) *Medicago sativa* Lb.

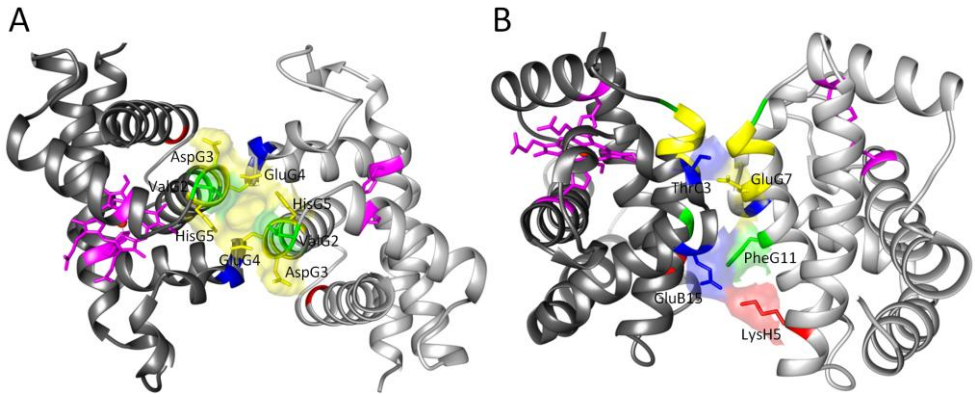

Figure 8. Dimer interface in class-1 BvHbs. The residues involved in the dimer formation are indicated in yellow (G-helix), blue (BC region), and red (H-helix). Residues that stabilize the dimer formation are in green. The two subunits are colored in dark and light grey. The proximal and distal histidines as well as the heme group are in pink. *A,* Upper view, the three consecutive amino acids at the G-helix responsible of dimerization are observed, AspG3, GluG4, and HisG5. A ValG2, from each subunit, stabilizes their interaction. *B,* Frontal view, the interaction between ThrC3 and GluG7 and between LysH5 and GluB15 are clearly observed. This last interaction is further stabilized by a PheG11 (green) **(Paper I)**

In paper IV we describe the the X-ray structure of PttHb1 which was solved to a final resolution of 2.5Å. In this structure the E-helix was shifted by one helix-turn taking an identical conformation to the one in barleyHb1 (Hoy *et al.*, 2007). PttHb1 was crystallized as a dimer and as expected, the crystal structure displays the highly conserved 3-on-3 globin fold. Another model of PttHb1 was constructed using the unliganded hexacoordinated tremaHb1 structure (PDB code 3QQQ). The structure shows that the distal His69 is shifted away from the iron atom in the haem group due to movement of the E-helix. The movement of the E-helix opens up a channel (Fig. 8 in **Paper IV**) that could fit small molecules such as NO. Since the *in vivo* data showed that PttHb1 in presence of PtthFNR is able to complement the absence of yeast flavoHb and confer NO resistance we investigated the interaction mechanism of PttHb1 and PtthFNR further by creating a three dimensional model of PttHb1 – PtthFNR complex (Fig. 9). The structures of the haemoglobin and the flavoreductase domain of yeast flavoHb are very similar to the poplar nsHb1 and hFNR structures, respectively. Intuitively, PtthFNR and PttHb1 could work together to perform the role of NO dioxygenase in a similar structural arrangement as flavoHbs. In this structural framework, the surface residues of both proteins do not interfere but make sensible contacts with each other. The modelled complex supports the idea that PtthFNR interacts with PttHb1 to transfer the required electron to reduce $HbFe^{3+}$ to $HbFe^{2+}$ and catalyze the NO dioxygenation in a manner similar to flavoHbs **(Paper IV)**.

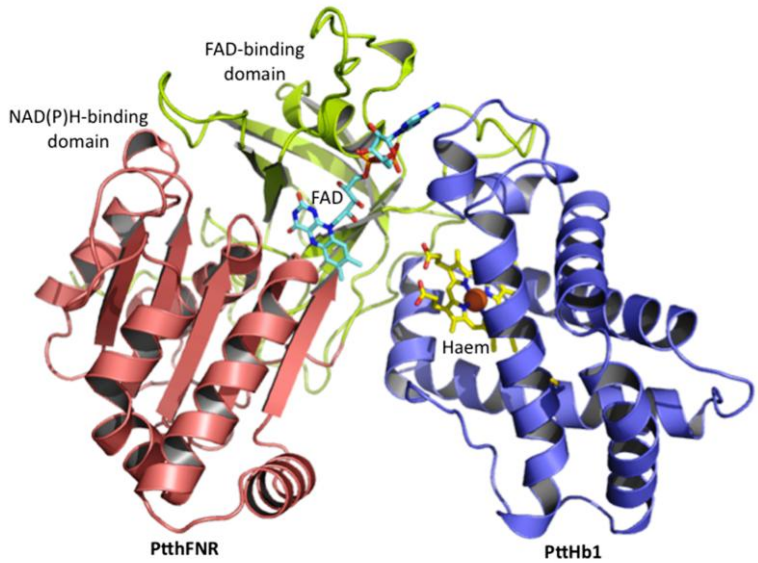

Figure 9. The cartoon representation of the model of the PtthFNR – PttHb1 complex. PttHb1, the globin domain, is in light blue with haem group in yellow, and iron is in orange sphere. The FAD binding domain of the PtthFNR model is in light green, and the NAD(P)H binding domain is in salmon. The FAD group is shown in cyan sticks **(Paper IV).**

II. Biochemical and Biophysical Properties of Plant Hemoglobins

2.1. Spectral properties

The heme prosthetic group is a chemical compound consisting of a Fe^{2+} ion contained in the centre of a porphyrin ring. This ring is made up of four pyrrolic groups joined together by methine bridges. The Fe^{2+} ion has six possible coordination bonds; four of them keep it bound to the porphyrin ring, leaving two other coordination positions available for bonding to the apoprotein. The mode of attachment and coordination of the heme group to the protein chain together with the environment of the surrounding heme pocket, have a great influence on the chemistry of Hbs (Kakar *et al.*, 2010).

The type of heme coordination has provided a source of classification for Hbs. In general, Hbs that transport oxygen have a pentacoordinate heme iron. In pentacoordinate Hbs, a proximal histidine coordinates the fifth site, leaving the sixth open for oxygen binding (Hoy & Hargrove, 2008). In plants, the pentacoordinate Hbs are represented by sHbs.

Later, the discovery of nsHbs revealed recombinant proteins with coordination states distinct from the pentacoordinate sHbs. NsHbs have their heme groups coordinated by two histidine side chains, one of which reversibly binds to the sixth coordination site of the heme iron to allow the stable binding of exogenous ligands like oxygen, CO, and NO (Fig. 6) (Arredondo-Peter *et al.*, 1997; Duff *et al.*, 1997; Bruno *et al.*, 2007a). The structure of six coordinate bonds to the heme iron resulted in these proteins being referred to as "hexacoordinate" Hbs (hxHbs) (Kakar *et al.*, 2010).

The number of bonds coordinating the heme group can be spectroscopically determined. The absorption spectrum of ferrous bis-histidyl heme of nsHbs resembles the spectrum of cytochrome b_5. It has two visible bands near 555 and 529 nm (the α and β bands, respectively) in their reduced deoxy form (Fig. 10A) (Arredondo-Peter *et al.*, 1997; Duff *et al.*, 1997). This spectrum is in contrast to the broad single band

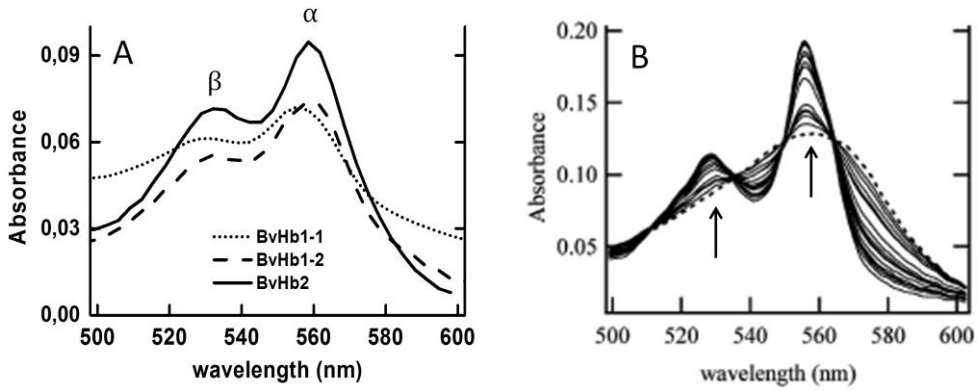

Figure 10. Hexacoordination in nsHbs. *A,* Absorption spectra of the three nsHbs from sugar beet with the the α and β bands at the visible region **(Paper II)**. *B,* Spectral transition from pentacoordinate (dotted line) to hexacoordinate heme (solid lines) upon imidazole addition to a solution containing a modified soybean Lb (LbaH61A) with a high affinity for imidazole. Graphic adapted from Smagghe *et. al.* (2006).

near 558 nm found in most pentacoordinated Hbs such as sHbs (Fig. 10B) (Antonini & Brunori, 1971)

When relevant redox states of nsHbs are to be analyzed, as well as their binding with ligands such as oxygen and CO, great amount of information can be obtained by observing their spectral characteristics (Arredondo-Peter *et al.*, 1997; Duff *et al.*, 1997). Like all other ferrous ligand-bound Hbs, when the three BvHbs and PttHb1 are mixed with CO or oxygen, species are generated with unmistakable and characteristic spectrum. As observed in Fig. 11, the HbCO complex show two visible absorption bands with their maxima near 537 and 566 nm. The soret band lies near 416 nm. The oxyHb forms have the two visible peaks around 539 and 574 nm while the soret band is around 410 nm **(Paper II and IV)**.

Another relevant redox state in Hbs is the ferric form which in solution will have a dark red brownish color. As for nsHbs, in this oxidation state the histidine coordination to the sixth axial position converts the heme iron of nsHbs to the low spin electronic configuration yielding stronger visible absorbance, and splitting of the ferrous visible absorbance band into two peaks near 560 and 530 nm (Fig. 11C). EPR is a particularly sensitive measure of the spin state in ferric Hbs, where low spin Hbs (like the hxHbs) exhibit weaker and more complex spectra dominated by a rhombic signal with features at g values of 3.0, 2.2, and 2.0 (Peisach *et al.*, 1969).

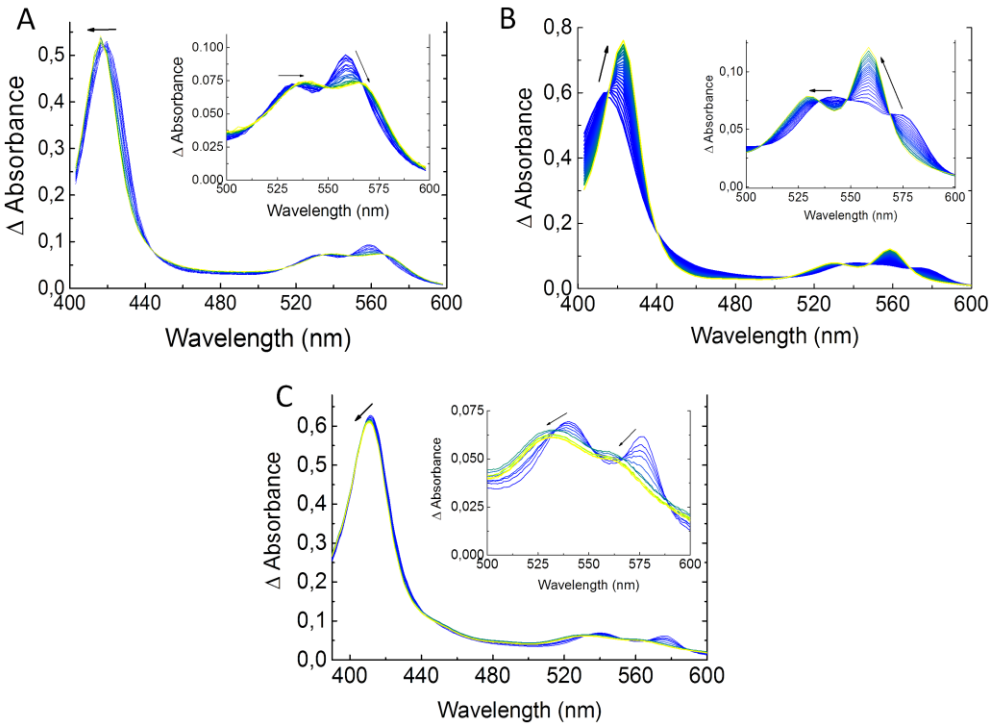

Figure 11. Optical spectra of BvHbs. *A,* Absorbance spectra associated with the binding of CO to ferrous BvHb2 (the direction of the spectra change is indicated by the arrows, from deoxyHb to HbCO). *B,* Absorbance spectra associated with the rebinding of oxygen to a deoxygenated BvHb2 (the direction of the spectra change is indicated by the arrows, from deoxyHb to oxyHb). C, Optical changes reflecting the progressive conversion of oxygenated BvHb1-2 to its oxidized MetHb form. Insets: Optical spectra in the visible regions.

2.2. Ligand binding kinetics

Unlike the simple bimolecular reaction that occurs in pentacoordinate Hbs, ligand binding to hxHbs is more complex due to competition between the binding of the exogenous ligand and the intramolecular coordination of the distal histidine. The first observations of ligand binding to nsHbs revealed limiting rates of bimolecular binding due to the coordination of the distal histidine that blocks the ligand binding site (Hargrove, 2000; Trent & Hargrove, 2002; Smagghe *et al.*, 2006). This complicates ligand binding reactions by preceding bimolecular interactions with a reversible first order event (Brancaccio *et al.*, 1994; Coletta *et al.*, 1996), as shown in Scheme 1:

$$\mathrm{Hb_{hex}} \underset{k_{\mathrm{H}}}{\overset{k_{-\mathrm{H}}}{\rightleftarrows}} \mathrm{Hb_{pent}} + \mathrm{L} \underset{k_{\mathrm{L}}}{\overset{k'_{\mathrm{L}}}{\rightleftarrows}} \mathrm{Hb_{L}} \qquad \text{(Scheme 1)}$$

In this scheme, Hb_{hex} and Hb_{pent} are the hexacoordinated and pentacoordinated forms of the Hb, and the rate constants for distal histidine coordination (H) and exogenous ligand (L) binding are written at the top and bottom of each step. This scheme has been previously used for other hxHbs by Brancaccio *et al.* (1994) and Coletta *et al.* (1996).

The degree to which distal histidine coordination affects ligand binding is influenced by two factors (Smagghe *et al.*, 2006; Smagghe *et al.*, 2008a): 1) the speed with which the coordinating histidine associates and dissociates from the heme iron, and 2) the equilibrium fraction of protein in the hexacoordinated state.

2.2.1. Hexacoordination

With the exception of sHbs, all plant Hbs show some degree of hexacoordination which can be determined by the analysis of the mechanism described in Scheme 1. Assuming that the concentration of Hb_{pent} remains in a steady state and that k_{CO} is very small, the following Equation 1 can be generated (Smagghe *et al.*, 2006):

$$k_{\mathrm{obs,CO}} = \frac{k_{-\mathrm{H}} k'_{\mathrm{CO}}[\mathrm{CO}]}{k_{\mathrm{H}} + k_{-\mathrm{H}} + k'_{\mathrm{CO}}[\mathrm{CO}]} \qquad \text{(Equation 1)}$$

This equation allows the measurement of rate constants for hexacoordination (k_H and k_{-H}) by using $k'_{CO,pent}$, determined with laser flash photolysis. It also predicts a limited rate of exogenous ligand binding when coordinated histidine dissociation is low (Sturms *et al.*, 2010). If ligand binding is very rapid, the time course for rebinding following flash photolysis is a single exponential process with $k_{obs} = k'_L[L]$. For this reason, the relatively slow reactivity of CO, compared to O_2, makes it a very convenient ligand to evaluate hexacoordination in nsHbs (Hargrove, 2000).

The individual k_H and k_{-H} parameters can be determined from the hyperbolic kinetic pattern observed when the rates are plotted against increasing CO concentrations after stopped-flow rapid mixing (Fig. 12B). These values are used to determine hexacoordination affinity ($K_H = k_H/k_{-H}$), which in turn is used to calculate the fraction of hexacoordinate proteins within a population by using the following equation: $F_H = K_H/(1+K_H)$ (Smagghe *et al.*, 2006). In general, class-2 nsHbs have the highest affinities for distal histidine coordination in the ferrous oxidation state, whereas the degree of coordination in class-1 nsHbs is much less (Bruno *et al.*, 2007a). However, in some cases, as with BvHb2, the fraction of hexacoordinate

proteins (83%) will be below the values reported for other class-2 nsHbs (~95%) (Table 1) (Smagghe *et al.*, 2009). Such a difference comes from the distinct k_H and k_{-H} rates when compared to other class-2 nsHbs **(Paper II)**.

Studies of Hbs from very ancient organisms, such as prokaryotes and archaea, have been carried out in order to determine if it was hexacoordinate or pentacoordinate Hbs which evolve first. These studies have concluded that all globins evolved from a common ancestor with a function unrelated to oxygen transport (Weber & Vinogradov, 2001). Whether the coordination state of this primordial Hb was pentacoordinate or hexacoordinate is still unknown (Kakar *et al.*, 2010). Certain is though that the progenitors of plant and animal Hbs are different. In plants, pentacoordinated oxygen transporters have evolved from hexacoordinated Hbs in both class-1 and class-2 nsHbs (Smagghe *et al.*, 2009; Sturms *et al.*, 2010), and there are no examples of hxHbs evolving from pentacoordinate proteins. By contrast, all animal hxHbs evolved from a pentacoordinate ancestor (Kundu *et al.*, 2003; Brunori & Vallone, 2007).

2.2.2. Stopped-flow rapid mixing

Stopped-flow rapid mixing is a common technique for the study of ligand binding reactions. In this method, protein and ligand are mixed together directly into an observation chamber at relatively high velocities. There, time courses for reactions can be measured at time scales as short as few milliseconds (Hargrove, 2005). Therefore, this technique has been extensively used for the characterization of ligand binding reactions in Hbs (Olson, 1981).

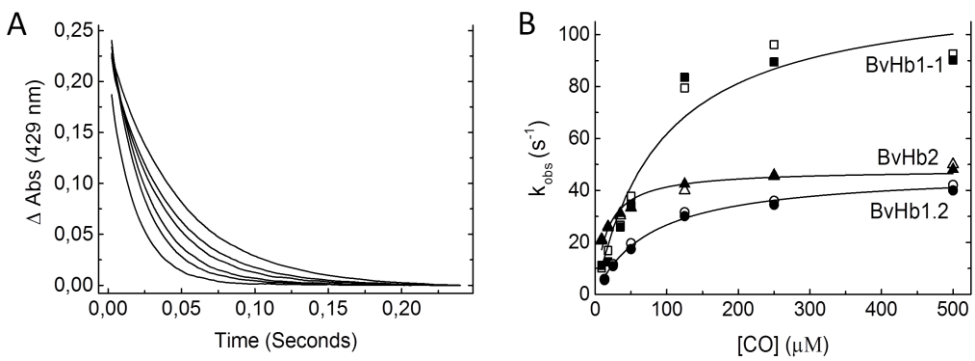

Figure 12. CO binding initiated by stopped-flow rapid mixing. *A*, Time courses for CO binding to BvHb2 following rapid mixing at different CO concentrations (from left to right: 500, 250, 125, 35, 18, and 9 μM) monitored at 429 nm. *B*, CO dependence of the rate constants for binding to the three BvHbs. The data was fitted to Equation 1 to extract the hexacoordination rates, k_H and k_{-H}. The closed and open symbols are k_{obs} values monitored at 430 and 555 nm for BvHb1-1, 430 and 560 nm for BvHb1-2, and 429 and 558 nm for BvHb2 **(Paper II)**

The effect of histidine coordination on CO binding is observed when deoxy-nsHb samples are rapidly mixed with increasing concentrations of CO in a stopped-flow apparatus. The rates of this reaction obey to a single exponential process conform to that expected from static spectra, namely formation of the CO adduct from the ferrous hexacoordinated species (Fig. 12A). The asymptotic values approached at high CO concentrations depend on the protein under study (Fig. 12B) **(Paper II)**. These data are consistent with the fact that CO binding to the hexacoordinated form is limited by the dissociation rate constant of the intrinsic distal histidine ligand (Scheme 1).

2.2.3. Laser flash photolysis

Together with stopped-flow rapid mixing, laser flash photolysis is another technique traditionally used to initiate reactions for the kinetic measurements of ligand binding to Hbs (Sawicki & Morris, 1981). In laser flash photolysis the reaction is initiated by photolysing the protein-ligand bond for a period of time long enough for the ligand to diffuse out of the protein matrix. When the light pulse is turned off, rebinding can be monitored on time scales much more rapid than those observed in stopped flow mixing.

In the case of nsHbs, when ligand rebinding kinetics is analyzed after photodissociation, the obtained time courses follow double exponential functions (Fig. 13A). Simple analysis of a two exponential fit to time courses for CO rebinding at varying CO concentrations yields rate constants for formation and dissociation of the hexacoordinate complex and the bimolecular rate constant for CO binding. Such rebinding kinetics was observed when BvHb1-1 and BvHb1-2 were analyzed and may adequately be described by Scheme 1 as discussed by Hargrove (2000). The fast phase represents binding of CO to the transient pentacoordinate form generated by photolysis while the slow process represents CO binding to the hexacoordinate specie formed by histidine rebinding to the iron. The slow phase of the rebinding kinetics in these proteins is essentially the same as reported by stopped-flow (Fig. 13).

However, BvHb2, while exhibiting a fast phase corresponding to CO binding to pentacoordiante form, displayed a slow phase the dependence of which on the concentration of CO was significantly faster than that given by stopped flow spectrometry. This phenomenon has also been described by Trent et al. (2001) who account for the discrepancy by proposing that the hexacoordinate form populated following flash photolysis is meta-stable and at longer times relaxes to the hexacoordinate form that is studied in stopped flow experiments. Thus, this result indicates that ligand binding to BvHb2 is not a simple combination of competing first order (hexacoordination) and second order (exogenous ligand binding) reactions **(Paper II)**.

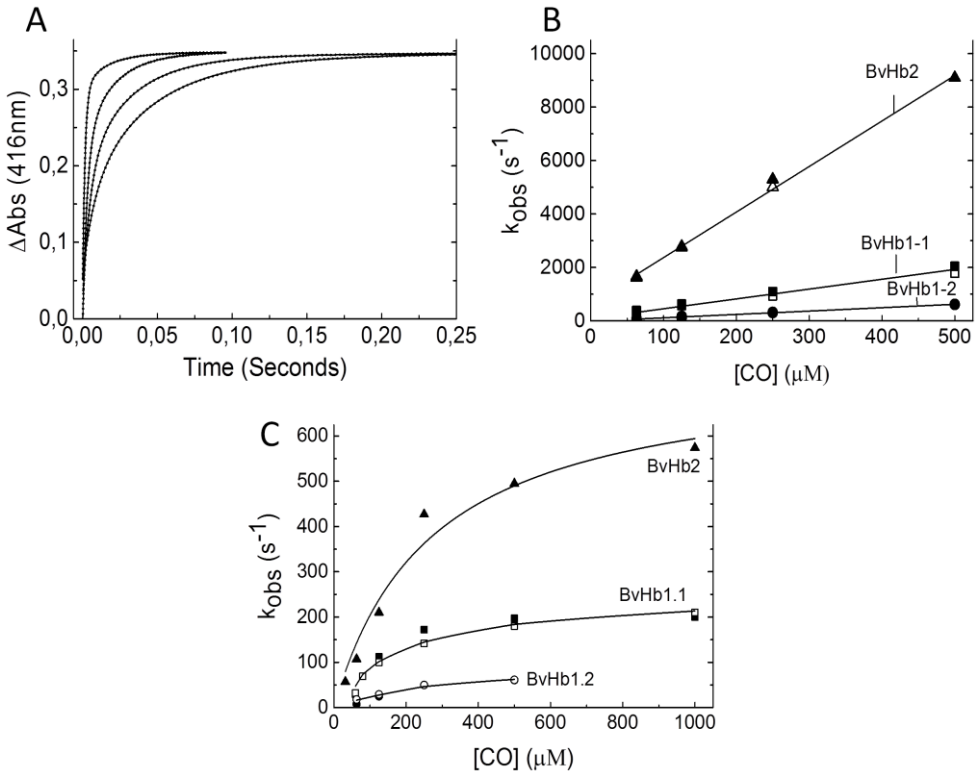

Figure 13. CO binding initiated by laser flash photolysis. *A,* Time courses for rebinding of CO, after flash photolysis, monitored at 416 nm. The binding is a double exponential process at all CO concentrations (from left to right: 500, 250, 125, and 63 μM). *B*, Dependences of the pseudo first order ligand rebinding rates at different CO concentrations yield the following bimolecular rate constants: 3.68 x 10^6, 1.27 x 10^6, and 17 x 10^6 M^{-1} s^{-1} for BvHb1-1, BvHb1-2, and BvHb2, respectively (Table 1). The closed and open symbols correspond to k_{obs} values monitored at 416 and 430 nm (429 nm for BvHb2), respectively. *C*, The slow phase of the rebinding of CO to the three BvHbs. For both class-1 BvHbs this is essentially the same as reported by stopped flow, 200 s^{-1} and 50 s^{-1} for BvHb1-1 and BvHb1-2 approximately. In the case of BvHb2, this slow phase is faster than that given by stopped flow spectrometry, with an approximate rate equal to 600 s^{-1} **(Paper II)**

2.3. CO binding

Biophysical studies analyzing the migration and binding of CO have mainly been carried out in the nsHbs of *A. thaliana*; AtHb1 and AtHb2. Those studies have revealed that pronounced differences exist in their active sites including both extension and volume of their internal cavities (Bruno *et al.*, 2007a; Bruno *et al.*, 2007b; Nienhaus *et al.*, 2010; Spyrakis *et al.*, 2013). Those differences are reflected in the different k'_{CO} values presented by AtHb1 and AtHb2 (Table 1). Ligand

TABLE 1. Ligand binding and hexacoordination rates of different plants Hbs. Plant species that have both sHbs and nsHbs have a pink background, Monocot plants have a grey background. Table adapted and modified from **Paper II.**

	$k'_{O2\ pent}$ [a]	k_{O2} [b]	$K_{O2\ pent}$ [c]	K_{O2} [d, c]	$k'_{CO\ pent}$ [a]	k_H [b]	k_{-H} [b]	K_H	F_H
Class-1 nsHbs									
AtHb1	74	0.12	617	200	0.55	230	110	2.1	68
BvHb1-1	59	0.075	787	271	3.68	220	118	1.9	66
BvHb1-2	57	0.158	361	157	1.27	59.5	47.4	1.3	57
Tomato Hb1	30	0.5	60	30	1	200	200	1	50
Soybean Hb1	59	0.013	4538	1512	2.7	160	80	2	67
Lotus Hb1-1	81	0.004	20250						
Lotus Hb1-2	300	0.27	1111						
Barley Hb1	50	0.027	1852	440	2	170	62	2,8	74
Maize 1a	44	0.054	820	430	1.4	22	25	0.9	47
Maize 1b	210	0.27	780	240	44	43	19	2.3	70
RiceHb1a	60	0.038	1579	540	6.8	75	40	1.9	66
RiceHb1b	40	0.1	400	280	1.8	6.7	15	0.45	31
Class-2 nsHbs									
AtHb2	86	0.14	614	21	77	330	12	28	97
BvHb2	72	0.094	766	132	17	232	48	4.8	83
Chicory Hb2a	50	0.11	455	1.7	54	2900	11	264	100
Chicory Hb2b	50	2.7	19	0.5	10	920	27	34	97
Tomato Hb2	45	0.4	113	1.9	26	1400	30	47	98
LotusHb2	77	0.86	90						

[a] $x10^{-6}\ M^{-1}\ s^{-1}$ [b] s^{-1} [c] $x10^{-6}\ M^{-1}$ [d] $K_{O2\ pent}/(1+K_H)$

binding studies have demonstrated that the higher values of k'_{CO} for AtHb2, as compared to AtHb1, are because the His ligation step is characterized by a lower enthalpic barrier, favoring the binding of the endogenous ligand (Bruno *et al.*, 2007a). Under high viscosity conditions, CO rebinding to AtHb1 appeared to be mostly regulated by ligand migration, while CO rebinding to AtHb2 is mostly governed by protein dynamics. Therefore, the migration pattern of the ligand within the protein matrix is much simpler in AtHb2 (Bruno *et al.*, 2007b). A higher accessibility of small gaseous molecules to AtHb2 has been ascribed to the presence of a Phe at position B9, as it alters the structural arrangement of residues in the distal cavity (Spyrakis *et al.*, 2013). Even though such a mutation also exists in several class-

1 and class-2 nsHbs, none of them has a CO binding rate as high as AtHb2, clearly implying that other amino acids should also be responsible for this property (Fig. 7).

It has been determined that the HisE7 side chain of AtHb1 interacts strongly with the heme-bound CO while in the case of AtHb2 such an interaction does not exist (Bruno *et al.*, 2007a; Nienhaus *et al.*, 2010). Such a lack of interaction has been demonstrated to be the result of a large shift of helix E along the helical axis that displaces the HisE7 side chain from the bound ligand by several Å (Nienhaus *et al.*, 2010). Concomitantly, it is at the E-helix where most of the structural rearrangements have been observed during the transition of hexacoordinate to ligand bound protein (Kakar *et al.*, 2010). In terms of variability, this region is highly conserved within the different types of plant Hbs; except for both class-1 BvHbs where some residues were replaced by amino acids usually found in class-2 nsHbs and sHbs (Fig. 7) **(Paper II)**.

Similar to previous studies, the CO binding rate of BvHb2 is higher when compared to the k'_{CO} values determined for class-1 BvHbs (Table 1). However, the differences between them are not as large as determined for *Arabidopsis* (Bruno *et al.*, 2007a) and tomato (Ioanitescu *et al.*, 2005) (Table 1). The low CO binding rate obtained for BvHb2 can be due to the AsnB1 and GluB4 residues localized at the same positions as in many class-1 nsHbs, including AtHb1 (Fig. 7). Another reason given to explain the high CO rebinding rate in AtHb2 is the flexibility of its helix B together with the deformability of the CD loop (Spyrakis *et al.*, 2013). Since the CD loop is highly conserved among class-2 nsHbs, we find the flexibility of B-helix more significant for the high CO rebinding rate of this group.

The analysis of more nsHbs is needed in order to clarify if the CO binding is the result of ligand migration, protein dynamics, or a combination of both. Undoubtedly, the crystal structure of class-2 nsHbs needs to be resolved in order to determine if additional structural features influence their binding to ligands as for example the observed rotation of the proximal HisF8 in BvHb2 **(Fig. 7 in Paper II)**.

2.4. Oxygen binding

In oxygen transport Hbs, the mechanism used to establish appropriate oxygen binding kinetics (and thus affinities) involves the combined efforts of the proximal and distal histidines (Olson & Phillips, 1996). As a result, their association equilibrium constants should be moderate, to enable them to bind oxygen when it is present, and their dissociation rate constants must be at least 1 s^{-1}, to release it when needed. Moreover, their concentration must be higher than that of oxygen in solution (millimolar concentrations) (Kundu *et al.*, 2003; Smagghe *et al.*, 2009)

In hexacoordinate Hbs, the affinity for oxygen can be determined by using the values of K_H, given in Table 1, together with Equation 2 (Smagghe *et al.*, 2009):

$$K_{O2} = \frac{K_{O2.pent}}{1 + K_H} \qquad \text{(Equation 2)}$$

The hexacoordination affinity (K_H) is calculated from the hexacoordination rate constants obtained from rapid mixing CO binding experiments (Table 1). The $K_{O2.pent}$ represents the affinity to the pentacoordinate form which is estimated from the ratio of the second rate constant for oxygen rebinding to the dissociation rate constant (k_{O2}). The rebinding of oxygen is assessed by laser flash photolysis (Fig. 14A and 13B) while the oxygen dissociation is determined by stopped-flow experiments using sodium dithionite as an oxygen scavenger (Fig. 14C) **(Paper II)**.

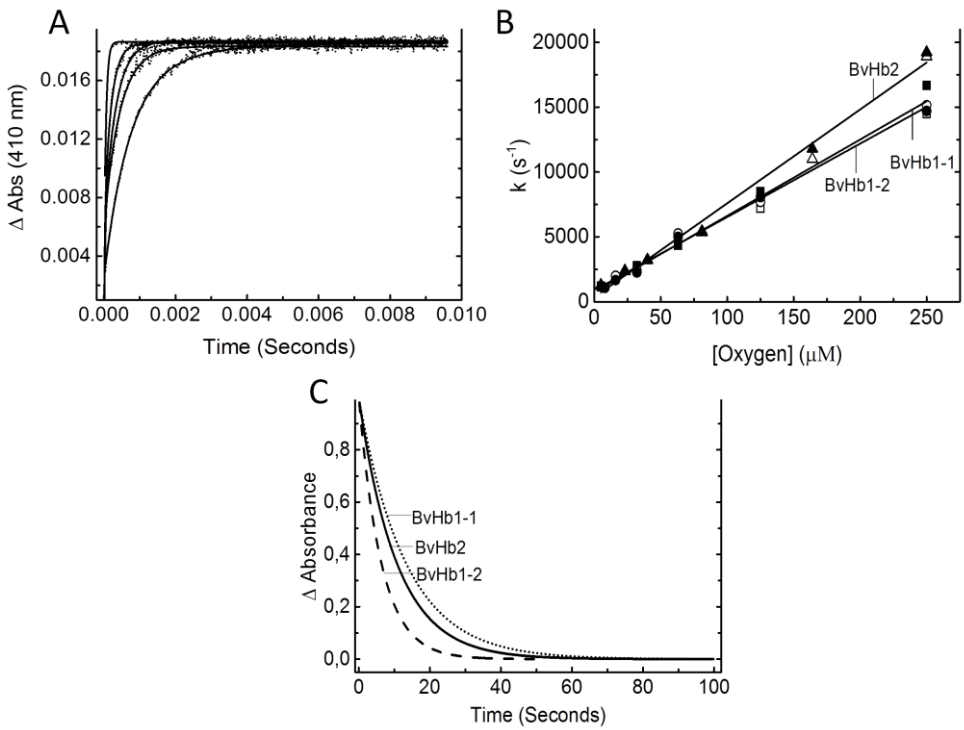

Figure 14. Oxygen binding to BvHbs. *A,* Time courses for rebinding of oxygen after flash photolysis at different oxygen concentrations (from left to right: 250, 81, 39, 23, and 5 μM) monitored at 410 nm. *B,* Dependences of the pseudo first order ligand rebinding rates on different oxygen concentrations, as measured by flash photolysis, provide $k'O_2$ values. These values are 59 x 10^6, 57 x 10^6, and 72 x 10^6 M^{-1} s^{-1} for BvHb1-1, BvHb1-2, and BvHb2, respectively. The closed and open symbols correspond to k_{obs} rates monitored at 410 and 430 nm, respectively. *C,* Time courses for the dissociation of oxygen, k_{O2}: 0.075, 0.158, and 0.094 s^{-1} for BvHb1-1, BvHb1-2, and BvHb2, respectively.

When oxygen association and dissociation rates from plant nsHbs are analyzed it is clear that, unlike the association rates, the dissociation rates are the most variable parameter influencing oxygen affinity. Since slow oxygen dissociation implies stabilization of the bound ligand, the variable rates observed in plant Hbs indicate variability also on the way the protein interacts with the bound oxygen. The results in **Paper II** show the impact k_{O2} has on the oxygen affinity of BvHb1-2 and BvHb2. Detailed analyses of the equilibrium constants of these two proteins and their amino acid sequences demonstrate that BvHb2 has retained some features of BvHb1-2 and vice versa. These results strengthened the possibility of BvHb2 taking over some BvHb1-2 functions in particular developmental stages and plant organs (Leiva-Eriksson *et al.*, 2014) as also observed in *Arabidopsis* (Hebelstrup *et al.*, 2006).

Another factor affecting oxygen affinity in nsHbs is their degree of hexacoordination. Due to the augmentation of affinity by hexacoordination (Equation 2), hxHbs could have affinity constants appropriate for oxygen transport. Thus, a high degree of hexacoordination was correlated to lower oxygen affinity. It was concluded that the strong hexacoordination of class-2 nsHbs allowed them to have P_{50} values for oxygen binding (~ 1 μM) similar to mammalian Mb, which is roughly 200-fold greater than those for class-1 nsHbs and ~10-fold greater than sHbs (Smagghe *et al.*, 2009). However, our results in **Paper II** indicate that the oxygen affinities of BvHb2 and BvHb1-2, in solution, are very similar even though BvHb2 presents a higher degree of hexacoordination (Table 1). Therefore not only the rate of binding of the distal His is of importance but also the interaction between the heme pocket and the oxygen molecule.

If the evolutionary origin of the plants is considered at the moment of comparing the oxygen affinity of class-1 nsHbs, it is evident that nsHbs from leguminous plants have the highest values. They are followed by the oxygen affinity values of monocot plants and then dicot plants which, in general, are not so different from the estimated values for class-2 nsHbs (Table 1). Such a selection seems to have followed the evolution and diversification of both plant Hbs and plant species (Vazquez-Limon *et al.*, 2012), which could also suggest selective pressure for different physiological functions.

III. Physiological and Functional Properties of NsHbs

3.1. Gene expression and localization *in vivo*

3.1.1. Expression under normal conditions

Information about the expression of nsHbs genes under normal growth conditions is limited as well as the number of species studied (Arredondo-Peter *et al.*, 1997; Trevaskis *et al.*, 1997; Duff *et al.*, 1998; Lira-Ruan *et al.*, 2001; Wang *et al.*, 2003; Bustos-Sanmamed *et al.*, 2011; Leiva-Eriksson *et al.*, 2014). Such expression is variable and depends on the plant species, developmental stage, organ, cell, and even organelle.

The expression of class-1 nsHb1 genes has usually been reported in organs different from those related to reproduction or reproductive stages (Nie & Hill, 1997; Trevaskis *et al.*, 1997; Parent *et al.*, 2008). They have been shown to be expressed in seeds of barley (Duff *et al.*, 1998), seeds and hypocotyl of sugar beet (Paper I), and germinating seedlings of *Arabidopsis* (Trevaskis *et al.*, 1997). It has been suggested that the expression of class-1 nsHbs genes during early seed germination and post-germination reflects a participation in maintaining energy and redox status within the embryo (Hebelstrup et al., 2007). Some exceptions, of course, exist. For instance, class-1 nsHbs from *Lotus japonicus* and tomato are expressed in roots of mature plants (Wang et al., 2003; Bustos-Sanmamed et al., 2011).

On the other hand, the expression of several other class-2 nsHbs genes has often been often detected in reproductive organs or in processes related to it such as embryogenesis and seed maturation (Hendriks *et al.*, 1998; Wang *et al.*, 2003; Ross *et al.*, 2004; Hebelstrup & Jensen, 2008). Thus, class-2 nsHbs have been found to be expressed in flowers of Arabidopsis, *L. japonicus*, tomato, and sugar beet **(Paper I)**. Changes in carbon translocation and photosynthetic rates indicate that the energy demand in reproductive organs is very high (Cooke & Scott, 1993). If class-2 nsHbs are directly involved in the maintenance of energy is unknown, but given the nature of these proteins, their participation in this process is almost certain.

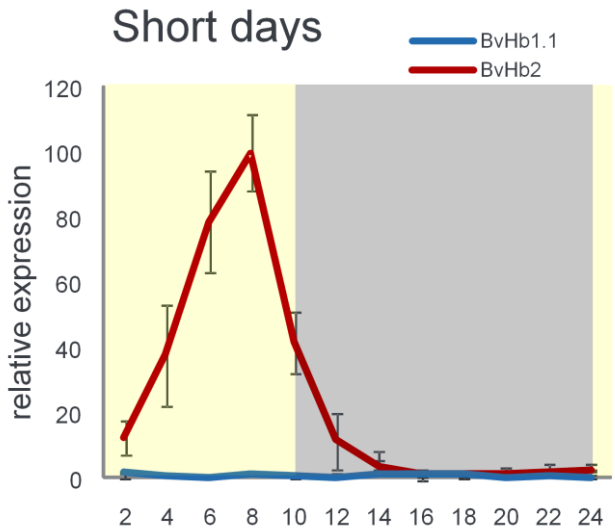

Figure 15. Relative expression of *BvHb1.2* and *BvHb2* under short days. Biennial sugar beet plants grown in SD with a temperature of 18 °C. Leaf samples were harvested every 2 hours. Data represents the mean ± SE (n=5).

To date, only nsHbs from annual plants have been studied. Therefore, no precedent is found regarding their expression under vernalization (period of cold before flowering). Similarly, there are no studies of their expression at different photoperiods, long (LD) and short (SD) days **(Paper I).**

Even though low temperature may cause stress in plants, for some species to spend some time in cold is an essential part of their development. In the case of sugar beet, when plants are grown in greenhouses it is necessary to mimic the winter conditions they are adapted to in order to make them flower. The expression of the three *BvHbs* during this period of cold was very different for each of the three proteins. This result reflects the different roles they have in the plant. Expression of *BvHb1-2* was basically absent during this period. The expression of *BvHb1-1* might be due to changes in the metabolism of the plant as a result of long-time exposure to cold. Another reason for the increment of its expression may be the scavenging of NO, as is has been demonstrated that its concentration increases with cold (Zhao *et al.*, 2009). Finally, the expression of *BvHb2* may be related to modifications in carbohydrate metabolism which is induced by low temperatures (Stitt & Hurry, 2002). Our results indicate that a possible role of BvHb2 is related to carbon metabolism or allocation. This possibility needs to be further investigated before any conclusions can be made.

In the different photoperiod experiments, the expression pattern of *BvHb1-1* and *BvHb2* were contrasting at LDs and SDs (Fig. 15). Our results indicate that *BvHb1-1* is unaffected by day light while, *BvHb2* is very responsive with a conserved expression amplitude regardless of the length of the day. An unpredicted result was the similarity

between the photoperiodic behavior of *BvHb2* and carbon allocation. Both starch accumulation and *BvHb2* expression decrease before the end of the day period and are independent of light and day length (Li *et al.*, 1992). The expression of BvHb1-1, on the other hand, was very low and invariable except for a sudden increase at the end of the dark period. Such a rise seems to respond to an endogenous signal since no external cause could be identified. It seems as if such a signal prepares the leaves for the time when the light will be on.

3.1.2. Expression under stress conditions

Hemoglobins are one of many alternative strategies that plants have evolved to overcome stress conditions and survive (Dordas, 2009). The expression of nsHb genes in response to hypoxia, cold, nutrient deprivation, darkness, and osmotic stress has been evaluated (Taylor *et al.*, 1994; Nie & Hill, 1997; Trevaskis *et al.*, 1997; Sowa *et al.*, 1998; Hunt *et al.*, 2001; Wang *et al.*, 2003; Bustos-Sanmamed *et al.*, 2011). Also, their reaction to signaling compounds such as NO and hormones has been studied (Hunt *et al.*, 2001; Ross *et al.*, 2004; Ohwaki *et al.*, 2005; Shimoda *et al.*, 2005; Qu *et al.*, 2006; Sasakura *et al.*, 2006; Bustos-Sanmamed *et al.*, 2011). In some cases it has been observed that the the expression of class-1 and class-2 nsHbs in response to stressing conditions is differential (Hunt *et al.*, 2001). For instance, the expression of tomato *Hb1* (*SOLlyGlb1*) increases in roots when the nutrients in the culture medium are modified. However, the levels of tomato Hb2 mRNA (*SOLly GLB2*) are not significantly altered. However, some overlapping can be found as in the case of *BvHb1-2* and *BvHb2* from sugar beet **(Paper I).** For details in these studies the review by Dordas (2009) is recommended.

Hypoxia is the most common stress condition used to analyze the expression of plant Hbs (Sowa *et al.*, 1998; Hunt *et al.*, 2002; Dordas *et al.*, 2003). Sometimes by itself (Trevaskis, 1997) or in combination with other stress condition such as hydrogen peroxide (Yang *et al.*, 2005). Usually the response is rapid, peaking within a few hours of plant exposure (Hill, 2012)

Another approach to examine plant stress involves the use of hormones. Many of them have been used with *Arabidopsis* (Trevaskis *et al.*, 1997; Hunt *et al.*, 2001) and *L. japonicus* (Bustos-Sanmamed *et al.*, 2011). So far, the results have been so variable that no trend or common denominator could be observed. However, it has been determined that a general relationship between NO and several hormones exists in order to regulate certain biological functions. Thus, auxins regulate lateral roots growth and delay flowering, jasmocid acid regulates wound inducible genes, ABA regulates stomatal closure and seed germination, and ethylene regulates iron uptake and root hair elongation. From this, a hypothesis has been proposed suggesting that nsHbs influence and alter the expression and site of action of auxins, jasmonates,

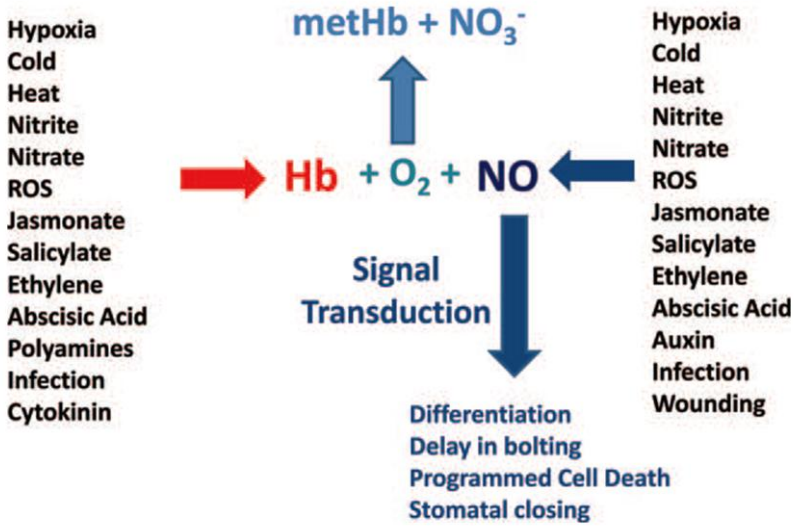

Figure 16. Diagram of the eliciting agents and mode of action of nsHbs and NO in plants. In the diagram a correlation is made between the factors that alter the expression of plant Hbs (left) and affect the levels of NO (right). In the middle there is a scheme of the reaction of Hb and NO, with the production of metHb. Below are listed the effect of NO production in plants. In order to simplify it, the different classes of nsHbs has not been differentiate it. Adapted from Hill RD (2012)

ethylene and abscisic acid (ABA) through modulation of NO levels within the cell (Fig. 16) (Hill, 2012).

3.1.3. Tissue and cellular localization

Until recently, nsHbs were expected to be found within the cytoplasm of the cell as no signal sequences that would target them to organelles or export them from the cell were ever identified. However, the genes of three different class-1 nsHbs, from sugar beet, grape, and spinach, were all found to carry information to be translocated into chloroplasts **(Paper I)**. Previously, only the truncated Hb from *Chlamydomonas* T1 was found to have a leading peptide for its translocation to chloroplasts (Couture *et al.*, 1994). However, nsHbs have previously been associated with this organelle when staining, and linear aggregates were observed colocalizing with the chloroplast during immunolocalization studies of somatic embryogenesis in chicory (*Cichorium intybus*) (Smagghe *et al.*, 2007). Chloroplasts are the major site of heme biosynthesis in higher plant cells (Cornah *et al.*, 2002), and studies on chloroplasts of *Arabidopsis* (Arnaud *et al.*, 2006; Jasid *et al.*, 2006) and soybean (Jasid *et al.*, 2006) have determined that this organelle contributes to NO synthesis *in vivo*. Therefore, a relationship between BvHb1.1 and NO might be plausible based on present

knowledge where class-1 nsHbs from different species are recognized as NO scavengers **(Paper IV)** (Gupta *et al.*, 2011; Spyrakis *et al.*, 2013).

NsHbs have also been reported to be in the nucleus of cells (Seregelyes *et al.*, 2000; Sainz *et al.*, 2013). In *L. japonicus*, members of the three classes of nsHbs were immunolocalized predominantly in the nuclei. There, flavins were quantified and their concentrations were sufficient to maintain Hbs in their functional state

By means of *in situ* hybridization, transcripts of nsHbs in *L. japonicus* were detected in vascular bundle cells (Fig. 17). These include phloem companion cells and xylem and phloem parenchyma cells, which exhibit high metabolic activity. In the vascular bundle cells, high respiratory rates are needed to sustain active transport of metabolites (Bustos-Sanmamed *et al.*, 2011). Therefore, it was proposed that nsHbs from *L. japonicus* may have functions aimed at ensuring adequate ATP synthesis, as reported for monocots (Igamberdiev & Hill, 2004). Phloem and xylem cells are able to generate NO at high rates (Gaupels *et al.*, 2008), which supports a role for LjGLB1-1 in NO metabolism in vascular bundles.

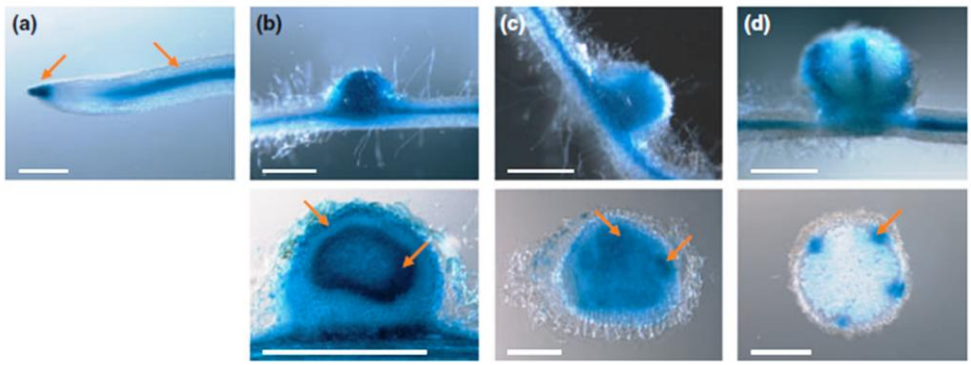

Figure 17. Localization of promoter activity of the class-2 nsHb gene from L. japonicus (LjGLB2). The lower panels correspond to half-cut longitudinal or transverse sections of the nodules shown in the upper panels. Staining for b-glucuronidase activity can be preferentially seen in: (a) root tips and vascular bundles; (b,c) cortex, vascular tissue, and infected zone of young nodules; and (d) vascular bundles of mature nodules. Bars, 500 lm. Figure adapted and modified from Bustos-Sanmamed et al. (2011)

3.2. Role of nsHbs in plants

Phylogenetic analyses have demonstrated that all Hbs are likely to have emerged from a bacterial single domain globin with one or more intrinsic enzymatic functions (Vazquez-Limon *et al.*, 2012). Their main recognized function as oxygen transporters, observed both in animal and plants, corresponds to recent evolutionary events. In animals, such a role has evolved around 500 to 600 million years ago (Goodman *et*

al., 1987). In plants, the transition occurred twice, the first one around 200 million years ago, resulting in the production of Lbs from class-2 nsHbs, which are ~40% identical in sequence. (Guldner *et al.*, 2004) (Sturms *et al.*, 2010). In the second event ParaHb1, an oxygen transport Hb, was originated from class-1 nsHbs leaving pairs of proteins with very high (>90%) sequence identity, but with different physiological functions (Appleby *et al.*, 1983).

To date, Hbs have been found to bind a broad range of ligands including small gaseous molecules (O_2, CO, NO), reactive compounds (H_2O_2 and H_2S), and biomolecules (lipids and other proteins). Thus, it is becoming increasingly apparent that in addition to the traditional functions of Hbs as carriers and stores of oxygen, they have a major role in controlling cellular levels of reactive oxygen species such as nitric oxide (NO) and hydroperoxides (H_2O_2) (Gardner, 2005b).

3.2.1. Nitric oxide

Even though the reaction of Hbs with oxygen is the most recognized, it is also very common for these proteins to react with NO. It has been demonstrated that blood cell Hb and Mb are likely scavengers of NO *in vivo (Eich et al., 1996)*, and that flavohemoglobins (flavoHbs) are scavengers of NO in bacteria and fungi (Gardner, 2005a). In plants, multiple studies have determined that nsHbs react with NO through the NO dioxygenase reaction, in a similar way as Hb in eritrocytes and Mb (Gardner, 2005a). In this reaction oxy-ferrous nsHbs react with NO yielding nitrate and ferric Hb, also known as metHb (Scheme 2) **(Paper IV)**:

$$HbFe^{2+}\text{-}O_2 + NO \rightarrow HbFe^{3+} + {}^{-}OONO \qquad \text{(Scheme 2)}$$

Even though the mechanisms of this reaction are not fully understood, evidence supporting the role of nsHbs as NO scavengers is increasing, mainly in favor of class-1 nsHbs. In alfalfa root cultures, cell lines expressing barley *Hb1* maintained root growth during hypoxia. The presence of NO/heme complexes in these cultures was determined by EPR analysis (Dordas *et al.*, 2003). Transgenic tobacco plants overexpressing alfalfa *Mhb1* were found to be less prone to necrosis after NO-treatment than the controls (Seregélyes *et al.*, 2003). The overexpression or silencing of *Hb1* genes has also been reported to affect NO emission in *Arabidopsis thaliana* (Perazzolli *et al.*, 2004; Cantrel *et al.*, 2011; Hebelstrup *et al.*, 2012) and *L.japonicus* (Shimoda et al., 2005; Bustos-Sanmamed et al., 2011).

However, the role of nsHbs as NO scavengers is still under discussion. Their low concentration in plants would not allow them to scavenge NO to a degree greater than Mb and Hb whose concentrations in mammals are much hgher (Kakar *et al.*, 2010). Therefore, the suggested biological role in hormone signal transduction (Hill, 2012) seems to be more likely than NO scavenging. Additionally, it has been

demonstrated that the ability of nsHbs to react with NO and scavenge it is limited by the rate of re-reduction of the resulting ferric Hb (Smagghe *et al.*, 2008b). Reports from alfalfa (Seregelyes *et al.*, 2004) and barley (Igamberdiev *et al.*, 2006) showed that the purification of their class-1 nsHbs to near homogeneity results in the loss of NO sensitivity. These results suggest that a reducing protein/agent is required for their NO scavenging activity.

The importance of the concentration of nsHbs has not been discussed in detail yet. Instead, research has been focused on the identification of a specific reductase that can support the catalytic scavenging of NO by reducing metHb into ferrous Hb (Scheme 3) **(Paper IV)**:

$HbFe^{3+}$ + e- (from FAD/NADH binding domain) → $HbFe^{2+}$ (Scheme 3)

The first reductase to be identified was a monohydroascorbate reductase (MDHAR) from barley roots (Igamberdiev *et al.*, 2006). Such a discovery allowed the authors to propose the Hb/NO cycle (Fig. 18) (Igamberdiev & Hill, 2004; Igamberdiev *et al.*, 2006). In this cycle, both Hb and MDHAR constitute a system metabolizing NO to nitrate. The metHb produced as a result of NO turnover is reduced via a coupled reaction in which ascorbate is oxidized and regenerated via an NADH-dependent cytosolic MDHAR. Since then, other reductases have been identified. In *L. japonicus*, it has been shown that nsHbs, immunolocalized in nuclei, were maintained functional by reduced flavins (Sainz *et al.*, 2013). In poplar, PttHb1, a class-1 nsHb, has been shown to relieve NO toxicity when expressed together with PtthFNR, a ferredoxin NADP+ oxidoreductase, **(Paper IV)**. Given the efficiency and versatility of the NAD(P)H + flavin systems, the possibility that flavins replace monodehydroascorbate reductase in the Hb/NO cycle has been raised (Sainz *et al.*, 2013). Especially after a study where it was demonstrated that the direct reduction of riceHb1 by ascorbic acid is unlikely to serve as a significant factor in NO metabolism, even in the presence of MDHAR (Wang & Hargrove, 2013).

In bacteria and yeast, the scavenging of NO is done by flavoHbs. The flavin domain of these proteins facilitates the reduction of the ferric globin at very high rates (Mills *et al.*, 2001). The coordination of the heme domain of flavoHbs in oxygen ligation is pentacoordinated and no examples of a hexacoordinate flavoHb have been found (Igamberdiev *et al.*, 2006). However, nature seems to have found other solutions. In **Paper IV** we demonstrate how PttHb1 interacts with PtthFNR in a similar way as the globin and flavoreductase domains interact in flavoHbs. When the three-dimensional PttHb1-PtthFNR complex was modeled it was found to be very similar to the flavoreductase domain of yeast flavoHb.

Regarding class-2 nsHbs, not much is known about their role in plants. The class-2 nsHb from *A. thaliana*, AtHb2, has been the most studied protein of this group and it has also been suggested to modulate NO. Those observations were made during somatic embryogenesis (Elhiti *et al.*, 2013), under hypoxic conditions (Hebelstrup *et*

al., 2006), and bolting (Hebelstrup & Jensen, 2008). However, indications that it would have a role as a sensor of oxygen concentration or as a carrier in oxygen transport have also been suggested. Indirect evidence for the second possibility has been obtained from *Arabidopsis* seeds. Their high oil content was attributed to the increased oxygen supply for mitochondrial respiration (Vigeolas *et al.*, 2011). In agreement with this result, analysis of the internal cavity of AtHb2 supported its role as an oxygen carrier (Spyrakis *et al.*, 2011). In sugar beet, the necessity of oxygen during vernalization was consistent with the increased expression of BvHb2 during this period. Therefore, a possible increment of oxygen availability by BvHb2 was not disregarded **(Paper I)**.

Since the oxygen affinity of these class-2 nsHbs is comparable to Lbs, a specific function of this protein in facilitating oxygen seems to be highly probable. However, more studies need to be done in order to clarify if class-2 nsHbs can have a role in NO modulation and/or oxygen supply.

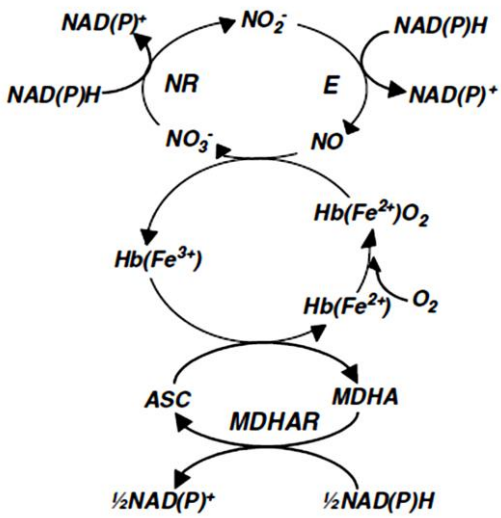

Figure 18. The Hb/NO cycle. NO is oxidized to nitrate by oxyhemoglobin [$Hb(Fe^{2+})O_2$], which turns to metHb [$Hb(Fe^{3+})$]. MetHb is reduced in the ascorbate (ASC)-mediated monodehydroascorbate reductase (MDHAR) reaction. *NR*, Nitrate reductase; *E*, NO sources including nitrate reductase and/or nitrite. Figure adapted from Igamberdiev *et al.* (2006)

3.2.2. Hydrogen peroxide

Hydrogen peroxide (H_2O_2) as a kind of reactive oxygen species is increased under different stresses and it has been reported to have multifaceted functions in plant stress responses (Neill *et al.*, 2002; Dat *et al.*, 2003). The involvement of H_2O_2 in the

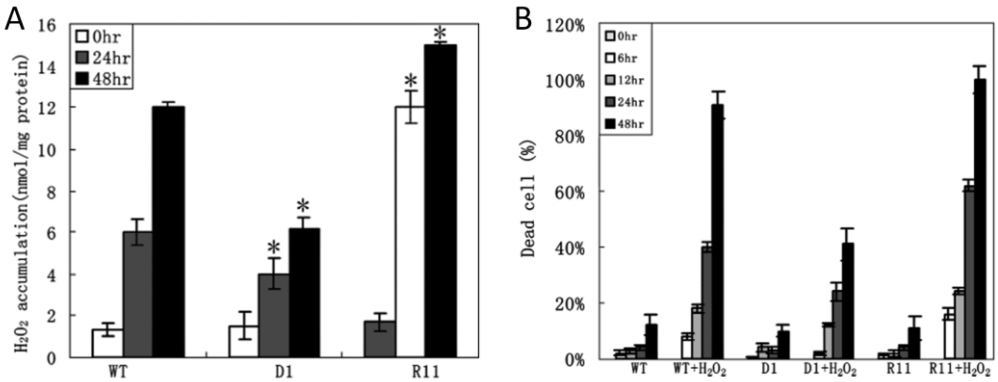

Figure 19. AtHb1 and H_2O_2. *A*, Production of H_2O_2 before and after hypoxic stress treatment in *Arabidopsis* seedlings. Asterisks indicate significant differences at the 5% level between transgenic plants and wild-type plants under the same conditions. *B*, Induction of cell death by exogenously supplied H_2O_2 (2 mM) in Arabidopsis protoplasts. Wild-type (WT), AtGLB1-overexpressing (D1) and AtGLB1-suppressed (R11) Arabidopsis cell lines. Figures adapted and modified from Yang *et al.* (2005)

response of plants to low oxygen stress has been verified. For example, a significant increase in the amount of H_2O_2 caused by anoxia was shown in anoxia-intolerant wheat (*Triticum aestivum*) root, but not in the anoxia-tolerant yellow flag iris (*Iris pseudacorus*) rhizome parenchyma suggesting that H_2O_2 might be a signal under low oxygen stress (Blokhina *et al.*, 2001).

Several studied have suggested that class-1 nsHbs may play a role against oxidative stress caused by H_2O_2. *Arabidopsis* lines overexpressing *AtHb1*, showed higher tolerance to hypoxic stress and relatively low endogenous H_2O_2 concentration when compared to wild type plants or in lines where the expression of *AtHb1* was suppressed by RNA interference (RNAi) (Fig. 19) (Yang *et al.*, 2005). In *L. japonicus*, nsHbs protected the cells against oxidative stress by intercepting superoxide and H_2O_2. The tolerance to oxidative stress has been correlated with the expression levels of both class-2 and class-3 nsHbs, indicating that protection against this type of stress is largely due to the heme groups rather than to intrinsic features of the globins.(Sainz *et al.*, 2013). Thus, some proteins would be more prone to react to H_2O_2 than others. Evidence of this is given in **Paper III** where it was demonstrated that BvHb2 modified electrodes could be used as mediatorless biosensors. In fact, the good stability of the modified sensors left open an interesting opportunity for practical applications in biosensing and bioengineering (Chekin *et al.*, 2010).

Similar to their role as NO dioxygenases, nsHbs that control cellular levels of hydroperoxides require that the resulting oxidized Hb (ferric Hb or metHb) should be effectively and rapidly reduced to sustain the reaction. Unfortunately, no reductase or reductant agent has been identified yet.

Hydrogen peroxide is able to oxidize globins up to their ferryl form. For instance, studies on Mb have demonstrated that when metMb reacts with additional H_2O_2, ferryl Mb is produced. Although ferryl Mb is stable for hours at room temperature (George & Irvine, 1952), the identity of the Mb residue(s) that form radicals in the presence of H_2O_2 has been the subject of some controversy. Globin· radicals have been localized to tyrosine and/or tryptophan residues (Tew & Ortiz de Montellano, 1988; Davies, 1991; Gunther *et al.*, 1998). Additionally, globin radicals may undergo subsequent chemistry and are capable of oxidizing a variety of biological molecules including proteins, nucleic acids, and lipids. (Kelman *et al.*, 1994). Similar results were obtained *in vitro* with BvHb1-2 and BvHb2 (Fig. 20). If the same oxidative reactions occur *in vivo* are still unknown, but the possible formation of ferryl BvHbs can be detrimental to the cell.

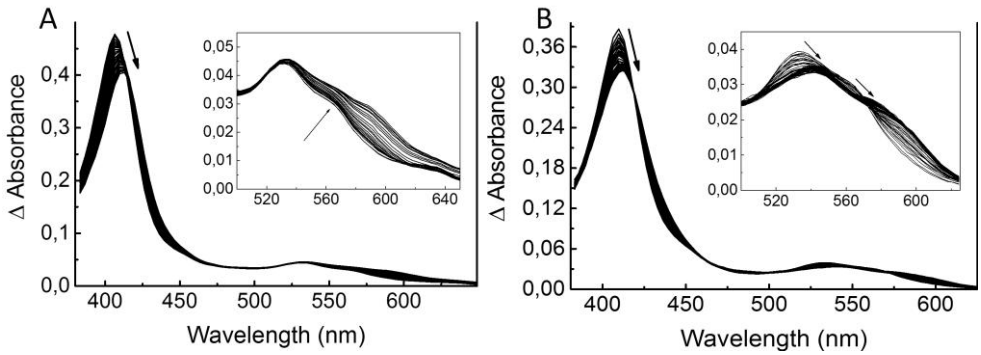

Figure 20. BvHbs Peroxidation. Spectral changes during ferryl formation from ferric BvHb1-2 (A) and BvHb2 (B). ***Insets.*** Magnification of the spectral changes observed in the visible region of the spectra.

3.3. Gene expression modifications and biotechnological applications

Genetic studies with the expression of nsHbs in plants, cell lines, and model systems such as yeast and *Arabidopsis* have been instrumental for improving the understanding of nsHbs' roles. Thus, one of the first reports altering the expression of nsHb genes was done in maize cells. There, the overexpression of barley *Hb1* enhanced their tolerance to hypoxia (Sowa *et al.*, 1998). Following studies with overexpression of *AtHb1* in *Arabidopsis* have shown that the synthesis of AtHb1 is essential for survival of seedlings under severe hypoxia (Hunt *et al.*, 2002). Similarly, it was observed that expression of a barley *Hb1* in alfalfa root cultures enhances growth and ATP/ADP ratio, whereas antisense suppression with the same gene tended to have the opposite effect (Dordas *et al.*, 2003). Additional studies

overexpressing or suppressing barley *Hb1* resulted in enhanced or reduced NADH-dependent reduction of NO to nitrate, respectively, not only in extracts from transgene roots (Igamberdiev *et al.*, 2004) but also in a suspension cell culture from transgenic maize (Dordas *et al.*, 2004). All together, these results have contributed to the formulation of the Hb/NO cycle (Fig.18). Furthermore, complementation experiments in yeast lacking the flavoHb gene have allowed us to demonstrate that PttHb1 and PtthFNR relieve the cells from NO toxicity in a concerted way, similar to flavoHbs **(Paper IV)**.

Arabidopsis was one of the first plants where transgenic methods were used to modify the expression of nsHbs. In one of those studies, the knockout of *AtHb2* was combined with the silencing of *AtHb1*. As a result, seedlings died at an early vegetative stage suggesting that both nsHbs played an essential role for their normal development. It was also suggested that some overlapping in their function exists so that the presence of at least one of them is essential for the survival of young non-stressed seedlings (Hebelstrup *et al.*, 2006).

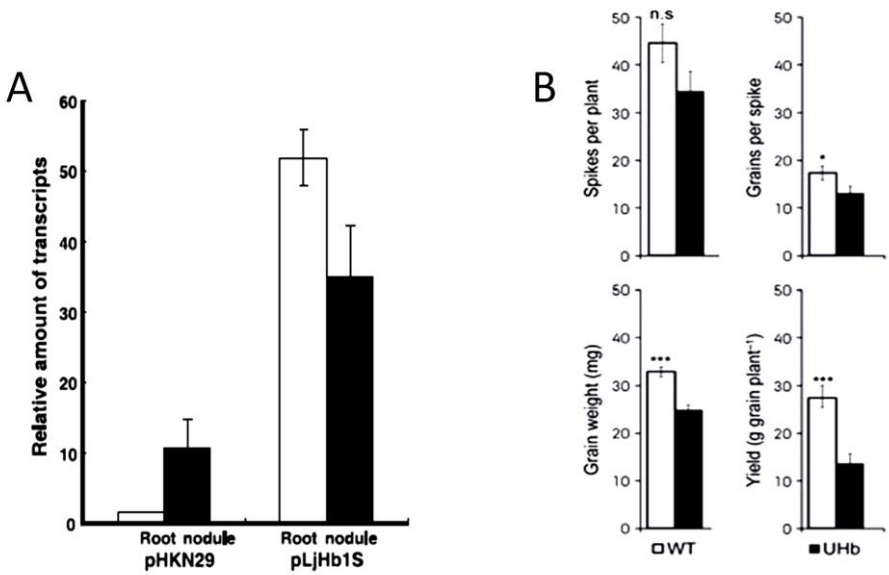

Figure 21. Contrasting results after the overexpression of class-1 nsHbs in *L. japonicus* and barley. *A,* Expression of LjHb1 and AfHb1 in the roots and nodules of transgenic hairy roots. Expression of LjHb1 is shown as the relative number of transcript compared with that in the GFP-positive hairy roots induced by vector control (pHKN29). Values represent the means of four biological replicates and the corresponding standard errors. *B,* Yield (g grain per plant) and yield components (number of spikes per plant, number of grains per spike and grain weight) of UHb plants in comparison with WT Control (Golden Promise). Significant differences according to Student's *t*-test are marked with either $*P < 0.05$, $***P < 0.001$ or n.s. (not significant). Figures adapted and modified from Shimoda et al. (2009) and Hebelstrup et. al. (2014)

As in the study described above, the alteration in the expression of nsHbs plants may result in evident phenotypes, some of them of biotechnological interest. In *L. japonicus*, the overexpression of *LjHb1* and *AfHb1* (a class-1 nsHb from *Alnus firma*) by transformed hairy roots caused changes in symbiosis with rhizobia. The number of nodules formed on hairy roots and nitrogenase activity increased enhancing symbiotic nitrogen fixation activity (Fig. 21A) (Shimoda *et al.*, 2009). In another study, changes in the expression of either *AtHb1* or *AtHb2* affected the time to bolting thereby reducing the time to flowering (Hebelstrup & Jensen, 2008). The specific overexpression of *AtHb2* in seeds can lead to an increase in their biosynthetic performance. As a result, the fatty acid content of mature seeds had an increment of 40% (Vigeolas *et al.*, 2011).

Even though the results described above seem to be very promising, the results obtained from the overexpression of barley *Hb1* in the same crop were not as successful. Unlike *Arabidopsis*, ectopic overexpression of barley *Hb1* delayed plant growth and development, and seed specific overexpression reduced seed yield (Fig. 21B). As a conclusion, the authors highlighted the necessity for using actual crop plants rather than laboratory model plants when assessing the effects of biotechnological approaches for crop improvement (Hebelstrup *et al.*, 2014).

During many years, researches have been working on the development of a new industrial oilseed crop. In **Paper V** we present very early data on the use of Hbs to manipulate the fatty acid production in seeds of *Arabidopsis* and *Lepidium. campestre*. *L. campestre* (field cress) of the Brassicaceae family has been selected among a number of earlier investigated candidates (Merker & Nilsson, 1995). It has several advantages for a production system based on undersowing in a spring cereal field. It has good agronomic features such as proper branching, suitable seed size, and high seed yield potential at >5 ton/ha (Merker et al., 2009). It also has an oil quality with high content of linolenic acid, which is an industrial quality as such (Nilsson et al., 1998). However, higher seed oil content is a key property in the efforts to make *L. campestre* a useful oil crop. The seed oil content of some accessions collected at various geographic locations is around 20% (Nilsson et al., 1998). To be economically competitive, it will be necessary to increase the seed oil content to at least 25-30%.

Given that *L. campestre* is also a Brassicaceae like *Arabidopsis*, we expect to be able to increase its oil content by overexpressing *Hb* in seeds. The preliminary results indicate that there is potential to increase the seed total fatty acid content, particularly in *L. campestre*. The results in *A. thaliana* also indicate that the level of desaturation may be affected. Further evaluation is necessary; however, the results demonstrate that the use of nsHbs gene expression in plants may provide us with a tool to enhance the seed oil quality in important oil crops.

Conclusions and Future Prospects

More than thirty years have passed since nsHbs were discovered. Still, there is a long way left before their role can be unmistakably determined. The ability these proteins have to bind different compounds make it very hard to assign them a single function. The most probable is that their roles are specie- and tissue-specific, maybe even cell-specific.

One of the first conclusions is that; even though, their primary structure divide nsHbs into class-1 and class-2 a very sharp dividing line between these two groups cannot be drawn without taking into account the phylogenetic classification of plants. This conclusion has been deduced from their expression pattern in sugar beet plants, and it can probably be extended to their sequence, structure, heme-pocket environment, and physiological roles. We suggest that the overlapping features observed in BvHbs are the result of selective pressure which should also be responsible that BvHb1-1 did not lose its N-terminus as other nsHbs from dicot plants **(Paper I)**. Evidently, the study of plant Hbs in a bigger variety of plant species is becoming mandatory. It is true that physiological studies in *A. thaliana* are fast and can answer many questions. However, it seems that in the case of plant Hbs, this plant model is not enough to cope with the broad diversity these proteins present. This is especially true if plant Hbs want to be considered as biotechnological targets.

The biophysical characterization of the three proteins confirmed the results and conclusions obtained in **Paper I**. The oxygen and CO binding rates of BvHb1-1 as well as its degree of hexacoordination are in concordance with the values given for other class-1 nsHbs. Therefore, it is highly probable that BvHb1-1 would have the role proposed for this group as a NO dioxygenase. However, different from other class-1 nsHbs, BvHb1-1 carries a leading peptide with information to be translocated to chloroplasts (Leiva-Eriksson *et al.*, 2014). How does it affect its role in sugar beet is something that needs further studies. One of the points to start would be to determine the localization *in vivo* not only of BvHb1-1 but also the class-1 nsHbs from grape and spinach. Regardless of the results, the findings would open up more research possibilities, not only in the field of plant Hbs but also photosynthesis and protein translocation,

Regarding BvHb1-2 and BvHb2, it was determined that their ligand binding rates and affinities are reciprocal. The same observation ´has been done when their amino

acid sequence was compared. Therefore, it is very likely that these two proteins have overlapping roles **(Paper II).** The study of more plant Hbs from dicot plants is quite necessary. As they have both class-1 and class-2 nsHbs, it would allow us to know if the reciprocity of their roles is a general feature.

In this thesis we have presented kinetic and ligand binding experiments with oxygen and CO. However, studies on the reaction of nsHbs with NO are highly necessary. The scarce number of kinetic studies with NO is due to the complexity of the experiment set up. NO is much more reactive than oxygen and the binding rates are very fast. In addition, the mechanism for this reaction is still under discussion. Great advances have been done with pentacoordinated proteins, but a lot more work needs to be done with hxHbs.

According to Kakar *et al.* (2010), one of the weakest sides of Hb research is the lack of physiological studies. Most of the roles proposed to them are usually the result of biophysical studies. In the laboratory, recombinant Hbs will readily adopt the ferrous and ferric oxidations states, and can even react with compounds that are not commonly found in cells. But without knowledge of function *in vivo*, it is difficult to judge the objective importance of these observations. In principle we agree with their statements. However, these types of studies are not completely wrong. On the bright side, these approaches allow the discovery of proteins with intrinsic potential for practical applications. Evidence of this is given in **Paper III** where it was demonstrated that BvHb2 modified electrodes could be used as mediatorless biosensors. The good stability of the modified sensors left open an interesting opportunity for practical applications in biosensing and bioengineering.

In **Paper IV** we present clear evidence of PttHb1 NO toxicity in mutant yeast and poplar roots together with PtthFNR in a concerted way in a similar way flavoHbs do. The interaction between the nsHb and the reductase were confirmed by bimolecular fluorescence complementation and complementation studies in yeast lacking flavoHb. In addition, for the first time a well-characterized chloroplast/plastid enzyme is found to be active as well in cytosol. A model of the interaction between PttHb1 and PtthFNR is also presented. However, the crystallization of this complex, would give much more information. For the proposed Hb/NO cycle to work the presence of a reductase in essential, and to date, more than one have been suggested. Based in our results and previous ones, it seems that the most effective reductase is a NADH-dependant, as the flavins. If there are one or several reductases for each plant Hbs is still unknown but is for sure very high in the list of questions to answer regarding nsHbs.

In **Paper V**, we present a summary of very early experiments where the genes of two class-2 nsHbs and a bacterial Hb are overexpressed in seeds of *Arabidopsis* and *Lepidium campestre*. After analysis of all the lines obtained after *Arabidopsis* transformation, we were able to pick lines significantly different from wild type. The nest step is to grow again those plants and increase the number of replicates. In the case of *L. campestre*, as it is a biennial plant, so far, we have produced the T2

generation and we are waiting for the third generation to grow. However, there seems to be potential to increase its seed total fatty acid content.

Given the number of potential roles nsHbs could have, they will probably become very common biotechnological targets. However, the risk exists that the results will vary from crop to crop as their role in plants seems to be very specific and fine-tuned.

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