# NONSYMBIOTIC PLANT HEMOGLOBINS: SYNOPSIS AND PERSPECTIVES OF THEIR STRUCTURE AND ENZYMATIC ACTIVITIES

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The first plant hemoglobins that were discovered were leghemoglobins which are highly expressed in the root nodules of legumes. Nonsymbiotic hemoglobins possess distinct primary structures and biochemical properties compared to them and are virtually distributed in all higher plants. Whereas leghemoglobins are nowadays well known both structurally and functionally, the function and mechanism of action of nonsymbiotic hemoglobins are still unclear. The main role of some nonsymbiotic hemoglobins was attributed to NO scavenging and thus increasing plant survival during hypoxia. Additionally, they may be involved in plant defence against pathogens and may be important players in other stress related responses since other enzymatic activities were discovered in some in vitro studies. Some structural aspects are also discussed.

Key words: globin, nonsymbiotic hemoglobin, plant stress response.

## INTRODUCTION

Hemoglobins (Hbs) are iron-containing heme proteins with a 'globin' polypeptide fold rich in  $\alpha$ -helices. They are among the most studied proteins due to their ease of investigation via spectroscopy and their importance in oxygen transport and storage in both medical and general biological contexts. In plants, Hbs have been known to exist for over 60 years. The first plant globins that were discovered were leghemoglobins (Lbs) which are highly expressed (millimolar range) in the root nodules of legumes and serve for oxygen storage and thereby creating an environment with an extremely low oxygen concentration. Thus, Lbs prevent the inhibition of the nitrogenase in nitrogen-fixing bacteria by oxygen (Garrocho-Villegas et al., 2007). Much later, in the early 1990s, nonsymbiotic hemoglobins (nsHbs) were discovered with distinct nucleotide sequences and only ~40% identity compared to Lbs (Gupta et al., 2011). Studies of these nsHbs classified them into three classes (1, 2 and 3) based on their primary sequence,

oxygen affinity and structural aspects (Garrocho-Villegas et al., 2007; Hoy and Hargrove, 2008; Smagge et al., 2009; Arredondo-Peter, 1998). Whereas Lbs are nowadays well known both structurally and functionally, the function and mechanism of action of nsHbs is still unclear (Smagge et al., 2009); class 3 in particular is extremely poorly characterized. Class 1 nsHbs possess an extremely high oxygen affinity; they are triggered by cold, hypoxia and fungal infection, and are involved in the redox balance of NO, a key component in stress related responses (Gupta et al., 2011; Dordas, 2003; Dordas, 2009). Class 2 nsHbs are much less studied then class 1; they are known to have lower oxygen affinity and they are thought to be involved in mediating oxygen diffusion to some tissues (Smagge et al., 2009). Class 3 Hbs are called truncated hemoglobins (TrHbs) due to their homology to bacterial Hbs which have a shorter sequence and compact '2-on-2' structures (Hoy and Hargrove, 2008; Pesce, 2013). Arabidopsis TrHb (AtHb3) has very unusual oxygen and NO binding kinetics; reports according to which the reduction of the ferric form leads to a hexacoordinate transient state which then slowly decays to a pentacoordinate species (Watts et al., 2001) were later questioned based on more detailed structural studies on AtHb3 (Reeder and Hough 2014) besides the need to explore what appears to be a set of complex and possibly unprecedented biochemical kinetic issues the physiological role of these plant TrHbs is still completely unknown, hence awaiting more detailed exploration (Smagge et al., 2009).

Briefly mentioned, nsHbs (mainly class 1) are thought to be biologically involved in stress control via Hb-NO/NO<sub>2</sub><sup>-</sup> interaction (triggered by hypoxia, cold, heat, infection, wounding or others), differentiation, delay in bolting and flowering, root hair elongation, programmed cell death, seed germination, stomatal closing, discussed by excellent reviews (Garrocho-Villegas et al., 2007; Hoy and Hargrove, 2008; Hill, 2012) and references therein, indication that these proteins are multifunctional with high importance in adaptation of land plants. Besides *Arabidopsis* nsHbs, homologs from other organisms were investigated, though few at the purified protein level; examples include rice, barley, maize, cotton, soy bean, *Parasponia, Alnus, Lotus japonicus, Trema tromentosa, Medicago*, and few others (Dordas, 2003). For a better understanding of their biological function, many other important plants with agricultural and other bioeconomic impact are candidate targets of detailed investigations. This short review aims to discuss current aspects regarding the role of the nonsymbiotic plant hemoglobins in stress related responses and the relations between these and their enzymatic activities.

#### Structural aspects of nsHbs

Hemoglobins have the typical fold of a myoglobin which consists of eight helices denoted by letters from A to H. They form a so called 3-on-3 architecture since the globin appears as a pack of three helices on top of other three helices. Class 1 and 2 of nsHbs have such a fold whereas class 3 present 2-on-2 fold due to some missing typical helices and some distortions in structure (Fig. 1). In addition to this, while class 1 and 2 present a hexacoordinated heme (bis-His), class 3 are described as pentacoordinated in deoxy state and the typical distal histidine is missing (Fig. 1) and have much lower oxygen affinity than the first two classes. Class 2 nsHbs have a stronger hexacoordinated character and thus lower oxygen affinity than class 1 but still much higher than class 3. These differences in structure, together with other structural aspects such as gating residues, quaternary structure, and solvent tunnelling and exposure surface are responsible of different reactivities and biological function.



Fig. 1. Plant hemoglobins fold for two representative ones, Hb1 from *Arabidopsis thaliana* (AtHb1, 3ZHW, pdb code) and Hb3 from the same organism (AtHb3, 4C0N, pdb code). Notice AtHb1 is hexacoordinated and has a 3-on-3 fold (helices A, E, F on B, G, H) while the latter one is pentacordinated and has a 2-on-2 fold (helices B, E on G, H).

# Involvement of the enzymatic activities of plant nonsymbiotic hemoglobins in stress responses

The main role of nsHbs (class 1) was attributed to NO scavenging and detoxification, increasing plant survival during hypoxia by acting as a nitric oxide dioxygenase coupled to NADH oxidation and thus maintaining the glycolysis at low oxygen concentration (during floods for example) (Dordas, 2003; Igamberdiev and Hill, 2004, Igamberdiev and Hill, 2004). This could happen since some nsHbs have characteristics of a true NO dioxygenase (very high dioxygen affinity, nanomollar level), see Fig. 2.



**Fig. 2.** A schematic representation of a nsHb involvement in NO scavenging during hypoxia. Oxidation of NADH in the process of nitrite reduction leads to NO which may trigger nsHbs overexpression. NO is oxidized to nitrate by the triggered nsHbs. Obtained nitrate is reduced to nitrite by cNR (cytosolic nitrate reductase) and part of nitrite is further reduced to ammonia by NiR (nitrite reductase). During these pathways, NAD(P)H equivalents are oxidized to NAD(P)<sup>+</sup>, maintaining glycolytic flux and thus cells viability. Additionally the redox status of the cytosol may be controlled.

Among others, by analogy with related flavohemoglobins from bacteria, this pathway would require a reducing system for keeping the Hb in the ferrous functional state. For this, several alternatives have been mentioned so far: direct reduction by NAD(P)H, FADH<sub>2</sub> or ascorbic acid which are less probable due to their low turnover rate and need of their high concentration. Another possible mechanism is the presence of a metHb reductase or other types of enzymes with reductase activity (Igamberdiev and Hill, 2004; Wang and Hargrove, 2013; Igamberdeiv et al., 2005) (Fig. 2). This issue may deserve separate investigation for each individual Hb since the known nsHbs display different patterns of expression and distribution and thus probably require different reaction partners.

Other enzymatic activities of nsHbs were observed *in vitro*, such as nitrite reductase (Sturms et al., 2011) Tiso et al., 2012), peroxidase (Violante-Mota, 2010; Sakamoto et al., 2004) hydroxylamine reduction to ammonia (Sturms et al., 2011) (Scheme 1). Nitric oxide might be produced as response to other stress factors such as osmotic stress, flood or cold (Lamotte et al., 2005) – and nsHbs are indeed upregulated by factors such as osmotic stress (Trevaskis et al., 1997), indicating that these proteins are used as responses to other abiotic stress factors (Dordas,

2009) this line of research might therefore lead to important findings in plant stress tolerance. In addition, Hbs are known to be involved in interaction with other reactive oxygen (ROS) or nitrogen species (RNS) such as superoxide, peroxide, peroxynitrite (Reeder, 2010) and sulfur-based ligands such as sulfide and sulfite in other organisms (Bagarinao and Vetter, 1992). These aspects are poorly studied for nsHbs and constitute an interesting and important research target. Potential interactions between nsHbs and plant hormones (jasmonic acid, indole acetic acid/auxin, abscisic acid, ethylene) as well as NO–plant hormone cross-talk are also research directions that need important attention since they have strong potential to lead to results with high impact in basic scientific and bioeconomic areas related to crop plants (Hill, 2012; Igamberdiev et al., 2005).



Scheme 1. Putative enzymatic activities of nsHbs, proven in vivo and/or in vitro.

## Involvement of plant nonsymbiotic hemoglobins in pathogenic stress

Nitric oxide is a key component in the plant-pathogen interaction (Romeo-Puertas, 2004); together with ROS, this triggers hypersensitivity and cell death, and induces defence genes (Delledonne et al., 1998). Important connections between nsHbs and pathogenic responses (via nitric oxide and/or other ROS) have been observed in transgenic tobacco plants (Seregélyes et al., 2003) as well as in Arabidopsis in interaction with Botrytis cinerea (Mur et al., 2012). Because various plant species respond differently when they interact with the same or different pathogens, several response mechanisms are expected and thus more plantpathogen systems need to be explored (Dordas, 2009), especially those with great economic impact. This high variability can nevertheless be tackled so that with proper strategies and control experiments important and reliable results can be found. Sclerotinia sclerotiorum (Lib.) de Bary is one of the most devastating and cosmopolitan necrotrophic fungal pathogen causing disease in numerous plants with great bioeconomic loss (Bolton et al., 2006). It is known that S. sclerotiorum corrupts its hosts by manipulation the redox environment (Volton et al., 2006; Williams et al., 2011: Zhou et al., 2013: Guo and Stotz, 2007), therefore nsHbs are expected to be involved in plant defence against this fungus, however this direction is completely unexplored.

In conclusion, nsHbs are a group of globins with interesting and important features regarding their structure, enzymatic reactivity and biological functions which

are still little explored though appears as important molecular players in plant stress responses with important putative scientific economic results.

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