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Non-symbiotic haemoglobins—What's happening beyond nitric oxide scavenging?

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Abstract

Background and aims Non-symbiotic haemoglobins have been an active research topic for over 30 years, during which time a considerable portfolio of knowledge has accumulated relative to their chemical and molecular properties, and their presence and mode of induction in plants. While progress has been made towards understanding their physiological role, there remain a number of unanswered questions with respect to their biological function. This review attempts to update recent progress in this area and to introduce a hypothesis as to how non-symbiotic haemoglobins might participate in regulating hormone signal transduction.

Principal results Advances have been made towards understanding the structural nuances that explain some of the differences in ligand association characteristics of class 1 and class 2 non-symbiotic haemoglobins. Non-symbiotic haemoglobins have been found to function in seed development and germination, flowering, root development and differentiation, abiotic stress responses, pathogen invasion and symbiotic bacterial associations. Microarray analyses under various stress conditions yield uneven results relative to non-symbiotic haemoglobin expression. Increasing evidence of the role of nitric oxide (NO) in hormone responses and the known involvement of non-symbiotic haemoglobins in scavenging NO provide opportunities for fruitful research, particularly at the cellular level.

Conclusions Circumstantial evidence suggests that non-symbiotic haemoglobins may have a critical function in the signal transduction pathways of auxin, ethylene, jasmonic acid, salicylic acid, cytokinin and abscisic acid. There is a strong need for research on haemoglobin gene expression at the cellular level relative to hormone signal transduction.

Introduction

Haem proteins are critically important proteins in self-replicating organisms, with the haem iron acting as an electron transfer component in many redox reactions and/or as a chelating agent for small, biologically important ligands. The class of haem proteins known as haemoglobins are perhaps the best known of these proteins because of their major role in the animal kingdom for the transport of oxygen. The animal haemoglobins are also among the most studied due most probably to the ready supply of experimental material. Haemoglobins, however, have significant roles in other organisms ranging from bacteria to higher plants. Their biological function in these organisms can be considerably different from that of some of the animal haemoglobins. Bacterial and yeast flavohaemoglobins, with an additional domain for binding FAD and NAD(P)H, have an enzymic function to degrade nitric oxide (NO), providing protection

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against nitrosative stress (Wu *et al.* 2003). Much of the early research on plant haemoglobins centred around leghaemoglobin, which serves to transport oxygen in a stabilized, extremely low free oxygen concentration to symbiotic nitrogen-fixing bacteria (Appleby 1992). In the late 1980s, evidence of haemoglobin in a non-nodulating plant, *Trema tomentosa*, suggested that there might be another form of plant haemoglobin, distinct from leghaemoglobin (Bogusz *et al.* 1988). A non-symbiotic haemoglobin, with a clearly distinct nucleotide sequence (~40 % identity) from leghaemoglobins, was isolated from barley in 1994 (Taylor *et al.* 1994). This haemoglobin, now classified as a class 1 non-symbiotic haemoglobin, is up-regulated by low cell oxygen tension and, although possibly not being its only function, scavenges NO (Dordas *et al.* 2003, 2004; Perazzolli *et al.* 2004). Unlike flavohaemoglobins, it has no flavin and NAD(P)H domain and, therefore, must function with a reductase to regenerate haemoglobin to maintain NO breakdown (Igamberdiev and Hill 2004). At least two classes of haemoglobins have been identified in several species (Arredondo-Peter *et al.* 1997; Trevaskis *et al.* 1997; Hunt *et al.* 2001; Shimoda *et al.* 2005), while only a class 1 haemoglobin has been identified in barley. Class 2 non-symbiotic haemoglobins have lower oxygen affinity and are induced by cold and cytokinins, but not hypoxia (Trevaskis *et al.* 1997; Hunt *et al.* 2001). A truncated haemoglobin, termed as class 3 (Hb3), has also been identified in arabidopsis (Watts *et al.* 2001). Hb3 has unusual concentration-independent O_2 and CO binding properties, with the association constants of the two ligands each being independent of their concentration. Hb3 is found throughout the plant and is not influenced by factors that influence other non-symbiotic haemoglobins.

There have been a number of excellent reviews over the last several years that have covered specific aspects of plant haemoglobin research. A review by Dordas (2009) emphasizes the metabolic and physiological research related to non-symbiotic haemoglobins, while Garrocho-Villegas *et al.* (2007) provide a retrospective overview of the last six decades of research, examining the literature on both leghaemoglobins and non-symbiotic haemoglobins. Smagghe *et al.* (2009) present a comprehensive, thoughtful look at the relationship between the structural properties of the plant haemoglobins, their oxygen binding properties and the likely physiological function of the molecule. Reviews from Hargrove's laboratory (Hoy and Hargrove 2008; Kakar *et al.* 2010) are an excellent resource for information on the structure and ligand binding characteristics of plant haemoglobins. In this review, we will attempt to focus, as much as possible, on recent advances in the area, ending on a hypothesis suggesting a biological role for plant haemoglobins in hormone signal transduction.

Structural considerations in relation to function and species divergence

A rice class 1 haemoglobin (Hb1) was the first non-symbiotic haemoglobin to be crystallized (Hargrove *et al.* 2000). The molecule exists as a dimer and has a weak hexacoordinate haem configuration in the ferrous state in comparison with class 2 haemoglobins (Smagghe *et al.* 2009). Class 1 haemoglobins exist as a mixture of the penta- and hexacoordinate forms, while class 2 haemoglobins are fully hexacoordinate (Bruno *et al.* 2007). The haem iron is coordinated to the two histidines in a manner similar to cytochrome *b5*, with an unusual bis-histidyl haem coordination for a haemoglobin reversibly binding oxygen (Hargrove *et al.* 2000). The distal histidine, however, is rapidly displaced by ligands, resulting in high-affinity oxygen binding. Phenylalanine B10 is a regulatory element in hexacoordination, facilitating stable oxygen binding to ferrous haemoglobin and promoting binding for ligands such as azide in the ferric state by preventing tight His^{E7} coordination (Smagghe *et al.* 2006). Studies with *Arabidopsis thaliana* (arabidopsis) mutants support these conclusions (Faggiano *et al.* 2009). Phenylalanine B10 is conserved among haemoglobins across several kingdoms and has a direct role in ligand-binding regulation (Smagghe *et al.* 2006).

There has been considerable interest in the characteristics of non-symbiotic haemoglobin ligand docking sites. There is evidence of the existence of temporary docking sites within the protein matrix for CO binding before re-binding to haem that may relate to the observed NO dioxygenase activity of the protein (Abbruzzetti *et al.* 2007). These docking sites are substantially different between arabidopsis Hb1 (AHb1) and non-symbiotic haemoglobin class 2 (AHb2), with stronger polar interactions and hydrogen bonding in AHb1 (Bruno *et al.* 2007). There are suggestions that the distal haem cavity in AHb1 is connected via a relatively open channel to the exterior, while temperature-dependent protein dynamics influence ligand migration from the distal cavity to the solvent in AHb2. Spectroscopic studies (Nienhaus *et al.* 2010) have identified two CO docking sites in AHb1, only one of which can be occupied in AHb2. Using this information, a mechanism has been proposed to account for NO dioxygenase activity by which binding of O_2 results in the formation of a channel through Hb1 from the distal cavity to the bulk solvent, permitting NO to occupy a docking site near the haem-bound O_2, which would facilitate the

reaction of the two ligands to form nitrate. Hb2 appears to lack this ability, leading to the suggestion that Hb2 is not involved in NO dioxygenase activity, at least via a mechanism that is dependent upon the supply of metabolite to internal storage sites. Two recent papers (Spyrakis *et al.* 2011; Vigeolas *et al.* 2011) suggest a possible role for AHb2 in oxygen transport.

There has been a long, continuing interest in the ancestral development of non-symbiotic haemoglobins (Smagghe *et al.* 2009). A caesalpinoid haemoglobin (ppHb) has been cloned that has an intermediate structure between a non-symbiotic haemoglobin and a leghaemoglobin, suggesting that it may be an ancestral leghaemoglobin (Gopalasubramaniam *et al.* 2008). There appears to have been a compaction of the CD loop in ppHb and decreased mobility of the distal histidine, leading to a pentacoordinate protein with a more compact globular structure. The moss, *Ceratodon purpureus,* haemoglobin (cerHb) possesses three introns, like higher plant non-symbiotic Hbs, indicating that this pattern has been retained during the ancestral development of the plant haemoglobins (Garrocho-Villegas and Arredondo-Peter 2008). CerHb is predicted to have a hexacoordinate structure with high affinity for O_2. It is suggested that the major evolutionary changes that have occurred in the transition from ancestral haemoglobins to non-symbiotic haemoglobins and leghaemoglobins are a hexacoordinate to pentacoordinate haem transition, decrease in the CD-loop and N- and C-termini, and protein compaction into a globular structure. Examination of haemoglobins in two closely related plants, *Trema* (non-nitrogen fixing) and *Parasponia* (nitrogen fixing), suggest distinct mechanisms for convergent evolution of oxygen transport in different phylogenetic classes of plant haemoglobins (Sturms *et al.* 2010).

Non-symbiotic haemoglobins, NO, reactive oxygen species and stress

Involvement in NO metabolism is a key component in the function of non-symbiotic haemoglobins (Dordas *et al.* 2003, 2004; Perazzolli *et al.* 2004). A mechanism by which this occurs has been proposed (Igamberdiev and Hill 2004; Igamberdiev *et al.* 2005) and some suggestions have been presented as to how this metabolism may be integrated in the cell (Igamberdiev *et al.* 2010). There is also evidence that these haemoglobins are involved in aspects of reactive oxygen metabolism (Igamberdiev *et al.* 2006). Alfalfa root cultures over-expressing barley Hb1 had substantially increased ascorbate levels, as well as elevated monodehydroascorbate reductase and ascorbate peroxidase activities, under both normoxic and anoxic conditions. Antisense Hb1 lines had increased levels of dehydroascorbate reductase and glutathione reductase activities under the same conditions. While there were observed decreases and increases in NO levels accompanying the changes in Hb1 expression, there was little effect on H_2O_2 levels. Aconitase, a reactive oxygen species (ROS)-sensitive enzyme, was protected by Hb1 expression apparently due to protection from NO. Research on rice Hb1 concluded that it was inefficient at scavenging H_2O_2 compared with a typical plant peroxidase and that these haemoglobins are unlikely to function as peroxidases (Violante-Mota *et al.* 2010).

How does the relationship between haemoglobin expression and ROS at the metabolic level relate to events at the cellular and physiological level? Specific, unequivocal evidence is limited, but there are a few examples that are worth noting. Reactive oxygen species and antioxidants are significant components in a number of plant responses (Mittler 2002). Antioxidants have been implicated in protecting plant cells from programmed cell death (Fath *et al.* 2002; Mittler 2002; Barth *et al.* 2006), as has NO (Beligni *et al.* 2002). Unpublished data from our laboratory have shown that maize somatic embryogenesis can be altered by modifying expression of maize Hb1 and maize Hb2. Down-regulating Hb2 enhances somatic embryogenesis, while down-regulating Hb1 represses embryo formation. Localization of NO differed in a cell-specific fashion between the two phenotypes and programmed cell death was evident in NO-containing cells. Reactive oxygen species and NO are involved in the self-incompatibility response in pollen–pistil interactions resulting in programmed cell death (Wilkins *et al.* 2011). Reactive oxygen species/NO scavengers interfere with this response. Haemoglobin is an NO scavenger and, as noted above, haemoglobin expression improves antioxidant status in the cell.

Changes in the expression of either AHb1 or AHb2 affect the time to bolting of arabidopsis (Hebelstrup and Jensen 2008). Silencing of haemoglobin expression delays bolting, while over-expression results in earlier bolting than in wild-type plants. Haemoglobin appears to be acting as a scavenger of NO in this process, as NO donors were antagonistic to the effects of haemoglobin on bolting. Nitric oxide has been shown to repress the arabidopsis floral transition (He *et al.* 2004), which would support the contention that haemoglobin expression reduces the time to bolting via NO scavenging. Nitric oxide and haemoglobin have also been proposed to be involved in gene regulation and lipid-based signalling during the response to chilling in arabidopsis (Cantrel *et al.* 2011). Nitric oxide levels are increased after a short exposure to chilling, an effect that is not observed in plants over-expressing AHb1. Two sphingolipids,

phytosphingosine phosphate and ceramide phosphate, are negatively regulated by NO during the chilling process.

Nitric oxide has been shown to up-regulate haemoglobin expression in a number of species (Ohwaki *et al.* 2005; Qu *et al.* 2006; Sasakura *et al.* 2006; Bustos-Sanmamed *et al.* 2011), some in relation to infection by micro-organisms. *Alnus* Hb1 is strongly induced in actinorhizal nodules by NO and cold stress, but not by hypoxia or osmotic stress (Sasakura *et al.* 2006). Acetylene reduction is strongly reduced by NO, suggesting that Hb1 may support nitrogen fixation ability by scavenging NO in members of the genus *Frankia*. Hb1 is also up-regulated in *Lotus japonicus* in root nodules by NO, cold and hypoxia (Bustos-Sanmamed *et al.* 2011). Because of the enhanced expression of all three classes of haemoglobin in *L. japonicus* symbiotic nodules, it has been suggested that they are required for symbiosis (Bustos-Sanmamed *et al.* 2011). Truncated haemoglobins (Hb3) are induced by infection of *Datisca glomerata* by *Frankia* (Pawlowski *et al.* 2007), leading these authors to suggest a role for the truncated haemoglobins in NO detoxification. Inoculation of *L. japonicus* with *Mesorhizobium loti* also transiently induced Hb1, while Hb2 was only induced by the application of sucrose (Shimoda *et al.* 2005). The same laboratory reported that over-expression of Hb1 enhances symbiotic nitrogen fixation in the same system, suggesting that this occurs via removal of NO as an inhibitor of nitrogenase (Shimoda *et al.* 2009).

Cotton Hb1 (GhHb1) is induced by NO, H_2O_2, ethylene, salicylate and methyljasmonate (Qu *et al.* 2006), agents that have been associated with plant disease responses (Mur *et al.* 2009). Ectopic expression of GhHb1 in arabidopsis increased the tolerance of the plants to NO. The transgenic plants also constitutively expressed the defence response genes, *PR-1* and *PDF1.2*. Expression of GhHb1 conferred enhanced disease resistance to *Pseudomonas syringae* and tolerance to *Verticillium dahliae*. GhHb1 is induced in cotton roots after infection by *V. dahliae* or exogenous H_2O_2 and is suggested to play a role in the defence responses against *V. dahliae* invasion (Qu *et al.* 2005). Over-expressing an alfalfa non-symbiotic haemoglobin in tobacco led to decreased necrosis from *P. syringae* or tobacco mosaic virus-infected leaves (Seregelyes *et al.* 2003). This was accompanied by increased ROS and salicylic acid in haemoglobin over-expressed plants.

A relationship between non-symbiotic haemoglobin gene expression and hypoxic stress has been evident since the first description of these molecules (Taylor *et al.* 1994). The response is rapid, peaking within a few hours of plant exposure to hypoxia. Altering the expression of the haemoglobin gene through constitutive expression of appropriate gene constructs in various plants provided further evidence of its role in hypoxic stress. Thus, over-expression of class 1 non-symbiotic haemoglobins has been shown to enhance tolerance to hypoxic stress (Sowa *et al.* 1998; Hunt *et al.* 2002; Dordas *et al.* 2003; Zhao *et al.* 2008), while under-expression of the gene reduced tolerance (Sowa *et al.* 1998; Dordas *et al.* 2003).

Various microarray studies of plants under hypoxic stress have produced mixed results on non-symbiotic haemoglobin gene expression and hypoxia. Loreti *et al.* (2005) demonstrated a rapid increase in *AHb1* expression by a northern blot analysis in arabidopsis seedlings exposed to anoxia, coincident with an increase in alcohol dehydrogenase, but found no differential expression of the same haemoglobin in a microarray analysis, comparing anoxic versus normoxic tissue. They did, however, observe an almost two-fold decrease in class 2 haemoglobin transcript expression under the same conditions. In another study of the transcriptome of arabidopsis, van Dongen *et al.* (2009) showed at least a two-fold increase in class 1 haemoglobin expression at 2 and 48 h after exposure of the roots to either 1, 4 or 8 % oxygen. Some puzzling results were found for non-symbiotic haemoglobin expression in an examination of the transcriptome patterns of *Oryza sativa* and arabidopsis under abiotic stress that, unfortunately, did not include hypoxia/anoxia as one of the stresses (Narsai *et al.* 2010). There were changes in haemoglobin expression in both species, but in two of the rice lines there was a strong up-regulation of class 1 haemoglobin upon imposition of the stress, while in the arabidopsis line there was a down-regulation. The authors attribute the difference in response of the two species to possible differences in NO signalling between them. Since there are five known non-symbiotic haemoglobins in rice (Lira-Ruan *et al.* 2002; Garrocho-Villegas *et al.* 2008) and three in arabidopsis (Garrocho-Villegas *et al.* 2007), this difference may also reflect an alternative function for one of the haemoglobins in rice. Using developmentally regulated proteins and transgenic arabidopsis expressing a FLAG-epitope tagged ribosomal protein, ribosome-associated mRNAs were immunopurified from specific cell populations of intact seedlings exposed to 2 h of anoxia to identify differentially expressed mRNAs in 21 cell populations (Mustroph *et al.* 2009). No up-regulation of non-symbiotic haemoglobins in any of the cell populations was observed. An analysis of the transcriptome of grey poplar (*Populus canescens*) under hypoxic stress (Kreuzwieser *et al.* 2009) found changes in only truncated haemoglobins (class 3), with up-regulation occurring after short

exposure (5 h) and down-regulation apparent after 24 h hypoxia or longer. There clearly are issues to be resolved in understanding why there is such a variation in response relative to non-symbiotic haemoglobins with these microarray analyses of plant stress responses. From the perspective of specific examination of class 1 non-symbiotic haemoglobin response to hypoxia by northern blot analysis, there is unequivocal evidence of up-regulation in barley (Taylor *et al.* 1994), arabidopsis (Hunt *et al.* 2002), oak (Parent *et al.* 2008) and rice (Lira-Ruan *et al.* 2001). An obvious explanation for the absence of these haemoglobins in microarray screens associated with hypoxia is that they may not undergo a strong up-regulation upon application of the stress, either as a result of the short time interval of the response or due to localization to specific cells. This lack of strong up-regulation is apparent when examining the gene profiles in the supplementary data accompanying some of the above-cited manuscripts and in qualitative estimations of the northern blots in these same manuscripts, where non-symbiotic haemoglobins have been specifically examined.

Several studies have examined the effect of oxygen limitation on seed development, some of which have involved the role of non-symbiotic haemoglobins in the stress response (Rolletschek *et al.* 2002, 2004; Borisjuk *et al.* 2007; Thiel *et al.* 2011; Vigeolas *et al.* 2011). Steep oxygen gradients are observed in the caryopsis during barley seed development, with highly hypoxic regions having low ATP concentrations and reduced capacity for storage starch accumulation (Rolletschek *et al.* 2004). Legume embryo development occurs in a hypoxic environment as well, with very young embryos being most hypoxic, having low ATP and energy charge levels (Rolletschek *et al.* 2002). Hypoxia induces a nitrite-dependent increase in NO levels in seeds (Borisjuk *et al.* 2007). The presence of NO results in a decrease in seed oxygen consumption, reduced ATP availability and biosynthetic activity, while increasing oxygen availability reduces NO and increases metabolic activity. This balancing of NO and oxygen is suggested to play a part in regulating seed storage activity (Borisjuk *et al.* 2007). To test this hypothesis, arabidopsis seed oxygen stress was manipulated by transformation to achieve seed-specific elevation of AHb1 (Thiel *et al.* 2011). The transformed seed did not accumulate NO under hypoxic stress and had higher respiratory activity and energy charge than the wild type. Transcript profiling revealed that AHb1 overexpression resulted in a re-arrangement of stress-related regulatory pathways under non-stress conditions. Transcription factors, such as *WRKY* and *AP2/EREBP*, and genes associated with hormones such as abscisic acid (ABA), salicylic acid and jasmonic acid were up-regulated. An auxin transporter (*AUX1*) and several auxin-induced genes (*GH3*, *SAUR*, *IAA*, *ARF1*) were strongly down-regulated. *WRKY* has been implicated in hypoxic responses in arabidopsis (Mustroph *et al.* 2009), while members of the *AP2/EREBP* family, having a significant role in conferring submergence tolerance to rice (Xu *et al.* 2006), are involved in the anaerobic response in arabidopsis (Hinz *et al.* 2010; Licausi *et al.* 2010) and have been suggested to be responsible for negative anaerobic regulation of the maize *GapC4* promoter in tobacco (Niemeyer *et al.* 2011). *SAUR* genes have been shown to be negative regulators of auxin synthesis and transport (Kant *et al.* 2009) and are up-regulated by anoxia in rice (Lasanthi-Kudahettige *et al.* 2007). The GH3 family of proteins catalyse the amino acid conjugation of auxins and jasmonates for hormone homeostasis during stress responses (Park *et al.* 2007). This all indicates a strong relationship between haemoglobin expression and cell auxin (jasmonate?) homeostasis, and suggests that this will be a fertile area for future research advances.

Tissue, cellular and sub-cellular localization

Earlier in the review, it was established that oxyhaemoglobins are effective scavengers of NO in many biological systems, including plants. The limited studies in which sites of cellular expression of NO and non-symbiotic haemoglobins have been examined support the conclusion that this reaction occurs *in situ*. Thus, regions in arabidopsis leaves in which NO is highly expressed show altered NO levels when class 1 haemoglobin levels are modified (Hebelstrup *et al.* 2006; Hebelstrup and Jensen 2008). Class 1 haemoglobins have been found in seed tissue (Duff *et al.* 1998; Hunt *et al.* 2001; Ross *et al.* 2001; Uchiumi *et al.* 2002), in the roots of herbaceous species (Hunt *et al.* 2001; Uchiumi *et al.* 2002; Larsen 2003; Wang *et al.* 2003; Qu *et al.* 2006) and trees (Jokipii *et al.* 2008; Parent *et al.* 2008), in leaves and flowers (Hebelstrup *et al.* 2006), and in meristematic tissue (Hebelstrup and Jensen 2008). Studies with a rice class 2 haemoglobin promoter:GUS fusion in arabidopsis found expression in roots, in young leaf vasculature, in flowers and at the pedicel/stem junction (Ross *et al.* 2004). All three classes of *L. japonicus* non-symbiotic haemoglobins are expressed at very high levels in the symbiotic nodules of the plant in comparison with levels in other plant organs (Bustos-Sanmamed *et al.* 2011). A sub-class of the class 1 haemoglobin had considerably higher expression in leaves than its counterpart, while a sub-class of the class 3 haemoglobin had only high expression in nodules relative to its

counterpart, which was roughly uniformly expressed throughout the plant.

It might be expected that non-symbiotic haemoglobins would be found within the cytoplasm of the cell as they lack gene signal sequences that would target them to organelles or export from the cell. They have, however, been reported in the nucleus of cells as well as in the cytoplasm (Seregélyes *et al.* 2000; Ross *et al.* 2001). This seems to be in keeping with results found for cytoglobin, a vertebrate haemoglobin having some similar properties to non-symbiotic haemoglobins (Schmidt *et al.* 2004). Cytoglobin is widely distributed in mammalian cells, but the degree of sub-cellular localization seems to be cell-type dependent, with some cell types having nuclear and cytoplasmic localization while others have it only in the cytoplasm.

Non-symbiotic haemoglobins and hormones

Hormone cross-talk is gaining increasing attention as a means of understanding how hormone action regulates plant growth and development. The term 'cross-talk' invokes the requirement of a mechanism by which the distinct hormone pathways can communicate with one another. In this section, the possible role of non-symbiotic haemoglobins and NO in hormonal cross-talk will be examined. The proposed hypothesis will suggest that non-symbiotic haemoglobins influence and alter the expression and site of action of auxins, jasmonates, ethylene and ABA through modulation of NO levels within the cell. The hypothesis derives from the following observations: non-symbiotic haemoglobins are widely distributed in the plant kingdom and are found in distinct locations in plant tissue; NO is found in plants and is degraded through reaction with oxyhaemoglobin; non-symbiotic haemoglobin and NO expression vary during plant development, and are affected by biotic and abiotic stress.

The transition to flowering is a specific example that potentially links effects of haemoglobin on NO to physiological responses. Nitric oxide represses the floral transition in arabidopsis, where either NO or a mutant over-expressing NO delays bolting (He *et al.* 2004). Silencing AHb1 expression, which increases cellular NO levels, also delays bolting in arabidopsis (Hebelstrup and Jensen 2008). The arabidopsis mutant over-expressing NO has an abnormal, serrated leaf phenotype (He *et al.* 2004), which is also seen in Hb1-silenced arabidopsis lines (Hebelstrup *et al.* 2006). In addition, the observed aerial rosettes observed in Hb1-silenced lines (He *et al.* 2004; Hebelstrup *et al.* 2006) suggest possible alterations in auxin transport (Galweiler *et al.* 1998).

Nitric oxide has been identified as a signalling component in the transduction of a number of plant hormones. Figure 1 gives an overview of the general relationship between NO and several hormones that regulate certain biological functions. It is difficult to completely delineate NO in relation to specific hormones as so many of the effects have potentially multiple intersecting points of interaction. Thus, ethylene, jasmonic acid and salicylic acid are frequently linked with respect to biotic stress and all of them have been linked to NO, in one way or another (Nurnberger and Scheel 2001). Jasmonic acid, through up-regulation of *MYC2*, a repressor of auxin, links the two hormones in regulating root meristem development (Chen *et al.* 2011). The relationships between auxin and ethylene are well known and have common ties with NO in regulating processes such as heavy metal uptake and root development. Numerous studies have linked NO to auxin-controlled processes. Lateral and adventitious root development have been strongly associated with NO and auxin in several species (Pagnussat *et al.* 2003, 2004; Pimpl *et al.* 2003; Correa-Aragunde *et al.* 2004; Guo *et al.* 2008; Lanteri *et al.* 2008; Negi *et al.* 2008; Yadav *et al.* 2010; Jin *et al.* 2011). There are also links with root hair development (Guo *et al.* 2009), root gravitropic bending (Hu *et al.* 2005), rhizobial nodule formation (Pii *et al.* 2007) and root responses to iron deficiency (Chen *et al.* 2010). Nitric oxide promotes root hair elongation and development, with ethylene and auxin both being involved in the process (Guo *et al.* 2009; Strader *et al.* 2010). Nitric oxide acts downstream of auxin and ethylene (Wilson *et al.* 2008), suggestive of a role as a signalling molecule in the process. Constitutive expression of AHb1 does, in fact, reduce root hair development (Hunt *et al.* 2002), which would be predicted if the haemoglobin was scavenging NO. Also, variation in the constitutive expression of a class 1 non-symbiotic haemoglobin in hairy root cultures under hypoxic conditions affects root growth and alters NO levels (Dordas *et al.* 2003).

Nitric oxide influences plant hormonal response in a number of other stress-related situations. Nitric oxide attenuates ozone-induced salicylic acid accumulation and elevates ethylene levels in arabidopsis (Ahlfors *et al.* 2009). Ethylene and NO have been shown mutually to influence each other in up-regulating Fe-acquisition genes with respect to root micronutrient deficiencies (Garcia *et al.* 2011). There is strong evidence linking NO, jasmonic acid and salicylic acid in the plant defence response to pathogens (Wendehenne *et al.* 2004; Hu *et al.* 2009). What are the potential connections with non-symbiotic haemoglobin? Class 1 non-symbiotic haemoglobins are induced in cotton by salicylic acid, methyl jasmonate, ethylene, hydrogen peroxide and NO

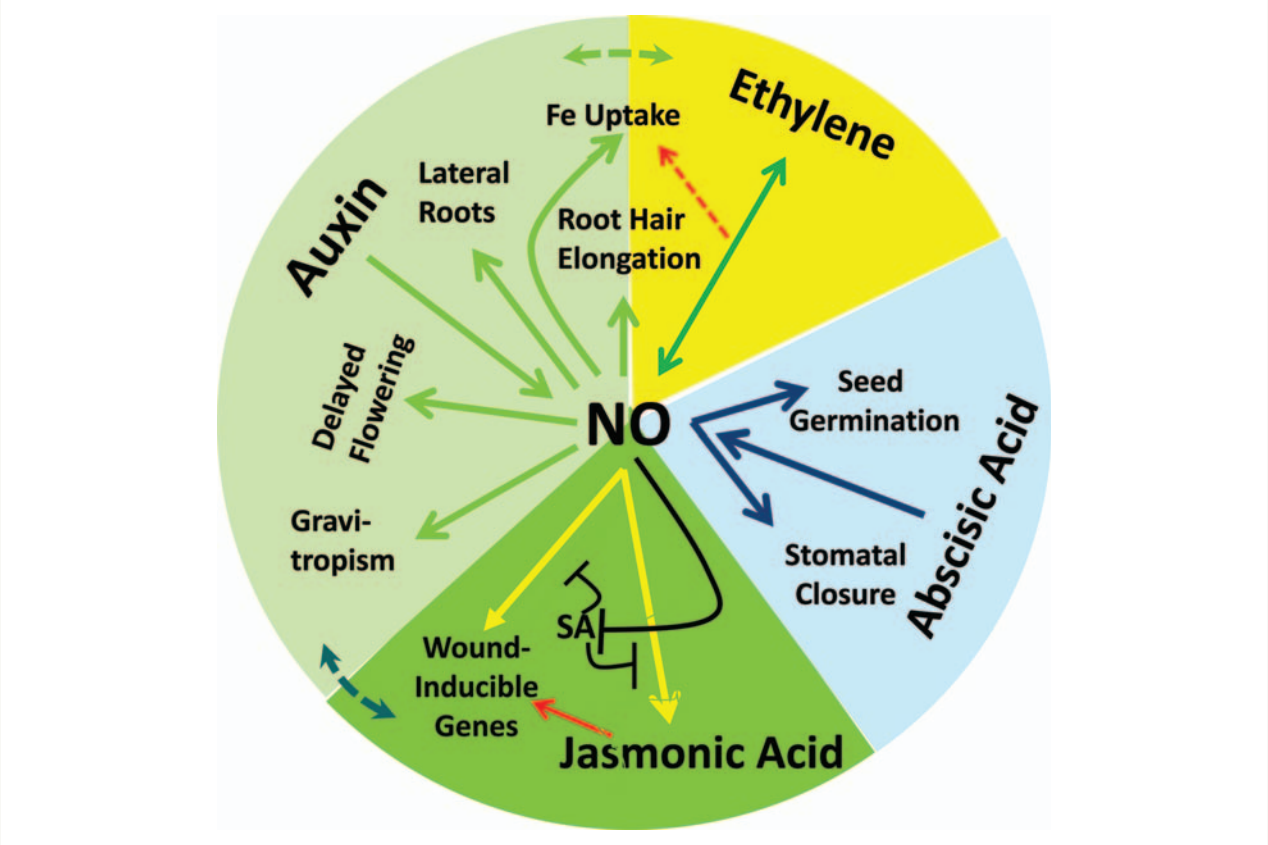

Fig. 1 **The relationship between NO, hormones and biological function.**

(Qu *et al.* 2006). Over-expression of the haemoglobin leads to constitutive expression of disease response genes and conferred enhanced disease resistance (Seregelyes *et al.* 2003, 2004; Qu *et al.* 2006). Suppression of class 1 haemoglobin expression in maize cell cultures leads to a substantial increase in ethylene production (Manac'h-Little *et al.* 2005), consistent with the expected response if the increase was due to elevated cell NO levels.

There is a potential role of non-symbiotic haemoglobins in ABA responses. Nitric oxide functions downstream of ABA in stomatal closure and abiotic stress responses (Neill *et al.* 2008), and there is reason to believe that ethylene also plays a part in the process (Wilkinson and Davies 2010). Non-symbiotic haemoglobins have been found in stomatal guard cells (Smagghe *et al.* 2007), indicating the possibility of their functioning in regulating stomatal closure. There is also evidence of the role of NO in ABA- and gibberellin (GA)-regulated events within seed tissue (Bethke *et al.* 2007), although the results are counter-intuitive to what one would expect relative to what is known about ABA, NO and stomatal closure. Nitric oxide acts as a downstream signal molecule in ABA-induced stomatal closure whereas, in seed tissue, it is suggested that it acts to reduce seed dormancy and promote germination, an action not normally associated with ABA. Regardless of the anomaly, NO is a factor in seed germination and dormancy. Non-symbiotic haemoglobins are expressed in seed tissue (Taylor *et al.* 1994; Duff *et al.* 1998; Arechaga-Ocampo *et al.* 2001; Ross *et al.* 2001), particularly during germination (Duff *et al.* 1998), making it highly probable that the protein is modulating NO levels during the germination process.

Cytokinin up-regulates class 2 non-symbiotic haemoglobin expression (Hunt *et al.* 2001). The effect of this expression would be to reduce NO levels, altering downstream signalling from auxin, ethylene, jasmonic acid or salicylic acid that might be present in the cells synthesizing the protein. There is evidence that non-symbiotic haemoglobins affect cytokinin signalling. Shoot organogenesis is suppressed in AHb2 knockout lines, while over-expressing AHb1 or AHb2 enhanced both shoot formation and altered expression of genes associated with cytokinin perception and signalling (Wang *et al.* 2011). Up-regulation of Hb1 or Hb2 activated *CKI1* and *AHK3*, genes encoding cytokinin receptors and altered

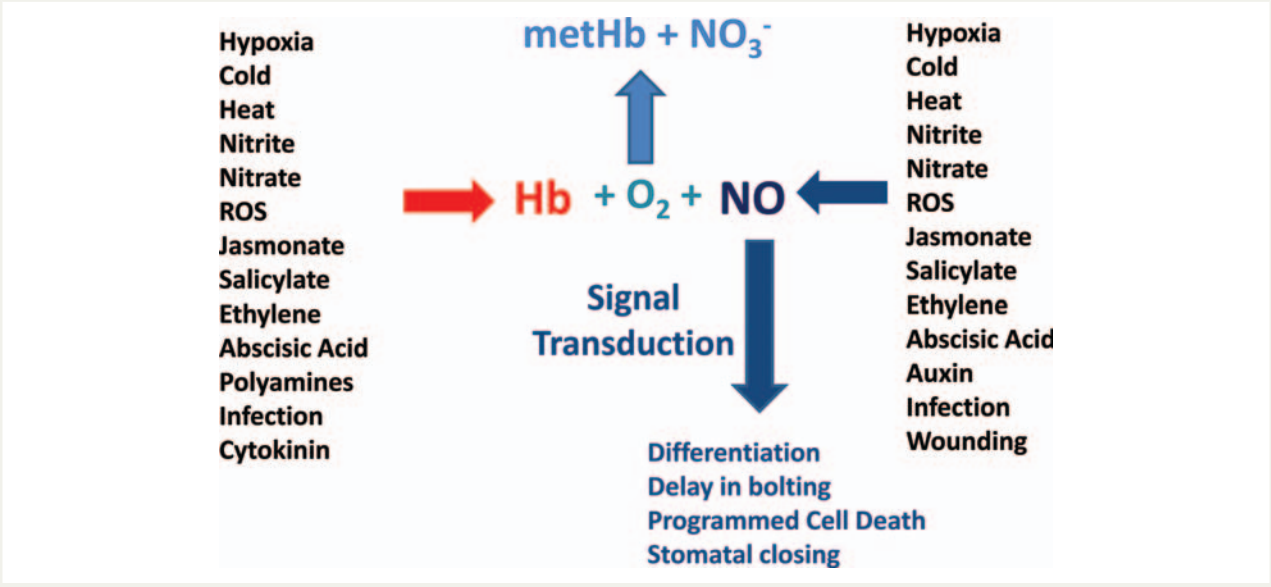

Fig. 2 **A schematic diagram of the eliciting agents and mode of action of non-symbiotic haemoglobins and NO in plants.**

expression of cytokinin response regulators (ARRs). Cytokinin feedback repressors (Type-A ARRs) were repressed in haemoglobin over-expressing lines, while cytokinin activators (Type-B ARRs) were up-regulated. There have been reports that cytokinin increases NO production (Tun *et al.* 2001, 2008), but there are also reports that the reverse occurs (Wilhelmova *et al.* 2006). In a study to determine whether NO is involved in cytokinin function, it was concluded that NO has no direct role in eliciting the primary cytokinin response in plants (Romanov *et al.* 2008).

Figure 2 provides a simplified schematic of the mechanism by which haemoglobin might participate in hormone regulation. To minimize the complexity of the diagram, no attempt has been made to differentiate between the class of haemoglobin induced by the eliciting agent but, undoubtedly, this aspect will determine the site and outcome of haemoglobin expression, due both to the differing promoter regions of the genes and the difference in the kinetics of ligand binding of the protein products. The mechanism is meant to apply to conditions in which NO is acting as a signalling molecule and not as an alternative electron carrier during hypoxia (Igamberdiev *et al.* 2007). The most striking feature of the diagram is the similarity in the agents that influence the expression of haemoglobin and NO. With a few exceptions, both products are elicited by the same agents, suggesting that their relative expression with respect to one another, to influence the biological outcome, may be highly dependent on the concentration of the eliciting agent. Nitric oxide, as proposed by others, acts as a signalling molecule in the appropriate signal transduction pathway, resulting in a specific biological outcome. If haemoglobin is induced as a result of the induction process, it has the potential to interact, in the oxyhaemoglobin form, with NO to produce metHb (Fe^{3+}) and nitrate, reducing the levels of NO and modulating the biological response.

Conclusions and forward look

While the relationship between non-symbiotic haemoglobins and plant hormone responses is largely theoretical at this stage, the hypothesis can be tested and there are sufficient avenues to pursue that would verify or defeat it. The most promising area of advancement would appear to be in auxin signalling where links between non-symbiotic haemoglobins, NO and responses known to be triggered by auxin already exist in the literature but require more detailed examination to establish the relationship. Shoot and root organogenesis, somatic embryogenesis and programmed cell death are fertile study areas. Nitric oxide is a significant factor in biotic stress responses and several reports have linked non-symbiotic haemoglobins with enhanced disease resistance. There remains the problem of determining how this is achieved. Examining the link between haemoglobin and jasmonic and salicylic acid signalling is a logical place to start. In the abiotic stress area, programmed cell death during aerenchyma

formation in anaerobic roots is an area of interest, as would be internodal elongation during flooding. Abscisic acid has already been linked to NO in germination and stomatal responses. Assessing a role for non-symbiotic haemoglobin has potential as certain haemoglobin classes have been detected in seed and leaf tissue.

There remain significant advances with respect to hormone signalling, NO and non-symbiotic haemoglobins that can be accomplished to enhance our understanding of the action of this molecule in the plant kingdom.

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Conflict of interest statement

None declared.

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