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 O2 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. Smaghe 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 (Smaghe 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 HisE7 coordination (Smaghe 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 (Smaghe 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 (Abruzzetti 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 O2 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 O2, 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 O2. 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 H2O2 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 H2O2 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 (Contral 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, H2O2, 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 H2O2 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 over-expression 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 oxyhaemoglobin 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 (Jokipi 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 (Seregelyes 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
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
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

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**Fig. 2** A schematic diagram of the eliciting agents and mode of action of non-symbiotic haemoglobins and NO in plants.
formation in anaerobic roots is an area of interest, as would be internodal elongation during flooding. Absciscic 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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None declared.

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