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Modulation of nitric oxide bioactivity by plant haemoglobins

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Abstract

Nitric oxide (NO) is a highly reactive signalling molecule that has numerous targets in plants. Both enzymatic and non-enzymatic synthesis of NO has been detected in several plant species, and NO functions have been characterized during diverse physiological processes such as plant growth, development, and resistance to biotic and abiotic stresses. This wide variety of effects reflects the basic signalling mechanisms that are utilized by virtually all mammalian and plant cells and suggests the necessity of detoxification mechanisms to control the level and functions of NO. During the last two years an increasing number of reports have implicated non-symbiotic haemoglobins as the key enzymatic system for NO scavenging in plants, indicating that the primordial function of haemoglobins may well be to protect against nitrosative stress and to modulate NO signalling functions. The biological relevance of plant haemoglobins during specific conditions of plant growth and stress, and the existence of further enzymatic and non-enzymatic NO scavenging systems, suggest the existence of precise NO modulation mechanisms in plants, as observed for different NO sources.

Key words: Nitric oxide, non-symbiotic haemoglobin, plant haemoglobin.

Introduction

NO is a small reactive molecule that rapidly diffuses and permeates cell membranes. Its broad chemistry involves an array of interrelated redox forms with different reactivities (Delledonne, 2005). Because of its unique chemistry, which permits its stability and reactivity, NO and its redox-activated forms are intra- and intercellular signalling molecules (Durner *et al.*, 1998).

In plants, NO has a role in several physiological processes including disease resistance and abiotic stress responses as well as growth and development (Neill *et al.*, 2003; Romero-Puertas *et al.*, 2004). The complexity of NO signalling involves various messenger molecules such as cGMP, cADP ribose, and Ca^{2+} (Durner *et al.*, 1998; Wendehenne *et al.*, 2001; Lamotte *et al.*, 2004; Romero-Puertas *et al.*, 2004), which both directly and indirectly modulate several physiological functions and alter the expression of specific genes (Polverari *et al.*, 2003; Parani *et al.*, 2004). Furthermore, the NO signalling pathways involve post-translational modification of target proteins such as NO-dependent tyrosine nitration and reversible cysteine *S*-nitrosylation, which can modulate the activity and function of different proteins (Sokolovski and Blatt, 2004; Feechan *et al.*, 2005; Lindermayr *et al.*, 2005). Although the existence of multiple mechanisms for NO action renders the dissection of specific pathways difficult, it may explain the incomplete inhibition observed when individual steps in specific NO-mediated pathways are blocked (Clarke *et al.*, 2000).

Both cytotoxic and cyto-protecting/stimulating properties of NO have been described in plants (Beligni and Lamattina, 1999). Therefore, the wide variety of sources of NO and its effects suggests the necessity of detoxification mechanisms to control its level, reactivity, and signalling functions. In this review, recent publications that have provided new insights into NO regulation are presented that

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Abbreviations: cADPR, cyclic ADP ribose; cGMP, cyclic GMP; flavoHbs, flavohaemoglobins; GSNO, *S*-nitroso-L-glutathione; GSNOR, GSNO reductase; H_2O_2, hydrogen peroxide; Hb, haemoglobin; HR, hypersensitive response; NO, nitric oxide; NO_2, nitrogen dioxide; NO_3^-, nitrate; NOS, nitric oxide synthase; NR, nitrate reductase, nsHb, non-symbiotic haemoglobin; O_2, oxygen; O_2^-, superoxide anion; $OH^·$, hydroxyl radical; $ONOO^-$, peroxynitrite; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance; SOD, superoxide dismutase; SNO, S- S-nitroso thiol.

aid in the elucidation of the specific role of haemoglobin (Hb)-based control of NO under different conditions in plants.

NO functions in plants

NO is a widespread signalling molecule that plays a crucial role in the modulation of several physiological processes during the entire life of the plant (Crawford and Guo, 2005) from germination (Bethke *et al*., 2004*b*; Simontacchi *et al.*, 2004; Zhang *et al*., 2005) to fruit maturation and senescence (Leshem *et al*., 1998; Beligni and Lamattina, 2001). In roots, NO operates in the auxin signalling pathway that leads to root organogenesis (Pagnussat *et al.*, 2003; Correa-Aragunde *et al.*, 2004) and it also plays an important role in modulation of the gravitropic response (Hu *et al*., 2005). In leaves, NO mediates abscisic acid-induced stomatal closure (Desikan *et al.*, 2002, 2004; Garcia-Mata and Lamattina, 2002), modulates the rate of leaf expansion (Leshem and Haramaty, 1996), and may affect metabolic processes such as photosynthesis (Hill and Bennett, 1970) and respiration (Zottini *et al.*, 2002). Furthermore, NO also seems to be involved in programmed cell death during xylem differentiation (Gabaldon *et al*., 2005) and is a key signal for the control of flowering timing (He *et al*., 2004) and for the orientation of pollen tube growth (Prado *et al.*, 2004).

NO is also involved as a control signal during several abiotic stress responses, including salinity and osmotic stress, temperature, UV light stress, and anoxia (Rockel *et al*., 2002; An *et al*., 2005; Shimoda *et al*., 2005). In particular, NO increases resistance to different abiotic stress such as drought, salt, and oxidative stress (Zhao *et al.*, 2004; Shi *et al*., 2005).

NO is produced during biotic stress in both pathological and non-pathological plant–microbe interactions (Romero-Puertas *et al*., 2004; Wendehenne *et al.*, 2004; Zeidler *et al*., 2004). Its accumulation under conditions that are concomitant with the avirulent gene-dependent oxidative burst has been linked to the induction of the hypersensitive cell death (Delledonne *et al*., 1998; de Pinto *et al.*, 2002) and to cell-to-cell spread of apoptotic signals (Zhang *et al.*, 2003; Tada *et al.*, 2004). The NO produced during biotic stress is also involved in the activation of systemic acquired resistance (Durner *et al*., 1998) through the up-regulation of defence genes (Polverari *et al*., 2003). Furthermore, NO production was recently observed during the response to necrotrophic pathogens and insect herbivores (Jih *et al*., 2003; Huang *et al*., 2004; Van Baarlen *et al*., 2004) in addition to a beneficial plant–microbe interaction, as demonstrated in *Lotus japonicus* with the symbiotic rhizobium *Mesorhizobium loti* (Shimoda *et al*., 2005).

The above observations indicate that NO plays a key role in plant metabolism, signalling, defence, and development, suggesting that control of the level of NO in plants is required in order to activate proper signalling functions. As for animal systems, where different nitric oxide synthases (NOS) have been identified (Stuehr *et al*., 2004), different NO sources in plants also seem to be involved in NO production during specific processes (Neill *et al*., 2003; Romero-Puertas *et al*., 2004). Recently, the presence of a plant NOS gene has been shown in *Arabidopsis* (*AtNOS1*; Guo *et al*., 2003). AtNOS1 plays an important role in plant growth, fertility, and hormone signalling (Guo *et al.*, 2003; He *et al*., 2004) and it is also involved in the plant–pathogen response (Zeidler *et al*., 2004).

Another enzymatic source of NO is nitrate reductase (NR), an enzyme with a fundamental role in nitrogen assimilation, which can produce NO from nitrite when photosynthetic activity is inhibited or when its substrate nitrite accumulates (Yamasaki and Sakihama, 2000; Rockel *et al*., 2002; Lamattina *et al*., 2003). NR is an important source of abscisic acid-induced NO synthesis in stomata guard cells (Desikan *et al*., 2002; García-Mata and Lamattina, 2003), although no significant effects of NR have been observed with regard to NO accumulation during pathogenic infection (Zhang *et al.*, 2003) or during wounding stress (Garces *et al*., 2001).

Recent studies have shown other nitrite-mediated NO sources in plants, including the enzymatic production of NO in mitochondria under anoxia (Planchet *et al*., 2005) and the membrane associated nitrite-NO oxidoreductase in roots (Stöhr *et al*., 2001). Other potential enzymatic NO sources such as xanthine oxidoreductase, horseradish peroxidase, or cytochrome P450 should be also considered (Corpas and del Rio, 2004), as well as non-enzymatic mechanisms, including the spontaneous liberation of NO from nitrite in the presence of acid pH and reducing agents (Yamasaki and Sakihama, 2000; Bethke *et al.*, 2004*a*) and the carotenoid-mediated NO production in the presence of nitrite and light (Cooney *et al*., 1994).

Therefore, different NO sources are most likely required to control the production of NO during specific processes, although the physiological significance of NO production in different instances and identification of the precise source of NO depending on the physiological process and its regulation or interrelation with other possible sources requires further investigation.

NO reactions

NO and its exchangeable redox-activated forms are well known as intra- and intercellular signalling molecules, since NO can rapidly diffuse across biological membranes and contribute to transient cell-to-cell signalling for brief periods of time (Beligni and Lamattina, 2001). As free NO is highly reactive, several reactions that are controlled by both the concentration and redox state of NO and the availability and reactivity of target groups can occur without enzyme

catalysis (Crawford and Guo, 2005). These reactions regulate the signalling and toxicity of NO and may also modulate its levels. The free radical NO has a half-life of just a few seconds and rapidly reacts with O_2 to form nitrogen dioxide (NO_2), which degrades to nitrite and nitrate in aqueous solutions (Neill *et al.*, 2003). NO can also react with the free radical superoxide (O_2^-) to form the reactive molecule peroxynitrite ($ONOO^-$), which can lead to the formation of NO_2 and the potent oxidant hydroxyl radical ($OH^{\cdot}$). $OH^{\cdot}$ is a very strong oxidizing specie that can rapidly attack biological membranes and all types of biomolecules such as DNA and proteins leading to irreparable damage, metabolic dysfunction, and cell death (del Rio *et al*., 2003). By itself, $ONOO^-$ is responsible for tyrosine nitration and oxidation of thiol residues to sulphenic and sulphonic acids (Lamattina *et al*., 2003) and it is considered the major toxic reactive nitrogen species in animal cells (Stamler *et al*., 1992), but not for plant cells (Delledonne *et al*., 2001).

As a consequence, the effect of NO on animal cells depends on many complex conditions, such as the rate of production and diffusion, the concentration of reactive oxygen species (ROS), and the level of enzymes involved in ROS scavenging, such as superoxide dismutase and catalase (Tamir *et al*., 1993). In plants, the accumulation of NO and H_2O_2 during the hypersensitive disease resistance response (HR) is responsible for the execution of the cell death program (Delledonne *et al*., 1998; Clarke *et al*., 2000; de Pinto *et al*., 2002). Since the independent increase of only one component of this binary system has little effect on the induction of cell death (Delledonne *et al*., 2001), the relative rates of production of NO and O_2^- are critical in modulating the effects of NO. This can paradoxically offer protection against oxidative damage by intercepting reactive species and converting them to less damaging and/or more easily detoxified products. Thus, whereas H_2O_2 is the noxious partner of NO to induce cell death, O_2^- may be considered as a chemical NO scavenger that controls the level of NO in plant cells through the formation of $ONOO^-$ (Delledonne *et al*., 2001).

NO can also react rapidly with thiol- and transition metal-containing proteins, including a wide functional spectrum of proteins such as receptors, transcription factors and cellular messengers (Stamler *et al.*, 2001). For example, the NO-dependent activation of guanylate cyclase by binding to the haem iron established a function for NO in signal transduction (Murad, 1986). NO can react with glutathione to form *S*-nitrosoglutathione (GSNO), which can function as a mobile reservoir of NO bioactivity (Feechan *et al*., 2005) or a *S*-nitrosylating agent. *S*-nitrosylation is represented by binding of a NO group to the thiol side chain of a cysteine residue. In animals, this modification is involved in a large part of the almost ubiquitous influence of NO on cellular signal transduction (Hess *et al*., 2005), and during the last few years over 100 proteins have been identified as targets of *S*-nitrosylation (Stamler *et al*., 2001). Temporal and spatial regulation of *S*-nitrosylation allows post-translational modification to function as a mechanism that conveys redox-based signalling (Hess *et al*., 2005). Very little is known about *S*-nitrosylation in plants, although numerous proteins have been obtained from the deduced *Arabidopsis* proteome that have a degenerate *S*-nitrosylation motif (Huber and Hardin, 2004). Recently, a proteomic approach using the biotin switch method (Jaffrey *et al.*, 2001) identified 63 proteins from *Arabidopsis* cell culture extracts treated with GSNO and 52 proteins from NO-treated *Arabidopsis* leaves as putative targets for *S*-nitrosylation in plants (Lindermayr *et al*., 2005). The characterization of mechanisms regulating *S*-nitrosylation/de-nitrosylation will undoubtedly aid in an improved understanding of the functional consequences and relevance of *S*-nitrosylation in plants, and might identify possible mechanisms to control the levels of NO and NO-related species.

Haemoglobins detoxify NO

The wide variety of NO sources and biological effects suggests the requirement of detoxification mechanisms in plants to control the levels of NO as well as its reactivity and signalling functions. Haemoglobins (Hbs) are most commonly recognized for their ability to act either as O_2 carriers or stores to facilitate O_2 delivery, even though they are also well known regulators of NO homeostasis. In humans, Hbs regulate the activity of NO through either detoxification (Joshi *et al*., 2002) or delivery through *S*-nitrosylation reactions (Gow *et al*., 1999). Bacterial flavohaemoglobins (flavoHbs) consume NO enzymatically through a NO reductase/denitrosylase activity (Gardner *et al*., 1998; Hausladen *et al*., 1998) to protect bacterial cells from NO, as also observed for truncated Hbs (Quellet *et al*., 2002). *Ascaris* Hb has a NO consumption activity that involves the intermediacy of *S*-nitrosylated Hb (Minning *et al*., 1999). Thus, the primordial function of Hbs, present not only in erythrocytes but also in microorganisms, invertebrates, and plants may well be to protect against nitrosative stress and modulate the signalling functions of NO.

In plants, there are at least three distinct types of Hbs that have been classified as symbiotic, non-symbiotic Hbs (nsHb), and truncated Hbs. The latter are the most recently discovered plant Hbs; they are characterized by a three-dimensional structure with a 2-on-2 arrangement of α-helices (Watts *et al.*, 2001) and appear to be ubiquitous in the plant kingdom (Vieweg *et al*., 2005). Symbiotic Hbs are found specifically in symbiotic legume root nodules where they scavenge and transport O_2 to protect *Rhizobium* nitrogenase from inactivation (Appleby, 1984). The nsHbs appear to be ubiquitous in the plant kingdom and are organized in two classes. Class-1 Hbs have an extremely

high affinity for O_2 and are induced in plants during hypoxic stress, whereas class-2 nsHbs have lower affinity for O_2, are induced by low temperature, and are expressed during plant development (Trevaskis *et al*., 1997; Hunt *et al*., 2001).

Although the presence of stress-induced nsHbs is widespread in the plant kingdom, their function has only been recently elucidated and their cellular localization is not clearly understood. In particular, alfalfa class-1 nsHb has been shown to localize in both the nucleus and cytosol (Seregélyes *et al*., 2000), although the main nsHbs activity involved in NO degradation was found to be localized in the soluble cytosolic fraction (Igamberdiev *et al*., 2004) in agreement with the previous protein sequence analysis of rice and *Arabidopsis* class-1 nsHb (Arredondo-Peter *et al*., 1997; Trevaskis *et al*., 1997). In contrast to symbiotic Hb, the nsHbs exhibit an iron hexacoordination in which the ligand binding site on the haem prosthetic group is occupied by a His residue similar to many Hbs (Kundu *et al*., 2003). For example, the human neuroglobins show a hexacoordinated structure, are up-regulated during hypoxia, and their expression is directly associated with protection against hypoxic challenge (Sun *et al*., 2001, 2003). Similarly, class-1 nsHbs are strongly expressed during hypoxia or similar stresses, and are required for survival of plants after a severe hypoxic challenge (Hunt *et al*., 2002; Dordas *et al*., 2003*b*). However, these nsHbs possess a high affinity for O_2 and slow O_2 dissociation rate constants and are, therefore, unlikely to function as O_2 transporters (Kundu *et al*., 2003). On the other hand, hypoxia is a stress condition that generates copious amounts of NO (Dordas *et al*., 2003*b*) suggesting other possible Hb-based cell-protection mechanisms.

Class-1 nsHbs from *Arabidopsis* (Perazzolli *et al*., 2004), barley (Igamberdiev *et al*., 2004), and alfalfa (Seregélyes *et al*., 2004) are now known to detoxify NO to nitrate in an NAD(P)H-dependent manner. Biochemical evidence indicates that rapid nitrate accumulation is accompanied by NO-dependent oxidation of oxygenated to oxidized nsHb (Dordas *et al*., 2004; Perazzolli *et al*., 2004). Furthermore, this Fe(III) intermediate of haem can be directly reduced by NADPH, as for nsHb from *Arabidopsis*, which catalyses an enzymatic cycle for NO metabolism with continuous nitrate accumulation in the presence of excess NO and NADPH (Fig. 1A; Perazzolli *et al*., 2004). Otherwise, oxidized nsHb might be reduced by a mixture of NADH and FAD, as for alfalfa nsHb (Seregélyes *et al*., 2004), or by a methaemoglobin reductase, as for barley nsHb (Igamberdiev *et al*., 2004).

The demonstration that class-1 nsHb from *Arabidopsis* can also metabolize GSNO through production of *S*-nitrosohaemoglobin (Fig. 1B; Perazzolli *et al*., 2004) suggests a conserved role of haemoglobin *S*-nitrosothiols in processing NO and *S*-nitroso compounds across humans, nematodes, and plants. Hexacoordinate plant Hbs appear to

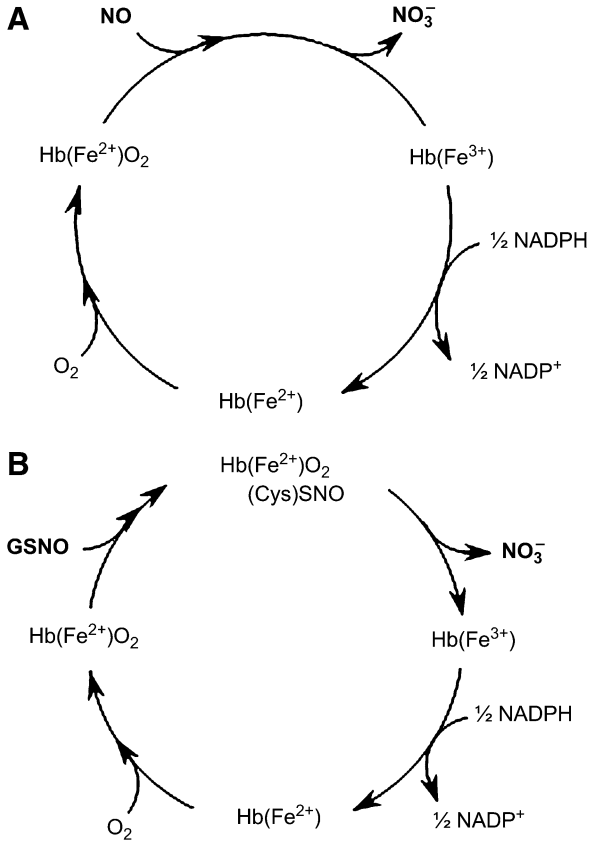

Fig. 1. Haemoglobin-based NO-detoxification reactions. (A) Reaction cycle for NO metabolism by *Arabidopsis* class-1 nsHb: oxygenated Hb is oxidized by NO with nitrate production. The recovery of oxygenated Hb for another NO degradation cycle is ensured by regeneration by NADPH and subsequent association with O_2. This reduction of nsHb may be augmented *in vivo* by specific interaction with a methaemoglobin reductase system. (B). Reaction cycle for GSNO metabolism by *Arabidopsis* class-1 nsHb: oxygenated Hb is oxidized by GSNO with nitrate production through the formation of an *S*-nirosoHb intermediate. The recovery of oxygenated Hb is ensured by regeneration by NADPH and subsequent association with O_2. The reduction of nsHb may be augmented *in vivo* by specific interaction with a methaemoglobin reductase system as well.

operate as *Ascaris* Hb (Minning *et al*., 1999), although they are structurally very different. Both *Arabidopsis* class-1 and *Ascaris* Hbs have evolved distal cysteine residues in the haem pocket, not present in human and bacterial Hbs, which are implicated in NO/SNO detoxification. *Arabidopsis* class-1 nsHb seem to retain the primitive function in NO/SNO detoxification by positioning cysteine residues in the distal haem pocket similar to *Ascaris* Hb, whereas NO delivery function in human haemoglobin is accomplished through the use of a proximal cysteine residue (Jia *et al*., 1996).

A role of nsHbs in NO modulation has been largely demonstrated *in vitro* and *in vivo* only for the high O_2 affinity class-1. Conversely, the function of the strongly different class-2 nsHbs has not been investigated. Class-2 nsHbs seem to be exclusive to dicots (Hunt *et al*., 2001), are induced by cold stress (Trevaskis *et al*., 1997), and are

expressed in specific tissues of mature flowering plants (Hunt *et al*., 2001). The *Arabidopsis* class-2 nsHb show lower O_2 affinity than class-1 nsHbs and might be involved in O_2 storage or transport (Trevaskis *et al*., 1997). A possible interaction with NO, particularly in NO biosynthesis from nitrite at low O_2 levels, has recently been proposed (Crawford and Guo, 2005).

Haemoglobin-based NO modulation during hypoxic stress

The main physiological function for the NO scavenging activity of nsHb appears to be protection against nitrosative stress associated with hypoxia (Dordas *et al*., 2003*b*, 2004; Perazzolli *et al*., 2004). Overexpression of class-1 nsHb results in a greater cell viability and stronger plant growth under hypoxia in *Arabidopsis* (Hunt *et al*., 2002; Perazzolli *et al*., 2004) and in alfalfa cultures expressing the barley nsHb (Dordas *et al*., 2003*b*), whereas the suppression of nsHb expression reduces organ growth under hypoxic stress (Dordas *et al*., 2003*b*; Perazzolli *et al*., 2004). This nsHb-mediated hypoxia tolerance depends on the high O_2 affinity of nsHbs and NO detoxification (Dordas *et al*., 2003*b*; Perazzolli *et al*., 2004), but it is not due to O_2 delivery (Hunt *et al*., 2002).

Hypoxic stress activates NR leading to copious amounts of NO synthesis and elevated NO emission from plant tissues that are measurable by chemiluminescence (Rockel *et al*., 2002; Perazzolli *et al*., 2004) and electron paramagnetic resonance (Dordas *et al*., 2003*b*, 2004). Hypoxia stimulated NO accumulation is dramatically suppressed in *Arabidopsis* plants expressing *Arabidopsis* class-1 nsHb (Fig. 2; Perazzolli *et al*., 2004), in alfalfa root cultures overexpressing barley class-1 nsHb (Dordas *et al*., 2003*b*) and in maize cell lines expressing the same barley nsHb (Dordas *et al*., 2004); whereas the transgenic lines suppressed for nsHbs expression have enhanced levels of NO (Fig. 2; Dordas *et al*., 2003*b*, 2004; Perazzolli *et al*., 2004).

NO is an effective inhibitor of cytochrome oxidase in the mitochondrial electron transport chain (Zottini *et al.*, 2002) and may further reduce cell respiration and energy production. Under hypoxia, nsHbs scavenge NO and may also help in maintaining the energy status of plant cells by an alternative mechanism to the classic fermentation pathways (Igamberdiev and Hill, 2004; Igamberdiev *et al*., 2005). The NR-dependent production of NO and its subsequent oxidation by nsHb seems to be a NAD(P)H-consuming mechanism. The overall system oxidizes 2.5 moles of NADH per 1 mol of nitrate recycled, leading to the maintenance of redox status during hypoxia (Dordas *et al*., 2003*a*; Igamberdiev and Hill, 2004). The lower NADH/NAD^+ and NADPH/$NADP^+$ ratios in alfalfa root cultures expressing barley nsHb compared with control cells, and the higher ratios in nsHb silenced lines upon low O_2 and antimycin treatment, support the existence of this alternative fermentation mechanism (Igamberdiev *et al*., 2004). Since alfalfa root cultures expressing barley nsHb (Dordas *et al*., 2003*a*) and maize-cultured cells expressing barley nsHb (Sowa *et al*., 1998) have lower alcohol dehydrogenase activity than control cultures under hypoxia, alternative fermentation based on nsHb could substitute alcohol dehydrogenase for recycling NADH. Furthermore, this nsHb cycle sustains glycolysis and the energy status of plant cells, maintaining a higher level of ATP in nsHb overexpressing lines under hypoxia (Sowa *et al*., 1998; Dordas *et al*., 2003*a*; Igamberdiev *et al*., 2005).

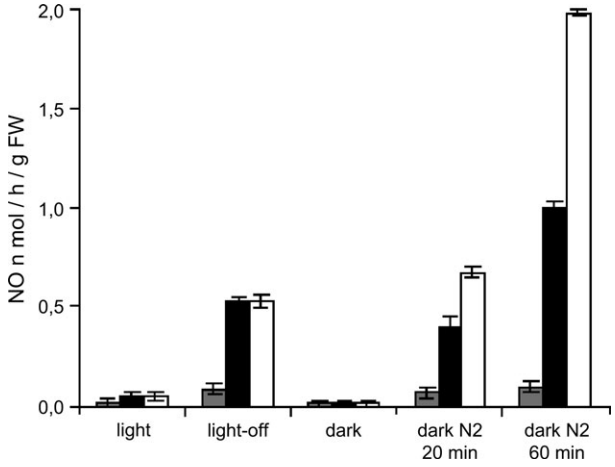

Fig. 2. Gas phase NO emission pattern of *Arabidopsis* wild-type plants (black bars) and transgenic *Arabidopsis* lines expressing the *Arabidopsis* class-1 nsHb gene in sense (grey bars) or antisense (white bars) orientation, 30 min after light exposure (light), after transfer of light-adapted plants to darkness (light-off), 30 min after dark adaptation (dark), and 20 min or 60 min after hypoxic stress by flushing with N_2 in the dark (dark N_2 20 min and 60 min; Perazzolli *et al*., 2004).

Haemoglobin-based NO modulation during plant growth conditions

NO accumulates under normal growth conditions as it is produced from nitrite either through light-mediated non-enzymatic conversion by carotenoids or by the action of NR (Klepper, 1990). In *Arabidopsis* lines overexpressing the class-1 nsHb, the NR–mediated emission of NO resulting from the accumulation of nitrite on transfer of light-adapted plants to darkness (Kaiser *et al*., 2002) is significantly lower compared with control plants (Fig. 2; Perazzolli *et al*., 2004), suggesting a function of nsHbs in the constant control of NO accumulation.

Arabidopsis class-1 nsHb is induced by nitrate (Wang *et al*., 2000), a class-1 nsHb of the *Lotus japonicus* is strongly induced by NO (Shimoda *et al*., 2005), and both rice class-1 nsHbs are strongly up-regulated by nitrite, nitrate, and NO (Ohwaki *et al*., 2005). Furthermore, treatment with

nitrate and nitrite failed to induce nsHbs in rice mutants defective in NR expression (Ohwaki *et al*., 2005) indicating that the induction of nsHb is closely associated with NR-dependent NO production. Since NR-derived NO is potentially dangerous, these data indicate that nsHbs may well protect against NO generated following nitrogen fertilization (Klepper, 1990) and during normal growth condition (Ohwaki *et al*., 2005). The effect of nsHbs in defence against nitrosative stress is observed during treatment with NO donors (Dordas *et al*., 2003*a*; Seregélyes *et al*., 2003). NO treatment causes a decline in the ATP levels and ATP/ADP ratios in transgenic alfalfa cultures underexpressing the nsHbs, whereas lines overexpressing the class-1 barley nsHb show protection against this nitrosative stress (Dordas *et al*., 2003a). Similarly, tobacco seedlings and leaves of transgenic lines overexpressing the alfalfa class-1 nsHb are less sensitive to NO than wild-type plants (Seregélyes *et al*., 2003).

Transient NO generation observed after symbiotic bacteria inoculation in *Lotus japonicus* roots indicates that NO also accumulates during beneficial plant–microbe interactions (Shimoda *et al*., 2005). NO could be produced in the infected cells of root nodules due to their high metabolic activity during the infection process, or in the release of NO by nitrogen-fixing bacteroids (Vieweg *et al*., 2005). Thus, NO may be involved in symbiotic interactions, either as a messenger molecule or as a by-product of the altered metabolism in root nodules (Herouart *et al*., 2002). Symbiotic rhizobium infection causes the up-regulation of a class-1 nsHb in *Lotus japonicus* (Shimoda *et al*., 2005) and the induction of the two truncated Hbs in root nodules and in roots of *Medicago truncatula* colonized by arbuscular mycorrhizal fungi (Vieweg *et al*., 2005). It is assumed that these Hbs could be involved in NO detoxification in specific root tissues during symbiosis (Shimoda *et al*., 2005; Vieweg *et al*., 2005), since it can inactivate nitrogenase (Cueto *et al*., 1996).

In other detrimental plant–microbe interactions, NO exerts a number of fundamental functions, such as those occurring during plant defence against pathogen attack, by contributing together with ROS to trigger hypersensitive cell death and induce defence genes (Delledonne *et al*., 1998). The observation that challenge of *Arabidopsis* plants overexpressing the *Arabidopsis* class-1 nsHb with an avirulent strain of the bacterial pathogen *Pseudomonas syringae* causes normal accumulation of NO and hypersensitive cell death, indicates that nsHbs do not interfere with NO bursts originated by acute responses when NO signalling functions through the hypersensitive resistance response are needed (Perazzolli *et al*., 2004). However, further experimentation is required to elucidate the role of nsHb during disease resistance, since the overexpression of alfalfa nsHb in tobacco results in fewer lesions in leaves when challenged with incompatible bacteria or tobacco necrosis virus compared with control plants (Serégelyes *et al*., 2003). Moreover, these lines have higher ROS, salicylic acid (SA), and pathogenesis-related protein 1 levels after pathogen infection (Serégelyes *et al*., 2003, 2004). The differences of nsHb expressing plants during plant–pathogen interactions might be explicable by the different rates of catalytic NO detoxification. The expression of *Escherichia coli* flavoHb, which possesses strong NO detoxification activity (Gardner *et al*., 1998), is able to attenuate the pathogen-induced NO burst when expressed in plants and reduces the development of hypersensitive cell death and the expression of defence genes (Zeier *et al*., 2004).

Further NO modulation mechanisms in plant

In addition to Hb-based NO detoxification and non-enzymatic NO scavenging by O_2^- or through thiols, plants may possess additional NO metabolizing mechanisms to control the levels of NO under different conditions. For example, some enzymes such as xanthine oxidase, glutathione peroxidase, and GSNO reductase (GSNOR) are reported to break down NO-related species (Sies *et al*., 1997; Trujillo *et al*., 1998; Díaz *et al*., 2003). GSNO has been shown to induce systemic resistance against TMV infection in tobacco (Song and Goodman, 2001) and to be a powerful inducer of plant defence genes (Durner *et al*., 1998). An enzyme that metabolizes GSNO has been identified in *E. coli* (Liu *et al*., 2001*a*) and the characterization of GSNOR-deficient yeast showed enhanced susceptibility to nitrosative stress (Liu *et al*., 2001*b*). A similar gene has also been identified in both pea and *Arabidopsis* (Shafqat *et al*., 1996; Sakamoto *et al*., 2002), suggesting that plants may be able to modulate the bioactivity and signalling function of this stabilized form of NO. A recent study has shown that mutation in *AtGSNOR1*, an *Arabidopsis thaliana* GSNOR, modifies cellular levels of SNO under both physiological and pathophysiological conditions (Feechan *et al*., 2005) indicating the existence of cross-reactions between free NO and GSNO that could control the level of NO and its signalling capacity. In the absence of GSNOR activity, the cellular level of SNO is higher compared with control *Arabidopsis* lines and the plant resistance response against avirulent pathogens and virulent pathogens are compromised. This suggests that whereas a reduction of NO accumulation leads to pathogen susceptibility, a decrease in the concentration of SNO promotes protection against microbial infection (Feechan *et al*., 2005). AtGSNOR is also required to maintain the non-host resistance to the wheat powdery mildew pathogen in *Arabidopsis* and is a positive regulator of the SA signalling network since AtGSNOR is required for normal *PR1* expression in pathogen-infiltrated and in SA-treated plants (Feechan *et al*., 2005). *Arabidopsis* plants overexpressing AtGSNOR show a reduced SNO level, accelerated

kinetics of *PR1* expression, and enhanced basal resistance against bacterial and oomycete pathogens (Feechan *et al*., 2005). These results confirm that an additional regulatory system depending on GSNO formation and AtGSNOR activity is involved in the control of NO signalling functions (Feechan *et al*., 2005). However, the physiological relevance of these enzymes in plant NO metabolism should be more thoroughly investigated under different stress conditions.

Conclusions

During the last two years large advances have been made in the field of NO regulation and metabolism. Recent publications have reported a crucial involvement of nsHbs in NO modulation during different plant growth and stress conditions, indicating that, in plants as well, the primordial function of Hbs is the detoxification of NO catalysing an O_2 and NAD(P)H-dependent nitrate-forming reaction. The *in vivo* results have demonstrated that the Hb-based NO detoxification plays a crucial role to protect plant cells against nitrosative stress and modulation of NO signalling functions. Furthermore, the existence of different NO modulation reactions indicates that specific NO detoxification mechanisms may be involved in specific plant conditions in the fine control of the level and functions of NO. However, the identification of the precise source of NO, depending on the physiological process and the associated NO modulation system that prevent unregulated NO accumulation, needs further investigation. In particular, the involvement of nsHb and truncated Hbs in NO modulation during pathogenic and symbiotic plant–microbe interactions needs clarification. Moreover, nsHb interactions with NO have been demonstrated only for the class-1 molecule and the function of the strongly different class-2 nsHb has not yet been investigated. While the low O_2 affinity of the class-2 nsHbs suggests their involvement in O_2 storage or transport, their possible activity in NO biosynthesis at low O_2 levels has recently been hypothesized. Furthermore, the reaction of NO with thiols and the subsequent SNO interaction with nsHb and other target proteins demonstrated in plants are a promising starting point to characterize enzymatic and non-enzymatic NO control more fully and the possible signal cascade by which NO operates in plant cells through protein *S*-nitrosylation.

Future prospectives

NO participates in the regulation of several physiological processes, and the identification of mechanisms controlling its level in plant cells indicates a fine-tuning between NO synthesis and detoxification. Recent reports suggest a crucial role of plant Hbs in NO metabolism, and these findings now raise questions about their main physiological functions. Whereas the role of high oxygen affinity class-1 nsHbs has been largely demonstrated in NO detoxification during plant growth and hypoxic stress (Dordas *et al*., 2003*b*, 2004; Igamberdiev *et al*., 2004; Perazzolli *et al*., 2004), other studies suggest a function in H_2O_2 metabolism (Sakamoto *et al*., 2004; Yang *et al*., 2005). Since during hypoxia copious amounts of H_2O_2 are generated, it has been proposed that the nsHb-dependent protection to hypoxia may be a result of the decreased cellular level of H_2O_2 (Yang *et al*., 2005). However, other evidence suggests that class-1 nsHbs overexpressing plants produce increased levels of H_2O_2 upon bacterial infection (Serégelyes *et al*., 2003). Further investigation of the balance between NO and ROS during hypoxia, together with a detailed characterization of the interaction between nsHb with H_2O_2 is expected to lead to a better understanding of the activities of nsHbs in ROS homeostasis. In addition a better biochemical characterization of plant Hbs can be expected. The structural features of haem iron coordination recently reported for tomato nsHb (Ioanitescu *et al*., 2005), and the kinetic binding properties of rice class-1 nsHb (Hargrove, 2000) have the potential to provide further information about the enzymatic properties of nsHbs. In particular, the effect of hexacoordination in the increase of the rate of iron reduction, reported for human and rice hexacoordinated Hbs (Weiland *et al*., 2005), could deepen the biochemical knowledge regarding mechanisms of nsHbs reduction through direct NAD(P)H processes (Perazzolli *et al*., 2004; Seregélyes *et al*., 2004) and through mediation of a specific reductase (Igamberdiev *et al*., 2004).

The evidence that bacterial flavoHbs (Zeier *et al*., 2004) and alfalfa class-1 nsHb (Serégelyes *et al*., 2003) affect SA accumulation in pathogen-infected plants, and the recent demonstration that the barley class-1 nsHb affects ethylene accumulation in NO- and hypoxia-treated maize cells (Manac'h-Little *et al*., 2005) indicates a role of nsHbs in controlling NO-dependent hormone-mediated signalling that deserves further investigation. The recent identification of *S*-nitrosylation of *Arabidopsis* class-1 nsHb (Perazzolli *et al*., 2004) and other plant proteins (Sokolovski and Blatt, 2004; Lindermayr *et al*., 2005), together with the evidence that *Arabidopsis* class-1 nsHb can mediate tyrosine nitration of itself and other proteins (Sakamoto *et al*., 2004), are important starting points from which to characterize the signal cascade by which NO operates in plant cells.

References

An L, Liu Y, Zhang M, Chen T, Wang X. 2005. Effects of nitric oxide on growth of maize seedling leaves in the presence or absence of ultraviolet-B radiation. *Journal of Plant Physiology* **162,** 317–326.

Appleby CA. 1984. Leghemoglobins and rhizobium respiration. *Annual Review of Plant Physiology* **35,** 443–478.

Arredondo-Peter R, Hargrove MS, Sarath G, Moran JF, Lohrman J, Olson JS, Klucas RV. 1997. Rice hemoglobins: gene

cloning, analysis and oxygen-binding kinetics of a recombinant protein synthesized in *Escherichia coli*. *Plant Physiology* **115,** 1259–1266.

Beligni MV, Lamattina L. 1999. Is nitric oxide toxic or protective. *Trends in Plant Science* **4,** 299–300.

Beligni MV, Lamattina L. 2001. Nitric oxide in plants: the history is just beginning. *Plant, Cell and Environment* **24,** 267–278.

Bethke PC, Badger MR, Jones RL. 2004*a*. Apoplastic synthesis of nitric oxide by plant tissues. *The Plant Cell* **2,** 332–341.

Bethke PC, Gubler F, Jacobsen JV, Jones RL. 2004*b*. Dormancy of *Arabidopsis* seeds and barley grains can be broken by nitric oxide. *Planta* **219,** 847–855.

Clarke A, Desikan R, Hurst RD, Hancock JT, Neill SJ. 2000. NO way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. *The Plant Journal* **5,** 667–677.

Cooney RV, Harwood PJ, Custer LJ, Franke AA. 1994. Light-mediated conversion of nitrogen dioxide to nitric oxide by carotenoids. *Environmental Health Perspectives* **102,** 460–462.

Corpas FJ, Barrosso JB, del Río LA. 2004. Enzymatic sources of nitric oxide in plant cells: beyond one protein–one function. *New Phytologist* **162,** 243–248.

Correa-Aragunde N, Graziano M, Lamattina L. 2004. Nitric oxide plays a central role in determining lateral root development in tomato. *Planta* **218,** 900–905.

Crawford NM, Guo FQ. 2005. New insights into nitric oxide metabolism and regulatory functions. *Trends in Plant Science* **4,** 195–200.

Cueto M, Hernandez-Perera O, Martin R, Bentura ML, Rodrigo J, Lamas S, Golvano MP. 1996. Presence of nitric oxide synthase activity in roots and nodules of *Lupinus albus*. *FEBS Letters* **398,** 159–164.

de Pinto MC, Tommasi F, De Gara L. 2002. Changes in the antioxidant systems as part of the signaling pathway responsible for the programmed cell death activated by nitric oxide and reactive oxygen species in tobacco Bright-Yellow 2 cells. *Plant Physiology* **2,** 698–708.

del Rio LA, Corpas FJ, Sandalio LM, Palma JM, Barroso JB. 2003. Plant peroxisomes, reactive oxygen metabolism and nitric oxide. *IUBMB Life* **2,** 71–81.

Delledonne M. 2005. NO news is good news for plants. *Current Opinion in Plant Biology* **8,** 390–396.

Delledonne M, Xia Y, Dixon RA, Lamb C. 1998. Nitric oxide functions as a signal in plant disease resistance. *Nature* **394,** 585–588.

Delledonne M, Zeier J, Marocco A, Lamb C. 2001. Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proceedings of the National Academy of Sciences, USA* **23,** 13454–13459.

Desikan R, Cheung M-K, Bright J, Henson D, Hancock JT, Neill SJ. 2004. ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells. *Journal of Experimental Botany* **55,** 205–212.

Desikan R, Griffiths R, Hancock J, Neill S. 2002. A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in A*rabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* **99,** 16314–16318.

Díaz M, Achkor H, Titarenko E, Martínez MC. 2003. The gene encoding glutathione-dependent formaldehyde dehydrogenase/GSNO reductase is responsive to wounding, jasmonic acid and salicylic acid. *FEBS Letters* **543,** 136–139.

Dordas C, Hasinoff BB, Igamberdiev AU, Manac'h N, Rivoal J, Hill RD. 2003*b*. Expression of a stress-induced haemoglobin affects NO levels produced by alfalfa root cultures under hypoxic stress. *The Plant Journal* **35,** 763–770.

Dordas C, Hasinoff BB, Rivoal J, Hill RD. 2004. Class-1 hemoglobins, nitrate and NO levels in anoxic maize cell-suspension cultures. *Planta* **219,** 66–72.

Dordas C, Rivoal J, Hill RD. 2003*a*. Plant haemoglobins, nitric oxide and hypoxic stress. *Annals of Botany* **91,** 173–178.

Durner J, Wendehenne D, Klessig DF. 1998. Defence gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proceedings of the National Academy of Sciences, USA* **17,** 10328–10333.

Feechan A, Kwon E, Yun BW, Wang Y, Pallas JA, Loake GJ. 2005. A central role for *S*-nitrosothiols in plant disease resistance. *Proceedings of the National Academy of Sciences, USA* **22,** 8054–8059.

Gabaldon C, Gomez Ros LV, Pedreno MA, Ros Barcelo A. 2005. Nitric oxide production by the differentiating xylem of *Zinnia elegans*. *New Phytologist* **165,** 121–130.

Garces H, Durzan D, Pedroso MC. 2001. Mechanical stress elicits nitric oxide formation and DNA fragmentation in *Arabidopsis thaliana*. *Annals of Botany* **87,** 567–574.

Garcia-Mata C, Lamattina L. 2002. Nitric oxide and abscisic acid cross talk in guard cells. *Plant Physiology* **128,** 790–792.

Garcia-Mata C, Lamattina L. 2003. Abscisic acid, nitric oxide and stomatal closure: is nitrate reductase one of the missing links? *Trends in Plant Science* **1,** 20–26.

Gardner PR, Gardner AM, Martin LA, Salzman AL. 1998. Nitric oxide dioxygenase: an enzymic function for flavohaemoglobin. *Proceedings of the National Academy of Sciences, USA* **95,** 10378–10383.

Gow AJ, Luchsinger BP, Pawloski JR, Singel DJ, Stamler JS. 1999. The oxyhaemoglobin reaction of nitric oxide. *Proceedings of the National Academy of Sciences, USA* **96,** 9027–9032.

Guo FQ, Okamoto M, Crawford NM. 2003. Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science* **302,** 100–103.

Hausladen A, Gow AJ, Stamler JS. 1998. Nitrosative stress: metabolic pathway involving the flavohaemoglobin. *Proceedings of the National Academy of Sciences, USA* **95,** 14100–14105.

He Y, Tang R-H, Hao Y, *et al.* 2004. Nitric oxide represses the *Arabidopsis* floral transition. *Science* **305,** 1968–1971.

Herouart D, Baudouin E, Frendo P, Harrison J, Santos R, Jamet A, Van de Sype G, Touati D, Puppo A. 2002. Reactive oxygen species, nitric oxide and glutathione: a key role in the establishment of the legume–*Rhizobium* symbiosis? *Plant Physiology and Biochemistry* **40,** 619–624.

Hargrove MS. 2000. A flash-photolysis method to characterize hexacoordinate hemoglobin kinetics. *Biophysical Journal* **79,** 2733–2738.

Hess DT, Matsumoto A, Kim SO, Marshall HE, Stamler JS. 2005. Protein *S*-nitrosylation: purview and parameters. *Nature Reviews Molecular Cell Biology* **2,** 150–166.

Hill AC, Bennet JH. 1970. Inhibition of apparent photosynthesis by nitrogen oxides. *Atmospheric Environment* **4,** 341–348.

Hu X, Neill SJ, Tang Z, Cai W. 2005. Nitric oxide mediates gravitropic bending in soybean roots. *Plant Physiology* **137,** 663–670.

Huang X, Stettmaier K, Michel C, Hutzler P, Mueller MJ, Durner J. 2004. Nitric oxide is induced by wounding and influences jasmonic acid signaling in *Arabidopsis thaliana*. *Planta* **218,** 938–946.

Huber SC, Hardin SC. 2004. Numerous post-translational modifications provide opportunities for the intricate regulation of metabolic enzymes at multiple levels. *Current Opinion in Plant Biology* **3,** 318–322.

Hunt PW, Klok EJ, Trevaskis B, Watts RA, Ellis MH, Peacock WJ, Dennis ES. 2002. Increased level of haemoglobin 1 enhances

survival of hypoxic stress and promotes early growth in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* **99,** 17197–17202.

Hunt PW, Watts RA, Trevaskis B, Llewelyn DJ, Burnell J, Dennis ES, Peacock WJ. 2001. Expression and evolution of functionally distinct haemoglobin genes in plants. *Plant Molecular Biology* **47,** 677–692.

Igamberdiev AU, Baron K, Manac'h-Little N, Stoimenova M, Hill RD. 2005. The haemoglobin/nitric oxide cycle: involvement in flooding stress and effects on hormone signalling. *Annals of Botany* **96,** 557–564.

Igamberdiev AU, Hill RD. 2004. Nitrate, NO and haemoglobin in plant adaptation to hypoxia: an alternative to classic fermentation pathways. *Journal of Experimental Botany* **55,** 2473–2482.

Igamberdiev AU, Seregélyes C, Manac'h N, Hill RD. 2004. NADH-dependent metabolism of nitric oxide in alfalfa root cultures expressing barley haemoglobin. *Planta* **219,** 95–102.

Ioanitescu AI, Dewilde S, Kiger L, Marden MC, Moens L, Van Doorslaer S. 2005. Characterization of non-symbiotic tomato hemoglobin. *Biophysical Journal* **89,** 2628–2639.

Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, Snyder SH. 2001. Protein *S*-nitrosylation: a physiological signal for neuronal nitric oxide. *Nature Cell Biology* **3,** 193–197.

Jia L, Bonaventura C, Bonaventura J, Stamler JS. 1996. *S*-nitrosohaemoglobin: A dynamic activity of blood involved in vascular control. *Nature* **380,** 221–226.

Jih PJ, Chen YC, Jeng ST. 2003. Involvement of hydrogen peroxide and nitric oxide in expression of the ipomoelin gene from sweet potato. *Plant Physiology* **132,** 381–389.

Joshi MS, Ferguson TBJ, Han TH, Hyduke DR, Liao JC, Rassaf T, Bryan N, Feelisch M, Lancaster JRJ. 2002. Nitric oxide is consumed, rather than conserved, by reaction with oxyhaemoglobin under physiological conditions. *Proceedings of the National Academy of Sciences, USA* **99,** 10341–10346.

Kaiser WM, Weiner H, Kandlbimder A, Tsai CB, Rockel P, Sonoda M, Planchet E. 2002. Modulation of nitrate reductase: some new insights, an unusual case and a potentially important side reaction. *Journal of Experimental Botany* **53,** 875–882.

Klepper L. 1990. Comparison between nox evolution mechanisms of wild-type and nr1 mutant soybean leaves. *Plant Physiology* **93,** 26–32.

Kundu S, Trent III JT, Hargrove MS. 2003. Plants, humans and haemoglobins. *Trends in Plant Science* **8,** 387–393.

Lamattina L, Garcia-Mata C, Graziano M, Pagnussat G. 2003. Nitric oxide: the versatility of an extensive signal molecule. *Annual Review of Plant Physiology* **54,** 109–136.

Lamotte O, Gould K, Lecourieux D, Sequeira-Legrand A, Lebrun-Garcia A, Durner J, Pugin A, Wendehenne D. 2004. Analysis of nitric oxide signaling functions in tobacco cells challenged by the elicitor cryptogein. *Plant Physiology* **135,** 516–529.

Leshem YY, Haramathy E. 1996. The characterization and contrasting effects of nitric oxide free radical in vegetative stress and senescence of *Pisum sativum* foliage. *Journal of Plant Physiology* **148,** 258–263.

Leshem YY, Willis RBH, Ku VVV. 1998. Evidence for the function of the free radical gas, nitric oxide (NO), as an endogenous maturation and senescence regulating factor in higher plants. *Plant Physiology and Biochemistry* **36,** 825–833.

Lindermayr C, Saalbach G, Durner J. 2005. Proteomic identification of *S*-nitrosylated proteins in *Arabidopsis*. *Plant Physiology* **137,** 921–930.

Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS, Steverding D. 2001*b*. Nitrosative stress: protection by glutathione-dependent formaldehyde dehydrogenase. *Redox Report* **4,** 209–210.

Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS. 2001*a*. A metabolic enzyme for *S*-nitrosothiol conserved from bacteria to humans. *Nature* **410,** 490–494.

Manac'h-Little N, Igamberdiev AU, Hill RD. 2005. Haemoglobin expression affects ethylene production in maize cell cultures. *Plant Physiology and Biochemistry* **43,** 485–489.

Minning DM, Gow AJ, Bonaventura J, Braun R, Dewhirst M, Goldberg DE, Stamler JS. 1999. Ascaris haemoglobin is a nitric oxide-activated 'deoxygenase'. *Nature* **401,** 497–502.

Murad F. 1986. Cyclic guanosine monophosphate as a mediator of vasodilation. *The Journal of Clinical Investigation* **78,** 1–5.

Neill SJ, Desikan R, Hancock JT. 2003. Nitric oxide signaling in plant. *New Phytologist* **159,** 11–35.

Ohwaki Y, Kawagishi-Kobayashi M, Wakasa K, Fujihara S, Yoneyama T. 2005. Induction of class-1 non-symbiotic haemoglobin genes by nitrate, nitrite and nitric oxide in cultured rice cells. *Plant Cell Physiology* **46,** 324–331.

Pagnussat GC, Lanteri ML, Lamattina L. 2003. Nitric oxide and cyclic GMP are messengers in the indole acetic acid-induced adventitious rooting process. *Plant Physiology* **132,** 1241–1248.

Parani M, Rudrabhatla S, Myers R, Weirich H, Smith B, Leaman DW, Goldman SL. 2004. Microarray analysis of nitric oxide responsive transcripts in *Arabidopsis*. *Plant Biotechnology Journal* **2,** 359–366.

Perazzolli M, Dominici P, Romero-Puertas MC, Zago ED, Zeier J, Sonoda M, Lamb C, Delledonne M. 2004. *Arabidopsis* non-symbiotic haemoglobin AHb1 modulates nitric oxide bioactivity. *The Plant Cell* **16,** 1–10.

Planchet E, Gupta KJ, Sonoda M, Kaiser WM. 2005. Nitric oxide (NO) emission from tobacco leaves and cell suspensions: rate-limiting factors and evidence for the involvement of mitochondrial electron transport. *The Plant Journal* **41,** 732–743.

Polverari A, Molesini B, Pezzotti M, Buonaurio R, Marte M, Delledonne M. 2003. Nitric oxide-mediated transcriptional changes in *Arabidopsis thaliana*. *Molecular Plant–Microbe Interactions* **16,** 1094–1105.

Prado AM, Porterfield DM, Feijo JA. 2004. Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. *Development* **131,** 2707–2714.

Quellet H, Ouellet Y, Richard C, Labarre M, Wittenberg B, Wittenberg J, Guertin M. 2002. Truncated haemoglobin HbN protects *Mycobacterium bovis* from nitric oxide. *Proceedings of the National Academy of Sciences, USA* **99,** 5902–5907.

Rockel P, Strube F, Rockel A, Wildt J, Kaiser WM. 2002. Regulation of nitric oxide (NO) production by plant nitrate reductase *in vivo* and *in vitro*. *Journal of Experimental Botany* **53,** 103–110.

Romero-Puertas MC, Perazzolli M, Zago ED, Delledonne M. 2004. Nitric oxide signalling functions in plant–pathogen interactions. *Cellular Microbiology* **9,** 795–803.

Sakamoto A, Sakurao S, Fukunaga K, Matsubara T, Ueda-Hashimoto M, Tsukamoto S, Takahashi M, Morikawa H. 2004. Three distinct *Arabidopsis* hemoglobins exhibit peroxidase-like activity and differentially mediate nitrite-dependent protein nitration. *FEBS Letters* **572,** 27–32.

Sakamoto A, Ueda M, Morikawa H. 2002. *Arabidopsis* glutathione-dependent formaldehyde dehydrogenase is an *S*-nitrosoglutathione reductase. *FEBS Letters* **515,** 20–24.

Seregélyes C, Igamberdiev AU, Maassen A, Hennig J, Dudits D, Hill RD. 2004. NO-degradation by alfalfa class-1 haemoglobin (Mhb1): a possible link to *PR-1a* gene expression in Mhb1-overproducing tobacco plants. *FEBS Letters* **571,** 61–66.

Seregélyes C, Barna B, Hennig J, Konopka D, Pasternack TP, Lukàcs N, Fehér A, Horvàth GV, Dudits D. 2003. Phytoglobin can interfere with nitric oxide functions during plant growth and

pathogenic responses: a transgenic approach. *Plant Science* **165,** 541–550.

Seregélyes C, Mustàrdy L, Ayaydin F, *et al.* 2000. Nuclear localization of a hypoxia-inducible novel nonsymbiotic haemoglobin in cultured alfalfa cells. *FEBS Letters* **482,** 125–1030.

Shafqat J, El-Ahmad M, Danielsson O, Martinez MC, Persson B, Pares X, Jornvall H. 1996. Pea formaldehyde-active class III alcohol dehydrogenase: common derivation of the plant and animal forms but not of the corresponding ethanol-active forms (classes I and P). *Proceedings of the National Academy of Sciences, USA* **11,** 5595–5599.

Shi S, Wang G, Wang Y, Zhang L, Zhang L. 2005. Protective effect of nitric oxide against oxidative stress under ultraviolet-B radiation. *Nitric Oxide* **13,** 1–9.

Shimoda Y, Nagata M, Suzuki A, Abe M, Sato S, Kato T, Tabata S, Higashi S, Uchiumi T. 2005. Symbiotic rhizobium and nitric oxide induce gene expression of non-symbiotic haemoglobin in *Lotus japonicus*. *Plant Cell Physiology* **46,** 99–107.

Sies H, Sharov VS, Klotz LO, Briviba K. 1997. Glutathione peroxidase protects against peroxynitrite-mediated oxidations. A new function for selenoproteins as peroxynitrite reductase. *Journal of Biological Chemistry* **272,** 27812–27817.

Simontacchi M, Jasid S, Puntarulo S. 2004. Nitric oxide generation during early germination of sorghum seeds. *Plant Science* **167,** 839–847.

Sokolovski S, Blatt MR. 2004. Nitric oxide block of outward-rectifying K^+ channels indicates direct control by protein nitrosylation in guard cells. *Plant Physiology* **136,** 4275–4284.

Song F, Goodman RM. 2001. Activity of nitric oxide is dependent on, but is partially required for function of, salicylic acid in the signaling pathway in tobacco systemic acquired resistance. *Molecular Plant–Microbe Interactions* **12,** 1458–1462.

Sowa AW, Du SMG, Guy PA, Hill RD. 1998. Altering haemoglobin levels changes energy status in maize cells under hypoxia. *Proceedings of the National Academy of Sciences, USA* **95,** 10317–10321.

Stamler JS, Lamas S, Fang FC. 2001. Nitrosylation: the prototypic redox-based signaling mechanism. *Cell* **6,** 675–683.

Stamler JS, Singel DJ, Loscalzo J. 1992. Biochemistry of nitric oxide and its redox-activated forms. *Science* **258,** 1898–1902.

Stöhr C, Strube F, Marx G, Ullrich WR, Rockel P. 2001. A plasma membrane-bound enzyme of tobacco roots catalyses the formation of nitric oxide from nitrite. *Planta* **212,** 835–841.

Stuehr DJ, Santolini J, Wang ZQ, Wei CC, Adak S. 2004. Update on mechanism and catalytic regulation in the NO synthases. *Journal of Biological Chemistry* **35,** 36167–36170.

Sun Y, Jin K, Mao XO, Zhu Y, Greenberg DA. 2001. Neuroglobin is up-regulated by hypoxia and protects neurons from hypoxic-ischemic injury. *Proceedings of the National Academy of Sciences, USA* **18,** 15306–15311.

Sun Y, Jin K, Peel A, Mao XO, Xie L, Greenberg DA. 2003. Neuroglobin protects the brain from experimental stroke *in vivo*. *Proceedings of the National Academy of Sciences, USA* **100,** 3497–3500.

Tada Y, Mori T, Shinogi T, *et al.* 2004. Nitric oxide and reactive oxygen species do not elicit hypersensitive cell death but induce apoptosis in the adjacent cells during the defence response of oat. *Molecular Plant–Microbe Interactions* **17,** 245–253.

Tamir S, Lewis RS, de Rojas Walker T, Deen WM, Wishnok JS, Tannenbaum SR. 1993. The influence of delivery rate on the chemistry and biological effects of nitric oxide. *Chemical Research In Toxicology* **6,** 895–899.

Trevaskis B, Watts RA, Andersson C, Llewellyn D, Hargrove MS, Olson JS, Dennis ES, Peacock WJ. 1997. Two haemoglobin genes in *Arabidopsis thaliana*: the evolutionary origins of leghaemoglobins. *Proceedings of the National Academy of Sciences, USA* **94,** 12230–12234.

Trujillo M, Alvarez MN, Peluffo G, Freeman BA, Radi R. 1998. Xanthine oxidase-mediated decomposition of *S*-nitrosothiols. *Journal of Biological Chemistry* **273,** 7828–7834.

Van Baarlen P, Staats M, Van Kan JAL. 2004. Induction of programmed cell death in lily by the fungal pathogen *Botrytis elliptica*. *Molecular Plant Pathology* **5,** 559–574.

Vieweg MF, Hohnjec N, Küster H. 2005. Two genes encoding different truncated haemoglobins are regulated during root nodule and arbuscular mycorrhiza symbioses of *Medicago truncatula*. *Planta* **220,** 757–766.

Wang R, Guegler K, LaBrie ST, Crawford NM. 2000. Genomic analysis of a nutrient response in *Arabidopsis* reveals diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate. *The Plant Cell* **12,** 1491–1509.

Watts RA, Hunt PW, Hvitved AN, Hargrove MS, Peacock WJ, Dennis ES. 2001. A haemoglobin from plants homologous to truncated haemoglobins of micro-organisms. *Proceedings of the National Academy of Sciences, USA* **98,** 10119–10124.

Weiland TR, Kundu S, Trent JT 3rd, Hoy JA, Hargrove MS. 2005. Bis-histidyl hexacoordination in haemoglobins facilitates haem reduction kinetics. *Journal of the American Chemical Society* **126,** 11930–11935.

Wendehenne D, Pugin A, Klessig DF, Durner J. 2001. Nitric oxide: comparative synthesis and signaling in animal and plant cells. *Trends in Plant Science* **4,** 177–183.

Wendehenne D, Durner J, Klessig DF. 2004. Nitric oxide: a new player in plant signalling and defence responses. *Current Opinion in Plant Biology* **7,** 449–455.

Yamasaki H, Sakihama Y. 2000. Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: *in vitro* evidence for the NR-dependent formation of active nitrogen species. *FEBS Letters* **1,** 89–92.

Yang LX, Wang RY, Ren F, Liu J, Cheng J, Lu YT. 2005. AtGLB1 enhances the tolerance of *Arabidopsis* to hydrogen peroxide stress. *Plant and Cell Physiology* **46,** 1309–1316.

Zeidler D, Zahringer U, Gerber I, Dubery I, Hartung T, Bors W, Hutzler P, Durner J. 2004. Innate immunity in *Arabidopsis thaliana*: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defence genes. *Proceedings of the National Academy of Sciences, USA* **44,** 15811–15816.

Zeier J, Delledonne M, Mishina T, Severi E, Sonoda M, Lamb C. 2004. Genetic elucidation of nitric oxide signaling in incompatible plant–pathogen interactions. *Plant Physiology* **136,** 2875–2886.

Zhang C, Czymmek KJ, Shapiro AD. 2003. Nitric oxide does not trigger early programmed cell death events but may contribute to cell-to-cell signaling governing progression of the *Arabidopsis* hypersensitive response. *Molecular Plant–Microbe Interactions* **16,** 962–972.

Zhang H, Shen WB, Zhang W, Xu LL. 2005. A rapid response of α-amylase to nitric oxide but not gibberellin in wheat seeds during the early stage of germination. *Planta* **220,** 708–716.

Zhao L, Zhang F, Guo J, Yang Y, Li B, Zhang L. 2004. Nitric oxide functions as a signal in salt resistance in the calluses from two ecotypes of reed. *Plant Physiology* **134,** 849–857.

Zottini M, Formentin E, Scattolin M, Carimi F, Lo Schiavo F, Terzi M. 2002. Nitric oxide affects plant mitochondrial functionality *in vivo*. *FEBS Letters* **515,** 75–78.