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Running title: *Redox regulation of symbiosis*

Redox control of the legume-*Rhizobium* symbiosis

Manuel A Matamoros*, Manuel Becana*

Departamento de Nutrición Vegetal, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), Apartado 13034, 50080 Zaragoza, Spain

*Corresponding authors: e-mail addresses: m.matamoros@csic.es, becana@eead.csic.es

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Abstract

The onset of the nitrogen-fixing legume-rhizobium symbiosis is a complex process that requires elaborate communication between the two partners. Nodule metabolism is very active and continuously generates reactive oxygen species (ROS) and reactive nitrogen species (RNS). During the evolution of aerobic life, ROS and RNS have been recruited as versatile signaling molecules due to their chemical properties and capacity to interact with enzymes and transcription factors. Numerous studies have demonstrated the presence of nitric oxide, hydrogen peroxide and other ROS and RNS at different stages of symbiosis, from early recognition between plant and bacteria to nodule senescence. Antioxidant metabolites and enzymes and other proteins such as phytoglobins finely regulate ROS and RNS concentrations, thereby allowing the beneficial and critical participation of these molecules as signals in many aspects of nodule physiology. Here we review the contribution of ROS, RNS and antioxidants to the redox control of the onset of symbiosis and subsequent nodule development, paying attention to both developmental (aging) and stress-induced senescence. Because of their importance in regulation and signaling, a substantial part of this review is devoted to post-translational redox modifications involving, among others, residues of methionine, cysteine and tyrosine of nodule proteins.

Abbreviations

Glb	phytoglobin
Gpx	glutathione peroxidase
Grx	glutaredoxin
GSH	glutathione
hGSH	homoglutathione
Lb	leghemoglobin
NTR	NADPH-thioredoxin reductase
Prx	peroxiredoxin
PTM	post-translational modification
RNS	reactive nitrogen species
ROS	reactive oxygen species
SOD	superoxide dismutase
SNF	symbiotic N_2 fixation
Trx	thioredoxin

1. Introduction

In nitrogen-poor soils many legumes are able to establish N_2-fixing symbioses with bacteria collectively known as rhizobia. Essentially, the mutual recognition of plant and bacteria relies on a complex exchange of molecular signals, involving flavonoids exudated from the roots and nodulation (Nod) factors produced by the rhizobia. In most crop legumes, as well as in model legumes, bacteria infection takes place through root hairs. The root hairs curl and trap the bacteria, which penetrate the epidermis and cortex through tubular structures known as infection threads. Bacteria are released from infection threads into the symbiosomes, organelle-like structures surrounded by a membrane, the symbiosomal or peribacteroid membrane, derived from the root cell plasma membrane. The cytoplasm of infected cells ultimately becomes filled with symbiosomes where bacteria differentiate into bacteroids that reduce (fix) N_2 into ammonia.

Legume nodules provide optimal conditions for the expression of bacterial nitrogenase, whose activity contributes fixed nitrogen to ecosystems and croplands (Oldroyd, 2013). In simple terms, nodules can be classified as indeterminate or determinate according to their growth pattern. Indeterminate nodules, formed by the model legume *Medicago truncatula* and crops such as pea, alfalfa and vetch, contain a persistent meristem and are generally elongated with a longitudinal gradient of age. Indeed, in a typical indeterminate nodule, four zones can be distinguished from the apex (distal) to the base (proximal) region: zone I (meristem), zone II (invasion), zone III (infected) and zone IV (senescent). Determinate nodules, formed by the model legume *Lotus japonicus* and crops such as soybean, common bean and cowpea, lack permanent meristems and are usually spherical. In this case, symbiotic N_2 fixation (SNF) takes place in the central infected zone, which contains also uninfected or interstitial cells and is surrounded by a multi-layered cortex or nodule parenchyma (Figure 1; Dupont et al., 2012; Minchin, James, & Becana, 2008).

In the last decades, the legume-rhizobium symbiosis has been the focus of intensive research. Most plant and bacterial genes essential for symbiosis have been characterized and the complete genome sequences of many rhizobial and ten legume species are available (Wang et al., 2017). Transcriptomic studies have greatly expanded our

knowledge of developmental and stress-induced nodule senescence (Pérez Guerra et al., 2010). Also, physiological, biochemical and molecular approaches have provided a wealth of information on the antioxidants of nodules (Becana, Matamoros, Ramos, Rubio, & Sainz, 2014; Becana, Matamoros, Udvardi, & Dalton, 2010; Dupont et al., 2012; Puppo et al., 2005) and on the metabolic exchange between the two symbiotic partners (Udvardi & Poole, 2013). Readers are referred to all those articles for useful information complementary to this review.

Reactive oxygen species (ROS), such as superoxide radicals and hydrogen peroxide (H_2O_2), and reactive nitrogen species (RNS), such as nitric oxide (NO), peroxynitrite ($ONOO^-$) and nitrosothiols, are produced at high concentrations in plant cells under severe stress. The excess of ROS and RNS may overwhelm the antioxidant defences of the plant, causing cellular damage and ultimately death. However, under physiological or mild stressful conditions, the spatio-temporal production and the concentrations of ROS and RNS are kept under control by a plethora of antioxidant enzymes and metabolites (Becana, Matamoros, Udvardi, & Dalton, 2010; Puppo et al., 2005). This tight regulation permits both types of reactive molecules to play essential roles as signals ('oxidative and nitrosative signaling') during plant development and in perception, adaptation and tolerance to stress (Umbreen et al., 2018; Waszczak, Carmody, & Kangasjärvi, 2018). Likewise, it has been shown that superoxide, H_2O_2 and NO are produced in all developmental stages of the legume-rhizobium symbiosis, from rhizobial infection to nodule maturation and senescence (Cárdenas, Martínez, Sánchez, & Quinto, 2008; Jamet, Mandon, Puppo, & Hérouart, 2007; Rubio et al., 2004; Santos, Hérouart, Sigaud, Touati, & Puppo, 2001). Here, we provide an update of findings related to the antioxidant system of legume nodules and then focus on the latest advances on redox-based post-translational modifications (PTMs) of nodule proteins. These PTMs can readily modulate protein activity in response to developmental or environmental cues and thus have a major impact on SNF.

2. Production of reactive oxygen and nitrogen species

2.1 Root infection and nodule primordia

Several studies have reported the rapid and transient generation of ROS in response to rhizobial Nod factors during the early stages of symbiosis (Bueno et al., 2001;

Cárdenas, Martínez, Sánchez, & Quinto, 2008; Santos, Hérouart, Sigaud, Touati, & Puppo, 2001). ROS act downstream of Nod factors in the signaling pathways that lead to the initiation of nodule primordia and are required for an adequate progression of infection threads (Cárdenas, Martínez, Sánchez, & Quinto, 2008; Jamet, Mandon, Puppo, & Herouart, 2007; Rubio et al., 2004; Santos, Hérouart, Sigaud, Touati, & Puppo, 2001). Although ROS can be generated from different sources (Becana et al., 2000), there is substantial evidence that points to cell membrane NADPH oxidases (also termed 'respiratory burst oxidase homologues', Rbohs) as the most important ROS-generating system during the first stages of symbiosis (for reviews see Marino, Dunand, Puppo, & Pauly, 2012; Montiel, Arthikala, Cárdenas, & Quinto, 2016). In common bean, down-regulation of *RbohA* and *RbohB* impairs infection thread formation and affects nodule development and function; conversely, *RbohB* overexpression increases the number of infection events and nodule number (Arthikala et al., 2017; Montiel, Arthikala, Cárdenas, & Quinto, 2016). In plants, small GTP-binding proteins are key regulators of ROS generation by Rbohs. In *M. truncatula*, knockdown plants in the small ρ-type GTPase MtROP9 do not generate ROS in response to *Sinorhizobium meliloti* and show major alterations in the infection process (Kiirika et al., 2012).

NO is a gaseous free radical that performs multiple signaling and regulatory functions in plants (Umbreen et al., 2018). During the early interaction between *L. japonicus* and *Mesorhizobium loti* a transient production of NO is observed after only four hours of inoculation. Remarkably, NO production is induced by rhizobial outer membrane lipopolysaccharides and occurs only in the presence of compatible bacteria, which suggests a role for NO in plant-bacteria recognition (Murakami et al., 2011; Nagata et al., 2008). Likewise, in the *M. truncatula-S. meliloti* symbiosis, NO is detected in infection threads and nodule primordia after two and four days of inoculation, respectively (del Giudice et al., 2011). In *M. truncatula* NO modulates the expression of numerous genes necessary for the onset of symbiosis and plays a crucial role in the repression of the plant's defence responses (Boscari et al., 2013).

2.2 Mature N_2-fixing nodules

SNF by nitrogenase consumes high amounts of ATP and reducing power that are provided by bacteroid respiration. Both the bacteroidal and mitochondrial electron transport chains inevitably generate ROS. Another potentially important source of ROS

in nodules is oxidation of heme and [Fe-S] clusters of proteins. Leghemoglobin (Lb) transports and delivers O_2 to the symbiosomes at a low steady concentration (Appleby, 1984) and is essential for SNF (Ott et al., 2005). Mutant nodules lacking Lb generate high concentrations of superoxide, probably as a result of activation of Rboh enzymes (Wang et al., 2019). Autoxidation of oxygenated Lb generates superoxide at significant levels, a process that is favored by the acid pH of senescent nodules (Becana & Klucas, 1992; Puppo, Rigaud, & Job, 1981). The nitrogenase components are irreversibly inactivated by O_2 probably because of the partial reduction of O_2 to ROS by [Fe-S] clusters (Robson & Postgate, 1980). Other abundant bacteroid proteins contributing to ROS production include ferredoxin, the proximal electron donor to nitrogenase, and hydrogenases, membrane-bound enzymes involved in the recycling of H_2 produced by nitrogenase activity (Becana et al., 2000). Rbohs participate also in ROS production in mature nodules. In *M. truncatula*, *RbohA* expression is induced in nodules and its down-regulation decreases SNF and expression of nitrogenase genes (Marino, Dunand, Puppo, & Pauly, 2012).

Several technical approaches have been used to localize NO production in mature nodules. The fluorescent dye 4,5-diaminofluorescein and a *S. meliloti* strain engineered as NO biosensor proved that NO is produced in zone III of *M. truncatula* nodules (Baudouin, Pieuchot, Engler, Pauly, & Puppo, 2006; Cam et al., 2012). Complementary studies with fluorescent probes and electron paramagnetic resonance spectroscopy of the nitrosyl-ferrous leghemoglobin (LbNO) complexes have shown NO production in a variety of legume nodules and treatment conditions (Calvo-Begueria et al., 2018; Horchani et al., 2011; Mathieu, Moreau, Frendo, Puppo, & Davies, 1998; Meilhoc, Blanquet, Cam, & Bruand, 2013; Sánchez et al., 2010). It should nevertheless be borne in mind that detection of NO by fluorescent dyes is prone to many artefacts, as concluded by Calvo-Begueria et al. (2018). Notably, these authors detected NO in the nodule parenchyma of soybean nodules, where the O_2 diffusion barrier is located (Minchin, James, & Becana, 2008). This observation suggests that NO is engaged in the control of O_2 entry into the infected zone. A few potential sources of NO in mature nodules have been proposed but most evidence supports the contribution of the nitrate reductase activities of both symbiotic partners (Horchani et al., 2011). The presence of an arginine-dependent (NO synthase-like) activity in nodules was also surmised on the basis of studies with inhibitors of animal NO synthases (Horchani et al., 2011; and references therein). However, as occurs with the detection of similar activities in other

plant systems, the protein(s) responsible for this reaction remain(s) elusive. The use of several inhibitors of plant NR and animal NO synthase activity suggested that in mature nodules the contribution of these enzymes to NO synthesis is secondary with respect to the bacteroid denitrification pathway (Calvo-Begueria et al., 2018).

2.3 Senescent nodules

Legume nodule senescence is a highly organized and regulated process characterized, among other factors, by the degradation of the two symbiotic partners and the switch of the nodule status from carbon sink to general nutrient source (Van de Velde et al., 2006). Extensive studies have been performed on both nodule developmental (aging) and stress-induced senescence (eg. Becana, Matamoros, Udvardi, & Dalton, 2010; Pérez Guerra et al., 2010; Puppo et al., 2005; Yuan et al., 2017; and references therein). The developmental and dark-stress induced senescence of *M. truncatula* nodules have been compared in detail at the structural and molecular levels (Pérez Guerra et al., 2010). Features shared by both processes include decreases of nitrogenase expression/activity and Lb content and up-regulation of cysteine proteinase transcripts. However, some interesting structural differences have been noted, such as degradation of symbiosomal membranes during aging but not during dark stress (Pérez Guerra et al., 2010). At the biochemical level, some studies have reported increases of H_2O_2, lipid peroxides and protein carbonyls in the two types of senescing nodules (Alesandrini, Mathis, Van de Sype, Hérouart, & Puppo, 2003; Evans et al., 1999; Loscos, Matamoros, & Becana, 2008). However, other studies found no evidence of oxidative stress associated to nodule aging (Groten et al., 2005). Most probably, alterations in redox homeostasis as a result of an increase in ROS production and a decrease in antioxidants may be perceived by redox sensors that, in turn, modify the signaling pathways that trigger senescence (Becana, Matamoros, Udvardi, & Dalton, 2010; Puppo et al., 2005). It is also conceivable that localized oxidative modifications, for example in the mitochondria, can be conveyed to the rest of the cell and regulate the senescence process (Matamoros et al., 2013).

Increasing evidence suggests that NO plays a key role in leaf and nodule senescence. It has been proposed that NO can act as either a positive or a negative regulator depending on the plant organ (Bruand & Meilhoc, 2019). In leaves NO delays senescence and the expression of an NO-scavenging protein (the bacterial

flavohemoglobin Hmp, which shows NO dioxygenase activity) induces leaf senescence in Arabidopsis (Mishina, Lamb, & Zeier, 2007). The underlying molecular mechanisms are still poorly defined, but NO-mediated transcriptional regulation or protein PTMs might be involved. On the contrary, NO triggers nodule senescence. Higher NO concentrations have been detected during the later stages of the symbiosis. As occurs in leaves, activation of specific NO-mediated signaling pathways may induce senescence. Alternatively, senescence might result from NO-mediated inactivation of key nodule proteins such as glutamine synthetase (GS) and Lb (see below), or from the inhibition of nitrogenase and the bacterial and mitochondrial respiratory chains (Bruand & Meilhoc, 2019, and references therein).

3. Antioxidant systems

3.1 Ascorbate, glutathione and associated enzymes in nodules

Ascorbate (vitamin C) and glutathione (GSH; γGlu-Cys-Gly) are major antioxidants and redox buffers of plants and are present at millimolar concentrations in legume nodules. Both metabolites act as antioxidants in their own right as ROS scavengers but also indirectly by being substrates of the enzymes of the ascorbate-GSH pathway (Foyer & Noctor, 2011). In this pathway, ascorbate peroxidase catalyzes the reduction of H_2O_2 by ascorbate, which is oxidized to monodehydroascorbate (ascorbate free radical) and dehydroascorbate. Ascorbate is then regenerated by NADH-dependent monodehydro-ascorbate reductase and GSH-dependent dehydroascorbate reductase. Finally, NADPH-dependent glutathione reductase reduces glutathione disulfide back to GSH. In nodules, the four enzymes are present in the cytosol and mitochondria and probably also in the plastids and peroxisomes (Dalton, Russell, Hanus, Pascoe, & Evans, 1986; Iturbe-Ormaetxe, Matamoros, Rubio, Dalton, & Becana, 2001; Matamoros et al., 2006) and their activities are positively correlated to SNF (Dalton, Langeberg, & Treneman, 1993) (Figure 2).

Ascorbate is synthesized primarily by the L-galactose pathway in leaf cells (Smirnoff, 2018) and probably also in nodules (Loscos, Matamoros, & Becana, 2008). Aging and stress-induced senescence entails a progressive decline of ascorbate biosynthesis and regeneration capacity in nodules (Loscos, Matamoros, & Becana, 2008). Increasing the ascorbate content in legumes has therefore biotechnological interest because it could render plants more nutritious and tolerant to environmental

stresses. Recent results show that *M. truncatula* plants overexpressing key genes of the L-galactose pathway have higher rates of SNF at the cost of a lower growth (Matamoros, Dalton, & Becana, 2017; Torres-Jerez, Huertas-Ruz, Lara-Dampier, Dalton, & Udvardi, 2017).

In plants GSH is involved in multiple physiological processes including redox homeostasis, regulation of cell cycle, responses to stress and heavy metal detoxification (Noctor et al., 2012). In legumes homoglutathione (hGSH; γGlu-Cys-βAla) may partially or completely replace GSH depending on the plant species and organ (Frendo et al., 1999; Klapheck, 1988; Matamoros, Moran, Iturbe-Ormaetxe, Rubio, & Becana, 1999). This can be illustrated by the thiol distribution in the two model legumes: *M. truncatula* produces GSH in all the plant and hGSH only in the roots and nodules (Frendo et al., 1999), whereas *L. japonicus* produces almost exclusively hGSH in the roots and leaves and both thiols only in the nodules (Matamoros et al., 2003). The synthesis of GSH and hGSH takes place by two sequential reactions. The first step is catalyzed by γ-glutamylcysteine synthetase and the second one by specific glutathione or homoglutathione synthetases (Klapheck, 1988; Frendo et al., 1999; Matamoros, Moran, Iturbe-Ormaetxe, Rubio, & Becana, 1999). Interestingly, in the two model legumes the homoglutathione synthetase gene arose from a duplication event and both genes are tandemly arranged in the chromosome. Homoglutathione synthetase has a higher affinity for β-alanine than for glycine (Frendo et al., 2001; Iturbe-Ormaetxe et al., 2002). To date there is not conclusive proof for specific functions of GSH or hGSH in legumes, although the differential transcriptional response of glutathione synthetase and homoglutathione synthetase to NO and hormones suggests that the functions of the two thiols do not completely overlap (Clemente et al., 2012; Innocenti et al., 2007). This issue will need to be settled by using gene-specific knockout mutants.

GSH and hGSH play essential roles in nodulation (Becana, Matamoros, Udvardi, & Dalton, 2010; Frendo et al., 2005). In *M. truncatula* the down-regulation of any of the three genes involved in GSH and hGSH synthesis resulted in plants with fewer nodules and lower rates of SNF. Conversely, the overexpression of γ-glutamylcysteine synthetase increased the GSH content and SNF (El Msehli et al., 2011). Bacteroids actively express the genes of the GSH biosynthetic pathway (Roux et al., 2014) and contain most of the GSH (~85%) of the nodules (Figure 2; Matamoros et al., 2013). The importance of bacterial GSH for the symbiosis was established with the use of mutants. Thus, the *S. meliloti gshA* strain lacking γ-glutamylcysteine synthetase did not nodulate

M. truncatula, whereas the *gshB* mutants deficient in glutathione synthetase formed nodules with reduced SNF (Cheng, Karunakaran, East, Muñoz-Azcarate, & Poole, 2017; Harrison et al., 2005; Muglia, Comai, Spegazzini, Riccillo, & Aguilar, 2008). The enzyme glutathione reductase of *S. melioti* bacteroids is also critical for symbiosis and nodule redox homeostasis because the corresponding *gor* mutant, which shows a decreased ratio of reduced to oxidized glutathione (GSH/GSSG), has a lower SNF efficiency (Tang, Li, Liu, Yu, Yan, & Luo, 2018).

3.2 Catalase

Catalases are tetrameric heme proteins localized to the peroxisomes that catalyze the decomposition of H_2O_2 to water and O_2. Because of their low affinity for H_2O_2 they may be efficient antioxidants only at high H_2O_2 concentrations. Unlike Arabidopsis, which has three catalase genes that are differentially regulated during development and in response to environmental factors (Mhamdi et al., 2010), a single catalase gene has been identified in *L. japonicus* (Becana, Matamoros, Ramos, Rubio, & Sainz, 2014). In white lupin nodules, catalase was immunolocalized in peroxisomes of infected cells and found to decrease during nitrate-induced senescence (Lorenzo, Lucas, Vivo, & de Felipe, 1990). As for the microsymbiont, *S. meliloti* contains three catalase genes encoding two monofunctional (KatA and KatC) and one bifunctional (KatB) catalase-peroxidase enzymes. Interestingly, the bacteroids of the single mutants behave similarly to those of the wild-type strain, but the nodules with bacteroids lacking both KatA and KatC enzymes show dramatic declines in SNF (Jamet, Sigaud, Van de Sype, Puppo, & Hérouart, 2003).

3.3 Superoxide dismutases

Superoxide dismutases (SODs) are metalloenzymes that catalyze the dismutation of superoxide radicals to H_2O_2 and O_2. They can be classified as CuZnSODs, MnSODs and FeSODs based on their metal cofactors. All of them have been identified in the nodule host cells: CuZnSOD and FeSOD in the cytosol, plastids and nuclei, and MnSOD in the mitochondria (Figure 2). In addition, bacteroids contain a MnSOD with significant homology to the plant protein (Becana, Matamoros, Ramos, Rubio, & Sainz, 2014; Rubio et al., 2007). The transcripts and proteins of SOD isoforms are differentially localized in determinate and indeterminate nodules, suggesting specific

roles for the three classes of SODs during nodule development. Also, co-localization studies suggest a role for CuZnSOD in cell wall growth and in the progression of infection threads by providing the H_2O_2 required for the cross-linking of extensins in the extracellular matrix and in the lumen of infection threads (Rubio et al., 2004; 2007).

3.4 Thiol peroxidases, thioredoxins and glutaredoxins

Peroxiredoxins (Prxs) and glutathione peroxidases (Gpxs) are ubiquitous non-heme enzymes involved in redox homeostasis. Prxs and Gpxs differ in protein sequence and structure but possess similar biochemical properties, functioning as thiol peroxidases in most organisms (Rouhier & Jacquot, 2005). They reduce H_2O_2 and organic peroxides with electrons donated by a catalytic peroxidatic cysteine residue, which results in the oxidation of the thiol group and the formation of a disulfide bond (Dietz, 2011; Passaia & Margis-Pinheiro, 2015). Vertebrate Gpxs contain selenocysteine in their active site and are reduced back to the active form by GSH, whereas plant Gpxs have catalytic cysteines that are generally reduced by thioredoxins (Trxs) and NADPH-thioredoxin reductase (NTR) in the cytosol and mitochondria, or by Trxs and ferredoxin-thioredoxin reductase in the chloroplasts. Prxs are classified in four groups based on the protein sequence and structure: 1-Cys Prx, PrxQ, PrxII and 2-Cys Prx (Dietz, 2011). Seven Prxs genes were identified in *L. japonicus* and all of them, except 1-Cys Prx, are expressed in nodules. Immunoblot and proteomic analyses detected mitochondrial PrxIIF, cytosolic PrxIIB, plastidic PrxIIE and 2-Cys Prx in the nodules (Figure 2; Dam et al., 2014; Tovar-Méndez et al., 2011). The genome of *L. japonicus* contains six *Gpx* genes, of which *LjGpx1* and *LjGpx3* are highly expressed in nodules, especially in the infected zone. LjGpx1 localizes to the plastids and nuclei and LjGpx3 to the cytosol and endoplasmic reticulum, and both enzymes seem to prevent lipid peroxidation under stress conditions (Matamoros et al., 2015).

Trxs are classified in seven groups based on protein sequence and localization: Trx*f*, Trx*m*, Trx*x*, Trx*y* and Trx*z* localize in the chloroplasts, Trx*h* in the cytosol and Trx*o* in the mitochondria and nuclei (Martí et al., 2009; Meyer, Buchanan, Vignols, & Reichheld, 2009). The nodules of *L. japonicus* contain functional NTR-Trx systems in the cytosol and mitochondria, and probably a ferredoxin-thioredoxin reductase-Trx system in the plastids. Moreover, NTRC, an enzyme that contains both NTR and Trx domains and may act as a complete NTR-Trx system, was detected in the plastids (Dam

et al., 2014; Tovar-Méndez et al., 2011). More recently, another type of Trx, termed Trx*s*, has been characterized in *M. truncatula* (Ribeiro et al., 2017). Two of the four isoforms, Trx*s1* and Trx*s2*, are predominantly expressed in nodules and have an atypical catalytic site, lack classical disulfide reductase activity and have an N-terminal signal peptide for targeting to the secretory pathway. Trx*s* have no orthologues in non-legumes or legumes with determinate nodules like *L. japonicus* or soybean (Alkhalfioui et al., 2008). The Trx*s1* isoform is targeted to the symbiosomes, where it modulates the redox state of nodule-specific cysteine-rich peptides. These plant peptides are involved in the terminal differentiation of *S. meliloti* into bacteroids (Ribeiro et al., 2017).

Glutaredoxins (Grxs) are small and ubiquitous redox regulators functionally related to Trxs which catalyze the reduction of disulfide bonds using GSH as preferred electron donor (Meyer, Buchanan, Vignols, & Reichheld, 2009). Grxs also participate in deglutathionylation reactions and in the assembly of [Fe-S] clusters (Moseler et al., 2015). They form a complex family with more than 30 isoforms identified in vascular plants that are subdivided into four classes. To our knowledge, only a few Grxs (class I and class II) have been identified so far in nodules (Dam et al., 2014; Tovar-Méndez et al., 2011). The class II Grxs detected in *L. japonicus* are homologues to Arabidopsis Grxs S16 and S17. Grx S16 plays a key role in the control of vegetative growth and Grx S17 is involved in development and associates with cytosolic [Fe-S] components (Knuesting et al., 2015; Iñigo et al., 2016; Rey, Becuwe, Tourrette, & Rouhier, 2017). If these proteins perform similar functions in the nodules awaits investigation. Regarding the bacterial partner, the *S. meliloti* genome encodes three Grxs. Mutation of *Smgrx1* had the strongest phenotype, resulting in nodule abortion and absence of bacteroid differentiation; in contrast, *SmGrx2* mutation impaired nodule development but not bacteroid differentiation and *SmGrx3* mutation had no effect on symbiotic performance (Benyamina et al., 2013). Interestingly, the same authors reported that the deficiency of SmGrx2, but not of the other SmGrxs, affects the iron homeostasis of bacteroids, reinforcing the view that each SmGrx isoform plays specific roles during symbiosis.

3.5 *S*-Nitrosoglutathione reductase

A major way by which NO signaling is transmitted is through *S*-nitrosylation of target proteins. This PTM may regulate protein function and has been involved in processes such as the response to stress, hormone signaling and development (Astier et al., 2012).

The extent of *S*-nitrosylation is in part modulated by the intracellular level of *S*-nitrosoglutathione (Begara-Morales et al., 2018). This is regulated by *S*-nitrosoglutathione reductase, which catalyzes the NADH-dependent reduction of *S*-nitrosoglutathione producing glutathione disulfide and ammonia (Lindermayr, 2017). Expression analysis unveiled the presence of one functional *S*-nitrosoglutathione reductase gene in leaves and roots and two functional genes in nodules (M. A. Matamoros & M. Becana, unpublished results). The function of these enzymes in the N_2-fixing symbiosis remains unknown.

3.6 Hemoglobins

Hemoglobins perform multiple functions in all organisms. In addition to Lbs, legumes, like other plants, contain non-symbiotic hemoglobins, now termed phytoglobins (Glbs; Hill, Hargrove, & Arredondo-Peter, 2016), that are expressed in all tissues. Glbs occur at micromolar concentrations and are grouped in three classes based on their amino acid sequences and phylogenetic analyses (Smagghe et al., 2007; 2009). Class 1 and class 2 Glbs are structurally similar to animal myoglobin and hemoglobin, with a tertiary structure based on a 3-on-3 (3/3) α-helical fold, whereas class 3 Glbs have homology to bacterial truncated hemoglobins and a 2-on-2 (2/2) α-helical sandwich structure (Wittenberg, Bolognesi, Wittenberg, & Guertin, 2002). Class 1 Glbs display very high O_2 affinities and are thus unsuitable for O_2 transport and delivery (Smagghe et al., 2009). They are induced by hypoxia and flooding in several species and confer stress tolerance, at least in part through the ability of the oxyferrous hemoglobins ($Glb^{2+}O_2$) to dioxygenate NO to NO_3^- by their NO dioxygenase activity (Gardner, 2012; Hebelstrup, Shah, & Igamberdiev, 2013; Hill, 2012). Class 2 Glbs have O_2 affinities similar to Lb and their functions are not well defined, although they are involved in plant development and organogenesis (Hebelstrup, Shah, & Igamberdiev, 2013; Hill, 2012). Class 3 Glbs have unknown functions in plants, although at least one class 3 Glb, THB1, of the unicellular green alga *Chlamydomonas reinhardtii* is able to scavenge NO *in vivo* (Sanz-Luque et al., 2015).

In Arabidopsis each type of Glb is represented by a single gene, whereas *L. japonicus* express two class 1 (LjGlb1-1 and LjGlb1-2), one class 2 (LjGlb2) and two class 3 (LjGlb3-1 and LjGlb3-2) Glbs (Bustos-Sanmamed et al., 2011). The expression of *LjGlb1-1*, *LjGlb2* and *LjGlb3-1* is very high in nodules relative to other plant organs,

suggesting that the respective proteins are required for symbiosis. Overexpression of *LjGlb1-1* increases nodulation and nitrogenase activity, reduces NO level in nodules and delays nodule senescence (Fukudome et al., 2019; Shimoda et al., 2009). Studies with knockout or knockdown mutants of *LjGlb1-1* demonstrated its involvement in the infection process and in the regulation of NO during the initial stages of symbiosis (Fukudome et al., 2016). These authors suggested that the duration and amplitude of the NO signal is regulated by Glbs and is crucial for the onset of symbiosis.

The bacterial partner of the symbiosis also contains hemoglobins. The flavohemoglobin Hmp of *S. meliloti* contributes to regulate NO concentration in nodules and is important for the establishment and function of symbiosis (Cam et al., 2012). The typical symbiont of soybean, *Bradyrhizobium japonicum*, contains a single-domain hemoglobin, Bjgb, that may be implicated also in NO detoxification (Sánchez et al., 2011). Besides hemoglobins, bacteroids contain other proteins that may control NO concentration. Respiratory nitric oxide reductases (Nor), which reduce NO to N_2O in the denitrification pathway, are important for NO homeostasis because mutants defective in Nor (norB in *S. meliloti* or norC in *B. japonicum*) accumulate NO, as detected by fluorescent probes or electron paramagnetic resonance of LbNO complexes (Calvo-Begueria et al., 2018; Meilhoc, Blanquet, Cam, & Bruand, 2013; Sánchez et al., 2011).

4. Protein post-translational modifications related to redox signaling

Proteins are exposed to enzymes and redox reactive compounds that can modify their chemical structures (Figure 3). These PTMs can be reversible (disulfide bonds, methionine sulfoxides, *S*-nitrosylation) or irreversible (carbonylation, glycation). Theoretically, redox modifications can influence protein activity, stability and localization. However, the effect on protein function is difficult to anticipate and the same modification may have contrasting effects in different proteins. Therefore, a case study approach is often necessary.

4.1 Methionine sulfoxidation

Methionine residues can be readily oxidized to a mixture of methionine-*S*-sulfoxide and methionine-*R*-sulfoxide. The oxidation can be reverted by two methionine sulfoxide reductases, MsrA and MsrB, that respectively reduce the *S* and *R* epimers and are ubiquitous in all organisms (Tarrago, Laugier, & Rey, 2009). Methionine oxidation may

alter protein structure and function making this modification of regulatory significance in redox signaling. For example, the *in vivo* oxidation of Met-538 in Arabidopsis nitrate reductase inhibits phosphorylation of Ser-534, and some protein kinases can be directly activated by methionine oxidation (Erickson et al., 2008; Hardin, Larue, Oh, Jain, & Huber, 2009). In humans, this modification has also been associated to protein aggregation and degradation during age-related diseases (Stadtman, Moskovitz, & Levine, 2003).

Little is known about methionine oxidation in legume nodules. In bean, the ratio of methionine to methionine sulfoxide did not change for most proteins in aging nodules. One exception was GS, a key enzyme of nodule carbon and nitrogen metabolism. The GS-N1 isoform contains two methionine residues that are more oxidized to sulfoxides in senescing nodules than in young nodules, but the *in vivo* relevance of this modification could not be determined (Matamoros et al., 2013). The rhizobial proteomes contains several proteins with homology to MsrA and MsrB. It is still uncertain, however, whether these proteins are able to reduce the methionine sulfoxides back to methionines in proteins.

4.2 Sulfenylation

Along with methionine, the chemical characteristics of the sulfur atom make cysteine residues major targets of oxidation in proteins. However, not all the cysteines show the same reactivity, which depends on the protein microenvironment and the residue pK_a value. Usually, only thiols with low pK_a play key roles in catalysis and serve as important sites for PTM (Go, Chandler, & Jones, 2015). Deprotonated thiolates are prone to oxidation to form disulfide bridges (S-S) and sulfenic (R-SOH), sulfinic (R-SO_2H) and sulfonic (R-SO_3H) acids. Sulfonic acid formation seems irreversible but the other modifications can be reversed by Trxs, Grxs and sulfiredoxins (Meyer, Buchanan, Vignols, & Reichheld, 2009; Sevilla et al., 2015).

Thiol redox modifications may alter the structure, localization and activity of enzymes and transcription factors (Waszczak et al., 2015). In legumes, the use of chemical and genetic probes combined with mass spectrometry analyses allowed the identification of sulfenylated proteins in inoculated roots and mature nodules of *M. truncatula* (Oger, Marino, Guigonis, Pauly, & Puppo, 2012). Most proteins (~80%) were of plant origin. Interestingly, the pattern of sulfenylation differed at different

stages of the symbiosis. Proteins involved in redox signaling constituted the largest group during the establishment of the symbiosis, whereas in mature nodules most proteins were related to amino acid and carbohydrate metabolism, protein synthesis, folding, modification and degradation. This is consistent with the well-recognized importance of redox regulation for nodule development (Frendo, Matamoros, Alloing, & Becana, 2013; Ramu, Peng, & Cook, 2002; Rubio et al., 2004). The sulfenylated proteins identified in the bacteroids include the iron protein (NifH) and the iron-molybdenum protein (NifK) of nitrogenase.

4.3 *S*-Nitrosylation

Thiol-containing proteins can also undergo *S*-nitrosylation. This is an important NO-dependent PTM in which NO is reversibly incorporated to a reactive cysteine residue (Astier et al., 2012). In Arabidopsis, a study aimed at identifying endogenously *S*-nitrosylated proteins showed that this PTM is a regulatory mechanism in photosynthesis, carbohydrate metabolism and stress responses (Hu et al., 2015). In mature nodules of *M. truncatula*, 80 proteins of bacterial or plant origin were identified as *S*-nitrosylated and 27 proteins were also sulfenylated (Puppo, Pauly, Boscari, Mandon, & Brouquisse, 2013). Most proteins susceptible to both PTMs participate in carbon and nitrogen metabolism and energy production, which underlines the importance of redox regulation in nodule metabolism.

Although the list of *S*-nitrosylated proteins is long, the effect of this PTM on protein function is known only in a few cases. For example, ascorbate peroxidase activity is up-regulated by *S*-nitrosylation of a cysteine residue located in the ascorbate binding site (Begara-Morales et al., 2014). In *L. japonicus*, LjGpx1 and LjGpx3 are highly expressed in nodules and are regulated by *S*-nitrosylation of the peroxidatic cysteine *in vitro* and *in vivo* (Matamoros et al., 2015). In contrast to ascorbate peroxidase, the modification of Gpxs inhibits their enzymatic activities. In *M. truncatula*, *in vitro* studies showed that GS isoenzymes can be differently regulated by NO. Plastid-located MtGS2a activity is inhibited by *S*-nitrosylation. MtGS1a is not affected by this PTM but is inactivated by tyrosine nitration (see below; Melo, Silva, Ribeiro, Seabra, & Carvalho, 2011), thus showing the versatility of NO-mediated regulation of protein function.

4.4 *S*-Glutathionylation

S-glutathionylation is the reversible addition of glutathione to a reactive cysteine residue of a protein. This PTM usually occurs in response to increases in ROS and NO levels and protects cysteine residues against further deleterious oxidation. As for other PTMs, *S*-glutathionylation may induce functional changes in the target protein and regulate signal transduction and metabolic pathways (Zaffagnini, Bedhomme, Lemaire, & Trost, 2012). Whereas this modification may occur via nonenzymatic mechanisms, deglutathionylation is usually carried out by Grxs (Meyer, Buchanan, Vignols, & Reichheld, 2009). *S*-glutathionylation has been widely studied in animal systems (Zhang, Ye, Singh, Townsend, & Tew, 2018) and is also emerging as an important mechanism of redox regulation in plants. In Arabidopsis, a number of *S*-glutathionylated proteins were identified in response to oxidative stress (Dixon, Skipsey, Grundy, & Edwards, 2005). This PTM regulates the activity of Trx*f* in chloroplasts and glycine decarboxylase in mitochondria and is therefore involved in the regulation of carbon fixation and photorespiration (Zaffagnini, Bedhomme, Lemaire, & Trost, 2012). *S*-glutathionylation is also expected to be a redox signaling mechanism in the onset and functioning of the rhizobium-legume symbiosis. This is suggested by the observations that SmGrx1 displays deglutathionylation activity and that the corresponding bacterial mutant strain has an impaired symbiotic phenotype (Benyamina et al., 2013). To our knowledge, there is no information about the occurrence of this PTM in nodules.

4.5 Persulfidation

Besides sulfenylation, nitrosylation and glutathionylation, the thiol group of cysteine residues may be modified in yet other ways. Hydrogen sulfide (H_2S) has emerged as a novel signaling molecule playing an important role in many physiological and pathological processes in plants and animals (Aroca, Gotor, & Romero, 2018). There are three main routes by which H_2S exerts its biological effects: metal centre interactions, ROS and RNS scavenging, and persulfidation. This PTM is accepted as the main mechanism by which H_2S transmits its signaling capacity. In this process, a thiol (R-SH) is converted into a perthiol (R-SSH, also called a persulfide), and this can alter protein structure and function because of the decrease in the pK_a and the increase in nucleophilicity of the persulfide group (Ono et al., 2014). In Arabidopsis, >2000

proteins involved in key processes of plant biology have been recently identified as targets of persufidation (Aroca, Gotor, & Romero, 2018). Of these, ~25% have been found to be also *S*-nitrosylated and ~3% are *S*-glutathionylated, showing that many proteins may be regulated by these redox-dependent mechanisms. To gain insight into the possible roles of protein persufidation in nodules, a proteomic study is underway with bean nodules at different stages of development (M. Matamoros & M. Becana, unpublished results). Preliminary data show that ~650 and ~350 proteins of nodule host cells and bacteroids, respectively, are persulfidated *in vivo* under physiological conditions.

4.6 Nitration

This PTM consists in the covalent addition of a nitro group (-NO_2) to one of the two equivalent *ortho* carbons in the aromatic ring of tyrosine residues to form 3-nitrotyrosine (NO_2–Tyr) (Kolbert, Feigl, Bordé, Molnár, & Erdei, 2017). Tyrosine nitration requires the presence of $ONOO^-$ or NO_2 because NO itself is not reactive enough to nitrate tyrosine residues. $ONOO^-$ is formed by the reaction of NO and superoxide radicals. Radicals derived from $ONOO^-$ breakdown oxidize tyrosine residues to tyrosyl radicals, which react with NO_2, produced also from $ONOO^-$ decomposition, to yield NO_2–Tyr. Alternatively, NO_2 may be generated by the oxidation of NO_2^- to NO in the presence of H_2O_2 and peroxidases (Radi, 2018). In nodules another mechanism has been described: H_2O_2 oxidizes Lb to ferryl Lb and this, in turn, oxidizes NO_2^- to NO_2 and tyrosine to tyrosyl radicals; then both radicals react to form NO_2–Tyr (Sainz et al., 2015).

Tyrosine nitration causes a decrease of the residue pK_a, enhances its hydrophobicity and provokes steric restrictions because NO_2–Tyr is larger than tyrosine. In plant cells tyrosine nitration generally leads to protein loss-of-function, although there are some exceptions. In nodules tyrosine nitration may have an important regulatory role because two key proteins for nodule functioning, GS and Lb, are targets of this PTM. MtGS1a is inactivated by tyrosine nitration and the level of nitrated protein increases under conditions in which SNF is impaired, such as in ineffective nodules or in nodules of plants fed with NO_3^- (Melo, Silva, Ribeiro, Seabra, & Carvalho, 2011). Lb is susceptible to nitration in both the heme and globin. Navascués et al. (2012) identified green Lb derivatives in senescing soybean nodules. These modified Lbs have identical globins to

the parent red Lbs but their hemes are nitrated in a vinyl group. In a follow-up study, it was found that one tyrosine residue located in the distal heme pocket was the major target of nitration of the globin moiety (Sainz et al., 2015). However, the amount of nitrated globin decreased during senescence, suggesting that heme and globin nitration occurs through different mechanisms and/or that globin nitration, but not heme nitration, makes the protein prone to degradation by nodule proteases as observed for other plant proteins (Castillo et al., 2015).

The significance of protein nitration in redox signaling is still poorly defined. This stable PTM was categorized as irreversible, but recent research in animals has identified denitrase mechanisms that could be also operative in plants (Kolbert, Feigl, Bordé, Molnár, & Erdei, 2017).

4.7 Carbonylation and glycation

In cells, metal-catalyzed oxidation occurs when free Fe^{2+} or Cu^{+} reacts with H_2O_2 and generates hydroxyl radicals through the Fenton reaction (Halliwell, 2006). These radicals can irreversibly oxidize amino acid side chains and introduce the carbonyl moiety in proteins (Møller, Rogowska-Wrzesinska, & Rao, 2011). Carbonyl groups may also be generated indirectly by Michael addition of lipid peroxidation decomposition products to arginine, cysteine, histidine and lysine residues (Matamoros et al., 2018; Møller, Rogowska-Wrzesinska, & Rao, 2011). Protein carbonylation contributes to cellular damage caused by stress conditions and age-associated diseases in animals (Höhn, König, & Grune, 2013) and plants (Matamoros et al., 2013; Sun et al., 2014). It is also likely, however, that irreversible protein carbonylation has a role in the regulation of protein function, thus contributing to redox signaling (Oracz et al., 2007; Winger, Taylor, Heazlewood, Day, & Millar, 2007).

Protein glycation occurs when arginine and lysine residues react with reducing sugars, generating Amadori and Heyns compounds (Heyns & Noack, 1962). These glycation products are readily oxidized, yielding relatively stable advanced glycation end products. Alternatively, these glycation products can be formed by the reaction of arginine and lysine residues with α-dicarbonyls (eg. glyoxal and methylglyoxal) generated by monosaccharide auto-oxidation under oxidative conditions. In humans, the formation of glycation end products accompanies atherosclerosis and diabetes (Höhn, König, & Grune, 2013). However, very little is known about protein glycation in plants.

The Arabidopsis proteome modified by advanced glycation products, as well as the age-dependent increase of glycation at specific sites, have been recently reported (Bilova et al., 2017).

An extensive study on protein oxidation in nodules allowed the identification of 238 and 131 plant and bacterial carbonylated proteins, respectively, and the carbonylation sites were determined. The study revealed that carbonylation occurs under normal growth conditions and that lipid peroxidation-derived products are the major contributors to protein carbonylation in nodule cells (Matamoros et al., 2018). The same study also uncovered major effects of carbonylation on two key nodule proteins, malate dehydrogenase and Lb. Malate dehydrogenase is essential for SNF because malate is the primary source of carbon transported to the bacteroids, and its activity is negatively correlated to the carbonylation level. Carbonylation also induced Lb aggregation, probably rendering the protein inactive and more susceptible to degradation by cell proteases. Other numerous glycated proteins were identified *in vivo*, including three nodule proteins that are central to carbon and nitrogen metabolism: sucrose synthase, GS and glutamate synthase. Label-free quantification identified 10 plant proteins and 18 bacterial proteins as age-specifically glycated, although the functional implications of these modifications are unknown.

5. Conclusions

Extensive transcriptomic work has been carried out to elucidate the mechanisms of redox control in legume nodules. This has not been in pace, however, with parallel studies at the proteomic and metabolomic levels. An increasing number of nodule proteins bearing redox-dependent PTMs have been identified, but in most cases the precise effects of such modifications on protein function remain unknown. Because the same PTM may cause distinct effects on various proteins, an individual study of each protein is necessary. The results available so far suggest that the selective redox-dependent modification of enzymes, transcriptional regulators and components of signaling pathways may constitute a major control mechanism of the infection process and of nodule metabolism, development and senescence. In the next years, considerable effort will be required to identify ROS and RNS sensors and to establish the protein interactions that convey specific signals for the functioning of symbiosis and its response to abiotic and biotic stress. Understanding redox signaling in plant and nodule

cells will constitute a solid base to manipulate nodule activity and improve adaptation of legumes to a changing environment.

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Figure legends

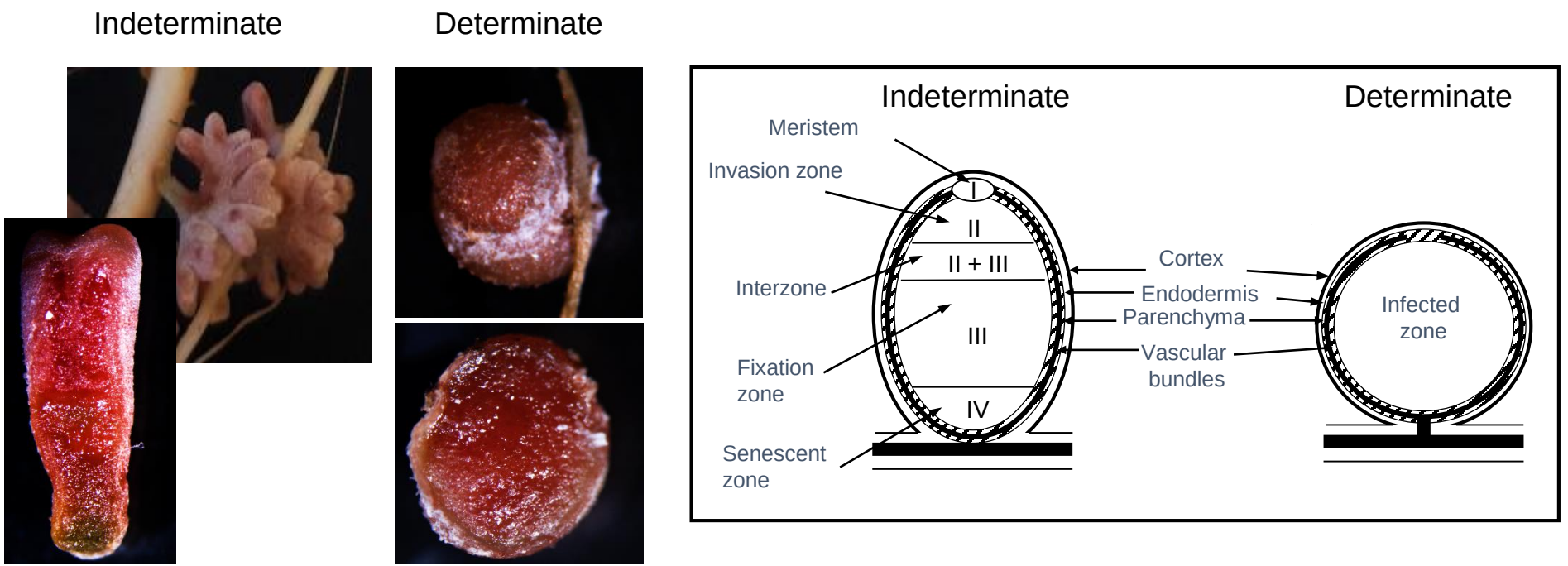
Figure 1. Main characteristics of indeterminate and determinate nodules. Both types differ structurally and biochemically. In the photographs, the red color in the fixation zone (III) of the *M. truncatula* indeterminate nodule and in the infected zone of the *L. japonicus* determinate nodule is due to the high concentration of Lb. The green-brownish color in the senescent zone (IV) of the indeterminate nodule is indicative of Lb degradation to biliverdin-like pigments.

Figure 2. Schematics showing the subcellular localization of antioxidant enzymes and metabolites in legume nodules. *Additional abbreviations*: ASC, ascorbate; γEC, γ-glutamylcysteine; γECS, γ-glutamylcysteine synthetase; ETC, electron transport chain; FTR, ferredoxin-thioredoxin reductase; GalLDH, L-galactono-1,4-lactone dehydrogenase; (h)GSHS, (homo)glutathione synthetase; Ox met, oxidative metabolism.

Figure 3. Redox-dependent PTMs of proteins from legume nodules. Oxidation of thiols of cysteine residues (-SH/-S^-) leads to the formation of sulfenic acid (-SOH), which may react with another -SH to form disulfides (-S-S-). This modification can be reverted by Trxs and Grxs. The -SOH group can be further oxidized to sulfinic acid (-SO_2H) and sulfonic acid (-SO_3H). The -SO_2H group may be reduced back to -SH by sulfiredoxins, whereas -SO_3H formation is irreversible. The -SH group can be also persulfidated to -SSH. *S*-nitrosylation (-SNO) may be mediated by NO, NO^+, nitrogen oxides and *trans*-nitrosylating agents such as *S*-nitrosoglutathione (GSNO). Glutathionylation occurs by three main mechanisms: reaction of the target protein with GSNO, -SH/-SS- exchange with glutathione disulfide and reaction of GSH with -SOH. $ONOO^-$-derived radicals modify tyrosine residues. Tyrosyl radicals generated by hydroxyl (·OH) and carbonate (·CO_3^-) radicals react with NO_2 yielding NO_2–Tyr. Methionine is typically oxidized to a

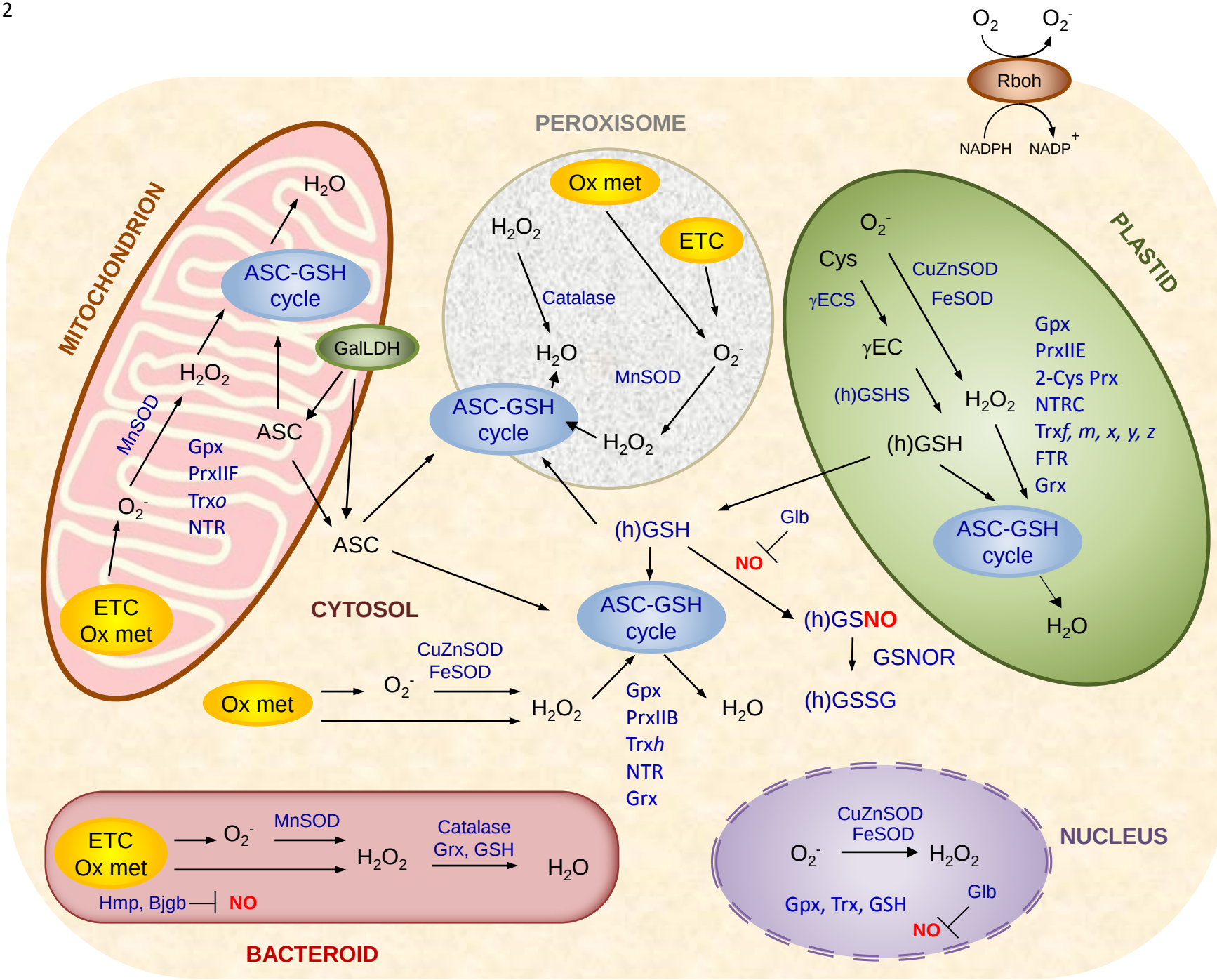
sulfoxide. The *S* and *R* stereoisomers are specifically reduced back to methionine by methionine sulfoxide reductases A and B, respectively. Direct oxidation of lysine, arginine, proline and threonine by ·OH incorporates the carbonyl moiety into proteins. Alternatively, oxidation of polyunsaturated fatty acid produces unstable lipid hydroperoxides that decompose to secondary products. These react with amino acid side chains and generate carbonyl derivatives. Moreover, arginine and lysine residues may react with reducing sugars or α-dicarbonyls such as glyoxal and methylglyoxal, generating glycation products that are readily oxidized yielding relatively stable advanced glycation end products (AGEs).

Figure 1



	Indeterminate	**Determinate**
Host plant	Alfalfa, pea, *Medicago truncatula*	Bean, soybean, *Lotus*
Geographic origin	Template	Tropical and subtropical
Nodule shape	Elongated	Spherical
Initial cell divisions	Inner cortex	Outer cortex
Nodule growth	Cell division. Persistent meristem	Cell expansion
Flavonoids inducing *nod* genes	Isoflavones	Flavones, flavonones
Export of assimilated nitrogen	Amides	Ureides (*Lotus* is an exception)

Figure 2


O2
O2-
Rboh
NADPH
NADP+
PEROXISOME
MITOCHONDRION
PLASTID
H2O
ASC-GSH cycle
GalLDH
H2O2
MnSOD
ASC
Gpx
PrxIIF
Trxo
NTR
O2-
ETC
Ox met
Ox met
H2O2
ETC
Catalase
H2O
O2-
MnSOD
ASC-GSH cycle
H2O2
O2-
Cys
γECS
CuZnSOD
FeSOD
γEC
(h)GSHS
H2O2
(h)GSH
Gpx
PrxIIE
2-Cys Prx
NTRC
Trxf, m, x, y, z
FTR
Grx
ASC-GSH cycle
H2O
ASC
(h)GSH
Glb
NO
CYTOSOL
ASC-GSH cycle
(h)GSNO
GSNOR
(h)GSSG
CuZnSOD
FeSOD
Ox met
O2-
H2O2
H2O
Gpx
PrxIIB
Trxh
NTR
Grx
ETC
Ox met
O2-
MnSOD
H2O2
Catalase
Grx, GSH
H2O
Hmp, Bjgb
NO
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CuZnSOD
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O2-
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Gpx, Trx, GSH
Glb
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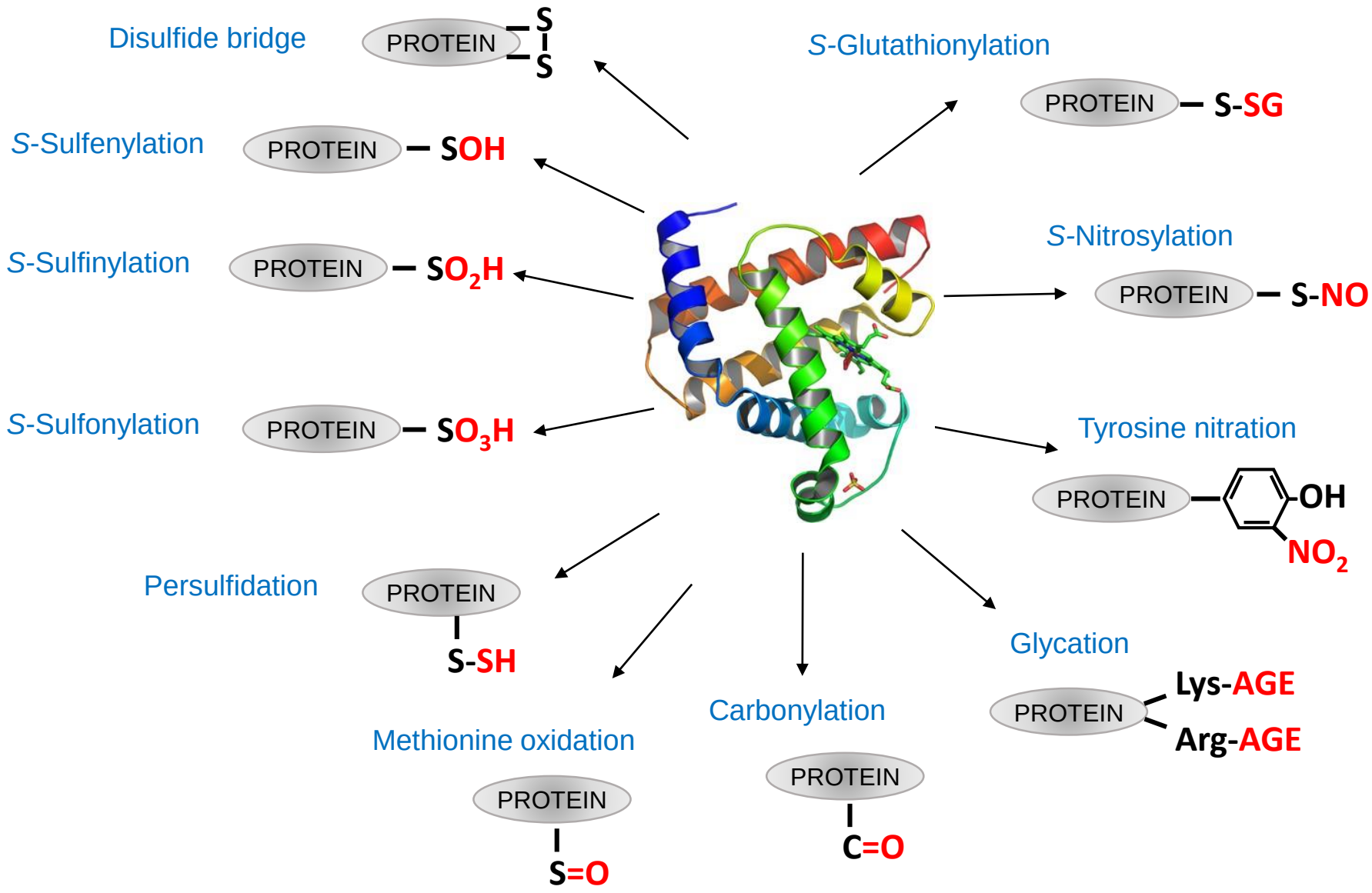
NUCLEUS

Figure 3


Disulfide bridge
PROTEIN
S
S
S-Glutathionylation
PROTEIN
S-SG
S-Sulfenylation
PROTEIN
SOH
S-Sulfinylation
PROTEIN
SO2H
S-Nitrosylation
PROTEIN
S-NO
S-Sulfonylation
PROTEIN
SO3H
Tyrosine nitration
PROTEIN
OH
NO2
Persulfidation
PROTEIN
S-SH
Glycation
PROTEIN
Lys-AGE
Arg-AGE
Carbonylation
PROTEIN
C=O
Methionine oxidation
PROTEIN
S=O