Tansley review

Recent insights into antioxidant defenses of legume root nodules

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Summary

Legume root nodules are sites of intense biochemical activity and consequently are at high risk from the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). These molecules can potentially give rise to oxidative and nitrosative damage but, when their concentrations are tightly controlled by antioxidant enzymes and metabolites, they also play positive roles as critical components of signal transduction cascades during nodule development and stress. Thus, recent advances in our understanding of ascorbate and (homo)glutathione biosynthesis in plants have opened the possibility of enhancing N_2 fixation through an increase of their concentrations in nodules. It is now evident that antioxidant proteins other than the ascorbate-glutathione enzymes, such as some isoforms of glutathione peroxidases, thioredoxins, peroxiredoxins, and glutathione *S*-transferases, are also critical for nodule activity. To avoid cellular damage, nodules are also endowed with several mechanisms for sequestration and homeostasis of Fenton-active metals (nicotianamine, phytochelatins, and metallothioneins) and for controlling ROS/RNS bioactivity (hemoglobins). The use of 'omic' technologies has expanded the list of known antioxidants in plants and nodules that participate in ROS/RNS/antioxidant signaling networks, although aspects of developmental variation and subcellular localization of these networks remain to be elucidated. To this end, a critical point will be to define the transcriptional and post-transcriptional regulation of antioxidant proteins.

I. Introduction

Antioxidant defenses are indispensable to all aerobic life, but they are especially important for N_2-fixing organisms, whether symbiotic (e.g. rhizobia in legume root nodules) or free-living (e.g. cyanobacteria). The reasons for this are not immediately obvious because the O_2 sensitivity of nitrogenase mandates that a very low concentration of free O_2 be maintained in the vicinity of the active enzyme. Nonetheless, several processes generate reactive oxygen species (ROS) in N_2-fixing systems. ROS include the superoxide radicals and H_2O_2, which are produced by the high rates of respiration required to support N_2 fixation, the autoxidation of the oxygenated form of leghemoglobin (Lb), and the oxidation of several proteins with strong reducing potential (e.g. nitrogenase, ferredoxin, and hydrogenase). Antioxidants in nodules include a host of enzymes and metabolites that function to eliminate ROS, generally by reducing them to less harmful forms and, in some cases, to water. However, when present at low, tightly-controlled concentrations, ROS also perform useful functions in plant and nodule development and in stress perception and signaling (*section XII*). Consequently, antioxidants not only prevent cellular damage ('oxidative stress'), but permit a fine tuning of ROS levels to optimize their functions in metabolism. Most of the antioxidants in legume nodules are also present in other plant organs or tissues, but the levels in nodules are generally higher, which suggests an important connection between N_2 fixation and antioxidants.

Reactive nitrogen species (RNS), such as nitric oxide (NO) and peroxynitrite, are also formed in nodules and other plant organs. However, much less is known about the *in vivo* sources of RNS compared with ROS. In fact, NO formation has been detected in the infected cells of functional nodules (Baudouin *et al.,* 2006), but the origin is unclear. In plants, there are many potential sources of NO, both enzymatic such as nitrate reductase and a NO synthase-like activity that still awaits identification, and nonenzymatic, such as the reduction of nitrite by ascorbate at acid pH (for a review see del Río *et al.,* 2004). As occurs for ROS, uncontrolled formation of RNS is potentially toxic and may cause cellular damage ('nitrosative stress'), but low levels of RNS, especially of NO, are critical in multiple processes of plants. These include, to name a few, seed germination, stomatal closure, root growth, nodule formation, and stress responses.

Antioxidants can modulate RNS levels avoiding nitrosative stress while allowing RNS to function in plant development, metabolism, and signaling.

Oxidative challenges and defenses have been reviewed comprehensively elsewhere for both nodules (e.g. Matamoros *et al.,* 2003; Puppo *et al.,* 2005) and plants in general (Dalton, 1995; Noctor & Foyer, 1998; Mittler, 2002; Mittler *et al.,* 2004). Readers are referred to those reviews for detailed background information. Here, we recapitulate briefly what is known on antioxidants in nodules and then provide an analysis of recent developments on dual nature of ROS/RNS, namely, as potentially toxic and essential signaling molecules. Although rhizobia produce their own antioxidants that clearly contribute to nodule function (e.g. Muglia *et al.,* 2008), the emphasis of this review is on the antioxidants of plant origin.

II. Ascorbate

Vitamin C (ascorbate) is an ubiquitous and abundant metabolite in plants. Ascorbate is present at concentrations of 1-2 mM in nodules (Dalton *et al*., 1986), 5-25 mM in leaves, and 25-50 mM in chloroplasts (Smirnoff, 2000), which are consistent with its multiple and essential functions. The steady-state concentrations of ascorbate are tightly controlled at many levels, including synthesis, degradation, transport, regeneration, and compartmentation. Ascorbate is a potent water-soluble antioxidant, acting both as a direct ROS scavenger and as a metabolite of the ascorbate-glutathione (GSH) pathway for H_2O_2 detoxification (*section IV*), but it is also a cosubstrate of several dioxygenases involved in proline hydroxylation and in flavonoid and hormone biosynthesis (for a review see Arrigoni & De Tullio, 2002). Furthermore, the ascorbate redox state, defined as the ratio of reduced to total ascorbate (ascorbate + dehydroascorbate), affects the progression of the cell cycle (Potters *et al.,* 2000) and is critical in the perception of stressful conditions in the apoplast (see below). The essentiality of ascorbate in plants is also supported by the absence of known mutants that are completely deficient in ascorbate synthesis (De Tullio & Arrigoni, 2004).

The delay in our understanding of ascorbate physiology was largely due to the difficulties in elucidating its biosynthetic pathway. A brief overview of the D-mannose/L-galactose (or Smirnoff-Wheeler) pathway is presented here as context

for the nodule-related issues to follow (Table 1). Detailed reviews are available elsewhere (Linster & Clarke, 2008; Ishikawa *et al*., 2006). Conversion of mannose-1-P to ascorbate is a six-step process that is apparently present in virtually all plant cells (De Tullio & Arrigoni, 2004). Mannose-1-P is readily available *via* a one-step isomerization of fructose-6-P from glycolysis. A critical breakthrough was made possible by the identification of ascorbate-deficient mutants of *Arabidopsis thaliana* (Conklin *et al*., 2000). These mutants were named '*vtc*' (for vitamin C) and the underlying gene functions gradually identified over the next decade. *VTC2*, which codes for a GDP-L-galactose phosphorylase, was the last of the *VTC* genes to be assigned a function (Laing *et al*., 2007; Linster *et al*., 2007; Table 1). Elucidation of the complete D-mannose/L-galactose pathway has opened the exciting prospect of using metabolic engineering to increase ascorbate production. This could have tremendous benefits in terms of enhanced stress tolerance in plants, nutritional value to humans and livestock, and even capacity for N_2 fixation.

Ascorbate may have also regulatory roles in nodules as a major contributor to the redox state of cells. Recently, Groten *et al.* (2005) hypothesized that pea (*Pisum sativum*) nodules are unable to synthesize ascorbate and have to import it from the shoot or root through the vascular system. They also observed that the leaves, and to a lesser extent the roots, accumulated ascorbate when supplied with galactose as a precursor. The capacity to accumulate ascorbate was retained in young nodules but was lost during development. This finding could imply that the plant might regulate key aspects of nodule metabolism through the transport of ascorbate from the shoot to the nodules (Groten *et al.*, 2005; Puppo *et al.*, 2005). However, GalLDH activity was subsequently found in mitochondrial membranes of bean (*Phaseolus vulgaris*) nodules (Matamoros *et al.*, 2006), as previously reported in other plant systems (Siendones *et al.*, 1999; Bartoli *et al.*, 2000). The expression of five genes of the Smirnoff-Wheeler pathway was likewise detected in *Lotus japonicus* and bean nodules (Colebatch *et al.*, 2002; Matamoros *et al.*, 2006; Loscos *et al.*, 2008), lending further support to the functionality of the ascorbate biosynthetic pathway in nodules. Recently, the transcript of GalLDH was localized in nodules of *L. japonicus* and alfalfa (*Medicago sativa*) by *in situ* RNA hybridization. High GalLDH expression (mRNA and activity) and ascorbate concentration were detected in the infected zone of both types of nodules

(Matamoros *et al.*, 2006). However, many key questions still remain to be solved. The characterization of genes and enzymes will be essential to understand how ascorbate synthesis is regulated in legumes. Further studies are also necessary to ascertain the functionality in nodules of alternative pathways of ascorbate biosynthesis, such as those described in animals or in ripening strawberry (*Fragaria x ananassa*) fruit (Valpuesta & Botella, 2004). These pathways involve the enzymes L-gulono-1,4-lactone dehydrogenase and D-galacturonate reductase, respectively, but their relative importance is uncertain because mutants affected in the corresponding genes are yet to be isolated. However, we could not detect D-galacturonate reductase protein in legume extracts using a polyclonal antibody raised against the strawberry enzyme.

The concentrations of ascorbate in cells are also regulated by the rates of oxidation and degradation. Many physiological roles of ascorbate imply its oxidation to monodehydroascorbate or dehydroascorbate. This occurs during peroxide removal by the ascorbate-GSH pathway in the cytosol, chloroplasts, and some other organelles (*section IV*), but also in the apoplast as a result of ascorbate oxidase (AO) activity, which catalyzes the oxidation of ascorbate to monodehydroascorbate. In the apoplast, ascorbate is present at millimolar concentrations (up to 10% of total ascorbate in leaf cells is in the apoplast) and AO activity controls the ascorbate redox state in such a way that this compartment becomes essential in the defense and stress response of plants to abiotic and biotic stresses (Pignocchi & Foyer, 2003). Several functions have been attributed to this enigmatic enzyme in plants because AO activity is regulated at multiple levels and is responsive to environmental and developmental cues (Pignocchi *et al.,* 2003; Pignocchi & Foyer, 2003). Nevertheless, the information on AO in plants in general and in nodules in particular is scant. Interestingly, treatment of bean plants with jasmonic acid, a well-known stress-related compound, caused transcriptional activation of AO and posttranslational inhibition of dehydroascorbate reductase (DR) in nodules (Loscos *et al.,* 2008). These authors proposed that the combination of the two effects would increase apoplast oxidation and that this may trigger a signal by which nodules perceive and respond to stress situations.

Ascorbate is usually regenerated from its oxidation products by monodehydroascorbate reductase (MR) and DR, which are present in several cellular compartments (*section IV*). However, dehydroascorbate is unstable and

can be further oxidized and hydrolyzed to many compounds, including oxalic, tartaric, and threonic acids (Hancock & Viola, 2005) if it is not rapidly reduced back to ascorbate by DR. The route for ascorbate degradation in plants is poorly known and therefore biochemical studies on the enzymes and metabolites involved are urgently needed, especially in leaves and nodules, which show high concentrations and rapid turnover of ascorbate.

III. Thiols

The thiol tripeptide GSH (γGlu-Cys-Gly) is a major water-soluble antioxidant and redox buffer in plants, performing critical functions in cell cycle regulation, development, sulfur transport and storage, stress responses, and heavy metal detoxification (Maughan & Foyer, 2006). In legumes, homoglutathione (hGSH; γGlu-Cys-βAla) may partially or completely replace GSH (Frendo *et al.,* 2001; Matamoros *et al.,* 2003).

The synthesis of GSH is accomplished in two sequential ATP-dependent reactions catalyzed by γ-glutamylcysteine synthetase (γECS) and glutathione synthetase (GSHS), whereas the synthesis of hGSH shares the same first enzyme and then requires a specific homoglutathione synthetase (hGSHS). The biochemical properties of the three thiol synthetases have been examined in several plants, but little is known about the regulation of the thiol biosynthetic pathway in legume roots and nodules. Interestingly, the *hGSHS* gene shows high sequence identity with the *GSHS* gene and probably derived from it by tandem duplication, at least in *Medicago truncatula* (Frendo *et al.,* 2001) and *L. japonicus* (Matamoros *et al.,* 2003). Despite this close relationship, the expression of the *GSHS* and *hGSHS* genes is strongly dependent on the legume species and tissue, and the two genes are also differentially regulated in response to signaling compounds or stress conditions. For example, in *M. truncatula*, hGSHS can be detected in the roots and nodules and GSHS throughout the plant (Frendo *et al.,* 1999), whereas in *L. japonicus* GSHS can be detected only in the nodules and hGSHS also in leaves and roots (Matamoros *et al.,* 2003). Moreover, in roots of *M. truncatula*, the expression of the *γECS* and *GSHS* genes, but not of the *hGSHS* gene, is induced by NO (Innocenti *et al.,* 2007). Bean plants treated with H_2O_2 showed upregulation of the *γECS* and *hGSHS* genes in nodules, whereas

treatments with Cd, NaCl, or jasmonic acid had no effect (Loscos *et al.,* 2008). These observations suggest the presence of gene-specific *cis*-regulatory elements in the *GSHS* and *hGSHS* promoters and/or additional distinct regulatory mechanisms for the two genes, but, most importantly, provide strong support for a different role of GSH and hGSH in nodules. Recent studies in *M. truncatula* using the γECS inhibitor buthionine sulfoximine or antisense constructs of GSHS and hGSHS have shown that GSH and/or hGSH play essential roles in nodulation; furthermore, the inhibition of nodule formation correlated with a decrease in the number of lateral roots, suggesting that thiol deficiency impairs meristem formation (Frendo *et al.,* 2005). We propose that GSH rather than hGSH is specifically required to promote meristematic activity in nodules, based on this study and on several lines of indirect evidence: GSH is required for cell division in root tips (Vernoux *et al.,* 2000); and GSH (and not hGSH) concentration is especially high in zones I+II of indeterminate nodules (Matamoros *et al.,* 1999). Further research is clearly needed to identify the specific functions of GSH and hGSH in the development and stress responses of nodules.

IV. Ascorbate-glutathione pathway

The ascorbate-GSH or Halliwell-Asada pathway (Fig. 1) involves the participation of the enzymes ascorbate peroxidase (Apx), MR, DR, and glutathione reductase (GR) in a coupled series of reactions that scavenge H_2O_2 by relying ultimately on the reducing power of NAD(P)H (Noctor & Foyer, 1998; Mittler *et al*., 2004). Isoforms of the four enzymes have been found in several cell compartments, including the cytosol, plastids, mitochondria, and peroxisomes, and therefore it is generally believed that the pathway is operative at multiple cellular sites. The genes of the ascorbate-GSH pathway are expressed at high levels in nodules, as well as in other tissues (Fig. 2).

The ascorbate-GSH pathway provides one of the chief antioxidant mechanisms in plants in general and was first described in nodules more than twenty years ago (Dalton *et al*., 1986). Since then, much evidence has amassed demonstrating its importance for N_2 fixation and the symbiotic association in general. For example, there is a close positive correlation between nodule effectiveness and the enzyme activities of the pathway (Dalton *et al.,* 1993), and

numerous parameters associated with N_2 fixation and antioxidants in nodules are increased in response to an increase in the nodule ascorbate content (Table 2). Because most of this evidence goes back 5-15 years, readers are referred to an earlier review for a comprehensive discussion (Matamoros *et al*., 2003). The pathway is certainly a very major factor in the antioxidant defenses in nodules but is not emphasized here because of space limitations and the goal of focusing on more emerging topics.

V. Superoxide dismutases and catalases

Superoxide dismutases (SODs) are metalloenzymes that catalyze the dismutation of superoxide radicals to H_2O_2 and O_2. They are classified in three groups based on their metal cofactors: CuZnSOD, FeSOD, and MnSOD. The three classes of enzymes occur in nodules, albeit at different subcellular locations: CuZnSOD in the cytosol, plastids, and (possibly) in the periplasmic space of bacteroids; FeSOD in the cytosol, plastids, and some bacteroids; and MnSOD in bacteroids, mitochondria, and (possibly) peroxisomes. Microarray data of *M. truncatula* nodules indicate that there is high expression of three CuZnSODs and mitochondrial MnSOD, with somewhat lower expression of the plastidial (FeSODp; TC148846) and cytosolic (FeSODc; TC148518) FeSOD isoforms (Fig. 2).

The transcripts and proteins of some SOD isoforms have been localized and their expression patterns examined in indeterminate and determinate nodules. In the indeterminate nodules of alfalfa, the expression of mitochondrial MnSOD is highest in the infected zone, whereas that of cytosolic CuZnSOD (CuZnSODc) was particularly abundant in the meristem and invasion zones, suggesting distinct roles of the enzymes during nodule development (Rubio *et al.,* 2004). In particular, colocalization of H_2O_2 and studies with inhibitors of CuZnSOD activity supported a role of CuZnSOD in providing H_2O_2 for cross-linking of highly-glycosylated glycoproteins (extensins) in the extracellular matrix and in the lumen of infection threads, which is required for cell wall growth and progression of infection threads (Wisniewski *et al.,* 2000). In determinate nodules of *L. japonicus*, the expression of four *SOD* genes, encoding CuZnSODc, MnSOD, FeSODp, and FeSODc, was investigated (Rubio *et al.,* 2007). The *CuZnSODc* and

MnSOD genes were found to be down-regulated during nodule development, whereas *FeSODc* was induced and *FeSODp* transcription was not affected. It was proposed that CuZnSODc and FeSODc may functionally compensate each other at the late stages of nodule development. The induction of FeSODc suggests a higher availability of Fe in old nodules, probably as a result of Lb degradation. In a recent study, enhanced levels of CuZnSOD, MnSOD, and superoxide production were found in the vascular bundle cells of *Sesbania rostrata* stem and root nodules, suggesting that these antioxidant enzymes participate in coping with superoxide radicals produced by mitochondrial respiration in these metabolically active cells (Rubio *et al.,* 2009).

Catalases have been studied extensively in many plants where the different isoforms and genes have been characterized (see review by Scandalios *et al.,* 1997). They are tetrameric hemeproteins (240 kDa) that catalyze the decomposition of H_2O_2 to O_2 and water, and are mainly localized in peroxisomes and glyoxysomes. Because of the low affinity of catalases for H_2O_2 (K_m in the molar range) compared with Apx (K_m in the micromolar range), it is believed that they are only efficient at high levels of H_2O_2 and are essential for maintaining the redox balance during oxidative stress (Willekens *et al.*, 1997). In white lupin (*Lupinus albus*) nodules, catalase has been immunolocalized in the peroxisomes of infected cells and found to decrease during senescence induced by nitrate (Lorenzo *et al.,* 1990). It is surprising that since this pioneering study almost no progress has been made in our understanding of catalases from nodule host cells. Instead, the regulation of the catalase genes of bacteroids has been examined in detail (*section X*).

VI. Thiol peroxidases: peroxiredoxins and glutathione peroxidases

Thiol peroxidases include two groups of closely related enzymes, Prxs and glutathione peroxidases (Gpxs), that are widespread in many organisms. Both peroxiredoxins (Prxs) and Gpxs are small proteins (17-24 kDa) that lack heme and hence rely on external electron donors for catalytic activity. They are encoded by multigene families and the corresponding isoforms are located at multiple subcellular locations, including the cytosol, plastids, and mitochondria. Although

expression levels for these classes of genes are generally higher in leaves than in nodules, the levels in nodules are still considerable (Fig. 2).

Prxs catalyze the reduction of H_2O_2 or alkyl hydroperoxides (ROOH) to water or the corresponding alcohols (ROH), respectively, using preferentially thioredoxin (Trx) as electron donor:

$$ROOH + Trx\text{-}(SH)_2 \rightarrow ROH + Trx\text{-}S_2 + H_2O$$

In plants there are four classes of Prxs, designated as 1C-Prx, 2C-Prx, PrxQ, and PrxII, based on the number of catalytic cysteine residues and amino acid sequences (Dietz, 2003). Essentially, the reaction of the Prxs containing two catalytic cysteines (2C-Prx, PrxQ, and PrxII) is as follows. A sulfhydryl group is oxidized by the peroxide to sulfenic acid, then a second sulfhydryl group attacks the sulfenic acid group forming a disulfide bridge, and finally this is reduced again to thiol groups by Trxs or alternative thiol active proteins such as glutaredoxin (Grx) or cyclophilin. Although well studied in *Arabidopsis*, Prxs have only recently been described in N_2-fixing nodules (Groten *et al.,* 2006). Pea (*Pisum sativum*) nodules contain cytosolic PrxII and mitochondrial PrxIIF. The levels of cytosolic PrxII increased with application of exogenous ascorbate in 9-week-old nodules, but those of PrxIIF remained unaffected (Groten *et al.,* 2006). Studies by these authors and in our laboratory failed to detect significant protein levels of the plastidic (2C-Prx, PrxQ) or nuclear (1C-Prx) isoforms in legume nodules.

The reaction catalyzed by Gpxs is usually described in the same way as that of Prxs but with GSH instead of a thiol protein as the reductant of peroxides. However, recent studies have shown that Gpxs use Trxs more efficiently, and in some cases exclusively, as electron donors (Herbette *et al.,* 2002). Consequently, Gpxs are more appropriately designated 'Trx peroxidases' (Rouhier & Jacquot, 2005) and are considered a fifth class of Prxs (Navrot *et al.,* 2006). A difference between Gpxs and Prxs is that some Gpxs are able to reduce fatty acid and lipid hydroperoxides (but not H_2O_2) using GSH (Herbette *et al.,* 2002), and this function is relevant *in vivo* because these enzymes protect membrane lipids from ROS-induced peroxidation.

Phylogenetic analysis of Gpxs has revealed that there are five distinct classes in vascular plants. Each of these classes is present in *L. japonicus*, the only N_2-

fixing plant that has been examined in this regard (Ramos *et al.,* 2009). Two genes, *LjGpx3* and *LjGpx6*, which putatively encode proteins located in the cytosol or secretory pathway and in the plastids, respectively, are highly expressed in nodules. One of such genes, *LjGpx6,* was highly induced by treatment of plants with the NO-releasing compound sodium nitroprusside, suggesting that NO can modulate the function of Gpxs and that these enzymes may be, in turn, mediating the effects of NO in metabolic signaling pathways. Surprisingly, immunogold studies showed that at least some Gpx isoforms are associated primarily with chloroplasts, proplastids, or amyloplasts in leaves, roots, or nodules. Furthermore, the enzyme was found to be associated to starch grains (Ramos *et al.,* 2009), a localization consistent with that reported previously for certain Trx and Prx isoforms (Balmer *et al.,* 2006; Barajas-López *et al.,* 2007). Because Trxs are substrates of both Gpxs and Prxs, the finding of Gpx associated to starch grains in amyloplasts suggests that H_2O_2 or other peroxides are formed during starch metabolism and that Gpxs may act not only as peroxide scavengers but also as ROS sensing molecules. In fact, a dual role of the *Arabidopsis* Gpx3 isoform, as a general ROS scavenger and specifically as an oxidative transducer in abscisic acid and drought stress signaling, has been recently demonstrated (Miao *et al.,* 2006).

VII. Protein disulfide reductases: thioredoxins and glutaredoxins

Thioredoxins (Trxs) are a family of ubiquitous small proteins (12-14 kDa) involved in redox regulation (Meyer *et al.,* 2005). They contain a conserved reactive site (Trp-Cys-Gly-Pro-Cys) which is able to reduce disulfide bridges in target proteins. After oxidation of the thiol groups, chloroplastic and cytosolic Trxs are regenerated by ferredoxin-Trx reductase and NADPH-Trx reductase, respectively. In plants, Trxs are classified in six classes according to their sequences and localizations in the chloroplasts (*m, f, x, y*), cytosol or phloem sap (*h*), and mitochondria (*o*).

The antioxidant roles of Trxs may be largely indirect as they primarily function through redox regulation of other proteins. The antioxidant properties of Trxs are not clearly understood, but it is likely that Trxs can repair other proteins that have been damaged by ROS (Vieira Dos Santos & Rey, 2006). Some of the strongest evidence supporting an antioxidant role for Trx is that transformation of

a Trx-deficient mutant of yeast with the soybean *Trx* gene confers tolerance to exogenous H_2O_2 (Lee *et al*., 2005). This gene appears to be required for nodulation in soybean as RNAi repression led to severely impaired nodule development, and its expression in nodules increases during nodule formation and is at its highest in the central infected zone of mature nodules (Lee *et al*., 2005). Furthermore, two novel Trx isoforms have been found in *M. truncatula* and were designated as 's' for 'symbiosis', as they function specifically in symbiotic interactions (Alkhalfioui *et al.,* 2008). Collectively, these observations indicate that Trx is an antioxidant that is essential for proper nodule development and function.

Glutaredoxins (Grxs) are also small proteins closely related to Trxs which are encoded by multigene families. They are present in the same tissue and cellular locations as Trxs, including the phloem sap, reinforcing the view that they have overlapping functions (Meyer *et al.,* 1999). Grxs participate in oxidative stress protection in several ways. They directly reduce peroxides and regenerate ascorbate from dehydroascorbate, act as electron donors for some Prxs, and are involved in the protection of thiol groups through glutathionylation/ deglutathionylation reactions. Therefore, Grxs may have an important role as redox regulators in plant tissues (Rouhier *et al.,* 2006). However, virtually nothing is known about the presence of Grxs in nodules.

VIII. Glutathione *S*-transferases

Glutathione *S*-transferases (GSTs) are ubiquitous enzymes best known for their role in detoxifying xenobiotics, especially herbicides such as atrazine. They accomplish this by conjugating the target molecules to (h)GSH, thus facilitating their metabolism, sequestration, or removal (Edwards *et al.,* 2000). However, GSTs may also act as antioxidants by at least two mechanisms. First, GSTs may act as a Gpx to directly scavenge peroxides. Second, lipid peroxidation end products such as alkenals, 4-hydroxynonenal, and other α,β-unsaturated aldehydes may be conjugated to GSH and targeted for removal (Edwards *et al*., 2000; Dalton *et al*., 2009).

GSTs constitute a large gene family in plants, with 25 members in soybean and 42 in maize (McGonigle *et al*., 2000). The importance of GSTs in N_2-fixing

nodules is indicated by the observation that soybean nodules contain at least 14 isoforms of GSTs with variable, though substantial, levels of expression (Dalton *et al.,* 2009). Thus, the mRNA level of the most prevalent isoform, GST9, was as much as 60% of that of Apx, one of the most abundant proteins in nodules. Down-regulation by RNAi technology of GST9 results in substantial decreases in nitrogenase (acetylene reduction) activity. The GST-suppressed nodules also showed increased oxidative damage of proteins. Furthermore, there was a marked organ specificity for GSTs in soybean as the relative abundance of isoforms is different in nodules compared to uninfected roots or leaves (Dalton *et al.,* 2009).

Elucidation of the role of GSTs in nodules is complicated not only by the abundance of different isoforms, but also by the fact that the normal target molecules have not been specifically identified and are likely to be equally diverse. Another factor to consider is that the host cells of nodules of some legume species, such soybean and bean, contain hGSH, which partially or completely replaces GSH, thus adding a further level of complexity. For instance, for some substrates, hGSH is conjugated more readily than GSH (McGonigle *et al*., 1998).

IX. Antioxidants and metal sequestration

Metal homeostasis is central in nodules because they contain abundant metalloproteins essential for N_2 fixation or ROS protection. However, the Fe or Cu of their prosthetic groups or cofactors can be released by proteases during senescence or under stress conditions. These metals are potentially prooxidants at trace ('catalytic') amounts, giving rise to highly oxidizing hydroxyl radicals ($^{\cdot}OH$) according to Fenton chemistry:

$$Fe^{2+}/Cu^{+} + H_2O_2 \rightarrow Fe^{3+}/Cu^{2+} + OH^{-} + {}^{\cdot}OH$$

Therefore, a strict control of the intracellular concentrations of Fe and Cu is critical to avoid oxidative damage (Halliwell & Gutteridge, 2007). Protection against metal-promoted toxicity is largely based on mechanisms to remove metals by sequestration into storage proteins or by chelation to specific polypeptides or metabolites. Both mechanisms operate in nodules and will be briefly described below.

Ferritin is a spherical protein complex of 24 subunits, capable of concentrating and storing up to 4500 atoms of Fe in the form of hydrated ferric oxide in a large central compartment (reviewed by Liu & Thiel, 2005). Not only is such stored Fe Fenton inactive, but the creation of the oxide also results in removal of O_2, further enhancing the antioxidant properties. Ferritin plays a critical role in nodules because of the high Fe requirement and the associated high risk of oxidative damage. Ferritin is localized primarily in the plastids and amyloplasts of nodules, as well as in the bacteroids, and is associated with effectiveness (Ko *et al.,* 1987; Lucas *et al.,* 1998). Ferritin mRNA and protein increase markedly early in maturation of soybean nodules, at the same stage that Lb synthesis starts (Ragland & Theil, 1993). Immunolabeling studies showed that ferritin decreased in the infected cells of senescing soybean and white lupin nodules and was also lower in the senescent zone of alfalfa nodules (Lucas *et al.,* 1998). Subsequent studies have shown that, in mature nodules of yellow lupin (*Lupinus luteus*), ferritin polypeptides accumulate in a layer of cells between the meristem and the bacteroid tissue, which is reminiscent of the interzone II/III of typical indeterminate nodules (Strozycki *et al.,* 2007). These authors also found that ferritin expression is correlated with development of yellow lupin nodules, suggesting that this protein is transcriptionally regulated and takes part of a mechanism by which nodule function is prolonged in indeterminate nodules (Strozycki *et al.,* 2007).

Another protective mechanism against metal toxicity is chelation by cysteine-rich polypeptides or proteins, which include two major groups: phytochelatins (PCs) and metallothioneins (MTs). Both of them are present in nodules. PCs have a general structure $(\gamma\text{Glu-Cys})_{2\text{-}11}$-Gly and are synthesized from GSH by phytochelatin synthase (Cobbett & Goldsbrough, 2002) according to the reaction:

$$\gamma\text{Glu-Cys-Gly} + (\gamma\text{Glu-Cys})_{n\text{-}1}\text{-Gly} \rightarrow (\gamma\text{Glu-Cys})_{n}\text{-Gly} + \text{Gly}$$

In some legumes hGSH can replace GSH, producing homophytochelatins (hPCs) of general structure $(\gamma\text{Glu-Cys})_{2\text{-}11}$-βAla (Grill *et al.,* 1986; Klapheck *et al.,* 1995). Both types of polypeptides, PCs and hPCs, are able to chelate certain metals (Cu, Zn, Cd, Hg, Pb) and metalloids (As). The resulting complexes are transported into the vacuoles, avoiding cellular toxicity. Exposure of *L. japonicus* plants to Cd

caused accumulation of PCs and hPCs in roots and nodules (Ramos *et al.,* 2007). This and a follow-up study (Ramos *et al.,* 2008) revealed that *L. japonicus* contain three functional *phytochelatin synthase* genes that are differentially regulated in response to metals and have different abundance in roots and nodules. However, phytochelatin synthases also fulfill other functions. In *Arabidopsis*, PC synthesis is required for the degradation of GS-conjugates (Blum *et al.,* 2007) and for the homeostasis of Zn (Tennstedt *et al.,* 2009). Besides the role of PCs in avoiding the prooxidant effects of metals, the high thiol content of these polypeptides suggests that they may interact with ROS/RNS levels and, in fact, nitrosylated-PCs have been recently detected *in vivo* (De Michele *et al.,* 2009). The possibility that these PC nitrosothiols modulate NO levels awaits detailed investigation.

Plants also contain small proteins (1-2 kDa), called MTs, that chelate metals and protect cells against oxidative stress. MTs are encoded by large families of closely-related genes and this complexity has precluded in-depth studies of their function (Cobbett & Goldsbrough, 2002). In yeast and mammals, MTs are involved in the homeostasis of essential metals (Cu, Zn) and the detoxification of heavy metals (Cd, Hg). There are also evidences supporting a role of MTs in Cu homeostasis and tolerance in plants. However, an additional feature of MTs is their ability to efficiently scavenge ROS, including superoxide and hydroxyl radicals (Kumari *et al*., 1998; Wong *et al.,* 2004). In this respect, it is worth noting that Clement *et al*. (2008) identified two genes, encoding ferritin and MT, that were markedly up-regulated in soybean nodules in response to drought, a common cause of oxidative stress. The mRNA levels of both genes were particularly high in the infected cells. This finding, which was somewhat expected in the case of ferritin, also lends further indirect support to an antioxidant role of MTs in nodules by chelating potentially Fenton-active Cu, by directly scavenging ROS, or by both mechanisms.

Another important metal chelator in plants is nicotianamine. This compound has a high binding affinity for Fe^{2+} and forms complexes that are poor Fenton reagents, which supports a role of nicotianamine in protecting cells from oxidative damage (von Wirén *et al*., 1999). Nicotianamine is synthesized by nicotianamine synthase (NAS) in a one-step reaction with three molecules of *S*-adenosyl-L-methionine as the sole substrate. Very little is known about nicotianamine or NAS in nodules beyond the recent, single report that there are two forms of NAS in

nodules of *L. japonicus* (Hakoyama *et al*., 2009). One of these (*LjNAS2*) was specifically expressed in nodules, whereas the other form (*LjNAS1*) was expressed mainly in leaves, stems, and cotyledons. Expression of *LjNAS2* in nodules was highest 24 d after inoculation with rhizobia. A mutant deficient in *LjNAS2* formed ineffective nodules. Although an antioxidant role for nicotianamine is still plausible, the observation that *LjNAS2* mRNA was detected only in vascular bundles suggests that Fe transport may account for the observed phenotype of this mutant. In *M. truncatula*, at least one *NAS* gene and one *MT* gene are highly active in nodules, underscoring the importance of metal transport and homeostasis in this plant organ (Fig. 2).

X. Antioxidants of nodule bacteroids

The antioxidants discussed up to this point are all of plant origin, but bacteroids also contain metabolites (GSH) and enzymes (MnSOD, catalase, Prx, GR) that fulfill antioxidant roles and may be involved in redox regulation. These functions are beyond the scope of this review but a few examples deserve mention, especially to the extent that they influence plant responses or processes. Although nodule host cells make their own GSH, some of this critical antioxidant needs to be produced by the bacterial partner to achieve optimal N_2 fixation, as evidenced by the observation that rhizobia deficient in glutathione synthetase formed nodules with early senescence and diminished symbiotic performance (Harrison *et al.,* 2005; Muglia *et al.,* 2008). Catalase is another interesting example of how alterations of antioxidant enzymes of bacteroids can affect dramatically N_2 fixation. In *S. meliloti* there are three catalase genes encoding two monofunctional (KatA and KatC) and one bifunctional catalase-peroxidase (KatB) enzymes (Jamet *et al.,* 2003). KatA is inducible by H_2O_2 and is constitutively expressed in bacteria and bacteroids, whereas KatB are KatC are expressed in bacteria within the infection threads. The single *katA*$^-$ or *katC*$^-$ mutants nodulate normally, but the *katA*$^-$*katC*$^-$ or *katB*$^-$*katC*$^-$ double mutants produce nodules with a drastic reduction in N_2-fixing activity. The *katA*$^-$*katB*$^-$ double mutant is not viable. Therefore, the three catalases are required for symbiosis, although possibly at different stages of infection and nodule development (Sigaud *et al.,* 1999; Jamet *et al.,* 2003). However, this situation may be different in other rhizobia species such as *R. etli*,

in which only one catalase gene, *katG*, encoding a dual catalase-peroxidase, is detectable (Vargas *et al.,* 2003).

Another case in which bacteria antioxidants can be manipulated deserves attention. The overexpression of flavodoxin, an antioxidant that is not normally present in either rhizobia or plants, in the bacteroids delays senescence of *M. truncatula* nodules, using as markers the decline in N_2-fixing activity and the structural alteration of nodule components (Redondo *et al.,* 2009). In this case, flavodoxin may promote a favorable redox balance or perhaps even detoxify ROS. A follow-up study demonstrated that the flavodoxin-expressing bacteroids even ameliorated Cd-induced damage in alfalfa nodules (Shvaleva *et al*., 2010).

XI. Other molecules of nodules with antioxidative properties

Nodules contain other metabolites and enzymes that can destroy, or modulate, ROS/RNS levels, at least when assayed *in vitro*. However, the biological significance of these molecules *in vivo* requires further investigation. Two examples are uric acid, an abundant metabolite of nodules and an efficient scavenger of peroxynitrite, and liposoluble antioxidants such as tocopherols, ubiquinol, or flavonoids, which protect membrane fatty acids from peroxidation. None of these compounds have been studied in nodules in connection with ROS/RNS metabolism. More information is available, although still clearly insufficient, with respect to other molecules of nodules with antioxidative properties. We will briefly describe some of them because of their considerable interest for future studies. These metabolites or enzymes can be also considered as 'antioxidants' in broad terms due to their abilities to modulate ROS/RNS levels, and include polyamines, heme oxygenase, and hemoglobins (Hbs). Because of their important roles in signaling, Hbs will be described in the next section.

Polyamines are polycationic compounds widespread in many organisms and particularly in plants, where they play as yet poorly defined roles in developmental processes and stress responses (Bouchereau *et al.,* 1999). Legume nodules accumulate polyamines to levels that are five to ten fold higher than in the roots or leaves (Fujihara *et al.,* 1994). In nodules of *L. japonicus*, the expression of genes involved in the synthesis of spermidine, spermine, and putrescine is induced early in nodule development and declines with aging, whereas polyamines accumulate

steadily during nodule maturation, suggesting that they are involved in nodule cell division and expansion, but also in other functions related to N_2 fixation (Flemetakis *et al.,* 2004; Efrose *et al.,* 2008). Exogenous addition of polyamines delays senescence (Lahiri *et al.,* 1992) and this effect may be ascribed at least in part to their ROS scavenging properties (Bors *et al.,* 1989; Bouchereau *et al.,* 1999). In addition, polyamines can give rise to H_2O_2 as substrates of diamine and polyamine oxidases (see next section) and there is strong evidence that they are also precursors of NO (Yamasaki & Cohen, 2006), further suggesting an important role of these compounds in ROS/RNS metabolism.

Heme oxygenase catalyzes the breakdown of heme according to the following reaction:

$$\text{heme} + \text{NADPH} + H^+ + 3\ O_2 \rightarrow \text{biliverdin} + Fe^{3+} + \text{CO} + NADP^+ + H_2O$$

Although the release of free Fe3+ could result in prooxidant consequences, the process is considered to provide a substantial defense against ROS due to the antioxidant properties of biliverdin (Ryter & Tyrrell, 2000; Yannarelli *et al.,* 2006). Once heme oxygenase opens up the porphyrin ring, biliverdin is reduced to bilirubin by a NADPH-dependent biliverdin reductase. This produces bilirubin, an antioxidant that scavenges ROS with the concomitant regeneration of biliverdin. The reductase then functions to regenerate bilirubin in a continuing cycle that protects cells from up to a 10,000-fold excess of H_2O_2. The operation of this cycle in nodules is still speculative because only the first enzyme, heme oxygenase, has been reported. Expression of the *HO1* gene was enhanced in nodules in comparison to leaves and uninfected roots, and was highest in mature nodules (Baudouin *et al.,* 2004). In contrast to the situation in mammals, prooxidants such as H_2O_2 and paraquat did not induce expression, an observation that suggests that heme oxygenase is not involved in antioxidant protection in nodules. By contrast, more recent studies have shown that, under oxidative conditions induced by Cd (Balestrasse *et al.,* 2005) or salt stress (Zilli *et al.,* 2008), there is a marked increase in heme oxygenase expression (mRNA and protein) in nodules, providing credence to an antioxidative role. Furthermore, both UV irradiation and application of exogenous H_2O_2 caused oxidative damage and upregulation of heme oxygenase in soybean leaves (Yannarelli *et al.,* 2006). These treatments also

increased Apx and catalase activities, making it tempting to include heme oxygenase amongst the list of antioxidants. The second enzyme of the heme degradation pathway, biliverdin reductase, has been found in *Arabidopsis* (Gisk *et al.,* 2010) and thus it is expected to occur also in legume nodules. The fact that heme oxygenase is encoded by a small gene family, with four putative members in *Arabidopsis*, argues further that the reactions of heme degradation have an importance in plant physiology that has not previously been appreciated (Gisk *et al.,* 2010).

XII. Antioxidants and oxidative/nitrosative signaling

In plants and other organisms, antioxidants prevent the potentially deleterious effects of ROS ('oxidative stress') and RNS ('nitrosative stress'). However, these reactive molecules also perform critical functions at low controlled concentrations by acting in certain cellular locations, developmental stages, or stressful conditions. Antioxidants are able to modulate ROS/RNS concentrations and thereby are likely to affect signaling transduction cascades. This has led to the concept of 'oxidative signaling', which emphasizes the multiple useful roles of ROS in plants, especially in redox signaling (Foyer & Noctor, 2005). This concept is very appropriate in the light of several facts: some ROS are second messengers implicated in signaling pathways that are activated in the plants in response to developmental and environmental cues; ROS can modify gene expression in a ROS-specific manner; and the production of ROS is, in many cases, genetically programmed. This can be exemplified in the nodulation process. In the early stages of infection, superoxide radicals and H_2O_2 are produced by the root cells in response to rhizobia, which suggests that the symbiotic bacteria are initially perceived as invaders (Santos *et al.,* 2001). Furthermore, H_2O_2 accumulation has been detected in the invasion zone of alfalfa and pea nodules, in association with infection threads (Santos *et al.,* 2001; Rubio *et al.,* 2004). This H_2O_2 is required for inter- and intra-molecular cross-linking of extensins (*section V*) and may be produced by CuZnSOD activity (Rubio *et al.,* 2004), diamine oxidase activity using putrescine as a substrate (Wisniewski *et al.,* 2000), and/or a germin-like protein with SOD activity (Gucciardo *et al.,* 2007). The concentration of H_2O_2 in the infection threads may be also modulated by the catalase activity of bacteroids,

as shown by experiments with *S. meliloti* mutants overexpressing KatB (Jamet *et al.,* 2007). Collectively, these data indicate that controlled ROS production is essential for the onset of symbiosis. However, how the plant's defense response is suppressed is not completely clear. Rhizobial mutant strains defective in exopolysaccharides, lipopolysaccharides, or cyclic β−glucans are unable to infect root cells and activate defense reactions, which is strong evidence for a signaling role of these complex carbohydrates during the symbiotic interaction (see review by Mithöfer, 2002). Similar experiments with incompatible rhizobia or with *S. meliloti nodC*$^-$ mutants have shown that Nod factors are implicated in suppressing the plant's defense response (Bueno *et al.,* 2001). Also, application of compatible Nod factors to *M. truncatula* slowed the rate of H_2O_2 efflux from excised root segments (Shaw & Long, 2003), and similar studies in bean showed a transient increase of ROS, within seconds, at the tip of actively growing root hair cells (Cárdenas *et al.*, 2008).

Redox signaling can be also mediated by RNS, for example, *via* posttranslational modification of antioxidant proteins or transcription factors. Thus, RNS can cause nitrosylation (addition of a NO group) or nitration (addition of a NO_2 group) of cysteine or tyrosine residues, respectively. For example, a list of proteins having nitrated tyrosine residues in sunflower (*Helianthus annuus*) hypocotyls has been published very recently (Chaki *et al.,* 2009), but similar studies in nodules are lacking. In this context, it would then appear logical to extend the concept of 'oxidative signaling' to the participation of RNS ('nitrosative signaling') in signal transduction pathways.

An important case of modulation and signaling by NO and other RNS is closely related to the function of some Hbs. Three types are known and may coexist in plants: nonsymbiotic, symbiotic, and truncated Hbs. The first group is classified, in turn, into class 1 (with very high O_2 affinity) and class 2 Hbs (with lower O_2 affinity and a primary sequence more similar to those of symbiotic Hbs). Class 1 Hbs are expressed under hypoxia, cold, and osmotic stress, upon treatment with NO, and during rhizobial infection. In hypoxic conditions, these Hbs are part of a NO dioxygenase system, converting NO to nitrate. This system consumes NAD(P)H and mantains ATP levels, allowing plant survival (Igamberdiev & Hill, 2004). In *L. japonicus*, a class 1 Hb controls the plant's defense response during

the early stages of the rhizobial interaction, by modulating NO concentration, and overexpression of this protein enhances symbiotic N_2 fixation (Shimoda *et al.,* 2009). In contrast, very little is known about class 2 and truncated Hbs, albeit recent data suggest that at least some of them can also modulate NO levels and are expressed in nodules (Vieweg *et al.,* 2005).

Symbiotic Hbs include Lbs and Hbs from some actinorhizal plants. Besides the role of Lbs in facilitating O_2 diffusion to symbiosomes, these abundant proteins can form complexes with NO and thus modulate NO bioactivity. The nitrosyl complexes (LbNO) are very stable and can be detected in intact nodules by electron paramagnetic resonance (Mathieu *et al.,* 1998; Meakin *et al.,* 2007). The NO bound to Lb may have originated in the host cells (Baudouin *et al.,* 2006), in the bacteroids (Meakin *et al.,* 2007), or in both nodule compartments. It can be argued that the presence of LbNO, decreasing O_2 buffering in the cytoplasm, is potentially detrimental to nitrogenase. However, LbNO complexes are most abundant at the early stages of nodule development, which suggests a beneficial role of Lb as a NO reservoir or as part of a mechanism to detoxify RNS or prevent rejection of symbiotic rhizobia. This hypothesis is supported by the enhanced expression of Lb prior to active N_2 fixation. Recent *in vitro* experiments have demonstrated that ferrous Lb (in the oxygenated form) can scavenge NO and peroxynitrite, and also that these RNS can reduce ferryl-Lb, an inactive form produced by oxidation of Lb with H_2O_2 (Herold & Puppo, 2005). Taken together, these observations suggest that nonsymbiotic Hbs and Lbs are involved in metabolism, transport, and signaling by RNS.

Apart from their funtion in controlling ROS/RNS concentration, antioxidants themselves may act as signals, as can be illustrated with two examples. Studies with *Arabidopsis* mutants with ascorbate deficiency (*vtc1*) have shown that ascorbate influences plant growth and development by modulating expression of genes involved in defense and abscisic acid signaling (Pastori *et al.,* 2003). Another major case of a signaling function for antioxidants follows from studies with animal systems and point to Prxs as components of redox signaling cascades in plants. In *Arabidopsis,* nitrosylation of PrxIIE inhibits its capacity to detoxify peroxynitrite (Romero-Puertas *et al.,* 2007). This posttranslational modification of PrxIIE causes a dramatic increase in nitrotyrosine formation, modulating tyrosine kinase signaling pathways, and is biologically relevant. Although similar

information does not exist in legume nodules, the involvement of redox signaling by Prxs in the first steps of symbiosis and in nodule operation could be anticipated.

XIII. Antioxidants and oxidative/nitrosative stress

The findings mentioned above clearly illustrate that ROS/RNS are produced in plants, and particularly in nodules, with useful purposes, a major of which is redox signaling. Other studies also favor the concept of 'oxidative/nitrosative signaling'. For example, using proteomic analysis and detection with an antibody against nitrotyrosine, only 21 nitrated proteins were identified in sunflower hypocotyls (Chaki *et al.,* 2009), suggesting that nitration is specifically targeted in cells rather than an indiscriminate phenomenon. However, this term may not apply to all circumstances, especially in nodules at the later stages of senescence or under stressful conditions. Nodule natural senescence (aging) is a complex and programmed process, which shares some features with stress-induced senecence, such as a decrease of N_2-fixing activity and Lb content and an increase of proteolytic activity and ROS production. In aging soybean nodules, Evans *et al.* (1999) found an increase of ROS (mainly organic peroxides), catalytic Fe, oxidized homoglutathione, and oxidatively modified proteins and DNA bases, but no changes in ascorbate or tocopherol, concluding that these nodules were suffering from oxidative stress. Lipid peroxidation was also found to be elevated in nodules of pigeonpea (*Cajanus cajan*) and bean with advancing age (Swaraj *et al.,* 1995; Loscos *et al.,* 2008).

Similarly, in nodules of several legumes exposed to drought (Gogorcena *et al.,* 1995), nitrate (De Lorenzo *et al.,* 1994; Escuredo *et al.,* 1996), prolonged darkness (Gogorcena *et al.,* 1997; Hernández-Jiménez *et al.,* 2002), or prooxidants such as Cd or H_2O_2 (Loscos *et al.,* 2008), there was accumulation of lipid peroxides or oxidized proteins concomitantly with a decline in antioxidant protection. In some cases, an increase in hydroxyl radical production and catalytic Fe was detected (Becana & Klucas, 1992; Gogorcena *et al.,* 1995). These observations were also interpreted in terms of oxidative damage in nodules as a result of an increase in ROS production and/or decrease in antioxidant defenses. Recent work indicated that the application to pea roots of paraquat, a compound

that exacerbates formation of superoxide radicals, caused similar effects to those produced by drought (Marino *et al.,* 2006), lending indirect support to the participation of ROS in the deleterious consequences of stress on N_2 fixation. Indeed, drought induced the expression of several antioxidant genes and caused oxidative damage in alfalfa nodules (Naya *et al.,* 2007). However, results were different in nodulated plants exposed to salt stress. In soybean or bean nodules exposed to high salinity, no symptoms of oxidative stress could be found, although antioxidant enzyme activities were induced (Comba *et al.,* 1998; Loscos *et al.,* 2008). The upregulation of antioxidant enzymes, and particularly of SOD, was also seen in several other studies, suggesting that plants are perceiving an increase in ROS production and that antioxidants contribute to salt tolerance (Tejera *et al.*, 2004; Jebara *et al.,* 2005; Nandwal *et al.,* 2007). Therefore, the data described so far indicate that the plant's response, in terms of antioxidants and oxidative damage, is dependent on the type of stress and the legume species. The complexity of the interaction between the two symbiotic partners, probably differing in stress tolerance, and the structural and biochemical differences between indeterminate and determinate nodules, make it difficult, if not impossible, to establish a general model for stress-induced nodule senescence.

XIV. Conclusions and perspectives

Nitrogen-fixing nodules have a high potential for production of ROS/RNS and hence require powerful antioxidant protection. Our knowledge of the most prominent of these defenses, the ascorbate-GSH pathway, has matured considerably since its initial description nearly 30 years ago. Indeed, nodules may not function without it, a situation similar to that in chloroplasts, which are also sites of concentrated biochemical activity prone to generation of ROS/RNS. In recent years, the prospects for enhancing the activity of the ascorbate-GSH pathway, and concomitantly N_2 fixation, have been raised by advances in our understanding of the ascorbate biosynthetic pathway. The goal of increasing N_2 fixation has been touted for many years as a sort of holy grail that has been used to justify countless grants and research careers without much practical success. Such a goal may now be within reach, especially considering the numerous studies in

which metabolic engineering has been used to enhance the ascorbate content, and thus stress tolerance, of non-fixing plants (see Ishikawa *et al.,* 2006).

It may be useful to consider the various strategies of plants to protect against potentially toxic ROS/RNS concentrations while allowing them to perform essential functions in growth and metabolism. These strategies may be used at several levels that vary temporally and functionally. An initial, preventative strategy is to minimize ROS/RNS formation by restricting levels of 'catalytic' iron, O_2, or NO by binding to ferritin, Lbs, or class 1 Hbs, respectively. A second line of defense includes ROS scavenging by enzymes such as SODs, peroxidases, and catalases, which provide a more conventional class of antioxidant protection. Finally, a third defense is provided by GSTs, which remove the toxic by-products of ROS action. Control of ROS/RNS concentrations by antioxidant enzymes and metabolites will permit the plant cells, for instance, to utilize these reactive molecules as components of a redox transduction pathways in which appropriate responses are mediated through regulation of gene expression.

The use of genomic and proteomic technologies has greatly expanded the list of known antioxidants in plants and, in some cases, in nodules. The list now includes dozens of new entries, including multiple isoforms of the enzymes of the ascorbate-GSH pathway, SODs, GSTs, Trxs, and Gpxs. It is now necessary that progress will focus on more precisely defining the physiological roles of the various components, their interactions ('antioxidant network'), and their posttranslational modifications (nitrosylation, nitration, glutathionylation, and others), which may modulate their antioxidant activities and signaling functions *in vivo*. New roles are also emerging for numerous metabolites or proteins, which may be considered as 'antioxidants' in broad terms because they operate by metal sequestration or by modulating ROS/RNS bioactivity. It is also critical to ascertain the contributions and interactions of ROS/RNS in signal transduction pathways ('signaling network') associated with the onset or breakdown of the rhizobial symbiosis and with the response of nodules to stressful conditions. Finally, but probably most importantly, these networks need to be placed into a spatio-temporal context (nodule tissues and cells, gene and protein expression, metabolite distribution) from rhizobial root infection to nodule senescence.

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Figure legends

Figure 1. Generalized scheme showing processes for generation and removal of ROS/RNS in legume root nodules. Additional abbreviations to those appearing in the text: ASC, ascorbate; CAT, catalase; DHA, dehydroascorbate; GSSG, oxidized glutathione; MDHA, monodehydroascorbate; TF, transcription factor(s).

Fig. 2. Heat map showing microarray expression levels of genes related to antioxidant properties in the model legume *Medicago truncatula.* Color scale is based on a log2 transformed expression value obtained by RMA (Robust Multichip Average). Numbers after the tissue designation indicate days after inoculation with rhizobia, except that Nod16* also received a high nitrate treatment for 2 d at day no. 14. Details of methods and conditions can be found in Benedito et al. (2008) and The Medicago truncatula Gene Expression Atlas (http://mtgea.noble.org/v2/index.php).

Table 1 Enzymes involved in the biosynthesis of ascorbate in plants *via* the Smirnoff-Wheeler pathway

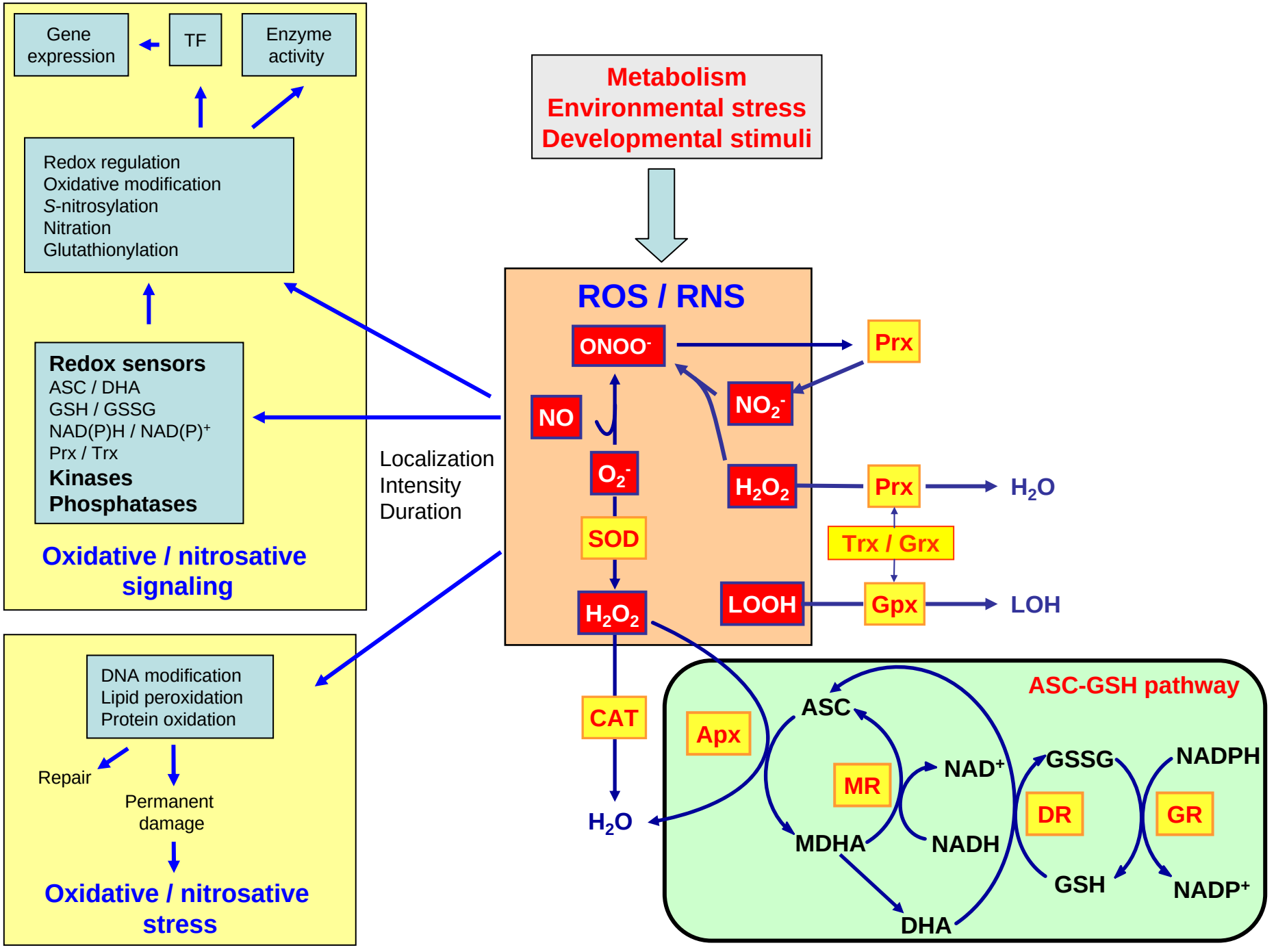
Step number	Substrates	Products	Enzyme	Abbreviation
1	D-fructose-6-P	D-mannose-6-P	phosphomannose isomerase	PMI
2	D-mannose-6-P	D-mannose-1-P	phosphomannose mutase	PMM
3	D-mannose-1-P, GTP	GDP-D-mannose, PP_i	GDP-D-mannose pyrophosphorylase	VTC1
4	GDP-D-mannose	GDP-L-galactose	GDP-D-mannose-3',5'-epimerase	GME
5	GDP-L-galactose, P_i	L-galactose-1-P, GDP	GDP-L-galactose phosphorylase	VTC2/VTC5
6	L-galactose-1-P	L-galactose, P_i	L-galactose-1-P phosphatase	VTC4
7	L-galactose, NAD^+	L-galactono-1,4-lactone, NADH	L-galactose dehydrogenase	GalDH
8	L-galactono-1,4-lactone*	L-ascorbate	L-galactono-1,4-lactone dehydrogenase	GalLDH

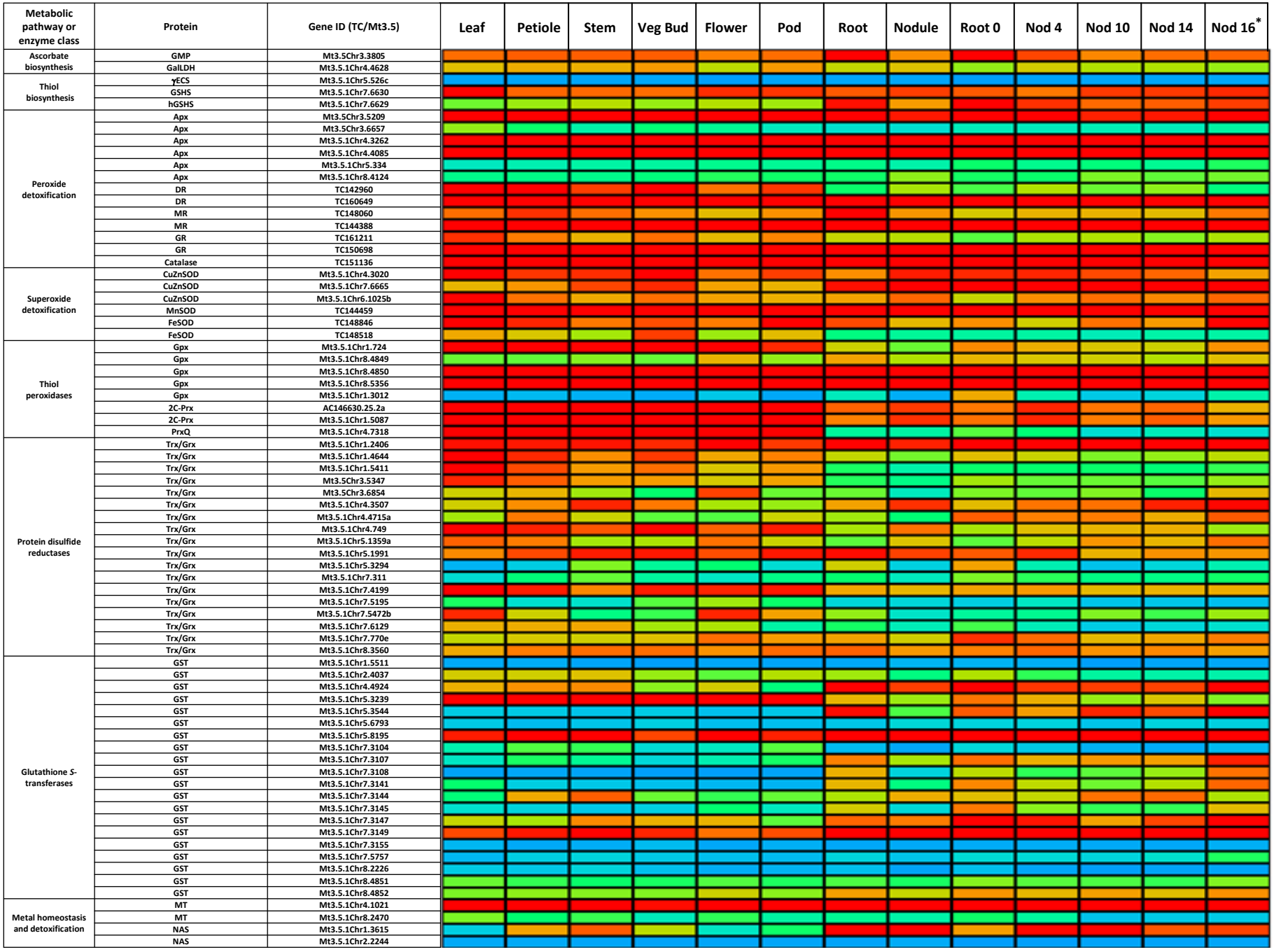
*Cytochrome *c* acts as an oxidant.

Table 2 Response of legume-rhizobia symbioses to enhanced ascorbate

Parameter	Maximum fold-change*		
	Stem infusion of intact plants	Exogenous application to intact plants or nodules	*In vitro* reconstitution system (including Apx)
Nitrogenase activity	4.1	2.3	5.0
Apx activity	1.5	1.9	nd†
Lipid peroxide content	0.7	nd	nd
Lb content	1.3	Increase‡	nd
Lb oxygenation	nd	nd	1.4
Nodule number	nd	2-3	nd
Nodule weight	nd	6-12	nd
Nodule N content	nd	3-5	
Plant total N content	nd	2	
PrxII (cytosolic) protein content	nd	Increase	

*Values taken from Chinoy (1985), Bashor & Dalton (1999), Ross *et al.* (1999), and Groten *et al.* (2006).
†Not determined.
‡No precise data are given.


Gene expression
TF
Enzyme activity
Redox regulation
Oxidative modification
S-nitrosylation
Nitration
Glutathionylation
Redox sensors
ASC / DHA
GSH / GSSG
NAD(P)H / NAD(P)+
Prx / Trx
Kinases
Phosphatases
Oxidative / nitrosative signaling
Metabolism
Environmental stress
Developmental stimuli
ROS / RNS
ONOO-
Prx
NO2-
NO
O2-
H2O2
Prx
H2O
SOD
Trx / Grx
LOOH
Gpx
LOH
H2O2
Localization
Intensity
Duration
CAT
H2O
ASC-GSH pathway
Apx
ASC
MR
NAD+
GSSG
NADPH
DR
GR
MDHA
NADH
GSH
NADP+
DHA
DNA modification
Lipid peroxidation
Protein oxidation
Repair
Permanent damage
Oxidative / nitrosative stress


Metabolic pathway or enzyme class
Protein
Gene ID (TC/Mt3.5)
Leaf
Petiole
Stem
Veg Bud
Flower
Pod
Root
Nodule
Root 0
Nod 4
Nod 10
Nod 14
Nod 16*
Ascorbate biosynthesis
GMP
Mt3.5Chr3.3805
GalLDH
Mt3.5.1Chr4.4628
Thiol biosynthesis
γECS
Mt3.5.1Chr5.526c
GSHS
Mt3.5.1Chr7.6630
hGSHS
Mt3.5.1Chr7.6629
Peroxide detoxification
Apx
Mt3.5Chr3.5209
Apx
Mt3.5Chr3.6657
Apx
Mt3.5.1Chr4.3262
Apx
Mt3.5.1Chr4.4085
Apx
Mt3.5.1Chr5.334
Apx
Mt3.5.1Chr8.4124
DR
TC142960
DR
TC160649
MR
TC148060
MR
TC144388
GR
TC161211
GR
TC150698
Catalase
TC151136
Superoxide detoxification
CuZnSOD
Mt3.5.1Chr4.3020
CuZnSOD
Mt3.5.1Chr7.6665
CuZnSOD
Mt3.5.1Chr6.1025b
MnSOD
TC144459
FeSOD
TC148846
FeSOD
TC148518
Thiol peroxidases
Gpx
Mt3.5.1Chr1.724
Gpx
Mt3.5.1Chr8.4849
Gpx
Mt3.5.1Chr8.4850
Gpx
Mt3.5.1Chr8.5356
Gpx
Mt3.5.1Chr1.3012
2C-Prx
AC146630.25.2a
2C-Prx
Mt3.5.1Chr1.5087
PrxQ
Mt3.5.1Chr4.7318
Protein disulfide reductases
Trx/Grx
Mt3.5.1Chr1.2406
Trx/Grx
Mt3.5.1Chr1.4644
Trx/Grx
Mt3.5.1Chr1.5411
Trx/Grx
Mt3.5Chr3.5347
Trx/Grx
Mt3.5Chr3.6854
Trx/Grx
Mt3.5.1Chr4.3507
Trx/Grx
Mt3.5.1Chr4.4715a
Trx/Grx
Mt3.5.1Chr4.749
Trx/Grx
Mt3.5.1Chr5.1359a
Trx/Grx
Mt3.5.1Chr5.1991
Trx/Grx
Mt3.5.1Chr5.3294
Trx/Grx
Mt3.5.1Chr7.311
Trx/Grx
Mt3.5.1Chr7.4199
Trx/Grx
Mt3.5.1Chr7.5195
Trx/Grx
Mt3.5.1Chr7.5472b
Trx/Grx
Mt3.5.1Chr7.6129
Trx/Grx
Mt3.5.1Chr7.770e
Trx/Grx
Mt3.5.1Chr8.3560
Glutathione S-transferases
GST
Mt3.5.1Chr1.5511
GST
Mt3.5.1Chr2.4037
GST
Mt3.5.1Chr4.4924
GST
Mt3.5.1Chr5.3239
GST
Mt3.5.1Chr5.3544
GST
Mt3.5.1Chr5.6793
GST
Mt3.5.1Chr5.8195
GST
Mt3.5.1Chr7.3104
GST
Mt3.5.1Chr7.3107
GST
Mt3.5.1Chr7.3108
GST
Mt3.5.1Chr7.3141
GST
Mt3.5.1Chr7.3144
GST
Mt3.5.1Chr7.3145
GST
Mt3.5.1Chr7.3147
GST
Mt3.5.1Chr7.3149
GST
Mt3.5.1Chr7.3155
GST
Mt3.5.1Chr7.5757
GST
Mt3.5.1Chr8.2226
GST
Mt3.5.1Chr8.4851
GST
Mt3.5.1Chr8.4852
Metal homeostasis and detoxification
MT
Mt3.5.1Chr4.1021
MT
Mt3.5.1Chr8.2470
NAS
Mt3.5.1Chr1.3615
NAS
Mt3.5.1Chr2.2244
0
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