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Reactive oxygen and nitrogen species and glutathione: key players in the legume–*Rhizobium* symbiosis

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Abstract

Several reactive oxygen and nitrogen species (ROS/RNS) are continuously produced in plants as by-products of aerobic metabolism or in response to stresses. Depending on the nature of the ROS and RNS, some of them are highly toxic and rapidly detoxified by various cellular enzymatic and non-enzymatic mechanisms. Whereas plants have many mechanisms with which to combat increased ROS/RNS levels produced during stress conditions, under other circumstances plants appear to generate ROS/RNS as signalling molecules to control various processes encompassing the whole lifespan of the plant such as normal growth and development stages. This review aims to summarize recent studies highlighting the involvement of ROS/RNS, as well as the low molecular weight thiols, glutathione and homoglutathione, during the symbiosis between rhizobia and leguminous plants. This compatible interaction initiated by a molecular dialogue between the plant and bacterial partners, leads to the formation of a novel root organ capable of fixing atmospheric nitrogen under nitrogen-limiting conditions. On the one hand, ROS/RNS detection during the symbiotic process highlights the similarity of the early response to infection by pathogenic and symbiotic bacteria, addressing the question as to which mechanism rhizobia use to counteract the plant defence response. Moreover, there is increasing evidence that ROS are needed to establish the symbiosis fully. On the other hand, GSH synthesis appears to be essential for proper development of the root nodules during the symbiotic interaction. Elucidating the mechanisms that control ROS/RNS signalling during symbiosis could therefore contribute in defining a powerful strategy to enhance the efficiency of the symbiotic interaction.

Key words: Glutathione, homoglutathione, *Medicago truncatula–Sinorhizobium meliloti* symbiosis, nitrogen fixation, reactive nitrogen species, reactive oxygen species.

Introduction

Reactive oxygen and nitrogen species (ROS/RNS) have been characterized as key actors in the response of plants to both biotic and abiotic stresses (for reviews, see Apel and Hirt, 2004; Delledonne, 2005). Initially, these species were only regarded as damaging to cells. Indeed, ROS and RNS are involved in degenerative processes associated with senescence and cell death. More recently, ROS and RNS species emerged as ubiquitous signalling molecules participating in the recognition of and the response to stress factors. For a signalling molecule to be effective, it needs to be produced quickly and efficiently on demand, to induce defined effects within the cell, and to be removed rapidly and effectively when no longer required. This is the case of ROS and RNS, which appear to be mainly generated by enzymes; hence, their rates and subcellular sites of production may be under metabolic control (Matamoros *et al.*, 2003; Neill *et al.*, 2003). It is now obvious that their role is not restricted to the stress response, but can encompass the whole lifespan of the plant, including normal growth and development stages. Moreover, ROS/RNS bioactivity is largely dependent upon their turnover, which not only includes ROS/RNS production, but also their detoxification. ROS/RNS detoxification relies on

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Abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; GSH, glutathione; hGSH, homoglutathione; AD, antioxidant defence

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the antioxidant defence (AD), which involves enzymatic activities (catalases, superoxide dismutases, peroxidases) and antioxidant molecules (glutathione, GSH, ascorbate). Together with ROS/RNS production, AD is actively regulated in the response to environmental cues and plant development. Taken together, ROS/RNS and AD contribute to the redox balance, the modulation of which is probably crucial for physiological regulation.

The symbiosis between legumes and rhizobia is a complex process relying on finely tuned infectious and developmental events. The initial step of the symbiotic interaction is a chemical cross-talk between the plant and the bacterial partners leading to the production of bacterial nodulation factors (NF) upon sensing of the flavonoids present in root exudates. NF not only participate in bacterial infection, but also trigger the initiation of a specific developmental programme ending in the formation of a new organ, the nodule (for a review see Oldroyd *et al.*, 2005). Nodule formation is assured by the dedifferentiation and division of root cortical cells and nodules are subsequently colonized by the bacteria released from the infection threads formed upon infection. During the last few years, significant progress has been made in understanding nod factor transduction pathways. In particular, several NF receptor candidates have been identified (Spaink, 2004). Moreover, a series of genes involved in signalling processes acting downstream of NF perception have been characterized (for a review, see Geurts *et al.*, 2005; Stacey *et al.*, 2006). Nevertheless, how those elements integrate to form the complex signalling network regulating symbiotic interaction is still unknown.

There is increasing evidence that ROS, RNS and/or GSH play an important role in legume–rhizobia symbiosis (Hérouart *et al.*, 2002). As for plant–pathogen interactions, they may be important actors in the initial interaction between the two partners, which leads to the recognition of the bacteria as a partner or as a foe. Indeed, 90% of the infection threads initially formed abort in *Medicago*. Such abortion is achieved by a hypersensitive-like response, a well-known ROS- and RNS-regulated process during plant pathogenesis (Vasse *et al.*, 1993; Penmetsa and Cook, 1997). The cell mitotic cycle is also tightly regulated by the cell redox balance (Vernoux *et al.*, 2000). Therefore, nodule organogenesis may be deeply dependent on the cellular redox conditions. More generally, nodulation efficiency is highly dependent on plant fitness (Oldroyd *et al.*, 2001), which may be modulated by ROS and RNS formation and the antioxidant content. This is important when considering that the active nodule metabolism involved in the nitrogen fixation may itself provoke the formation of ROS, which will impair nodule functioning (Puppo *et al.*, 2005).

In this framework, current knowledge on where, when, and how ROS, RNS, and GSH participate in symbiosis regulation is summarized, and new fields are suggested for future investigations.

Reactive oxygen species

ROS, which are formed in numerous cellular processes, were first described as deleterious, as they can provoke cellular damage (Halliwell and Gutteridge, 1986). It is now largely admitted that they can play a signalling role in various cellular mechanisms (Neill *et al.*, 2002). It has been demonstrated that ROS are key players in the plant's defence system against pathogens ('oxidative burst'; for a review see Apel and Hirt, 2004), and also in fundamental processes such as cellular growth (Foreman *et al.*, 2003), stomatal closure (Pei *et al.*, 2000), and in the regulation of gene expression (Neill *et al.*, 2002; Vranova *et al.*, 2002).

More recently, the involvement of ROS in the establishment of the legume–*Rhizobium* symbiosis has also been underlined, supporting the hypothesis that symbiosis and pathogeny are variations on a common theme (Baron and Zambryski, 1995). However, the situation differs according to the infection time-course. In the early stages of the symbiotic interaction, the oxidation of nitro blue tetrazolium (NBT) can be detected in infection threads, indicating that O_2^- is produced during the infection process (Santos *et al.*, 2001; Ramu *et al.*, 2002). Moreover, this production was not observed when *M. truncatula* plants were inoculated with a *S. meliloti nodD1ABC* mutant that was unable to produce Nod factors, suggesting that they have a role in the oxidative burst (Ramu *et al.*, 2002). By contrast, in the very early stages of the symbiotic interaction, the production of H_2O_2 appears to be inhibited by the Nod factors (Shaw and Long, 2003); in the same way, a *S. meliloti nodC*$^-$ mutant, defective in Nod factor biosynthesis, showed an increase in H_2O_2 accumulation (Bueno *et al.*, 2001). Moreover, the compatible interaction between *M. sativa* and *S. meliloti* is linked, at least in part, with an increase in antioxidant defence (particularly catalase and lipoxygenase) during the preinfection period (up to 12 h) (Bueno *et al.*, 2001). Simultaneous treatment of an alfalfa suspension culture with yeast elicitors and *S. meliloti* lipopolysaccharides (LPS) was unable to induce an alkalinization of the culture medium or an oxidative burst that is systematically observed when the cells are treated with yeast elicitors alone (Albus *et al.*, 2001). Thus, *S. meliloti* LPS released from the bacterial surface might function as a specific signal molecule, promoting the symbiotic interaction and suppressing a pathogenic response in the host plant, alfalfa (Albus *et al.*, 2001).

In the later stages of the nodulation process, ROS (H_2O_2, O_2^-) can be observed in infected cells of young nodules, revealing the prolonged production of these radicals during nodule development. H_2O_2 production was detected in ultrathin sections of mature 6-week-old nodules as an electron-dense precipitate stained with cerium chloride (Santos *et al.*, 2001; Rubio *et al.*, 2004). Extensive cerium labelling was observed in the cell walls of infected cells and in some infection threads all around bacteria. In functioning

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nodules, leghaemoglobin autoxidation appears to be an important source of ROS. To avoid any deleterious effect, nitrogen-fixing nodules are fitted with a very efficient antioxidant defence (Matamoros *et al.*, 2003). Moreover, a strong cerium precipitate can also be observed around peribacteroid and bacteroid membranes of senescent infected cells, strongly suggesting that H_2O_2 is involved in the senescence process (Alesandrini *et al.*, 2003; Rubio *et al.*, 2004).

However, it should be noted that ROS have not been detected in the micro-organisms progressing within the threads, suggesting that the rhizobia have an efficient antioxidant defence (Santos *et al.*, 2001; Rubio *et al.*, 2004). To detoxify ROS, symbiotic bacteria display a multiple enzymatic antioxidant defence that is required for the development and the functioning of symbiosis. *S. meliloti* possesses two superoxide dismutases that convert O_2^- to O_2 and H_2O_2 (Santos *et al.*, 2000; Hérouart *et al.*, 2002) and three haem *b*-containing catalases, which are able to scavenge H_2O_2 (Hérouart *et al.*, 1996; Ardissone *et al.*, 2004). Two catalases are monofunctional hydroperoxidases and are regulated mainly at the transcriptional level: in the presence of H_2O_2 or in low-phosphate conditions for KatA or in the presence of paraquat (a superoxide-generating compound) and in response to environmental stresses such as heat, osmotic and ethanol shocks for KatC (Sigaud *et al.*, 1999; Yuan *et al.*, 2005). The H_2O_2-dependent expression of *katA* involves the redox-sensitive transcriptional regulator OxyR (Jamet *et al.*, 2005), while the PhoB response regulator controls the *katA* transcription at a second promoter in phosphate-starved cells (Yuan *et al.*, 2005). By contrast, KatB is a bifunctional catalase-peroxidase constitutively expressed in free-living cells, such as *in planta* (Jamet *et al.*, 2003). In addition to these well-characterized enzymes, *S. meliloti* genome analysis suggests that this bacterium contains three alkyl hydroperoxide reductases, at least one of which is encoded by an *ahpC*-like gene and might use H_2O_2 in addition to alkyl hydroperoxide as a substrate (Seaver and Imlay, 2001). Other non-enzymatic scavengers such as glutathione (see below) or other enzymatic systems might account for the antioxidant machinery of rhizobia. For example, chloroperoxidase is secreted after exposure to H_2O_2 or organic hydroperoxides in *S. meliloti* (Barloy-Hubler *et al.*, 2004) and a microaerobiosis-induced 2-cys peroxiredoxin (PrxS) expressed during the symbiotic interaction has been characterized in *R. etli* (Dombrecht *et al.*, 2005). Moreover, it has recently been demonstrated that the levels of peroxiredoxins are decreased during nodule development and are redox regulated during nodule senescence (Groten *et al.*, 2006).

The whole antioxidant defence is essential for optimal nodule development and maintenance. The three catalases are differentially expressed during nodule formation. The *katB* gene is expressed during all steps of symbiosis, whereas *katC* expression is only detectable in the infection threads, at the early stage of infection, and in the infection zone of the mature nodule (Jamet *et al.*, 2003). The *katA* expression is restricted to the fixation zone of the mature nodule, despite a clear production of H_2O_2 around bacteria inside the infection threads (Jamet *et al.*, 2005; Yuan *et al.*, 2005). The presence of KatB and KatA in the bacteroid is probably related to high internal H_2O_2 generation as a consequence of the high aerobic metabolism required to produce the ATP necessary to sustain nitrogenase activity. Whereas a single mutation in one catalase gene of *S. meliloti* does not lead to any phenotype, a feature also observed for *katG* mutant strains of *R. etli* (Vargas Mdel *et al.*, 2003) and *B. japonicum* (Panek and O'Brian, 2004), the double *katB/katC* and *katA/katC* mutants of *S. meliloti* are strongly impaired in nodule formation and in nitrogen-fixation activity, respectively (Jamet *et al.*, 2003). Interestingly, the nodule formed by the *katB/katC* double mutant contains many infection threads. However, the bacteria are released into plant cells without peribacteroid membranes, preventing differentiation into bacteroids and leading to bacteria that directly undergo senescence. The nodules induced with the *katA/katC* mutant strain display a very thin fixation zone. In fact, the bacteria are correctly released from the infection threads, but the newly differentiated bacteroids rapidly become senescent. The impact of ROS production on the microsymbiont was also shown by Dombrecht *et al.* (2005) in the case of *R. etli–Phaseolus vulgaris* symbiosis, where a *katG/prxS* double mutant exhibited a significant reduction in nitrogen fixation.

ROS production might also have a signalling role during symbiosis. Indeed, a *S. meliloti* mutant overexpressing the constitutive catalase, KatB, which exhibits the highest affinity for H_2O_2 among the three catalases (Ardissone *et al.*, 2004), was constructed in this laboratory. The $katB^{++}$ resulting strain was able to degrade H_2O_2 very rapidly and displayed an intracellular H_2O_2 concentration below that of the wild-type strain (A Jamet *et al.*, unpublished data). *In planta*, the mutant displayed a delayed nodulation phenotype, Nod^d, associated with cytological modifications which are currently under investigation. These last results clearly indicate that the presence of H_2O_2 is essential for optimal symbiosis development. It still remains to define more precisely the role of H_2O_2 in the infection thread development including bacterial proliferation.

An open question concerns the enzymes responsible for enhanced ROS formation during infection and nodule organogenesis, which have not been identified yet. The superoxide radicals are formed in the infection threads (Santos *et al.*, 2001) possibly by a membrane-bound NADPH oxidase, as observed in activated neutrophils. Indeed, inhibition of the oxidative burst with DPI (a specific suicide inhibitor of the NADPH oxidase) corroborates this hypothesis (Ramu *et al.*, 2002; Shaw and Long, 2003; Rubio *et al.*, 2004). Moreover, other possible sources for

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H_2O_2 are cell wall peroxidases, germin-like oxalate oxidases, and diamine oxidases (Wisniewski *et al.*, 2000).

In conclusion, these data clearly show that ROS accumulation is observed during the infection process and during the degeneration of the symbiotic association. It is, however, important to note that in the first hours of the infection, the production of ROS is inhibited. This clearly indicates the complexity of the process. Moreover, as ROS are produced by the plant partner, it would also be of interest to analyse the consequences of modifying plant ROS-scavenging or ROS-producing activities on the symbiotic capacities. Furthermore, the availability of the complete sequence of the *S. meliloti* genome and of large collections of *Medicago truncatula* ESTs, as well as microarray chips for both partners, will provide the opportunity to identify target genes regulated by ROS during symbiosis in both partners.

Reactive nitrogen species

Together with ROS, RNS, and in particular nitric oxide (NO), are now considered as major components of oxidative burst and redox state regulation. Indeed, NO has been shown to be a ubiquitous signalling molecule in plants, controlling physiological processes as diverse as flowering, iron homeostasis, drought response, or resistance against pathogens (for review see Neill *et al.*, 2003). Several studies have also illustrated the major role of NO as a signal in controlling primary and adventitious root organogenesis, a developmental process which shares common features with nodule formation (Pagnussat *et al.*, 2002; Correa-Aragunde *et al.*, 2004). Recent advances point out that it may also be involved in legume–rhizobia symbiotic interactions.

Several studies have reported direct or indirect evidence for the production of NO during plant–symbiont interactions. Data from Shimoda *et al.* (2005) suggest that a rapid and transient NO production, detected with the permeant NO-sensitive probe 4,5 diaminofluorescein diacetate, occurs in *Lotus japonicus* roots inoculated with *Mesorhizobium loti* (Shimoda *et al.*, 2005). Using the same approach, such production was not observed during *M. truncatula–S. meliloti* interaction. The NO production in *L. japonicus* may reflect a specificity of this symbiotic model. Whereas the presence of NO during the early stages of symbiosis remains puzzling, its production in mature nodules has been clearly shown. A direct detection of NO using the DAF-2DA probe has been performed by confocal microscopy in *M. truncatula/S. meliloti* nodules (E Baudouin *et al.*, unpublished data). The NO production, which can be impaired with the NO scavenger carboxyPTIO, is localized in the bacteroid-containing cells of the fixing zone. These data corroborate previous studies on soybean nodules, in which the presence of $NO^{\bullet}$ complexed with leghaemoglobin (Lb), a haemoprotein ensuring the oxygen flux to the bacteroids, had been detected in soybean nodules using Electron Paramagnetic Resonance (Mathieu *et al.*, 1998).

Several mechanisms by which NO could be produced during plant/rhizobia interactions have been proposed. The bacterial denitrification pathway could be a first candidate. Indeed, rhizobia being denitrifying bacteria can generate NO as an intermediate of the reduction of NO_3^- to N_2. Mesa *et al.* (2004) showed that *Bradyrhizobium japonicum* denitrification genes are expressed in the nodule fixation zone (Mesa *et al.*, 2004). Nevertheless, using *S. meliloti* mutants of the denitrification pathway, it was observed that NO accumulation was not modified in nodules, which makes the participation of the denitrification process to NO production in nodules unlikely (E Baudouin *et al.*, unpublished data). NO could also be produced by the plant partner. Recent studies have identified plant NO synthases, which can generate NO from L-arginine (Guo *et al.*, 2003). In this respect, Cueto *et al.* (1996) reported the presence of NO synthase activity in the fixation zone of lupin nodules, which was impaired by the mammalian NOS inhibitor N(G)-monomethyl-L-arginine (Cueto *et al.*, 1996). This compound also inhibited NO production observed in *Medicago truncatula* nodules, further implicating a NO synthase-like enzyme in NO production during symbiosis (E Baudouin *et al.*, unpublished results). A gene encoding a putative NO synthase in *M. truncatula* has recently been isolated in this laboratory and its study during the symbiotic interaction is in progress (N Pauly, unpublished results). In addition to NO synthases, nitrate reductases also participate to NO production in plants. In this view, the induction of a nitrate reductase independently of nitrate in *L. japonicus* nodules has been reported and it would be interesting to analyse its possible involvement in NO production (Kato *et al.*, 2003). Future studies will have to clarify whether and when these putative NO generating systems operate during symbiosis.

The question of the possible role of NO in nodules is raised. As NO has been shown to inhibit nitrogenase (Trinchant and Rigaud, 1982; Kanayama *et al.*, 1990), its steady-state concentration should be kept low at the bacteroid level. This is also true for the RNS peroxynitrite which is formed by the combination of NO with oxygen superoxide. In this context, the presence of large amounts of the O_2 carrier leghaemoglobin, which has a high affinity for NO and can act as a NO scavenger (Herold and Puppo, 2005), may modulate NO bioactivity. This may also be a function of non-symbiotic haemoglobins, which scavenge NO in plants (Romero-Puertas *et al.*, 2004) and which are rapidly induced upon symbiotic infection and accumulate in fixing nodules (Shimoda *et al.*, 2005; Vieweg *et al.*, 2005). It has recently been shown that haemoglobin overexpression in alfalfa roots efficiently prevented the inhibition of aconitase, a NO-sensitive enzyme, by exogenous and endogenous NO generation (Igamberdiev *et al.*, 2005).The low level of free NO could

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function in signalling processes in nodules, where it may participate in the low oxygen response of the fixing cells. In this way, Mesa *et al.* (2003) showed that, in *B. japonicum*, NO can regulate specific transcription factors such as NnrR which control denitrification gene expression. This activation would involve the FixLJ pathway, which is activated by low oxygen concentrations and can readily fix NO (David *et al.*, 1988; McGongile *et al.*, 2000). Moreover, a series of genes related to the plant response to hypoxia was recently isolated in a screen for *M. truncatula* genes induced upon NO treatment (M De Stefano, unpublished data). As these hypoxia-related genes are also regulated in fixing *L. japonicus* nodules (Colebatch *et al.*, 2004), this may extend the hypothesis of NO regulating the low oxygen response to both symbiotic partners. The availability of symbiotic partners, modified for NO production or scavenging, will open new opportunities to unravel the molecular mechanisms regulated by NO during symbiotic interactions.

Glutathione

One of the major antioxidant molecules in eukaryotes is GSH, a low-molecular mass thiol implicated in antioxidant defence mainly through the ascorbate/GSH cycle. It plays a crucial role in plant defence against abiotic and biotic stresses and is also involved in heavy metal tolerance and xenobiotic detoxification. The presence of homoglutathione (γ-glutamylcysteine-β-alanine; hGSH), a homologue of GSH, is one of the characteristics of leguminous plants (Frendo *et al.*, 1999; Matamoros *et al.*, 1999; Moran *et al.*, 2000).

The importance of GSH and hGSH during the first steps of symbiosis between *M. truncatula* and *S. meliloti* has been examined. Using both buthionine sulphoximine (BSO), a specific inhibitor of GSH and hGSH synthesis, and transgenic roots expressing GSH synthetase and hGSH synthetase in an antisense orientation, the deficiency in GSH and hGSH synthesis appeared to inhibit the formation of the root nodules (Frendo *et al.*, 2005). This inhibition was not correlated to a modification in the number of infection events or to a change in the expression of the Rhizobium Induced Peroxidase *rip1*, indicating that the low level of GSH or hGSH did not alter the first steps of the infection process. By contrast, a strong diminution in the number of nascent nodules and in the expression of the early nodulin genes, *Mtenod12* and *Mtenod40*, was observed in GSH and hGSH-depleted plants suggesting that GSH is involved in the nodule meristem formation. Thus, GSH and hGSH appear to be essential for the proper development of the root nodules resulting from the symbiotic interaction (Frendo *et al.*, 2005).

The genes regulated by GSH/hGSH need to be identified in order to understand the role of GSH/hGSH in nodule formation. For this purpose, a transcript profiling analysis in *M. truncatula* plants inoculated with *S. meliloti* was performed using a cDNA-Amplified Fragment Length Polymorphism (AFLP) protocol. The effect of GSH depletion by BSO was studied to investigate whether there are patterns of gene regulation that require GSH. A collection of 306 gene tags regulated at different time points during the first 4 d of the nodulation process was obtained. Of these, 91 gene tags classifiable in two clusters corresponding, respectively, to up-regulated and down-regulated genes, showed GSH-dependent expression changes. Sequence analysis and functional characterization of these gene tags which is currently being undertaken will lead to a better understanding of the role of GSH/hGSH in nodule formation (C Pucciariello *et al*., unpublished data).

Nodules have a strong capacity to produce ROS due to their high respiration rates, the strong reducing conditions required to reduce N_2, and the leghaemoglobin autoxidation process (Becana *et al.*, 2000; Hérouart *et al.*, 2002). Moreover, although this may not be true in all symbiotic systems (Groten *et al.*, 2005), ROS have been shown to accumulate during nodule senescence (Becana and Klucas, 1992; Alesandrini *et al.*, 2003). In this framework, the GSH/hGSH pool may have an important antioxidant role in nitrogen-fixing nodules. These organs have a high thiol content with an active glutathione-ascorbate cycle (Dalton *et al.*, 1986; Groten *et al.*, 2005). However, legume root nodules are characterized by an early senescence. During senescence of nodules, GSH and hGSH are found to decline (Evans *et al.*, 1999; Matamoros *et al.*, 2003; Groten *et al.*, 2005). Indeed, ageing causes a 50% decrease in hGSH in soybean and pea nodules and an 82% decrease in GSH in pea nodules (Evans *et al.*, 1999; Matamoros *et al.*, 2003; Groten *et al.*, 2005) with a concomitant accumulation of catalytic Fe and oxidation of thiols, lipids, proteins, and DNA (Evans *et al.*, 1999). Moreover, this age-related decline in thiol content can be observed in mature nodules where Matamoros *et al.* (1999) reported the senescent zones of pea nodules having 50% less GSH and 25% less hGSH than the meristematic and infected zones. Finally, there is a strong correlation between the N-fixation capacity and the nodule GSH content (Groten *et al.*, 2005). Taken together these data strongly suggest a key involvement of thiols in the N_2-fixation efficiency. This is consistent with recent data indicating that antioxidants such as thioredoxin are essential to lower reactive oxygen species levels during nodule development (Lee *et al.*, 2005).

Studies have shown that both thiols (GSH and hGSH) are especially abundant in the meristematic and infected zones of *P. sativum* nodules (Matamoros *et al.*, 1999). The high concentration of thiols in nodules (Frendo *et al.*, 1999; Matamoros *et al.*, 1999), along with the finding that concentration of thiol peptides are 3–4-fold higher in effective than in ineffective nodules (Dalton *et al.*, 1993) strongly suggest that part of GSH and hGSH may be synthesized by the microsymbiont. Thus, it has been tested

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whether part of the GSH present in the nodule may be synthesized by the bacteria and whether the bacterial GSH pool may modify the nodulation and nitrogen-fixation processes. *In silico* analysis of the bacterial genome was performed to find γ-glutamylcysteine synthetase (γECS) and glutathione synthetase (GSHS), the genes involved in GSH synthesis. Then, γECS- and GSHS-defective mutant strains (*SmgshA* and *SmgshB*, respectively) derived from wild-type *S. meliloti* Rm1021 were constructed. The *SmgshA* mutant strain, which is unable to synthesize GSH due to a gene disruption in *gshA*, encoding the enzyme for the first step in the biosynthesis of GSH, was unable to grow under non-stress conditions, precluding any nodulation. By contrast, *SmgshB* was able to grow, indicating that γ-glutamylcysteine, the dipeptide intermediate, can substitute for GSH under non-stress conditions. However, the *SmgshB* strain showed a delayed-nodulation phenotype coupled with a 75% reduction in the nitrogen fixation capacity. This phenotype was linked to abnormal nodule development with an early senescence process. Both the *SmgshA* and *SmgshB* mutant strains exhibited higher catalase activity than the wild-type *S. meliloti* strain, suggesting that both mutant strains are under oxidative stress. Taken together, these results show that the bacterial GSH pool plays a critical role in the growth of *S. meliloti* and during its interaction with the plant partner (Harrison *et al.*, 2005).

Conclusion

The recent data reviewed in this article point to possible new functions for ROS, RNS, and GSH during legume–rhizobia interactions. These overcome the deleterious and protective roles classically associated with such compounds in nodules. In particular, it is now obvious that key steps in the symbiotic process, such as infection thread development or nodule formation, are deeply impaired by the modulation of ROS or GSH content. Recent advances also underline that the relationship between redox-related molecules and symbiosis is much more subtle than first envisaged. For instance, H_2O_2, the production of which is inhibited during the very early steps of legume-*rhizobia* interactions, is subsequently produced in infection threads. Moreover, if bacterial strains with impaired capacity to detoxify H_2O_2 have deficiencies in their symbiotic capacities, bacterial strains with enhanced detoxification potential will also show symbiotic defects. Therefore, the outcome of ROS/GSH/RNS production during symbiosis would be a matter of where, when, and to what level they accumulate. Moreover, as these molecules interact and finally influence the cellular redox state, it has to be discovered whether ROS/GSH/RNS regulate the symbiotic process through a modulation of the redox state or, instead, act as signalling molecules on their own.

A major step towards an understanding of the mechanisms regulated by ROS/GSH/RNS during symbiosis relies on the identification of their molecular targets. Strategies are currently being developed that rely on cDNA-AFLP and microarray technologies to isolate and analyse H_2O_2, GSH, and NO targets. Together with the use of symbiotic partners modified in ROS/GSH/RNS production, the study of these targets will provide information on the nature of the processes regulated by such molecules during symbiosis. It will also give an opportunity to investigate how ROS/GSH/RNS production overlaps with the regulation of specific genes at particular stages of the symbiotic process. Finally, it will give clues on whether and how ROS/GSH/RNS cross-talk during symbiosis. As ROS/GSH/RNS not only modify gene expression, but also impact plant physiology through post-translational modification of protein targets, future prospects will be aimed at comparing glutathionylated and nitrosylated protein patterns during symbiosis. The identification of differentially modified proteins will provide clues to the early targets of ROS/GSH/RNS sensing and signalling. Taken together, these data should show how ROS/GSH/RNS participate in the symbiotic process at the molecular level. Legume–rhizobia symbiosis is a remarkable model for infectious and developmental processes and their integration, and this information will be of general interest for the plant biologists.

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