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Review

Redox regulation of differentiation in symbiotic nitrogen fixation ☆

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ABSTRACT

Background: Nitrogen-fixing symbiosis between *Rhizobium* bacteria and legumes leads to the formation of a new organ, the root nodule. The development of the nodule requires the differentiation of plant root cells to welcome the endosymbiotic bacterial partner. This development includes the formation of an efficient vascular tissue which allows metabolic exchanges between the root and the nodule, the formation of a barrier to oxygen diffusion necessary for the bacterial nitrogenase activity and the enlargement of cells in the infection zone to support the large bacterial population. Inside the plant cell, the bacteria differentiate into bacteroids which are able to reduce atmospheric nitrogen to ammonia needed for plant growth in exchange for carbon sources. Nodule functioning requires a tight regulation of the development of plant cells and bacteria.
Scope of the review: Nodule functioning requires a tight regulation of the development of plant cells and bacteria. The importance of redox control in nodule development and N-fixation is discussed in this review. The involvement of reactive oxygen and nitrogen species and the importance of the antioxidant defense are analyzed.
Major conclusions: Plant differentiation and bacterial differentiation are controlled by reactive oxygen and nitrogen species, enzymes involved in the antioxidant defense and antioxidant compounds.
General significance: The establishment and functioning of nitrogen-fixing symbiosis involve a redox control important for both the plant–bacteria crosstalk and the consideration of environmental parameters. This article is part of a Special Issue entitled Redox regulation of differentiation and de-differentiation.

1. Introduction

Plants have particular growth characteristics, developmental patterns and structural architecture. Plant development takes place largely after embryogenesis occurring during seed formation and maturation. Indeed, plant growth is correlated to the postembryogenic formation of new organs such as roots, leaves, stems and flowers. It is first linked to cell division which is restricted to meristems and to cellular differentiation which occurs in a second step [1,2]. Differentiation changes the meristematic cells into non-dividing cells with specific functions such as vascular tissues [3]. Nevertheless, most of the cells keep their potentiality to dedifferentiate into dividing cells depending on the biotic and abiotic environment [4]. Finally, the plant architecture is partially governed by the search for essential elements involved in plant growth such as light, water or minerals. This has lead to specific development of leaves necessary for light energy reception and gas exchange, or roots that draw nutrients required for plant development [5,6].

Redox regulation plays a major role in plant development and adaptation to biotic and abiotic environment [7–10]. In this context, plants have developed a large number of redox systems essential for the sessile life style. Reactive oxygen and nitrogen species (ROS and RNS) such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) or nitric oxide (NO) are redox signaling molecules actively produced by plants in response to their environment. On the other hand, plant antioxidant defense is very efficient with numerous enzyme families such as ascorbate peroxidases, glutathione peroxidases, peroxiredoxins, catalases or superoxide dismutases. Moreover, thioredoxin and glutaredoxin families, which are involved in the redox control of protein activity, are also large multigenic families with more than twenty members in each of them [11,12]. Non-enzymatic antioxidant compounds like NAD(P)H, glutathione and ascorbate are present in the millimolar range in plant cells and allow a reducing environment as well as the efficient functioning of the antioxidant defense [13–15].

Plants often use symbiotic interactions with fungi or bacteria to allow a more efficient nutrition process. Interaction with symbiotic fungi improves plant nutrition in water, phosphate and other nutrients [16]. In exchange, the plants provide carbohydrates to the fungi through photosynthate supply. Similarly, plants from the legume family, including alfalfa, soybean and pea, perform a symbiotic interaction with soil bacteria of the *Rhizobium* family to increase their nitrogen

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amount needed for their growth when mineral nitrogen source is scarce.

Nitrogen fixing symbiosis (NFS) involves multiple processes. The molecular cross-talk between the plant and the bacteria allows the entry of the bacteria into the plant [17]. Plant exudates, such as flavonoids, attract rhizobia and trigger the production of nodulation (Nod) factors by the bacteria. Nod factors (NF) are essential for the recognition of the bacteria by the plants. They enable the entry of the bacteria in the plant as well as the formation of nodule meristem *via* cortical cell dedifferentiation [18,19]. Rhizobia bound to curled root hairs of the infection zone induce cell wall degradation and enter into the root through the infection thread (IT), a channel shaped structure which defines the intracellular path of the bacteria through the epidermis and the cortex (Fig. 1B). The plant infection may also occur *via* an intercellular infection process called crack entry which occurs at lateral root bases [20]. Thereafter, the bacteria are internalized in the cells and form a new organelle-like structure called symbiosome. In the symbiosomes, the bacteria divide and differentiate into the nitrogen-fixing bacteroids. The bacterial nitrogenase reduces atmospheric nitrogen (N_2) into ammonia which is exported to the plant cell cytoplasm and integrated into ureides or asparagine. In return, the plant supplies the energy needed for this reduction (16 ATP for one N_2). The nitrogen/carbon exchange between the nodules and the roots requires the presence of a vascular tissue called vascular bundle for the metabolite transport. Moreover, as the nitrogenase is strongly sensitive to oxygen, a specific oxygen barrier is formed by a cell layer around the infected cells which reduces the level of oxygen (O_2) in the nodule cortex.

Nodules are classified as indeterminate and determinate according to their mode of development [21]. In indeterminate nodules, such as those formed by pea, alfalfa or *Medicago truncatula*, the nodule meristem derives from the inner-cortex dedifferentiation and is persistent during the whole lifetime of the nodule, giving elongated nodules (Fig. 1C). Consequently, the meristematic, infection and nitrogen-fixing zones are present at the same time in the nodule. In determinate nodules, such as the ones from soybean or *Lotus japonicum*, the nodule meristem dedifferentiates from outer cortex cells and is only transiently active. This results in spherical nodules, containing cells in a similar developmental state.

Nodule meristematic cells differentiate into multiple cellular types. The peripheral cell layers contain the epidermis, the cortex, the endodermis and the parenchyma. These cell layers participate in the protection of the internal N_2 fixation zone which contains the infected cells and contribute to the root–nodule exchange with the presence of the vascular bundles. The central zone of the nodule contains the meristematic, infection and nitrogen-fixing zones (Fig. 1C). As already mentioned, these different zones are present, respectively, at the same time in indeterminate nodules and successively in determinate ones. The infection of plant cells by the bacteria requires differentiation of the nodule meristematic cells with DNA endoreduplication cycles. The infected nodule cells enlarge and reach ploidy levels of 32C and 64C, and are 80-fold larger than diploid meristematic cells [22,23]. The endoreduplication and the expansion of the infected cells go together with changes in cellular metabolism which allows the reception of the bacteria and nitrogen assimilation by the plant.

During NFS, differentiation also occurs for the bacterial partner which is converted into nitrogen-fixing bacteroids. As for nodule development, bacteroid differentiation is divergent depending on the host plant [24]. In some legumes, bacteroid morphology is unaffected compared to the free living bacteria with a small rod-like shape whereas in other legumes, bacteroids present an extreme morphological change with an elongated phenotype (5 to 10-fold longer) and sometime a Y-shaped form. The bacteroid enlargement is coupled to the endoreduplication of the bacterial genome and a terminal differentiation which is irreversible and prevents further reproduction. In contrast, the bacteroids which do not show these morphological changes have a similar DNA content to free-living bacteria and are able to divide when extracted from nodules. The different bacteroid phenotype is linked to the plant host as a *Rhizobium* strain 32H1 has a terminal differentiation when colonizing peanut and a reversible differentiation with cowpea.

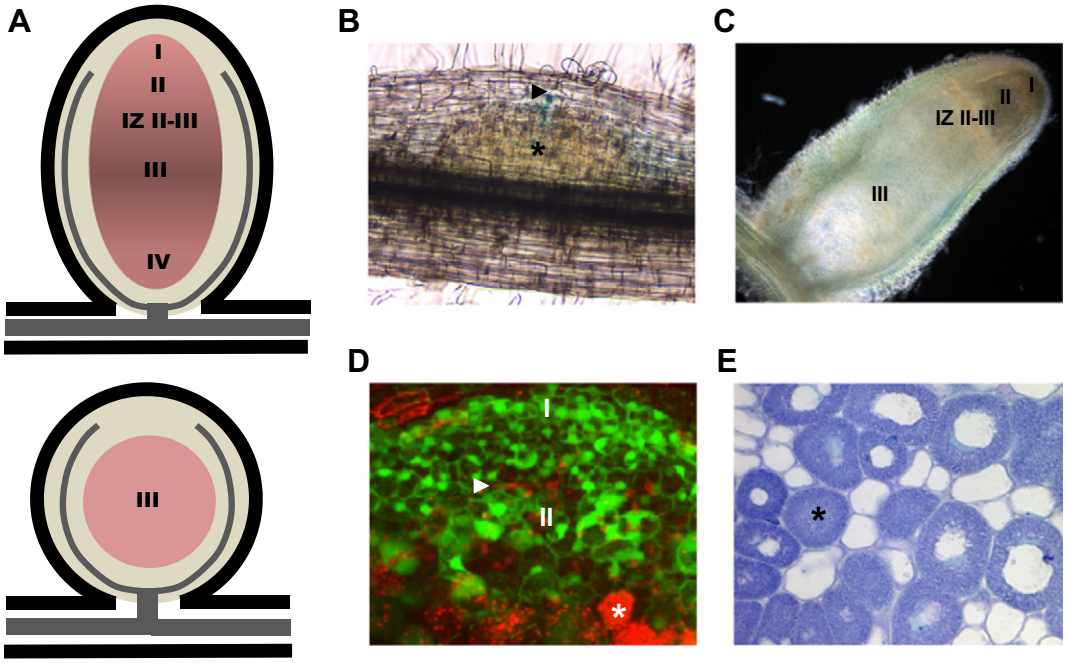

Fig. 1. The different steps of the root nodule formation and root nodule structure. (A) Structure of indeterminate with the apical meristem (I), the infection zone (II), the interzone II–III (IZ II–III), the nitrogen-fixing zone (III) and the senescent zone (IV) and determinate nodules with the nitrogen-fixing zone (III). (B) Development of root nodule with the root nodule meristem (⁎) and infection thread (►) in blue. (C) Picture of an indeterminate root nodule. (D) Picture of the nodule meristematic zone (I) and the infection zone (II); the plant cell cytosol which increases during cellular differentiation is labeled in green, the infection threads (►) and the infected cells (⁎) are labeled in red. (E) The nitrogen-cell is fully packed with numerous endosymbiotic bacteria called symbiosomes (⁎).

The comparison of transcriptomes from plant hosts inducing bacterial terminal differentiation (*M. truncatula*) or not (*L. japonicum*) allowed the identification of plant factors mediating terminal differentiation in legumes. These plant factors are antimicrobial-like peptides called nodule-specific cysteine-rich (NCR) peptides. This peptide family was extensively described in *M. truncatula* and homologs were found in *Medicago sativa*, *Pisum sativum*, *Vicia faba* and *Astragalus sinicus*. All these leguminous species have a bacteroid terminal differentiation in their nodules. The NCR peptides produced by the host plant are targeted to the bacteroids through the secretory pathway. Moreover, it was shown that the *M. truncatula dnf1-1* mutant, deficient in the nodule secretory pathway, produces nodule with infected cells in which bacteroids are not able to differentiate. *In vitro* treatment of free living *Sinorhizobium meliloti* bacteria with some NCR peptides induces membrane permeabilization, inhibition of cell division and genome duplication linked to bacterial elongation. These phenotypic characteristics correlate with the *in vivo* bacterial modifications observed in end-differentiated bacteroids compared to free living bacteria.

NFS requires multiple steps of differentiation in both partners to allow the formation of functional root nodules. This review summarizes the results showing that these differentiation steps are under the control of the cellular redox status. The involvement of reactive oxygen and nitrogen species as well as the importance of the antioxidant defense in both symbiotic partners are described during nodule establishment, functioning and developmental senescence.

2. Redox regulation of plant partner

2.1. Importance of ROS and RNS

Early differentiation during the NFS involves the structural modification of root hairs and IT formation which allows root infection by the bacteria. In parallel, root cortex cells dedifferentiate to produce the nodule meristem. The molecular communication between plant and bacteria involves the modification in ROS and RNS production by the plant partner. Changes of ROS and RNS accumulation have been detected during the symbiotic interaction from the first hours following the initial interaction up to the rupture of the interaction during nodule senescence [25].

Lohar et al. [26] suggest that NF-induced root hair deformation is partially regulated by a transient reduction of ROS accumulation in *M. truncatula*. In contrast, Peleg-Grossman et al. [27] showed that ROS accumulation is involved in the establishment of symbiosis as pharmacological treatment with diphenyleneiodonium (DPI) suppresses both ROS accumulation and root hair curling. In the semiaquatic legume *Sesbania rostrata* which produces lateral root base nodules, the formation of intercellular infection pockets is associated with the production of H_2O_2. Pharmacological experiments showed that ROS mediate NF responses and are required for nodule initiation [28]. Functional analysis of the *Phaseolus vulgaris* NADPH oxidase [respiratory burst oxidase homolog (Rboh)] PvRbohB showed that RNAi-mediated *PvRbohB* down-regulation in transgenic roots reduced ROS production and strongly impaired IT and nodule formation [29]. The structure of PvRbohB-RNAi nodules was also altered compared to the control nodules. ITs were more abundant, irregular in shape and wider, and the number of infected cells was reduced and contained less bacteroids. Finally the structure of the bacteroids was also affected with a significant increase of the space between the bacteroid and the symbiosome membrane compared to the control bacteroids. This phenotype is correlated to a 10-fold decrease in the nitrogen fixation efficiency in PvRbohB-RNAi nodules compared to control ones. The correlation between ROS accumulation and the intracellular infection process observed in the nodule has been also pointed out by Andrio et al. [30] in *M. truncatula*. Using a fluorescent protein probe specific for H_2O_2 [31,32], an *in vivo* accumulation of this ROS was observed in the nodule infection zone compared to the meristematic zone and the epidermis. Interestingly, this H_2O_2 accumulation was correlated to the upregulation of a gene encoding a putative protein kinase *MtSpk1* which is regulated by H_2O_2 and NF treatments and is involved in nodule formation. Finally, in *M. truncatula*, the reduction of ROS production in transgenic RNAi roots with a lower expression of *MtROP9*, a small G protein potentially involved in RBOH regulation, was correlated to an impaired infection process and a reduced nodule formation [33]. Taken together, these results showed a link between ROS production and nodule development. Functional analysis of the *M. truncatula* NADPH oxidase *MtRbohA* gene showed that its expression is restricted to the nitrogen-fixing zone of the functional nodule. *MtRbohA* expression was largely enhanced under hypoxic conditions and reduced expression of *MtRbohA* provoked a decrease in the nitrogen fixation activity, correlated to a down regulation of the expression of the microsymbiont nitrogenase gene [34]. These results suggest that hypoxia, prevailing in the nodule-fixing zone, induces *MtRbohA* expression, which would, in turn, lead to the regulation of nodule functioning.

During the last decade, NO has been shown to play a major role in plant development and plant interaction with its biotic and abiotic environment [35,36]. In this context, NO production has been detected during nitrogen-fixing symbiosis [25]. In contrast to animals, the biosynthesis pathway of NO is unclear and may be linked to enzymatic or non-enzymatic biological reactions [37]. However, pharmacological and genetic approaches leading to the modification of NO content modify the nodulation process. In *L. japonicum*, the overexpression of non-symbiotic hemoglobins (nsHb; *LjHb1* and *AfHb1*), which are able to convert NO into nitrate, reduced the NO level in nodules, and increased the number of nodules on transgenic roots compared to control roots [38]. Moreover, nitrogenase activity was increased in transgenic roots overexpressing nsHb. These results were strengthened by pharmacological analyses which showed that NO scavenger enhanced and NO donor inhibited nitrogenase activity, respectively. In contrast, in the *M. truncatula–S. meliloti* interaction, NO scavenger and overexpression of Hmp, a bacterial hemoprotein able to convert NO into nitrate (aerobically) or nitrous oxide (anaerobically) [39], reduced NO level and delayed nodule formation [40]. The reduced level of NO in *MtNoa1/Rif1*- RNA_i roots was also correlated to a reduced number of nodules after *Rhizobium* inoculation [41]. In mature *M. truncatula* nodules, NO seems to have a metabolic role involved in hypoxia adaptation and nodule energetic status maintenance [25,42]. NO content is also correlated with the induction of nodule senescence in *M. truncatula* as the modification of NO accumulation by *S. meliloti* modulates the occurrence of nodule senescence [43]. Taken together, these results showed that NO is involved in nodule formation and functioning, but suggest that its physiological effects can be different depending on the level and the site of production [25].

In conclusion, ROS and RNS are involved in the regulation of the nodulation process. However, they may have various roles as they are involved in the establishment and the functioning of the nodule on the one hand and in the regulation of the nodule number and the onset of senescence on the other hand.

2.2. Importance of the plant partner antioxidant defense

The involvement of the plant antioxidant defense in nitrogen-fixing symbiosis has been analyzed during the last two decades (Table 1). The presence of glutathione and ascorbate at the millimolar concentration and the positive correlation between their content and nitrogen fixation efficiency were the first evidences, suggesting their importance in the nitrogen-fixing symbiosis [44–47]. Similarly, the efficiency of the enzymes involved in the ascorbate–glutathione pathway is also linked to nitrogen fixation efficiency. The importance of low molecular thiols, glutathione (γ-glutamyl-cysteine–glycine; GSH) and homoglutathione (γ-glutamyl-cysteine–β-alanine; hGSH), a legume GSH homolog, was demonstrated in nodule development using pharmacological and genetic approaches. Using both buthionine sulfoximine, a specific

Table 1
Functional characterization of genes involved in redox regulation during nitrogen-fixing symbiosis.

Redox molecules	Proteins	Organisms	Symbiotic phenotypes	Ref.
Plants				
NO	MtNOA1	*Medicago truncatula*	Decreased nodule number	[41]
NO	NR1/2	*M. truncatula*	Reduced nodule size	[42]
NO	LjnHB1	*Lotus japonicus*	Increased nodule number	[38]
H_2O_2	MtRbohA	*M. truncatula*	Decreased nitrogenase activity	[34]
H_2O_2	MtROP9	*M. truncatula*	Impaired infection and decreased nodule number	[33]
H_2O_2	PvRbohB	*Phaseolus vulgaris*	Decreased nodule number and nitrogenase activity	[29]
GSH	GSHS1/2	*M. truncatula*	Decreased nodule number	[48]
GSH	γECS	*M. truncatula*	Decreased nodule size and nitrogenase activity	[49]
Trx	GmTrx	*Glycine max*	Decreased nodule number	[52]
GST	GST9	*G. max*	Decreased nitrogenase activity	[58]
Bacteria				
NO	NirK	*Sinorhizobium meliloti*	Reduced nodule size and nitrogenase activity	[42]
NO	Hmp	*S. meliloti*	Decreased nitrogenase activity, early nodule senescence Delayed nodulation, delayed nodule senescence (*hmp*++)	[40,43]
H_2O_2	KatB	*S. meliloti*	Delayed nodulation, aberrant ITs (*katB*++)	[59]
H_2O_2	KatB/KatC	*S. meliloti*	Impaired bacteroid differentiation	[65]
H_2O_2	KatA/KatC	*S. meliloti*	Early nodule senescence	[65]
H_2O_2	KatE	*Mesorhizobium loti*	Decreased nitrogenase activity	[97]
H_2O_2	KatG/PrxS	*Rhizobium etli*	Decreased nitrogenase activity	[98]
$O_2^{\bullet-}$	SodAB	*S. meliloti*	Depending on the strain used	[62]
$O_2^{\bullet-}$	SodA	*M. loti*	Depending on the *L. japonicus* cultivar used	[95]
GSH	GshB	*S. meliloti*, *Rhizobium tropici*, *R. etli*	Early nodule senescence	[101–103]
Grx	Grx1	*S. meliloti*	Impaired bacteroid differentiation	[68]
Grx	Grx2	*S. meliloti*	Decreased nitrogenase activity	[68]
Trx	TrxL	*S. meliloti* CF52G	Decreased nitrogenase activity	[99]

inhibitor of GSH and hGSH synthesis, and transgenic roots expressing GSH synthetase and hGSH synthetase transcripts in an antisense orientation, Frendo et al. [48] showed that decreased GSH and hGSH synthesis inhibited the formation of root nodules. This inhibition was correlated to a strong diminution in the number of nodule meristems and in the expression of the early nodulins *Mtenod12* and *Mtenod40*, which are gene markers of nodule formation. To analyze the importance of GSH and hGSH in nitrogen-fixing nodules, genetic approaches using the nodule nitrogen-fixing zone-specific nodule cysteine rich (NCR001) promoter were used to modify the GSH/hGSH level in *M. truncatula* nodules [49]. γ-Glutamylcysteine synthetase (γECS) overexpression resulted in a higher GSH content which was correlated with increased nitrogen fixation efficiency. Conversely, down-regulation of the *γECS* gene by RNA interference resulted in decreased nitrogen fixation efficiency and a lower (h)GSH content. This lower (h)GSH content was correlated to a reduction in the nodule size suggesting that the lower (h)GSH content and/or nitrogen fixation efficiency in the nitrogen-fixing zone impair nodule development which occurs mainly in zone I (meristematic zone in which cells actively divide) and in zone II (infection zone in which cellular enlargement occurs with endoreduplication and cellular infection). Taken together, these results showed the importance of plant (h)GSH content in nitrogen-fixing symbiosis.

The presence of redoxins (glutathione peroxidase, peroxiredoxins, glutaredoxins and thioredoxins) and the NADPH dependent reducing systems have been reported in root nodules [50,51]. Their importance has been reported in nodule development. The gene *GmTrx* corresponding to a thioredoxin (Trx) h was shown to be upregulated in the pericycle of 2-day-old nodules and in the infected cells of mature nodules in soybean [52]. This thioredoxin is able to protect a Trx yeast mutant against H_2O_2 treatment. RNA interference-mediated repression of the thioredoxin gene under the control of the leghemoglobin promoter severely impaired nodule development. This shows that the expression of *GmTrx* is required for optimal nodule growth. Nodulin-35, a subunit of uricase which is involved in the metabolism of ureides used for transport and storage of nitrogen during biological nitrogen fixation in soybean, is a target of GmTrx [53]. These data suggest that the regulation of nitrogen assimilation through the redox control of uricase may contribute to nodule development in soybean. Whereas their roles in the nodulation process have not been clearly identified, two thioredoxins, specifically expressed during nitrogen-fixing symbiosis and called thioredoxins s, were identified in *M. truncatula* [54]. As no orthologs were found in other plant genomes like *Arabidopsis*, rice (*Oryza sativa*) or poplar (*Populus* spp.), these novel isoforms are thought to be specific to legumes. These proteins have redox potential values similar to those of the classical Trxs, but they possess atypical putative catalytic sites (LCSPC for Trx s1 and WCGQNC for Trx s2) and lack disulfide reductase activity with insulin. They are targeted to the endoplasmic reticulum and their pattern of expression suggests that these novel isoforms function specifically in symbiotic nitrogen fixation in legumes. *In silico* analysis of the *Trx s1* and *Trx s2* expression in different organs and under different growth conditions using the *M. truncatula* gene expression atlas [55,56] showed that *Trx s1* is mainly expressed during the nodulation process and that *Trx s2* seems to be less specific (Fig. 2A). Analysis of spatial localization of *Trx s1* and *Trx s2* expression using the symbimics website which gives access to the laser-capture microdissection coupled to RNA sequencing in *M. truncatula* root nodules [57] showed that the two thioredoxin s are mainly expressed in the meristematic and infection zones of the nodule (Fig. 2B and C). The expression pattern of the thioredoxins s suggests that they are involved in nodule development and the cellular differentiation which occurs in zone II.

The importance of the glutathione-S-transferase (GST) in nitrogen-fixing symbiosis has been demonstrated in soybean [58]. As for other enzymatic antioxidant families, this family consists of a large number of isoforms. The soybean contains at least 14 forms of GSTs, with GST9 being the most prevalent, as measured by both real-time reverse transcription-polymerase chain reaction and identification of peptides by proteomic analysis. Levels of GST9 increased with nodule aging and down regulation of GST9 by RNA interference on composite plants led to a decrease in nitrogenase activity and an increase in oxidatively damaged proteins. These results indicate that GSTs provide antioxidant defenses that are critical to support nitrogen fixation. In *M. truncatula*, more than 15 putative GSTs are also expressed in the nodules [57]. However, *in silico* expression analysis using the *M. truncatula* gene expression atlas and the symbimics website does not allow the identification of specific root nodule GST isoforms as observed in soybean.

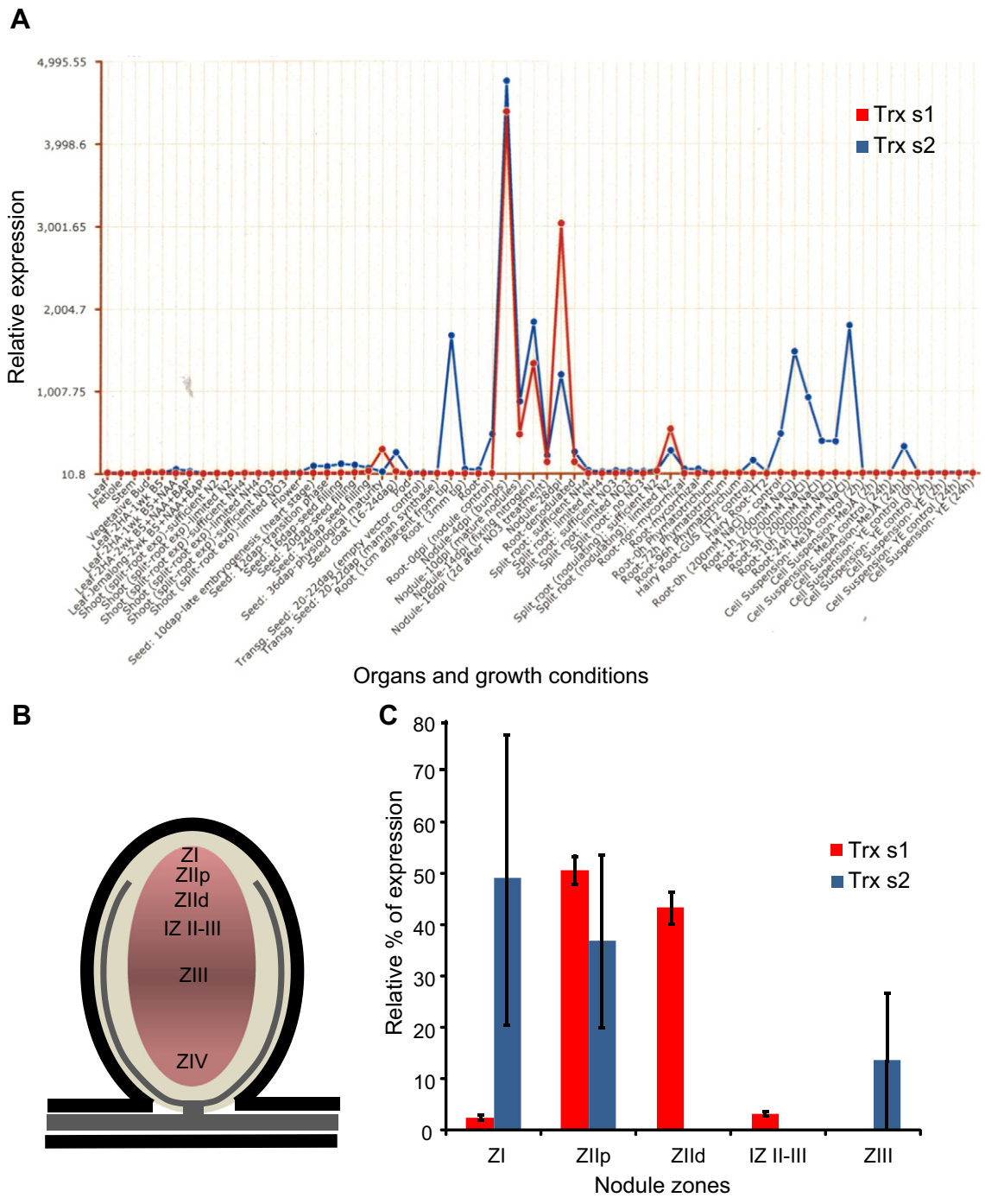

Fig. 2. *In silico* analysis of thioredoxins s expression in *M. truncatula*. (A) Analysis of Trx s1 and s2 expression in different organs and plant growth conditions (Fig. S1 summarizes the various conditions). (B) Nodule regions corresponding to the microdissected nodule zones. (C) Analysis of *Trx s1* and *s2* expression in meristematic zone (ZI), proximal infection zone (ZIIp), distal infection zone (ZIId), infection–fixation interzone (IZ II–III) and nitrogen fixing zone (ZIII). Error bars indicate the SEM.

3. Redox regulation of the bacterial partner

During plant colonization, rhizobia have to cope with the fluctuant accumulation of ROS and RNS mainly produced by the plant partner. They have evolved various strategies to modulate the level of reactive species (RS), including inhibition of RS production, RS detoxification, protection and regulation of their own enzyme activities. The redox signaling involves a fine regulation of the antioxidant defense during plant colonization and bacterial differentiation, and is crucial for the outcome of the interaction. Later, during nodule functioning, ROS are accumulated due to the high respiration rate of nitrogen fixing bacteroids and are involved in the aging of the microsymbiont.

3.1. Redox-control during the infection process and bacteroid differentiation

Following their progression in the IT, the bacteria are released inside the plant cell by a process similar to endocytosis, being entrapped inside a peribacteroid membrane of plant origin. In the proximal infection zone, the bacteria divide few times before initiating their differentiation into nitrogen fixing bacteroids. In developing nodules, an *S. meliloti*

mutant overexpressing a catalase gene, thereby acting as a sink for external H_2O_2 inside the IT, displayed a delayed nodulation phenotype associated with aberrant IT [59]. Thus, the presence of H_2O_2 has a positive effect on IT elongation. NO was also detected during root hair infection and IT growth, by using the NO specific DAF-2DA fluorescent probe, suggesting that NO could be involved in redox signaling [40].

S. meliloti possesses two superoxide dismutases (SOD) and three-heme b-containing catalases [60–63]. Depending on the strain genotype, a *sodB* null mutant displays a differentiation phenotype *in* planta [62,64]. An *S. meliloti* mutant inactivated in the catalases KatB and KatC displayed a drastic phenotype during symbiosis [65]. The aborted nodule contains many IT releasing bacteria inside the plant cell, but the bacteroids undergo senescence just after their release. Peroxiredoxins (Prx) may also contribute to redox equilibrium in the infection zone. The *S. meliloti* genome contains two bacterial specific genes, *ohr1* and *ohr2*, encoding Organic Hydroperoxide Resistant proteins, and the *prxC* gene encoding a peroxidase belonging to the ubiquitous TSA/AhpC peroxiredoxin subfamily [25,66]. *S. meliloti ohr1* and *prxC* genes were shown to be expressed in the IT and in the infection zone (Mandon et al., unpublished observations). The Ohr peroxidases play a central role in the response against long chain fatty acid peroxide damage, while members of peroxiredoxins detoxify a wide variety of small alkyl peroxides and may act as the primary scavenger of H_2O_2 under low peroxide concentration [66,67]. Inactivation of one of the three glutaredoxins of *S. meliloti*, SmGRX1, also leads to nodule abortion. Numerous symbiosomes are visible inside the plant cells infected with the *Smgrx1* mutant, but the bacteria are spherical and show a general lack of bacteroid differentiation [68]. SmGRX1 has been shown to contribute to protein deglutathionylation and to defense against H_2O_2 in free-living bacteria. Altogether, these observations suggest that a tight control of the ROS balance is required for bacteroid differentiation and that thiol-based post-translational modifications, such as S-glutathionylation, may play a key role in modulating the function of proteins essential for this process.

Protein targets of this redox regulation remain largely undefined. However the NCR peptides, which control the terminal differentiation of bacteroids, contain 4 or 6 cysteine residues potentially sensitive to glutathionylation and/or RS post-translational modifications. It has been shown that the oxidation state of the cysteine residues influences the NCR activity on free-living *S. meliloti* [69]. SmGRX1 could react with NCRs to regulate their activity, as demonstrated for Trx with human defensins [70]. Moreover, the expression of *Smgrx1* and *SmtrxA* is induced in free *S. meliloti* treated with NCR peptides, suggesting that they play a role in the bacterial response to NCR during symbiosis [71,72]. Another potential target for these cysteine-based modifications, is the essential enzyme ribonucleotide reductase (RNR) that contains a redox active thiol group for catalysis and is a substrate of the Trx/Grx pathways [73]. Interestingly, *S. meliloti* encodes a cobalamin-dependent class II RNR, which is specifically required for bacteroid differentiation. The *Escherichia coli* Class Ia RNR cannot be substituted for *S. meliloti* RNR in nodules, probably due to its inactivation by ROS and/or inadequate oxygen availability [74].

During the late stage of differentiation, microaerobic conditions and cellular redox state contribute to the induction of mature nodule specific metabolic enzymes. Particularly, ROS-dependent post-translational modifications modulate the activity of main regulators of nitrogen fixation. These modifications constitute important components of the signaling cascade that controls the expression of nitrogen fixation genes in response to oxygen pressure. In rhizobacteria, the genes involved in nitrogen fixation and in the microaerobic respiratory pathways are controlled *via* the FixLJ/K and NifA cascades (Fig. 3), NifA is the ubiquitous regulator of *nif*/*fix* genes of diazotrophic bacteria [75]. The heme protein kinase FixL is an oxygen sensor which induces *fixK* expression in a FixJ-dependent manner. In *S. meliloti*, both *nifA* and *fixK* genes are directly FixJ-dependent, while in *Bradyrhizobium japonicum nif*/*fix* genes and symbiosis-related genes depend on two interlinked regulatory cascades FixLJ–FixK2 and RegSR–NifA. The FixK2 in turn induces the expression of the three transcriptional regulators *fixK1*, *rpoN1* (the NifA-dependent sigma factor) and *nnrR*, leading to the overexpression of microaerobic respiratory genes, NifA-dependent *nif*/*fix* genes and the nitrate alternative respiratory pathway [76,77]. The NifA-oxygen sensitivity correlates with the presence of an invariant Cys-X4-Cys motif, most likely a redox-target [75]. FixK2 can be reversibly inactivated by the formation of an intermolecular disulfide bridge and irreversibly inactivated by the formation of sulfenic or sulfonic acid derivatives [78]. In parallel, RegSR is a member of two-component redox-sensitive systems well characterized in *Rhodobacter* species. In *Rhodobacter capsulatus*, the redox sensor RegB contains a highly conserved redox-active cysteine that can form intermolecular disulfide bridges under oxidizing conditions, thus converting active RegB dimers into inactive tetramers [79,80]. The redox state of the ubiquinone pool has been shown to regulate the transmembrane protein RegB kinase activity [81].

NO also constitutes a signaling molecule for the microsymbiont, which modulates both the production and degradation of this RNS, using the flavohemoglobin Hmp to transform NO into nitrate or nitrous oxide [82]. In the *M. truncatula* nodules, around one-third of the NO-generated in nodules is produced by the rhizobial denitrification pathway [42]. This pathway depends on the *napEDABC*, *nirK*, *norCBQD*, and *nosRZDYFLX* genes that encode periplasmic nitrate reductase (NR), periplasmic nitrite reductase (NiR), NO reductase (NOR), and nitrous oxide (N_2O) reductase (NOS), respectively [83,84]. In rhizobial species, denitrification gene expression depends on low oxygen conditions and the presence of nitrate or oxide derivatives *via* the regulator NnrR first identified in *Rhodobacter sphaeroides* [85].

In *B. japonicum*, the maximal induction of the *nnrR* gene requires the activation of both FixLJ–FixK2 and RegSR–NifA pathways [76,86]. In *S. meliloti*, the FixLJ–FixK and NnrR are also the main regulators of the NO response [87,88]. However, FixK and NnrR belong to two independent signaling pathways that respond to NO [87,89]. The ability of FixL to bind NO in addition to O_2 and direct activation of FixJ-dependent genes in the presence of an NO donor was reported leading to an overlap between the microoxic energetic and the denitrification pathways [87,90]. Thus, a maximal expression of the denitrification genes may occur in zone III where NO and microoxia occur. Although *Rhizobium etli* is unable to use nitrate for respiration, the bacteria contain a nitrite reductase (NirK) and a NO reductase (NorC), which are important during symbiosis. Similarly to soybean nodules induced by a *B. japonicum napA* mutant, a mutation in *R. etli norC* or *nirK* genes modulates significantly the level of nitrosylleghemoglobine (LbNO) in bean nodules [84,91].

3.2. Redox-control in nodule functioning

In addition to the ROS produced by the plant partner, the high respiratory rate of nitrogen fixing bacteria contributes significantly to ROS levels and bacteroids contain numerous ROS-scavenging enzymes. Their role is essential for nodule functioning, as the nitrogenase activity depends on a reducing environment and many enzyme activities may be influenced by the redox state. Thus, the regulation of protein function *via* oxidative modification has been highlighted for various proteins of nitrogen-fixing bacteroids. Twenty sulfenylated enzymes have been detected in bacteroids, including proteins related to carbohydrate and nitrogen metabolism, showing that sulfenylation may regulate the activity of proteins playing a major role in nodule functioning [92].

A fine-tune control of the redox state is crucial for bacteroid persistence. Proteomic analyses showed the presence of SOD in both bacteroids and free-living cells of *S. meliloti* and *B. japonicum* [93,94]. In addition, a *sodA* mutant of *Mesorhizobium loti* displays a variable symbiotic defect depending on the *L. japonicus* cultivar used [95]. The *Rhizobium leguminosarum* OxyR-dependent Fe/Mn Sod is present in the cytosol of bacteroids and is secreted in the cytosol of infected and uninfected cells of pea nodules [96]. Oxidative stress responsive catalases are

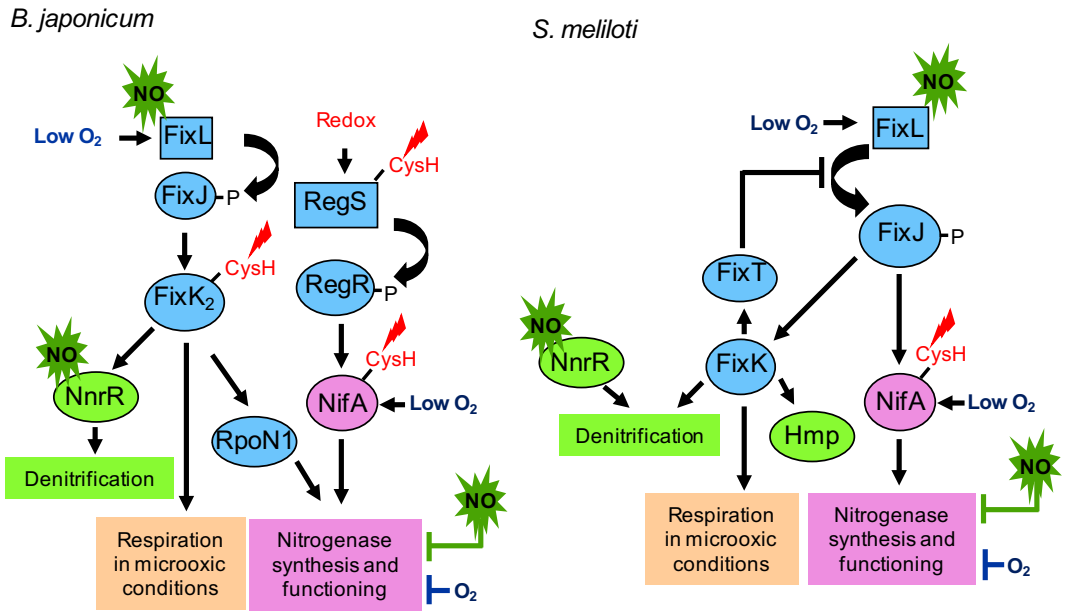

Fig. 3. Redox signaling in regulatory pathways of nitrogen fixation under microoxic conditions in *S. meliloti* and *B. japonicum*. The low oxygen tension activates the *S. meliloti* FixLJ–FixK and the *B. japonicum* FixLJ–FixK2/RegSR–NifA regulatory cascades. NO regulates the transduction pathways at transcriptional and post-translational levels. The redox state modifications regulate the activities of important components of the pathways.

important to sustain the nitrogenase activity. The *S. meliloti katA*/*katC* double mutant displayed a drastic reduction in nitrogen fixation capacity and early senescence [65]. In *M. loti*, only the *katE* mutant showed a decrease (50%) of the nitrogenase activity [97]. Members of peroxiredoxins detoxify a wide variety of small alkyl peroxides and may display a very high affinity towards H_2O_2. Due to the functional redundancy of catalases and peroxiredoxins, some rhizobacteria use both types of enzymes to scavenge H_2O_2. The *katG–prxS* double mutant of *R. etli* is affected in its nitrogen fixation capability with a 40% loss of nitrogenase activity [98]. The *ohr* and *prxC* genes are fully expressed in *S. meliloti* bacteroids [66,93]. In contrast, neither catalase nor peroxiredoxin has been detected in bacteroid extracts from *B. japonicum* [94]. In *S. meliloti*, the presence of TrxB, a NADPH-dependent thioredoxin reductase involved in protein reduction, was also reported [93]. The Trx-like protein TrxL from *S. meliloti* (CE52G) was shown to be required for optimal nitrogen fixation efficiency. In free-living bacteria a *trxL* mutant was affected in its response to oxidative stress induced by paraquat, suggesting that TrxL may modulate the bacteroid redox potential during nitrogen fixation [99].

GSH is present in infected cells of *P. sativum* more particularly in bacteroids, where total glutathione (reduced + oxidized) increases with nodule progression from the young up to mature stage [100]. These finding suggest that bacterial GSH is important for nodule functioning, a hypothesis that was confirmed in different symbiotic interactions by inactivating the GSH synthetase encoding gene *gshB*. During interaction with their respective hosts, nodules induced by different *gshB* strains presented an abnormal development and early senescence [101–103]. The early senescent pattern of *P. vulgaris* nodules infected with a *Rhizobium tropici* mutant was correlated to increased levels of superoxide accumulation, whereas *gshB* expression increased in mature and early senescent nodules infected with the WT strain. These different observations suggest that the bacterial GSH contributes to the protection of N_2 fixing bacteroids against ROS, and thus to their persistence. In *R. etli*, a new metabolic relationship between GSH and glutamine has been identified, that may also be important for nitrogen fixation efficiency and nodule maintenance [103]. The GSH biosynthetic pathway is positively regulated by the LysR-like regulator LsrB, which also controls the expression of the redox sensor OxyR [104]. Whereas *oxyR* inactivation did not affect symbiosis efficiency [105], an ls*rB*-mutant induced ineffective nodules in alfalfa, that could partly result from GSH depletion [106]. An *S. meliloti* glutaredoxin, SmGRX2, involved in the regulation of iron metabolism, also contributes to bacteroid persistence in *M. truncatula* nodules. Sm*grx2* inactivation decreased nodule development and N_2 fixation capacity of bacteroids, without affecting bacteroid differentiation, highlighting the importance of iron homeostasis in nodule functioning [68]. Nitrogen reduction could be directly regulated by SmGRX2 activity as the nitrogenase activity involves the interaction of two major components, the iron (Fe) protein containing a Fe–S cluster and the MoFe protein containing Fe–S and Fe–Mo clusters.

NO is also an essential determinant for the maintenance of a functional nitrogen fixing zone and the *hmp* gene of *S. meliloti* is required for optimal nitrogen fixation [87]. As the increase of ROS and RNS in nodules correlates with aging of the bacteroids, overexpression of the antioxidant defense enzymes should delay the onset of senescence. Indeed, an *S. meliloti* mutant overexpressing *hmp* showed a clear delay in nodule senescence [43]. These authors proposed that NO is a non-systemic signal that triggers nodule senescence. As ROS might also contribute to initiate senescence, overexpression of antioxidant enzymes such as bacterial catalases, might contribute to the persistence of functional nodules.

4. Conclusion

During the last years, the characterization of ROS and RNS metabolism and the analysis of the antioxidant defense in the nodule have demonstrated the importance of redox regulation in nitrogen-fixing symbiosis [25,44,107]. The results obtained showed the complexity of the regulation which involves spatial (root epidermis and cortex; nodule zones) and temporal factors (infection process, cellular differentiation process, nitrogen fixation process) as well as a plant–microbe crosstalk during the entire life time of the nodule. The characterization of these different elements is essential to define the precise roles of the different components involved in the redox processes. In this context, the production of biological tools to detect *in vivo* specific, quantitative, dynamic modifications of ROS, RNS and antioxidant molecules is needed to analyze the cellular modifications occurring during the nitrogen symbiosis. H_2O_2 specific fluorescent probes and

redox-sensitive proteins are good examples of these tools [108,109]. The generation of mutant and/or transgenic lines allowing the modification of gene expression would help to define the importance and the roles of genes involved in the production of ROS/RNS and in the antioxidant defense. The generation of mutant libraries from *M. truncatula* [110,111] and *L. japonicus* [112] will allow the screening of mutant lines needed to establish the roles of the different genes. Although the analysis of mutant lines is important to establish the function of individual proteins or metabolites, the redox regulation is strongly dependent on the post-translational regulation based on reversible redox-modifications such as oxidation, nitrosylation and glutathionylation of protein targets. The recent reports on oxidation [92] and nitrosylation [25] of proteins during the symbiotic process are first steps to identify the proteins targets *via* redox proteomics approaches [113]. These analyses will help to define the early signaling events involved in redox regulation. The redox regulatory network involves a huge number of metabolites and proteins which should be coordinated to define a final signal. Moreover, these redox molecules are located in different cellular compartments (cytosol, nucleus, mitochondria, plastids and symbiosomes) which should also communicate to allow a good coordination of the multiple metabolic pathways involved in the symbiotic nitrogen fixation [114]. Analysis of this supracellular crosstalk that involves the plant metabolic status as well as its biotic and abiotic environment sensing promises to provide exciting results which should lead to an extension of our knowledge on redox regulation in plants.

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