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Review

Nodules and oxygen

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Abstract In root nodule symbioses, bacterial microsymbionts are hosted inside plant cells and supply the host plant with the products of biological nitrogen fixation, rendering it independent of soil nitrogen sources. Two types of such interactions are known, legume/rhizobia symbioses involving several alpha- and beta-proteobacterial genera, collectively called rhizobia, and members of the Leguminosae (Fabaceae) family, and actinorhizal symbioses involving members of the Gram-positive actinomycetous genus *Frankia* and a diverse group of plants from 25 genera from eight different families, collectively called actinorhizal plants, with one exception trees or woody shrubs.

Key words: Actinorhiza, Chitinase, *Frankia*, rhizobia, root nodule.

The oxygen dilemma of nitrogen fixation

N_2 fixation is catalyzed by the enzyme nitrogenase which is highly sensitive to O_2 and can only function in an O_2-free environment. For aerobic bacteria like rhizobia and *Frankia* strains, this leads to the so-called oxygen dilemma of nitrogen fixation: a high O_2 flux to the respiratory chain is required adjacent to a vanishingly low O_2 concentration at the sites of N_2 fixation. In order to solve this problem, external O_2 barriers are combined with high O_2 utilization at the nitrogenase site to obtain a steep O_2 gradient. Only a few prokaryotes can create their own O_2 diffusion barrier that enables them to perform N_2 fixation in air. This group includes *Frankia* strains which produce specialized vesicles whose cell walls show high O_2 diffusion resistance. Rhizobia rely on the plant host to provide oxygen protection for nitrogenase. Legume nodules are stem-like organs with peripheral vascular bundles in the so-called nodule cortex and the rhizobia-containing cells in the inner tissue. The nodule endodermis, a part of the nodule cortex that is interrupted at the nodule apex, forms the O_2 diffusion barrier, protecting the bacteria-containing tissue while leaving the vascular system well aerated. O_2 concentrations in the inner tissue are very low, in the range of 50 nM (Bergersen 1996; Hunt and Layzell 1993). In order to provide a high O_2 flux for respiration, an O_2-binding protein, leghemoglobin (Lb), is formed in the infected cells in mM concentrations (Appleby 1984; Hargrove et al. 1997). Lb facilitates O_2 diffusion to the N_2 fixing bacteria (bacteroids) and plant mitochondria (Wittenberg et al. 1974).

Plant hemoglobins

There are three classes of plant hemoglobins (Hbs). The symbiotic Lbs of legumes belong to class II; members of this class are not present in all plant genera, and no function could be assigned to non-symbiotic class II hemoglobins (Trevaskis et al. 1997). Class I Hbs are spread throughout the plant kingdom and differ from class II in that their affinity to O_2 is very high due to a very low dissociation constant (Trevaskis et al. 1997). Their function seems to lay in modulating levels of nitric oxide (NO), an inhibitor of mitochondrial electron transport that is also an important second messenger in plants, involved in a broad range of developmental processes and in pathogen defense signaling (Lamattina et al. 2003). Class I Hbs are supposed to be involved not only in NO scavenging, but also in connecting NO turnover and the maintenance of the redox balance in the plant cell (Igamberdiev et al. 2004; Hebelstrup et al. 2007). The sequence of reactions required for NO scavenging under hypoxic conditions has been defined as the Hb/NO cycle (Igamberdiev and Hill 2004; Figure 1). The third class of plant Hbs is represented by homologues of microbial truncated Hbs (2-on-2 Hbs; reviewed by Wittenberg et al. 2002) which seem to have evolved independently of class I and class II Hbs (Garrocho-Villegas et al. 2007). Their function has been suggested to be linked to NO scavenging as well (Milani et al. 2003; Vieweg et al. 2005), but proof has yet to be provided. Truncated hemoglobins are formed in infected cells of legume nodules (Vieweg et al. 2005).

Interestingly, *Parasponia*, the only non-legume genus

whose members can enter a root nodule symbiosis with rhizobia, contains a class I hemoglobin that is present at high levels in infected cells, if also at low levels in roots, and has the oxygen binding kinetics of symbiotic, i.e. class II hemoglobins (Appleby et al. 1983; Wittenberg et al. 1986; Bogusz et al. 1988; Trinick et al. 1989). Since class II hemoglobins are not present in all higher plants, it seems that here a class I hemoglobin was recruited for the function of facilitation of O_2 diffusion. The activity of the promoter of the class I hemoglobin from *P. andersonii* was examined in transgenic legumes (Andersson et al. 1997) and non-symbiotic plants (Bogusz et al. 1990) and found to resemble that of non-symbiotic class I hemoglobins. Hence, it seems likely that the *Parasponia* class I Hb can fulfill both functions, facilitation of O_2 diffusion and NO detoxification. However, since the NO detoxification function of class I hemoglobins was not known when the *Parasponia* Hb was discovered, this was never analysed.

Apart from class I and class II Hbs, Hb sequences similar to microbial so-called truncated (2-on-2) Hbs were found in plants (trHbs; Watts et al. 2001; Wittenberg et al. 2002). Their sequences are highly conserved, and phylogenetic analysis has shown that they evolved through a lineage independent of that of class I and class II Hbs (reviewed by Garrocho-Villegas et al. 2007). TrHbs have been found in vegetative and embryonic plant organs as well as in nodules.

Nodule oxygen protection mechanisms lead to oxidative stress and NO production

In spite of their relevance for O_2 transport, class II Hbs are generally believed to contribute to oxidative stress in nodules, i.e., to the production of reactive oxygen species (ROS) like superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2; Becana et al. 1998; 2000). This has recently been confirmed by Ott et al. (2005) who showed that RNAi inhibition of *leghemoglobin* (*lb*) gene transcription in nodules of *L. japonicus* led not only to an increase in free O_2 and the loss of nitrogenase and nitrogen fixation in nodules, but also to reduced H_2O_2 contents (Günther et al. 2007). The mechanism of ROS production by symbiotic hemoglobins is as follows: Lb (Fe^{2+}) that has bound O_2 can undergo spontaenous autooxidation to inactive Lb (Fe^{3+}) (Becana et al. 2000), while the O_2 molecules are reduced and released as O_2^-. Lb (Fe^{2+}) is then regenerated by the action of ferric-Lb reductase (Ji et al. 1992). O_2^- is used by superoxide dismutase (SOD; Rubio et al. 2004) to form O_2 and hydrogen peroxide (H_2O_2). ROS like O_2^- and H_2O_2, while serving also as second messengers (Torres and Dangl 2005), can cause oxidative damage to membranes and various other cellular components and have to be detoxified quickly. The main pathway for ROS detoxification in plant tissues including nodules, is the ascorbate-glutathione cycle (Figure 1; Noctor and Foyer 1998; Matamoros et al. 2003). Ascorbate levels, the activities of enzymes of the ascorbate-glutathione pathway, or both use to be enhanced in legume nodules compared to roots (summarized by Günther et al. 2007).

Class II Hbs are not the only source of ROS in nodules. Due to the O_2 diffusion barrier, the inner tissue of legume nodules is hypoxic, i.e. respiratory activity will easily exceed O_2 availability. Plant mitochondria react to hypoxia with the production of ROS (Fukao and Bailey-Serres 2004). Furthermore, O_2 can be reduced directly by nitrogenase, hydrogenase and ferredoxin in the bacteroids (Dalton 1995). Not surprisingly, legume nodules contain high activities of the ascorbate-glutathione cycle enzymes (Matamoros et al. 1999a; 2003) and millimolar concentrations of ascorbate (Matamoros et al. 1999b).

Hypoxia also leads to the production of NO (Neill et al. 2008). Baudouin et al. (2006) have shown that NO is produced in rhizobia-containing cells of nodules of the legume *Medicago truncatula*. Consistent with their function of lowering internal NO levels, class I hemoglobins are formed in the infected cells of *Lotus japonicus* nodules (Uchiumi et al. 2002; Shimoda et al. 2005). The reconstitution of class I Hb after NO scavenging requires the oxidation of ascorbate (Figure 1). So the detoxification of ROS and NO is linked via ascorbate.

Limitations of legume nodule research

Understanding the role of oxidative stress in nodules is of critical importance since it seems to be responsible for the drought-induced inhibition of nitrognenase in legume nodules. Research is hampered by the fact that the roles of the different components of this complex system cannot easily be disentangled *in vivo*. While nodules of *L. japonicus* deficient in Lb displayed reduced H_2O_2 contents (Günther et al. 2007), it was not clear in how far the lack of nitrogenase activity, Lb protein and reduced respiratory activities as denoted by a two- to threefold decrease in the ATP/ADP ratio compared to wild type nodules, contributed to this reduction in oxidative stress. Hence, in understanding the contribution of the different compounds of the oxygen protection system to oxidative stress in legume root nodules, comparison between root nodule types where nitrogen-fixation does not involve the production of a plant hemoglobin, would be helpful.

Oxygen protection of nitrogenase in actinorhizal nodules

In actinorhizal root nodules, oxygen protection mechanisms are far more diverse than in legumes, which

is probably a reflection of the diversity of plant families entering a root nodule symbiosis with *Frankia*. Furthermore, as mentioned above, *Frankia* strains can perform oxygen protection of nitrogenase themselves by forming specialized cells, vesicles, surrounded by multilayered envelopes containing bacterial steroid lipids (hopanoids) at the ends of hyphae or short side branches, where nitrogenase can be active under aerobic conditions (Berry et al. 1993). The thickness of the envelope depends on the oxygen tension (Harris and Silvester 1992). Vesicles are always round and septate in culture, but in symbiosis, their shape and their location inside the infected cell depends on the host plant species (Silvester et al. 1990).

In contrast to legume nodules (Figure 2A), actinorhizal nodules are coralloid organs consisting of multiple modified lateral roots with central vascular tissue and infected cells in the expanded cortex (Figure 2B–2F). They are surrounded by a superficial periderm which can be interrupted by lenticels (*Alnus*, *Datisca*, *Coriaria*) to allow gas access or, in case of species often exposed to flooding, upward growing roots with large air spaces in the cortex, so-called nodule roots (*Casuarina*, *Myrica*, *Datisca*). In actinorhizal nodules, an O_2 diffusion barrier surrounding the entire infected tissue as found in legume nodules, would cut off the central vascular system from O_2 supply. Nevertheless, one actinorhizal symbiosis exists where oxygen protection mechanisms resemble those found in legumes: in nodules of *Casuarina glauca*, a class II hemoglobin is present in high concentrations in infected cells (Fleming et al. 1987; Jacobsen-Lyon et al. 1995), and *Frankia* does not form vesicles (Berg and McDowell 1988). O_2-microelectrode analysis has shown that the infected cells of *C. glauca* nodules are in a low O_2 environment (Tjepkema 1979), presumably due to the fact that their cell walls, and the walls of adjacent uninfected cells, are impregnated with a very hydrophobic lignin (Berg and McDowell 1987).

The situation in *Myrica* nodules appears similar on first view: they are aerated by nodule roots, the walls of nodule cortical cells become lignified upon infection (Tjepkema and Asa 1987), and this modification, while not examined in detail as for *C. glauca* nodules, is likely to play a role in slowing the diffusion of O_2 into the infected cells which show low pO_2 (Zeng and Tjepkema 1994) and are not penetrated by India ink. However, in contrast with *C. glauca*, apart from the pockets of infected cells the cortex seems well-aerated, and *Frankia* does form vesicles in *Myrica* nodules (Tjepkema 1979;

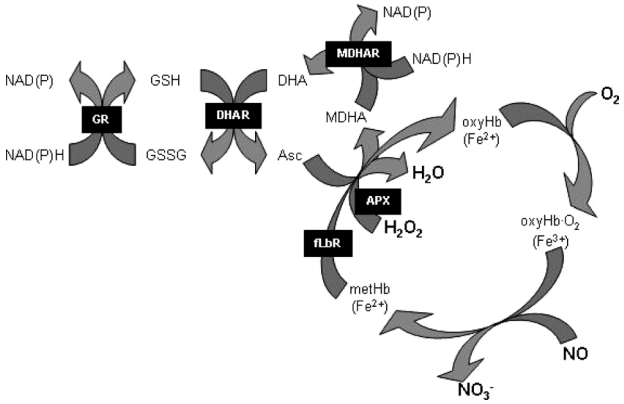

Figure 1. Detoxification of H_2O_2 in the ascorbate-glutathione cycle and detoxification of NO via class I Hb in the Hb/NO cycle. H_2O_2 is produced from the superoxide anion, O_2^-, by superoxide dismutase (not shown). Asc, ascorbate; APX, ascorbate peroxidase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; fLbR, ferric-Lb reductase; GR, glutathione reductase; GSH, glutathione; metHb, methemoglobin; oxyHb, oxyhemoglobin; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase. Ascorbate serves as electron donor for the detoxification of H_2O_2 via APX and for the reconstitution of oxyHb from metHb formed during detoxification of NO. Based on Gossett et al. (1996) and Igamberdiev et al. (2006).

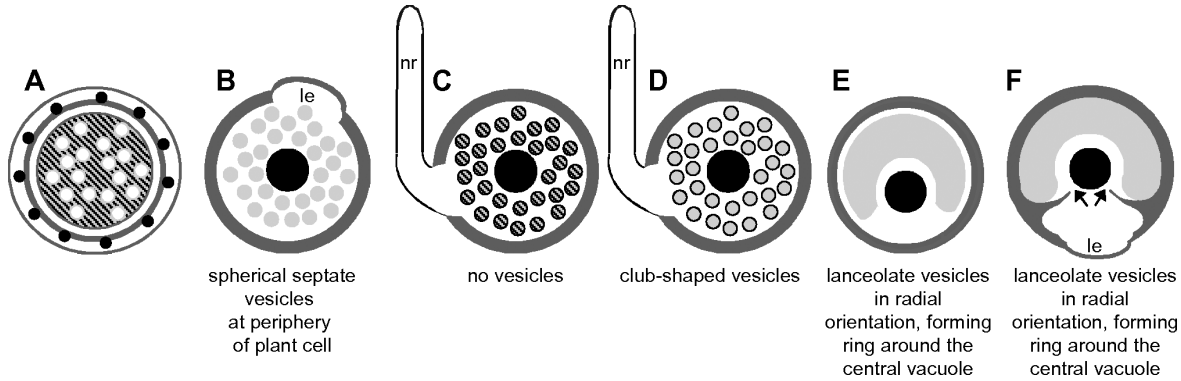

Figure 2. Schematic cross sections through (A) legume nodule and actinorhizal nodule lobes from (B) *Alnus*, (C) *Casuarina*, (D) *Myrica*, (E) *Datisca* and (F) *Coriaria* and description of the vesicles formed by the N_2-fixing microsymbionts in B–F. Vascular bundles are depicted in black, oxygen diffusion barriers (nodule endodermis in legume nodule, periderm in actinorhizal nodules, around infected cells in *Casuarina* and hypothetically in *Myrica*) in dark grey. Infected cells are depicted in grey; infected cells containing a class II Hb are hatched. le, Lenticel; nr, nodule root. The arrows in (F) point at the narrow areas where O_2 has to diffuse through in order to reach the infected cells.

Tjepkema 1983) with envelopes of similar thickness as in the well-aerated nodules of *Alnus* (Berg 1994). Furthermore, while *Myrica* nodules, like *C. glauca* nodules, contain high concentrations of a hemoglobin (Pathirana and Tjepkema 1995), this is not a class II hemoglobin (Heckmann et al. 2006).

In contrast, actinorhizal nodules of *Alnus*, *Datisca* and *Coriaria* are well aerated with continuous air spaces leading from lenticels in the surrounding periderm to the surfaces of infected cells as shown by O_2-microelectrode analysis and India-ink infiltration (Silvester and Harris 1989; Tjepkema 1979; Tjepkema et al. 1988). In *Alnus* nodules, *Frankia* forms spherical septate vesicles with thick envelopes at the periphery of the infected cells, in form similar to the vesicles formed in the free-living state (Newcomb and Wood 1987). In nodules of *Datisca* and *Coriaria*, *Frankia* forms lanceolate vesicles oriented towards the cell center, positioned in around the central vacuole in radial orientation (Newcomb and Pankhurst 1982; Hafeez et al. 1984). The tight packaging of these vesicles leads to a very low surface exposure (Silvester et al. 1999). Plant mitochondria are clustered at the vesicle base, presumably to contribute to oxygen scavenging by plant respiration (Silvester et al. 1999). In spite of the well aerated nodule cortex, the thin vesicle envelopes indicate that they exist in a low oxygen environment.

Oxidative stress and NO in actinorhizal nodules

While a class II hemoglobin has only been found in nodules of the actinorhizal genus *Casuarina*, two close relatives of *Casuarina* contain elevated amounts of a class I hemoglobin in their nodules. Hemoglobins were purified from the nodules of *Alnus glutinosa* (Suharjo and Tjepkema 1995) and *Myrica gale* (Pathirana and Tjepkema 1995). The *Alnus* hemoglobin was not present in nodules in amounts comparable to that of the symbiotic hemoglobin from *Casuarina* nodules, but the *Myrica* hemoglobin was. Cloning of the corresponding genes from *A. firma* (Sasakura et al. 2006) and *M. gale* (Heckmann et al. 2006) revealed that both proteins represented class I hemoglobins, and for the *A. firma* hemoglobin a function in NO scavenging could be demonstrated experimentally.

The *hbI* gene of *M. gale* was expressed at high levels in nodules and at low levels in non-symbiotic organs, and the expression pattern of a promoter-GUS fusion in Arabidopsis had similarities with those of the endogenous class I and class II hemoglobin genes (Heckmann et al. 2006). It was induced by hypoxia and by ethylene. The *A. firma* HbI gene, however, was induced by nitrate, nitrite and NO and also by cold stress, but not by hypoxia (Sasakura et al. 2006).

So in two close relatives of *Casuarina*, class I hemoglobins were present at enhanced levels in nodules compared to roots. Since the kinetic properties of both hemoglobins were not examined, it cannot be excluded that they have, or one of them has, acquired a function in the facilitation of O_2 diffusion, comparable to the class I hemoglobins of *Parasponia* sp., but it would be quite surprising if within one phylogenetic branch of actinorhizal plants that presumably goes back to a common ancestor (Swensen and Mullin 1997), different hemoglobins were recruited for the O_2 diffusion facilitation function. In legumes, class II hemoglobins have been found in nodules throughout, although it is not clear how often the symbiotic syndrome evolved independently within the legume family (Doyle and Luckow 2003). However, it is possible that the common symbiotic ancestor of *Casuarina*, *Alnus* and *Myrica* had a symbiosis where *Frankia* was solely responsible for O_2 protection of nitrogenase within the nodule, and that hemoglobins were recruited at a later step. This would not have been possible in legumes since rhizobia cannot fix nitrogen aerobically.

The other explanation for the presence of elevated levels of class I hemoglobins in nodules of *Alnus* and *Myrica* would be that they are not involved in the facilitation of O_2 diffusion, but in modulating NO levels (Igamberdiev et al. 2004; Hebelstrup et al. 2007). The ascorbate-glutathione pathway has not been examined in actinorhizal nodules, except that ascorbate peroxidase (APX) levels were analysed in *Alnus rubra* and found to be at least an order of magnitude higher than in legume nodules (Dalton et al. 1987). Glutathione reductase (GR) levels, however, were similar in *A. rubra* and legume nodules. The high APX activities in *A. rubra* nodules might indicate a high turnover of metHb in the detoxification of NO, reflecting the high concentrations of a class I hemoglobin in these nodules (Figure 1).

Truncated hemoglobins in nodules

The functions of microbial truncated hemoglobins (TrHbs; three subgroups, TrHbOs, TrHbNs and TrHbPs) seem to be diverse and to include the detoxification of NO (TrHbN; Ouellet et al. 2002) and the facilitation of oxygen diffusion (TrHbO; Liu et al. 2004). One organism can contain trHbs of more than one group, but all plant trHbs represent one subgroup of TrHbNs (Wittenberg et al. 2002; Vuletich and Lecomte 2006). Truncated hemoglobins of the TrHbO and TrHbN type have been found in rhizobia and also in *Frankia* (summarized by Pawlowski et al. 2007).

In the legume *M. truncatula*, two *trHb* genes were found to be expressed at elevated levels in nodules compared to roots, one of them specifically in infected cells (Vieweg et al. 2005). Expression of plant *trHb*

genes in nodules could also be shown in other legume species (*Phaseolus vulgaris*, GenBank accession CV537139; soybean, GenBank accession no. AI988677). Elevated plant *trHb* gene expression was also found in actinorhizal nodules from *Datisca glomerata* where their expression was confined to the infected cells (Pawlowski et al. 2007). *Frankia trHbO* was found to be expressed during symbiosis with *D. glomerata* nodules (Pawlowski et al. 2007), but no data are available about the expression microsymbiont *trHb* genes in other root nodule symbioses.

The function of rhizobial and *Frankia trHbO* and *trHbN* genes has not yet been determined unequivocally. However, studies on oxygen dissociation rates and regulation of gene expression in culture have been performed (Beckwith et al. 2002; Schwintzer and Tjepkema 2005), and for *Frankia* trHbO, a role in the facilitation of oxygen diffusion has been suggested based on nodule oximetry data (Pawlowski et al. 2007). Earlier studies on CO-reactive heme contents of actinorhizal nodules demonstrate that if a class I hemoglobin is present in *D. glomerata* nodules as shown for *A. firma* (Sasakura et al. 2006) and *M. gale* (Heckmann et al. 2006), it is only present at extremely low levels.

Conclusions

When comparing the presence of the three different types of hemoglobins in root nodules, and particularly in infected cells, from legumes and actinorhizal plants (Table 1), it becomes obvious that nodules that contain elevated levels of a TrHb compared to roots, do not contain significant amounts of a class I Hb. Two functions have been suggested for TrHbs from plants: NO detoxification (Vieweg et al. 2005) similar to that of class I Hbs, and a function in O_2 transport (Garrocho-Villegas et al. 2007).

Assuming a function of TrHbs in NO detoxification, it appears plausible that either class I Hbs or TrHbs could be recruited for NO scavenging in nodules since NO is formed during N_2 fixation (Cueto et al. 1996). The fact that even relatively closely related legumes, *M. truncatula* and *L. japonicus*, differ in which NO-scavenger is formed in their infected cells (Uchiumi et al. 2002; Vieweg et al. 2005), suggests that the need for NO scavenging was not a basal feature in the evolution of root nodules. Maybe in the beginning, microsymbiont NO scavenging systems (TrHbN?) were sufficient. In this context, it is not surprising that plants from closely related actinorhizal genera, namely *Casuarina*, *Alnus* and *Myrica*, should also differ with regard to their type of NO-scavenger.

It also appears that in nodules where the infected cells are located in a well-aerated environment, as is the case for *Alnus*, *Myrica* and *Datisca*, the need for NO scavenging—i.e., the concentrations of the class I Hb or TrHb—is higher than in nodules where the infected cells are in a microaerobic environment (legumes, *Casuarina*). This is not consistent with the fact that hypoxia increases NO production and that the transcription of many plant class I *hb* genes, e.g., the one from *M. gale*, is induced by hypoxia (Heckmann et al. 2006). Yet, it would be consistent with the fact that the transcription of the Arabidopsis *trHb* gene, encoding another putative NO scavenger, is reduced by hypoxia (Watts et al. 2001). On the other hand, assuming a function of trHbs in O_2

Table 1. D121ifferent types of plant hemoglobins in root nodules from legumes and actinorhizal plants. Plants with infected cells with well-aerated infected cells are shaded in grey. 'High levels' or 'low levels' refers to gene expression levels or protein levels, depending on the type of analysis performed in the cited paper.

	Medicago truncatula	*Lotus japonicus*	*Alnus glutinosa*	*Casuarina glauca*	*Myrica gale*	*Datisca glomerata*
class I Hb	**not expressed in nodules** (www.tigr.org)	**yes,** low levels, cellular localization unknown (Uchiumi et al. 2002)	**yes,** relatively high levels (Sasakura et al. 2006)	**yes,** low levels, not in infected cells (Jacobsen-Lyon et al. 1995)	**yes,** high levels, cell-specific localization unknown (Heckmann et al. 2006)	**(no)** possible only at extremely low levels (Asa and Tjepkema 1987)
class II Hb	**yes,** high levels, infected cells (Carvalho et al. 2003)	**yes,** high levels, infected cells (Kapranov et al. 1997)	**no** (Suharjo and Tjepkema 1995; Sasakura et al. 2006)	**yes,** high levels, infected cells (Jacobsen-Lyon 1995)	**no** (Pathirana and Tjepkema 1995; Heckmann et al. 2006)	**(no)** possible only at extremely low levels (Asa and Tjepkema 1987)
2-on-2 Hb	**yes,** low levels, infected cells and vascular system (Vieweg et al. 2005)	**not expressed in nodules** (www.tigr.org)	**unknown**	**unknown**	**unknown**	**yes,** high levels, infected cells (Pawlowski et al. 2007)

transport instead of NO detoxification, it would seem that there is no plant NO scavenging system in nodules of *M. truncatula* and of *D. glomerata*. Furthermore, there would be no plant NO scavenging system in infected cells of *C. glauca*. Altogether, a function of plant trHbs in NO scavenging seems to fit better with the available data.

In seeds, NO has been suggested to mediate the integration of O_2 uptake, respiratory control and ATP availability (Borisjuk et al. 2007). Even well-aerated nodules, like roots, will be frequently challenged with limited O_2 supply (Drew 1997). It seems plausible that the basic levels of NO required for the integration of O_2 uptake and respiratory control are higher in a well aerated tissue than in an microaerobic tissue, and that these higher basic levels of NO also necessitate a higher capacity of the NO scavenging system.

Altogether, we are still far from understanding the interplay of oxygen protection of nitrogenase and ROS control in root nodules. An aspect that deserves further examination is the presence and function of bacterial Trhbs in symbiosis.

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