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Hemoglobins in the legume-rhizobium symbiosis

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Dedication: In memory of Professor Tomás Ruiz-Argüeso, an excellent scientist, family man and friend.

Summary

Legume nodules have two types of hemoglobins: symbiotic or leghemoglobins (Lbs) and nonsymbiotic or phytoglobins (Glbs). The latter are categorized into three phylogenetic classes differing in heme coordination and O_2 affinity. This review is focused on the roles of Lbs and Glbs in the symbiosis of rhizobia with crop legumes and the model legumes for indeterminate (*Medicago truncatula*) and determinate (*Lotus japonicus*) nodulation. Only two hemoglobin functions are well established in nodules: Lbs deliver O_2 to the bacteroids and act as O_2 buffers, preventing nitrogenase inactivation; and Glb1-1 modulates nitric oxide concentration during symbiosis, from the early stage, avoiding the plant's defense response, to nodule senescence. Here, we critically examine early and recent results, update and correct the information on Lbs and Glbs with the latest genome versions, provide novel expression data, and identify targets for future research. Crucial unresolved questions include the expression of multiple Lbs in nodules, their presence in the nuclei and in uninfected nodule cells, and, intriguingly, their expression in nonsymbiotic tissues. RNA-sequencing data analysis show that Lbs are expressed as early as a few hours after inoculation and that their mRNAs are also detectable in roots and pods, which clearly suggests that these heme proteins play additional roles unrelated to nitrogen fixation. Likewise, issues awaiting investigation are the functions of other Glbs in nodules, the spatiotemporal expression profiles of Lbs and Glbs at the mRNA and protein levels, and the molecular

mechanisms underlying their regulation during nodule development and in response to stress and hormones.

Key words: leghemoglobins, model legumes, nitric oxide, nitrogen fixation, oxygen transport, phytoglobins, rhizobium, symbiosis

Introduction

Legumes are second only to cereals in terms of agronomic importance as a prime source of protein and oil for humans and animals. They establish symbioses with bacteria, collectively known as rhizobia, forming nodules on the roots and occasionally on the stems (Sprent & James, 2007). Inside the nodules bacteria differentiate into bacteroids that reduce atmospheric N_2 to ammonia for use by the plant. The onset and development of this symbiosis requires the participation of various types of hemoglobins that are described in detail in this review.

The first plant hemoglobin was identified in 1939 by Kubo in soybean (*Glycine max*) nodules. This was followed by the demonstration by Keilin and Wang that this hemoglobin forms a 'perfectly reversible compound with molecular oxygen'. Simultaneously, Virtanen drew a similar conclusion for pea (*Pisum sativum*) hemoglobin and found that its content in nodules was correlated to N_2 fixation. Some year later Virtanen & Laine (1946) coined the name 'leghemoglobin' (Lb) for the hemoglobin of legume nodules, but it was not until 1974 when Wittenberg, Bergersen, Appleby and Turner established its function as a protein that 'facilitates O_2 diffusion' to the bacteroids (for this and other early references see Appleby, 1992).

Another breakthrough was the discovery of hemoglobin in nodules of the non-legume *Parasponia andersonii* (Cannabaceae) formed in symbiosis with bradyrhizobia (Appleby *et al.*, 1983), as well as in nodules of the actinorhizal plants *Casuarina glauca*, *Myrica gale* and *Alnus*

glutinosa, formed in symbiosis with the actinobacterium *Frankia* (Silvester *et al.*, 2008). These findings unveiled the presence in plants of symbiotic hemoglobins other than Lbs. Furthermore, hemoglobins were also found in roots of nodulating and non-nodulating plants (Bogusz *et al.*, 1988). These results led these authors to speculate that hemoglobins have a general function in all plant roots, possibly related to O_2 sensing under hypoxic conditions (Appleby *et al.*, 1988).

The increasing availability of molecular biology tools during the 1990s paved the way for major discoveries in the plant hemoglobin field. These include the finding of 'nonsymbiotic hemoglobins' or 'phytoglobins' (Glbs) in virtually all plant tissues, and the subsequent categorization of Glbs into two phylogenetic classes having distinct expression profiles during plant development and in response to hormones and stress (Andersson *et al.*, 1996; Trevaskis *et al.*, 1997; Hunt *et al.*, 2001). Shortly after, a third class of Glb was discovered and initially named 'truncated' because of their homology with the truncated hemoglobins of prokaryotes (Watts *et al.*, 2001; Wittenberg *et al.*, 2002). Currently, thanks to extensive sequencing of plant genomes, hundreds of sequences of the three Glb classes, Lbs and other symbiotic hemoglobins are easily accessible. The hemoglobin sequences belong to virtually all major lineages of plants, from microalgae to dicots, allowing detailed phylogenetic analyses (Vázquez-Limón *et al.*, 2012; Becana *et al.*, 2020).

The focus of this review is on the roles of hemoglobins (Lbs and Glbs) in the legume-rhizobium symbiosis, from infection to nodule senescence. Research on hemoglobins of crop legumes is described but most recent progress has been made with model symbioses and these are given special attention. We critically examine early and novel results, updating information on the hemoglobin genes and proteins of the two model legumes, identifying gaps of knowledge and highlighting avenues for future research. This review assumes the readers are familiarized with the structure and reactivity of plant hemoglobins, but otherwise information may be sought in other references (Hoy *et al.*, 2007; Becana *et al.*, 2020).

Hemoglobins in nodules of cultivated legumes

Crop legumes produce two major types of nodules, indeterminate and determinate, that greatly differ in structure and metabolism. Some of their relevant features are compared in Fig. 1. Widely cultivated legumes with indeterminate nodulation include pea, alfalfa (*Medicago sativa*), white

clover (*Trifolium repens*) and broad bean (*Vicia faba*), whereas determinate nodulation occurs in soybean, common bean (*Phaseolus vulgaris*) and mungbean (*Vigna radiata*). In this section we will describe several unresolved questions regarding Lbs from legumes of agronomic interest.

First, we do not know why nodules contain multiple Lbs (Becana & Sprent, 1989 and references therein; Appleby, 1992). In soybean nodules there are four major Lbs (*a*, c_1, c_2 and c_3) and four minor Lbs (*b*, d_1, d_2 and d_3) that are produced by N-terminal acetylation of the major components. The Lb*a*/Lbc_3 ratio increases dramatically during nodule maturation, suggesting that Lb*a* is mainly regulating O_2 when the nodule structure is complex (Fuchsman & Appleby, 1979). In pea nodules there are two major Lbs (LbI and LbIV) and three minor Lbs, and the LbI /LbIV ratio decreases with nodule age (Uheda & Syõno, 1982). The O_2-binding affinities of Lb*a* and LbIV are higher than those of Lb*c* and LbI, respectively, which suggests that changes in Lb proportions during nodule development tend to maximize the efficiency of N_2 fixation (Uheda & Syõno, 1982; Appleby, 1992). Further work on pea Lbs by Kawashima *et al.* (2001) showed that LbIV mRNA is evenly distributed throughout the nodules, except in the meristem, whereas LbI mRNA is restricted to the region from zone II to the distal part of zone III (tissue zonation of indeterminate nodules is shown in Fig. 1). This indicates that the expression of Lbs is spatiotemporally regulated, yet their individual role(s) remain(s) to be elucidated. The effect of conditions other than aging on the expression of *Lb* genes has been seldom addressed. Nitrate supply to mungbean plants caused an inversion in the abundance of LbI and LbII (Becana & Sprent, 1989). These variations, along with those observed during soybean and pea nodule development, may reflect different rates in the biosynthesis of Lbs.

A second issue that merits deeper investigation is the pathway of Lb degradation (Fig. 2). It is most surprising that so little progress has been made ever since a 'green pigment' was described in soybean and pea nodules (Virtanen & Laine, 1946) or biliverdin isomers were found to be generated *in vitro* by reaction of Lbs with ascorbate under aerobic conditions (Lehtovaara & Perttilä, 1978). Ferrous and ferric Lb are oxidized to ferryl Lb when treated with H_2O_2 (Aviram *et al.*, 1978), but ferric Lb also produces a green derivative with an intramolecular heme-protein cross-link and a Lb dimer with an intermolecular cross-link between a tyrosine and another amino acid residue (Moreau *et al.*, 1995a). Ferryl Lb can be reduced back to ferric Lb by ascorbate, which is present at high concentrations in the nodule cytosol (Moreau *et al.*, 1995b). As is the case of ferric Lb, all these Lb derivatives are non-functional. However, the information on Lb green pigments *in vivo* is scarce. The green Lb discovered in soybean nodules (Jun *et al.*, 1994) was

identified almost twenty years later as nitri-Lb (Navascués *et al.*, 2012). The protein bears a nitro group (NO_2) on the 4-vinyl of the heme as a result of the reaction of Lb with nitrite in the mild acid pH of senescent nodules. The presence of nitri-Lb reveals the production of nitrating reactive species, a conclusion confirmed by the finding of Lbs with nitrated tyrosines (Sainz *et al.*, 2015). These are formed by a mechanism requiring H_2O_2 and nitrite and, unlike nitri-Lb, are more abundant in young nodules. Also interestingly, nitric oxide (NO) interacts rapidly with Lb^{2+} forming nitrosyl-leghemoglobin ($Lb^{2+}NO$), which can be used as a probe for NO production in intact nodules by using electron paramagnetic resonance spectroscopy (Calvo-Begueria *et al.*, 2018 and references therein). This Lb complex is obviously inactive for O_2 transport but it is unknown if it can act as an NO carrier. Because of the key role of NO as a signal molecule in symbiosis (see next sections), it is worth to carefully assess the presence of $Lb^{2+}NO$ in intact indeterminate and determinate nodules during developmental and stress induced senescence. The homeostasis of NO in nodules is essential not only because it fulfills signaling roles but also because it is a precursor of other reactive nitrogen species, such as peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2), that can nitrate proteins, lipids and DNA (Radi, 2004). In this respect, Lbs may act as sinks of these nitrating molecules to protect N_2 fixation, as suggested by experiments *in vitro* (Herold & Puppo, 2005) and *in vivo* (Sainz *et al.*, 2015). However, it is unclear whether nitrated Lbs mark the protein for degradation or are simple by-products. Nodule proteases may release heme from Lb (Pfeiffer *et al.*, 1983) and heme oxygenase breaks down heme to biliverdin-IXα, carbon monoxide and Fe^{2+} (Shekhawat & Verma, 2010). In alfalfa, the isoform 1 of heme oxygenase is more abundant in nodules than in roots or leaves, suggesting that it contributes to Lb catabolism (Baudouin *et al.*, 2004). This enzymatic function is relevant because free heme is toxic and may arise from the rapid turnover of Lbs, which occurs in approximately two days in the case of pea Lbs (Bisseling *et al*., 1980).

A third not yet fully resolved issue is the mechanism that maintains Lb in the ferrous form (Lb^{2+}) to transport O_2 (Fig. 2). In nodules, Lb^{2+} and its oxygenated form ($Lb^{2+}O_2$) are prone to (auto)oxidation to the inactive ferric form (Lb^{3+}). Two studies from Klucas' lab using mainly soybean nodules are central. In the first one, these authors induced formation of Lb^{3+} by treating nodule slices with hydroxylamine, and observed its reduction to Lb^{2+} in the presence of nicotinate to detect the Lb^{2+} nicotinate complex (Lee & Klucas, 1984). In the second study, they monitored spectroscopically the Lb state in intact nodules (Lee *et al.*, 1995). They were able to detect Lb^{3+} in young nodules exposed to 100% O_2 or in unexposed senescent nodules. Therefore, Lb^{3+} is formed

in nodules under certain conditions and is reduced back to Lb^{2+}. What is then the mechanism for Lb^{3+} reduction? Diverse flavoproteins catalyze the electron transfer from NADH to the flavin cofactor and then to the heme. Free flavins (riboflavin, FMN and FAD) and a flavoenzyme, characterized as a ferric leghemoglobin reductase, reduce Lb^{3+} at the physiological concentrations of NADH, flavins and Lb^{3+} in nodules (Becana & Klucas, 1990). Free flavins are present throughout the nodule but a flavin reductase showing spatiotemporal coexpression with Lb is still missing. A similar consideration applies in the case of Glbs, for which a 'metphytoglobin reductase' has been speculated to exist in the cytosol of other plant cells, but with no experimental basis to date (Gupta & Igamberdiev, 2016). Thus, the search for such an enzyme still continues.

Hemoglobins in indeterminate nodules: *Medicago truncatula*

Molecular analyses of cultivated legumes were hampered by their large genomes and recalcitrance to transformation and/or regeneration. This prompted the scientific community to adopt two model legumes for molecular genetics and genomics: *Medicago truncatula* (barrel medic) for indeterminate nodulation (Barker *et al.*, 1990) and *Lotus japonicus* (bird's-foot trefoil) for determinate nodulation (Handberg & Stougaard, 1992). They are autogamous plants with small diploid genomes (520 Mb, $2n=2x=16$ and 470 Mb, $2n=2x=12$, respectively) and short generation times, produce high fruit set and seed numbers, and are easy to transform and regenerate. The genomes of the two species and their bacterial microsymbionts, *Sinorhizobium meliloti* and *Mesorhizobium loti*, have been completely sequenced. Also, for both legumes and bacteria there is a wealth of mutants and transcriptomic data, including expressed sequence tags, DNA microarrays and RNA sequencing (RNA-seq) profiles. As detailed in the next sections, these powerful molecular tools have contributed in a decisive manner to increase our knowledge on Lbs and Glbs.

The first studies on Lbs in *M. truncatula* were carried out by Barker's group, who cloned two *Lb* genes in this legume and its tetraploid relative, alfalfa (de Billy *et al.*, 1991; Gallusci *et al.*, 1991). The expression of *Lb1* and *Lb2* was triggered simultaneously during nodule development five days post-inoculation (dpi) with *S. meliloti*. The *Lb1* to *Lb2* mRNA ratio remained constant during nodule development. This was also the case after treating the plants with 5 mM KNO_3 for 2-3 days, when N_2 fixation had decreased by *c.* 75%. Microscopic observation showed that

transcription of *Lb* genes in alfalfa nodules is triggered in a single cell layer in the interzone II-III in mature nodules and in young immature nodules where zone III has not developed yet, demonstrating that Lb is transcribed prior to N_2 fixation (de Billy *et al.*, 1991).

To gain insight into the hemoglobin genes and proteins of *M. truncatula* ecotype Jemalong A17, we searched in the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi), Phytozome 12 (Mt4.0v1; https://phytozome.jgi.doe.gov/pz/portal.html), the Symbimics database (https:// iant.toulouse.inra.fr/symbimics), the *M. truncatula* Gene Expression Atlas (MtGEA; https://mtgea.noble.org/v3), and the *M. truncatula* Small Secreted Peptide database (MtSSPdb; https://mtsspdb.noble.org/database). Moreover, we used our RNA-seq data of early symbiotic gene expression (Larrainzar *et al.*, 2015), now remapped to the latest version of the *M. truncatula* genome (Mt5.0). We found a total of 17 genes, encoding 12 Lbs, 3 class 1 Glbs and 2 class 3 Glbs, which confirms the gene numbers reported by Berger *et al.* (2020). Here, we would like to complete the information on *M. truncatula* hemoglobins as follows. First, we have updated the gene identifiers (IDs) according to Mt5.0 (Pecrix *et al.*, 2018), although still include those of Mt4.0 (Tang *et al.*, 2014) for cross-reference (Fig. 3a). In addition, we propose to revise the nomenclature of Lbs by following an increasing order of ID numbers. This is done for two reasons. First, Mt5.0 has better quality than Mt4.0, which made it possible to assign most genes to chromosomes. This provides a solid reference and reduces potential issues related to gene redundancy or misnaming. Second, having the reference to the locus in the chromosome permits to easily infer putative endoduplication events, usually clustering together in the same chromosome. Renaming *Glb* genes seems unnecessary because there are only two or three members of each class. Second, available expression data, including ours, strongly suggest that *Lb9* and *Lb12* are not expressed. This is consistent with the prediction that Lb9 and Lb12 may be truncated proteins based on their small sizes compared with those of typical Lbs (146-153 amino acid residues) (Fig. 3a). Third, phylogenetic analysis shows that Lb3 is distant from the cluster that includes all other Lbs, hinting at a different function for this particular protein (Fig. 3b). Symbimics is an excellent resource for RNA-seq gene expression data across different nodule tissues (Roux *et al.*, 2014). Inspection of that database reveals that all *Lb* genes (except *Lb9* and *Lb12*) are preferentially expressed in the interzone II-III and zone III. This leaves us again with the intriguing question of why there are so many Lbs in *M. truncatula* nodules.

The best studied Glb in legumes is Glb1-1. In *M. truncatula* this protein is involved in the regulation of nodulation and N_2 fixation by modulating NO concentration during nodule development (Berger *et al.*, 2020). These authors made very interesting observations and opened the door for further experiments, some of which we would like to suggest here. First, NO inhibits the expression of *Lb* genes. This observation was made on only one *Lb* gene (Medtr1g011540) and may well hold true for the others. This possibility should be checked and its biological relevance examined. Second, all *Glb* genes, except *Glb3-2*, are greatly induced in eight-week-old senescent nodules. Quite likely, not all these inductions are related to changes in NO levels and hence further investigation on the role(s) of Glbs is warranted. Finally, the 'hemoglobin-NO cycle' could be functioning in nodules, much like in hypoxic roots, as the nodule infected zone is under microoxic conditions. This suggestion merits special attention, but it is first necessary to explain briefly how this cycle is proposed to operate in plants (Fig. 2). Under low O_2, nitrite is reduced to NO at complex III (cytochrome bc_1) and complex IV (cytochrome oxidase) in the mitochondria; NO diffuses to the cytosol, where it is converted to nitrate by the NO dioxygenase activity of oxyferrous phytoglobin ($Glb^{2+}O_2$) producing ferric phytoglobin (Glb^{3+}); nitrate is reduced to nitrite by nitrate reductase in the cytosol; and Glb^{3+} is reduced back to Glb^{2+}/$Glb^{2+}O_2$ by a metphytoglobin reductase also in the cytosol (Gupta & Igamberdiev, 2016). As a result, the hemoglobin-NO cycle would maintain the redox and energy status of cells, which is required for hypoxic survival of the plant (Gupta & Igamberdiev, 2016). In principle, class 1 Glbs are involved in the cycle because only these proteins are inducible by hypoxia and NO, and because plants overexpressing this class of Glbs have better cell energetics and hypoxia tolerance (Sowa *et al.*, 1998; Hunt *et al.*, 2002). However, all three classes of Glbs as well as nodule Lbs have NO dioxygenase activity (Herold & Puppo, 2005; Rubio *et al.*, 2019) and hence all of them could, theoretically, contribute to the cycle. The data of Berger *et al.* (2020) using transgenics do support the cycle in nodules but, as it occurs with other plant systems, the existence of two key components, namely, a mitochondrial nitrite transporter and a cytosolic metphytoglobin reductase, still needs to be demonstrated.

In sharp contrast to Glb1-1, the functions of Glb1-2 or Glb1-3 are unknown. The *Glb1-2* gene is in tandem with *Glb1-1* on chromosome 4, whereas *Glb1-3* is located on chromosome 2 according to Mt5.0 (Fig. 3a). *Glb1-1* displays a tissue profile similar to that of *Lb* genes, whereas *Glb1-2* is expressed in zone I (meristem) and *Glb1-3* has negligible expression levels (see Symbimics database). Also, we know little about the two class 3 Glb genes. They are very

divergent and located on different chromosomes, but each of them is highly conserved across legumes (Becana *et al.*, 2020). *Glb3-1* is predominantly expressed in the interzone II-III and in the infected cells of zone III, whereas *Glb3-2* is expressed in the vascular bundles and at the base of the nodule (Vieweg *et al.*, 2005). Based on *in silico* analysis, these two hemoglobins have been suggested to lack NO dioxygenase activity (Berger *et al.*, 2020), but this activity is inherent to heme proteins and indeed has been demonstrated for Glb3-1, Glb3-2 and other Glbs of *L. japonicus* (Rubio *et al.*, 2019). It is therefore necessary to characterize the proteins in order to draw reliable conclusions. In Fig. 3a, we have also indicated the alternative spliced forms of *Lb* and *Glb* genes which are supported by expressed sequence tags or RNA-seq data. As many as seven genes may give rise to spliced forms: *Lb3*, *Lb4*, *Lb6*, *Lb7*, *Glb1-1*, *Glb1-2* and *Glb1-3*. The *Glb1-2* gene is a very rare case because it may give rise to four alternative spliced variants, predictably encoding proteins with one or two heme domains. Elucidation of a role for alternative splicing in hemoglobin genes will require additional studies at the mRNA and protein levels.

To identify transcriptional responses of hemoglobin genes at very early symbiotic stages, we surveyed RNA-seq data of expression in roots from 30 min to 48 hours post-inoculation (hpi) (Fig. 4). These experiments were conducted with plants of the wild-type A17 genotype and three symbiotic mutants grown in aeroponic culture: *Nodulation factor perception* (*nfp*), which is Nod factor-insensitive; *Lysine motif domain-containing receptor-like kinase 3* (*lyk3*), which has diminished Nod factor-sensitivity; and *sickle* (*skl*), which is ethylene insensitive, hyperinfective and hypernodulating (Larrainzar *et al.*, 2015 and references therein). Intriguingly, the expression of four *Lb* genes and four *Glb* genes (Fig. 4) was detected at this initial stage of infection. There was a symbiotic-dependent induction of *Lb* genes starting at 12–24 hpi, as observed in the A17 genotype and especially in the *skl* mutant. The presence of significant transcript levels of these genes is quite surprising because at this stage nodule primordia have not yet been formed. In contrast, the expression of the four *Lbs* in the *nfp* and *lyk3* mutants remained very low throughout the whole period, indicating that Nod factor perception and signaling are required for *Lb* induction (Fig. 4). With regard to Glbs, the most salient feature was the transient up-regulation of *Glb1-1* with a peak at 1–3 hpi in the A17 genotype (Fig. 4). In the three mutants, the *Glb1-1* mRNA level decreased after 30 min and increased thereafter as for A17. Because the increase in expression occurred before the biosynthesis and/or perception of Nod factor (estimated at *c.* 3 hpi) in all the genotypes tested, the increase in the *Glb1-1* mRNA level may be part of a generic response to the rhizobia, independent of the Nod factor signaling cascade. Likewise, the induction does not

require an active ethylene signaling pathway because it also occurred in the *skl* mutant (Fig. 4). Clustering analysis based on gene expression profiles showed that *Glb1-1* is included in a group of genes related to plant defense responses such as *MtERF1-1* (Medtr4g100380; Anderson *et al.*, 2010). This observation fits well with a role of Glb1-1 in controlling NO levels during infection (Nagata *et al.*, 2008; Fukudome *et al.*, 2016; Berger *et al.*, 2020).

Next, we screened the RNA-seq data of the genome-wide MtSSPdb, recently made available by Boschiero *et al.* (2020), and extracted two datasets with novel information on the *Lb* and *Glb* genes. The first one provided the expression profiles across plant organs (Fig. 5). As expected, all *Lb* genes, especially *Lb6*, are highly expressed in nodules at 10–28 dpi and are down-regulated with nitrate supply (Fig. 5a). Most interestingly, however, *Lb1* is expressed in root bumps at 4 dpi, *Lb4* in roots of 5-week-old plants, and *Lb6* in large pods of 10 week-old-plants. There is also a very low but detectable level of *Lb11* mRNA in roots, leaves, petioles and large pods. Like Lbs, *Gb1-1* and *Glb3-1* are consistently expressed in nodules but, unlike Lbs, their expression is further enhanced with nitrate supply (Fig. 5b). The expression level of *Glb1-1* is very high only in roots, whereas *Glb3-2* is expressed throughout plant organs (Fig. 5b). The second dataset provided very relevant and abundant information on the effects of macronutrient deficiencies on hemoglobin gene expression (Fig. 6). In the first place, these results proved that *Lb* genes are expressed in the roots. Thus, *Lb4* is highly expressed in the nitrogen (N) and sulfur (S) treatments, whereas *Lb6* is preferentially expressed in the potassium (K) treatments. Notably, *Lb6* is strongly up-regulated by phosphorus (P) deficiency. *Lb4* expression decreases with N deficiency, whereas *Lb6* expression decreases with low or high K but is activated under P and S deficiencies (Fig. 6). In the shoot only *Lb6*, *Lb7* or *Lb8* mRNAs are detectable, albeit at very low levels. As for the *Glb* genes, *Glb1-1* and *Glb3-2* were clearly the most expressed ones in the roots and shoots, respectively; however, *Glb1-1* is down-regulated under P, K and S deficiencies, whereas *Glb3-2* remains unaltered (Fig. 6). Taken together, the results of Figures 4, 5 and 6 evidence that the *Lb* genes are active in plant organs other than nodules and that the expression levels depend on the specific gene and plant organ. It will, therefore, be necessary to determine whether the mRNAs are translated into functional proteins and that these follow a similar distribution within the plant.

Hemoglobins in determinate nodules: *Lotus japonicus*

Three *Lb* genes have been identified and mapped on chromosome 5 in *L. japonicus*: *Lb1* and *Lb2* in tandem and *Lb3* at *c*. 3 centimorgans from them (Uchiumi *et al.*, 2002; Fig. 3a). A few years later, Udvardi's lab used RNA interference (RNAi) to down-regulate the transcript levels of the three Lbs below 3% of those in wild-type plants (Ott *et al.*, 2005). The RNAi lines served to demonstrate that Lbs are essential for plants relying exclusively on N_2 fixation (nodulated) but not for plants grown on nitrogen fertilization (non-nodulated). The resulting white nodules showed an increase in the internal free O_2 concentration, proving the function of Lb as an O_2 buffer. These nodules also exhibited a decrease in the ATP/ADP ratio and loss of the NifD protein (molybdenum-iron α chain of nitrogenase). Subsequent work showed extensive alterations in ultrastructure and bacteroid differentiation, as well as major changes of gene expression in the bacteroids and host cells in nodules of the RNAi lines (Ott *et al.*, 2009). Recently, Duanmu's group in China used CRISPR/Cas9 technology to selectively knockout the Lbs of *L. japonicus*, thus obtaining single, double and triple mutants (Wang *et al.*, 2019). The authors found a similar expression pattern and tissue localization of the three Lbs during nodule development, and concluded that Lbs have a synergistic effect to maximize N_2 fixation. The nodules of the triple mutant (*lb123*) lacking the three Lbs show vacuolization of infected cells, accumulation of poly-β-hydroxybutyrate in bacteroids and disruption of mitochondria. The *lb123* mutant nodules also have enhanced production of superoxide radicals and H_2O_2, probably related to activation of NADPH oxidase and changes in superoxide dismutase isoforms. Notably, the study also provided RNA-seq data of more than 20000 genes of the mutant nodules (Wang *et al.*, 2019).

The first *Glb* gene (*Hb1*) identified in *L. japonicus* encodes a class 1 Glb and was mapped on chromosome 3 (Uchiumi *et al.*, 2002). Subsequently, these authors reported a second *Glb* gene (*Hb2*), situated in tandem with *Hb1* but sharing only 74% similarity in amino acid sequence with the Hb1 protein (Shimoda *et al.*, 2005). Both genes encode class 1 Glbs and were thus later on renamed *Glb1-1* and *Glb1-2* to avoid confusion with class 2 Glbs (Bustos-Sanmamed *et al.*, 2011; Fig. 3a). Uchiumi's group made the very relevant observation that *Glb1-1* expression and NO production are concomitantly and transiently induced in roots at 4 hpi of *L. japonicus* roots by *M. loti* but not by incompatible rhizobia or pathogenic bacteria (Shimoda *et al.*, 2005; Nagata *et al.*, 2008). These authors also found that *Glb1-1* is mainly expressed in nodules and strongly induced by hypoxia, NO and cold stress, whereas *Glb1-2* is mainly expressed in leaves and induced by sucrose (Shimoda *et al.*, 2005). Overexpression of *Glb1-1* in hairy roots increased nodule number and N_2 fixing activity, which was attributed to a higher capacity of nodules to scavenge NO and

protect nitrogenase (Shimoda *et al.*, 2009). These results were confirmed by using stable overexpressing lines, which had reduced NO levels and enhanced N_2 fixation in mature and senescent nodules (Fukudome *et al.*, 2019). The use of knockout lines of the *LORE1* collection (https://lotus.au.dk) proved the key role of Glb1-1, but not of Glb1-2, in regulating NO levels during infection and hence in the onset of symbiosis (Fukudome *et al.*, 2016).

Extensive work by our group identified three other functioning *Glb* genes in *L. japonicus* (Bustos-Sanmamed *et al.*, 2011). Two of the genes encode class 3 Glbs and were designated *Glb3-1* and *Glb3-2*, whereas another gene, termed *Glb2*, was found to encode an unusual hemoglobin. This has been renamed here *Glb2-1* because there is a second gene, *Glb2-2*, in tandem with *Glb2-1*, according to the genome v1.2 of *L. japonicus* ecotype Gifu (Kamal *et al.*, 2020). Phylogenetic analysis revealed that Glb2-1 and Glb2-2 form a cluster with *M. truncatula* Lb3 (Fig. 3b). However, Glb2-1 (and predictably Glb2-2) differs from Lbs in that it is pentacoordinate as Glb^{2+} and hexacoordinate as Glb^{3+} (Calvo-Begueria *et al.*, 2017), whereas Lbs are pentacoordinate in both oxidation states (Becana *et al.*, 2020). Two additional observations support that Glb2-1 is not a genuine Lb: the *Glb2-1* gene is expressed in root tips and vascular bundles (Bustos-Sanmamed *et al.*, 2011) and the *lb123* mutant nodules are completely white (Wang *et al.*, 2019). In a previous work we examined the expression of *Glb* genes in nodules and roots of nodulated *L. japonicus* plants grown in hydroponic culture for 45 days and then treated with 50 μM hormones for two days (Bustos-Sanmamed *et al.*, 2011). We have now conducted an experiment using both nodulated plants (20-days old; nodules 15 dpi) and non-nodulated plants (15-days old) treated for 24 h with 50 μM of hormones (Fig. 7). Although conditions are hardly comparable, some interesting observations are shared by the two sets of experiments: *Glb1-1* expression was enhanced by polyamines and 1-aminocyclopropane-1-carboxylic acid (ACC; the immediate precursor of ethylene) in roots; *Glb1-2* expression was decreased by cytokinins in roots; and *Glb2-1* expression was drastically down-regulated by cytokinins in nodules. Notably, in our recent experiments we also found that ACC decreased the expression of the four genes in nodules, but up-regulated *Glb1-1* and *Glb1-2* in roots (Fig. 7). These results strongly suggest differential regulation of *Glb* genes in the hormonal signaling pathways and plant organs.

Hemoglobins in rhizobia

The existence of hemoglobins in rhizobia, although largely suspected, was not proved until complete genome sequencing became readily available. Three types of hemoglobins occur in rhizobia. Flavohemoglobins (Hmp), which have flavin and heme domains, are known to act as NO scavengers in prokaryotes and yeast. They are present in *S. meliloti* and *M. loti* (Fig. 2). The role of Hmp in NO detoxification has been suggested by experiments with a *hmp* null mutant (Meilhoc *et al.*, 2010; Cam *et al.*, 2012) and with a strain (*hmp*$^{++}$) overexpressing the *hmp* gene (del Giudice *et al.*, 2011). Compared with the wild-type strain, the *hmp* mutant displays higher sensitivity toward NO and produces nodules with better N_2 fixation efficiency and with delayed senescence. - Conversely, the *hmp*$^{++}$ strain is able to significantly reduce NO levels in nodules, is less infective than the wild-type, and causes a delay in nodule formation. Taken together, these experiments provide sound evidence that NO is a key player in nodule formation and senescence. The function of Hmp in *M. loti* remains unknown but, quite likely, it may play a similar role in NO regulation during symbiosis.

In contrast, *Bradyrhizobium diazoefficiens* has a single-domain hemoglobin (Bjgb). This protein plays also a role in NO detoxification in the free-living bacteria during nitrate-dependent growth (Cabrera *et al.*, 2016). Very recently, it has been shown that soybean plants inoculated with a *bjgb* mutant strain have more tolerance to flooding than those inoculated with the wild-type strain (Salas *et al*., 2020). Because flooding causes hypoxia in roots and nodules and thereby triggers NO production, the beneficial effect of a mutant lacking Bjgb was unexpected, assuming that the protein scavenges NO. These authors showed that Bjgb-deficient bacteroids have enhanced expression and activity of the denitrifying enzyme NO reductase, which in turn prevents NO accumulation. Like other prokaryotes, rhizobia contain 'truncated' hemoglobins but, to our knowledge, there are no studies on these proteins; therefore, their possible roles, linked (or not) to NO detoxification, as is the case of other hemoglobins of this type, deserve investigation (Becana *et al.*, 2020).

Future prospects

We have reviewed data demonstrating the major roles of Lbs as a transporter and buffer of O_2 and of Glb1-1 in regulating NO concentration during symbiosis. The best studied hemoglobin in legumes is symbiotic Lb, but even in this case many unanswered questions remain. We need to know why there are so many Lbs, especially in indeterminate nodules and whether they play a role in protecting nodule activity against reactive molecules; how they are maintained in the functional reduced state; how their expression is regulated; why they also occur in the nuclei and in uninfected cells of nodules; and, even more importantly, why they are expressed in nonsymbiotic plant organs. Much less is known about other class 1 Glbs and about class 2 and 3 Glbs in nodules. To ascertain their functions in symbiosis, it will be necessary to combine exhaustive biochemical information on their structure and reactivities with studies addressing their spatiotemporal regulation in roots and nodules in response to a number of factors such as NO, mineral nutrition, stress conditions and hormonal signaling.

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References

Anderson JP, Lichtenzveig J, Gleason C, Oliver RP, Singh KB. 2010. The B-3 ethylene response factor MtERF1-1 mediates resistance to a subset of root pathogens in *Medicago truncatula* without adversely affecting symbiosis with rhizobia. *Plant Physiology* **154:** 861–873.

Andersson CR, Jensen EO, Llewellyn DJ, Dennis ES, Peacock WJ. 1996. A new hemoglobin gene from soybean: a role for hemoglobin in all plants. *Proceedings of the National Academy of Sciences, USA* **93:** 5682-5687.

Appleby CA, Tjepkema JD, Trinick MJ. 1983. Hemoglobin in a nonleguminous plant, *Parasponia*: possible origin and function in nitrogen fixation. *Science* **220:** 951-953.

Appleby CA, Bogusz D, Dennis ES, Peacock WJ. 1988. A role for haemoglobin in all plant roots? *Plant Cell & Envirnoment* **11:** 359-367.

Appleby CA. 1992. The origin and functions of haemoglobin in plants. *Science Progress* **76:** 365-398.

Arrighi J-F, Godfroy O, de Billy F, Saurat O, Jauneau A, Gough C. 2008. The *RPG* gene of *Medicago truncatula* controls *Rhizobium*-directed polar growth during infection. *Proceedings of the National Academy of Sciences, USA* **105:** 9817-9822.

Aviram I, Wittenberg BA, Wittenberg JB. 1978. The reaction of ferrous leghemoglobin with hydrogen peroxide to form leghemoglobin (IV). *Journal of Biological Chemistry* **253:** 5685-5689.

Barker DG, Bianchi S, Blondon F, Dattee Y, Duc G, Essad S, Flament P, Gallusci P, Genier G, Guy P, *et al.* 1990. *Medicago truncatula*, a model plant for studying the molecular genetics of the rhizobium-legume symbiosis. *Plant Molecular Biology Reports* **8:** 40-49.

Baudouin E, Frendo P, Le Gleuher M, Puppo A. 2004. A *Medicago sativa* haem oxygenase gene is preferentially expressed in root nodules. *Journal of Experimental Botany* **55:** 43-47.

Becana M, Sprent JI. 1989. Effect of nitrate on components of nodule leghaemoglobins. *Journal of Experimental Botany* **40:** 725-731.

Becana M, Klucas RV. 1990. Enzymatic and nonenzymatic mechanisms for ferric leghemoglobin reduction in legume root nodules. *Proceedings of the National Academy of Sciences, USA* **87:** 7295-7299.

Becana M, Yruela I, Sarath G, Catalán P, Hargrove MS. 2020. Plant hemoglobins: a journey from unicellular green algae to vascular plants. *New Phytologist* doi: 10.1111/nph.16444.

Berger A, Guinand S, Boscari A, Puppo A, Brouquisse R. 2020. *Medicago truncatula* phytoglobin 1.1

controls symbiotic nodulation and nitrogen fixation via the regulation of nitric oxide. *New Phytologist* doi: 10.1111/nph.16462.

Bisseling T, Van Straten J, Houwaard F. 1980. Turnover of nitrogenase and leghemoglobin in root nodules of *Pisum sativum*. *Biochimica et Biophysica Acta* **610:** 360-370.

Boschiero C, Dai X, Lundquist PK, Roy S, de Bang TC, Zhang S, Zhuang Z, Torres-Jerez I, Udvardi MK, Scheible WR *et al.* 2020. MtSSPdb: the *Medicago truncatula* small secreted peptide database. *Plant Physiology* **183:** 399-413.

Bogusz D, Appleby CA, Landsmann J, Dennis ES, Trinick MJ, Peacock WJ. 1988. Functioning haemoglobin genes in non-nodulating plants. *Nature* **331:** 178-180.

Bustos-Sanmamed P, Tovar-Mendez A, Crespi M, Sato S, Tabata S, Becana M. 2011. Regulation of non-symbiotic and truncated hemoglobin genes of *Lotus japonicus* in plant organs and in response to nitric oxide and hormones. *New Phytologist* **189:** 765-776.

Cabrera JJ, Salas A, Torres MJ, Bedmar EJ, Richardson DJ, Gates AJ, Delgado MJ. 2016. An integrated biochemical system for nitrate assimilation and nitric oxide detoxification in *Bradyrhizobium japonicum*. *Biochemical Journal* **473:** 297-309.

Calvo-Begueria L, Cuypers B, Van Doorslaer S, Abbruzzetti S, Bruno S, Berghmans H, Dewilde S, Ramos J, Viappiani C, Becana M. 2017. Characterization of the heme pocket structure and ligand binding kinetics of non-symbiotic hemoglobins from the model legume *Lotus japonicus*. *Frontiers in Plant Science* **8:** article 407.

Calvo-Begueria L, Rubio MC, Martínez JI, Pérez-Rontomé C, Delgado MJ, Bedmar EJ, Becana M. 2018. Redefining nitric oxide production in legume nodules through complementary insights from electron paramagnetic resonance spectroscopy and specific fluorescent probes. *Journal of Experimental Botany* **69:** 3703-3714.

Cam Y, Pierre O, Boncompagni E, Hérouart D, Meilhoc E, Bruand C. 2012. Nitric oxide (NO): a key player in the senescence of *Medicago truncatula* root nodules. *New Phytologist* **196:** 548-560.

de Bang TC, Lundquist PK, Dai X, Boschiero C, Zhuang Z, Pant P, Torres-Jerez I, Roy S, Nogales J, Veerappan V, *et al*. 2017. Genome-wide identification of *Medicago* peptides involved in macronutrient responses and nodulation. *Plant Physiology* **175:** 1669-1689.

de Billy F, Barker DG, Gallusci P, Truchet G. 1991. Leghaemoglobin gene trascription is triggered in a single cell layer in the indeterminate nitrogen-fixing root nodule of alfalfa. *Plant Journal* **1:** 27-35.

del Giudice J, Cam Y, Damiani I, Fung-Chat F, Meilhoc E, Bruand C, Brouquisse R, Puppo A, Boscari A. 2011. Nitric oxide is required for an optimal establishment of the *Medicago truncatula-Sinorhizobium meliloti* symbiosis. *New Phytologist* **191:** 405-417.

Fuchsman WH, Appleby CA. 1979. Separation and determination of the relative concentrations of the homogeneous components of soybean leghemoglobin by isolectric focusing. *Biochimica et Biophysica*

Acta **579:** 314-324.

Fukudome M, Calvo-Beguería L, Kado T, Osuki K, Rubio MC, Murakami E, Nagata M, Kucho K, Sandal N, Stougaard J *et al.* 2016. Hemoglobin LjGlb1-1 is involved in nodulation and regulates the level of nitric oxide in the *Lotus japonicus-Mesorhizobium loti* symbiosis. *Journal of Experimental Botany* **67:** 5275-5283.

Fukudome M, Watanabe E, Osuki K, Imaizumi R, Aoki T, Kucho K, Becana M, Uchiumi T. 2019. Stably transformed *Lotus japonicus* plants overexpressing phytoglobin LjGlb1-1 show decreased nitric oxide levels in roots and nodules as well as delayed nodule senescence. *Plant and Cell Physiology* **60:** 816-825.

Gallusci P, Dedieu A, Journet EP, Huguet T, Barker DG. 1991. Synchronous expression of leghaemoglobin genes in *Medicago truncatula* during nitrogen-fixing root nodule development and response to exogenously supplied nitrate. *Plant Molecular Biology* **17:** 335-349.

García-Calderón M, Pérez-Delgado CM, Credali A, Vega JM, Betti M, Márquez AJ. 2017. Genes for asparagine metabolism in *Lotus japonicus*: differential expression and interconnection with photorespiration. *BMC Genomics* **18:** 781.

Gupta KJ, Igamberdiev AU. 2016. Reactive nitrogen species in mitochondria and their implications in plant energy status and hypoxic stress tolerance. *Frontiers in Plant Science* **7:** 369.

Handberg K, Stougaard J. 1992. *Lotus japonicus*, an autogamous, diploid legume species for classical and molecular genetics. *Plant Journal* **2:** 487-496.

Herold S, Puppo A. 2005. Oxyleghemoglobin scavenges nitrogen monoxide and peroxynitrite: a possible role in functioning nodules? *Journal of Biological Inorganic Chemistry* **10:** 935-945.

Hirsch AM. 1992. Developmental biology of legume nodulation. *New Phytologist* **122:** 211-237.

Hoy JA, Robinson H, Trent III JT, Kakar S, Smagghe BJ, Hargrove MS. 2007. Plant hemoglobins: a molecular fossil record for the evolution of oxygen transport. *Journal of Molecular Bioloy* **371:** 168-179.

Hunt PW, Watts RA, Trevaskis B, Llewelyn DJ, Burnell J, Dennis ES, Peacock WJ. 2001. Expression and evolution of functionally distinct haemoglobin genes in plants. *Plant Molecular Biology* **47:** 677-692.

Hunt PW, Klok EJ, Trevaskis B, Watts RA, Ellis MH, Peacock WJ, Dennis ES. 2002. Increased level of hemoglobin 1 enhances survival of hypoxic stress and promotes early growth in *Arabidopsis thaliana. Proceedings of the National Academy of Sciences, USA* **99:** 17197-17202.

Jun H-K, Sarath G, Wagner FW. 1994. Detection and purification of modified leghemoglobins from soybean root nodules. *Plant Science* **100:** 31-40.

Kamal N, Mun T, Reid D, Lin J, Akyol TY, Sandal N, Asp T, Hirakawa H, Stougaard J, Mayer KFX *et al.* 2020. A chromosome-scale *Lotus japonicus* Gifu genome assembly indicates that symbiotic

islands are not general features of legume genomes. *BioRxiv* doi: 10.1101/2020.04.17.042473.

Kawashima K, Suganuma N, Tamaoki M, Kouchi H. 2001. Two types of pea leghemoglobin genes showing different O_2-binding affinities and distinct patterns of spatial expression in nodules. *Plant Physiology* **125:** 641-651.

Larrainzar E, Riely BK, Kim SC, Carrasquilla-Garcia N, Yu HJ, Hwang JH, Oh M, Kim GB, Surendrarao AK, Chasman D *et al.* 2015. Deep sequencing of the *Medicago truncatula* root transcriptome reveals a massive and early interaction between nodulation factor and ethylene signals. *Plant Physiology* **169:** 233-265.

Lehtovaara P, Perttilä U. 1978. Bile-pigment formation from different leghaemoglobins. Methine-bridge specificity of coupled oxidation. *Biochemical Journal* **176:** 359-364.

Lee KK, Klucas RV. 1984. Reduction of ferric leghemoglobin in soybean root nodules. *Plant Physiology* **74:** 984-988.

Lee KK, Shearman LL, Erickson BK, Klucas RV. 1995. Ferric leghemoglobin in plant-attached leguminous nodules. *Plant Physiology* **109:** 261-267.

Li H, Jiang F, Wu P, Wang K, Cao Y. 2020. A high-quality genome sequence of model legume *Lotus japonicus* (MG-20) provides insights into the evolution of root nodule symbiosis. *Genes* **11:** 483 (doi:10.3390/genes11050483).

Meilhoc E, Cam Y, Skapski A, Bruand C. 2010. The response to nitric oxide of the nitrogen-fixing symbiont *Sinorhizobium meliloti*. *Molecular Plant-Microbe Interactions* **23:** 748-759.

Moreau S, Davies MJ, Puppo A. 1995a. Reaction of ferric leghemoglobin with H_2O_2: formation of heme-protein cross-links and dimeric species. *Biochimica et Biophysica Acta* **1251:** 17-22.

Moreau S, Puppo A, Davies MJ. 1995b. The reactivity of ascorbate with different redox states of leghaemoglobin. *Phytochemistry* **39:** 1281-1286.

Nagata M, Murakami E, Shimoda Y, Shimoda-Sasakura F, Kucho K, Suzuki A, Abe M, Higashi S, Uchiumi T. 2008. Expression of a class 1 hemoglobin gene and production of nitric oxide in response to symbiotic and pathogenic bacteria in *Lotus japonicus*. *Molecular Plant-Microbe Interactions* **21:** 1175-1183.

Navascués J, Pérez-Rontomé C, Gay M, Marcos M, Yang F, Walker FA, Desbois A, Abián J, Becana M. 2012. Leghemoglobin green derivatives with nitrated hemes evidence production of highly reactive nitrogen species during aging of legume nodules. *Proceedings of the National Academy of Sciences, USA* **109:** 2660-2665.

Ott T, van Dongen JT, Günther C, Krusell L, Desbrosses G, Vigeolas H, Bock V, Czechowski T, Geigenberg P, Udvardi M. 2005. Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. *Current Biology* **15:** 531-535.

Ott T, Sullivan J, James EK, Flemetakis E, Günther C, Gibon Y, Ronson C, Udvardi M. 2009.

Absence of symbiotic leghemoglobins alters bacteroid and plant cell differentiation during development of *Lotus japonicus* root nodules**.** *Molecular Plant-Microbe Interactions* **22:** 800-808.

Pecrix Y, Staton SE, Sallet E, Lelandais-Brière C, Moreau S, Carrère S, Blein T, Jardinaud MF, Latrasse D, Zouine M *et al.* 2018. Whole-genome landscape of *Medicago truncatula* symbiotic genes. *Nature Plants* **4:** 1017-1025.

Pfeiffer NE, Torres CM, Wagner FW. 1983. Proteolytic activity in soybean root nodules. Activity in host cell cytosol and bacteroids throughout physiological development and senescence. *Plant Physiology* **71:** 797-802.

Radi R. 2004. Nitric oxide, oxidants, and protein tyrosine nitration. *Proceedings of the National Academy of Sciences, USA* **101:** 4003-4008.

Robinson MD, Oshlack A. 2010. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biology* **11:** R25.

Roux B, Rodde N, Jardinaud MF, Timmers T, Sauviac L, Cottret L, Carrère S, Sallet E, Courcelle E, Moeau S *et al.* 2014. An integrated analysis of plant and bacterial gene expression in symbiotic root nodules using laser-capture microdissection coupled to RNA sequencing. *Plamt Journal* **77:** 817-837.

Rubio MC, Calvo-Begueria L, Díaz-Mendoza M, Elhiti M, Moore M, Matamoros MA, James EK, Díaz I, Pérez-Rontomé C, Villar I *et al.* 2019. Phytoglobins in the chloroplasts, nuclei and cytoplasm modulate nitric oxide and interact with abscisic acid. *Plant Journal* **100:** 38-54.

Sainz M, Calvo-Begueria L, Pérez-Rontomé C, Wienkoop S, Abián J, Staudinger C, Bartesaghi S, Radi R, Becana M. 2015. Leghemoglobin is nitrated in functional legume nodules in a tyrosine residue within the heme cavity by a nitrite/peroxide-dependent mechanism. *Plant Journal* **81:** 723-735.

Salas A, Tortosa G, Hidalgo-García A, Delgado A, Bedmar EJ, Richardson DJ, Gates AJ, Delgado MJ. 2020. The hemoglobin Bjgb from *Bradyrhizobium diazoefficiens* controls NO homeostasis in soybean nodules to protect symbiotic nitrogen fixation. *Frontiers in Microbiology* **10:** article 2915.

Silvester WB, Berg RH, Schwintzer CR, Tjepkema JD. 2008. Oxygen response, hemoglobin, and the structure and function of vesicles. In: Pawlowski K, Newton WE, eds. *Nitrogen-fixing Actinorhizal Symbioses*. Dordrecht, The Netherlands: Springer, 105-146.

Shekhawat GS, Verma K. 2010. Haem oxygenase (HO): an overlooked enzyme of plant metabolism and defence. *Journal of Experimental Botany* **61:** 2255-2270.

Shimoda Y, Nagata M, Suzuki A, Abe M, Sato S, Kato T, Tabata S, Higashi S, Uchiumi T. 2005. Symbiotic *Rhizobium* and nitric oxide induce gene expression of non-symbiotic hemoglobin in *Lotus japonicus*. *Plant and Cell Physiology* **46:** 99-107.

Shimoda Y, Shimoda-Sasakura F, Kucho K, Kanamori N, Nagata M, Suzuki A, Abe M, Higashi S, Uchiumi T. 2009. Overexpression of class 1 plant hemoglobin genes enhances symbiotic nitrogen fixation activity between *Mesorhizobium loti* and *Lotus japonicus*. *Plant Journal* **57:** 254-263.

Sowa AW, Duff SMG, Guy PA, Hill RD. 1998. Altering hemoglobin levels changes energy status in maize cells under hypoxia. *Proceedings of the National Academy of Sciences, USA* **95:** 10317-10321.

Sprent JI, Ardley J, James EK. 2017. Biogeography of nodulated legumes and their nitrogen-fixing symbionts. *New Phytologist* **215:** 40-56.

Tang H, Krishnakumar V, Bidwell S, Rosen B, Chan A, Zhou S, Gentzbittel L, Childs KL, Yandell M, Gundlach H *et al.* 2014. An improved genome release (version Mt4.0) for the model legume *Medicago truncatula*. *BMC Genomics* **15:** 312.

Trevaskis B, Watts RA, Andersson CR, Llewellyn DJ, Hargrove MS, Olson JS, Dennis ES, Peacock WJ. 1997. Two hemoglobin genes in *Arabidopsis thaliana*: the evolutionary origin of leghemoglobins. *Proceedings of the National Academy of Sciences, USA* **94:** 12230-12234.

Uchiumi T, Shimoda Y, Tsuruta T, Mukoyoshi Y, Suzuki A, Senoo K, Sato S, Kato T, Tabata S, Higashi S *et al.* 2002. Expression of symbiotic and nonsymbiotic globin genes responding to microsymbionts on *Lotus japonicus*. *Plant and Cell Physiology* **43:** 1351-1358.

Uheda E, Syõno K. 1982. Physiological role of leghaemoglobin heterogeneity in pea root nodule development. *Plant & Cell Physiolgy* **23:** 75-84.

Vázquez-Limón C, Hoogewijs D, Vinogradov SN, Arredondo-Peter R. 2012. The evolution of land plant hemoglobins. *Plant Science* **191-192:** 71-81.

Vieweg MF, Hohnjec N, Küster H. 2005. Two genes encoding different truncated hemoglobins are regulated during root nodule and arbuscular mycorrhiza symbioses of *Medicago truncatula*. *Planta* **220:** 757-766.

Virtanen AI, Laine T. 1946. Red, brown and green pigments in leguminous root nodules. *Nature* **157:** 25-26.

Wang L, Rubio MC, Xin X, Zhang B, Fan Q, Wang Q, Ning G, Becana M, Duanmu D. **2019.** CRISPR/Cas9 knockout of leghemoglobin genes in *Lotus japonicus* uncovers their synergistic roles in symbiotic nitrogen fixation. *New Phytologist* **224:** 818-832.

Watts RA, Hunt PW, Hvitved AN, Hargrove MS, Peacock WJ, Dennis ES. 2001. A hemoglobin from plants homologous to truncated hemoglobins from microorganisms. *Proceedings of the National Academy of Sciences, USA* **98:** 10119-10124.

Wittenberg JB, Bolognesi M, Wittenberg BA, Guertin M. 2002. Truncated hemoglobins: a new family of hemoglobins widely distributed in bacteria, unicellular eukaryotes, and plants. *Journal of Biological Chemistry* **277:** 871-874.

Figure legends

Fig. 1 Major characteristics differentiating 'classical' indeterminate and determinate nodules (modified from Hirsch, 1992). Some features should not be considered universal because there are exceptions. Thus, *Lotus japonicus* (*) thrives in temperate climates and exports asparagine from the nodules to the leaves (García-Calderón *et al.*, 2017). Also, many (sub)tropical legumes produce indeterminate nodules. There are also other nodule types not considered in this review, such as those of *Lupinus albus* ('lupinoid' type) and *Aeschynomene evenia* ('aeschynomenoid' type). For details of biogeographical distribution of nodulation (**) and on 'non-classical' types of nodules, see Sprent *et al.* (2017).

Fig. 2 Redox reactions of hemoglobins and some other relevant reactions involving nitric oxide (NO), nitrite (NO_2^-) and nitrate (NO_3^-) in nodules. In the host cell cytosol, reactions in red are those known so far that may occur with both leghemoglobin (Lb) and phytoglobin (Glb), but it does not necessarily mean that those in black are exclusive of Lbs. *(1)* Oxygenation of Lb^{2+}/Glb^{2+}. *(2)* NO binding to Lb heme to form nitrosyl-leghemoglobin ($Lb^{2+}NO$). *(3)* Nitrite reductase activity of Lb^{2+}/Glb^{2+}, which takes place under anoxia. *(4)* Isomerization of peroxynitrite ($ONOO^-$) to NO_3^- by $Lb^{2+}O_2$. (*5)* Nitric oxide dioxygenase (NOD) activity of $Lb^{2+}O_2$/$Glb^{2+}O_2$. *(6)* NAD(P)H-dependent nitrate reductase. *(7)* Putative mitochondrial nitrite transporter, specific or shared by other anions. *(8)* Reduction of NO_2^- to NO by the mitochondrial electron transport chain (ETC) under hypoxic conditions. *(9)* Reduction of Lb^{3+}/Glb^{3+} by a putative flavoprotein reductase or by other mechanisms such as NAD(P)H-reduced free flavins. *(10)* Oxidation of Lb^{3+} by H_2O_2 to form ferryl Lb (Lb^{4+}) and two Lb derivatives: a Lb with a heme-globin cross-link and a Lb dimer. *(11)* Reduction of Lb^{4+} to Lb^{3+} by ascorbate. *(12)* Autoxidation of $Lb^{2+}O_2$ generating superoxide anion radicals. *(13)* Oxidation of Lb^{2+} by H_2O_2 to Lb^{4+}. *(14)* Nitration of Lb heme by NO_2^- under mild acid conditions to form nitri-Lb. (*15)* Nitration of tyrosine (Tyr) residues on Lb by H_2O_2/nitrite. *(16)* Degradation of Lb heme by NADPH-dependent heme oxygenase, producing biliverdin, carbon monoxide (CO) and free iron. *(17)* NOD activity of the single-domain hemoglobin (Bjgb) of *Bradyrhizobium diazoefficiens* bacteroids. *(18)* Regeneration of the ferrous form of Bjgb, probably by a flavin reductase. *(19)* NOD activity of the flavohemoglobin (Hmp) of *Sinorhizobium meliloti* or *Mesorhizobium loti* bacteroids. *(20)* Regeneration of the ferrous form of Hmp. This protein contains a flavin cofactor that is able to accept electrons from NAD(P)H and to transfer them to the heme. Therefore, Hmp does not need (theoretically) a reductase to keep the protein in the functional reduced state. Reactions in dashed lines still need further experimental support.

Fig. 3 Hemoglobin genes and proteins of the model legumes *Medicago truncatula* ecotype Jemalong A17 (*marked on blue background*) and *Lotus japonicus* ecotypes MG-20 and Gifu B-129 (*marked on red background*). (a) For *M. truncatula*, the first column includes the gene identifiers (IDs) of genome version Mt4.0 and the second column those of v5.0, in which the prefix 'MtrunA17Chr' has been omitted for simplicity. For example, the ID for *Lb1* is MtrunA17Chr1g0148751. For *L. japonicus*, the first column includes the gene IDs of genome version v3.0 (MG-20) and the second column those of v1.2 (Gifu). In the latter, the prefix 'LotjaGi' has been omitted for simplicity. For example, the ID for *Lb1* is LotjaGi5g1v0024700. For both legumes, the protein lengths are given in amino acids (aa) and the alternative spliced forms (AS) are indicated where applicable. Because a new version of the *L. japonicus* ecotype MG-20 genome (LjPB_ver1.0) has just been published (Li *et al.*, 2020), although the website is not publicly available yet, we provide the new gene IDs for further reference: *Lb1* and *Lb2* (Lj5g0024797; still not given separate gene IDs); *Lb3* (Lj5g000269); *Glb1-1* (Lj3g0017143); *Glb1-2* (Lj3g0010114); *Glb2-1* (Lj5g0004398); *Glb2-2* (Lj5g0019740); *Glb3-1* (Lj1g0018927); and *Glb3-2* (Lj1g0018426). (b) Phylogenetic tree of Lbs and Glbs of *M. truncatula (names in blue)* and *L. japonicus (names in red)*. The proteins are encoded by the gene IDs indicated in (a). The main groups are circled in colors: *L. japonicus* Lbs (*red*); *M. truncatula* Lb1 + Lb10 and Lb2 + Lb4 (*orange*); class 1 Glbs from both legumes (*blue*); *M. truncatula* Lb3 + *L. japonicus* class 2 Glbs (*green*); and two groups of class 3 Glbs (*violet*). The tree was constructed from a ClustalW alignment of full-length protein sequences with Geneious software (https://www.geneious.com) using Jukes-Cantor as the genetic distance model and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) as the tree building method. Branch support was estimated based on 1000 bootstrap replicates; branches with support >60% are shown and those below that threshold are collapsed. The bar indicates substitutions per site.

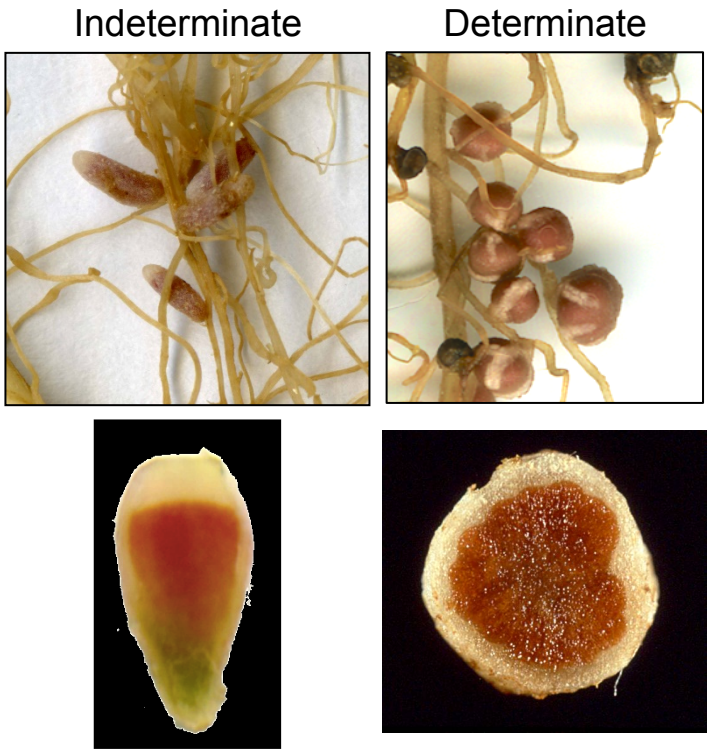
Fig. 4 Expression profiles of *Lb* and *Glb* genes in *Medicago truncatula* (A17) roots from 30 min to 48 hours post-inoculation (hpi) with *Sinorhizobium meliloti* ABS7. Four genotypes were analyzed: A17 (wild-type); the nodulation factor receptor mutants *nfp* and *lyk3*; and the hypernodulator mutant *skl*. The figure shows only the data of genes with meaningful expression levels. The rest of the genes are either expressed below the detection limit or not expressed in the roots at the early time points examined in these experiments. Detailed description of the three mutants and experimental procedures are given in Larrainzar *et al.* (2015) and references therein. RNA-sequencing data were generated and remapped to genome version Mt5.0 (Pecrix *et al.*, 2018). Data are expressed as Normalized Trimmed Mean of M component (NTMM) counts (Robinson & Oshlack, 2010) and represent the average of four biological replicates with standard errors.

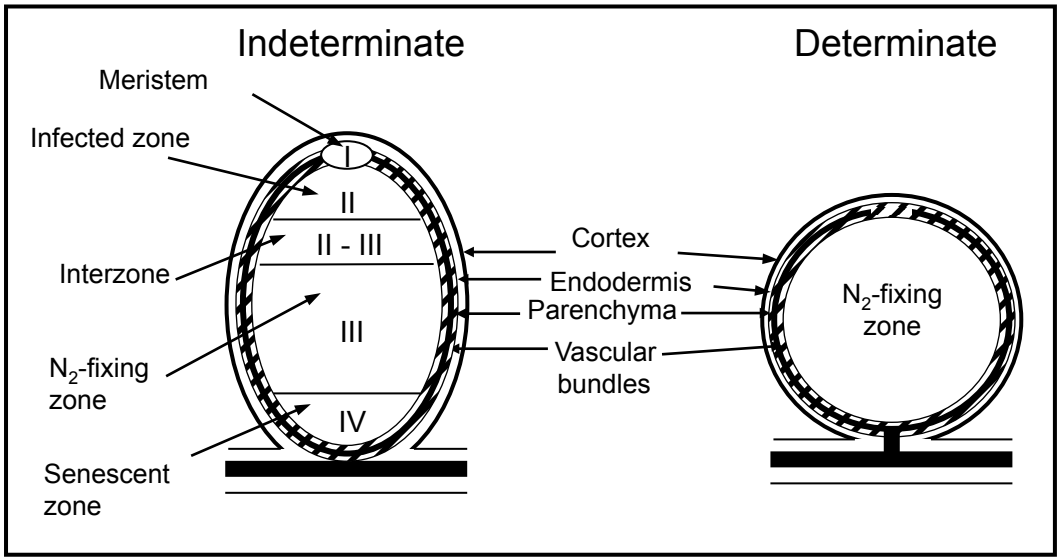
Fig. 5 Expression profiles of *Lb* and *Glb* genes in various organs of *Medicago truncatula* (A17) plants. (a) *Upper plots*. Expression levels of *Lb* genes in roots and nodules after 0 to 28 days post-inoculation (dpi) of plants with *Sinorhizobium meliloti* ABS7. Nod 0 are roots before inoculation; Nod 4 are root bumps at 4 dpi; Nod 10, Nod 14 and Nod 28 are nodules at 10, 14 and 28 dpi. Re-supply treatments with 10 mM KNO_3 were applied to Nod 14 for 12 h and 48 h before sampling. *Lower plots*. Expression profiles of *Lb* genes in different organs. Five-week-old plants were used to sample roots, stems, leaves, petioles and buds, whereas 8- to 10-week-old plants were used to sample medium and large pods. (b) Expression profiles of *Glb* genes in the same organs and conditions described in (a). RNA-sequencing data are given as fragments per kilobase of transcript per million reads mapped (FPKM) and represent the average of three biological replicates with standard errors. Further details are given in de Bang *et al.* (2017), Boschiero *et al.* (2020) and the *M. truncatula* SSP database.

Fig. 6 Expression profiles of *Lb* and *Glb* genes of *Medicago truncatula* (A17) plants with different macronutrient treatments. Plants were grown on a mixture of sand and silica (3:1 v/v) under controlled greenhouse conditions for three weeks. Sets of plants were watered every 2 d with diverse nutrient conditions as follows: Nitrogen [1X, 2 mM N; 1/10X, 0.2 mM N], Phosphorus [1X, 0.6 mM P; 1/100X, 6.0 μM P]; Potassium [1X, 2.45 mM K; 1/1000X, 2.45 μM K]; and Sulfur [1X, 0.6 mM S; 1/100X, 6.0 μM S]. For each treatment, [1X] is the control condition. Re-supply treatments [+1X] with the corresponding nutrients were applied 6 h before sampling. RNA-sequencing data are given in fragments per kilobase of transcript per million reads mapped (FPKM) and represent the average of three biological replicates with standard errors. Statistical analysis was performed only in relevant Lb or Glb expression levels. Asterisks denote significant differences from the control based on the Student's *t*-test ($p< 0.05$). RNA-sequencing analyses, experimental procedures and nutrient solution composition are given in de Bang *et al.* (2017), Boschiero *et al.* (2020) and the *Medicago truncatula* SSP database.

Fig. 7 Expression profiles of *Glb* genes in nodules and roots of *Lotus japonicus* (Gifu) treated with hormones. Seeds were germinated for three days in the dark and two more days with a 16-h photoperiod. Seedlings to be nodulated were then transferred to nitrogen-free Jensen plates, inoculated with *Mesorhizobium loti* R7A and grown for 15 days. Seedlings not to be nodulated were transferred to Jensen plates supplemented with 2 mM NH_4NO_3 and were grown for 10 days. Plants were incubated for 24 h on plates containing 50 μM of 1-aminocyclopropane-1-carboxylic acid (ACC), cytokinins (CK; an equimolar mixture of kinetin and 6-benzyl-aminopurine), or polyamines (PA; an equimolar mixture of spermine, spermidine and putrescine). Hormone stock solutions (100 mM) were prepared in ethanol (ACC and PA) or

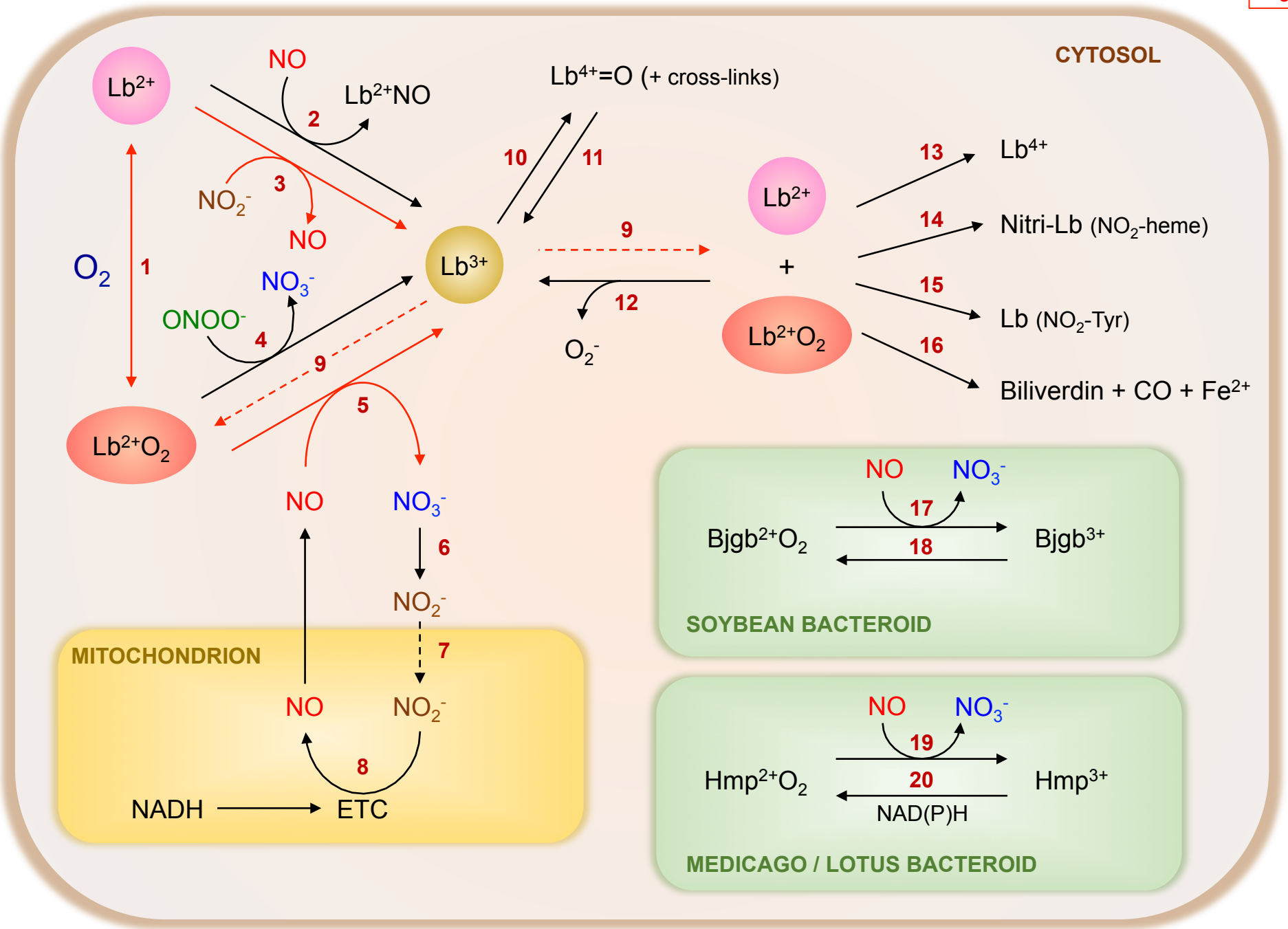
1 M NaOH (CK). Control (mock) plants were transferred to plates containing ethanol (1:2000) or 0.5 mM NaOH for 24 h. Transcript levels of *Glb* genes were determined by real-time quantitative-PCR. Normalized relative quantities (NRQs) were calculated with *ubiquitin* as reference gene (Rubio *et al.*, 2019). The stability of this gene was tested across all samples by using *ATP synthase* as a second reference gene. At least three biological replicates per treatment were used and reactions were performed in triplicate. Values are means ± SE. Asterisks denote significant differences from the control based on the Student's *t*-test ($p < 0.05$).





	Indeterminate	**Determinate**
Host plant	Alfalfa, pea, *Medicago truncatula*	Bean, soybean, *Lotus japonicus**
Geographic origin**	Temperate regions	Tropical and subtropical regions
Nodule shape	Elongate	Spherical
Initial cell divisions	Inner cortex	Outer cortex
Nodule meristem	Persistent meristem	Transient meristem
Nodule growth	Cell division	Cell expansion
Flavonoids inducing *nod* genes	Flavones, flavanones	Isoflavones (mainly)
Export of assimilated nitrogen	Amides	Ureides

Figure 2


CYTOSOL
Lb2+
NO
Lb2+NO
2
NO2-
3
NO
O2
1
NO3-
ONOO-
4
9
5
Lb2+O2
Lb3+
10
11
Lb4+=O (+ cross-links)
9
12
O2-
Lb2+
+
Lb2+O2
13
Lb4+
14
Nitri-Lb (NO2-heme)
15
Lb (NO2-Tyr)
16
Biliverdin + CO + Fe2+
NO
NO3-
6
NO2-
7
MITOCHONDRION
NO
NO2-
8
NADH
ETC
NO
NO3-
17
18
Bjgb2+O2
Bjgb3+
SOYBEAN BACTEROID
NO
NO3-
19
20
Hmp2+O2
Hmp3+
NAD(P)H
MEDICAGO / LOTUS BACTEROID

(a)

Gene IDs (old)	Gene IDs (new)	Globin	Length	Comments
Medtr1g011540	1g0148751	Lb1	146	Lb2 in Gallusci *et al.* (1991)
Medtr1g049330	1g0170851	Lb2	148	
Medtr1g090810	1g0197501	Lb3	155, 174	Two AS? AS1 (155 aa) Arrighi *et al.* (2008); AS2 (174 aa) XP_ 013469303
Medtr1g090820	1g0197531	Lb4	106, 107, 148	Three AS
Medtr5g041610	5g0417631	Lb5	147	
Medtr5g066070	5g0427351	Lb6	106, 147	Lb1 in Gallusci *et al.* (1991). Two AS
Medtr5g080400	5g0435611	Lb7	111, 153	Two AS
Medtr5g080440	5g0435621	Lb8	147	
Medtr5g080900	Not annotated	Lb9	132	Incomplete? No expression detected
Medtr5g081000	5g0435981	Lb10	146	Annotated as Lb3 in GenBank (accession no. XP_003616492)
Medtr5g081030	5g0435991	Lb11	147	
Medtr7g110180	7g0270491	Lb12	109	Incomplete? No expression detected
Medtr4g068860	4g0034301	Glb1-1	160, 133	Functionally characterized by Berger *et al.* (2020). Two AS
Medtr4g068870	4g0034311	Glb1-2	215, 298 344, 351	Four AS with one or two hemes
Medtr0026s0210	2g0305541	Glb1-3	173, 342	Two AS? It has 93% identity with Glb1-2
Medtr3g109420	3g0139391	Glb3-1	170	Predominantly in infected cells of nodules
Medtr1g008700	1g0147401	Glb3-2	169	Expressed throughout the plant. In vascular tissue and at the base of nodules
Lj5g3v0035290.2	5g1v0024700	Lb1	146	Mis-annotation in MG-20. Lb1 and Lb2 are different genes. Uchiumi *et al.* (2002)
Lj5g3v0035290.1	5g1v0024900	Lb2	146	Lb2 has 99% identity with Lb1. Uchiumi *et al.* (2002)
Lj5g3v0465970	5g1v0046500	Lb3	147	Lb3 has 84% identity with Lb1 or Lb2. Uchiumi *et al.* (2002)
Lj3g3v3338170	3g1v0504500	Glb1-1	161	Lj4g3v0353440 is artificial duplication. Called Hb1, characterized by Uchiumi's group
Lj3g3v3338180	3g1v0504600	Glb1-2	161	Lj4g3v0353430 is artificial duplication. Called Hb2 by Shimoda *et al.* (2005)
Lj5g3v1699110	5g1v0253250	Glb2-1	152	Unusual globin. Pentacoordinate as ferrous and hexacoordinate as ferric
Mis-annotated	5g1v0253200	Glb2-2	163	In tandem with Glb2-1 but only 63% identity. Probably, heme coordination as Glb2-1
Lj1g3v2035270	1g1v0398700	Glb3-1	169	Highly expressed in nodules
Lj1g3v0948590	1g1v0172000	Glb3-2	168	Expressed throughout the plant

(b)

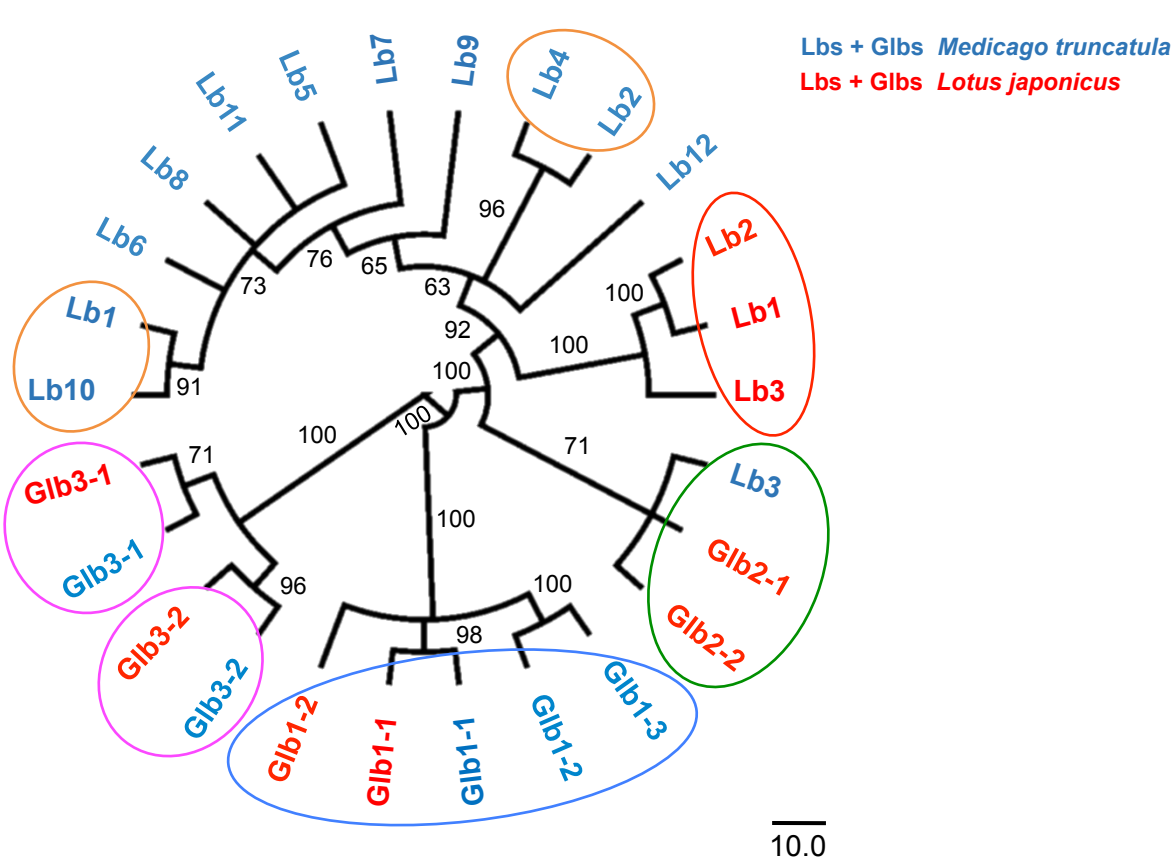
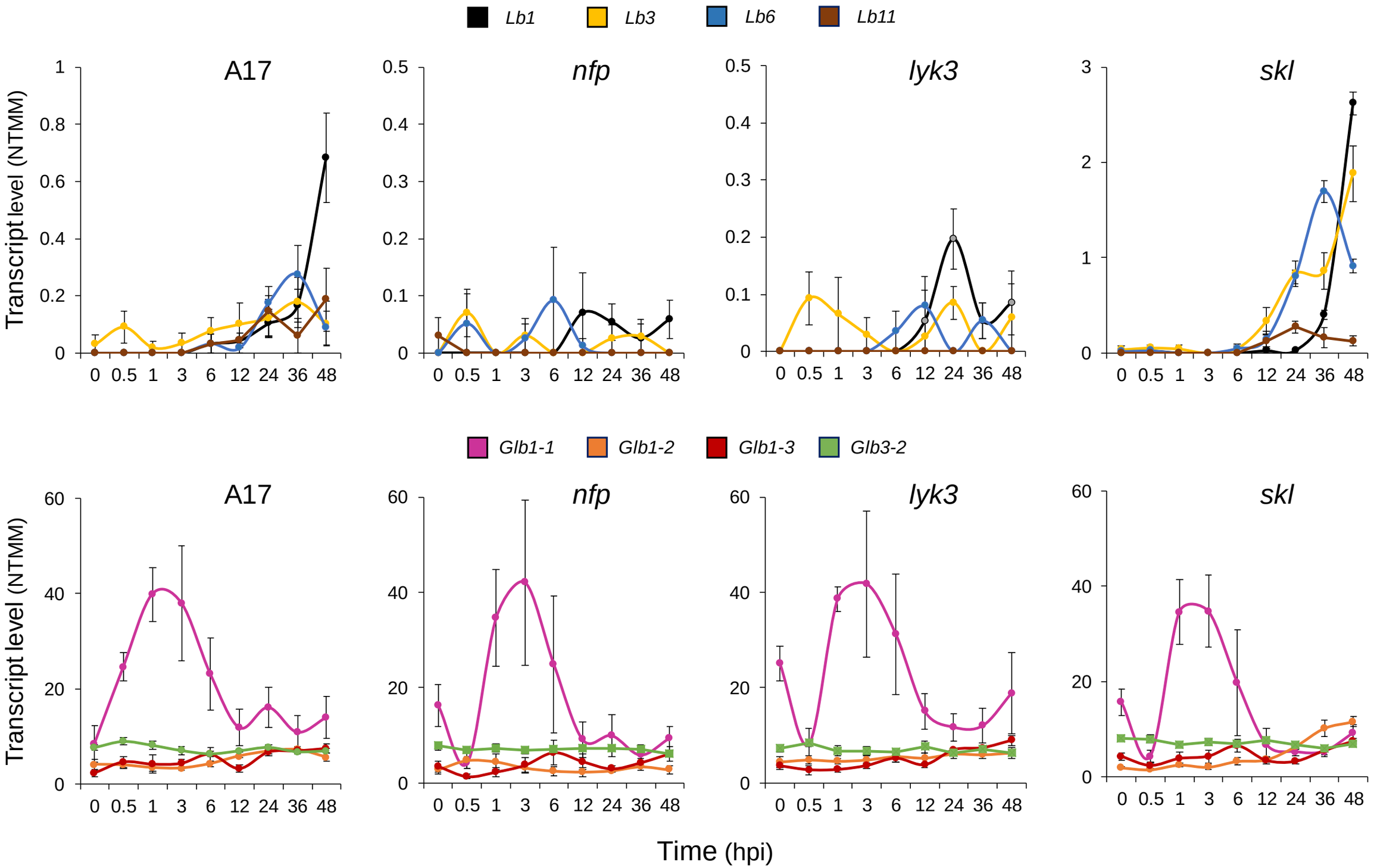


Figure 4


Lb1
Lb3
Lb6
Lb11
A17
nfp
lyk3
skl
Transcript level (NTMM)
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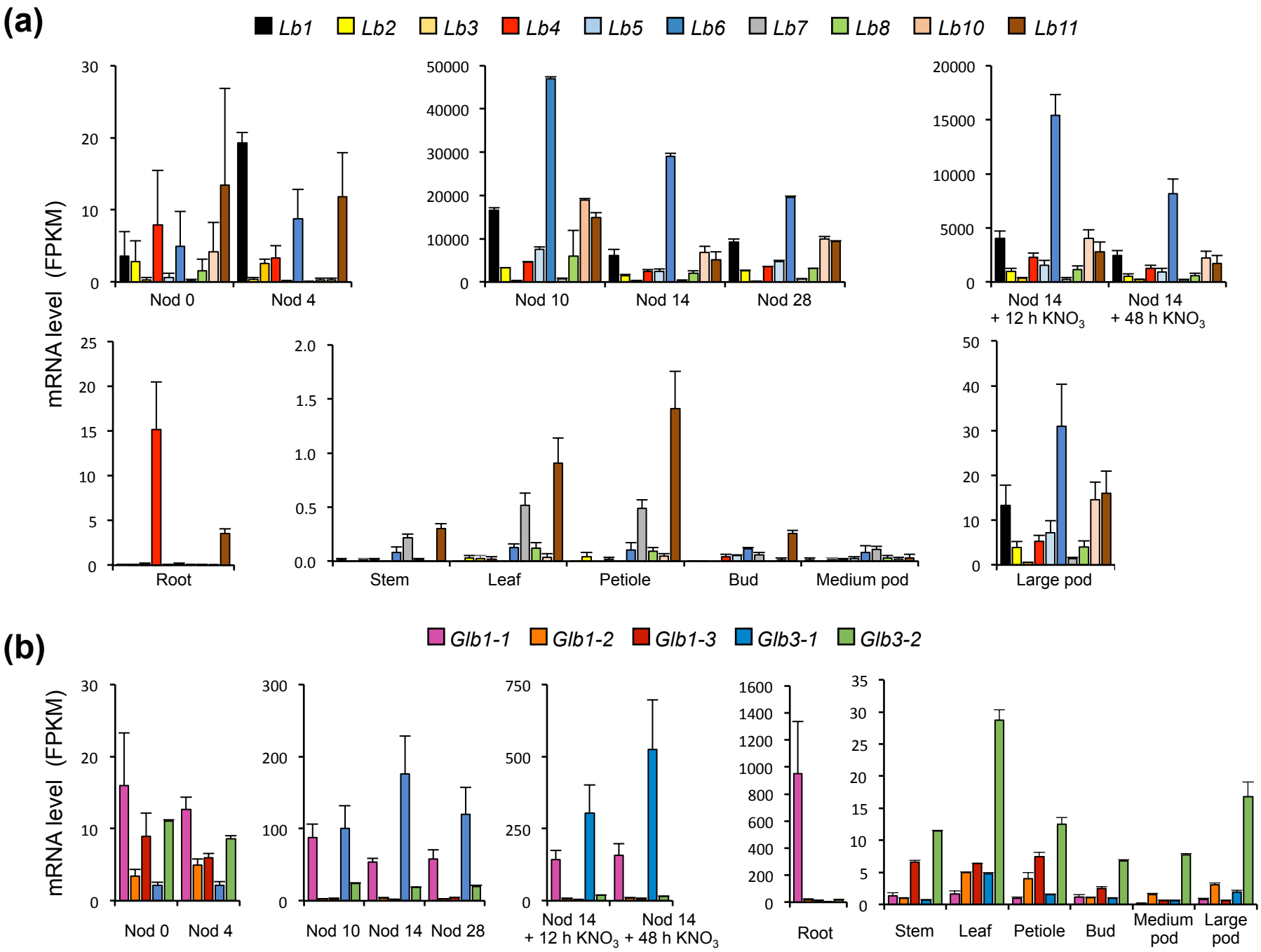
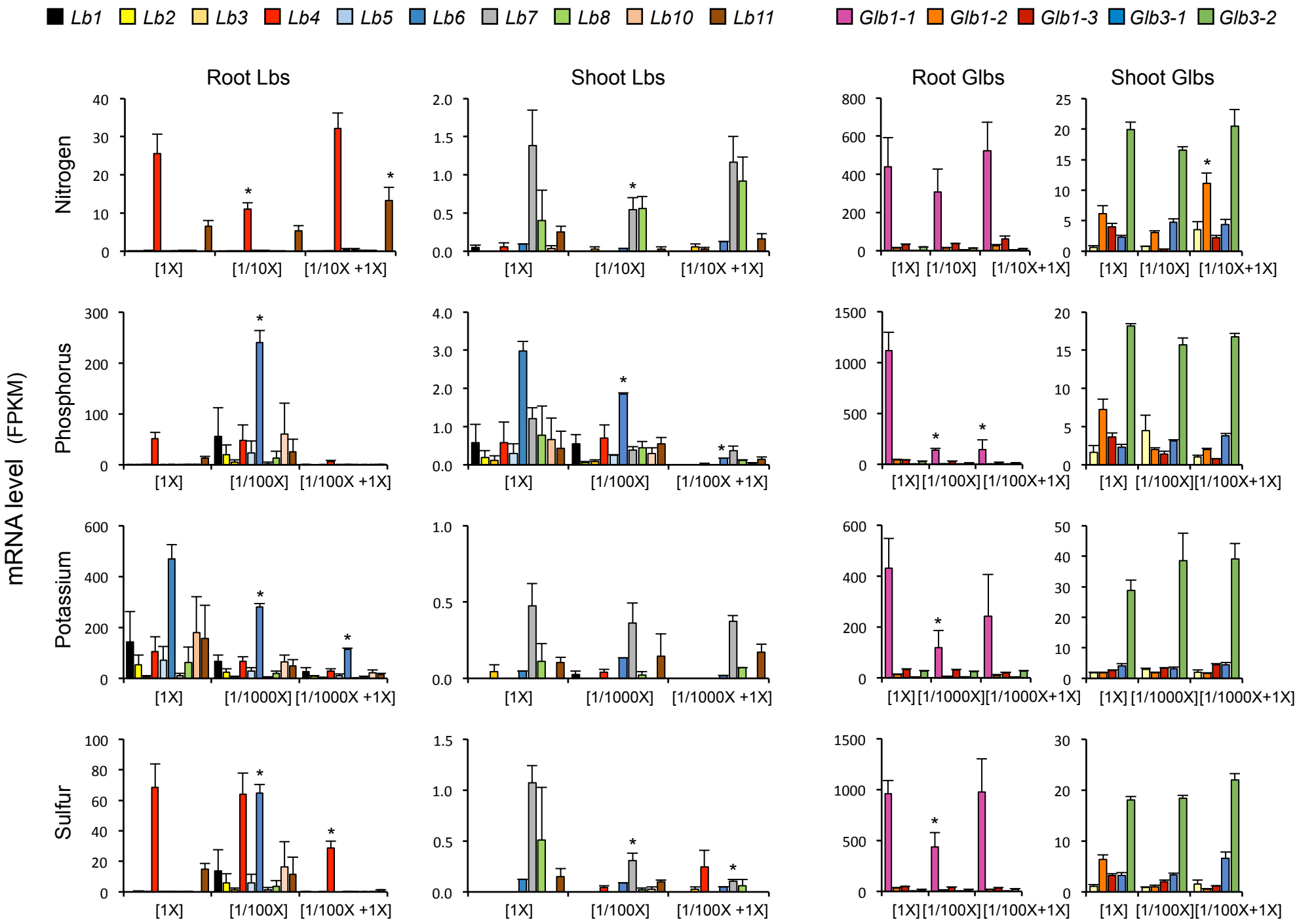
Glb1-1
Glb1-2
Glb1-3
Glb3-2
Time (hpi)


(a)
Lb1
Lb2
Lb3
Lb4
Lb5
Lb6
Lb7
Lb8
Lb10
Lb11
mRNA level (FPKM)
Nod 0
Nod 4
Nod 10
Nod 14
Nod 28
Nod 14 + 12 h KNO3
Nod 14 + 48 h KNO3
Root
Stem
Leaf
Petiole
Bud
Medium pod
Large pod
(b)
Glb1-1
Glb1-2
Glb1-3
Glb3-1
Glb3-2
mRNA level (FPKM)
Nod 0
Nod 4
Nod 10
Nod 14
Nod 28
Nod 14 + 12 h KNO3
Nod 14 + 48 h KNO3
Root
Stem
Leaf
Petiole
Bud
Medium pod
Large pod


Lb1
Lb2
Lb3
Lb4
Lb5
Lb6
Lb7
Lb8
Lb10
Lb11
Glb1-1
Glb1-2
Glb1-3
Glb3-1
Glb3-2
Root Lbs
Shoot Lbs
Root Glbs
Shoot Glbs
Nitrogen
Phosphorus
Potassium
Sulfur
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[1/100X]
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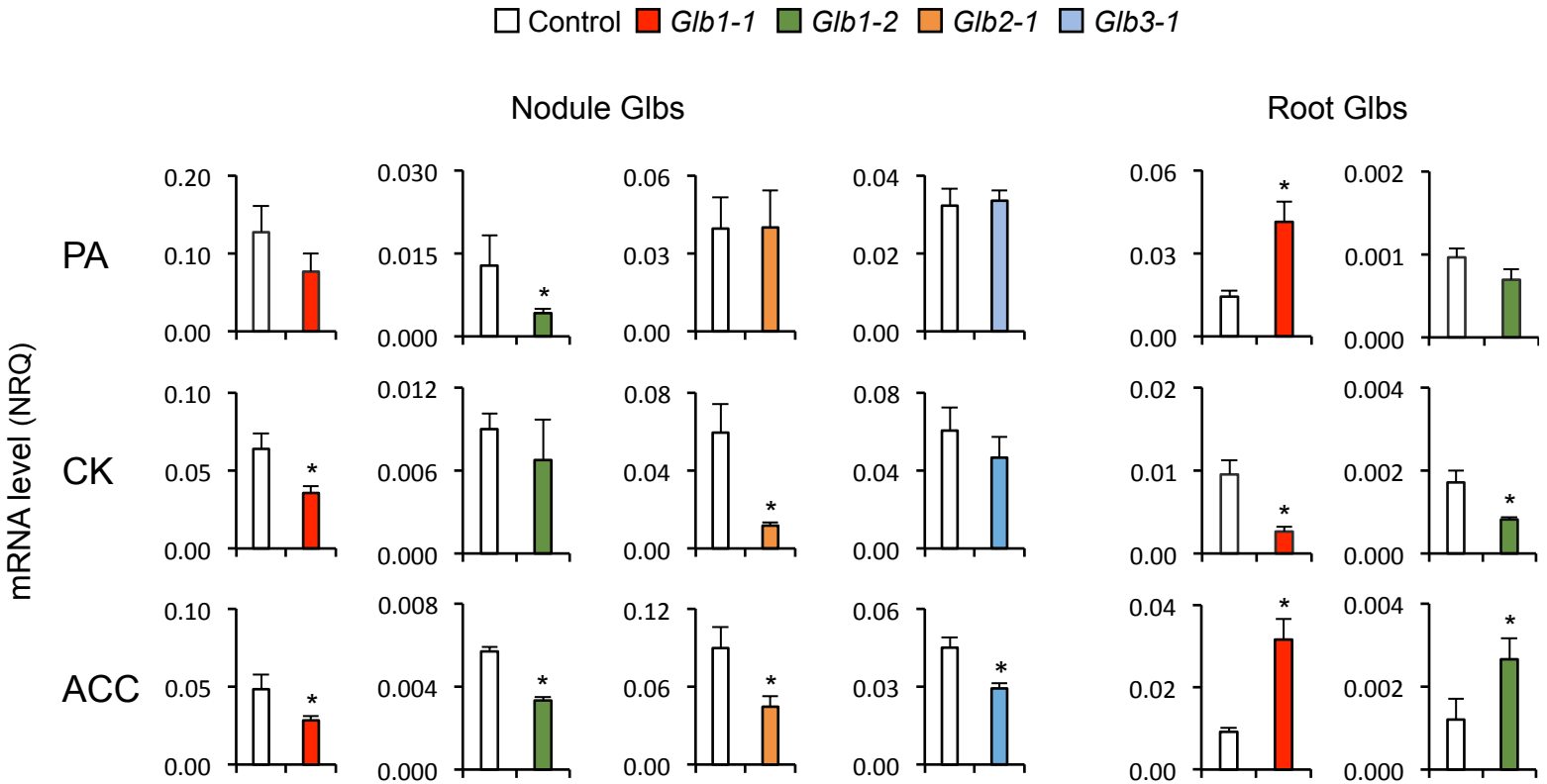
Figure 7


Control
Glb1-1
Glb1-2
Glb2-1
Glb3-1
Nodule Glbs
Root Glbs
mRNA level (NRQ)
PA
CK
ACC
0.20
0.10
0.00
0.030
0.015
0.000
0.06
0.03
0.04
0.02
0.012
0.006
0.08
0.12
0.008
0.004
0.002
0.001
0.05
0.01
*