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Research

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Medicago truncatula Phytoglobin 1.1 controls symbiotic nodulation and nitrogen fixation via the regulation of nitric oxide concentration

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Summary

- In legumes, phytoglobins (Phytogbs) are known to regulate nitric oxide (NO) during early phase of the nitrogen-fixing symbiosis and to buffer oxygen in functioning nodules. However, their expression profile and respective role in NO control at each stage of the symbiosis remain little-known.
- We first surveyed the *Phytogb* genes occurring in *Medicago truncatula* genome. We analyzed their expression pattern and NO production from inoculation with *Sinorhizobium meliloti* up to 8 wk post-inoculation. Finally, using overexpression and silencing strategy, we addressed the role of the Phytogb1.1-NO couple in the symbiosis.
- Three peaks of *Phytogb* expression and NO production were detected during the symbiotic process. NO upregulates *Phytogbs1* expression and downregulates *Lbs* and *Phytogbs3* ones. *Phytogb1.1* silencing and overexpression experiments reveal that Phytogb1.1-NO couple controls the progression of the symbiosis: high NO concentration promotes defense responses and nodular organogenesis, whereas low NO promotes the infection process and nodular development. Both NO excess and deficiency provoke a 30% inhibition of nodule establishment. In mature nodules, Phytogb1.1 regulates NO to limit its toxic effects while allowing the functioning of Phytogb-NO respiration to maintain the energetic state.
- This work highlights the regulatory role played by Phytogb1.1–NO couple in the successive stages of symbiosis.

Introduction

The symbiotic interaction between legumes and Rhizobium bacteria results in the formation of a new root organ, the nodule, whose main function is the reduction and fixation of atmospheric nitrogen (N_2). The process starts with the mutual recognition of both the plant and the bacterial partners. Bacteria enter the root hairs via a specific structure, the infection thread, while some cells of the root cortex divide to form the nodule (Long, 2001). Inside the infection thread that progresses and reaches the cortical cells, bacteria divide and are released into the host cells of the developing nodule. Bacteria then differentiate into bacteroids that reduce N_2 via nitrogenase activity (Oldroyd & Downie, 2008). Indeterminate nodules such as those of alfalfa, clover or pea possess a persistent meristem and comprise four distinct zones: zone I, the meristematic cells; zone II, where the bacteria enter the host cells and differentiate into bacteroids; zone III, where bacteroids reduce N_2 to ammonia (NH_3); and zone IV, characterized by the breakdown of the symbiosis and the onset of senescence (Timmers *et al.*, 2000). As nitrogenase is irreversibly inhibited by traces of oxygen (O_2), N_2 fixation requires the microaerophilic conditions found in nodules (Appleby, 1992).

Nitric oxide (NO) is a bioreactive gaseous molecule found in living organisms. In plants, it participates in the regulation of developmental stages, from germination to senescence (Bruand & Meilhoc, 2019; González-Gordo *et al.*, 2019; Stasolla *et al.*, 2019), and in the response to many abiotic stresses, including hypoxia (Simontacchi *et al.*, 2015). NO is produced during symbiotic interactions, and many studies report its presence during legume–rhizobia symbiosis. NO production is transiently induced in the roots of *Lotus japonicus* and *Medicago sativa* a few hours post-inoculation (hpi) with their bacterial partners (Nagata *et al.*, 2008; Fukudome *et al.*, 2016). NO is also produced in shepherd's crooks of root hairs, infection threads, and nodule primordia (del Giudice *et al.*, 2011). In mature nodules, NO was found complexed with leghemoglobin (Lb) (Maskall *et al.*, 1977; Mathieu *et al.*, 1998; Sánchez *et al.*, 2010) and its presence was mainly associated with the N_2-fixing zone (Baudouin *et al.*, 2006). Cam *et al.* (2012) observed that NO is also produced between the N_2-fixing and senescence zones at the end of the symbiotic process. Considered together, these observations mean that NO is present at various timepoints of the symbiotic process (Hichri *et al.*, 2015, 2016; Meilhoc *et al.*, 2015) and the question is raised as to its physiological

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roles in different times and spaces of symbiotic interaction (Hichri *et al.*, 2015; Berger *et al.*, 2019).

The toxic, signaling or metabolic roles of NO depend on its concentration at the action site (Mur *et al.*, 2013). Therefore, its concentration must be tightly controlled. Several NO sources have been identified in plants, including reductive and oxidative pathways (Mur *et al.*, 2013). The turnover of NO metabolism and messaging depends on the activity of S-nitrosoglutathione reductase that controls the S-nitrosoglutathione pool, a major reservoir of NO (Leterrier *et al.*, 2011; Yun *et al.*, 2016; Astier *et al.*, 2018). NO removal was mainly ascribed to hemoglobins (Hbs) (Gupta *et al.*, 2011). Plant Hbs, renamed phytoglobins (Phytogbs; Hill *et al.*, 2016), have been classified into six categories, including: Phytogb0 – nonsymbiotic hemoglobin (nsHb); Phytogb1 – class 1 nonsymbiotic hemoglobin (nsHb-1); Phytogb2 – class 2 nonsymbiotic hemoglobin (nsHb-2); SymPhytogb – symbiotic hemoglobin (symHb); Lb – leghemoglobin (Lb); and Phytogb3 – class 3 truncated hemoglobin (trHb) (Hill *et al.*, 2016). Three types of Hbs were described in legumes and are expressed during N_2-fixing symbiosis: Phytogb1, Lb and Phytogb3 (Bustos-Sanmamed *et al.*, 2011). Owing to their very high affinity for O_2 and NO, Phytogb1 are capable of recovering traces of O_2 and NO to convert them to nitrate at very low O_2 concentrations (Gupta *et al.*, 2011; Igamberdiev *et al.*, 2011). Phytogb1 scavenge NO and, in return, NO functions as an inducer of Phytogb1 (Nagata *et al.*, 2009; Hill, 2012). Thus, the 'Phytogb1–NO' couple forms a feedback loop allowing a rapid NO concentration regulation. Such a regulation was shown to occur during early steps of N_2-fixing (Nagata *et al.*, 2009; Murakami *et al.*, 2011) and mycorrhizal (Martinez-Medina *et al.*, 2019) symbiosis. In *L. japonicus* nodules, the overexpression of *LjPhytogb1* reduces NO content and enhances N_2 fixation (Shimoda *et al.*, 2009; Fukudome *et al.*, 2018), suggesting that reversible inhibition of nitrogenase is relieved by the scavenging of NO by Phytogb1. Functional nodules are characterized by a microoxic environment. In many root systems under microoxic conditions, NO increases and is scavenged by Phytogb1 to generate ATP in a Phytogb–NO respiratory cycle (Igamberdiev & Hill, 2004). This cycle contributes to the preservation of NAD(P)H/$NAD(P)^+$ and ATP : ADP ratios in hypoxic cells and keeps their viability (Igamberdiev *et al.*, 2010). Accumulating data support the existence of a Phytogb–NO cycle in legume nodules: a strong increase of LbNO complex formation is observed in nodules of soybean plants submitted to hypoxia (Meakin *et al.*, 2007; Sanchez *et al.*, 2010), and the inhibition of the Phytogb–NO cycle strongly decreases the ATP : ADP ratio in *M. truncatula* nodules (Horchani *et al.*, 2011). Lbs accumulate at a millimolar concentration in the cytoplasm of infected nodular cells (Appleby, 1992). They are considered as markers of N_2-fixing symbiosis and their protein abundance correlates with the N_2-fixation activity of the nodules. Lbs buffer free O_2 in the nanomolar range, thus avoiding the inactivation of nitrogenase while maintaining a high flux of O_2 for respiration (Appleby, 1992). It has been shown that deoxy-Lb binds to NO with high affinity to form stable complexes in soybean and that Lb could act as a NO and peroxynitrite scavenger (Herold & Puppo, 2005). Phytogb3 are induced in *M. truncatula* (Vieweg *et al.*, 2005) and *Frankia* (Niemann & Tisa, 2008; Coats *et al.*, 2009) N_2-fixing symbiosis and have been proposed to be involved in NO scavenging.

Although analyzed at specific time-points of the N_2-fixing symbiosis, neither *Phytogbs* expression nor NO production have been investigated in respect of the entire symbiotic process. In this work, we first survey *Phytogb* genes in the *M. truncatula* genome. Then, we analyze Phytogbs expression and NO production from the first hours of symbiotic interaction up to 8 wk post-inoculation (wpi), when the interaction breaks down. Using overexpression and silencing strategy, we investigate further the role of Phytogb1.1 in the regulation of NO concentration during the first days of symbiosis establishment and in N_2-fixing nodules. Based on our data, we discuss the roles of Phytogb1.1 and NO during the different stages of symbiosis.

Materials and Methods

Plants growth and inoculation conditions

Medicago truncatula (cv Jemalong A17) were scarified, sterilized and germinated as in del Giudice *et al.* (2011). Seedlings were cultivated and inoculated with *Sinorhizobium meliloti* 2011 strain either in Petri dishes as in del Giudice *et al.* (2011), or in planters as in Horchani *et al.* (2011). A basic intake of 0.2 mM KNO_3 is provided to crops on Petri dishes and planters. Cultures in Petri dishes were used for short-term experiments up to 14 d post-inoculation (dpi), while those in planters were used for long-term experiments up to 8 wpi. Roots and/or nodules were harvested at various times of the kinetics. For short-term experiments, 2-cm-long root segments corresponding to the infection zone (del Giudice *et al.*, 2011) were harvested for gene expression and NO production; for long-term experiments only nodules were used.

Plasmid constructions

For overexpression constructions, the complete cDNA of *M. truncatula Phytogb1.1* was amplified by PCR and cloned in pDONR207 vector. This sequence was introduced either in the pK7WG2D vector under the control of the 35S promoter (named 35s::*Phytogb1.1*) by simple Gateway reaction or in pKm43GWrolDGFP by multiple Gateway reaction according to the manufacturer's instructions (Invitrogen). For the multiple Gateway reaction, *Phytogb1.1* open reading frams was placed under the control of the NCR001 gene promoter (Mergaert *et al.*, 2003) (named NCR::*Phytogb1.1*). For RNAi constructions, a common region of *c.* 200 bp found in the complete cDNA of *M. truncatula Phytogb1.1* was amplified by PCR using the couple primers RNAi–Phytogb1.1. This sequence was introduced into either the pK7GWIW2D vector (Karimi *et al.*, 2002) (named RNAi::*Phytogb1.1*) or the pK7GWIWG5D(II) vector (Horchani *et al.*, 2011) (named NCR-RNAi::*Phytogb1.1*). Primer sequences are provided in Supporting Information Table S1.

Roots transformation by *Agrobacterium* rhizogenes

The different constructions were introduced into *A. rhizogenes* strain *Arqua1* (Quandt & Hynes, 1993). *Medicago truncatula*

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plants were transformed with *A. rhizogenes* according to Boisson-Dernier *et al.* (2001). Control plants were transformed with *A. rhizogenes* containing either the pK7GWIGW2D or the pK7WG2D empty vectors. Transgenic roots were selected under a Leica MZ FLIII fluorescence stereomicroscope (Leica, Wetzlar, Germany) based on the green fluorescence protein signal at 2 wk after germination. After the removal of nontransgenic roots, composite plants were transferred to new plates containing Fahräeus medium supplemented with 0.2 mM NH_4NO_3 and without antibiotic. For the construction under the control of NCR001 promoter, *M. truncatula* plants were transformed with *A. rhizogenes* according to Vieweg *et al.* (2005). After the selection of transformed hairy roots based on the fluorescent marker at 21 d after transformation, composite plants harboring transgenic roots were placed in planters and inoculated 3 d later with *S. meliloti* strain.

Measurement of NO production

Nitric oxide detection was performed as in Horchani *et al.* (2011) using the 4,5-diaminofluorescein probe (DAF-2; Sigma-Aldrich) with the following changes. Either nodules (20–30 mg FW) or root segments (50–100 mg FW) were incubated in 1 ml of detection buffer (10 mM Tris-HCl pH 7.4, 10 mM KCl) in the presence of 10 μM DAF-2. As a control, NO production was measured in the same experimental system through the use of the Cu(II) fluorescein (CuFL) fluorescent probe (Strem Chemicals, Bischheim, France) instead of DAF-2 in the detection buffer as described in Horchani *et al.* (2011). Similar results were obtained with both probes. The production of NO was measured with a spectrofluorimeter-luminometer (Xenius, Safas, Monaco).

RNA isolation, reverse transcription and genes expressions

RNAs were isolated from 100 mg of frozen material ground in liquid N_2 using the RNAzol following the manufacturer's recommendations (Sigma-Aldrich). RNA quality was checked and DNase treatment was carried out before the synthesis by GoScript reverse transcriptase (Promega) of the cDNAs. The quantitative reverse transcription polymerase chain reaction (RT-qPCR) was done with the Go-Taq qPCR master Mix kit according to the manufacturer's instructions (Promega). RT-qPCR data analyses were carried out using RqPCRBase, an R package working in the R computing environment for analysis of quantitative real-time PCR data (Hilliou & Tran, 2013). The expression of the different genes was normalized against two housekeeping genes, *Mtc27* (Van de Velde *et al.*, 2006) and *Mta38* (del Giudice *et al.*, 2011). RT-qPCR analyses were carried out in triplicate, using the primers reported in Table S1.

NO donor treatments

Plants were treated with 0.5 mM of either diethylamine-NONOate (DEA-NO) or DEA solutions. Two hundred microliters of solution were added along the whole length of the roots at 2 h before inoculation with *S. meliloti* and then every 24 h for 4 d. Control plants were treated with water. After 4 d, plants were either analyzed for gene expression or transferred to a NO donor free medium and grown for an 10 additional days before measurement of nodule number.

Nitrogen-fixing capacity measurement

Nitrogenase activity of nodules was determined *in vivo* by measuring acetylene reducing activity (ARA; Hardy *et al.*, 1968). Nodulated roots were harvested and incubated at 30°C for 1 h in rubber-capped tubes containing a 10% acetylene atmosphere. Ethylene concentrations were determined by GC (Agilent GC 6890N; Agilent Technologies, Les Ulis, France) equipped with a GS-Alumina (Agilent Technologies) separating capillary column. Three independent biological replicates have been performed with five technical replicates per biological assay.

Extraction and measurement of nodule adenine nucleotides

Adenine nucleotides were extracted and measured as in Horchani *et al.* (2011). Adenine nucleotides were measured in a Xenius spectrofluorimeter-luminometer using the ATPlite one-step assay system (Perkin-Elmer, Villebon-sur-Yvette, France) according to the manufacturer's instructions.

Phylogeny

The phylogeny data were obtained using the one-click mode of the website (http://www.phylogeny.fr; Dereeper *et al.*, 2008) which includes a sequence alignment using the Muscle and Gblocks programs. Phylogenetic reconstruction was done with the PHyML program using the maximum likelihood method. Nodes with a robustness < 80% were pooled in the same phylogenetic subgroup.

Results

Medicago truncatula phytoglobin family

Research in genomic and protein libraries (JCVI, https://www.jcvi.org/medicago-truncatula-genome-database; NCBI, https://www.ncbi.nlm.nih.gov/; UniProt, https://www.uniprot.org/) revealed that the *M. truncatula* genome contains 17 Phytogb genes. Phylogenetic analysis of protein sequences of *M. truncatula* Phytogbs, compared with *Glycine max*, *L. japonicus* and *Arabidopsis thaliana* Phytogbs, confirmed the presence of three Phytogb classes (Fig. S1). Three Phytogb1, two Phytogb3 and 12 Lbs were identified in *M. truncatula*, whereas *G. max* and *L. japonicus* possess two Phytogb1, and only four and six Lbs, respectively (Fig. S1). This large number of Lbs with distinct protein sequences (Fig. S2) and Affymetrix expression patterns (Fig. S3) highlights the still unresolved but different roles and locations of each of them within the *M. truncatula* nodule. *Medicago truncatula Lbs* genes are found in chromosomes 1, 5 and 7 (Fig. S4a). Five *Lbs* genes are located close to each other in a 265 kb region of chromosome 5. This cluster of genes could be the origin of gene duplication events and explain the large number

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of *Lbs* in *M. truncatula* (Storz, 2016). *Phytogb1.1* and *Phytogb1.2* are located in a restricted area in chromosome 4 (no information is available on the chromosomal location of *Phytogb1.3*). The two *Phytogb3* genes previously identified by Vieweg *et al.* (2005) are located in the chromosomes 1 and 3. The 'exon-intron' structure analysis shows that most of the *Phytogbs* contain four exons and three introns (Fig. S4b). This structure, already described in the *Phytogbs* of *L. japonicus* (Bustos-sanmamed *et al.*, 2011), is representative of the ancestral hemoglobin gene (Hardison, 1998). The MEME analysis tool (http://meme-suite.org) was used to identify conserved motifs in the protein sequences of *M. truncatula* Phytogbs (Fig. S5). This analysis identified four highly conserved motifs, one of which is involved in heme binding and another responsible for NO dioxygenase activity (Fig. S5) (Smagghe *et al.*, 2008). The protein sequence of Phytogb1.1 is similar to that of Lbs, except for Lb8 and Lb11. Phytogb1.2 and 1.3 have a sequence twice as long and a repetition of the four protein motifs (Figs S4b, S5) that correspond to the same repetitions of exons in the gene sequence. This doubling of the gene and protein sequence is not observed in Phytogbs of *A. thaliana* or legumes such as *G. max*, *L. japonicus* and *P. sativum*, but it is found in *Trifolium subterraneum* and *Vicia faba* (http://www.coolseasonfoodlegume.org/). Interestingly, the two *Phytogb3* genes have only the heme binding domain (Fig. S5), which raises the question of whether they possess NO dioxygenase activity.

Considering the confusion in the name of the *M. truncatula Phytogb* genes in the literature, and based on Mt4.0 database classification, we propose to homogenize their nomenclature. Nomenclature, Affymetrix, gene (Mt4.0 genome version from Noble database), and Symbimics accession codes (Roux *et al.*, 2014) of the 17 *MtPhytogb* genes are listed in Table 1.

Phytoglobin genes expression during the symbiotic process

Medicago truncatula Phytogb expression patterns were analyzed from 0 to 8 wpi. Two types of *M. truncatula* cultures were used: a short-term culture from 0 to 14 dpi, and a long-term culture up to 8 wpi. As compared with its expression level in noninoculated roots, *Phytogb1.1* expression exhibited first a 75% drop at 4 hpi with *S. meliloti*, and two transient peaks at 10 hpi and 4 dpi (Fig. 1a). It then increased progressively up to 5 wpi and strongly at 7–8 wpi, at the onset of nodule senescence (Fig. 1b). After a 50% decrease during the first hours of the interaction, *Phytogb1.2* expression transiently peaked at 4 dpi and strongly increased at 7–8 wpi in senescent nodules (Fig. 1c,d). After a transient decrease at 1–2 dpi, the expression of *Phytogb1.3* changed only slightly up to 6 wpi and then peaked at 7 wpi when senescence is initiated (Fig. 1e,f). *Phytogb3.1* expression, undetectable in noninoculated roots, was rapidly induced at 10 hpi. Its expression remained steady up to 9 dpi, then increased to reach a plateau between 3 and 7 wpi, and finally increased strongly at 8 wpi (Fig. 1g,h). Except for a peak at 4 dpi, the expression of *Phytogb3.2* fluctuated only moderately and remained stable during the whole symbiotic process (Fig. 1i,j).

The analysis of Affymetrix and Symbimics data (Fig. S3; Table S1) showed that the 12 *Lb* genes exhibit a similar expression pattern and are expressed in the nodule interzone II–III and zone III. Therefore, to avoid analyzing the expression of the 12 *Lb* genes, we used *Lb4* and *Lb3*, whose expression is average among the different *Lb*, as representative *Lb* markers (Fig. S3). Their expression remained close to the detection limit up to 4 dpi (Fig. 1k). Then, it strongly increased to reach a maximum between 3 and 5 wpi, when the N_2-fixing activity of nodules is maximal, and finally decreased when the nodules enter in senescence between 6 and 8 wpi (Fig. 1l).

The expression levels of *Phytogb* as compared with each other, before inoculation and at four time-points in the symbiosis, are reported in Fig. 2. Several features emerged from this analysis: predictably, *Lb4* was more highly expressed than *Phytogb1* and *Phytogb3* in 4 wpi N_2-fixing nodules; whereas *Phytogb1.1*, *1.2*, *1.3* and *3.2* were constitutively expressed in roots and nodules, *Phytogb3.1* became one of the most highly expressed in mature nodules, suggesting a particular role in N_2 fixation; with the exception of *Phytogb3.2*, all the *Phytogbs* analyzed in this study were highly expressed in the senescent nodules at 8 wpi; in noninoculated roots, *Phytogb1.1* was the most strongly expressed *Phytogb* and it remained highly expressed throughout the symbiotic process.

NO production during the symbiotic process

Nitric oxide production was followed at the same time-points as those chosen for *Phytogbs* expression analysis. As reported in Fig. 3, three production peaks were detected: the first at 10 hpi during the first hours of the interaction between the plant and the bacteria, the second at 4 dpi during the early development of the nodule, and the third at 3–4 wpi when nodule reaches maturity. In view of the repetitions, a fourth peak is possible at 6 wpi, but this needs further investigation. When expressed as a function of protein mass (Fig. S6), the amount of NO production in the nodules is close to that in the roots, but the NO peak pattern remains the same. Such a pattern, which does not exclude the possibility of other production peaks on shorter time steps, highlights the fact that NO production presents wide fluctuations during the symbiotic process which coincide with the expression pattern of *Phytogb* genes, particularly *Phytog1.1*, suggesting their involvement in NO regulation.

NO regulates phytoglobin gene expression

Previous reports showed that *Phytogb1* genes are responsive to NO in *L. japonicus* and *Alnus firma* (Shimoda *et al.*, 2005; Sasakura *et al.*, 2006; Bustos-Sanmamed *et al.*, 2011), but information was missing for most of the other *Phytogb* genes. To fill this gap, the effects of NO were analyzed on *M. truncatula Phytogb* gene expression in roots inoculated with *S. meliloti* and treated for 4 d with 0.5 mM of either the NO-donor DEA-NO, or its control DEA. DEA-NO treatment was found to upregulate the expression of *Phytogb1.1*, *Phytogb1.2* and *Phytogb1.3* genes (Fig. 4a–c). As a positive control, the effects of DEA-NO

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Table 1 Nomenclature, access codes and Symbimics expression of *Medicago truncatula* phytoglobins.

					DESEQ MEAN				
Formal name	New nomenclature	Code affymetrix	Code gene Mt4.0	Mt20120830-LIPM	FI	FIID	FIIP	IZ	ZIII
Leghemoglobins	Lb1	Mtr.27725.1.S1_s_at	Medtr5g080400	Mt0010_10133	18.8	65.8	515.5	126 988.2	36 232.2
	Lb2	Mtr.40141.1.S1_at	Medtr5g066070	Mt0008_10574	112.8	905.6	4010	136 478.7	137 042.5
	Lb3	Mtr.38572.1.S1_at	Medtr1g090810	Mt0039_00029	18.2	92.3	477	1413.4	1908.7
	Lb4	Mtr.40134.1.S1_x_at	Medtr1g011540	Mt0002_10177	104.9	261.2	605.7	66 480.1	38 108.6
	Lb5	Mtr.40138.1.S1_at	Medtr5g041610	Mt0012_10297	13.3	33.7	181.8	48 344.6	24 958.1
	Lb6	Mtr.51231.1.S1_x_at	Medtr5g080440	Mt0010_10135	14.5	157.6	2343.9	111 612	26 444.4
	Lb7	Mtr.31441.1.S1_at	Medtr5g081000	Mt0010_00186	104.1	1478.2	14585.5	185 977	71 579.4
	Lb8	Mtr.8284.1.S1_s_at	Medtr1g090820	Mt0039_00027	21.2	197.8	5311.1	49 348.4	14 757.8
	Lb9	Mtr.36094.1.S1_at	Medtr5g081030	Mt0010_00186	104.1	1478.2	14585.5	185 977	71 579.4
	Lb10	Mtr.43465.1.S1_at	Medtr1g049330	Mt0002_11840	11.7	29.4	2188.5	52 581.5	14 066.4
	Lb11	Mtr.36091.1.S1_at	Medtr7g110180	Mt0067_00016	1.5	1	84	766.9	145.9
	Lb12	NC	Medtr5g080900	Mt0010_00174	0	0	0	19	4.8
ns-Hb	Phytogb1.1	Mtr.10462.1.S1_at	Medtr4g068860	Mt0003_10767	2.4	1	2.2	152.1	167.5
	Phytogb1.2	Mtr.29072.1.S1_s_at	Medtr4g068870	Mt0003_10768	20.6	5.1	1.8	7.9	11.7
	Phytogb1.3	NC	Medtr0026s0210	Mt0218_10008	0	0.2	0	0	0
tr-Hb	Phytogb3.1	Mtr.47990.1.S1_at	Medtr3g109420	Mt0007_00981	3.4	18.9	139.9	278.2	583.4
	Phytogb3.2	Mtr.10341.1.S1_at	Medtr1g008700	Mt0002_00088	76.8	75.6	182.8	280.3	260.8

Affimetrix gene codes were on https://mtgea.noble.org/v3/; Mt4.0 gene codes were on https://www.jcvi.org/medicago-truncatula-genome-database; Symbimics data were in Roux *et al*. (2014). NC, no communicated; DESEQ MEAN corresponds to the expression value of the RNA-sequencing analysis in the different zones of the nodule; FI, meristematic zone; FIID, distal infection zone; FIIP, proximal infection zone; IZ, interzone; ZIII, N_2-fixing zone. Shades of red highlight the highest expression value for each gene.

and DEA were analyzed on two plant defense marker genes, glutathione S-transferase (*MtGST*, Medtr7g071380) (Gullner *et al*., 2018) and chalcone synthase (*MtCS*, Medtr1g124600) (Dao *et al*., 2011), which are induced by NO in 4 dpi roots (Boscari *et al*., 2013). Their induction in response to DEA-NO confirmed the efficiency of the treatment (Fig. S7). Conversely, DEA-NO treatment was found to downregulate the expression of *Phytogb3.1*, *Phytogb3.2* and *Lb4* genes (Fig. 4d–f). These results indicate that the six *Phytogb* genes are responsive to high NO concentration. Considered together, the data dealing with NO production pattern (Fig. 3), *Phytogb* expressions (Fig. 1) and NO-donor effects (Fig. 4) indicate that a close relationship exists between NO concentration and *Phytogb1.1* expression.

Phytogb1.1 expression regulates NO concentration during symbiosis establishment and nodule organogenesis

To analyze the potential involvement of Phytogb1.1 in the modulation of NO concentration during early symbiosis steps, we generated two types of *M. truncatula* transformed roots. 35s::*Phytogb1.1* overexpressed *Phytogb1.1*, and RNAi::*Phytogb1.1* silenced *Phytogb1.1* expression, both under the control of the constitutive CaMV 35s promoter (Fig. S8). At 4 dpi, 35s::*Phytogb1.1* roots showed a 4.5-fold enhanced expression of *Phytogb1.1* as compared with control plants, whereas RNAi::*Phytogb1.1* roots showed a 2.5-fold decrease in *Phytogb1.1* expression (Fig. 5a). The expression of *Phytogb 1.2, 1.3, 3.1* and *3.2* genes was not modified in *35s::Phytogb1.1* and RNAi*::Phytogb1.1* roots (Fig. S9a). NO concentrations were decreased 1.6-fold and increased 1.3-fold in 35s::*Phytogb1.1* and RNAi::*Phytogb1.1* roots, respectively (Fig. 5b), confirming that Phytogb1.1 regulates the concentration of NO in inoculated roots. No growth phenotype was visible on the transformed roots.

Then, the role of Phytogb1.1 in the nodulation process was investigated. As reported in Table 2, the nodule number per plant was lower both in 35s::*Phytogb1.1* and in RNAi::*Phytogb1.1* roots compared with control roots (Table 2). Interestingly, the treatment of nontransformed roots with 0.5 mM DEA-NO during the 4 d following inoculation also resulted in a decreased nodule number per plant at 14 dpi without any other visible phenotype change on plant growth (Table 3). This indicates that both an excess and a lack of NO (± 30%) result in the inhibition of nodule establishment.

To explore further the role of Phytogb1.1 and NO in the early stages of symbiosis, we analyzed the expression of various marker genes in control and transformed roots at 4 dpi. Both *GST* and *CS* genes were found to be induced in RNAi::*Phytogb1.1*, while their expression was unchanged in 35s::*Phytogb1.1* (Fig. 6a,b), indicating that their expression, and consequently the plant defense response, is upregulated by increased NO concentration, but not repressed under low NO. *Enod20* is a marker of rhizobia infection (Greene *et al*., 1998; Vernoud *et al*., 1999). Its expression was upregulated in 35s::*Phytogb1.1* roots, and downregulated in RNAi::*Phytogb1.1* roots (Fig. 6c), indicating that the infection process is negatively regulated by NO. *Cre1* is a marker of nodule organogenesis (Frugier *et al*., 2008). Its expression was downregulated in 35s::*Phytogb1.1* roots and upregulated in RNAi::*Phytogb1.1* roots (Fig. 6d), indicating a positive regulation of organogenesis by NO. *Lb4* was chosen as a representative marker of early nodule

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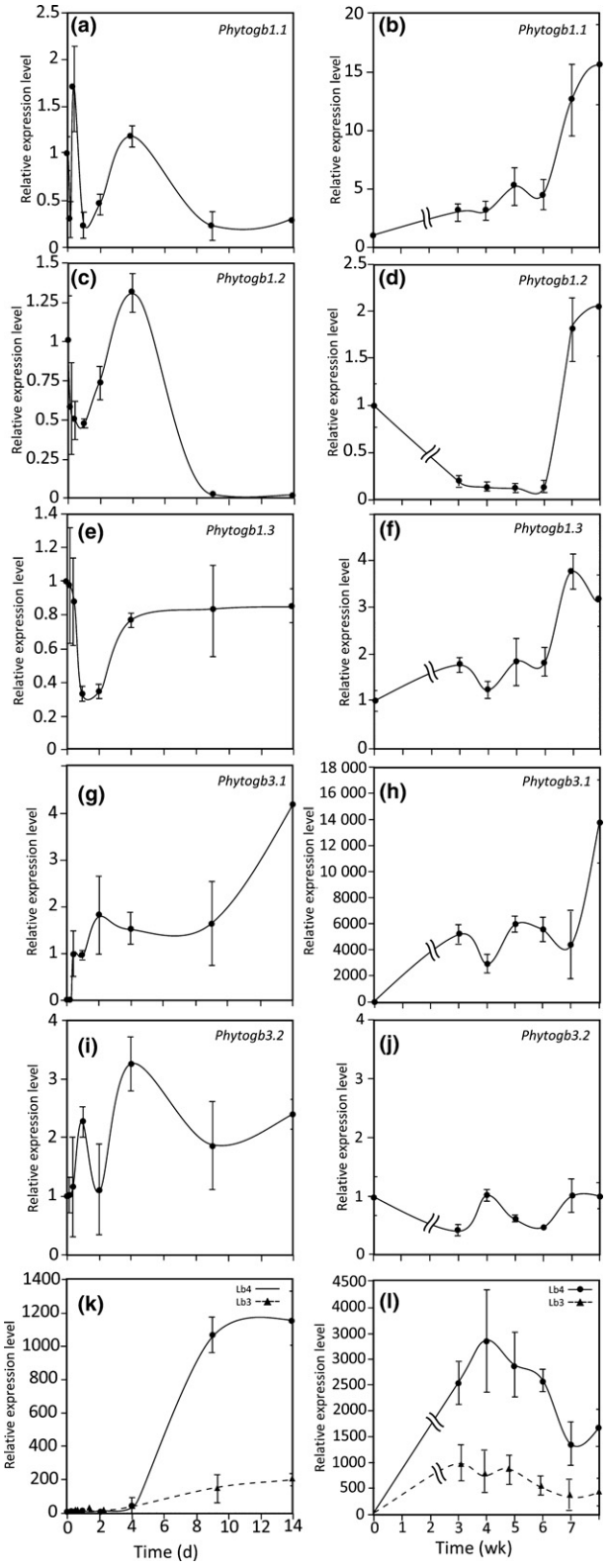

Fig. 1 Expression of *Medicago truncatula Phytogb1*, *Phytogb3* and *Lb* genes during the symbiotic process: short-term kinetic 14 d post-inoculation (dpi) (a, c, e, g, i, k); long-term kinetic 8 wk post-inoculation (wpi) (b, d, f, h, j, l). Expression of *Phytogb1.1* (a, b), *Phytogb1.2* (c, d), *Phytogb1.3* (e, f), *Phytogb3.1* (g, h), *Phytogb3.2* (i, j), and *Lb3* and *4* (k, l). Data are means ± SE ($n = 3$). Each measurement was done in triplicate.

development and N_2-fixation machinery acquisition (Appleby, 1992). Its expression was higher in 35s::*Phytogb1.1* and lower in RNAi::*Phytogb1.1* compared with the control roots, supporting the idea that the early development of the nodule may be negatively regulated by NO (Fig. 6e).

Phytogb1.1 expression regulates NO concentration and nitrogen fixation in mature nodules

To analyze the role of *Phytogb1.1* specifically in mature nodules, new constructions either overexpressing (NCR::*Phytogb1.1*) or silencing (NCR-RNAi::*Phytogb1.1*) *Phytogb1.1* were designed using the nodule zone III specific promoter NCR001 (Mergaert *et al.*, 2003). These constructs present the advantage of modifying the expression of *Phytogb1.1* in the N_2-fixing zone without impacting the formation and development of the nodule. The result was that *Phytogb1.1* was 3.3-fold more and three-fold less expressed in 3 wpi NCR::*Phytogb1.1* and NCR-RNAi::*Phytogb1.1* nodules, respectively, compared with their respective controls (Fig. 7a). The expression of *Phytogb 1.2*, *1.3*, *3.1* and *3.2* genes was not modified in NCR*::Phytogb1.1* and NCR-RNAi*::Phytogb1.1* nodules (Fig. S9b). NO concentrations were 1.5-fold decreased and 1.4-fold increased in the NCR::*Phytogb1.1* and the NCR-RNAi::*Phytogb1.1* nodules, respectively, compared with their control (Fig. 7b), indicating that Phytogb1.1 regulates the concentration of NO in mature nodules. No growth phenotype was visible on the transformed roots.

The functional state of 3 wpi nodules was assessed through the measurement of the *in vivo* nitrogenase activity (measured as ARA) to evaluate their N_2-fixing capacity and the ATP : ADP ratio to evaluate their energy state (Table 4). Compared with their control, NCR*::Phytogb1.1* nodules exhibited a 34% higher ARA, while RNAi-NCR::*Phytogb1.1* ones exhibited a 30% reduced ARA. Similarly, when compared with control nodules, ATP : ADP ratios were found to be higher in NCR*::Phytogb1.1* nodules (8.0 ± 0.3) and lower in RNAi-NCR::*Phytogb1.1* nodules (4.8 ± 0.1). These data clearly indicate that Phytogb1.1 is able to modulate the energy and N metabolism of mature nodules, presumably through the regulation of the concentration of NO.

To go further in the understanding of the role of Phytogb1.1 in these processes, the expression of genes involved in N_2-reduction and assimilation, hypoxia and senescence was analyzed in control and transformed nodules (Fig. 8). The expression of glutamine synthetase 1a (GS1a), involved in the assimilation of N (Groat & Vance, 1981), and of *Lb4* was found to be clearly induced in NCR::*Phytogb1.1* nodules and reduced in NCR-RNAi::*Phytogb1.1* nodules as compared with their respective controls (Fig. 8a,b), indicating a negative regulation of N_2 fixation by NO. As mature nodules exhibit a microoxic environment, the expression of alcohol dehydrogenase (*ADH*) and pyruvate decarboxylase (*PDC*), two marker genes of hypoxia (Bailey-Serres & Voesenek, 2008), was analyzed. Their expression was increased in NCR-RNAi::*Phytogb1* nodules, whereas it was unchanged in NCR::*Phytogb1.1* nodules (Fig. 8c,d), indicating that a rise of NO concentration in the nodule activates the expression of hypoxia-responsive pathway. Finally,

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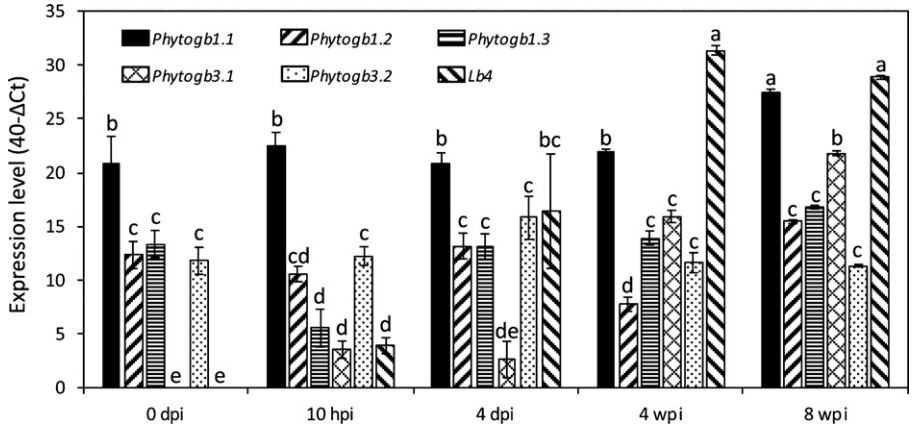

Fig. 2 Expression of *Medicago truncatula Phytogb* genes at various times of the symbiotic process. The reference value '1' was attributed to the first time when the cycle threshold (*Ct*) of the analyzed gene was significantly detectable. Comparative expression levels between genes are given on a logarithmic scale expressed as $40 - \Delta Ct$, where ΔCt is the difference in quantitative reverse transcription polymerase chain reaction threshold cycle number between the respective gene and the reference gene; the number 40 was chosen because the PCR run stops after 40 cycles (Bari *et al.*, 2006; Truong *et al.*, 2015). Data are means ± SE ($n = 3$). Values followed by different letters are significantly different according to one-way ANOVA analysis followed by a Fisher test ($P < 0.05$). dpi, d post-inoculation; hpi, h post-inoculation; wpi, wk post-inoculation.

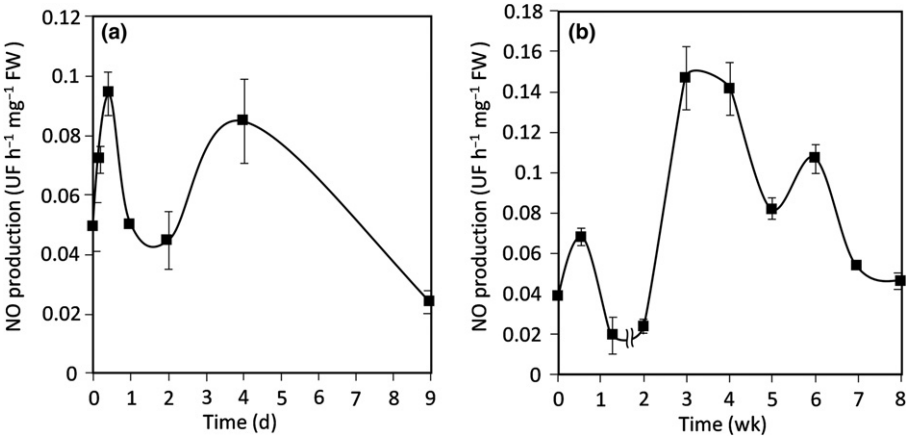

Fig. 3 Nitric oxide (NO) concentration during the *Medicago truncatula* symbiotic process: (a) short-term kinetic 14 d post-inoculation (dpi); (b) long-term kinetic 8 wk post-inoculation (wpi). The fluorescence intensity of the NO production was measured using the 4,5-diaminofluorescein probe (DAF-2; Sigma-Aldrich). Data are means ± SE ($n = 3$). Each measurement was done in triplicate.

we analyzed the expression of the cysteine protease 6 (*CP6*) gene, a reliable marker of senescence in *M. truncatula* nodules (Van de Velde *et al.*, 2006; Pierre *et al.*, 2014). *CP6* was found to be downregulated in NCR::*Phytogb1.1* nodules, and strongly upregulated in NCR-RNAi::*Phytogb1.1* nodules (Fig. 8e), indicating that overexpression of *Phytogb1.1* delayed the senescence, while its downregulation promoted it.

Discussion

In this study we identified 17 *M. truncatula Phytogb* genes composed of 12 *Lb*, three *Phytogb1* and two *Phytogb3*. *Lbs* are close to each other, but phylogenetically (Fig. S1) and structurally (Fig. S2) different from the other *Phytogb* types (Vinogradov *et al.*, 2006). Most *MtLbs* are strongly expressed in the nodule interzone II–III, whereas *MtLb2* is equally expressed in the interzone and zone III, and *MtLb3* is mainly expressed in zone III (Table S1), confirming that several Lb classes exist with different locations within the nodule. The role of Lb diversity in legumes plants is not fully figured out, even if it was proposed that, in addition to their role as oxygen-carriers (Appleby, 1992), the abundance of Lbs could be one of the cornerstones necessary for the functioning of a Phytogb–NO cycle in microaerobic conditions, such as that prevailing in nodules (Hichri *et al.*, 2015; Berger *et al.*, 2019).

The main objective of this study was to analyze the expression pattern of the different *Phytogbs* and the NO production throughout the symbiotic process, and to identify the Phytogbs potentially involved in NO regulation. Our results show that three Phytogb expression and NO production peaks can be considered (Figs 1–4): during the first hours of the symbiotic interaction (10 hpi); during the early development of the nodule (4 dpi); and when the nodule becomes mature (3–4 wpi). The most salient feature emerging from this analysis is the high expression of *Phytogb1.1*, which fits particularly with NO variation pattern from the beginning of the interaction to the N_2-fixing nodule step (Figs 1–3). This led us to generate *Phytogb1.1*-overexpressing and -silencing plants to investigate in greater

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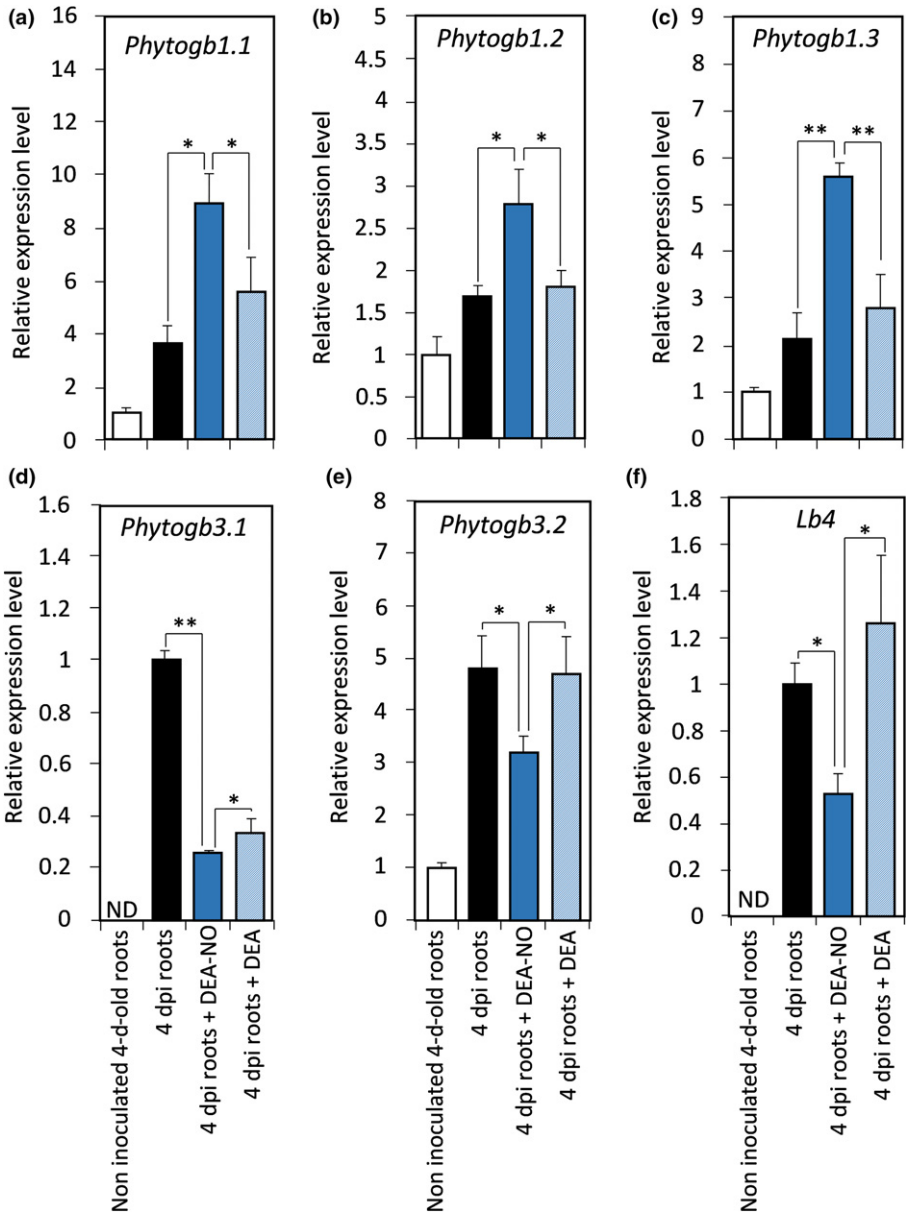

Fig. 4 *Medicago truncatula Phytogb* genes expression after 4 d of nitric oxide (NO) donor treatment. Plant roots were either inoculated with *Sinorhizobium meliloti* in the presence or absence of 0.5 mM diethylamine (DEA)/diethylamine NONOate (DEA-NO), or not inoculated (control), and grown for 4 d before RNA extraction and analysis. Data are means ± SE ($n = 3$). Each measurement was done in triplicate. *, $P < 0.05$; **, $P < 0.01$, according to the Student's *t*-test. dpi, day post-inoculation; ND, not detected.

detail the connection between Phytogb1.1 and NO during the symbiotic process.

The Phytogb1.1-NO couple regulates symbiosis establishment and nodule organogenesis

The 'Phytogb1-NO' couple forms a feedback loop that allows NO concentration to be quickly regulated (Hill, 2012). Here, we demonstrate that the overexpression of *Phytogb1.1* decreases NO production while its silencing increases it (Fig. 5b), confirming that Phytogb1.1 negatively regulates NO concentration as previously reported in *L. japonicus* (Nagata *et al.*, 2008; Shimoda *et al.*, 2009; Fukudome *et al.*, 2016). Both higher and lower NO concentrations inhibit the nodulation (Table 2). These results are consistent with a previous report showing that high NO concentration inhibits the nodulation by affecting the formation of an infection thread (Fukudome *et al.*, 2016), but they also confirm the observations that nodulation is inhibited by a decrease in NO concentration (Pii *et al.*, 2007; del Giudice *et al.*, 2011). This means that an excess as well as a lack of NO impair nodule establishment and growth, and that NO concentration needs to be tightly regulated at the site of nodule initiation for a successful establishment of the symbiotic relationship.

Based on the feedback mechanism of the Phytogb1.1–NO couple, the first transitory NO production peak observed at 10 hpi (Fig. 3) may be linked to the sharp and transient decrease in *Phytogb1.1* at 4 hpi (Fig. 1). The subsequent upregulation of *Phytogb1.1* at 10 hpi may be linked to the NO production peak as observed in the *L. japonicus* root surface when inoculated with its symbiont *Mesorhizobium loti* (Nagata *et al.*, 2008). This NO peak may be related to the defense mechanisms established by the plant in response to the rhizobium. In *G. max* (Libault

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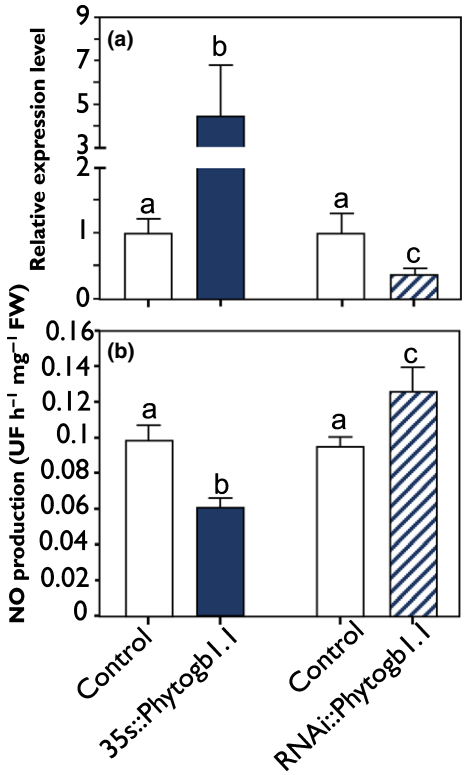

Fig. 5 Relative expression level of *Medicago truncatula Phytogb1.1* and nitric oxide (NO) concentration in control and *Phytogb1.1*-transformed roots at 4 d post-inoculation (dpi). (a, b) Expression analysis of *Phytogb1.1* genes (a) and analysis of NO concentration (b) in control and transformed plant roots either overexpressing (35s::*Phytogb1.1*) or silencing *Phytogb1.1* (RNAi::*Phytogb1.1*) at 4 dpi. Data are means ± SE ($n = 3$). Each measurement was done in triplicate. Values followed by different letters are significantly different according to one-way ANOVA analysis followed by a Fisher test ($P < 0.05$). UF, unit of fluorescence.

Table 2 Number of nodules in control and *Phytogb1.1*-transformed *Medicago truncatula* plants at 14 d post-inoculation (dpi).

Construct	Nodule number per plant
Control 35s	14.3 ± 1.1 a
35s::Phytogb1.1	6.7 ± 0.5 b
Control RNAi	13.9 ± 0.8 a
RNAi::Phytogb1.1	6.6 ± 0.4 b

Data are means ± SE ($n = 3$). Each measure was done with 12–18 plants. Values followed by different letters are significantly different according to one-way ANOVA analysis followed by a Fisher test ($P < 0.05$).

Table 3 Number of *Medicago truncatula* nodules after 4 d of nitric oxide (NO) donor treatment.

Condition	Nodule number per plant
Control	7.7 ± 0.4 a
DEA-NO	5.6 ± 0.5 b
DEA	7.8 ± 0.3 a

Plant roots inoculated with *Sinorhizobium meliloti* were treated with either 0.5 mM diethylamine NONOate (DEA-NO) or 0.5 mM diethylamine (DEA). After 4 d, plants were transferred to a NO donor free medium and grown for an additional 10 d before measurement of nodule number. Data are means ± SE ($n = 3$). Each measure was done with more than 50 plants. Values followed by different letters are significantly different according to one-way ANOVA analysis followed by a Fisher test ($P < 0.05$).

et al., 2010), *L. japonicus* (Stacey *et al.*, 2006) and *M. truncatula* (Jones *et al.*, 2008) roots, a large number of plant defense genes have been shown to be induced within 12 hpi with their symbiotic rhizobia, and their expression gradually returned to background levels within 24 hpi when the infection process was initiated. Increased *GST* and *CS* gene expression in both RNAi::*Phytogb1.1 M. truncatula* roots (Fig. 6) and roots treated with NO-donor (Fig. S7) were in agreement with the literature and indicate that the induction of plant defense mechanisms is linked to higher NO concentration resulting from *Phytogb1.1* downregulation.

The upregulation of *Phytogb1.1* at 10 hpi triggers the decrease in NO concentration to its basal value for 2 d (Fig. 3). In *M. truncatula*, *Enod20*, a marker of root infection and cortical cell activation, was shown to be mainly expressed during the formation of the infection thread and the initiation of the nodule primordium (Greene *et al.*, 1998; Vernoud *et al.*, 1999) which take place between 1 and 3 dpi (Timmers *et al.*, 1999; Xiao *et al.*, 2014). In 35s::*Phytogb1.1* roots with low NO concentration, *Enod20* is highly expressed (Fig. 6c), whereas it is weakly expressed in RNAi::*Phytogb1.1* roots with high NO concentration. These observations indicate that the infection of the plant and the activation of cortical cells require a low NO concentration and a decreased plant defense response. This explanation is consistent with observations made in a NR-deficient double mutant *A. thaliana* plant line (Vitor *et al.*, 2013). This mutant, exhibiting a low NO concentration, is prone to infection by pathogens.

The second NO production peak observed at 4 dpi (Fig. 1) suggests that NO is involved in the onset of nodule organogenesis that starts from 3 to 4 dpi in the *M. truncatula–S. meliloti* symbiosis (Oldroyd & Downie, 2008; Xiao *et al.*, 2014). Such an involvement is consistent with the observation that, in 4 dpi *M. truncatula* roots, NO scavenging resulted in the downregulation of many cell division and growth-related genes (Boscari *et al.*, 2013). *Cre1*, which encodes for a cytokinin receptor, regulates the symbiotic interaction and is considered as a nodule organogenesis marker upregulated by NO (Ferrarini *et al.*, 2008; Frugier *et al.*, 2008; del Giudice *et al.*, 2011). During the first days following inoculation with symbiotic rhizobia, a specific production of NO was reported in the pericycle, endodermis and dividing cortical root cells, where *Cre1* is expressed and the nodule primordium is initiated (del Giudice *et al.*, 2011; Plet *et al.*, 2011). The induction and repression of *Cre1* in RNAi::*Phytogb1.1* and 35S::*Phytogb1.1* roots (Fig. 6c), respectively, means that high NO promotes nodule development, while low NO inhibits it. This suggests that *Cre1* induction and the onset of nodule organogenesis are under the control of NO and Phytogb1.1. Lbs, whose expression in young developing *M. truncatula* nodules starts strongly from 5 dpi (Gallusci *et al.*,

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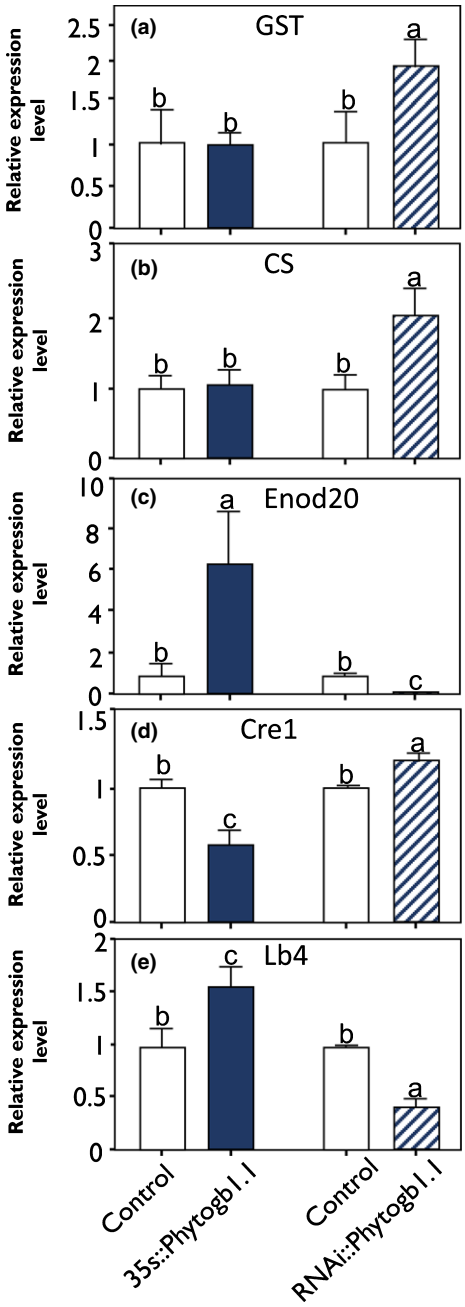

Fig. 6 Relative gene expression level in control and *Phytogb1.1 Medicago truncatula*-transformed roots at 4 d post-inoculation (dpi). (a–e) Expression analysis of *GST* (a), *CS* (b), *Enod20* (c), *Cre1* (d), and *Lb4* genes (e) in control and transformed plant roots either overexpressing (35s::*Phytogb1.1*) or silencing *Phytogb1.1*(RNAi::*Phytogb1.1*) at 4 dpi. Data are means ± SE ($n = 3$). Each measurement was done in triplicate. Values followed by different letters are significantly different according to one-way ANOVA analysis followed by a Fisher test ($P < 0.05$).

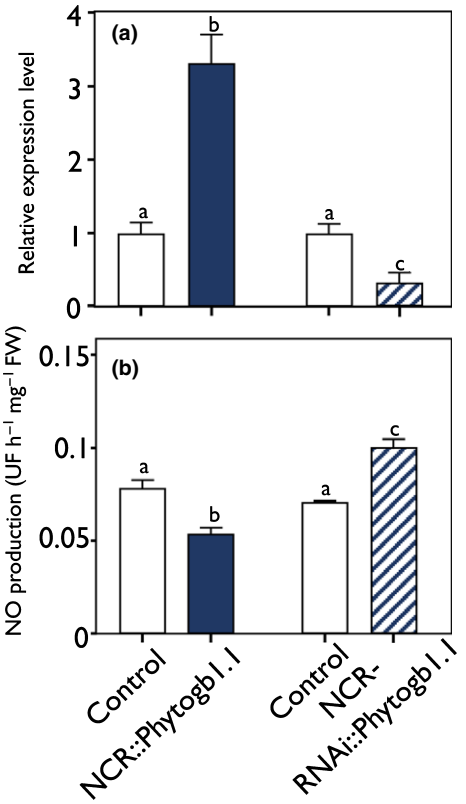

Fig. 7 Relative expression level of *Medicago truncatula Phytogb1.1* and nitric oxide (NO) concentration in 3 wk post-inoculation (wpi) nodules of control and *Phytogb1.1*-transformed plants. (a, b) Expression analysis of *Phytogb1.1* gene (a) and analysis of NO concentration (b) in control and transformed plant nodules either overexpressing (NCR::*Phytogb1.1*) or silencing *Phytogb1.1* (NCR-RNAi::*Phytogb1.1*) at 3 wpi. Data are means ± SE ($n = 3$). Each measurement was done in triplicate. Values followed by different letters are significantly different according to one-way ANOVA analysis followed by a Fisher test ($P < 0.05$). UF, unit of fluorescence.

Table 4 Nitrogenase activity and energy state in control and *Phytogb1.1*-transformed *Medicago truncatula* nodules at 3 wk post-inoculation (wpi).

Construct	ARA (nmol ethylene h^{-1} mg^{-1} nodule)	ATP : ADP ratio
Control 35s	15.8 ± 1.1 a	6.9 ± 0.1 a
35s::Phytogb1.1	21.2 ± 1.4 b	8.1 ± 0.3 b
Control RNAi	16.7 ± 1.6 a	7.0 ± 0.1 a
RNAi::Phytogb1.1	11.0 ± 0.8 c	4.8 ± 0.1 c

Nitrogenase activity (estimated as ARA) was normalized per nodule FW. Energy state was measured as ATP : ADP ratio. Data are means ± SE ($n = 3$). Each measurement was done in triplicate. Values followed by different letters are significantly different according to one-way ANOVA analysis followed by a Fisher test ($P < 0.05$).

1991), are markers of N_2 fixation (Appleby, 1992). Our results show that the high expression of *Lb4-Lb3* after 4 dpi (Fig. 1) is correlated with a decrease in NO concentration between 4 and 14 dpi (Fig. 3), and that NO represses the expression of *Lb4* (Figs 4, 6). This means that after the onset of nodule organogenesis, a decrease in NO concentration is necessary for the development and growth of the nodule.

Considered together, our results led us to propose a scenario in which the Phytogb1.1–NO couple plays a role of symbiosis regulator. First, within hours after inoculation, the low level of *Phytogb1.1* (4 hpi) allows an increase of NO. The increase in NO concentration (at 4 and 10 hpi) allows the establishment of plant defense reactions (induction of *GST*, *CS*) as well as the

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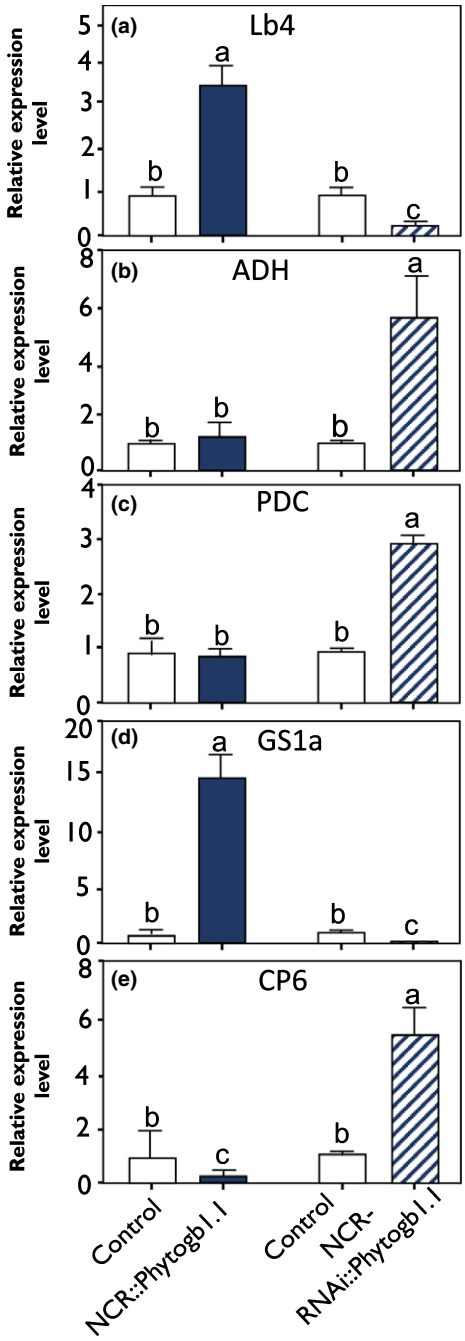

Fig. 8 Relative gene expression level in 3 wk post-inoculation (wpi)-old nodules of control and Phytogb1.1 *Medicago truncatula* transformed plants. (a–e) Expression analysis of *Lb4* (a), *ADH* (b), *PDC* (c), *GS1a* (d), and *CP6* genes (e) in control and transformed plant nodules either overexpressing (NCR::*Phytogb1.1*) or silencing *Phytogb1.1* (NCR-RNAi:: *Phytogb1.1*) at 3 wpi. Data are means ± SE ($n = 3$). Each measurement was done in triplicate. Values followed by different letters are significantly different according to one-way ANOVA analysis followed by a Fisher test ($P < 0.05$).

induction of *Phytogb1.1* (10 hpi). Second, increased Phytogb1.1 activity reduces NO to its initial concentration (1–2 dpi), which, in turn, lowers the defense reactions, allowing the infection and the reception of the symbiont (induction of *Enod20*) and subsequently downregulation of *Phytogb1.1* expression (1–2 dpi). Third, low Phytogb1.1 triggers a new rise in NO concentration (between 2 and 4 dpi) which allows the initiation of nodule organogenesis (induction of *Cre1*) and, again, the induction of *Phytogb1.1* (4 dpi). Finally, once the organogenesis is initiated, the subsequent decrease in NO concentration (between 9 and 14 dpi) accompanies the nodule development and growth (induction of *Lb4*). It is therefore easy to understand the reduction in nodule number under both high and low NO concentrations (Tables 2, 3): a high NO concentration inhibits the infection process, whereas a low NO concentration inhibits nodule organogenesis.

A recent study on mycorrhizal symbiosis between *Solanum lycopersicum* and *Rhizophagus irregularis* also shows two peaks of NO production, in the hours following inoculation and then at 48 hpi, under the control of SlPhytogb1 (Martinez-Medina *et al.*, 2019). Such similar behavior suggests that the establishment of the interaction and the symbiotic organogenesis are controlled by the Phytogb1.1–NO couple in both N_2-fixing and mycorrhizal symbiosis.

The Phytogb1.1–NO couple modulates energy and N_2-fixing metabolism

At 3–4 wpi, nodules reach their mature N_2-fixing state. This period is characterized by a strong and a moderate increase in *Lbs* and *Phytogb1.1* expression (Figs 1, 2), respectively, and a high NO concentration (Fig. 3). The question of Lbs and Phytogb1.1 functions with regard to NO may be raised. The presence of Lb–NO complexes, detected by electron paramagnetic resonance (EPR), in soybean and *L. japonicus* nodules *in vivo* (Maskall *et al.*, 1977; Mathieu *et al.*, 1998; Sánchez *et al.*, 2010) shows that Lbs are involved in the complexation of NO. It may be noted that the higher level of *Lb* gene expression observed in the interzone II–III rather than in zone III (Table S1) is consistent with the fact that NO represses *Lb* gene expression (Fig. 4) and that it is mainly produced in zone III (Baudouin *et al.*, 2006). The ability of Lbs to bind O_2 and NO to produce NO_3^- (Herold & Puppo, 2005) makes them good candidates to detoxify NO which is present in high concentrations in the mature nodules (Baudouin *et al.*, 2006).

Although less expressed than *Lbs*, the significant expression of *Phytogb1.1* in nodules (Fig. 2) suggests that Phytogb1.1 has its own function in the N_2-fixing metabolism. In *L. japonicus* nodules, *LjHb1*overexpression results in decreased NO content and increased ARA (Shimoda *et al.*, 2009; Fukudome *et al.*, 2019), whereas *LjGlb1.1* mutants nodules show higher NO content and lower ARA (Fukudome *et al.*, 2016). These authors suggested that the role of *Phytogb1.1* is to scavenge NO to avoid the inhibition of the nitrogenase and the N_2 fixation. Here, lower NO concentration in *Phytogb1.1*-overexpressing nodules resulted in higher *Lb4* and *GS1a* expression, and higher ARA and energy state, while opposite effects were observed in NCR-RNAi:: *Phytogb1.1* nodules (Figs 7, 8; Table 4), indicating that high NO concentration inhibits N_2-fixing metabolism, whereas low NO concentration favors it. This regulation occurs both at post-translational and transcriptional levels. First, NO is a potent inhibitor of nitrogenase (Trinchant & Rigaud, 1982; Kato *et al.*, 2010)

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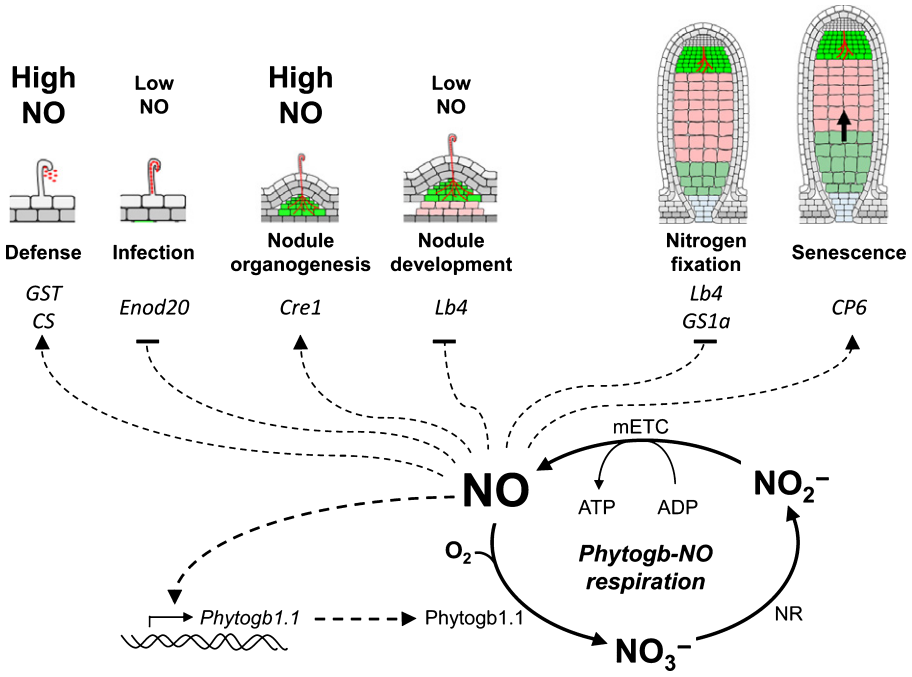

Fig. 9 Synoptic representation of Phytogb1.1 and nitric oxide (NO) function during the symbiotic process. Depending on the steps of the symbiotic process, the NO concentration either increases (high NO) or decreases (low NO) and regulates the transition between the different stages of the symbiosis. The NO content is regulated by the Phytogb1.1-NO loop. In microoxic mature nodules, Phytogb–NO respiration regulates the concentration of NO and ensures the regeneration of energy (ATP) necessary for the functioning of plant cell metabolism. The arrow indicates the direction of increase of senescent tissue. mETC, mitochondrial electron transfer chain; NR, nitrate reductase.

and disrupts Lb and GS1a activities after nitration of their tyrosine moieties by peroxynitrite, a NO derivative (Melo *et al.*, 2011; Navascues *et al.*, 2012). At the gene level, NO represses the expression of the bacterial *nifH* and *nifD* in soybean nodules (Sánchez *et al.*, 2010), and present data show that it also represses the expression of key genes in the N_2-fixing metabolism (*Lb4* and *GS1a*).

However, it should be noted that ARA is more substantial in *L. japonicus* nodules in the presence of 0.1 mM single nucleotide polymorphism (SNP; NO donor) than in either the absence or presence of higher (1 mM) concentrations of SNP, indicating that low but significant NO concentration is beneficial to N_2 fixation (Kato *et al.*, 2010). The microoxic environment prevailing in nodules raises the question of energy supply. Accumulated data support the functioning of Phytogb–NO respiration in nodules (Horchani *et al.*, 2011). Both Lbs and Phytogb1.1 have the ability to bind O_2 and NO to produce NO_3^- (Herold & Puppo, 2005), which makes them good candidates to participate in the regeneration of ATP through the functioning of the Phytogb–NO respiration. In the present study, the silencing of *Phytogb1.1* and the increase in NO concentration trigger the overexpression of *ADH* and *PDC* (Fig. 8), which mimics a situation of hypoxia. The decrease in ATP : ADP ratio and ARA in *Phytogb1.1*-silenced nodules, and their increase in *Phytogb1.1*-overexpressing nodules (Table 4) indicate that Phytogb1.1 participates in NO turnover, but is also involved (alongside Lb?) in the functioning of Phytogb–NO respiration and the maintenance of the nodules' energy state. The very recent elucidation of the role of NO and Phytogb1 in the perception of hypoxia in *A. thaliana* (Hartman *et al.*, 2019) makes it possible to hypothesize that the Phytogb1.1–NO couple is also involved in the regulation of the nodule metabolism. This hypothesis is a promising challenge for future investigations.

In conclusion, this work highlights the regulatory role of Phytogb1.1 in the regulation of NO during the early stages of symbiosis (defense response, infection, nodule organogenesis), and in the N_2-fixing nodule, as well as the close relationship between NO production and the expression of the other Phytogb genes (Fig. 9). However, the control of NO in the nodule cannot be done only by the plant partner. Indeed, the *S. meliloti* flavohemoglobin was shown to be involved in NO degradation and is essential in maintaining efficient N-fixing symbiosis (Meilhoc *et al.*, 2010; Cam *et al.*, 2012). Otherwise, the bacterial NO reductase and the nnrS system were also shown to regulate NO concentration in N_2-fixing nodules (Meilhoc *et al.*, 2013; Blanquet *et al.*, 2015). How the regulatory systems of the plant and the bacterial partners are coordinated to control NO is one of the main issues to decipher the toxic, signaling, and metabolic functions of NO at each stage of the symbiotic interaction.

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Author contributions

ABerger, ABoscari and RB planned and designed the research. ABerger and SG performed the experiments. ABerger, ABoscari and RB analyzed the data. ABerger, ABoscari, AP and RB interpreted the data and wrote the manuscript.

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References

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

Astier J, Gross I, Durner J. 2018. Nitric oxide production in plants: an update. *Journal of Experimental Botany* **69**: 3401–3411.

Bailey-Serres J, Voesenek LACJ. 2008. Flooding stress: acclimations and genetic diversity. *Annual Review of Plant Biology* **59**: 313–339.

Bari R, Pant BD, Stitt M, Scheible W-R. 2006. PHO2, MicroRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiology* **141**: 988–999.

Baudouin E, Pieuchot L, Engler G, Pauly N, Puppo A. 2006. Nitric oxide is formed in *Medicago truncatula–Sinorhizobium meliloti* functional nodules. *Molecular Plant–Microbe Interactions* **19**: 970–975.

Berger A, Boscari A, Frendo P, Brouquisse R. 2019. Nitric oxide signaling, metabolism and toxicity in nitrogen-fixing symbiosis. *Journal of Experimental Botany* **70**: 4505–4520.

Blanquet P, Silva L, Catrice O, Bruand C, Carvalho H, Meilhoc E. 2015. *Sinorhizobium meliloti* controls nitric oxide-mediated post-translational modification of a *Medicago truncatula* nodule protein. *Molecular Plant–Microbe Interactions* **28**: 1353–1363.

Boisson-Dernier A, Chabaud M, Garcia F, Bécard G, Rosenberg C, Barker DG. 2001. *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogenfixing and endomycorrhizal symbiotic associations. *Molecular Plant–Microbe Interactions* **14**: 695–700.

Boscari A, Del Giudice J, Ferrarini A, Venturini L, Zaffini A-L, Delledonne M, Puppo A. 2013. Expression dynamics of the *Medicago truncatula* transcriptome during the symbiotic interaction with *Sinorhizobium meliloti*: which role for nitric oxide? *Plant Physiology* **161**: 425–439.

Bruand C, Meilhoc E. 2019. Nitric oxide in plants: pro- or anti-senescence. *Journal of Experimental Botany* **70**: 4419–4427.

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

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.

Coats V, Schwintzer CR, Tjepkema JD. 2009. Truncated hemoglobins in Frankia CcI3: effects of nitrogen source, oxygen concentration, and nitric oxide. *Canadian Journal of Microbiology* **55**: 867–873.

Dao TTH, Linthorst HJM, Verpoorte R. 2011. Chalcone synthase and its functions in plant resistance. *Phytochemistry Reviews* **10**: 397–412.

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.

Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, Guindon S, Lefort V, Lescot M *et al.* 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research* **36**: 465–469.

Ferrarini A, De Stefano M, Baudouin E, Pucciariello C, Polverari A, Puppo A, Delledonne M. 2008. Expression of *Medicago truncatula* genes responsive to nitric oxide in pathogenic and symbiotic conditions. *Molecular Plant–Microbe Interactions* **21**: 781–790.

Frugier F, Kosuta S, Murray JD, Crespi M, Szczyglowski K. 2008. Cytokinin: secret agent of symbiosis. *Trends in Plant Science* **13**: 115–120.

Fukudome M, Calvo-Begueria L, Kado T, Osuki K-I, Rubio MC, Murakami E-I, Nagata M, Kucho K-I, 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 KI, Imaizumi R, Aoki T, 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 & Cell Physiology* **60**: 816–825.

Gallusci P, Dedieu A, Journet E-P, 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.

González-Gordo S, Bautista R, Claros MG, Cañas A, Palma JM, Corpas FJ. 2019. Nitric oxide-dependent regulation of sweet pepper fruit ripening. *Journal of Experimental Botany* **70**: 4557–4570.

Greene EA, Erard M, Dedieu A, Barker DG. 1998. MtENOD16 and 20 are members of a family of phytocyanin-related early nodulins. *Plant Molecular Biology* **36**: 775–783.

Groat RG, Vance CP. 1981. Root nodule enzymes of ammonia assimilation in Alfalfa (*Medicago sativa*). *Plant Physiology* **67**: 1198–1203.

Gullner G, Komives T, Király L, Schröder P. 2018. Glutathione S-transferase enzymes in plant-pathogen interactions. *Frontiers in Plant Science* **9**: 1836.

Gupta KJ, Hebelstrup KH, Mur LAJ, Igamberdiev AU. 2011. Plant hemoglobins: important players at the crossroads between oxygen and nitric oxide. *FEBS Letters* **585**: 3843–3849.

Hardison R. 1998. Hemoglobins from bacteria to man: evolution of different patterns of gene expression. *Journal of Experimental Biology* **201**: 1099–1117.

Hardy RW, Holsten RD, Jackson EK, Burns RC. 1968. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. *Plant Physiology* **43**: 1185–1207.

Hartman S, Liu Z, van Veen H, Vicente J, Reinen E, Martopawiro S, Zhang H, van Dongen N, Bosman F, Bassel GW *et al.* 2019. Ethylene-mediated nitric oxide depletion pre-adapts plants to hypoxia stress. *Nature Communication* **10**: 4020.

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.

Hichri I, Boscari A, Castella C, Rovere M, Puppo A, Brouquisse R. 2015. Nitric oxide: a multifaceted regulator of the nitrogen-fixing symbiosis. *Journal of Experimental Botany* **66**: 2877–2887.

Hichri I, Meilhoc E, Boscari A, Bruand C, Frendo P, Brouquisse R. 2016. Nitric oxide: Jack-of-all-trades of the nitrogen-fixing symbiosis? In: Wendehenne D, ed. *Nitric oxide and signaling in plants. Advances in Botanical Research, Vol. 77*. London, USA, & New York, USA: Academic Press, 193–218.

Hill RD. 2012. Non-symbiotic haemoglobins-What's happening beyond nitric oxide scavenging? *AoB plants* **2012**: pls004.

Hill R, Hargrove M, Arredondo-Peter R. 2016. Phytoglobin: a novel nomenclature for plant globins accepted by the globin community at the 2014 XVIII conference on oxygen-binding and sensing proteins. *F1000Research* **5**: 212.

Hilliou F, Tran T. 2013. RqPCR analysis: analysis of quantitative real-time PCR data. In: *Proceedings of the International Conference on Bioinformatics Models, Methods and Algorithms (BIOINFORMATICS-2013)*, pp. 202–211.

Horchani F, Prévot M, Boscari A, Evangelisti E, Meilhoc E, Bruand C, Raymond P, Boncompagni E, Aschi-Smiti S, Puppo A *et al.* 2011. Both plant and bacterial nitrate reductases contribute to nitric oxide production in *Medicago truncatula* nitrogen-fixing nodules. *Plant Physiology* **155**: 1023–1036.

Igamberdiev AU, Bykova NV, Hill RD. 2011. Structural and functional properties of class 1 plant hemoglobins. *IUBMB Life* **63**: 146–152.

Igamberdiev AU, Bykova NV, Shah JK, Hill RD. 2010. Anoxic nitric oxide cycling in plants: participating reactions and possible mechanisms. *Physiologia Plantarum* **138**: 393–404.

Igamberdiev AU, Hill RD. 2004. Nitrate, NO and haemoglobin in plant adaptation to hypoxia: an alternative to classic fermentation pathways. *Journal of Experimental Botany* **55**: 2473–2482.

Jones KM, Sharapova N, Lohar D, Zhang J, VandenBosch K, Walker GC. 2008. Differential response of the plant *Medicago truncatula* to its symbiont

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Sinorhizobium meliloti or an exopolysaccharide-deficient mutant. *Proceedings of the National Academy of Sciences, USA* **105**: 704–709.

Karimi M, Inzé D, Depicker A. 2002. GATEWAY™ vectors for *Agrobacterium*-mediated plant transformation. *Trends in Plant Science* **7**: 193–195.

Kato K, Kanahama K, Kanayama Y. 2010. Involvement of nitric oxide in the inhibition of nitrogenase activity by nitrate in Lotus root nodules. *Journal of Plant Physiology* **167**: 238–241.

Leterrier M, Chaki M, Airaki M, Valderrama R, Palma JM, Barroso JB, Corpas FJ. 2011. Function of S-nitrosoglutathione reductase (GSNOR) in plant development and under biotic/abiotic stress. *Plant Signaling & Behavior* **6**: 789–793.

Libault M, Farmer A, Brechenmacher L, Drnevich J, Langley RJ, Bilgin DD, Radwan O, Neece DJ, Clough SJ, May GD *et al.* 2010. Complete transcriptome of the soybean root hair cell, a single-cell model, and its alteration in response to *Bradyrhizobium japonicum* infection. *Plant Physiology* **152**: 541–552.

Long SR. 2001. Genes and signals in the rhizobium–legume symbiosis. *Plant Physiology* **125**: 69–72.

Martinez-Medina A, Pescador L, Fernandez I, Rodríguez-Serrano M, García JM, Romero-Puertas MC, Pozo MJ. 2019. Nitric oxide and phytoglobin PHYTOGB1 are regulatory elements in the *Solanum lycopersicum*–*Rhizophagus irregularis* mycorrhizal symbiosis. *New Phytologist* **223**: 1560–1574.

Maskall CS, Gibson JF, Dart PJ. 1977. Electron-paramagnetic-resonance studies of leghaemoglobins from soya-bean and cowpea root nodules. Identification of nitrosyl-leghaemoglobin in crude leghaemoglobin preparations. *The Biochemical Journal* **167**: 435–445.

Mathieu C, Sophie M, Frendo P, Puppo A, Davies M. 1998. Direct detections of radicals in intact soybean nodules: presence of nitric oxide-leghemoglobin complexes. *Free Radical Biology & Medicine* **24**: 1242–1249.

Meakin GE, Bueno E, Jepson B, Bedmar EJ, Richardson DJ, Delgado MJ. 2007. The contribution of bacteroidal nitrate and nitrite reduction to the formation of nitrosylleghaemoglobin complexes in soybean root nodules. *Microbiology* **153**: 411–419.

Meilhoc E, Blanquet P, Cam Y, Bruand C. 2013. Control of NO level in rhizobium-legume root nodules: not only a plant globin story. *Plant Signaling & Behavior* **8**: e25923.

Meilhoc E, Boscari A, Brouquisse R, Bruand C. 2015. Multifacted roles of nitric oxide in legume-rhizobium symbioses. In: de Bruijn FJ, ed. *Biological nitrogen fixation, Vol. 2.* Chichester, UK: John Wiley & Sons, 637–647.

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.

Melo PM, Silva LS, Ribeiro I, Seabra AR, Carvalho HG. 2011. Glutamine synthetase is a molecular target of nitric oxide in root nodules of *Medicago truncatula* and is regulated by tyrosine nitration. *Plant Physiology* **157**: 1505–1517.

Mergaert P, Nikovics K, Kelemen Z, Maunoury N, Vaubert D, Kondorosi A, Kondorosi E. 2003. A novel family in *Medicago truncatula* consisting of more than 300 nodule-specific genes coding for small, secreted polypeptides with conserved cysteine motifs. *Plant Physiology* **132**: 161–173.

Mur LAJ, Mandon J, Persijn S, Cristescu SM, Moshkov IE, Novikova GV, Hall MA, Harren FJM, Hebelstrup KH, Gupta KJ. 2013. Nitric oxide in plants: An assessment of the current state of knowledge. *AoB Plants* **5**: 1–17.

Murakami EI, Nagata M, Shimoda Y, Kucho KI, Higashi S, Abe M, Hashimoto M, Uchiumi T. 2011. Nitric oxide production induced in roots of *Lotus japonicus* by lipopolysaccharide from *Mesorhizobium loti*. *Plant and Cell Physiology* **52**: 610–617.

Nagata M, Hashimoto M, Murakami EI, Shimoda Y, Shimoda-Sasakura F, Kucho KI, Suzuki A, Abe M, Higashi S, Uchiumi T. 2009. A possible role of class 1 plant hemoglobin at the early stage of legume–rhizobium symbiosis. *Plant Signaling and Behavior* **4**: 202–204.

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.

Navascues J, Perez-Rontome C, Gay M, Marcos M, Yang F, Walker FA, Desbois A, Abian 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.

Niemann J, Tisa LS. 2008. Nitric oxide and oxygen regulate truncated hemoglobin gene expression in Frankia strain CcI3. *Journal of Bacteriology* **190**: 7864–7867.

Oldroyd GED, Downie JA. 2008. Coordinating nodule morphogenesis with rhizobial infection in legumes. *Annual Review of Plant Biology* **59**: 519–546.

Pierre O, Hopkins J, Combier M, Baldacci F, Engler G, Brouquisse R, Hérouart D, Boncompagni E. 2014. Involvement of papain and legumain proteinase in the senescence process of *Medicago truncatula* nodules. *New Phytologist* **202**: 849–863.

Pii Y, Crimi M, Cremonese G, Spena A, Pandolfini T. 2007. Auxin and nitric oxide control indeterminate nodule formation. *BMC Plant Biology* **7**: 21.

Plet J, Wasson A, Ariel F, Le Signor C, Baker D, Mathesius U, Crespi M, Frugier F. 2011. MtCRE1-dependent cytokinin signaling integrates bacterial and plant cues to coordinate symbiotic nodule organogenesis in *Medicago truncatula*. *The Plant Journal* **65**: 622–633.

Quandt J, Hynes MF. 1993. Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. *Gene* **127**: 15–21.

Roux B, Rodde N, Jardinaud MF, Timmers T, Sauviac L, Cottret L, Carrère S, Sallet E, Courcelle E, Moreau 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. *The Plant Journal* **77**: 817–837.

Sánchez C, Gates AJ, Meakin GE, Uchiumi T, Girard L, Richardson DJ, Bedmar EJ, Delgado MJ. 2010. Production of nitric oxide and nitrosylleghemoglobin complexes in soybean nodules in response to flooding. *Molecular Plant–Microbe Interactions* **23**: 702–711.

Sasakura F, Uchiumi T, Shimoda Y, Suzuki A, Takenouchi K, Higashi S, Abe M. 2006. A class 1 hemoglobin gene from *Alnus firma* functions in symbiotic and nonsymbiotic tissues to detoxify nitric oxide. *Molecular Plant–Microbe Interactions* **19**: 441–450.

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 KI, 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*. *The Plant Journal* **57**: 254–263.

Simontacchi M, Galatro A, Ramos-Artuso F, Santa-María GE. 2015. Plant survival in a changing environment: the role of nitric oxide in plant responses to abiotic stress. *Frontiers in Plant Science* **6**: 1–19.

Smagghe BJ, Hoy JA, Percifield R, Kundu S, Hargrove MS, Sarath G, Hilbert JL, Watts RA, Dennis ES, Peacock WJ *et al.* 2009. Correlations between oxygen affinity and sequence classifications of plant hemoglobins. *Biopolymers – Peptide Science Section* **91**: 1083–1096.

Stacey G, McAlvin C, Kim S-Y, Olivares J, Soto M. 2006. Effects of endogenous salicylic acid on nodulation in the model legumes *Lotus japonicus* and *Medicago truncatula*. *CMS Symbols – Symposia on Communication for Social Development* **141**: 1473–1481.

Stasolla C, Huang S, Hill RD, Igamberdiev AU. 2019. Spatio-temporal expression of phytoglobin – a determining factor in the NO specification of cell fate. *Journal of Experimental Botany* **70**: 4365–4377.

Storz JF. 2016. Gene duplication and evolutionary innovations in hemoglobin-oxygen transport. *Physiology* **31**: 223–232.

Timmers AC, Auriac MC, Truchet G. 1999. Refined analysis of early symbiotic steps of the *Rhizobium–Medicago* interaction in relationship with microtubular cytoskeleton rearrangements. *Development* **126**: 3617–3628.

Timmers ACJ, Soupene E, Auriac M-C, de Billy F, Vasse J, Boistard P, Truchet G. 2000. Saprophytic intracellular rhizobia in alfalfa nodules. *Mol Plant-Microbe Interact* **13**: 1204–1213.

Trinchant JC, Rigaud J. 1982. Nitrite and nitric oxide as inhibitors of nitrogenase from soybean bacteroids. *Applied and Environmental Microbiology* **44**: 1385–1388.

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Truong HN, Thalineau E, Bonneau L, Fournier C, Potin S, Balzergue S, Van Tuinen D, Jeandroz S, Morandi D. 2015. The *Medicago truncatula* hypermycorrhizal B9 mutant displays an altered response to phosphate and is more susceptible to *Aphanomyces euteiches*. *Plant, Cell & Environment* **38**: 73–88.

Van de Velde W, Guerra JCP, De Keyser A, De Rycke R, Rombauts S, Maunoury N, Mergaert P, Kondorosi E, Holsters M, Goormachtig S. 2006. Aging in legume symbiosis. A molecular view on nodule senescence in *Medicago truncatula*. *Plant Physiology* **141**: 711–20.

Vernoud V, Journet EP, Barker DG. 1999. MtENOD20, a Nod factor-inducible molecular marker for root cortical cell activation. *Molecular Plant–Microbe Interactions* **12**: 604–614.

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.

Vinogradov SN, Hoogewijs D, Bailly X, Arredondo-Peter R, Gough J, Dewilde S, Moens L, Vanfleteren JR. 2006. A phylogenomic profile of globins. *BMC Evolutionary Biology* **6**: 31.

Vitor SG, Duarte G, Saviani E, Vincentz M, Oliveira HC, Salgado I. 2013. Nitrate reductase is required for the transcriptional modulation and bactericidal activity of nitric oxide during the defense response of *Arabidopsis thaliana* against *Pseudomonas syringae*. *Planta* **238**: 457–486.

Xiao TT, Schilderink S, Moling S, Deinum EE, Kondorosi E, Franssen H, Kulikova O, Niebel A, Bisseling T. 2014. Fate map of *Medicago truncata* root nodules. *Development* **141**: 3517–3528.

Yun B-W, Skelly MJ, Yin M, Yu M, Mun B-G, Lee S-U, Hussain A, Spoel SH, Loake GJ. 2016. Nitric oxide and S-nitrosoglutathione function additively during plant immunity. *New Phytologist* **211**: 516–526.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Phylogenetic tree of *Medicago truncatula*, *Lotus japonicus*, *Glycine max* and *Arabidopsis thaliana* phytoglobins.

Fig. S2 Multiple sequence alignment of *Medicago truncatula* phytoglobins.

Fig. S3 Microarray data for *Medicago truncatula* phytoglobins.

Fig. S4 (a) Chromosomal localization and (b) exon-intron structure of *Medicago truncatula Phytogb* genes.

Fig. S5 MEME model of primary sequences of *Medicago truncatula* Phytogb proteins.

Fig. S6 Variations of NO concentration during the symbiotic process expressed as a function of protein content.

Fig. S7 Defense gene (*GST* and *CS*) expression after 4 d of NO donor treatment.

Fig. S8 Pictures of control, *35s::Phytogb1.1* and *RNAi::Phytogb1.1* transgenic hairy root growth on Petri dishes at 2 d after inoculation with *Sinorhizobium meliloti*.

Fig. S9 Relative expression level of class 1 and 3 *Phytogb* genes in *Phytogb1.1*-transformed roots.

Table S1 Primer sequences for quantitative RT-PCR analysis.

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