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Excess nitrate induces nodule greening and reduces transcript and protein expression levels of soybean leghaemoglobins

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• **Background and Aims** Efficient biological nitrogen fixation (BNF) requires leghaemoglobin (Lb) to modulate oxygen pressure in nodules. Excess N supply severely inhibits BNF through effects on Lb during nodulation. As yet, a systematic identification and characterization of Lb-encoding genes in soybean has not been reported.
• **Methods** The effects of N on BNF were studied in soybean plants inoculated with rhizobia and exposed to excess or low N availability in hydroponic cultures. To identify soybean Lb proteins, BLAST searches were performed on the Phytozome website. Bioinformatic analysis of identified *GmLb*s was then carried out to investigate gene structure, protein homology and phylogenetic relationships. Finally, quantitative real-time PCR was employed to analyse the expression patterns of soybean *Lb* genes in various tissues and in response to high N availability.
• **Key Results** Excess N significantly accelerated nodule senescence and the production of green Lb in nodules. In total, seven haemoglobin (*Hb*) genes were identified from the soybean genome, with these *Hb* genes readily split into two distinct clades containing predominantly symbiosis-associated or non-symbiotic Hb members. Expression analysis revealed that all of the symbiosis-associated *Lb*s except *GmLb5* were specifically expressed in nodules, while the non-symbiotic *GmHb*s, *GmHb1* and *GmHb2*, were predominantly expressed in leaves and roots, respectively. Among identified *GmLb*s, *GmLb1–4* are the major *Lb* genes acting in soybean nodulation, and each one is also significantly suppressed by exposure to excess N.
• **Conclusions** Taken together, the results show that excess N inhibits BNF by reducing nodule formation, Lb concentration and nitrogenase activity. The characteristics of the entire Hb family were analysed, and we found that *GmLb1–4* are closely associated with nodule development and N_2 fixation. This works forms the basis for further investigations of the role of Lbs in soybean nodulation.

Key words: Soybean, leghaemoglobin, excess N, biological nitrogen fixation, phylogenetic analysis, expression patterns, nodule development.

INTRODUCTION

Biological nitrogen fixation (BNF) is a prominent natural source of nitrogen (N) in agro-ecosystems. In leguminous plants, BNF occurs in nodules, highly specialized organs in which N_2 is fixed into biologically available ammonia through symbiotic interactions with rhizobia (Oldroyd and Downie, 2008; Oldroyd *et al.*, 2011). Soybean (*Glycine max*), the most commonly cultivated legume species, fixes ~16.4 million tons of N_2 annually, which represents about 77 % of the N_2 fixed by the leguminous crop species (Herridge *et al.*, 2008). Due to its high BNF capacity, soybean serves as a central player in sustainable agriculture.

Within the symbiosome that houses N_2-fixing rhizobia, the nitrogenase complex is sensitive to oxygen (O_2) and must be kept under anaerobic conditions. Legumes have evolved to meet this condition by making leghaemoglobin (Lb) an essential component of N_2 fixation in order to regulate O_2 pressure in bacterial symbionts (Appleby and Bergersen, 1980; Hoy and Hargrove, 2008). Therefore, identification and characterization of Lb protein family members in soybean might prove to be

an important component of developing new approaches to enhance biological N_2 fixation.

Haemoglobins (Hbs) are a superfamily of haem-containing proteins that contain a conserved three-on-three or two-on-two arrangement of α-helical globin fold structures that are involved in binding and/or transporting oxygen via a haem prosthetic group (Garrocho-Villegas *et al.*, 2007; Hoy and Hargrove, 2008). Haemoglobin was originally identified and studied in mammals, but now has been found to exist in nearly all organisms, including Archaeobacteria, Eubacteria and eukaryotes (Vinogradov *et al.*, 2005, 2006). Plants have evolved to harbour up to three types of Hb, delimited as symbiotic, non-symbiotic and truncated Hbs (Gupta *et al.*, 2011). Symbiotic Hbs are known as leghaemoglobins and were first isolated from the red pigment of soybean nodules (Baulcombe and Verma, 1978). The primary function of these Lbs is to deliver O_2 to bacteroids at low but steady rates that allow efficient bacterial respiration while also preventing O_2-mediated inactivation of nitrogenase in infected cells (Appleby and Bergersen, 1980; Hoy and Hargrove, 2008). Non-symbiotic Hbs are found in a

large variety of plants other than legumes and their main function seems to be related to scavenging nitric oxide (NO), which is an important molecule in responses to abiotic and biotic stresses in plants (Baudouin, 2011; Gupta and Igamberdiev, 2011). The third type of plant Hbs is a set of truncated molecules that are 20–40 amino acid residues shorter than normal Hbs (Vuletich and Lecomte, 2006). As yet, the functions of truncated Hbs remain unclear, though there is some evidence that they may be involved in regulating oxygen delivery under high O_2 concentration conditions (Watts *et al.*, 2001).

In mature nodules, Lb is the most abundant protein. Yet, despite this abundance and the vital role Lb plays in BNF, Lb-encoding genes in legumes remain poorly characterized. In *Lotus japonicus*, three symbiotic and two non-symbiotic *Lb*s have been identified, with the knockdown of symbiotic *Lb*s via RNA interference resulting in increased free O_2 concentrations within nodules, significant declines in bacterial nitrogenase activity, and complete loss of BNF (Ott *et al.*, 2005, 2009). In *Medicago*, accumulation of MtCAS31 (cold-acclimation-specific 31) delays drought-induced nodule senescence and maintains nitrogenase activity through a direct protein–protein interaction that protects the Lb MtLb120-1 (Li *et al.*, 2018*a*). An Lb gene in *Vicia faba*, *VfLb29*, is a late nodulin that is specifically activated both in infected root nodule cells and in arbuscule-containing cortical cells in mycorrhizal roots, which suggests that Lb might play diverse roles in plant–microbe symbioses (Vieweg *et al.*, 2004). Soybean nodules contain four major *Lb* genes, known as *Lba* (Glyma.10G199100), *Lbc1* (Glyma.10G199000), *Lbc2* (Glyma.20G191200) and *Lbc3* (Glyma.10G198800) (Brisson and Verma, 1982; Marcker *et al.*, 1984). Accumulation of *Lbc1* and *Lbc3* mRNA has been found in very young nodules on soybean roots prior to *Lba* expression (Marcker *et al.*, 1984). This indicates that differentially expressed *Lb*s might fulfil distinct functions during nodule formation and development. However, the precise nature of these physiological functions remains to be determined. In many legumes, pink- and/or red-pigment Lbs perform critical functions in N_2 fixation. Later, during nodule senescence, whether natural or stress-induced, green Lbs are associated with decreased N_2 fixation capacity (Navascués *et al.*, 2012).

In agriculture, the application of N fertilizer or the presence of a high level of residual N severely limits both the formation of nodules and N_2 fixation (Streeter, 1998; Miller *et al.*, 2007). More importantly, excess N accelerates nodule senescence, which is accompanied by the generation of green Lb proteins in bacteroids. This observation of green Lb in nodules has been associated with the presence of divalent iron, and might be a product of the breaking of the tetrapyrrole ring in Lb complexes (Virtanen and Laine, 1946). Moreover, ferrous, ferric and ferryl Lbs can also react with reactive nitrogen species. Green Lbs and red Lbs have identical globins, but the haem groups of green Lbs are nitrated with a nitro (NO_2) group on the 4-vinyl of the haem complex (Navascués *et al.*, 2012). Beyond this, little is known about how green Lbs are involved in N-induced nodule senescence.

Although Hbs are widespread in the plant kingdom, little is known about their genes and gene products in most legumes, including soybean. The objectives of this research were to conduct a systematic identification and characterization of soybean *Hb* genes, and to use this information to identify soybean *Lb* family members involved in the development of nodules and

regulation of N_2 fixation. Firstly, we investigated BNF capacity during nodule development as affected by high N supply, and in the process we were further able to conduct a systematic investigation of the entire *Hb* gene family. Analysis included the identification of *Hb* gene and protein sequences in soybean, along with comparison of soybean Hbs with Hbs from other species in phylogenetic and gene structural analysis. In addition, tissue-specific expression patterns and responses to excess N supply were also assessed using quantitative real-time PCR (qPCR). In the end, many avenues for continued research were opened, many of which might impact nutrient management strategies and efforts to more efficiently maintain soybean yields under a range of N availability conditions.

MATERIALS AND METHODS

Identification and bioinformatics analysis of GmLb genes

A BLAST search was performed with the known soybean (*Glycine max*) symbiotic (accession number V00453) and non-symbiotic (accession number U47143) Hb (Gopalasubramaniam *et al.*, 2008) amino acid sequences at the Phytozome website (http://www.phytozome.net). This yielded a total of seven predicted *Hb* genes, including five symbiotic *Hb*s known as *Lb*s, named *GmLb1* to *GmLb5*, and two non-symbiotic Hbs named *GmHb1* and *GmHb2* based on amino acid sequence similarity. General information for each *Hb* member (e.g. number of exons and introns, length of open reading frame) was also retrieved from Phytozome. Calculation of protein size was performed using the Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html). A phylogenetic tree of Hbs from soybean (GmLb1–5/GmHb1–2), common bean (PvLbs/PvHbs), *Medicago truncatula* (MtLbs/MtHbs), *Arabidopsis thaliana* (AtHbs), rice (OsHbs) and maize (ZmHbs) was constructed using the neighbour-joining method with 1000 bootstrap replicates in the MEGA 6.1 program. The corresponding locus names derived from the above plant species are shown in Supplementary Data Table S1. The genomic structures of soybean Hb genes were analysed at the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/index.php).

Plant materials and growth conditions

Seeds from the soybean genotype HN66 were surface-sterilized in 10 % H_2O_2 for 1 min, rinsed with distilled water, and germinated in sand-irrigated with a low-N (LN) nutrient solution containing 530 μM N (Li *et al.*, 2018*b*). One week after germination, uniform seedlings were inoculated with rhizobium strain *Bradyrhizobium* sp. (BXYD3) by immersing roots in a rhizobial suspension for 2 h prior to transplanting into LN hydroponic cultures as previously described (Li *et al.*, 2018*b*). Plants were reared in growth chambers (day/night: 14 h/10 h, 26 °C/24 °C) for 7, 14, 20, 30, 40 and 50 d after inoculation (dai). Moreover, the soybean plants were transferred to high-N (HN, 20 mM N added) or a refreshed LN solution at their peak of N_2 fixation (30 dai) for 9 d. The nodules at different growth stages or under the HN condition were separately harvested and further classified into two groups based on nodule diameter (*D*), specifically large (*D* ≥ 2 mm) and small (*D* < 2 mm) nodules,

and then the number and weight of nodules were determined for each group. Large nodules were also used for RNA extraction, determination of Lb concentration, nitrogenase activity analysis and infected cell surveys.

For expression analysis of *Hb* members in various tissues, seeds of the soybean genotype Williams 82 were germinated in sand and pre-treated as described above. After inoculation with BXYD3, seedlings were grown in a low-N solution for 30 d prior to harvesting roots, nodules, stems, leaves and flowers. Pods ~1 cm long and immature seeds were harvested separately 40 and 50 d after inoculation. All samples were stored at −80 °C for RNA extraction and qPCR analysis.

Observation of infected cells in nodules and nitrogenase activity

To better evaluate how N supply affects nodule growth and development, nodules were embedded in paraffin and sectioned transversely to a thickness of 18 μm with a microtome (Leica RM2235). After dewaxing, nodule sections were stained with 0.1 % toluidine blue and observed by light microscopy (Axio Imager A2m; Carl Zeiss). Ten nodules from LN or HN treatments were selected randomly and measured. Three cross-sections were made on each nodule, which led to a total of 30 cross-sections being observed for each treatment in determination of the number of infected cells per mm^2 and the surface area of 100 infected cells in ImageJ (https://imagej.nih.gov/ij/).

The nitrogenase activity of nodules was analysed using an acetylene reduction assay (David *et al.*, 1980). Nodules were separately sampled in 8-mL airtight glass bottles from which 1 mL of air was extracted prior to the injection of an equal volume of acetylene. After 2 h, the reaction was terminated by adding 0.5 м NaOH. Other research has found that downregulation of O$_2$ concentration in the infected cells of nodules is able to cause an O$_2$-related limitation of nitrogenase activity (Carroll *et al.*, 1987; Kaiser *et al.*, 1997). Because the O$_2$ barrier will close down after 30 min or so in isolated nodules, a short (30 min) reaction assay could be appropriate for nitrogenase activity analysis. Given the equal treatment in all cases, subsequently in this study 0.3 mL of gas was analysed in gas chromatography with a flame ionization detector (GC-2014AF). Nitrogenase activity was calculated as the amount of acetylene reduced by nitrogenase in nodules per hour and per gram of nodule fresh weight.

Measurement of Lb concentration

For the measurement of Lb concentration in nodules, about 50 mg of fresh nodules was first ground and homogenized in 16-fold volumes of 0.1 м precooled PBS (Na$_2$HPO$_4$-NaH$_2$PO$_4$ buffer at 5 °C, pH 6.8). The resulting slurry was then centrifuged at 12 000 *g* for 15 min prior to assaying the supernatant by spectrophotometry at a wavelength of 540, 520 and 560 nm. The Lb, including red and green pigment, was calculated separately as described previously (Larue and Child, 1979). Bovine Hb was used as a protein standard in this study.

RNA extraction and qPCR

High-quality total RNA was isolated from soybean roots, nodules, stems, leaves, flowers, young pods and seeds using the RNAiso Plus reagent (TaKaRa Japan) according to the manufacturer's instructions. Subsequently, RNA samples were treated with RNase-free DNase I (TaKaRa, Japan) to remove genomic DNA contamination. First-strand cDNA was synthesized using oligo(dT), deoxyribonucleotide triphosphates and MMLV reverse transcriptase (Promega, USA) based on protocols from the supplier. Quantitative real-time PCR was performed in 20-μL volumes containing 2 μL of 1:50 diluted cDNA, 0.6 μL of specific primers, 6.8 μL of distilled, deionized water and 10 μL of TransStart Top Green qPCR SuperMix (Trans) using a LightCycler 96 (Roche Diagnostics) PCR system. Reaction conditions for thermal cycling were: 95 °C for 1 min, 40 cycles of 95 °C for 15 s, 56–60 °C for 15 s and 72 °C for 30 s. The annealing temperature (56–60 °C) was adjusted to suit the amplification of individual *Hb* genes. The elongation factor *1α* gene (*TefS1*, accession number X56856) from soybean was used as an endogenous control to evaluate relative transcript abundance. Relative expression values were calculated as the ratio of the expression value of the target gene to that of *TefS1* using the $2^{-\Delta\Delta CT}$ method. The specific primers used for detecting target genes are listed in Supplementary Data Table S2.

Data analysis

All of the data were analysed statistically using Sigma Plot (version 12.0) for calculating means and standard errors. Comparisons between groups were performed using Student's *t*-test or univariate analysis in SPSS (version 16.0).

RESULTS

Analysis of the relationship between nodule development and BNF capacity

Because soybean nodules belong to the determinate type, the size of nodules could represent the age of nodules. Therefore, we harvested the biggest nodules at 7, 14, 20, 30, 40 and 50 dai to investigate the relationship between nodule development and BNF capacity under LN conditions (530 μm N added). The colour of the nodules changed incrementally from white (7 dai) to pink (14, 20 and 40 dai) and red (30 dai), then to green (50 dai) at different growth stages (Fig. 1A). Consistently, the red Lb concentration increased significantly with nodule growth, peaked at 30 dai, and subsequently declined with age, while the green Lb concentration was increased at 40 dai and significantly elevated at 50 dai (Fig. 1B); interestingly, nitrogenase activity showed similar changes (Fig. 1C). These results indicated that the green derivatives of Lbs generated during nodule senescence, and the development of nodules with pink or red Lbs, are closely related to soybean BNF capacity.

Effects of excess N supply on BNF capacity

To gain insight into the effect of excess N on biological nitrogen fixation capacity, rhizobia-inoculated soybeans were grown in hydroponic cultures for 30 d, at which point the nodules had their highest N$_2$-fixation capacity, and were then transferred to either HN (20 mm N added) or LN solution for
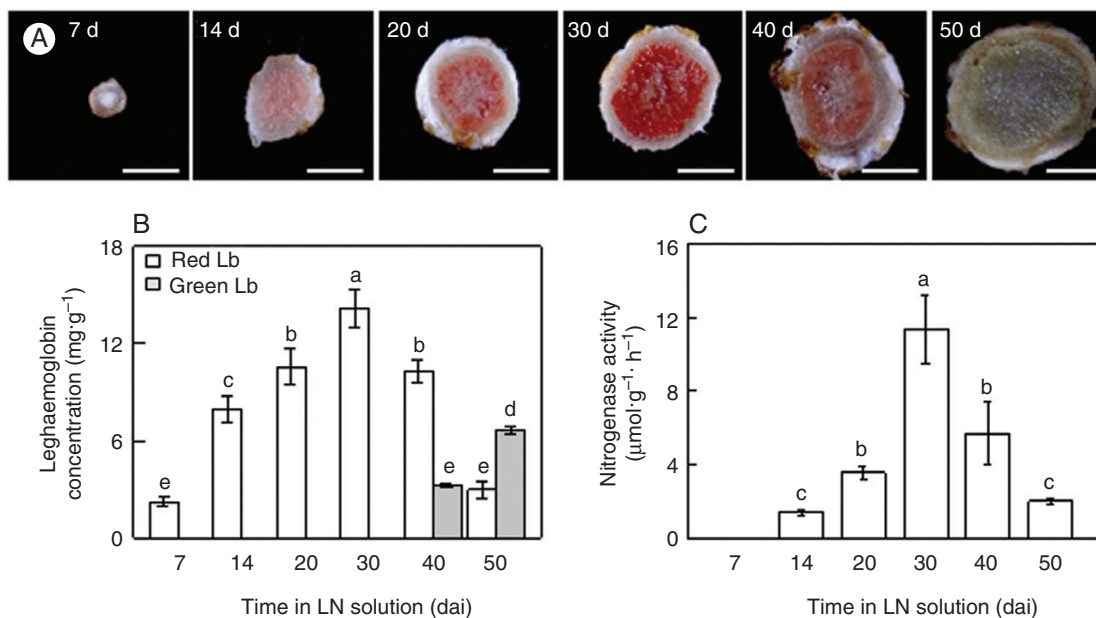
Fig. 1. Leghaemoglobin concentration and nitrogenase activity in nodules at different growth stages. (A) Nodule colour at different growth stages. Scale bars = 2 mm. (B) Leghaemoglobin concentration. (C) Nitrogenase activity. Seven-day-old soybean seedlings were inoculated with rhizobia and grown in LN (530 µM N added) nutrient solution for 7, 14, 20, 30, 40 and 50 dai. Values are mean ± s.e. ($n = 5$). Different letters indicate a significant difference at the 0.05 level.
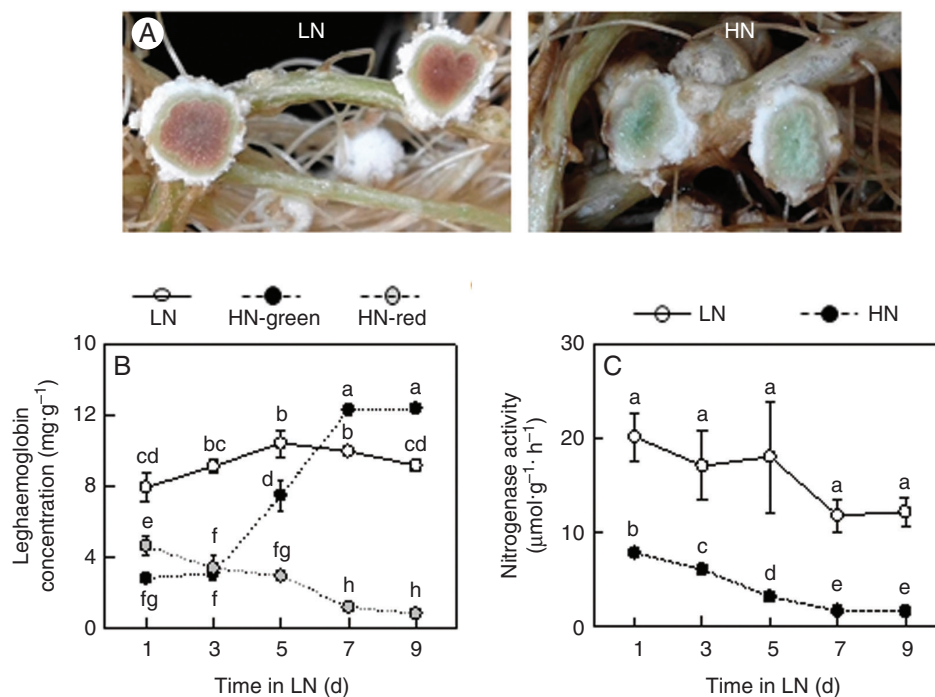

Fig. 2. Leghemoglobin concentration and nitrogenase activity as affected by excess N supply in nodules on soybean roots reared in hydroponic culture. (A) Photographs showing nodule growth. (B) Leghemoglobin concentration. (C) Nitrogenase activity. Seven-day-old soybean seedlings were inoculated with rhizobia and grown in LN (530 µM N added) nutrient solution for 30 d and the inoculated plants were then transferred into LN or HN (20 mM N added) solution for 9 d. Values are mean ± s.e. ($n = 10$). Different letters indicate a significant difference at the 0.05 level.

9 d. In these conditions, excess N application resulted in the production of large amounts of green Lb derivatives in nodules (Fig. 2A). These green Lbs are typically generated during nodule senescence, when they severely inhibited BNF. Consistent with

this role, in this study we found that the green Lb concentration increased significantly with increasing HN treatment duration, and its highest concentration was 12.37 mg g^{-1} FW at 9 d (Fig. 2B). However, the red Lb concentration and nitrogenase

activity were significantly reduced 1.70- and 2.56-fold, respectively, in HN treatments compared with LN treatments as soon as 1 d. Then, they gradually declined with additional HN days (Fig. 2B, C). This indicates that excess N may accelerate nodule senescence, which means that appropriate N supplies are quite important for proper management of soybean nodulation.

Nodule growth and development of infected cells are affected by excess N

In order to evaluate the effects of excess N supply on nodule growth, nodules were harvested separately and classified into two groups based on nodule diameter (*D*) under LN and HN conditions. The results showed that excess N supply not only limited nodule development, particularly the formation of big nodules, but also suppressed nodule initiation (Fig. 3A). The number of large nodules (*D* ≥ 2 mm), nodule mass and individual nodule size were 30.7 %, 45.2 % and 30.3 % less in the HN than in the LN condition, respectively (Fig. 3B–D). In addition, toluidine blue staining was employed in observations of infected cells. As shown by the morphology of typical nodule slices, infected cells within nodules were bigger and more numerous under LN conditions than under HN conditions, which inhibited infected cell growth enough to produce declines in infected cell size and number (Fig. 3E). Quantitatively, the number of infected cells (Fig. 3F) and the surface area per 100 infected cells (Fig. 3G) were 21.2 % and 52.7 % less, respectively, in HN than in LN. These results suggest that HN

suppression of BNF might be caused by inhibition of nodule formation and infected cell development in nodules through acceleration of nodule senescence.

Identification of GmLb members in soybean

Upon observing the effects of excess N on nodule colour and Lb concentration as described above (Fig. 2), we attempted to investigate the soybean *Lb* gene family more comprehensively. In total, five putative *Lb* and two non-symbiotic *Hb* genes were identified according to BLASTP searches of the protein database of soybean in the Phytozome web portal (http://www.phytozome.net/). General information on the distinguishing characteristics of *Lb*s and *Hb*s is summarized in Table 1. They were located on chromosomes 10, 11 and 20, with four found on chromosome 10, two on chromosome 11 and one on chromosome 20. The open reading frames of identified *Lb*s/*Hb*s ranged from 435 to 507 bp in length and were predicted to encode proteins containing 144–168 amino acids (Table 1).

Phylogenetic analysis and classification of soybean Hbs

To determine evolutionary relationships among Hb and similar proteins from other species, a phylogenetic tree was constructed by neighbour-joining analysis in MEGA 6.1 using the full Hb protein sequences from soybean, common bean, *M. truncatula*, *A. thaliana*, rice and maize. As shown
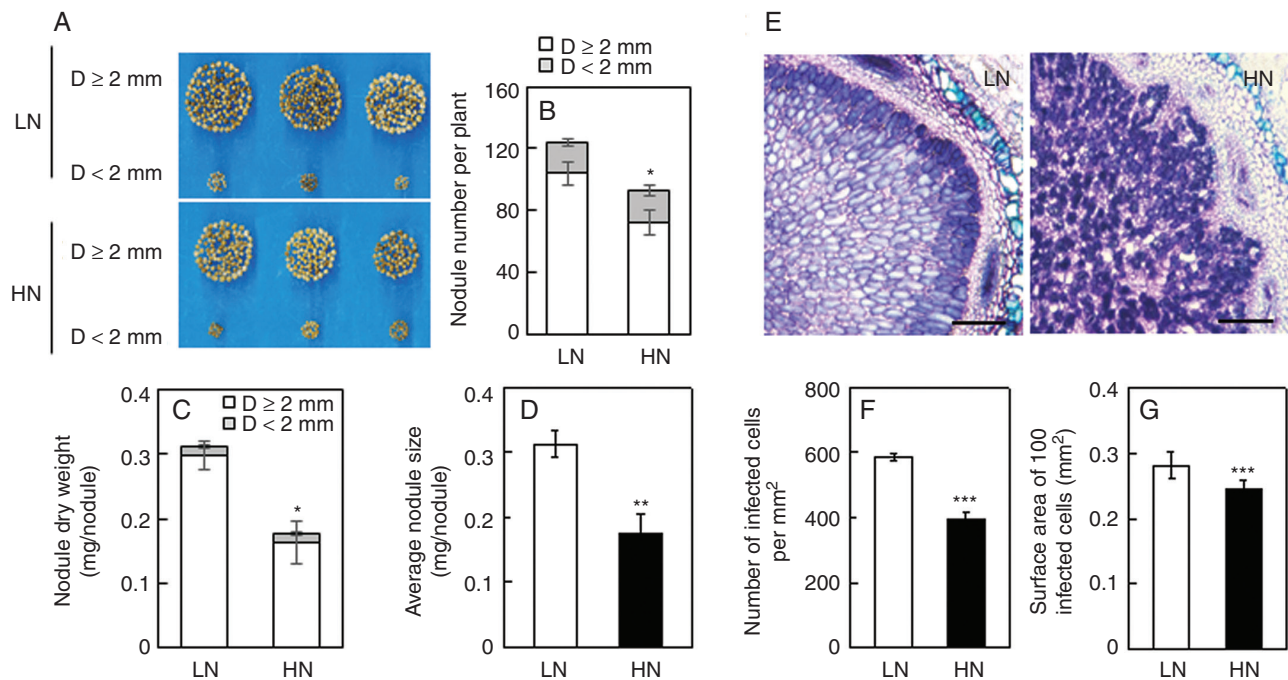

FIG. 3. Effects of excess N supply on nodule growth and infected cell development. (A) Phenotypes of nodules. (B) Nodule number. (C) Nodule dry weight. (D) Average nodule size. (E) Toluidine blue-stained nodule cross-sections. Scale bars = 200 μm. (F) Number of infected cells. (G) Surface area of 100 infected cells. Seven-day-old soybean seedlings were inoculated with rhizobia and grown in LN (530 μM N added) nutrient solution for 30 d prior to transferring inoculated plants into LN or HN (20 mM N added) solution for 9 d. Nodules were classified into two groups according to diameter (*D*): large (*D* ≥ 2 mm) and small (*D* < 2 mm). Values are mean ± s.e. (*n* = 3). Ten nodules were selected from each replicate for infected cell analysis. Asterisks represent significant differences in a single trait between plants grown in LN and HN treatments: *0.01 < *P* ≤ 0.05; **0.001< *P* ≤0.01; ***P* ≤ 0.001 (Student's *t*-test).

TABLE 1. *General information for the seven identified Hb members*

Genes	Locus	Chromosomal location	Exon/intron number	Length of ORF (bp)	Number of amino acids	Protein size (kDa)
GmLb1	Glyma.10G198800.1	10	4/3	438	145	15.6
GmLb2	Glyma.10G199000.1	10	4/3	435	144	15.4
GmLb3	Glyma.10G199100.1	10	4/3	435	144	15.4
GmLb4	Glyma.20G191200.1	20	4/3	438	145	15.5
GmLb5	Glyma.10G198900.1	10	4/3	507	168	18.1
GmHb1	Glyma.11G121700.1	11	4/3	474	157	17.6
GmHb2	Glyma.11G121800.1	11	4/3	486	161	18.0

Gene locus, exon and intron number and open reading frame (ORF) length were determined from sequences deposited at the Phytozome website (http://www.phytozome.net). Protein size was calculated using the Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html).
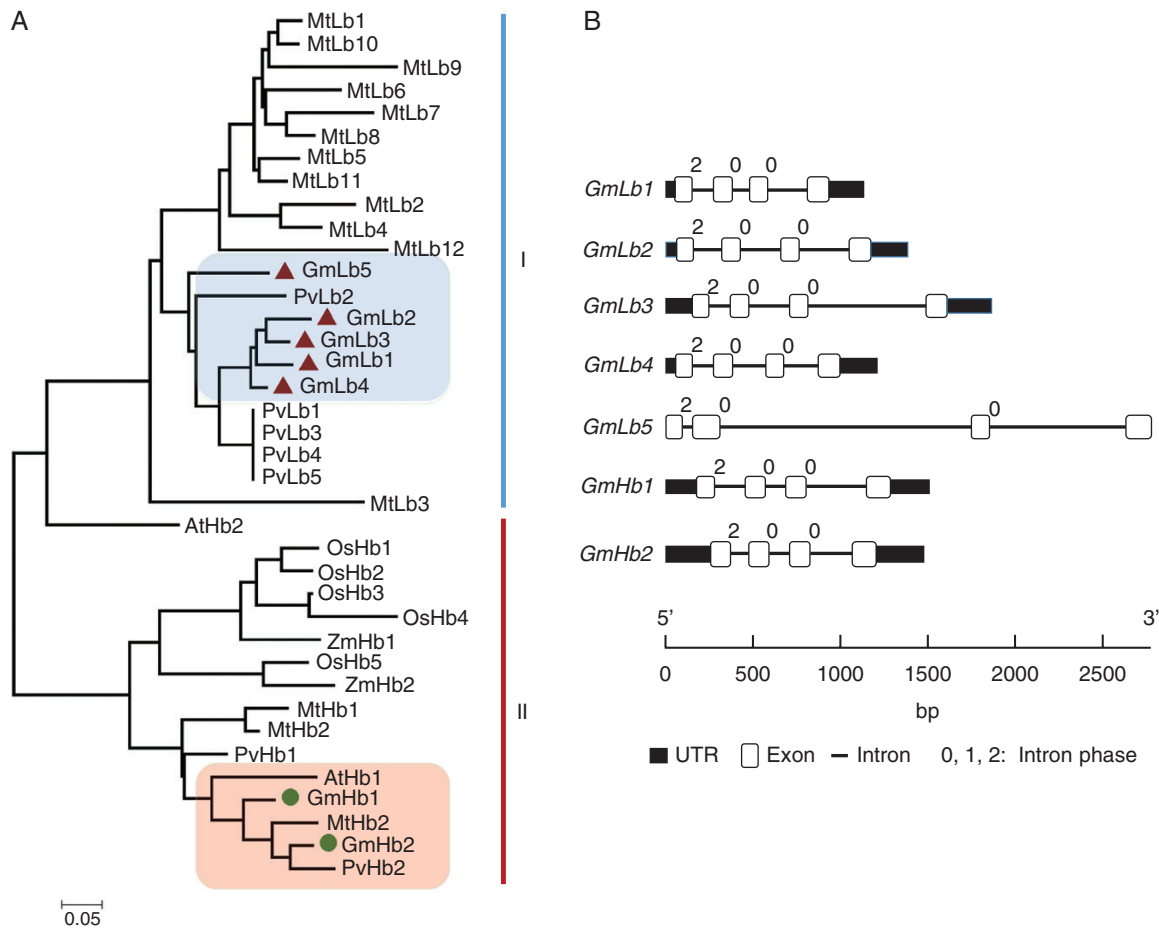

FIG. 4. General characteristics of the five identified *GmLb*s and two non-symbiotic *GmHb*s in soybean. (A) Phylogenetic analysis of Hb from different plant species. This phylogenetic tree includes seven Hbs from soybean (*Glycine max*), seven from common bean (*Phaseolus vulgaris*), 15 from *Medicago truncatula*, two from *Arabidopsis thaliana*, five from rice (*Oryza sativa*) and two from maize (*Zea mays*). It was constructed by the neighbour-joining method with 1000 bootstrap replicates in the MEGA 6.1 program. Haemoglobin proteins sorted into two clades, labelled clade I and clade II. Five GmLbs are highlighted by red triangles in the blue box, and two GmHbs are indicated by green circles in the red box. (B) Gene structures of symbiotic *Lb*s and non-symbiotic *Hb*s from soybean. White boxes represent exons, thin lines represent introns and black boxes represent 5′-/3′-UTRs. Numbers above introns indicate intron phases. Phase 0, 1 and 2 introns were defined as introns starting before the first, after the first and after the second nucleotide of a codon, respectively.

in Fig. 4A, the analysed plant Hb proteins can be divided into two clades, labelled clade I and clade II. Clade I contains most of the tested legume Lbs and includes five GmLbs and PvLbs along with 12 *Medicago* Lb members. Within clade I, four soybean Lbs, GmLb1, GmLb2, GmLb3 and GmLb4, aligned very closely, while GmLb5 sorted into a subgroup with common bean PvLb2. Other than AtHb2 aligning closely with other clade I Lbs, the remaining legume Hbs and the Hbs from non-legume plant species all belonged to clade II (Fig. 4A).

Phylogenetic relationships among soybean Lbs and Hbs were further distinguished by variation in amino acid identity between members of this family (Supplementary Data Table S3). Clade

I GmLbs all exhibited 69–95 % amino acid identity, with alignments between GmLb1, GmLb2, GmLb3 and GmLb4 displaying the highest identities (92–95 %). The two non-symbiotic GmHbs in clade II were 86 % identical in their amino acid sequences. Between clade I and clade II, sequence identity dropped to 41–45 %. Taking sequence identity and phylogenetic tree analysis together, these results suggest that symbiotic Lbs (clade I) have diverged significantly from non-symbiotic Hbs (clade II) through the course of soybean evolution.

Full-length cDNAs and genome sequences of *GmLbs*/*GmHbs* were used to investigate gene structures on the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/index.php). In this analysis, all *Hb*s were found to have four exons and three introns, with an intron phase pattern of 2, 0 and 0 from the 5′ to the 3′ end (Table 1, Fig. 4B). The symbiotic *Lb*s in clade I, namely *GmLb1*, *GmLb2*, *GmLb3*, *GmLb4* and *GmLb5*, each contain one or more long introns, especially *GmLb5*, which has a total intron length of 2415 bp (Fig. 4B and Supplementary Data Table S4). This gene structure pattern stands in contrast to the gene structure in the non-symbiotic clade II members *GmHb1* and *GmHb2*, which have similar gene structures with total intron lengths of 769 and 596 bp, respectively. It is also notable that the first three exons of clade I *GmLb*s are of equal length, while both clade II *GmHb*s have the same second and third exon and second intron lengths (Supplementary Data Table S4). This difference in gene structure indicates that the symbiosis-related *Lb* genes evolved from these non-symbiotic *Hb*s.

Conserved amino acids of GmLbs

Multiple sequence alignment was conducted to examine amino acid sequence similarity and to identify conserved motifs across families of plants, with identical and similar amino acid sequences being shaded black and grey, respectively, in Fig. 5. All GmLb/GmHb members possess a typical globin domain (http://www.phytozome.net/). Symbiotic Lbs play important roles in BNF through modulation of O_2 diffusion into nodules (Appleby, 1992). During senescence, these same Lbs tend to generate the green derivatives observed within nodules, which is caused by haem nitration (Navascués *et al.*, 2012). Further study revealed that Tyr[30] located in the haem distal pocket is the major target of nitration. Otherwise, Tyr[25] and a small proportion of C-terminal Tyr[133] were also capable of being converted into the nitrated species NO_2-Tyr[25] and NO_2-Tyr[133] in GmLbs (Sainz *et al.*, 2015). Here, multiple sequence alignment revealed that the symbiotic GmLb1, 2, 3 and 4 with the highest protein homology to each other contain Tyr[25] and Tyr[30] amino acid residues (red box in Fig. 5 and Supplementary Data Table S3). Excepting GmHb1, other Hbs have a Tyr[133] residue, suggesting that these residues are conserved in soybean symbiotic Lbs, which imparts the potential to form NO_2-Tyr[25], NO_2-Tyr[30] and/or NO_2-Tyr[133] in nodules during senescence.

Tissue-specific expression of GmLbs/GmHbs

To clarify *GmLb*/*GmHb* expression patterns among soybean tissues, qPCR analysis was performed to determine relative transcript abundances in seven tissues, namely roots, nodules, stems, young leaves, flowers, pods and young seeds (Fig. 6). The results showed that four symbiotic *Lb* genes, *GmLb1* to *GmLb4*, were most abundant in nodules, while *GmLb5* was also highly expressed in flowers, indicating that *GmLb1*, *2*, *3* and *4* might be the key *Lb* genes involved in BNF in soybean. In contrast, the non-symbiotic *Hb*s in clade II, *GmHb1* and *GmHb2*, were predominantly expressed in leaves and roots, respectively, suggesting other roles, possibly still modulating O_2 levels, for clade II genes in response to environmental stress during plant growth and development.

Transcription of GmLbs in nodules at different growth phases and in response to excess N application

To evaluate the expression of soybean *Lb* genes in nodules at different growth stages and how it might respond to excess N application, we first investigated the transcription of all *GmLb* members in nodules, with the result that *GmLb1* to *GmLb4* were the most abundantly expressed *GmLb*s in nodules (Fig. 7A). Therefore, *GmLb1* to *GmLb4* were then selected to be analysed separately in further experiments. During nodule growth and development, the transcripts of *GmLb1*, *2*, *3* and 4 all gradually increased and peaked at 30 dai, except *GmLb3*, which was most abundant in nodules at 20 and 30 dai; all transcripts then declined with nodule senescence (Fig. 7B). In the HN treatment, expression levels of *GmLb1*, *2*, *3* and 4 were all downregulated in nodules by more than 2-fold at 3 d in comparison with expression in nodules produced under LN conditions (Fig. 7C). Then, the transcription of four *GmLb*s obviously decreased with increasing HN duration. At 9 d, *GmLb1–4* exhibited a 13.1, 13.5, 12.5 and 8.3-fold decrease, respectively, in transcript level in response to HN. Taken together, these results suggest that *GmLb1* to *GmLb4* are the major Lb genes required for the binding and release of oxygen in order to maintain oxygen gradients during N_2 fixation in nodules.

DISCUSSION

Soybean, the most widely grown legume crop globally, possesses a superior capacity to fix N_2. A large proportion of the N_2 fixed by soybean nodules is also available for the growth of neighbouring or subsequent crops in intercropping/rotation systems, and thereby contributes to increases in grain production while also reducing the demand for N fertilizers in agro-ecosystems (Meng *et al.*, 2015; Wang *et al.*, 2016). However, several environmental factors can restrict BNF in legumes, most notably the common practice of applying high rates of N to soils. Excess N facilitates nodule senescence through the production of green Lb derivatives (Navascués *et al.*, 2012; Saito *et al.*, 2014). In legumes, the absence of red Lb in bacteroids of root nodules precedes the abortion of BNF (Ott *et al.*, 2005, 2009). To better understand the possible functions and mechanism of Lb activity in BNF, members of the *Lb* gene family were identified and investigated in a systematic fashion.

Nitrogen has been shown to inhibit BNF through decreases in nodule number, along with declines in nodule mass and N_2 fixation activity, as well as acceleration of nodule senescence in green nodules (Saito *et al.*, 2014; Ferguson *et al.*, 2018). This
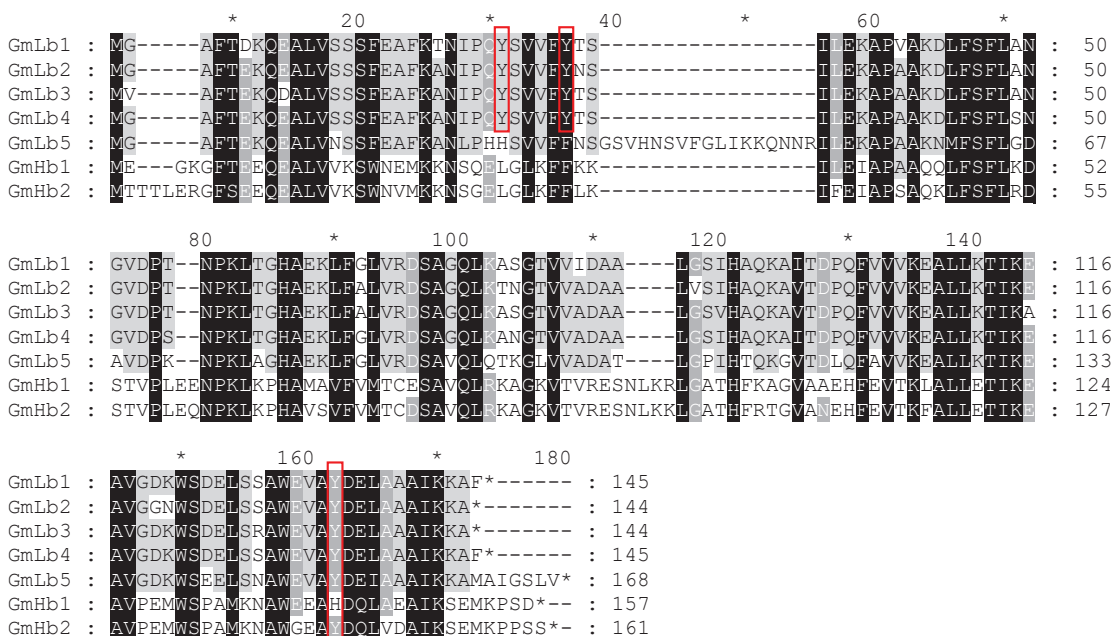
FIG. 5. Alignment of GmLb and GmHb amino acid sequences. Sequence alignment was performed with the ClustalW and GeneDoc programs. Identical and similar amino acids are shaded in black and grey, respectively. The red box represents the tyrosine amino acid residue primarily targeted for nitration to form 3-nitrotyrosine (NO_2-Tyr), which then generates the green derivatives of Lbs during nodule senescence.
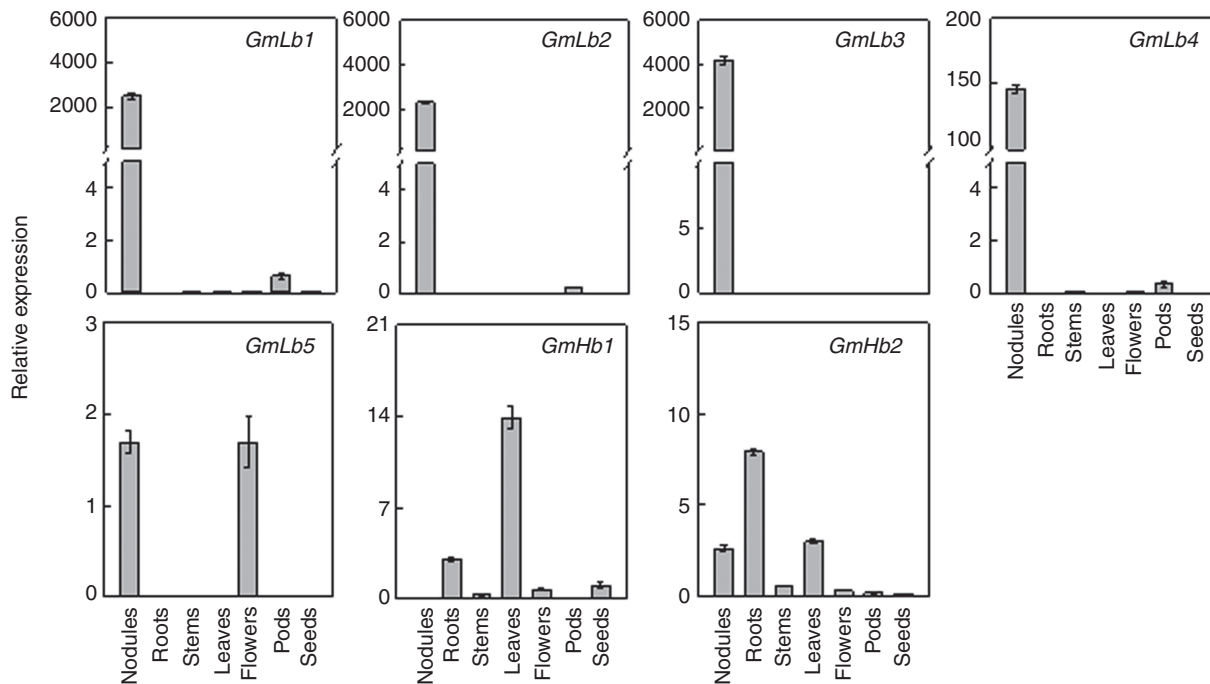

FIG. 6. Tissue-specific expression patterns of *GmLb* and *GmHb* members. Seven-day-old seedlings were inoculated with rhizobia and transplanted into LN (530 μm N added) nutrient solution. qPCR was conducted with transcripts extracted from nodules, roots, stems, leaves and flowers sampled after 30 d of soybean growth, along with pods and young seeds sampled after 40 and 50 d, respectively. Relative expression values were calculated as the ratio of *GmLb* or *GmHb* expression to that of the soybean housekeeping gene *TefS1* (accession number X56856). Values are mean ± s.e. ($n = 4$).

homeostatic activity presumably allows soybeans to balance the high cost of N_2 fixation with tight modulation of O_2 levels, while also impacting the translocation of carbon among root sinks capable of contributing to N acquisition from soils or symbiotic

relationships with mycorrhizal fungi or nodulating rhizobial species (Fujikake *et al.*, 2003). Previous work demonstrated that high NO_3^- availability not only leads to Lb degradation, as reflected by declines in both tissue Lb concentrations and the ratio
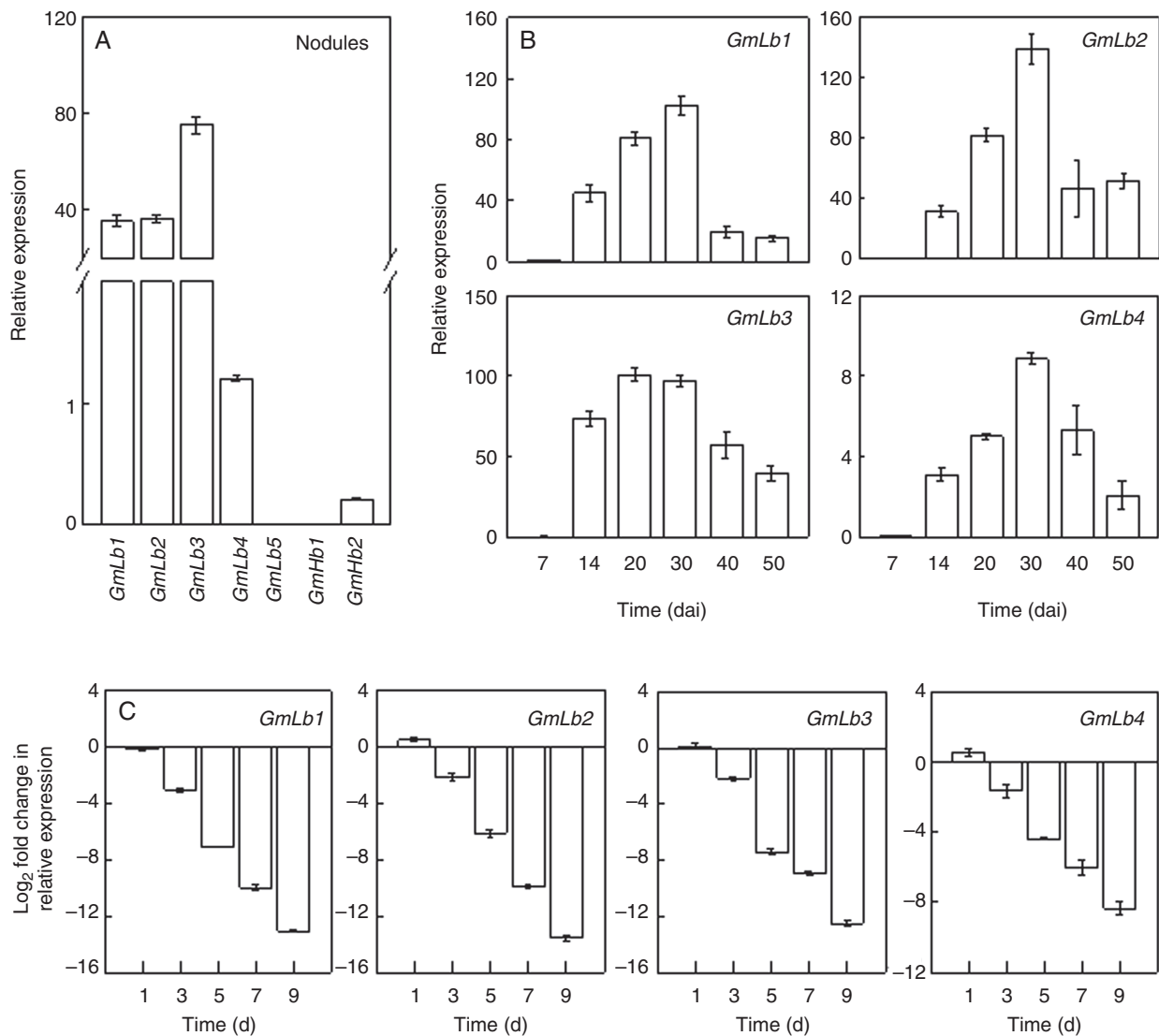
FIG. 7. Transcription of symbiotic *GmLb*s and non-symbiotic *GmHb*s in nodules. (A) Relative expression of *GmLb*s/*GmHb*s in soybean nodules under LN conditions. (B) Expression of nodule-specific *GmLb* genes in nodules at 7, 14, 20, 30, 40 and 50 dai. Relative expression values were calculated as the ratio of *GmLb* expression to that of the soybean housekeeping gene *TefS1* (accession number X56856). Relative expression was normalized against the mean of each *GmLb* transcription level at 7 dai. (C) Expression of nodule-specific *GmLb* genes in response to excess N supply, shown as the binary logarithm of fold changes in relative expression of *GmLb* members under HN (20 mM N added) compared with LN (530 µM N added) conditions. Values are mean ± s.e. (*n* = 4).

of Lb to soluble proteins, but also represses the expression of the *Lb* genes (Bisseling *et al.*, 1978; Becana and Sprent, 1989; de Billy *et al.*, 1991; Gallusci *et al.*, 1991; Sainz *et al.*, 2015). In the current work, we found that red Lb biosynthesis is closely correlated with soybean BNF capacity and that vigorous nodules with red Lb pigments turn green during nodule senescence (Fig. 1). Furthermore, our results also revealed that excess N inhibited BNF, as indicated by limited nodule formation and development, and reduced Lb levels as well, which was consistent with the previous reports (Saito *et al.*, 2014). Furthermore, high N supply also accelerated nodule senescence and the production of green Lb in nodules (Figs 2 and 3). Green Lbs usually contain nitrated haems, the Tyr[30] located in the haem distal pocket being the major target of nitration in soybean, followed by Tyr[25] (Navascués *et al.*, 2012; Sainz *et al.*, 2015).

Previous analysis has also identified four major *Lb* genes associated with symbiotic BNF and which encode products possessing remarkably high affinities for O_2, specifically Lba (Glyma.10G199100), Lbc1 (Glyma.10G199000), Lbc2 (Glyma.20G191200) and Lbc3 (Glyma.10G198800) (Brisson and Verma, 1982; Marcker *et al.*, 1984). Here, we confirmed the production of these soybean Lbs and renamed them as GmLb1, GmLb2, GmLb3 and GmLb4 (Table 1). Multiple sequence alignment revealed that Tyr[25] and Tyr[30] are only found in the four major Lbs associated with symbiosis, GmLb1 to GmLb4 (Fig. 5), which, together with the potential for tyrosine to be nitrated, suggests that Tyr[25] and Tyr[30] might be key residues in BNF. Furthermore, *GmLb1*, *2*, *3* and *4* were all severely downregulated during nodule senescence as well as in nodules exposed to excess N (Fig. 7B, C). This led to significant declines

in Lb concentration and thus limited infected cell development and nitrogenase activity in nodules (Figs 1–3). For further details of the mechanisms of Lb biosynthesis and their individual functions in nodules and responses to various environmental stresses during nodulation, we require further study focused on these specific topics.

In the current study, a total of seven soybean *Hb* genes were isolated in a genome-wide search based on sequence similarity. These *Hb*s could then be categorized into two distinct clades in a phylogenetic analysis of Hb proteins from monocot and dicot species (Fig. 4A), which was further supported by distinct patterns in gene structures among the two *Hb* clades, along with protein identities that were higher within clades than between clades (Fig. 4B, Supplementary Data Table S3). Interestingly, clade I includes GmLb1 to GmLb5, which appear active in root tissues involved in symbiotic interactions, along with most of the other tested legume Lbs. In contrast, clade II contains the soybean Hbs not involved in symbiosis, GmHb1 and GmHb2, as well as all of the tested monocot Hbs from rice and maize and Hbs from the non-symbiotic plant *Arabidopsis* (Fig. 4A). On the whole, these results indicate that Hbs can be found in monocot and dicot plant species, though those participating in symbiotic interactions have diverged significantly from other Hbs. This conclusion is further supported by expression profiles in which all symbiosis-associated clade I *GmLb*s except *GmLb5* were specifically expressed in nodules (Figs 4 and 6). In short, it appears that specific biological functions associated with *GmLb*s/*GmHb*s might be grouped into categories that are in accordance with gene classification, though further experiments are required to test this hypothesis.

The qPCR results returned for the *GmLb*s/*GmHb*s observed in various soybean tissues in this study confirm the tissue-specific expression patterns reported previously in global transcription studies using next-generation sequencing (Libault *et al.*, 2010). Similarly, the symbiosis-associated *GmLb1*, *2*, *3* and *4* are specifically expressed in nodules (Figs 6 and 7A). Beyond the expression of *GmLb1–4*, transcripts of the symbiosis-associated *GmLb5* were also detected in both nodules and flowers at approximately equal levels of expression, and the non-symbiotic *GmHb2* was most abundant in roots (Fig. 6). This result stands in contrast to the report authored by Libault *et al.* (2010), in which *GmLb5* was not detected in flowers and *GmHb2* was highly expressed both in nodules and roots. These differences in expression patterns observed for the two *Hb*s between the two studies might result from differences between the tested genotypes, variations in growth conditions, including N availability, differences in sampling procedures, or differences in the timing of sampling relative to developmental stage. Notwithstanding these specific differences between studies, *GmLb* expression patterns appear to be stable and reproducible, and have been largely confirmed in three independent studies.

Expression profiles for *GmLb*s suggest potentially specific roles for individual members. Transcripts of the symbiosis-associated members of the *GmLb* family, *GmLb1* to *GmLb4*, were found exclusively in nodules, where they were also the most highly expressed *GmLb*s (Fig. 7A), suggesting that these four *GmLb*s are the genes primarily responsible for *Lb* expression in nodules, which is consistent with previous studies (Brisson and Verma, 1982; Marcker *et al.*, 1984). An implication of this conclusion is that symbiosis-associated *GmLb*s play vital roles in BNF.

Other *Hb* genes not associated with symbiotic interactions have been isolated from a number of both legume and non-leguminous plant species. These *Hb* genes exhibit a variety of O_2-binding properties and gene expression patterns, many of which include responses to numerous stimuli. For example, the clade-I non-symbiotic *Hb*s from barley are expressed in aleurone and root tissues subjected to low oxygen stress (Taylor *et al.*, 1994). Another *Hb* gene in cotton, *GhHb1*, is induced in roots challenged with the *Verticillium* wilt fungus. Moreover, ectopic overexpression of *GhHb1* in *Arabidopsis* leads to constitutive expression of the defence genes *PR-1* and *PDF1.2*, which confers enhanced disease tolerance on treated plants (Qu *et al.*, 2006). In *Lotus japonicus*, the non-symbiotic Hb *LjHb1* has been strongly induced in response to hypoxia, cold stress, and exposure to an exogenous NO donor. Observations using fluorescence microscopy demonstrated that induction of *LjHb1* expression corresponds to the generation of NO (Shimoda *et al.*, 2005). Altogether, these results suggest that non-symbiotic *Hb* genes are involved in various biotic and abiotic stress responses. In this study, the expression of two non-symbiotic Hbs, *GmHb1* and *GmHb2*, varied among the seven tissues observed, with the highest expression occurring in leaves and roots (Fig. 6). This result indicates that each of these two genes might function in the development of organ-specific responses to different stresses. Further experiments with forward and reverse genetic approaches are needed to conclusively test this hypothesis and to determine specific roles for each GmLb and GmHb.

Conclusions

In this study, we found that red Lb biosynthesis is closely associated with soybean BNF, and excess N application significantly inhibited BNF and accelerated nodule senescence and production of green pigmented Lb. Subsequent systematic isolation and characterization of soybean GmLb protein family members was then performed, along with assessment of *GmLb* molecular features and expression patterns in vegetative and reproductive tissues. The results reveal the existence of seven *Hb* members that can be divided into two distinct subgroups based on sequence similarity and gene structural features, which is further supported by the observation of distinct expression profiles between the two subgroups. The first group, clade I, includes symbiotic-associated Lbs, *GmLb1* to *GmLb4* being the four major nodulation Lbs, each of which was further observed to be suppressed in nodules developing under excess N availability conditions. The non-symbiotic *GmHb*s, *GmHb1* and *GmHb2*, sorted into clade II and are predominantly expressed in leaves and roots, respectively, which suggests that *Hb* genes fill diverse roles in plant growth and development beyond acting in nodulation on soybean roots. Further understanding of the molecular mechanisms of Lb biosynthesis may lead to insights that can be applied to improving BNF and, thus, soybean production.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following.

Table S1: *Hb* gene loci from different plant species included in phylogenetic tree analysis.

Table S2: gene-specific primers used for qPCR analysis.

Table S3: amino acid sequence identity within the coding regions of haemoglobin protein family members.

TableS4: length of exons and introns in each soybean *GmLb* and *GmHb* gene.

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