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Symbiotic Leghemoglobins Are Crucial for Nitrogen Fixation in Legume Root Nodules but Not for General Plant Growth and Development

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Summary

Hemoglobins are ubiquitous in nature and among the best-characterized proteins [1–9]. Genetics has revealed crucial roles for human hemoglobins [10], but similar data are lacking for plants. Plants contain symbiotic and nonsymbiotic hemoglobins [11]; the former are thought to be important for symbiotic nitrogen fixation (SNF). In legumes, SNF occurs in specialized organs, called nodules, which contain millions of nitrogen-fixing rhizobia, called bacteroids [12, 13]. The induction of nodule-specific plant genes, including those encoding symbiotic leghemoglobins (Lb), accompanies nodule development [14, 15]. Leghemoglobins accumulate to millimolar concentrations in the cytoplasm of infected plant cells prior to nitrogen fixation and are thought to buffer free oxygen in the nanomolar range, avoiding inactivation of oxygen-labile nitrogenase while maintaining high oxygen flux for respiration [16]. Although widely accepted, this hypothesis has never been tested in planta. Using RNAi, we abolished symbiotic leghemoglobin synthesis in nodules of the model legume *Lotus japonicus*. This caused an increase in nodule free oxygen, a decrease in the ATP/ADP ratio, loss of bacterial nitrogenase protein, and absence of SNF. However, LbRNAi plants grew normally when fertilized with mineral nitrogen. These data indicate roles for leghemoglobins in oxygen transport and buffering and prove for the first time that plant hemoglobins are crucial for symbiotic nitrogen fixation.

Results and Discussion

Legume genomes typically contain several symbiotic and nonsymbiotic leghemoglobin (Lb) genes (Figure 1A). For instance, *Lotus japonicus* contains three symbiotic leghemoglobins, *LjLb1*, *LjLb2*, and *LjLb3*, which are expressed exclusively in nodules, and two nonsymbiotic leghemoglobins, *LjNSG1* and *LjNSG2* [17]. Expressed-sequence-tag (EST) data for *L. japonicus* indicate that the three symbiotic leghemoglobin genes are transcribed at similar levels in nodules and together account for more than 10% of all plant messenger RNA (mRNA) in this organ. In contrast, the nonsymbiotic leghemoglobin genes are expressed at very low levels in nodules (see http://www.tigr.org/). Expression of multiple symbiotic leghemoglobin genes in nodules has stymied attempts to use classical forward genetics to address the physiological role(s) of the corresponding proteins during symbiotic nitrogen fixation (SNF). However, the high degree of DNA sequence identity between the three symbiotic leghemoglobin genes of *L. japonicus* (Figure 1B) enabled us to take a reverse-genetics approach to eliminate the expression of all three genes simultaneously. Using RNA interference (RNAi), we succeeded in reducing the levels of symbiotic leghemoglobin mRNA in two of five independent, stable transgenic lines of *L. japonicus*. Expression of all three symbiotic leghemoglobins was practically eliminated in these two LbRNAi lines: Transcript levels of all three genes were below 3% of those in wild-type nodulated roots three weeks after inoculation with rhizobia (Figure 2A). In contrast, transcript levels of the two nonsymbiotic leghemoglobin genes, *LjNSG1* and *LjNSG2*, were only slightly reduced in the LbRNAi lines in comparison to in the wild-type controls (Figure 2A). Leghemoglobin protein was undetectable in LbRNAi nodules by Western-blot analysis (Figures 2A and 2B), and intact nodules on LbRNAi plants were white instead of the leghemoglobin-pink of wild-type nodules (Figure 3A).

The LbRNAi lines lacking leghemoglobin protein allowed us to answer the long-standing question: Are these prominent nodulins required for SNF? Under symbiotic conditions in which plants were completely dependent upon rhizobia for fixed nitrogen, LbRNAi lines exhibited the classic symptoms of extreme nitrogen limitation: severely stunted growth, an increase in the root/shoot ratio, and delayed flowering compared to the wild-type. They also exhibited yellow chlorotic leaves and anthocyanin accumulation in the stems (Figure 3B). In contrast, wild-type control plants thrived under these conditions. Consistent with their nodule-specific expression pattern, the three symbiotic leghemoglobins of *L. japonicus* were not required for plant growth and development under nonrestrictive nutrient conditions. LbRNAi lines grew normally and flowered at the same time as wild-type control plants when grown on soil containing mineral and organic nitrogen (Figure 3C). Thus, the symbiotic leghemoglobins have evolved a truly symbiosis-specific set of functions that are required for SNF.

Although LbRNAi lines were defective in SNF, nodules developed normally on these plants for the first three weeks after inoculation. In particular, nodule morphology and the extent of central, infected tissue were unchanged in LbRNAi lines compared to the wild-type (Figure 3A). Furthermore, bacterial population density was similar in nodules of LbRNAi and wild-type plants: The bacterial density in three-week-old nodules of LbRNAi plants was 87% (±11% standard deviation (SD), n = 6) that of the wild-type nodules, as measured by real-time polymerase chain reaction (PCR) of nodule DNA; primers to the bacterial genes *nifH* and *sigA* were

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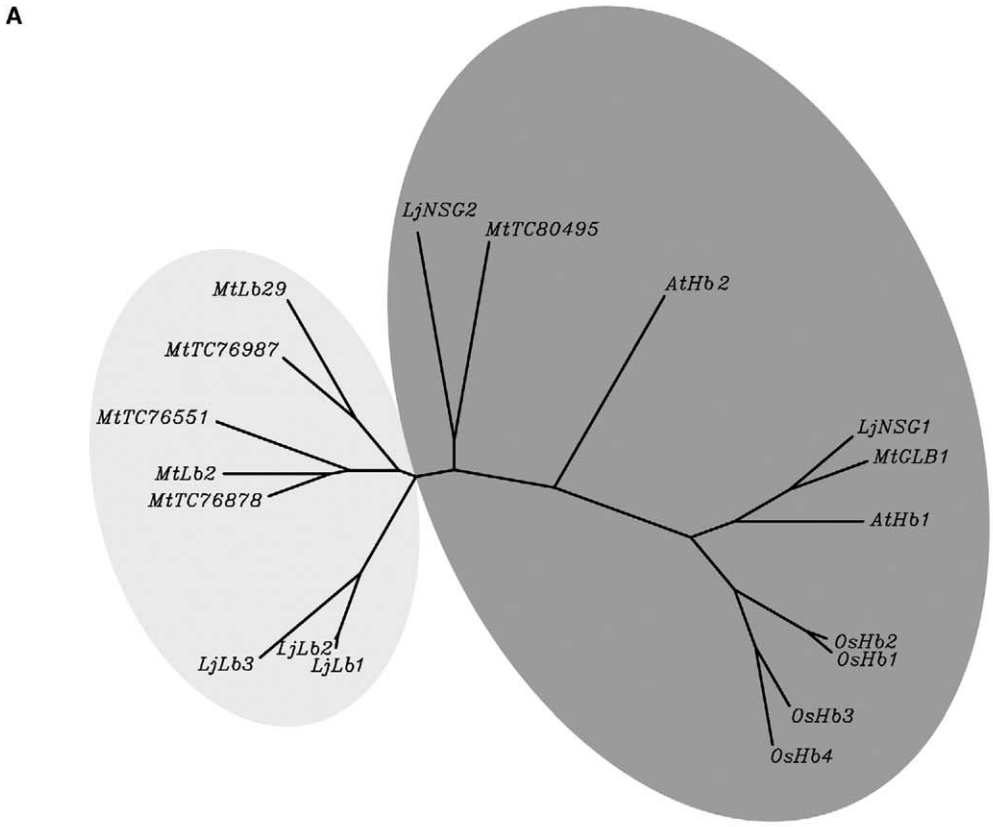

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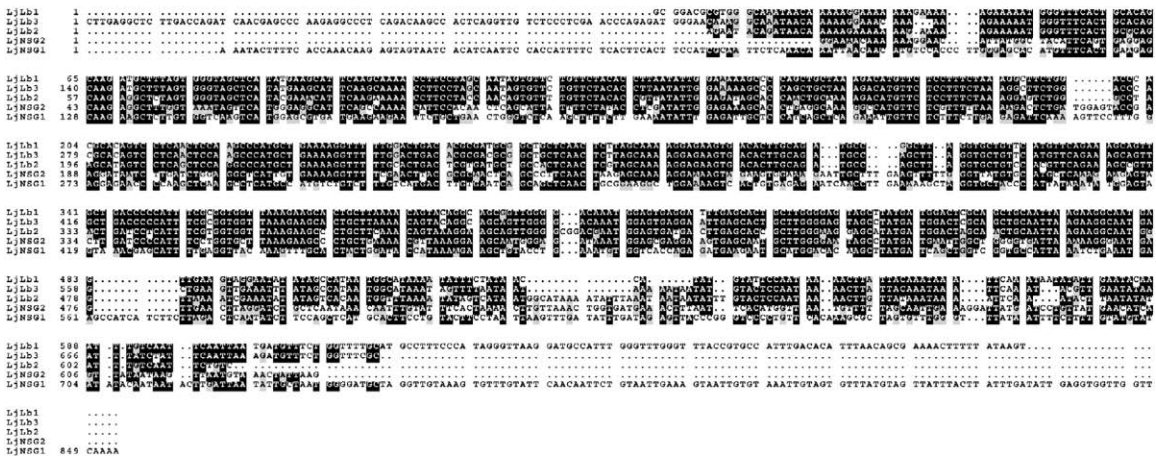

Figure 1. Phylogenetic Tree and Multiple-Sequence Alignment of Hemoglobins from *L. japonicus* and Other Plant Species

Included in the unrooted phylogenetic tree are the *Arabidopsis* hemoglobins AtHb1 (TIGR TC211680) and AtHb2 (TC199105); the rice hemoglobins OsHb1 (TC231324), OsHb2 (TC224250), OsHb3 (TC229216), and OsHb4 (TC246382); the *Medicago* hemoglobins MtLb2 (TC76879), MtTC76878, MtTC76551, MtLb29 (TC77993), MtTC76987, MtTC80495, and MtGLB1 (TC76971); and the *L. japonicus* hemoglobins LjLb1 (AB042716), LjLb2 (AB042717), LjLb3 (AB008224), LjNSG1 (TC16046), and LjNSG2 (TC10713). Phylogenetic analyses based on protein sequence (A) and DNA-sequence alignment (B) were performed via the GenomeNet CLUSTALW server (Koyoto Centre: http://clustalw.genome.jp). The coding sequence of LjLb2 was used for RNAi experiments.

used. Differentiation of rhizobia into nitrogen-fixing bacteroids involves induction of many genes, including the nitrogenase structural genes, *nifHDK*. Measurement, using real-time reverse-transcription PCR (RT-PCR), of *nifH* transcript levels in LbRNAi nodules showed that rhizobia induce expression of *nif* genes in these nodules, although *nifH* transcript levels were lower than in nodules of wild-type plants (21 ± 4% of wild-type, n = 6). Reduced *nifH* transcript levels in LbRNAi nodules may have resulted from increased levels of free oxygen

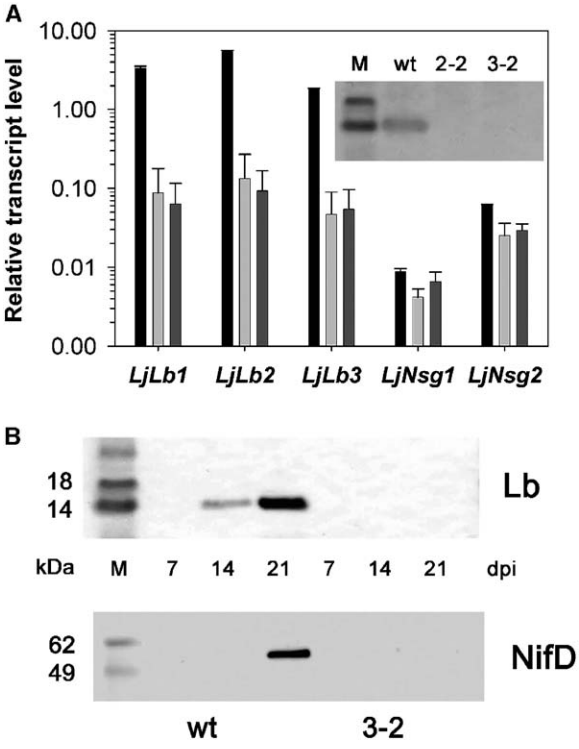

Figure 2. Molecular Characteristics of LbRNAi Lines

(A) Leghemoglobin transcript levels in nodulated roots 3 weeks after inoculation of wild-type (left, in black) and LbRNAi lines 2-2 (middle) and 3-2 (right). Transcript levels are expressed relative to those of the constitutive gene ubiquitin [21]. The inset depicts Western-blot detection of leghemoglobins in isolated nodules of the wild-type (wt) but not of the two LbRNAi lines (2-2 and 3-2). Lane M shows markers of 14 and 18 kDaA.

(B) Western-blot detection of leghemoglobins (Lb, above) and nitrogenase NifD (below) in nodulated roots of wild-type (wt) and LbRNAi line 3-2, from 7 to 21 days post-inoculation (dpi). Western-blot analyses were performed after SDS-PAGE of 20 μg protein.

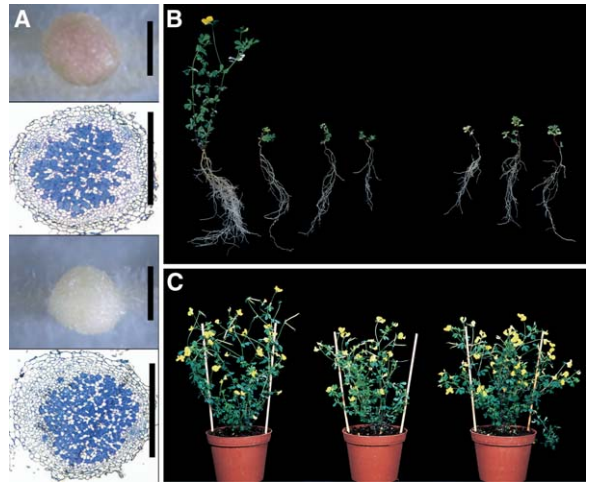

Figure 3. Symbiotic and Nonsymbiotic Phenotypes of LbRNAi Lines

(A) Typical nodules of wild-type and LbRNAi plants 14 days after inoculation with rhizobia. The top two images show whole and sectioned wild-type nodules, respectively, and the bottom pair depicts the same for LbRNAi line 3-2. Note the absence of leghemoglobin pigmentation in the LbRNAi nodule but the similar extent of dark-staining, infected cells in the central zone of the nodule section. The scale bars indicate 1 mm.

(B) Ten-week-old rhizobia-inoculated plants. Three individuals of two independent LbRNAi lines (2-1, 2-2, 2-3 and 3-1, 3-2, 3-3) are compared with a typical wild-type control (far left). Plants were grown in sand without added mineral nitrogen.

(C) Ten-week-old noninoculated plants grown in soil with nitrogen fertilizer. Two LbRNAi individuals are compared to a typical wild-type plant (left).

in these nodules (see below). Expression of *nif* genes in rhizobia is negatively regulated by oxygen [18, 19]. Despite the presence of *nif* gene transcripts in LbRNAi nodules, NifD protein was undetectable by Western-blot analysis, in contrast to its abundance in wild-type nodules (Figure 2B). This suggests that nitrogenase proteins are unstable in LbRNAi nodules, and that this instability accounts for the phenotype of symbiotic LbRNAi plants (Figure 3B).

The elimination of leghemoglobin protein in LbRNAi lines enabled us to test, for the first time ever, the physiological role of these proteins in nodules. Using a needle-type fiberoptic oxygen microsensor, we found that steady-state levels of free oxygen throughout nodules were higher for the LbRNAi lines than for wild-type controls (Figure 4). Wild-type nodules exhibited a steep oxygen gradient from the surface toward the center of the nodule, with levels less than 20% of ambient oxygen within 0.2 diameters of the surface and below the limit of detection (1% ambient oxygen) for much of the central, infected zone (Figure 4). In contrast, nodules from LbRNAi lines exhibited a shallower oxygen gradient, with more than 50% of ambient oxygen at 0.2 diameters and never less than 4.5%, even at the center of nodules (Figure 4). Thus, leghemoglobins help to establish very low free-oxygen concentrations throughout much of a wild-type nodule. Interestingly, despite the increase in free oxygen for oxidative phosphorylation,

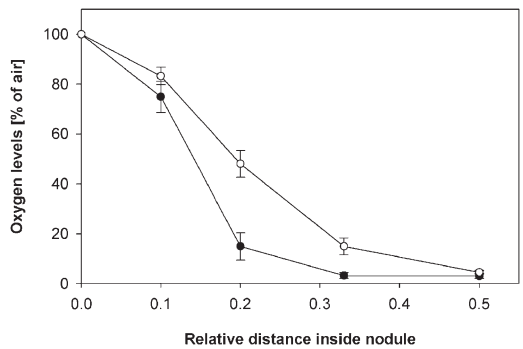

Figure 4. Transects of Free Oxygen in Nodules of Wild-Type and LbRNAi Lines

Oxygen levels are expressed as a percentage of the concentration in air, and the surface and center of nodules are indicated on the *x*-axis by 0.0 and 0.5, respectively. Wild-type is represented by filled circles and LbRNAi lines are represented by open circles. Data points represent mean ±SD of between 11 and 13 independent measurements.

and despite the loss of a major sink for ATP in the form of bacteroid nitrogenase in nodules of LbRNAi lines, the nodule ATP/ADP ratio was more than 4-fold lower in these lines than in the wild-type. The concentrations of ATP and ADP in LbRNAi nodules were 56.9 ± 10.9 and 105.4 ± 26.3 nmol/g fresh weight (±SD, n = 3), respectively, whereas in wild-type nodules the corresponding values were 97.8 ± 15.7 and 39.9 ± 10.5 nmol/g fresh weight, respectively. Despite the reduced cellular energy status (ATP/ADP) in LbRNAi lines compared to that in the wild-type, the total amount of ATP plus ADP was not significantly different between the two types of nodules. Thus, high concentrations of leghemoglobins enhance energy metabolism in nodule cells, presumably by increasing the overall amount of oxygen (free plus protein bound) and thereby the flux of oxygen to sites of respiration.

The results presented here have positive implications for the functional analysis of other nodulins. We have demonstrated that RNAi is a potent way to simultaneously reduce or eliminate transcripts and proteins derived from multiple highly expressed homologous genes in legumes. Thus, in the future, RNAi should help to resolve the symbiotic functions of other nodulins, many of which are members of multigene families.

In summary, we have shown that the most prominent of legume nodulins, the leghemoglobins, are required for SNF, but not for plant growth and development in the presence of an external source of fixed nitrogen. Physiological analysis of nodules from LbRNAi plants revealed the crucial contribution of leghemoglobins to establishing low free-oxygen concentrations but high energy status in nodules, conditions that are necessary for effective SNF. This work represents the first demonstration of a role for hemoglobins in plants and helps to close the gap in the understanding of hemoglobin functions in plants versus animals.

Experimental Procedures

Plant Cultivation

Lotus japonicus ecotype GIFU (*B-129*) seeds were scarified in 98% sulfuric acid for 15 min, sterilized in 2% sodium hypochlorite containing 0.01% Tween 20 for 15 min, rinsed five times with sterile distilled water, and germinated on moist, sterile filter paper for 3 days. Germinated seedlings were transferred to agarose plates containing 25% B&D medium [20] and 0.1 mM KNO_3. Plants were grown in a 16/8 hr day/night cycle with a light intensity of 150 $\mu Em^{-2}s^{-1}$ at 21°C in a controlled-environment chamber (York International, model P-U201AF). Plants were inoculated at 2 weeks of age with a 1:50 dilution of a 2-day-old liquid culture of *Mesorhizobium loti* strain NZP2235. Alternatively, plants were grown in a greenhouse in coarse quartz sand or soil, as described previously [21].

RNAi Construct and Plant Transformation

A 401 base pair DNA fragment of *LjLb2* (AB042717) was PCR amplified from cDNA via the following primers:

LjLbEntry
forward: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGGGTTTCACTGCACAGCAAG-3′
reverse: 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCATAGGCTACTCCCCAAGCA-3′

These primers contained recombination sites for GATEWAY cloning (Invitrogen). pBINAR-RNAi (pBIN19 backbone with a converted GATEWAY cassette from vector pJawohl8, obtained from Imre Somssich) was used as a binary vector containing a kanamycin-resistance gene for selection of plant transformants. *Lotus japonicus* plants were transformed as described previously [22].

RNA Extraction and Real-Time RT-PCR

RNA was extracted from 100 mg plant tissue with the RNeasy Kit (QIAGEN). DNaseI treatment of 1 μg total RNA, cDNA synthesis, and real-time RT-PCR were carried out as described previously [23]. The following primers were used to amplify the different leghemoglobins:

LjLb1
forward: 5′-TTTGAGCACTGCTTGGGGAGTAGCT-3′
reverse: 5′-CTGCAATTAAGAAGGCAATG-3′
LjLb2
forward: 5′-ATTGAGCACTGCTTGGGGAGTAGCC-3′
reverse: 5′-CTGCAATTAAGAAGGCAATG-3′
LjLb3
forward: 5′-CTTGAGCACCGCTTGGGAAGGAGCA-3′
reverse: 5′-CTGCAATTAAGAAGGCAATG-3′

Plant-gene transcript levels were normalized to those of ubiquitin, as described previously [21]. After reverse transcription of nodule RNA in the presence of 1 pM of the primers FeNif1R and MlsigAr1, we measured rhizobial *nifH* and *sigA* transcript levels by using the following PCR primers:

nifH
forward: 5′-TCCAAGCTCATCCACTTCGTG-3′
reverse: 5′-AGTCCGGCGCATACTGGATTA-3′
sigA
forward: 5′-GCCCTCTGCTCGACCTTTCC-3′
reverse: 5′-AGCATCGCCATCGTGTCCTC-3′

Levels of *nifH* transcript were normalized to those of the constitutive gene, *sigA*.

DNA Extraction and Real-Time-PCR Quantification

DNA was isolated from nodules via a published protocol [24]. Plant genomic DNA was quantified with primers to ubiquitin under standard real-time-PCR-reaction conditions [21, 23]. Rhizobial DNA was quantified with the primers to *nifH* and *sigA*, described above.

Immunoblotting

Twenty milligrams of nodulated roots were homogenized in a buffer containing 10 mM Tris (pH 7.5), 140 mM NaCl, 5 mM Ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1 mM Phenylmethylsulphonylfluoride (PMSF), and 1 mM Dithiothreitol (DTT). After centrifugation for 10 min in a microcentrifuge, supernatant-protein concentration was determined via the Bradford method (Biorad). Extracted protein was separated by SDS-PAGE on a commercial 10% Bis-Tris gel (Invitrogen) according to the manufacturer's instructions (100V, 1.5 hr). We blotted proteins onto a Protran® nitrocellulose membrane (Schleicher und Schuell) by using the XCellII Blot Module (Invitrogen). Equal loading of lanes and quality of transfer were checked by Ponceau staining of membranes. Antiserum against soybean leghemoglobin was kindly provided by Norio Suganuma (Aichi University of Education, Japan), and antiserum against the NifD subunit of nitrogenase was provided by Ton Bisseling (University of Wageningen, The Netherlands).

Oxygen Measurements

We measured free-oxygen concentrations in intact 3-week-old nodules by using a needle-type fiber-optic oxygen microsensor (MicroxTX2, Presens, Regensburg, Germany) with a tip diameter of less than 50 μm [25]. The location of the tip within the nodule was derived from the scaling of the micromanipulator with which the sensor was driven through the tissue. The entire procedure was performed under a binocular microscope.

Measurement of ATP and ADP

ATP and ADP were measured with a cycling assay, as described previously [26].

Nodule Cross-Sections

Nodules were fixed with Technovit 7100 (Heraeus). Embedded plant material was cut to a thickness of 5 μm with a microtome (Leica). Dried sections were stained in a solution of 1% Toloudine Blue for 2 min and then rinsed with water.

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References

1. Kubo, H. (1939). Über Haemoprotein aus den Wurzelknöllchen von Leguminosen. Acta Phytochim. *11*, 195–200.
2. Perutz, M.F., and Weisz, O. (1947). Crystal structure of human carboxyhaemoglobin. Nature *160*, 786–787.
3. Vainshtein, B.K., Harutyunyan, E.H., Kuranova, I.P., Borisov, V.V., Sosfenov, N.I., Pavlovsky, A.G., Grebenko, A.I., and Konareva, N.V. (1975). Structure of leg-hemoglobin from Lupin Root Nodules at 5 a Resolution. Nature *254*, 163–164.
4. Wakabayashi, S., Matsubara, H., and Webster, D.A. (1986). Primary sequence of a dimeric bacterial hemoglobin from Vitreoscilla. Nature *322*, 481–483.
5. Appleby, C.A., Tjepkema, J.D., and Trinick, M.J. (1983). Hemoglobin in a nonleguminous plant, Parasponia: Possible genetic origin and function in nitrogen fixation. Science *220*, 951–953.
6. Bogusz, D., Appleby, C.A., Landsmann, J., Dennis, E.S., Trinick, M.J., and Peacock, W.J. (1988). Functioning hemoglobin genes in non-nodulating plants. Nature *331*, 178–180.
7. Trevaskis, B., Watts, R.A., Andersson, C.R., Llewellyn, D.J., Hargrove, M.S., Olson, J.S., Dennis, E.S., and Peacock, W.J. (1997). Two hemoglobin genes in Arabidopsis thaliana: The evolutionary origins of leghemoglobins. Proc. Natl. Acad. Sci. USA *94*, 12230–12234.
8. Watts, R.A., Hunt, P.W., Hvitved, A.N., Hargrove, M.S., Peacock, W.J., and Dennis, E.S. (2001). A hemoglobin from plants homologous to truncated hemoglobins of microorganisms. Proc. Natl. Acad. Sci. USA *98*, 10119–10124.
9. Czelusniak, J., Goodman, M., Hewettemmett, D., Weiss, M.L., Venta, P.J., and Tashian, R.E. (1982). Phylogenetic origins and adaptive evolution of avian and mammalian hemoglobin genes. Nature *298*, 297–300.
10. Stamatoyannopoulos, G. (1972). The molecular basis of hemoglobin disease. Annu. Rev. Genet. *6*, 47–70.
11. Andersson, C.R., Jensen, E.O., Llewellyn, D.J., Dennis, E.S., and Peacock, W.J. (1996). A new hemoglobin gene from soybean: A role for hemoglobin in all plants. Proc. Natl. Acad. Sci. USA *93*, 5682–5687.
12. Hellriegel, H., and Wilfarth, H. (1888). Untersuchungen über die Stickstoffnährung der Gramineen und Leguminosen. Zeitschrift des Vereins für die Rübenzuckerindustrie des Deutschen Reiches *93*, 863–877.
13. Brewin, N.J. (1991). Development of the legume root nodule. Annu. Rev. Cell Biol. *7*, 191–226.
14. Legocki, R.P., and Verma, D.P.S. (1980). Identification of "nodule-specific" host proteins (nodulins) involved in the development of Rhizobium-Legume symbiosis. Cell *20*, 153–163.
15. Franssen, H., Mylona, P., Pawlowski, K., Vandesande, K., Heidstra, R., Geurts, R., Kozik, A., Matvienko, M., Yang, W.C., Hadri, A.E., et al. (1995). Plant genes involved in root-nodule development on legumes. Proc. R. Soc. Lond. B. Biol. Sci. *350*, 101–107.
16. Appleby, C.A. (1984). Leghemoglobin and rhizobium respiration. Annu. Rev. Plant Physiol. Plant Mol. Biol. *35*, 443–478.
17. Uchiumi, T., Shimoda, Y., Tsuruta, T., Mukoyoshi, Y., Suzuki, A., Senoo, K., Sato, S., Kato, T., Tabata, S., Higashi, S., et al. (2002). Expression of symbiotic and nonsymbiotic globin genes responding to microsymbionts on Lotus japonicus. Plant Cell Physiol. *43*, 1351–1358.
18. Batut, J., and Boistard, P. (1994). Oxygen control in Rhizobium. Antonie Van Leeuwenhoek *66*, 129–150.
19. Fischer, H.M. (1996). Environmental regulation of rhizobial symbiotic nitrogen fixation genes. Trends Microbiol. *4*, 317–320.
20. Broughton, W., and Dilworth, M.J. (1971). Control of leghaemoglobin synthesis in snake beans. Biochem. J. *125*, 1075–1080.
21. Colebatch, G., Desbrosses, G., Ott, T., Krusell, T., Montanari, O., Kloska, S., Kopka, J., and Udvardi, M.K. (2004). Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in Lotus japonicus. Plant J. *39*, 487–512.
22. Thykjaer, T., Finnemann, J., Schauser, L., Christensen, L., Poulsen, C., and Stougaard, J. (1997). Gene targeting approaches using positive-negative selection and large flanking regions. Plant Mol. Biol. *35*, 523–530.
23. Czechowski, T., Bari, R.P., Stitt, M., Scheible, W.R., and Udvardi, M.K. (2004). Real-time RT-PCR profiling of over 1400 Arabidopsis transcription factors: Unprecedented sensitivity reveals novel root- and shoot-specific genes. Plant J. *38*, 366–379.
24. Doyle, J.J., and Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry Bulletin *19*, 11–15.
25. van Dongen, J.T., Schurr, U., Pfister, M., and Geigenberger, P. (2003). Phloem metabolism and function have to cope with low internal oxygen. Plant Physiol. *131*, 1529–1543.
26. Gibon, Y., Vigeolas, H., Tiessen, A., Geigenberger, P., and Stitt, M. (2002). Sensitive and high throughput metabolite assays for inorganic pyrophosphate, ADPGlc, nucleotide phosphates, and glycolytic intermediates based on a novel enzymic cycling system. Plant J. *30*, 221–235.