Sainz, M.; Pérez-Rontomé, C.; Ramos, J.; Mulet Salort, JM.; James, EK.; Bhattacharjee, U.; Petrich, JW.... (2013). Plant hemoglobins may be maintained in functional form by reduced flavins in the nuclei, and confer differential tolerance to nitro-oxidative stress. The Plant Journal. 76(5):875-887. doi:10.1111/tpj.12340.

Biological Sciences - Plant Biology

Plant hemoglobins can be maintained in functional form by flavins in the nuclei and plastids and confer differential tolerance to oxidative and nitrosative stress

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Author contributions: M.S., C.P-R., J.R., and J.M.M. performed research; E.K.J., J.M.M., U.B., and J.W.P. contributed new reagents/analytic tools; J.M.M., J.W.P., and M.B. analyzed data; M.B. designed research and wrote the paper.

The authors declare no conflict of interest.

Bacteria, plant, and animal Hbs need to have their heme in the ferrous state to bind O_2 and other ligands of physiological interest. Here, we have characterized the full set of nonsymbiotic (class 1 and 2) and 'truncated' (class 3) Hbs of the model legume *Lotus japonicus*. Class 1 Hbs are hexacoordinate but class 2 and 3 Hbs are pentacoordinate. Three globins, GLB1-1, GLB2, and GLB3-1, are nodule-enhanced proteins. The O_2 affinity of GLB1-1 (~50 pM) was the highest known for any plant or animal Hb and the protein may function as an O_2 scavenger. The five globins were reduced by free flavins, which transfer electrons from NAD(P)H to the heme iron in aerobic and anaerobic conditions. Class 1 Hbs were reduced at very fast rates with FAD, class 2 Hbs at slower rates with both FMN and FAD, and class 3 Hbs at intermediate rates with FMN. The three globin classes were immunolocalized predominantly in the nuclei and plastids. Flavins were quantified in legume nodules and nuclei and their concentrations were sufficient to maintain Hbs in their functional state. All Hbs, except GLB1-1, could be expressed in a flavohemoglobin-deficient yeast mutant and found to confer tolerance to oxidative stress induced by methyl viologen, copper, or low temperature, indicating an antioxidative role of the hemes. However, only GLB1-2 and GLB2 afforded protection from nitrosative stress induced by *S*-nitrosoglutathione. Because this compound is specifically involved in transnitrosation reactions with thiol groups, our results point to a contribution of the single cysteine residues of the proteins in the stress response.

flavins / immunogold localization / legume nodules / nitrosative stress / oxidative stress / plant hemoglobins

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Originally, Hbs were thought to be present only in the red blood cells of vertebrates and in the infected cells of legume root nodules and their functions restricted to the transport and delivery of O_2 (1, 2). However, the last three decades have witnessed the discoveries of Hb genes and proteins in all kingdoms of life (3). To name a few, flavohemoglobins (flavoHbs) occur in some bacteria and fungi, nonsymbiotic Hbs in plants, truncated Hbs in bacteria, unicellular eukaryotes, and plants, and neuroglobin and cytoglobin in animals (2, 3). Additional roles for some of these Hbs have been also demonstrated. FlavoHbs contain heme and FAD-reductase domains and can detoxify nitric oxide (NO) using O_2 to produce NO_3^- by functioning as NO dioxygenase enzymes (2, 4). The functions of other prototypical Hbs are also related to NO metabolism. The Hb of *Ascaris suum* uses NO to scavenge O_2 producing NO_3^- and thus provides this parasitic nematode with an anaerobic niche in the intestinal tract, whereas human Hb can be specifically nitrosylated in Cys^{93} of the β-chains, preserving and delivering NO to the vascular endothelium in an O_2-dependent manner (2).

Plants can express up to three classes of Hbs: nonsymbiotic, symbiotic, and 'truncated' (1, 5-7). Nonsymbiotic Hbs occur at nanomolar or micromolar concentrations in many plant tissues and can be futher categorized into class 1 and 2 based on phylogenic relationships, gene expression profiles, and O_2-binding properties (7). Class 1 Hbs display high O_2 affinities (7) and modulate NO concentration in stressful conditions (8). Class 2 Hbs have O_2 affinities that ressemble those of symbiotic Hbs (5, 7) and their functions are largely unknown. Recent studies suggest a role of class 2 Hbs in improving O_2 availability in developing seeds (9) and of both class 1 and 2 Hbs in shoot organogenesis (10). Symbiotic Hbs are found at millimolar concentrations in nodules of legumes (leghemoglobins, Lbs) and some actinorhizal plants, where they facilitate a steady low O_2 supply to the bacterial microsymbionts (1, 7). Class 3 Hbs have a 2-on-2 α-helical sandwich secondary structure instead of the canonical 3-on-3 structure of other Hbs. Although virtually nothing is known about their function in plants, some of their bacterial counterparts have been implicated in tolerance to nitrosative stress (2, 4).

Two biochemical properties of plant and other Hbs are central for their biological functions: the coordination and the redox state of the heme iron. Typical Hbs such as Hb, Mb, and Lbs are pentacoordinate because they have the fifth position of the iron coordinated to one His (proximal) and the sixth position open for ligand binding, whereas neuroglobin, cytoglobin, and plant nonsymbiotic Hbs are hexacoordinate because they have both positions (proximal and distal) coordinated to His residues (11). The binding of gaseous ligands to the heme iron occurs predominantly (NO) or exclusively (O_2, CO) when it is in ferrous form. However, many factors in cells, including acid pH or trace amounts of metals, are conducive for heme oxidation and hence mechanisms should exist in vivo to maintain Hb iron in the reduced state. In plants, several flavoproteins have been proposed as Hb reductants (12, 13). For any of these mechanisms to be germane, Hbs and their putative reductants should colocalize within plant cells. The subcellular localizations of globins other than Lbs in vascular plants are nevertheless far from clear. Thus, some class 1 Hbs have been located to both the nuclei and cytosol (8, 14, 15), only the cytosol (16), or only the plastids (17).

In previous work, we identified the nonsymbiotic and truncated Hb genes of the model legume *Lotus japonicus* and found that their mRNAs are abundant in nodules (18). The genes encode two class 1 Hbs (GLB1-1 and GLB1-2), one class 2 Hb (GLB2), and two class 3 Hbs (GLB3-1 and GLB3-2). This gene profile may be extensive to other legumes because two class 3 Hbs are expressed in *Medicago truncatula* (19) and soybean (20), but is in contrast with *Arabidopsis thaliana*, which only contains one globin of each class (5, 6). Here we investigate several crucial features of plant Hbs by characterizing the five globins of *L. japonicus*. The proteins differ in ligand binding kinetics, are mainly localized to the nuclei and plastids, and afford protection against oxidative and nitrosative stress in yeast. In vitro reduction assays of globins and assessment of flavin concentrations in plants and yeast lead us to propose that flavins are involved in the maintenance of all three classes of plant globins in the functional state.

Results

The Five Globins Differ in Biochemical Properties. Nonsymbiotic and truncated Hbs are present at very low concentrations in plant tissues and hence the five globins of *L. japonicus* were heterologously produced for biochemical characterization. Only GLB1-2 and GLB3-2 could be expressed at reasonable yields in *E. coli* BL21, whereas the other globins had to be expressed in its mutant C41 derivative. In particular, GLB3-1 was produced solely in C41 cells. The purification of GLB1-1 at high yield required addition of 50-150 mM NaCl to the buffers. Nevertheless, to make uniform comparisons of biochemical properties, all proteins were prepared at the final stage in 50 mM phosphate buffer (pH 7.0). The Soret/A_{280} ratios of the proteins were ~2.7, indicating purification to near homogeneity.

The Soret-visible spectra of globins provided important structural information (Figs. S1 and S2). The two class 1 Hbs showed hexacoordination in the deoxyferrous (α band at ~557 nm and β band at ~530 nm) and ferric (α band at ~531 nm and shoulder at ~560 nm) forms. The spectrum of deoxyferrous GLB1-1 had split α and β bands, reflecting slow interconversion between two protein conformations, each probably corresponding to a different ligand environment of the heme (Fig. S1). Quite surprisingly for a class 2 Hb, which was expected to be hexacoordinate in the ferric and ferrous states (7, 11), GLB2 showed hexacoordination in the ferric form but pentacoordination in the deoxyferrous form (Fig. S3). The two class 3 Hbs displayed typical spectra of pentacoordinate globins, in both the deoxyferrous (α band at ~560 nm but no β band) and ferric (poorly defined bands at ~500, 540, and 580 nm) forms (Figs. S4 and S5).

All the globins were able to bind O_2, CO, and NO in the ferrous state and cyanide in the ferric state (Figs. S1 to S5). Flash photolysis (20) and stopped-flow (21) experiments were set up to measure O_2 affinity constants (K^{O2}). These values were found to be distinctly different for each globin class and also for the proteins within the same class (Table 1). Notably, GLB1-1 has an extremely high O_2 affinity (K^{O2} ~50 pM) due to a very slow O_2 dissociation rate (k_{off}^{O2} ~0.005 s^{-1}). The K^{O2} of GLB1-2 is also very high (~0.9 nM) but on-par with

monocot Hbs. By contrast, GLB2 has a moderate K^{O2} (~11 nM), which results from a relatively fast O_2 dissociation rate (k_{off}^{O2} ~1.23 s^{-1}) and is in the range of soybean Lb*a* (Table 1). GLB3-1 and GLB3-2 showed O_2 and CO binding kinetics that are independent of substrate concentration and consequently bimolecular rate constants cannot be calculated. However, the observed O_2 binding constants (k_{on}^{O2}) of GLB3-1 and GLB3-2, measured by flash photolysis, were ~240 s^{-1} and ~6540 s^{-1}, respectively.

Flavins Reduce the Three Globin Classes but at Distinctly Different Rates. Some reports indicating that class 1 Hbs are, at least in part, located to the nucleus (8, 14, 15), which appear to lack Hb reductases, prompted us to test the hypothesis that free flavins can mediate heme reduction in the three globin classes. To this purpose, we used physiological concentrations of pyridine nucleotides and flavins in legume tissues, estimated in the range of 0.2-2 mM and 5-50 μM, respectively (12, 22, 23).

Reduction assays of the five globins were performed systematically with FMN and FAD as both flavins are very soluble and cofactors of flavoproteins. Neverheless, assays with riboflavin, an abundant flavin of nodules (12, 22), were also found to reduce globins as did the flavin coenzymes. For clarity, Figure 1 only shows the data obtained using 0.2 mM NAD(P)H for class 1 and 2 Hbs, 1 mM NAD(P)H for class 3 Hbs, and 20 μM of the most active flavin (FAD or FMN) for each globin. These results can be summarized as follows. *(i)* NADH or NADPH alone did not reduce any globin at significant rates. *(ii)* Flavins catalyzed heme reduction at rates that were dependent on the pyridine nucleotide, flavin coenzyme, and globin class. *(iii)* The two class 1 Hbs showed important differences in their reduction kinetics with FAD and FMN. With 0.2 mM NAD(PH, GLB1-1 was reduced quite rapidly (60-100 min) by NADH + FAD (~92%), NAD(P)H + FMN (~95%), and to a lower extent by NADPH + FAD (~33%). GLB1-2 was reduced very fast (20 min) by NADH + FAD (97%) and NADPH + FAD (75%), and much less efficiently by NAD(P)H + FMN (20%). Rising NAD(P)H to 1 mM caused complete reduction of GLB1-1 with both coenzymes and ~90% reduction of GLB1-2 with FAD, but only ~36% reduction with FMN. *(iv)* The two class 3 Hbs did not significantly differ in the reduction rates. GLB3-1 and GLB3-2 required 1 mM

NAD(P)H for nearly complete reduction (60-100 min) with FMN (87-94%), although reduction levels were still low with FAD (38-47%). With 0.2 mM NAD(P)H, reduction of both globins was ~16% with FMN and ~40% with FAD. *(v)* GLB2 was the most slowly reduced globin. After 180 min with 0.2 mM NAD(P)H, reduction was 67% with NADH + FAD or NADH + FMN, 58% with NADPH + FMN, and only 26% with NADPH + FAD. Increasing NAD(P)H to 1 mM caused 82% reduction of GLB2 with NADH + FAD or NADH + FMN. We can thus conclude that class 1 Hbs are reduced very fast and equally well with FAD or FMN in the case of GLB1-1 but only at substantial rates with FAD in the case of GLB1-2; class 2 Hb was reduced slowly compared to the other globins, with a preference for NADH over NADPH but with no preference for the flavin; and class 3 Hbs require high NAD(P)H concentrations that the other globins and were reduced more efficiently with FMN than with FAD.

Root cells may experience episodic hypoxia triggered by stresses such as drought or flooding and nodule infected cells have a free O_2 concentrations of ~30 nM to avoid nitrogenase inactivation (1). We thus determined whether globin reduction could occur under nearly anaerobic conditions similar to those that can be encountered in some plant tissues. Figure 2 shows that four out of the five globins were completely reduced to their deoxyferrous forms by the NAD(P)H + flavin system when virtually all O_2 was removed. The exception was GLB1-1, which still retained traces of O_2 as the oxyferrous complex. The singular behavior of this globin was already noticed during the reduction assays in CO-saturated buffer, as CO could not displace O_2 from the ferrous heme, which can be explained by the extremely high O_2 affinity of GLB1-1 (Table 1). Only dithionite could reduce GLB1-1 to the deoxyferrous form because this potent reducing compound also provokes complete depletion of O_2 in the reaction medium (Fig. S1).

The Three Globin Classes are Predominantly Localized to the Nuclei and Plastids. The precise and sensitive immunolocalization of the low abundant globins with transmission electron microscopy required a combination of affinity-purified polyclonal antibodies with

the preparation of plant material for microscopy using cryotechniques, such as high-pressure freezing/freeze-substitution and/or low temperature fixation and resin embedding (24), as these preserve sensitive epitopes better than conventional methods of fixation and dehydration (25). Immunoblot analyses of purified recombinant proteins showed that the antibodies were specific for each globin class but cross-reacted with the other protein within the same class (Fig. S6*A*). Immunogold microscopy of *L. japonicus* nodules revealed that the three classes of globins are predominantly localized in the nuclei (Fig. 3*A*, *E*, and *G*) and, to a lower extent, in the plastids (Fig. 3*C, F*, and *H*). Low labeling was occasionally found on the cytosol but no labeling was seen on the mitochondria or peroxisomes. The same localization pattern was observed in *L. japonicus* roots (Fig. 3*B*) and leaves (Fig. 3*D* and *I*) and in *M. truncatula* nodules (Fig. 3*J*). In chloroplasts, however, immunolabeling was almost undetectable for GLB2, weak and mainly located on the thylakoids for GLB3 (Fig. 3*I*), and more intense and mainly associated with starch grains for GLB1 (Fig. 3*D*).

Globin Expression in Yeast Confers Differential Tolerance to Oxidative and Nitrosative Stress. Yeast is an optimal eukaryotic system for screening and characterization of plant genes (26). We transformed the yeast *yhb1* mutant, defective in flavoHb, with the episomal plasmid pVV214 bearing each of the globins or *L. japonicus* Lb1 under the control of the strong constitutive promoter *PGK1*, which enables the expression of plant genes in yeast. Immunoblots confirmed that all the proteins were expressed, except GLB1-1. This may be ascribed to instability of the GLB1-1 mRNA or protein. Consequently, data of stress tolerance are presented only for the other four globins. Because yeast flavoHb is implicated in NO detoxification (2, 4), we selected several inducers of oxidative and nitrosative stress. Methyl viologen (MV) is a potent generator of superoxide radicals by redox cycling and its effects on yeast mitochondria are well documented (27). Addition of 1.5 mM MV to the YPD medium caused toxicity to yeast, as evidenced by the retarded growth relative to the untreated control, but cells expressing GLB2, GLB3-1, GLB3-2, or Lb1 were more tolerant than the flavoHb-deficient mutant (Fig. 4*A*). Oxidative stress was also induced in yeast by treatment with

$CuSO_4$. Extracellular Cu^{2+} is reduced to Cu^{+} by the Ftr1/2 iron reductase system and Cu^{+} is then transported to the cytoplasm, where it can participate in Fenton reactions giving rise to hydroxyl radicals (28). Cells expressing any of the four globins or Lb1 were more tolerant to 10 mM $CuSO_4$ than the mutant strain, albeit the effect was more conspicuous for GLB3-2 (Fig. 4*A*). Cold stress alters membrane functionality and triggers oxidative stress in yeast cells, as evidenced by the increase in H_2O_2 and the induction of superoxide dismutase and catalase (29). Cells expressing GLB2, GLB3-1, GLB3-2, and Lb1 were more tolerant than the mutant to a downshift of the growth temperature from 30°C to 10°C (Fig. 4*A*). Yeast cells were also exposed to *S*-nitrosoglutathione (GSNO), a physiological NO donor that triggers nitrosative stress at millimolar concentrations (30). This treatment was performed in liquid YPD medium because GSNO was relatively unstable during the 2-day incubation in solid medium, and GSH was used as a parallel control because GSNO decomposition may generate GSH in addition to NO (Fig. 4*B*). Mutant cells treated with 5 mM GSNO showed a 50% growth inhibition relative to wild-type cells, indicating toxicity in the absence of the flavoHb. Quite unexpectedly, only expression of GLB2, and to a much lower extent of GLB1-2, could complement flavoHb deficiency. No effects could be observed for yeast cells supplied with 5 mM GSH (Fig. 4*B*).

Flavin Concentrations In Vivo are Consistent with a Role in Globin Reduction. For the purpose of this work, we have quantified free flavins in roots and nodules of *L. japonicus* and in nuclei of soybean nodules. These measurements required improved methods for flavin determination by HPLC with fluorescence detection and for nuclei purification in percoll gradients. Nuclei preparations were found to be highly pure and free of contamination with cytosol, bacteroids, or symbiosomes by using antibodies to histone H3, Lb, and NifH. Flavin concentrations were estimated to be ~5 μM in roots and ~30 μM in nodules of *L. japonicus*, similar to those reported for other legumes (12, 22, 23). However, nuclei of soybean nodules contain flavins at levels of ~25 nmol/mg protein, whereas those estimated for whole nodules are ~7 nmol/mg protein, indicating that nuclei are particularly rich in flavins. In all cases, the

most abundant flavin was riboflavin, with lower amounts of FMN and FAD. Free flavins were also quantified in yeast cells expressing plant Hbs after 48 h of growth to find out whether they could be contributing to maintain globin activity. Using published conversion factors for cellular volumes, yeast cells were found to contain 4 μM riboflavin, 12 μM FMN, and 19 μM FAD. The flavin levels in roots, nodules, nuclei, and yeast are in the range needed to sustain the operation of the NAD(P)H + flavin system in vitro.

Discussion

The genome of *L. japonicus* encodes three Lbs and five other globins (18, 31). The latter are much less known and have been the focus of our investigation. We previously found that the GLB1-1, GLB2, and GLB3-1 mRNA levels were ~15-, 2-, and 7-fold, respectively, greater in nodules than in roots and were low or absent in other plant organs (18). The same situation probably occurs in other legumes, based on a transcript analysis in the *M. truncatula* Gene Expression Atlas (http://mtgea.noble.org/v3/). Therefore, these three globins are nodule-enhanced proteins and their functions should be suited for the peculiar microaerobic environment and metabolism of nodules. To characterize all the globins and compare GLB1-1 and GLB3-1 with the other members of the same class, we first measured their K^{O2} under identical assay conditions (Table 1). Class 1 Hbs usually have high K^{O2}, but GLB1-1 truly makes an exceptional case. The K^{O2} of GLB1-1 is 50-fold greater than that of the octameric O_2-scavenging Hb of the nematode *A. suum* (~2.7 nM), the highest O_2 affinity known to date for any plant or animal Hb (2, 32). Because of its extremely high K^{O2}, GLB1-1 would remain oxygenated and active even in the presence of CO. This may be important in nodules, where CO can be formed in significant amounts from Lb degradation by heme oxygenases (33). The K^{O2} of GLB1-2 is still too high for O_2 transport, but the moderate O_2 affinity of GLB2 and the unusual pentacoordination of its deoxy form are compatible with a role of this protein in O_2 sensing (7) and/or transport (34). Moreover, our results with yeast provide evidence for an additional function of GLB2 in NO detoxification, a role so far contemplated only for plant

class 1 Hbs (see below). The observed k_{on}^{O2} for GLB3-1 was similar to the value of 400 s^{-1} reported for *A. thaliana* GLB3 (6), but that of GLB3-2 was 30-fold higher, which is an indication that the two 'truncated' Hbs of *L. japonicus* show distinct O_2 affinities and are not functionally redundant, as occurs for GLB1-1 and GLB1-2.

The heme iron of Hbs must be in the ferrous state to bind O_2 and mechanisms should exist to reduce ferric Hbs in vivo. Several FAD-containing enzymes have been proposed to mediate reduction of plant Hbs. Ferric Lb reductase and dihydrolipoamide dehydrogenase are closely related enzymes that can reduce Lb in vitro, but they appear to be exclusively located to the mitochondria (35) and hence are unlikely to play a meaningful role in Hb reduction. Monodehydroascorbate reductase is abundant in the plastids, peroxisomes, and cell walls, and more sparse in the cytosol (25, 36). A cytosolic isoform of this antioxidant enzyme copurified with barley GLB1 when fractions were assayed for NO-scavenging activity, suggesting that it could sustain at least some Hb functions in vivo (13). By using an antibody raised against monodehydroascorbate reductase of soybean nodules (25), we found that most of the enzyme in *L. japonicus* nodules is associated with cell walls, especially in the vascular bundles, and failed to detect it in the nuclei. Nevertheless, we cannot exclude the possibility that specific monodehydroascorbate reductase isoforms play a role as Hb reductants in the cytosol and plastids, or that other flavoproteins with Hb reductase activity occur in the nuclei.

Previous work showed that NAD(P)H is a poor direct reductant of soybean Lb (12) but can significantly reduce *A. thaliana* GLB1 (30). Here, we found that the direct reduction of Hbs by NAD(P)H was physiologically irrelevant and inhibited by catalase, indicating involvement of H_2O_2. In sharp contrast, we demonstrate that all three classes of plant globins can be reduced by flavins acting as intermediate electron carriers (Fig. 1) and that this reaction does not involve H_2O_2 or superoxide and also occurs under anaerobic conditions (Fig. 2). Hence, it would not be inhibited in vivo by ascorbate peroxidase or superoxide dismutases, which are abundant in the cytosol, plastids, or nuclei (24, 25, 36). The heme reduction rates vary with the globin and flavin coenzyme, being very fast for hexacoordinate class 1 Hbs. The reduction rate for GLB2 was slow relative to class 1 or 3 Hbs but similar to

that seen for soybean Lb (12), which lends further biochemical support to an evolutionary link between class 2 Hbs and Lbs (5, 7). We thus conclude that, unlike pyridine nucleotides alone or some flavoproteins, the NAD(P)H + flavin system reduces in vitro all three globin classes in aerobic and anaerobic conditions, is not potentially inhibited by antioxidant enzymes, and is operative at the flavin concentrations estimated in plant cells and isolated nuclei.

In the present study, we show for the first time that all three globin classes are simultaneously present at high levels in the nuclei and at low levels in the cytosol of plant cells (Fig. 3). Because significant amounts of monocot Hbs have been detected in the cytosol (16), our finding suggests that there may be differences among plant species and/or movement of the proteins between the two compartments in response to developmental or stress stimuli. We also found substantial amounts of all globins in chloroplasts, consistent with two scattered reports of Hb in chloroplasts of a green alga (37) and wheat (38). In *L. japonicus* chloroplasts, GLB1 is predominantly associated with starch grains and GLB3 with thylakoids, which lends support to distinct roles for these two globin classes. However, the proteins also occur in root and nodule amyloplasts, which indicate that their functions are beyond photosynthesis and probably include metabolic regulation. The finding that GLB1-2 and GLB2 confer protection from nitrosative stress (Fig. 4) suggests that these proteins could also be involved in regulation of NO in plastids, which are a cellular source of NO (39). By modulating NO levels in the nuclei and plastids, globins could prevent formation of toxic peroxynitrite or regulate gene expression through nitrosylation of transcription factors in response to hormones or other regulatory compounds.

Our results that plant globins protect against oxidative stress (Fig. 4) are unprecedented and provide evidence that they can intercept superoxide and H_2O_2, which are cytotoxic if produced in excess and are not offset by antioxidant defenses (25, 36). The tolerance to oxidative stress is correlated with the expression levels of the proteins (high for GLB3-1 and GLB3-2, intermediate for GLB2, and weak for GLB1-2), indicating that the protection against this type of stress is largely due to the heme groups rather than to intrinsic features of

the globins. In sharp contrast, the selective tolerance to nitrosative stress afforded by GLB1-2 and GLB2 indicates that detoxification of GSNO, which does not react with hemes but directly participates in transnitrosation reactions with thiol groups, may involve nitrosylation of the single Cys residue of the proteins. This is supported by the observation that Lb1, which shares a 74% similarity with GLB2 but lacks Cys, does not protect against GSNO (Fig. 4). Furthermore, a mutation in which Cys^{65} is replaced by Ser causes a drastic decrease in the yield of the recombinant protein, suggesting that the single Cys residue of GLB2 is also important for protein stability, as has been reported for barley GLB1 (40). Further studies will be needed to establish the roles of hemes and Cys residues in NO scavenging by all three classes of globins, which is a subject of current intense debate in human Hb (2, 41).

Materials and Methods

Plant Material. Seedlings of *Lotus japonicus* 'MG20' and *Medicago truncatula* 'Jemalong' were inoculated with *Mesorhizobium loti* R7A and *Sinorhizobium meliloti* 2011, respectively, and were grown in controlled environment cabinets (18). Soybean (*Glycine max* cv. Williams) was inoculated with *Bradyrhizobium japonicum* USDA110 and grown in the field. Nodules, roots, and leaves to be used for microscopy were cut into small pieces and directly immersed in fixative as indicated below. Other plant material was used fresh or after short storage at -80ºC.

Production and Purification of Globins. Total RNA was extracted from nodules of *L. japonicus* and cDNA was synthesized following conventional protocols. The complete ORFs of the five globins and Lb1 were obtained by PCR using KAPA HiFi DNA polymerase (KAPA Biosystems; Woburn, MA) and gene-specific primers (Table S1), and were cloned in Champion pET200/D-TOPO expression vectors (Invitrogen). Details of methods used for cloning of the PCR products and for the expression and purification of the five proteins are provided in *SI Materials and Methods*.

Ligand Binding Kinetics. Laser flash photolysis (20) and stopped-flow (21) reactions were used to measure the bimolecular O_2 association (k_{on}) and dissociation (k_{off}) rate constants, respectively. For flash photolysis experiments, globins were prepared as described (20) in air-saturated and O_2-saturated 100 mM phosphate buffer (pH 7), and for determination of k_{on}, oxyferrous globins in air-

equilibrated 100 mM phosphate buffer (pH 7) were mixed with 100 mM phosphate buffer (pH 7) containing dithionite and saturated with 1 mM CO. Details of the procedures and instruments used are provided in *SI Materials and Methods*.

Spectroscopic Analyses and Reduction Assays. Spectra (350-650 nm) were obtained with 70 μM ferric globins in 50 mM potassium phosphate buffer (pH 7.0). The cyanoferric, deoxyferrous, and nitrosyl complexes were produced by adding a few crystals of potassium cyanide, sodium dithionite, and nitrite + dithionite, respectively. The CO-ferrous complexes were obtained by adding dithionite in CO-saturated buffer and the oxyferrous complexes by reducing the ferric globins with 20 μM FAD and 1 mM NADH in oxygenated buffer. The reduction assays were carried out as indicated in the legends to Figures 1 and 2. All assays with flavins were performed in the absence and presence of 5 μg of catalase (25,000 units/mg protein; Sigma) with similar results.

Antibody Production and Immunolocalization. Purified GLB1-2, GLB2, and GLB3-2 were dialyzed exhaustively in PBS buffer and injected in white rabbits to produce polyclonal monospecific antibodies using conventional immunization procedures (Bio-Genes; Berlin, Germany). Immunolocalization was performed as described (24). Nodules, roots, and leaves of *L. japonicus* and *M. truncatula* were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde, then dehydrated in ethanol by progressively lowering the temperature from 0 to -20°C. Further details of antibody affinity purification and sample preparation are given in *SI Materials and Methods.*

Yeast Strains, Transformation, and Stress Treatments. Standard methods for yeast manipulation were used (42). Wild-type *Saccharomyces cerevisiae* strain BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0)* and its flavoHb-deficient mutant derivative (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 yhb1::kanMX4*) were obtained from the Euroscarf collection (Frankfurt, Germany) and grown in YPD medium or in SD synthetic minimal medium supplemented with the strain nutritional requirements (42). For plasmid construction, the ORFs were cloned into the pENTR/D-TOPO vector and then into pVV214 via the Gateway method (Invitrogen). The mutant strain was transformed with the constructs by the lithium acetate-PEG method (43). Stress treatments of yeast cells in solid (MV, $CuSO_4$, and cold) or liquid (GSNO) YPD media were performed as indicated in the legend to Figure 4. GSNO was synthesized by reaction of acidified $NaNO_2$ with GSH and its concentration standardized using an extinction coefficient of 0.767 mM^{-1} cm^{-1} at 334 nm.

Flavin Determination in Plants and Yeast. Nodules were ground at 0°C in the dark with a pestle in 10 mM ammonium acetate (pH 6.0) containing 10% methanol. Yeast cells were grown in liquid YPD medium to OD_{660}~0.7, washed twice with PBS, and broken by vortexing with stainless steel beads (3

mm) in a MM301 mixer mill (Retsch; Haan, Germany). Nuclei were purified from soybean nodules in 30%/ 60% percoll density gradients by a modification of the protocol described by Folta and Kaufman for *A. thaliana* (44). Details are given in *SI Materials and Methods.*

ACKNOWLEDGMENTS. We thank Laura Calvo, Ana Castillo, and Ana Alvarez for help with protein purification, nuclei isolation, and HPLC-MS analysis, respectively. This work was funded by MINECO-FEDER (grant AGL2011-24524) and Gobierno de Aragón-FSE (group A53). M.S. was supported by a predoctoral contract from Junta de Ampliación de Estudios-CSIC.

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Figure Legends

Fig. 1. Reduction of globins with NAD(P)H and flavins. The assays were performed in a medium comprising 50 mM phosphate buffer (pH 7.0), 50 μM diethylenetriaminepentaacetic acid, 30 μM ferric globins, 20 μM flavins, and 200 μM (GLB1-1, GLB1-2, GLB2) or 1 mM (GLB3-1, GLB3-2) of NAD(P)H. All reduction assays were carried out in CO-saturated buffer to trap the ferrous forms as CO-complexes, except GLB1-1, which was reduced in aerobic buffer. Data are expressed in percent of reduced globins, as either O_2-complex (GLB1-1) or CO-complex (all others). Values are the means ± SE of 3-5 replicates, corresponding to at least two different protein preparations.

Fig. 2. Anaerobic reduction of globins with NAD(P)H and flavins. Assays were performed by purging the buffer and reagents with N_2 inside a rubber stopped cuvette, in which the protein was subsequently injected with a needle. Representative reactions are shown to demonstrate complete reduction of four globins to the deoxyferrous forms. Under these reaction conditions, GLB1-1 still retained O_2 as the oxyferrous complex.

Fig. 3. Immunogold localization of globins in legume tissues. The figure shows only some representative micrographs to avoid redundancy because similar subcellular localizations were found for the three classes of globins. For GLB1, labeling (arrows) was predominantly observed on the nuclei of nodule (*A*) and root (*B*) cells, on nodule plastids (*C*), and on leaf chloroplasts (*D*) of *L. japonicus*. Likewise, significant labeling (arrows) was also found for GLB2 and GLB3 on nuclei (*E* and *G*, respectively) and plastids (*F* and *H*, respectively) of nodules. Weak labeling was seen also for GLB3 on chloroplasts (*I*). Shown is also, for comparison, GLB1 labeling on nuclei of *M. truncatula* nodule host cells (*J*). Negative controls, in which non-immune sera have replaced the antibodies, are shown, for example, for nuclei of *M. truncatula* nodules (*K*) and chloroplasts of *L. japonicus* leaves (*L*). c, cytosol; ch, chloroplast; is, intercellular space; m, mitochondrion; n, nucleus; nu, nucleolus; w, cell wall. Bars, 1 μm.

Fig. 4. Tolerance of yeast cells expressing globins to oxidative and nitrosative stress. *(A)* Drop tests were performed by growing transformed cells until saturation in SD medium. Cell cultures were then diluted 1:10, 1:100 and 1:1000, spotted onto plates of YPD medium containing MV (1.5 mM) or $CuSO_4$ (10 mM). These compounds were added after autoclaving, but prior to gelification, and growth was recorded after 4-5 d. Cold stress was applied by exposing YPD plates to 10°C for four weeks. Three independent complete experiments, each one using a different plate and two biological replicates per plate, were set with similar results. *(B)* Liquid cell cultures were grown until saturation in SD medium, then diluted to an

initial OD_{660} of 0.01 in YPD medium containing 5 mM GSNO (left) or 5 mM GSH (right). Growth was monitored in microtitre plates using the Bioscreen C microbiological workstation (Thermo Fisher Scientific) with automatic recording of OD_{600} every 30 min. Three independent complete experiments, each one with three biological replicates, were set with similar results.