Plant hemoglobins can be maintained in functional form by reduced flavins in the nuclei and confer differential tolerance to nitro-oxidative stress

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Received 22 July 2013 ; revised 9 September 2013

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Running title: Plant hemoglobins

Keywords: *Lotus japonicus*, plant hemoglobins, flavins, legume nodules, nitrosative stress, oxidative stress, plant cell nuclei, yeast complementation.

Total word count (7306). Summary (249), Introduction (1222), Results (2124), Discussion (1358), Experimental procedures (1675), Acknowledgments (89), Table titles (10), Figure legends (579).

Not included in total word count: References (1623).

SUMMARY

Bacteria, plant and animal hemoglobins (Hbs) need to have their hemes in the ferrous state to bind O_2 and other physiological ligands. Here we have characterized the full set of nonsymbiotic (class 1 and 2) and 'truncated' (class 3) Hbs of *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 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. 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.

INTRODUCTION

Originally, hemoglobins (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 (Appleby, 1984; Angelo *et al.,* 2008). However, the last three decades have witnessed the discoveries of Hb genes and proteins in all kingdoms of life (Vinogradov *et al.,* 2005). 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 (Vinogradov *et al.,* 2005; Angelo *et al.,* 2008). 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 (Angelo *et al.,* 2008; Gardner, 2012). 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 (Angelo *et al.,* 2008).

Plants can express up to three classes of Hbs: nonsymbiotic, symbiotic and 'truncated' (Appleby, 1984; Trevaskis *et al.,* 1997; Watts *et al.,* 2001; Smagghe *et al.,* 2009). Nonsymbiotic Hbs occur at nanomolar or micromolar concentrations in many plant tissues and can be futher categorized into class 1 (Glb1) and class 2 (Glb2), based primarily on analyses of their amino acid sequences and phylogenic relationships, which in turn have been found to be correlated with differences in their O_2-binding properties (Smagghe *et al.,* 2009). The Glb1 proteins display very high O_2 affinities, which makes them unsuitable for O_2 transport and delivery (Smagghe *et al.,* 2009), and their functions have been extensively studied in cereals, alfalfa (*Medicago sativa*) and *Arabidopsis thaliana* (reviews by Dordas *et al.,* 2003; Hill, 2012; Hebelstrup *et al.,* 2013). Seminal work in 1994 by Hill's group with barley (*Hordeum vulgare*) had shown that *Glb1* is strongly induced by hypoxia and flooding.

Consistent with this, overexpression of *Glb1* in *A. thaliana* enhances survival to hypoxic conditions (Hunt *et al.,* 2002; Hebelstrup *et al.,* 2007). A number of studies with alfalfa transgenic roots and maize (*Zea mays*) suspension cell cultures showed that the tolerance to hypoxia mediated by Hb entails NO scavenging and led to the proposal of a Hb/NO cycle (reviews by Dordas *et al.,* 2003; Igamberdiev *et al.,* 2010; Hebelstrup *et al.,* 2013). Essentially, this cycle encompasses the oxygenation of NO to NO_3^- by the NO dioxygenase activity of ferrous Glb1 and the re-reduction of the resulting ferric Glb1 by a reductase with concomitant oxidation of NAD(P)H. Nitrate can then be reduced to NO_2^- by cytosolic nitrate reductase and NO_2^- can be reduced to NO also by nitrate reductase (low-affinity binding), the mitochondrial electron transport chain and other redox systems (Gupta *et al.,* 2011). The reduction of Glb1 is probably the rate-limiting step of the cycle and the regeneration of $NAD(P)^+$ may contribute to maintain cellular energetics and redox balance under hypoxic conditions (Dordas *et al.,* 2003; Igamberdiev *et al.,* 2010).

The Glb2 proteins have O_2 affinities that ressemble those of symbiotic Hbs and are induced by cold and cytokinins but not by hypoxia (Trevaskis *et al.,* 1997; Hunt *et al.,* 2001; Smagghe *et al.,* 2009). However, their functions are less defined than those of Glb1. Recent studies with *A. thaliana* have shown that changes in expression of *Glb1* or *Glb2* affect the time to bolting (Hebelstrup and Jensen, 2008), and that repression of *Glb2* inhibits shoot organogenesis whereas overexpression of *Glb1* or *Glb2* enhances the number of shoots (Wang *et al.,* 2011). In the same model plant, overexpression of *Glb2* specifically in developing seeds increases adenylate charge, oil content and polyunsaturated fatty acids (Vigeolas *et al.,* 2011). Unpublished results, cited by Hill (2012), indicate that somatic embryogenesis in maize can be altered by manipulating expression of *Glb1* and *Glb2*. Taken together, the above data indicate tha Glb1 and Glb2 play major roles in plant development and organ morphogenesis. Although the underlying mechanisms still await elucidation, a participation of the two globins *via* modulation of hormone signal transduction is a most attractive hypothesis (Hill, 2012).

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 (Appleby, 1984; Smagghe *et al.,* 2009). Class 3 Hbs (Glb3) 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 the function of Glb3 proteins in plants, some of their bacterial counterparts have been implicated in tolerance to nitrosative stress (Angelo *et al.,* 2008; Gardner, 2012).

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 globins such as Hb, myoglobin 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 (Kakar *et al.,* 2010). 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 (Becana and Klucas, 1990; Igamberdiev *et al.,* 2006). 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 (Hebelstrup *et al.,* 2007; Seregélyes *et al.,* 2000; Qu *et al.,* 2005), only the cytosol (Ross *et al.,* 2001), or only the plastids (Smagghe *et al.,* 2007).

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 (Bustos-Sanmamed *et al.,* 2011). The genes encode two class 1 Hbs (Glb1-1 and Glb1-2), one class 2 Hb (Glb2) and two class 3 or 'truncated' Hbs (Glb3-1 and Glb3-2). This gene profile may be extensive to other legumes because two class 3 Hbs are expressed in soybean (*Glycine max*)

(Lee *et al.,* 2004) and *Medicago truncatula* (Vieweg *et al.,* 2005), but is in contrast with *A. thaliana*, which only contains one globin of each class (Trevaskis *et al.,* 1997; Watts *et al.,* 2001). Here we characterize all nonsymbiotic and 'truncated' globins of *L. japonicus*. The proteins differ in ligand binding kinetics, are mainly localized to the nuclei 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 100-150 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. They showed that the two class 1 Hbs (Figures S1 and S2) are mainly hexacoordinate both in the deoxyferrous (α band at ~557 nm and β band at ~530 nm) and ferric (α band at ~530 nm and shoulder at ~560 nm) forms. The spectrum of deoxyferrous Glb1-1 has a well-defined Soret band (425 nm) and split α (554 and 563 nm) and β (527 and 534 nm) bands (Figure S1), and is identical (± 0.5 nm) to that found for barley Glb1 (Duff *et al.,* 1997). The narrow Soret band suggests a single species and the four-banded visible spectra might reflect slow

interconversion between two protein conformations, each probably corresponding to a different ligand environment of the heme (Duff *et al.,* 1997). Quite surprisingly for a class 2 Hb, which is expected to be hexacoordinate in the ferric and ferrous states (Smagghe *et al.,* 2009; Kakar *et al.,* 2010), Glb2 shows typical spectra of hexacoordinate globin in the ferric form but pentacoordinate in the deoxyferrous form (Figure S3). The two class 3 Hbs displayed typical spectra of pentacoordinate globins in the deoxyferrous (α band at ~560 nm but no β band) and ferric (poorly defined bands at ~500, 540, and 580 nm) forms (Figures 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 (Figures S1 to S5). Flash photolysis (Hargrove, 2000) and stopped-flow (Trent *et al.,* 2001; Sturms *et al.,* 2010) 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 (Hebelstrup *et al.,* 2007; Seregélyes *et al.,* 2000; Qu *et al.,* 2005), 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 to be in the range of 0.2-2 mM and 5-50 μM, respectively (Pankhurst *et al.,* 1974; Becana and Klucas, 1990; Rodríguez-Celma *et al.,* 2011).

Reduction assays of the five globins were performed systematically with FMN and FAD as both flavins are very soluble and cofactors of flavoproteins. Nevertheless, riboflavin, an abundant flavin of nodules (Pankhurst *et al.,* 1974; Becana and Klucas, 1990), was 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(P)H, 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)* 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. *(v)* 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. 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 (Appleby, 1984). 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 (Figure S1).

The three globin classes are predominantly localized to the nuclei

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 (Rubio *et al.,* 2009), as these preserve sensitive epitopes better than conventional methods of fixation and dehydration (Dalton *et al.,* 1993). 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 (Figure 3a). Immunogold microscopy of *L. japonicus* nodules revealed that the three classes of globins are predominantly localized in the nuclei (Figure 4a-d) and, to a much lower extent, in the nodule plastids or leaf chloroplasts (Figure 4e) and in the cytosol, whereas no labeling was seen on the mitochondria or peroxisomes. Immunolocalization of Lb, with an antibody raised against Lb1, one of the three Lbs of *L. japonicus*, was also carried out for comparison. This symbiotic globin was found not only in the cytosol, as expected for a protein that transports O_2 in the cytosol and delivers it to the

symbiosomes (Appleby, 1984), but also abundantly in the nuclei of the nodule infected cells (Figure 4f).

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 (Mulet *et al.,* 2004). 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 (Figure 3b), 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 (Angelo *et al.,* 2008; Gardner, 2012), 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 (Cochemé and Murphy, 2008). 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 (Figure 5a). 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 (Ríos *et al.,* 2013). 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 (Figure 5a). 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 (Zhang *et al.,* 2003). 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 (Figure 5a). Yeast cells were also exposed to *S*-nitrosoglutathione (GSNO), a physiological NO donor

that triggers nitrosative stress at millimolar concentrations (Perazzolli *et al.,* 2004). 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 (Figure 5b). 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 (Figure 5b).

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 (Pankhurst *et al.,* 1974; Becana and Klucas, 1990; Rodríguez-Celma *et al.,* 2011). However, nuclei of soybean nodules contain flavins at levels of ~25 nmol mg^{-1} protein, whereas those estimated for whole nodules are ~7 nmol mg^{-1} 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 (Uchiumi *et al.,* 2002; Bustos-Sanmamed *et al.,* 2011). 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 (Bustos-Sanmamed *et al.,* 2011). 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 (Weber and Vinogradov, 2001; Angelo *et al.,* 2008). 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 (Baudouin *et al.,* 2004). 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 (Smagghe *et al.,* 2009) and/or transport (Spyrakis *et al.,* 2011). However, for Glb2 to act by facilitating O_2 diffusion, the protein should be present at high (millimolar) concentrations, which is unlikely to occur for any nonsymbiotic or 'truncated' Hbs in plant tissues (Mur *et al.,* 2012; Hebelstrup *et al.,* 2013). The observed k_{on}^{O2} for Glb3-1 was similar to the value of 400 s^{-1} reported for *A. thaliana* Glb3 (Watts *et al.,* 2001), 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 (Moran *et al.,* 2002) 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 (Miyake *et al.,* 1998; Dalton *et al.,* 1993; Becana *et al.,* 2010). 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* (Igamberdiev *et al.,* 2006). By using an antibody raised against monodehydroascorbate reductase of soybean nodules (Dalton *et al.,* 1993), 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, 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 (Becana and Klucas, 1990) but can significantly reduce *A. thaliana* Glb1 (Perazzolli *et al.,* 2004). 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 (Figure 1) and that this reaction does not involve H_2O_2 or superoxide and also occurs under anaerobic conditions (Figure 2). Hence, it would not be inhibited *in vivo* by ascorbate peroxidase or superoxide dismutases, which are abundant in the cytosol, plastids or nuclei (Dalton *et al.,* 1993; Rubio *et al.,* 2009; Becana *et al.,* 2010). 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 (Becana and Klucas, 1990), which lends further biochemical support to an

evolutionary link between class 2 Hbs and Lbs (Trevaskis *et al.,* 1997; Smagghe *et al.,* 2009). 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. Our results that flavins can actively reduce plant Hbs, and that this reduction is particularly fast for Glb1 proteins (Figure 1), raise the intriguing possibility that flavins can replace monodehydroascorbate reductase in the Hb/NO cycle originally postulated by Hill and coworkers (Dordas *et al.,* 2003; Igamberdiev *et al.,* 2006). Given the efficiency and versatility of the NAD(P)H + flavin system, however, we anticipate that reduced flavins will sustain NO dioxygenase activity of Glb1 proteins by reducing their ferric to oxyferrous/deoxyferrous forms in aerobic/nearly anaerobic conditions, which will be a subject of further research.

In the present study, we show that all three globin classes are simultaneously present at high levels in the nuclei and at low levels in the cytosol and plastids of plant cells (Figure 4). Because significant amounts of Hbs have been detected in the cytosol (Ross *et al.,* 2001) and plastids (Kim *et al.,* 2013) of monocots, as well as in chloroplasts of the green alga *Chlamydomonas eugametos* (Couture *et al.,* 1994), our finding suggests that there may be differences among plant species and/or movement of the proteins among these compartments in response to developmental or stress stimuli. By modulating NO levels in the nuclei, 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 (Figure 5a) 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 (Dalton *et al.,* 1993; Becana *et al.,* 2010). 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, only expression of Glb1-2 or

Glb2 protected yeast cells against nitrosative stress (Figure 5b), which is consistent with the observation that *A. thaliana* Glb1 and Glb2 are able to scavenge NO *in vivo* (Hebelstrup and Jensen, 2008). The selective tolerance afforded by Glb1-2 or Glb2 in yeast strongly suggests that detoxification of GSNO, which primarily participates in transnitrosylation reactions with thiol groups, involves *S*-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 (Figure 5b). 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 reported for barley Glb1 (Bykova *et al.,* 2006). Further studies will be needed to establish the relative 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 (Gladwin *et al.,* 2000; Angelo *et al.,* 2008).

EXPERIMENTAL PROCEDURES

Plant material

Seedlings of *Lotus japonicus* 'MG20' were inoculated with *Mesorhizobium loti* R7A and grown in controlled environment cabinets (Bustos-Sanmamed *et al.,* 2011). 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, USA) and gene-specific primers (Table S1), and were cloned in Champion pET200/D-TOPO expression vectors (Invitrogen, http://www.invitrogen.com). The fragments containing the complete ORFs were amplified by PCR using a program consisting of an initial denaturation step at 95ºC for 5 min, followed by 25 amplification cycles (98ºC for 20 s, 50-60ºC gradient for 30 s and 72ºC for 30 s) and a final elongation step at 72ºC for 3 min. The PCR products (500-600 bp) were then cloned into the Champion pET200 Directional TOPO vector (Invitrogen) and were fully sequenced to verify the absence of mutations. This plasmid encodes an N-terminal poly-His tag. Glb1-2 and Glb3-2 were

expressed in *Escherichia coli* BL21 Star (DE3) cells (Invitrogen), and Glb1-1, Glb2 and Glb3-1 in *E. coli* C41 (DE3) cells (Lucigen, http://www.lucigen.com). Cells were cultured at 37ºC for 16 h in 10 ml of LB medium. Then, 5 ml of the culture was added to 1 l of TB medium and cells were incubated at the same conditions until an OD_{660}=1 was reached. Finally, recombinant proteins were expressed by incubation of cells at 37ºC for 4-6 h with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), except in the case of Glb1-2, which was overexpressed with no added IPTG. Transformed cells were washed in PBS and stored at -80ºC for no longer than three weeks. Cells were resuspended in PBS with 40 mM imidazole, sonicated (6 x 4 min), and cleared by centrifugation. The supernatant was fractionated with 30-75% ammonium sulfate and loaded on a Ni affinity column (HiTrap Chelating HP; GE Healthcare, http://www3.gehealthcare.com). The column was washed with five volumes of the same medium and the recombinant proteins were eluted with the buffer containing 250 mM imidazole. The proteins were desalted, oxidized with ferricyanide, concentrated and further purified by chromatography on a weak-anion exchange resin (DE-52; Whatman, now part of GE Healthcare). Globins were quantified using the absorbance of the Soret band of the hemes.

Ligand binding kinetics

Laser flash photolysis (Hargrove, 2000) and stopped-flow (Trent et al., 2001; Smagghe et al., 2009) 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 (Hargrove, 2000) in air-saturated and O_2-saturated 100 mM phosphate buffer (pH 7.0). The instrument was a 10-20 Hz Nd-YAG Surelite II laser (Continuum; Santa Clara, CA, USA) that provided a 5 ns-excitation pulse at 532 nm. A 75-w xenon lamp (400–750 nm) was used as the probe beam. The spectra from the laser pulse were collected using a synchronized intensified charge coupled device (ICCD) camera from iStar (Andor Technology; South Windsor, CT, USA). The time-resolved spectra were then used to construct the decay curves from the intensities at 434 nm, which were fitted to single exponentials to get the constants using OriginPro (OriginLab; Northampton, MA, USA). Time courses were monitored within a window of 100 ns–100 ms. Bimolecular rate constants were determined from the ligand dependence of the rebinding time courses, although little geminate recombination was observed for either CO or O_2 compared to that observed for *A. thaliana* Hbs (Bruno *et al.,* 2007). Previsously described methods (Hargrove, 2000) were used to extract the ligand dependence of the rebinding time courses to obtain bimolecular rate constants.

For stopped-flow experiments, rapid mixing experiments were performed with a 1:1 mixture of air-equilibrated 100 mM phosphate buffer (pH 7.0) and a 1 mM CO solution in the same buffer as the displacing ligand (for further details see Trent *et al.,* 2001). For reasons that are not understood, Glb3-

2 required the presence of 100 μM sodium dithionite in the CO solution to displace O_2. The observed time course is thus equal to k_{off} for this protein. A control with myoglobin collected under the same conditions provided the appropriate value for k_{off} for myoglobin as well. The observed rates were collected using a SFM-400 stopped-flow reactor (Bio-Logic; Knoxville, TN, USA) coupled to a Bio-Logic MOS-500 spectrophotometer. All kinetic traces (average of five) were fit to exponential decays using IGOR Pro (WaveMetrics; Lake Oswego, OR, USA). The k_{off} values for O_2 were then calculated using the equation $k_{off}=k_{obs}[1+(k_{on}$ for O_2/k_{on} for CO).([O_2]/[CO])].

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^{-1} protein; Sigma-Aldrich, http://www.sigmaaldrich.com) 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 antibodies using conventional immunization procedures (BioGenes, http://www.biogenes.de). Polyclonal monospecific antibodies were affinity purified. The Glbs were coupled to CNBr-activated Sepharose 4 Fast Flow (GE Healthcare Life Sciences, http://www.gelifesciences.com) and the monospecific IgGs were eluted with 200 mM Gly-HCl buffer containing 250 mM NaCl (pH 2.2). The eluate was immediately adjusted to pH 7.5 with 2 M Tris-HCl.

Immunolocalization was performed as described (Rubio *et al.,* 2009). Nodules, roots and leaves of *L. japonicus* were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde, and dehydrated in ethanol under a progressively reduced temperature (0 to -20°C). The samples were then infiltrated in LR White resin over 2 days at -20°C, after which the samples were polymerized using UV light at -20°C for 24 h and 16°C for 24 h. All dehydration and resin-embedding steps were performed in a Leica AFS2. Ultrathin sections were collected on pyroxylin-coated Ni-grids and were incubated in each affinity-purified antibody diluted 1:10 in blocking/diluting buffer (Rubio *et al.,* 2009) for 1 h, washed and then incubated in 15 nm gold conjugated to protein A (BBInternational; Cardiff, UK) (diluted 1:100 in the same buffer) for 1 h. The sections were viewed and digitally photographed using a JEM 1400 transmission electron microscope (JEOL; Tokyo, Japan).

Yeast strains, transformation and stress treatments

Standard methods for yeast manipulation were used (Guthrie and Fink, 1991). 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 (Guthrie and Fink, 1991). 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 (Gietz and Woods, 2002). 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 5. 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 for *A. thaliana* by Folta and Kaufman (2000). Five grams of nodules were ground softly in an ice-cold mortar with 15 ml of extraction buffer (1 M hexylene glycol, 0.5 M PIPES-KOH pH 7.0, 10 mM $MgCl_2$). The homogenate was filtered through eight layers of cheesecloth and 10% Triton X-100 was added dropwise to reach a final concentration of 0.75% to lyse organelle membranes. The suspension was left to stand at 4°C for 10 min with gentle stirring and centrifuged at 300 ***g*** for 5 min and at 2000 ***g*** for 10 min. The pellet was resuspended in 1.5 ml of gradient buffer (0.5 M hexylene glycol, 0.5 M PIPES-KOH pH 7.0, 10 mM $MgCl_2$, 0.5% Triton X-100) and loaded onto two percoll layers (5 ml of 30% at top and 7 ml of 60% at bottom, both prepared in gradient buffer). The density gradient was generated by centrifugation at 4000 ***g*** for 30 min. The pellet was washed twice with gradient buffer to remove percoll, and the final pellet containing most of the nuclei was resuspended in flavin extraction medium (50% methanol, 10 mM ammonium acetate, pH 6.0).

Samples were centrifuged, filtered, and injected on the HPLC equipped with a Nova-Pak C_{18} column (3.9 x 150 mm; Waters) and with a fluorescence detector (model 474, Waters; excitation 445 nm, emission 520 nm). Flavin separation was performed, at a flow rate of 1 ml min^{-1}, using a gradient of buffers A (8% methanol, 1 mM ammonium acetate, pH 6.0) and B (90% methanol, 1 mM

ammonium acetate, pH 6.0): 0-5 min, 95% buffer A; 5-30 min, decreasing buffer A to 80%; 30-35 min, increasing buffer A to 95%. Flavin identification was verified by HPLC coupled to electrospray ionization-TOF MS (Rodríguez-Celma *et al.,* 2011).

ACKNOWLEDGMENTS

We are grateful to Ryan Sturms and Mark Hargrove (Iowa State University, USA) for help with stopped-flow measurements and to Raúl Arredondo-Peter (Universidad Autónoma del Estado de Morelos, Mexico) and two anonymous reviewers for helpful comments on the manuscript. Thanks are also due to 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.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Soret-visible spectra of ferric and ferrous Glb1-1 and representative complexes.

Figure S2. Soret-visible spectra of ferric and ferrous Glb1-2 and representative complexes.

Figure S3. Soret-visible spectra of ferric and ferrous Glb2 and representative complexes.

Figure S4. Soret-visible spectra of ferric and ferrous Glb3-1 and representative complexes.

Figure S5. Soret-visible spectra of ferric and ferrous Glb3-2 and representative complexes.

Table S1. Oligonucleotides used in this study.

SHORT LEGENDS FOR MAIN FIGURES

Figure 1. Reduction of globins with NAD(P)H and flavins.

Figure 2. Anaerobic reduction of globins with NAD(P)H and flavins.

Figure 3. Immunoblots showing antibody specificity and globin expression in yeast cells.

Figure 4. Immunogold localization of globins in legume tissues.

Figure 5. Tolerance of yeast cells expressing globins to oxidative and nitrosative stress.

REFERENCES

Angelo, M., Hausladen, A., Singel, D.J. and Stamler, J.S. (2008) Interactions of NO with hemoglobin: from microbes to man. *Methods Enzymol.* **436,** 131-168.

Appleby, C.A. (1984) Leghemoglobin and *Rhizobium* respiration. *Annu. Rev. Plant Physiol.* **35,** 443-478.

Baudouin, E., Frendo, P., Le Gleuher, M. and Puppo, A. (2004) A *Medicago sativa* haem oxygenase gene is preferentially expressed in root nodules. *J. Exp. Bot.* **55,** 43-47.

Becana, M. and Klucas, R.V. (1990) Enzymatic and nonenzymatic mechanisms for ferric leghemoglobin redution in legume root nodules. *Proc. Natl. Acad. Sci. USA,* **87,** 7295-7299.

Becana, M., Matamoros, M.A., Udvardi, M. and Dalton, D.A. (2010) Recent insights into antioxidant defenses of legume root nodules. *New Phytol.* **188,** 960-976.

Bruno, S., **Faggiano, S., Spyrakis, F., Mozzarelli, A., Abbruzzetti, S., Grandi, E., Viappiani, C., Feis, A. et al.** (2007) The reactivity with CO of AHb1 and AHb2 from *Arabidopsis thaliana* is controlled by the distal HisE7 and internal hydrophobic cavities. *J. Am. Chem. Soc.* **129,** 2880-2889.

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

Bykova, N.V., Igamberdiev, A.U., Ens, W. and Hill, R.D. (2006) Identification of an intermolecular disulfide bond in barley hemoglobin. *Biochem. Biophys. Res. Commun.* **347,** 301-209.

Cochemé, H.M. and Murphy, M.P. (2008) Complex I is the major site of mitochondrial superoxide production by paraquat. *J. Biol. Chem.* **283,** 1786-1798.

Couture, M., Chamberland, H., St-Pierre, B., Lafontaine, J. and Guertin, M. (1994) Nuclear genes encoding chloroplast hemoglobins in the unicellular green alga *Chlamydomonas eugametos*. *Mol. Gen. Genet.* **243,** 185-197.

Dalton, D.A., Baird, L.M., Langeberg, L., Taugher, C.Y., Anyan, W.R., Vance, C.P. and Sarath, G. (1993) Subcellular localization of oxygen defense enzymes in soybean (*Glycine max* [L.] Merr.) root nodules. *Plant Physiol.* **102,** 481-489.

Dordas, C., Rivoal, J. And Hill, R.D. (2003) Plant haemoglobins, nitric oxide and hypoxic stress. *Ann. Bot.* **91,** 173-178.

Duff, S.M.G., Wittenberg, J.B. and Hill, R.D. (1997) Expression, purification, and properties of recombinant barley (*Hordeum* sp.) hemoglobin. *J. Biol. Chem.* **272,** 16746-16752.

Folta, K.M. and Kaufman, L.S. (2000) Preparation of transcriptionally active nuclei from etiolated *Arabidopsis thaliana*. *Plant Cell Rep.* **19,** 504-510.

Gardner, P.R. (2012) Hemoglobin: a nitric-oxide dioxygenase. *Scientifica*, **2012,** 1-34.

Gietz, R.D. and Woods, R.A. (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol.* **350,** 87-96.

Gladwin, M.T., Ognibene, F.P., Pannell, L.K., Nichols, J.S., Pease-Fye, M.E., Shelhamer, J.H., and Schechter, A.N. (2000) Relative role of heme nitrosylation and β-cysteine 93 nitrosation in the transport and metabolism of nitric oxide by hemoglobin in the human circulation. *Proc. Natl. Acad. Sci. USA,* **97,** 9943-9948.

Gupta, K.J., Fernie, A.R., Kaiser, W.M. and van Dongen, J.T. (2011) On the origins of nitric oxide. *Trends Plant Sci.* **16,** 160-168.

Guthrie, C. and Fink, G.R. (1991) *Guide to Yeast Genetics and Molecular and Cell Biology.* New York: Academic Press.

Hargrove, M.S. (2000) A flash photolysis method to characterize hexacoordinate hemoglobin kinetics. *Biophys J.* **79,** 2733-2738.

Hebelstrup, K.H., Igamberdiev, A.U. and Hill, R.D. (2007) Metabolic effects of hemoglobin gene expression in plants. *Gene,* **398,** 86-93.

Hebelstrup, K.H. and Jensen, E.Ø. (2008) Expression of NO scavenging hemoglobin is involved in the timing of bolting in *Arabidopsis thaliana*. *Planta* **227,** 917-927.

Hebelstrup, K.H., Shah, J.K. and Igamberdiev, A.U. (2013) The role of nitric oxide and hemoglobin in plant development and morphogenesis. *Physiol. Plant.* **148,** 457-469.

Hill, R.D. (2012) Non-symbiotic haemoglobins – What's happening beyond nitric oxide scavenging? *AoB Plants* **2012,** pls004; doi10.1093/aobpla/pls004.

Hunt, P.W., Watts, R.A., Trevaskis, B., Llewelyn, D.J., Burnell, J., Dennis, E.S. and Peacock, W.J. (2001) Expression and evolution of functionally distinct haemoglobin genes in plants. *Plant Mol. Biol.* **47,** 677-692.

Hunt, P.W., Klok, E.J., Trevaskis, B., Watts, R.A., Ellis, M.H., Peacock, W.J. and Dennis, E.S. (2002) Increased level of hemoglobin 1 enhances survical of hypoxic stress and promotes early growth in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA,* **99,** 17197-17202.

Igamberdiev, A.U., Bykova, N.V. and Hill, R.D. (2006) Nitric oxide scavenging by barley hemoglobin is facilitated by a monodehydroascorbate reductase-mediated ascorbate reduction of methemoglobin. *Planta,* **223,** 1033-1040.

Igamberdiev, A.U., Bykova, N.V., Shah, J.K. and Hill, R.D. (2010) Anoxic nitric oxide cycling in plants: participating reactions and possible mechanisms. *Physiol. Plant.* **138,** 393-404.

Ioanitescu, A.I., Dewilde, S., Kiger, L., Marden, M.C., Moens, L. and Van Doorslaer, S. (2005) Characterization of nonsymbiotic tomato hemoglobin. *Biophys J.* **89,** 2628-2639.

Kakar, S., Hoffman, F.G., Storz, J.F., Fabian, M. and Hargrove, M.S. (2010) Structure and reactivity of hexacoordinate hemoglobins. *Biophys. Chem.* **152,** 1-14.

Kim, D.Y., Hong, M.J., Lee, Y.J., Lee, M.B. and Seo, Y.W. (2013) Wheat truncated hemoglobin interacts with photosystem I PSK-I subunit and photosystem II subunit PsbS1. *Biol. Plant.* **57,** 281-290.

Lee, H., Kim, H. and An, C.S. (2004) Cloning and expression analysis of 2-on-2 hemoglobin from soybean. *J. Plant Biol.* **47,** 92-98.

Miyake, C., Schreiber, U., Hormann, H., Sano, S. and Asada, K. (1998) The FAD-enzyme monodehydroascorbate radical reductase mediates photoproduction of superoxide radicals in spinach thylakoid membranes. *Plant Cell Physiol.* **39,** 821-829.

Moran, J.F., Sun, Z., Sarath, G., Arredondo-Peter, R., James, E.K., Becana, M. and Klucas, R.V. (2002) Molecular cloning, functional characterization, and subcellular localization of soybean nodule dihydrolipoamide reductase. *Plant Physiol.* **128,** 300-313.

Mulet, J.M., Alemany, B., Ros, R., Calvete, J.J. and Serrano, R. (2004) Expression of a plant serine *O*-acetyltransferase in *Saccharomyces cerevisiae* confers osmotic tolerance and creates an alternative pathway for cysteine biosynthesis. *Yeast,* **21,** 303-312.

Mur, L.A.J., Mandon, J., Persijn, S., Cristescu, S.M., Moshkov, I.E., Novikova, G.V. et al. (2012) Nitric oxide in plants: an assessment of the current state of knowledge. *AoB Plants* **5,** pls052; doi:10.1093/aobpla/pls052.

Pankhurst, C.E., Schwinghamer, E.A., Thorne, S.W. and Bergersen, F.J. (1974) The flavin content of clovers relative to symbiosis with a riboflavin-requiring mutant of *Rhizobium trifolii*. *Plant Physiol* **53,** 198-205.

Perazzolli, M., Dominici, P., Romero-Puertas, M.C., Zago, E., Zeier, J., Sonoda,M., Lamb, C. and Delledonne, M. (2004) Arabidopsis nonsymbiotic hemoglobin AHb1 modulates nitric oxide bioactivity. *Plant Cell,* **16,** 2785-2794.

Qu, Z.L., Wang, H.Y. and Xia, G.X. (2005) *GhHb1:* a nonsymbiotic hemoglobin gene of cotton responsive to infection by *Verticillium dahliae*. *Biochim. Biophys. Acta,* **1730,** 103-113.

Ríos, G., Cabedo, M., Rull, B., Yenush, L., Serrano, R. and Mulet, J.M. (2013) Role of the yeast multidrug transporter Qdr2 in cation homeostasis and the oxidative stress response. *FEMS Yeast Res.* **13,** 97-106.

Rodríguez-Celma, J., Vázquez-Reina, S., Orduna, J., Abadía, A., Abadía, J., Álvarez-Fernández, A. and López-Millán, A.F. (2011) Characterization of flavins in roots of Fe-deficient strategy I plants, with a focus on *Medicago truncatula*. Plant Cell Physiol. **52:** 2173-2189.

Ross, E.J.H., Shearman, L., Mathiesen, M., Zhou, Y.J., Arredondo-Peter, R., Sarath, G. and Klucas, R.V. (2001) Nonsymbiotic hemoglobins in rice are synthesized during germination and in differentiating cell types. *Protoplasma,* **218,** 125-133.

Rubio, M.C., Becana, M., Kanematsu, S., Ushimaru, T. and James, E.K. (2009) Immunolocalization of antioxidant enzymes in high-pressure frozen root and stem nodules of *Sesbania rostrata*. *New Phytol.* **183,** 395-407.

Seregélyes, C., Mustárdy, L., Ayaydin, F., Sass, L., Kovács, L., Endre, G., Lukács, I., Vass, I., et al. (2000) Nuclear localization of a hypoxia-inducible novel non-symbiotic hemoglobin in cultured alfalfa cells. *FEBS Lett.* **482,** 125-130.

Smagghe, B.J., Blervacq, A.S., Blassiau, C., Decottignies, J.P., Jacquot, J.P., Hargrove, M.S. and Hilbert, J.L. (2007) Immunolocalization of non-symbiotic hemoglobins during somatic embryogenesis in chicory. *Plant Sign. Behavior,* **2,** 43-49.

Smagghe, B.J., Hoy, J.A., Percifield, R., Kundu, S., Hargrove, M.S., Sarath, G., Hilbert, J.L., Watts, R.A., et al. (2009) Correlations between oxygen affinity and sequence classifications of plant hemoglobins. *Biopolymers,* **91,** 1083-1096.

Spyrakis, F., Bruno, S., Bidon-Chanal, A., Luque, F.J., Abbruzzetti, S., Viappiani, C., Dominici, P. and Mozzarelli, A. (2011) Oxygen binding to *Arabidopsis thaliana* AHb2 nonsymbiotic hemoglobin: evidence for a role in oxygen transport. *IUBMB Life,* **63,** 355-362.

Sturms, R, Kakar, S., Treent, III J. and Hargrove, M.S. (2010) *Trema* and *Parasponia* hemoglobins reveal convergent evolution of oxygen transport in plants. *Biochemistry,* **49,** 4085-4093.

Trent, J.T., Watts, R.A. and Hargrove, M.S. (2001) Human neuroglobin, a hexacoordinate hemoglobin that reversibly binds oxygen. *J. Biol. Chem.* **276,** 30106-30110.

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.

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

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

Vigeolas, H., Hühn, D. and Geigenberger, P. (2011) Nonsymbiotic hemoglobin-2 leads to an elevated energy state and to a combined increase in polyunsaturated fatty acids and total oil content when overexpressed in developing seeds of transgenic Arabidopsis plants. *Plant Physiol.* **155,** 1435-1444.

Vinogradov, S.N., Hoogewijs, D., Bailly, X., Arredondo-Peter, R., Guertin, M., Gough, J., Dewilde, S., Moens, L. et al. (2005) Three globin lineages belonging to two structural classes in genomes from the three kingdoms of life. *Proc. Natl. Acad. Sci. USA,* **102,** 11385-11389.

Wang, Y., Elhiti, M., Hebelstrup, K.H., Hill, R.D. and Stasolla, C. (2011) Manipulation of hemoglobin expression affects *Arabidopsis* shoot organogenesis. *Plant Physiol. Biochem.* **49,** 1108-1116.

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.

Weber, R.E. and Vinogradov, S.N. (2001) Nonvertebrate hemoglobins: functions and molecular adaptations. *Physiol. Rev.* **81,** 569-628.

Zhang, L., Onda, K., Imai, R., Fukuda, R., Horiuchi, H. and Ohta, A. (2003) Growth temperature downshift induces antioxidant response in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **307,** 308-314.

Table 1 Rate and equilibrium constants[a] of O_2 binding for representative globins

Globin	$k_{on}^{O_2}$ (μM^{-1} s^{-1})	$k_{off}^{O_2}$ (s^{-1})	K^{O2} (nM)	Reference
High O_2-affinity globins				
Lotus Glb1-1	81	0.004	0.05	This study
Lotus Glb1-2	300	0.27	0.90	This study
Rice Glb1	68	0.038	0.56	Smagghe *et al.* (2009)
Barley Glb1	50	0.027	0.54	Smagghe *et al.* (2009)
Tomato Glb1	30	0.50	15	Ioanitescu *et al.* (2005)
Trema Hb	210	0.38	1.82	Sturms *et al.* (2010)
Lotus Glb2	77	0.86	11	This study
Arabidopsis Glb1	74	0.12	1.61	Smagghe *et al.* (2009)
Arabidopsis Glb2	86	0.14	2.0	Smagghe *et al.* (2009)
Arabidopsis Glb2	150	2.00	13	Ioanitescu *et al.* (2005)
Ascaris Hb	1.5	0.004	2.73	Weber and Vinogradov (2001)
Human neuroglobin	300	0.4	1.3	Ioanitescu *et al.* (2005)
O_2 transporters				
Soybean Lb*a*	130	5.6	43	Smagghe *et al.* (2009)
Parasponia Hb	165	15.0	90	Sturms *et al.* (2010)
Sperm whale Mb	14	12	857	Weber and Vinogradov (2001)

[a] k_{on}, association rate; k_{off}, dissociation rate; K, equilibrium constant (k_{off}/k_{on}).

FIGURE LEGENDS

Figure 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.

Figure 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.

Figure 3. Immunoblots showing antibody specificity and globin expression in yeast cells.

(a) Recombinant proteins challenged with the antibodies generated against the three globin classes. Protein loaded was 0.1 μg and dilutions of primary and secondary antibodies were 1:2,000-1:3,000 and 1:20,000, respectively.

(b) Extracts of yeast cells expressing the globins. Protein loaded was 10-40 μg and dilutions of primary and secondary antibodies were 1:250 and 1:20,000, respectively. Labels underneath the lanes indicate the globin name or the yeast wild-type (*wt*) and flavoHb-deficient (*yhb1*) mutant strains.

Figure 4. Immunogold localization of globins in legume tissues.

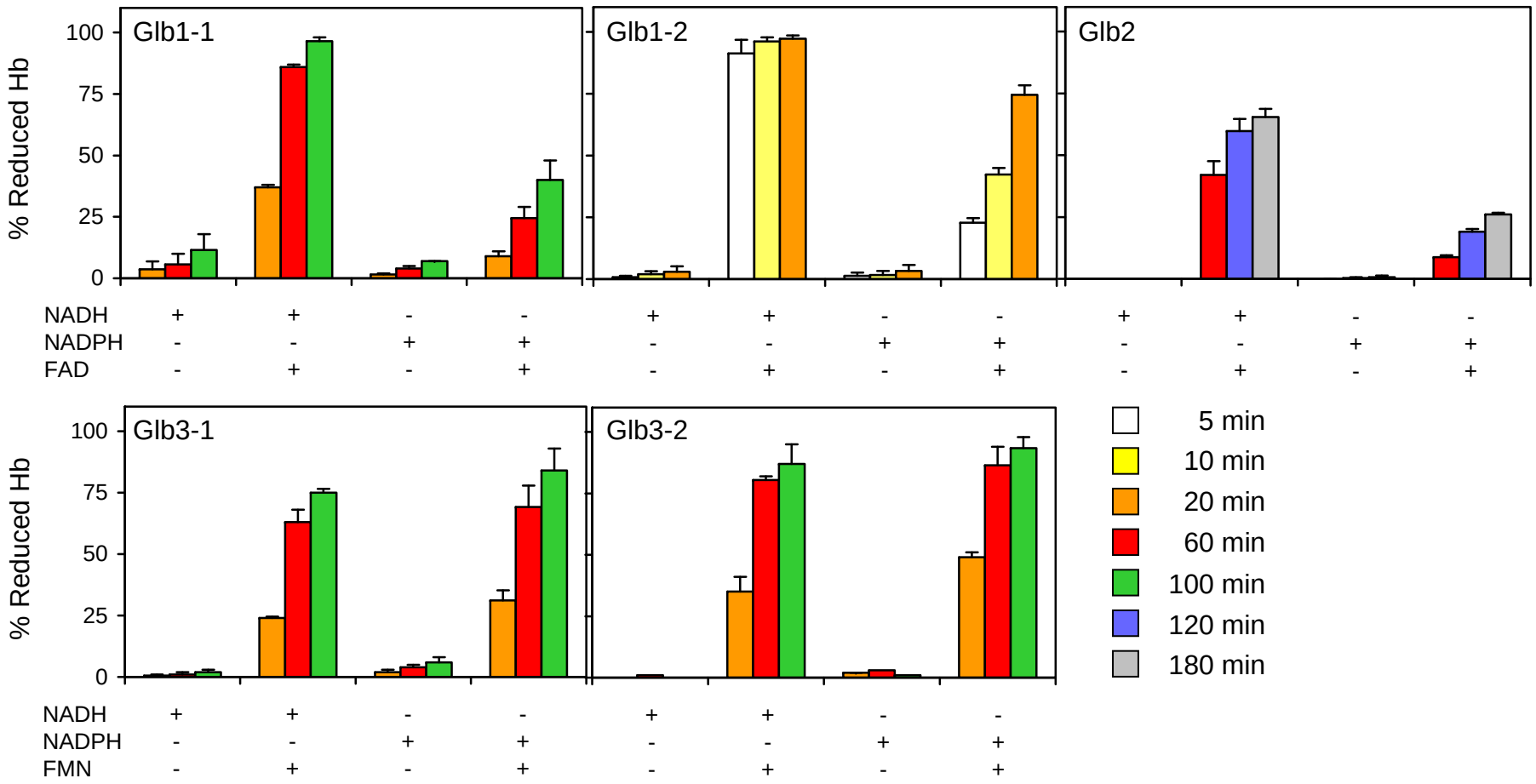
The figure shows only some representative micrographs to avoid redundancy because similar subcellular localizations were found for the three classes of globins. For Glb1, labeling (arrows) was predominantly observed on the nuclei of nodule (a) and root (b) cells. Likewise, significant labeling (arrows) was found for Glb2 (c) and Glb3 (d) on nuclei of nodule host cells. Weak labeling was seen for Glb3 on chloroplasts (e). Shown is also, for comparison, labeling for Lb on nuclei (arrows) and cytosol (double arrowheads) of nodule host cells (f). Negative controls, in which non-immune sera have replaced the antibodies, are shown, for example, for nuclei of nodule cells (g) and chloroplasts (h). Symbols: b, bacteroid; cyt,

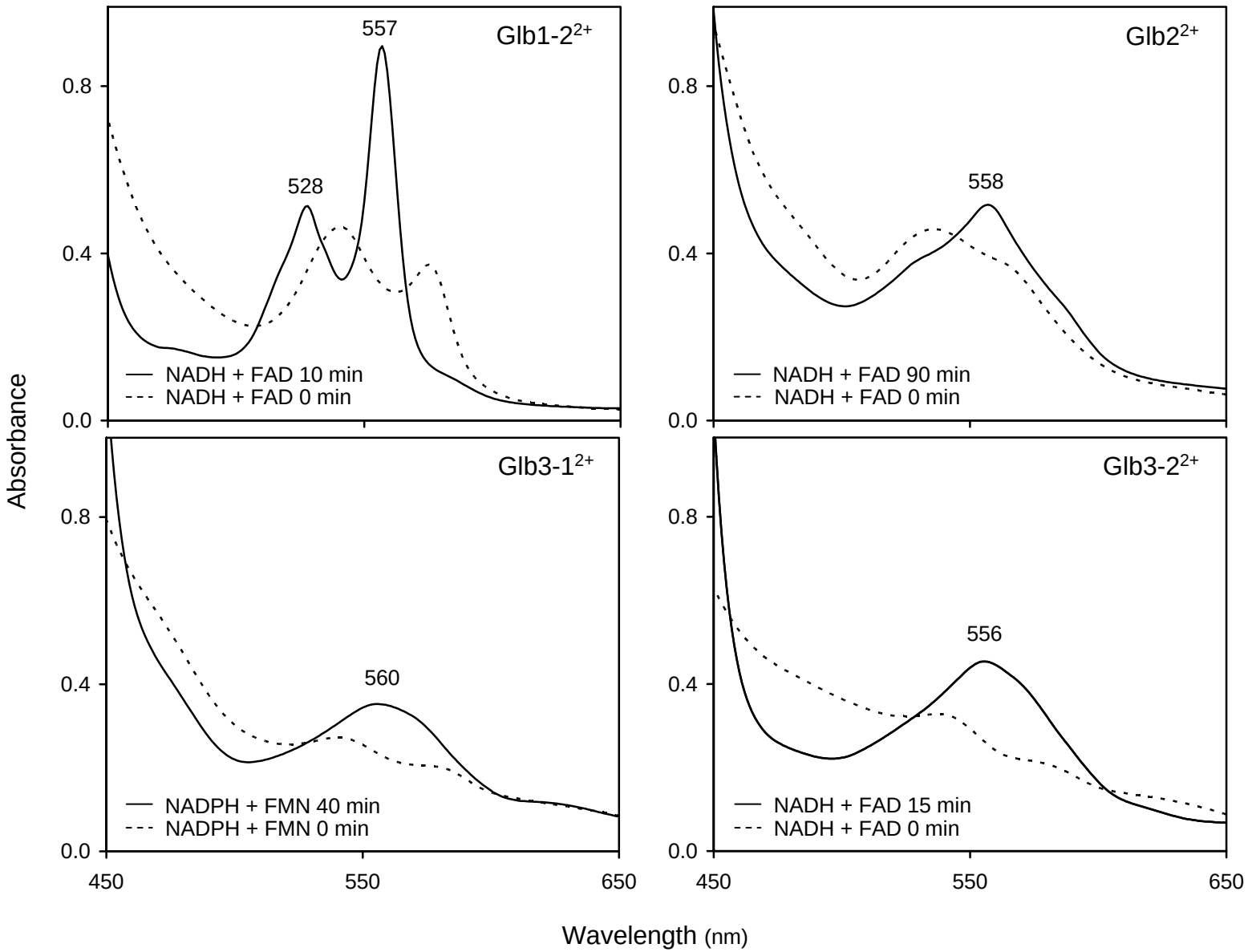
cytosol; ch, chloroplast; is, intercellular space; m, mitochondrion; n, nucleus; nu, nucleolus; s, starch grain; w, cell wall. Bars, 1 μm.

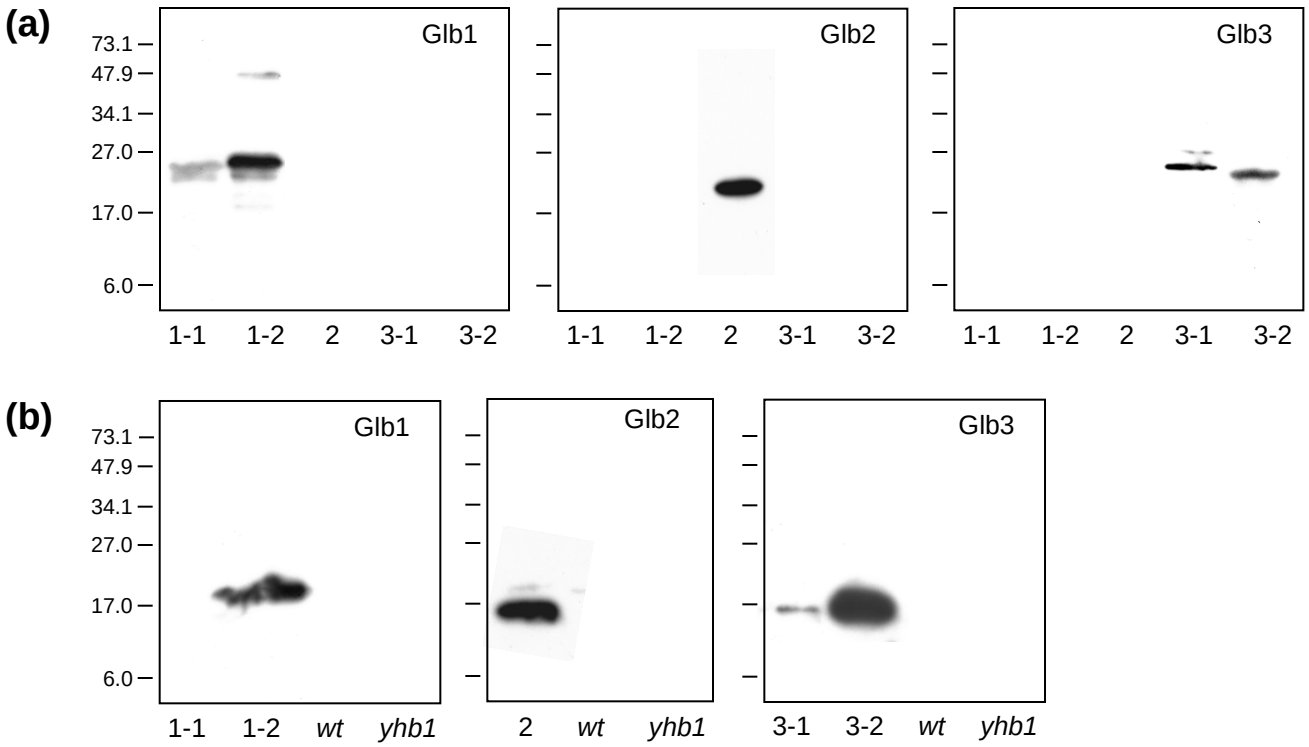
Figure 5. Tolerance of yeast cells expressing globins to oxidative and nitrosative stress.

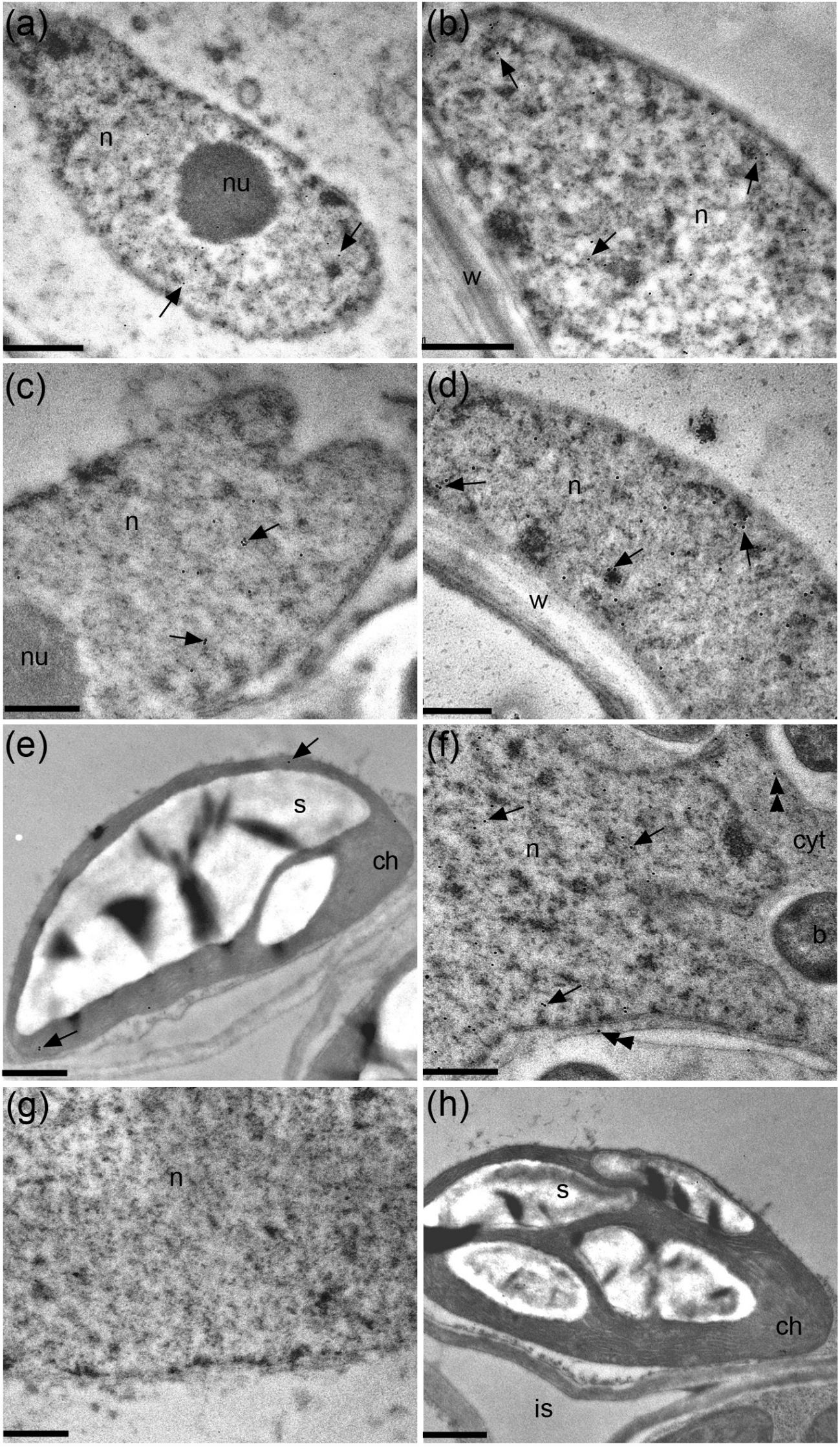
(a) Drop tests were performed by growing transformed cells until saturation in SD medium. Cell cultures were then diluted 1:10, 1:100 and 1:1000, spotted onto plates of YPD medium containing MV (1.5 mM) or $CuSO_4$ (10 mM). These compounds were added after autoclaving, but prior to gelification, and growth was recorded after 4-5 days. 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.

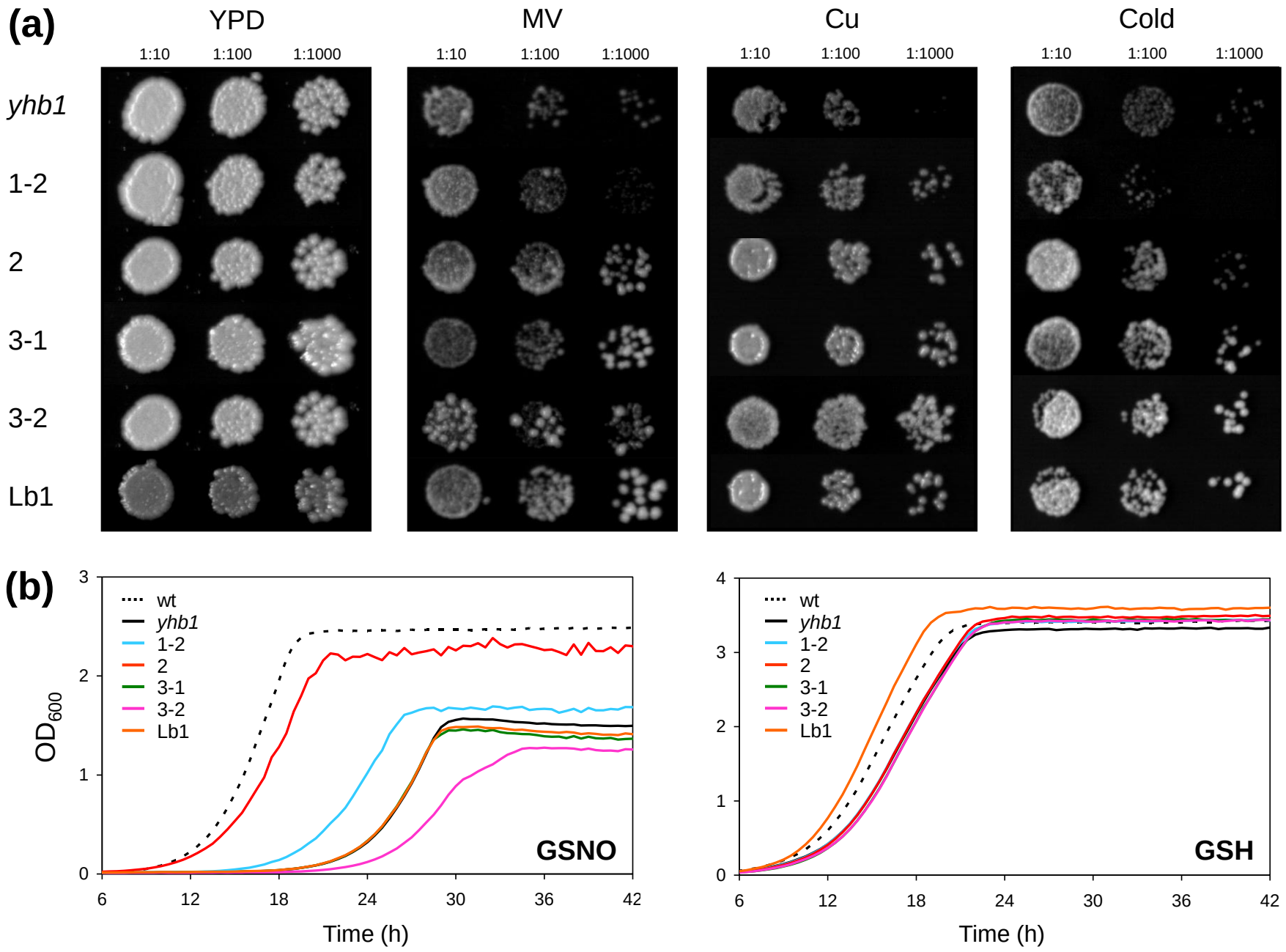
(b) Liquid cell cultures were grown until saturation in SD medium, then diluted to an initial OD_{660} of 0.01 in YPD medium containing 5 mM GSNO (left) or 5 mM GSH (right). Growth was monitored in microtitre plates using the Bioscreen C microbiological workstation (http://www.bioscreen.fi) with automatic recording of OD_{600} every 30 min. Each point represents the mean of three replicates with SE<2% in most cases (error bars are not depicted for clarity). The experiment was reproduced independently three times with similar results.


% Reduced Hb
100
75
50
25
0
Glb1-1
Glb1-2
Glb2
NADH + + - - + + - - + + - -
NADPH - - + + - - + + - - + +
FAD - + - + - + - + - + - +
Glb3-1
Glb3-2
NADH + + - - + + - -
NADPH - - + + - - + +
FMN - + - + - + - +
5 min
10 min
20 min
60 min
100 min
120 min
180 min


Glb1-2^{2+}
557
528
NADH + FAD 10 min
NADH + FAD 0 min
Glb2^{2+}
558
NADH + FAD 90 min
NADH + FAD 0 min
Glb3-1^{2+}
560
NADPH + FMN 40 min
NADPH + FMN 0 min
Glb3-2^{2+}
556
NADH + FAD 15 min
NADH + FAD 0 min
Absorbance
0.0
0.4
0.8
450
550
650
Wavelength (nm)


(a)
Glb1
Glb2
Glb3
73.1
47.9
34.1
27.0
17.0
6.0
1-1
1-2
2
3-1
3-2
(b)
Glb1
Glb2
Glb3
wt
yhb1


(a)
n
nu
(b)
n
w
(c)
n
nu
(d)
n
w
(e)
s
ch
(f)
n
cyt
b
(g)
n
(h)
s
ch
is


(a)
YPD
MV
Cu
Cold
1:10
1:100
1:1000
yhb1
1-2
2
3-1
3-2
Lb1
(b)
wt
yhb1
1-2
2
3-1
3-2
Lb1
OD600
Time (h)
GSNO
GSH

Legends of Supplementary Figures

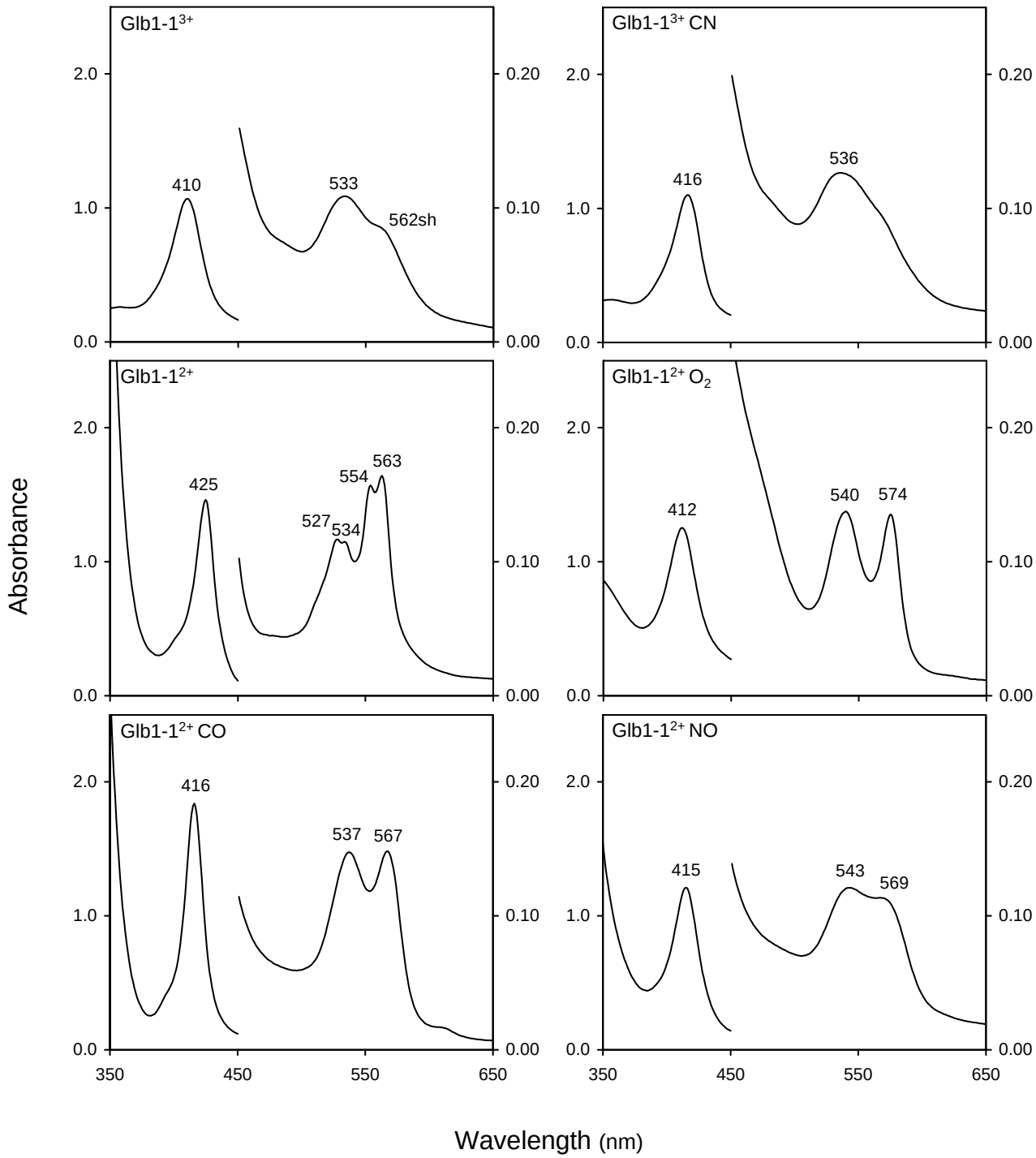
Figure S1. Soret-visible spectra of ferric and ferrous Glb1-1 and representative complexes.

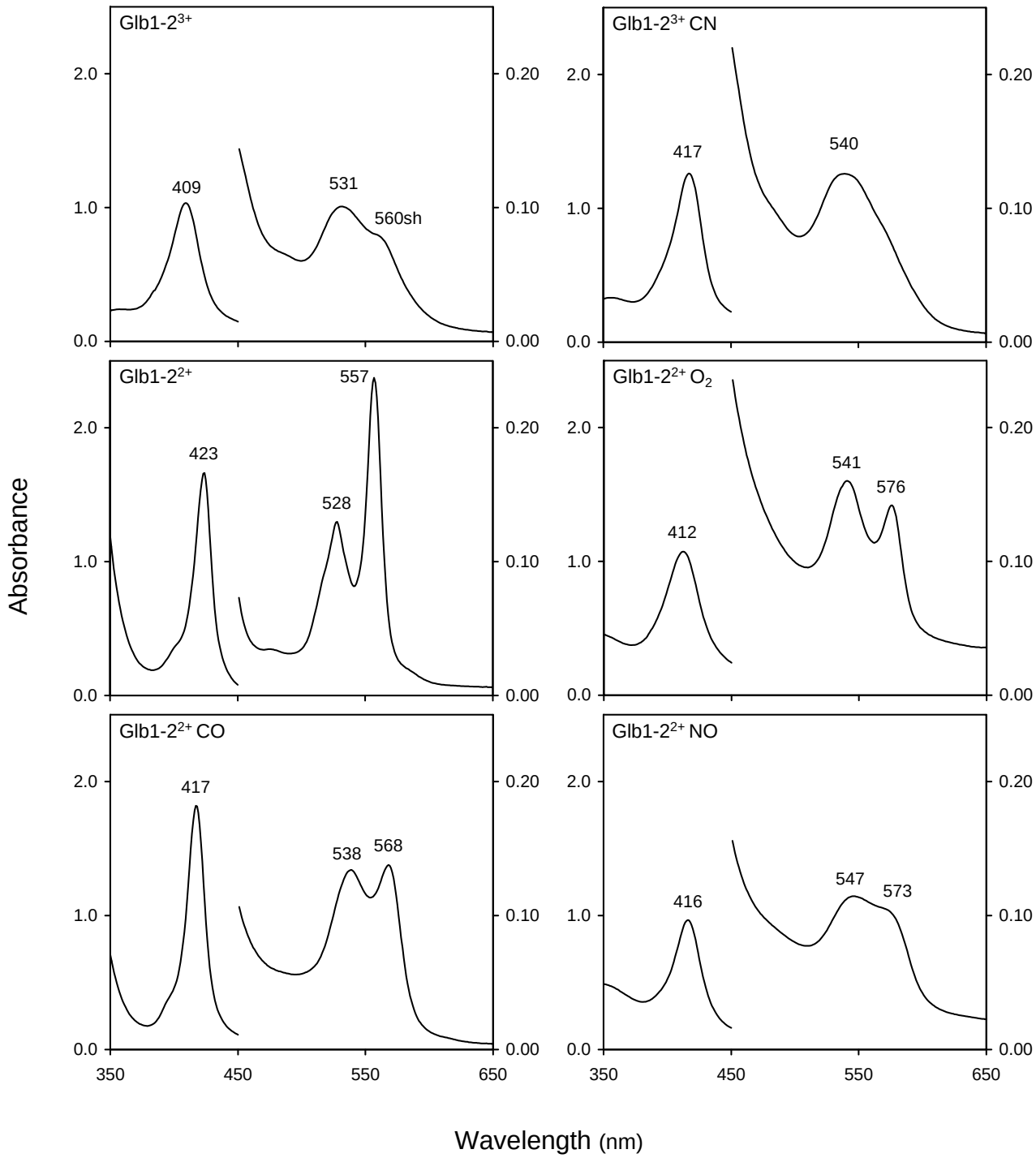
Figure S2. Soret-visible spectra of ferric and ferrous Glb1-2 and representative complexes.

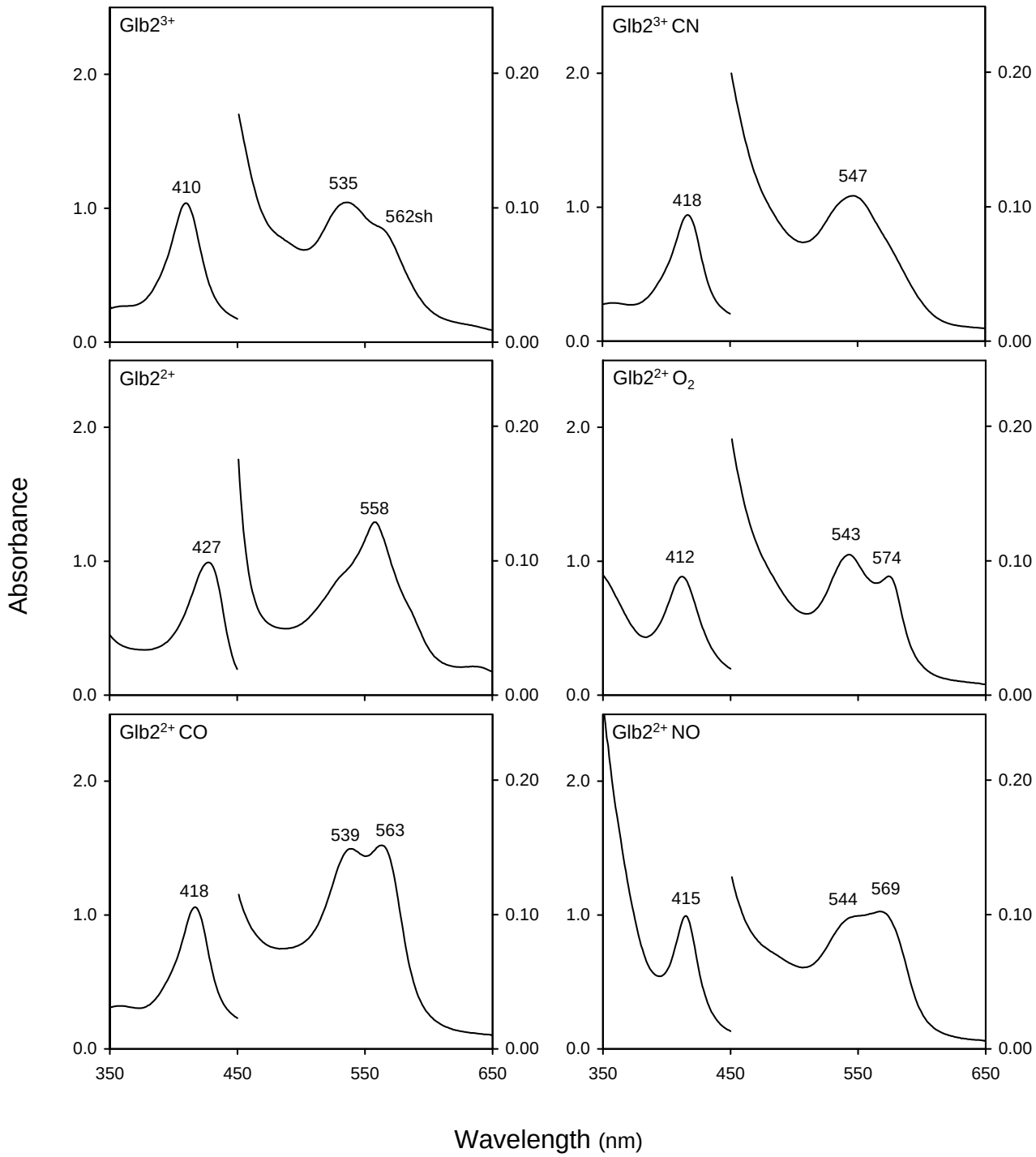
Figure S3. Soret-visible spectra of ferric and ferrous Glb2 and representative complexes.

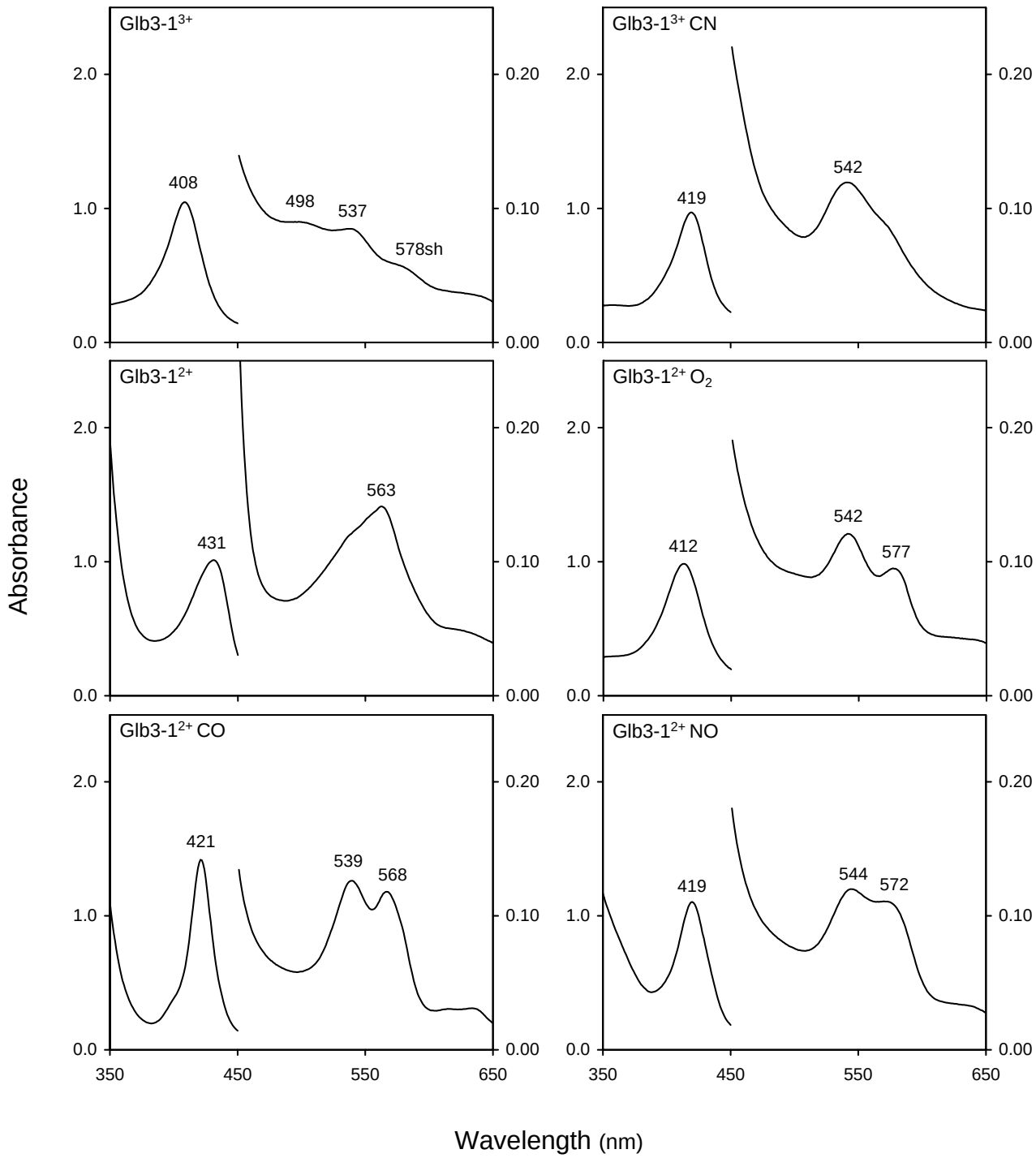
Figure S4. Soret-visible spectra of ferric and ferrous Glb3-1 and representative complexes.

Figure S5. Soret-visible spectra of ferric and ferrous Glb3-2 and representative complexes.


Glb1-1^{3+}
410
533
562sh
Glb1-1^{3+} CN
416
536
Glb1-1^{2+}
425
527
534
554
563
Glb1-1^{2+} O_2
412
540
574
Glb1-1^{2+} CO
416
537
567
Glb1-1^{2+} NO
415
543
569
Absorbance
Wavelength (nm)


Glb1-2^{3+}
409
531
560sh
Glb1-2^{3+} CN
417
540
Glb1-2^{2+}
423
528
557
Glb1-2^{2+} O_2
412
541
576
Glb1-2^{2+} CO
417
538
568
Glb1-2^{2+} NO
416
547
573
2.0
1.0
0.0
0.20
0.10
0.00
350
450
550
650
Absorbance
Wavelength (nm)


Glb2^{3+}
410
535
562sh
Glb2^{3+} CN
418
547
Glb2^{2+}
427
558
Glb2^{2+} O_2
412
543
574
Glb2^{2+} CO
418
539
563
Glb2^{2+} NO
415
544
569
2.0
1.0
0.0
0.20
0.10
0.00
350
450
550
650
Absorbance
Wavelength (nm)


Glb3-1^{3+}
408
498
537
578sh
Glb3-1^{3+} CN
419
542
Glb3-1^{2+}
431
563
Glb3-1^{2+} O_2
412
542
577
Glb3-1^{2+} CO
421
539
568
Glb3-1^{2+} NO
419
544
572
2.0
1.0
0.0
0.20
0.10
0.00
350
450
550
650
Absorbance
Wavelength (nm)

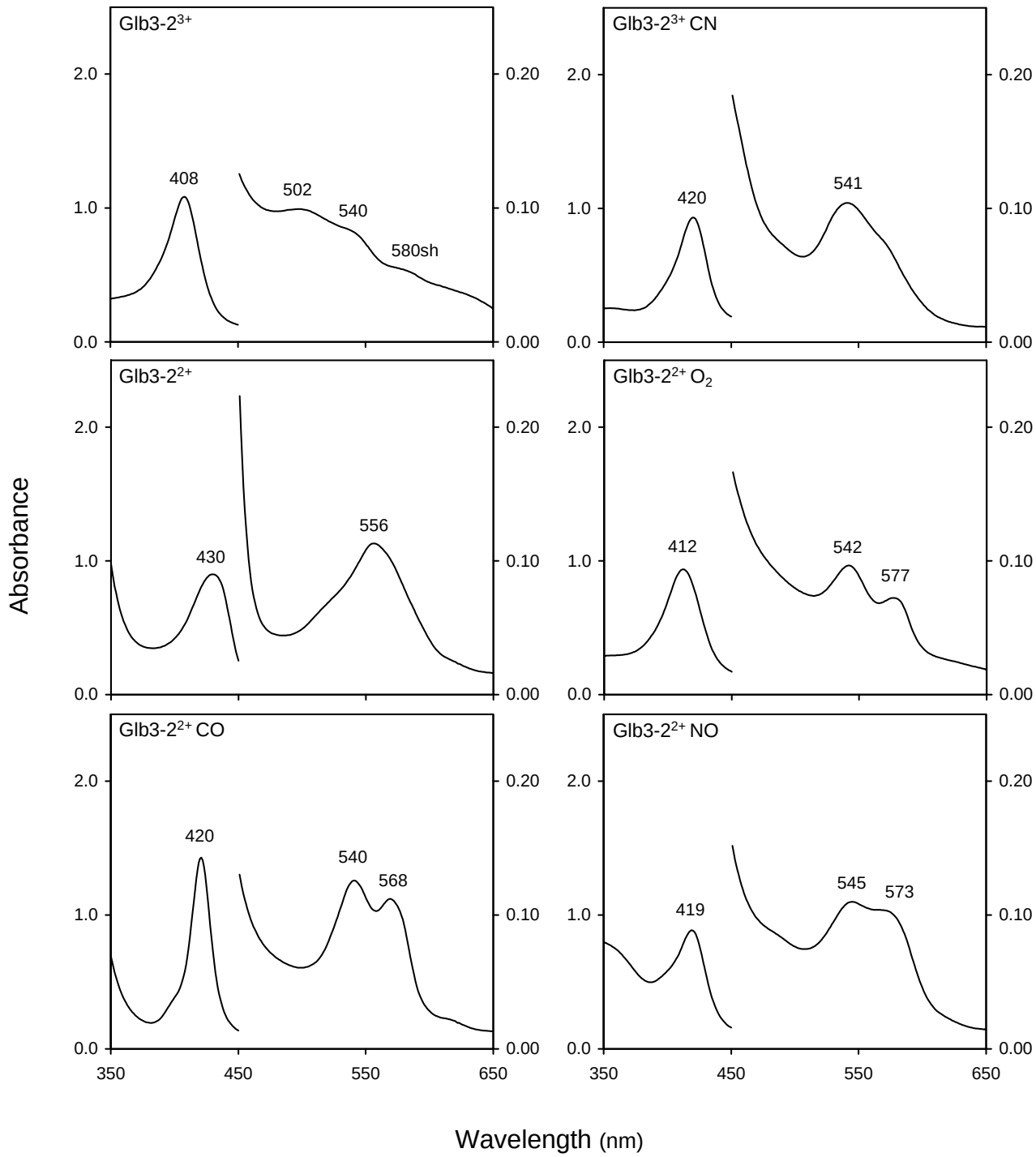

Glb3-2^{3+}
408
502
540
580sh
Glb3-2^{3+} CN
420
541
Glb3-2^{2+}
430
556
Glb3-2^{2+} O_2
412
542
577
Glb3-2^{2+} CO
420
540
568
Glb3-2^{2+} NO
419
545
573
Absorbance
Wavelength (nm)
0.0
1.0
2.0
0.00
0.10
0.20
350
450
550
650

Table S1 Oligonucleotides used in this study

Gene	Forward	Reverse
Glb1-1	CACCATGTCCACCCTTGGGAG	GGGGACCCGGGTAACTCTATC
Glb1-2	CACCATGGCAGAAAACACAACCA	AGCCCCAAAGCAACAATAAGTAG
Glb2	CACCATGGCTACATTCAGTGAG	GTTTAACAAGTTTTAGTGAAATAC
Glb3-1	CACCATGCAGAGTCTTCAACACAA	CTCATTCTTCTTTGCTATCATTCA
Glb3-2	CACCATGCAAAGCCTGCAGCAT	GTGCAGCGTACGGGTAAAAACA
Lb1	CACCATGGGTTTCACTGCACAG	CCTACTTCAACTCATTGC