the plant journal

SEB Society for Experimental Biology

The Plant Journal (2015) **81,** 467–479 doi: 10.1111/tpj.12744

THB1, a truncated hemoglobin, modulates nitric oxide levels and nitrate reductase activity

Emanuel Sanz-Luque[1,†], Francisco Ocaña-Calahorro[1,†], Amaury de Montaigu[2], Alejandro Chamizo-Ampudia[1], Ángel Llamas[1], Aurora Galván[1] and Emilio Fernández[1,*]

[1]*Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Córdoba, Campus de Rabanales, Campus de excelencia internacional (CeiA3), Edif. Severo Ochoa, 14071 Córdoba, Spain, and*

[2]*Department of Plant Developmental Biology, Max-Planck-Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, D-50829 Köln, Germany*

Received 18 June 2014; revised 21 November 2014; accepted 2 December 2014; published online 11 December 2014.
*For correspondence (e-mail bb1feree@uco.es)
†These authors contributed equally to this work.

SUMMARY

Hemoglobins are ubiquitous proteins that sense, store and transport oxygen, but the physiological processes in which they are implicated is currently expanding. Recent examples of previously unknown hemoglobin functions, which include scavenging of the signaling molecule nitric oxide (NO), illustrate how the implication of hemoglobins in different cell signaling processes is only starting to be unraveled. The extent and diversity of the hemoglobin protein family suggest that hemoglobins have diverged and have potentially evolved specialized functions in certain organisms. A unique model organism to study this functional diversity at the cellular level is the green alga *Chlamydomonas reinhardtii* because, among other reasons, it contains an unusually high number of a particular type of hemoglobins known as truncated hemoglobins (THB1–THB12). Here, we reveal a cell signaling function for a truncated hemoglobin of *Chlamydomonas* that affects the nitrogen assimilation pathway by simultaneously modulating NO levels and nitrate reductase (NR) activity. First, we found that *THB1* and *THB2* expression is modulated by the nitrogen source and depends on NIT2, a transcription factor required for nitrate assimilation genes expression. Furthermore, *THB1* is highly expressed in the presence of NO and is able to convert NO into nitrate *in vitro.* Finally, THB1 is maintained on its active and reduced form by NR, and *in vivo* lower expression of THB1 results in increased NR activity. Thus, THB1 plays a dual role in NO detoxification and in the modulation of NR activity. This mechanism can partly explain how NO inhibits NR post-translationally.

Keywords: nitric oxide, truncated hemoglobin, nitrogen metabolism, NIT2, *Chlamydomonas reinhardtii*, nitrate reductase.

INTRODUCTION

Hemoglobins (Hbs) belong to a large and diverse family of proteins found in all kingdoms of life. They were primarily studied as O_2-transporting and -storing molecules both in animal erythrocytes, and in nitrogen-fixing root nodules of plants. However, evidence supports that ancestral Hbs appeared as enzymes that detoxify NO through their NO dioxygenase activity (NOD) (Gardner, 2008). NOD activity, which produces NO_3^- from NO and O_2, is considered to be a ubiquitous activity for Hbs *in vitro* albeit they show different efficiencies. *In vivo* reduction of Hbs seems to limit their activities (Smagghe *et al.*, 2008). Flavohemoglobins from fungi and bacteria have a reductase domain responsible for reduction (Ermler *et al.*, 1995; Schinko *et al.*, 2010). Some Hbs, like AHb1 from *Arabidopsis,* are able to take electrons directly from NAD(P)H (Perazzolli *et al.*, 2004) and others use FAD as an electron carrier between NAD(P)H and Hb (Sainz *et al.*, 2013). Despite their ubiquity, the physiological functions of many Hbs remain unclear.

NO is a molecule implicated in the regulation of diverse physiological processes in all living beings. In photosynthetic organisms, it plays essential roles in plant growth, development and defence (Corpas *et al.*, 2004; He *et al.*, 2004; Villarreal and Martínez, 2009; Fernández-Marcos *et al.*, 2011). NO has also been shown to be an important regulator of the nitrate assimilation pathway in plants and algae (Du *et al.*, 2008; Jin *et al.*, 2009; de Montaigu *et al.*, 2010; Rosales *et al.*, 2011; Sanz-Luque *et al.*, 2013). In the unicellular alga *Chlamydomonas reinhardtii,* main genes

of the nitrate assimilation pathway are under the control of the master regulator NIT2 and are downregulated by ammonium (Fernandez and Galvan, 2008) in an NO- and cGMP-dependent manner (de Montaigu *et al.*, 2010, 2011). In addition, NO inhibits nitrate reductase activity (NR) and ammonium and nitrate uptakes (Sanz-Luque *et al.*, 2013). Furthermore, it is known that NO can be produced by NR when nitrite accumulates in the medium (Sakihama *et al.*, 2002). In fact, NR is the main source of NO reported in photosynthetic organisms where NO synthases have not been described with exception of the green alga *Ostreococcus tauri* (Foresi *et al.*, 2010). Thus, the regulation of NO levels is an important task for cells and Hbs are emerging as important players in NO detoxification.

Three types of hemoglobins are found in photosynthetic organisms and are categorized as symbiotic (sHb), nonsymbiotic (nsHb), and truncated hemoglobins (trHb). Symbiotic Hbs are found in nodules and scavenge O_2 to avoid the inactivation of nitrogenase (Hoy and Hargrove, 2008). Nonsymbiotic Hbs are categorized into class 1 (GLB1) and class 2 (GLB2) based on phylogenetic analysis and on their O_2 binding properties (Hoy and Hargrove, 2008; Smagghe *et al.*, 2009; Gupta *et al.*, 2011). Proteins from GLB1 class, with high O_2 affinity, are the best studied Hbs and are important players in NO detoxification in plants (Perazzolli *et al.*, 2004; Hoy and Hargrove, 2008; Gupta *et al.*, 2011). Processes in which these proteins control NO homeostasis are being currently reported (Hebelstrup and Jensen, 2008; Wang *et al.*, 2011; Mur *et al.*, 2012; Hebelstrup *et al.*, 2013). Truncated hemoglobins were the last to be identified in plants (Watts *et al.*, 2001), and their functions remain poorly understood. These trHbs have the typical 3-on-3 α-helical globin fold truncated to a 2-on-2 α-helical 'sandwich', although the protein length is not necessarily shorter (Pesce *et al.*, 2000; Wittenberg *et al.*, 2002; Hoy and Hargrove, 2008). Phylogenetic analysis categorizes them into three groups: I (trHbN), II (trHbO), and III (TrHbP). Group I is the most diverse (Vuletich and Lecomte, 2006), with members in eubacteria, cyanobacteria, protozoa, algae and plants, and has mostly been studied in bacteria. NOD activity has been thoroughly characterized for the trHb of group I GlbN from *Mycobacterium* (Ouellet *et al.*, 2002; Lama *et al.*, 2006) and it has been recently reported in plants (Kim *et al.*, 2014). Residues in GlbN important for the structure and NOD activity have been extensively characterized (Pesce *et al.*, 2000; Ouellet *et al.*, 2002; Wittenberg *et al.*, 2002; Milani *et al.*, 2003; Vuletich and Lecomte, 2006; Bidon-Chanal *et al.*, 2009; Daigle *et al.*, 2009; Stewart and Coyne, 2011). In this trHb a tunnel/cavity network formed by hydrophobic residues appears to be conserved. Oxygen and NO diffuse by different tunnel branches. Oxygen is first bound to the heme group and diffuses through a short tunnel branch. Then, a Phe residue (PheE15) in the long tunnel branch changes its conformation and allows the diffusion of NO to the heme cavity where it is converted to nitrate (Milani *et al.*, 2003; Bidon-Chanal *et al.*, 2009; Oliveira *et al.*, 2012). Knowledge on the physiological roles of trHbs is scarce. In *Mycobacterium,* trHb protects against NO damage and could play an important function in mycobacterial infection persistence (Ouellet *et al.*, 2002). In two species of Raphidophytes, a gene coding a trHb is fused to the NR gene, and a possible participation in the conversion of NO to nitrate followed by nitrate reduction is suggested (Stewart and Coyne, 2011). In *Chlamydomonas* a large family of 12 trHbs has recently been reported. A physiological role has been described only for one of them (THB8). THB8 is required for hypoxic survival in a process involving NO, but its activity and specific role are unclear (Hemschemeier *et al.*, 2013).

In the present work we have reported two trHb from *Chlamydomonas* (THB1 and THB2) that belong to group I and are regulated by the nitrogen source and the regulatory gene *NIT2.* Furthermore, we show that THB1, which is upregulated by NO, has NOD activity and is maintained in its active and reduced form through a mechanism that takes electrons from NR and alters NR activity. Thus, we report a physiological role for THB1 in removing excess NO and modulating NR activity in *Chlamydomonas.*

RESULTS

THB1 and THB2 are typical truncated hemoglobins

In *Chlamydomonas* 12 genes containing globin domains are present (THB1–THB12). All of them have the typical truncated hemoglobin organization of a 2-on-2 α-helical 'sandwich' (Hemschemeier *et al.*, 2013). However, only THB1, THB2, THB3 and THB4 have sizes and structures similar to other trHbs identified to date. *THB1* and *THB2* genes are contiguous on chromosome 14, and *THB3* and *THB4* are adjacent on chromosome 4. *THB1-4* cDNAs were isolated by PCR and their sequences annotated (accession numbers KC992719, KC992720, KC992718, and KC992721, respectively). Deduced proteins sequences showed different degrees of conservation with other trHbs identified in bacteria and plants (Figure S1). THB1, THB2, and THB4 are highly conserved and show 32.1, 32.6 and 30.9% identity with GlbN of *Mycobacterium tuberculosis,* respectively. In fact, these three THBs show a close phylogenetic relationship with trHbs of group I (Figure 1a), some of which are reported as enzymes with NOD activity. THB3 is more atypical and it does not cluster clearly with groups I or II. The sequence identity between THB1 and THB2 is 63.1%. This high conservation and their contiguous localization on chromosome 14 suggest a gene duplication event.

Alignment of these THBs with GlbN and other truncated hemoglobins showed that important residues for NOD activity, interaction with the heme group, structure, and tunnel network formation (Pesce *et al.*, 2000; Daigle *et al.*,

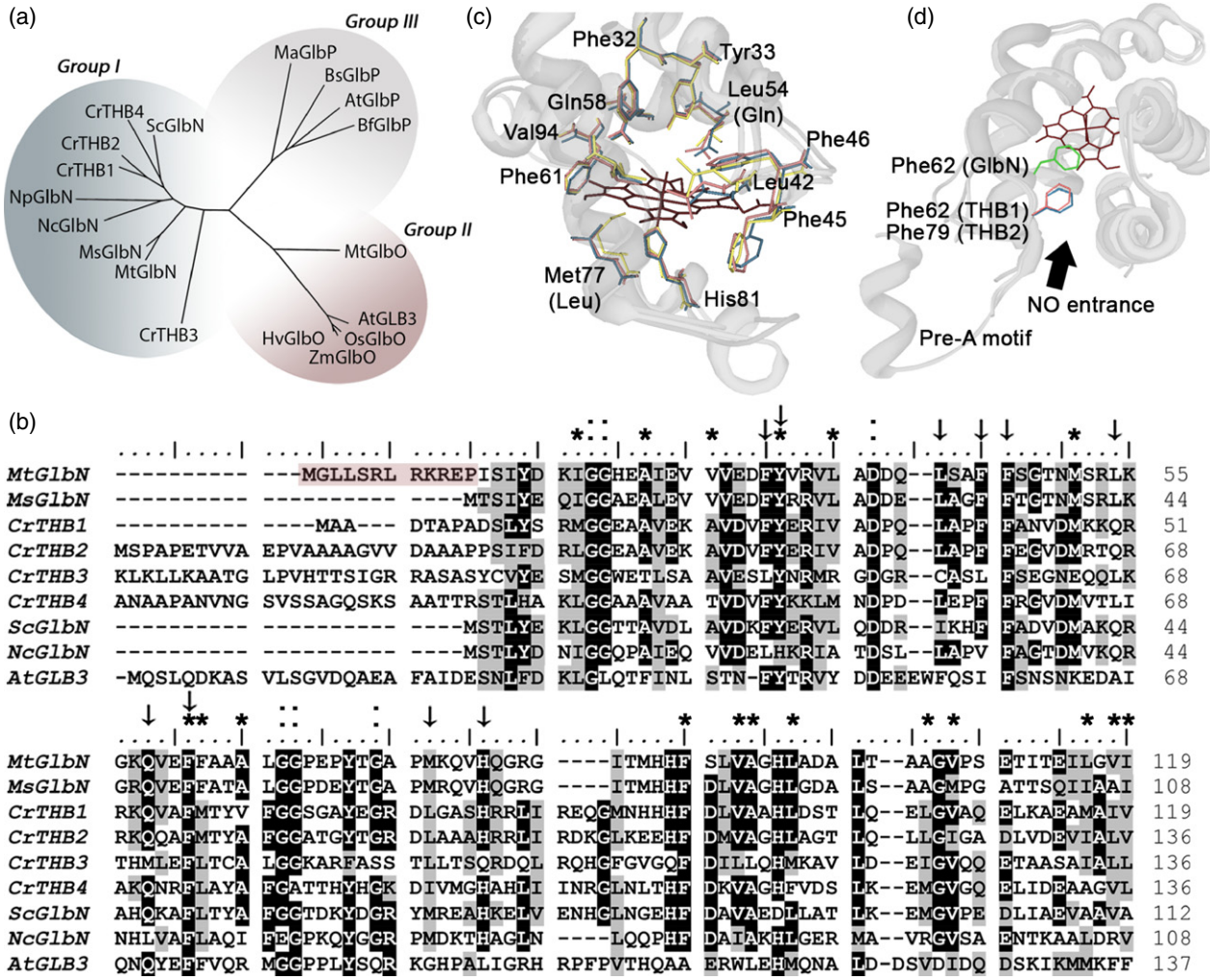

Figure 1. Analysis of truncated hemoglobin sequences and structures.
(a) Phylogenetic tree of THB1-4 and truncated hemoglobins from groups I, II and III (Genious software, neighbour-joining tree).
(b) Alignment of THB1–THB4 and several trHb from group I. Residues related to hydrophobic tunnels are indicated with '*', those important for NOD activity and heme interaction are marked with '↓'and residues relevant for maintaining the proper structure are indicated with ':'. The 12 residues of the Pre-A sequence in MtGlbN are highlighted with a red box. Sequences were aligned by using the ClustalW method and Bioedit software. Accession numbers are listed in Table S1. Alignment of complete sequences is represented in Figure S2.
(c) Tridimensional position of important residues for NOD activity in MtGlbN (yellow), CrTHB1 (red) and CrTHB2 (blue). These residues correspond to '↓'in (b). Amino acids are labeled for MtGlbN, and the residues that differ in CrTHB1 and CrTHB2 are annotated in brackets.
(d) Spatial localization of Phe acting as the NO entry gate in the long tunnel branch in MtGlbN and CrTHB1 and CrTHB2. CrTHB1 and CrTHB2 structures were generated using Swiss-model (http://swissmodel.expasy.org/). Crystal structure of MtGlbN was taken from the Protein Data Bank (reference 1IDR). (c) and (d) images were produced with Swiss-Pdb Viewer software.

2009; Stewart and Coyne, 2011) were conserved in THB1, THB2 and THB4 (Figure 1b), suggesting that these THBs could function as NOD enzymes. Although THB3 also shows significant similarity with other trHbs, this protein lacks important residues such as the histidine implicated in heme coordination. *In silico* predicted structures for THB1 and THB2 showed that positions and orientations of essential residues for NOD activity were conserved respect to GlbN from *M. tuberculosis* (Figure 1c). In addition, although the *Chlamydomonas* THB sequences show different residues at some important positions, this is compensated by the appearance of amino acids at other positions that can play a similar role. For example, Phe62 from GlbN that works as an NO gate to the heme group could correspond to Phe62 and Phe79 in THB1 and THB2, respectively, supporting a similar mechanism for NO entry (Figure 1d). Nonetheless, there is an N-terminal 12-residue Pre-A motif in GlbN from *Mycobacterium tuberculosis* that is important for NOD activity (Lama *et al.*, 2009) and is not conserved in the *Chlamydomonas* THBs (Figures 1 and S2).

THB1 and *THB2* are regulated by the nitrogen source in a NIT2-dependent way

It is known that intracellular NO production is increased in cells growing in several nitrogen sources (de Montaigu *et al.*, 2010; H., Wang *et al.*, 2012; Wei *et al.*, 2014), but the mechanisms implicated in the regulation of NO levels are unknown. Thus, we have studied the expression patterns of *THB1-4* in ammonium (A), nitrate plus ammonium (NA), nitrate (N) and N-free medium (–N) to elucidate a possible role for some of these THBs (Figure 2a). *THB1* and *THB2* were strongly regulated by nitrogen but not *THB3* and *THB4. THB1* showed highest expression levels in NA medium, even at short time points (30 min). This gene also showed high expression in N medium with an increase of transcript levels after 3 h of induction, whereas the lowest expression was detected in –N. *THB2* was preferentially expressed in N medium with maximum levels of transcript after 1 h of induction. *THB2* also showed a significant upregulation in NA after 3 h and showed lowest expression in A medium.

THB2 showed a similar expression pattern to genes belonging to the nitrate assimilation cluster in *Chlamydomonas* (Quesada *et al.*, 1993; Loppes *et al.*, 1999; Fernandez and Galvan, 2008; de Montaigu *et al.*, 2010). These cluster genes are under the control of the nitrate-specific regulatory transcription factor NIT2 (Fernandez *et al.*, 1989; Quesada *et al.*, 1998; Camargo *et al.*, 2007). A previously characterized nit2 insertional mutant (89.87) was used (Gonzalez-Ballester *et al.*, 2005; Camargo *et al.*, 2007) to study whether NIT2 modulates *THB1* and *THB2* expression. Expression of these two genes was not significantly affected by nitrogen in the 89.87 mutant (Figure 2b). Interestingly, NIT2 was important not only for the positive response to N but also for NA upregulation of *THB1* and *THB2.* In the nit2 mutant, both *THB* genes showed a basal expression. These data point to a NIT2 and nitrogen-dependent regulation mechanism of *THB1* and *THB2,* and suggest that these genes could play an important role in the inorganic nitrogen assimilation pathway. These results were similar in both Tris–acetate–phosphate (TAP) medium

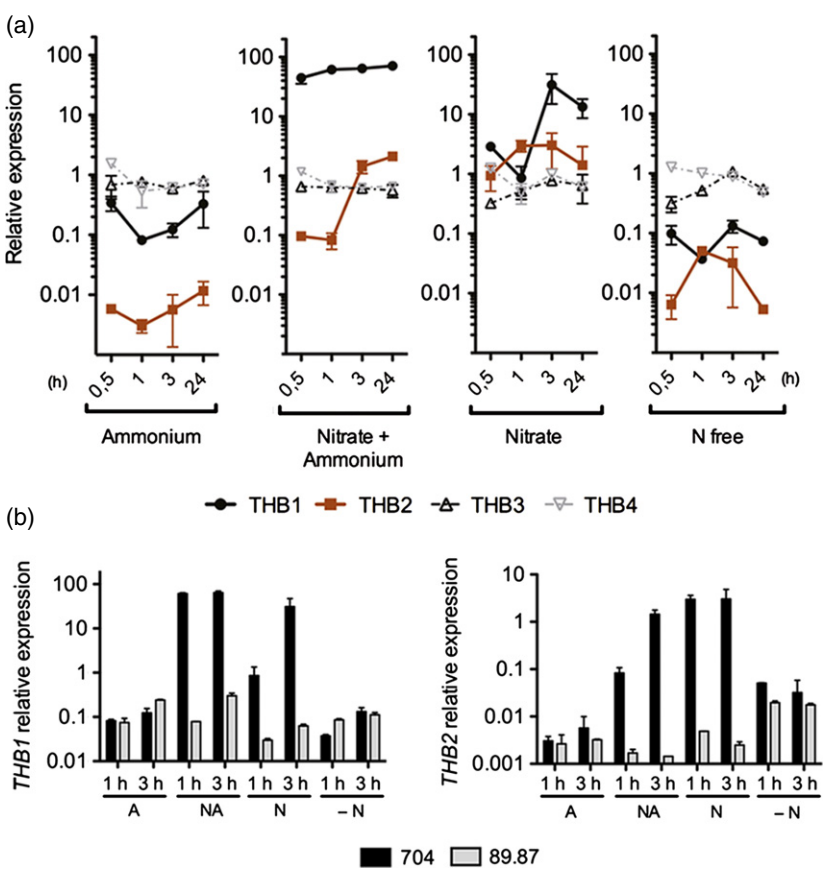

Figure 2. *THBs* expression in different nitrogen sources in 704 (wt) and 89.87 (nit2^{-} mutant) strains.
Cells were grown in TAP medium containing 8 mM NH_4Cl until the exponential growth phase, and cells were washed and incubated in the indicated medium. Ammonium and nitrate concentrations were 8 and 4 mM, respectively.
(a) *THB1-4* expression in the 704 (wt) strain.
(b) *THB1* and *THB2* expression in 89.87 (nit2^{-} mutant) strain. The housekeeping gene corresponds to a ubiquitin ligase that shows constitutive expression under all the experimental conditions. The error bars represent the standard deviation (SD) of three biological replicates.

and minimal medium with CO_2 as the carbon source (Figure S3).

THB1 and *THB2* are differentially expressed in the presence of NO

Given the common capacity of Hbs to function as NODs (Smagghe *et al.*, 2008), it has been proposed that transcriptional induction of Hb genes by NO should be considered as strong evidence for their NO metabolic function (Gardner, 2012). Thus, we have studied if NO modulated *THB1* and *THB2* expression. We have used DEA-NONOate (50 and 100 μM) and GSNO (100 and 200 μM) as NO donors. The first one releases NO in minutes and the second one has a longer half-lifetime (Floryszak-Wieczorek *et al.*, 2006; Li and Lancaster, 2009). Both NO donors caused up- and downregulation of *THB1* and *THB2,* respectively, in −N and N media (Figure 3). Nitrite, used as control, at the same concentration of the NO donor did not produce any effect. Importantly, DEA-NONOate and GSNO have different chemical structures but had similar effects on the transcription of both genes (Figure 3). Upregulation of *THB1* points to a possible physiological role as NOD enzyme. *THB2* downregulation supports the idea that *THB2* is transcriptionally regulated by the same mechanisms than *NIA1,* which is repressed by NO (de Montaigu *et al.*, 2010). From here on, our studies on THB1 are focused in understanding the role of this hemoglobin in nitrogen metabolism.

THB1 can be reduced to its ferrous and active form by NADH in the presence of flavins (FAD) and has NOD activity *in vitro*

NO dioxygenation appears as a general activity of hemoglobins *in vitro* but it is limited *in vivo* by protein reduction (Smagghe *et al.*, 2008). Here, we have studied the THB1 reduction process. While some hemoglobins are able to take electrons directly from nicotinamide adenine dinucleotide (NADH) as AHb1 from *A. thaliana* (Perazzolli *et al.*, 2004), others such as trHbs from *L. japonicus* need the presence of flavin cofactors (Sainz *et al.*, 2013). THB1 was expressed in *E. coli* and purified in aerobic conditions. THB1 showed absorption spectra typical of the ferric form, with a Soret peak at 411 nm and a broad peak at 538 nm with a weak shoulder at 565 nm. After the addition of sodium dithionite (anaerobic conditions) the THB1 reduction was performed and THB1(Fe^{2+}) exhibited a shifted Soret peak at 425 nm and two resolved bands at 528 and 558 nm (Figure 4a). Under aerobic conditions, NADH alone was unable to reduce the heme but, in the presence of FAD (2 μM), heme reduction and subsequent oxygenation were catalyzed. Spectra obtained showed two peaks at 544 and 579 nm similar to other reported hemoglobins (Perazzolli *et al.*, 2004; Sainz *et al.*, 2013). This reduction was slow with approximately 90% of maximal observed reduction reached in 60 min and a slight increase until 80 min (Figure 4b). Using 20 μM of FAD, the same levels of reduction were obtained in approximately 10 min (Figure 4b). These data demonstrate that THB1 needs FAD as an electron carrier to take reducing power from NADH. However, this kinetics seems to be too slow to have a significant physiological role.

Most, if not all, hemoglobins are expected to exhibit NOD activity *in vitro,* however, this issue cannot be considered as granted for THB1 solely on the basis of conserved amino acids residues in the protein and its transcriptional upregulation by NO. Thus, we have studied the capacity of THB1 to produce nitrate from NO. As expected, THB1

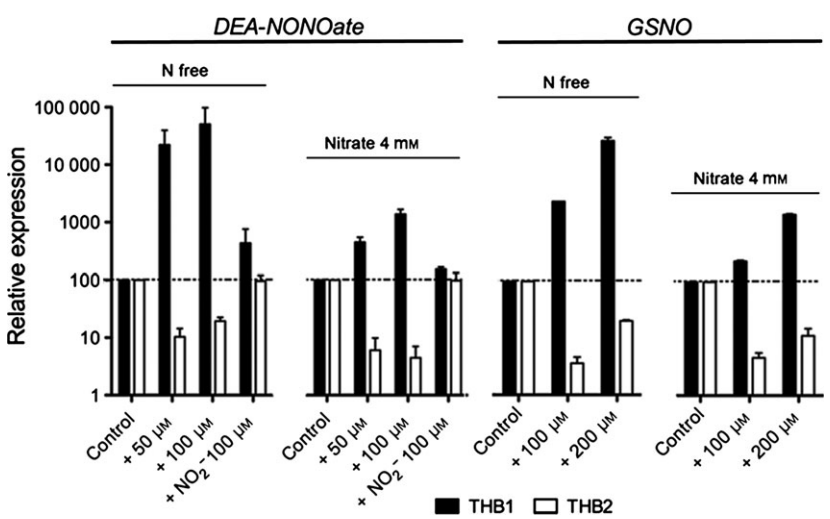

Figure 3. Differential regulation of *THB1* and *THB2* expression by nitric oxide.
Cells grown in ammonium medium were washed and induced for 1 h in the indicated medium. The effect of DEA-NONOate and GSNO on *THB1* and *THB2* expression was determined by real-time PCR. Nitrite 100 μM was used as a control. The expression levels without the NO donor were considered as 100%. The error bars represent the standard deviation (SD) of three technical replicates. These experiments were repeated at least three times with similar results.

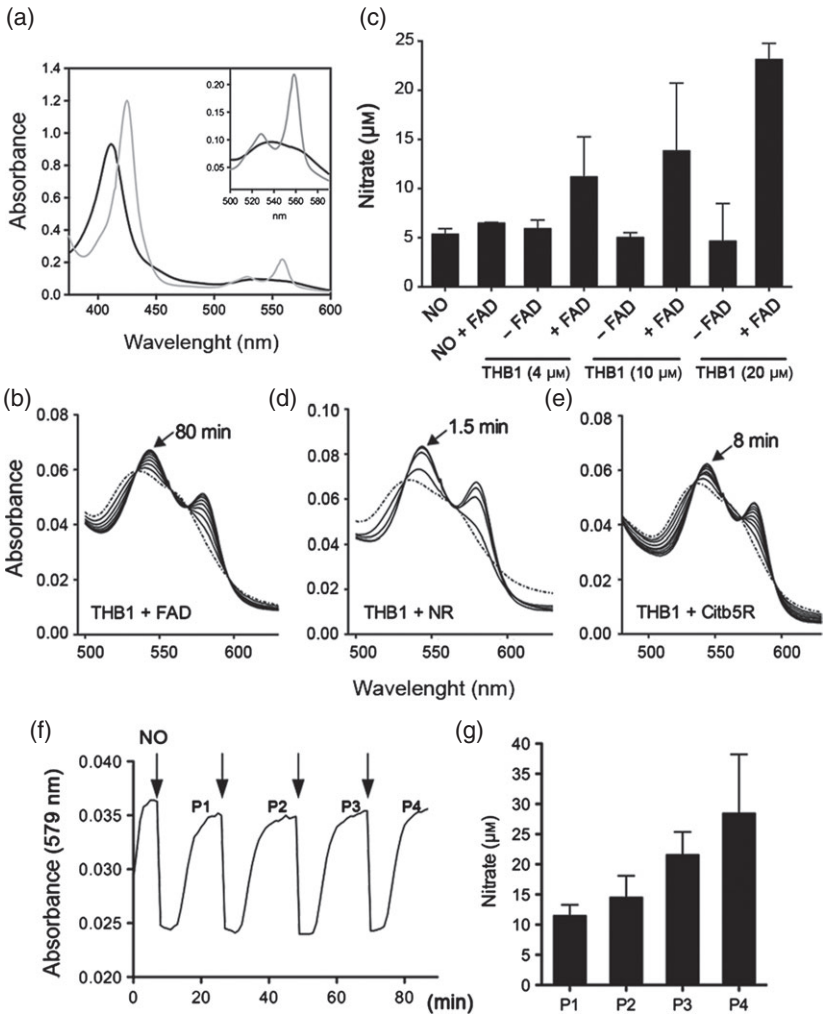

Figure 4. Absorbance spectra, NOD activity and reduction of THB1.
(a) THB1 was purified in aerobic conditions (black solid line). THB1-(Fe^{2+}) was produced by adding an excess of solid sodium dithionite (gray line). Two resolved bands at 528 and 558 nm (see inset) and shifted Soret peak at 425 nm appeared in these conditions.
(b) Conversion of 20 μM THB1-(Fe^{3+}) (dotted line) to THB1-(Fe^{2+}-O_2) the reduced and oxygenated state (black lines) with free cofactors (NADH 300 μM and FAD 2 μM). Each line represents 10 min of incubation. NADH alone, without FAD, was unable to reduce THB1-(Fe^{3+}) (dotted line) after 80 min. The spectrum recorded with free cofactors as indicated above with FAD 20 μM after 10 min was identical to that obtained with FAD 2 μM after 80 min shown in this Figure.
(c) THB1 NOD activity. Nitrate produced with different concentrations of THB1 was determined. NADH 300 μM and the NO donor DEA-NONOate 50 μM were used in all assays. Where indicated 20 μM FAD was used. Nitrate was measured by HPLC as detailed in Experimental procedures.
(d, e) THB1 reduction (20 μM) with (d) NR (2 μM) and (e) Cytb5R (2 μM) was carried out with NADH as electron donor (300 μM). NR and Cytb5R were added to measure the blank, thus these enzymes did not contribute to the spectra. To start the experiment, THB1 was added and a spectrum was immediately recorded (dotted line). Each black line represents 30 sec of incubation in (d) and 1 min in (e). All experiments were performed in Tris–HCl 50 mM pH 7.5 and spectra at about 15°C.
(f) NOD activity of THB1 was determined by the absorbance at 579 nm recording the changes between THB1-(Fe^{2+}-O_2) and THB1-(Fe^{3+}) forms as indicated in Experimental procedures .
(g) Nitrate production by THB1 at the different time points (P1–P4). A control without THB1 was used to subtract the nitrate produced in a THB1-independent way.

showed an FAD-dependent NOD activity but was unable to oxygenate NO as metHb (Fe^{3+}) when FAD was omitted from the reaction (Figure 4c).

NR efficiently catalyzes THB1 reduction

As indicated above the THB1 reduction by NADH and FAD as free cofactors is relatively slow, so we tested if THB1 reduction could be an enzyme-dependent process. NR is a homodimeric enzyme, which uses NAD(P)H as an electron donor. Each monomer contains FAD, heme (cytochrome b557) and molybdenum cofactor (MoCo), and it has been reported that NR can donate electrons from FAD to acceptors as cytochrome *c* or ferricyanide (diaphorase activity) (Fernandez and Cardenas, 1982; Franco *et al.*, 1984). As *NIA1* is expressed in N medium, we have studied if NR was able to reduce THB1 to its active form *in vitro.* When

using equivalent amounts of NR than FAD, THB1 was similarly reduced in 1.5 min, 40–50 times faster than using free cofactors (Figure 4d). These data suggest that NR could be catalyzing the reduction and maintenance of THB1 in its active form *in vivo.* We have also evaluated if THB1 could be reduced by the cytochrome *b5* reductase (Cytb5R), an homologous enzyme to the reductase domain of NR in *Chlamydomonas* (Figure S4). Cytb5R completely reduced THB1 in 8 min, five times slower than NR (Figure 4e). FNR1, an FAD-dependent reductase with low similarity to NR (Figure S4), led to a slight and incomplete reduction of THB1 even after 45 min (Figure S5).

In addition, the role of NR in the THB1 reduction was studied using cell-free extracts of four different strains (Figure S6). We used the 305 mutant (NR^-), affected in NAD(P)H-NR activity and without diaphorase-NR activity, its parental strain 6145c (wt), the 104 mutant, affected in the molybdenum cofactor synthesis ($CnxE^-$), which has diaphorase-NR activity but not NR activity, and its parental strain 21gr (wt). Cell-free extracts of the 305 mutant were able to reduce THB1 protein significantly slower (≈5 min) than those of its wt (≈2 min). This experiment showed that NR has an important role in the reduction of THB1 but there existed other diaphorase activities in the cell that can reduce THB1, at least *in vitro.* Nevertheless, THB1 reduction with extracts from mutant 104 was even faster (≈3 min) than with those of its wt (21gr) (≈7 min). This result supports the hypothesis that THB1 reduction is carried out by the NR diaphorase activity. The higher activity observed in 104 compared with the wild type can be explained by the overexpression of *NIA1* in this mutant (Quesada and Fernandez, 1994). These data support that THB1 is preferentially reduced by an enzyme-dependent mechanism, and that NR is an important enzyme for THB1 reduction *in vivo.*

THB1 NOD activity with NR

To measure the NOD activity of THB1 with NADPH–NR as electron donor and to determining the nitrate production, we have used a partially purified NR without MoCo cofactor, which only has diaphorase and not nitrate reductase activity. We observed that the diaphorase activity of NR can maintain THB1 on its reduced form after several NO additions (Figure 4f). We analyzed absorbance changes at the 579 nm characteristic peak that appears when THB1 is reduced and oxygenated (Fe^{2+}-O_2) and disappears when the protein is oxidized (Fe^{3+}) (Figure 4d). We observed that the NO addition oxidized THB1 and when NO disappeared NADPH–NR reduced it again. This process was performed several times showing the redox cycles of this system (Figure 4f). After each NO addition the nitrate produced was determined (Figure 4g). We observed that nitrate concentration increased after each NO addition. With this experiment we show that THB1 can take electrons from NR (diaphorase activity) and was able to produce nitrate from NO.

THB1 inhibits NR activity and scavenges NO *in vivo*

THB1 is reduced by NR, therefore we might expect that the deviation of electrons to THB1 would result in less reduction of nitrate. Thus, we investigated the effect of THB1 silencing on NR activity *in vivo.* With this aim, an artificial microRNA (Molnár *et al.*, 2009) was designed for *THB1.* Wild type strain (704) was transformed with the plasmid pChlamiRNA3-THB1 and several transformants were obtained with different levels of *THB1* expression. In these strains, *THB1* deficiency led to higher NR activity (Figure 5a). These results support the hypothesis that THB1 would inhibit NR by uncoupling the electron transfer from NAD(P)H to nitrate. In addition, *in vitro* experiments showed that high THB1 concentrations were able to partially inhibit NR activity from extracts even in the absence of NO (Figure 5b). This inhibition was observed with low amounts of THB1 when recombinant NR was used (Figure S7). Altogether, these data indicate that THB1 activity can regulate nitrate reduction by NR.

To confirm that THB1 was able to scavenge NO *in vivo,* cells from wt, the nit2 mutant 89.87 (<1% THB1 expression), amiTHB1-1 (15%) and amiTHB1-4 (47%) were induced in NA and loaded with 1 μM DAF-FM DA. Cells were then incubated with different concentrations of DEA-NONOate and probe-derived fluorescence was measured. NO determination with DAF probes is widely reported in the bibliography and it has been reported that this methodology is not always specific to NO, meaning that DAF measurements should be combined with other techniques to measure intracellular NO (Planchet and Kaiser, 2006; Rümer *et al.*, 2012). However, in this experiment we are not using DAF-FM to detect NO produced in the cells but the NO released by the donors and not scavenged. If THB1 has NOD activity in our conditions, fluorescence should correlate inversely with THB1 expression. As expected, the fluorescence signal was highest in 89.87 followed by amiTHB1-1, amiTHB1-4 and the 704 control strain (Figure 5c). The results described above support that THB1 has NOD activity *in vivo* and regulates NR activity. Finally, we compared the scavenging capacity of different NR mutants. We used the 701 strain lacking a cell wall (Loppes *et al.*, 1999) and the mutant 305 obtained from the 6145c strain; 701 has not been deeply characterized, however it has been reported that 305 strain lacks the NR diaphorase activity (Sosa *et al.*, 1978). As expected, these mutants showed higher levels of fluorescence than their corresponding wild type strains.

DISCUSSION

Truncated hemoglobins play an important role in reducing NO levels in bacteria (Ouellet *et al.*, 2002), but in eukaryotes

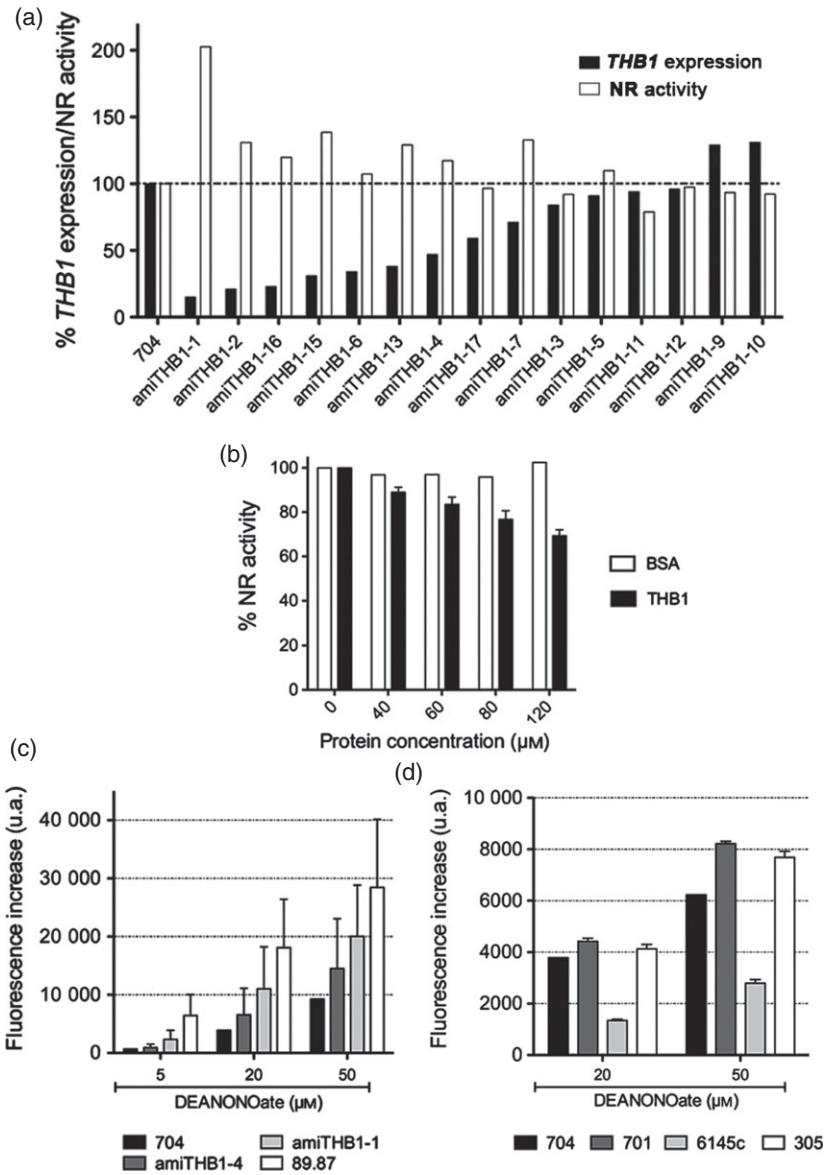

Figure 5. Effects of reduced *THB1* expression on NR activity *in vivo,* inhibition of NR by THB1 *in vitro* and cellular NO scavenging capacity.
(a) 704 and amiTHB1 strains were grown in TAP ammonium and induced in nitrate medium for 3 h. In each strain, THB1 expression and NR activity were determined. Inverse correlation was confirmed with the Spearman test ($r = -0.75$, $P < 0.001$ with a 95% confidence interval) calculated using GraphPad Prism software.
(b) Increasing concentrations of recombinant THB1 partially inhibit NR activity from cell-free extracts; 704 cells were grown in ammonium and induced in 4 mM nitrate medium for 3 h, then NADH-NR activity was determined from extracts. Same amounts of bovine serum albumin (BSA) were used as control. In this NR assay, nitrate 250 μM and NADH 500 μM were used. The error bars represent the standard deviation (SD) of three biological replicates. The NADPH-NR activity corresponding to 100% in (a) and (b) was 20.5 ± 1.9 mU mg of chlorophyll^{-1}.
(c) Wt and strains with different levels of THB1 expression (amiTHB1-4 → 47%, amiTHB1-1 → 15%, and 89.87 → 1%) were grown in TAP ammonium, concentrated (45 μg chlorophyll ml^{-1}) and induced in nitrate plus ammonium (NA) for 3 h. Cells were incubated with 1 μM of DAF-FM DA for the last 30 min. DEANONOate was added at the indicated concentrations and fluorescence was measured 10 min later. The error bars represent the standard deviation (SD) of at least three biological replicates.
(d) Fluorescence increase by NO treatment was measured in two mutants (701 and 305) with no NADH-NR activity. 701, a cell wall mutant was compared with 704 and 305 with 6145c with the aim to compare strains with similar genetic backgrounds. Cells were induced in nitrate 4 mM medium and the experiment was carried out as indicated above. The error bars represent the SD of three technical replicates and similar results were obtained in three biological replicates. In (c) and (d) fluorescence without NO donor is considered as control and the increase for each NO concentration is shown.

their function remains unclear. In this work we have identified two trHbs, which are regulated by the nitrogen source (THB1 and THB2). These hemoglobins have a high similarity with GlbN, the trHb from *Mycobacterium* responsible for NO scavenging and produced by macrophages as a defense mechanism. *Chlamydomonas* THB1 and THB2

belong to the same phylogenetic group as GlbN (Group I) (Figure 1a) and conserve the main residues implicated in the structure and formation of the network of tunnels and cavities needed for ligand diffusion and NOD activity (Figure 1b,c), which overall supports that these proteins have similar activities. However, THB1 and THB2 do not conserve the unusual short motif of 12 residues present in GlbN from *M. tuberculosis,* which is important for its high NOD activity. This domain does not affect the structural integrity of the protein and is located near the NO gate. This domain has not been described in others truncated hemoglobins, however the insertion at the N-terminus of Pre-A in GlbN of *M. smegmatis* (MsGlbN), which lacks this domain, improves its NOD activity (Lama *et al.*, 2009). We propose that trHbs without the Pre-A motif might require interactions with other proteins to be totally active.

Expression studies revealed that both trHbs were highly expressed in N medium (Figure 2a). *THB1* showed highest transcription levels in NA medium at short (30 min) and long (24 h) time points and in N medium after 3 h of induction. Both conditions are suitable for an NO burst inside the cell. In NA medium, nitrate assimilation genes are repressed by ammonium through an NO and cGMP-dependent mechanism (de Montaigu *et al.*, 2010). In N, the NR can produce NO from nitrite in conditions in which nitrite accumulates (i.e. by uncoupling between NR and NiR activities) (Mallick *et al.*, 1999; Yamasaki and Sakihama, 2000; Sakihama *et al.*, 2002). *THB1* expression patterns suggest that this hemoglobin could be implicated in NO metabolism in these specific conditions. In addition, *THB1* upregulation by NO (Figure 3) can be considered as strong evidence that this protein has NOD activity (Gardner, 2012).

THB2 showed a similar expression pattern to *NIA1,* i.e. low in ammonium, intermediate in mixed nutrition, high in nitrate, and strongly repressed in the presence of NO (Loppes *et al.*, 1999; Fernandez and Galvan, 2008; de Montaigu *et al.*, 2010, 2011). In this case, *THB2* inhibition by NO (Figure 3) does not necessarily discard its NOD activity but supports co-regulation with *NIA1* and suggests that THB2 could work closely with NR. Hemoglobin co-expression with NR has been described in *Zea mays* (Trevisan *et al.*, 2011) or Raphidophytes algae (Stewart and Coyne, 2011). We propose that THB1 and THB2 have the same activity because of their high sequence identity. Nevertheless, higher expression levels of *THB1* compared with *THB2* in all conditions suggest that THB1 could act as the principal scavenger of NO produced by nitrogen assimilation.

The function of these proteins is associated with the nitrate assimilation pathway for two reasons: first, the transcriptional upregulation in several nitrate sources and second, the dependence on the transcriptional factor for this pathway, NIT2 (Figure 2b). Our data support the known function of NIT2 in N and reveal a less studied role of NIT2 in NA conditions in which *NIT2* expression levels are much lower than in N (Camargo *et al.*, 2007). NIN-like proteins (NLPs) are homologues of NIT2 in plants, and the mechanisms of NLPs binding to DNA and activation of nitrate-responsive gene expression have been described recently (Castaings *et al.*, 2011; Konishi and Yanagisawa, 2013). However, if either one or several plant hemoglobin genes are regulated by NLPs is unknown.

THB1 was purified as metHb (Fe^{3+}), but the heme iron must be in the ferrous state to bind O_2 and carry out the NOD enzymatic reaction. In contrast with the non-symbiotic hemoglobin Glb1 from *Arabidopsis* (Perazzolli *et al.*, 2004), THB1 was unable to take electrons from NADH directly. However, it could use FAD as an electron carrier between NADH and the heme iron, similarly to *L. japonicus* globins (Sainz *et al.*, 2013) (Figure 4b). In plants, it has been reported that concentrations of free flavin cofactors in roots and nodules are high enough to maintain globins in the ferrous state (Sainz *et al.*, 2013). Nonetheless, according to the time needed to get a complete reduction (>60 and 10 min with 2 and 20 μM FAD, respectively for THB1, being even slower than for truncated Hbs from *L. japonicus*), we consider this mechanism too slow to be physiologically relevant. Herein we have shown that THB1 is very efficiently reduced by NR, which contains FAD as a cofactor (Figure 4d). It is noteworthy that GlbN from *Mycobacterium tuberculosis* can be reduced *in vitro* at a concentration and velocity similar to those of CrTHB1 by the NADH ferredoxin/flavodoxin reductase system from *E. coli* (Singh *et al.*, 2014). NR takes electrons from NAD(P)H to reduce nitrate or alternatively to transfer these electrons to other acceptors from FAD (diaphorase activity). Our data points out that NR reduces THB1 through its diaphorase activity maintaining THB1 on its active form (Figures 4f,g and S6). Consequently, THB1 NOD activity may be associated to NR which would result in the partial inhibition of its activity of nitrate reduction due to the draining of NAD(P)H electrons. Thus, when a burst of NO is produced, THB1 takes electrons from NR and nitrite production is consequently decreased. According to this, we suggest that THB1 could participate in maintaining coupled nitrate and nitrite reduction. When intracellular nitrite increases by low NiR activity (i.e. low photosynthetic rate), it can be excreted and/or used by NR to generate NO (Navarro *et al.*, 2000; Sakihama *et al.*, 2002). THB1 would then act by removing the very reactive NO and simultaneously inhibiting NR by redirecting the electrons from FAD to THB1. This mechanism would eventually prevent the loss of nitrogen produced by the nitrite excretion and the generation of additional nitrite because of NR inhibition. Furthermore, it has been reported that NO inhibits NR *in vivo* but not *in vitro,* suggesting the requirement of another cellular component (Sanz-Luque *et al.*, 2013), and our data support a

mechanism by which THB1 could at least partially be implicated in this inhibition.

We have also shown that Cytb5R, an enzyme with high identity to NR, could mediate THB1 reduction *in vitro* (Figure 4e). However, its efficiency was lower than NR, supporting the connection between THB1 and the nitrogen assimilation pathway. However, THB1 interactions with other reductases cannot be discarded.

Conversely, THB1 lacks the N-terminal Pre-A motif, which is important for NOD activity of GlbN from *M. tuberculosis* (Lama *et al.*, 2009), so it is therefore possible that THB1 interaction with a reductase enzyme (NR in N medium) would be necessary to obtain optimal NOD activity. In addition, the predicted THB1 structure supports the hypothesis of this interaction because surface residues at the entrance of the heme cavity are positively charged, suggesting that this position could interact with the reductase domain of NR, which has a high density of negative charge in the surface amino acids near to the FAD cofactor. However, Cytb5R, which reduces THB1 at a slower rate than NR, showed a smaller complementarity of charge with THB1 (Figure 6). Nevertheless, the implication of these residues in the interaction will have to be studied in detail.

The relationship between NR and THB1 is supported by the data obtained with strains that express artificial micro RNA, which suppresses *THB1* gene expression (amiTHB1). THB1 downregulation correlates with a higher NR activity *in vivo* (Figure 5a), further supporting that THB1 affects NR activity probably by acting as an electron sink. In addition, high THB1 concentrations were able to inhibit NR activity *in vitro* (Figures 5b and S7) supporting this interaction. How this interaction could modify the flexible NR domains structure leading to the redirection of electrons and to a less active enzyme is not known. Thus, the reduction of THB1 levels results in an increased NR activity because more electrons can be used for nitrate reduction. In the same way, the opposite effect occurs when THB1 concentration increases.

Finally, NO scavenging by THB1 was observed *in vivo* from the fluorescence produced by the DAF-FM probe after addition of different concentrations of DEA-NONOate to several strains expressing THB1 at different levels and in various NR mutants. Thus, strains with lower expression levels of THB1 presented higher fluorescence levels (Figure 5c). This supports that THB1 plays a significant role as an NO detoxifier *in vivo.* Furthermore, according to the higher fluorescence levels and the strongly affected

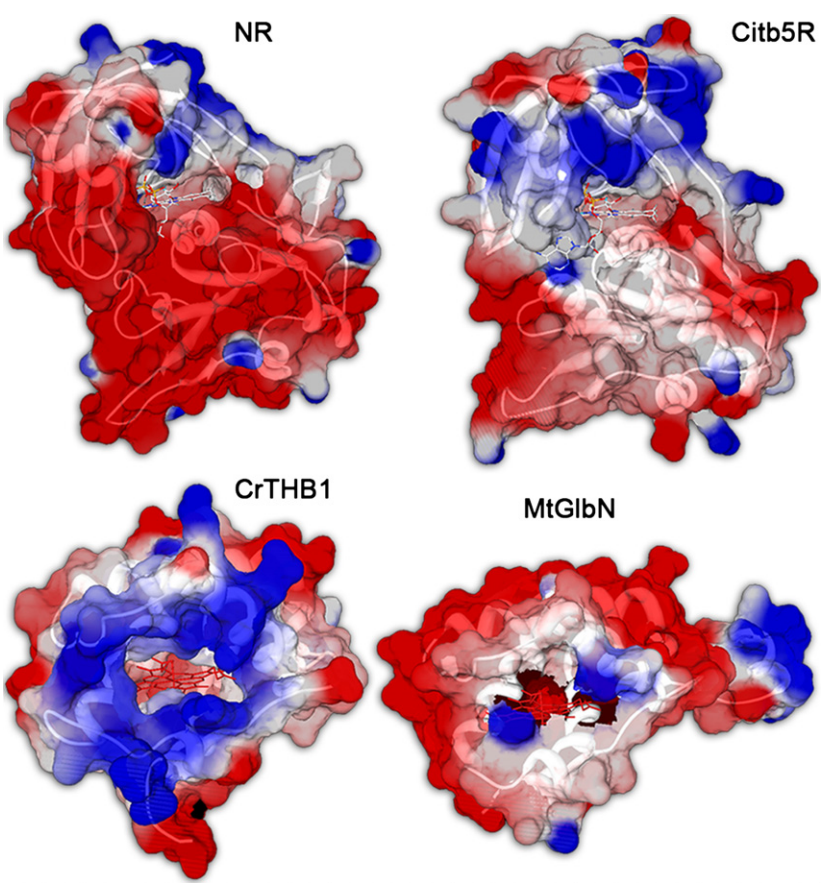

Figure 6. Electrostatic potential of reductase domain of NR, Cytb5R, CrTHB1 and MtGlbN.
Crystal structure of MtGlbN was taken from the Protein Data Bank with reference 1IDR. Reductase domain of NR, Cytb5R and CrTHB1 structures were generated using Swiss-model (http://swissmodel.expasy.org/). All images were produced with Swiss-Pdb Viewer software. Blue and red colours represent residues positively and negatively charged, respectively. Parameters to calculate electrostatic potential were the standard used by the software.

expression of *THB1* and *THB2* in the nit2 mutant, we suggest that probably both THBs work as NO scavengers, although further studies on THB2 will be required. Our data are supported by the results of Wei *et al.* (2014) that detected higher NO levels in a nit2 strain compared with wild type (wt). In any case, special care must be taken if a nit2 mutant is going to be used to study NO level fluctuations, particularly when NO donors are used. As expected, NR mutants, lacking diaphorase activity, showed higher fluorescence levels than wt strains (Figure 5d). These facts support that both NR and THB1 work together as an NO detoxifier system in the alga.

In conclusion, this work contributes to clarifying the role of truncated hemoglobins and how these proteins are maintained in their active form *in vivo.* We have demonstrated that two truncated hemoglobins are closely related to the nitrogen assimilation pathway and that THB1 controls NO levels in the cell by working with NR and regulating nitrate reduction.

EXPERIMENTAL PROCEDURES

Strains and reagents

Algae and bacteria strains used are listed in Table S2. Cells were grown under mixotrophic conditions with TAP medium (Harris, 2009). MAHMA-NONOate (6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-hexanamine), DEA-NONOate (2-(*N*,*N*-diethylamino)-diazenolate 2-oxide sodium salt), GSNO (*S*-nitrosoglutathione), and DAF-FM DA (diaminofluorescein-FM diacetate) were obtained from Sigma-Aldrich, Inc (www.sigmaaldrich.com).

Quantitative real-time PCR

Quantification of *THB1-4* transcripts was performed by real-time polymerase chain reaction (PCR). Reverse transcription of 1 μg of total RNA was carried out using the iScript Selected cDNA Synthesis Kit (Bio-Rad, www.bio-rad.com) and following manufacturer instructions. Quantitative real-time PCR was performed on an iCycler MyiQ2 real-time PCR detection system (Bio-Rad) using SsoFast™ EvaGreen Supermix (Bio-Rad). RNA levels were normalized using the ubiquitin ligase gene as internal standard (Gonzalez-Ballester *et al.*, 2004). Primers sequences are listed in Table S3.

Recombinant protein expression and purification

NR and Cytb5R were purified as described previously (Chamizo-Ampudia *et al.*, 2011; Sanz-Luque *et al.*, 2013). NR without MoCo cofactor was purified with the same protocol and plasmid but using the chlM *E. coli* strain (Johnson and Rajagopalan, 1987). *THB1* and *FNR1* cDNAs were amplified by PCR (primers in Table S1) and were cloned in pQE80L (Qiagen, www.qiagen.com). *THB1* was expressed in the C41 *E. coli* strain. THB1 induction was performed overnight at 37°C with 100 μM isopropyl-beta-D-thiogalactopyranoside (IPTG), and purification was carried out with a Ni–nitrilotriacetic acid (Ni–NTA) matrix, as recommended by the supplier (Qiagen), under native conditions at 4°C. FNR1 expression was performed in *E. coli* BL21. Cells were grown aerobically in LB medium to an A_{550} of 0.1 before induction. *E. coli* BL21 cells were induced with 100 μM IPTG to start expression. After induction, the cells were grown for an additional 16 h at 37°C. Purification was carried out following the same protocol than for THB1.

NOD activity and nitrate determination

Reactions were performed in Tris–HCl 50 mM pH 7.5 with 300 μM NADH and 50 μM DEA-NONOate. When indicated 20 μM FAD and different concentrations of THB1 were added. Samples were incubated for 30 min at 30°C. Nitrate produced was separated and quantified by HPLC. To assay NOD activity with NR, reactions were performed in the same buffer with 10 μM NADPH and a NADPH-generating system. As NO source MAHMA-NONOate 10 μM was used. THB1 (7.5 μM) was reduced with 2 nmol of $MoCo^{-}$–NR. At the different times (P1–P4) nitrate was determined using *Chlamydomonas* NR (Sanz-Luque *et al.*, 2013) and the Griess assay (Snell and Snell, 1949). Supporting experimental details are shown in Methods S1.

Artificial miRNA constructs and fluorescence measurement with DAF-FM DA

We prepared vector pChlamiRNA3-THB1 following the protocol described by Molnár *et al.* (2009). Specific oligos used for plasmid construction are listed in Table S1. This vector was used to transform the 704 strain. For DAF-FM DA experiments, ammonium grown cells were concentrated (45 μg ml^{-1} chlorophyll) and induced in nitrate 4 mM plus ammonium 8 mM for 3 h. In the last 30 min, 1 μM of DAF-FM DA was added to each culture. DEA-NONOate was added at different concentrations and cells were incubated for 15 min before measurement. Then, cultures were placed in triplicate in OptiPlate Black Opaque 96-well Microplate from PerkinElmer (perkinelmer.com), and fluorescence was measured in a Tecan SpectraFluor Plus spectrophotometer. Excitation and emission wavelength were 485 and 510 nm, respectively.

Nitrate reductase assays

Chlamydomonas cells were grown in ammonium 8 mM and induced in 4 mM KNO_3 medium for 3 h. Samples of 50 μl extracts, prepared from 10 ml of culture, were used to determine NADPH-NR in Tris–HCl 50 mM pH 7.5 buffer, by following the protocol described (Fernandez and Cardenas, 1982).

ACKNOWLEDGEMENTS

This work was funded by MINECO (Ministerio de Economia y Competitividad, Spain, Grant no. BFU2011-29338) with support of European Fondo Europeo de Desarrollo Regional (FEDER) program, Junta de Andalucía (P08-CVI-04157 and BIO-502), and Plan Propio de la Universidad de Córdoba. Authors thank Maribel Macías for skillful technical assistance. One of us (F. O-C) thanks MINECO for a 'Formación de Personal Investigador' fellowship.

NOTE ADDED UNDER REVIEWING

During the process of reviewing this paper, THB1 from *Chlamydomonas* was also shown to be regulated by nitrogen and NIT2 and to have NOD activity (Johnson *et al.*, 2014).

SUPPORTING INFORMATION

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

Figure S1. Degree of identity among compared hemoglobins sequences in Figure 1(b).

Figure S2. Alignment of the complete sequences of several trHbs from group I (extended version of Figure 1b).

Figure S3. Expression of *THBs* in response to the nitrogen source in 704 (wt) and 89.87 (nit2$^-$) mutant strains.

Figure S4. Alignment of reductase enzymes used for THB1 reduction.

Figure S5. THB1 reduction with FNR1.

Figure S6. THB1 reduction with cell-free extract from NR mutants with and without diaphorase activity.

Figure S7. NR activity in presence of THB1.

Table S1. Accession numbers of proteins used in Figure 1.

Table S2. Algal and bacterial strains.

Table S3. List of primers used.

Methods S1. NOD activity and nitrate determination.

REFERENCES

Bidon-Chanal, A., Martí, M.A., Estrin, D.A., Luque, F.J. (2009) Exploring the nitric oxide detoxification mechanism of *Mycobacterium tuberculosis* truncated haemoglobin N. In *Self-Organization of Molecular Systems: From Molecules and Clusters to Nanotubes and Proteins* (Russo, N., Antonchenko, V.Y. and Kryachko, E., eds) Dordrecht, the Netherlands: Springer, NATO Science for Peace and Security Series A: Chemistry and Biology, pp. 33–47.

Camargo, A., Llamas, A., Schnell, R.A., Higuera, J.J., Gonzalez-Ballester, D., Lefebvre, P.A., Fernandez, E. and Galvan, A. (2007) Nitrate signaling by the regulatory gene *NIT2* in *Chlamydomonas*. *Plant Cell*, **19**, 3491–3503.

Castaings, L., Marchive, C., Meyer, C. and Krapp, A. (2011) Nitrogen signalling in *Arabidopsis*: how to obtain insights into a complex signalling network. *J. Exp. Bot.* **62**, 1391–1397.

Chamizo-Ampudia, A., Galvan, A., Fernandez, E. and Llamas, A. (2011) The *Chlamydomonas reinhardtii* molybdenum cofactor enzyme crARC has a Zn-dependent activity and protein partners similar to those of its human homologue. *Eukaryot. Cell*, **10**, 1270–1282.

Corpas, F.J., Barroso, J.B., Carreras, A. *et al.* (2004) Cellular and subcellular localization of endogenous nitric oxide in young and senescent pea plants. *Plant Physiol.* **136**, 2722–2733.

Daigle, R., Guertin, M. and Lagüe, P. (2009) Structural characterization of the tunnels of *Mycobacterium tuberculosis* truncated hemoglobin N from molecular dynamics simulations. *Proteins*, **75**, 735–747.

Du, S., Zhang, Y., Lin, X., Wang, Y. and Tang, C. (2008) Regulation of nitrate reductase by nitric oxide in Chinese cabbage pakchoi (*Brassica chinensis* L.). *Plant Cell Environ.* **31**, 195–204.

Ermler, U., Siddiqui, R.A., Cramm, R. and Friedrich, B. (1995) Crystal structure of the flavohemoglobin from *Alcaligenes eutrophus* at 1.75 Å resolution. *EMBO J.* **14**, 6067–6077.

Fernandez, E. and Cardenas, J. (1982) Regulation of the nitrate-reducing system enzymes in wild-type and mutant strains of *Chlamydomonas reinhardtii*. *Mol. Gen. Genet.* **186**, 164–169.

Fernandez, E. and Galvan, A. (2008) Nitrate assimilation in *Chlamydomonas*. *Eukaryot. Cell*, **7**, 555–559.

Fernandez, E., Schnell, R., Ranum, L.P., Hussey, S.C., Silflow, C.D. and Lefebvre, P.A. (1989) Isolation and characterization of the nitrate reductase structural gene of *Chlamydomonas reinhardtii*. *Proc. Natl Acad. Sci. USA*, **86**, 6449–6453.

Fernández-Marcos, M., Sanz, L., Lewis, D.R., Muday, G.K. and Lorenzo, O. (2011) Nitric oxide causes root apical meristem defects and growth inhibition while reducing PIN-FORMED 1 (PIN1)-dependent acropetal auxin transport. *Proc. Natl Acad. Sci. USA*, **108**, 18506–18511.

Floryszak-Wieczorek, J., Milczarek, G., Arasimowicz, M. and Ciszewski, A. (2006) Do nitric oxide donors mimic endogenous NO-related response in plants? *Planta*, **224**, 1363–1372.

Foresi, N., Correa-Aragunde, N., Parisi, G., Calo, G., Salerno, G. and Lamattina, L. (2010) Characterization of a nitric oxide synthase from the plant kingdom: NO generation from the green alga *Ostreococcus tauri* is light irradiance and growth phase dependent. *Plant Cell*, **22**, 3816–3830.

Franco, A.R., Cárdenas, J. and Fernandez, E. (1984) Heteromultimeric structure of the nitrate reductase complex of *Chlamydomonas reinhardtii*. *EMBO J.* **3**, 1403–1407.

Gardner, P.R. (2008) Assay and characterization of the NO dioxygenase activity of flavohemoglobins. *Methods Enzymol.* **436**, 217–237.

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

Gonzalez-Ballester, D., Camargo, A. and Fernandez, E. (2004) Ammonium transporter genes in *Chlamydomonas*: the nitrate-specific regulatory gene *Nit2* is involved in Amt1;1 expression. *Plant Mol. Biol.* **56**, 863–878.

Gonzalez-Ballester, D., de Montaigu, A., Higuera, J.J., Galvan, A. and Fernandez, E. (2005) Functional genomics of the regulation of the nitrate assimilation pathway in *Chlamydomonas*. *Plant Physiol.* **137**, 522–533.

Gupta, K.J., Hebelstrup, K.H., Mur, L.A.J. and Igamberdiev, A.U. (2011) Plant hemoglobins: important players at the crossroads between oxygen and nitric oxide. *FEBS Lett.* **585**, 3843–3849.

Harris, E. (2009) *The Chlamydomonas Sourcebook*. New York: Academic Press.

He, Y., Tang, R.-H., Hao, Y. *et al.* (2004) Nitric oxide represses the *Arabidopsis* floral transition. *Science*, **305**, 1968–1971.

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.

Hemschemeier, A., Düner, M., Casero, D., Merchant, S.S., Winkler, M. and Happe, T. (2013) Hypoxic survival requires a 2-on-2 hemoglobin in a process involving nitric oxide. *Proc. Natl Acad. Sci. USA*, **110**, 10854–10859.

Hoy, J.A. and Hargrove, M.S. (2008) The structure and function of plant hemoglobins. *Plant Physiol. Biochem.* **46**, 371–379.

Jin, C.W., Du, S.T., Zhang, Y.S., Lin, X.Y. and Tang, C.X. (2009) Differential regulatory role of nitric oxide in mediating nitrate reductase activity in roots of tomato (*Solanum lycocarpum*). *Ann. Bot.* **104**, 9–17.

Johnson, M.E. and Rajagopalan, K.V. (1987) Involvement of chlA, E, M and N loci in *Escherichia coli* molybdopterin biosynthesis. *J. Bacteriol.* **169**, 117–125.

Johnson, E.A., Rice, S.L., Preimesberger, M.R. *et al.* (2014) Characterization of THB1, a *Chlamydomonas reinhardtii* truncated hemoglobin: linkage to nitrogen metabolism and identification of lysine as the distal heme ligand. *Biochemistry*, **53**, 4573–4589.

Kim, D.Y., Hong, M.J. and Seo, Y.W. (2014) Role of wheat trHb in nitric oxide scavenging. *Mol. Biol. Rep.* **41**, 5931–5941.

Konishi, M. and Yanagisawa, S. (2013) *Arabidopsis* NIN-like transcription factors have a central role in nitrate signalling. *Nat. Commun.* **4**, 1617.

Lama, A., Pawaria, S. and Dikshit, K.L. (2006) Oxygen binding and NO scavenging properties of truncated hemoglobin, HbN, of *Mycobacterium smegmatis*. *FEBS Lett.* **580**, 4031–4041.

Lama, A., Pawaria, S., Bidon-Chanal, A. *et al.* (2009) Role of Pre-A motif in nitric oxide scavenging by truncated hemoglobin, HbN, of *Mycobacterium tuberculosis*. *J. Biol. Chem.* **284**, 14457.

Li, Q. and Lancaster, J.R. (2009) Calibration of nitric oxide flux generation from diazeniumdiolate *NO donors. *Nitric Oxide*, **21**, 69–75.

Loppes, R., Radoux, M., Ohresser, M.C. and Matagne, R.F. (1999) Transcriptional regulation of the *Nia1* gene encoding nitrate reductase in *Chlamydomonas reinhardtii*: effects of various environmental factors on the expression of a reporter gene under the control of the Nia1 promoter. *Plant Mol. Biol.* **41**, 701–711.

Mallick, N., Rai, L.C., Mohn, F.H. and Soeder, C.J. (1999) Studies on nitric oxide (NO) formation by the green alga *Scenedesmus obliquus* and the diazotrophic cyanobacterium *Anabaena doliolum*. *Chemosphere*, **39**, 1601–1610.

Milani, M., Pesce, A., Ouellet, H., Guertin, M. and Bolognesi, M. (2003) Truncated hemoglobins and nitric oxide action. *IUBMB Life*, **55**, 623–627.

Molnár, A., Bassett, A., Thuenemann, E., Schwach, F., Karkare, S., Ossowski, S., Weigel, D. and Baulcombe, D. (2009) Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*. *Plant J.* **58**, 165–174.

de Montaigu, A., Sanz-Luque, E., Galvan, A. and Fernandez, E. (2010) A soluble guanylate cyclase mediates negative signaling by ammonium on

expression of nitrate reductase in *Chlamydomonas*. *Plant Cell*, **22**, 1532–1548.

de Montaigu, A., Sanz-Luque, E., Macias, M.I., Galvan, A. and Fernandez, E. (2011) Transcriptional regulation of CDP1 and CYG56 is required for proper NH^{4+} sensing in *Chlamydomonas*. *J. Exp. Bot.* **62**, 1425–1437.

Mur, L.A.J., Sivakumaran, A., Mandon, J., Cristescu, S.M., Harren, F.J. and Hebelstrup, K.H. (2012) Haemoglobin modulates salicylate and jasmonate/ethylene-mediated resistance mechanisms against pathogens. *J. Exp. Bot.* **63**, 4375–4387.

Navarro, M.T., Guerra, E., Fernandez, E. and Galvan, A. (2000) Nitrite reductase mutants as an approach to understanding nitrate assimilation in *Chlamydomonas reinhardtii*. *Plant Physiol.* **122**, 283–290.

Oliveira, A., Singh, S., Bidon-Chanal, A., Forti, F., Martí, M.A., Boechi, L., Estrin, D.A., Dikshit, K.L. and Luque, F.J. (2012) Role of PheE15 gate in ligand entry and nitric oxide detoxification function of *Mycobacterium tuberculosis* truncated hemoglobin. N P. Carloni, ed. *PLoS ONE*, **7**, e49291.

Ouellet, H., Ouellet, Y., Richard, C., Labarre, M., Wittenberg, B., Wittenberg, J. and Guertin, M. (2002) Truncated hemoglobin HbN protects *Mycobacterium bovis* from nitric oxide. *Proc. Natl Acad. Sci. USA*, **99**, 5902–5907.

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.

Pesce, A., Couture, M., Dewilde, S. and Guertin, M. (2000) A novel two-over-two [alpha]-helical sandwich fold is characteristic of the truncated hemoglobin family. *EMBO J.* **19**, 2424–2434.

Planchet, E. and Kaiser, W.M. (2006) Nitric oxide production in plants: facts and fictions. *Plant Signal. Behav.* **1**, 46.

Quesada, A. and Fernandez, E. (1994) Expression of nitrate assimilation related genes in *Chlamydomonas reinhardtii*. *Plant Mol. Biol.* **24**, 185–194.

Quesada, A., Galván, A., Schnell, R.A., Lefebvre, P.A. and Fernandez, E. (1993) Five nitrate assimilation-related loci are clustered in *Chlamydomonas reinhardtii*. *Mol. Gen. Genet.* **240**, 387–394.

Quesada, A., Hidalgo, J. and Fernandez, E. (1998) Three Nrt2 genes are differentially regulated in *Chlamydomonas reinhardtii*. *Mol. Gen. Genet.* **258**, 373–377.

Rosales, E.P., Iannone, M.F., Groppa, M.D. and Benavides, M.P. (2011) Nitric oxide inhibits nitrate reductase activity in wheat leaves. *Plant Physiol. Biochem.* **49**, 124–130.

Rümer, S., Krischke, M., Fekete, A., Mueller, M.J. and Kaiser, W.M. (2012) DAF-fluorescence without NO: elicitor treated tobacco cells produce fluorescing DAF-derivatives not related to DAF-2 triazol. *Nitric Oxide*, **27**, 123–135.

Sainz, M., Pérez-Rontomé, C., Ramos, J., Mulet, J.M., James, E.K., Bhattacharjee, U., Petrich, J.W. and Becana, M. (2013) Plant hemoglobins may be maintained in functional form by reduced flavins in the nuclei, and confer differential tolerance to nitro-oxidative stress. *Plant J.* **76**, 875–887.

Sakihama, Y., Nakamura, S. and Yamasaki, H. (2002) Nitric oxide production mediated by nitrate reductase in the green alga *Chlamydomonas reinhardtii*: an alternative NO production pathway in photosynthetic organisms. *Plant Cell Physiol.* **43**, 290–297.

Sanz-Luque, E., Ocana-Calahorro, F., Llamas, A., Galvan, A. and Fernandez, E. (2013) Nitric oxide controls nitrate and ammonium assimilation in *Chlamydomonas reinhardtii*. *J. Exp. Bot.* **64**, 3373–3383.

Schinko, T., Berger, H., Lee, W. *et al.* (2010) Transcriptome analysis of nitrate assimilation in *Aspergillus nidulans* reveals connections to nitric oxide metabolism. *Mol. Microbiol.* **78**, 720–738.

Singh, S., Thakur, N., Oliveira, A. *et al.* (2014) Mechanistic insight into the enzymatic reduction of truncated hemoglobin N of Mycobacterium tuberculosis: role of the CD loop and Pre-A motif in electron cycling. *J. Biol. Chem.* **289**, 21573–21583.

Smagghe, B.J., Trent, J.T. and Hargrove, M.S. (2008) NO dioxygenase activity in hemoglobins is ubiquitous in vitro, but limited by reduction *in vivo*. H. H. H. W. Schmidt, ed. *PLoS ONE*, **3**, e2039.

Smagghe, B.J., Hoy, J.A., Percifield, R. *et al.* (2009) Correlations between oxygen affinity and sequence classifications of plant hemoglobins. *Biopolymers*, **91**, 1083–1096.

Snell, F.D. and Snell, C.T. (1949) *Colorimetric Methods of Analysis*, 3rd edn, vol **2**, Princeton, NJ: Van Nostrand.

Sosa, F.M., Ortega, T. and Barea, J.L. (1978) Mutants from *Chlamydomonas* reinhardii affected in their nitrate assimilation capability. *Plant Sci. Lett.* **11**, 51–58.

Stewart, J.J. and Coyne, K.J. (2011) Analysis of raphidophyte assimilatory nitrate reductase reveals unique domain architecture incorporating a 2/2 hemoglobin. *Plant Mol. Biol.* **77**, 565–575.

Trevisan, S., Manoli, A., Begheldo, M., Nonis, A., Enna, M., Vaccaro, S., Caporale, G., Ruperti, B. and Quaggiotti, S. (2011) Transcriptome analysis reveals coordinated spatiotemporal regulation of hemoglobin and nitrate reductase in response to nitrate in maize roots. *New Phytol.* **192**, 338–352.

Villarreal, N. and Martínez, G. (2009) Influence of plant growth regulators on polygalacturonase expression in strawberry fruit. *Plant Sci.* **176**, 749–757.

Vuletich, D.A. and Lecomte, J.T.J. (2006) A phylogenetic and structural analysis of truncated hemoglobins. *J. Mol. Evol.* **62**, 196–210.

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.

Wang, H., Lu, Y., Liu, P., Wen, W., Zhang, J., Ge, X. and Xia, Y. (2012) The ammonium/nitrate ratio is an input signal in the temperature-modulated, SNC1-mediated and EDS1-dependent autoimmunity of nudt6-2 nudt7. *Plant J.* **73**, 262–275.

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.

Wei, L., Derrien, B., Gautier, A. *et al.* (2014) Nitric oxide-triggered remodeling of chloroplast bioenergetics and thylakoid proteins upon nitrogen starvation in *Chlamydomonas reinhardtii*. *Plant Cell*, **26**, 353–372.

Wittenberg, J., Bolognesi, M. and Wittenberg, B. (2002) Truncated hemoglobins: a new family of hemoglobins widely distributed in bacteria, unicellular eukaryotes, and plants. *J. Biol. Chem.* **277**, 871–874.

Yamasaki, H. and Sakihama, Y. (2000) Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. *FEBS Lett.* **468**, 89–92.

